

Circadian Regulation of Human Immunodeficiency Virus Type 1 Replication

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Thesis submitted for the degree of:
Doctor of Philosophy (DPhil)

Trinity 2023

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Acknowledgments

Firstly, I would like to express my gratitude to my supervisor, Jane. You ignited my passion for circadian rhythms and pathogen research, and your consistent support throughout my entire PhD has been exceptional. You have been a great mentor providing invaluable support during one of the most important stages of my career. I am immensely grateful to Alan, who was as a fantastic day-to-day supervisor and provided invaluable guidance, especially in the face of challenging results. Thank you to my co-supervisor Sri, whose circadian expertise has been of great value. I would also like to thank our collaborator Seph, my rotation supervisor Jan, IITM director Chris and administrators Dawn and Louise for their continuous help.

This work would not have been possible without the great help of my fellow lab members, Peter Balfe (and his funny emails), James, Andrea, Peter Wing, Claudia, Mirjam, Senko, Rita, Görkem, Olivia, Rory. I am sincerely thankful to all of you for cultivating such a warm and inclusive lab environment.

My experience at Oxford has been truly remarkable, largely due to the incredible friends I have had the pleasure of sharing my time with. From the start of my internship with Clelia, Joana, Chiara, and Flavia before even beginning my PhD, reuniting with Fabi from Bonn and then meeting one of my best friends Linnea through IITM. I have been fortunate to cross paths with so many amazing individuals: to Laura, Luna, Amanda, Nina, Cristian, Maria, Fede and Henry, I am immensely grateful to have met you, and the lifelong friendships we have formed hold a special place in my heart.

To Dana and Freya, you are the best friends I could have asked for, and the significance of our friendship is immeasurable. The trust and openness we share, regardless of any distance has always provided me with immense support. I am deeply grateful to my closest friends, Scheki, Nadine and Vanessa, for their unwavering support and love from Germany.

Big thank you to Papa, who has already sparked my scientific interest more than 2 decades ago. Starting with experiments in our small chemistry laboratory at home, to endless patience when helping with my chemistry homework in high school and statistics lessons during my undergrad. You have always provided the biggest motivation and support to pursue my scientific curiosity. To Mama, who has taught me resilience, to never lose sight of the bigger picture and switch to 'Google Earth Perspective' in challenging situations. Your reflective view on the world and remarkable emotional intelligence have taught me countless qualities that have equipped me to face any challenge. You are the most amazing parents and I know that I can count on your support in any circumstance - words cannot express how thankful I am for that. Family has always been my safe harbour, and I am incredibly fortunate to have the best siblings Tamara and Benjamin. All your support, together with the rest of the family has been a constant source of strength and emotional stability. The same applies to Cyril's family: Joëlle, Laurent, Marine, and Olivia. Your warm welcome, generosity and the sense of home I felt every time we visited you in London has meant a lot to me.

I cannot express enough how grateful I am to my partner, Cyril. Without you, I would not have applied for this PhD, and from the very beginning you have stood by my side with the kindest and most loving support. Every single day you were contributing to my happiness, inspiring and motivating me, and always putting a smile on my face. From philosophical conversations, to venting about failed experiments, and simply cherishing the joys of life together – there is nothing I cannot share with you, and I could not think of a better person by my side, not only throughout this PhD journey, but for the rest of my life.

Publications (obtained during DPhil)

Journals

1. Zhuang, X.; Gallo, G.; Sharma, P.; Magri, A.; **Borrmann, H.**; ... McKeating, J. A. (2023): Hypoxia inducible factors inhibit respiratory syncytial virus infection by modulation of nucleolin expression, *iScience*
2. **Borrmann, H.**; Ismed, D.; Kliszczak, A. E.; ... McKeating, J. A. (2023): Inhibition of salt inducible kinases reduces rhythmic HIV-1 replication and reactivation from latency, *Journal of General Virology*
3. **Borrmann, H.**; Ulkar, G.; Kliszczak, A. E.; ... McKeating, J. A. (2023): Molecular components of the circadian clock regulate HIV-1 replication, *iScience*
4. Zhuang, X.; Wang, W.; **Borrmann, H.**; Balfe, P.; ... E.; McKeating, J. A. (2022): Time-of-day variation in SARS-CoV-2 RNA levels during the second wave of COVID-19 infection, *Viruses*
5. Zhuang, X.; Tsukuda, S.; Wrensch, F.; Wing, P. A. C.; Schilling, M.; Harris, J. H.; **Borrmann, H.**; ... McKeating, J. A. (2021): The circadian clock component BMAL1 regulates SARS-CoV-2 entry and replication in lung epithelial cells, *iScience*
6. Zhuang, X.; Forde, D.; Tsukuda, S.; D'Arienzo, V.; Mailly, L.; Harris, J.; Wing, P. A. C.; **Borrmann, H.**; ... McKeating, J. A. (2021): Circadian control of hepatitis B virus replication, *Nature Communications*
7. Wing, P. A.; Lui, P. J.; Harris, J. M.; Magri, A.; Michler, T.; Zhuang, X.; **Borrmann, H.**; ... McKeating, J. A. (2021): Hypoxia inducible factors regulate hepatitis B virus replication by activating the basal core promoter, *Journal of Hepatology*
8. Sengupta, S.; Ince, L.; Sartor, F.; **Borrmann, H.**; Zhuang, X.; Naik, A.; Curtis, A.; McKeating, J. A. (2020): Clocks, Viruses, and Immunity: Lessons for the COVID-19 Pandemic, *Journal of Biological Rhythms*
9. **Borrmann, H.**; McKeating, J. A.; Zhuang, X. (2020): The circadian clock and viral infections, *Journal of Biological Rhythms*
10. **Borrmann, H.**; Davies, R.; Dickinson, M.; ... Zhuang, X. (2020): Pharmacological activation of the circadian component REV-ERB inhibits HIV-1 replication, *Scientific Reports*
11. **Borrmann, H.**; Rigby, R. E. (2020): A versatile mouse model of COVID-19, *Nature Reviews Immunology*

Conference presentations

1. "Circadian Regulation of Viruses", Podium Presentation and Poster Presentation, **Gordon Research Conference Chronobiology**, Maine, US (06/2023)
2. "Molecular Components of the Circadian Clock Regulate HIV-1 Replication", Podium Presentation, **Gordon Research Seminar Chronobiology**, Maine, US (06/2023)
3. "Circadian Regulation of Viruses: The Clock is Ticking for HIV-1 and SARS-CoV-2", Podium Presentation, **Oxford-Kumamoto Universities Joint Annual Symposium**, University of Oxford, UK (03/2023)
4. "Circadian Regulation of Viruses: The Clock is Ticking for HIV-1", Podium Presentation, **Oxford Life Science Association Symposium**, University of Oxford, UK (02/2023)
5. "The Clock is Ticking for HIV-1", Podium Presentation and Poster Presentation, **European Biological Rhythms Society**, University of Zürich, CH (07/2022) – *Excellence Award*
6. "The Clock is Ticking for HIV-1", Data Dash and Poster Presentation, **Society for Research on Biological Rhythms**, Amelia Island Florida, US (05/2022)
7. "Circadian Regulation of HIV-1", Podium Presentation, **Centre for Biological Timing Symposium**, University of Manchester (02/2022) – *Best Presentation Award*
8. "The Clock is Ticking for HIV - Pharmacological activation of the circadian system inhibits HIV-1 replication", (Online) Podium Presentation, **European Society of Clinical Virology Conference** (09/2021)
9. "Pharmacological Activation of the Circadian Component REV-ERB Inhibits HIV-1 Replication", Poster Presentation, **Circadian Summer School**, Charité University Berlin (09/2021) – *Best Poster Award*
10. "Pharmacological Activation of the Circadian Component REV-ERB Inhibits HIV-1 Replication", (Online) Podium Presentation, **UK Clock Club**, University of Bristol (11/2020)

Scientific Blog Posts

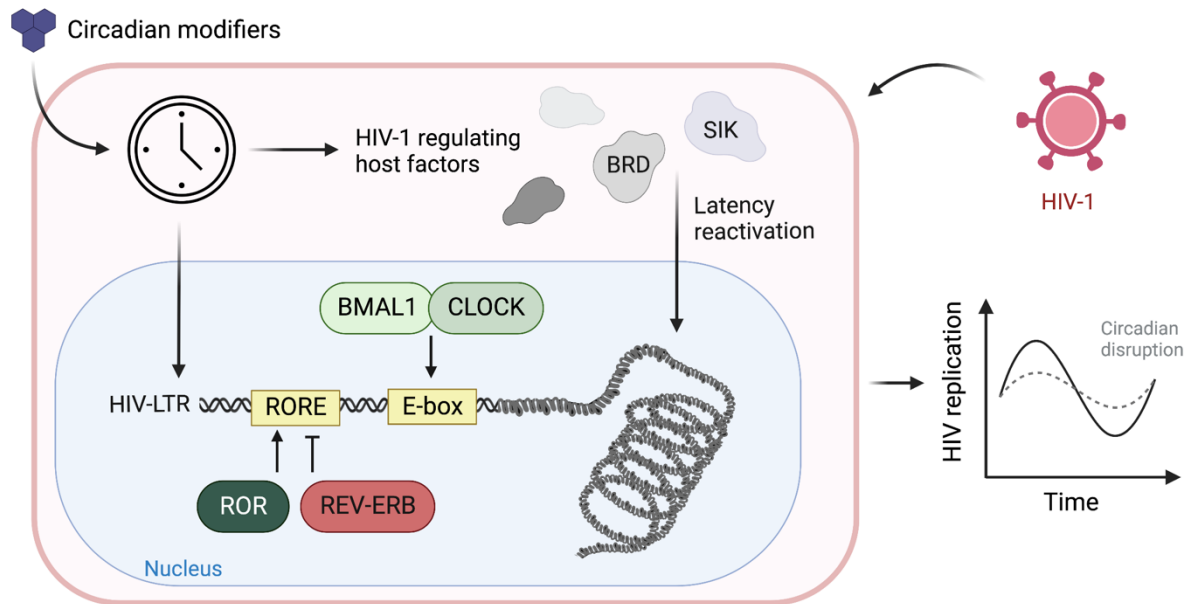
1. Zhuang, X.; **Borrmann, H.**; McKeating J. A. (2020): Wrestling with time: viruses vs. the host circadian clock, *Behind the paper - Nature Microbiology Community*
2. **Borrmann, H.**; Wilson, T. S.; McKeating, J. A.; Zhuang, X. (2020): It's virus o'clock, *After the paper – Nature Microbiology Community*

Abstract

Human immunodeficiency virus (HIV) causes significant health problems globally, and despite improvements in therapy there remains no cure. Viral replication is reliant on the host and many physiological processes are influenced by endogenous 24 h oscillations, called circadian rhythms. On a cellular level, circadian transcription factors generate daily oscillations in gene expression. As there is an emerging role for clock components in regulating viral replication, we studied the interplay between the circadian clock and HIV-1.

Using a cellular circadian model system, we demonstrate rhythmic HIV-1 replication, which has a period of ~24 hours and is regulated by the cell-intrinsic clock. Pharmacological modulation of circadian transcription factors altered HIV-1 replication across multiple HIV-1 subtypes, indicating pan-genotypic anti-viral potential. Genetic disruption of the circadian activator BMAL1 reduced HIV-1 replication and blunted rhythmicity in transcription. In contrast, knockdown of the circadian repressor REV-ERB enhanced viral replication. We show binding of clock factors to the viral genome and reveal time differential binding of circadian nuclear receptors REV-ERB and ROR, which compete for binding to a ROR response element in the HIV-1 promoter. We demonstrate circadian regulation of HIV-1 host factors, which will influence rhythmic HIV replication. Moreover, we uncover a role for the circadian machinery in regulating latent HIV-1 infection. Bromodomain proteins and salt inducible kinases are both involved in circadian networks, and our findings indicate that they regulate reactivation from latency.

Our work provides novel insights in the circadian regulation of HIV-1 replication by molecular components of the clock. Circadian modifiers with anti-viral properties could uncover novel drug targets, which may augment existing treatments and will help to inform HIV therapy and management.



Graphical abstract. HIV-1 replication is rhythmic and regulated by the molecular clock. BMAL1 binds to E-boxes and REV-ERB competes with ROR for binding to a ROR response element (RORE) in the HIV-LTR. Circadian modifiers disturb cellular circadian rhythms and alter HIV-1 replication. The cellular clock regulates host factors important for HIV replication, including salt inducible kinases (SIKs) and bromodomain proteins (BRDs) which modify HIV-1 reactivation from latency. *Figure generated with Biorender.com.*

List of abbreviations

Abbreviation	Name
ACE2	Angiotensin-converting enzyme 2
AIDS	Acquired immunodeficiency syndrome
AMV	Avian myeloblastosis virus
APOBEC3	Apolipoprotein B mRNA editing enzyme catalytic subunit 3
ARNT	Aryl hydrocarbon receptor nuclear translocator
ART	Antiretroviral therapy
BET	Bromodomain and extra-terminal domain
BMAL1	Brain and muscle ARNT-like 1
<i>Bmal1-luc</i>	<i>Bmal1</i> promoter luciferase
BRD	Bromodomain
CA	Capsid protein
CCL21	C-C chemokine ligand 21
CCR5	C-C chemokine receptor type 5
CDK9	Cyclin-dependent kinase 9
ChIP	Chromatin immunoprecipitation
CLOCK	Circadian locomotor output cycles kaput
COVID-19	Coronavirus disease 2019
CRY	Cryptochrome
CT	Circadian time
Ct	Cycle threshold
CXCL2	C-X-C motif chemokine ligand 2
CXCR4	C-X-C motif chemokine receptor 4
DBP	D-box binding protein
DC	Dendritic cell
DENV	Dengue virus
DMSO	Dimethyl sulfoxide
E-box	Enhancer box
EC50	Half maximal effective concentration
Env	Envelope
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FFT-NLLS	Fast Fourier Transform Non-linear Least Squares
FSC	Forward scatter
Gag	Group specific antigen
GO	Gene ontology
Gp	Glycoprotein
HAART	Highly reactive ART
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDAC	Histone deacetylases

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLF	Hepatic leukaemia factor
HOMER	Hypergeometric optimisation of motif enrichment
HSV	Herpes simplex virus
IAV	Influenza A virus
IC50	Half maximal inhibitory concentration
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iPSCs	Human induced pluripotent stem cells
ISG	Interferon stimulated genes
KD	Knockdown
KO	Knockout
LD	Live-dead
LDH	Lactate dehydrogenase
LPA	Latency promoting agent
LPS	Lipopolysaccharide
LRA	Latency reversing agent
LTR	Long terminal repeat
Luc	Luciferase
MFI	Mean fluorescent intensity
MS	Multiple sclerosis
MuHV	Murid herpesvirus
NCoR	Nuclear co-repressor
Nef	Negative factor
NF- κ B	Nuclear factor kappa B
NFAT	Nuclear factor of activated T cells
NIFL3	Nuclear factor interleukin-3 regulated
NL4.3- <i>luc</i>	NL4.3 R-E-luciferase
NLRP3	NLR family pyrin domain containing 3
NR1D	Nuclear receptor subfamily 1 group D
Nuc	Nucleosome
OE	Overexpression
p-TEFb	Positive transcription elongation factor-b
PBMC	Peripheral blood mononuclear cell
PER	Period
PERT	PCR-based product-enhanced reverse transcription assay
PLWH	People living with HIV
PMA	Phorbol 12-myristate 13-acetate
Pol	Polymerase
qPCR	Quantitative polymerase chain reaction
Rev	Regulator of expression of virion proteins

REV-ERB	Nuclear receptor subfamily 1 group D, opposite strand of ERBA oncogene
ROR	Retinoic acid–related orphan receptor
RORE	ROR response element
RRE	Rev responsive element
RT	Reverse transcription
SAMHD1	SAM domain and HD domain-containing protein 1
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCN	Suprachiasmatic nucleus
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SIK	Salt inducible kinase
SIV	Simian immunodeficiency virus
SP1	Specificity protein 1
SSC	Side scatter
TAR	Transactivation-responsive region
Tat	Transactivator of transcription
TBP	TATA box binding protein
TCR	T cell receptor
TEF	Thyrotrope embryonic factor
TF	Transcription factor
Th17	T helper 17 cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSS	Transcriptional start site
TTFL	Transcriptional translational feedback loop
UNAIDS	United Nations Programme on HIV/AIDS
Vif	Viral infectivity factor
Vpu	Viral protein U
Vrp	Viral protein R
VSV-G	Vesicular stomatitis virus encoded G protein
WHO	World Health Organisation
ZIKV	Zika virus
ZT	Zeitgeber time

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1. Introduction

1.1. Human immunodeficiency virus type 1

1.1.1. Prevalence, incidence, and epidemiology

Human immunodeficiency virus (HIV) is a major global health problem that has resulted in the loss of 40.1 million lives to date. In 2021 alone, 650 000 people died from HIV-related causes, 1.5 million new HIV infections were reported, and an estimated 38.4 million people are living with HIV (World Health Organisation, 2023). The incidence of new infections reached its highest point in 1999, while global HIV-related deaths peaked in 2006 and have been declining thereafter (**Fig.1.1.A**). Among all age groups, younger adults (aged 25-49 years) account for a significant proportion of HIV-related deaths and geographically Sub-Saharan Africa has the highest prevalence of HIV infection (**Fig.1.1.B**) (GBD-Collaborators, 2019).

HIV transmission continues to occur in all countries worldwide, and while there have been reductions in HIV incidence rates, these have not been consistent across all regions and populations at risk (GBD-Collaborators, 2019). HIV is transmitted through sexual contact involving mucosal surfaces, as well as through maternal-infant exposure and percutaneous inoculation (Kourtis et al., 2006; Shaw and Hunter, 2012). There are two primary species of the virus: HIV-1 and HIV-2, the latter of which associates with reduced disease progression and has lower transmission rates compared to HIV-1 (Nyamweya et al., 2013). Heterosexual transmission of HIV-1 is responsible for nearly 70% of infections globally, and the viral load is a significant factor in determining the risk of HIV-1 transmission (Shaw and Hunter, 2012). This is supported by the observation that individuals in the acute or early phase of infection show a higher risk of transmission (Miller et al., 2010; Powers et al., 2011).

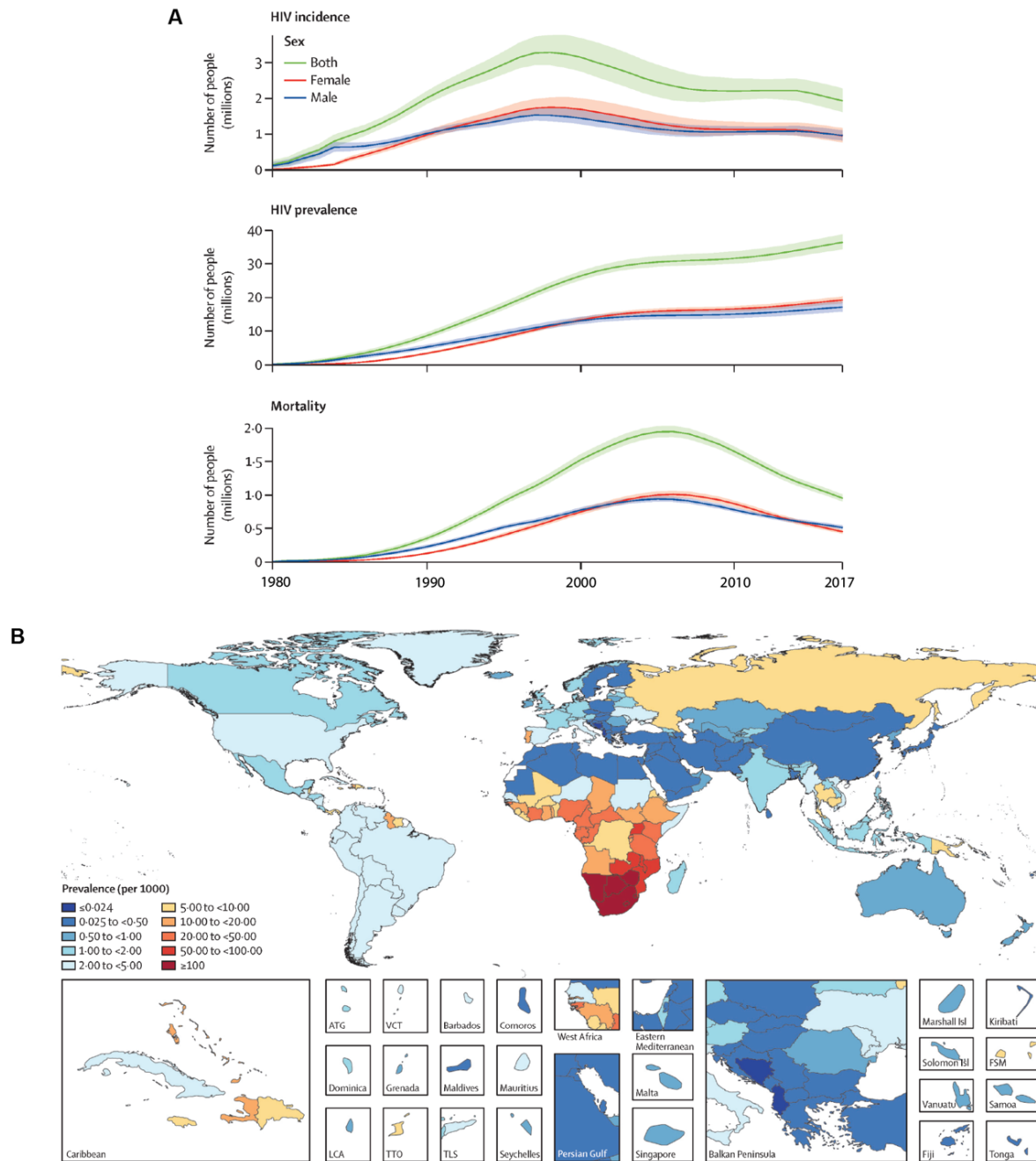


Figure 1.1. Temporal development and geographical distribution of HIV infection. A) Global HIV incidence, prevalence, mortality, by sex for all ages, 1980-2017. Shaded areas are 95% uncertainty intervals. **B)** Age-standardised HIV prevalence for both sexes in 2017. ATG=Antigua and Barbuda. VCT=Saint Vincent and the Grenadines. Isl=islands. FSM=Federated States of Micronesia. LCA=Saint Lucia. TTO=Trinidad and Tobago. TLS=Timor-Leste. *Figures taken from Frank et al., 2019.*

HIV-1 infection can be classified in clinical stages (**Fig.1.2**), however, the natural course of infection can vary in HIV-positive individuals (Sabin and Lundgren, 2013). The period from infection to the first detection of virus in the blood, is referred to as the eclipse phase and typically lasts between 7-21 days (Clark et al., 1991; Lindbäck et al., 2000). This clinically silent period is followed by three stages of infection: acute, chronic and acquired immunodeficiency syndrome (AIDS) (Maartens et al., 2014).

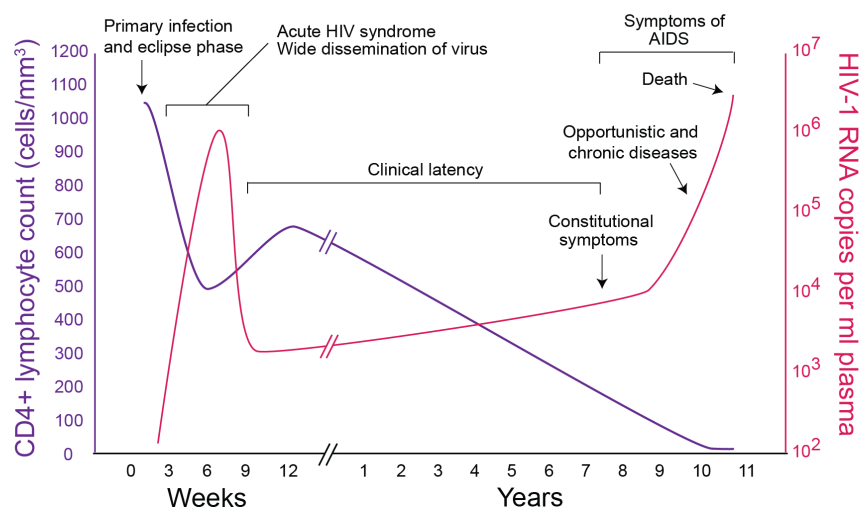


Figure 1.2. Stages of HIV infection. Changes in CD4 lymphocyte count and HIV-1 RNA copies are shown throughout progression of HIV-1 infection. The period from primary infection to first detection of HIV-1 RNA is called eclipse phase. HIV-1 RNA levels increase and CD4 T cell counts decrease during the acute phase, which is followed by clinical latency. After multiple years, opportunistic diseases and symptoms of AIDS increase, often resulting in death. *Figure adopted from Gentaur.co.uk.*

The acute stage of infection reflects a time when HIV multiplies rapidly and spreads throughout the body, which can associate with flu-like symptoms (Henn et al., 2017). The virus infects and can deplete CD4 T cells, leading to a decline in the number of immune cells (Holmes et al., 2006). The chronic phase of infection, also known as asymptomatic HIV infection or clinical latency, is where the virus persists and replicates in the body, albeit at significantly reduced levels (Maartens et al., 2014). Chronic infection typically progresses to AIDS within approximately 10 years or longer, although some individuals may experience a faster disease progression. AIDS is the most severe stage of infection reflecting the extensive damage to the immune system that leaves individuals at risk of diseases (Joshi et al., 2011; Freiberg et al.,

2013). The body is unable to effectively combat opportunistic infections and if untreated, people with AIDS typically do not survive more than 3 years. However, due to the widespread use of antiretroviral therapy (ART) HIV is now recognised as a manageable chronic health condition and people living with HIV (PLWH) can remain in a chronic phase of infection for decades (Deeks et al., 2013a). Nonetheless, it continues to pose significant health challenges globally despite its discovery 40 years ago (Barré-Sinoussi et al., 1983), which emphasises the need for continued research on this pathogen.

1.1.2. Origin and genotypes of HIV-1

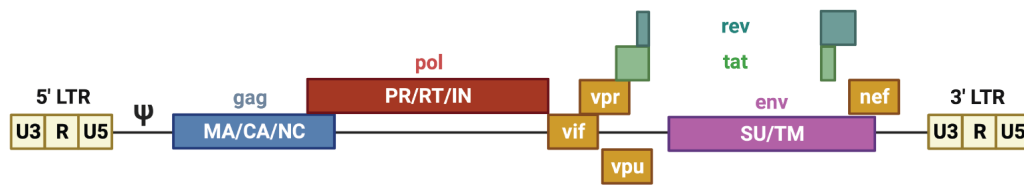
The origin of HIV is zoonotic, meaning it originated from non-human primates and was transmitted to humans (Hahn et al., 2000). HIV-1, the predominant strain responsible for the global HIV epidemic, originated from a chimpanzee virus (simian immunodeficiency virus, SIV) that crossed over to humans in Central Africa in the early 20th century (Sharp and Hahn, 2011). Separate transmission events occurred independently that gave rise to four distinct HIV-1 groups: the major group (M), the outlier group (O), the non-major and non-outlier group (N) and the recently discovered putative group (P) (Korber et al., 2000; Keele et al., 2006; Vallari et al., 2011). Only group M underwent pandemic spread, making it the most prevalent HIV-1 group that was further classified into subtypes (also called clades) represented by letters, and sub-subtypes represented by numbers: A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K (Taylor et al., 2008). The distribution of HIV-1 subtypes and circulating recombinant forms varies globally, reflecting the nature of HIV-1 molecular epidemiology. Subtype B, predominantly found in first-world countries, has been extensively studied but represents only 12% of global HIV infections, whereas subtype C is responsible for 50% of prevalent HIV infections (Hemelaar et al., 2006). Distinctive features of each viral subtype and their interactions with the human host can impact HIV transmission and disease progression (Cao et al., 2020), emphasizing the importance of testing anti-viral drugs against all subtypes.

1.1.3. HIV-1 genome and virion structure

HIV-1 has a RNA genome of 9,200-9,600 nucleotides length and is flanked by 5' and 3' long terminal repeats (LTRs), which are considered identical and contain three regions: the unique 3' end (U3), repeated (R) and unique 5' end (U5) that encode regulatory motifs. The viral genome encodes nine genes that express 15 proteins: three polyproteins necessary for virion structure and six accessory proteins (**Fig.1.3.A**). The three essential structural genes include the group specific antigen (*gag*), polymerase polyprotein (*pol*) and envelope (*env*). *Gag* encodes for matrix, capsid and nucleocapsid; *pol* for reverse transcriptase, integrase and protease; and *env* encodes the proteins that form the viral envelope. The six accessory proteins include the transactivator of transcription (*tat*) and regulator of expression of virion proteins (*rev*) which control viral replication. Additional regulatory genes include: negative factor (*nef*), viral infectivity factor (*vif*), viral protein R (*vpr*), and viral protein U (*vpu*) that modulate viral infectivity and immune evasion (Feinberg and Greene, 1992; Engelman and Cherepanov, 2012).

HIV-1 particles contain two copies of a single-stranded RNA genome, which have 5' cap and 3' poly-A tails (Watts et al., 2009). The genetic material is assembled with the viral-encoded enzymes reverse transcriptase, integrase and protease within a conical capsid structure composed of repeating units of the viral p24 protein, also known as the capsid protein (CA). Proteomic analyses have revealed the presence of multiple cellular host proteins within HIV-1 particles (Munoz et al., 2022). Virions are formed by a plasma membrane derived from the host cell, which contains viral envelope proteins contributing to the structure and function, and measures approximately 120 nm in diameter (**Fig.1.3.B**).

A



B

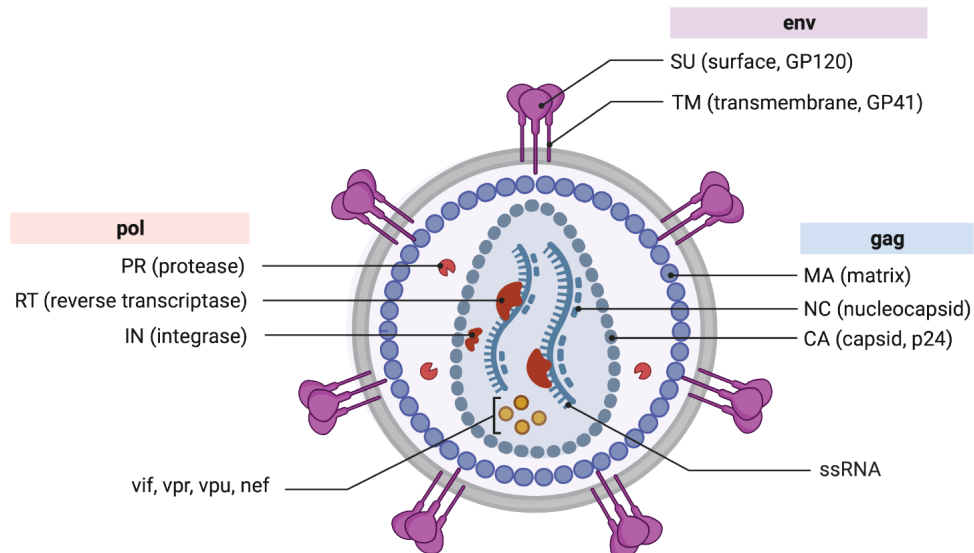


Figure 1.3. HIV-1 genome and virion structure. A) Structure of the HIV-1 genome, which encodes genes for 3 essential proteins (*gag*, *pol*, *env*) and 6 accessory proteins (*vpr*, *vpu*, *vif*, *rev*, *tat*, *nef*) and is flanked by the 5' and 3' LTRs. The LTR is subdivided into 3 regions: U3, R and U5 and followed by the packaging signal psi (Ψ). **B)** The HIV-1 virion is composed of a lipid membrane containing HIV-1 surface and transmembrane envelope proteins. The cone-shaped p24 capsid contains two copies of the single-stranded RNA genome, integrase, reverse transcriptase, *vif*, *vpr*, *vpu* and *nef*. It is surrounded by the viral matrix. *Figures obtained from Biorender.com.*

1.1.4. HIV-1 life cycle

HIV-1 mainly infects CD4 T lymphocytes, macrophages or dendritic cells (DCs), and viral entry is a multi-step process mediated by viral encoded Env and host cell receptors. The trimeric envelope proteins glycoprotein (gp) 120 and gp41 bind to CD4 (Dalgleish et al., 1984; Moore et al., 1990) initially with low affinity, and induce structural rearrangements of gp120 to increase interaction (Wilén et al., 2012). These conformational changes expose co-receptor binding sites which bind to C-C chemokine receptor type 5 (CCR5) (Dragic et al., 1996) or C-X-C motif chemokine receptor 4 (CXCR4) (Feng et al., 1996) on the cell surface. Viruses that

utilise CCR5 as a co-receptor are classified as R5 tropic, while those that infect via CXCR4 are classified as X4 tropic (Berger et al., 1998). Upon binding of gp120 to the co-receptor, it triggers the exposure of a hydrophobic gp41 fusion peptide. This fusion peptide inserts into the plasma membrane, creating a connection between the viral and cell membranes. Subsequent structural changes lead to the formation of a fusion pore and the virion contents can enter the cell (Melikyan, 2008) (**Fig.1.4**).

Within hours after viral entry, reverse transcription converts the viral genome into DNA which is influenced by the abundance of nucleotide precursors. The first step of reverse transcription is primed through binding of a cellular tRNA^{Lys3} to the viral RNA near the 5' end. It initiates formation of the reverse transcription complex containing viral and host proteins. The reverse transcription step lacks proofreading activity and has a mutation rate of approximately 3.4×10^{-5} mutations per base per replication cycle, which results in the genesis of viral variants or quasispecies within an infected person (Perelson et al., 1996). Reverse transcription happens inside the viral core, but the timing and location at which the capsid disassembles is under debate. Recent research shows, that the viral core remains intact and is imported into the nucleus, where uncoating happens near the integration site (Burdick et al., 2020) just before the viral DNA integrates into the host genome (Li et al., 2021).

Upon completion of reverse transcription and entry into the nucleus, the pre-integration complex is formed and the proviral double-stranded DNA is integrated into the host genome, regulated by the viral integrase and various cellular proteins. Millions of integration sites across the human genome have been revealed through large scale sequencing (Wang et al., 2007), but integration occurs preferentially into introns of actively transcribing genes (Schröder et al., 2002; Dahabieh et al., 2015). Apart from integration into the host genome, the viral DNA has three alternative fates: the formation of a 2-LTR circle by joining ends, homologous recombination resulting in a single LTR circle, or auto-integration leading to a rearranged

circular structure (Sloan and Wainberg, 2011). However, none of these alternatives yield infectious virus.

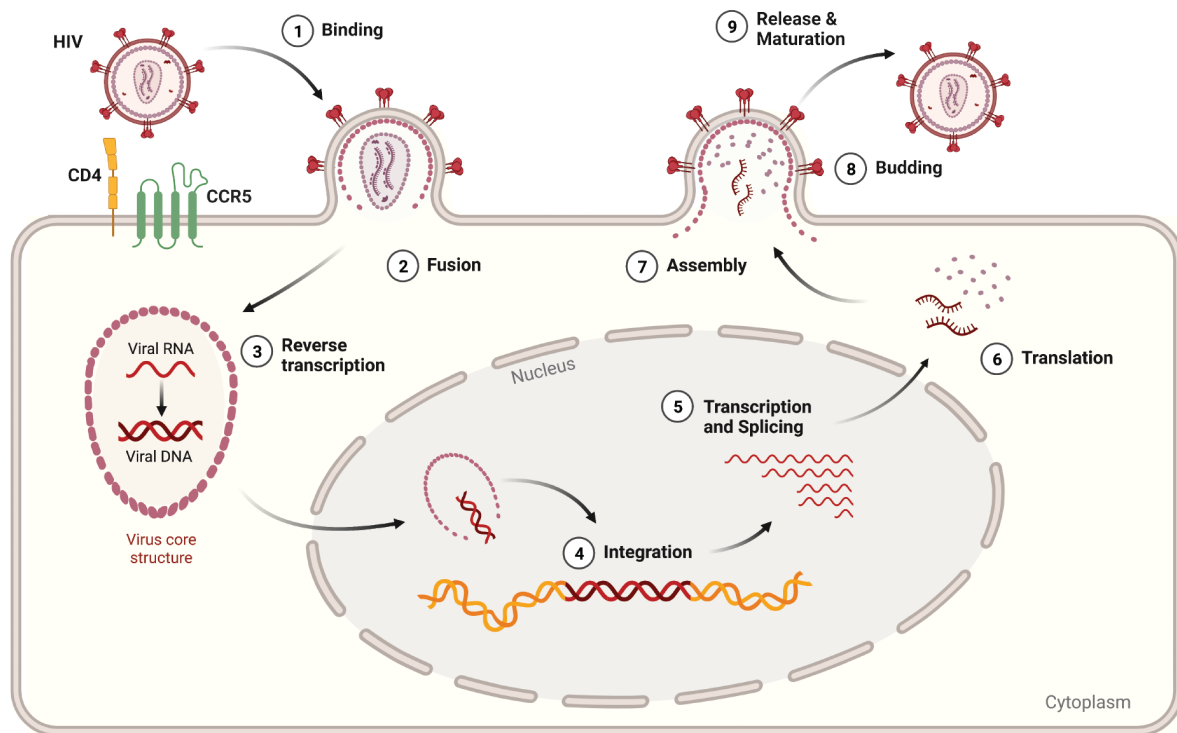


Figure 1.4. HIV-1 replication cycle. HIV-1 infects immune cells via binding to the CD4 receptor and a secondary chemokine receptor (CCR5 or CXCR4), followed by fusion with the cell membrane. Viral material is released, RNA is reverse transcribed within the core and imported into the nucleus where uncoating happens near the integration site. After integration, transcription of the provirus and pre-RNA is generated which is spliced and exported into the cytoplasm. Viral proteins are synthesised and new particles assemble at the plasma membrane. New virus is released and undergoes a morphological transformation known as maturation. *Figure modified from Biorender.com.*

Following integration of viral DNA into the host genome, the provirus can undergo productive replication or enter a state of latency (described in 1.1.5). Transcription of viral genes is regulated by the 5' LTR, for which host transcription factors and viral accessory proteins are essential. TATA box binding protein (TBP), Nuclear factor kappa B (NF- κ B), Nuclear factor of activated T cells (NFAT), and Specificity protein 1 (SP1) play a crucial role in facilitating the recruitment of RNA Polymerase (Pol) II to the HIV-LTR. They bind to the corresponding motifs in the HIV-LTR: a TATA box, three SP1 sites (Taube et al., 1999), and two NF- κ B binding sites and initiate viral transcription. Early HIV-1 transcription is inefficient due to its dependency on

Tat, but once sufficient Tat is transcribed, it initiates a robust positive feedback loop. Tat associates with cyclin T1, binds the stem-loop transactivation-responsive region (TAR) (Wei et al., 1998) and the Tat-Cyclin-T1-TAR complex recruits Cyclin-dependent kinase 9 (CDK9). CDK9 phosphorylates the C-terminal domain of RNA Pol II (Price, 2000), initiating transcription. RNA Pol II pauses immediately downstream the transcription initiation site and is released by phosphorylation of positive transcription elongation factor-b (p-TEFb) which is recruited by Tat and enhances efficient RNA elongation (Karn and Stoltzfus, 2012). Transcription of the provirus generates pre-RNA which contains multiple splice sites and allows a single viral gene to encode many proteins.

Unspliced mRNAs are incorporated into the virion as genomic RNA, partially spliced mRNAs are responsible for encoding the Env, Vif, Vpu, and Vpr proteins, whereas fully spliced mRNAs encode the Rev, Tat, and Nef proteins (Karn and Stoltzfus, 2012). Most multiple spliced HIV-1 mRNAs can exit the nucleus utilising the host machinery, however, the remaining ones require the virus nuclear-shuttling protein Rev. It binds to Rev responsive elements (RRE) in the viral RNA and mediates export of unspliced or partially spliced mRNA into the cytosol.

Viral proteins are translated, and the Gag precursor protein is synthesised which translocates to the plasma membrane and initiates the assembly of new virions. For packaging of the HIV RNA genome into capsids, a stem loop rich region psi (Ψ) which is located at the 5' end of the genome is essential (McBride and Panganiban, 1997; Kuzembayeva et al., 2014). Virus assembly proceeds and particles become enveloped during a budding process (Sundquist and Kräusslich, 2012). Aside of HIV-1 RNA and viral proteins, host proteins are incorporated, but their precise significance is not fully understood (Burnie and Guzzo, 2019; Dicker et al., 2021). New particles are released and the viral protease is activated which cleaves the Gag polyprotein, resulting in the generation of mature proteins. This cleavage event triggers a morphological change defined as 'maturation' (Freed, 1998) that is essential for the final assembly and structural integrity of the virus (Sakuragi, 2011).

Successful HIV-1 replication is dependent on host cellular proteins, underscoring the importance of exploring cellular processes and host proteins in the viral life cycle.

1.1.5. HIV-1 latency

HIV-1 can remain present within host cells without actively replicating or generating infectious particles, a state called latency. This allows the virus to evade detection by the immune system and persist in a dormant form. The precise mechanisms underlying the establishment of HIV-1 latency are partially understood, but numerous theories suggest a connection to the activation status and physiology of CD4 T cells, where resting cells support low level viral replication (Zack et al., 1990; Siliciano and Greene, 2011). Following activation, CD4 T cells experience a rapid phase of cellular proliferation and differentiation, leading to the generation of effector cells which are highly susceptible to HIV-1 infection. Effector cells have a limited lifespan, and infected cells tend to die at an accelerated rate due to virus-induced cytopathic effects (Ho et al., 1995). However, a subset of these cells can survive, transition to a resting state and become memory cells. These memory cells can persist for extended time periods and express less activating host transcription factors. HIV transcription is dependent on positive regulation by host factors, and their absence results in a stably integrated, but transcriptionally silent forms of the virus in a long-lived cell (Jordan et al., 2003).

A latent state is maintained in these cells through epigenetic changes in chromatin, which can lead to more condensed chromosomal DNA, termed heterochromatin. This restricts the accessibility of transcriptional machinery to genes, resulting in reduced HIV and host gene expression (Battivelli et al., 2018). Nucleosomes, which consist of histone proteins wrapped by 147 base pairs of DNA, serve as the fundamental structural units of chromatin (Bai and Morozov, 2010). The nucleosomes *nuc-0* and *nuc-1* are present in the 5' LTR and regulate the basal transcriptional activity as they overlap with the binding sites for essential transcription factors. The acetylation state of histones correlates with the preservation of

nucleosomes, and reactivation from latency is associated with a remodeling of nucleosomes. Hence, histone deacetylase (HDAC) inhibitors which facilitate nucleosome remodeling, were identified to activate HIV-1 transcription (Van Lint et al., 1996; Shirakawa et al., 2013).

Another contributing factor to differences in HIV-1 transcription and latency formation is the orientation with which HIV integrates relative to host genes. Convergent orientation can lead to premature transcription termination as collisions of RNA Pol II occur (Han et al., 2008), however, further studies are required to understand this phenomenon. HIV latency is likely to be a complex mechanism involving multiple pathways, including DNA methylation or changes in RNA splicing and export. Similarly, multifactorial mechanisms reactivate latent HIV genomes. For instance, once host cells are activated through antigen encounter this can re-initiate viral transcription (Siliciano and Greene, 2011).

Overall, HIV-1 latency takes advantage of a fundamental aspect of the immune system, which is the immunological memory found in long-lived resting lymphocytes.

1.1.6. Host pathways and immune responses shaping HIV-1 infection

The HIV-1 life cycle is dependent and influenced by host pathways and proteins which can either support (dependency factors) or suppress (restriction factors) viral replication (Brass et al., 2008; Harris et al., 2012; Hiatt et al., 2022). Hundreds of dependency factors have been identified which are important for various stages of the viral replication cycle. Interestingly, expression of dependency factor genes was enriched in immune cells, which highlights that HIV-1 has adapted to host cells that are specialised to support the necessary functions for their life cycle (Brass et al., 2008).

In contrast to dependency factors, HIV-1 restriction factors are less abundant and are characterised by their ability to suppress HIV replication (Harris et al., 2012). Some restrictions include hypermutation of viral DNA by Apolipoprotein B mRNA Editing Enzyme Catalytic

Subunit 3 (APOBEC3) deaminases, dNTP hydrolysis by SAM Domain and HD Domain-Containing Protein 1 (SAMHD1) and inhibition of virion release by tetherin (Simon et al., 2015). HIV has evolved multiple counter-restriction mechanisms to interact with repression factors and surpass their limitations: Vif counteracts the packaging of APOBEC3 into virions, Vpr alleviates the SAMHD1-mediated inhibition of reverse transcription, and Vpu, Env, and Nef sequester tetherin (Malim and Bieniasz, 2012). The virus evolves to continually adapt, and the mutation and recombination rate for HIV-1 exceeds most other viruses. Around 20% of the circulating HIV-1 variants are recombinants formed from multiple subtype variants (Hemelaar et al., 2006), which are often capable to evade the immune system (Streeck et al., 2008; Manel and Littman, 2011).

Differences in gene expression in immune cell types can alter their susceptibility to HIV-1 infection. T helper 17 cells (Th17) are most susceptible to HIV-1 infection (Wacleche et al., 2017), as they express a panel of HIV dependency factors, including high levels of NF- κ B (Cleret-Buhot et al., 2015). Additionally, the Th17 cell master transcription factor retinoid-related orphan receptor gamma (RORC2) enhances HIV-1 gene expression via binding to the HIV-LTR (Wiche Salinas et al., 2021). Th17 cells do not express proteins from the RNase superfamily that would usually limit HIV-1 replication, further enhancing their permissiveness to infection (Christensen-Quick et al., 2016). Clinical studies show a complex perturbation in Th17 subsets during HIV infection which are most likely a consequence of virus-dependent changes in their immune activation status (El Hed et al., 2010).

The immune response to HIV infection involves a complex interplay between various components of the immune system. The acute phase of infection triggers the activation of cytotoxic CD8 T lymphocytes. These cells recognise virus peptides presented on infected cells by human leukocyte antigen (HLA) class I molecules (McMichael and Rowland-Jones, 2001), which initiate the secretion of anti-viral factors including interferon gamma (IFN γ) and tumour necrosis factor alpha (TNF α). Elimination of infected cells caused by the cytopathic response

contributes to the fast decline in CD4 T cells (Doitsh et al., 2010) and only partially recovers during the clinical latent phase when viral replication is reduced. There is a continuous depletion of CD4 T cells from mucosal tissues, decreased T cell proliferation and increased apoptosis. The exhaustion of the immune system combined with chronic inflammation causes an accelerated immune ageing (Appay and Kelleher, 2016; Chauvin and Sauce, 2022). It is characterised by the deterioration of the immune system beyond what is expected with chronological age and leads to increased susceptibility to infections and age-related complications (Rodés et al., 2022).

Overall, the immune response to HIV involves a diverse array of immune cell types that interact in complex ways, and our understanding of these interactions is still incomplete.

1.1.7. Model systems for HIV-1 research

Since HIV-1 replication is dependent on species-specific cellular host proteins to support infection, it has a narrow host range and does not replicate in conventional rodent models. Mice which have a reconstituted human immune system (humanised mice), or macaques infected with SIV are used as surrogate models to study HIV-1 infection (Hatzioannou and Evans, 2012). However, these models have limitations in their capacity to replicate fundamental aspects of HIV-1 disease in humans, which is why human cell culture systems are invaluable to study HIV molecular biology.

To reflect the natural composition of HIV-1 target cells *in vitro*, it is common to isolate human peripheral blood mononuclear cells (PBMCs) that includes 70–90% lymphocytes, 10-20% monocytes and 1-2% dendritic cells (Sen et al., 2017). Depleting CD8 T cells from the PBMCs can mimic the interaction between HIV and CD4 T cells without cytotoxic effects caused by these cells. To ensure robust infection of CD8 depleted PBMCs, it is possible to pool cells

from multiple donors. While this reduces donor to donor variability, it can potentially mask specific effects that may or may not be beneficial, depending on the research objectives.

Immortalised cell lines provide a convenient tool for HIV-1 studies as they are readily available, robust, and offer consistent characteristics compared to primary cells, which can be challenging to obtain and exhibit inherent variability. Jurkat cells are immortalized human T lymphocyte cells, originating from the peripheral blood of a 14-year-old boy with T cell leukaemia, that support HIV-1 infection (Schwenk and Schneider, 1975; Schneider et al., 1977). Jurkat cells were used to establish a model of HIV-1 latency, J-Lat cells, which contain an integrated copy of HIV-1 where the *nef* gene is replaced by *Gfp* (Jordan et al., 2003). HIV-1 is transcriptionally silent in these cells, but can be activated by treatment with stimulants such as $\text{TNF}\alpha$. To study HIV-1 infection of macrophages, monocytic THP-1 cells can be differentiated into cells that resemble mature macrophages using stimulants like phorbol 12-myristate-13-acetate (PMA) (Daigneault et al., 2010; Richter et al., 2016). Other cell types, such as HeLa (Brass et al., 2008; Gordon et al., 2020) or HEK293T (Chang et al., 2018), have been widely used to study HIV molecular biology as these cells are easy to genetically manipulate and provide robust data especially in large experimental settings like screening assays. HeLa cells were used to generate TZM-bl cells, that were engineered to express CD4 and CCR5 receptors as well as the luciferase gene under control of the HIV-1 promoter, thereby providing a practical HIV-1 reporter system (Platt et al., 1998).

To study HIV-1 replication *in vitro*, cells can be infected with authentic virus that undergoes full replication. Molecular clones isolated from patients serve as one of the most physiologically relevant models, specifically transmitted founder virus clones which were obtained from acute early infections (Ochsenbauer et al., 2012; Ashokkumar et al., 2020). Alternatively, modified viral constructs which lack essential components to produce progeny virus can be used. They integrate into the host genome, but do not spread to infect other cells, thereby initiating a single-cycle infection. This is particularly useful to study the early stages of

the viral life cycle, without the confounding factors associated with multiple rounds of viral replication. Multiple single-cycle constructs are available that encode fluorescent or luminescent markers, allowing a quantitative read-out of viral replication. To bypass the HIV the natural HIV entry receptors and ensure high infectivity of a broad range of cells, viral pseudoparticles can be produced by complementing HIV-1 genetic material with vesicular stomatitis virus encoded G protein (VSV-G). This facilitates efficient entry as particles can enter into cells via the endocytic pathway (Aiken, 1997).

The selection of a suitable HIV-1 model is critical and depends on the scientific questions being asked. Diverse cell types exhibit distinct properties, and variations in the viruses used can influence research findings.

1.1.8. HIV-1 therapy

A variety of antiretroviral therapies have been developed, targeting distinct stages of the viral life cycle and can be divided into six classes of drugs. Viral entry inhibitors block the interaction between the HIV-1 envelope with CD4, co-receptors CCR5 and CXCR4, or inhibit fusion (Lobritz et al., 2010). Reverse transcription can be inhibited by nucleoside and nucleotide analogues, which are incorporated into nascent HIV DNA and prevent the subsequent incorporation of other nucleosides. Alternatively, reverse transcription can be inhibited by non-nucleoside reverse-transcriptase inhibitors which bind to the allosteric site of the HIV reverse transcriptase and inactivate the enzyme (Das and Arnold, 2013). Integrase inhibitors prevent integration of viral DNA into the host genome (Jóźwik et al., 2020), and Raltegravir was the first one to be approved in 2007 (Evering and Markowitz, 2008). Drugs targeting the viral protease inhibit the production of mature virions during the budding process and prevent the cleavage of precursor proteins, such as Gag/Pol (Wensing et al., 2010; Lv et al., 2015). Typically, a combination of multiple drugs is employed which includes a minimum of two drug classes and is referred to as highly active ART (HAART).

ART has contributed to significant improvements for patients as it reduces viral replication, prevents transmission, and increases CD4 T cell counts, thereby improving immune function. A decreased risk of opportunistic infections and delayed disease progression leads to overall prolonged survival (Deeks et al., 2013b). However, HIV can develop drug resistance and ART is not curative, as the virus persists in latently infected, long-lived resting T cells (Siliciano et al., 2003).

There are two major strategies to tackle latent HIV infection which aim to either completely eliminate or immunologically control persistent HIV (Yeh and Ho, 2021). In order to eliminate viral reservoirs, HIV transcription needs to be activated to expose the virus to the immune system, enabling immune cells or anti-HIV drugs to target and combat it effectively. This approach is often referred to as 'shock and kill' (Thorlund et al., 2017). HDAC inhibitors are the most extensively studied pharmaceutical agents for reactivating the latent reservoir. They modify the acetylation status of histones, leading to chromatin remodeling and activation of HIV transcription (Shirakawa et al., 2013). Alternatively, viral transcription can be activated through the release of sequestered cellular transcription factors which are essential for viral replication (e.g., NF- κ B, NFAT, p-TEFb, AP-1) (Thorlund et al., 2017). However, most latency reversing agents have shown a modest impact on viral reservoirs (Ait-Ammar et al., 2019). An alternative concept is to 'block and lock' the virus and to achieve long-term suppression of viral replication. This approach uses latency-promoting agents (LPAs), such as HIV Tat inhibitors or HIV-specific transcriptional inhibitors (Mousseau et al., 2012), and aims to suppress the HIV promoter through epigenetic mechanisms (Ahlenstiel et al., 2020). Ultimately, this could lead to a lasting inhibition of HIV transcription even in the absence of ongoing therapy (Vansant et al., 2020).

Unfortunately, none of the current treatment options achieves a fully effective cure, which highlights the need for new therapeutic strategies. The WHO and UNAIDS announced the 95-95-95 target to end the HIV epidemic by 2030, which requires that 95 % of all PLWH have a

diagnosis, 95% should be taking ART and 95% of patients on treatment should achieve a suppressed viral load (Frescura et al., 2022). However, it is unclear if these ambitious aims will be reached and further research is essential (Stover et al., 2021; Godfrey-Faussett et al., 2022).

1.2. Circadian rhythms

1.2.1. Circadian rhythms from macroscale to microscale

The Earth's rotation causes day/night cycles, and all life on earth has evolved to these rhythmically changing environmental conditions. The diurnal cycle of light and darkness plays a crucial role in driving the daily rhythm of energy harvesting and storage, and solar irradiation imposes a cyclical pattern of DNA damage followed by recovery. Circadian rhythms follow a roughly 24 h cycle and have been widely observed across all domains of life. 'Circadian' comes from 'circa' 'dies' (Latin for 'about' 'a day'), but not all daily rhythms are circadian rhythms. To be classified as such, a biological rhythm must fulfil the following three criteria: (1) It must have an endogenous free-running period of ~24 hours (± 2 h), even in the absence of environmental cues. (2) Exposure to external stimuli, called Zeitgebers, readjusts the timing (phase and period) of the oscillations causing entrainment. (3) The rhythm has to be temperature compensated and maintain circadian periodicity over a range of physiological temperatures (Tsuchiya et al., 2003).

Circadian rhythms enable organisms to anticipate and adapt to regular environmental changes, allowing them to optimize the use of available resources such as light and food. This ability provides a selective advantage to organisms with circadian rhythms and enhances their evolutionary fitness (Woelfle et al., 2004; Dodd et al., 2005). Circadian rhythms can persist even without transcription through oxidation-reduction cycles of peroxiredoxin proteins, which constitute a universal marker for clocks in all domains of life (O'Neill et al., 2011; Edgar et al.,

2012). One of the simplest known circadian rhythms are those of cyanobacteria, which function with just three proteins (Golden et al., 1997). More complex organisms like fungi, plants and animals all exhibit circadian rhythms, which are orchestrated through intricate regulatory networks that play a crucial role in regulating physiological functions (Dunlap, 1999; Young and Kay, 2001).

1.2.2. The mammalian circadian clock system

In mammals, the primary external time cue that regulates circadian rhythms is the light/dark cycle. The retina contains specialised photosensitive ganglion cells which transmit signals through the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) in the brain, often referred to as the 'central clock'. The SCN serves as the coordinator to synchronise circadian rhythms in each tissue throughout the body, enabling organisms to anticipate and adjust to their dynamic environment (Astiz et al., 2019). It comprises a complex neuronal network with extensive connections throughout the brain, transmitting signals to the autonomic nervous and endocrine systems (Schibler et al., 2015). This modulates the sensitivity of peripheral tissues to hormones, resulting in time-dependent variations in responses to hormone stimulation (Buijs et al., 2006; Becker et al., 2019). The importance of the SCN as the main pacemaker becomes evident as circadian rhythms in genetically arrhythmic mice were restored through the transplantation of a functional SCN (MR Ralph, 1990; Sujino et al., 2003). Additionally, other environmental stimuli, such as temperature (Buhr et al., 2010; Burt et al., 2021) or nutrition (Wehrens et al., 2017), can independently synchronise peripheral tissues. For instance, restricted nutrient supply can uncouple circadian oscillators in peripheral tissues from the SCN (Damiola et al., 2000), and the circadian clock in the liver can be entrained by feeding (Stokkan et al., 2001).

The hierarchical structure of the circadian system with central and peripheral clocks is further extended to molecular clocks within individual cells (**Fig.1.5**). Molecular clocks operate

autonomously within each cell, enabling the expression of circadian genes and the generation of circadian oscillations in gene expression (Takahashi, 2017). These molecular clocks are governed by self-sustained transcriptional translational feedback loops (TTFLs), consisting of activators and repressors. The two main activators of a core feedback loop are the basic helix-loop-helix transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1). They form heterodimers that bind to specific genomic regulatory elements known as enhancer boxes (E-boxes), with a consensus sequence of 'CANNTG', while the canonical motif is 'CACGTG'. CLOCK:BMAL1 thereby regulate the expression of circadian genes, including their repressors Period (PER) and Cryptochrome (CRY), which dimerize and form a negative feedback loop (Partch et al., 2014). Per and Cry have multiple isoforms (Per1, Per2, Per3 and Cry1, Cry2) all of which play a role in circadian regulation (Takahashi, 2017). A second interlocked TTFL comprises the nuclear receptors REV-ERB α/β (encoded by nuclear receptor subfamily 1 group D, NR1D1/NR1D2) and retinoic acid-related orphan receptors RORA/RORB/RORC, which also contain E-boxes in their promoters. They bind ROR response elements (RORE, 'RGGTCA') in the promoter regions of genes, including the *Bmal1* promoter (Guillaumond et al., 2005). REV-ERBs and RORs are often competing for binding to these motifs, while REV-ERB represses and ROR activates gene expression, respectively. These two feedback loops are further enhanced by a third regulatory arm that includes the D-box binding protein (DBP), thyrotrope embryonic factor (TEF) and hepatic leukaemia factor (HLF). They interact with D-boxes in promoter regions of genes, including the repressor nuclear factor interleukin-3 regulated (NFIL3).

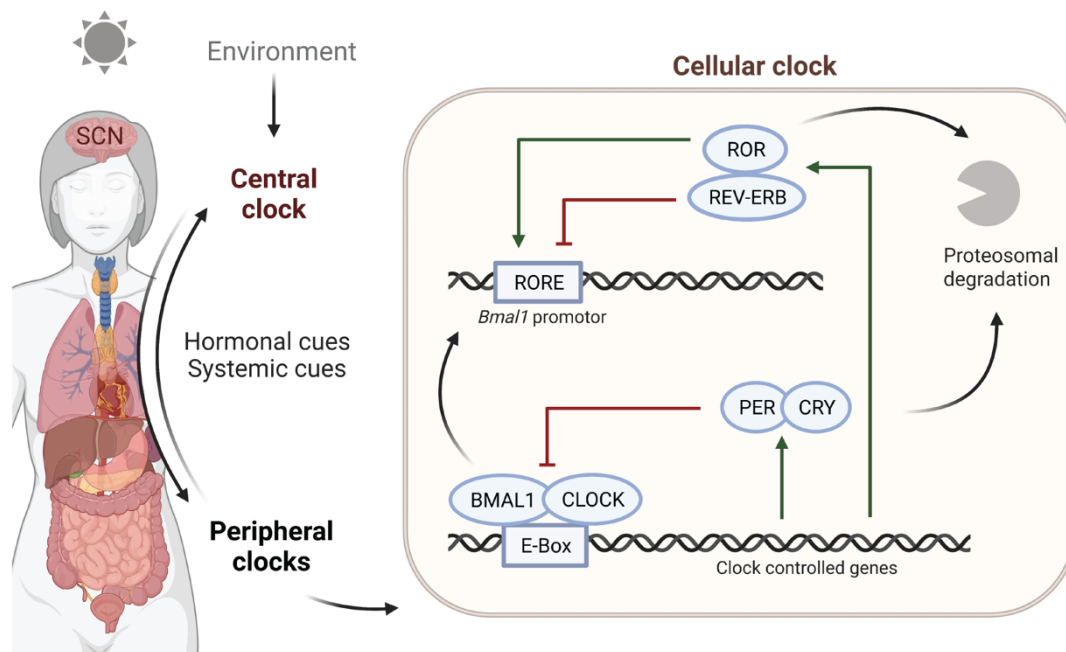


Figure 1.5. Circadian regulation of the human body. External signals from the environment are integrated in the body's central clock, the SCN in the brain, which synchronises peripheral organ clocks across the body. Each cell has a cellular clock, which consists of transcriptional/translational feedback loops that coordinate rhythmic gene expression, and proteasomal degradation to restart the system. The activators BMAL:CLOCK bind to E-boxes in promoter regions of genes and activate the expression of REV-ERB, ROR, PER, and CRY. PER:CRY inhibit Bmal1 activity, and ROR and REV-ERB compete for binding to ROREs in the Bmal1 promoter. *Figure generated with Biorender.com.*

Additional to transcriptional regulation, post-transcriptional and post-translational cellular processes such as RNA splicing, regulation through microRNAs, and protein transport are under circadian control and contribute to cellular oscillations (Gallego and Virshup, 2007; Preußner and Heyd, 2016). The stability of the core clock proteins is an important determining factor for circadian dynamics and ubiquitination-dependent degradation of circadian transcription factors resets the TTFL, establishing 24 h rhythms of RNA and protein products (Lück et al., 2014). Beyond the regulation through the core circadian machinery, other protein families such as kinases or histone modifying enzymes have identified as regulators of the cellular clock (Takahashi, 2017). Molecular clocks can be synchronised *in vitro* using different entrainment cues, including a glucocorticoid stimulus or incubation with high percentages of serum (Nagoshi et al., 2004).

Overall, the mammalian circadian clock functions as a multi layered system, where central cues are integrated in the brain, communicated with peripheral clocks and transcriptional feedback loops within cells generate cycles with different phases of gene expression. These circadian circuits share a common design principle which is a network of negative feedback with delay.

1.2.3. Clock regulation of host immunity

To anticipate environmental changes and minimize the risk of infection, immune function depends on the cellular clock and many immune parameters oscillate throughout the day (Wang et al., 2022a). Immune cells, just like most other cells in the body, have a circadian machinery that regulates rhythmic gene expression (Bollinger et al., 2011). Immune cell rhythms in mice were sustained in constant darkness which indicates their endogenous circadian nature (Druzd et al., 2017), and immune cell activity is generally higher during the active phase, which is when pathogen encounter is expected.

The innate immune response, which acts as the initial defence against invading pathogens, involves various cell types such as macrophages, dendritic cells, and neutrophils which are regulated by the circadian clock on multiple levels, including their motility and secretion of signalling molecules (Curtis et al., 2014; Baxter and Ray, 2019). *Bmal1* deletion was shown to enhance macrophage phagocytosis and thereby increased antibacterial immunity in a murine model of *Streptococcus pneumoniae* infection (Kitchen et al., 2020). Deficiency of *Bmal1* in myeloid cells reduced rhythmic recruitment to arteries and veins (de Juan et al., 2019) and abrogated time-of-day differences in a CNS autoimmune disease model (Sutton et al., 2017). Moreover, REV-ERB α mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines (Gibbs et al., 2012). REV-ERB α repressed diurnal expression of the NLR family pyrin domain containing 3 (NLRP3) inflammasome (Pourcet et al., 2018; Wang et al., 2018a), and inhibited production of the cytokine interleukin

1 β (IL-1 β) in macrophages in a model of lipopolysaccharide (LPS) induced pulmonary inflammation (Yu et al., 2019). Cytokines and chemokines are further regulated by the circadian clock in neutrophils and dendritic cells, where BMAL1 controls the expression of C-X-C motif chemokine ligand 2 (CXCL2) (Adrover et al., 2019). Rhythms in C-C chemokine ligand 21 (CCL21) caused increased DC migration into skin lymphatic vessels in mice 7 hours after light onset, referred to as Zeitgeber time (ZT) 7 (Holtkamp et al., 2021). Additionally, DCs from mice lacking Rev-erb showed enhanced expression of proinflammatory cytokines (Amir et al., 2020).

Similar to the innate immune response, the adaptive immune response comprised by lymphocytes is also influenced by the circadian clock, and exhibits circadian rhythmicity persisting for weeks following an initial challenge (Labrecque and Cermakian, 2015; Cermakian et al., 2022; Ince et al., 2023). There is conflicting data with regards to the role of the circadian clock in T cell development. T cells with an intrinsic deletion of Bmal1 show normal thymic development (Hemmers and Rudensky, 2015), however, several clock genes are involved in the differentiation of T helper cell subtypes and RORC is considered a master regulator of Th17 development (Yang et al., 2008; Yu et al., 2013; Chang et al., 2019). T cells showed a circadian variation in proliferation after T cell receptor (TCR) stimulation across 24 h, which was blunted in Clock mutant mice (Fortier et al., 2011). Moreover, oscillations in Th17 differentiation were disrupted in mice with perturbed light cycles which caused higher susceptibility to colitis (Yu et al., 2013). There was a loss of rhythmic T cellularity in blood and lymph nodes in mice without the input from adrenergic nerves, which highlights the importance of neural inputs for the diurnal variation of lymphocyte recirculation (Suzuki et al., 2016). This was observed for B cells and caused additional loss of diurnal variation in immunoglobulin M (IgM) and IgG1 antibody production (Suzuki et al., 2016). Rhythmic B cell effector responses were further regulated through clock-driven Toll-like receptor (TLR) 9 protein expression (Silver et al., 2012). Phenotype and activity of both innate and adaptive immune cells is

influenced by metabolic pathways and mitochondrial function, which are linked to BMAL1 activity, and highlight the importance of rhythmicity of immunometabolism (Early and Curtis, 2016; Carroll et al., 2019).

In summary, all arms of the immune system are regulated by the circadian clock, and rhythmicity of immune responses can contribute to circadian regulation of various illnesses.

1.2.4. Circadian rhythms in disease

A disrupted circadian clock has been linked to increased susceptibility to several diseases, including pathogen-associated infections, autoimmune disorders, sleep related pathologies and cancer (Rijo-Ferreira and Takahashi, 2019).

The vulnerability to bacterial infection can exhibit time-of-day dependence, as evidenced by elevated rates of *Salmonella* (Bellet et al., 2013), *Streptococcus pneumoniae* (Gibbs et al., 2012), or *Chlamydia* (Lundy et al., 2019) infection in mice during the early morning hours.

Many parasitic infections show rhythmic daily patterns, which are regulated by host clocks and parasite intrinsic clocks. Malaria parasites (*Plasmodium*) have intrinsic clocks (Rijo-Ferreira et al., 2020) and are synchronised to their hosts on a populational level, likely influenced by the rhythmic availability of nutrients in the host environment (O'Donnell et al., 2022). The replication of *Plasmodium* in red blood cells takes 24 hours (or multiples of 24 hours), followed by bursting of the cells which causes rhythmic fevers in the host (Mideo et al., 2013; Hunter et al., 2022). Parasitic infection with *Leishmania* is regulated by nocturnal melatonin pineal synthesis (Laranjeira-Silva et al., 2015) and immune cell clocks (Kiessling et al., 2017). Infection of mice with *Leishmania* parasites at different times of day influenced the magnitude of the inflammatory responses, causing increased parasite burden in mice infected at night (Kiessling et al., 2017). Various species of *Trypanosoma* display circadian rhythms that align with the activity patterns of their respective hosts, and this synchronisation is often reflected

in the rhythmic fluctuations of parasite numbers in the bloodstream (Cornford et al., 1976; Hawking, 1978). Recent studies have revealed that *Trypanosoma brucei* exhibit inherent circadian rhythms in gene expression when cultured *in vitro* (Rijo-Ferreira et al., 2017). *Trypanosoma brucei* causes sleeping sickness, manifested by behavioural changes and sleep cycle disturbances, suggesting a complex association between the pathogen and the circadian clock of the host (Rijo-Ferreira et al., 2018; Rijo-Ferreira and Takahashi, 2020). In addition to the rhythmicity observed in parasites and hosts, the circadian clock of vectors involved in transmission plays a crucial role, as their rhythmic behaviour can significantly impact the success of infection (Prior et al., 2020).

Autoimmune diseases frequently display circadian oscillations and circadian rhythms are involved in the initiation and progression of these conditions. Patients with rheumatoid arthritis experience painful joint stiffness in the morning, which was associated with increased levels of pro-inflammatory cytokines (Herold and Günther, 1987; Cutolo and Straub, 2008). Circadian disruption increased the incidence of multiple sclerosis (MS) (Sutton et al., 2017) and genetic variations of core clock genes contribute to a higher risk of MS (Lavtar et al., 2018). REV-ERB α enhanced Th17-mediated expression of pro-inflammatory cytokines in a MS model (Amir et al., 2018) and Th17 cell responses are generally known to contribute to the circadian regulation of various autoimmune conditions (Ivanov et al., 2006; Park et al., 2019).

Other diseases associated with circadian clock deregulation are cancer and sleep disorders. Circadian rhythms can contribute to tumour growth and metastasis through modulation of tumour microenvironment, including angiogenesis, tumour-promoting inflammation, and immune escape (Xuan et al., 2021; Zhang et al., 2021b). Moreover, the molecular machinery of the circadian clock is interconnected with cellular processes involved in cell division, DNA repair, apoptosis, and metabolism (Sancar et al., 2010). Disturbances in these processes due to circadian disruption can lead to genomic instability, impaired DNA repair, and altered cell

proliferation, all of which are factors that contribute to cancer development. Additionally, circadian clock genes have been shown to regulate the expression of genes involved in tumour growth (Ramsey and Ellisen, 2011). Increased risk of certain types of cancer is associated with disruptions in circadian rhythms, such as shift work, irregular sleep patterns, and exposure to artificial light at night (Sulli et al., 2019). Moreover, these clock disturbances can lead to various sleep disorders, some of which have a genetic basis and are characterised by delayed or advanced sleep phase patterns (Meyer et al., 2022).

It is crucial to recognize that the relationship between circadian rhythms and diseases is bidirectional: the circadian clock can influence disease outcomes, and conversely, disease conditions can also impact the clock. It is important to distinguish between effects driven by circadian transcription factors at the cellular level and those influenced by the overall function of the organisms circadian clock (Man, 2016; Scheiermann et al., 2018). This interaction is complex and multifactorial, making it challenging to establish direct cause-and-effect relationships and extensive research is needed to better understand the interplay between circadian rhythms and diseases.

1.2.5. Circadian pathways shape viral infections

As obligate parasites, viruses are completely reliant on their host for replication. More than 80% of protein coding genes in various tissues show daily rhythmic expression in primates (Mure et al., 2018), and given the dependency of virus replication on cellular pathways, it is unsurprising that host clock components have been reported to influence virus replication (Zhuang et al., 2017; Zhuang et al., 2022). The viral life cycle varies depending on the type of virus, but most viruses follow a series of steps that include viral entry, replication of genetic material, synthesis of viral proteins, budding, and release. These processes and many others can, at least in part, be influenced by the cellular circadian clock (**Fig.1.6**).

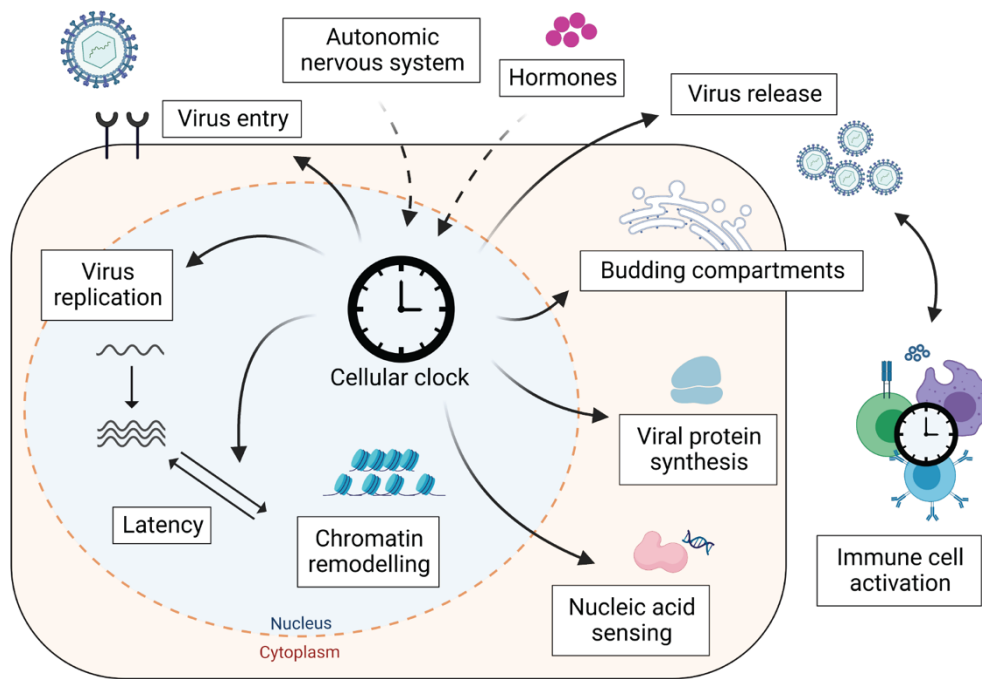


Figure 1.6. The cellular clock can modulate the virus life cycle. Circadian rhythms of cellular processes can influence viral infection at multiple steps in the virus life cycle, from the regulation of entry receptors to lipid-dependent pathways for particle genesis. Additionally, immune responses combating viral infections, such as nucleic acid sensing and the activity of immune cells, are regulated by cellular circadian clocks. *Figure generated with Biorender.com.*

It is interesting to consider the role of the endocrine system in regulating viruses, since it plays a crucial role in synchronising the SCN with the peripheral clocks. One of the best-studied examples of this synchronisation is through the expression of glucocorticoids (Oster et al., 2006; Pezuk et al., 2012; Oster et al., 2017). The administration of therapeutic glucocorticoids, such as cortisol, has been used to treat viral infections, and reduced the severity of COVID-19 (Matsuyama et al., 2020; Horby et al., 2021) while increasing reactivation of HBV (Hatano et al., 2019). Melatonin, a hormone produced by the pineal gland, plays a role in regulating sleep patterns and has been shown to exhibit broad ranging anti-viral effects in virus-induced diseases (Anderson and Reiter, 2020). One of its mechanisms of action includes the inhibition of the NLRP3 inflammasome (Zhang et al., 2016b; Cao et al., 2017; Ma et al., 2018). Certain viruses have the ability to infect the central nervous system (Koyuncu, 2013), which is influenced by circadian rhythms, however, there is no evidence to

suggest a direct interaction between viruses, the circadian clock, and the autonomic nervous system.

This provides an overview of how various circadian regulated host pathways can influence viral infections, which plays a role for diverse classes of viruses and will be further explored in the subsequent section.

1.2.6. Clock regulation of different virus classes

Several respiratory viruses were shown to be regulated to the circadian clock, for example, Bmal1 deficiency increased the susceptibility to Respiratory Syncytial Virus and Human Parainfluenza Virus Type 3 infection (Majumdar et al., 2017). Moreover, multiple independent studies assessed the circadian regulation of Influenza virus infection. Bmal1 KO mice showed worse acute viral bronchitis and enhanced asthma-like airway changes (Ehlers et al., 2018), and survival was higher when mice were infected with Influenza virus before their active phase compared to the resting phase (Sengupta et al., 2019). No differences in viral load were observed between mice infected at different time points, and infection at the start of the active phase promoted lung inflammation independent of the viral burden (Sengupta et al., 2019). Bmal1 was shown to regulate diurnal oscillation of inflammatory monocytes (Ly6c^{hi} monocytes) (Nguyen et al., 2013), which together with natural killer cells play an important role in the time-of-day dependency of Influenza infection (Sengupta et al., 2019). In conjunction with innate immune cell function, loss of lymphocyte circadian clocks ablated rhythmic adaptive immune responses essential for combating Influenza A virus (Druzd et al., 2017). This suggests that severe outcomes of Influenza infection are mediated by time-dependent regulation of host tolerance and immune activation. Additionally, viral replication was increased in arrhythmic Bmal1 KO cells *in vitro*, demonstrating a role for cellular circadian pathways to regulate Influenza A virus infection (Edgar et al., 2016). Interestingly, a recent study reported loss of circadian protection against Influenza infection in adult mice exposed

to hyperoxia as neonates. This dysregulation was mediated by the lung intrinsic clock, independent of viral burden through host tolerance pathways (Issah et al., 2021). Latest studies are aiming to elucidate the role of circadian rhythms in SARS-CoV-2 infection. Shift work has been associated with worse disease outcomes in COVID-19 infection (Liu et al., 2021; Maidstone et al., 2021), and circadian disruption altered the lung transcriptome which predisposed to viral infection (Taylor et al., 2023). At the molecular level, the entry of SARS-CoV-2 into cells relies on the Angiotensin-converting enzyme 2 (ACE2) receptor, whose expression levels were shown to oscillate regulated by post-transcriptional circadian mechanisms. This caused differences in SARS-CoV-2 entry depending on the time of day. Furthermore, SARS-CoV-2 replication of multiple strains was reduced in Bmal1 KO cells, which is likely regulated through an enhanced interferon stimulated gene response (Park and Iwasaki, 2020; Zhuang et al., 2021b).

Viruses infecting the liver are prone to be affected by the clock, since it is one of the most circadian regulated organs of the body, where 20% of the transcriptome shows rhythmic expression (Zhang et al., 2014). Hepatitis C virus (HCV) re-infection kinetics following liver transplantation were enhanced when the surgery was conducted in the morning compared to the afternoon (Zhuang et al., 2018). This clinical finding is consistent with further *in vitro* analysis, which showed that rhythmic expression of viral receptors regulated HCV infection in circadian-synchronised hepatocytes (Zhuang et al., 2019). Additionally, genetic knockout of Bmal1 or the pharmacological activation of REV-ERB inhibited HCV replication, as well as related Flaviviruses such as Dengue and Zika, through the repression of lipid pathways crucial for their replication (Zhuang et al., 2019).

Numerous studies analysed the circadian regulation of Herpesvirus infection. Infection of mice with Herpes simplex virus (HSV) during the rest phase associated with reduced symptoms compared to infection during the active phase. This is likely regulated through the entry receptor Nectin1, which shows rhythmic expression and is decreased upon Clock silencing *in*

vitro (Matsuzawa et al., 2018). Bmal1 KO caused enhanced replication of Murid Herpesvirus 4 (MuHV-4) and HSV-1 *in vivo* and *in vitro*, and viral DNA levels were higher when inoculation occurred at the beginning of the resting phase (Edgar et al., 2016). The HSV encoded protein ICP0 interacts with CLOCK:BMAL1 and silencing or mutating Clock reduced viral replication (Kalamvoki and Roizman, 2010; Kalamvoki and Roizman, 2011). HSV can establish latent infections with periodical reactivation (Nicoll et al., 2012), and chronic sleep disruption with an acute inflammatory challenge caused reactivation of Murine Herpesvirus in latently infected mice (Trammell and Toth, 2016). Reactivation from latency is often modulated through epigenetic and chromatin conformational changes. Interestingly, CLOCK has histone-deacetylase activity (Doi et al., 2006) and the chromatin state is influenced by time dependent binding of circadian transcription factors (Nobuya Koike, 2012; Pacheco-Bernal et al., 2019). Dynamic changes in histone marks suggest daily remodeling of the epigenetic landscape, which could influence reactivation of HSV, as well as other latent viruses.

Overall, there is clear evidence for diverse ways in which different viruses are regulated by circadian rhythms, and an overview of the molecular interactions is provided in **Table 1**.

Table 1. Overview of interactions of different viruses with the cellular circadian clock.

Virus	Clock component	Effects and proposed mechanism	References
Dengue Virus	BMAL1, REV-ERB	<ul style="list-style-type: none"> Modulation of host lipid pathway essential for replication REVERB inhibits stearyl-CoA-desaturase expression (rate limiting for infection) Dengue infection increases the locomotor activity of vector <i>Aedes aegypti</i> females 	Lima-Camara et al., 2011; Zhuang et al., 2019
Hepatitis B Virus	REV-ERB, BMAL1, PER1-3, CRY1,2	<ul style="list-style-type: none"> Over expression of HBx protein disrupts expression of circadian genes Lower mRNA levels of Per1-3 and Cry2 in HCC tissue REV-ERB regulates entry into host cells BMAL1 binds to viral promoter region 	Yang et al., 2014; Zhuang et al., 2021
Hepatitis C Virus	BMAL1, REV-ERB	<ul style="list-style-type: none"> HCV infection upregulates miR-10 which downregulates Bmal1 via suppressing ROR Overexpression of Per2 counteracts HCV replication Downregulation of Per2 and Cry2 in HCV patients Modulation of host lipid pathway essential for replication REVERB inhibits stearyl-CoA-desaturase expression (rate limiting for infection) 	Benegiamo et al., 2013; Zhuang et al., 2018; Zhuang et al., 2019; Hori et al., 2019
Herpesvirus	CLOCK, BMAL1	<ul style="list-style-type: none"> CLOCK activates expression of entry receptor via binding to its promoter Virus induces BMAL1 expression, low levels of BMAL1 increase infection HSV viral protein IPC0 interacts with CLOCK:BMAL1, remodelling of viral chromatin 	Kalamvoki and Roizman, 2010; Kalamvoki and Roizman, 2011; Edgar et al., 2016; Matsuzawa et al., 2018
Human Immunodeficiency Virus	CLOCK, BMAL1, REV-ERB, PER2	<ul style="list-style-type: none"> Variation in HIV RNA in patients associates with BMAL1 HIV RNA has a circadian cycle in patients on ART CLOCK/BMAL1 drive HIV transcription via E-box in LTR Tat decreases amplitude of circadian rhythm SNPs in CLOCK and Per2 genes alter sleep behaviour 	Clark et al., 2005; Duncan et al., 2008; Wang et al., 2014; Lee et al., 2015; Chang et al., 2018; Stern et al., 2022
Influenza Virus	BMAL1, PER1	<ul style="list-style-type: none"> Virus induced Bmal1 expression IAV advances Bmal1, Clock and Rev-erb expression and increases Per2 amplitude low levels of BMAL1 increase viral load regulation of infection through time-dependant host tolerance/immune activation circadian protection against influenza infection is lost in adult mice exposed to hyperoxia as neonates 	Sundar et al., 2015; Edgar et al., 2016; Ehlers et al., 2018; Sengupta et al., 2019; Issah et al., 2021
Parainfluenza Virus Type 3	BMAL1	<ul style="list-style-type: none"> BMAL1 decreases viral replication 	Majumdar et al., 2017
Respiratory Syncytial Virus	BMAL1	<ul style="list-style-type: none"> BMAL1 decreases viral replication 	Majumdar et al., 2017
SARS-CoV-2	BMAL1, REV-ERB	<ul style="list-style-type: none"> BMAL1 enhances virus entry and replication REV-ERB decreases virus entry, cell-cell fusion and replication BMAL1 regulates anti-viral interferon response Sleep disruption enhances the susceptibility to COVID-19 	Zhuang et al., 2021; Liu et al., 2021; Taylor et al., 2023
Simian Immunodeficiency Virus	Unknown	<ul style="list-style-type: none"> Infection impairs amplitude of circadian rhythm of body temperature and locomotor activity 	Huitron-Resendiz et al., 2007
Zika Virus	BMAL1, REV-ERB	<ul style="list-style-type: none"> Modulation of host lipid pathway essential for replication REVERB inhibits stearyl-CoA-desaturase expression (rate limiting for infection) 	Zhuang et al., 2019

1.2.7. Viruses dictating time

Similar to how the circadian rhythm of the host can impact virus replication, viral infection can disrupt the circadian system of the host.

Liver biopsies from chronic HCV infected patients had elevated levels of microRNA miR-10a, which has been shown to reduce *Bmal1* expression in hepatocytes (Horii et al., 2019). A separate study found reduced *Per2* and *Cry2* expression in liver biopsies from HCV infected patients or in cell models expressing the HCV core protein (Benegiamo et al., 2013). Moreover, the HBV encoded regulatory protein HBx perturbed circadian regulated gene transcripts *in vitro* (Yang et al., 2014) and viral-dependent disruption of circadian signaling pathways may contribute to liver disease (Mukherji et al., 2019).

Herpesvirus infection of mice with MuHV-4 induced *Bmal1* expression regardless of the time of infection, suggesting that Herpesviruses can perturb cellular circadian cycling. Similar observations were reported in HSV-1 and Influenza A infection (Edgar et al., 2016). Influenza virus reduced the amplitude of *Per2* expression and altered the phase of *Bmal1*, *Clock* and *Rev-erb β* expression in the lungs of infected mice (Sundar et al., 2015).

These examples demonstrate that viruses can influence circadian gene expression levels by altering the activity of clock transcription factors. Additionally, viral infections can alter circadian rhythms on a behavioural level, as simian immunodeficiency virus was reported to impair the amplitude of the circadian rhythm of body temperature and reduced locomotor activity in monkeys (Huitron-Resendiz et al., 2007). Other pathways, such as changes in metabolism or disruptions in overall homeostasis caused by infection, are likely to contribute and warrant further investigation.

1.2.8. Circadian and circannual rhythms affect viral transmission

The link between host oscillation and viral infections goes beyond the 24 h period as seasonal changes can affect susceptibility to infections (Dowell, 2001). Epidemic diseases often exhibit an annual cycle, influenced by a combination of environmental factors, exogenous influences, host behavior, and phenology (Martinez, 2018).

Seasonal variations influence activity and abundance of immune cells in the blood, with decreased neutrophil activity (Klink et al., 2012), but increased total blood monocyte numbers during the winter (Dopico et al., 2015). Expression of disease risk markers like C-reactive protein and IL-6 receptor are increased during winter, which coincides with lower Bmal1 expression (Dopico et al., 2015). Although the precise factors underlying these seasonal variations remain uncertain, it is believed that changes in hormone levels contribute to these fluctuations. Notably, the expression of glucocorticoid receptors is at its lowest during the winter season (Wood and Loudon, 2014; Dopico et al., 2015).

Moreover, seasonality and circadian rhythms of vectors can determine if a virus will encounter the host in its active or resting phase (Meireles-Filho and Kyriacou, 2013). Several viruses are transmitted by insect vectors which have their own circadian rhythm and exhibit different activity patterns depending on the time of day. For instance, *Aedes aegypti* mosquitoes, which transmit Dengue virus (DENV), are most active during the daytime and at dusk/dawn. Interestingly, virus infection can enhance mosquito activity, further facilitating viral transmission (Lima-Camara et al., 2011). The transmission of Zika virus (ZIKV) is thought to be dependent on seasonal climate changes (Petersen et al., 2016) and implementing strategic pregnancy planning measures could help mitigate the risk of vertical transmission (Martinez, 2016). Rice irrigation during the summer months is associated with increased incidence of Japanese encephalitis virus infection (Tian et al., 2015) and light pollution during winter is linked to increased risk of West Nile virus infection (Kernbach et al., 2019). This highlights

how human agricultural and technological interventions can further contribute to the seasonal spread of viral diseases.

1.2.9. Circadian approaches for therapy

Identifying host factors that associate with viral proteins can uncover new drug targets, and the circadian machinery of the host offers numerous potential targets for anti-viral interventions.

Many small molecule modulators targeting core clock proteins (mainly CRYs, REV-ERBs or RORs) have been discovered that have potential for therapeutic avenues (Ribeiro et al., 2021). Synthetic REV-ERB agonists can alter immune responses by selectively regulating inflammatory cytokines (Gibbs et al., 2012) and were reported to inhibit HCV entry, RNA replication and release of infectious particles (Zhuang et al., 2019). This is likely regulated through repression of fatty acid biosynthesis which is an essential pathway for HCV replication (Lyn et al., 2014; Nio et al., 2016), as well as DENV and ZIKV (Paul, 2015). Moreover, REV-ERB agonists exhibited anti-influenza virus activity *in vitro* (Cheng et al., 2022). Additional pharmacological agents, including CRY stabilizers (Hirota T., 2012; Lee et al., 2015a) and ROR inverse agonists (Kumar et al., 2011) could have similar effects on viral replication. These studies indicate clinical potential, but therapeutic exploitation of circadian compounds for viral infection and their potential systemic effects on the host circadian rhythm requires further evaluation.

In addition to direct therapeutic targeting of the circadian machinery, optimizing the time of day for medication or clinical interventions can lead to improved disease outcomes, a field called chronotherapy (Smith et al., 2019). Drugs with short half-lives and rhythmically expressed targets can be sensitive to time of day of administration (Ruben et al., 2019). For

instance, it has been shown that the effective dose of acyclovir required to treat HSV infection in mice is four times higher during the active phase than during rest (Matsuzawa et al., 2018). Diagnostic tests for SARS-CoV-2 showed variation in the probability of a positive test depending on the time of day of testing (McNaughton et al., 2021). Moreover, the time of day of vaccination can largely influence vaccine induced immune responses and protection. Morning administration of an inactivated SARS-CoV-2 vaccine showed higher antigen specific B cell responses (Zhang et al., 2021a), while the afternoon administration of an mRNA based vaccine led to enhanced anti-body levels (Wang et al., 2022b). Influenza vaccination resulted in higher antibody responses when administered in the morning compared to the afternoon (Long et al., 2016). The circadian clock of CD8 T cells modulates their response to vaccination, and higher T cell activation was observed when mice were immunized with ovalbumin peptide pre-loaded dendritic cells during the day (Nobis et al., 2019). Moreover, the month of vaccine administration in countries with seasonally changing environments can affect protection against a range of viruses, including rabies vaccines (Moore et al., 2006) or yellow fever virus vaccination (Querec et al., 2009). These findings emphasize the variations observed in different vaccine types, highlighting the importance to carefully consider immunological readouts and take caution when extrapolating conclusions.

An improved understanding of the viral-circadian interplay can inform these simple measures of timing drug or vaccine administration, to increase drug efficiency and reduce side effects (Ruben et al., 2019; Zhao et al., 2020). Overall, circadian rhythms impact viral infections and disease outcomes at multiple levels, which holds significant translational potential (**Fig.1.7**).

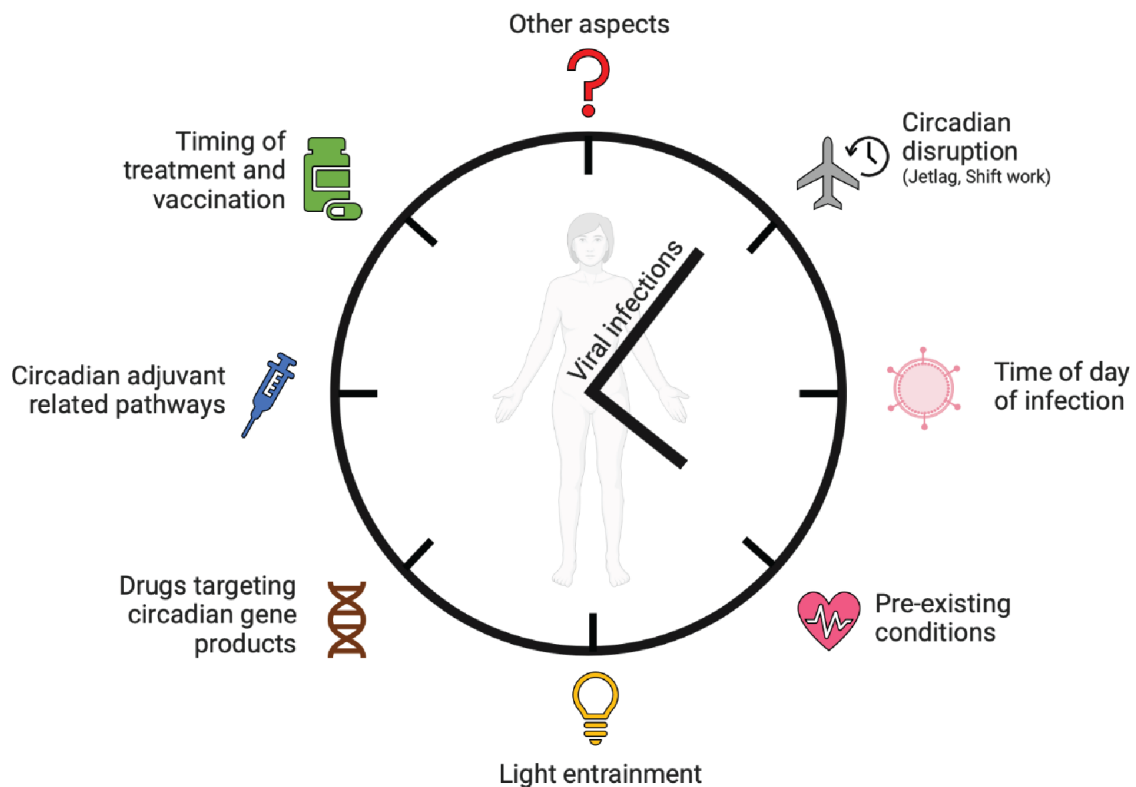


Figure 1.7. Translational effects of circadian rhythms on viral infections. Circadian rhythms affect multiple aspects of viral infections, reaching from the molecular level to clinical application. Considering these interactions may provide new perspectives for therapy and enhance disease outcomes.

1.3. Implications for circadian regulation of HIV-1 infection

When examining the interaction between HIV and circadian rhythms, it is crucial to recognize the reciprocal nature of this relationship, where HIV can impact the clock, and circadian rhythms can affect the virus.

Multiple studies analysed how HIV infection can modulate circadian rhythms, and demonstrate a role for the HIV Tat protein in circadian regulation. Tat reset the murine circadian clock through influencing light entrainment pathways (Clark et al., 2005; Ying et al., 2018) and chronic overexpression of Tat in the brain decreased the amplitude of locomotor activity in mice (Duncan et al., 2008). In HIV-1 infected patients the Tat protein was shown to promote melatonin expression, which may alter sleep (Wang et al., 2014), as people living with HIV

often experience sleep disturbances (Wang et al., 2014; Lee et al., 2015b; O'Brien et al., 2022). There is an association between genetic variability in circadian genes and sleep patterns in adults with HIV, where polymorphism in *Clock* and *Per2* genes associated with poor sleep maintenance and increased total sleep time (Lee et al., 2015b). Delayed circadian rhythms were observed in older Africans living with HIV (Redman et al., 2023), and HIV-1 infection has been linked to attenuated diurnal blood pressure rhythms (Baekken et al., 2009; Borkum et al., 2014; Kent et al., 2016; Manner et al., 2017). These studies suggest that HIV-1 can modulate the host circadian system, however, Stern et al. reported intact circadian transcriptional machinery in T cells from HIV infected subjects (Stern et al., 2022), illustrating the need for future studies.

In contrast, there are limited reports studying how the circadian clock influences HIV replication. The first studies on the circadian regulation of HIV-1 were published 25 years ago, but not followed up. While two of these studies did not observe daily rhythms in HIV-1 replication (Pachl et al., 1995; Deeks et al., 1997), Zeichner et al. observed diurnal patterns of viral RNA in patients (Zeichner et al., 1996) and Ou et al. identified E-boxes in the HIV-LTR as important motifs for viral gene expression (Ou et al., 1994). A recent clinical study found that increased levels of cell-associated, unspliced HIV RNAs associated with BMAL1 expression in HIV-1 patients (Chang et al., 2018). The authors validated these observations and observed that CLOCK and BMAL1 overexpression induced HIV transcription via an E-box motif in the HIV-LTR (Chang et al., 2018). A follow up report by the same group found that HIV-1 RNA in males living showed a circadian cycle, despite receiving ART (Stern et al., 2022). While this illustrates the clinical importance of HIV-circadian interplay, it demonstrates how little is known about the underlying molecular mechanisms.

1.4. Objectives and aims

Objective 1: To elucidate whether HIV-1 replication is rhythmic on a cellular level.

HIV-1 replication has been observed to exhibit diurnal oscillations in patients, but this could be regulated through systemic cues and rhythmic immune cell responses at the organism level. We will address if HIV-1 replication is rhythmic at a cellular level and whether this regulation is driven by the intrinsic clock machinery of the cell, independent of external cues. We will establish circadian HIV-1 model systems which allow real-time detection of HIV-1 replication kinetics in synchronised cells *in vitro*. Circadian analysis tools will help to evaluate the rhythmic properties of replication.

Objective 2: Identify circadian modifiers that alter HIV-1 replication.

Small molecule modulators that target key circadian transcription factors have been identified. We will focus on REV-ERB drugs and test these agents for their ability to modulate HIV-1 replication. Furthermore, we will test novel ROR inhibitors for their potential to alter rhythmic circadian and HIV-1 gene expression. Pan-genotypic activity will be evaluated by using different HIV-1 subtypes.

Objective 3: Uncover the molecular mechanisms underlying the circadian regulation of HIV-1 replication.

Knockdown and overexpression of core circadian transcription factors in infected cells will elucidate their importance for HIV-1 replication. We will study the interaction of BMAL1, REV-ERB and ROR with the HIV-1 genome, to assess whether regulation occurs through direct binding. The kinetics of these interactions will be inferred by analysing the temporal dynamics of transcription factor binding. Moreover, experimental and bioinformatical analysis will determine if the expression of host factors known to regulate HIV-1 replication are circadian regulated.

Objective 4: Investigate the role of circadian rhythms in HIV-1 latency.

HIV-1 establishes latent infection which can be reactivated through pharmacological modulation of diverse host pathways. We aim to characterize the role of the circadian machinery in latency reactivation. Therefore, we will test a library of circadian modifiers for their potential to alter HIV-1 reactivation and will characterise potential hits.

2. Materials and methods

2.1. Chemicals and reagents

2.1.1. Reagents

Chemical/Reagent	Supplier	Product Code
Agarose	Sigma	A9539
Amershan Hybond P PVDF membrane	Merck	GE10600023
AMV Reverse Transcriptase	NEB	M0277S
Bionic buffer	Sigma	B6185
Bovine Serum Albumin	Sigma	A7906-100G
cOmplete protease inhibitor	Roche	1169749001
DMSO	Santa-Cruz	Sc-202581
EDTA	Invitrogen	AM 9260G
Ethanol Absolute (99–100%)	Sigma	32221-2.5-m
Formaldehyde	Sigma	F8775-500mL
Fugene SI	Strattech Scientific	SI-1000-FUG
Glycine	Sigma	G8898-1KG
IGEPAL CA-630	Sigma	18896-100mL
Live Dead Fixible Aqua stain	Life Technologies	50-112-1526
Methanol Absolute (99–100%)	Sigma	32213-2.5-m
Nonidet P-40	Abcam	ab142227
Passive Lysis Buffer	Promega	E1941
Phorbol 12-myristate 13-acetate (PMA)	Sigma	P8139
Polyethylenimine (PEI) 25kD linear	Polyscience	29366-100
Propanol Absolute (99–100%)	Sigma	34863
Proteinase K	Invitrogen	AM2546
RNase A	Fisher	EN0531
SDS	Fisher	10265153
Sodium acetate buffer	Sigma	S7899-100mL
Sodium Azide	Sigma	S2002
Sodium deoxycholate	Sigma	L7026-100mL
Sodium hydrogen carbonate	Sigma	S6297-250G
SuperSignal West Dura Extended Duration Substrate	Thermo Scientific	34076
SYBR Safe DNA stain	Thermo Fisher	S33102
Tris-EDTA Buffer Solution	Sigma	93283-100mL
Triton X-100	Sigma	T8787-100mL
Trypan Blue	Sigma	T8154-100mL
Tween 20	Sigma	P7949-100mL
ViaFect	Promega	E4981
VivoGlo Luciferin	Promega	P1041

2.1.2. Kits

Kit	Supplier	Product Code
CD4 T Cell Isolation, human	Miltenyi Biotec	130-096-533
CD8 T Cell Isolation MicroBeads, human	Miltenyi Biotec	130-045-201
cDNA Synthesis Kit	PCR Biosystems Ltd	PB30.11-10
CytoTox 96 Non-Radioactive Cytotoxicity Assay	Promega	G1780
Firefly Luciferase Assay kit	Promega	E1501
GenElute HP Plasmid Midiprep Kit	Sigma	NA0200-1KT
MycoAlert Mycoplasma Detection Kit	Lonza Biologicals PLC	LT07-318
PCR Purification	Qiagen	28004
QIAprep Spin Miniprep Kit	Qiagen	27104
qPCRBIO SyGreen Blue Mix	PCR Biosystems Ltd	PB20.17-20
QuickChange II XL kit	Agilent	200524
RNeasy Mini Kit	Qiagen	74106
SensiFAS SYBR No-ROX One-Step Kit	Bioline	BIO-72005
TURBO DNA free kit	Life Technologies	AM1907

2.1.3. Drugs and compounds

Compound	Supplier	Product Code
ARN-3236	MedChemExpress	HY-120856
GSK2667	University of Birmingham	N/A
GSK2981278	Cambridge Bioscience	20974
GSK805	Cambridge Bioscience	9002444
Raltegravir	Cambridge Bioscience	HY-10353
SR8278	Calbiochem	554718
SR9009	Calbiochem	554726
TNF α	Miltenyi Biotec	130-094-014
YKL-05-099	MedChemExpress	HY-101147

2.1.4. Antibodies

Antibody	Supplier	Product Code
human anti-CD28	Life Technologies	clone CD28.2
human anti-CD3	R&D	clone UCHT1
Mouse anti- β -actin (WB)	Sigma	A5441
p24 (KC57-FITC)	Beckman Coulter	6604665
Rabbit anti-BMAL1 (ChIP)	Abcam	Ab3350
Rabbit anti-BMAL1 (WB)	Abcam	Ab93806

Rabbit anti-NR1D1 (ChIP)	Proteintech	14506-I-AP
Rabbit IgG (ChIP)	Sigma	NI01
Rat anti-RORC (WB and ChIP)	eBioscience	AFKJS-9

2.2. Primer sequences

All oligonucleotides were ordered from Life Technologies.

Gene expression primers (human)	Forward 5' – 3'	Reverse 5' – 3'
Ambra1	CTCTTCCTCAGACAACCAGGGT	TCCAAGCGAAGGTGCAGACATC
Atp2A2	GGACTIONTTGAAGGCGTGGATTGTG	CTCAGCAAGGACTGGTTTTTCGG
B2M	CTACACTGAATTACCCCCACTG	ACCTCCATGATGCTGCTTACATG
Arntl (Bmal1)	GCTCAGGAGAACCCAGGTTATC	GCATCTGCTTCCAAGAGGCTCA
Ccnt1	TGTTGACGCCACTGTGACCTTG	GTTTTCTTGGCAGCCTCGCATG
Cntnap1	GCCTTGTACTIONGCAACTGTGACG	CTCAGAAGTGGAGCGGTTTCGTA
Cops2	CTGATGTGGAGAGCTTGCTGGT	GGTTGGTCCATTTATCTAGTGCAG
Cry1	GCAGTTGCTTGCTTCCTGACAC	GACAGCCACATCCAACTTCCAG
Cry2	AGGAGAACCACGACGAGACCTA	CCGTTCCAAGTGCTTATCCAGG
Csde1	GTAGTTTTGTGCCATGAAGGAGGC	CCACATCATCGCCAGGCTGTAA
Cxcr4	CTCCTCTTTGTCATCACGCTTCC	GGATGAGGACACTGCTGTAGAG
Ddb1	CATTCCTCGCTCCATCCTGATG	CCTTCTTACGGTCGCTCAACAG
Ddx6	CCGAAATGGCTTATGCCGCAATC	GGAGATAGGTCTCTGCCAGCTT
Eif2Ak2	GAAGTGGACCTCTACGCTTTGG	TGATGCCATCCCGTAGGTCTGT
Eif3E	CTTTCTGTGCGATCCACCACTG	TCAATCTTGGCATCCAGTCTTGC
Eif3M	GAGAACTCGGAAGGTGGACTTC	CCAGGATCAAGAGTAGGGATACC
Emc1	TGGTAACAGCCTCAGGCAAGCT	GAGCAGTAGTTCTCTGGACCATC
Erp44	AGTAGTGTTTGCCAGAGTTGATTG	CTGCCAATGCTTTCACTGATCGC
Glg1	GGTAGAAGACTGTGAACACCGTC	CACTGGTCTCATTCCAACCGTG
Gps1	ACTACTGCACCAGCGCCAAACA	ACTCAGCCTTGCTGACGTAGCT
Hdac3	GAGTTCTGCTCGCGTTACACAG	CGTTGACATAGCAGAAGCCAGAG
Hexim1	GAGGACAGTAGGTGGCAATCGA	AGGCAGCTAGATTCTGGACAGG
Hgh1	AGCCTACCTGATCCTTCGAGAG	GCAGGTTTTCCATGCCACGTTT
Hist1H3l	GGATACCAACCTGTGCGCCATT	GGACAGACTTCTTGGGCTGATAG
Hnrnp3	GGAGGTTTTGATGACTATGGTGG	CCTGAACTTGATCACCAGCTC
Hyou1	CTTCAACCTGGATGAGAGTGGC	ACAGGCTGGAAATGGTGTTGCC
Itga4	GCATACAGGTGTCCAGCAGAGA	AGGACCAAGGTGGTAAGCAGCT
Larp7	AGAGCCTCTACCTGGCAGGAAA	ACTGCTTGAGCATCCTCAGGAG
Mepce	TGGAGCGGACACATCAGTCTTC	CCCAGTTCAGATGCACCCACTT

P4Hb	TCACCAAGGAGAACCTGCTGGA	GGCAAGAACAGCAGGATGTGAG
Per1	TCAACTGCCTGGACAGCATCCT	TCAGAGGCTGAGGAGGTGGTAT
Per2	AGCTGCTTGGACAGCGTCATCA	CCTTCCGCTTATCACTGGACCT
Phb	AAGCGGTGGAAGCCAAACAGGT	GCCAGTGAGTTGGCAATCAGCT
Preb	AAGTCGTCTCCTGCCTCGATGT	CACATCCGTCACCACAATGCCA
Psm7	GATGTGAAGCCGAAGGACCTAG	TCCTCAGCTTCCTCTGCTCCAA
Ranbp1	ACCATGACCCTCAGTTTGAGCC	AGTGCCTCGCTCCTTCCATTCT
Rars	GAAACAGTGCGCCTCATGGATC	AGCCATACGCAACGGATGTCTG
Nr1d1 (Rev-erb α)	CTGCCAGCAATGTCGTTCAAG	TGGCTGCTCAACTGGTTGTTGG
Sars	CCCAGAGAATGTGCTGAGTTTCG	CTCAAACCGCTCTGCTTCCAAC
Sgpl1	GAACACTGCCATGCTCGTCTGT	GATGAGGAAGCCTCCCAGACAA
Sik1	GATGCCACCAAAGCAGCTACAG	CCTGCGTGAAATCCACAGTCTTG
Sik2	GAGCAGGTGAAAGTGCAGATCG	TGTAGCGGTCCATGCACATGGC
Sik3	CGAAGTTTGTGAACTGGCAGGTG	AAGGCGTGATGCTCTGGAGGTA
Skp1	TCCTGAGGAGATTCGCAAGACC	ACTTCTCTTCACACCACTGGTTC
Slirp	TTCCTTGGACTGCGGCGTCGA	CCAAACCTCTGTGAAAGCCAGTC
Spcs3	GAGAGGTGATAATCCGAAGCTGC	TGGTACGACGTTCCAAGACAGG
Strap	GATGCCACCAAAGCAGCTACAG	CCTGCGTGAAATCCACAGTCTTG
Timm13	GAGCAGGTGAAAGTGCAGATCG	TGTAGCGGTCCATGCACATGGC
Tomm40	CGAAGTTTGTGAACTGGCAGGTG	AAGGCGTGATGCTCTGGAGGTA
Uba1	TCCTCACAGAGGACAAGTGCCT	CTTGAGCAGCTCACAGCCAATG

HIV-1 primers	Forward 5' – 3'	Reverse 5' – 3'
NL4.3 Gag	CGAGAGCGTCGGTATTAAGC	CTGAAGGGATGGTTGTAGCTG
CH185 TF Gag	CGAGAGCGTCAGTATTAAGA	CTTAAGAGCTGATTGAAGCTG

ChIP primers	Forward 5' – 3'	Reverse 5' – 3'
HIV-LTR E-box 1&2	ACCAGAGAGACCCAGTACAGGCA	GCTGGGGACTTTCCAGGGAGGT
HIV-LTR E-box 3	TAGTACTCCGGATGCAGCTCTCGG	AGCATGGGATGGAGGACCCGGA
HIV-LTR E-box 4	GCTGTTGTTCTCTCCTTCATTGGCC	CACACCAGGGCCAGGGATCAGA
<i>Per1</i> promoter E-box	GTCAAGGAAAATCCCCAGCTTCTG	CCAAGATTGGTGACGTAAATGCCA
HIV-LTR RORE	TCCATGCAGGCTCACAGGGTGT	CACCAGGGCCAGGGGTCAGATA
<i>Bmal1</i> promoter RORE	TTGGGCACAGCGATTGGT	GTAAACAGGCACCTCCGTCC

Mutagenesis primers	Forward 5' – 3'	Reverse 5' – 3'
E-box deletion	ATGCAGCTCTCGGGCATGAAATGC TAGGCG	CGCCTAGCATTTTCATGCCCCGAGAG CTGCAT
E-box mutant 1	ATGCAGCTCTCGGGCCATATGATG AAATGCTAGGCG	CGCCTAGCATTTTCATCATATGGCC CGAGAGCTGCAT
E-box mutant 2	CCGGATGCAGCTCTCGGGCTCAC GGATGAAATGCTAGGCGGCTG	CAGCCGCCTAGCATTTTCATCCGTG AGCCCCGAGAGCTGCATCCGG
E-box mutant 3	GCATTTTCATCACGTCGCCCCGAGAG CTGCA	TGCAGCTCTCGGGCGACGTGATG AAATGC

Other primers	Forward 5' – 3'	Reverse 5' – 3'
MS2 PERT primers	TCCTGCTCAACTTCCTGTCGAG	CACAGGTCAAACCTCCTAGGAATG

2.3. Cells and tissue culture

2.3.1. Culture media and supplements

Culture media and supplements	Supplier	Product Code
DMEM, high glucose, GlutaMAX	Gibco	31966047
DMEM, no phenol red	Gibco	31053028
RPMI-1640	Gibco	21875034
Fetal Bovine Serum, heat inactivated	Gibco	10500064
Penicillin/streptomycin	Gibco	15140122
MEM-Non essential amino acids (NEAA)	Gibco	11140050
Puromycin	Life Technologies	A1113803
Recombinant human M-CSF	Gibco	PHC9501
HEPES	Life Technologies	15630056
L-Glutamine	Life Technologies	25030024
beta-mercaptoethanol	Life Technologies	31350010
GlutaMAX	Life Technologies	35050038
Sodium pyruvate	Life Technologies	11360039
Opti-MEM	Thermo Fisher	31985070
Proleukin (IL-2)	Novartis	N/A

2.3.2. Cell lines and culture conditions

Cell line	Description	Media	Source
HEK293T	Human embryonic human kidney cells	DMEM	ATCC
TZM-bl	HeLa cell line with integrated copies of the firefly luciferase gene under control of the HIV-1 promoter	DMEM	Prof Bill Paxton University of Liverpool, UK
U-2 OS	Human bone osteosarcoma epithelial cells	DMEM	ATCC
Jurkat	Immortalised human T lymphocyte cells	RPMI	Prof Xiaoning Xu, Imperial College, UK
J-Lat	Jurkat based cell line with integrated copies of full-length HIV-1 with non-functional <i>env</i> due to a frameshift, and <i>Gfp</i> in place of the <i>nef</i> gene.	RPMI	NIBSC AIDS Repository
THP-1	Human leukaemia monocytic cell line	RPMI	ATCC

All cells were cultured in the presence of 10% FBS, 1% Penicillin-Streptomycin and DMEM without phenol red (for real-time monitoring of luminescence) was additionally supplemented with 1% NEAA, 1% L-Glutamine. Cells were maintained at 37°C, 5% CO₂ in a standard cell culture incubator (New Brunswick). Mycoplasma tests were performed using MycoAlert Mycoplasma Detection Kit to ensure that none of the cells were contaminated.

To differentiate THP-1 into M0 macrophages, cells were incubated in the presence of 100 nM PMA for 2 days, recovered in normal media for one day and subsequently used for experiments.

Stable cell line	Generation	Selection
U-2 OS <i>Bmal1</i> knockdown	Transduction with lentivirus delivering sh <i>Bmal1</i> plasmid	Puromycin 2 µg/ml
U-2 OS <i>Bmal1-luc</i>	Transduction with lentivirus delivering pABpuro-BlucF	Puromycin 2 µg/ml
Jurkat <i>Bmal1-luc</i>	Transduction with lentivirus delivering pABpuro-BlucF	Puromycin 2 µg/ml
THP-1 <i>Bmal1-luc</i>	Transduction with lentivirus delivering pABpuro-BlucF	Puromycin 2 µg/ml

To generate cells stably expressing the *Bmal1* promoter in front of luciferase, U-2 OS cells, Jurkat T cells and differentiated THP-1 cells were transduced with lentivirus delivering the pABpuro-BlucF plasmid for 24 h. Cells were recovered for 2 days, media was subsequently supplemented with 2 µg/ml puromycin and cells were used for experiments after at least one week of selection. For *Bmal1* KD experiments, U-2 OS cells were transduced with lentivirus delivering sh*Bmal1* for 24 h, media was changed and cells recovered for 2 days. Cells

expressing the construct were selected by incubation with 2 µg/ml puromycin and knockdown was confirmed by western blot after at least one week of selection.

2.3.3. Primary cells

Leukapheresis cones were purchased from NHS Blood and Transplant (Oxford, UK) and PBMCs were isolated. CD8 T cells were depleted (CD8 MicroBeads, Miltenyi Biotec) or CD4 T cells isolated (CD4 T cell isolation kit, Miltenyi Biotec) and cells cultured in RPMI-1640 containing 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% Glutamax, 1% NEAA and 2 mM beta-mercaptoethanol. Cells were stimulated using 50 IU/ml IL-2, 0.01 µg/ml soluble human anti-CD3 and 0.1 µg/ml soluble human anti-CD28 for 3 days. Afterwards, cells were used for experiments where media was supplemented with 50 U/ml IL-2. *Primary T cell experiments were performed in collaboration with Prof Borrow's group.*

Human induced pluripotent stem cells (iPSC) derived macrophages were differentiated from the human iPSC line OX1-6136 and cultured in DMEM/F12 supplemented with 1% penicillin/streptomycin, GlutaMAX (2 mM), stabilised Insulin (5 µg/ml), HEPES pH 7.4 (15 mM), M-CSF (100 ng/ml). *iPSC derived macrophages were obtained and cultured by Dr. Schilling.*

All work with primary human cells was compliant with institutional guidelines, with written informed consent from all donors and ethical approval for research use.

2.4. Plasmids and constructs

2.4.1. Plasmids preparation

Plasmid	Supplier	Product Code
pABpuro-BluF	Addgene	46824
pMD2G	Addgene	12259
psPAX2	Addgene	12260
pcDNA3.1	Thermo Fisher	V79020
HIV-LTR subtype luciferase constructs	Prof Bill Paxton University of Liverpool, UK	
Bmal1 expression plasmid	Prof Ximing Qin, Anhui University, China	
Clock expression plasmid	Prof Ximing Qin, Anhui University, China	
shBmal1 (ARNTL lentivector)	Abmgood	12431091
shRev-Erb α (NR1D1 lentivector)	Prof Benedetto Grimaldi, Genoa University, Italy	
shRev-Erb β (NR1D2 lentivector)	Prof Benedetto Grimaldi, Genoa University, Italy	

All plasmids were transformed into NEB 10-beta Competent *E. coli* (C3019I) according to manufacturer's instructions, and transformation mixtures were plated on Luria Broth (LB, Sigma, L2897) agar plates containing the appropriate antibiotic for plasmid selection. For cultivation, *E. coli* was grown at 37°C under agitation at 180 rpm in LB medium supplemented with the appropriate antibiotic. Plasmids were isolated using QIAprep Spin Miniprep Kit or GenElute HP Plasmid Midiprep Kit and concentration determined using NanoDropOne (Thermo Fisher).

2.4.2. Agarose gel electrophoresis

To check plasmid preparations and PCR products, agarose gels with the appropriate percentage depending on fragment size (usually 1.0-2.5% agarose) were prepared in bionic buffer, supplemented with 1:10.000 SYBR safe reagent. Appropriate DNA ladder was loaded along with samples supplemented with 5x loading dye, electrophoresis was performed at 75 V for 45 min and gels were imaged with a G:Box Mini (Syngene).

2.4.3. Mutagenesis

Site directed mutagenesis of the Luci-B-Lai subtype LTR plasmid was performed using the QuickChange II XL kit according to manufacturer's instructions. Primers for mutagenesis were designed using the QuickChange online tool and are listed above. E-box mutation 2 (Yang et al., 2002) and E-box mutation 3 (Ohno et al., 2003) were based on previous publications. *This work was performed by Dr. Magri.*

2.4.4. siRNAs

siRNA	Supplier	Product Code
SilencerSelect Negative Control	Thermo Fisher	4390844
siARNTL	Thermo Fisher	4392420, ID: s1616
siSIK1	Thermo Fisher	4427038, ID: s45377
siSIK2	Thermo Fisher	4427037, ID: s23355
siSIK3	Thermo Fisher	4427037, ID: s225956

2.5. Transfections

2.5.1. PEI transfection of adherent cells

Transfection of adherent cells using PEI was performed as reverse transfection to maximise the transfection efficiency. Appropriate amount of plasmid DNA (as detailed below) was diluted in Opti-MEM and PEI was added at a 1 μ l/ μ g ratio, typically at a 3:1 or 4:1 dilution. Transfection mixes were incubated at room temperature for 30 min, added to trypsinised cells resuspended in DMEM without antibiotics and seeded into the required plate format. The medium containing transfection reagents was changed to fresh DMEM 24 h post transfection.

Culture format	Cell Number	Reaction Volume
96 well plate	10,000	100 μ l
24 well plate	50,000	500 μ l
12 well plate	100,000	1 ml
6 well plate	300,000	2 ml
10cm dish	2,000,000	7 ml

Culture format	Opti-MEM	DNA	PEI (3/4:1)
96 well plate	10 μ l	100 ng	0.3 - 0.4 μ l
24 well plate	50 μ l	500 ng	1.5 - 2.0 μ l
12 well plate	100 μ l	1 μ g	3.0 - 4.0 μ l
6 well plate	200 μ l	3 μ g	9.0 - 12.0 μ l
10cm dish	700 μ l	7-8 μ g	24.0 - 32.0 μ l

2.5.2. ViaFect transfection of suspension cells

Transfection of suspension cells was performed using ViaFect. ViaFect was mixed with 500 ng of DNA in a ratio of 2:1 (transfection reagent:DNA) in a 50 μ l reaction in RPMI. After 20 min incubation at room temperature, 5 μ l of the transfection mix were added to 2×10^4 cells in 95 μ l media in a 96 well plate and incubated overnight. Media was changed to remove the transfection reagent and cells were used for each experiment, respectively.

2.5.3. FugeneSI transfection of siRNA

To transiently silence gene expression in suspension cells (Jurkat and J-Lat), transfection mixes were prepared by adding 1 pmol siRNA to 0.3 μ l FugeneSI per well of a 96-well plate. Briefly, 7.5 μ l of FugeneSI reagent were diluted in 117.5 μ l of serum free RPMI and 2.5 μ l of 10 μ M siRNA stock added to 122.5 μ l serum free media. Both solutions were mixed, incubated at room temperature for 5 min and 10 μ l added to each well of a 96 well plate, containing 2×10^4 cells in 95 μ l media. 48 h post transfection, media was changed and cells processed according to each experiment.

2.6. Virus production

Virus	Plasmids	Ratio
NL4.3 R-E- <i>luc</i> VSV-G	NL4.3 R-E- <i>luc</i> + VSV-G	1:1
GKO no envelope	GKO + pcDNA3.1	1:1
GKO-envX4	GKO + envX4	1:1
GKO-VSV-G	GKO + VSV-G	1:1
shBmal1 lentivirus	shBmal1 + psPAX2 + pMD2G	4:2:1
BlucF lentivirus	pABpuro-BlucF + psPAX2 + pMD2G	4:2:1

HEK293T cells were transfected using PEI with the specified plasmid combinations and ratios to generate each respective virus. Medium was replaced 4 h post transfection with DMEM medium without antibiotics supplemented with 10 mM HEPES and the supernatant harvested 48 h later. Virus containing supernatants were centrifuged at 1800 rpm for 5 min to remove leftover cells, supernatants filtered with a 0.45 µm filter, aliquoted and stored at -80°C. Virus concentrations were determined using a qPCR-based product-enhanced reverse transcriptase (PERT) assay and new aliquots of virus were defrosted for each experiment.

HIV-1 transmitted founder virus stocks (CH185 and CH058) were produced by Dr. Kliszczyk using Lipofectamine or Fugene 6 and virus concentrations determined using a colorimetric reverse transcriptase assay (Roche).

2.7. Virus quantification via PERT assay

Concentrations of viral stocks were quantified by measuring the reverse transcriptase activity with a qPCR-based product-enhanced RT assay (Vermeire et al., 2012). Viral particles were lysed 1:1 in PERT lysis buffer (100 mM Tris-HCL pH 7.5, 50 mM KCL, 0.25% triton X, 40% glycerol) for 10 min at room temperature to release HIV-1 RT. A one-step 15 µl qPCR reaction with exogenous MS2 bacteriophage RNA (Merck, 10165948001) as a template, 100 nM forward and 100 nM reverse MS2 primers, Sensifast RiboSafe RNase inhibitor and Sensifast One-Step SYBR buffer was mixed. A dilution series of either the Avian myeloblastosis virus

(AMV) RT control in AMV buffer supplemented with 20% glycerol and 10 mg/ml BSA or HIV-1 RT lysate was prepared and added to PCR reactions. Real-time qPCR was carried out using a LightCycler 96 (Roche) with the following amplification protocol: 10 min at 45°C, 2 min at 95°C, 45 cycles of 10 s at 95°C and 15 s at 60°C, followed by sample dissociation melt peak. A standard curve based on the AMV RT concentrations was plotted (**Fig.2.1.A**) and HIV-1 RT activity was estimated by determining the amount of RNA that is converted to cDNA (NL4.3 R-E-*luc* VSV-G example shown in **Fig.2.1.B**).

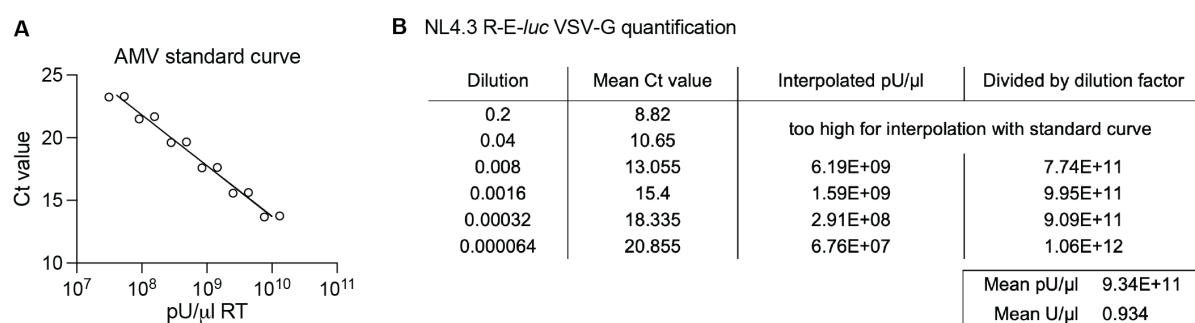


Figure 2.1. Virus quantification via PERT assay. A) Reverse transcriptase activity of the AMV reverse transcriptase was determined with a qPCR-based product-enhanced RT assay. Different amounts of AMV RT were used to determine the cycle threshold (Ct) values at each concentration. A curve was interpolated and used as standard (representative for n=4). **B)** Quantification of NL4.3 R-E-*luc* VSV-G concentration using the PERT assay. Concentrations were determined by comparing Ct values of different virus dilution with the AMV standard curve, divided by dilution factor and average concentration is shown as units per μl.

2.8. Transcript expression analysis

2.8.1. Nucleic acid extraction

All RNA extractions were performed using the RNeasy kit, following manufacturer's instructions where cells were lysed in 350 μl RLT lysis buffer and stored at -80°C. Samples were processed by adding 1 volume of 70% ethanol, applied to MinElute Spin Columns and washed with RW1 and RPE wash buffers. The optional spin to remove remaining liquid was carried out in all instances and samples eluted in 30 μl of nuclease free water.

Cells which had been transfected with plasmid or infected with virus were subjected to TURBO DNase digestion to remove any DNA contaminant. Reactions were carried out according to manufacturer's instructions in 50 µl with up to 2 µg of input RNA, and 2 units (1 µl) of TURBO DNase reagent, or reactions were downscaled proportionately to 20 µl reactions. Samples were incubated at 37°C for 30 min, inactivated with 0.1 volumes DNase inactivation buffer for 5 min at room temperature, centrifuged at 10,000 x g for 90 s, supernatant aspirated, and pellets discarded.

RNA levels were quantified using NanoDropOne or with the LVis plate and Clariostar Plate Reader (BMG Labtech), and reverse transcribed for gene expression analysis by qPCR.

2.8.2. Reverse transcription

RNA was reverse transcribed in a 20 µl cDNA synthesis reaction using the PCR BioSystems cDNA synthesis kit. Briefly, 0.25-1 µg RNA was mixed with 5x master mix containing random hexamers and oligodT and 20x reverse transcriptase. Samples were incubated at 42°C for 30 min and 85°C for 10 min to denature RTase, and stored at -20°C.

2.8.3. qPCR

Following cDNA synthesis, samples were diluted appropriately in nuclease free water (usually 1:3 or 1:4), and 2x SYBR green buffer and 0.5 µM primers were added to the template in a total reaction volume of 10 µl. Real-time PCRs were carried out using a LightCycler 96 (Roche) with the following amplification protocol: 2 min at 95°C, 45 cycles of 5 s at 95°C and 30 s at 60°C, followed by sample dissociation melt peak. Melting curves were analysed following every qPCR to ensure that amplification of the target sequence was specific.

2.9. Western blotting

When cells were processed for RNA extraction, the cell lysates could be purified separately and used for western blotting. Cells were lysed using RLT lysis buffer, precipitated in 70% ethanol, and samples applied to MinElute Spin Columns which retain nucleic acids on their membrane. The column flow through containing cellular proteins was then processed separately, and 2 volumes of ice-cold acetone were added. Samples were incubated on ice for 30 min or kept at -20°C, followed by centrifugation at >16,000 g for 30 min at 4°C. Protein pellets were washed in ice cold 100% ethanol, centrifuged for 10 min, and the dried pellet was resuspended in appropriate lysis buffer or 1x Laemmli sample buffer (Tris-HCL pH 8, 0.0625 M; 2% SDS; 10% Glycerol, 5% 2-mercapto ethanol and bromophenol blue).

Alternatively to acetone precipitation, cells were lysed using RIPA buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% NP40, and 1% sodium deoxycholate, supplemented with a protease inhibitor cocktail), and viscous samples were sonicated for 5 min. 4x Laemmli sample buffer was added, samples incubated at 95°C for 5 min and allowed to cool down. Proteins were separated on a 10% polyacrylamide gel and transferred to a methanol activated polyvinylidene difluoride membrane. Membranes were blocked in 5% skimmed milk in PBST (PBS/0.1% Tween-20), followed by incubation with primary antibodies in 5% skimmed milk overnight shaking at 4°C. After 3 washes in PBST, the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were added in in 5% skimmed milk, incubated shaking for 1 h at room temperature and membranes washed again. WestDura chemiluminescence substrate was used to visualise proteins using a G:Box Mini (Syngene).

2.10. HIV-1 infection endpoint experiments

2.10.1. NL4.3-*luc* VSV-G infections

To study single-cycle HIV-1 replication using NL4.3 R-E-*luc* VSV-G, reporter virus was generated as described in section 2.6. U-2 OS, Jurkat cells, activated CD8 depleted PBMCs or iPSC derived macrophages were infected with NL4.3-*luc* VSV-G (100 U RT/ 10^6 cells, unless stated differently) for 24 h. Next, cells were either treated with drugs or used for overexpression/knockdown experiments as described below. At the end of each experiment, cells were lysed in 60 μ l passive lysis buffer, and luciferase activity quantified as readout for viral replication using a Firefly Luciferase Assay kit and Glomax luminometer (Promega).

2.10.2. Transmitted founder virus infections

Activated CD8 depleted PBMCs were infected with transmitted founder virus from patient CH185 or CH058 by spinoculation of 0.25 ng RT / 10^6 cells in 100 μ l of media for 2 h (2200 rpm, 25°C). Cells were washed with RPMI, seeded into 96 well plates and cultured in medium supplemented with or without indicated drugs for 7 days. After 3 days, media was changed and fresh drug added. Cells were harvested throughout the experiment at day 2, 4, 7 post infection, followed by lysis and Gag RNA detection via qPCR.

2.10.3. GKO dual reporter virus infections

For initial experiments, Jurkat cells (5×10^5 cells/ml) were infected with 5 ng p24/ml of GKO-no envelope, GKO-envX4 or GKO-VSV-G for 6 days. GKO-VSV-G infections were then optimised by performing a time course where cells were incubated with virus for 1, 2, 3, 6 or 14 days (media and virus were replaced every 3 days). Lastly, a range of concentrations of GKO-VSV-G (0.125 – 32 ng p24/ml) was tested. The optimal infection conditions of 20 ng p24/ml GKO-VSV-G for 48 h were used for further experiments.

2.10.4. Genetic modulation of Rev-erb, Bmal1 and Clock expression

TZM-bl cells were transduced with lentivirus delivering short hairpin control, shRev-erb α or shRev-erb β which encode *Gfp*. Transduction was confirmed 48 h later by acquiring fluorescent images and detecting transcripts of each isoform by qPCR. HIV-1 LTR activity was quantified by measuring luciferase 48 h after transduction using the Firefly Luciferase Assay kit.

Jurkat cells were transfected with *Bmal1* and *Clock* expression plasmids, or a pcDNA3.1 control. To generate transient Bmal1 knockdown in Jurkat T cells, Bmal1 siRNA or a scrambled control were transfected using FugeneSI. 48 h post transfection cells were harvested for western blotting, transcript expression analysis by qPCR and luminescence detection using the Firefly Luciferase Assay kit.

2.10.5. Drug treatment and toxicity determination

TZM-bl cells harbouring an integrated copy of HIV-1, Jurkat cells infected with NL4.3-*luc* VSV-G or GKO-VSV-G, or J-Lat cells were incubated with different drug concentrations as indicated and for most cases, drugs were administered for 24 hours. However, in experiments investigating the duration of drug treatment, cells were collected after 4, 8, or 24 h of treatment. DMSO treatment was used as a control. Post treatment, supernatants were collected to determine cytotoxicity and cells were lysed for luminescence readout as described above. For GKO-VSV-G infections, active and latent viral replication was analysed by flow cytometry.

Cytotoxicity was evaluated using the CytoTox 96 Non-Radioactive kit which measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis and death. Briefly, supernatants were centrifuged (1800 rpm, 5 min) to remove any leftover cells, CytoTox reagent was added (1:1) and incubated at room temperature in darkness for 30 min. Additionally, full cell lysates were included as positive control. Stop solution was added,

absorbance was measured at 490 nm and percent cytotoxicity was calculated by dividing the sample LDH release with the maximum cell lysate control LDH release.

Alternative to cytotoxicity measurement using the CytoTox kit, cell viability was determined using a live-dead stain and flow cytometry as described below.

2.11. Time course experiments

2.11.1. Real-time luciferase measurement

Real-time luciferase was measured to analyse rhythmic *Bmal1* promoter activity in U-2 OS cells or THP-1 cells expressing *Bmal1-luc*, and to detect rhythmic viral replication in U-2 OS cells infected with NL4.3-*luc* VSV-G (100 U RT/ 10⁶ cells) for 24 h. Cells were synchronised via serum shock with 50% FBS for 1 h, and medium was changed to DMEM lacking phenol red 24 h post synchronisation, supplemented with or without drugs and 100 µM VivoGlo luciferin. Luciferase activity was measured every 30 min for a period of 48 h using a CLARIOstar luminometer (BMG Labtech), where cells were kept at 37°C and 5% CO₂. Alternatively, media was changed directly after serum shock and luminescence readout started, and the first 24 h of data were discarded.

Cycling datasets were analysed with BioDare2 (Zielinski et al., 2014) using empirical JTK_cycle (eJTK cycle (Hutchison et al., 2015)) and Fast Fourier Transform Non-linear Least Squares (FFT-NLLS (Straume et al., 2002)) analysis to estimate the period, phase and amplitude of cycling transcripts. All data was normalised to control peak expression and curves fitted using Prism 9 (GraphPad). It is important to note that this normalisation sets the baseline of all data to 0. Representative raw data for each experiment can be found in **Figure**

2.2.

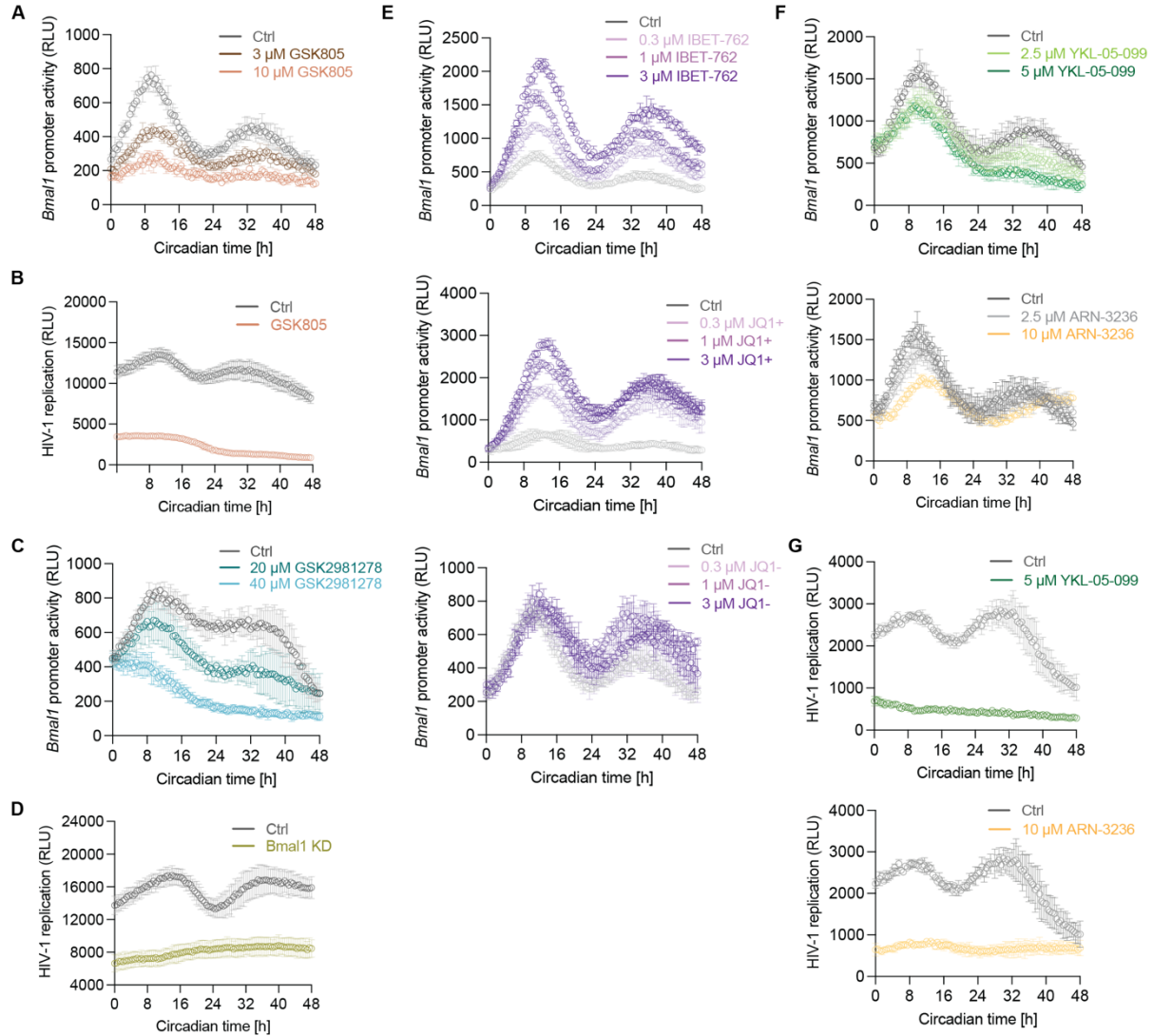


Figure 2.2. Raw luminescence values for real-time measurements. **A)** U-2 OS cells stably expressing *Bmal1-luc* were synchronised by serum shock, incubated with GSK805 and luciferase measured starting 24 h post synchronisation (representative of $n=3$, mean \pm S.D., related to Figure 4.4.C). **B)** U-2 OS cells were infected with NL4.3-*luc* VSV-G, synchronised and treated with 10 μ M GSK805. 24 h after synchronisation luciferase signal was acquired every 30 min as readout for viral replication (representative of $n=3$, mean \pm S.D., related to Figure 4.5.A). **C)** U-2 OS *Bmal1-luc* cells were synchronised, incubated with or without GSK2981278 and *Bmal1* promoter activity measured in 30 min intervals (representative of $n=4$, mean \pm S.D., related to Figure 4.6.A). **D)** *Bmal1* was silenced in U-2 OS cells using shRNA mediated knock-down, cells were synchronised, infected with NL4.3-*luc* VSV-G and luciferase measured at 30 min intervals (representative of $n=3$, mean \pm S.D., related to Figure 5.2.D). **E)** U-2 OS cells expressing *Bmal1-luc* were synchronised, treated with IBET-762, JQ1+ or JQ1- and luminescence recorded (representative of $n=3$, mean \pm S.D., related to Figure 6.5). **F)** U-2 OS cells expressing *Bmal1-luc* were synchronised, incubated with or without ARN-3236 or YKL-05-099 and luciferase activity quantified (representative of $n=3$, mean \pm S.D., related to Figure 6.6.B). **G)** U-2 OS cells infected with NL4.3-*luc* VSV-G were synchronised, treated with ARN-3236 or YKL-05-099 and HIV-1 replication measured starting 24 h post serum shock (representative of $n=3$, mean \pm S.D., related to Figure 6.6.C).

2.11.2. Rhythmic transcript detection

To detect rhythmic transcript levels, U-2 OS cells, Jurkat cells or activated CD8 depleted PBMCs were infected with HIV-1 NL4.3-*luc* VSV-G (100 U RT/ 10^6 cells) for 24 h (or non-infected cells as control), synchronised via serum shock with 50% FBS for 1 h and 24 h later cells were harvested at 4 h intervals, followed by RNA extraction and transcript detection by qPCR. Data was detrended with BioDare2 and curves fitted using Prism 9.

2.12. Chromatin immunoprecipitation

2.12.1. Cell preparation, fixation and harvesting

Jurkat cells were adjusted to 1×10^6 cells/ml in 30 ml medium, infected with NL4.3-*luc* VSV-G (100 U RT/ml) for 24 h and then incubated in the presence of 10 μ M GSK805 (or untreated control) for 24 h.

U-2 OS cells were adjusted to 1×10^5 cells/ml in 15 ml medium, NL4.3-*luc* VSV-G (100 U RT/ml) was added and cells were seeded into 15 cm dishes. 24 h post infection, virus was removed, cells were synchronised by serum shock (50% FBS for 1 h), and harvested 24 h (CT0) or 36 h (CT12) post synchronisation.

Cells were fixed and crosslinked with 1% formaldehyde for 10 min on ice, 1 M glycine in PBS was added at a final concentration of 125 mM glycine and incubated for 10 min at room temperature with gentle rocking. Cells were washed twice with cold PBS. Jurkat cell suspensions were handled in falcon tubes throughout these steps, while U-2 OS cells were now scraped into 5 ml of ice-cold PBS (using a rubber cell scraper) and transferred to 15 ml falcon tubes. Suspensions were pelleted by centrifugation (1000 rpm, 10 min, 4°C) and pellets lysed in SDS lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 1% NP-40) supplemented with freshly prepared protease inhibitor cocktail. Lysates were stored at -80°C until experiment continuation.

2.12.2. Sonication, pre-clear and immunoprecipitation

Samples were diluted 1:1 with ChIP dilution buffer (0.01% SDS, 1.1% Triton, 0.2 mM EDTA; 16.7 mM Tris pH 8.1, 167 mM NaCl), transferred to Diagenode Bioruptor tubes and sonicated using a Bioruptor sonicator (high, 30 min, 15 s on, 15 s off). Lysates were clarified by centrifugation at 13,000 rpm for 10 minutes, at 4°C and a 2% input control was taken and stored at -20°C. For pre-clear and immunoprecipitation, proteinase A agarose beads were washed twice in 800 µl of ChIP dilution buffer (5 min mixing at 4°C on over-end rotator, centrifugation at 2000 rpm for 3 min at 4°C). Sonicated samples were diluted 1:6 with ChIP dilution buffer, washed protein A agarose beads (40 µl per condition) were added and incubated for 1 h on an over-end rotator at 4°C. Beads were pelleted (1000 rpm, 5 min, 4°C) and supernatants immunoprecipitated with 3-5 µg of antibodies of interest or IgG control, rotating overnight at 4°C.

2.12.3. Pull-down, washing and DNA preparation

90 µl of washed proteinase A agarose beads were added to the immunoprecipitate and incubated at 4°C for 90 min on an over-end rotator. Beads were collected (380 g, 8 min, 4°C), washed in low salt buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris pH 8.1, 500 mM NaCl), LiCl Buffer (1% Igepal, 1 mM EDTA, 10 mM Tris pH 8.1, 250 mM LiCl, 1% sodium deoxycholate) and finally twice in TE wash buffer (10 mM Tris pH 8.0, 1 mM EDTA). For each wash, samples were mixed for 5 min on an over-end rotator at 4°C and pelleted at 2000 rpm for 3 min at 4°C. Complexes were released from the beads using 240 µl elution buffer (0.1 M NaHCO₃, 1% SDS), 240 µl elution buffer was added to input controls and all samples were reverse crosslinked overnight at 65°C, shaking at 1400 rpm in the presence of 200 mM NaCl. Proteins were digested with 2 µl of 20 mg/ml proteinase K for 4 h shaking at 45°C, followed by treatment with 1 µl RNaseA for 30 min shaking at 37°C and 1400 rpm. DNA was purified

with MiniElute PCR Purification columns, transcription factor binding assessed by qPCR. The % input was calculated for each sample and normalised to IgG, allowing the calculation of fold enrichment of binding.

2.13. J-Lat latency reactivation experiments

J-Lat cells were adjusted to 2×10^5 cells/ml, seeded in 96 well plates and incubated with 100 ng/ μ l TNF α in combination with each drug, respectively. After 24 h drug treatment, cells were stained, fixed and analysed by flow cytometry as described below. To generate SIK KD cells, J-Lat cells were transfected with siRNAs using FugeneSI for 48h and cells were activated for 24 h with 100 ng/ μ l TNF α , followed by staining, fixation and analysis by flow cytometry.

2.14. Flow cytometry

Flow cytometry was used to determine viability, GFP and mKO2 expression of Jurkat cells, CD8 depleted PBMCs, J-Lat cells. At the end of each experiment, cells were washed with PBS, and incubated with LIVE/Dead fixable Aqua (1:500 dilution) for 10 min in complete darkness. After washing cells with PBS, the cell pellet was resuspended in 2 % formaldehyde and incubated for 10 min without light exposure. Cells were washed again, resuspended in PBS and fluorescence was measured using a Cyan ADP flow cytometer (Beckman Coulter) or Attune NxT flow cytometer (Thermo Fisher).

Additionally, flow cytometry was used to measure intracellular p24 levels of NL4.3-*luc* VSV-G infected U-2 OS cells. Here, live-dead staining was conducted as described above, but fixation was performed by incubating cells with 20 μ g/ml lysolecithin in 4% formaldehyde for 2 min at room temperature. Next, cells were resuspended in cold 50% methanol and incubated on ice for 15 min. After centrifugations supernatants were discarded, 0.1% NP-40 added, and incubated on ice for 5 min. Finally, cells were stained with 2 μ l KC57-FITC antibody in 150 μ l

FACS buffer (0.5 % BSA and 0.05 % Sodium Azide (NaN₃) in 1x PBS) per well for 15 min at room temperature, washed with FACS buffer, and samples acquired as described before. All data was analysed using FlowJo (TreeStar).

2.15. Bioinformatic analysis

Program	Software	Source
Prism 9.4.1	GraphPad	https://www.graphpad.com
FlowJo 10.9	BD	https://www.flowjo.com
eJTK_cycle	BioDare2	https://biodare2.ed.ac.uk
Fast Fourier Transform Non-linear Least Squares (FFTL)	BioDare2	https://biodare2.ed.ac.uk
Hypergeometric Optimisation of Motif EnRichment (HOMER)	N/A	http://homer.ucsd.edu/homer/

For RORC binding motif identification in *Bmal1*, *Rev-erb α* , *Per1* and *Cry2* promoters, the Eukaryotic Promoter Database (Dreos et al., 2014) was used to analyse regions up to 1 kb downstream of the transcriptional start site (TSS).

A list of HIV-1 host factors was obtained from Hiatt et al. (Hiatt et al., 2022) and cycling genes were identified by analysing the human datasets on the Circa Database (Pizarro et al., 2013). BMAL1 regulated genes were identified from a published ChIP-seq dataset of mouse liver (Beytebiere et al., 2019). REV-ERB α (Chang et al., 2019) and RORC (Ciofani et al., 2012) target genes were obtained from ChIP-seq datasets of mouse T cells. BMAL1, REV-ERB α and RORC were manually compared to HIV-1 host factors using Excel and overlaps identified. HOMER, a tool for Hypergeometric Optimisation of Motif EnRichment (Heinz et al., 2010) was used to inspect promoter regions of HIV-1 host factors for the presence of circadian motifs. Sequences up to -1kb upstream of the TSS were analysed and E-box ('CANNTG', canonical 'CACGTG') or RORE ('RGGTCA') identified.

To assess molecular function and connectivity of circadian regulated HIV-1 host factors, gene ontology (GO) analysis was performed using ShinyGO (Ge et al., 2019), whereby each node represents an enriched GO term and related GO terms are connected by lines, whose thickness reflects the percentage of overlapping genes.

2.16. Statistics

All data was analysed using GraphPad Prism version 9.4.1 and non-parametric Mann-Whitney or Kruskal-Wallis tests were performed. Significance values are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, all data are presented as mean value \pm SEM or mean value \pm S.D. Please see individual figure legends for further details.

3. HIV-1 replication is rhythmic

3.1. Introduction

Diurnal fluctuations of peripheral HIV-1 RNA levels have been observed in people living with HIV-1 (Stern et al., 2022), however, the underlying mechanisms remain to be elucidated. Many physiological parameters oscillate throughout the day, and it has been shown that immune functions change diurnally (Baxter and Ray, 2019; Wang et al., 2022a), which could result in the rhythmic expression of HIV RNA (Mazzocchi et al., 2020). In addition to changes reflected by external and systemic rhythmic cues, it is possible that the cellular intrinsic clock is sufficient to regulate rhythmic HIV replication.

To investigate if the cell intrinsic clock plays a role in HIV replication, the use of circadian synchronised cell culture systems is essential. Cells which are propagated under constant conditions in a laboratory setting lack rhythmic stimuli, hence, their endogenous cellular clocks are asynchronous (Welsh et al., 2004). It is possible to synchronise the circadian clocks of cells by providing an artificial entrainment cue, after which the cell population will cycle coherently for several days in the absence of stimuli (**Fig.3.1**). Cells are usually left to recover 24 hours after synchronisation to minimise any immediate effects of the synchronisation agent, and time thereafter is referred to as circadian time (CT). Multiple synchronising cues have been discovered, including the glucocorticoid dexamethasone (Balsalobre et al., 2000) or treating cells to a high concentration of serum ('serum shock') (Balsalobre et al., 1998). While dexamethasone is one of the strongest entrainment cues, we avoided using this agent as it has been reported to have anti-viral and immunomodulatory properties (Liu et al., 2021). It is not fully understood how serum shock synchronises cellular clocks, but it has been shown to induce Per2 expression which resets the cellular circadian machinery (Balsalobre et al., 1998).

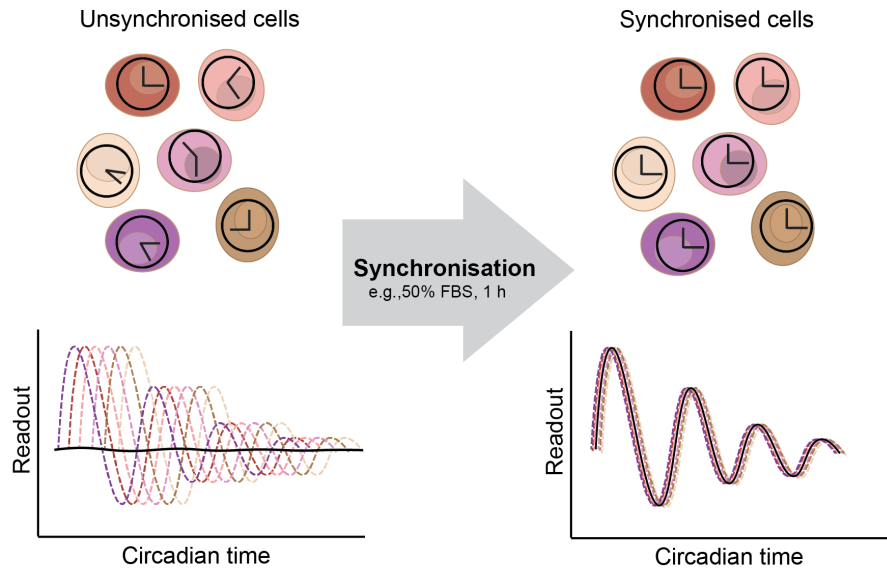


Figure 3.1. Schematic of circadian synchronisation *in vitro*. Almost all cells have an intrinsic clock machinery which regulates rhythmic gene expression. In the absence of entrainment cues in an *in vitro* cultured population, the clock of each cell has a slightly different period and is in a different phase, hence no rhythms can be detected. Treating cells with 50% FBS for 1 h resets all cellular clocks and leads to a circadian entrained population of cells which cycle synchronously.

As HIV-1 primarily infects T cells and macrophages, they would provide ideal cell types for our studies, however, the synchronisation of T cells *ex vivo* has proven challenging as these cells have weak endogenous oscillations which are difficult to detect with conventional methods (Bollinger et al., 2011). The human osteosarcoma cell line U-2 OS is one of the most commonly used and reliable circadian model systems for *in vitro* studies (Zhang et al., 2009). This chapter aimed to identify and characterise a suitable model system to study the circadian regulation of HIV-1. We demonstrated that U-2 OS cells support HIV-1 replication and can be synchronised by serum shock. Infection of U-2 OS cells with a reporter virus encoding luciferase allowed real-time monitoring of HIV-1 replication and revealed rhythmic HIV-1 replication across 48 hours. Additionally, harvesting infected cells in 4 h intervals to detect HIV-1 transcripts confirmed this phenotype. Overall, this indicates that rhythmicity in HIV-1 replication is a result of cell-intrinsic clock regulation.

3.2. Results

3.2.1. Validation of systems for HIV-1 circadian research

To establish a circadian model system for HIV studies, we attempted to synchronise Jurkat CD4 T cells and primary CD8 depleted PBMCs from multiple donors by serum shock. Activated CD8 depleted PBMCs and Jurkat cells were harvested every 4 hours starting 24 h after synchronisation, *Bmal1* expression was quantified by qPCR and did not show robust oscillations in either cell type (**Fig.3.2.A**). As HIV-1 can infect macrophages, we tested a different approach and differentiated the THP-1 monocyte cell line into M0 macrophages using PMA treatment. We transduced cells with a lentivirus delivering a construct of *Bmal1* promoter-driven luciferase (*Bmal1-luc*) to monitor real-time *Bmal1* promoter activity following synchronisation by serum shock. However, no rhythmicity in *Bmal1* promoter activity was detected (**Fig.3.2.B**). Next, we tested U-2 OS cells and show robust oscillations in circadian gene expression, as evidenced by the qPCR analysis of *Bmal1*, *Rev-erb α* , *Per1*, *Per2*, *Cry1* and *Cry2* transcripts (**Fig.3.2.C**). Transcripts show the expected expression patterns which have been extensively characterised in the literature, for instance, *Bmal1* and *Rev-erb α* are anti-phase to each other (Vollmers et al., 2008; Hughes et al., 2009).

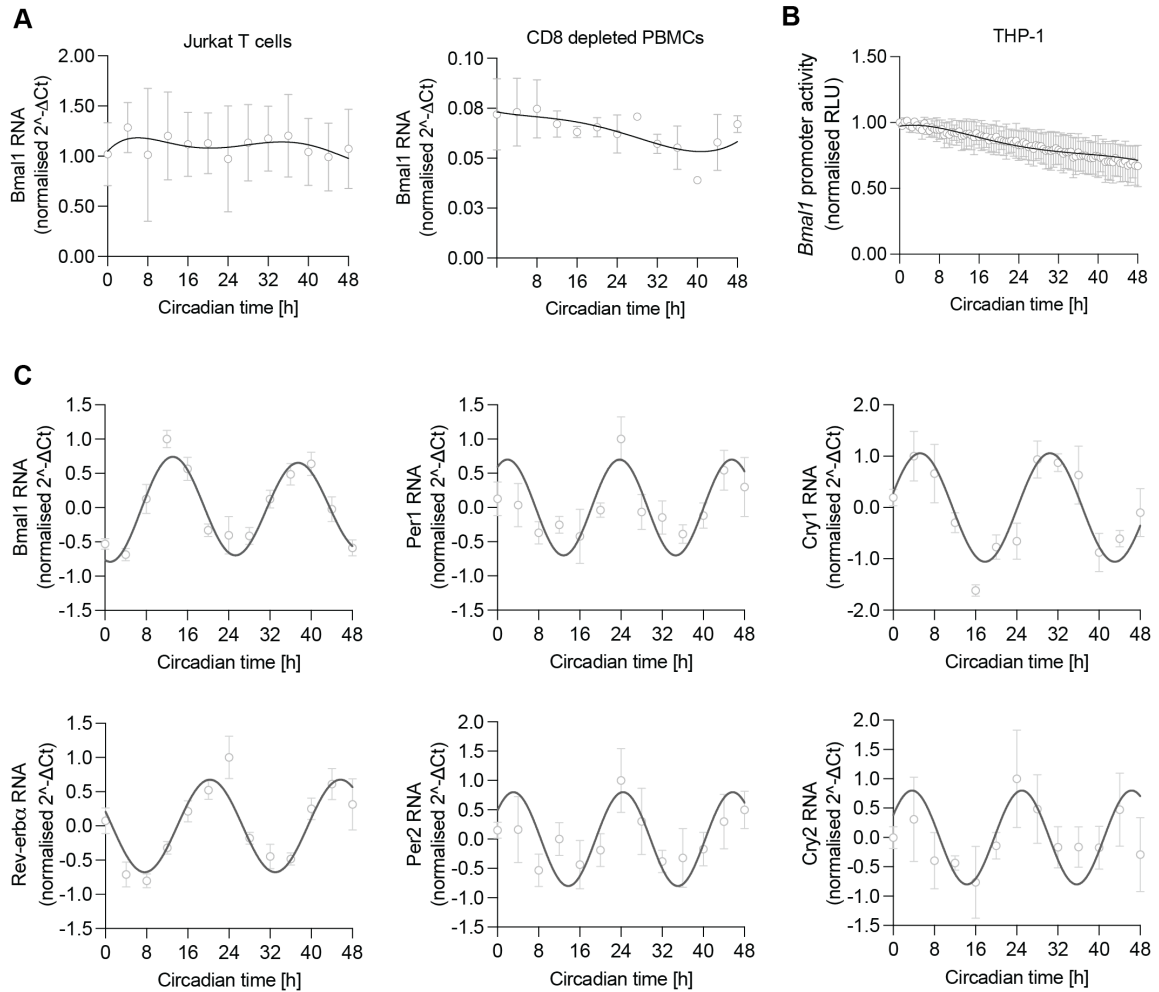


Figure 3.2. Circadian synchronisation of cells. **A)** Jurkat T cells or CD8 depleted PBMCs (activated for 3 days with anti-CD3/CD28) were synchronised by serum shock for 1 h using 50% FBS. 24 h post synchronisation, cells were harvested at 4 h intervals, RNA was extracted and expression of *Bmal1* relative to *B2M* housekeeper was quantified by qPCR (mean \pm S.D., $n=2$). **B)** THP-1 cells were differentiated using 100 nM PMA for 2 days, recovered for one day in normal media, and transduced with a lentivirus that expresses luciferase under the control of the *Bmal1* promoter. Cells were synchronised by serum shock, and luminescence recorded at 30 min intervals starting 24 h post synchronisation (mean \pm S.E.M., $n=3$). **C)** U-2 OS cells were incubated with 50% serum for 1 h and 24 h post serum shock cells were harvested at 4 h intervals. RNA was extracted and expression of *Bmal1*, *Rev-erb α* , *Per1*, *Per2*, *Cry1* and *Cry2* RNAs relative to *B2M* housekeeper was measured by qPCR (mean \pm S.E.M., $n=4$, normalised to peak).

We tested if U-2 OS cells support HIV-1 infection by using a single-cycle HIV-1 reporter NL4.3 R-E-*luc* (NL4.3-*luc*), which allows quantification of luciferase as a readout for viral replication without generating progeny virus and secondary reinfection events (Connor et al., 1995) (**Fig.3.3.A**). This strain is extensively used due to its well-defined genetic sequence and the

availability of detailed information about its biological properties. U-2 OS do not express CD4 or chemokine receptors, hence, we generated pseudoparticles by complementing the glycoprotein-defective NL4.3-*luc* with vesicular stomatitis virus encoded G protein (VSV-G) to bypass the natural HIV entry receptors (Aiken, 1997). Infection of U-2 OS cells led to a dose dependent increase in luminescence (**Fig.3.3.B**), demonstrating that these cells support viral replication. To ensure that HIV-1 replication is based on HIV-1 integration into the host genome of these cells, we pre-treated U-2 OS cells with the clinically licensed integrase inhibitor Raltegravir (Evering and Markowitz, 2008) and showed reduced HIV-1 replication (**Fig.3.3.C**). To quantify the rate of infection of U-2 OS cells we measured the HIV antigen p24 by flow cytometry. We revealed a rate of infection of ~10% (**Fig.3.3.D**), which confirms successful infection of a substantial number of cells.

In summary, these data show that U-2 OS cells support HIV-1 replication which requires integration into the host genome and can be used for HIV-circadian studies.

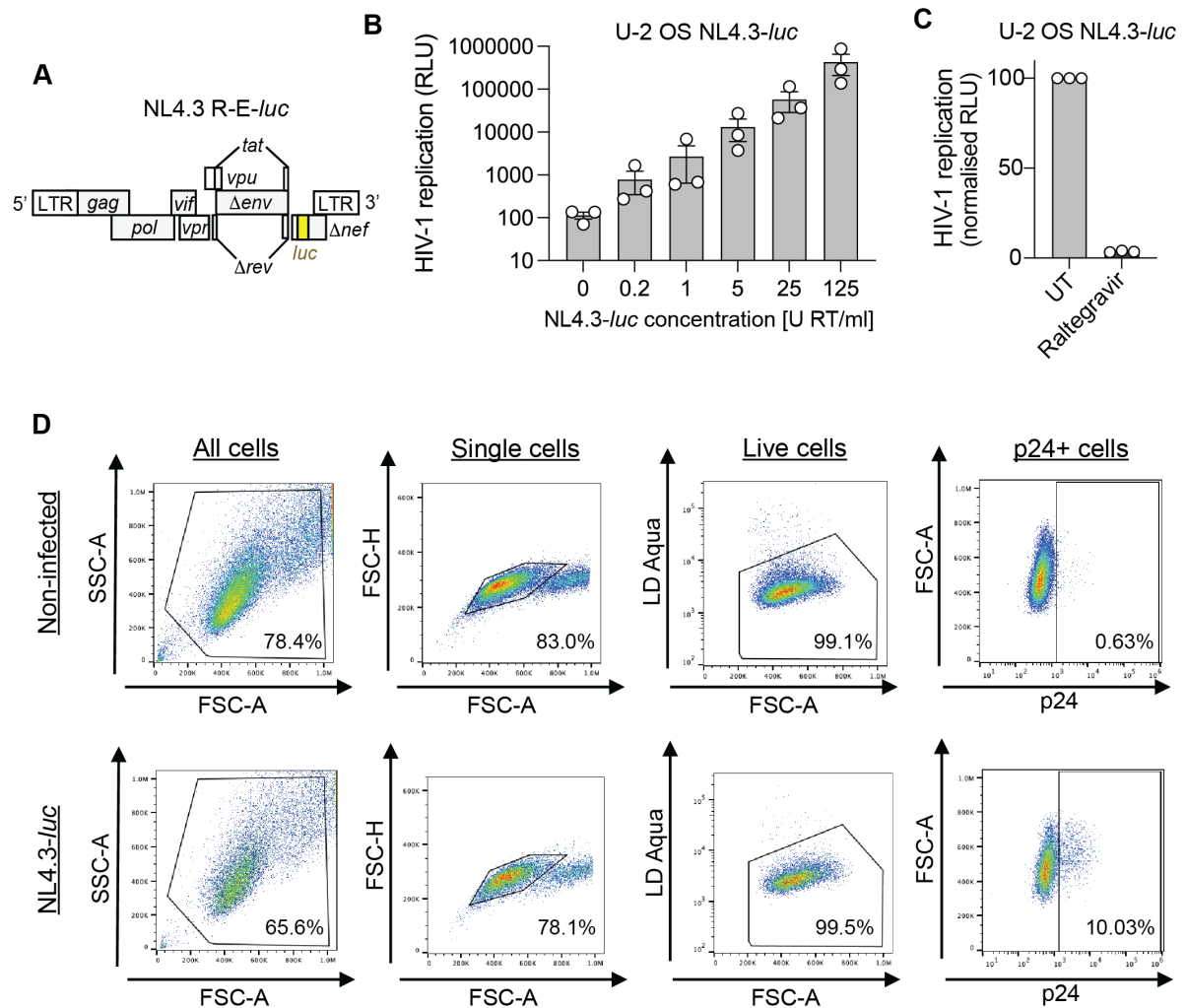


Figure 3.3. U-2 OS cells support HIV-1 infection. **A)** Cartoon of the HIV-1 NL4.3 R-E-luc (NL4.3-luc) reporter, which encodes HIV genes with flanking LTRs, including defective envelope (Δenv) and regulator of expression of virion proteins (Δrev). The luciferase gene is inserted into the negative regulator factor gene (Δnef) and allows readout of viral replication. **B)** U-2 OS cells were infected with NL4.3-luc VSV-G for 24 h, cells were lysed and luminescence measured as a readout for HIV-1 replication (mean \pm S.E.M., $n=3$). **C)** U-2 OS cells were pre-treated with the integrase inhibitor Raltegravir (30 μ M) for 24 h. Cells were then infected with 100 U RT/ml NL4.3-luc VSV-G for 24 h in the presence of Raltegravir and luciferase activity measured 24 h post infection (mean \pm S.E.M., $n=3$). **D)** U-2 OS cells were infected with 100 U RT/ml NL4.3-luc VSV-G for 24 h (or non-infected control) and fixed with paraformaldehyde. Cells were stained, and viability and p24 expression assessed by flow cytometry (representative dot plots for $n=3$. SSC = Side Scatter, FSC = Forward Scatter, LD = life-dead). All data are expressed relative to the control cells.

3.2.2. Real-time monitoring of HIV-1 reporter virus replication and HIV-1 transcription

To assess whether HIV-1 replication is rhythmic and to study post-integration transcriptional events, U-2 OS cells were first infected with NL4.3-*luc* VSV-G and then synchronised by serum shock (**Fig.3.4.A**). HIV-1 replication was either monitored real-time by measuring luminescence every 30 minutes, or cells were harvested at 4 h intervals to quantify HIV-1 Gag RNA by qPCR. We demonstrated rhythmic HIV-1 replication (**Fig.3.4.B**) with a period of 24.7 h and peak viral replication at circadian time 12.2 h ($p < 0.00001$, Fast Fourier Transform Non-linear Least Squares analysis FFT-NLLS (Straume et al., 2002) using BioDare2 (Zielinski et al., 2014)). This is consistent with an endogenous free-running period of ~24 hours (± 2 h) which is characteristic for circadian rhythms.

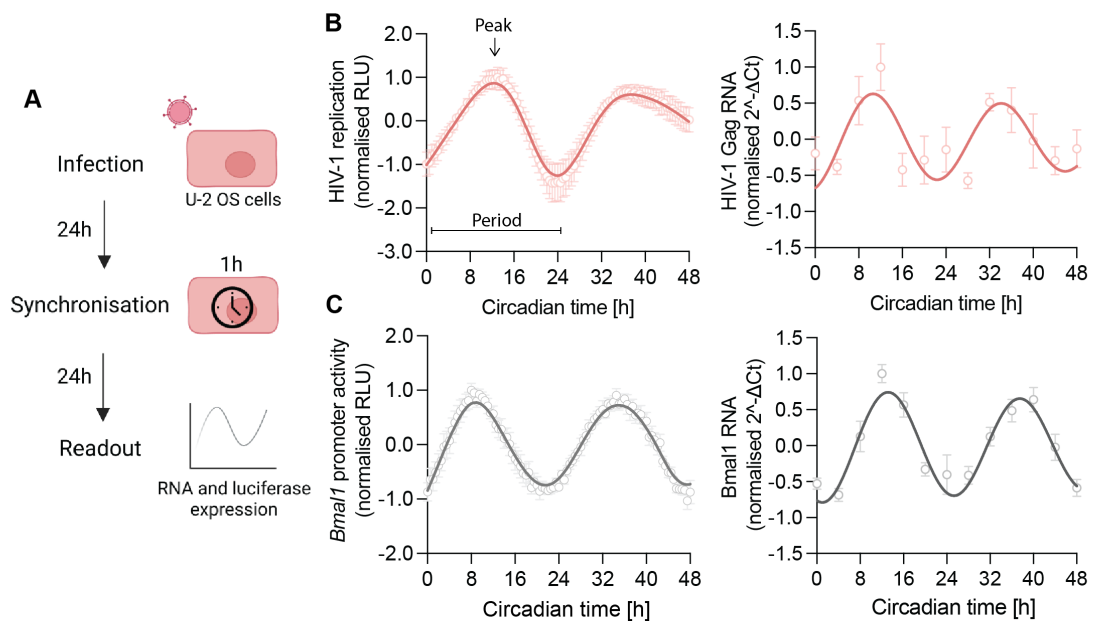


Figure 3.4. HIV-1 replication is rhythmic. A) Schematic of experiment, where U-2 OS cells were infected with NL4.3-*luc* VSV-G for 24 h followed by serum shock. 24 h post synchronisation, luciferase activity was measured or cells harvested for RNA extraction. **B)** U-2 OS cells were infected with NL4.3-*luc* VSV-G, synchronised and HIV-1 replication measured by luciferase activity (mean \pm S.E.M., $n=6$). Cells were harvested at 4 h intervals and HIV-1 Gag transcripts were quantified relative to B2M housekeeper by qPCR (mean \pm S.E.M., $n=4$). Analysis of luciferase data: eJTK cycle $p < 0.00001$, period=24.7 h, peak expression=12.2 h (FFT-NLLS analysis, BioDare2). **C)** U-2 OS cells stably expressing *Bmal1* promoter-driven luciferase were synchronised and promoter activity measured at 30 min intervals (mean \pm S.E.M., $n=7$). Wild-type U-2 OS cells were synchronised, harvested every 4 h, followed by qPCR detection of *Bmal1* RNA relative to B2M (mean \pm S.E.M., $n=4$). All data are normalised to peak expression.

To compare HIV-1 and Bmal1 rhythmic gene expression we quantified Bmal1 transcripts by qPCR or transduced U-2 OS cells with a *Bmal1* promoter-driven luminescence reporter. Notably, *Bmal1* promoter activity and transcript levels displayed a similar period and peak expression to HIV-1 replication (**Fig.3.4.C**).

Overall, we showed rhythmic HIV-1 replication in a circadian synchronised model system, suggesting a role for the host cellular circadian machinery in regulating HIV-1 infection.

3.3. Discussion

In this chapter we showed that U-2 OS cells are a suitable *in vitro* model system for HIV-circadian studies and demonstrated rhythmic viral transcription in synchronised cells. Our attempts to synchronise Jurkat T cells did not result in rhythmic expression of the key circadian activator Bmal1. Previous studies have also failed to synchronise Jurkat cells in conventional 2D tissue culture (Bollinger et al., 2011), however, one study achieved clock driven secretion of luciferase in a continuous flow cell system (Tamayo et al., 2020). The authors used bioengineered cells that secrete a luciferase reporter, combined with an artificial continuous flow cell culture system and synchronisation with dexamethasone, which makes this model suboptimal for our use. However, this indicates that the failure by us and others to detect rhythmic circadian transcript expression in Jurkat T cells *in vitro* does not reflect the absence of circadian rhythms. It is likely that the artificial nature of immortalised immune cell lines causes dampened circadian rhythms which requires sophisticated protocols and equipment for detection. We hypothesise that a similar explanation holds true for the absence of detectable rhythms in the THP-1 monocytic cell line.

Contrary to results in immortalised immune cell lines, it has been shown that immune cells isolated from mice or humans have a cell intrinsic clock which can be detected *in vitro*. Dendritic cells or CD8 T cells isolated from bone marrow of mice expressing a circadian

reporter showed rhythmicity in bioluminescence after serum shock, however, with low amplitude (Nobis et al., 2019). Another study analysed peripheral blood CD4 T cell samples from human volunteers collected at 3 h intervals and showed cycling of key clock gene expression across 24 hours (Bollinger et al., 2011). Thereafter the authors noted a dampening in amplitude that most likely reflects their desynchronisation. Hence, primary T cells are only able to maintain synchrony for a short amount of time *in vitro*, and since our protocol studying rhythmic HIV-1 replication takes 4 days we might have exceeded this time window. An additional explanation for why we did not detect rhythmic Bmal1 expression could be the heterogeneity of the CD8 depleted PBMC population which includes CD4 T cells, macrophages and dendritic cells. We opted to use this population (instead of a single cell type like CD4 T cells only) to maximise infection and to reflect a natural composition of all HIV-1 target cells. However, circadian rhythms in each cell type may vary in their period which leads to desynchronisation of the population in the absence of entrainment signals and could have complicated the detection of rhythmic gene expression.

U-2 OS cells are a well-established model for circadian research and once these cells grow to form confluent monolayers, contact inhibition reduces circadian rhythms in cell cycle activity. While U-2 OS cells have been reported to support HIV infection (Di Primio et al., 2013; Zhang and Bieniasz, 2020), they are not a commonly used model and we first confirmed that they support viral infection. We observed rhythmic HIV-1 replication when measuring luciferase activity of a reporter virus and detecting HIV-1 Gag RNA transcripts. We selected to quantify Gag since it is an essential HIV-1 protein and hence indicative of functional viral replication (Freed, 1998; Bell and Lever, 2013).

Peripheral HIV-1 RNA levels showed oscillations in patients (Stern et al., 2022). Similarly, recent reports have indicated rhythmicity of Influenza and Herpesvirus in animal models (Edgar et al., 2016; Sengupta et al., 2019). However, on a whole organism level the virus is subjected to oscillations of the host immune system, rhythms of physiological parameters and

external cues which can influence viral replication dynamics. We showed oscillations in viral replication in a simple system of synchronised cells in tissue culture, which demonstrates that the cell intrinsic clock is sufficient for rhythmic HIV-1 replication in absence of external stimuli.

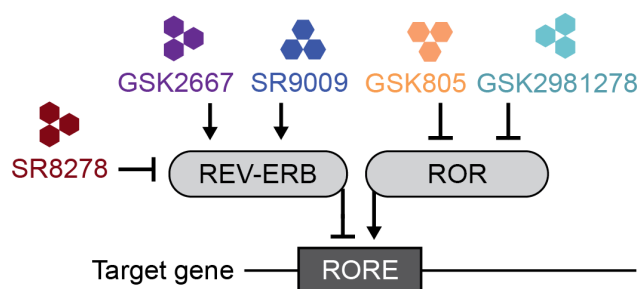
To our knowledge, this is the first time rhythmic viral transcription has been monitored real-time *in vitro*, demonstrating the importance of the cell intrinsic clock and highlighting a role for the host circadian machinery in regulating HIV-1.

4. Pharmacologically modulating ROR and REV-ERB alters HIV-1 replication

4.1. Introduction

Intrigued by the finding of rhythmic HIV-1 replication *in vitro*, we hypothesised a role for circadian transcription factors in regulating viral transcription. Pharmacological agents targeting core circadian factors provide tools to study the underlying mechanism(s). Several synthetic ligands targeting core clock proteins (mainly CRYs (Hirota et al., 2012), REV-ERBs (Solt et al., 2012; Wang et al., 2020) or RORs (Kojetin and Burris, 2014)) have been reported (Ribeiro et al., 2021), while BMAL1 is currently considered undruggable.

The physiological ligand of REV-ERB is heme which is required for recruiting co-repressors to regulate gene expression (Raghuram et al., 2007), and multiple synthetic ligands targeting REV-ERB have been developed (**Fig.4.1**). The first REV-ERB small molecule ligand was GSK4112, which enhanced the recruitment of Nuclear Co-Repressor 1 (NCoR1) and induced circadian phase shifts in cellular assays (Meng et al., 2008). However, due to poor pharmacokinetic properties this compound is not suitable for *in vivo* work. Another agonist, SR9009, was developed based on the structure of GSK4112 and acts in a similar manner by enhancing the recruitment of NCoR1 (Solt et al., 2012). It is one of the best characterised small molecules targeting REV-ERB: *In vitro* it causes a decrease in amplitude of key clock genes like *Per2* and disrupts the cellular circadian machinery. *In vivo*, a reduction in locomotor activity and increased energy expenditure was observed when treating mice with the agonist (Solt et al., 2012). A further compound is GSK2667, which showed similar pharmacokinetic profiles to that of SR9009, but with improved potency and selectivity (Trump et al., 2013). A REV-ERB antagonist, SR8278, was reported to activate REV-ERB target genes in cellular assays by blocking interaction with the endogenous REV-ERB ligand heme (Kojetin et al., 2011).



Compound	Target	Effect	Mechanism	Reference
SR8278	REV-ERB α/β	Inhibition	Blocks interaction with endogenous ligand heme	Kojetin et al., 2011
SR9009	REV-ERB α/β	Activation	Increases recruitment of of NCoR	Solt et al., 2012
GSK2667	REV-ERB α	Activation	Increases recruitment of of NCoR	Trump et al., 2013
GSK2981278	RORC	Inhibition	Inhibits interaction and recruitment of co-activators	Smith et al., 2016
GSK805	RORC	Inhibition	Interaction with ligand binding domain blocks transcriptional effects	Xiao et al., 2014

Figure 4.1. Small molecule ligands targeting REV-ERB and ROR. The antagonist SR8278 inhibits REV-ERB α/β activity, while the agonists GSK2667 and SR9009 activate REV-ERBs. The inverse agonists GSK805 and GSK2981278 both inhibit RORC activity, respectively. REV-ERB and ROR compete for binding to ROREs in promoter regions of target genes, whereby REV-ERB suppresses and ROR activates transcription.

REV-ERB and ROR have similarities in their DNA-binding domains (Kojetin and Burris, 2014), however, their ligand domains interact with different molecules: ROR binds oxysterols, cholesterol and cholesterol sulphate (Jetten, 2009). Various small ligands targeting different ROR isoforms have been identified. RORC is the master transcription factor regulating Th17 cell differentiation, and compounds specific for RORC have been shown to modulate Th17 cell transcriptional networks (Solt et al., 2011). The small molecule GSK805 was identified to directly and reversibly interact with the ligand binding domain of RORC2 to block its transcriptional activity by disrupting its interaction with currently unidentified ligands. It is thought to affect recruitment of coregulators to the RNA polymerase machinery, thereby modulating the Th17 cell transcriptome (Xiao et al., 2014). Interestingly, Th17 cells are highly permissive to HIV-1 infection, and GSK805 was shown to suppress viral replication *in vitro* (Wiche Salinas et al., 2021). Another RORC inhibitor, GSK2981278, was identified for topical

treatment of psoriasis (Smith et al., 2016) and evaluated as safe in a stage I clinical trial (Kang et al., 2018). Moreover, GSK2981278 interfered with binding of RORC2 to the IL-17 α promoter that was induced by gut bacterial extracts of patients with arteriosclerotic cerebral small vessel disease (Cai et al., 2021). Despite the extensive characterisation of RORC inhibitors in the immune-modulatory context (Xiao et al., 2014; Withers et al., 2016; López-Posadas et al., 2019), these compounds have not been studied for their regulation of the cellular circadian machinery. Generally, our knowledge of how ROR inverse agonists impact the circadian clock or perturb rhythmic HIV replication is limited (Takeda et al., 2012; Ribeiro et al., 2021).

In this chapter we tested the REV-ERB modulators GSK2667, SR9009, SR8278 and ROR inverse agonists GSK805 and GSK2981278 (**Fig.4.1**) for their ability to alter clock gene expression and HIV-1 replication. The REV-ERB antagonist increased HIV-1 transcription while agonists inhibited viral replication and HIV-LTR activity of multiple HIV-1 clades. We characterised the perturbation of the cellular circadian machinery caused by RORC inverse agonists and demonstrated inhibition of rhythmic viral replication. Altogether, this highlights a role of the circadian machinery in regulating HIV-1 replication and provides a rationale for future work to identify novel circadian modulators with anti-viral properties.

4.2. Results

4.2.1. Pharmacological modulation of REV-ERB alters HIV-1 replication

We first sought to investigate the effect of REV-ERB modulators on HIV-LTR activity and to define the kinetics of drug treatment. Initial experiments were performed with the TZM-bl HeLa cell line, which encodes integrated copies of HIV-LTR that drive luciferase gene expression in the absence of Tat (Aguilar-Cordova et al., 1994). The REV-ERB agonists SR9009 and GSK2667 inhibited HIV-LTR activity, whereas the antagonist SR8278 increased promoter activity in a dose dependent manner (**Fig.4.2.A**). To evaluate the optimal timeframe of drug exposure, treatment length of 4 h, 8 h, and 24 h were evaluated and demonstrated peak

activity after 24 hours for all compounds (**Fig.4.2.B**). Combined treatment of agonists SR9009 or GSK2667 with the antagonist SR8278 had a modest effect compared to single treatments (**Fig.4.2.C**). This validates the specificity of these drugs and demonstrates that the compounds are targeting a common molecular pathway as predicted.

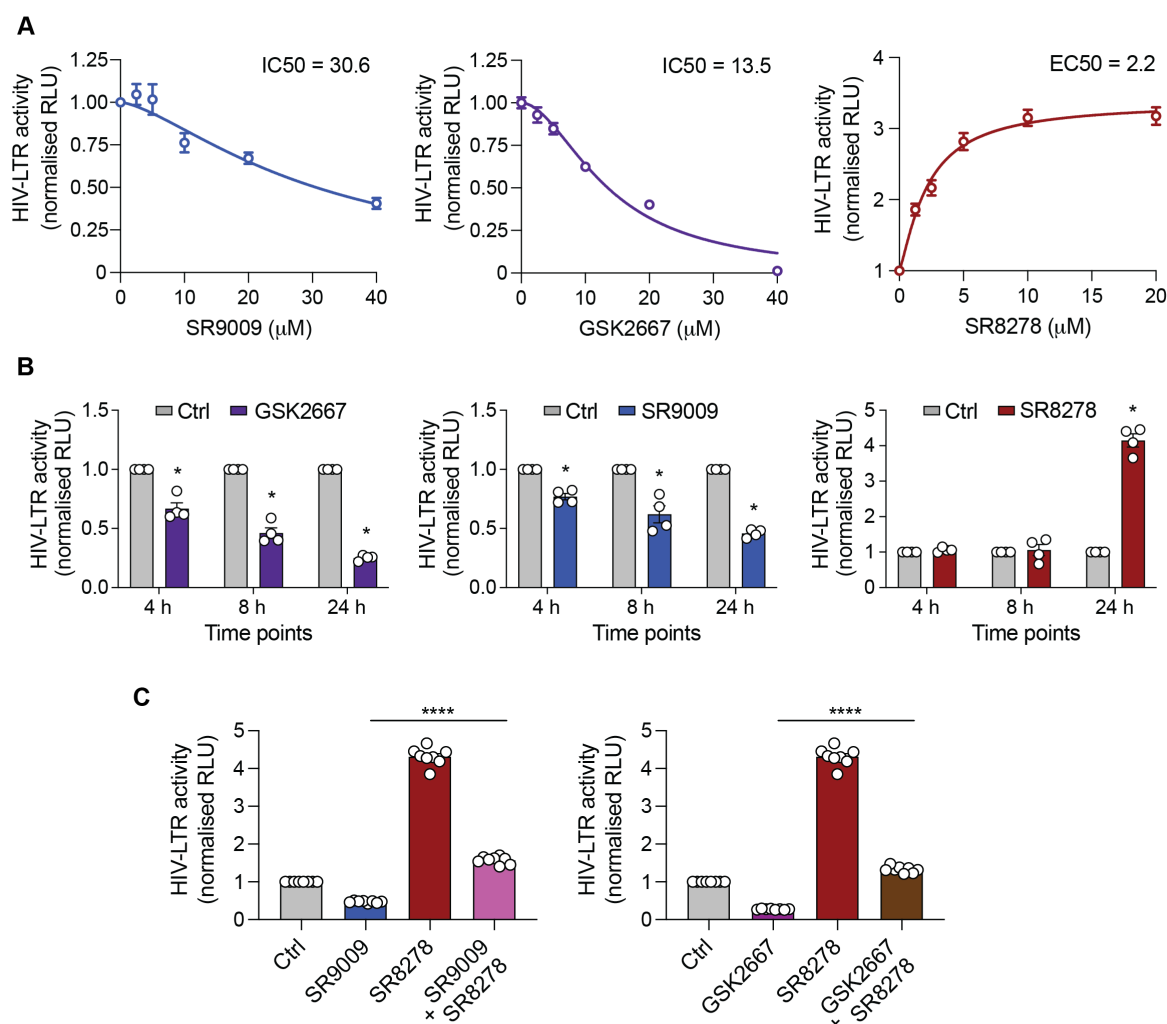


Figure 4.2. REV-ERB agonists and antagonist modulate HIV-LTR activity. **A)** TZM-bl cells were treated with REV-ERB agonists SR9009, GSK2667 or antagonist SR8278 for 24 h and luciferase activity was measured as readout for HIV-1 promoter activity (mean \pm S.E.M., $n=4$). Half maximal inhibitory concentration (IC_{50}) and effective concentration (EC_{50}) was quantified. **B)** TZM-bl cells were incubated with 20 μM of each drug for 4, 8, or 24 h and HIV-LTR activity was quantified (mean \pm S.E.M., $n=4$, Mann-Whitney test). **C)** TZM-bl cells were incubated for 24 h with 20 μM SR9009, GSK2667 or SR8278 alone, or combined treatment of antagonist with each agonist was performed. HIV-1 promoter activity was measured by quantifying luciferase activity (mean \pm S.E.M., $n=8$, Kruskal-Wallis ANOVA). All data are expressed relative to the control untreated cells. *Experiments and analysis performed in collaboration with Dr. Zhuang.*

To extend these observations to more physiologically relevant cell types we used Jurkat T cells infected with VSV-G complemented HIV-1 NL4.3-*luc*. The antagonist SR8278 increased HIV-1 replication, whereas SR9009 lead to a dose dependent reduction in luciferase activity (**Fig.4.3.A**). As a control, we show that the synthetic ligands regulate *Bmal1* promoter activity in these cells as expected (**Fig.4.3.B**). Reassuringly, these findings were repeated in activated primary human CD4 T cells (**Fig.4.3.C**) and macrophages derived from human induced pluripotent stem cells (iPSC, **Fig.4.3.D**).

To investigate the drug potency to reduce authentic HIV infection, Jurkat cells and activated primary CD4 T cells were infected with a patient-derived subtype B HIV (CH058). Following treatment with SR9009, steady-state HIV-1 RNA levels were reduced compared to the control (**Fig.4.3.E**). HIV-1 replication in CD8 depleted PBMCs was assessed by quantifying secreted p24 antigen, and SR9009 reduced antigen levels over the observed period of 10 days (**Fig.4.3.F**).

Collectively, our findings showed that pharmacological activation of REV-ERB inhibit HIV replication in multiple cell types, whereas REV-ERB inhibition enhances viral replication, respectively.

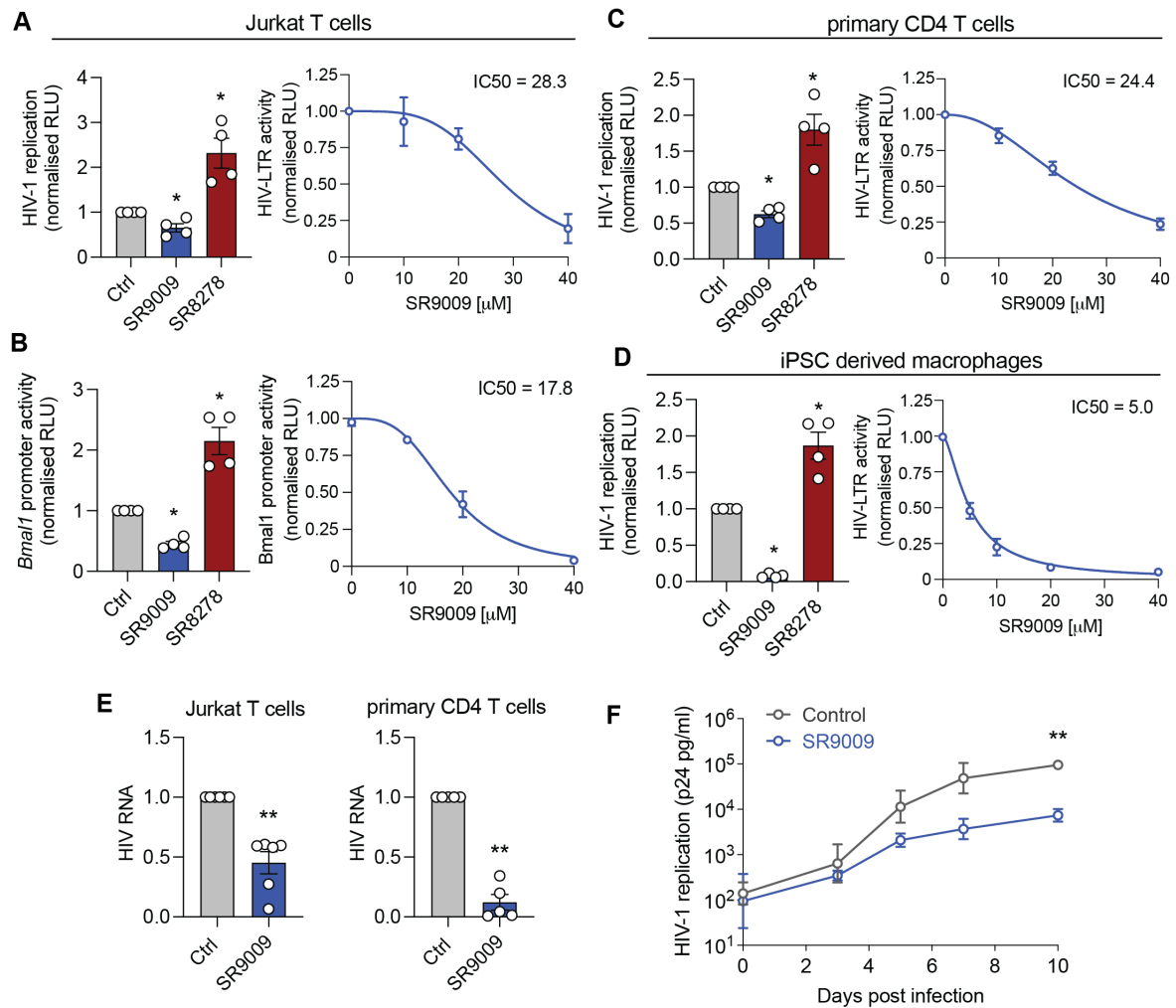


Figure 4.3. Pharmacological modulation of REV-ERB alters HIV-1 replication in immune cells. **A)** Jurkat cells were infected with NL4.3-*luc* VSV-G, treated with 20 μ M SR9009 or SR8278 for 24 h and luciferase was measured as readout of HIV-1 replication. The IC50 of SR9009 was determined 24 h post treatment using a range of doses (mean \pm S.E.M., n=4, Mann-Whitney test). **B)** Jurkat T cells expressing *Bmal1-luc* were treated with 20 μ M SR9009 or SR8278, or a range of doses of SR9009 for 24 h, *Bmal1* promoter activity was quantified and the IC50 of SR9009 calculated (mean \pm S.E.M., n=4, Mann-Whitney test). **C)** Primary CD4 T cells were isolated from PBMCs, activated for 3 days with anti-CD3/CD28, infected with NL4.3-*luc* VSV-G and incubated with 10 μ M (or indicated concentrations) of SR9009 or SR8278 for 24 h. Luciferase activity was quantified as readout for viral replication and the IC50 was calculated (mean \pm S.E.M., n=4, Mann-Whitney test). **D)** Human iPSC derived macrophages were infected with NL4.3-*luc* VSV-G and treated with SR9009 (10 μ M) or SR8278 (5 μ M) for 24 h. Luciferase was measured and the IC50 of SR9009 was determined 24 h post treatment using a range of doses (mean \pm S.E.M., n=4, Mann-Whitney test). **E)** Jurkat cells or activated primary CD4 T cells were infected with patient derived HIV-1 for 72 h. Infected cells were cultured in medium with or without SR9009 and viral RNA quantified 48 h later (mean \pm S.E.M., n=4, Mann-Whitney test). **F)** Activated CD8 depleted PBMCs were infected with patient derived HIV-1 and cultured in medium with or without SR9009. Extracellular p24 levels were measured at intervals across 10 days (mean \pm S.E.M., n=3, Paired t test). All data are expressed relative to UT control. *Experiments and analysis performed in collaboration with Dr. Zhuang.*

4.2.2. ROR inhibition disturbs rhythmicity of host clock factors and HIV-1 replication

RORC is an important transcription factor for circadian regulation, and analysing the promoter regions of core circadian genes *Bmal1*, *Rev-erb α* , *Per1*, and *Cry2* revealed the presence of RORC specific binding motifs (**Fig.4.4.A**). Several drugs targeting ROR have been described, and we aimed to characterise the clock modulating properties of RORC inverse agonist GSK805.

We first established that the RORC isoform is expressed in U-2 OS and Jurkat T cell lines (**Fig.4.4.B**). Treating synchronised U-2 OS cells expressing *Bmal1-luc* luciferase with different doses of GSK805 showed a dose-dependent dampening of rhythmic *Bmal1* promoter activity. Analysis revealed a 36.1% reduction in amplitude for 3 μ M and 63.9% reduction at 10 μ M compared to control cells, respectively (**Fig.4.4.C**). To validate the perturbation of clock genes on a transcriptional level, synchronised U-2 OS cells were treated with the inverse agonist and harvested at 4 h intervals. Gene expression analysis revealed the reduction in the rhythmic expression of *Bmal1*, *Rev-erb α* , *Per1*, and *Cry2*, consistent with the RORC binding elements in their promoter regions (**Fig.4.4.D**). Reassuringly, GSK805 treatment showed a dose-dependent inhibition of *Bmal1* promoter activity and protein expression in Jurkat cells (**Fig.4.4.E**). To extend our observations to primary cells, we treated CD8 depleted PBMCs with the RORC inhibitor and showed a reduction in *Bmal1*, *Rev-erb α* , *Per1*, and *Cry2* transcripts (**Fig.4.4.F**).

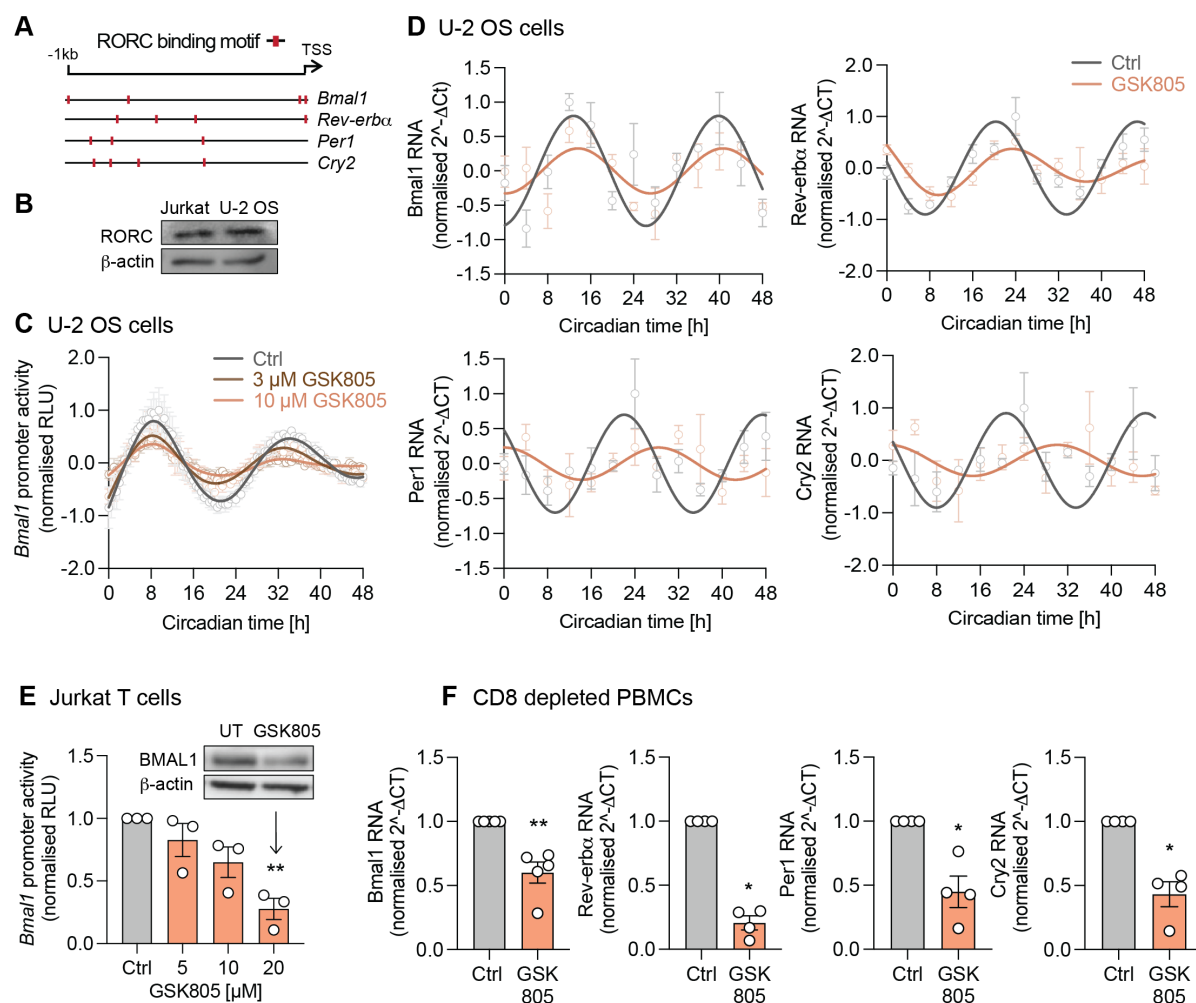


Figure 4.4. ROR inverse agonist GSK805 perturbs the cellular clock. **A)** Presence and location of RORC binding motif in *Bmal1*, *Rev-erbα*, *Per1* and *Cry2* promoter (1kb downstream of TSS) was identified with the Eukaryotic Promoter Database and is indicated by red symbols. **B)** Jurkat and U-2 OS cells were lysed and RORC or β -actin expression was determined by western blotting (representative of $n=2$). **C)** U-2 OS cells expressing *Bmal1-luc* were synchronised by serum shock and incubated with GSK805 at indicated concentrations. Luminescence as readout for *Bmal1* promoter activity was recorded at 30 min intervals starting 24 h after serum shock (mean \pm S.E.M., $n=3$, raw data in Figure 2.2.A). Amplitude reduction compared to control: 36.1% for 3 μ M and 63.9% for 10 μ M, as determined by FFT-NLLS analysis using BioDare2. **D)** U-2 OS cells were synchronised and treated with GSK805 (10 μ M). 24 h post synchronisation, cells were harvested at 4 h intervals and transcript levels of *Bmal1*, *Rev-erbα*, *Per1* and *Cry2* relative to B2M housekeeper were quantified by qPCR (mean \pm S.E.M., $n=3$). **E)** Jurkat cells expressing *Bmal1-luc* were treated with different doses of GSK805 for 24 h, and luciferase activity was quantified (mean \pm S.E.M., $n=3$, Kruskal-Wallis ANOVA). Jurkat cells were treated with GSK805 (20 μ M), and BMAL1 and β -actin protein expression was assessed by western blotting (representative of $n=3$). **F)** Activated CD8 depleted PBMCs were treated with GSK805 (2.5 μ M) for 7 days and *Bmal1*, *Rev-erbα*, *Per1*, or *Cry2* RNA levels were detected by qPCR relative to B2M housekeeper (mean \pm S.E.M., $n=4-5$, Mann-Whitney test). All data are expressed relative to UT control or normalised to peak expression.

Intrigued by the disruption of the cellular circadian system caused by GSK805, we hypothesised this compound would inhibit rhythmic HIV-1 replication. Quantifying luciferase as a readout for viral replication and detection of viral RNA at 4 h intervals revealed dampened rhythmic HIV-1 replication in drug treated U-2 OS cells (**Fig.4.5.A**).

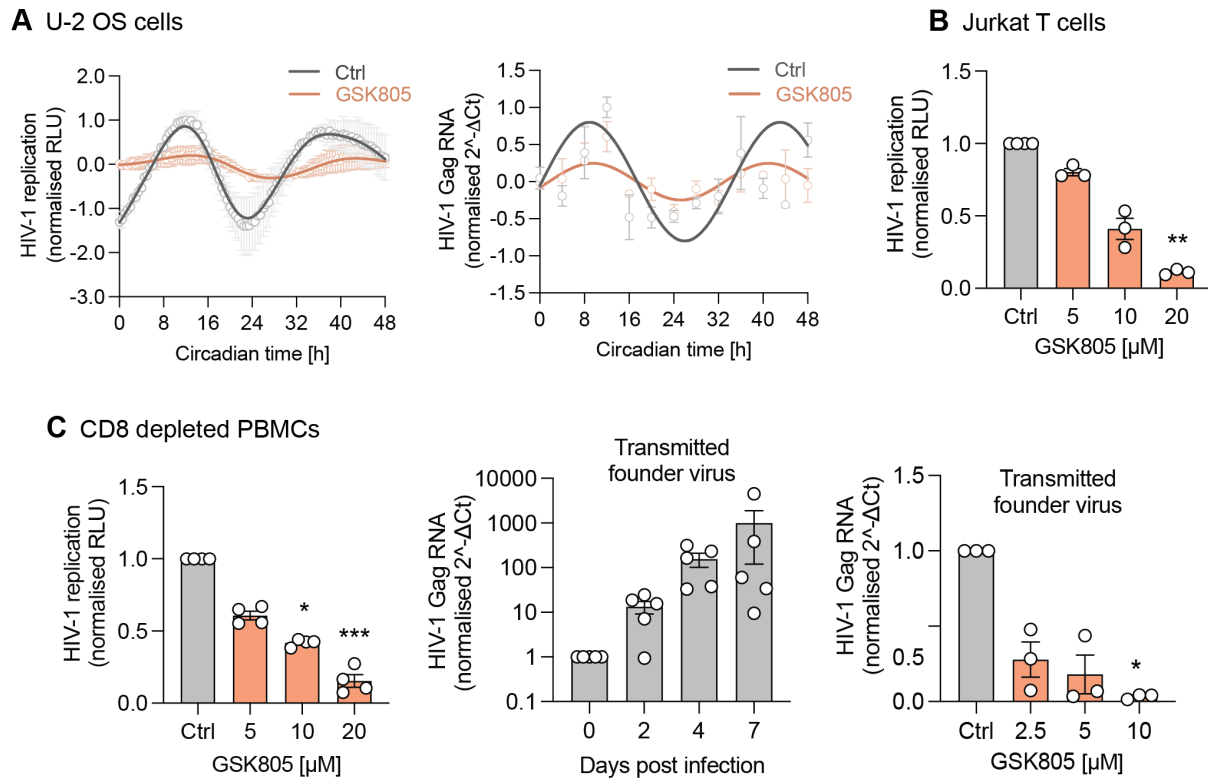


Figure 4.5. Rhythmic HIV-1 replication is reduced by ROR inverse agonist. A) U-2 OS cells were infected with HIV-1 NL4.3-*luc* VSV-G, synchronised by serum shock and media supplemented with 10 μM GSK805. 24 h post synchronisation, luciferase was measured every 30 min or cells were harvested at 4 h intervals to quantify HIV-1 Gag RNA relative to housekeeper B2M (mean \pm S.E.M., $n=3$, normalised to peak expression, raw data in Figure 2.2.B). **B)** Jurkat cells were infected with NL4.3-*luc* VSV-G, treated with different doses GSK805 and HIV-1 replication was quantified by luciferase measurement (mean \pm S.E.M, $n=3-4$, Kruskal-Wallis ANOVA). **C)** Activated CD8 depleted PBMCs were infected with either NL4.3-*luc* VSV-G or patient derived transmitted founder virus. HIV-1 replication of NL4.3-*luc* infected cells was quantified after 24 h treatment with GSK805 at different concentrations (mean \pm S.E.M, $n=3-4$, Kruskal-Wallis ANOVA). Transmitted founder virus replication was measured by quantification of HIV-1 Gag RNA levels relative to B2M housekeeper across 7 days in untreated cells (mean \pm S.E.M, $n=5$). Transmitted founder virus infected cells were cultured in the presence of GSK805 and HIV-1 Gag RNA was quantified at day 7 post infection (mean \pm S.E.M., $n=3$, Kruskal-Wallis ANOVA). All data are expressed relative to UT control.

Importantly, the inverse agonist also inhibited HIV-1 NL4.3-*luc* replication in Jurkat cells (**Fig.4.5.B**) and CD8 depleted PBMCs (**Fig.4.5.C**). CD8 depleted PBMCs were infected with

a patient-derived founder strain of HIV-1 (CH185) and the anti-viral activity of GSK805 was reproduced (**Fig.4.5.C**).

Overall, we demonstrate that the RORC inverse agonist GSK805 perturbs rhythmic gene expression of several key circadian genes and HIV-1 replication.

4.2.3. An additional ROR inhibitor reduces Bmal1 expression and HIV-1 replication

To validate our findings with a chemically different ROR inverse agonist we selected GSK2981278, which has not been studied in the context of circadian biology or HIV-1 replication. GSK2981278 treatment inhibited rhythmic *Bmal1* promoter activity in synchronised U-2 OS cells and reduced the amplitude by 63.9% (20 μ M) and 88.9% (40 μ M) compared to the controls (**Fig.4.6.A**). We confirmed that the drug reduced *Bmal1* promoter activity and protein expression in Jurkat T cells (**Fig.4.6.B**). Importantly, we observed a reduction in the level of Bmal1 transcripts in activated CD8 depleted PBMCs (**Fig.4.6.C**).

To investigate the effect of GSK2981278 on HIV-1 replication, Jurkat cells or activated CD8 depleted PBMCs were infected with NL4.3-*Luc* VSV-G and incubated with the compound, which resulted in decreased HIV-1 replication (**Fig.4.6.D**). HIV-1 RNA levels were likewise reduced in CD8 depleted PBMCs infected with a patient derived transmitted founder virus (**Fig.4.6.E**).

Collectively, these findings demonstrate that the RORC inhibitor GSK2981278 disrupts the cellular clock and inhibits HIV-1 replication.

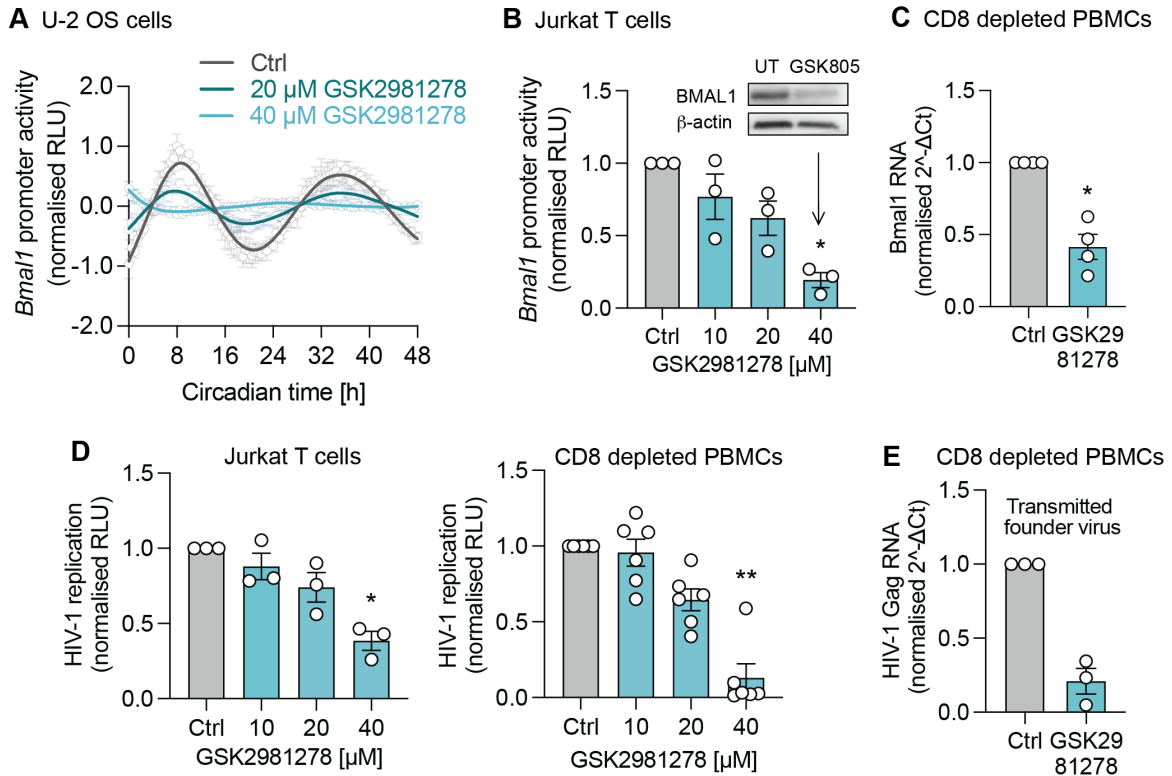


Figure 4.6. Validation of an alternative ROR inhibitor in reducing *Bmal1* expression and HIV-1 replication. **A)** U-2 OS cells expressing the *Bmal1* promoter in front of luciferase were synchronised by serum shock. 24 h later, media was supplemented with GSK2981278 and luciferase recorded at 30 min intervals (mean \pm S.E.M., $n=4$, normalised to peak expression, raw data in Figure 2.2.C). **B)** Jurkat T cells expressing *Bmal1-luc* were treated with different doses GSK2981278 for 24 h and luciferase quantified as readout for *Bmal1* promoter activity. BMAL1 protein levels were analysed by western blotting of Jurkat cells treated with 40 μ M GSK2981278 for 24 h. **C)** Activated CD8 depleted PBMCs were incubated with 20 μ M GSK2981278 for 7 days, RNA was extracted and *Bmal1* expression relative to B2M housekeeper quantified by qPCR (mean \pm S.E.M., $n=4$, Mann-Whitney test). **D)** Jurkat T cells (mean \pm S.E.M., $n=3$, Kruskal-Wallis ANOVA) or activated CD8 depleted PBMCs (mean \pm S.E.M., $n=6$, Kruskal-Wallis ANOVA) were infected with NL4.3-*luc* for 24 h, followed by GSK2981278 treatment for 24 h and lysis for luciferase quantification as readout for viral replication. **E)** Activated CD8 depleted PBMCs were infected with a patient derived transmitted founder virus, cultured in medium with or without 20 μ M GSK2981278 for 7 days, followed by RNA extraction and quantification of HIV-1 Gag RNA levels relative to housekeeper B2M (mean \pm S.E.M., $n=3$). All data are expressed relative to UT control.

4.2.4. REV-ERB and ROR synthetic ligands have pan-subtype anti-viral activity

REV-ERBs and RORs are known to regulate gene expression through binding ROR response elements in promoter regions of genes. We and others detected a RORE motif in the HIV-LTR which is highly conserved among many HIV-1 sequences (**Fig.4.7.A**) (Orchard et al., 1993; Ladas, 1994; Lee et al., 1994). To analyse pan-subtype anti-viral activity of REV-ERB and

ROR drugs, we selected a panel of HIV LTR-*luc* reporter plasmids of representative strains for the major HIV-1 clades A-G which encode a RORE in their promoter region (**Fig.4.7.B**). The representative REV-ERB agonist SR9009 (**Fig.4.7.C**) and RORC inverse agonist GSK2981278 (**Fig.4.7.D**) both inhibited HIV-LTR activity across all stains in Jurkat cells, demonstrating pan-subtype anti-viral activity.

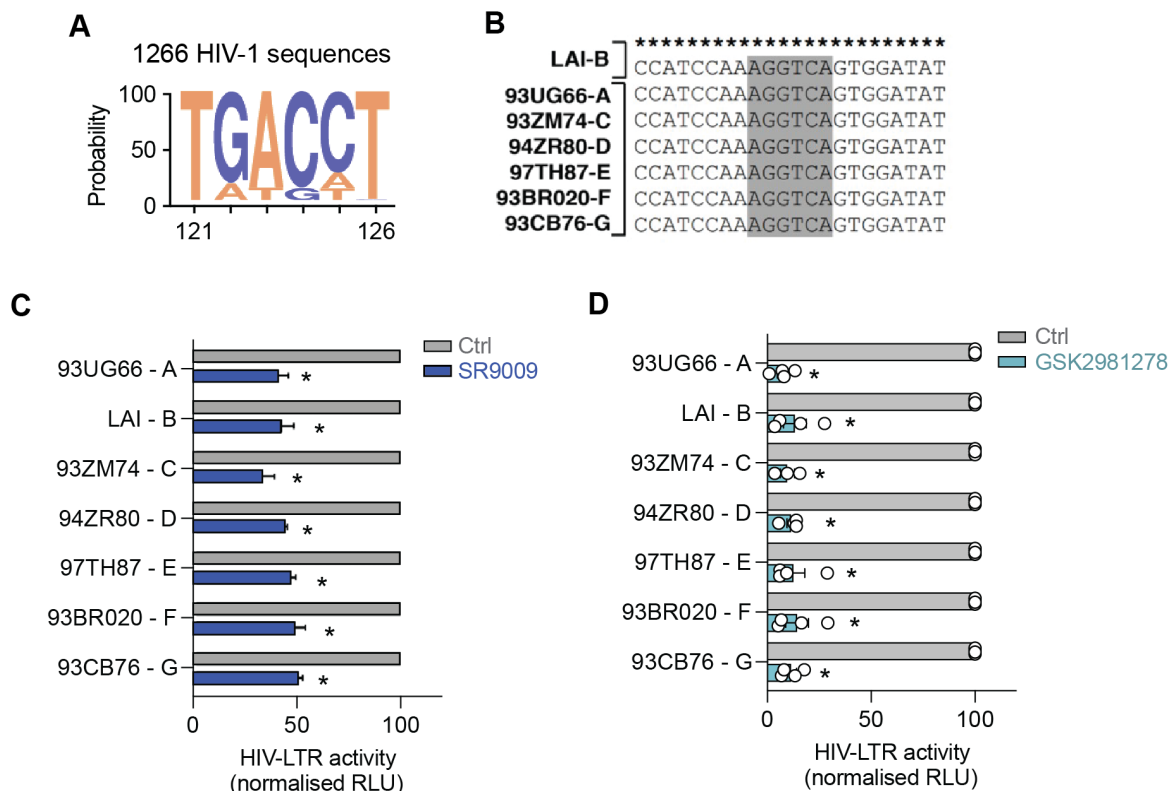


Figure 4.7. REV-ERB agonist and ROR antagonist have pan-subtype anti-viral activity. **A)** Conservation of nucleotides reflected by the height of the bases (y axis 0–100%) in position 121 to 126 of 1266 HIV-1 sequences deposited in the Los Alamos Database (coordinates are from the HXB2 reference). The consensus plot shows bases of the coding strand corresponding to nucleotides 'AGGTCA' on the antisense strand, thereby representing the RORE 'RGGTCA'. **B)** Conserved RORE in representative strains of HIV clades A-G. **C)** Jurkat T cells were transfected with HIV-LTR constructs from HIV-1 subtypes A-G, treated with SR9009 (20 μ M) for 24 h and LTR activity was quantified by measuring luciferase (mean \pm S.E.M., n=4, Mann-Whitney test). **D)** Jurkat T cells transfected with constructs of HIV-LTR sequences from different HIV-1 subtypes were treated with GSK2981278 for 24 h, followed by quantification of luciferase as LTR activity readout (mean \pm S.E.M., n=4, Mann-Whitney test). *Experiments and analysis panel B and C performed by Dr. Zhuang.*

4.3. Discussion

In summary, we showed that REV-ERB modulators, which are well characterised for their effects on the circadian clock, altered HIV-1 replication in TZM-bl, Jurkat T cells, iPSC derived macrophages and primary T cells. We revealed that RORC inverse agonists disturb rhythmicity of circadian host factors and HIV-1 replication in synchronised U-2 OS cells, and inhibit HIV-1 replication in immune cells. The anti-viral properties of REV-ERB agonists and RORC inverse agonists were observed across representative strains for the major HIV-1 clades A-G. Importantly, none of the compounds showed detectable cytotoxicity in any of the cell types used (**Fig.4.8.** and (Borrmann et al., 2020)).

Interestingly, REV-ERB agonists have been shown to inhibit the replication of Flaviviruses (Zhuang et al., 2019). SR9009 inhibited HCV entry into cells and reduced HCV replication as well as Dengue and Zika virus infection. The authors identified that microRNA miR-122 most likely contributes to the anti-viral activity of REV-ERB agonists through regulating cholesterol and lipid metabolism. Additionally, pharmacological activation of REV-ERB inhibited HBV uptake and transcription *in vitro* and in mice models. SR9009 was shown to regulate core clock gene transcripts in human and mouse hepatocytes which contributes to reduced HBV infection (Zhuang et al., 2021a). Moreover, a recent study demonstrated that SR9009 and related analogues inhibited Influenza virus replication by interfering with early stages of viral life cycle and upregulating key anti-viral interferon stimulated genes (ISGs) (Cheng et al., 2022). SR9009 treatment of cells pre and post infection with SARS-CoV-2 reduced viral RNA, suggesting that entry and post-entry steps in the viral life cycle are influenced by the circadian system. Expression of the SARS-CoV-2 receptor ACE2 was reduced upon SR9009 treatment, and the REV-ERB agonist induced the expression of ISGs (Zhuang et al., 2021b). In addition to these studies that highlight the direct anti-viral properties of REV-ERB agonists, multiple other reports demonstrate reduced inflammatory responses (Wang et al., 2021), prevention of cytokine production (Wang et al., 2018a; Guo et al., 2019) and altered autoimmune

responses (Chang et al., 2019). Overall, these studies highlight the anti-viral properties of REV-ERB agonists which are regulated through multiple cellular pathways, reaching from lipid metabolism to inflammatory responses. As with most other viruses, HIV-1 replication relies on these cellular processes. Therefore, research findings in the context of other viruses may also be relevant to the regulation of HIV-1, and vice versa.

However, SR9009 has REV-ERB-independent effects on cell proliferation and metabolism (Dierickx et al., 2019) which highlights that phenotypes observed upon SR9009 treatment should be interpreted with caution. Moreover, although the pharmacological effects of REV-ERB ligands have been well-established in animals, there has been no advancement in their translation to clinical trials.

In contrast, many ROR modulators are in clinical development for their effectiveness in treatment of mainly autoimmune diseases (Sun et al., 2019). The ROR inverse agonist GSK2981278 has been proven safe in a phase I trial against psoriasis (Kang et al., 2018) and GSK805 has been used in mice suggesting that pre-clinical animal models could be developed (Withers et al., 2016). While this indicates general clinical relevance, it is important to note that the therapeutic exploitation of circadian compounds to modulate viral replication needs extensive evaluation and the drugs were used here primarily to probe the mechanism. Small molecules targeting ROR are usually designed to target one of the ROR isoforms, however, all isoforms show high similarities in their structure (Zhang et al., 2015). Our work has focussed on drugs inhibiting RORC since it is most important for Th17 development and HIV infection, and there are limited studies for its effects on circadian rhythms. We revealed that GSK805 perturbs the cellular circadian machinery and our results are consistent with Takeda et al. who observed inhibition of RORC mediated activation of clock gene promoters by other ROR inhibitors (Takeda et al., 2012). Moreover, RORC knockout in mice was shown to decrease the amplitude of circadian oscillations in target genes without changing the phase or the period (Liu et al., 2008), which is also reflected by our findings. We confirmed our results using an

independent RORC inhibitor, GSK2981278, which reduced BMAL1 protein expression, NL4.3 reporter virus and a patient derived founder virus very potently. This suggest that it could be a promising compound to investigate further in the context of both HIV and circadian rhythms.

REV-ERB and ROR are both implicated in cellular processes beyond regulating circadian rhythms, which presents a double-edged sword. While their broad actions are advantageous for treating diseases that involve multiple pathways, pharmacological modulation of these proteins can result in undesired and unforeseen effects (Wang et al., 2020). Given the inversed activity-rest cycle between diurnal humans and nocturnal mice there are concerns that small molecules modulating circadian rhythms may not be efficacious at all in humans although they are in animals. All of this emphasizes the challenges of implementing circadian modifiers in clinical settings, nevertheless, they remain a valuable resource for investigating the circadian regulation in HIV.

Overall, these findings discover novel clock and HIV-1 small molecule regulators which can guide future studies to dissect the underlying mechanisms of circadian regulation of HIV-1.

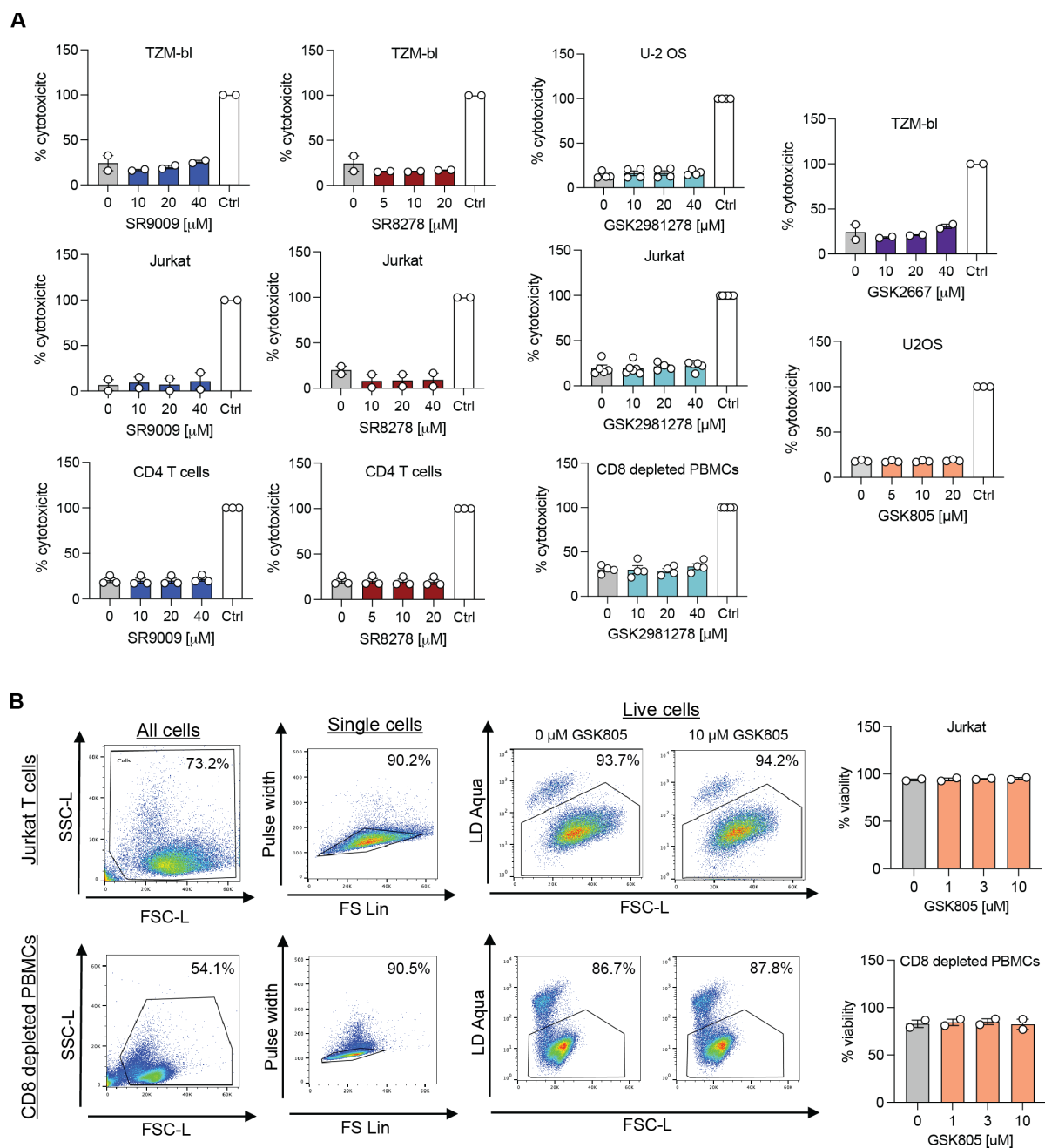


Figure 4.8. Non-cytotoxicity of REV-ERB and ROR compounds for various cell types. A) Toxicity of a range of doses of SR9009, SR8278, GSK2667, GSK805 and GSK2981278 was determined in different cell types 24 h post treatment using an LDH release assay (mean \pm S.E.M., $n=2-6$). Data are expressed relative to the positive control representing total cell lysate (100% cytotoxicity). **B)** Jurkat cells or activated CD8 depleted PBMCs were treated with GSK805 for 24 h, and viability assessed by flow cytometry using Aqua live-dead stain (mean \pm S.E.M., $n=2$). Representative dot plots illustrating gating strategy and analysis (mean \pm S.E.M., $n=2$). SSC = Side Scatter, FSC = Forward Scatter.

5. Circadian transcription factors regulate HIV-1 replication

5.1. Introduction

The observation that compounds targeting the circadian factors REV-ERB and ROR altered HIV-1 replication suggests a role for the cellular circadian machinery in regulating viral transcription, however, it does not explain how. REV-ERB and ROR compete for binding to ROREs in the promoter regions of genes and pharmacological modulation of these factors can directly alter circadian gene expression (**Fig.5.1.A**). Bmal1 expression is regulated by ROREs in its own promoter and altered REV-ERB/ROR activity can modulate Bmal1 expression, which can then modify E-box regulated transcription (**Fig.5.1.B**). HIV-1 transcription is regulated through its long terminal repeat, and interestingly this region harbours multiple circadian motifs. The LTR of the NL4.3 strain encodes one RORE and four E-boxes (**Fig.5.1.C**), which indicates that REV-ERB, ROR and BMAL1 could bind the HIV-LTR and regulate viral replication.

Generally, all viral life stages depend on a myriad of host proteins, and cellular transcription factors which interact with the HIV-LTR are essential to regulate viral transcription (Duttilleul et al., 2020). Several studies have identified factors that positively or negatively influence HIV replication (König et al., 2008; Jäger et al., 2011). Considering the role of circadian factors to regulate the cellular transcriptome and proteome, it is plausible that a number of HIV host factors are subject to regulation by the circadian clock.

This chapter aimed to dissect the role of REV-ERB, ROR and BMAL1 in regulating rhythmic viral replication. We revealed that genetic modulation of REV-ERB or BMAL1 altered viral gene expression and mutation of an E-box motif in the HIV-LTR reduced HIV-1 replication. Chromatin immunoprecipitation demonstrated binding of BMAL1 to the HIV-LTR and we observed differential binding of REV-ERB and ROR to the same motif at different times. We show that HIV-1 host factors are under circadian regulation and likely contributing to

rhythmicity in viral replication. In summary, these findings reveal multiple mechanisms by which circadian transcription factors regulate rhythmic replication of HIV-1.

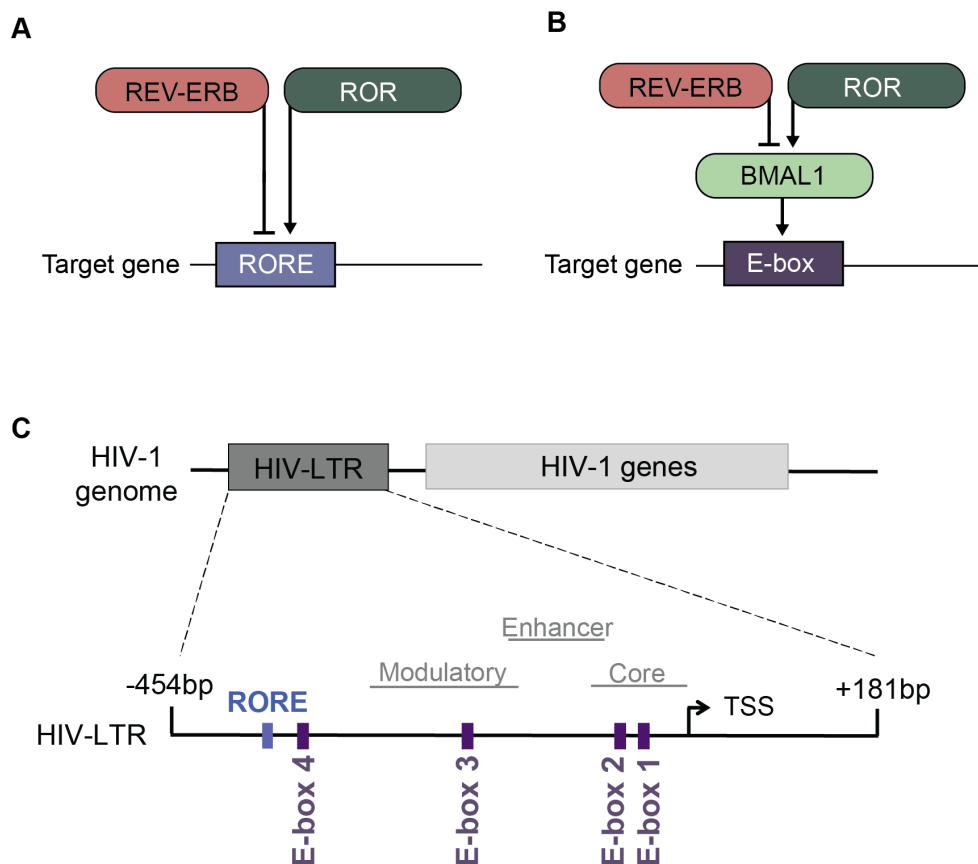


Figure 5.1. Cartoon of transcriptional regulation through circadian motifs in the HIV-LTR. **A)** REV-ERB and ROR are competing for binding to RORE in target genes, whereby REV-ERB is repressing and ROR is activating gene expression. **B)** REV-ERB reduces and ROR enhances *Bmal1* expression through a RORE in the *Bmal1* promoter. BMAL1 is then activating target gene expression through binding to E-box motifs. **C)** The HIV-1 5' LTR is regulating expression of HIV-1 genes, and contains multiple regulatory motifs. Location of base pairs (bp) is shown relative to the TSS in the NL4.3 strain: E-box 1: CAGCTG, E-box 2 & 3: CAGATG, Ebox 4: CAGTTG. RORE: AGGTCA.

5.2. Results

5.2.1. Silencing Rev-erbs and overexpressing Bmal1 activates the HIV-1 promoter

To further evaluate the role of REV-ERB in HIV-1 replication we silenced both isoforms using lentiviral transduction of short hairpin RNAs (shRNAs) in TZM-bl cells. The lentiviral vector encodes *Gfp* which allowed us to confirm transduction efficiency (**Fig.5.2.A**) and knockdown

(KD) of Rev-erb α and Rev-erb β was measured by qPCR (**Fig.5.2.B**). We observed an increase in LTR activity following KD of either REV-ERB isoform (**Fig.5.2.C**), suggesting that REV-ERBs suppress the HIV-1 promoter activity, consistent with our previous findings with REV-ERB agonists and antagonists.

Curious about the presence of E-boxes in the HIV-LTR, we hypothesised a role for BMAL1 in regulating HIV-1 replication and while pharmacologically targeting BMAL1 is not possible, genetic silencing provides a tool to test this. Bmal1 was silenced in U-2 OS cells and KD was confirmed by western blotting. Cells were infected with NL4.3-*luc* VSV-G and real-time monitoring of reporter gene expression showed blunted viral replication in Bmal1 KD cells (**Fig.5.2.D**). To extend this to more physiological cell types, Jurkat T cells were infected with NL4.3-*luc* VSV-G, Bmal1 was silenced or Bmal1 and Clock were overexpressed, and luciferase was measured as readout for viral replication. Consistently, we observed reduced HIV-1 replication in Bmal1 KD cells and increased luciferase activity in Bmal1 OE cells (**Fig.5.2.E**).

Altogether, this demonstrates that REV-ERBs suppress HIV-LTR activity while BMAL1 promotes viral replication.

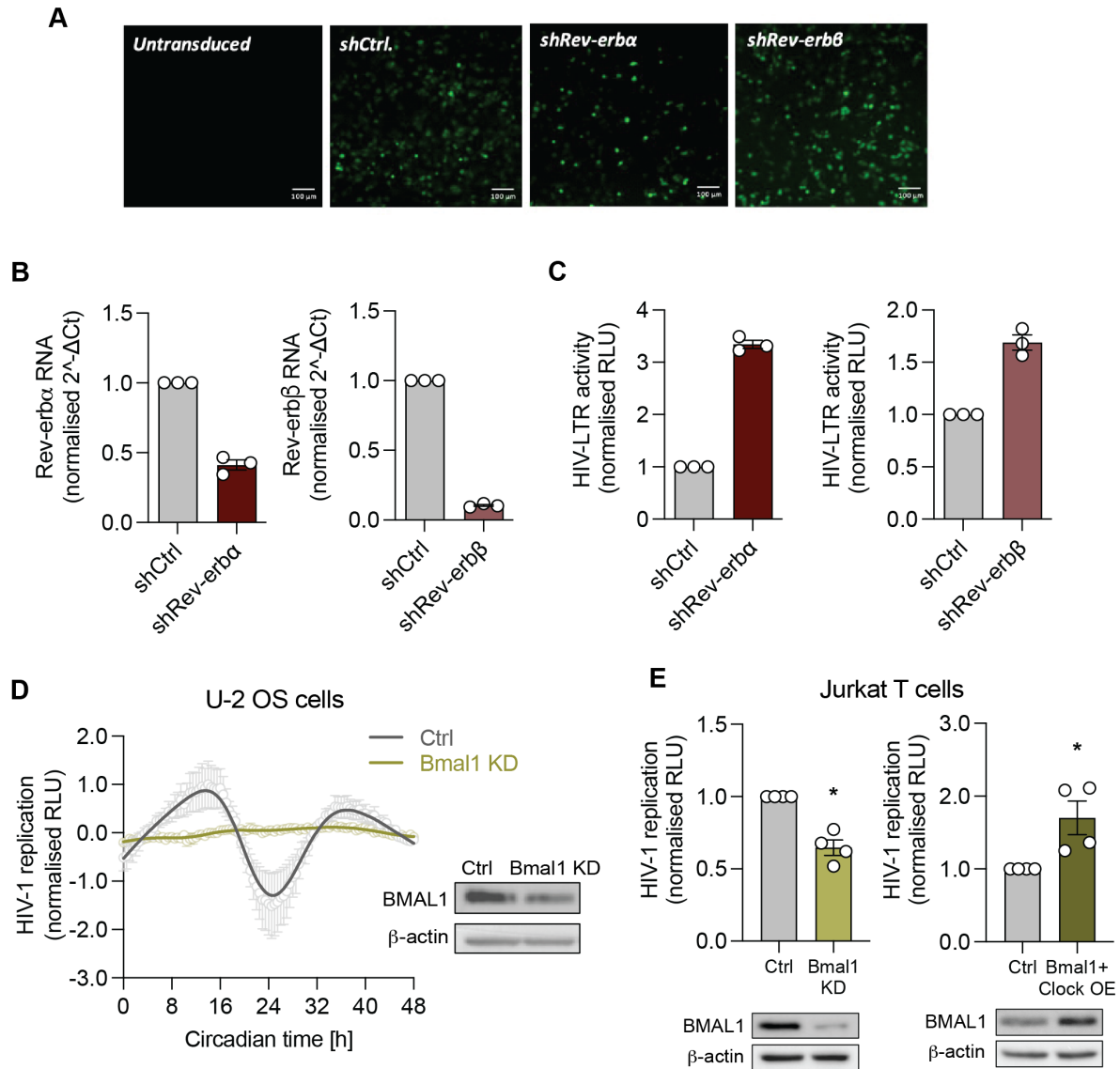


Figure 5.2. Genetic modulation of Rev-erb and Bmal1 expression alters HIV-1 replication.

A) TZM-bl cells were transduced with lentivirus delivering shCtrl, shRev-erb α or shRev-erb β which encode *Gfp*, and transduction was confirmed by acquiring fluorescent images. **B)** Silencing of Rev-erbs was confirmed by detecting transcripts of each isoform by qPCR 48 h post transduction (mean \pm S.E.M., $n=3$). **C)** HIV-LTR activity in TZM-bl cells was quantified by measuring luciferase 48 h after transduction (mean \pm S.E.M., $n=3$). **D)** U-2 OS Bmal1 KD cells were generated by shRNA mediated silencing and KD confirmed by western blotting (representative of $n=2$). Cells were infected with NL4.3-*luc* VSV-G for 24 h, synchronised by serum shock and luciferase measured at 30 min intervals starting 24 h post synchronisation (mean \pm S.E.M., $n=3$, normalised to peak expression, raw data in Figure 2.2.D). **E)** Jurkat T cells were infected with NL4.3-*luc* VSV-G for 24 h. Bmal1 was silenced or Bmal1 and Clock were overexpressed via transfection of respective plasmids. Protein levels were assessed by western blotting (representative of $n=2$) and luciferase activity was measured to detect viral replication 48 h post transfection (mean \pm S.E.M., $n=4$, Mann-Whitney test). All data are expressed relative to the control. Experiments and analysis panel A-C performed by Dr. Zhuang.

5.2.2. BMAL1 binds to E-boxes in HIV-1 promoter

Intrigued by the reduction of rhythmic HIV-1 replication upon *Bmal1* silencing, we investigated the importance of the E-box motifs in the HIV-LTR. We generated constructs encoding the HIV-LTR driving luciferase with 3 different mutations and full deletion of E-box 3, which is highly conserved across 897 HIV-1 sequences available in the LANL repository (**Fig.5.3.A**). Mutation and deletion of the E-box reduced HIV-LTR activity (**Fig.5.3.B**), demonstrating its importance for HIV-1 replication. We hypothesised that BMAL1 can directly bind the HIV-LTR and performed chromatin immunoprecipitation (ChIP) of NL4.3-*luc* VSV-G infect Jurkat cells with BMAL1 antibody or unspecific IgG control. DNA from the immunoprecipitate was amplified by qPCR using primers for the regions encoding E-box motifs in the HIV-LTR and in the known *Per1* promoter control. BMAL1 showed binding to E-boxes in the HIV-LTR and the *Per1* promoter compared to non-specific IgG (**Fig.5.3.C**).

In summary, these findings demonstrate that BMAL1 binds the HIV-LTR, which could contribute to rhythmic HIV-1 replication.

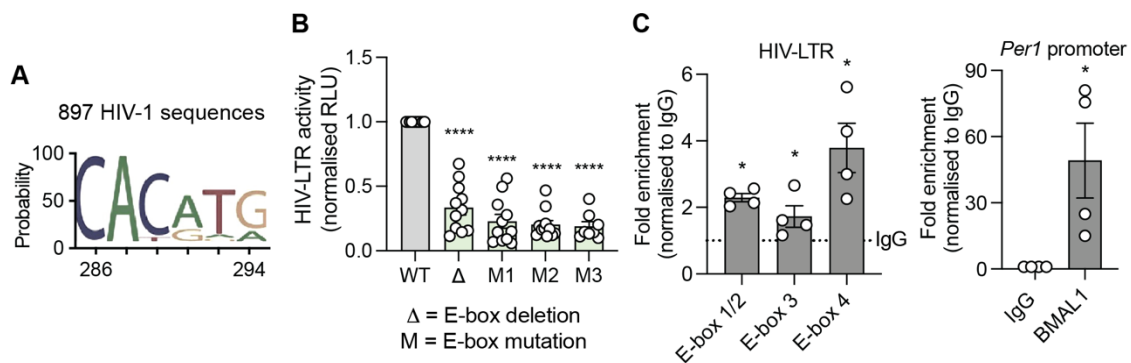


Figure 5.3. BMAL1 binds to E-box motifs in the HIV-LTR. **A)** Conservation of bases in position 286-294 (E-box 3) in the HIV-1 LTR based on the 897 HIV-1 sequences available in the LANL repository (coordinates are from the HXB2 referent, y axis 0–100%). **B)** Jurkat T cells were transfected with and HIV-LTR luciferase reporter construct with mutation or deletion of E-box 3, and luciferase detected 24 h later (mean \pm S.E.M., $n=8-11$, Mann-Whitney test). **C)** Jurkat T cells were infected with NL4.3-*luc* VSV-G for 24 h and chromatin immunoprecipitated with BMAL1 antibody or non-specific IgG control. Fold enrichment of binding to either the E-boxes in the HIV-LTR or to the *Per1* promoter control was detected by qPCR and is shown compared to the IgG control (mean \pm S.E.M., $n=4$, Mann-Whitney test). All data are expressed relative to the control. *Experiments and analysis panel A and B performed by Dr. Zhuang and Dr. Magri.*

5.2.3. Time differential binding of REV-ERB α and RORC to the HIV-LTR

In addition to the presence of E-boxes in the HIV-1 promoter, there is one RORE and it was previously reported to bind RORC (Wiche Salinas et al., 2021). In a circadian context, ROR can compete with REV-ERB for binding to ROREs (Guillaumond et al., 2005), leading us to speculate that REV-ERB may bind the HIV-LTR (**Fig.5.4.A**). We isolated chromatin from HIV-1 infected Jurkat T cells and ChIP-qPCR analysis revealed REV-ERB α binding to the HIV-LTR and its host target *Bmal1* promoter (**Fig.5.4.B**). We hypothesised that if RORC and REV-ERB α compete for binding to the HIV-1 LTR, inhibiting ROR should increase REV-ERB α binding. Infected Jurkat T cells were treated with RORC inverse agonist GSK805 for 24 h, followed by immunoprecipitation and detection of REV-ERB α binding, and we observed an increase in binding to both the HIV-LTR and the *Bmal1* promoter control compared to untreated cells (**Fig.5.4.C**).

To elucidate temporal dynamics of REV-ERB α and RORC binding, we harvested chromatin from infected synchronised U-2 OS cells at CT0 or CT12 and confirmed synchronisation by measuring *Bmal1* and *Rev-erb α* transcripts as well as HIV-1 replication (**Fig.5.4.D**). Binding of REV-ERB α to the HIV-LTR and endogenous *Bmal1* promoter was higher at CT0 than CT12, whereas RORC showed the opposite phenotype (**Fig.5.4.E**). These data align with the observed pattern of rhythmic HIV-1 replication, exhibiting trough viral replication at CT0 and peak at CT12, respectively. We propose a model where binding of the circadian repressor REV-ERB α reduces HIV-1 replication, while RORC promotes HIV-1 replication at alternate circadian times, contributing to rhythmic HIV-1 replication (**Fig.5.4.F**).

Altogether, our findings reveal a temporal interplay between REV-ERB α and RORC binding to the HIV-LTR that plays a crucial role in regulating rhythmic replication of HIV-1.

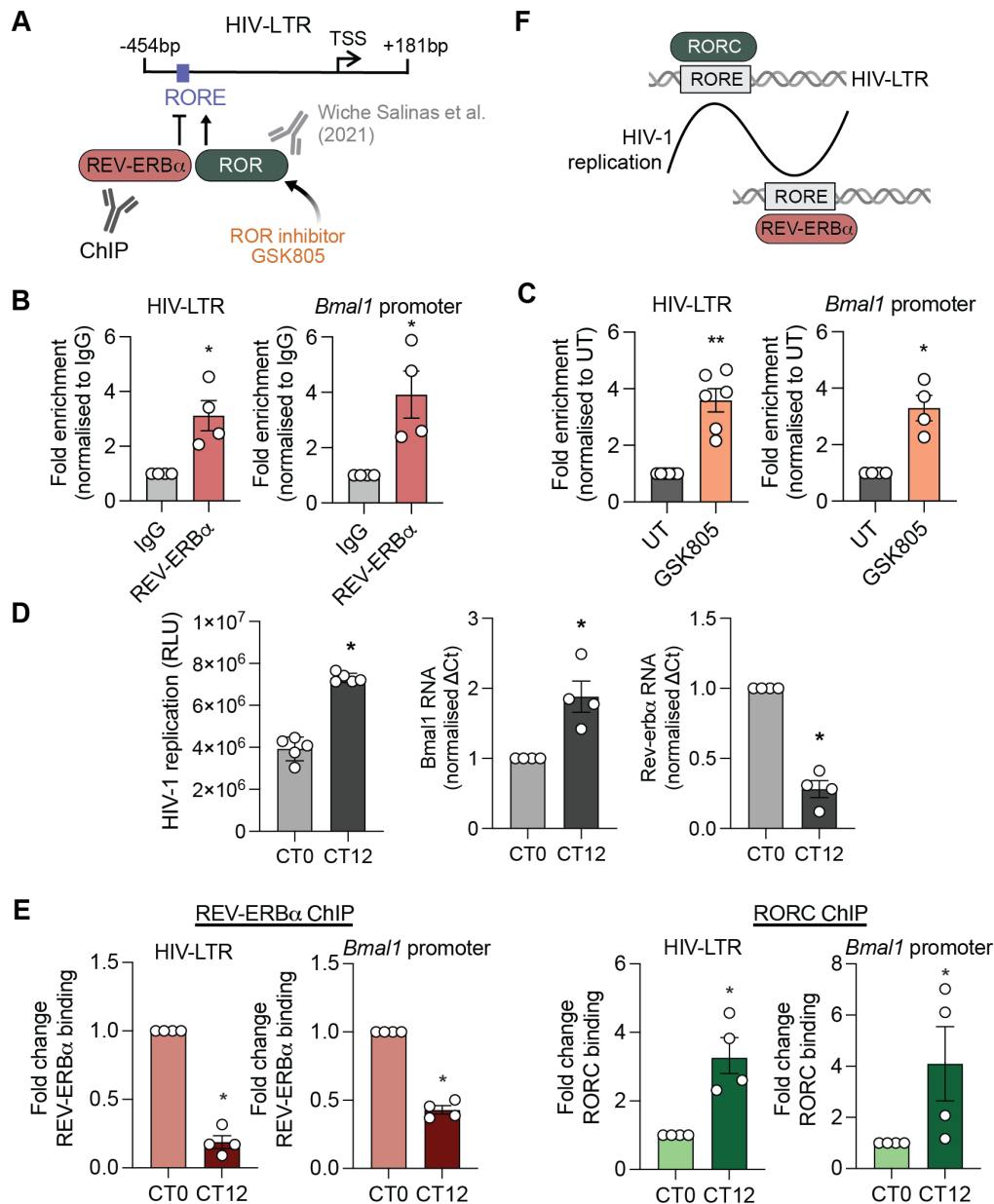


Figure 5.4. Time differential binding of REV-ERB α and RORC to the HIV-LTR. **A)** Model where REV-ERB α and RORC compete for binding to the HIV-LTR RORE. RORC binding was shown by Wiche Salinas et al., 2021. **B)** Jurkat cells were infected with NL4.3-*luc* VSV-G and REV-ERB α binding to the RORE in HIV-LTR or *Bmal1* promoter control was assessed relative to non-specific IgG (mean \pm S.E.M., n=4, Mann-Whitney test). **C)** Jurkat cells infected NL4.3-*luc* VSV-G were incubated with ROR inhibitor GSK805 for 24 h, followed by REV-ERB α ChIP. Binding to the RORE in HIV-LTR or *Bmal1* promoter control is shown relative to the UT control (mean \pm S.E.M., n=4, Mann-Whitney test). **D)** U-2 OS cells infected with NL4.3-*luc* VSV-G, were synchronised and cells lysed 24 h (CT0) and 36 h (CT12) post synchronisation. Luciferase was measured as readout of HIV-1 replication (representative for n=4, \pm S.D, Mann-Whitney test) and *Bmal1* and *Rev-erb α* transcripts quantified by qPCR (mean \pm S.E.M., n=4, Mann-Whitney test). **E)** Synchronised, NL4.3-*luc* VSV-G infected U-2 OS were harvested at CT0 and CT12 for REV-ERB α or RORC ChIP. Fold enrichment of binding was quantified by qPCR (mean \pm S.E.M., n=4, Mann-Whitney test). **F)** Model in which REV-ERB α binding to the HIV-LTR suppresses HIV-1 replication, while RORC binding promotes viral replication at different circadian times, resulting in rhythmic HIV-1 replication (mean \pm S.E.M., n=4, Mann-Whitney test).

5.2.4. HIV-1 host factors are regulated by the cellular circadian machinery

HIV-1 depends on a myriad of host factors for replication (König et al., 2008; Jäger et al., 2011) and we hypothesised that many of these are clock regulated. A recent study identified 90 host factors that regulate HIV-1 replication in human CD4 T cells (Hiatt et al., 2022), and we assessed whether the expression of these factors is rhythmic using the Circa Database (Pizarro et al., 2013). Interestingly, 43% of HIV host factor genes are expressed rhythmically in multiple human tissues (**Fig.5.5.A**).

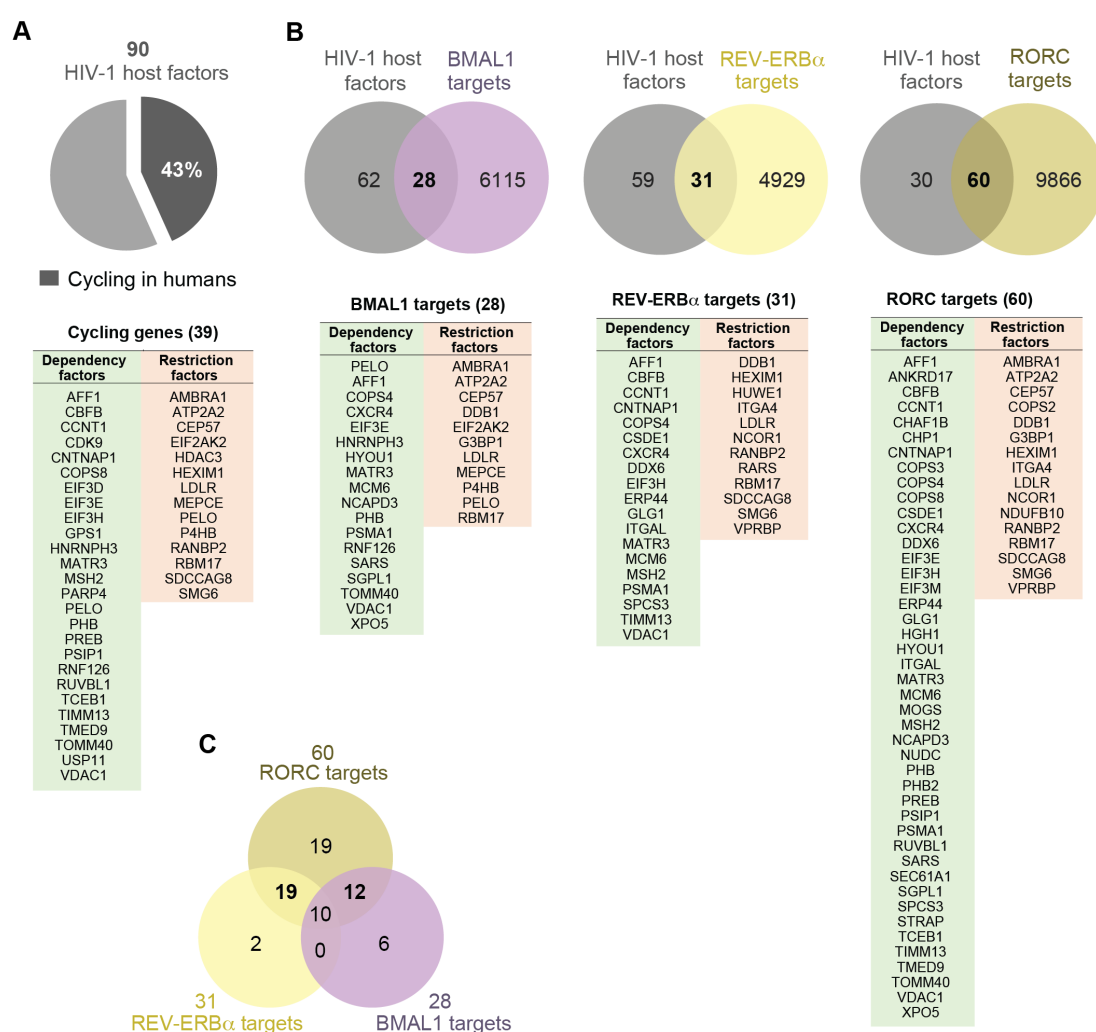


Figure 5.5. HIV-1 host factors are BMAL1, REV-ERB α and RORC targets. A) HIV-1 host factors known to activate or repress HIV-1 replication (dependency factors or restriction factors, Hiatt et al., 2022) were analysed for their rhythmic expression using Circa Database, and 43% of genes were identified as cycling in humans. **B)** HIV-1 host factors (Hiatt et al., 2022) were compared to experimentally validated BMAL1 targets (Beytebiere et al., 2019), REV-ERB α targets (Chang et al., 2019) and RORC targets (Ciofani et al., 2012). **C)** Overlap of HIV-1 host factors that are BMAL1, REV-ERB α or RORC targets.

To extend these conclusions we analysed several experimentally validated ChIP-seq data sets. We analysed the overlap of HIV-1 host factors with BMAL1 regulated genes from the mouse liver (Beytebiere et al., 2019) and REV-ERB α (Chang et al., 2019) or RORC (Ciofani et al., 2012) regulated genes from murine Th17 cells, and revealed 28 BMAL1, 31 REV-ERB α and 60 RORC regulated HIV host factors (**Fig.5.5.B**). Reassuringly, many REV-ERB targets were also regulated by ROR, and there was some overlap with BMAL1 target genes (**Fig.5.5.C**).

To identify those genes that are potentially regulated by BMAL1, REV-ERB, or ROR through the presence of circadian motifs, we analysed the promoter regions of HIV-1 associated host factors using HOMER (Hypergeometric Optimisation of Motif EnRichment tool (Heinz et al., 2010)). We identified 35 genes encoding a RORE ('RGGTCA'), and 74 genes harbouring an E-box ('CANNTG'), out of which 10 genes had the canonical E-box motif ('CACGTG', **Fig.5.6.A**). Since 5 promoters encoded both RORE and E-box motifs, this adds to a total of 40 genes (**Fig.5.6.B**). We hypothesised that anti-viral ROR inverse agonists would reduce expression of HIV-1 dependency factors, and we screened all 40 genes for their response to GSK805 and GSK2981278 in Jurkat T cells. Both compounds reduced the expression of 7 genes: *Csde1*, *Eif3m*, *Ranbp1*, *Skp1*, *Slirp*, *Spcs3* and *Timm13* (**Fig.5.6.C**). Gene ontology analysis was performed to assess their molecular function and connectivity (Ge et al., 2019), and illustrates that these genes are involved in multiple cellular processes, ranging from transcriptional control and protein transport to ubiquitinylation (**Fig.5.6.D**).

Overall, our bioinformatic and experimental analysis demonstrates the circadian regulation of HIV-1 host factors which can be modulated by ROR inhibition and are likely contributing to rhythmicity in HIV-1 replication.

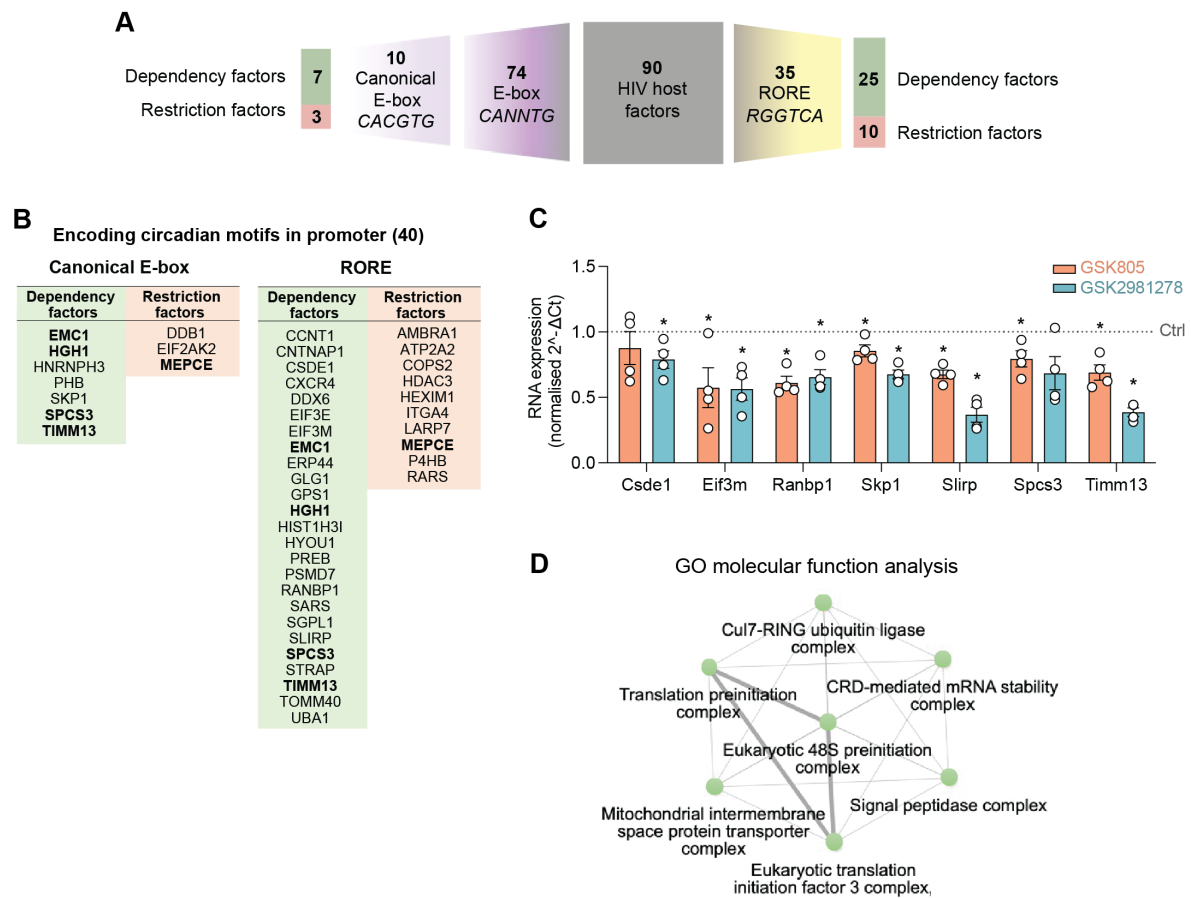


Figure 5.6. HIV-1 host factors encode circadian motifs and expression is modulated by ROR inhibition. **A)** Promoter regions of HIV-1 host factors (Hiatt et al., 2022) were analysed for the presence of the circadian motifs E-box and RORE using HOMER up to -1kb from the TSS. **B)** Gene promoters of HIV-1 host factors encoding a canonical E-box motif 'CACGTG' or RORE 'RCGTCA'. Promoter regions containing both E-box and RORE are written in bold. **C)** Jurkat cells were treated with ROR inverse agonist GSK805 (20 μ M) or GSK2981278 (40 μ M) for 24 h and expression of HIV-1 dependency factors quantified by qPCR. Data are expressed relative to the control (mean \pm S.E.M., $n=4$, Mann-Whitney test). **D)** Gene ontology (GO) molecular function analysis. Each node shows an enriched GO term, and lines connect related GO terms with thickness indicating the percentage of overlapping genes.

5.3. Discussion

In this chapter we showed that REV-ERBs are suppressive while BMAL1 activates HIV-1 transcription through direct binding to the HIV-LTR. We showed temporal dynamics in binding of REV-ERB α and RORC, suggesting competition for binding to the RORE motif in the HIV-

LTR. Moreover, bioinformatic analysis showed that several host factors required for HIV-1 replication are regulated by the circadian clock and may contribute to rhythmic viral replication. To complement our pharmacologically targeting of circadian factors in the previous chapter, we silenced these genes that may result in fewer off-target effects. Silencing of Rev-erb α or Rev-erb β in TZM-bl cells enhanced HIV-LTR activity in the absence of Tat. This indicates a role for REV-ERBs in suppressing HIV-1 promoter activity which is consistent with our earlier observations using REV-ERB antagonists. We did not perform RORC silencing, as it was previously shown to reduce HIV-1 replication in Jurkat and primary CD4 T cells, and the extent of RORC depletion correlated with the reduction of HIV-1 replication which indicates specificity (Wiche Salinas et al., 2021). Consistently, exogenous expression of RORC in HEK293T cells enhanced HIV-1 infection (Wiche Salinas et al., 2021). Overall, these findings are in line with our results of REV-ERB and ROR inhibitors and demonstrate that REV-ERBs repress, and RORC activates HIV-1 replication.

While it is not possible to target BMAL1 with drugs, the genetic approach used in this chapter allowed us to modify Bmal1 expression and revealed that Bmal1 KD reduced rhythmic HIV-1 replication in synchronised U-2 OS cells. Silencing Bmal1 in non-synchronised Jurkat T cells confirmed this phenotype. Bmal1 knockdown is sufficient to cause circadian disruption (Bunger et al., 2000; McDearmon et al., 2006), but to exert its function it requires heterodimerisation with CLOCK (Gekakis et al., 1998). Therefore, we co-overexpressed Bmal1 and Clock together which enhanced viral replication, supporting a positive role for BMAL1 in driving HIV-1 transcription. Our results are in line with an earlier study that demonstrated enhanced HIV promoter activity upon overexpression of BMAL1/CLOCK in HEK293T cells (Chang et al., 2018). As REV-ERB, ROR and BMAL1 exert multiple cellular functions besides regulating circadian rhythms, knockdown or overexpression of these factors may have wide-ranging effects on other cellular pathways. Consequently, it is challenging to

establish a definite connection between the observed phenotypes following Rev-erb and Bmal1 knockdown or overexpression to the cellular clock.

We aimed to further define the importance of Bmal1 by mutating circadian motifs in the HIV-LTR. The number of E-boxes in different HIV-1 strains varies, with the HxB2 strain having three E-boxes and NL4.3, which we primarily used in our research, encodes four E-boxes in the 5' LTR. A recent report identified that mutation of the second E-box in the HxB2 strain resulted in the most significant reduction in HIV-LTR activity in HEK293T cells (Chang et al., 2018). The corresponding E-box in the NL4.3 strain is E-box number 3, which we chose to mutate/delete to assess its importance for LTR activity in Jurkat T cells. Interestingly, this motif encodes for a canonical E-box in the HIV 3' LTR and overlaps with a hypoxia response element, which could further highlight its importance. Different mutations or deletion of this E-box reduced HIV-LTR activity, however, the presence of multiple transcription factor binding motifs within the HIV-LTR implies that mutating six base pairs can affect the binding of other factors besides BMAL1. This makes it challenging to conclude that reduced LTR activity is solely due to less BMAL1 activity, and instead of mutating further motifs, we proceeded to investigate BMAL1 binding to the HIV-1 promoter directly through chromatin immunoprecipitation. We show BMAL1 binding to the E-boxes in the HIV-LTR, however, it is important to interpret direct mapping cautiously as the sonication protocol employed in the ChIP assay shears chromosomal DNA into approximately 200-300bp fragments (D'Arienzo et al., 2021) and the distance between each E-box is less than 200bp.

To gain a better understanding of how REV-ERBs and ROR regulate HIV-1 replication, we conducted chromatin immunoprecipitation experiments and focussed on the REV-ERB α and RORC isoforms. This is due to the premise that REV-ERB α is more important for the circadian clock machinery, although REV-ERB α and REV-ERB β are functionally redundant for maintaining circadian oscillations (Liu et al., 2008). The RORC isoform is particularly relevant for HIV-1 replication as it serves as the master transcription factor for Th17 development, and

has been reported to bind to the HIV-LTR (Wiche Salinas et al., 2021). We showed REV-ERB α binding to the RORE in the HIV-LTR, which was enhanced upon ROR inhibition, suggesting competition between these nuclear receptors for binding the HIV-LTR, similar to their competition for *Bmal1* and other clock-controlled genes (Guillaumond et al., 2005). We observed temporal differences in REV-ERB α and RORC binding, which suggest that circadian pathways could influence the dynamics of nuclear receptor binding to the HIV-1 genome, thereby regulating rhythmic viral replication. However, due to the presence of binding sites for BMAL1, REV-ERB/ROR and various other transcription factors, the temporal regulation of HIV-1 replication is likely to be a very complex process.

An earlier study examined the expression of host factors that are required for HIV-1 replication and found that Th17 cells had higher levels of *Rorc* and *Bmal1* transcripts compared to Th1 cells, and there was an enrichment of canonical pathways associated with REV-ERB α in Th17 cells (Cleret-Buhot et al., 2015). We hypothesised that numerous cellular host factors required for HIV-1 replication are subject to circadian regulation, and focussed our analysis on genes identified by Hiatt et al (Hiatt et al., 2022). This study used a high-throughput CRISPR–Cas9 gene editing approach in primary T cells and targeted genes which have been previously identified to interact with HIV-1 through protein interaction (Jäger et al., 2011). While this limits the identification of novel HIV-1 regulating proteins, it centred our analysis on experimentally validated HIV-1 host factors which have functional impact on HIV replication. We reveal that expression of nearly half of the HIV-1 host factors display circadian oscillations in humans. Nonetheless, it is important to note that this does not consider tissue-specific gene expression patterns and rhythmicity at the transcriptome level does not necessarily translate into diurnal changes in protein expression (Wong and O'Neill, 2018). We identified HIV-1 host factors which are likely to be targets of BMAL1, REV-ERB and RORC regulation and encode circadian motifs in their promoter regions. However, the experimentally validated datasets which we analysed were obtained from studies of mice, and given species specific differences in

circadian gene expression (Ruben et al., 2018; Yeung et al., 2018) this reflects a limitation of insufficient circadian studies using human immune cells.

The existence of a circadian motif in a promoter region does not guarantee the gene is regulated by the circadian clock as only ~1% of E-boxes in the human genome were shown to be bound by BMAL1 (Beytebiere et al., 2019). Moreover, a more distant mechanism such as the 3D-chromatin structure could be important (Takahashi, 2017; Yeung et al., 2018). We evaluated our bioinformatic analysis experimentally and hypothesised a reduction of HIV-1 dependency expression upon treatment with ROR inverse agonists. We identified seven hits: *Csde1*, *Elf3m*, *Ranbp1*, *Skp1*, *Slirp*, *Spcs3* and *Timm13* and there is further evidence for their role in HIV-1 replication or circadian rhythms. CSDE1, an RNA-binding protein, showed co-localisation with HIV-1 RNA in HeLa cells that were infected with the virus (Knoener et al., 2021) and the eukaryotic translation initiation factor 3 (EIF3) has been implicated to limit HIV-1 replication (Jäger et al., 2011; Gordon et al., 2020). Our observations are consistent with the importance of RANBP1 in the post-transcriptional regulation of HIV-1 (Zolotukhin and Felber, 1997) and its silencing has been shown to inhibit viral replication (Brass et al., 2008). SKP1 impacts cellular rhythmicity as it is a component of a ubiquitin ligase complex that participates in the degradation of CRY (Yumimoto et al., 2013). There is limited evidence for SPSC3 or TIMM13 in regulating the cellular clock, however, SPSC3 has been reported to play a role in Flavivirus genesis (Zhang et al., 2016a).

Overall, the identification of these circadian regulated host factors lays the foundation for further research aimed at understanding the underlying mechanisms that govern rhythmic HIV-1 replication.

6. HIV-1 latency is influenced by the circadian machinery

6.1. Introduction

Our previous findings on the circadian regulation of HIV-1 used model systems that supported active viral replication. However, HIV-1 can establish latent infection where integrated viral genomes are not transcribed and allow viral persistence with minimal detection by the immune system (Dahabieh et al., 2015). Multiple strategies have been developed, which pharmacologically reactivate latent HIV genomes to expose it to immune cells and anti-HIV drugs (Thorlund et al., 2017). However, the majority of latency reversing agents have limited efficacy to reduce viral reservoirs (Ait-Ammar et al., 2019). Other approaches have taken an alternative goal to suppress the reactivation of latent viral genomes to ‘block and lock’ the virus (Ahlenstiel et al., 2020). HIV-1 latency is regulated by complex processes including epigenetic modifications causing chromatin conformation changes (Battivelli et al., 2018; Verdikt et al., 2021), environmental oxygen tension (Zhuang et al., 2020) and cellular metabolism (Crater et al., 2022). Acquiring a comprehensive understanding of the pathways that regulate HIV-1 latency and reactivation is necessary to develop new therapeutic approaches. Many of the host pathways proposed to regulate HIV-1 latency and reactivation play a role in the rhythmic expression of circadian genes (Dahabieh et al., 2015; Pacheco-Bernal et al., 2019; Jefferys et al., 2021). The wide range of pharmacological agents available today provides a valuable opportunity to investigate the impact of these agents on the cellular clock and HIV-1 latency. It may provide insights for repurposing these drugs for novel applications in the field.

A library of clinically safe drugs with prior human use or drugs with well-annotated drug targets was screened for their effect on the cellular clock by the Vasudevan laboratory (Department of Pharmacology, Oxford) and a number of hits were identified. We tested these compounds for their potential to alter HIV-1 latency reactivation using a HIV-1 latency reporter system

(Battivelli et al., 2018) that allows the detection of early transcription events and discriminates active versus latent infected cells. We identified a role for drugs targeting the bromodomain and extra-terminal (BET) protein family to reactivate latent HIV and active viral replication. In contrast, salt inducible kinase (SIK) inhibitors reduced reactivation of latent reservoirs and suppressed HIV-1 replication. In summary, this chapter identified that BET and SIK inhibitors, which both contribute to regulation of circadian regulatory networks, play a role in regulating HIV-1 latency.

6.2. Results

6.2.1. Establishing HIV-GKO dual reporter virus to study HIV-1 latency

There are a range of cellular model systems and reporter viruses available to study latent HIV-1 infections, however, most assays cannot detect latently infected cells that do not express HIV proteins. Hence, one common approach is to study the reactivation of established latent pro-viral genomes which can underestimate the frequency of latently infected cells (Calvanese et al., 2013). A recent study generated a dual HIV reporter model 'GKO', which expresses GFP (along with HIV encoded genes) under control of the 5' LTR, and a distinct, unrelated fluorescent protein mKO2 controlled by the constitutive promoter of elongation factor 1 alpha ($EF1\alpha$, **Fig.6.1.A**) (Battivelli et al., 2018; Battivelli and Verdin, 2018). This allows the simultaneous measurement of active HIV replication (GFP+) and latently infected cells (GFP-, mKO2+) via flow cytometry. Importantly, $EF1\alpha$ expression is not clock regulated (Tamayo et al., 2020) which makes this reporter virus suitable for circadian studies.

To establish the GKO model in our laboratory, we produced virus by transfecting HEK293T cells with the HIV-GKO plasmid either in combination with plasmid encoding VSV-G, the HIV envelope X4, or a no-envelope control. Jurkat T cells were infected with the different viruses

and analysed for expression of GFP and mKO2 via flow cytometry. GKO-VSV-G pseudotyped virus resulted in the highest number of active or latent infected cells (~28%), whereas virus expressing the HIV envelope X4 only established infection in a minority of cells (~3%). Infection with the no envelope control virus resulted in a negligible number of GFP or mKO2 positive cells (~1%), which likely reflects non-specific endocytic particle uptake and can be considered as background (**Fig.6.1.B**).

Next, we aimed to identify the optimal timeframe for readout. Infection of Jurkat cells with VSV-G complemented HIV-GKO was measured 1, 2, 3, and 6 days post infection, and the highest number of active or latent infected cells was observed after 2 days (**Fig.6.1.C**). The percentage of GFP+ cells decreased afterwards, which may be explained by cell death and/or the reduced proliferation of infected cells. The amount of viral inoculum used to infect the cells was evaluated and reassuringly showed a dose-dependent relationship in reporter expression. Infection with 20 ng p24/ml of GKO-VSV-G resulted in the optimal ratio of active versus latent infected cells and an overall infection of ~80%, without impacting cell viability (**Fig.6.1.D**).

In summary, we established the HIV-GKO dual reporter system and identified the optimal parameters for latency analysis in Jurkat T cells, which are 48 hours of infection with 20 ng p24/ml GKO-VSV-G.

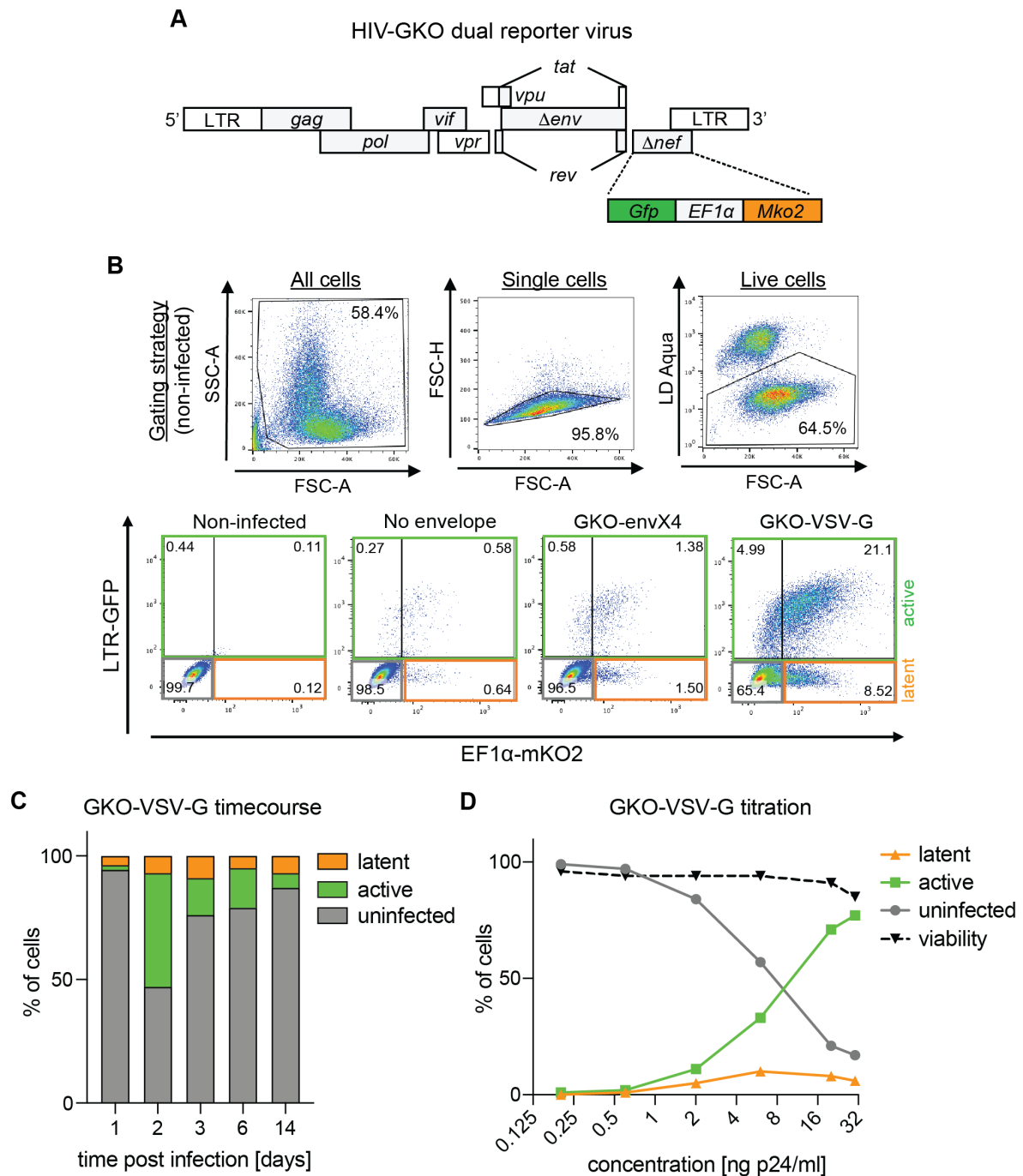


Figure 6.1. Optimisation of the HIV-GKO reporter system in Jurkat T cells. **A)** Schematic representation of the HIV-GKO reporter system where the envelope was removed, and *Gfp* and the *EF1α* promoter in front of *Mko2* were inserted into the negative regulator factor (*nef*) gene. **B)** Flow cytometry gating strategy (non-infected ctrl) and analysis of Jurkat T cells infected with 5 ng p24/ml of different GKO viruses (no envelope control, GKO-envX4 or GKO-VSV-G) for 6 days. GFP+ cells have active HIV-1 replication and mKO2+ GFP- cells harbour latent HIV-1. **C)** Jurkat T cells were infected with 5 ng p24 GKO-VSV-G for 1, 2, 3, 6 or 14 days and changes in the percentage of active versus latent HIV infection were measured by flow cytometry. **D)** Jurkat T cells were infected with different concentrations of GKO-VSV-G and cells were analysed by flow cytometry 48 h post infection. Representatives for n=2.

6.2.2. A screen for circadian drugs to modify latent HIV-1 infection

After setting up the GKO-VSV-G HIV reporter system in our laboratory, we used this assay to screen 45 compounds which were provided by co-supervisor Sridhar Vasudevan. They were identified to alter the amplitude or period of *Bmal1* promoter activity in U-2 OS cells or immortalised murine hepatocytes (MMHD3), and many are clinically licensed compounds or supplements from natural sources (unpublished data by Vasudevan et al., **Table 2**).

Table 2. Overview of screen of circadian modifiers.

Nr	Licenced	Drug	U-2 OS cells		MMDH3 cells		
			Period	Amplitude	Period	Amplitude	
1	Yes	Ampiroxicam	-	↑	-	↑	↑ Increase ↓ Decrease - No effect No data
2	Yes	b-eoestradiol			-	↑	
3	Supplement	Berberine					
4	Yes	Bexarotene	↑	-			
5	No	Bio					
6	Yes	Brimonidine	↑	↑	-	-	
7	No	CGS-15943	↑	-	↑	↑	
8	No	CSNKtd					
9	Yes	Deracoxib	-	↑	-	↑	
10	Veterinary use	Diclazuril					
11	Yes	Doxorubicin	↓	↓	↓	↓	
12	No	Ebselen	↑	-	↑	-	
13	Yes	Flutamide	-	↑	-	↑	
14	No	GSK1362					
15	No	GSK2667		↓			
16	No	GSK4112					
17	Supplement	Harmaline	↑	↑	↑	↑	
18	No	IB-MECA	↑	↑	↑	↑	
19	No	IBET-762					
20	No	(-) JQ1	-	-			
21	No	(+) JQ1	↑	↑			
22	No	Linopridine	↑	↑	↑	↑	
23	Yes	Nialamide	-	↑	-	↑	
24	No	NIBR-0213	↑	↑	↑	↑	
25	Yes	Niflumic acid	-	↑	-	↑	
26	Supplement	Nobiletin	-	↑	-	↑	
27	Yes	Olmesartan	↓	-	↓	↓	
28	Yes	Oxaprozin	-	↑	-	↑	
29	Yes	Ozagrel	-	↑	-	↑	
30	Yes	Pinacidil	-	↑	-	↑	
31	Yes	Progesterone	↑	↑	↑	↑	
32	Yes	Roziglitazone			↑	↑	
33	No	S1P					
34	Yes	Siponimod	-	-	-	-	
35	No	SMTC	arhythmic				
36	No	SR1078	-	↑	-	↑	
37	No	SR202					

38	No	SR8278		↑		↑
39	No	SR9009		↓		↓
40	Yes	Telmisartan	-	↓	↑	↑
41	No	U46619				
42	Yes	Vitamin D	↑	↑		
43	Yes	Withaferin	↑	-		
44	No	Zaprinast	↑	↑	-	↑
45	No	Zebularin	-	↑		

Jurkat T cells were infected with GKO-VSV-G using previously optimised conditions (48 h, 20 ng p24/ml) and incubated with TNF α for 24 h, which is known to reactivate latent HIV-1 (Duh et al., 1989; Folks et al., 1989). Reassuringly, we observed a ~50% reduction in silent latently infected cells (mKO2+) (**Fig.6.2.A**). Next, Jurkat infected with GKO-VSV-G were incubated with 2 μ M or 20 μ M of each drug for 24 h, and analysed by flow cytometry. The strongest increase of cells harbouring latent HIV was caused by the compound 6-bromoindirubin-30-oxime (BIO), whereas Doxorubicin led to the strongest reduction in mKO2+ GFP- cells in both concentrations tested. Additionally, Berbine, IBET-762, JQ1+, Telmisartan and Withaferin reduced latently infected cells in one of the concentrations tested (**Fig.6.2.B**).

Intrigued by the high fluorescence signals caused by some of the compounds, we treated uninfected cells with the drugs to check for autofluorescence. Indeed, we detected fluorescence activity in the absence of virus for BIO, Berbine, Doxorubicin, Telmisartan and Withaferin, suggesting that the earlier proposed changes in latently infected cells were an artefact due to autofluorescence (representatives shown in **Fig.6.2.C**).

Nonetheless, the compounds JQ1+ and IBET-762 were identified to reduce latently infected cells, whereas the inactive form JQ1- did not have any effects. These agents target the BET protein family and were studied further.

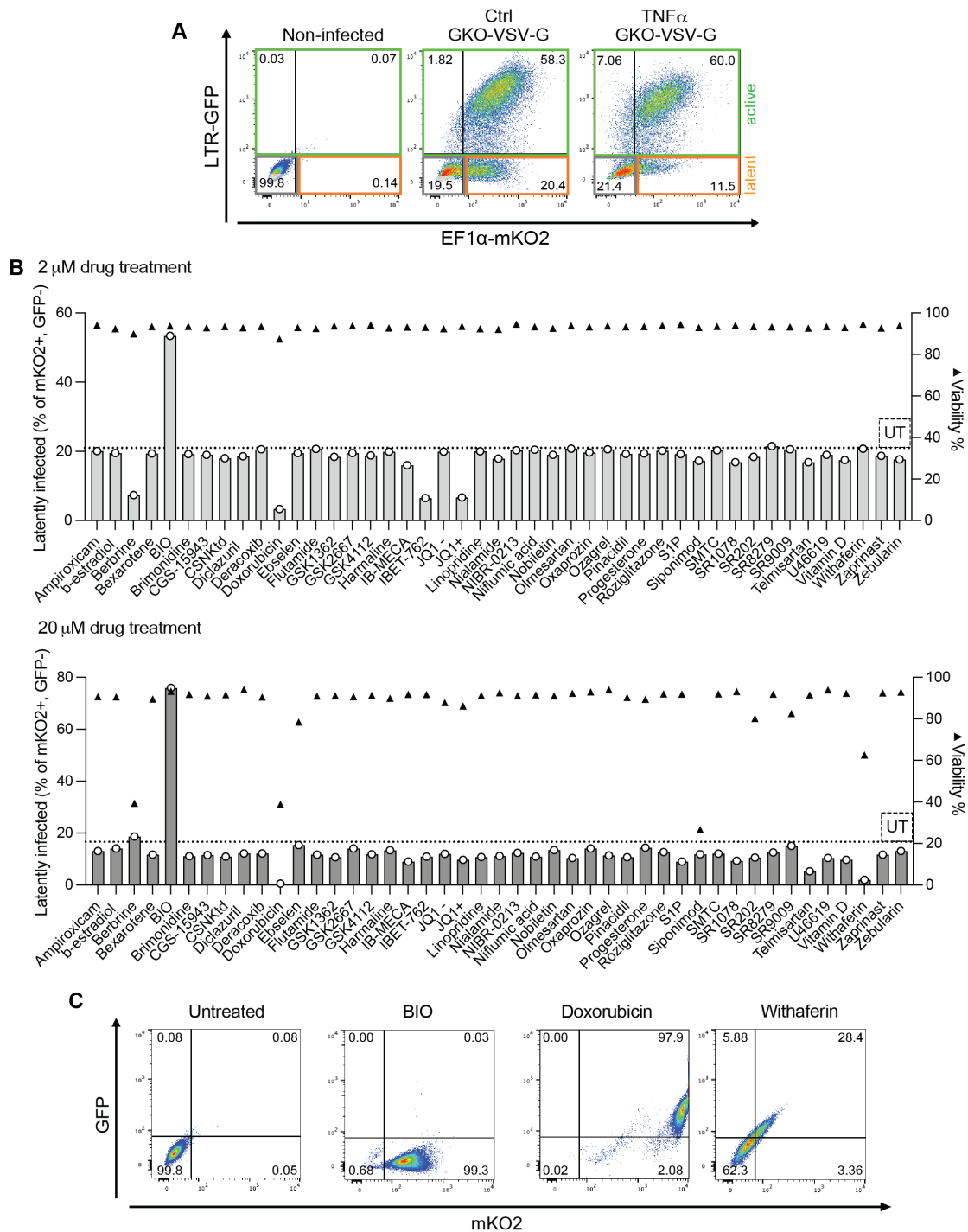


Figure 6.2. A screen of circadian drugs for modifying HIV-1 latency. **A)** Jurkat T cells were infected with 20 ng p24/ml GKO-VSV-G for 48 h, followed by treatment with or without 200 ng/ μ l TNF α . Percentage of active (GFP+) versus latent (GFP- mKO2+) HIV-1 infection was quantified by flow cytometry. **B)** GKO-VSV-G infected Jurkat T cells were treated with 2 μ M or 20 μ M of each drug and the percentage of latently infected cells (mKO2+, GFP-) and cell viability was determined by flow cytometry. **C)** Uninfected Jurkat T cells were treated with 10 μ M BIO, Doxorubicin, or Withaferin and expression of GFP and mKO2 was assessed by flow cytometry. Given the large scale, experiments were performed as n=1.

6.2.3. Characterisation of BET bromodomain inhibitors for reactivation from latency

We identified that treatment with 2 μM of JQ1+ and IBET-762 reduced the percentage of latently infected cell in GKO-VSV-G treated Jurkat cells, and we next aimed to further define the characteristics of reactivation from latency. Analysis of different doses of each drug (0.3 μM , 1 μM , 3 μM) in GKO-VSV-G infected Jurkat cells revealed a dose dependent increase in the frequency and mean fluorescent intensity (MFI) of GFP+ actively transcribing cells, and a concomitant reduction in the number and MFI of latent infected cells (mKO2+, GFP-). Reassuringly, cell viability was not impacted and JQ1-, the inactive form of JQ1+, did not alter viral infection (**Fig.6.3**).

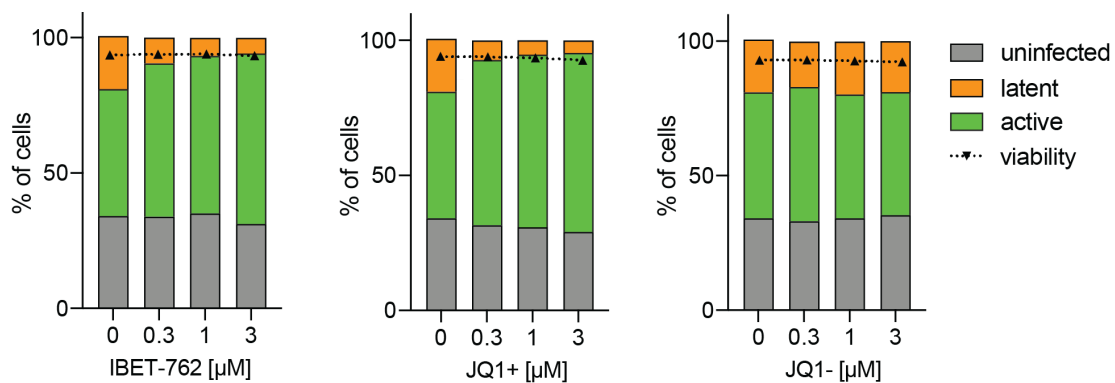


Figure 6.3. IBET-762 and JQ1+ decrease the frequency of latent vs active GKO-VSV-G infected Jurkat cells. Jurkat T cells were infected with GKO-VSV-G for 48 h, followed by treatment with different concentrations of IBET-762, JQ1+ and JQ1- for 24 h. Flow cytometry analysis revealed the percentage of uninfected (GFP-, mKO2-), latently infected (GFP-, mKO2+) and actively infected (GFP+) cells and determined viability. Representatives for n=3.

To confirm these observations in a separate model of active and latent infection, we used the NL4.3 R-E-*luc* system (**Fig.3.3.A**) to examine active infection in Jurkat cells, and J-Lat cells were used as a latency-specific model system (Jordan et al., 2003). J-Lat is a Jurkat based line which contains integrated copies of HIV-1, where the *nef* gene is replaced by *Gfp* (**Fig.6.4.A**) and reactivation from latency can be measured via GFP expression. J-Lat cells

were selected for null GFP expression, and are commonly treated with $\text{TNF}\alpha$ as a first stimulus to reactivate HIV (**Fig.6.4.B**) in combination with drugs of interest.

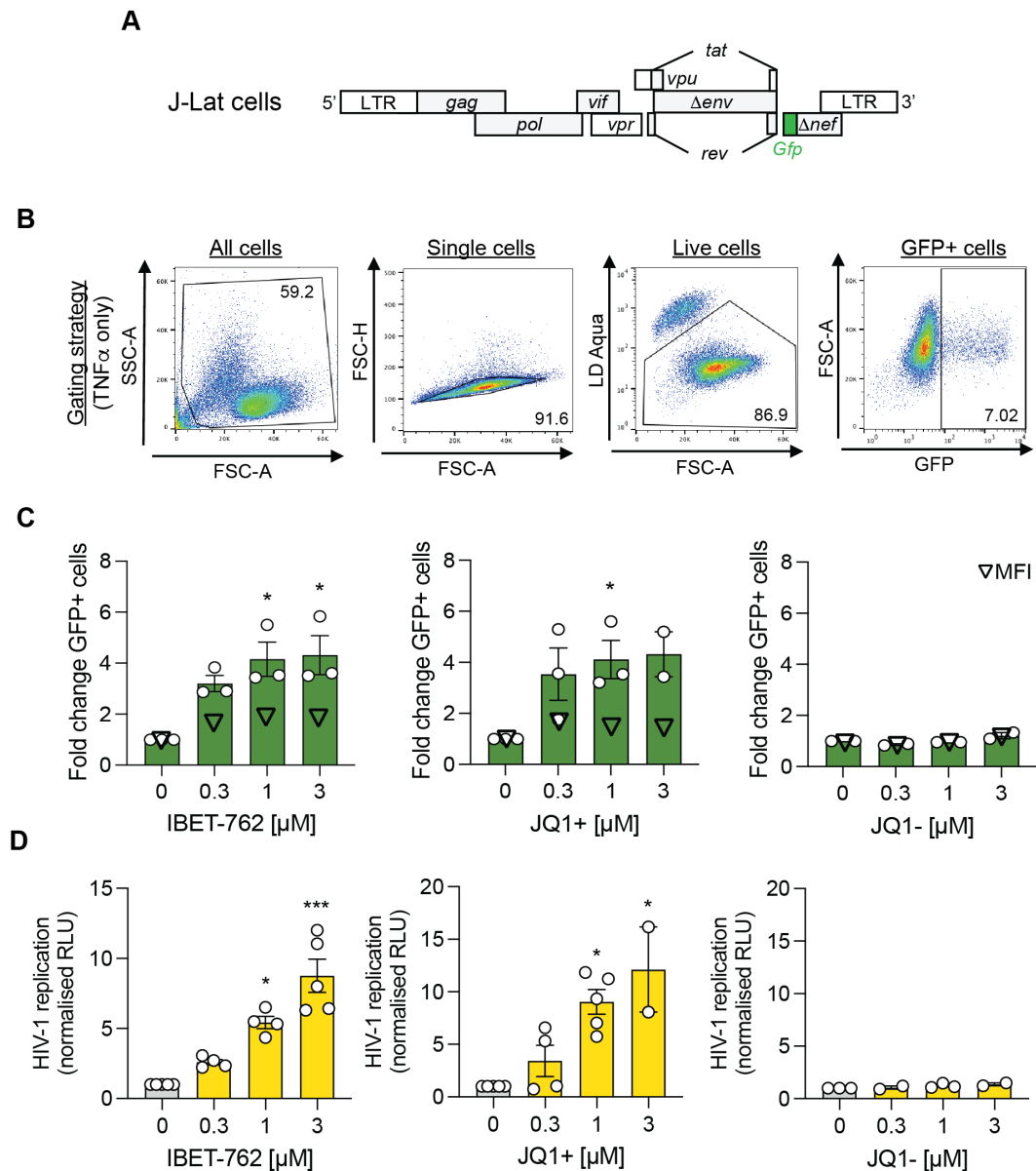


Figure 6.4. Characterisation of BET bromodomain inhibitors to alter latent and active HIV-1 infection. **A)** Schematic representation of the HIV-1 reporter construct integrated in the Jurkat based J-Lat cell line, where *env* was removed, and *Gfp* was inserted into the *nef* gene. **B)** Flow cytometry gating strategy to analyse J-Lat cells for viability and expression of GFP. Example of cells treated with 100 ng/ μl $\text{TNF}\alpha$ for 24 h before readout. **C)** J-Lat cells were treated with 100 ng/ μl $\text{TNF}\alpha$ in combination with IBET-762, JQ1+ or JQ1- at indicated concentrations. Percentage of GFP expressing cells and MFI was acquired using flow cytometry and is shown as fold change normalised to control (mean \pm S.E.M., $n=2-3$, Kruskal-Wallis ANOVA). **D)** Jurkat T cells were infected with NL4.3-*luc* VSV-G for 24 h, followed by treatment with different concentrations of IBET-762, JQ1+ or JQ1- for 24 h. HIV-1 replication was quantified by measuring luciferase signal and is shown normalised to untreated cells (mean \pm S.E.M., $n=2-5$, Kruskal-Wallis ANOVA).

JQ1+ and IBET-762 reactivated HIV-1 in a dose-dependent manner in J-Lat cells, as evidenced by an increased percentage of GFP+ cells and MFI (**Fig.6.4.C**), and the negative control JQ1- had no effect (**Fig.6.4.C**). Active HIV-1 replication was assessed by measuring luciferase in NL4.3-*luc* VSV-G infected Jurkat cells, and was increased when treating with the BET bromodomain inhibitors (no change for JQ1- control, **Fig.6.4.D**). Overall, this is in line with our previous observation of increased active versus decreased latent infection in the GKO model system and confirms the activating effect of the compounds on HIV-1 replication.

Finally, we confirmed the circadian effect of these drugs observed by Vasudevan et al. U-2 OS cells stably expressing *Bmal1* promoter-driven luciferase were synchronised by serum shock and treated with IBET-762 and JQ1+ (or JQ1- control). We observed a dose dependent increase in *Bmal1* promoter activity amplitude (**Fig.6.5**).

In summary, we show that the BET bromodomain inhibitors IBET-762 and JQ1+ reactivate latent HIV-1, enhance viral replication and increase the amplitude of *Bmal1* promoter cycling.

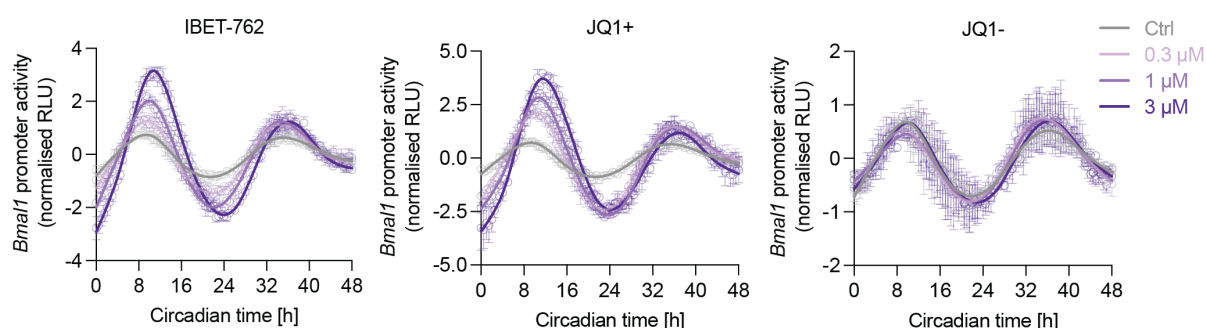


Figure 6.5. JQ1+ and IBET-762 increase the amplitude of rhythmic *Bmal1* promoter activity. U-2 OS cells stably expressing luciferase under the control of the *Bmal1* promoter were synchronised via serum shock. 24 post synchronisation cells were treated with BET bromodomain inhibitors IBET-762, JQ1+ and JQ1- and luciferase activity measured in 30 min intervals for 48 h (mean \pm S.E.M., n=3). All data are normalised to peak expression, raw data in Figure 2.2.E.

6.2.4. A role for salt inducible kinases in regulating latent HIV-1

A number of reports show a role for salt inducible kinases in regulating circadian regulatory networks, however, there is limited evidence for SIKs impacting HIV-1 infection. SIKs convey

signals through phosphorylation of multiple targets, including cAMP-regulated transcriptional coactivators (CRTC) (Wein et al., 2018; Darling and Cohen, 2021) and class IIa HDACs which have been implicated to control HIV-1 regulation (Ma et al., 2021). We hypothesised that SIKs regulate rhythmic HIV-1 replication and reactivation of latent infection and tested this by pharmacological inhibition of SIK *in vitro*.

We selected to test the two structurally unrelated SIK inhibitors YKL-05-099 (Sundberg et al., 2016) and ARN-3236 (Tesch et al., 2021) and first evaluated their effect on cellular circadian rhythms. U-2 OS cells stably expressing *Bmal1* promoter-driven luciferase were synchronised, incubated in the presence of either YKL-05-099 or ARN-3236 and luminescence recorded in real-time. We observed a reduction in the amplitude and a phase shift of *Bmal1* promoter cycling, indicating a disruption of the cellular clock (**Fig.6.6.A**). To test the effect of each compound on rhythmic HIV-1 replication, we infected U-2 OS cells with NL4.3-*luc* VSV-G followed by synchronisation and drug treatment, and show reduced rhythmic HIV-1 replication (**Fig.6.6.B**). Reassuringly, neither treatment caused cytotoxic effects in U-2 OS cells (**Fig.6.6.C**).

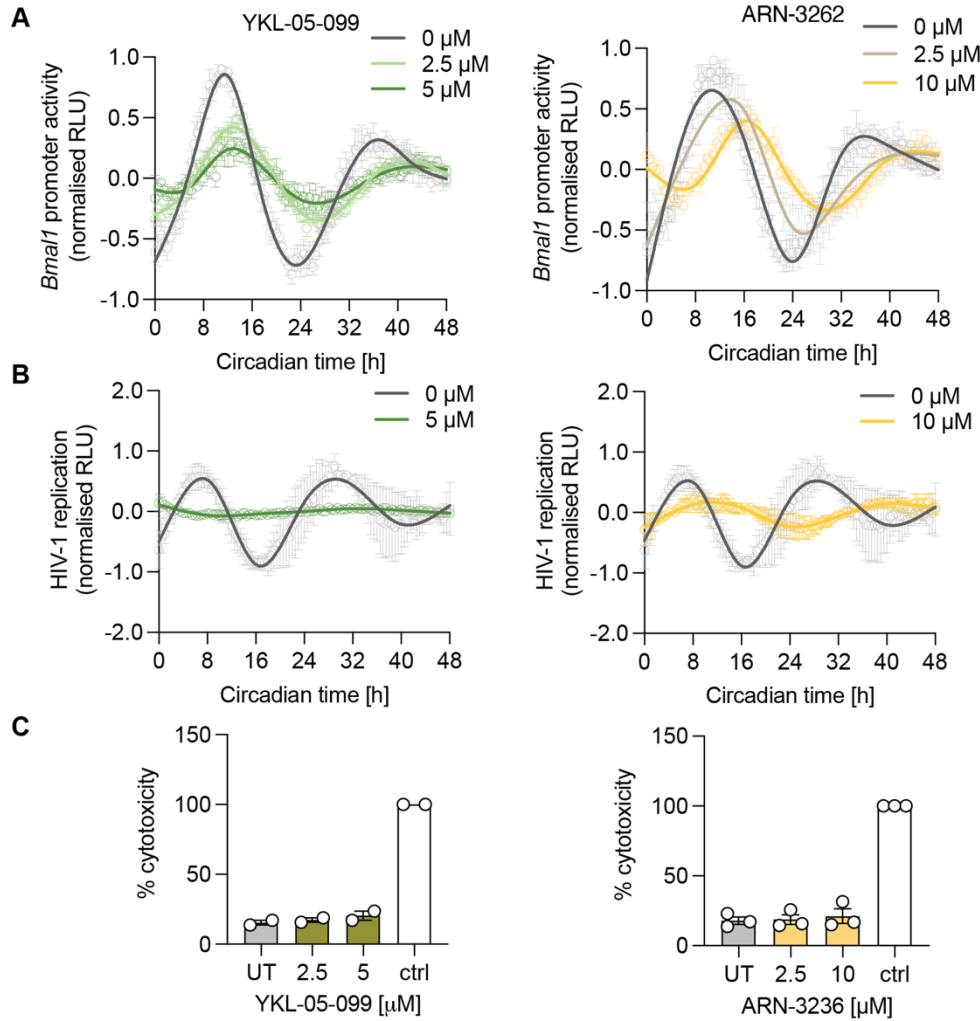


Figure 6.6. Salt inducible kinase inhibitors perturb rhythmic *Bmal1* promoter activity and HIV-1 replication. **A)** U-2 OS cells stably expressing *Bmal1-luc* were synchronised by serum shock, treated with different concentrations of YKL-05-099 or ARN-3236 and luciferase measured every 30 min for 48 h, starting 24 h post synchronisation (mean \pm S.E.M., $n=3$, normalised to peak expression, raw data in Figure 2.2.F). **B)** U-2 OS cells were infected with NL4.3-*luc* VSV-G for 24 h, incubated in the presence of YKL-05-099 (5 μ M) or ARN-3236 (10 μ M) and HIV-1 replication was measured by quantifying luciferase activity (mean \pm S.E.M., $n=3$, normalised to peak expression, raw data in Figure 2.2.G). **C)** Cytotoxicity was measured with an LDH release assay (mean \pm S.E.M., $n=2-3$) and data are expressed relative to the positive control representing total cell lysate (100% cytotoxicity).

To test SIK inhibitors in a more physiological context, activated CD8 depleted PBMCs were infected with NL4.3-*luc* VSV-G and treated with YKL-05-099, ARN-3236 and another SIK inhibitor HG-9-91-01 for 24 h. HIV-1 replication was reduced in a dose dependent manner with all treatments (**Fig.6.7.A**), with minimal cellular toxicity (**Fig.6.7.B**).

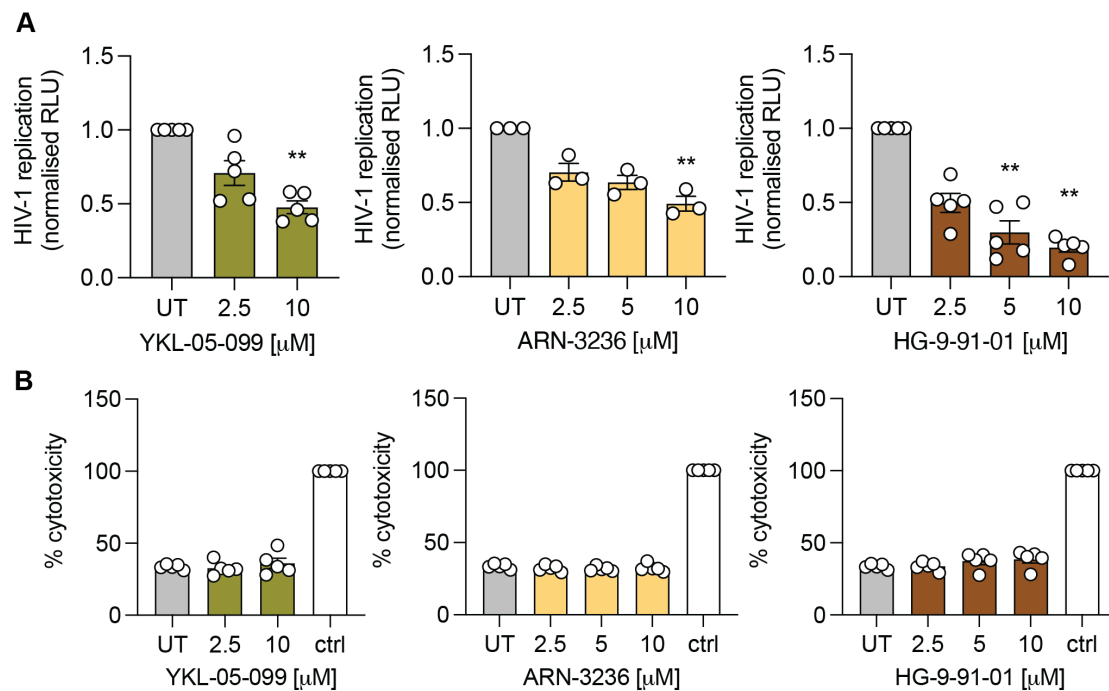


Figure 6.7. Pharmacological modulation of SIKs reduces HIV-1 replication. **A)** CD8 depleted PBMCs were stimulated with IL-2, anti-CD3 and anti-CD28 for 3 days. After infection with HIV NL4.3-*luc* VSV-G for 24 h, cells were treated with different concentrations of YKL-05-099, ARN-3236 or HG-9-91-01 for 24 h and HIV-1 replication was quantified by luciferase readout (mean \pm S.E.M., $n=3-5$, Kruskal-Wallis ANOVA). **B)** Toxicity was determined using an LDH release assay (mean \pm S.E.M., $n=2-6$) and data are expressed relative to the positive control representing total cell lysate (100% cytotoxicity).

Intrigued by the anti-viral activity of these compounds, we speculated that SIK inhibitors could reduce the reactivation of latent HIV-1 and tested this using the J-Lat latency model (**Fig.6.4.A-B**). Treatment of J-Lat cells with $\text{TNF}\alpha$ in combination with the most specific compound, ARN-3236, reduced both the percentage and MFI of GFP expressing cells without effecting cell viability (**Fig.6.8.A**).

Due to the potential off-target effects commonly observed with small molecule inhibitors and the ability of SIK inhibitors to modulate the activity of various kinases (Tesch et al., 2021), we augmented our findings with a genetic approach. There are three SIK family members (SIK1, SIK2 and SIK3), and we intended to silence their expression separately by transfecting J-Lat cells with siRNA targeting each isoform. After 24 hours of $\text{TNF}\alpha$ activation, RNA was extracted

and analysis revealed that the siRNAs, which were reported to target a specific isoform, reduced the expression of all isoforms (**Fig.6.8.B**). Hence, a pool of siRNAs was used to ensure silencing of all Sik isoforms and knockdown efficiency was verified by qPCR. Quantification of HIV-1 RNA levels showed a reduction in viral transcripts compared to the control, supporting a pro-viral role for SIKs in regulating the abundance of HIV-1 transcripts (**Fig.6.8.C**).

Collectively, these findings demonstrate that inhibition of salt inducible kinases reduces rhythmic HIV-1 replication and reactivation from latency.

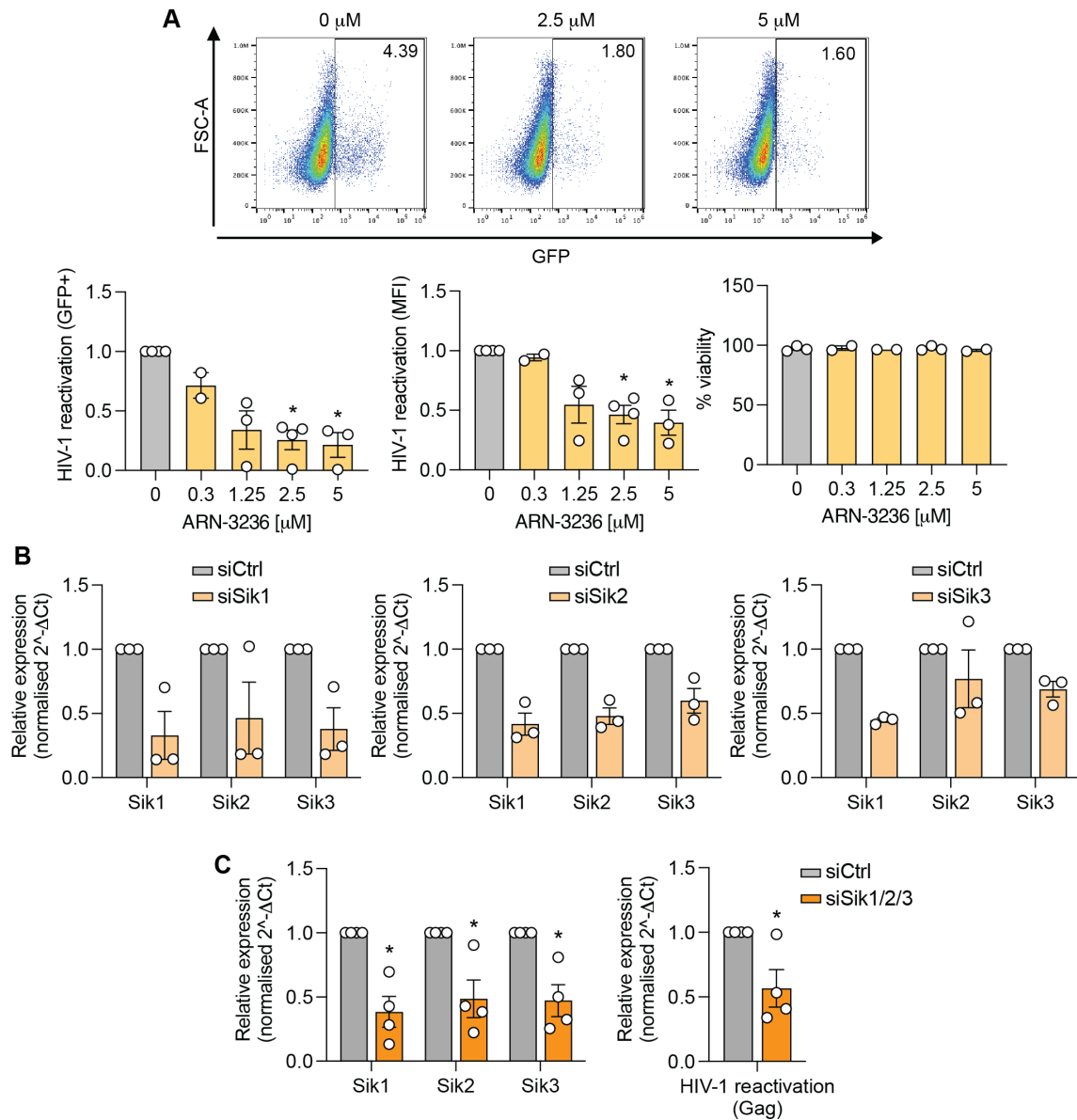


Figure 6.8. ARN-3236 or genetic silencing of SIKs decreases HIV-1 latency reactivation. A) J-Lat cells were treated with 100 ng/μl TNF α alone, or in combination with ARN-3236 at indicated concentrations. Cell viability, percentage of cells expressing GFP and the MFI were determined by flow cytometry (mean \pm S.E.M., $n=2-4$, Kruskal-Wallis ANOVA). Dot plots of one representative experiment are shown. **B)** J-Lat cells were transfected with siRNAs targeting Sik1, Sik2 or Sik3, cells were treated with TNF α and transcripts analysed by qPCR 24 h post treatment (mean \pm S.E.M., $n=3$). **C)** Sik1/2/3 were silenced in J-Lat cells using a pool of siRNA for 48 h, cells were treated with TNF α for 24 h and RNA extracted to quantify Sik1, Sik2, Sik3 and HIV-1 Gag transcripts by qPCR (mean \pm S.E.M., $n=4$, Mann-Whitney test). All data are normalised to controls.

6.3. Discussion

In this chapter, we established a dual reporter virus system which allows a simultaneous readout of active and latent HIV-1 infection for the screening of circadian modifying drugs. We identified that BET bromodomain inhibitors IBET-762 and JQ1+ reactivate latent HIV-1 and increase viral replication. Additionally, we demonstrated a pro-viral role for salt inducible kinases to regulate HIV-1 replication and latency.

The GKO-VSV-G reporter virus was originally established in primary CD4 T cells (Battivelli et al., 2018) and to facilitate the screening of a large number of drugs in a practical manner, we set-up infection of the Jurkat T cell line. We observed an increase of productive and latent infected cells proportional to the input of virus, which is in line with the previous report. Battivelli et al. sorted cells to obtain a population of latently infected cells without active viral replication to analyse latency reversing agents. We were interested in the detection of early transcriptional events and selected to use unsorted cells for our experiments. Our initial compound screen aimed to identify changes in latently infected cells, however, altered active HIV-1 replication was studied in the subsequent characterisation of drugs. Although we treated cells with two concentrations of each drug, it is important to note that we did perform multiple repeats or timepoints of the screen. Consequently, there is a possibility that we may have missed potential hits, for instance due to the kinetics of drug action. The fluorescence based viral readout is not suitable to test drugs which are autofluorescence, another limitation which could have decreased the identification of relevant compounds in our screen.

We identified that the BET bromodomain inhibitors reduce latent infection and increase the frequency of productively infected cells, and we confirmed this observation in 3 separate models (GKO-VSV-G, J-Lat, NL4.3-*luc* VSV-G). Bromodomain (BRD) proteins, particularly BRD2 and BRD4, are important for the recruitment of transcription factors to promoter regions and inhibit transcription via p-TEFb (Bartholomeeusen et al., 2012). Importantly, HIV-1 transcription following integration is dependent on the activity of p-TEFb (Zhu et al., 1997).

Previous studies have demonstrated that BET bromodomain inhibitors reactivate latent HIV-1 and highlight multiple potential mechanisms of action. JQ1+ reactivated HIV transcription in J-Lat cells and allowed recovery of replication-competent HIV from pools of resting CD4 T cells isolated from HIV-infected, ART-treated patients (Banerjee et al., 2012). This was likely mediated through chromatin reorganisation, as JQ1+ upregulated histone modification genes, inducing HDACs and suppressed histone methyltransferases. Additionally, JQ1+ treatment downregulated T cell activation genes. Reassuringly, the authors observed a reactivation of J-Lat cells treated with 0.5 μ M JQ1+ for 24 h, which is similar to the effective dose range and kinetics in our experiments (Banerjee et al., 2012). Boehm et al. treated Jurkat and J-Lat cells with BET bromodomain-targeting compounds, including JQ1+ and IBET-151, and observed reactivation of latent HIV-1 via a Tat-independent mechanism (Boehm et al., 2013). In contrast, Li et al. showed that BRD4 inhibits Tat transactivation and JQ1+ dissociates BRD4 from the HIV promoter, allowing Tat recruitment (Li et al., 2013). Knockdown of BRD4 or treatment with JQ1+ reduced HIV-1 latency reactivation and the authors propose a model where BRD4 competes with the virus for HIV-1 dependency factors (Zhu et al., 2012).

These reports along with other studies (Laird et al., 2015; Dai et al., 2022), demonstrate that research with the same inhibitors and model systems we used has been published. Similarly, the role of BET bromodomain inhibitors in regulating circadian rhythms has been studied in detail. Acetylation of BMAL1:CLOCK leads to the recruitment of BRD4 and p-TEFb, which promotes the elongation of circadian transcripts (Petkau et al., 2019). BRD4 binds to regulatory regions in a circadian manner and is evicted by REV-ERB, providing dynamic modulation of chromatin looping (Kim et al., 2018), which can be enhanced through the recruitment of histone modifying enzymes via BRD2 (Boehm et al., 2013). While it is reassuring that our findings align with these studies, we chose to not further study the BET inhibitors in the context of HIV-1 and circadian rhythms due to a lack of novelty.

Instead, we focussed on exploring the importance of salt inducible kinases as there is limited evidence regarding the role of SIKs in regulating HIV-1 infection. It is well-established that SIKs are involved in the regulation of circadian networks. The CRTC1-SIK1 pathway is important for the entrainment of circadian rhythms and suppresses the effects of light on the clock (Jagannath et al., 2013). On a molecular level, SIK3 destabilises the key circadian factor PER2 (Hayasaka et al., 2017), and several studies have shown that pharmacological inhibition of SIKs can modify circadian rhythmicity and sleep homeostasis. The SIK inhibitor indirubin-3-monoxime (I3M) caused a phase shift of circadian rhythms in fibroblasts (Jagannath et al., 2013), and HG-9-91-01 altered phosphorylation of synaptic phosphoproteins relevant for sleep homeostasis in mice (Wang et al., 2018b). YKL-05-099 and ARN-3236 have not been investigated for their effect on the cellular circadian network, which is why we tested their impact on *Bmal1* promoter rhythmicity. We identified a dampening and phase shift of *Bmal1* promoter activity, which indicates perturbed cellular rhythms. As we previously demonstrated that the circadian machinery regulates rhythmic HIV-1 replication, we hypothesised that altered cellular oscillations due to SIK inhibition could impact HIV-1 infection.

Multiple kinases have been reported to regulate HIV-1 infection (Duverger et al., 2014; De Martini et al., 2019), and SIK1 was identified through an ingenuity pathway analysis as a kinase potentially involved in regulating HIV-1, but it was not investigated further (De Martini et al., 2019). We experimentally demonstrated that SIK inhibition reduces rhythmic HIV-1 replication and reactivation of latent infection, highlighting a pro-viral role for SIKs in regulating HIV-1 infection. We showed that SIK inhibitors reduced HIV-1 infection of cell lines and primary cells using laboratory adapted virus reporter strains, and further studies with primary viruses are merited. Our attempts to individually silence Sik1, Sik2, and Sik3 using commercially available siRNAs were unsuccessful, however, it is plausible that custom-designed siRNAs and additional efforts could have achieved successful silencing of these isoforms independently. There is some redundancy in SIK function (Sun et al., 2020), which is why we

decided to not study the role of each isoform and performed a combined knockdown of all isoforms instead. We observed a reduction in the reactivation of latent HIV-1 in SIK silenced cells, but it is unclear if the effect is mediated directly through SIK silencing, or if SIK knockdown and pharmacological inhibition causes broad cellular changes that reduced HIV-1 infection.

CRTCs are one of the main SIK targets, and their phosphorylation by SIKs causes their retention in the cytoplasm. SIK inhibitors induce the translocation of CRTC from the cytoplasm to the nucleus, enabling it to exert its functional role. Interestingly, it was reported that CRTC2 acts as a suppressor of HIV-1 transcription by inhibiting the binding of RNA Pol II to the HIV-1 LTR (Ma et al., 2021). This could suggest a mechanism by which SIK inhibitors reduce RNA Pol II binding to the HIV-LTR, thereby leading to a CRTC2-mediated anti-viral effect. However, additional studies would be required to validate this hypothesis.

Moreover, the inhibition of SIK increased nuclear localisation and activity of class IIa HDAC (Jagannath et al., 2023). HDAC inhibitors have been thoroughly investigated in the reactivation of latent HIV, as they enhance the accessibility of integrated viral genomes to transcription factors, thereby promoting viral transcription (Shirakawa et al., 2013). Most treatment regimens aim to target a wide spectrum of HDAC classes, however, primarily recruitment of class I HDACs to the HIV-1 LTR causes chromatin remodelling through histone deacetylation (Archin et al., 2009). Consequently, class I specific HDAC inhibitors are considered more effective (Archin et al., 2009; Zaikos et al., 2018). Current understanding suggests that class IIa HDACs exhibit minimal or no deacetylase activity, but they can act as adaptors by recruiting class I HDACs and other transcription factors (Parra, 2015). As SIK inhibitors enhance the nuclear localisation of HDAC class IIa, they have the potential to recruit HDAC class I proteins, which could in turn repress HIV-1 transcriptional activity and result in the anti-viral phenotype. Experimental validation is necessary to confirm this speculative

mechanism, which could provide valuable insights into the regulation of HIV-1 infection by HDACs.

Apart from CRTC and HDACs, SIKs are implicated in multiple pathways that regulate diverse cellular processes such as metabolism (Sun et al., 2020) and inflammation (Sundberg et al., 2014). Whether direct regulation by SIKs altered HIV-1 replication, or whether these changes are mediated through the circadian clock or other pathways remains to be investigated. ARN-3236 is currently undergoing phase I clinical trials for ovarian, peritoneal, and other solid tumours, and several SIK inhibitors are being developed for potential clinical use (Jagannath et al., 2023), which highlights potential therapeutic applications in the future.

In conclusion, our findings in this chapter underscore the significance of BET bromodomain proteins and SIKs in HIV-1 replication and reactivation from latency, providing valuable insights for further investigations into the underlying mechanisms. Ultimately, these findings have the potential to enhance and complement current therapeutic strategies for HIV-1 treatment.

7. Discussion, limitations, future work

Across result chapters 3-6, we have explored the contribution of the cellular circadian machinery to HIV-1 replication. We demonstrated that HIV-1 replication is rhythmic and regulated by the cell intrinsic circadian clock that can be perturbed by pharmacological targeting or genetic silencing of clock factors. Small molecules targeting REV-ERB and ROR showed pan-genotypic anti-viral activity. REV-ERB repressed viral replication, while BMAL1 and ROR enhanced HIV-1 transcription, and all factors bind conserved circadian motifs in the viral promoter region. REV-ERB α and RORC compete for binding to the same region, and differentially bind to viral DNA depending on the time of day. HIV-1 host factors are BMAL1, REV-ERB α and RORC targets, which contributes to rhythmic viral regulation. We identified that BET bromodomain inhibitors activate HIV-1 replication and reactivation from latency, while SIK inhibitors had the opposite effect. Overall, this work demonstrates the importance of the cellular circadian clock to regulate active and latent HIV-1 replication. Most specific points were discussed in earlier chapters, and this final discussion will focus on the broader context, limitations, and future directions.

It is important to note that all our experiments were performed with cell lines or primary cells isolated from healthy donors and infected with HIV-1 *in vitro*. Infected cells isolated from people living with HIV would likely behave differently, however, as 77% of PLWH are receiving ART it is difficult to study early infection dynamics in samples from human participants. In contrast to clinical and epidemiological studies, this thesis primarily aimed to gain mechanistic insights into HIV-1 infection, but it is essential to acknowledge the limitations imposed by the artificial nature of *in vitro* infection. Nevertheless, the results offer valuable new concepts and approaches that can be further explored to study HIV-1 infection *in vivo* and it is crucial to expand on these findings in future studies.

We previously reported that BMAL1 binds E-box motifs in the HBV genome (Zhuang et al., 2021a), and hypothesised a model where viruses have evolved to act in concert with host

circadian transcription factors. Here, we show binding of BMAL1, REV-ERB α and RORC to the HIV-LTR, supporting this hypothesis. It is important to consider that the immunoprecipitated proteins may bind as part of a complex and our ChIP-qPCR assay does not elucidate the significance of co-factors. REV-ERB usually exerts its function through recruitment of NCoR1 which was increased following SR9009 treatment (Solt et al., 2012; Chang et al., 2019), and RORs can also interact with NCoR (Jetten, 2009). Interestingly, a previous study showed that NCoR is bound to the HIV-LTR in unstimulated macrophages and released after TLR activation, indicating a role for NCoR in repressing HIV-1 transcription (Hanley and Viglianti, 2011). This is further supported by findings that NCoR gene polymorphisms affect susceptibility to HIV infection in exposed individuals (Chinn et al., 2010). It is plausible that the inhibitory effects of NCoR are interconnected with those of REV-ERB, forming a complex with additional proteins. To gain insights into the composition of binding complexes involving circadian transcription factors and their association with the HIV-LTR, it would be interesting to perform mass spectrometry and protein-protein interaction analysis.

The ability of BMAL1 to activate viral replication was observed for both HIV-1 and HBV, suggesting a conserved mechanism across these diverse viral families (Zhuang et al., 2021a). In contrast, Bmal1 silencing enhanced Herpesvirus and Influenza A virus infections (Edgar et al., 2016). Another study found that Bmal1 KO mice had more asthma-like airway changes than control animals, which associated with less control of viral replication and worse acute viral bronchitis caused by Sendai virus and IAV (Ehlers et al., 2018). Circadian control of IAV was not mediated by direct effects on viral replication and associated with lung inflammation regulated through temporal gating by lung and myeloid clocks (Sengupta et al., 2019). Similarly, Bmal1 KO mice infected with IAV had increased lung inflammation, which highlights a role for Bmal1 in maintaining normal pulmonary function during viral infection (Sundar et al., 2015). It is important to recognize that studying viral dynamics in whole animals *in vivo* may yield different results compared to examining circadian regulation of viral replication in cells *in*

vitro. Our study did not account for the complex *in vivo* interactions between the rhythmicity of viral replication and physiological oscillations induced by factors such as immune cell clocks. This limits the insights on interconnectivity of body clock and HIV-1 replication, nonetheless, our findings underscore the importance of the cell intrinsic clock in governing the rhythmicity of HIV-1 replication, emphasizing that the cellular clock alone can regulate the temporal patterns of HIV-1 replication.

Our bioinformatic analysis identified that multiple host factors known to influence HIV-1 replication are regulated by the circadian clock. Remarkably, ~9.5% of human protein coding genes are implicated in HIV replication (Tough and McLaren, 2018), and over 40% of genes in the mouse genome are rhythmically expressed in at least one tissue (Zhang et al., 2014). There is limited transcriptomic data on immune cell clocks, but one study showed that at least 8% of transcripts in murine macrophages oscillate with circadian rhythm (Keller et al., 2009). This illustrates the likelihood of circadian regulation of processes important for HIV-1 replication and supports the notion of viral adaptation to the host circadian clock. Our bioinformatic analysis and experimental validations only provide a glimpse into the complex interplay between HIV-1, host factors, and circadian rhythms, and further exploration through global analyses would be insightful. This could help to generate a functional map of HIV-host interactions from a circadian perspective.

During the intracellular HIV-1 life cycle, multiple cellular proteins and small cellular RNAs can be incorporated into new virions. Some well-studied examples incorporated into the viral envelope include tRNAs, which are necessary for the initiation of reverse transcription (Kleiman et al., 2010), and proteins that facilitate virion adherence to host cells (Ott, 2008). Exploring the potential inclusion of cellular circadian factors or small regulatory RNAs that affect cellular machinery would be an intriguing area of study to determine their role in HIV-1 infection.

Our research only focussed on steps in the HIV-1 life cycle after integration into the host genome, hence drug treatments in all our experiments were performed after infection. We did not investigate viral entry, however, expression of one of the HIV-1 entry receptor CXCR4 was reported to show diurnal variation and was regulated through glucocorticoids (Shimba et al., 2018). This could result in time-of-day dependent viral entry, similar to reports from our laboratory showing oscillations in cellular uptake of HBV (Zhuang et al., 2021a), HCV (Zhuang et al., 2019) and SARS-CoV-2 (Zhuang et al., 2021b). We primarily investigated the transcriptional regulation of HIV-1 by BMAL1, REV-ERB and ROR. This does not account for circadian regulation of post-transcriptional mechanisms like splicing, m6A methylation of viral RNAs or particle assembly, and does not evaluate the role of other core clock factors like PER or CRY. A Per1 short isoform was shown to inhibit HIV-1 transcription in resting CD4 T cells and Per1 silencing upregulated viral transcription (Zhao et al., 2018). It is likely that multiple cellular clock factors contribute to the circadian regulation of HIV-1 which is worthy of further investigation.

Interestingly, the HIV-1 replication cycle is approximately 24 h, starting with viral entry into cells to release of infectious particles (Mohammadi et al., 2013). This observation is intriguing from a circadian perspective, as it could demonstrate that the virus has evolved to adopt to 24 hour scheduled host biology. Analysis of the host transcriptome throughout one viral replication cycle of 24 hours revealed that 52% of overall variability in host gene expression could be explained by viral progression through the life cycle (Mohammadi et al., 2013). These findings demonstrate the substantial impact of HIV on host cell homeostasis and the question arises if HIV-1 can perturb the host clock.

While our work has investigated how the clock regulates HIV replication, other studies suggest a reciprocal influence of the virus infection on circadian regulated pathways. Synaptic transport of HIV Tat protein caused neurotoxicity and neuroglial dysfunction at sites distant from viral replication in rats (Bruce-Keller et al., 2003). Intraparenchymal injection of Tat in the

SCN of mice altered the phase of the central clock, and delayed the onset of running wheel activity by mimicking effects of light exposure on the circadian timing system (Clark et al., 2005). In contrast, Duncan et al. did not observe altered entrainment of the SCN in a mice model with chronic brain expression of Tat, however, animals had decreased locomotor activity and amplitude of circadian rhythms (Duncan et al., 2008). These observations were further validated by intracerebroventricular injection of Tat expression plasmid in mice, which decreased Clock expression and increased levels of Cry1 transcripts. Moreover, decreased locomotor activities and increased melatonin levels were observed (Ying et al., 2018), which suggests that HIV-1 infection may alter host rhythms. Conversely, an intact circadian machinery was observed in T cells from PLWH on ART, as evidenced by oscillations in gene expression of core clock genes (Stern et al., 2022). Clinical studies have documented a reduction in diurnal blood pressure rhythms among HIV-infected individuals, which may contribute to a higher cardiovascular disease risk (Kent et al., 2016). The decreased nocturnal decline in systolic blood pressure correlated with lower CD4 T cells counts, which could be attributed to the compromised immune status in PLWH (Manner et al., 2017). HIV patients experience an increase in premature immune aging and non-AIDS related diseases as they age (Appay and Kelleher, 2016). Importantly, increasing age generally leads to a dampening of circadian oscillations which can associate with changes in rhythmic activities such as sleep/wake patterns (Hood and Amir, 2017). Polymorphisms in circadian genes have been linked with sleep disturbances and altered rhythms in adults living with HIV (Lee et al., 2015b), and delayed circadian rhythms were detected in older HIV+ Africans (Redman et al., 2023). These studies highlight the interplay between the clock, aging and sleep in HIV-1 infection. Given the interconnected yet distinct nature of sleep and circadian rhythms, unravelling the underlying mechanisms is challenging. Our study contributes by elucidating the cellular-level circadian regulation of HIV-1, shedding light on the molecular basis of this relationship.

In the context of sleep, it is worth discussing the role of the endocrine system and specifically melatonin in the circadian regulation of HIV-1. Melatonin levels increase at night and impact various physiological processes, most importantly sleep and the body internal clock. Melatonin is associated with anti-inflammatory immune responses (Yu et al., 2017; Hardeland, 2018), and melatonin treatment has been reported to provide potential benefits against a range of viral infections (Anderson and Reiter, 2020). It resets immune cell activity during night/rest time by inducing Bmal1 expression (Anderson and Reiter, 2020) and since our work has demonstrated a role for Bmal1 to regulate HIV-1 replication, it would be interesting to analyse this relationship further. This pathway did not play a role in our tissue culture experiments which did not contain melatonin, however, there are multiple clinical studies indicating a potential interplay. Levels of melatonin in blood (Nunnari et al., 2003) or saliva (Ahmadi-Motamayel et al., 2017) were lower in HIV-1-infected individuals compared to age matched uninfected subjects. PLWH expressed higher plasma melatonin levels in the morning (Wang et al., 2014), and a later dim light melatonin onset than uninfected individuals (Redman et al., 2023), suggesting a possible delay in the circadian phase. Further investigation into the relationship between melatonin, Bmal1, and HIV-1 replication could provide valuable insights, especially considering the ready availability and low cost of melatonin.

Another hormone implicated in both HIV-1 infection and circadian regulation is estradiol. The previously mentioned study by Stern et al. found that circulating estradiol rhythms were associated and highly predictive of the circadian variation in HIV-1 RNA in patients (Stern et al., 2022). Notably, this study focused on male participants to mitigate potential sex-based differences, however, it is recognised that the impact of estradiol on HIV transcription is relevant for both males and females (Das et al., 2018). Estradiol induces a complex formation mediated through estrogen receptor alpha, which binds the HIV-LTR and represses HIV-1 transcription (Szotek et al., 2013). Additionally, estrogen receptor alpha is a regulator of latency which further expands the importance of circadian oscillations in estradiol to

reactivation of HIV-1 from latency. Estradiol inhibited HIV-1 transcription, while estrogen receptor modulators enhanced the activity of latency reversing agents in a previous study (Das et al., 2018). We tested estradiol for its effect on active and latent HIV infection in the GKO-VSV-G dual reporter system, but did not see any alterations in the frequency of latent infected cells. These variations could be due to differences in cell types, reporter virus, kinetics, and dosage of drugs.

HIV-1 infection persists over a host's lifetime, and one would hypothesise that it is advantageous for the virus to adapt to host physiological processes, including endogenous circadian rhythms. This is evident in the delicate equilibrium between the host immune response and strategies for the virus to evade immune surveillance. Humans generally exhibit reduced inflammatory responses during the night, which corresponds to the resting phase when the likelihood of encountering pathogens is low (Wang et al., 2022a). We hypothesise that HIV adaptation to host circadian regulated pathways confers an advantage by maximising viral replication during periods when the host anti-viral defences are low. Stern et al. showed elevated Bmal1 and HIV-1 gene expression in CD4 T cells collected during night-time in individuals receiving ART (Stern et al., 2022). This is consistent with our findings which demonstrate a correlation between HIV-1 replication and peak Bmal1 expression, and further strengthens our hypothesis.

ART should suppress most active HIV-1 replication and have a minimal effect on latent infected cells that lack viral encoded RNAs or proteins (Chun et al., 1999; Siliciano et al., 2003). However, there is a persistent detection of low-level cell-associated HIV-1 RNA in peripheral blood CD4 T cells in almost all PLWH despite receiving therapy, consistent with ongoing viral transcription (Lewin et al., 1999; Palmer et al., 2008). Multiple studies aim to reactivate latent HIV-1 to expose infected cells to immune responses, and a clinical study found that short-term administration of disulfiram increased cell-associated unspliced HIV RNA (Elliott et al., 2015). Before treatment, baseline viral RNA levels were measured on three different days, while the

third sample was collected immediately before the first dose of disulfiram and was earlier in the day compared to the previous timepoints. Interestingly, HIV-1 RNA levels were significantly higher at this third timepoint, and the authors speculate that this could result from changes in HIV transcription influenced by circadian rhythm or anticipatory stress (Elliott et al., 2015). Individuals with higher viral baseline levels at the third timepoint had significant increases in plasma HIV RNA after disulfiram treatment, which indicates the potential influence of circadian rhythms on latency reactivation (Ait-Ammar et al., 2019).

HIV transcription is enhanced by the acetylation of histones bound to the HIV-LTR (Van Lint et al., 1996; Archin et al., 2012), and it is intriguing to note that the circadian factor CLOCK possesses intrinsic histone acetyl transferase activity (Doi et al., 2006). König et al. performed genome-wide siRNA analyses to identify host factors regulating HIV-1 replication, and although not explicitly mentioned in the main text, a closer examination of supplementary tables revealed that CLOCK silencing led to a ten-fold decrease of HIV proviruses per cell (König et al., 2008). As discussed above, REV-ERB recruits NCoR to repress transcription, which is implicated in negative regulation of HIV-1 transcription. Interestingly, NCoR1 was associated with repressed HIV-LTR in a complex with HDAC3, and binding of NCoR1 was diminished upon activating latent infected cells with PMA, suggesting its potential involvement in the regulation of latency (Natarajan et al., 2013).

In addition to factors involved in direct circadian feedback loops, other proteins within the broader circadian network may contribute to the regulation of HIV-1 latency. To this end we identified a role for BET bromodomain and salt inducible kinase inhibitors to enhance or reduce activation of latent HIV-1, respectively. As discussed previously, BRD proteins have been implicated in HIV-1 latency reactivation by various other studies (Zhu et al., 2012; Boehm et al., 2013). In contrast, salt inducible kinases have not been studied in the context of HIV-1 infection and we showed that SIK inhibition reduced activation of latent HIV in J-Lat cells. J-Lats require $\text{TNF}\alpha$ as first stimulus to reactivate latency, which is standard practice in

the field (Jordan et al., 2003; Fujinaga and Cary, 2020) and was used in our experiments. However, it is important to note, that $\text{TNF}\alpha$ can suppresses the expression of clock genes by interfering with E-box-mediated transcription (Cavadini et al., 2007). Therefore, additional studies using different latency models are necessary to confirm the role of SIKs in reactivating latency.

SIKs regulate gene expression through CRTC and we speculate that SIK inhibition modulates viral replication through a CRTC associated mechanism (Ma et al., 2021). Interestingly, cAMP response element-binding protein (CREB) was shown to bind to a CRE motif in the HIV-LTR and has intrinsic histone acetyltransferase (Marzio et al., 1998). Reactivation from latency required demethylation of CREB sites in the LTR (Tanaka et al., 2003), which further points towards a complex mechanism involving SIKs, CRTC, CREB, HDACs in regulating HIV-1 latency. Overall, these findings indicate that circadian rhythms can influence latency at a molecular and clinical level. Hence, the time of administration and blood collection could affect potency of latency reversing agents, which should be considered in clinical trials.

Our work analysed various small molecule modulators which regulate circadian rhythms and perturb HIV-1 replication. While compounds were primarily used to probe molecular mechanisms, their clinical relevance could be evaluated in future experiments. It is striking that 82% of the genes encoding proteins targeted by therapeutic drugs in clinical practice exhibit rhythmic expression in at least one tissue (Mure et al., 2018). The majority of the 100 bestselling drug in the United States target circadian genes and have a half-life of <6 h (Zhang et al., 2014), however, only 4 of the 50 most prescribed drugs have time-of-day dosing recommendations (Marc D. Ruben, 2019). From a translational perspective, this underscores the significance of research such as ours, which explores the connection between circadian regulation of diseases and clock-modulating drugs.

In conclusion, our study sheds light on the interplay between HIV-1 and the circadian system, offering valuable insights into the host pathways governing viral transcription. These findings contribute to our fundamental understanding of the molecular mechanisms underlying HIV-1 replication and provide a basis for further exploration of therapeutic interventions targeting circadian regulation in HIV infection. We hypothesise that viruses have developed mechanisms to hijack and utilise the cellular circadian machinery, underscoring the need for further investigation into the interplay between viruses and the circadian system. By unravelling this complex relationship, we can pave the way for innovative approaches to combat viral infections and improve patient outcomes.

8. References

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