UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of *high mobility Group AT-Hook 1 (HMGA1)* Gene Expression in HIV Reactivation

A Thesis submitted in partial satisfaction of the
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by

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The Thesis of Hosiana Abewe is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

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DEDICATION

First and foremost, I would like to thank and dedicate all my success to the almighty GOD; without his love, grace, guidance and protection, I would not be who I am today. I dedicate this thesis work to my parents, Jean Baptist Nkuba and Marie Agnes Mukandashimye, who have loved me unconditionally and have supported me since the day I was born. I thank you for raising me into a strong, hard working woman I am today, and for being my role models. This thesis is also dedicated to my siblings for being my source of inspiration and encouragement. Last but not least, I dedicate this thesis work to my best friend, Clement Muhayimana, who has been my rock throughout this journey. I am truly thankful for having you in my life.

EPIGRAPH

For the LORD gives wisdom;

from His mouth come knowledge and understanding.

—Proverbs 2:6

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ABSTRACT OF THE THESIS

The Role of *high mobility Group AT-Hook 1 (HMGA1)* Gene Expression in HIV Reactivation

by

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Professor Douglas Richman, Chair

In the present era of combination anti-retroviral therapy (ART), the latent cellular reservoir of HIV is recognized as the major barrier to a cure. HIV latency may be reversed by a controlled induction of virus reactivation in the presence of ART to reveal latently infected cells for immune system recognition and destruction. Histone deacetylase inhibitors (HDACis) have been used as potential latency reversing agents for HIV. Suberoylanilide hydroxamic acid (SAHA), the most widely tested HDACi, has effectively induced expression of HIV RNA

in patients on suppressive ART, but has failed to reduce the size of the latent reservoir. The secondary effects of SAHA on host gene and protein expression are among the possible explanations of why SAHA has failed to deplete the latent reservoirs. SAHA upregulates *the high mobility group AT-hook 1 (HMGA1)* gene, which encodes a protein that represses reporter transcription from HIV promoter, long terminal repeat (LTR) in primary CD4 T cells. In this thesis study, we investigated the role of *HMGA1* in HIV reactivation by SAHA. Latently infected CD4 T cells from 11 different blood donors were generated through *in vitro* model of HIV latency and were each treated with 1uM SAHA or its solvent dimethyl sulfoxide (DMSO). The expression of *HMGA1* and HIV reactivation were assessed using droplet digital PCR (ddPCR). The degree of *HMGA1* upregulation by SAHA negatively correlated with levels of HIV reactivation. Knockdown experiments were conducted using GapmeR technology in primary CD4 T cells. *HMGA1* GampeRs were used to test whether *HMGA1* played a role in HIV reactivation by SAHA. Latently infected CD4 T cells from 5 seronegative blood donors were generated through *in vitro* model of HIV latency and were each treated with 1 uM SAHA or DMSO. The expression of *HMGA1* and HIV multiply spliced transcripts was measured using ddPCR. Although, a modest knockdown of *HMGA1* was achieved, the improvement of HIV reactivation by SAHA in three samples suggested that *HMGA1* presents a potential target for improvement of HIV reactivation by SAHA. However, further studies that optimize *HMGA1* knockdown are needed to fully understand the role of *HMGA1* in HIV reactivation by SAHA.

1 Introduction

1.1 Cellular reservoirs of latent infection are the major barrier for an HIV cure

Although the mortality and morbidity in human immunodeficiency virus (HIV) infected individuals has decreased impressively over the past decades, HIV and acquired immunodeficiency syndrome (AIDS) remain among the world's greatest public health challenges. According to the Joint United Nations Programme on HIV and AIDS (UNAIDS) 2016 report, nearly 36.7 million people were living with HIV, including 2.1 million new infections, at the end of 2015 (Global AIDS update, 2016). Chronic HIV infection is currently a major health burden. The introduction of antiretroviral therapy (ART) has prevented the progression of HIV infection to AIDS by inhibiting ongoing virus replication and thereby reducing patients' plasma viral load to undetectable levels by standard assays (<50 copies of HIV/ml) (Blankson et al., 2002). ART has thus prolonged the life expectancy of individuals infected with HIV and reduced the risk of HIV transmission. Never-

theless, ART does not eliminate the virus in infected individuals; residual viremia persists in ART treated patients, thus requiring a lifelong treatment. Interruption of ART results in increased levels of viremia from HIV-1 latent reservoirs, which consists of a small population of cells bearing replication-competent provirus that persists during therapy (Darcis et al., 2017). As a chronic disease, HIV infection is potentially associated with inflammation and immunodeficiency complications, such as cardiovascular and liver diseases (Shahbaz et al., 2015; Bica et al., 2001). Because of side effects of ART, its cost and poor adherence in some cohorts, researchers have focused on finding a cure for HIV, whereby the latent reservoirs can be either inactivated or eliminated.

HIV integration into the host genome can result in two types of infections, productive or latent. HIV preferentially infects CD4 T cells and integrates into their genomes as part of the replication cycle. The viral integration is usually followed by productive infection, in which the viral genome is transcribed and virions are produced causing lysis of the infected cell due to their cytopathic effect (Archin et al., 2014) or recognition by the immune system of viral proteins produced by infected cells, resulting in destruction of the cells (Blankson et al., 2002). However, in rare occasions, latency occurs instead of productive infection (Kumar et al, 2015). In these cases, latency can arise from direct resting cell infection, during which resting CD4 T cells subsets, mostly memory and stem central memory cells are infected (Chun et al., 1997; Chomont et al., 2009), or from infection during deactivation, in which infection occurs in activated effector cells that are transi-

tioning to a resting state during generation of memory cells (Chun et al., 1997; Archin et al., 2014; Donahue & Wainberg, 2013). During latent infection, the viral genome remains stably integrated and transcriptionally silent, due to host cellular mechanisms, such as histone and DNA modifications, the absence of necessary transcriptional activators or the presence of transcriptional repressors, which allow them to escape immune system detection (Duh et al., 1989; He & Margolis, 2002). The residual plasma viremia in HIV infected individuals under ART is believed to predominantly correlate with the size of latent reservoir in resting CD4 T subset cells; the CD4 T memory stem cells in particular may present a favorable escape for the virus because of their abilities for self-renewal, resistance to apoptosis and long lifetime (Buzon et al 2013).

The viral reservoirs are established in the early days of infection usually before diagnosis. Hence, by the time ART is administered the size can be limited, but the establishment can't be prevented (Strain et al.,2005; Whitney; J.B. et al., 2014). Targeting HIV latent reservoirs using ART presents several limitations, including the fact that they exist in very small numbers (<50copies/ml) (Blankson et al., 2002) and are predominantly located in organs, such as the brain, lymph nodes, gut, female reproductive tract and other tissues, where ART accessibility may be low (Fletcher et al., 2014). The viral reservoirs are capable of producing virions upon various cellular stimuli; expression of viral RNA and protein is activated at the encounter of an antigen or expose to specific cytokines or chemokines, leading to productive infection. Thus, there is a need to eliminate the HIV latent

reservoirs to prevent resumption of viral replication on withdrawal of ART (Davey et al., 1999) and to provide a cure to eliminate the need for lifelong ART.

1.2 Strategies to eliminate the cellular reservoirs of latent HIV infection

Several therapeutic strategies targeting the HIV latent reservoirs are currently being tested to reduce or eliminate the latent reservoir or render the virus inactive. Studying HIV latency has been challenging, since latently infected cells are rare, and there are complex molecular mechanisms involved, which are not fully understood yet. Despite the challenges, transcriptional regulation is likely the key approach to latency (Van Lint et., 2013). Different cellular mechanisms implicated in latency establishment operate at post-transcriptional level, but mostly at the pre-transcriptional level (Darcis et al., 2017). Sequential elements have to occur to allow a successful transcription. The initial stages of transcription involve cell signaling that promotes chromatin decondensation and activates transcription factors, which assemble on the provirus promoter. If one of these initial stages is hindered, transcription does not occur. Important mechanisms involved in inhibition of HIV transcription include histone modifications affecting chromatin condensation (Van Lint et al., 1996; Van Lint et al., 2013), absence of inducible host transcription factors (Duh et al., 1989; He and Margolis, 2002) and the blockage of the cellular transcription elongation factor (P-TEFb) (Eilebrecht et al., 2014). To this end,

the potential proposed curative approaches aim either at reactivating the latent proviruses and revealing the latently infected cells for host immune system recognition and destruction or at locking them out permanently to avoid reactivation and viral rebound (Darcis et al., 2017).

Deep understanding of several cellular pathways involved in viral transcription is the core for successful implementation of the previously mentioned curative approaches. The most extensively studied pathways are associated with inhibition of viral transcription. In this study, we will limit our discussion to the role of chromatin packaging in HIV latency. Chromatin packaging greatly influences gene expression. The loosely packaged form of chromatin named euchromatin favors transcription, whereas the tightly packaged form called heterochromatin reduces transcriptional activity. HIV proviruses are subjected to the same chromatin remodeling like any other host genes (Kumar et al., 2015). One of mechanisms associated with chromatin remodeling and HIV latency is histone reversible acetylation. This modification is mainly governed by the activity of two types of enzymes: histone acetyltransferases (HATs), which decondense the chromatin by adding acetyl group to the amino group of lysine residues in histone tails, and histone deacetylases (HDACs), which compact the chromatin by removing the acetyl groups (Van Lint et al., 2013). HDACs are classified into groups based on homology: class I HDACs (HDAC1,2,3 & 8), class II HDACs (HDAC 4,5,6,7,9 & 10), class III deacetylases (SIRT1-7) and class IV HDAC (HDAC11); all groups are zinc-dependent except class III, which is NAD dependent (Keedy et al., 2009; Xing

and Siliciano, 2012). Among these groups, only three class I HDACs (HDAC1, 2 and 3) have been located at the promoter of integrated HIV, 5' long terminal repeat (LTR) (Xing and Siliciano, 2012). In latent HIV reservoirs, these HDACs are recruited to the HIV 5' LTR by host factors including late SV40 factor (LSF), Ying Yang 1(YY1), NF-kappaB p50 (Williams et al.,2005), where they reduce the accessibility of DNA templates by removing acetyl groups, therefore inhibiting transcriptional activity (Kumar et al., 2015).

The "shock and kill" treatment strategy proposes the use of latency reversing agents (LRAs) to promote transcription of silent proviruses, upon which cells harboring these proviruses are lysed by the viral cytopathic effects or cytolytic effector mechanisms of the host immune system detecting induced viral proteins (Darcis et al., 2017). LRAs, including protein kinase C (PKC) agonists, HDAC inhibitors (HDACis), histone methylation inhibitors (HMTi), DNA methylatransferase inhibitors (DNMTi), inhibitors of bromodomain and extraterminal (BET) domain proteins (BETi), and disulfiram, target different cellular pathways involved in viral transcription, particularly the ones associated with inhibition of viral promoter activity (Van Lint., 2013; Darcis et al., 2017). For example, hexamethylene bisacetamide (HMBA), BETi (Contreras et al., 2007; Bartholomeeusen et al., 2013) as well PKC agonists such as bryostantin-1 (Mehla et al. 2010) activate P-TEFb production and its recruitment to the HIV LTR, thereby promoting viral transcription. The shock and kill strategy also uses LRAs that interfere with enzymes implicated in reversible chromatin modifications, such as HATs, HDACs,

and histone methyltransferases, leading to alterations in the chromatin structure in a way that prevents transcription factors to access the viral genome, thus promoting viral silencing. Our discussion is restricted to the histone modifying enzymes, HDACs, responsible to HIV latency and how they are targeted using shock and kill strategy.

Histone deacetylase inhibitors (HDACis) are among the well characterized LRAs *in vitro* and ex *vivo*, and are used to promote a transcriptionally favorable environment for integrated HIV proviruses (Darcis et al., 2017). HDACis have been applied to cancer therapies and are currently investigated to induce latent proviruses by inhibiting HDACs. More than 10 HDACis are tested in different phases of clinical trials for various human diseases (Delagreverie et al., 2016). Vorinostat, also called suberoylanilide hydroxamic acid (SAHA), has been the most widely tested HDACi in clinical trials for HIV activation (Contreras et al., 2009; Darcis et al., 2017). Initially, SAHA was approved by the Food and Drug Administration (FDA) for the treatment of cutaneous T cell lymphoma (Mann et al., 2006). SAHA's hydroxamic acid moiety is highly efficient zinc chelator and makes SAHA a potent inhibitor of zinc-dependent HDACs 1,2, 3, and 6 (Wagner et al., 2013), which has resulted in induction of HIV RNA expression in patients on ART (Contreras et al., 2009; Xing and Siliciano, 2012). Archin and colleagues demonstrated that upon administration of SAHA, in each of eight patients studied, a single-dose of SAHA induced acetylation of histone H3 and transcription of HIV RNA in resting CD4 T cells simultaneously (2012). Although SAHA has effectively

reactivated latent virus in patients, it has failed to reduce the size of the latent reservoir (Elliott et al., 2014). It is possible that the net effect of SAHA results in induction of gene expression from the HIV promoter at insufficient levels to kill the infected cells (Margolis and Hazuda, 2013). The fact that SAHA binds not only to HDACs, but other proteins (Bantscheff et al. 2011), opens the possibility of secondary effects, which may add to or interfere with its primary effects.

Indeed, using combined proteomic and transcriptomic profiling, White and colleagues demonstrated that SAHA had mixed effects on host genes and proteins with a role in regulation of HIV transcription, in addition to the primary mechanism of chromatin unwinding (White et al., 2015). For example, up-regulation of transcriptional repressors, such as genes related to negative regulation of chromatin silencing and nuclear euchromatin, as well as down-regulation of transcriptional activators, including genes associated with histone acetylation and histone acetyltransferase, by SAHA (Beliakova-Bethell et al., 2013). A number of genes with a role in HIV reactivation are significantly affected at the RNA and protein levels by SAHA (White et al., 2015). Further analysis confirmed that three genes, including *the High mobility group AT-hook 1 (HMGA1), Anti-silencing function 1A histone chaperone (ASF1A) and Amino-terminal enhancer of split (AES)* were modulated by SAHA at both the RNA and protein levels. *AES*, which has a positive effect on HIV transcription, was down-regulated by SAHA, whereas both *HMGA1* and *ASF1A*, which negatively affect HIV transcription, were up-regulated by SAHA (White et al. 2015). Further analyses are needed to gain insights on

how up-regulation or down-regulation of each of these proteins correlates with HIV transcription in primary cells, and how they can be targeted for HIV cure.

1.3 *HMGA1*, a potential target for improvement of SAHA's efficacy

Also called architectural transcription factors, *HMGA1* proteins play an essential role in gene expression. They are members of the high mobility group superfamily, which consists of non-histone proteins involved in chromatin remodeling (Benecke and Eilebrecht, 2015). All of these proteins share an acidic carboxyl-terminus and differ from each other by unique functional motifs that confer distinct DNA binding domains and biologic activities (Shah et al., 2013). The *HMGA* family, which consists of *HMGA1* and *HMGA2*, is characterized by AT-hook binding motifs. This thesis study particularly focuses on *HMGA1* gene. The *HMGA1* gene encodes two protein isoforms, *HMGA1a* and *HMGA1b*, resulting from alternatively spliced messenger RNA (Shah et al., 2013). Using the three AT-hook domains, *HMGA1* protein binds the minor groove of chromosomal DNA at A/T-rich regions, thus inducing conformational changes in DNA structure creating a more favorable DNA conformation for factor binding, which promotes transcription (Eilebrecht et al, 2014; Benecke and Eilebrecht, 2015). Moreover, *HMGA1* in concert with other factors regulates gene expression; upon binding the A/T rich DNA sequences in gene promoter or enhancer regions, *HMGA1* recruits additional

transcription factors, with which it forms a higher order transcriptional complex or enhanceosome (Shah et al., 2013). Consequently, *HMGA1* is highly expressed in rapidly proliferating cells, including early embryonic cells, neoplastic tissues (Henderson et al., 2000) and cancer cells (Benecke et al., 2015).

Apart from being involved in tumor progression, *HMGA1* is implicated in gene expression of several types of viruses, such as herpes simplex virus (HSV) (French et al., 1996), human papovavirus JC (Leger et al., 1995), Epstein – Barr virus (EBV) (Schaefer et al., 1997) and HIV. Previous studies have revealed the role of *HMGA1* as a host-specific factor required for the integration of HIV-1 pre-integration complexes or for the integration of HIV pre-integration complexes (Miller et al., 1997; Henderson et al., 2000). Moreover, the identification of different low- and high-affinity *HMGA1* binding sites within the HIV LTR have suggested a role of *HMGA1* during HIV transcription (Henderson et al., 2000; Eilebrecht et al., 2013). Eilebrecht and colleagues have demonstrated two mechanisms by which *HMGA1* interferes with HIV reactivation. First, *HMGA1* specifically binds to the viral transactivating response element (TAR), thus competing with the viral transactivator of transcription (Tat) for TAR binding (2013). Secondly, in the absence of Tat, TAR-bound *HMGA1* prevents the binding of transcription activating host co-factors to TAR, thus diminishing HIV-1 promoter activity (2014). Droplet digital PCR (ddPCR) and RNA-seq analysis have suggested that significant up-regulation of *HMGA1* by SAHA can be potentially associated with the failure of SAHA to induce enough virions to kill latent reservoirs (Beliakova-Bethell et

al., unpublished observations). In the present study, two hypotheses are tested in primary CD4 T cells:

1. The degree of *HMGA1* upregulation by SAHA negatively correlates with levels of HIV reactivation.

2. Knocking down *HMGA1* expression improves the ability of SAHA to reactivate latent HIV.

2 Methods

2.1 *In Vitro* model of HIV latency

An *in vitro* model of HIV latency is the method used to generate latently infected CD4 T cells. Peripheral blood from HIV seronegative donors were collected by venipuncture according to institutional review board approved protocols. Peripheral blood mononuclear cells (PBMC) were isolated from blood using the density gradient centrifugation strategy. CD4 T cells were isolated from PBMCs using EasySep^{TM} Human CD4 T Cell Isolation Kit through the fully automated RoboSep. A portion (∼80 %) of the freshly isolated CD4 T cells (bystander) was set aside and incubated at 37°C for four days. The remaining portion (∼20%) of CD4 T cells were stained with the organic dye Alexa Fluor 700 (eFluor 700) and incubated overnight at 37°C. On the next day, the stained cells were infected with HIV for 4 hours, stimulated on plates coated with anti-CD3/CD28 and incubated for three days at 37°C. On day four, the unstimulated, unstained bystander cells were mixed with the fully activated, productively infected, stained, cells at a ratio of 4:1 in the presence of interleukins IL 2 and IL15. Bystander cells were infected

via cell to cell HIV transmission from the productively infected cells over the next three days. On day seven, the mixed cells were sorted using a MoFlo instrument to separate unstained resting cells, which contained latent provirus. The cells were retained in culture for three more days, and on day ten, the latent cells were ready for assays.

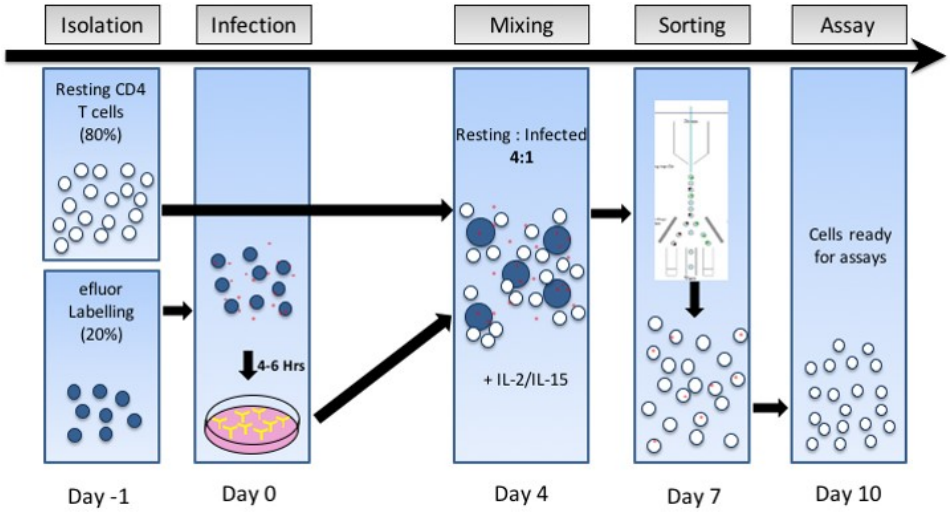

Figure 2.1: ***In vitro*** **model of HIV latency used to generate latently HIV infected primary CD4 T cells.** Day -1: Staining of 20% of freshly isolated CD4 T cells with viable dye eFluor; 80% of cells (resting cells) are maintained unstained and incubated until day 4. Day 0: eflour stained cells are HIV infected for 4 hours and stimulated on αCD 28 & αCD 3 coated plates for 3 days. Day 4: resting cells are mixed with stained, infected, stimulated cells at a ratio 4:1 and incubated until day 7. Day 7: small unstained cells from the mixture are sorted by flow cytometry. Day 10: cells are ready for treatment.

The *in vitro* model of HIV latency was used both in the analysis of the relationship between *HMGA1* upregulation by SAHA and HIV reactivation, and *HMGA1* gene expression knockdown studies.

2.2 Assessment of CD4 T cell purity, activation, and HIV infection by flow cytometry during the model set up

To assess the purity of the freshly isolated CD4 T cells, CD4 T cells were stained with anti-CD4 fluorescein isothiocyanate (anti-CD4-FITC). An antibody against anti - human leukocyte antigen -D related (HLA – DR) labeled with phycoerythrin (PE) was used to check the levels of activation in CD4 T cells and to ensure that it is low (<10%), since mostly resting cells were required for our experiments. Cells were incubated with antibodies for 30 minutes at 4°C, washed with phosphate buffered saline (PBS) and fixed with 1% formaldehyde. On days 4 and 7, aliquots of mixed cells along with controls, including bystander cells, uninfected stimulated unstained cells, infected unstained unstimulated cells, infected unstained stimulated cells and infected stained stimulated cells, were washed with PBS, fixed and permeabilized for 20 min. for intracellular p24 staining. The cells were incubated with the mouse monoclonal HIV-1 p24 antibody for 30 min at 4°C; finally, they were washed to remove unattached antibodies and fixed with 1% formaldehyde. Percentages of CD4+, HLA-DR+ and p24+ cells were assessed using BD Accuri C6 flow cytometer.

2.3 GapmeR-mediated gene expression knock-down

GapmeRs manufactured by Exiqon Inc, is an emerging technology used to silence the expression of a specific gene. GapmeR is a type of antisense oligonucleotide comprised of a synthetic single strand containing central block of DNA nucleotides flanked by locked nucleic acids on each side, which increase the stability of the strand in the biological fluid. GapmeRs are taken up by macropinocytosis (Fazil et al., 2016) and bind to the complementary sequence of the mRNA of interest. The host RNase H, a ubiquitous enzyme located in the nucleus as well as in the cytoplasm, recognizes RNA-DNA hybrids formed after sequence-specific binding of antisense oligonucleotides to their target mRNA and degrades the mRNA. GapmeRs present advantages over other commonly used synthetic molecules for knockdown, such as small interfering RNA (siRNA), because of their small size, stability, and ability to enter cells without the need of transfection, resulting in increased cell viability (Crooke et al., 2017).

To optimize usage of GapmeRs in primary CD4 T cells, cells from HIV seronegative donors were used. Positive control GapmeR developed by Exiqon, Inc., which targets host gene *MALAT1*, encoding a long non-coding RNA, was used for the initial optimization studies. Cellular uptake was monitored using a fluorescein-labeled GapmeR; and knockdown efficiency was determined using ddPCR. Different GapmeR concentrations (100-500 nM) were used over a time

course to determine optimal conditions for the knockdown of gene expression. The optimal knockdown conditions (300 nM for 3 days) were then used to test experimental *HMGA1* GapmeRs (1-8), designed by the Exiqon, Inc. online tool. GapmeRs 1-4 were designed against exonic regions of *HMGA1*, while GapmeRs 5-8 were designed against intronic regions to target RNA as it is synthesized.

2.4 Reactivation of HIV with SAHA following *HMGA1* gene expression knockdown using GapmeR

Latently infected cells were generated using the *in vitro* model of HIV latency, treated with GapmeR (300uM), plated and incubated for three days at 37°C. Untreated cells were used for background reference to determine the level of gene expression knockdown. On day three, cells that were incubated in the absence or presence of the *HMGA1*-specific GapmeR were treated with SAHA or its solvent dimethylsulfoxide (DMSO). The next day, cells from all treatment conditions were counted using Accuri flow cytometer and were collected for RNA extraction.

2.5 RNA extraction

At the end of the experiment, cells were collected in 1.5 ml centrifuge tubes and spun at 300g for 8 min in a tabletop centrifuge. Upon removal of supernatant,

the cell pellets were lysed with lysis buffer from the Qiagen RNeasy micro kit (RLT buffer containing β-mercaptoethanol). Lysates were frozen at 80°C, and were processed for RNA extraction when all samples were collected for an experiment. RNA was extracted using the Qiagen RNeasy micro kit according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from the extracted RNA using qScript manufactured by Quanta Bio Inc.

2.6 Droplet digital PCR (ddPCR)

DdPCR, is a novel technology that is used to precisely quantify the target nucleic acids in a sample. The ddPCR workflow starts like any other quantitative PCR assay; water or buffer, primers, DNA polymerase, deoxynucleotide triphosphates, and cDNA template are required to set up the reaction. In addition, ddPCR, in particular, requires fluorescently labeled internal hybridization probes (TaqMan probes) for detection of the amplification product (Mazaika and Homsy, 2014). We used the following TaqMan gene expression assays by Applied Biosystems: HS00852949-g1 for *HMGA1* and Hs03044961-g1 for *RPL27*. HIV MS assay was a custom assay from Integrated DNA Technologies, Inc. ddPCR Supermix for probes (No dUTP) (Bio-Rad, Inc., Hercules, CA) was used for cDNA amplification. The reactions were partitioned into 20,000 droplets using the Bio-Rad QX-100 Droplet Generator (BioRad Inc., Hercules, CA) following the manufacturer's instructions.

Upon transferring the droplets in the 96-well plate, which was heat sealed with foil, cDNA was amplified to endpoint using PCR cycles. For *HMGA1* and *RPL27* assays, the following cycle was used: 95°C 10 minutes (1 cycle), 94°C 30 seconds and 60°C 1 minute (45 cycles), 98°C 10 minutes (1 cycle), 4°C until droplet reading. For HIV MS assay, the following cycle was used: 95°C 10 minutes (1 cycle), 94°C 30 seconds and 62°C 1 minute (50 cycles), 98°C 10 minutes (1 cycle), 4°C until droplet reading. The data were processed and acquired using the Bio-Rad droplet reader, which measures concentration of target cDNA as copies per microliter from the fraction of positive droplets using Poisson statistics, from which total number of copies per reaction can be obtained.

2.7 *HMGA1* knockdown calculations

HMGA1 expression in both *HMGA1* GapmeR treated and untreated samples was first normalized to a host gene normalizer, *RPL27*, which is a gene that encodes a member of the L27e family of ribosomal proteins and is a component of the 60S subunit. *RPL27* expression is not modulated by SAHA, and thus is used to normalize *HMGA1* expression to account for technical errors during the experiment. The percent expression was then calculated by taking the ratio of *HMGA1* expression in cells treated with *HMGA1* GapmeR to *HMGA1* expression in cells not treated with *HMGA1* GapmeR. Percent knockdown was calculated by taking the difference between 100% and percent *HMGA1* expression.

2.8 HIV reactivation calculations

HIV expression data was normalized to the number of cells participating in each ddPCR reaction to generate the number of the multiply spliced (MS) HIV transcripts per cell. The ratio of MS/cell in samples treated with SAHA to DMSO treated samples was taken to calculate the fold changes of HIV expression in the absence or presence of *HMGA1*-specific GapmeR. The two fold changes were then divided (*HMGA1* GapmeR/no *HMGA1* GapmeR) to determine percent improvement of the ability of SAHA to activate the latent provirus.

2.9 Statistical analyses

The relationship between the degree of *HMGA1* upregulation by SAHA and HIV reactivation was assessed using Pearson correlation in Bioconductor R. To compare HIV reactivation by SAHA in the *HMGA1* GapmeR-treated vs untreated cells, the paired t test was used.

3 Results

3.1 The degree of *HMGA1* up-regulation by SAHA negatively correlates with levels of HIV reactivation

Several studies have led to important observations about the variability in host response to SAHA at the level of gene expression, as well as HIV reactivation out of latency (Elliott et al., 2014; Bantscheff et al. 2011; White et al., 2015). Based on previous studies by Eilebrecht and colleagues (Eilebrecht et al. 2014), the effect of SAHA on *HMGA1* expression is predicted to be inhibitory for HIV reactivation. Because *HMGA1* was upregulated by SAHA, we hypothesized that this effect of SAHA on the host might be inhibitory with respect to HIV reactivation. Here we tested whether the degree of upregulation of *HMGA1* expression by SAHA was negatively correlated with the degree of induction of HIV RNA. To test this hypothesis, the *in vitro* model of HIV latency was used. RNA samples

from 11 different blood donors, treated with 1 uM SAHA or its solvent DMSO were previously generated in the lab. These samples were used to measure expression of *HMGA1*, the host gene normalizer *RPL27* and the HIV MS transcript by ddPCR. Fold changes of the normalized expression values were used for the correlation analysis (please see Methods for details). A significant negative correlation (R=-0.87; p=0.0005) was observed between up-regulation of *HMGA1* by SAHA with induction of HIV multiply spliced transcripts (figure 3.1). In other words, the more up-regulation was *HMGA1*, the less HIV activation was observed.

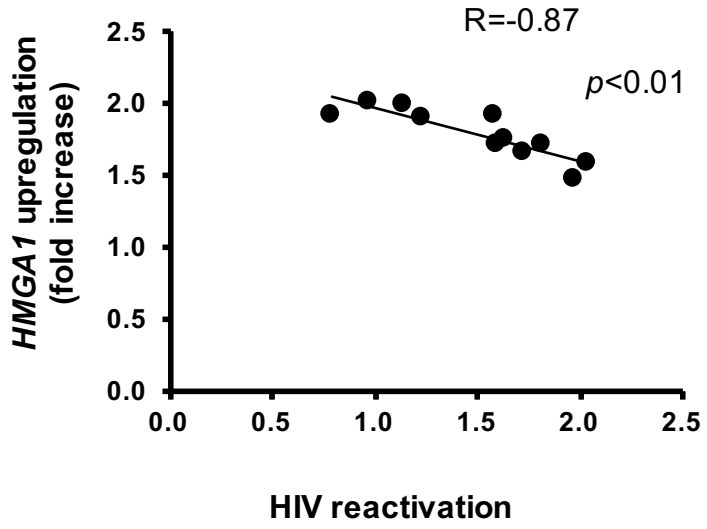

Figure 3.1: **The degree of HIV reactivation by SAHA negatively correlated with the degree of upregulation of the host gene *HMGA1* (R=-0.87; p=0.0005)**. Gene expression was measured by ddPCR in samples from N=11 independent blood donors. Upregulation of *HMGA1* and HIV are expressed as fold change in SAHA- treated relatively to DMSO-treated samples. Bioconductor R was used for Pearson correlation analysis.

As an alternative approach to analyze these data, donors were classified

into responders or non-responders. The technical variation of the MS assay in ddPCR was 1.5, which was the difference that could be seen with repetitive ddPCR reactions from the same RNA sample. Thus, any donor with HIV MS fold change of less than 1.5 following SAHA treatment was denoted a "non-responder", while any donor with fold change greater than 1.5. was denoted a "responder". Up-regulation of *HMGA1* was higher in non-responders, average *HMGA1* up-regulation of 2-fold in responders vs 1.7-fold in non-responders, (p=0.0056) (figure 3.2). These results were consistent with the idea that *HMGA1* was a potential HIV transcriptional repressor in primary CD4 T cells.

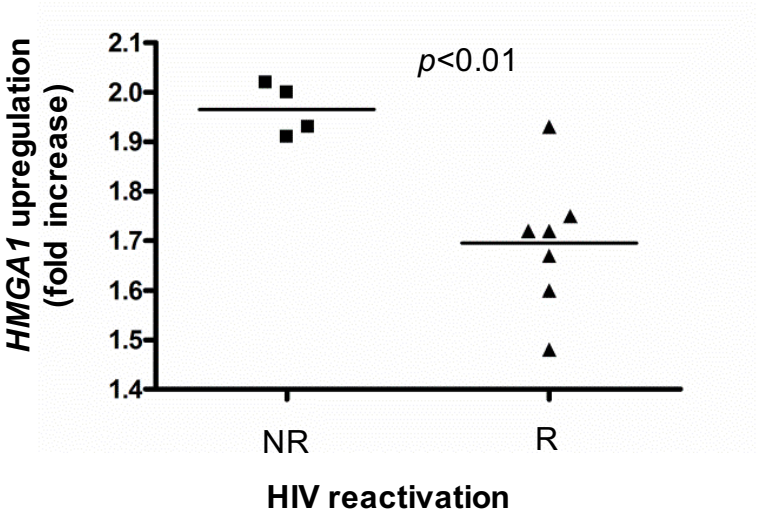

Figure 3.2: ***HMGA1* was upregulated by SAHA the most in samples with no HIV reactivation**. Donors were classified into non-responders (*NR*, HIV multiply spliced HIV transcript (MS) fold change <1.5) and responders (*R*, HIV MS fold change >1.5). Fold change cut off was selected based on the technical variation of the HIV MS assay in ddPCR. Differences between fold changes was assessed using Student's t-test.

3.2 GapmeRs induce gene expression knockdown in primary CD4 T cells

3.2.1 Assessment of knockdown with GapmeR in primary CD4 T cells

To perform a more direct assessment of *HMGA1* function in HIV reactivation from latency, we employed the novel GapmeR technology for knockdown of gene expression. Most of the tests and experiments with GapmeRs thus far was performed using cell lines. Therefore, our first goal was to determine whether GapmeRs could induce knockdown of gene expression in human primary CD4 T cells.

To establish conditions that promote efficient knockdown of gene expression in primary CD4 T cells, a dose response experiment was conducted using the positive control GapmeR specific for long-noncoding RNA *MALAT1*. CD4 T cell samples from three HIV seronegative donors were separately treated with different doses (100-500 nM) of *MALAT1*-specific GapmeR labeled with a fluorescent dye (fluorescein, or FAM), to enable visualization of its uptake by cells by flow cytometry. As depicted on figure 3.3 (right), *MALAT1*-specific GapmeR was taken up by nearly 99.9% of cells, evident from their shift in fluorescence intensity.

All tested concentrations of *MALAT1*-specific GapmeR were efficiently taken up by cells (>90% GapmeR+), except for the 100 nM concentration, with

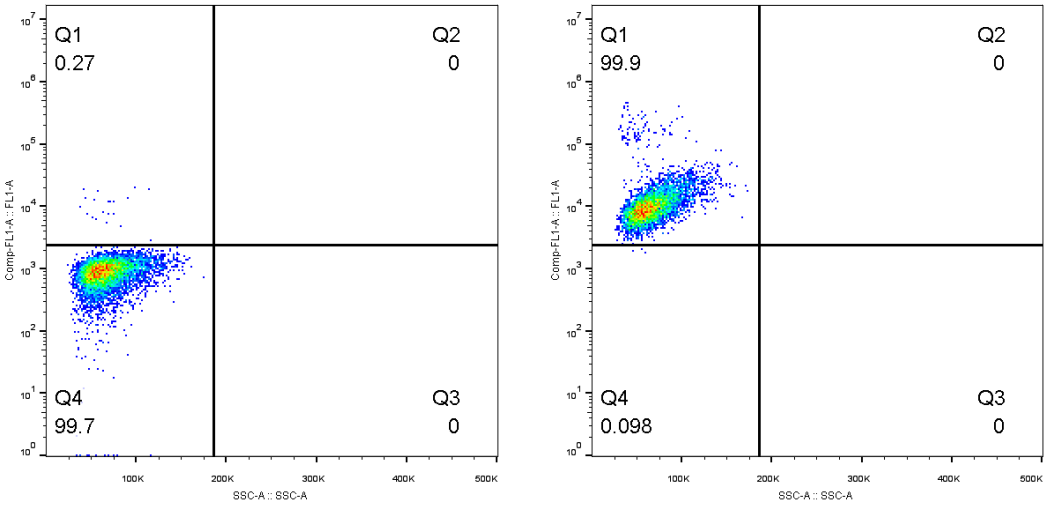

Figure 3.3: **Positive control, *MALAT1*-specific GapmeR, was efficiently taken up by primary CD4 T cells.** Cellular uptake of *MALAT1* GapmeR was assessed using Accuri flow cytometer. *MALAT1*-specific GapmeR was labeled with fluorescein (FAM). (Left) cells without *MALAT1*-specific GapmeR treatment; (Right) cells with *MALAT1*-specific GapmeR treatment.

which only 50% of GapmeR was taken up by cells (figure 3.4).

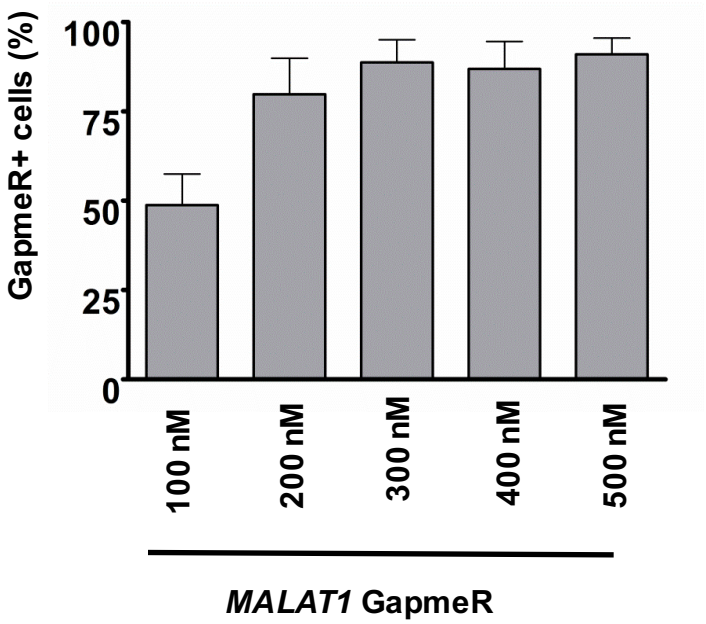

Figure 3.4: ***MALAT1*-specific GapmeR was efficiently taken up by uninfected primary CD4 T cells at multiple concentrations.** Flow cytometry was used to assess cellular uptake of *MALAT1*-specific GapmeR labeled with fluorescein (FAM) in samples from N=3 HIV seronegative blood donors. Cellular uptake of MALAT1-specific GapmeR at 200-500nM was >75%, whereas it was ∼50% at 100 nM.

Next, the expression of *MALAT1* was measured using ddPCR to assess the level of knockdown achieved by different doses of *MALAT1*-specific GapmeR. Samples treated with 100 nM GapmeR were excluded as the uptake by cells was suboptimal. A dose response was observed; however, the largest improvement of knockdown was seen between 200nM and 300nM (percent *MALAT1* expression compared to untreated dropped from 53% with 200 nM GapmeR to 37% with 300 nM GapmeR (figure 3.5). Since 300, 400, and 500 nM doses exhibited relatively

similar percentages of knockdown (37%, 32%, 26% respectively), the 300 nM dose of GapmeR was selected for further studies to reduce cost (figure 3.5).

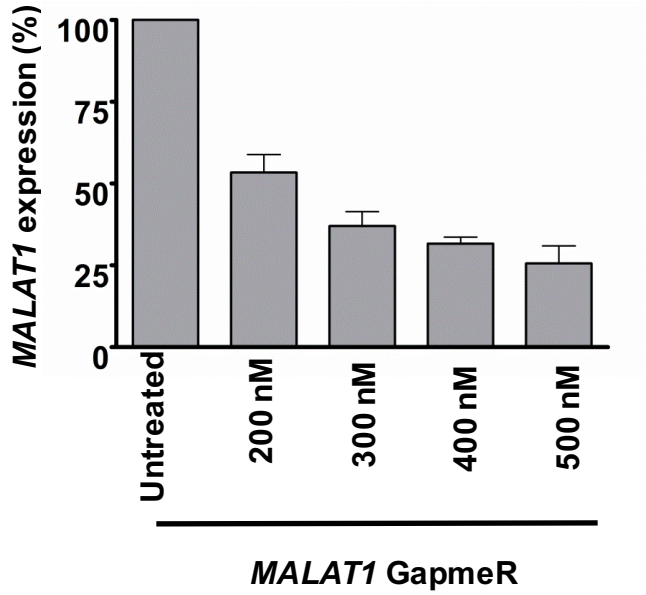

Figure 3.5: ***MALAT1*-specific GapmeR induced *MALAT1* expression knockdown in a dose-responsive manner**. *MALAT1* expression was measured using ddPCR in samples from N=3 HIV seronegative blood donor. A dose response was observed with major difference between 200 and 300nM concentrations. There was no major knockdown improvement among 300, 400, 500nM doses. .

3.2.2 Knockdown of *HMGA1* with GapmeR in primary CD4 T cells

With the established conditions, four different top predicted GapmeR sequences within the coding region of *HMGA1* (*HMGA1*-specific GapmeRs 1-4) were tested in primary cells for their ability to induce *HMGA1* knockdown. CD4 T cells from two seronegative donors were separately treated with *HMGA1* GapmeRs 1-

4. The expression of *HMGA1* and *RPL27* was measured using ddPCR. *HMGA1* GapmeR 4 induced a modest knockdown with a reduction in expression of 13% and 45%, whereas other GapmeRs did not exhibit any knockdown activity (figure 3.6). Because *HMGA1* GapmeR-induced knockdown was modest as compared to knockdown induced by *MALAT1*-specific GapmeR, further experiments were performed to increase *HMGA1* GapmeR – 4 concentration (500 nM) and time of treatment (5 days). However, no improvement of knockdown was observed under these conditions.

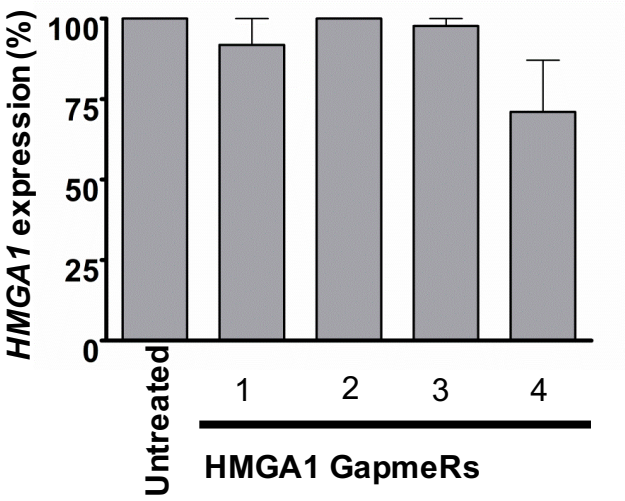

Figure 3.6: ***HMGA1*-specific GapmeR 4 induced a modest knockdown of *HMGA1* in uninfected primary CD4 T cells**. *HMGA1* expression was measured using ddPCR in samples from N=2 HIV seronegative blood donors. *HMGA1*-specific GapmeRs 1-4 were designed based on four top predicted sequences within *HMGA1* coding area. *HMGA1*-specific GapmeR 4 induced 13% knockdown in Donor 1 and 45% knockdown in Donor 2, whereas the remaining GapmeRs did not demonstrate any knockdown activity.

Because the positive control *MALAT1*, which was knocked down well by

a GapmeR, was a long non-coding RNA localized exclusively in the nucleus, we hypothesized that GapmeR-mediated knockdowns might be more efficient when an RNA was targeted in the nucleus, as opposed to the cytoplasm. Therefore, GapmeRs against intronic *HMGA1* sequences were designed to target *HMGA1* RNA in the nucleus as the transcript was being synthesized, before it is spliced and exported into the cytoplasm. These GapmeRs, 5-8, were tested in CD4 T cells from three different HIV seronegative donors, along with the original GapmeR 4 as a point of reference. GapmeRs against intronic *HMGA1* sequences demonstrated less ability to induce knockdown than *HMGA1* GapmeR 4 (figure 3.7). *HMGA1* GapmeR 4 was therefore used in the following studies to evaluate the general effect of *HMGA1* knockdown on HIV reactivation by SAHA despite its modest knockdown activity.

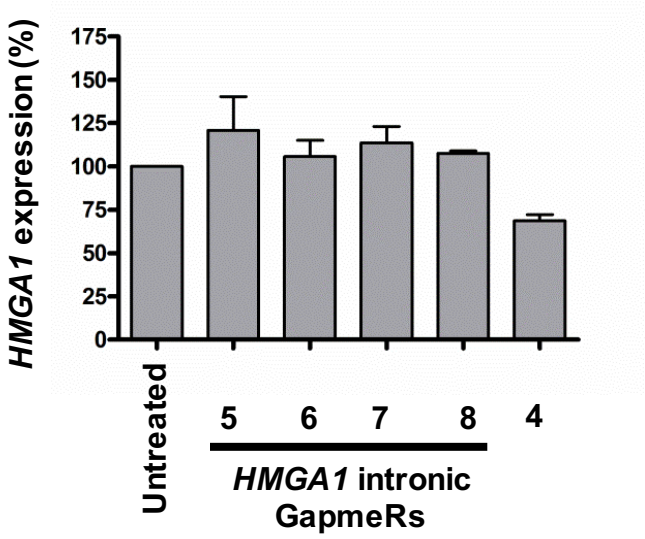

Figure 3.7: ***HMGA1*-specific GapmeRs 5-8 designed against intronic region of *HMGA1* gene demonstrated no knockdown activity.** *HMGA1* expression was measured using ddPCR in samples from N=3 HIV seronegative blood donors. *HMGA1*-specific GapmeRs 5-8 consisted of four top predicted sequences within *HMGA1* intronic region. *HMGA1* Gapmer 4 was included as benchmark for testing. *HMGA1*-specific GapmeR 4 induced 33% knockdown, and remained the only GapmeR with any knockdown activity among all GapmeRs tested.

3.3 *HMGA1* knockdown increases HIV reactivation by SAHA

3.3.1 Knockdown of *HMGA1* with *HMGA1*-specific GapmeR 4 in HIV infected primary cells

For this experiment, the *in vitro* model of HIV latency was used with cells from five different blood donors. The purity of CD4 T cells after isolation was >95%, and less than 10% of cells expressed HLA-DR activation marker. Cells

from all donors were successfully infected with HIV, as assessed by p24 staining during the set up of the latency model. Following establishment of latency, cells were treated first with *HMGA1*-specific GapmeR to induce knockdown (or left untreated). Following three days of incubation with GapmeR, both GapmeR-treated and untreated cells were treated with either SAHA or its solvent DMSO for 24 h, to assess HIV reactivation by SAHA with or without *HMGA1* knockdown.

Cells were collected and counted 24h following SAHA treatment and 4 days following addition of GapmeR to the culture. *HMGA1* knockdown was assessed using samples which were not treated with SAHA, but treated with DMSO, by comparing expression in cells that were incubated in the presence of *HMGA1* GapmeR 4 to cells incubated without GapmeR. The average *HMGA1* knockdown in 5 experiments was 30% (70% expression level, range 62-86%). The difference in expression between GapmeR-treated and untreated cells was significant (figure 3.8).

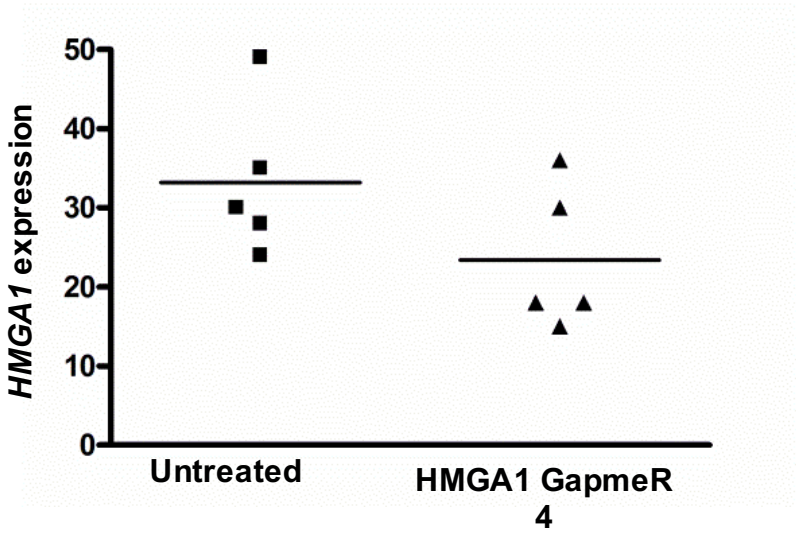

Figure 3.8: **Incubation in the presence of *HMGA1*-specific GapmeR 4 prior to and during SAHA treatment improves HIV reactivation by SAHA**. The *in vitro* model of HIV latency from N=5 blood donors were incubated for 3 days in the absence or presence of *HMGA1*-specific GapmeR 4. *HMGA1* knockdown was assessed using samples which were not treated with SAHA and then treated with either SAHA or its solvent DMSO for additional 24 h. The expression of *HMGA1* and *RPL27* was measured using ddPCR. *HMGA1* expression is calculated as copies of *HMGA1* RNA molecules per thousand *RPL27* RNA molecules. *HMGA1* GapmeR 4 induced 30 % knockdown ($p<0.01$) in all 5 samples.

3.3.2 HIV reactivation with SAHA following *HMGA1* knockdown

HIV MS RNA copy numbers obtained by ddPCR were normalized to cell counts, and fold changes between SAHA and DMSO treated cells were calculated for both GapmeR-treated and untreated cells. On average, *HMGA1* knockdown resulted in a modest improvement of HIV reactivation by SAHA (average 36%, $p=0.048$; range 7-76%) (figure 3.9). However, among the five donors tested, two

donors, the ones with the highest (75%) and lowest (7%) improvement of HIV reactivation in the presence of *HMGA1* knockdown could not be assessed with certainty, because the absolute fold changes of HIV expression in SAHA to DMSO treated samples were less than 1.5 (-1.27 vs 1.39 and 1.28 vs 1.37), below the technical variation of the assay. Excluding these donors, HIV reactivation by SAHA was increased, on average, by 33% (p=0.03) in the presence of *HMGA1* GapmeR. These results are consistent with the idea that even a modest reduction of *HMGA1* expression results in the improvement, albeit also modest, of SAHA to reactivate the latent HIV provirus, and with a role for *HMGA1* in the regulation of HIV transcription in primary CD4 T cells.

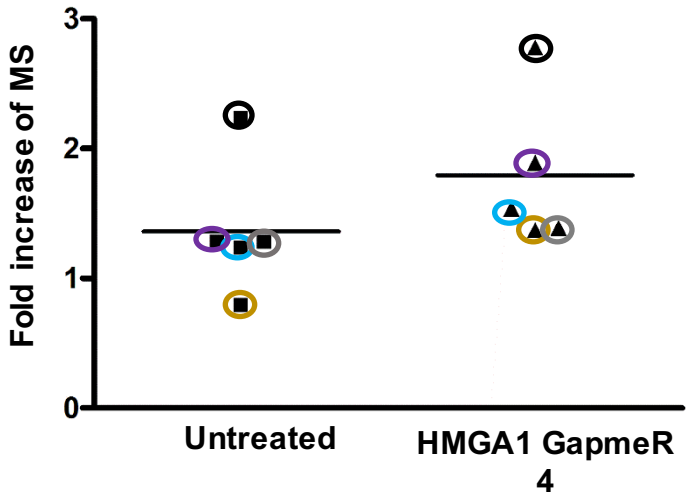

Figure 3.9: **Incubation in the presence of *HMGA1*-specific GapmeR 4 prior to and during SAHA treatment improves HIV reactivation by SAHA.** The in vitro model of HIV latency from N=5 blood donors were incubated for 3 days in the absence or presentce of *HMGA1*-specific GapmeR 4 and then treated with either SAHA or its solvent DMSO for additional 24 h. *HMGA1* and HIV MS expression was measured using ddPCR. Circle colors indicate samples from the same blood donor. On average, a higher increase of MS expression following SAHA treatment was observed in cells incubated in the presence of *HMGA1*-specific GapmeR 4.

Chapter 3. section 1, in full, include the material as it appears in the following abstract and any subsequent publication of this study: Abewe H, Mukim A, Deshmukh S, Spina CA, Richman DD, Beliakova-Bethell N. *Role of High-Mobility Group A1 (HMGA1)* Gene Expression in Reactivation of Latent HIV. Conference of Retroviruses and Opportunistic Infections. Seattle, Washington 2017. The thesis author was the primary author of this abstract.

4 Discussion

Studies to eradicate the latent reservoir are in their early stages; the tools, assays and cure approaches currently used remain limited. SAHA, the most characterized HDACi for reversing HIV latency, has induced transcription of proviruses from HIV infected individuals; however, it has failed to deplete latent reservoirs to a measurable level (Archin et al., 2017). Several reasons have been proposed to explain why HIV reactivation by SAHA has not led to measureable reductions of this reservoir. SAHA can bind to HDACs and other host proteins, which are implicated in HIV replication; however, the effects of SAHA on the host gene expression are broad.

There are additional mechanisms of action resulting from the expression of genes upregulated by SAHA as the result of chromatin unwinding. Numerous genes are up- or down-regulated as the result of SAHA treatment (White et al., 2015). It is crucial to understand if their up- or down-regulation interferes with HIV reactivation by SAHA and whether they can be experimentally and therapeutically modulated to improve the efficacy of other HDACi. With a long-term goal of

studying different host genes modulated by SAHA at RNA and protein levels, we conducted a pilot study on one of the genes up-regulated by SAHA at both RNA and protein levels, *HMGA1*.

Published studies have shown that *HMGA1* inhibits viral transcription in cell lines (Henderson et al., 2000; Eilebrecht., 2013); however, it has not yet been clarified if similar inhibition is observed in primary CD4 T cells, and if there is a negative relationship between *HMGA1* up-regulation and HIV reactivation by SAHA. The present study has conducted experiments using a primary CD4 T cell model of HIV latency to evaluate the role of *HMGA1* in regulating HIV expression and the ability of SAHA to reactivate the latent provirus. The results from this study are consistent with the idea that *HMGA1* may have a role in regulation of HIV expression in primary CD4 T cells, and a modest reduction of *HMGA1* expression results in the improvement, albeit also modest, of SAHA to induce reactivation of latent HIV provirus.

Gene knockdown in primary cells using common techniques have shown limitations. The knockdown using the RNA interfering (RNAi) technique has been ineffective due to the fact that primary T cells are hard to transfect and possibly lack an efficient RNAi machinery (Yin, J. et a., 2006; Oberdoerffer, P. et al., 2005). SiRNA transfection uses electroporation, which, however, involves high electrical pulses that can lead to significant loss of cell viability (Freeley, M. Long, A., 2013). To avoid the issues associated with delivery of complex molecules into primary T cells, we used the novel GapmeR technology, which should be more

easily taken up by cells by macropinocytosis, without the need for transfection (Fazil et al., 2016).

We used an online tool developed by the manufacturer of GapmeRs, Exiqon Inc., to design HMGA1-specific GapmeRs (1-4). A single Gapmer 4, demonstrated an ability to induce *HMGA1* knockdown, while the remaining GapmeRs had no activity. The knockdown was modest, and varied among different donors tested, between 13 and 45%, averaging 30% among all experiments. There are possible reasons why there was no knockdown activity in most of the GapmeRs. It was possible that the GapmeRs were not internalized properly by cell-surface proteins, thus resulting in inefficient knockdown. Studies have revealed two main steps through which GapmeR are taken up by primary T cells: adsorption and internalization (Crooke et al., 2017). During adsorption, GapmeRs bind to cell surface proteins with no energy requirement and can saturate the cell surface (Yu et al., 2014). Once bound to the cell surface, cell - surface proteins direct their internalization through multiple pathways, and depending on cell surface proteins, the internalization can be productive, that is leading to knockdown of gene expression, or non-productive, which does not result in knockdown of the gene (Juliano et al., 2014).

Even though macropinocytosis has been reported as the main mechanism of GapmeR internalization by primary T cells, that study was performed using activated T cells (Fazil et al., 2016). It is possible that the mechanism of GapmeR uptake differs in resting and activated primary cells. Compared to the 30%

knockdown achieved with *HMGA1*-specific GapmeR, an average knockdown of 63% could be achieved with the control *MALAT1*-specific GapmeR under the same conditions. These results demonstrate that GapmeR-mediated knockdowns may be dependent on the gene or localization of the targeted RNA of interest. To test localization hypothesis, we used *HMGA1*-specific GapmeRs designed against intronic regions of the gene to target pre-mRNA (GapmeRs 5-8); but no *HMGA1* knockdown was achieved using these GapmeRs.

Despite achieving only a modest reduction of *HMGA1* expression, knocking down *HMGA1* using *HMGA1*-specific GapmeR 4 improved HIV reactivation by SAHA by 36% (or 33% when excluding donors who were non-responders). The best responder (SAHA/DMSO=2.23 in the absence of GapmeR) had the least improvement with the GapmeR (25%), suggesting that the *HMGA1* effect on the knockdown of HIV reactivation by SAHA may be less pronounced in samples that respond well to SAHA. The small sample size in the knockdown experiment presents a limitation of our study, and we were not able to correlate the degree of *HMGA1* knockdown with the improvement of HIV reactivation by SAHA, or stratify samples based on responder vs non-responder groups. A larger sample size is being collected to perform both types of analyses.

The negative correlation observed between *HMGA1* up-regulation and HIV reactivation by SAHA, and the improvement of HIV reactivation by SAHA after *HMGA1* knockdown in three donors suggest that *HMGA1* may contribute to regulation of HIV expression and reactivation in primary CD4 T cells. However, given

the limitations encountered in this study, investigations to improve knockdown is necessary. We plan to either optimize *HMGA1* GapmeR by studying and improving their cellular uptake or to use alternate knockdown methods to validate improvement of HIV reactivation by SAHA. Moreover, we are currently increasing the number of samples to demonstrate how *HMGA1* knockdown affects the ability of SAHA to reactivate latent HIV. Even though the trend was consistent with the five donors, data from the two donors were inconclusive due to technical variation of the assay.

Ultimately, if the role of *HMGA1* in HIV reactivation is confirmed in the future studies, this gene would present a potential target for improvement of HIV reactivation, when using SAHA as a baseline treatment, which may potentially lead to depletion of the latent reservoirs. Overall, the approach of identifying host genes whose expression is perturbed by HDACis in a manner that may be inhibitory to HIV reactivation may lead to finding novel candidate treatments for HIV cure.

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