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MASTER OF DRUG DEVELOPMENT

Role of BRD4 and MLL1 in HIV transcription and reactivation

THESIS

submitted by

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List of abbreviations

Ac	Acetylated
AD	Aqua destillata
AIDS	Acquired immune deficiency syndrome
Amp	Ampicillin
ASK	Activator of S-phase kinase complex
B-HIVE	Barcoded HIV ensembles
BCA	Bicinchoninic acid protein assay
BD	Bromodomain
bDNA	Branched DNA
BET	Bromodomain and extra terminal domain motif
BnAbs	Broadly neutralizing antibodies
BRD4	Bromodomain containing protein 4
CAR	Chimeric antigen receptors
cART	Combination anti-retroviral therapy
CBP	CREB-binding protein
CC_{50}	50% cytotoxic concentration
CDK9	Cyclin-dependent kinase 9
ChIP	Chromatin immunoprecipitation
CKII	Casein kinase II
Cntrl	Control
Co-IP	Co-immunoprecipitation
CPSF6	Cleavage and polyadenylation specificity factor 6
CRISPR/cas9	Clustered regularly interspaced short palindromic repeats - cas9
CTD	Carboxy terminal domain
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco Modified Eagle Medium
DSIF	DRB sensitivity-inducing factor
ECs	Elite controllers
ELISA	Enzyme-linked immuno sorbent assay
ELL2	Elongation factor for RNA polymerase II
ET	Extra terminal
FBS	Fetal bovine serum
Gp	Glycoprotein
HDGF	Hepatoma-derived growth factor
HEK293T	Human embryonic kidney cells

HIV	Human immunodeficiency virus
HRP-2	Hepatoma-derived growth factor related protein
IBD	Integrase binding domain
IN	Integrase
IWS1	Interacts-with-Spt6
LEDGF/p75	Lens epithelium-derived Growth Factor of 75kDa
LPAs	Latency promoting agents
LRAs	Latency reversing agents
LV	Lentivirus
MED1	Mediator complex subunit 1
miRNA	Micro RNA
MLL1	Mixed lineage leukemia 1
MLLr	Mixed lineage leukemia-rearranged
mRNA	Messenger RNA
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
NELF	negative elongation factor
NF- κb	Nuclear factor that binds the immunoglobulin K light chain gene
Ns	Non-significant
P-TEFβ	Positive transcription elongation factor β
PAF1	Poll II-associated factor 1
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PogZ	Pogo transposable element with zinc finger domain
Pol	Polymerase
PWWP	Pro-Trp-Trp-Pro
qPCR	Quantitative PCR
RLU	Relative light units
RPMI	Roswell park memorial institute
RT	Reverse transcription
SAHA	Suberoylanilide Hydroxamic Acid
SEC	Super elongation complex
STI	Structured treatment interruption
T-ALL	T- cell acute lymphoblastic leukemia
TAR	Trans-activation response element
Tat	Trans-activator of transcription

TF	Transcription factors
TNF-α	Tumor Necrosis factor α
vDNA	Viral DNA
vRNA	Viral RNA
7SK snRNP	7SK small nuclear ribonucleoprotein

Summary

Infection with the human immunodeficiency virus (HIV) leads to acquired immune deficiency syndrome. Persistence of integrated viral DNA that has entered a transcriptionally silent mode in cellular reservoirs, remains a major hurdle to cure HIV infection. Currently, HIV infected patients are treated with combination antiretroviral therapy, which reduces the viral load but cannot cure HIV infection. My research group focuses on a block-and-lock cure strategy with the goal to generate a cellular reservoir resistant to reactivation and unable to rebound after treatment interruption. In this context, LEDGINs, small molecules that inhibit the interaction between viral integrase and lens epithelium-derived growth factor (LEDGF/p75), were developed. LEDGINs reduce viral integration and retarget the residual provirus to regions resistant to reactivation. Unfortunately, LEDGINs are insufficient to completely block HIV transcription. Therefore, a more in-depth investigation into other co-factors important in HIV gene regulation, such as bromodomain containing protein 4 (BRD4) and mixed lineage leukemia 1 (MLL1), are of importance to increase the therapeutic potential for a block-and-lock strategy. With the branched DNA (bDNA) technique, the viral DNA and viral RNA can be determined on single cell level. Therefore, this technique represents an elegant tool to study the impact of the addition of a compound on the transcriptional state of the provirus and to identify novel targets for a block-and-lock strategy.

JQ1 is a well-known BRD4 inhibitor currently used in the shock-and-kill strategy, which aims to eradicate the latent reservoir after reactivation. Several studies showed that HIV replication is induced by JQ1. In our hands the bDNA technique revealed that addition of JQ1 promotes basal transcription and tumor necrosis factor α (TNF-α) induced reactivation of HIV on single cell level with an optimal concentration of 1 μM JQ1. Moreover, apart from BRD4 inhibitors used in the shock-and-kill strategy, Niu *et al.* recently discovered the first BRD4 inhibitor known to repress HIV replication, ZL0580. Unfortunately, the high toxicity and lack of effect on HIV expression observed in this study, hamper the potential of ZL0580 in a block-and-lock strategy. Moreover, it was previously shown that LEDGINs do not affect the proximity of integrated provirus to enhancer regions in the genome that are dependent on BRD4. Therefore, it was hypothesized that BRD4 inhibition by JQ1 may silence the residual high vRNA expressors of LEDGIN-retargeted provirus by interfering with the enhancer regions. In contrast with this hypothesis, addition of JQ1 did not contribute to a more efficient silencing of HIV gene expression. Interestingly, this study showed that JQ1 had a different impact on TNF-α induced reactivation of LEDGIN-retargeted provirus compared to provirus with LEDGF/p75-mediated integration.

Finally, Gao *et al.* reported a role of MLL1 in latency reversal of HIV. In this thesis, the impact of MLL1 depletion on HIV replication was studied. Interestingly, the results indicated that MLL1 depletion severely impairs HIV infectivity. However, several questions remain about the underlying molecular mechanism and further research is needed to provide the evidence for MLL1 as a target in a block-and-lock cure strategy.

Samenvatting

Het humaan immunodeficiëntievirus (HIV) veroorzaakt het acquired immune deficiency syndrome. De persistentie van geïntegreerd viraal DNA in een transcriptioneel stille modus in cellulaire reservoirs is de belangrijkste barrière voor genezing van HIV infectie. Momenteel worden HIV-patiënten behandeld met combinatie antiretrovirale therapie, die de virale lading vermindert maar de ziekte niet geneest. Mijn onderzoeksgroep richt zich op een block-and-lock genezingsstrategie met als doel een cellulair reservoir te genereren resistent tegen reactivatie na onderbreking van de behandeling. Zo werden LEDGINs ontwikkeld, kleine moleculen die de interactie tussen het viraal integrase en lens epithelium-derived growth factor (LEDGF/p75) inhiberen en zo de virale integratie inhiberen en het residuele provirus omleiden naar regio's resistent tegen reactivatie. Helaas zijn LEDGINs onvoldoende om de transcriptie van HIV volledig te blokkeren. Daarom is diepgaand onderzoek nodig naar andere co-factoren die belangrijk zijn in de regulatie van HIV expressie, zoals bromodomain containing protein 4 (BRD4) en mixed lineage leukemia 1 (MLL1), om het potentieel voor een block-and-lock strategie te verhogen. De branched DNA (bDNA) techniek laat toe om het het viraal DNA en viraal RNA niveau per cel te analyseren. Bijgevolg is deze techniek een elegante methode om het effect van een inhibitor op de transcriptionele toestand van het provirus te bestuderen, om zo nieuwe doelwitten te identificeren voor een block-and-lock strategie.

JQ1 is een bekende BRD4 remmer, gebruikt in de shock-and-kill strategie, die gericht is op het uitroeien van het latente reservoir na reactivatie. Verschillende studies toonden aan dat JQ1 de HIV replicatie stimuleert. In deze studie gaf de bDNA-techniek aan dat JQ1 de basale transcriptie en de tumor necrosis factor α (TNF-α) geïnduceerde reactivatie van HIV bevorderde op ‘single cell’ niveau met een optimum bij 1 μM van JQ1. Bovendien rapporteerde Niu *et al.*, naast het gebruik van BRD4 remmers in de. shock-and-kill strategie, onlangs de eerste BRD4 remmer die HIV replicatie onderdrukt, ZL0580. Helaas belemmerde de hoge toxiciteit en het gebrek aan effect op HIV expressie in deze studie het potentieel van ZL0580 in een block-and-lock strategie. Bovnendien, is het eerder aangetoond dat LEDGINs geen invloed hebben op integratie in de nabijheid van enhancer regio’s, die afhankelijk zijn van BRD4. Daarom werd onderzocht of JQ1-gemedieerde inhibitie van BRD4 de residuele vRNA expressie na LEDGIN-gemedieerde retargeting zou inhiberen door te interfereren met de enhancer regio's. In tegenstelling, inhibeerde JQ1 niet de residuele HIV replicatie. Interessant is dat deze studie wel aantoonde dat JQ1 een andere invloed had op TNF-α geïnduceerde reactivatie van provirus met LEDGIN-geheroriënteerde integratie in vergelijking met LEDGF/p75-gemedieerde integratie.

Tenslotte rapporteerden Gao *et al.* een rol van MLL1 in reactivatie van HIV. Daarom was het doel om in deze thesis verder het effect van MLL1 depletie op HIV replicatie te onderzoeken. De resultaten tonen aan dat MLL1 depletie sterk de HIV-infectiviteit vermindert. Er blijven echter nog verschillende vragen over het moleculaire mechanisme achter deze bevindingen en verder onderzoek is nodig om MLL1 als een doelwit in een block-and-lock genezingsstrategie te identificeren.

1. Introduction

1.1 Pathophysiology of HIV

Human immunodeficiency virus (HIV) is an incurable infection which results in the acquired immune deficiency syndrome (AIDS) (1). HIV is a lentivirus, which is a member of the *Retroviridae* family (2). HIV is transmitted through blood or other secretions of the body like vaginal fluid. The HIV virus mainly attacks CD4+ T cells, dendritic cells and macrophages. These cells are essential for the functioning of the immune system and therefore HIV infection leads to an impaired immune system (2,3). As a consequence, HIV infected patients have a high risk for opportunistic infections. The clinical course of the HIV infection contains three phases. First, there is an acute symptomatic phase with flu-like symptoms, characterized by a dramatic decrease in CD4+T cells and a high viremia. The next phase is the asymptomatic phase. During this process, the immune response hinders the viral replication, which leads to a low viremia and an increase in CD4+ T cells. However, due to the ongoing replication of HIV, a slow drop in CD4+T cells occurs. The third 'AIDS' phase is reached when the CD4+ T cells drop to a critically low level (2,3).

1.2 HIV life cycle

The envelope of HIV contains two glycoproteins (gp), gp120 and gp41. Gp120 binds the CD4 receptor, and this interaction leads to a conformational change in gp120 whereby gp120 is able to interact with the HIV co-receptor, CXCR4 or CCR5. Next, due to insertion of the gp41 fusion peptides in the cell membrane, the viral and cellular membranes fuse (4,5,6). After virus entry into the cell, the viral capsid is disassembled by a process called uncoating (7). HIV is a member of the *Retroviridae* family, a family of viruses that typically convert their RNA into DNA via reverse transcription (RT). Afterwards, the viral DNA (vDNA) is integrated into the chromosome of the host cell, a reaction catalyzed by the viral enzyme integrase (IN) (8). Subsequently, the HIV virus uses host factors for transcription and translation of its viral proteins (6,8). In the next step the essential viral proteins and the viral genome are packaged into a virion, a process called viral assembly. This virion crosses the plasma membrane and acquires a lipid envelope, during budding. After budding, proteolytic maturation converts the virion into an infectious viral particle **(Figure 1.1)** (9,10).

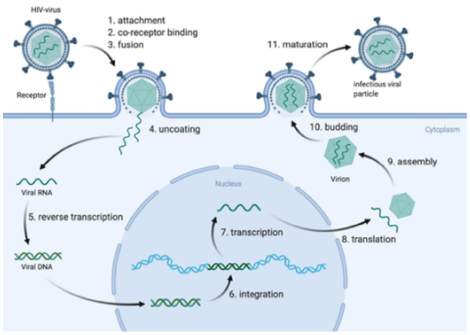


Figure 1.1. Life cycle of HIV virus: The virus attaches to the cell [1], followed by co-receptor binding [2] and fusion of the viral membrane with the cellular membrane of the host cell [3]. In the host cell, viral particles are able to uncoat [4]. Next, there is a RT of the vRNA to vDNA [5]. Afterwards, the vDNA integrates in the chromosome of the host cell, catalyzed by IN [6]. After transcription [7] and translation [8], the viral proteins and viral genome are packed into virions during assembly [9]. Consequently, the virion crosses the plasma membrane and acquires a lipid envelope during budding [10]. Finally, the virion is converted into a viral particle due to viral proteases during maturation [11]. HIV, human immunodeficiency virus; RT, reverse transcription; vRNA, viral RNA; vDNA, viral DNA; IN, integrase. (Adapted from: MG. Atta et al, Clin J Am Soc Nephrol., 2019, **14**(3), 435–444. And created with BioRender.com)

1.3 HIV latent reservoir

HIV latency is defined as the persistence of integrated vDNA in a transcriptionally silent mode (11). Due to this phenomenon the virus remains invisible for the host immune system and thus persists life-long (12,13). Since many (intact) latent viruses remain replication-competent, they are able to reactivate spontaneously or after treatment interruption. Therefore, the latent reservoir is the main hurdle towards a cure for HIV (13).

There are 2 main types of latency, namely pre-integration and post-integration latency. In pre-integration latency, the complementary DNA is synthesized during RT, but is not integrated into the cellular genome (13). More studies are necessary to determine its exact contribution to the latent reservoir. However, post-integration latency has a greater contribution to the latent reservoir (13). It refers to cells containing integrated provirus that expresses a significant lower amount of proteins without reactivation, but have the potential to be reactivated during treatment interruption of antiretroviral therapy or *in vitro* by the addition of cytokines or antigens (13,14).

The latent reservoir is created within days after infection (13) and is extremely stable, as its half-life is estimated to be approximately 44 months (15). The latent reservoir is mainly formed by infection of active CD4 + cells that undergo a transition to resting memory CD4 + T cells. Other cell types such as stem cell like memory T cells, follicular helper T cells, macrophages, monocytes and dendritic cells contribute to the latent reservoir as well (13,16). Nonetheless, the extent of their contribution to the reservoir remains unknown (13,17). Moreover, the latent reservoir is maintained by homeostatic proliferation and clonal expansion of latently infected cells (18,19). The reservoir is mainly found in the blood and other tissues such as lymph nodes and the gut-associated lymphoid tissue (13). Several studies suggest that limited drug penetration into these sanctuaries contributes to the persistence of HIV (20,21). However, the absence of sequence diversification and the lack of viral load reduction during treatment intensification, which refers to the addition of a fourth drug to triple combination therapy, is at odds with this explanation as a sole cause of persistence (22).

To conclude, HIV latency is considered as a complex phenomenon which is regulated by various mechanisms such as the integration site selection, availability of transcription factors (TF), RNA polymerase (pol) II pausing, the influence of surrounding genes, the relative orientation of the provirus, the presence of a microRNA (miRNA) and the efficacy of RNA export from nucleus to cytoplasm (13). Understanding the regulatory mechanisms of HIV latency is of paramount importance to find a cure for HIV. In this Master's thesis, an attempt to answer several key questions about the role of integration site selection in latency and its link to transcription and reactivation will be made.

1.4 Role of integration site selection and the epigenetic landscape in HIV replication

Integration is an essential step in the life cycle of the HIV provirus (23); still, the role of integration and the epigenetic landscape adjacent to the HIV integration site in HIV latency remains poorly understood (14,24). During integration, the HIV virus integrates the reverse transcribed vDNA into the chromosome of the host cell, a reaction catalyzed by the viral enzyme IN (8,24,25). It is known that integration affects HIV replication (8). Moreover, recent studies indicate that the integration site and the chromatin landscape surrounding the integration site are linked with the transcriptional state of the provirus (24,27). Furthermore, the site of HIV integration is not arbitrary, instead integration is targeted to transcriptionally active units within gene dense regions of the chromatin (14,27). Lens epithelium-derived growth factor of 75 kDa (LEDGF/p75) plays an important role in integration site selection and targets HIV integration into active genes (14,24,26).

The importance of integration site selection in a functional block-and-lock cure approach was further emphasized in a study on a specific group of patients, called elite controllers (ECs) (29–32). This small group of patients (0.2-0.5% of all HIV-infected patients) can control HIV replication without combination anti-retroviral therapy (cART). Recently, Jiang *et al.* discovered that the latent viral reservoirs of ECs are in a state of deep and long-lasting latency which is related to their integration site. They further suggested that the deep latent reservoir of ECs may be explained by a cell-mediated immune selection of latent provirus over time (29).The presence of deep latent provirus in patients that spontaneously control HIV infection underscored the potential of a functional block-and-lock cure where the provirus is still present but in such a deep latent state that it is unable to reactivate.

1.4.1 Epigenetic landscape at HIV integration site

Chromatin is made out of nucleosomes, which in turn consists out of a pair of 4 histones that form an octamer (33). The structure of the chromatin surrounding the HIV integration site affects transcription (23,24,27). Heterochromatin has a condensed structure, causing repression of transcription by hindering the accessibility of the HIV promoter region from transcriptional cofactors (34–36). Euchromatin refers to a less compact and more open structure of the chromatin, which ensures that the promoter region is accessible, and that transcription is therefore possible (34–36). Marini *et al*. showed that HIV prefers integration into a subset of genes, called HIV recurrent integration genes, which are dominantly located at the nuclear periphery (34). In contrast, HIV is less likely to integrate into heterochromatin regions such as lamin-associated domains and regions located at the center of the nucleus (34).

The transcriptional state can be regulated by epigenetic modifications as well, such as histone acetylation or DNA methylation. Acetylation of histone results in a more loosen chromatin and thereby induces the transcriptional activity (37). On the other hand, methylation of DNA is associated with a repression of the transcriptional activity due to a reduced accessibility of the chromatin to proteins (38).

1.4.2 Role of LEDGF/p75 in integration site selection

LEDGF/p75 is a transcriptional co-activator and is a member of the hepatoma-derived growth factor family (14). It contains an N-terminal PWWP domain (named after Pro-Trp-Trp-Pro), which recognizes the methylated histone mark (H3K36me2/3), an epigenetic mark associated with active gene expression. Furthermore, LEDGF/p75 contains an integrase binding domain (IBD) that undergoes interaction with IN **(Figure 1.2)** (39).

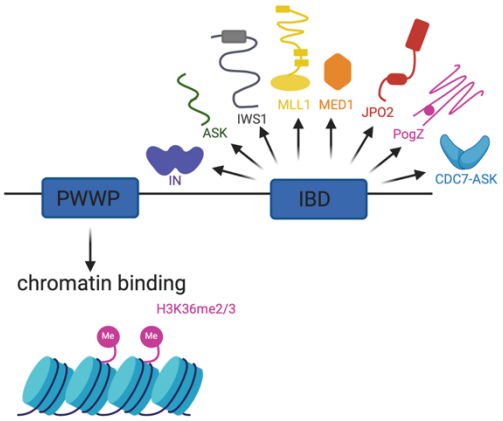


Figure 1.2. Schematic representation of LEDGF/p75: LEDGF/p75 contains a PWWP domain and an IBD domain. The PWPP domain binds the chromatin at the H3K36me2/3 mark. The IBD undergoes interaction with lentiviral IN, JPO2, PogZ, IWS1, ASK, CDC7- ASK, MED1, MLL1. LEDGF/p75, lens epithelium-derived growth factor; PWWP, Prp-Trp-Trp-Pro; IBD, integrase binding domain; IN, integrase; PogZ, pogo transposable element with zinc finger domain; IWS1, interacts-with Spt6; ASK, activator of S-phase kinase complex; MED1, mediator complex subunit 1; MLL1, mixed lineage leukemia 1. (Adapted from: P. Tesina et al, Nat Commun., 2015, **6**:7968, 1-14. And created with Biorender.com)

The interaction of IN with LEDGF/p75 is a unique and conserved property of the lentiviruses, such as HIV-1, HIV-2, simian immuno-deficiency virus, equine infectious anemia virus and feline immunodeficiency virus (40,41). However, it was later discovered that different cellular proteins are able to bind to LEDGF/p75 via the IBD, such as JPO2, pogo transposable element with zinc finger domain (PogZ), interacts-with-Spt6 (IWS1), activator of S-phase kinase complex (ASK), CDC7-ASK, mediator complex subunit 1 (MED1) and mixed lineage leukemia 1 (MLL1) proteins **(Figure 1.2)** (42–48). The hepatoma-derived growth factor related protein (HRP2) can take over the function of LEDGF/p75 after its depletion, because it contains an IBD and a PWWP domain as well (41,49,50). However, LEDGF/p75 is considered as the most important determinant in integration site selection, whereby integration is targeted to transcriptionally active genes and the nuclear periphery. Additionally, LEDGF/p75 promotes the catalytic activity of IN and prevents proteolytic degradation (14,51–53).

A knockdown of LEDGF/p75 resulted in an inhibition of HIV replication (54). Furthermore, overexpression of the IBD of LEDGF/p75 led to a competition of the IBD with LEDGF/p75, which results in an inhibition of HIV integration (55). These findings corroborated the LEDGF/p75-IN interaction as a therapeutic target for antiretroviral therapy (43).

LEDGINs have been discovered by structure-based drug design based on the interface between HIV IN and LEDGF/p75. This resulted in derivatives of 2- (quinolin-3yl) acetic acid, small molecules that inhibit the interaction between IN and LEDGF/p75 **(Figure 1.3)** (14,56,57).

LEDGINs have several effects on the viral replication cycle. First, LEDGINs allosterically inhibit the catalytic activity of IN (the so-called early effect) (14,43,58,59). Furthermore, LEDGINs enhance IN oligomerization resulting in defective progeny virions (the so-called late effect) (26,58,60). The ribonucleoprotein of the viral particles is moved outside the capsid core, while other particles do not even contain a core (58). Moreover, the particles produced in the presence of LEDGINs display less efficient RT, nuclear import and integration (14).

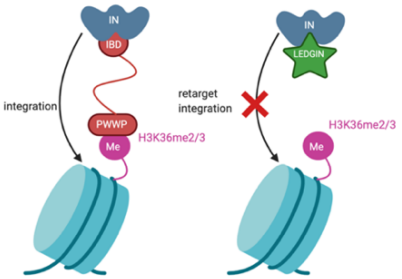


Figure 1.3. LEDGINs are small molecule inhibitors of IN-LEDGF/p75 interaction: LEDGF/p75, consisting out of an IBD, which interacts with IN, and a PWWP domain, which interacts with the chromatin, targets integration. LEDGINs inhibit the interaction between LEDGF/p75 and IN and thereby retarget integration to regions further away from the H3K36me2/3 mark. IN, integrase; LEDGF/p75, lens epithelium-derived growth factor; IBD, integrase binding domain; PWWP, Pro-Trp-Trp-Pro. (Figure created with Biorender.com)

In addition to the inhibition of HIV replication, Vranckx *et al.* showed that upon LEDGIN-treatment, the residual provirus shifts out of active transcriptional units and moves towards intergenic regions and genes that are less transcriptionally active. In addition, the integration site is retargeted away from H3K36me3, the mark recognized by LEDGF/p75 (61). Furthermore, the integrated provirus is located more in the center of the nucleus instead of in the periphery (61) and the integration occurrs more in the inverse orientation as well (61). This resulted in a residual provirus that is more latent and less sensitive to reactivation (62). These findings further imply the important role of LEDGF/p75 in targeting integration sites and its link with latency. Therefore, LEDGINs can be considered as a tool to study the link between HIV latency and integration. (14,50). Furthermore, these findings indicated that LEDGF/p75 is not only a target for antiretroviral therapy, but also for a functional block-and-lock cure strategy.

Moreover, with the barcoded HIV-ensembles (B-HIVE) technology, which tags the HIV genome with a unique barcode to trace insert-specific HIV expression, my host lab further confirmed the LEDGF/p75-independent phenotype after LEDGIN-treatment (62). This showed that LEDGIN-treatment enlarges the distance of integration to the marker recognized by LEDGF/p75 (H3K36me3), reduced the viral RNA (vRNA) expression per residual vDNA copy and increased the proportion of silent provirus (62).

However, it has to be mentioned that besides LEDGF/p75, HIV uses other cellular co-factors to regulate integration as well. Cleavage and polyadenylation specificity factor 6 (CPSF6) interacts with the viral capsids to promote nuclear entry which is suggested to influence the HIV integration pattern (63). The key role of CSPF6 in integration site selection was reported in several studies based on either loss of CPSF6 (63–67) or mutations at the CPSF6 binding site of the viral capsid (N74D) (67); in these studies the HIV integration site was shifted out of active genes. However, in contrast to LEDGF/p75, CPSF6 knockdown has no significant influence on HIV replication (24,68,69).

1.5 Towards a cure for HIV

1.5.1 Need for a cure of HIV infection

According to the World Health Organization, 76 million people have been infected with HIV and 33 million people have died since the beginning of the pandemic (70). However, significant progress in terms of HIV treatment led to an increased life expectancy of people living with HIV (70). Nevertheless, it remains challenging for the scientific community to find a cure for HIV. HIV patients are currently treated with cART. This treatment significantly increases the life expectancy of HIV infected patients. However, cART has some crucial drawbacks. First, there are serious side effects associated with these drugs (e.g. central fat accumulation, renal toxic effects, liver toxicity, hypersensitivity reactions and osteopenia) (71). Secondly, the treatment requires strict adherence to impede emergence of strains resistant to cART (72–74). From an economic perspective, the cost of cART therapy is not sustainable (75). Furthermore, the delivery of lifelong treatment to all infected patients remains an operational and logistical challenge (11). Therefore a limited number of infected people have access to cART, especially in low income countries (11). Finally, cART reduces the viral load but is not curative and therefore must be taken life-long to keep the HIV virus under control (11). This emphasizes that a persistent effort in academic research is required to broaden our basic understanding of the molecular virology of HIV, which is of significant importance to discover novel approaches and targets to cure HIV infection.

1.5.2 HIV cure strategies

Despite all the barriers on the road towards an HIV cure, several promising strategies are under development. Like discussed before, the co-receptors CCR5 and CXCR4 are involved in the entry of the HIV virus in the host cell by membrane fusion (4,5,6). Previous studies showed that people with a 32 base pair deletion in the CCR5 co-receptor gene are resistant to HIV infection (76). The Berlin patient, who was infected with HIV and diagnosed with acute myeloid leukemia, received two bone-marrow transplantations from a CCR-5 Δ32-donor, which cured his HIV infection (76,77). Unfortunately, the bone-marrow transplantation is not suitable to cure all HIV infected patients because it is a life-threatening procedure and challenging to find major histocompatibility complex matched CCR5 Δ32 donors. Moreover, the same transplantation in other patients resulted in a switch to CXCR4 tropic viruses and thereby failed to cure HIV (78). Still, all these findings fueled the interest of scientists in the mutation of this co-receptor and several attempts followed, as illustrated by the Boston patient and the Essen patient. Unfortunately, in these cases viral replication rebounded (78,79). Recently, the London patient received a bone-marrow transplantation from a CCR5Δ32 donor to treat his Hodgkin's lymphoma. The cART of the London patient was interrupted for 16 months after transplantation and since then HIV remission for more than 18 months has been achieved (80).

Gene editing strategies like zinc finger nucleases, transcription activator like effector nucleases and clustered regularly interspaced short palindromic repeats (CRISPR/cas9) are utilized to achieve a disruption of the HIV coreceptors CCR5 and CXCR4 (81,82). However, a careful selection of targets for gene editing is needed since disrupting host genes may have severe implications. For instance CXCR4 is not a valid target because of its role in hematopoietic development (83). Another major drawback of gene editing approaches in general is the unexpected off-target effects and the insufficiency to target all infected cells (82).

Broadly neutralizing antibodies (bnAbs) represent an alternative promising therapeutic avenue as well. They recognize conserved epitopes on the HIV surface envelope trimer. There are attempts for the development of bnAbs for the treatment of HIV infection. Additionally, bnAbs are used to guide HIV vaccine design as well (84,85).

Transplantation with differentiated T cells, which are genetically altered to bring chimeric antigen receptors (CAR) to expression that recognize HIV specific epitopes (e.g. HIV envelope glycoproteins such as gp120 and gp160 (86)), represent an alternative treatment strategy for HIV infection. These CAR-T cells specifically recognize HIV specific epitopes and generate a cytotoxic T cell response in the HIV infected cells (87). The anti-HIV CAR T cell therapies have shown success in the cancer field, but several obstacles for using CAR T cell therapies in HIV infection occurred such as off target effects, severe cytokine release syndrome, susceptibility to HIV infection and neurological toxicity (88).

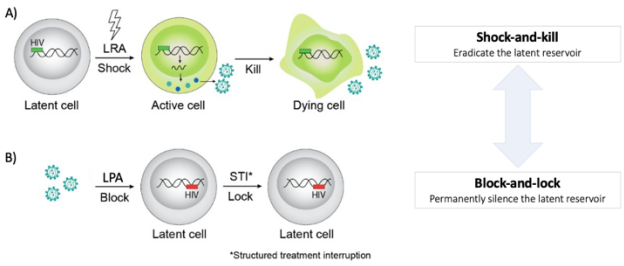


Figure 1.4. HIV cure strategies: A) The shock-and-kill approach, with the aim to eradicate the latent reservoir, is shown on the top. LRAs are added and lead to the production of viral proteins. These can be recognized and killed by the immune system or destroyed by the cytopathic effect of the viral protein. **B)** The block-and-lock strategy, with the goal to permanently silence the latent reservoir, is shown at the bottom. First, LPAs are administered to lock the provirus in a deep latent state, even after STI. HIV, human immunodeficiency virus; LRA, latency reversing agent; LPA, latency promoting agent; STI, structured treatment interruption. (Adapted from: Z. Debyser et al, Viruses., 2019, **11**(1), 1–12.)

Furthermore, the sterilizing shock-and-kill strategy has been widely investigated. Sterilizing strategies aims to eliminate the HIV virus completely (36). In the shock-and-kill strategy, the provirus is reactivated with latency reversing agents (LRA), which leads to the production of viral proteins. As a result, the virus can be eliminated by immune mediated clearance and the cytopathic effect of the reactivated virus **(Figure 1.4.A)** (89). Notwithstanding significant research on this strategy, this shock-and-kill strategy is still not successful in the clinic. First, many LRA's only reactivate CD4 + T cells and are not efficient for other cell types, such as macrophages. Another major drawback is the toxicity caused by the modulation of the gene expression. Additionally, reinfection can occur by activating uninfected HIV target cells. Finally, the cytopathic effect and the cytotoxic T lymphocytes are not always potent enough (13,89).

The limited success of eradication strategies resulted in a shift to investigate alternative approaches, such as a functional cure. A functional cure is defined as a long-term virological control or remission in the absence of treatment, despite the persistence of replication-competent virus (90). The aim of the block-and-lock functional cure is to create a cellular reservoir resistant to reactivation, which is unable to rebound after treatment interruption (14) . This can be achieved by blocking the transcription of the provirus and locking the reservoir in a deep latent state **(Figure 1.4.B)**. Different latency promoting agents (LPAs) can be used in such a strategy (57), such as the previously discussed LEDGINs (61), didehydrocortistatin A (91), curaxin CBL0100 (92), heat shock protein 90 inhibitors (93), Janus kinase signal transducer and activator of transcription pathway inhibitors (94) and mammalian target of rapamycin signaling inhibitors (95). In this Master's thesis the role of LEDGINs in a block-and-lock phenotype will be further investigated.

1.6 Role of BRD4 and enhancers in residual HIV transcription

1.6.1 Residual high vRNA expressors due to enhancer-mediated transcription

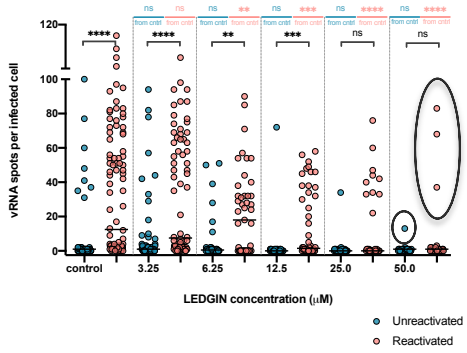


Figure 1.5. Residual high vRNA expressors after LEDGIN-treatment: With bDNA imaging, it is shown that increasing concentrations of LEDGINs reduce vRNA expression per residual DNA copy (blue spots). Moreover, LEDGINs reduce reactivation of vRNA expression after treatment with TNF-α, as shown by a reduction in the median number of vRNA spots per infected cell (red spots). Still, out of 100 cells, one high vRNA expressor persists in the unreactivated condition and three persist in the reactivated condition (black circles). Bars represent the median number vRNA spots per infected cell. Statistical significance was calculated with the Kruskal-Wallis test (ns=non-significant, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and the reactivated cells and between each concentration of LEDGIN compared to the control (0 µM LEDGIN). vRNA, viral RNA; bDNA, branched DNA; TNF- α, tumor necrosis factor α; ns, non-significant; cntrl, control (Unpublished results from PhD student Julie Janssens).

Using branched DNA (bDNA) imaging, which allows to simultaneously detect the vDNA and vRNA in single cells, my host lab recently confirmed the transcriptional silent phenotype after LEDGIN-mediated retargeting (96). LEDGINs reduce basal transcription and reactivation of HIV **(Figure 1.5)**. Still, few residual high vRNA expressors persist after treatment with high concentrations of LEDGINs **(Figure 1.5)**. The key question of this Master's thesis is to unravel the mechanisms behind these residual high vRNA expressors after LEDGIN-treatment.

Interestingly, the proximity of integration sites to (super-)enhancers is not altered by LEDGINs (62). Enhancers are DNA elements characterized by a high level of acetylation, which are able to interact with TF and promotors to enhance transcription (97,98). Super-enhancers are genomic regions with clusters of several enhancers characterized by increased interaction with TF, mediators and RNA pol II (97).

Additionally, super enhancers have the potential to bind a higher level of co-activators of transcription, such as bromodomain containing protein 4 (BRD4) and MED1 (99). The proximity of the integration site to enhancers is associated with a higher transcriptional activity of the provirus (100). Moreover, latent provirus is located further away from enhancers, which correlates with the fact that latent provirus has less transcriptional activity (100).

Because transcription is stimulated by enhancers and LEDGINs do not affect the proximity of integration sites to enhancers, it is postulated that the residual high vRNA expressors after LEDGIN-mediated retargeting are caused by integration close to (super-)enhancers. This hypothesis will be investigated during my Master's thesis by an experiment in which LEDGINs will be combined with enhancer antagonists. If residual high vRNA expressors are mediated by enhancers, blocking the enhancers will silence the residual high vRNA expressors. Because of the role of BRD4 in enhancer biology, BRD4 inhibitors will be used to investigate this hypothesis.

1.6.2 Role of BRD4 in HIV transcription and reactivation

BRD4 belongs to the family of the bromodomain and extra terminal motif (BET) proteins. All members of this family have the common feature of two conserved N-terminal tandem bromodomains (BD), BDI and BDII, and one conserved extra-terminal (ET) domain. The bromodomain binds acetylated lysine residues on histones and thereby modulates HIV-transcription (101). The ET-domain has a shorter sequence and is responsible for the recruitment of multiple proteins to regulated genes (101,102).

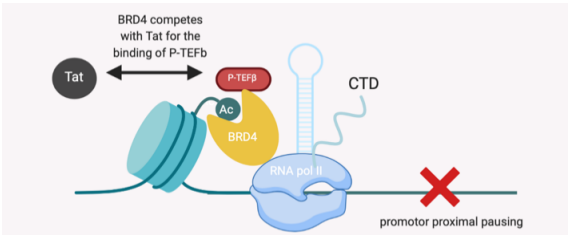


Figure 1.6. Promotor proximal pausing of RNA pol II: BRD4 is bound to acetylated (Ac) histones and competitively blocks the interaction of Tat with P-TEFβ and thereby prevents the formation of the SEC. This leads to promotor proximal pausing of RNA pol II. Pol, polymerase; BRD4, bromodomain containing protein 4; Ac, acetylated; Tat, trans-activator of transcription; P-TEFβ, positive transcription elongation factor β; SEC, super elongation complex. (Adapted from: Z. Li et al, Nucleic Acids Res., 2013, **41**(1), 277–287. And created with www.Biorender.com)

After initiation of transcription, promotor-proximal pausing of RNA pol II, caused by negative elongation factors, negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF), is known to block HIV transcription. The viral Trans-activator of transcription (Tat) protein antagonizes this block by bringing the super elongation complex (SEC) to the paused RNA pol II (103). The SEC consists of elongation factor for RNA polymerase II (ELL2) and positive transcription elongation factor β (P-TEFβ). Furthermore, P-TEFβ is composed of cyclin T and cyclin-dependent kinase 9 (CDK9). CDK9 is a serine/threonine kinase that phosphorylates and thereby inhibits two negative elongation factors NELF and DSIF. CDK9 phosphorylates the RNA pol II as well, which promotes HIV transcription (103). The other component of SEC, ELL2 directly promotes the activation of the RNA pol II as well (103).

BRD4, when bound to the acetylated histones, plays an important role in this mechanism by competing with Tat for P-TEFβ. Therefore it inhibits the Tat activation and the formation of the SEC , which results in promotor-proximal pausing of RNA pol II **(Figure 1.6)** (103).

In a first part of this Master's thesis, the role of BRD4 in HIV transcription and reactivation from LEDGF/p75-dependent provirus will be investigated by inhibiting BRD4 with two BRD4 inhibitors that will be discussed later, namely JQ1 and ZL0580. This mechanism relates to the effect of BRD4 on LEDGF/p75-dependent provirus and thus is unrelated to the residual high vRNA expressors after LEDGIN-mediated retargeting.

In contrast, BRD4 plays a role in the enhancer biology as well. The genomic region of the (super-)enhancers contain a high level of acetylated lysine residues and the BD domain of BRD4 is able to bind these acetylated histones (99). Therefore, BRD4 promotes HIV transcription by binding the (super-)enhancer regions (99). Inhibition of BRD4 can lead to a reduction of transcription of genes controlled by (super-)enhancers (99). Super-enhancers are more vulnerable to the reduced levels of BRD4 than typical enhancers, probably due to their higher level of BRD4-binding (104). It is postulated that the residual high vRNA expressors after LEDGIN-mediated retargeting are caused by integration close to (super-)enhancers (62). If transcription of the residual high vRNA expressors of LEDGIN-mediated retargeted provirus is indeed caused by enhancer-mediated transcription, blocking the enhancers with BRD4 inhibitors will silence these residual high vRNA expressors.

1.6.3 BET-inhibitors

BET-inhibitors can be classified based on their selectivity (105). First, the non-selective pan-inhibitors (e.g. JQ1) inhibits all BET proteins. Second, a particular BET protein such as BRD4 can be inhibited specifically. The third group selectively inhibits either the BDI or BDII domains of BRD4, for instance ZL0580 (106). Currently, BRD4 inhibitors are proposed to reactivate latent HIV infection and contribute to the shock-and-kill strategy (103). However, an exception is ZL0580, which suppresses HIV transcription and can be used in the block-and-lock strategy (106).

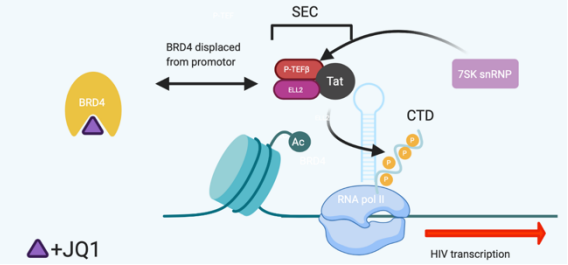


Figure 1.7. Mechanism of action of JQ1: JQ1 dissociates BRD4 from the Acetylated (Ac) histones, which results in increased P-TEFβ levels. Furthermore, JQ1 releases P-TEFβ from the inactive state, bound to 7SK snRNP. These two mechanisms promote the binding of Tat to P-TEFβ and thus the formation of the SEC. This results in a binding of the SEC to the TAR stem-loop structure and a phosphorylation of the Pol II CTD. BRD4, bromodomain containing protein 4; Ac, acetylated; P-TEFβ, positive transcription elongation factor β; 7SK snRNP, 7SK small nuclear ribonucleoprotein; Tat, trans-activator of transcription; SEC, super elongation complex; TAR, trans-activation response element; pol, polymerase; CTD, carboxy terminal domain. (Adapted from: Z. Li et al, Nucleic Acids Res., 2013, **41**(1), 277–287. And created with www.Biorender.com)

1.6.3.1 JQ1

JQ1 is one of the first discovered BET-inhibitors. It is a small-molecule pan-inhibitor of the BET bromodomain (105), which has been proposed as a latency reactivator with the potential to be used in the shock-and-kill strategy (103). JQ1 reactivates proviral expression by counteracting the inhibitory effect of BRD4 on Tat activation. As shown in figure 1.7, JQ1 displaces BRD4 from the promotor, relieving the competition between BRD4 and Tat for P-TEFβ. Accordingly, Tat can bind P-TEFβ and induce transcriptional elongation (103). P-TEFβ has an unbound active state and an inactive state in which it is bound to 7SK small nuclear ribonucleoprotein (7SK snRNP) (107). Moreover, JQ1 releases P-TEFβ from the inactive state which results in more availability of P-TEFβ to interact with Tat (103,107).When the SEC is formed, it can associate with the trans-activation response element (TAR) stem-loop structure and phosphorylate the pol II carboxy terminal domain (CTD) **(Figure 1.7)** (103).

1.6.3.2 ZL0580

ZL0580 is a small molecule discovered via structure guided-drug design (106). Niu *et al.* showed that ZL0580 silenced basal HIV transcription and reduced reactivation of latent HIV in Jurkat cells , CD4+ T cells and in peripheral blood mononuclear cell (PBMCs) from HIV infected patients (106). Co-immunoprecipitation (Co-IP) analysis showed that ZL0580 reduced the binding of CDK9, a component of P-TEFβ, to Tat and increased the binding of CDK9 to BRD4. Further Co-IP analysis demonstrated that ZL0580 reduced the protein stability of ELL2, a catalytic factor of the SEC. Moreover, Niu *et al.* reported that ZL0580 enhanced the transport of BRD4 and decreased the transport of Tat to the HIV promotor. Thereby ZL0580 reduced RNA pol II activation. They also claimed that ZL0580 induced a repressive chromatin structure at the HIV promotor **(Figure 1.8)** (106).

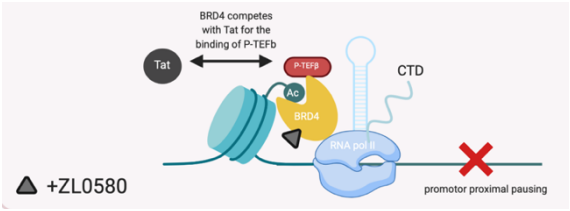


Figure 1.8. Mechanism of action of ZL0580: ZL0580 promotes the binding of BRD4 to the acetylated (Ac) histones, which results in a competition of BRD4 and Tat for P-TEFβ. This hampers the formation of the SEC and therefore inhibits HIV transcription. BRD4, bromodomain containing protein 4; Ac, acetylated; Tat, trans-activator of transcription; P-TEFβ, positive transcription elongation factor β; SEC, super elongation complex; HIV, human immune deficiency virus (Adapted from Q. Niu et al, J Clin Invest., 2019, **129**(8), 3361–3373. And created with Biorender.com)

Most studies of BRD4 -inhibitors focused on the relieve of competition between BRD4 and Tat for P-TEFβ, which allows the construction of the SEC again and promotes HIV transcription (103). However, other studies claimed that BRD4 can have different effects on HIV transcription, depending on the interaction with the acetylated histones (108). In line, Niu *et al.* suggested that different bindings of small molecules to BRD4 induce conformational changes in BRD4 and thereby alters its interactions with partnering proteins, which results in differential regulation of HIV transcription. This can be a possible explanation for the opposing effects of JQ1 and ZL0580 on Tat activation (106).

1.7 MLL1: from leukemia to HIV

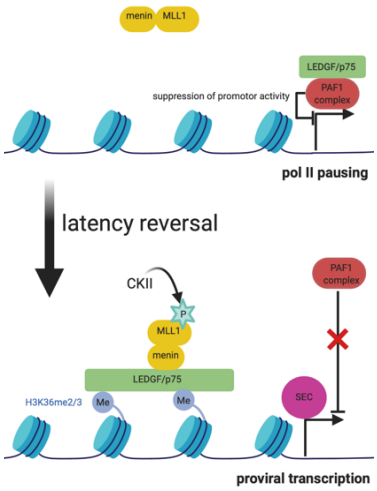


Figure 1.9. Opposing roles of LEDGF/p75 in HIV latency and latency reversal: During latency, LEDGF/p75 suppresses HIV transcription via pol II pausing by recruiting the PAF1-complex to the promotor. After, latency reversal, MLL1 competitively displaces the PAF1-complex from the promotor by CKII-dependent phosphorylation of MLL1. This relieves the obstacle for pol II elongation and allows the recruitment of SEC to the HIV promotor which induces proviral transcription. LEDGF/p75, lens epithelium-derived growth factor; HIV, human immunodeficiency virus; pol, polymerase; PAF1, pol II-associated factor; MLL1, mixed lineage leukemia 1; CKII, casein kinase II; SEC, super elongation complex. (Adapted from R. Gao R et al, Sci Adv., 2020, **6**(20), 1-14. And created with BioRender.com)

MLL1, is a histone methyltransferase, which stimulates the proliferation of immature hematopoietic progenitor cells during development by activating the homeobox gene expression (109). However, MLL1 can undergo gene rearrangements that result in fusion with oncogenic proteins. These oncogenic complexes can be tethered to the chromatin by a menin-mediated interaction with LEDGF/p75 (42,46). This results in a conversion of hematopoietic progenitor cells into leukemic stem cells by activation of the homeobox genes (109). This is the mechanism behind the development of Mixed lineage leukemia-rearranged (MLLr) (109).

Previously the role of LEDGF/p75 in HIV integration was discussed (110) and the function of LEDGF/p75 as a tether for MLL1 in MLLr (46). Interestingly, Goa *et al.* indicated that LEDGF/p75 has contrasting functions in HIV transcription during the post-integration steps of the HIV life cycle (111). LEDGF/p75 causes pausing of RNA pol II and thus stimulates latency, by associating and recruiting the poll II-associated factor 1 (PAF1)-complex to the HIV promotor. Moreover, LEDGF/p75 can switch its function during latency reversal and activate HIV transcription. Reactivation induces the expression of casein kinase II (CKII) kinase which phosphorylates the serine residues of the integrase binding motif domain of MLL1, which consequently induces the binding of MLL1 to LEDGF/p75. As a result, MLL1 displaces the PAF1-complex from the viral promotor by competition for binding to LEDGF/p75 (111). The clearance of PAF1-complex promotes proviral transcription by relieving the obstacle for transcription elongation and the barrier for the formation of the SEC at the proviral promotor **(Figure 1.9)**. These findings increase the understanding of the opposing regulatory functions of LEDGF/p75 at distinct steps of the HIV life cycle.

In this project, the role of MLL1 in HIV replication will be studied by comparing HIV expression in backbone lentivirus (LV) transduced cells with cells depleted for MLL1 using specific miRNA-based LV vectors. If silencing of HIV expression can be achieved, these proteins are possible future targets for a block-and-lock functional cure approach. Moreover, they could be combined with LEDGINs to further optimize the efficiency of the block-and-lock functional cure strategy.

2. Objectives

2.1 Objective 1: Role of BRD4 and enhancers in residual HIV transcription

2.1.1 Objective 1.1: Role of BRD4 in HIV transcription and reactivation

Using bDNA imaging of vDNA and vRNA and the luciferase reporter assay, the effect of BRD4 inhibition on basal HIV transcription and HIV reactivation from latency will be studied by using JQ1, a non-selective BRD4 inhibitor. In a normal context, without inhibition of LEDGF/p75, it is expected that JQ1 activates transcription and reactivation. In addition, the effect of ZL0580, a BRD4 inhibitor known to inhibit transcription (106), on basal transcription and reactivation of HIV will be investigated as well.

2.1.2 Objective 1.2: Role of BRD4 in a block-and-lock phenotype after LEDGIN-mediated retargeting

Paradoxically to the first objective where JQ1 activates transcription, JQ1 has potential functionality in a block-and-lock functional cure strategy after LEDGIN-treatment due to inhibition of (super-)enhancers. It will be investigated whether (super-)enhancers are linked with residual high vRNA expressors of LEDGIN-retargeted provirus by inhibiting the enhancers via BRD4. Therefore, the BRD4 inhibitor, JQ1, can be added to an experiment with LEDGINs to determine the effect on basal HIV transcription and HIV reactivation after LEDGIN-mediated retargeting. If JQ1 has no effect on the residual high vRNA expressors, ZL0580, a selective BRD4 inhibitor that is known to induce transcriptional and epigenetic suppression of HIV (106), will be investigated as an alternative. The vDNA and vRNA levels will be detected with the bDNA imaging technique. The luciferase reporter assay will be used an additional read-out.

2.2 Objective 2: Role of MLL1 in HIV replication

The role of MLL1 in HIV replication will be investigated by comparing the HIV expression in LV transduced cells with cells depleted for MLL1 using specific miRNA-based LV vectors. It is expected that MLL1 depletion will silence HIV expression (111). The luciferase reporter assay will be used as a read-out to investigate this hypothesis.

3. Material and methods

3.1 Cell culture

SupT1, Jurkat and MT-4 (T lymphoblast) cells were cultured in Roswell Park Memorial Institute (RPMI) medium (GIBCO BRL) in the presence of 10% (v/v) fetal bovine serum (FBS) (FBS, GIBCO) and 0.01% (v/v) gentamicin (GIBCO). The cells were cultured in the presence of 0.05% (v/v) blasticidin to select for MLL1 knockdown cell lines after lentiviral transduction. HeLaP4 cells (cervical cancer) were cultured using Dulbecco Modified Eagle Medium (DMEM) (DMEM, GIBCO, Dublin, Ireland) with 5% (v/v) FBS (FBS, GIBCO), 0.01% (v/v) gentamicin (GIBCO) and 1% (v/v) geneticin (GIBCO). To select for HeLaP4 cells depleted for MLL1, 0.05% (v/v) blasticidin was added. Cells were incubated at 37°C in an atmosphere of 5% CO2. All cells were tested to be free from mycoplasma contamination.

3.2 Generation of MLL1 knockdown cell lines

The role of MLL1 in HIV replication will be examined by comparing the HIV expression in LV transduced cells with cells depleted for MLL1 using specific miRNA-based LV vectors. To generate MLL1 knockdown cell lines, oligos were generated by annealing of a miRNA30 (112) loop together with a silencing RNA targeting MLL1 miRNA (Sigma-Aldrich database, miRNA 1-5, see table 3.1). Afterwards, the oligos were cloned by Esp3I-mediated insertion of the annealing products into the pGAE-SFFV-BsdR plasmid backbone containing Ampicillin (Amp) and blasticidin resistance fragments. Bacteria were transformed with the newly cloned MLL1 targeting miRNA (miRNA1-5) and incubated overnight at 37°C in the presence of Amp. The next day, the colonies were picked and grown in 5 mL of luria-bertani medium with Amp (0.1 mg/mL). Miniprep DNA extraction was done and insertion of the oligos was verified via gel electrophoresis. Plasmids were submitted for sequencing to exclude that mutations occurred in the miRNA sequence during cloning.

Afterwards, SIV-based vectors with specific MLL1 targeting miRNAs were produced by the Leuven Viral Vector Core. These vectors were used to transduce SupT1 cells, Jurkat cells and HeLaP4 cells. Briefly, human embryonic kidney cells (HEK293T) cells were transfected with a mixture of transfer plasmid encoding for the MLL1 targeting miRNAs (miRNA 1-5), SIV packaging plasmid and the VSVG envelope plasmid. Vectors were harvested 48h and 72h after transfection. Further, the vectors were concentrated by centrifugation with Vivaspin 50 kDa filters and 1:3 diluted and incubated with the cells. The following day, vectors were removed and selected with blasticidin (5 μg/mL). The phenotype was analyzed with RT-quantitative PCR (qPCR).

Table 3.1: sequences of sense and antisense oligo of MLL1 targeting miRNA 1-5.

miRNA 1	Sense oligo: GAGCGCGATTATGACCCTCCAATTAAATAGTGAAGCCACAGATGTATTTAATTGGAGGGTCATAATCCT AntiSense oligo: AGGCAGGATTATGACCCTCCAATTAAATACATCTGTGGCTTCACTATTTAATTGGAGGGTCATAATCGC
miRNA 2	Sense oligo: GAGCGGGCACTGTTAAACATTCCACTTTAGTGAAGCCACAGATGTAAAGTGGAATGTTTAACAGTGCTT AntiSense oligo: AGGCAAGCACTGTTAAACATTCCACTTTACATCTGTGGCTTCACTAAAGTGGAATGTTTAACAGTGCCC
miRNA 3	Sense oligo: GAGCGCCCTCCATCAACAGAAAGGATATAGTGAAGCCACAGATGTATATCCTTTCTGTTGATGGAGGCT AntiSense oligo: AGGCAGCCTCCATCAACAGAAAGGATATACATCTGTGGCTTCACTATATCCTTTCTGTTGATGGAGGGC
miRNA 4	Sense oligo: GAGCGGCGCCTAAAGCAGCTCTCATTTTAGTGAAGCCACAGATGTAAAATGAGAGCTGCTTTAGGCGGT AntiSense oligo: AGGCACCGCCTAAAGCAGCTCTCATTTTACATCTGTGGCTTCACTAAAATGAGAGCTGCTTTAGGCGCC
miRNA 5	Sense oligo: GAGCGCCCCATCCAGAACCAGAAGTATTAGTGAAGCCACAGATGTAATACTTCTGGTTCTGGATGGGAT AntiSense oligo: AGGCATCCCATCCAGAACCAGAAGTATTACATCTGTGGCTTCACTAATACTTCTGGTTCTGGATGGGGC

3.3 RT-qPCR

To further validate the knockdown efficiency of MLL1, RT-qPCR analysis was performed to determine the messenger RNA (mRNA) expression level. By using the Aurum Total RNA Mini Kit (Bio-Rad), total RNA was extracted. The RNA concentrations were measured with a spectrophotometer (Nanophotometer, Implen). The RNA was reverse transcribed to DNA with the use of a High-capacity vDNA Archive Kit (Applied Biosystems). Next, qPCR was conducted with 2 µL of the RNA sample (10 ng/µL) and 18 µL of a PCR mix consisting of 0.6 µL of each primer (300 nM) **(Table 3.2)**, 10 µL LightCycler 480 SYBR Green I master mix (Roche) and 6.8 µL aqua destillata (AD). The qPCR with LightCycler480 (Roche) detection system started with 1 cycle of 95°C for 10 minutes and was followed by 50 cycles of a two-step PCR with 10 sec of 95°C and 30 sec of 55°C. GADPH and β -actin were used as housekeeping genes to normalize the mRNA expression.

Table 3.2: Forward and reverse sequences of primers for RT-qPCR

MLL1 primer	Forward: 5'-AAC ATT GAT GCA GGT GAG AT -3' Reverse: 5'- ATT GAT GAC CCG AGA ATA GC -3'
β-actin primer	Forward: 5'-CAC TGA GCG AGG CTA CAG CTT- 3' Reverse: 5'-TGG ATG TCG CGC ACG ATT T -3'
GADPH primer	Forward: 5'GTCTCCTCTGACTTCAACAGCG-3' Reverse: 5'- ACCACCCTGTTGCTGTAGCCAA -3'

3.4 Latency reactivation experiments

For objective 1, SupT1 cells or Jurkat cells were transduced with a pNL4.3 based HIV FLUC construct. After three days, the virus was washed away. The cells were kept in culture for another seven. At day ten post-transduction, half of the samples were reactivated with 10 ng/mL tumor necrosis factor α (TNF-α) and the other half stayed non-reactivated. Additionally, the cells were treated with varying concentrations of JQ1 or ZL0580 to determine which inhibitor can block HIV transcription and reactivation. The basal transcriptional state and reactivation were measured 24 hours post-reactivation by a luciferase reporter assay or by the bDNA analysis in fixed cells. For objective 1.2, LEDGINs were added to the experiment during the transduction with the pNL4.3 based HIV FLUC construct to determine whether residual high vRNA expressors can be blocked by BRD4 inhibition.

For objective 2, the role of MLL1 in HIV transcription was investigated by comparing pNL4.3 based HIV FLUC transduced cells with cells depleted for MLL1 using specific miRNA-based LV obtained through the Leuven Viral Vector Core. Briefly, 20 000 HeLaP4 cells, wild type and MLL1 depleted HeLaP4 cells, were transduced with different dilutions of the pNL4.3 based HIV FLUC construct (1/100, 1/300, 1/900). Three days later, samples were taken for a luciferase reporter assay.

3.5 Luciferase assay

The luciferase assay is an average-based method, which was used to examine the effect of a small molecule (JQ1, ZL0580, LEDGINs) or a protein (MLL1 knockdown) on the HIV gene expression. Cells were transduced with a pNL4.3 based HIV FLUC construct, in which the *nef* position of the HIV genome is replaced with a luciferase reporter gene. Therefore, when the small molecule or protein promotes or inhibits viral replication, the cell will produce more or less of the luciferase reporter enzyme. To harvest cells for the luciferase assay, the cells were washed twice with phosphate buffered saline (PBS) and lysed with 80 µL of a lysis buffer (50 mM Tris, 200 mM NaCl, 0.2% (v/v) NP40 and 5% (v/v) glycerol). Afterwards, the cell lysates were centrifuged (1250 × g, 10 minutes) to force them to the bottom of the plate. Further, 5 µL of the sample and 25 µL of a FLUC assay reagent (ONE-Glo; Promega GMBH, Mannheim, Germany) were mixed in a 96 well multiplate (Corning). The bioluminescent signal as a result of luciferase activity, was measured with the Envision 2105 (PerkinElmer). Finally, the luciferase levels were normalized for the total protein content per well by the bicinchoninic acid (BCA) assay (see section 3.6).

3.6 Bicinchoninic acid assay

To measure the total protein concentration, a BCA assay was conducted (BCA Protein assay kit; Thermo Scientific). The assay relies on the biuret reaction, which is the reduction of Cu^{2+} to Cu^{+} by proteins in alkaline medium (113). As a reference, an albumin standard (0.2 mg/mL) was used in a 5-fold dilution series.

To reduce the influence of the lysis buffer, the same volume of lysis buffer as sample was added to the albumin standard. Further, PBS was added to the standard until a final volume of 100 µL was reached. Next, 5 µL of the sample and 95 µL of PBS were added in duplicate in the 96 well plate. The BCA Protein Assay Reagent B was diluted 1:50 (v/v) with the BCA Protein Assay Reagent A (BCA Protein assay kit; Thermo Scientific) and 100 µL of this solution was added to each well. Afterwards, the cells were incubated at 37°C for one hour to allow the colorimetric reaction to take place. Absorbance was measured at 540 nm by infinite M1000 (Tecan) and the albumin standard was used to calculate the exact protein concentration.

3.7 MTT test

The 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test, which relies on the mitochondrial dehydrogenase-mediated reduction of the yellow MTT substrate to the blue formazan, was used to estimate the toxicity of the compounds (114). Briefly, MT-4 cells were transduced with a pNL4.3 based HIV FLUC construct. After five days, the cells were supplemented with 20 µL of the MTT substrate. One hour after the addition of the MTT substrate, an acidized Triton-X in isopropanol solution was added and mixed by pipetting up and down to dissolve the blue formazan crystals. Consequently, the absorbance was measured by the infinite M1000 (Tecan). First, the background was measured at 690 nm and thereafter, the absorbance of formazan was measured at 540 nm. The absorbance value at 690 nm was subtracted from the absorbance value of 540 nm.

3.8 Branched DNA imaging

bDNA is a commercial, but in-house optimized signal amplification method for fluorescent in situ hybridization to visualize vDNA and vRNA simultaneously on single cell level. It is used as a tool to provide a link between the effect of addition of a compound (LEDGINs, JQ1, ZL0580) and the transcriptional state of the provirus. The collaborator of my host lab, Dr. Sarafianos (U. Missouri, Columbia), recently optimized this method allowing identification of both actively transcribing and latently infected cells (115). In contrast to average-based readouts, bDNA imaging allows to discriminate between single cells with low vRNA transcription or high vRNA transcription (high vRNA expressors). My host lab has optimized this technology in multiple cell lines such as Jurkat cells, SupT1 cells and primary blood lymphocytes. The latter cells are routinely isolated from buffy coats and infected by expert technicians of the lab **(Figure 3.1)**.

3.8.1 bDNA hybridization

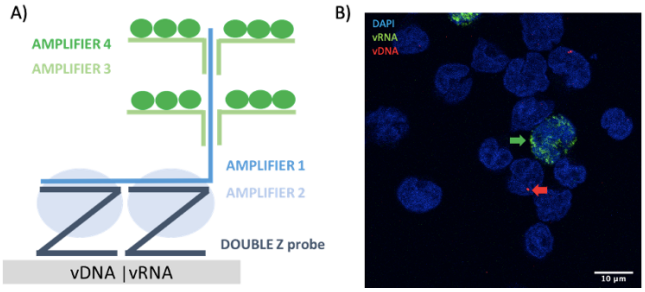


Figure 3.1. Branched DNA imaging technique: A) Cells are fixed and permeabilized to give the Z-probes access to the vDNA and vRNA. Target specific probes hybridize in pairs, which allows the binding of the pre-amplifier. Signal amplification is achieved by subsequent binding of different amplifiers and probes. The fluorescent signal is detected by confocal microscopy. **B)** SupT1 or Jurkat cells were transduced with a single round HIV pNL4.3 based HIV FLUC construct. After the bDNA hybridization, the vRNA (pseudocolored green) and vDNA (pseudocolored) can be detected with confocal microscopy. The nucleus is visualized with DAPI staining (pseudocolored blue). Scale bar represents 10 µm. vDNA, viral DNA; vRNA, viral RNA; HIV, human immunodeficiency virus; DAPI, 4′,6-diamidino-2-phenylindole. (Adapted from F. Wang et al, J Mol Diagnostics., 2012, **14**(1), 22–29. And confocal images obtained during the master internship)

Prior to bDNA hybridization, the cells were fixed on a cover slip with a 4% perfluoroalkyl solution (Z22C046, Alfa Aesar). After fixation, the cells were washed twice with PBS and dehydrated for five minutes using increasing concentrations of ethanol (50%, 70% and 100% (v/v) ethanol solution in PBS). The cover slips were stored in a 100% (v/v) ethanol solution at -20°C. Next, the cells were rehydrated with 100%, 70% and 50% (v/v) ethanol solutions diluted in PBS, respectively, with an incubation period of two minutes. The cells were washed twice with PBS and permeabilized with 0.1% (v/v) tween in PBS for 10 minutes on room temperature. After permeabilization, the cells were washed twice with PBS and the cover slips were immobilized on a microscopic slide (Fisherbrand colorfros plus microscope slides precleaned REF12-550-17/18/19, Fisher Scientific) with the use of a small drop of nail polish. Using an ImmEdge hydroscopic barrier pen (Cat No H-4000, Vector Laboratories), a circle was drawn around the cover slip and PBS was added to keep the cells hydrated. Cells were incubated with 100 µL of a 10% (v/v) target retrieval solution in PBS for 30 minutes at 67°C and afterwards cooled to room temperature for 15 minutes. All incubation steps were performed in a humidified HybEz oven. After discarding the target retrieval solution, a drop of 100% ethanol was added to the cover slip, followed by two washes with PBS. To give the Z-probes access to the vRNA and VDNA, the proteins were digested with moderate protease III (REF 322337, ACD Bio) for 15 minutes at 40°C.

After washing the cells twice with PBS, 25 µL of the vRNA probe targeting the non-GagPol region of the HIV genome (REF317711-C2, Advanced Cell Diagnostics) was added to the cells, diluted in a 1:50 ratio (v/v) with probe diluent (REF 300041, ACD Bio). To allow the probes to hybridize to the target vRNA, the cover slips were incubated for 2 hours at 40°C. Subsequently, the probes were discarded and washed three times with a wash buffer (1:50 diluted (v/v) in AD) (REF 320058, ACD Bio). All wash steps were performed by submerging the slides in the diluted wash solution and agitating them for two minutes.

After vRNA detection, the vDNA detection took place. A sense HIV-GagPol-C1 probe (REF 317701, ACD BIO) was diluted in a 1:2 ratio in a hybridization buffer. The hybridization buffer contained 500 µL 50% dextrane sulfate (0198, Amresco), 300 µL of a solution with 5 M NaCl, 125 µL of a 200 mM sodium citrate solution (pH 6.2), 375 mg of ethylene carbonate (E26258, Sigma), 25 µL of 10% tween-20 and 1550 µL nuclease free water (AM9937, Ambion). Afterwards, the solution was vortexed. The cover slips with the diluted DNA probes were incubated for two hours at 40°C. After decanting the probes, the cover slips were washed twice with washing buffer.

To visualize the fluorescent signal more clearly, a set of amplifiers were added. First, one drop of amplifier 1 was added on top of the cover slips and incubated for 30 minutes at 40°C. After decanting the first amplifier and washing the cover slips twice with washing buffer, the second amplifier 2 was added and incubated for 15 minutes at 40°C. Next, the cover slips were washed twice with washing buffer and a third amplifier 3 was added and incubated for 30 minutes at 40°C. Finally, after washing the cover slips twice with washing buffer, a fourth amplifier 4, which is conjugated with a fluorophore (ATTO 555/635) was added and incubated for 15 minutes at 40°C. In Figure 3.1 A, the target specific probes and the set of amplifiers can be seen. Further, to visualize the nucleus, a 4′,6-diamidino-2-phenylindole (DAPI) staining was performed. The cover slips were washed twice with PBS and afterwards incubated for five minutes at room temperature with one drop of DAPI. After the staining with DAPI, the cover slips were washed twice with PBS and the nail polish was removed with tweezers. Finally, the cover slips were mounted on a microscope slide with the use of Prolong Gold Antifade (P36930, Invitrogen) to protect the fluorescent dyes from bleaching.

3.8.2 Imaging, quantification and statistical analysis

After the bDNA hybridization, the fluorescent signal of DAPI, the vRNA probe (647 fluorophore) and vDNA probe (555 fluorophore) can be detected with fluorescence confocal microscopy (FLUOVIEW FV1000 or FV2000) with a 60x water objective. The excitation wavelengths of the lasers for DAPI, the vRNA probe, and the vDNA probe were set at 405, 532, and 635 nm, respectively. The range of emission wavelengths were 415-500, 550-600 and 655-700 for DAPI, the vRNA probe and the vDNA probe, respectively. 3D stacks were acquired with a 0.3 µm step size and 4 µs/pixel sampling speed **(Figure 3.1B)**. After taking the images of the DAPI, vRNA and vDNA signal, the images were converted to tiff files via Fiji using a home written Fiji routine. These tiff files were further used in the MATLAB routine to quantify the vDNA and vRNA spots on single cell level. Statistical significance was calculated by using the Kruskal-Wallis test, using Graphpad Prism (version 9.0) (GraphPad Software, La Jolla California USA, www.graphpad.com).

4. Results

4.1 Role of BRD4 and enhancers in residual HIV transcription

4.1.1 JQ1 promotes basal transcription and reactivation of HIV

The BRD4 inhibitor, JQ1, is a well-known LRA used in the shock-and-kill eradication strategy. Previous studies showed that JQ1 promotes HIV replication (103,107,116–118) and modestly promotes LRA-induced reactivation (116,117). However, all these studies used average-based read-outs. In this Master's thesis, the bDNA technique was used; it gives the opportunity to study the effect of JQ1 on the vDNA and vRNA expression of HIV on single cell level.

Briefly, SupT1 cells were transduced with a pNL4.3 based HIV FLUC construct (virus dilution 1/7000). Three days later the virus was washed away, and a sample was taken for a luciferase reporter assay to confirm the infection. On day ten post-transduction, the cells were treated with a dilution series of JQ1, and half of the cells were additionally reactivated with 10 ng/mL TNF-α. Finally, 24 hours post-reactivation, samples were taken for the luciferase reporter assay and the bDNA analysis **(Figure 4.1.A)**.

Although no statistically significant results were obtained, the luciferase reporter assay showed that JQ1 modestly increases HIV replication (orange bars) with an optimal concentration of 1 μM JQ1. As expected, treatment with TNF-α enhances HIV expression (blue bar compared to orange bar). Interestingly, addition of low concentrations of JQ1 slightly inhibited the TNF-α induced reactivation of HIV (blue bars). However, this inhibition was not significant and leveled-off when the concentration of JQ1 increased **(Figure 4.1.B)**.

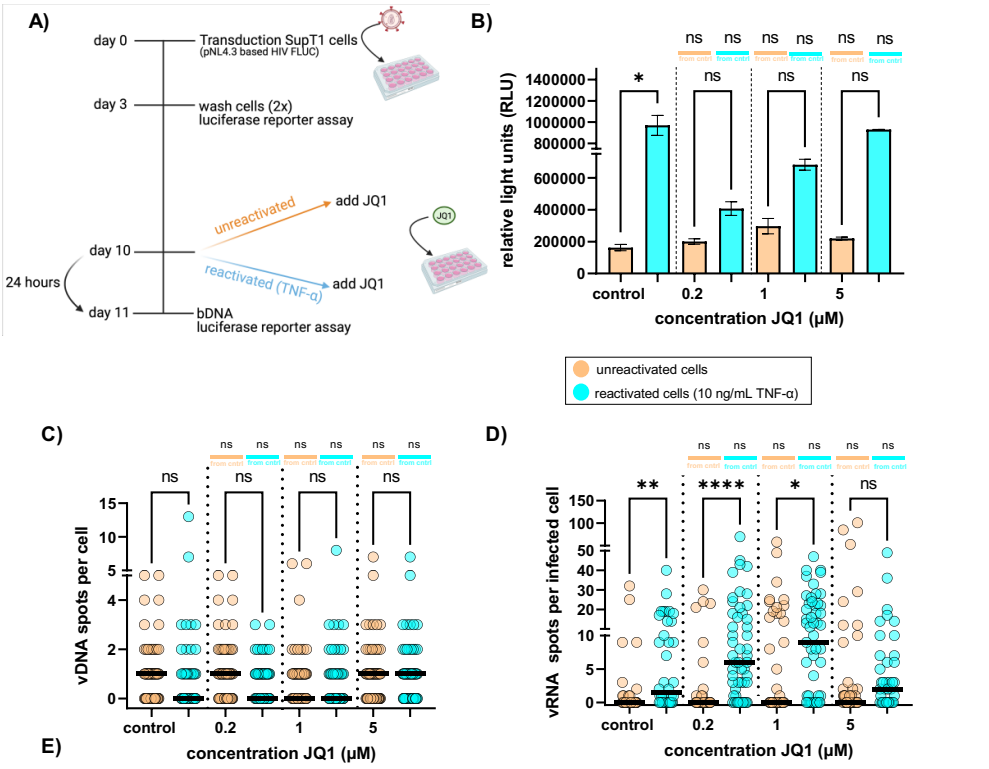
To further explore the impact of JQ1 on HIV transcription on single cell level, the bDNA analysis was performed in parallel to the luciferase reporter assay. When analyzing the vDNA expression with the bDNA technique, JQ1 had no impact on the number of vDNA spots per cell in the unreactivated cells, nor in the reactivated cells. Moreover, addition of TNF-α did not significantly alter the median number of vDNA spots per cell **(Figure 4.1.C)**. Next, the vRNA expression level was determined with the bDNA analysis. Non-infected cells were not counted when determining the vRNA expression per residual DNA copy. JQ1 had no significant effect on vRNA expression in the unreactivated cells (orange spots), although several cells showed a higher number of vRNA spots per cell. Furthermore, in line with previous research (119,120) and the luciferase assay, addition of TNF-α significantly enhanced the HIV expression (blue spots compared to orange spots). In addition, a dose dependent increase in the median number of vRNA spots was observed in the reactivated cells (blue spots) supplemented with JQ1, except for the highest concentration of JQ1 (5 μM), for which the median number of vRNA spots decreased to control-like levels (blue spots). Although the result was not significant, it suggests that JQ1 enhances TNF-α induced viral gene expression with an optimal concentration of 1 μM **(Figure 4.1.D)**.

For a more in-depth comparison, the vRNA expression level was normalized for the vDNA expression by calculating the ratio between the total number of vRNA spots and the total number of vDNA spots, after the bDNA analysis. For both the unreactivated and reactivated cells, this ratio increased with an optimal concentration of 1 μM JQ1. At a higher concentration of JQ1 (5 μM), the ratio of the unreactivated cells modestly decreased, while an even more pronounced decrease was shown in the reactivated cells **(Figure 4.1.F)**.

Additionally, the effect of JQ1 on HIV transcription and reactivation was investigated by calculating the relative fold-increase in vRNA expression induced by JQ1 (total number of vRNA spots of JQ1-treated cells/ total number of vRNA spots of control cells). The data showed a four-fold increase in the basal transcription at the optimal concentration of JQ1 (1 μM), which modestly leveled-off at higher concentrations of JQ1 **(Figure 4.3.A)**. In the reactivated cells, a two-fold increase of the TNF-α induced reactivation by JQ1 was shown, which decreased to 0.5-fold at the higher concentrations of JQ1 (5 μM) **(Figure 4.3.A)**. When the relative fold reactivation induced by TNF-α was calculated (total number of vRNA spots of TNF-α reactivated cells/ total number of vRNA spots of unreactivated cells), an optimal synergistic effect of JQ1 and TNF-α was observed at 0.2 μM of JQ1. However, at higher concentration of JQ1, JQ1 was shown to inhibit the TNF-α induced reactivation compared to the control (0 μM JQ1) **(Figure 4.3.B)**.

It has to be mentioned that the effect of JQ1 on basal transcription could be underestimated due to the high number of cells that do not express vRNA. It is possible that due to a heterogeneity in viral replication, these cells do not support viral transcription. Therefore, the experiment shown in Figure 4.1.D was reanalyzed and all the cells that did not contain vRNA spots were left out. Hereafter we refer to these vRNA expressors as RNA expressing cells (RNA^+) cells **(Figure 4.4.A)**. When only looking at the RNA^+ cells, the median number of vRNA spots in the unreactivated cells (orange spots) clearly increased until 1 μM of JQ1 followed by a pronounced decrease at higher concentrations of JQ1 (5 μM) to control-like levels (orange spots). Although not statistically significant, these data corroborate that JQ1 promotes basal transcription of RNA^+ cells with an optimal concentration of 1 μM JQ1 **(Figure 4.4.A)**. Of note, when reactivating the cells with TNF-α (blue spots), the same trend was observed in the vRNA expression level of the bDNA analysis as when analyzing all the infected cells (RNA^{all}), such as in Figure 4.1.D. More specifically, an increase in the median number of vRNA spots per infected cell was seen with an optimal concentration of 1 μM JQ1, followed by a decrease to a control-like levels at 5 μM JQ1 **(Figure 4.4.A)**. Next, the analysis using RNA^{all} cells **(Figure 4.1.A)** was compared to the analysis using RNA^+ cells **(Figure 4.4.A)** for both the unreactivated and reactivated cells. For the unreactivated cells, it was clear for the analysis using RNA^{all} cells that treatment with JQ1 did not alter the vRNA expression compared to the control **(Figure 4.4.B)**.

In contrast, the analysis using the unreactivated RNA^+ cells, corroborated that treatment with JQ1 increased the median number vRNA spots per infected cell compared to the control with an optimal concentration of 1 µM, followed by a pronounced decrease to control-like levels. Therefore, the analysis of RNA^{all} cells significantly differed from the analysis of RNA^+ cells in the unreactivated cells **(Figure 4.4.B)**. Further, the comparison indicates that in the reactivated cells, no significant difference between the analysis using RNA^{all} cells and RNA^+ cells occurred, expect for 1 µM of JQ1 **(Figure 4.4.B)**.



	median vDNA spots (unreactivated)	median vDNA spots (reactivated)
control	1.0	0.0
0.2 µM JQ1	1.0	0.0
1 µM JQ1	0.0	0.0
5 µM JQ1	1.0	1.0

	median vRNA spots (unreactivated)	median vRNA spots (reactivated)
control	0.0	1.5
0.2 µM JQ1	0.0	6.0
1 µM JQ1	0.0	9.0
5 µM JQ1	0.0	2.0

unreactivated cells

	total vDNA spots	total vRNA spots	normalized (vRNA/vDNA)
control	65.0	84	1.29
0.2 µM JQ1	70.0	118	1.69
1 µM JQ1	53.0	368	6.94
5 µM JQ1	66.0	352	5.33

reactivated cells

	total vDNA spots	total vRNA spots	normalized (vRNA/vDNA)
control	53.0	266	5.02
0.2 µM JQ1	43.0	597	13.88
1 µM JQ1	47.0	678	14.43
5 µM JQ1	68.0	229	3.37

Figure 4.1. Effect of JQ1 on basal transcription and reactivation of HIV: A) Methodology of infection experiment: SupT1 cells were transduced with a pNL4.3 based HIV FLUC construct (virus dilution 1/7000). On day ten post-transduction, the cells were treated for 24 hours with a dilution series of JQ1 (0 µM (control), 0.2 µM, 1 µM, 5 µM), in the presence or absence of 10 ng/mL TNF-α. After reactivation, samples were taken to perform the luciferase reporter assay and the bDNA analysis. **B)** The luciferase counts of the unreactivated (orange bars) and reactivated (blue bars) cells, normalized for the total amount of protein (determined by BCA assay), were plotted for each concentration of JQ1 and the control (0 µM JQ1). The error bars represent the standard deviation from technical duplicates. Statistical significance was calculated with a Kruskal-Wallis test (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and the reactivated cells and between the JQ1-treated cells compared to the control (0 µM JQ1).

C) The number of vDNA spots per cell and **D)** the number of vRNA spots per infected cell, after the bDNA analysis, were plotted for 72 cells for both the unreactivated (orange spots) and reactivated (blue spots) cells treated with varying concentrations of JQ1. Each dot represents the number of vDNA and vRNA spots for a single cell and the bar represents the median number vDNA spots per cell or the median number vRNA spots per infected cell. Statistical significance was calculated with a Kruskal-Wallis test (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and the reactivated cells and between the JQ1-treated cells compared to the control (0 µM JQ1). **E)** The table represents the median number of vDNA spots per cell or the median number of vRNA spots per infected cell of the unreactivated (orange spots) and reactivated (blue spots) cells. **F)** The total number of vDNA and vRNA spots, after the bDNA analysis, is presented for each concentration of JQ and the control (0 µM JQ1). Furthermore, the vRNA expression is normalized for the vDNA expression by dividing the total number of vRNA spots by the total number of vDNA spots. These experiments were conducted three times in SupT1 cells, results from one representative experiment are shown. HIV, human immunodeficiency syndrome; TNF-α, tumor necrosis factor α; bDNA, branched DNA; BCA, bicinchoninic acid protein assay; ns, non-significant; vDNA, viral DNA; vRNA, viral RNA; RLU, relative light units; cntrl, control.

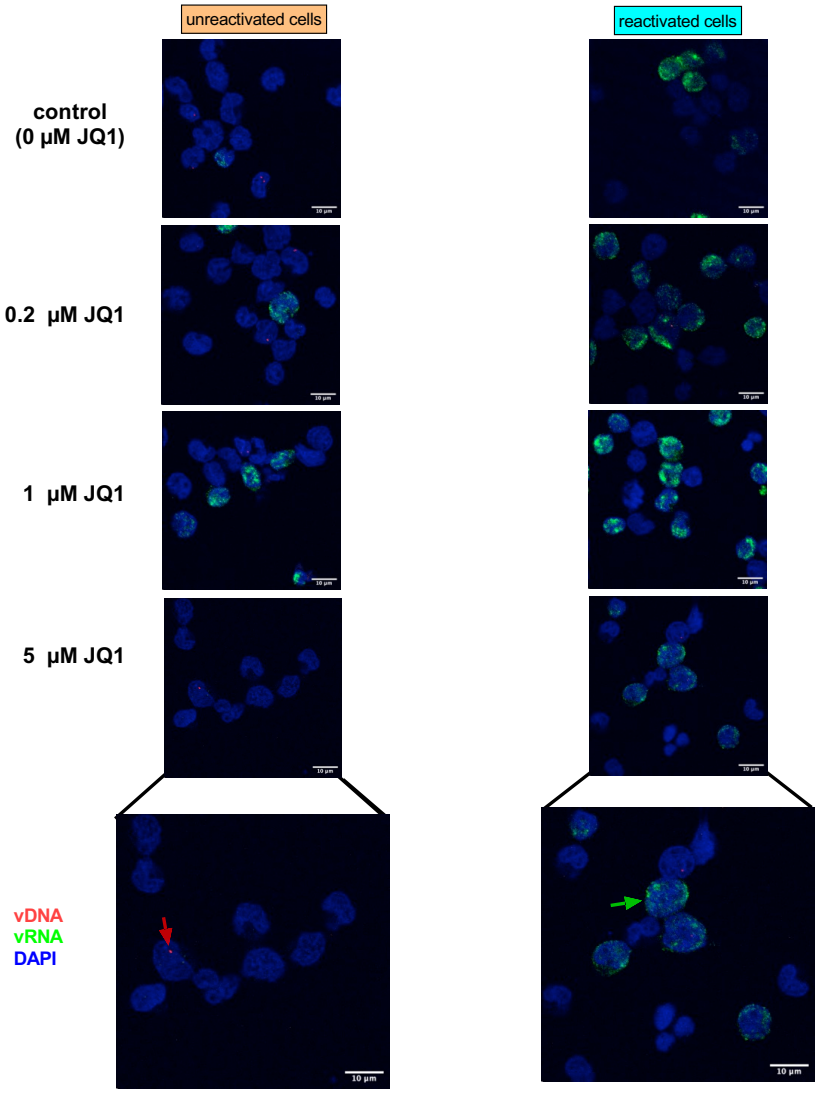


Figure 4.2. Confocal fluorescence microscopy of unreactivated and TNF-α reactivated cells, treated with a dilution series of JQ1 (0 µM (control), 0.2 µM, 1 µM, 5 µM): Infection experiments have been conducted as described in Figure 4.1.A. Cells were fixed, permeabilized and stained with probes targeting vDNA (pseudocolored red) and vRNA (pseudocolored green) according to the bDNA protocol described in the methodology (section 3.8.1). DAPI was used for visualization of the nucleus (pseudocolored blue). Linear adjustment of brightness and contrast was applied separately on DNA, RNA and DAPI channel on the entire images using Fiji. The scale bar represents 10 µM. TNF-α, tumor necrosis factor α; vDNA, viral DNA; vRNA, viral RNA; DAPI, 4′,6-diamidino-2-phenylindole.

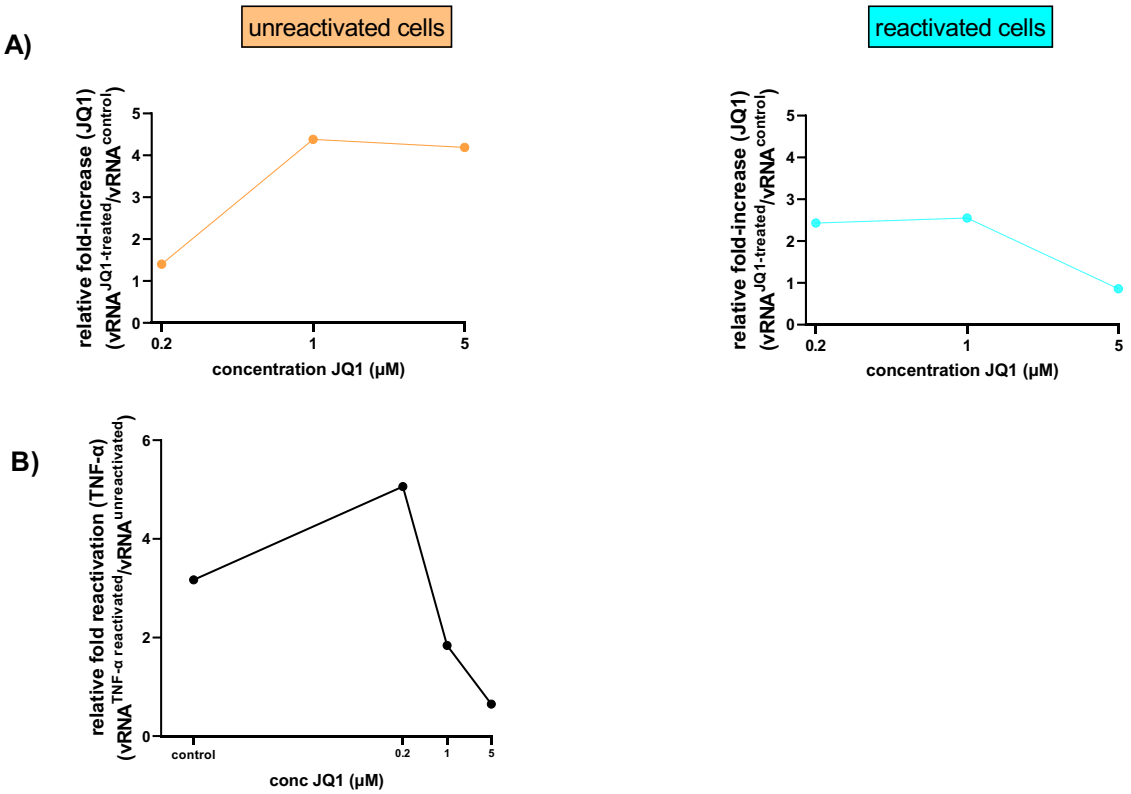
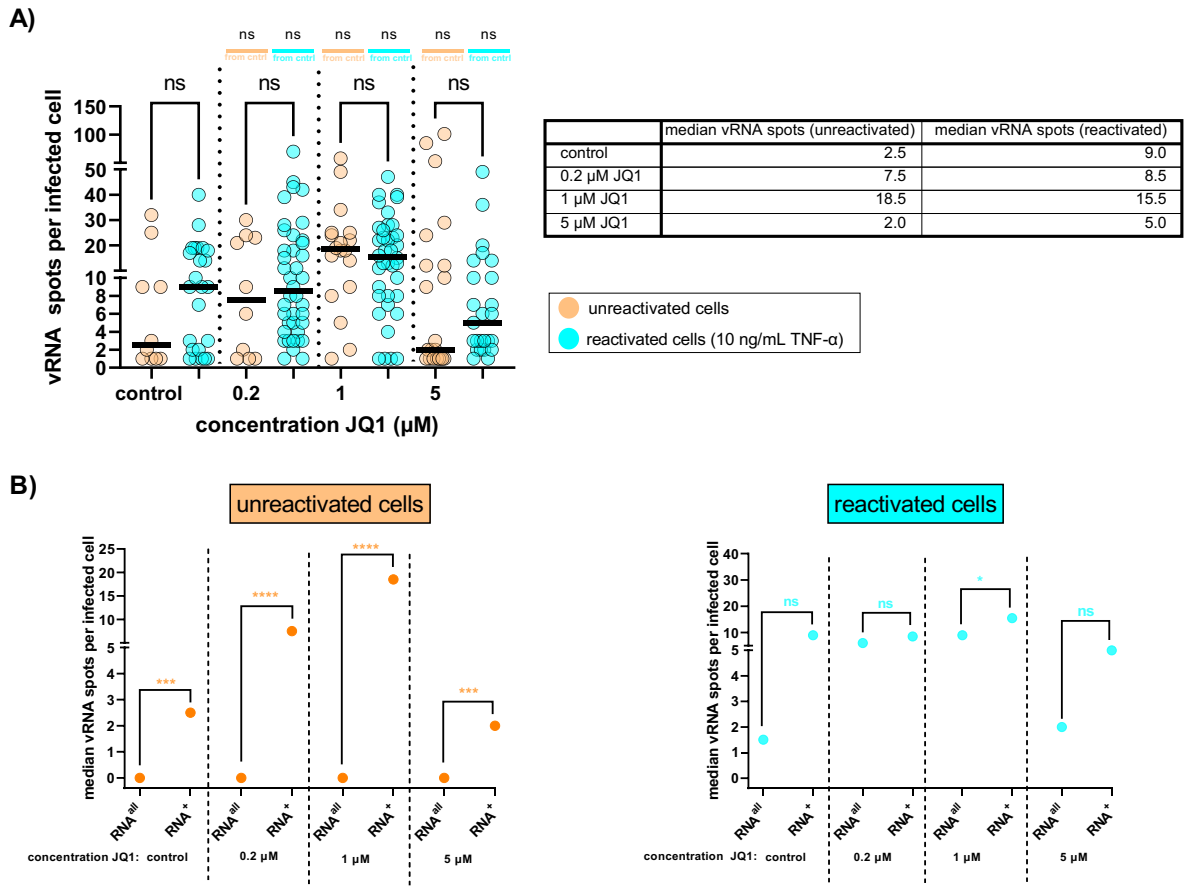


Figure 4.3. Relative fold-increase in transcription induced by JQ1 and TNF-α: A) The relative fold-increase in vRNA expression induced by JQ1 was calculated by dividing the total number of vRNA spots of the JQ1-treated cells by the total number of vRNA spots of the control cells (0 μM JQ1). The relative fold-increase in vRNA expression inducted by JQ1 is represented for each concentration of JQ, for both the unreactivated and the reactivated cells. **B)** The relative fold reactivation induced by TNF-α was calculated by dividing the total number of vRNA spots of the TNF-α reactivated cells by the total number of vRNA spots of the unreactivated cells. The relative fold reactivation induced by TNF-α is presented for each concentration of JQ1 and the control (0 μM JQ1). TNF-α, tumor necrosis factor α; vRNA, viral RNA.



	median vRNA spots (unreactivated)	median vRNA spots (reactivated)
control	2.5	9.0
0.2 µM JQ1	7.5	8.5
1 µM JQ1	18.5	15.5
5 µM JQ1	2.0	5.0

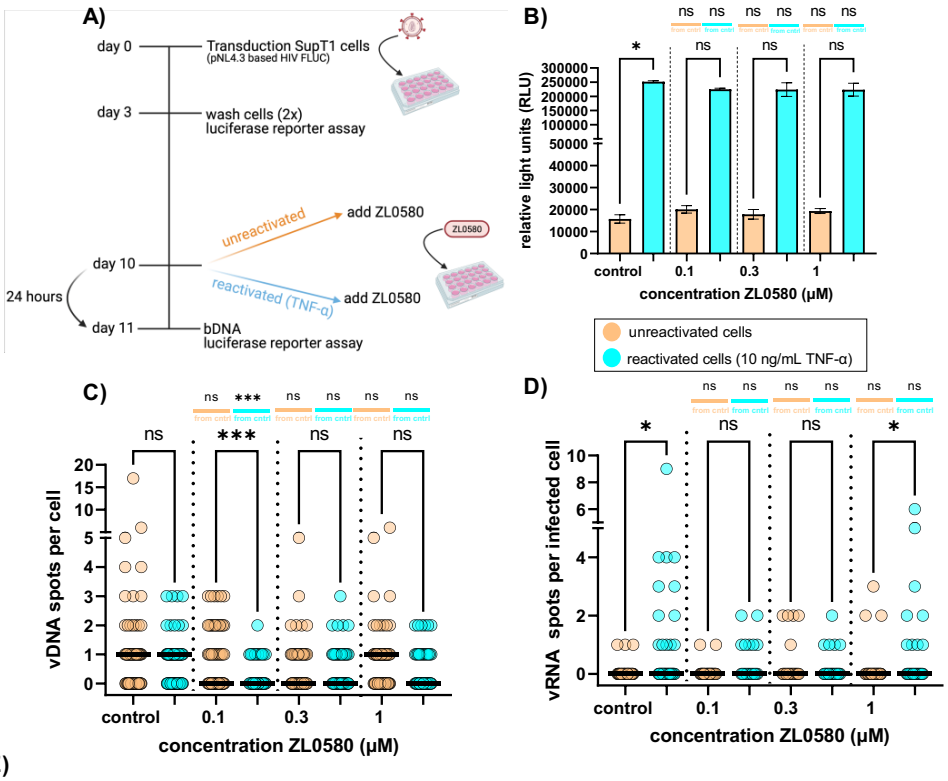
Figure 4.4. Effect of JQ1 on RNA expressing cells: A) All cells that did not contain vRNA spots were left out of the analysis. Hereafter we refer to these vRNA expressors as RNA expressing cells (RNA^+) cells. The number of vRNA spots per infected cell in the RNA^+ cells was plotted for both the unreactivated (orange spots) and reactivated (blue spots) cells treated with varying concentrations of JQ1. Each dot represents the number of vRNA spots for a single cell and the bar represents the median number of vRNA spots per infected cell. Next to the graph, a table is represented with the median number of vRNA spots per infected cell of the unreactivated (orange spots) and reactivated (blue spots) cells. Statistical significance was calculated with a Kruskal-Wallis test (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and the reactivated cells and between the JQ1-treated cells compared to the control (0 µM JQ1). **B)** The median number of vRNA spots per infected cell was compared between all infected cells (RNA^{all}; analysis of Figure 4.1.A) and the infected RNA expressing cells (RNA^+; analysis of Figure 4.4.A) for each concentration of JQ1 and the control (0 µM JQ1), in both the unreactivated and the reactivated cells. vRNA, viral RNA; RNA^+ cells, RNA expressing cells; ns, non-significant; RNA^{all}, all cells; cntrl, control.

4.1.2 ZL0580 does not affect the basal transcription and reactivation of HIV

In contrast to the pan-BRD4 inhibitor JQ1 that activates HIV gene expression (103,107,116–118), ZL0580, a unique and more selective BRD4 inhibitor that suppresses HIV expression, has recently been reported (106). Intriguingly, blocking BRD4 could be a new approach to a block-and-lock cure strategy instead of the current use of BRD4 inhibitors in the shock-and-kill strategy. However, ZL0580 is the first BRD4 inhibitor known to suppress HIV expression and is investigated by only one research group. Therefore, results need to be confirmed by independent research groups.

First, an MTT-tests was carried out in Jurkat cell lines to estimate the toxicity of ZL0580. This resulted in a mean 50% cytotoxic concentration (CC_{50}) of 2.347 µM. Second, in order to investigate the effect of ZL0580 on HIV transcription, infection experiments were conducted. Briefly, Jurkat cells were transduced with a pNL4.3 based HIV FLUC construct (virus dilution 1/7000). Three days later, the virus was washed away, and a sample was taken for a luciferase reporter assay to confirm the infection. On day ten post-transduction, the cells were treated with increasing concentrations of ZL0580, and half of the cells were additionally reactivated with 10 ng/mL TNF-α. Finally, 24 hours post-reactivation, samples were taken for a luciferase reporter assay and the bDNA analysis **(Figure 4.5.A)**.

The luciferase reporter assay showed that ZL0580 had no impact on the replication of HIV (orange bars) nor on the TNF-α induced reactivation of HIV (blue bars) **(Figure 4.5.B)**. In addition to the luciferase reporter assay that measures an averaged read-out, the more sensitive bDNA analysis was conducted to explore the effect of ZL0580 on HIV expression on single cell level. As expected, ZL0580 had no significant effect on the number of vDNA spots per cell **(Figure 4.5.C)**. Moreover, ZL0580 did not significantly alter the median number of vRNA spots per infected cell in the unreactivated (orange spots) and reactivated (blue spots) cells **(Figure 4.5.D)**. This suggests that ZL0580 has no influence on the transcriptional state of HIV. Collectively, both independent techniques indicate that ZL0580 has no effect on basal transcription nor on TNF-α induced reactivation of HIV.



E)

	median vDNA spots (unreactivated)	median vDNA spots (reactivated)
control	1.0	1.0
0.1 µM ZL0580	0.0	0.0
0.3 µM ZL0580	0.0	0.0
1 µM ZL0580	1.0	0.0

	median vRNA spots (unreactivated)	median vRNA spots (reactivated)
control	0.0	0.0
0.1 µM ZL0580	0.0	0.0
0.3 µM ZL0580	0.0	0.0
1 µM ZL0580	0.0	0.0

Figure 4.5. ZL0580 has no effect on basal transcription and TNF-α induced reactivation of HIV: A) Methodology of infection experiment: Jurkat cells were transduced with a pNL4.3 based HIV FLUC construct (virus dilution 1/7000). On day ten post-transduction, the cells were treated for 24 hours with a dilution series of ZL0580 (0 μM (control), 0.1 μM, 0.3 μM, 1 μM), in the presence or absence of 10 ng/mL TNF-α. After reactivation, samples were taken to perform the luciferase reporter assay and the bDNA analysis. **B)** The luciferase counts of the unreactivated (orange bars) and reactivated (blue bars) cells, normalized for the total amount of protein (determined by BCA assay), were plotted for each concentration of ZL0580 and the control (0 μM ZL0580). The error bars represent the standard deviation from technical duplicates. Statistical significance was calculated with a Kruskal-Wallis (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and the reactivated cells and between the ZL0580-treated cells compared to the control (0 μM ZL0580). **C)** The number of vDNA spots per cell and **D)** the number of vRNA spots per infected cell, after the bDNA analysis, were plotted for 73 cells for both the unreactivated (orange spots) and reactivated (blue spots) cells treated with varying concentrations of ZL0580. Each dot represents the number of vDNA and vRNA spots for a single cell and the bar represents the median number of vDNA spots per cell or the median number of vRNA spots per infected cell of the unreactivated (orange spots) and reactivated (blue spots) cells. Statistical significance was calculated with a Kruskal-Wallis test (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and the reactivated cells and between the ZL0580-treated cells compared to the control (0 μM ZL0580). **E)** A table is represented with the median number of vDNA spots per cell or the median number of vRNA spots per infected cell of the unreactivated (orange spots) and reactivated (blue spots) cells. These experiments were performed once in SupT1 cells and once in Jurkat cells, the representative experiment performed in the Jurkat cells is shown. TNF-α, tumor necrosis factor α; HIV, human immunodeficiency virus; bDNA, branched DNA; BCA, bicinchoninic acid protein assay; vDNA, viral DNA; vRNA, viral RNA; ns, non-signficant; RLU, relative light units; cntrl, control.

4.1.3 JQ1 does not block the basal transcription of LEDGIN-retargeted provirus but interferes with reactivation

Next analysis is based on the hypothesis that the residual high vRNA expressors after LEDGIN-treatment are linked to an integration in proximity to enhancer regions driven by BRD4. It was reasoned that JQ1, which is an inhibitor of the interaction between BRD4 and the acetylation marks (103), could block enhancer-driven expression after LEDGIN-treatment. However, since JQ1 also affects Tat-mediated HIV transcription the overall effect was unpredictable.

To experimentally verify the hypothesis, SupT1 cells were transduced with a pNL4.3 based HIV FLUC construct (virus dilution 1/7000). Meanwhile LEDGINs (CX014442) were added at different concentrations (0 μM, 2.35 μM, 4.7 μM, 9.42 μM, 18.83 μM). Three days later, the virus and LEDGINs were washed away, and a sample was taken for a luciferase reporter assay to confirm the infection and the inhibitory effect of LEDGINs on HIV infection. This luciferase reporter assay showed that LEDGINs induced a dose dependent reduction in HIV replication **(Figure 4.6.B)**. Next, on day ten post-transduction, the cells were treated with JQ1 (0 μM (control), 0.01 μM, 2 μM), and half of the cells were additionally reactivated with 10 ng/mL TNF- α. Finally, 24 hours post-reactivation samples were taken to perform the luciferase reporter assay and the bDNA analysis to determine the effect of JQ1 on LEDGIN-retargeted provirus **(Figure 4.6.A)**.

With bDNA imaging, the dose dependent decrease in vDNA spots after LEDGIN-treatment was further confirmed. As expected, JQ1 did not significantly alter the level of vDNA spots per cell. Therefore, the conditions with different amount of JQ1 (0 µM (control), 0.01 µM, 2 µM of JQ1) but the same amount of LEDGINs were pooled to clearly control the effect of LEDGINs on the vDNA expression level. LEDGINs significantly reduced the vDNA spots per cell, confirming the inhibition of integration by LEDGINs **(Figure 4.6.C)**.

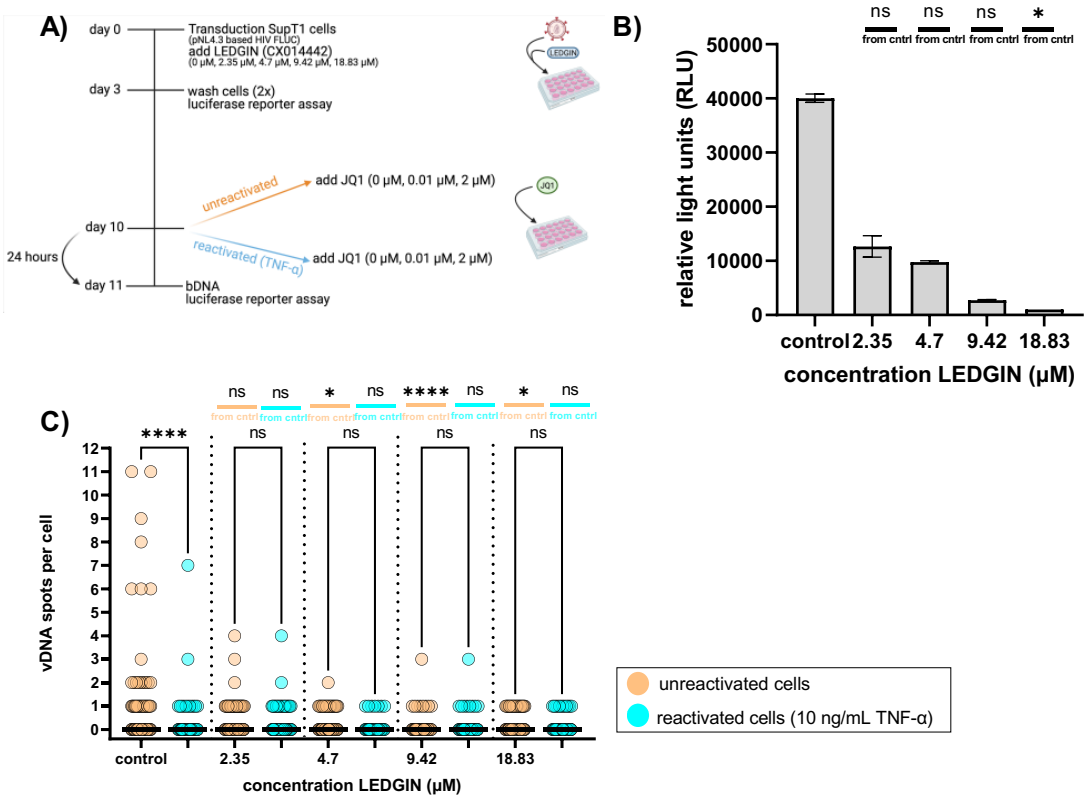


Figure 4.6. LEDGINs inhibit integration of HIV: A) Methodology of infection experiment: During the transduction (virus dilution 1/7000) of the SupT1 cells, LEDGINs (CX014442) were added at different concentrations (0 µM (control), 2.35 µM, 4.7 µM, 9.42 µM, 18.83 µM). Ten days post-transduction, the cells were treated with JQ1 (0 µM (control), 0.01 µM, 2 µM) in the presence or absence of 10 ng/mL TNF-α for 24 hours. After reactivation, samples were taken to perform the luciferase reporter assay and bDNA analysis. **B)** The luciferase counts on day three, before the addition of JQ1, normalized for the total amount of protein (determined by BCA assay), were plotted for each concentration of LEDGIN. The error bars represent the standard deviation from technical duplicates. Statistical significance was calculated with Krushkal-Wallis test (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the LEDGIN-treated cells compared to the control (0 µM LEDGIN). **C)** The vDNA spots per cell, after bDNA analysis 24-hours post reactivation, were plotted for 254 cells for both the unreactivated (orange spots) and reactivated (blue spots) cells treated with varying concentrations of LEDGINs. Each dot represents the number of vDNA spots for a single cell and the bar represents the median number of vDNA spots per cell. Statistical significance was calculated with a Kruskal-Wallis test (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and the reactivated cells and between the LEDGIN-treated cells compared to the control (0 µM LEDGIN). HIV, human immunodeficiency virus; TNF-α, tumor necrosis factor α; bDNA, branched DNA; BCA, bicinchoninic acid protein assay; vDNA, viral DNA; ns, non-significant; RLU, relative light units; cntrl, control.

After confirming the LEDGIN-induced inhibition on HIV integration, the effect of JQ1 on LEDGIN-retargeted provirus was examined by analyzing the vRNA expression level 24 hours post-reactivation. For each concentration of LEDGIN, a graph with the luciferase assay and the bDNA analysis is presented, with the increasing concentrations of JQ1 (0 μM (control), 0.01 μM, 2 μM) plotted on the x-axis **(Figure 4.7)**. It was investigated in particular whether at high concentrations of LEDGINs, JQ1 would reduce the residual basal transcription and TNF-α induced reactivation to achieve a complete silencing of HIV expression.

In the cells treated with 0 μM of LEDGIN, the luciferase reporter assay showed no significant difference when supplemented with JQ1, although addition of JQ1 resulted in a slight increase in HIV replication (orange bars) and TNF-α induced reactivation (blue bars) **(Figure 4.7.A)**. The bDNA analysis on the other hand, showed no significant difference of basal transcription (orange spots), while a dose dependent promotion of TNF-α induced reactivation was show, after addition of JQ1 (blue spots). However, it has to be pointed out that compared to the luciferase reporter assay and the vDNA detection, the highest number of vRNA spots was expected in the cells treated with 0 μM of LEDGIN. Still fewer vRNA spots were present compared to the cells treated with 2.35 μM LEDGINs. Therefore, it is likely that too few vRNA spots were detected in the cells treated with 0 μM of LEDGINs (in both unreactivated and reactivated cells) **(Figure 4.7.A)**. This may be due to a poor vRNA probe hybridization **(Figure 4.7.A)**.

Furthermore, the luciferase assay of the cells treated with 2.35 μM of LEDGINs indicated no significant difference when supplemented with JQ1, although addition of JQ1 resulted in a modest increase in HIV replication (orange bars) and TNF-α induced reactivation (blue bars) **(Figure 4.7.B)**. In addition, the bDNA analysis of the 2.35 μM LEDGIN-treated cells corroborated no significant promotion of basal transcription (orange spots), although the median number of vRNA spots increased in the JQ1-traeted cells with an optimal concentration of 0.01 μM JQ1. Moreover, JQ1 significantly promoted TNF-α induced reactivation (blue spots) with an optimal concentration at 0.01 μM JQ1 **(Figure 4.7.B)**.

When analyzing the cells treated with 4.7 μM and 9.42 μM of LEDGINs, the luciferase assay showed no significant difference of HIV replication (orange bars) and TNF-α induced reactivation (blue bars) **(Figure 4.7.C and Figure 4.7.D)**. However, a modest increase of basal transcription and TNF-α induced reactivation at 2 μM of JQ1 was shown. The bDNA analysis of the cells treated with 4.7 μM and 9.42 μM of LEDGINs corroborated an absence of a significant effect of JQ1 on basal transcription (orange spots) and TNF-α induced reactivation (blue spots) as well. However, also in this case a modest increase of basal transcription and TNF-α induced reactivation at 2 μM of JQ1 was shown **(Figure 4.7.C and Figure 4.7.D)**.

Although the result was not statistically significant, the cells treated with 18.83 μM of LEDGINs showed a dose dependent promotion of HIV replication (orange bars) and TNF-α induced reactivation (blue bars) in the luciferase assay after addition of JQ1 **(Figure 4.7.E)**. When analyzing the results with the bDNA analysis, JQ1 had no significant impact on the basal transcription (orange spots) and TNF-α induced reactivation (blue spots). Although not statistically different, the number of vRNA spots of several unreactivated cells increased when supplemented with JQ1, with an optimal concentration of 0.01 μM JQ1. Interestingly, the promotion of basal transcription seemed to be more pronounced compared to the stimulation of the TNF-α induced reactivation in the cells treated with 18.83 μM of LEDGINs **(Figure 4.7.E)**.

For a more in-depth overview of the effect of JQ1 on TNF-α induced reactivation after LEDGIN-treatment, the relative fold reactivation induced by TNF-α (total number of vRNA spots of TNF-α reactivated cells / total number of vRNA spots of unreactivated cells) was calculated. It is clear that JQ1 promotes TNF-α induced reactivation compared to the control (0 μM JQ1) at the optimal concentration of 0.01 μM JQ1, in the cells not treated with LEDGINs or with low concentrations of LEDGINs (2.35 μM). Interestingly, this pattern shifted when the cells were treated with higher concentrations of LEDGIN (4.7 μM, 9.42 μM, 18.83 μM). The optimal promotion of TNF-α induced reactivation by JQ1 at 0.01 μM was lost and addition of both 0.01 μM and 2 μM of JQ1 inhibited the TNF-α induced reactivation compared to the control (0 μM) **(Figure 4.8.A)**.

In addition, the relative fold-increase in vRNA expression induced by JQ1 was calculated by dividing the total number of vRNA spots of the JQ1-treated cells by the total number of vRNA spots of the control cells (0 μM JQ1). This graph showed that the relative fold -increase in vRNA expression induced by JQ1 is less pronounced in reactivated compared to unreactivated cells, indicating no synergistic effect between TNF-α and JQ1. Moreover, LEDGINs have no influence on this effect **(Figure 4.8.B)**.

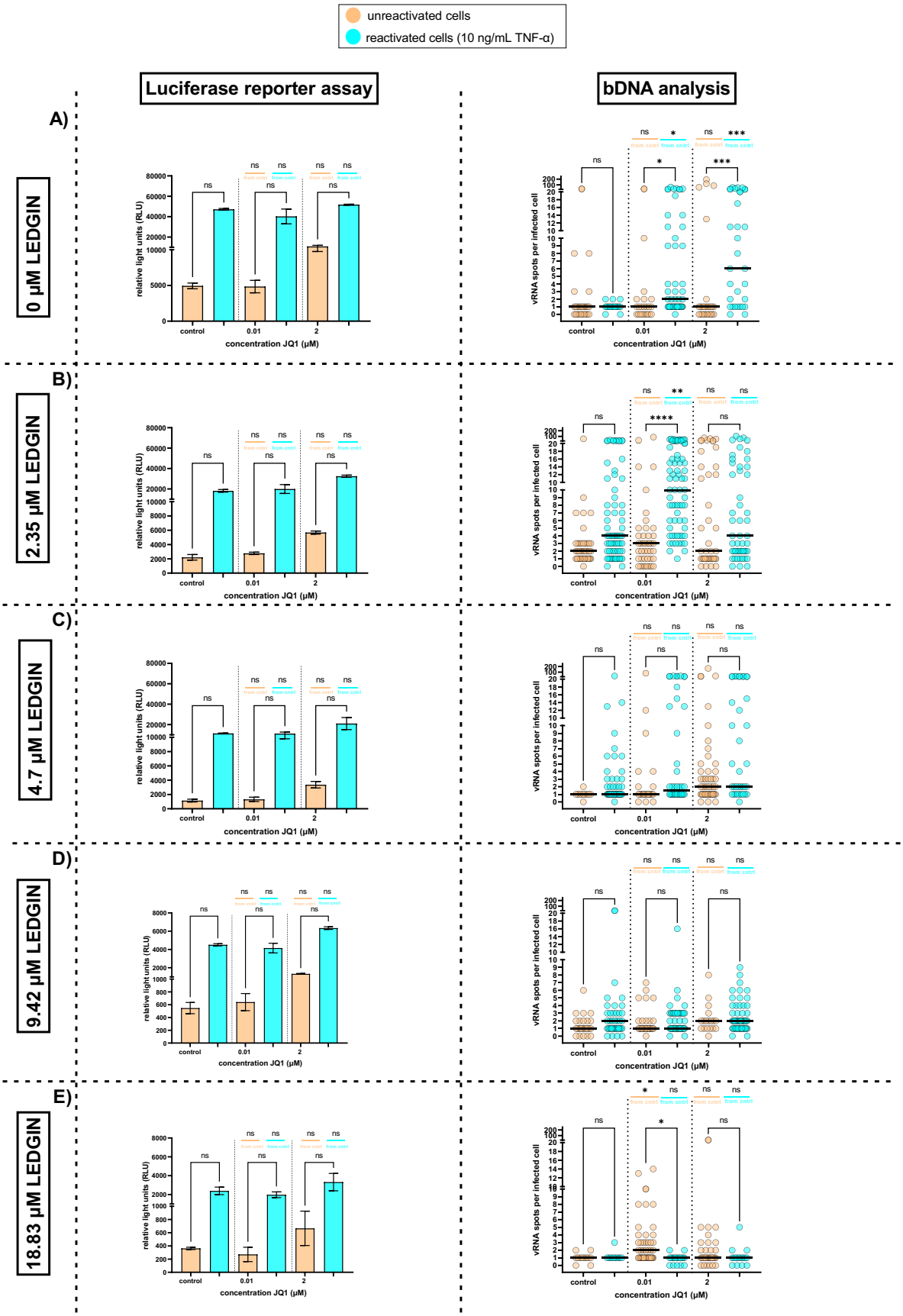

unreactivated cells
reactivated cells (10 ng/mL TNF-α)
Luciferase reporter assay
bDNA analysis
A)
0 µM LEDGIN
B)
2.35 µM LEDGIN
C)
4.7 µM LEDGIN
D)
9.42 µM LEDGIN
E)
18.83 µM LEDGIN
relative light units (RLU)
vRNA spots per infected cell
control
0.01
2
concentration JQ1 (µM)
ns
from cntrl

Figure 4.7. JQ1 promotes basal transcription of LEDGIN-retargeted provirus: Cells treated with varying concentrations of LEDGIN (0 µM, 2.35 µM, 4.7 µM, 9.42 µM, 18.83 µM) during transduction. Ten days post-transduction, the cells were treated with varying concentrations of JQ1 (0 µM (control), 0.01 µM, 2 µM) in the presence or absence of 10 ng/mL TNF-α for 24 hours. After reactivation, samples were taken to perform a luciferase reporter assay (left graph) and a bDNA analysis (right graph). Each graph represents cells treated with a different concentration of LEDGIN (A: 0 µM, B: 2.35 µM, C: 4.7 µM, D: 9.42, E: 18.83 µM). On the left side, the luciferase counts of the unreactivated (orange bars) and reactivated (blue bars) cells, normalized for the total amount of protein (determined by BCA assay), were plotted for each concentration of JQ1. The error bars represent the standard deviation from technical duplicates. Statistical significance was calculated with a Kruskal-Wallis test (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and reactivated cells and between the JQ1-treated cells compared to the control (0 µM JQ1). On the right side, the vRNA spots per infected cell,were plotted for 76 cells for both the unreactivated (orange spots) and reactivated (blue spots) cells treated with varying concentrations of JQ1. Each dot represents the number of vRNA spots for a single cell and the bar represents the median number of vRNA spots per infected cell. Statistical significance was calculated with a Kruskal-Wallis test (ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) between the unreactivated and the reactivated cells and between the JQ1-treated cells compared to the control (0 µM JQ1). TNF-α, tumor necrosis factor α; bDNA, branched DNA; BCA, bicinchoninic acid protein assay; ns, non-significant; vRNA, viral RNA; RLU, relative light units; cntrl, control.

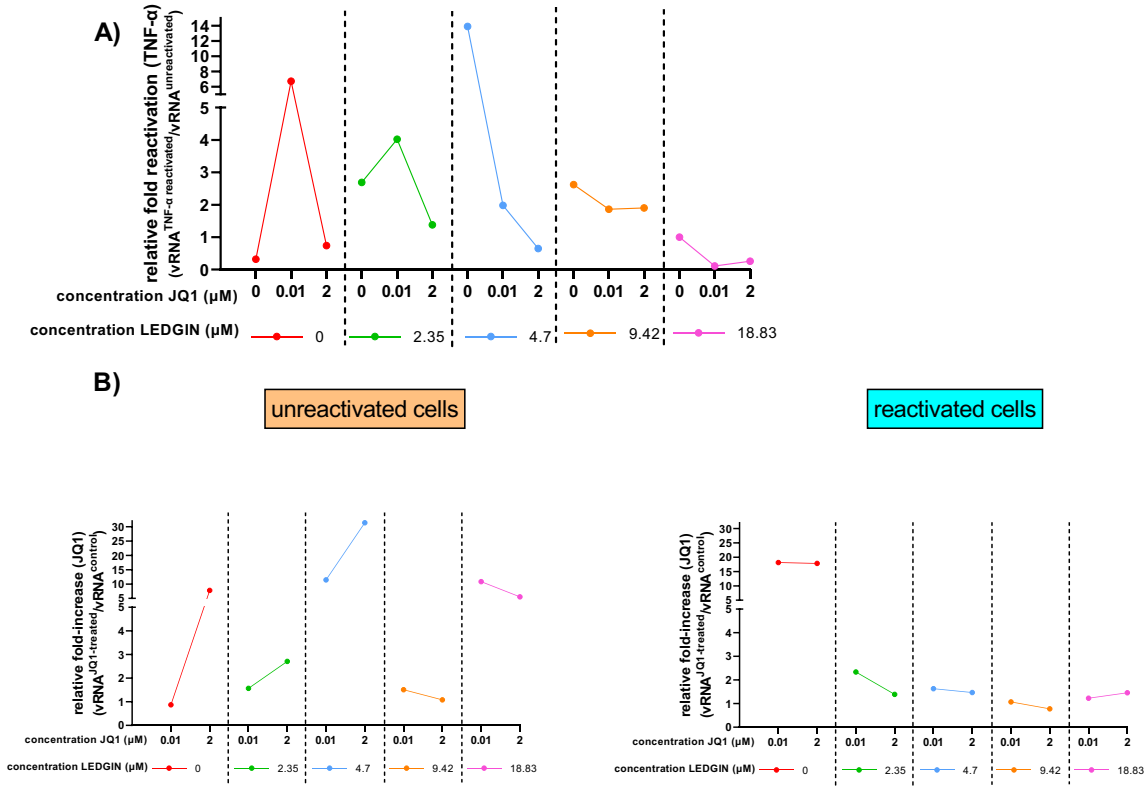
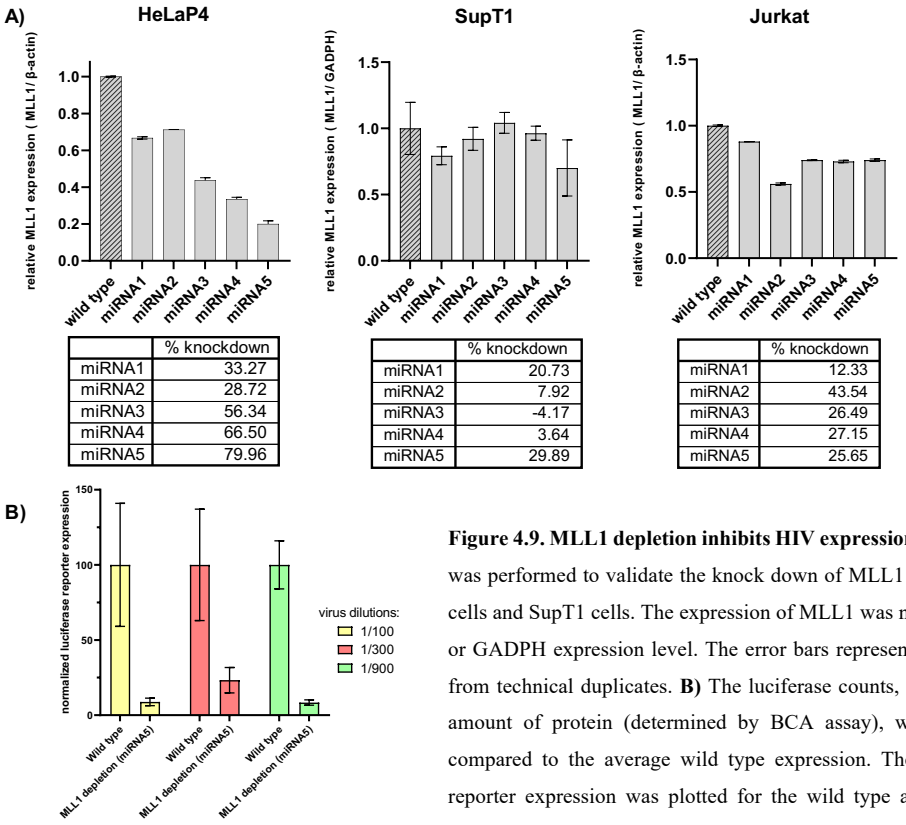


Figure 4.8. JQ1 inhibits TNF-α induced reactivation of LEDGIN-retargeted provirus. **A)** The relative fold reactivation induced by TNF-α was calculated by dividing the total number of vRNA spots of the TNF-α reactivated cells by the total number of vRNA spots of the unreactivated cells. The relative fold reactivation induced by TNF-α is represented for each concentration of LEDGIN in a different color**. B)** The relative fold-increase in vRNA expression induced by JQ1 was calculated by dividing the total number of vRNA spots in the JQ1-treated cells by the total number of vRNA spots in the control cells (0 µM JQ1). The relative fold-increase in vRNA expression induced by JQ1 is presented for each concentration of LEDGIN in a different color, for both the unreactivated and reactivated cells in a separate graph. TNF-α, tumor necrosis factor α; vRNA, viral RNA.

4.2 MLL1 inhibits HIV replication

Polyclonal MLL1 knockdown cell lines were generated by transducing HeLaP4, SupT1 and Jurkat cell lines with an SIV-based vector encoding specific MLL1 targeting miRNA30s. Five distinct miRNA30s **(Table 3.1)** were used to increase the likelihood of an efficient knockdown. To validate which miRNA induced the most potent knockdown, RT-qPCR analysis was performed. The RT-qPCR analysis revealed that miRNA5 resulted in the most potent MLL1 knockdown in the HeLaP4 cells, since it reduced the MLL1 expression with 80% compared to the wild type, which was transduced with the backbone LV vector **(Figure 4.9.A)**. Unfortunately, no strong knockdown was achieved in the SupT1 and Jurkat cells **(Figure 4.9.A)**. Therefore, further experiments were conducted with the HeLaP4 MLL1 knockdown cell line generated with miRNA5.

Infection experiments were conducted to compare the HIV expression in backbone LV transduced cells (wild type) and cells depleted for MLL1 (with the use of miRNA5). Briefly, 20 000 HeLaP4 cells, wild type and MLL1 depleted HeLaP4 cells, were transduced with different dilutions of a replication-deficient pNL4.3 based HIV FLUC construct (1/100, 1/300, 1/900). Three days later, samples were taken for a luciferase reporter assay. MLL1 depletion severely impaired single round HIV infectivity, as indicated by a drop between 77% and 92% of the normalized luciferase reporter expression in the distinct virus dilutions, of the MLL1 depleted cells compared to the wild type **(Figure 4.9.B)**.



	% knockdown
miRNA1	33.27
miRNA2	28.72
miRNA3	56.34
miRNA4	66.50
miRNA5	79.96

	% knockdown
miRNA1	20.73
miRNA2	7.92
miRNA3	-4.17
miRNA4	3.64
miRNA5	29.89

	% knockdown
miRNA1	12.33
miRNA2	43.54
miRNA3	26.49
miRNA4	27.15
miRNA5	25.65

Figure 4.9. MLL1 depletion inhibits HIV expression: A) RT-qPCR analysis was performed to validate the knock down of MLL1 in HeLaP4 cells, Jurkat cells and SupT1 cells. The expression of MLL1 was normalized to the β-actin or GADPH expression level. The error bars represent the standard deviation from technical duplicates. **B)** The luciferase counts, normalized for the total amount of protein (determined by BCA assay), were further normalized compared to the average wild type expression. The normalized luciferase reporter expression was plotted for the wild type and the MLL1 depleted HeLaP4 cell line (with the use of miRNA5), for different virus dilutions (1/100, 1/300,1/900). Each color represents a different virus dilution, and the error bars represent the standard deviation from technical duplicates.

5. Discussion

The main hurdle towards an HIV cure is the persistence of the latent reservoir (11,12,13). The shock-and-kill eradication strategy aims to completely eradicate the latent reservoir by the use of LRAs such as JQ1 (89). However, after several unsuccessful attempts to translate the shock-and-kill strategy in a clinically relevant therapy, the interest in a block-and-lock functional cure emerged. LEDGINs, antivirals that inhibit the LEDGF/p75-IN interaction, were developed by my research group and have additionally been shown to silence HIV expression by retargeting the HIV provirus to less transcriptionally active sites in the genome. Therefore, these small molecules represent a promising candidate for a block-and-lock cure strategy (14,62). However, it remains challenging to completely block HIV transcription, because few high vRNA expressors remain present in the bDNA analysis, even after treatment with high concentration of LEDGINs **(Figure 1.5)**. Therefore, a combination approach may be necessary to establish an optimal 'pro-latency cocktail'. Although JQ1 is known to promote HIV transcription (103,107,116–118), it can be a suitable candidate to suppress the residual high vRNA expressors of LEDGIN-retargeted provirus by interfering with enhancer-mediated transcription of HIV. This because, BRD4, which is inhibited by JQ1, is known to play a prominent role in enhancer biology (99) and because LEDGINs do not influence the proximity of integration sites to enhancers (62). Intriguingly, a recent study reported a BRD4 inhibitor, ZL0580, that suppressed HIV transcription without the addition of LEDGINs (106,121). It may be used as an alternative strategy to block the residual high vRNA expressors of LEDGIN-retargeted provirus. Instead of only relying on average-based read-outs to investigate this hypothesis, the bDNA analysis was used in this study to measure the vDNA and vRNA expression on a single cell level.

5.1 JQ1 promotes basal transcription and TNF-α induced reactivation of HIV with an optimal concentration

First, the effect of the BRD4 inhibitor, JQ1, on HIV basal transcription and TNF-α mediated reactivation, was investigated without the addition of LEDGINs. Two independent techniques, the luciferase reporter assay and the bDNA analysis, were used to study this objective. The luciferase reporter assay showed no significant effect of JQ1 on HIV replication although a modest increase of HIV replication was observed in the unreactivated cells after addition of JQ1 with an optimal concentration of 1 μM. Moreover, the luciferase assay indicated that JQ1 had no significant effect on TNF- α induced reactivation of HIV. Interestingly, addition of JQ1 did show a trend of inhibition of the TNF- α induced reactivation of HIV at low concentrations of JQ1(blue bars), while this inhibition leveled-off when the concentration of JQ1 increased **(Figure 4.1.B)**.

Besides the luciferase reporter assay that measures an average read-out over many cells, the bDNA analysis was conducted to obtain a clearer overview of the effect of JQ1 on basal transcription and TNF-α induced reactivation on single cell level. JQ1 had no significant effect on the median number of vDNA spots per cell, which further supports the evidence that JQ1 has no direct effect on HIV integration **(Figure 4.1.C)**. Overall, a promotion of basal transcription and TNF-α induced reactivation was observed by JQ1 with an optimal concentration of 1 µM JQ1. However, at higher concentrations of JQ1, the promotion of basal transcription and TNF-α induced reactivation leveled-off to control-like levels **(Figure 4.1.D, Figure 4.1.F, Figure 4.3.A, Figure 4.4.A)**.

Several studies showed with the use of average-based methods, such as flowcytometry, qPCR, p24 capsid enzyme-linked immuno sorbent assay (ELISA) and the luciferase reporter assay, that JQ1 activates HIV replication (103,107,116–118). Besides the SupT1 cells used in my thesis, JQ1 has been proven to induce HIV expression in several post-latency cell line models such as Ach2T cells, U1 promonocytes, J-Lat T cells, Jurkat cells and even more relevant cell lines such as primary CD4+ T cells and PBMCs (103,107,116–118). In line with the optimal concentration of JQ1 observed in this study, Bartholomeeusen e*t al.* showed that JQ1 reached a maximum stimulation of HIV transcription as well. However the maximum stimulation was reached at 5 µM of JQ1 instead of the 1 µM of JQ1 that was observed in this study (107). The difference of optimal concentration could possibly be explained by two reasons. First, Bartholomeeusen *et al.* used Jurkat cell lines, while this study was performed in SupT1 cells. Moreover, the concentrations that Bartholomeeusen *et al.* used in his experiments jumped from 0.5 µM to 5 µM of JQ1, and thus excluded concentrations around 1 µM of JQ1.

Besides the effect on basal transcription, the impact on TNF-α mediated reactivation has been examined as well in my thesis. Less research groups examined the combination of JQ1 with TNF-α. Boehm *et al.* indicated that combining JQ1 with TNF-α only modestly increased the promotion of HIV expression induced by JQ1 (117). However, they used a five times lower concentration of TNF-α compared to the concertation used in this thesis, which may explain why their TNF-α induced reactivation was less pronounced (117). Moreover, JQ1 has been combined with other LRAs in the study of Boehm *et al*. JQ1 had an additive effect with prostatin, while no additive effect and even an inhibition was reached with suberoylanilide hydroxamic acid (SAHA) (117). Furthermore, Zhu *et al.* investigated the combination of JQ1 with different LRAs such as phytohemagglutinin (PHA), prostatin and TNF-α in primary patient samples of people on long-term cART. In some patient samples, the combination of JQ1 with a LRA (PHA, prostatin, TNF-α) resulted in an enhanced HIV reactivation, while in other patient samples the LRA-induced HIV reactivation was inhibited by JQ1 (116).

A major strength of the analysis in this thesis compared to previous research is that the bDNA technique elucidates the impact of JQ1 on HIV gene regulation on single cell level. This increases the sensitivity of the analysis and gives the opportunity to exclude non-infected cells and cells that do not contain vRNA, thereby giving a more in-depth representation. A limitation is that this technique is time-consuming and expensive. Moreover, it is a random selection of cells. Imaging more cells will contribute to the power of this technique but on the other hand makes this analysis even more time and money consuming

BRD4 is considered as a negative regulator of HIV in a model where it contributes to promotor proximal pausing of HIV transcription. Briefly, BRD4 competes with Tat for the binding of P-TEFβ. JQ1 blocks BRD4 and thereby increases the availability of P-TEFβ for Tat. The P-TEFβ levels are further increased by addition of JQ1 because JQ1 releases P-TEFβ from its inactive state bound to 7SK snRNP **(Figure 1.7)**. A part of my results are in line with this model since JQ1 did not alter the vDNA copy number and since JQ1 promotes basal transcription and TNF-α induced reactivation of HIV in a dose dependent manner until 1 μM. Moreover, several studies have provided evidence that supports this hypothesis. First, Bartholomeeusen *et al*. reported that JQ1 increased the levels of BRD4 and P-TEFβ (107). Second, BRD4 depletion has been shown to abrogate the impact of JQ1 on HIV gene expression (116). Moreover, addition of JQ1 promoted Tat-dependent transcriptional activity and the interaction between Tat and P-TEFβ (116). In addition, with quantitative chromatin immunoprecipitation (ChIP) analysis, it was shown that JQ1 suppressed the binding of BRD4 to the acetylated lysine residues at the histones and promotes the binding of Tat to the HIV promotor (103).

Besides this strong evidence, some research groups still question this model since they noticed the same effect of JQ1 in cell lines lacking Tat (117). In addition, my results question this model as well, since it cannot clarify the leveling-off in promotion of basal transcription and TNF-α induced reactivation of HIV at the higher concentrations of JQ1. Besides the role of BRD4 as a negative regulator of Tat-dependent HIV transcription in promotor-proximal pausing, some research groups reported that BRD4 is a positive regulator of Tat-independent basal HIV transcription (122–125). This second model claims that BRD4 is able to recruit P-TEFβ to the viral promotor irrespective of Tat, which increases the phosphorylation of the pol II CTD and consequently promotes basal transcription of HIV (122,124). In addition, a third model has been proposed whereby BRD4 acts as an inhibitor of enhancer-mediated transcription (99). Chen *et al.* used the B-HIVE technique to detect a positive correlation between proximity of integration sites to enhancers and HIV gene expression (100). Secondly, BRD4 is known to play a role in enhancer-mediated transcription by binding the acetylated lysine residues at genomic enhancer regions (99,104). Third, Lovén *et al.* reported that inhibition of BRD4 by JQ1 decreased expression of enhancer related genes and even decreased the level of BRD4, MED1 and TF at the genomic enhancer regions (104).

Further, it has to be mentioned that several articles claim a role of TNF-α, a well-known LRA (119), in the enhancer biology as well. First, Zou *et al*. reported that BRD4 induces the nuclear factor that binds the immunoglobulin K light chain gene (NF- κb) light chain enhancers and that JQ1 was able to inhibit this induction (126,127). Moreover, Brown *et al.* reported that TNF-α was able to modify the enhancer environment in endothelial cells by recruiting BRD4, which resulted in pro-inflammatory activation. Moreover, this activation was abrogated by BRD4 inhibition (127,128).

The contrasting effects of JQ1 on HIV transcription, may be the result of these three distinct mechanisms that take place upon addition of JQ1: 1) JQ1 bound to BRD4 decreases the competition between BRD4 and Tat for P-TEFβ which alleviates promotor-proximal pausing and actives HIV transcription. 2) JQ1 bound to BRD4 suppresses the recruitment of P-TEFβ to the viral promotor in general and thereby blocks HIV transcription, irrespective of Tat. 3) JQ1 inhibits genomic enhancer-mediated transcription **(Figure 5.1)**. It is likely that at low concentrations of JQ1 the first mechanism is activated, while at higher concentrations of JQ1 the second and third mechanism become more pronounced which overall leads to inhibition of HIV transcription. As a result, the stimulatory effect of JQ1 levels-off at higher concentrations of JQ1. However, several studies critically question the positive regulatory function of BRD4 in HIV gene regulation (118). Still, since other independent research groups also see an optimal concentration of JQ1 (107) or even inhibition of LRA-induced reactivation by JQ1 (116), it is worth to investigate the contribution of these three mechanisms to the overall effect of JQ1 on HIV transcription.

To conclude, this thesis indicates that the role of BRD4 in HIV gene regulation could be more complex than originally thought. Since viral transcription is a crucial part of the molecular virology of HIV (129) and since the intriguing role of BRD4 in control of viral replication, it is a promising target for an HIV cure. Further understanding of the role of BRD4 in HIV regulation could open the doors to a new target for a block-and-lock cure strategy or further contribute to the use of BRD4 as a target in the shock-and-kill strategy.

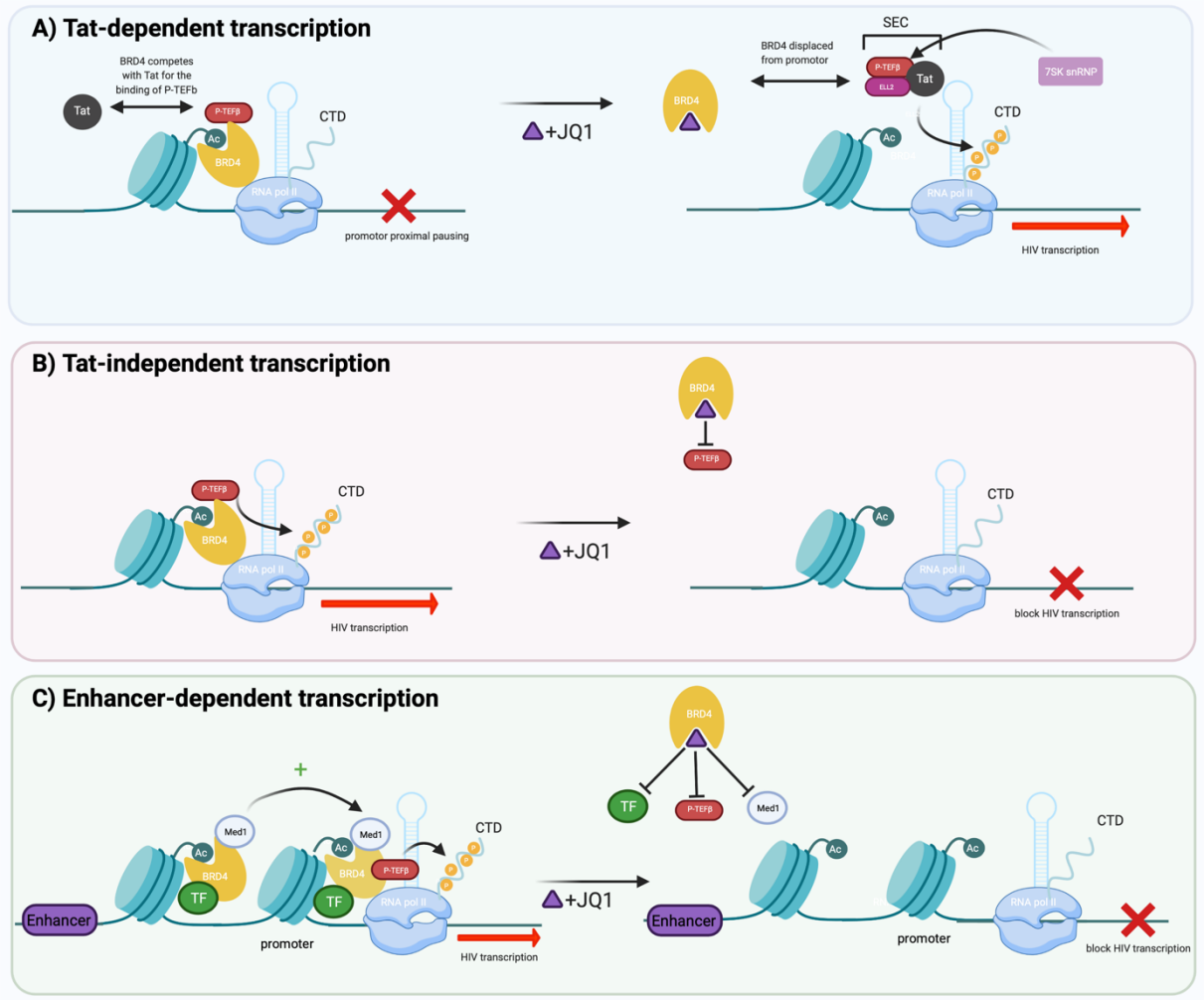


Figure 5.1. Role of BRD4 in distinct steps of HIV transcription: **A) BRD4 as a negative regulator of Tat-dependent transcription.** During promotor proximal pausing, BRD4 is bound to acetylated (Ac) histones and competitively blocks the interaction of Tat with P-TEFβ and thereby prevents the formation of the SEC. This leads to promotor proximal pausing of RNA pol II. JQ1 dissociates BRD4 from the acetylated (Ac) histones, which results in increased P-TEFβ levels. Furthermore, JQ1 releases P-TEFβ from the inactive state, bound to 7SK snRNP. These two mechanisms promote the binding of Tat to P-TEFβ and thus the formation of the SEC. This results in a promotion of HIV transcription by binding of the SEC to the TAR stem-loop structure and a phosphorylation of the Pol II CTD. **B) BRD4 as a positive regulator of Tat-independent transcription**. BRD4 is able to recruit P-TEFβ to the viral promotor irrespective of Tat, which increases the phosphorylation of the pol II CTD and consequently promotes HIV transcription. Supplementation of JQ1 suppresses the recruitment of P-TEFβ to the viral promotor, which blocks HIV transcription. **C) BRD4 as a regulator of enhancer-dependent transcription *in trans*.** The genomic region of the (super-)enhancers contains a high level of acetylated lysine residues that BRD4 is able to bind. This leads to the further recruitment of P-TEFβ, Med1 and TF, which results in a promotion of HIV transcription by a phosphorylation of the pol II CTD at the proviral promotor. JQ1 is able to displace BRD4 from the acetylated enhancer region, which blocks HIV transcription. BRD4, bromodomain containing protein 4; Tat, trans-activator of transcription; Ac, acetylated; P-TEFβ, positive transcription elongation factor β; SEC, super elongation complex; pol, polymerase; 7SK snRNP, 7SK small nuclear ribonucleoprotein; HIV, human immunodeficiency virus; TAR, trans-activation response element; pol, polymerase; CTD, carboxy terminal domain; Med1 mediator complex subunit 1; TF, transcription factors. (Adapted from: Z. Li et al, Nucleic Acids Res., 2013, **41**(1), 277–287. Q. Niu et al, J Clin Invest., 2019, **129**(8), 3361–3373. K. Klein et al, RMD Open., 2018, **4**(2), 1–10. And created with Biorender.com)

5.2 ZL0580 does not affect the basal transcription and reactivation of HIV

Besides JQ1, several other BRD4 inhibitors were developed to activate HIV transcription such as I-BET151, I-BET and MS417 (117). In general, all BRD4 inhibitors are known to promote HIV gene expression and therefore are useful in the shock-and-kill eradication strategy. In contrast, Niu *et al.* discovered a unique BRD4 inhibitor, ZL0580, that reduced HIV gene expression and thus is a promising tool in the block-and-lock functional cure strategy (106,121). However, since only one research group examined the effect of ZL0580 on HIV and since the detailed mechanisms of action remains a burning question further studies by independent research group are required. It remains controversial why inhibiting the same protein by ZL0580 or JQ1 can differently regulate HIV gene expression. Niu *et al.* suggested that ZL0580 has a different binding mode to BRD4 and therefore affects interaction partners distinct from JQ1 resulting in an opposite HIV gene regulation. This hypothesis is supported by binding assays which reveal that in contrast to JQ1, ZL0580 selectively binds BRD4 over other BET proteins and selectively interacts with the BD1 domain of BRD4 over the BD2 domain. Moreover, instead of JQ1 which binds to the KAc binding site of BRD4, ZL0580 binds to a non-KAc binding region of BRD4 (106,121). In line with this hypothesis, a previous study revealed that BRD4 can differentially regulate HIV expression by undergoing distinct interactions with AcH4 and AcH3 (108).

Although the promising potential of this small molecule, the suppression of HIV replication by ZL0580 could not be confirmed in our study. A first hurdle was the toxicity of ZL0580. The MTT-tests in Jurkat cell lines resulted in a mean CC_{50} of 2.347 µM, which was surprisingly lower than the toxicity reporter by Niu *et al.* in J-Lat cells (40 µM in J-Lat cells). However, since Jurkat cells are derived from T- cell acute lymphoblastic leukemia (T-ALL) patients (130), the toxicity of ZL0580 could possibly be explained by the anti-tumor effect of BRD4 inhibitors. Several studies proved that BRD4 inhibitors inhibit the cell proliferation in human T-ALL cell line by reducing the expression of Myc dependent genes. (131–134). However, it remains unclear why the toxicity of ZL0580 in the Jurkat cells differed significantly from the toxicity in the J-Lat cell line that Niu . *et al.* used, since the J-Lat cell line is a subclone from the Jurkat cell line (135).

Secondly, the bDNA analysis corroborated that ZL0580 did not alter the vDNA copy number, which indicates that ZL0580 has no impact on HIV integration **(Figure 4.5.C)**. Next, ZL0580 had no impact on the basal transcriptional level and the TNF-α induced reactivation of HIV in both the luciferase reporter assay and the bDNA analysis **(Figure 4.5.B, Figure 4.5.D)**. Overall, the high toxicity level and the lack of impact of ZL0580 on HIV transcription hamper the potential of BRD4 inhibitors as suppressors of HIV and contradict with the results from Niu *et al.* Several hypotheses could explain the opposing results between Niu *et al.* and this study. First, it has to be mentioned that Niu *et al.* only used average-based read-outs such as flow cytometry and qPCR, while the bDNA technique used in this study is the first technique that studies the effect of ZL0580 on HIV gene expression on single cell level.

Another possibility is that due to the high toxicity, the active range of ZL0580 could not be reached in my assays. Furthermore, the potential of BRD4 inhibitors as suppressors of HIV does not need to be completely excluded since, my data and those from other independent research groups see an optimal concentration of JQ1 for promotion of basal HIV transcription a (107) or even an inhibition of LRA-induced reactivation by JQ1 (116). Still, my results with ZL0580 need to be repeated in the Jurkat cell line to address fully reproducibility.

5.3 JQ1 antagonizes TNF-α induced reactivation of LEDGIN-retargeted provirus.

LEDGINs silence HIV expression by retargeting the HIV provirus to less transcriptionally active sites in the genome. However, several high vRNA expressors remain present **(Figure 1.5)**, possibly due to an integration in proximity to enhancer regions since LEDGINs do not influence the proximity of integration to these enhancer regions (62). To investigate this hypothesis, an infection experiment was conducted in which LEDGIN-treatment was combined with JQ1-treatment. It was hypothesized that because JQ1 inhibits BRD4 and BRD4 is involved in the enhancer biology, it may inhibit the enhancer-mediated transcription of the high vRNA expressors and contribute to a more complete block of HIV gene expression. Unfortunately, both the luciferase reporter assay as the bDNA read-out did not support this hypothesis since no additional block of HIV gene expression could be achieved by combining LEDGINs with JQ1 **(Figure 4.7.E)**. Of note, at the highest concentration of LEDGINs (18.83 μM) no residual high vRNA expressors were present in both the unreactivated (orange spots) and reactivated (blue spots) cells supplemented with 0 μM JQ1. However, in contrast with our hypothesis, JQ1 still promoted basal transcription (orange spots) and reactivation (blue spots) of HIV. This suggests that the residual high RNA expressors are not linked to integration in proximity to enhancer regions or that JQ- treatment alone is not sufficient to block HIV transcription controlled by enhancers.

Still several alternative explanations can be given. It could also be possible that the residual high vRNA expressors after LEDGIN-treatment are simply not retargeted by LEDGINs out of the active genes and thus are still tethered to the epigenetic mark, H3K36me3 by LEDGF/p75. Alternatively, it could be possible that JQ1 does not sufficiently block the enhancer regions. It is also possible that the previously reported Tat-dependent or independent activation of the HIV promotor, surpasses the inhibition of BRD4-mediated enhancer activation, since this mechanism is also irrespective of LEDGF/p75-mediated integration. Therefore, before completely excluding the enhancer hypothesis other options to block the enhancers-mediated transcription need to be explored. Since BRD4 knockdown enhances the effect of JQ1 (103), a combination approach between BRD4 knockdown and BRD4 inhibition with JQ1 could be used in this experiment. Furthermore, instead of using BRD4 as a target to block the enhancer-mediated transcription, other co-activators of transcription involved in the enhancer biology could be investigated as well. Vasant *et al.* showed that treatment with LEDGINs did not alter the proximity to the MED1 and CREB-binding protein (CBP) super-enhancer markers (62).

A knockdown of MED1 could therefore be an alternative strategy worth to examine (99). Moreover, due to the critical role of the CBP in the enhancer activity, the small molecule CBP/P300 bromodomain inhibitor, GNE-049 could potentially be used to block the enhancer activity as well (136–138).

Still, besides the somewhat disappointing results for the enhancer hypothesis, this experiment remains remarkably interesting to further clarify the characteristics of LEDGIN-retargeted provirus. It has been shown that treatment with LEDGINs reduces the integration and retargets the HIV provirus out of active genes (61). My data further confirm the inhibition of integration by LEDGINs **(Figure 4.6.B, Figure 4.6.C)**. However, several questions were raised about the chromatin landscape surrounding the retargeted integration site and how it affects the impact of LRAs on HIV gene expression. Provirus not retargeted by LEDGINs showed a more pronounced TNF-α induced reactivation with addition of 0.01 μM JQ1, which leveled off at the higher concentration of JQ1 **(Figure 4.7.A, Figure 4.8.A)**. This is in line with my previous experiment shown in Figure 4.3.B. Moreover, this pattern was still present in cells treated with low concentrations of LEDGINs (2.35 μM), probably because the low concentration of LEDGINs was not sufficient to retarget a significant amount of provirus out of the active genes **(Figure 4.7.B, Figure 4.8.A)**. Interestingly, at higher concentrations of LEDGINs (4.7 μM, 9.42 μM, 18.83 μM), JQ1 seemed to have a different impact on the TNF-α induced reactivation of HIV **(Figure 4.8.A)**. More specifically, the data showed that at a high concentration of LEDGIN, both concentrations of JQ1 inhibited TNF-α induced reactivation of HIV. **(Figure 4.8.A)**. Moreover, Figure 4.7.E clearly showed as well that the TNF-α induced reactivation is less pronounced at the highest concentration of LEDGINs (18.83 μM) compared to the lower concentrations of LEDGINS **(Figure 4.7).**

It remains unclear why JQ1 antagonized the TNF-α induced reactivation of LEDGIN-retargeted provirus. Vansant *et al.* showed with the B-HIVE technology that the chromatin landscape surrounding the integration sites determines the HIV gene expression. Therefore, it could be possible that the changed chromatin environment of LEDGIN-retargeted provirus changed the sensitivity to JQ1 and TNF-α. In the future, it would be interesting to use the B-HIVE technique for this infection experiment. This technique, which tags the HIV genome with a unique barcode to trace insert-specific HIV expression (62), could link a single provirus treated with a combination of LEDGINs and JQ1 to their chromatin features. It would be interesting as well to check if retargeting with LEDGINs influences the sensitivity to other LRAs such as interleukine-2, histone deacetylase inhibitors, protein kinase C agonists, etc.

Another possibility can be found by interpreting Figure 5.1. Without LEDGINs, JQ1 is known to promote HIV expression via a Tat-dependent mechanism **(Figure 5.1.A)** with an optimal concentration of 1 μM JQ1. If JQ1 still stimulates HIV transcription of LEDGIN-retargeted provirus, this indicates that LEDGIN-retargeted provirus is still dependent on Tat.

Therefore, the Tat-inhibitor, dedihydrocortistatin A (139), could be added to this experiment to form a more efficient 'pro-latency cocktail' containing: LEDGINs, JQ1 and dedihydrocortistatin A.

Moreover, since JQ1 acts synergistically with TNF-α at the optimal concentration of JQ1 in the absence of LEDGINs, but acts antagonistically in the presence of LEDGINs, this suggest that TNF-α works independent of Tat. Still, it remains unclear via which mechanism JQ1 inhibits the TNF-α induced reactivation of LEDGIN-retargeted provirus. However, due to the BRD4-dependent role of TNF-α in the enhancer biology, reported by Zou *et al.* and Brown *et al.* (126–128), and due to the observed changes in the effect of JQ1 on TNF-α induced reactivation of HIV transcription, it is possible that enhancers do play a role in the transcription LEDGIN-retargeted provirus after all.

Collectively, the role of BRD4 and enhancers in transcription of LEDGIN-retargeted provirus remains unclear and complex and further experiments with the B-HIVE technology and dedihydrocortistatin A could gain more insights and potentially promote the efficiency of the block-and-lock cure strategy.

5.4 MLL1 depletion silences HIV replication

MLL1 is known to play a prominent role in MLLr leukemia due to translocations in the gene encoding for this protein (140). LEDGF/p75 acts as a tether for MLL1 in MLLr leukemia (42,46). Moreover, LEDGF/p75 plays an important role in HIV integration (14,96). As a follow-up to these findings, Gao *et al.* reported that MLL1 and LEDGF/p75 play a role in post-integrative regulation of HIV transcription (111). They propose a model whereby LEDGF/p75 hampers transcriptional elongation of HIV by recruiting the PAF1-complex to the promotor. However, after latency reversal LEDGF/p75 takes the opposing role as a positive regulator of HIV transcription by interacting with MLL1 via a CKII-dependent phosphorylation and thereby displacing the PAF1-complex from the proviral promotor **(Figure 1.9)** (111).

To further elucidate the role of MLL1 in HIV replication, polyclonal MLL1 knockdown cell lines were generated by transducing HeLaP4, SupT1 and Jurkat cell lines with an SIV-based vector, each with one of five distinct MLL1 targeting miRNA30s. The knockdown efficiency was validated via RT-qPCR analysis. However, only miRNA5 sufficiently reduced the MLL1 expression, this only in the HeLaP4 cells. Unfortunately, it was difficult to validate the MLL1 knockdown on protein level via western blot analysis due to technical reasons, such as is the high molecular weight of MLL1 (N-terminal fragment of 320 kDa) (141).

In further infection experiments, the HIV replication was compared between LV transduced cells (wild type) and cells depleted for MLL1 (with miRNA 5). The luciferase reporter activity was reduced up to a range of 77% to 92%, indicating that MLL1 depletion severely impairs HIV infectivity **(Figure 4.9.B)**. Unfortunately, due to the difficulties to reactivate HeLaP4 cells, no latency reversal could be initiated and therefore only the effect of MLL1 depletion under unreactivated circumstances was examined.

Moreover, to prove a causal link between the observed phenotype and MLL1 knockdown, MLL1 back complemented cells should be generated in the future to rescue the phenotype. In addition, it has to be mentioned that this experiment was repeated using the same cell lines after several passages of cell culture, which resulted in a more modest reduction of the luciferase reporter expression of the MLL1 depleted cells compared to the wild type ranging between 18 % and 61%. However, this is probably caused by a reduced knockdown efficiency of the MLL1 depleted cell line, which was confirmed with RT-pPCR (% knockdown of HeLaP4 cells depleted from MLL1 with the use of miRNA5 decreased from 80% in the first experiment to 31% in the second experiment, after several cell passages). This suggested that despite the use of selection antibiotics in the cell culture (blasticidin, geneticin), a natural selection of cells with a higher MLL1 expression level may have occurred. Generation of a monoclonal cell lines would solve this problem for future research.

Gao *et al*. reported no change in pol II pausing at the proviral promotor in the absence of MLL1 in unreactivated circumstances (111). However, they found a decreased reactivation of HIV after MLL1 depletion, thus indicating a role of MLL1 during latency reversal. In contrast, this study seems to suggest a role of MLL1 in basal HIV replication, irrespective of latency reversal **(Figure 4.9.B)**. However, since this is a novel discovery, the mechanism behind the reduction in HIV infectivity after MLL1 depletion is unknown. Additional experiments may clarify the molecular mechanism behind the block in HIV replication after MLL1 depletion. Based on the model of *Gao et al.,* CKII-dependent phosphorylation enhances the interaction between LEDGF/p75 and MLL1. A supplementary strategy could be to add the commercially available CKII inhibitor, CX-4945, to the infection experiment (142). In addition, our lab recently created small molecule inhibitors of MLL1 and LEDGF/p75 in the context of the ongoing MLLr leukemia research. These molecules could be used to investigate if LEDGF/p75 that recruits MLL1 to the viral promotor, is responsible for the reduction in HIV replication after MLL1 knockdown. In an alternative strategy, LEDGF/p75 knockdown cell lines could be used to investigate the role of LEDGF/p75 in this phenotype. However, the pleiotropic effect of LEDGF/p75 on integration and integration site analysis complicates this approach.

Interestingly, to still achieve an optimal ‘’pro-latency cocktail’’, MLL1 depletion could be added to experiments where LEDGINs have been added during infection to achieve a more efficient silencing of HIV expression. The bDNA technique and luciferase reporter assay could be used here as well to investigate this hypothesis. Collectively, several critical questions remain about the role of MLL1 in HIV transcription and lots of promising research is still necessary. This research suggests that MLL1 may provide a valid target for a block-and-lock functional cure strategy.

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