HHS Public Access

Author manuscript

Virology. Author manuscript; available in PMC 2016 October 25.

Published in final edited form as:

Virology. 2016 October ; 497: 11–22. doi:10.1016/j.virol.2016.06.023.

Cellular minichromosome maintenance complex component 5 (MCM5) is incorporated into HIV-1 virions and modulates viral replication in the newly infected cells

Steven Santos[a], **Yuri Obukhov**[b,c], **Sergei Nekhai**[b,c], **Tatiana Pushkarsky**[a], **Beda Brichacek**[a], **Michael Bukrinsky**[a,*], and **Sergey Iordanskiy**[a]

[a]George Washington University School of Medicine and Health Sciences, Department of Microbiology, Immunology and Tropical Medicine, 2300 I Street NW, Ross Hall, Washington, DC 20037, USA

[b]Howard University College of Medicine, Department of Medicine, Center for Sickle Cell Disease, 1840 7th Street N.W., Washington DC 20001, USA

[c]Howard University College of Medicine, RCMI Proteomics Core Facility, 1840 7th Street N.W., Washington DC 20001, USA

Abstract

The post-entry events of HIV-1 infection occur within reverse transcription complexes derived from the viral cores entering the target cell. HIV-1 cores contain host proteins incorporated from virus-producing cells. In this report, we show that MCM5, a subunit of the hexameric minichromosome maintenance (MCM) DNA helicase complex, associates with Gag polyprotein and is incorporated into HIV-1 virions. The progeny virions depleted of MCM5 demonstrated reduced reverse transcription in newly infected cells, but integration and subsequent replication steps were not affected. Interestingly, increased packaging of MCM5 into the virions also led to reduced reverse transcription, but here viral replication was impaired. Our data suggest that incorporation of physiological amounts of MCM5 promotes aberrant reverse transcription, leading to partial incapacitation of cDNA, whereas increased MCM5 abundance leads to reduced reverse transcription and infection. Therefore, MCM5 has the properties of an inhibitory factor that interferes with production of an integration-competent cDNA product.

*Corresponding author. mbukrins@gwu.edu (M. Bukrinsky).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SS participated in the design of experiments, carried out most of the experiments, prepared samples, analyzed data and contributed to manuscript preparation. YO performed LC-MS/MS data collection and analyzed raw mass spectrometry data. SN participated in the design of the study, supervised LC-MS/MS experiments and contributed to drafting of the manuscript. TP performed MCM5 knockdown experiments. BB performed confocal imaging experiments. MB participated in the study design and coordination and prepared the manuscript. SI conceived the study, designed and coordinated experiments, participated in data analysis and contributed to manuscript preparation. All authors read and approved the final manuscript.

Keywords

HIV-1; Minichromosome maintenance complex; DNA helicase; MCM5; Reverse transcription; Integration; Viral replication

1. Introduction

The pre-integration events of HIV infection (reverse transcription, cytoplasmic trafficking, and nuclear import of viral DNA) occur within intracellular subviral particles called reverse transcription complexes (RTCs), which become pre-integration complexes (PICs) when cDNA processing is accomplished. These particles are derived from the viral capsid cores entering the target cell. Certain host cell proteins hijacked by virions from the virus-producing cells or recruited from the newly infected cell have been shown to stimulate [cyclophilin A (Briones et al., 2010; Luban, 2007; Luban et al., 1993; Schaller et al., 2011), heat shock protein 70 (Gurer et al., 2002), clathrin (Popov et al., 2011; Zhang et al., 2011), RNA helicase A (Jeang and Yedavalli, 2006; Roy et al., 2006), INI1/hSNF5 and Sin3a-HDAC1 complex (Sorin et al., 2009, 2006; Yung et al., 2004), importins α1 (Gallay et al., 1997) and α3 (Ao et al., 2010), importin 7 (Fassati et al., 2003; Zaitseva et al., 2009), TNPO3/transportin-SR2 (Christ et al., 2008; Krishnan et al., 2010), lens epithelium-derived growth factor (LEDGF) p75 (Ciuffi and Bushman, 2006; Llano et al., 2006; Maertens et al., 2003), barrier-to-autointegration factor (BAF) (Lin and Engelman, 2003), protein kinase A (PKA) (Giroud et al., 2013), eukaryotic elongation factor 1 (eEF1) (Warren et al., 2012)] or inhibit [apolipoprotein B mRNA editing enzyme catalytic (APOBEC) 3G (Bishop et al., 2008; Holmes et al., 2007b), APOBEC3F (Holmes et al., 2007a), Sterile alpha motif domain- and HD domain-containing protein (SAMHD) 1 (Hrecka et al., 2011; Laguette et al., 2011), Moloney leukemia virus (MOV) 10 (Furtak et al., 2010), RNA-associated early-stage anti-viral factor (REAF) (Marno et al., 2014)] the early events of HIV replication. Our published results of the proteomic analysis of HIV-1 cores (Santos et al., 2012) showed incorporation of multiple RNA- and DNA-binding proteins into the cores of virions assembled in $CD4^+$ T cell (Sup-T1) and macrophage (PMA and vitamin D_3 activated THP-1) lines. Some of the identified cellular proteins incorporated preferentially from one of the producer cell types, while the others, like the subunit 5 of the mini-chromosome maintenance complex (MCM5), displayed incorporation into the cores of virions assembled in both T cells and macrophage lines, although the ratio of incorporation varied depending on the cell type (Santos et al., 2012).

The $MCM_{2–7}$ heterohexamer is an essential replicative DNA helicase that contains six related ATPase associated subunits (MCM 2–7) required for both initiation and elongation of DNA replication (Bailis et al., 2008; Bochman and Schwacha, 2009). Within the $MCM_{2–7}$ hexamer, the MCM4/6/7 components are the “core” helicase, whereas the MCM3 and MCM5 subunits provide the regulatory function (Chuang et al., 2012; Schwacha and Bell, 2001). The $MCM_{2–7}$, a component of DNA pre-replicative complex, is normally active in the nucleus during the late G1 and S phases of the cell cycle and is essential for both the formation and maintenance of the replicative fork structure, as well as the reparation of double-strand breaks (Bailis et al., 2008). Insufficient amounts of the MCM complex have

been shown to impair the cell cycle and lead to genomic instability (Bochman and Schwacha, 2009; Chuang et al., 2012).

Previous studies have implicated the MCM helicase's involvement in life cycles of Epstein-Barr Virus (EBV) (Frappier, 2012) and Influenza Virus (Kawaguchi and Nagata, 2007). In the case of EBV, EBNA1 (a viral protein that binds to the origin of replication) specifically recruits the MCM helicase to assist in replication of viral DNA during S phase, which is critical for the maintenance of latent phase of infection. Influenza virus engages cellular MCM helicase for the replication of viral RNA during the infection. Kawaguchi and Nagata (2007) showed that binding of the MCM complex to the viral RNA-dependent RNA polymerase is required for the formation of a productive complex. They also demonstrated binding of the whole hexameric MCM complex to viral RNA and to the PA subunit of the viral polymerase complex, probably through the MCM2 subunit. They proposed that MCM acts as a "scaffold" between the viral polymerase and the RNA, which is necessary for elongation of RNA synthesis.

In the present study, we demonstrate a novel role for the monomer MCM5 of the MCM complex in the HIV-1 life cycle. We show association of MCM5 subunit with HIV-1 Gag polyprotein during the virion assembly and with viral cores in mature viral particles. Our results suggest that MCM5 stimulates non-productive reverse transcription and, when overexpressed in HIV virions, inhibits viral replication in the infected cells.

2. Results

2.1. Detection of the monomers of MCM_{2-7} replication complex within viral cores

Viral cores were isolated using the method of "spin-thru" equilibrium density gradient sedimentation (Aiken, 2009; Kewalramani and Emerman, 1996; Kotov et al., 1999) from the suspensions of HIV-1 NL4-3 virions harvested from T cells (Sup-T1), macrophages (PMA-treated THP-1), and epithelial HEK293T cells infected with the virus pseudotyped with amphotropic MLV envelope. LC-MS/MS analysis of in-solution trypsin digested core samples revealed all components of cellular MCM_{2-7} complex in cores of the virus produced from Sup-T1 and CEM_{NKR} cells (Table 1). This result is consistent with a report by Brégnard and coauthors who have recently detected monomers of the MCM complex via proteomic analysis of the whole virions of HIV-1 NL4-3 produced by transfected HEK293T cells (Bregnard et al., 2013). However, our analysis of the virus from HEK293T cells, as well as from the monocytic cells THP-1, revealed only certain subunits, and MCM5 was the only subunit detected in all preparations (Table 1). To estimate incorporation of MCM5 into the virions, we compared the ratio of MCM5- and p24-derived peptides in virions produced from Sup-T1 cells to the ratio in virions from THP-1 cells. This analysis demonstrated that MCM5/p24 ratio was 10-fold lower in Sup-T1-produced virions relative to the virions produced by THP-1 cells, indicating that MCM5 incorporation varies dependent on producer cell, and the number of MCM5 molecules per virion is likely much smaller than the number of Gag molecules. Based on these data, we suggest that MCM5 subunit of the replicative helicase complex is incorporated in varying numbers into HIV-1 virions from all types of virus producing cells.

2.2. Incorporation of MCM5 into HIV-1 virions depends on its interaction with Gag polyprotein

Since all MCM subunits possess DNA-binding activity (Schwacha and Bell, 2001), and at least some of them have been shown to bind RNA and RNA polymerase of influenza A virus (Kawaguchi and Nagata, 2007), we asked whether HIV-1 RNA or viral proteins are involved in the MCM5 packaging into the virion. Anti-HA immunoprecipitation (IP) from cytoplasmic lysates of the virus-producing HEK293T cells transfected with the vector expressing hemagglutinin (HA)-tagged MCM5 revealed association of MCM5 subunit with unprocessed HIV-1 Gag polyprotein (Fig. 1A). Interestingly, while many processed Gag molecules can be detected within the cytoplasm of producer cells (CA p24 and MA p17 bands in Fig. 1A, right panel), MCM5 co-immunoprecipitated mostly with unprocessed ($Pr55^{Gag}$) and C-terminally processed ($Pr41^{Gag}$ lacking p6 and NC p7 domains) polyprotein-precursor, but not with processed p17 or p24, suggesting localization of MCM at the sites of virion assembly. However, analysis of the virion suspensions equalized by p24 content, lysed with 0.5% Triton X-100 and immunoprecipitated with anti-HA antibody (to pull down MCM5) identified stable association of MCM5 with CA p24 and especially MA p17 Gag-derived viral proteins (Fig. 1B), suggesting that these Gag molecules were responsible for interaction of the Gag precursor with MCM5. Treatment of immunocomplexes with RNase A did not affect association of MCM5 with the Gag products, suggesting that MCM5 binding with Gag is not mediated by the RNA (Fig. 1B).

To test whether MCM5 is incorporated into the virions as a component of $MCM_{2–7}$ complex or as a separate protein via direct interaction with Gag, we overexpressed MCM5 in the virus-producing cells. Indeed, upon overexpression of MCM5 in HEK293T virus-producing cells, more MCM5 was present in the HIV-1 virions (Fig. 1C, top panels). To exclude the possibility of non-specific precipitation of MCM5 from overexpressing cells, we performed virus-precipitating procedure on mock-infected 293T cells. No MCM5 band was detected in the precipitate (not shown). The levels of MCM2 and MCM3 appeared unchanged in the virions collected from MCM5-transfected cells (Fig. 1C, right middle panel). This observation suggests that MCM5 might be incorporated into the HIV-1 virus particles independent of other subunits of the MCM complex, the notion consistent with results in Table 1.

To confirm the association of MCM5 with HIV-1 Gag, HEK293T cells were transfected with pNL4-3, immunostained with fluorescently labeled antibodies, and analyzed by confocal microscopy. Nuclei were counterstained with DAPI. As shown in Fig. 1D, in cells not expressing HIV-1 Gag, MCM5 is localized primarily to the nucleus (cells marked by yellow arrows in panel a). A cell in the process of mitosis (marked by red arrow in panel a) shows MCM5 localization outside of the DAPI-stained area, consistent with MCM5 release from the nucleus. In cells expressing HIV-1 Gag (marked by white arrows in panel a), MCM5 is distributed throughout the cell (see panels a and c) and is enriched in the area of Gag localization. In contrast, a similar analysis of MCM3 (Fig. 1E) demonstrated that localization of this protein did not change in the presence of HIV-1 protein expression, and it remained in the nuclei of cells regardless of Gag expression (cells expressing Gag are marked by white arrows and Gag-negative cell - by yellow arrow in panel a). Quantification

of images using Volocity software demonstrated redistribution of MCM5-specific staining from nucleus to cytoplasm, significantly decreasing the nucleus to cytoplasm ratio of fluorescence intensity in HIV Gag-positive cells (Fig. 1F). No such redistribution of fluorescence was observed for MCM3-specific staining. To demonstrate that the observed redistribution of MCM5 was occurring in a natural HIV target cell type, macrophage, we analyzed MCM5 in monocyte-derived macrophages infected with VSV-G-pseudotyped HIV-1 NL4-3. As shown in panel G of Fig. 1, MCM5 localized exclusively to the nucleus in the uninfected macrophages, but was distributed throughout the cell in the Gag-positive macrophage. Taken together, these results indicate that HIV-1 infection leads to exit of MCM5 from the nucleus and its association with the Gag protein.

2.3. MCM5 deficient virus displays decrease in cDNA accumulation but not in nuclear import and integration in newly infected T cells

To test whether the lack of MCM DNA helicase in HIV-1 virions affects the next round of infection, we used the virus derived from cells treated either with MCM5 siRNA (MCM5 knockdown) or with genistein (GST) and/or trichostatin A (TSA). GST and TSA are two anticancer drugs that have been shown to affect MCM2 gene expression and reduce the functional activity of the whole MCM complex (Majid et al., 2010). Of note, genistein has been reported to inhibit HIV-1 replication in macrophages through both entry-and post-entry effects due to its tyrosine kinase inhibitory activity (Stantchev et al., 2007), while TSA is a known activator of HIV transcription through its HDAC inhibitory activity (Quivy et al., 2002). However, no information is available regarding the effect of these drugs on viruses produced from drug-treated cells.

To determine the effect of these two drugs on MCM5 virion incorporation, we treated infected SupT1 cells with the MCM helicase inhibitors and analyzed MCM5 incorporation after isolating the newly produced virions. The GST concentration used in our study (50 μM) was previously shown to be non-toxic to HEK293 cells (Kolokoltsov et al., 2012), and the TSA concentration (100 ng/ ml or 0.3 nM) was about 300-fold lower than the 100 nM concentration used in other HIV studies (e.g., (Quivy et al., 2002)). Accordingly, no cytotoxic effect was observed and virus production was not affected (not shown). As shown in Fig. 2A (two upper panels), MCM5 protein levels remained unchanged or even increased within cell lysates in the presence of either one or both drugs. However, MCM5 was absent from the virions produced from drug-treated cells (Fig. 2A – two lower panels). Taken together, these data suggest that the MCM5 monomer is incorporated into HIV-1 viral particles, and the functional activity of MCM5, or possibly the whole MCM helicase, is important for MCM5 incorporation into HIV-1 virions. Utilizing these manipulated virus stocks, we then infected Jurkat cells and analyzed how the absence of MCM5 within HIV-1 virions affects the early stages of replication. Of note, the viruses were treated with DNAse I to remove contaminating plasmid DNA.

Unchanged levels of the viral RNA measured within the target cells harvested 2 h after incubation with the virus from drug-treated cells indicated that the virion attachment and entry events remained unaffected (Fig. 2B). This assay also indicated that the levels of HIV-1 RNA incorporated into the virions were not affected by MCM5 manipulation, an

important possibility to exclude given the MCM5 affinity for RNA. However, we observed a two- to threefold decrease in accumulation of late cDNA copies (Fig. 2C). To verify that this effect was due to reduced virion incorporation of MCM5, and not to non-specific activity of the drugs, we utilized siRNA to knockdown MCM5 within virus-producing HEK293T cells. As shown in Fig. 2D, successful knockdown of MCM5 was accomplished in 293T cells, and did translate to reduced MCM5 incorporation in virions. As expected, a similar trend to that observed with virus produced by cells treated with MCM-inhibiting drugs was seen when the virus from MCM5 silenced cells was used for infection: accumulation of late cDNA copies was reduced (Fig. 2E). Unexpectedly, the effects of MCM5 inactivation on reverse transcription did not translate into inhibition of post-reverse transcription steps of HIV-1 replication: the number of integrated proviral DNA copies in cells infected with the virus collected from cells treated with GST or TSA was reduced only insignificantly relative to control cells, and only virus from cells treated by a combination of TSA and GST produced the number of integrated DNA copies which was significantly reduced, by 60% (Fig. 2F). For cells infected with the virus produced by cells where MCM5 was knocked down, we did not detect significant differences in accumulation of 2-LTR circles (Fig. 2G) or integrated provirus (Fig. 2H) relative to infection with control virus, although there was a trend to increased integration of MCM5-depleted virus. Given that the decrease of MCM5 in virions was similar between different treatments (Fig. 1A), the effect of the drug combination on virus integration was likely due to an as yet uncharacterized activity of the drugs unrelated to their effect on MCM5. Taken together, these data suggest that virion-incorporated MCM5 stimulates reverse transcription and accumulation of cDNA products in target cells, but most of these products are unable to translocate into the nucleus and integrate into the host chromatin.

2.4. Increased MCM5 incorporation into HIV-1 virions leads to reduced reverse transcription and viral integration in the infected cells

Since the above described experiments showed that depletion of MCM5 in HIV-1 producing cells resulted in decreased incorporation and reduced reverse transcription, we then investigated the effect of MCM5 overexpression on the next round of infection. Fig. 3A demonstrates successful MCM5 overexpression within HEK293T cells which did translate to increased levels of MCM5 in HIV-1 virions. Analysis of viral particles collected from 5×10^6 MCM5- or empty vector-transfected cells using HIV-IG demonstrated that protein composition of the virions was very similar, although less virus was produced by MCM5-transfected cells (Fig. 3B). Our data indicate that increased incorporation of MCM5 into the virions did not affect viral entry (Fig. 3C), but resulted in approximately a 3-fold reduction of early reverse transcription products (strong-stop cDNA, Fig. 3D) and a 5-fold decrease of the late reverse transcription products (positive strand cDNA, Fig. 3E) in infected Jurkat cells. The same trend was observed in viral DNA integration (Fig. 3F), where a 3-fold decrease of integrated HIV-1 provirus was observed. To test whether the high level of MCM5 in the virion affects initial stages of reverse transcription, we analyzed dynamics of strong-stop cDNA accumulation in isolated and permeabilized virions using endogenous reverse transcription (ERT) assay as described earlier (Warrilow et al., 2008). We found that within the first two hours, the cores of virions from cells with overexpressed MCM5 accumulated 2-fold fewer early reverse transcription products than the cores of virions from

control cells (Fig. 3G), consistent with the inhibitory effect of virion-incorporated MCM5 on reverse transcription.

2.5. Virus with increased level of MCM5 incorporation displays reduced replication in the next round of infection

To test whether the differences in cDNA accumulation and integration induced by changes in abundance of MCM5 in the virions lead to effects at later stages of HIV-1 life cycle, we assessed replication of the viruses using single-round infection. The HIV-1 unspliced RNA was quantified in the infected Jurkat cells and activity of LTR-controlled luciferase was measured in TZM-bl reporter cells. Quantitative RT-PCR analysis of mRNA demonstrated that reduction of MCM5 incorporation into the virions did not significantly change the level of HIV-1 RNA transcription (Fig. 4A). In contrast, increased MCM5 incorporation into the virions resulted in decreased transcription in infected Jurkat cells, evidenced by a two-fold reduction in levels of HIV-1 RNA. A similar trend was observed in infected TZM-bl cells, which carry luciferase gene under control of HIV LTR. Small differences in the replication were detected between viruses produced from control, MCM5 knocked down cells or cells treated with MCM helicase inhibitor, but the virus from cells overexpressing MCM5 demonstrated a 3–4-fold lower level of luciferase activity (Fig. 4B). These data indicate that increased abundance of MCM5 protein in HIV-1 virions leads to a reduced efficiency of reverse transcription and integration, resulting in the reduced level of total viral replication.

3. Discussion

Results presented in this report indicate that the MCM5 monomer of the $MCM_{2–7}$ DNA helicase is incorporated into HIV-1 virions from the virus-producing cells and regulates early events of the next round of infection, specifically the step of reverse transcription. Earlier studies suggested that MCM5 may act independently of other MCM subunits, for example, it directly binds the transcription activator Stat1 in response to IFN-γ (DaFonseca et al., 2001). Interestingly, both reduction and overabundance of MCM5 in the virions inhibit reverse transcription. Given that reduction of MCM5 in the virions reduced the abundance of reverse transcription products but did not reduce integration, it appears likely that MCM5 promotes reverse transcription, but the excess of reverse transcription products produced with participation of MCM5 is composed of the molecules defective for integration. This suggests that MCM5 impairs reverse transcription. Such interpretation is consistent with the inhibitory effect on reverse transcription of MCM5 overexpression, which may be explained by a threshold hypothesis for the MCM5 effects: low (physiological) MCM5 concentrations accelerate a portion of RT-driven reactions at the expense of quality control, resulting in integration-defective products, whereas levels of MCM5 above the threshold lead to general impairment of reverse transcription and integration. This effect of virion-incorporated MCM5 on reverse transcription suggests interaction of this protein either with RT or with viral genomic RNA (similar to influenza virus (Kawaguchi and Nagata, 2007)) and/or newly synthesized DNA product. Thus, similar to MCM activity in influenza virus replication, the MCM5 monomer in the HIV-1 RTC/PIC might assist in forming a scaffold between nascent DNA and the RT molecule during initiation or elongation of reverse transcription. Somehow, this scaffold, while promoting reverse transcription, interferes with its completion, resulting

in accumulation of integration-defective cDNA copies. This effect on completion of reverse transcription is further exacerbated when MCM5 is overexpressed in the virions: increased number of MCM5 molecules may inhibit elongation of early reverse transcripts by direct binding to viral genomic RNA and sterically hindering the RT movement. A similar deamination independent mechanism was earlier described for APOBEC3G (Bishop et al., 2008).

Our experiments do not fully exclude the possibility that manipulation of MCM5 levels in producer cells, rather than levels of MCM5 incorporated into the virions, is responsible for the observed effects on HIV infectivity. However, several lines of evidence argue against this mechanism. First, the most likely target of MCM5 is viral RNA, but we did not detect any effects of MCM5 manipulation in producer cells on the amount of viral RNA entering the target cell (Fig. 2B). This result also suggests that binding, fusion and entry proceeded normally. Second, although MCM5 overexpression inhibited virus production, the ratio of HIV proteins (RT, Env, and Gag) in virions was not changed when MCM5 was overexpressed in producer cells (Fig. 3B). Third, the behavior of the virus produced by cells with MCM5 knocked down or inhibited (decrease in reverse transcription that does not translate to decreased nuclear import or integration) is hard to explain without invoking virion-associated protein. And fourth, it is hard to imagine how both down- and upregulation of MCM5 in producer cells can exert the same effect on HIV reverse transcription in the target cells unless accepting the proposed model.

An important question for the future studies is the amount of MCM5 molecules incorporated into HIV-1 virions. Our mass-spec analysis did not establish absolute numbers, but demonstrated that ratio of MCM5 to p24 is at least 10-fold lower in virions produced from CD4+ T cells than from myeloid cells. It appears that MCM5 incorporation varies dependent on producer cell, and the number of MCM5 molecules per virion is likely much smaller than the number of Gag molecules. It remains to be determined whether MCM5 incorporation into HIV virions can be stimulated by some physiological agent (e.g. cytokine), which would suggest a novel anti-HIV therapeutic strategy.

The observed activities of MCM5 closely resemble those described for another helicase, MOV10. Similar to MCM5, MOV10 is incorporated into HIV-1 virions via interaction with the Gag polyprotein, however, in contrast to MCM5 that co-precipitates with CAp24 and MAp17, nucleocapsid region of Gag was required for MOV10 incorporation (Abudu et al., 2012; Burdick et al., 2010; Wang et al., 2010). MOV10 overexpression in virus producing cell suppresses viral replication by inhibiting the early steps of reverse transcription (Arjan-Odedra et al., 2012; Wang et al., 2010), again closely resembling the effect of MCM5 overexpression. And knockdown of endogenous MOV10 in virus producing cells did not affect HIV-1 infectivity (Arjan-Odedra et al., 2012), similar to MCM5 knockdown, although effects on reverse transcription have not been investigated in that study. These similarities between MOV10 and MCM5 are intriguing. Given that MOV10 is an RNA helicase, whereas MCM5 is a DNA helicase, it seems reasonable that the common target of their activity is reverse transcription. Although endogenous levels of both these helicases do not alter HIV-1 replication, the results of overexpression studies suggest an intriguing possibility that helicases may function as inhibitors of HIV reverse transcription. It remains to be tested

whether the concerted action of endogenous DNA and RNA helicases (MCM5 and MOV10, respectively) incorporated into the virions provides a noticeable restrictive effect on HIV replication.

4. Conclusions

Taken together, our data suggest that the incorporation of physiological levels of MCM5 into HIV-1 virions facilitates viral cDNA accumulation in newly infected cells, probably by modifying nucleic acid configuration during reverse transcription. However, cDNA produced with participation of MCM5 is mostly defective for subsequent integration, so depletion of MCM5 from the virions does not lead to decreased integration or transcription. When MCM5 in the virions is overabundant, it downregulates HIV-1 reverse transcription, integration, and replication. In this case, it is likely due to further alteration of nucleic acid configuration, leading to a block in reverse transcription. Our data complements a number of reports showing that the cellular proteins incorporated into the virions from producer cells can significantly alter the infectivity of the virions ((Serquiña et al., 2013), reviewed in Iordanskiy et al. (2013)). MCM5 has the properties of an inhibitory factor that limits HIV-1 replication by interfering with production of an integration-competent cDNA product.

5. Materials and methods

5.1. Cells and viruses

The acute monocytic leukemia cell line THP1 (from S. Tsuchiya), T lymphoblastoma Sup-T1 cells (from James Hoxie) and TZM-bl cells were provided by the NIH AIDS Research & Reference Reagent Program. The human kidney fibroblasts HEK293T and T lymphoblasts Jurkat were purchased from ATCC (Manassas, VA). All cells were maintained at 37 °C and 5% CO_2 in 75 cm^2 tissue culture flasks with RPMI-1640 culture media supplemented with 10% Fetal Bovine Serum, penicillin/streptomycin (100 μg/ml), and L-Glutamine.

The stocks of the HIV-1 virus were prepared by transfection of HEK293T cells with HIV-1 NL4-3 provirus-encoding plasmid (Westervelt et al., 1992) at a 1:4 ratio using Metafectene transfection reagent (Biontex, Planegg, Germany). To generate the HIV-1 pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G), the HEK293T cells were co-transfected with pNL4-3 and pCMV-VSVG construct encoding VSV-G under control of CMV promoter. After 4 h incubation with the transfection complex, the media was changed and the cells were cultured in fresh media for 48 or 72 h at 37 °C and 5% CO_2. Then, the supernatants were harvested, filtered through a 0.45 μm filter and stored on wet ice at 4 °C for 1–4 days. Viruses were concentrated by centrifugation at 100,000 ×*g* and 4 °C for 3 h through 2 ml cushions of 30% sucrose in PBS in a Beckman SW-41 rotor. The pellets were re-suspended in 500 μl of fresh culture media and used for infection. CD45-depletion was performed using Human CD45 magnetic microbeads from Miltenyi Biotec (catalog no. 130-045-801) and performed according as previously described (Chertova et al., 2006).

5.2. Infection

The viral suspensions were normalized according to their RT activity, treated with 0.25 mg/ml DNase I RNase-free (Roche, Mannheim, Germany) for 60 min in the presence of 5

mM $MgCl_2$ at room temperature, mixed with Polybrene (Sigma) to a final concentration of 8 μg/ml and used for infection. Infection was performed in 6-well plates (2.5×10^6 cells per well). After 2 h incubation at 37 °C and 5% CO_2, the cells were washed from the virus-containing media, re-suspended in RPMI-1640 (pre-warmed to 37 °C) and incubated from 24 to 72 h.

5.3. Concentration of virus and "spin-thru" isolation of viral cores

The pellets of concentrated virus were re-suspended in 300 μl of STE buffer and the viral cores were then isolated by "spin-thru" purification as described earlier (Aiken, 2009; Kewalramani and Emerman, 1996; Kotov et al., 1999; Shah and Aiken, 2011). Briefly, 3.8 ml of a 30–50% linear density gradient of sucrose in STE buffer was overlaid with 1 ml of 15% sucrose containing 1% Triton X-100 and then covered with a 0.4 ml cushion of 7.5% sucrose in STE. The HIV-1 positive and negative samples, concentrated through 30% sucrose and resuspended in STE (0.3 ml) were carefully layered on top of the 7.5% sucrose layer and centrifuged in a Type 100 Ti rotor (Beckman Coulter) at 100,000 ×*g* at 4 °C for 16–18 h. The pellets were re-suspended in 26 μl of STE buffer and placed into poly-propylene non-siliconized Eppendorf microtubes; 4 μl aliquots were set aside for the p24 CA ELISA assay. The CA p24Gag–normalized suspensions of HIV-1 cores and control suspensions were subjected to SDS-PAGE protein separation for subsequent LC-MS/MS analysis, Western blotting, or to in-solution protein digestion with trypsin for the LC-MS/MS analysis of unseparated protein samples.

5.4. Gel separation of proteins, in-gel protein digestion and peptide extraction

The volumes of viral core suspensions, each containing 400 ng of p24 CA protein, and control suspensions taken in twofold excess were mixed with equal volumes of Laemmli Sample Buffer (BioRad, Hercules, CA) containing 5% β mercaptoethanol, heated in boiling water for 2 min and applied for SDS-PAGE protein separation. Separation of proteins was performed in 12.5% Tris–HCl Criterion Precast Gel (BioRad) at 100 V and 4 °C for 2–2.5 h. The gel was stained in 0.1% (wt/v) Coomassie (BioRad) solution (40% methanol (v/v), 10% acetic acid (v/v) in water with 1 g/L of Brilliant Blue R-250) for 1 h at room temperature. After 7–8 washes in de-staining solution (contains the same components, as staining solution, except Brilliant Blue R-250) the gel was placed into water, and each lane was sectioned into 10 contiguous pieces, which were subjected to proteolysis according to the modified previously published protocol (Formolo et al., 2011). Briefly, acetonitrile (ACN) dehydrated gel pieces were rehydrated in 10 mM DTT and incubated at 60 °C for 1 h. After cooling at room temperature, the gel slices were incubated with 50 mM iodacetamide for 1 h at room temperature in the dark for alkylation of proteins. After the second dehydration, a 15 μl dose of Trypsin Gold (Promega, Madison, WI) solution (20 μg/ml) in 40 mM NH_4HCO_3/10% ACN was added to each of the gel pieces. After 1 h saturation at 4 °C, the pieces were incubated at 37 °C overnight. The resulted peptides were extracted three times: (1) with 25 mM of NH_4HCO_3:ACN (1:1); (2) 5% formic acid (FA); (3) 5% FA:ACN (1:1). After pooling all the extracts together, samples were purified through ZipTip pipette tips C18 (Millipore), eluted with 30 μl of 0.1% trifluoroacetic acid (TFA) in 80% ACN and subjected to HPLC separation and MS/MS analysis.

5.5. In-solution protein digestion

The suspensions of HIV-1 cores after “spin-thru” centrifugation were treated with 10 mM DTT (60 °C for 1 h) and 150 mM iodacetamide (1 h at room temperature in the dark) in 20 l of STE buffer. The protein samples were then mixed with 100 μl of 200 mM ammonium bicarbonate and treated with 200 ng of Trypsin Gold (Promega) at 37 °C overnight. The resulted peptides were dried in SpeedVac, resuspended in water, purified through ZipTip pipette tips C18 as described above and then subjected to HPLC separation and MS/MS analysis.

5.6. HPLC-MS/MS of tryptic digests and database search

The peptides in each sample were separated by microcapillary reversed-phase liquid chromatography (HPLC), coupled online to an ion trap mass spectrometer Thermo LTQ Orbitrap XL. The mass spectrometer was operated in a data-dependent MS/MS mode using normalized collision-induced dissociation (CID) energy of 35%. The CID spectra were compared against those of the EMBL non-redundant protein database. Only peptides having cross-correlation (X_{corr}) cutoffs of 2.6 for $[M+2H]^{2+}$, 3.0 for $[M+3H]^{3+}$ and higher charge state were considered. These SEQUEST criteria thresholds resulted in a 1–2% of False Discovery Rate. The proteome analysis of the spectra was made by Proteome Discoverer 1.2 software (Thermo Fisher Scientific). The protein profiles of the samples of viral cores were compared with identically prepared samples from non-infected cells. The sub-cellular localization and function of each filtered protein was determined using gene ontology (GO) information obtained from cross-referencing each protein’s Swiss-Prot accession number to the GO localization information available on the NCBI protein database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein) and The Human Protein Atlas database (www.proteinatlas.org). The involvement of the proteins in known cellular pathways associated with major biological processes such as cell cycle, intracytoplasmic transport, cytoplasm organization, nuclear transport, chromatin structure maintenance/regulation, RNA splicing and reorganization, transcription, apoptosis, proteasomal degradation, etc. were assessed using NCBI RefSeq database (www.ncbi.nlm.nih.gov/RefSeq/) and DAVID Bioinformatics Resources 6.7 (NIAID NIH) (http://david.abcc.ncifcrf.gov).

5.7. Western blot analysis

The aliquots of the lysates of HIV-1 infected cells and the virus samples were subjected to SDS-PAGE, transferred to a PVDF membrane and then blotted using the following antibodies: anti-HIV-1 p24 (24-3) mouse monoclonal antibody, human HIV immunoglobulin (HIV-IgG), and for integrase - a combination of rabbit antiserum for integrase (aa 1–16, 23–34 or 276–288) from NIH AIDS Research & Reference Reagent Program; monoclonal rabbit GAPDH (G9545) from Sigma-Aldrich, anti-MCM5 (G-1) mouse monoclonal and anti-MCM3 (G-19) goat polyclonal antibodies from SantaCruz Biotechnology (Santa Cruz, CA). Specific bands were visualized by ECL (Thermo Scientific, Rockford, IL). Quantification of the Western blotting results was performed using ImageJ software.

5.8. Plasmid preparation

The MCM5 plasmid was ordered from the Thermo Scientific's Mammalian Gene Collection (clone ID: 2900229). Hemagglutinin (HA)-tagged MCM5 was created using HindIII and BamHI and the following primers: 5′-CTCCCCTGGTTTGTGAAGCTTGGAAAA-3′ and 5′-CAGTGAGGCGGCGCGGATCCCTTGAGGCGGTAGAG-3′. The amplified MCM5 gene was then inserted into a phCMV3 HA TAG vector using the Fast-Link Ligation System/Protocol.

5.9. Transfections

Plasmids were transfected into 293T cells to overexpress MCM5 with/without the HA tag using Metafectene according to the manufacturer's recommendations. To knockdown MCM5, siRNA was utilized and the SMARTpool of siRNA for MCM5 was ordered from Dharmacon (Thermo Fisher Scientific, Waltham, MA). To transfect siRNA into the cells, Dharmacon's specialized lipid reagent (Dharmafect 1) was used according to manufacturer specification. For siRNA controls, scrambled siRNA ordered from Dharmacon was used as control. We have observed that in the context of these experiments no difference was detected between cells transfected with scrambled siRNA and mock-transfected cells.

5.10. RNA purification and RT reaction

RNA was purified from samples using TRI Reagent-LS (MRC, Cincinnati, OH) according to the manufacturer's protocol. A total of 0.5 μg of RNA from the RNA fraction was treated with 0.25 mg/ml DNase I RNase-free (Roche, Mannheim, Germany) for 60 min in the presence of 5 mM $MgCl_2$, followed by the heat inactivation at 65 °C for 15 min. A 250 ng aliquot of total RNA was used to generate cDNA with the GoScript Reverse Transcription System (Promega, Madison, WI) using oligo-dT reverse primers.

5.11. DNA isolation and quantitative real-time PCR

Lysates of HIV-1 infected cells were normalized to the total protein count using DC Protein Assay (BioRad) following manufacturer's protocol. The total DNA was isolated using Wizard Genomic DNA Purification Kit (Promega, Madison, WI) following manufacturer's recommendations. After isolation, the cellular DNA samples were analyzed by quantitative TaqMan real-time PCR to quantify chromosomal DNA. Set of primers specific for the β-globin gene has been used: forward primer BGF1 (5′-CAACCTCAAACAGACACCATGG-3′), reverse primer BGR1 (5′-TCCACGTTCACCTTGCCC-3′), and probe BGX1 (5′-FAM-CTCCTGAGGAGAAGTCTGCCGTTACTGCC-TAMRA-3′). PCR reactions were performed with PerfeCTa qPCR FastMix, UNG (Quanta Biosciences, Gaithersburg, MD) using 300 nM of each primer and 200 nM of probe according to the manufacturer protocol. To quantitate viral cDNA, total DNA samples were analyzed by real-time PCR using two sets of primers. The first set detects the negative-strand "strong-stop" DNA (the early reverse transcription product) and consists of forward primer M667 (5′-GGCTAACTAGG-GAACCCACTG-3′), reverse primer AA55 (5′-CTGCTAGAGATTTTCCA-CACTGAC-3′), and probe Er-LTR (5′-FAM-GTCACACAACA-GACGGGCACACACTA-TAMRA-3′) specific for the R-U5 region of the HIV-1 LTR. The second set recognizes the positive-strand

DNA (late reverse transcription product) and consists of primers: FOR-LATE (5′-TGTGTGCCCGTCTGTTGTGT-3′), REV-LATE (5′-GAGTCCTGCGTC-GAGAGATC-3′), and probe Lt-LTR-Prb (5′-FAM-CAGTGGCGCCCGAA-CAGGGA-TAMRA-3′) specific for the U5-Ψ LTR region (Butler et al., 2001). Quantitative analysis of 2-LTR circles was performed according to published protocol (Butler et al., 2001) using the forward primer MH535 (5′-AACTAGGGAACCCACTGCTTAAG-3′), reverse primer MH536 (5′-TCCACAGATCAAGGATATCTTGTC-3′), and probe MH603 (5′-FAM-ACACTACTTGAAGCACTCAAGGCAAGCTTT-TAMRA-3′). Viral 2-LTR circles were detected from 500 ng total cellular DNA. Two-step nested PCR assays were used for quantitative HIV-1 DNA integration analysis. The first round PCR was performed in a 25 μl reaction mix as described in (Butler et al., 2001; O'Doherty et al., 2002). Briefly, 100 nM of the genomic *Alu* forward primer, Alu-F (5′-GCCTCAATAAAGCTTGCCTTGA-3′), 600 nM of HIV-1 *gag* reverse primer, Gag-R (5′-GCTCTCGCACCCATCTCTCTCC-3′), and 100 ng of cellular genomic DNA were mixed with 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.05 U of Platinum *Taq* DNA polymerase (Invitrogen) and *Taq* polymerase reaction buffer (Invitrogen). The conditions were 2 min hot start at 94°C, then 30 s at 93 ° C, 1 min at 50 °C, and 2 min at 70 °C for 20 cycles. The second round was performed with 5 μl of the material from the first round in 20 μl of reaction mix. The primer set and reaction conditions were the same as for quantitative detection of the positive-strand HIV-1 DNA, as described above. The 2 μl aliquots of RT reaction mixtures of the RNA samples (see above) were diluted 10-fold and 100-fold and subjected to quantitative real-time PCR analysis with the set of primers specific for HIV-1 gag. The primers Gag1483-F (5′-AAGGGGAAGTGACATAGCAG-3′) and Gag1625-R (5′-GCTGGTAGGGCTATACATTCTTAC-3′) were used for PCR reaction with iTaq Universal SYBR Green Supermix (BioRad,) according to the manufacturer protocol. Serial dilutions of DNA from 8E5 cells (CEM cell line containing a single copy of HIV-1 LAV provirus per cell) were used as the quantitative standards. Real-time PCR reactions were carried out at least in triplicate using the PTC-200 Peltier Thermal Cycler with Chromo4 Continuous Fluorescence Detector (both from MJ Research) and Opticon Monitor 2.03 software.

5.12. Endogenous reverse transcription

The assay was performed as described (Zhang et al., 1996) with some modifications. Prepared virus was resuspended in PBS to a p24 CA concentration of 1.5 ng/μl (200 μl of virus suspension required). Each virus (100 μl) was mixed with 2 × ERT reaction buffer (300 μl PBS, 50 μl of 50 mM $MgCl_2$, 50 μl of 300 μ/ml melittin [Sigma], 100 μl of 10 mM dNTP Mix [Promega]), and separately with 2 × ERT control buffer (275 μl PBS, 50 μl of 50 mM $MgCl_2$, 50 μl of 300 μ/ml melittin [Sigma], 125 μl of 20 μM nevirapine [NIH AIDS Reagent Program]). The mixtures were then aliquoted and frozen. After all time points were collected, DNA was isolated and quantified using above described PCR methods for early cDNA accumulation.

5.13. Luciferase assay

TZM-bl cells were plated in 96-well plates a day prior to infection. The following day, cells were infected with normalized virus and left for approximately 4–5 h, after which cells were washed with PBS three times and cultured in DMEM. At 48 h post-infection, cells were

lysed using the cell culture lysis reagent provided by the Promega Luciferase kit, from which point the manufacturer's protocol was followed.

5.14. Co-immunoprecipitation

Anti-HA Beads were ordered from Sigma Aldrich and were utilized to immunoprecipitate HA-tagged proteins (MCM5). The immunoprecipitation was done on either cell lysates or the virions lysed in 0.5% Triton X-100 according to the protocol provided by the manufacturer at 4 °C overnight. To check if binding was RNA-related, the lysates were treated with RNase A at a final concentration of 10 μg/ml (Sigma Aldrich) during the overnight incubation.

5.15. Drug treatment of cells to inhibit the MCM helicase

Genistein (GST) and Trichostatin A (TSA) were both ordered from Sigma Aldrich. Approximately 24 h prior to transfection with NL4-3 plasmid to produce virions, HEK293T cells were freshly plated and incubated with 50 μM GST and/or 100 ng/ml TSA. The next day, NL4-3 viral plasmid was transfected into the same cells to produce virus as described above.

5.16. Confocal imaging

Cell culture preparation, transfections, immunostaining, and imaging were performed as described previously with some modifications (Jennelle et al., 2014). Briefly, HEK293T cells were grown on coverslips, transfected with pNL4-3 and/or pCMV-MCM5 as described above. Two days post-transfection, cells were fixed with 3.5% paraformaldehyde (room temperature, 7 min), permeabilized with 0.1% Triton X-100 (room temperature, 5 min), blocked with 1% BSA in PBS (+4 °C, overnight), immunostained (see below), and mounted on microscopic glass slides using Fluoromount G. HIV-1 Gag was visualized using mouse monoclonal anti-HIV Gag antibody (NIH AIDS Research Program) followed by DyLight 550 goat anti-mouse IgG antibody (MyBiosource), and for staining of endogenous MCM5 or MCM3, rabbit monoclonal anti-MCM5 or anti-MCM3 antibody (Abcam) was followed by goat anti-rabbit IgG antibody (Invitrogen) conjugated to Alexa Fluor^{s} 647 or GFP, respectively. Images were captured with Cell Observer Spinning Disk fluorescent microscope (Carl Zeiss) using a Glycerine immersion lens 150 × / 1.35 or with Carl Zeiss LSM 710 confocal microscope using a Plan Apochromat 63 × /1.4 Oil DIC lens (Zeiss). Further image processing was performed using Volocity and ImageJ software.

5.17. Statistical analysis

All results are presented for one representative experiment out of at least two performed. Quantitative data were analyzed by two-way ANOVA (OriginPro v. 8.0) and Student's *t*-test (Microsoft Excel). Standard deviation was calculated in all quantitative experiments for at least three independent preparations. The difference was considered to be statistically significant when $P<0.05$.

Acknowledgments

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Sup-T1 cells from Dr. J. Hoxie, THP1 from Dr. S. Tsuchiya, 8E5 cells from Dr. T. Folks, anti-HIV-1 p24 mouse monoclonal antibody from Dr. M. Malim, antiserum to HIV-1 Integrase from Dr. D. P. Grandgenett, HIV-IG (catalog #3957) from NABI and NHLBI. The HIV-1 proviral clone NL4-3 was a gift from Dr. L. Ratner; pcDNA-Env (MLV) was kindly provided by Dr. N. Landau. Authors are grateful to Dr. A. Popratiloff for help with confocal microscopy, Dr. F. Kashanchi for advices and to Dr. T. Ammosova for instructions for sample preparations for LC-MS/MS. This work was supported in part by the District of Columbia Center for AIDS Research (DC CFAR), an NIH-funded program (grant P30 AI117970), and the NIH grant from Research Centers in Minority Institutions (RCMI) Program of the Division of Research Infrastructure, National Center for Research Resources (RCMI-NIH 2G12RR003048). SS was a predoctoral student in the Microbiology and Immunology Program of the Institute for Biomedical Sciences at the George Washington University. This work is from a dissertation presented to the above program in partial fulfillment of the requirements for the Ph.D. degree.

References

Abudu A, Wang X, Dang Y, Zhou T, Xiang SH, Zheng YH. Identification of molecular determinants from Moloney leukemia virus 10 homolog (MOV10) protein for virion packaging and anti-HIV-1 activity. J Biol Chem. 2012; 287:1220–1228. [PubMed: 22105071]

Aiken C. Cell-free assays for HIV-1 uncoating. Methods Mol Biol. 2009; 485:41–53. [PubMed: 19020817]

Ao Z, Danappa Jayappa K, Wang B, Zheng Y, Kung S, Rassart E, Depping R, Kohler M, Cohen EA, Yao X. Importin alpha3 interacts with HIV-1 integrase and contributes to HIV-1 nuclear import and replication. J Virol. 2010; 84:8650–8663. [PubMed: 20554775]

Arjan-Odedra S, Swanson CM, Sherer NM, Wolinsky SM, Malim MH. Endogenous MOV10 inhibits the retrotransposition of endogenous retro-elements but not the replication of exogenous retroviruses. Retrovirology. 2012; 9:53. [PubMed: 22727223]

Bailis JM, Luche DD, Hunter T, Forsburg SL. Minichromosome maintenance proteins interact with checkpoint and recombination proteins to promote s-phase genome stability. Mol Cell Biol. 2008; 28:1724–1738. [PubMed: 18180284]

Bishop KN, Verma M, Kim EY, Wolinsky SM, Malim MH. APOBEC3G inhibits elongation of HIV-1 reverse transcripts. PLoS Pathog. 2008; 4:e1000231–e1000231. [PubMed: 19057663]

Bochman ML, Schwacha A. The Mcm complex: unwinding the mechanism of a replicative helicase. Microbiol Mol Biol Rev. 2009; 73:652–683. [PubMed: 19946136]

Bregnard C, Zamborlini A, Leduc M, Chafey P, Camoin L, Saib A, Benichou S, Danos O, Basmaciogullari S. Comparative proteomic analysis of HIV-1 particles reveals a role for Ezrin and EHD4 in the Nef-dependent increase of virus infectivity. J Virol. 2013; 87:3729–3740. [PubMed: 23325686]

Briones MS, Dobard CW, Chow SA. Role of human immunodeficiency virus type 1 integrase in uncoating of the viral core. J Virol. 2010; 84:5181–5190. [PubMed: 20219923]

Burdick R, Smith JL, Chaipan C, Friew Y, Chen J, Venkatachari NJ, viks-Frankenberry KA, Hu WS, Pathak VK. P body-associated protein Mov10 inhibits HIV-1 replication at multiple stages. J Virol. 2010; 84:10241–10253. [PubMed: 20668078]

Butler SL, Hansen MS, Bushman FD. A quantitative assay for HIV DNA integration in vivo. Nat Med. 2001; 7:631–634. [PubMed: 11329067]

Chertova E, Chertov O, Coren LV, Roser JD, Trubey CM, Bess JW Jr, Sowder RC, Barsov E, Hood BL, Fisher RJ, Nagashima K, Conrads TP, Veenstra TD, Lifson JD, Ott DE. Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages. J Virol. 2006; 80:9039–9052. [PubMed: 16940516]

Christ F, Thys W, De RJ, Gijsbers R, Albanese A, Arosio D, Emiliani S, Rain JC, Benarous R, Cereseto A, Debyser Z. Transportin-SR2 imports HIV into the nucleus. Curr Biol. 2008; 18:1192–1202. [PubMed: 18722123]

Chuang CHH, Yang D, Bai G, Freeland A, Pruitt SC, Schimenti JC. Post-transcriptional homeostasis and regulation of MCM2-7 in mammalian cells. Nucleic Acids Res. 2012; 40:4914–4924. [PubMed: 22362746]

Ciuffi A, Bushman FD. Retroviral DNA integration: HIV and the role of LEDGF/p75. Trends Genet. 2006; 22:388–395. [PubMed: 16730094]

DaFonseca CJ, Shu F, Zhang JJ. Identification of two residues in MCM5 critical for the assembly of MCM complexes and Stat1-mediated transcription activation in response to IFN-gamma. Proc Natl Acad Sci USA. 2001; 98:3034–3039. [PubMed: 11248027]

Fassati A, Gorlich D, Harrison I, Zaytseva L, Mingot JM. Nuclear import of HIV-1 intracellular reverse transcription complexes is mediated by importin 7. EMBO J. 2003; 22:3675–3685. [PubMed: 12853482]

Formolo CA, Mintz M, Takanohashi A, Brown KJ, Vanderver A, Halligan B, Hathout Y. Time series proteome profiling. Methods Mol Biol. 2011; 694:365–377. [PubMed: 21082445]

Frappier L. EBNA1 and host factors in Epstein–Barr virus latent DNA replication. Curr Opin Virol. 2012; 2:733–739. [PubMed: 23031715]

Furtak V, Mulky A, Rawlings SA, Kozhaya L, Lee K, Kewalramani VN, Unutmaz D. Perturbation of the P-body component Mov10 inhibits HIV-1 infectivity. PLoS One. 2010; 5:e9081. [PubMed: 20140200]

Gallay P, Hope T, Chin D, Trono D. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc Natl Acad Sci USA. 1997; 94:9825–9830. [PubMed: 9275210]

Giroud C, Chazal N, Gay B, Eldin P, Brun S, Briant L. HIV-1-associated PKA acts as a cofactor for genome reverse transcription. Retrovirology. 2013; 10:157. [PubMed: 24344931]

Gurer C, Cimarelli A, Luban J. Specific incorporation of heat shock protein 70 family members into primate lentiviral virions. J Virol. 2002; 76:4666–4670. [PubMed: 11932435]

Holmes RK, Koning FA, Bishop KN, Malim MH. APOBEC3F can inhibit the accumulation of HIV-1 reverse transcription products in the absence of hypermutation. Comparisons with APOBEC3G. J Biol Chem. 2007a; 282:2587–2595. [PubMed: 17121840]

Holmes RK, Malim MH, Bishop KN. APOBEC-mediated viral restriction: not simply editing? Trends Biochem Sci. 2007b; 32:118–128. [PubMed: 17303427]

Hrecka K, Hao C, Gierszewska M, Swanson SK, Kesik-Brodacka M, Srivastava S, Florens L, Washburn MP, Skowronski J. Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. Nature. 2011; 474:658–661. [PubMed: 21720370]

Iordanskiy S, Santos S, Bukrinsky M. Nature, nurture and HIV: the effect of producer cell on viral physiology. Virology. 2013; 443:208–213. [PubMed: 23747196]

Jeang KT, Yedavalli V. Role of RNA helicases in HIV-1 replication. Nucleic Acids Res. 2006; 34:4198–4205. [PubMed: 16935887]

Jennelle L, Hunegnaw R, Dubrovsky L, Pushkarsky T, Fitzgerald ML, Sviridov D, Popratiloff A, Brichacek B, Bukrinsky M. HIV-1 protein Nef inhibits activity of ATP binding cassette transporter A1 by targeting endoplasmic reticulum chaperone calnexin. J Biol Chem. 2014; 289:28870–28884. [PubMed: 25170080]

Kawaguchi A, Nagata K. De novo replication of the influenza virus RNA genome is regulated by DNA replicative helicase, MCM. EMBO J. 2007; 26:4566–4575. [PubMed: 17932485]

Kewalramani VN, Emerman M. Vpx association with mature core structures of HIV-2. Virology. 1996; 218:159–168. [PubMed: 8615019]

Kolokoltsov AA, Adhikary S, Garver J, Johnson L, Davey RA, Vela EM. Inhibition of Lassa virus and Ebola virus infection in host cells treated with the kinase inhibitors genistein and tyrphostin. Arch Virol. 2012; 157:121–127. [PubMed: 21947546]

Kotov A, Zhou J, Flicker P, Aiken C. Association of Nef with the human immunodeficiency virus type 1 core. J Virol. 1999; 73:8824–8830. [PubMed: 10482638]

Krishnan L, Matreyek KA, Oztop I, Lee K, Tipper CH, Li X, Dar MJ, Kewalramani VN, Engelman A. The requirement for cellular transportin 3 (TNPO3 or TRN-SR2) during infection maps to human immunodeficiency virus type 1 capsid and not integrase. J Virol. 2010; 84:397–406. [PubMed: 19846519]

Laguette N, Sobhian B, Casartelli N, Ringeard M, Chable-Bessia C, Segeral E, Yatim A, Emiliani S, Schwartz O, Benkirane M. SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. Nature. 2011; 474:654–657. [PubMed: 21613998]

Lin CW, Engelman A. The barrier-to-autointegration factor is a component of functional human immunodeficiency virus type 1 preintegration complexes. J Virol. 2003; 77:5030–5036. [PubMed: 12663813]

Llano M, Saenz DT, Meehan A, Wongthida P, Peretz M, Walker WH, Teo W, Poeschla EM. An essential role for LEDGF/p75 in HIV integration. Science. 2006; 314:461–464. [PubMed: 16959972]

Luban J. Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. J Virol. 2007; 81:1054–1061. [PubMed: 16956947]

Luban J, Bossolt KL, Franke EK, Kalpana GV, Goff SP. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell. 1993; 73:1067–1078. [PubMed: 8513493]

Maertens G, Cherepanov P, Pluymers W, Busschots K, De CE, Debyser Z, Engelborghs Y. LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. J Biol Chem. 2003; 278:33528–33539. [PubMed: 12796494]

Majid S, Dar AA, Saini S, Chen Y, Shahryari V, Liu J, Zaman MS, Hirata H, Yamamura S, Ueno K, Tanaka Y, Dahiya R. Regulation of mini-chromosome maintenance gene family by microRNA-1296 and genistein in prostate cancer. Cancer Res. 2010; 70:2809–2818. [PubMed: 20332239]

Marno KM, Ogunkolade BW, Pade C, Oliveira NM, O'Sullivan E, McKnight A. Novel restriction factor RNA-associated early-stage anti-viral factor (REAF) inhibits human and simian immunodeficiency viruses. Retrovirology. 2014; 11:3. [PubMed: 24410916]

O'Doherty U, Swiggard WJ, Jeyakumar D, McGain D, Malim MH. A sensitive, quantitative assay for human immunodeficiency virus type 1 integration. J Virol. 2002; 76:10942–10950. [PubMed: 12368337]

Popov S, Strack B, Sanchez-Merino V, Popova E, Rosin H, Gottlinger HG. Human immunodeficiency virus type 1 and related primate lentiviruses engage clathrin through Gag-Pol or Gag. J Virol. 2011; 85:3792–3801. [PubMed: 21289110]

Quivy V, Adam E, Collette Y, Demonte D, Chariot A, Vanhulle C, Berkhout B, Castellano R, de Launoit Y, Burny A, Piette J, Bours V, Van Lint C. Synergistic activation of human immunodeficiency virus type 1 promoter activity by NF-kappaB and inhibitors of deacetylases: potential perspectives for the development of therapeutic strategies. J Virol. 2002; 76:11091–11103. [PubMed: 12368351]

Roy BB, Hu J, Guo X, Russell RS, Guo F, Kleiman L, Liang C. Association of RNA helicase a with human immunodeficiency virus type 1 particles. J Biol Chem. 2006; 281:12625–12635. [PubMed: 16527808]

Santos S, Obukhov Y, Nekhai S, Bukrinsky M, Iordanskiy S. Virus-producing cells determine the host protein profiles of HIV-1 virion cores. Retrovirology. 2012; 9:65. [PubMed: 22889230]

Schaller T, Ocwieja KE, Rasaiyaah J, Price AJ, Brady TL, Roth SL, Hue S, Fletcher AJ, Lee K, KewalRamani VN, Noursadeghi M, Jenner RG, James LC, Bushman FD, Towers GJ. HIV-1 capsid-cyclophilin interactions determine nuclear import pathway, integration targeting and replication efficiency. PLoS Pathog. 2011; 7:e1002439. [PubMed: 22174692]

Schwacha A, Bell SP. Interactions between two catalytically distinct MCM subgroups are essential for coordinated ATP hydrolysis and DNA replication. Mol Cell. 2001; 8:1093–1104. [PubMed: 11741544]

Serquiña AKP, Das SR, Popova E, Ojelabi OA, Roy CK, Göttlinger HG. UPF1 is crucial for the infectivity of human immunodeficiency virus type 1 progeny virions. J Virol. 2013; 87:8853–8861. [PubMed: 23785196]

Shah VB, Aiken C. *In vitro* Uncoating of HIV-1 Cores. J Vis Exp. 2011

Sorin M, Cano J, Das S, Mathew S, Wu X, Davies KP, Shi X, Cheng SW, Ott D, Kalpana GV. Recruitment of a SAP18-HDAC1 complex into HIV-1 virions and its requirement for viral replication. PLoS Pathog. 2009; 5:e1000463. [PubMed: 19503603]

Sorin M, Yung E, Wu X, Kalpana GV. HIV-1 replication in cell lines harboring INI1/hSNF5 mutations. Retrovirology. 2006; 3:56. [PubMed: 16945155]

Stantchev TS, Markovic I, Telford WG, Clouse KA, Broder CC. The tyrosine kinase inhibitor genistein blocks HIV-1 infection in primary human macrophages. Virus Res. 2007; 123:178–189. [PubMed: 17030448]

Wang X, Han Y, Dang Y, Fu W, Zhou T, Ptak RG, Zheng YH. Moloney leukemia virus 10 (MOV10) protein inhibits retrovirus replication. J Biol Chem. 2010; 285:14346–14355. [PubMed: 20215113]

Warren K, Wei T, Li D, Qin F, Warrilow D, Lin MH, Sivakumaran H, Apolloni A, Abbott CM, Jones A, Anderson JL, Harrich D. Eukaryotic elongation factor 1 complex subunits are critical HIV-1 reverse transcription cofactors. Proc Natl Acad Sci USA. 2012; 109:9587–9592. [PubMed: 22628567]

Warrilow D, Meredith L, Davis A, Burrell C, Li P, Harrich D. Cell factors stimulate human immunodeficiency virus type 1 reverse transcription in vitro. J Virol. 2008; 82:1425–1437. [PubMed: 18045931]

Westervelt P, Trowbridge DB, Epstein LG, Blumberg BM, Li Y, Hahn BH, Shaw GM, Price RW, Ratner L. Macrophage tropism determinants of human immunodeficiency virus type 1 in vivo. J Virol. 1992; 66:2577–2582. [PubMed: 1548783]

Yung E, Sorin M, Wang EJ, Perumal S, Ott D, Kalpana GV. Specificity of interaction of INI1/hSNF5 with retroviral integrases and its functional significance. J Virol. 2004; 78:2222–2231. [PubMed: 14963118]

Zaitseva L, Cherepanov P, Leyens L, Wilson SJ, Rasaiyaah J, Fassati A. HIV-1 exploits importin 7 to maximize nuclear import of its DNA genome. Retrovirology. 2009; 6:11. [PubMed: 19193229]

Zhang F, Zang T, Wilson SJ, Johnson MC, Bieniasz PD. Clathrin facilitates the morphogenesis of retrovirus particles. PLoS Pathog. 2011; 7:e1002119. [PubMed: 21738476]

Zhang H, Dornadula G, Pomerantz RJ. Endogenous reverse transcription of human immunodeficiency virus type 1 in physiological microenviroments: an important stage for viral infection of nondividing cells. J Virol. 1996; 70:2809–2824. [PubMed: 8627755]

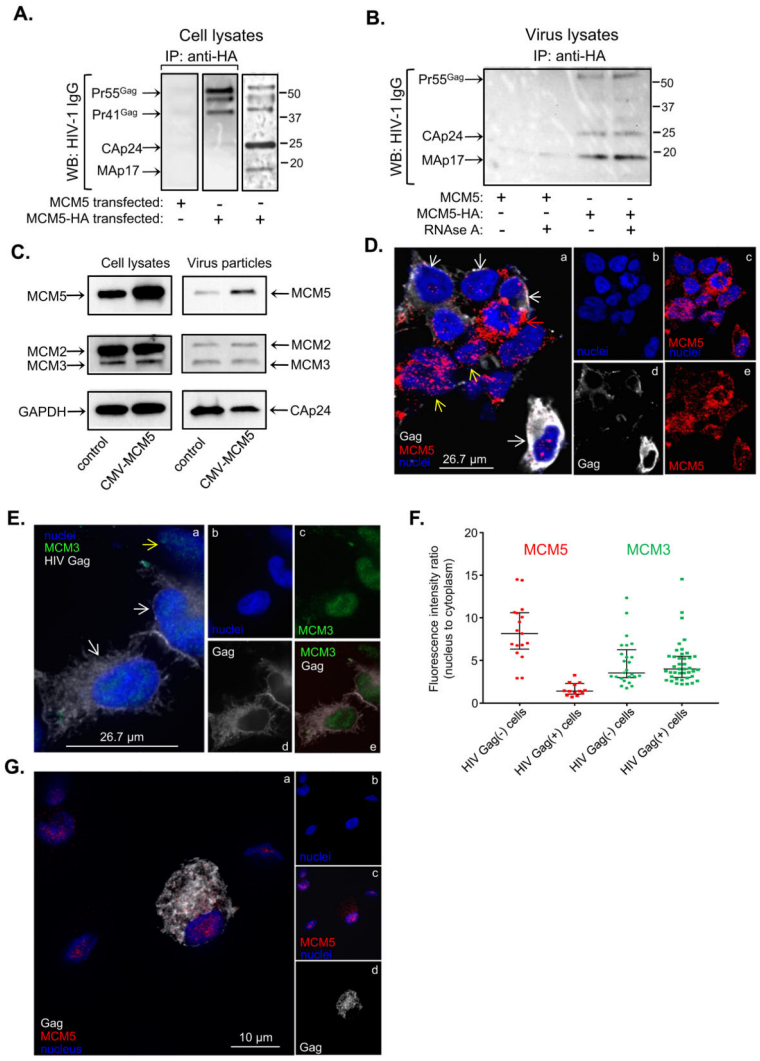

Fig. 1.

MCM5 is incorporated into HIV-1 virions via interaction with Gag polyprotein. (A) Co-immunoprecipitation of MCM5 and Gag from cytoplasmic lysates of virus-producing cells. HEK293T cells were transfected either with pCMV-MCM5 plasmid or pCMV-MCM5-HA, expressing HA-tagged MCM5. 24 h later the cells were transfected with pNL4-3; cells and culture supernatants were harvested 48 h after second transfection. The cell lysates were subjected to IP with anti-HA monoclonal antibody, and the immune complexes were analyzed by SDS-PAGE and Western blot. (B) IP of HA-tagged MCM5 from HIV lysates. Virus particles were concentrated from culture supernatants described in A, equalized by p24 and lysed. The lysates were incubated or not with RNase A, immunoprecipitated with anti-HA monoclonal antibody, and the immune complexes were analyzed by SDS-PAGE and Western blot. (C) The HEK293T cells were transfected with pNL4-3, and one set was transfected with MCM5, while the other was transfected with empty vector. The cells and culture supernatants were harvested, viral particles were concentrated and p24 normalized. The viral and cell lysates were subjected to SDS-PAGE and Western blot to test expression of MCM2, MCM3 and MCM5. (D) HEK293T cells transfected with pNL4-3 were

immunostained for MCM5 (red) and HIV-1 Gag (white). Cell nuclei were stained by DAPI (blue). Panel **a** shows a composite image of all three channels. White arrows indicate cells expressing Gag, yellow arrows – cells not expressing Gag, and red arrow points to dividing cell. Single channel images or a combination of two selected channels are shown in panels **b**, **c, d**, and **e.** (E) Experiment was performed as in panel **D**, but staining was for MCM3 (green) and HIV-1 Gag (white). Cells expressing Gag in panel **a** are marked by white arrows and Gag-negative cell – by yellow arrow. (F) Quantification of images in panels **D** and **E** was performed using the Volocity software. 14 HIV Gag-positive and 19 HIV Gag-negative cells were analyzed for MCM5 distribution, 41 Gag-positive and 26 Gag-negative cells were analyzed for MCM3 distribution. The graph presents mean±SEM of the ratio of fluorescence intensity in the nucleus to that in cytoplasm. The difference in the ratio of MCM5 fluorescence between Gag-positive and Gag-negative cells was highly significant ($p<0.001$ by Student's unpaired *t*-test). (G) Monocyte-derived macrophages were infected with HIV-1 and immunostained for MCM5 (red) and HIV-1 Gag (white) as in panel **D**.

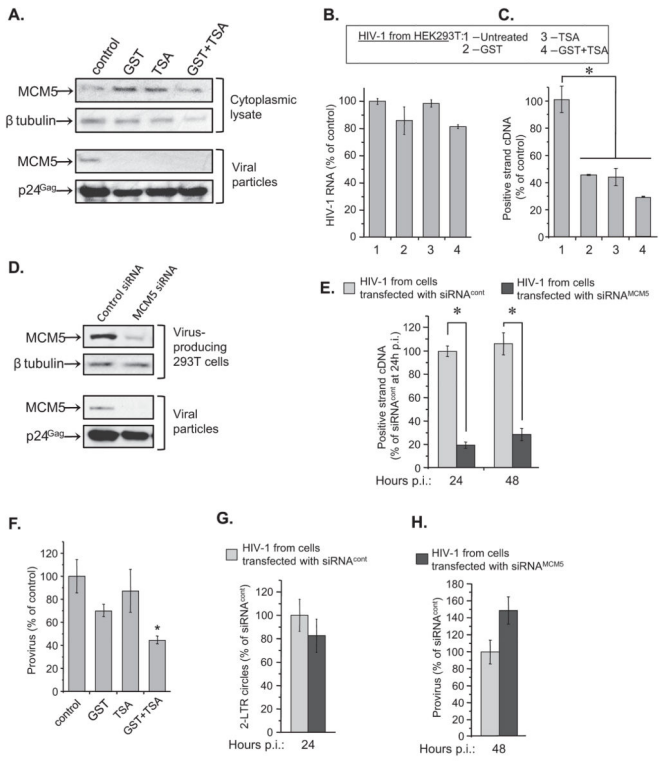

Fig. 2.

The effect of MCM5 knockdown or MCM inactivation on HIV reverse transcription and integration in the newly infected cells. (A) Sup-T1 cells were infected for 48 h with NL4-3 virus, washed and treated with genistein, TSA, or genistein and TSA together. The virions and cells were harvested 72 h after treatment, p24 normalized and analyzed by SDS-PAGE and Western blot. (B) Jurkat cells were infected with the MCM5-deficient NL4-3 produced by HEK293T cells treated with GST, TSA or GST and TSA together or with the control virus from untreated cells. All virus inoculums were equalized by p24. Cells were treated with subtilisin 2 h post-infection to remove bound virus that has not entered, RNA was isolated and quantitated by qRT-PCR with the *gag*-specific primers. Results are shown as mean of three independent experiments±SEM. (C) Jurkat cells infected as described in B were harvested at 24 h post-inoculation, total DNA was isolated and subjected to real-time PCR with primers and probe specific for positive strand HIV-1 cDNA. Results are shown as mean of three independent experiments±SEM; asterisk indicates $p<0.05$. (D) HEK293T cells were transfected with MCM5 siRNA or scrambled (control) siRNA and 24 h later were co-transfected with pNL4-3 plasmid. The virions were concentrated from culture supernatants, normalized by p24 and subjected to SDS-PAGE and Western blot. Cytoplasmic extracts of producer cells were also analyzed by Western blot. (E) Analysis of reverse transcription in Jurkat cells infected with HIV-1 produced from HEK293T cells treated with MCM5 siRNA or scrambled (control) siRNA. Reverse transcription efficiency was analyzed as in C. Results are shown as mean of four independent experiments±SEM; double asterisk indicates $p<0.01$. (F) Analysis of integration of the virus produced from genistein and TSA treated cells. Jurkat cells from the experiment described in B and C were harvested at 48 h post-virus inoculation, genomic DNA was isolated from nuclear extracts

and subjected to a two-step *Alu*-based nested PCR assay. Results are shown as mean of three independent experiments±SEM; asterisk indicates $p<0.05$. (G) Analysis of nuclear import in Jurkat cells infected with HIV-1 produced from HEK293T cells treated with MCM5 siRNA or scrambled (control) siRNA. Nuclear import was assessed by quantitation of 2-LTR circles at 24 post-inoculation. Results are shown as mean of three independent experiments±SEM. (H) Analysis of integration in Jurkat cells infected with HIV-1 produced from HEK293T cells treated with MCM5 siRNA or scrambled (control) siRNA. Integration was quantified as described in F. Results are shown as mean of three independent experiments±SEM; asterisk indicates $p<0.05$ (MCM5 siRNA-treated relative to control).

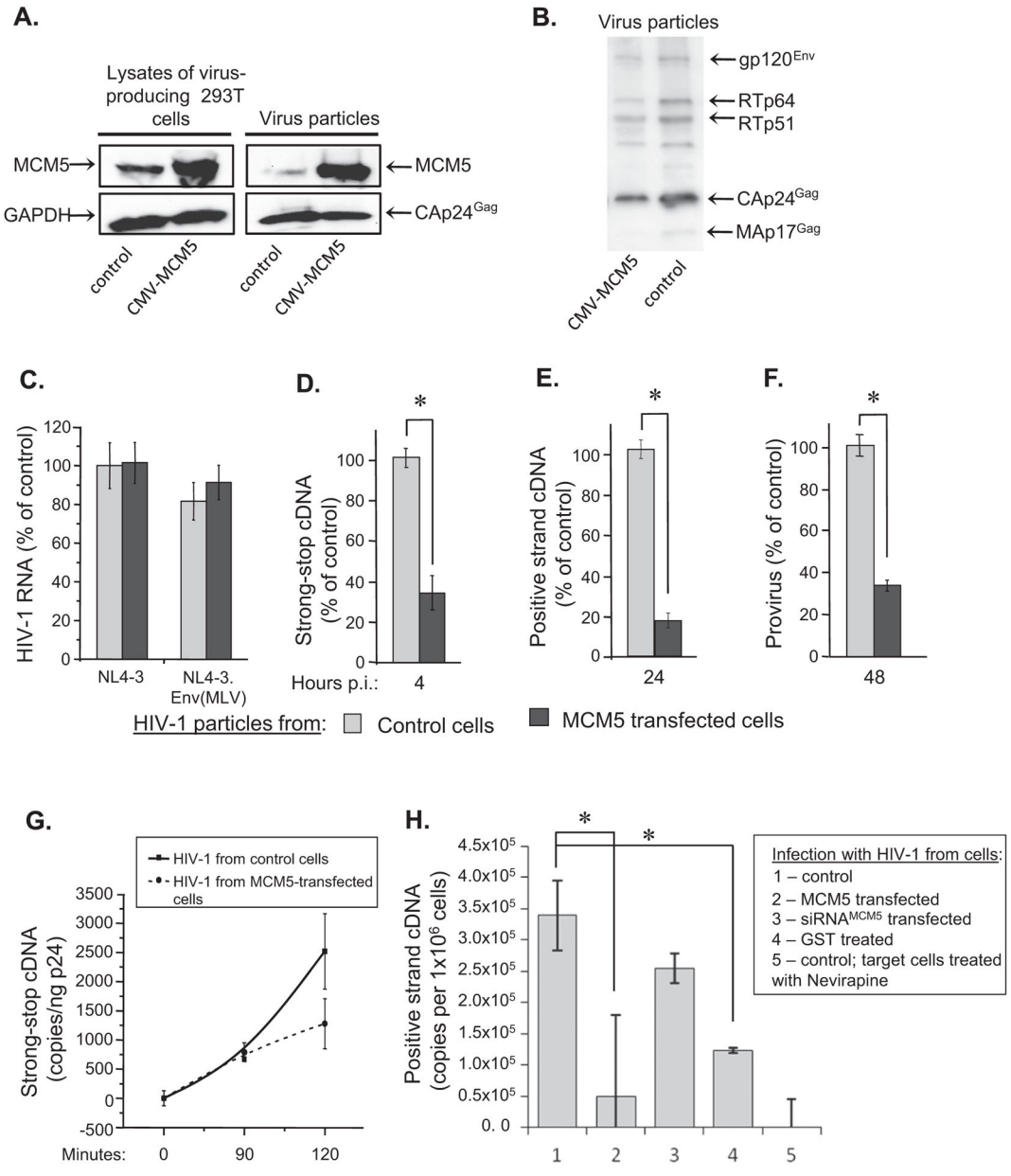

Fig. 3.

High level of MCM5 expression in virus-producing cells determines elevated MCM5 incorporation into the virions and reduced reverse transcription and integration of HIV-1 genome in target cells. (A) HEK293T cells were transfected with pNL4-3 plasmid, and one set was transfected with CMV-MCM5 and the other with empty vector (control). Viral particles were harvested at 72 h post-transfection, concentrated and p24 normalized. The viral and cell lysates were subjected to SDS-PAGE and Western blot to test expression of MCM5, GAPDH and p24. (B) Viral particles harvested from 5 $\times 10^6$ HEK293T cells transfected with CMV-MCM5 or empty vector (control) were analyzed by Western blot using pooled sera from HIV-positive subjects. (C) Jurkat cells were inoculated with NL4-3 produced by HEK293T cells transfected with pCMV-MCM5 or with control virus from cells

transfected with empty vector. Both viruses were pseudotyped or not with the Env from amphotropic MLV and normalized by p24. RNA was isolated from infected cells 2 h post infection and HIV-1 RNA was quantified by qRT-PCR. Results are shown as mean of three independent experiments±SEM. (D) Jurkat cells were infected with non-pseudotyped HIV-1 and 4 h post-inoculation DNA was isolated and subjected to qPCR with the primers specific for first strong-stop cDNA. Results are shown as mean of three independent experiments ±SEM; asterisk indicates $p \leq 0.05$. (E) Jurkat cells infected as in C were harvested at 24 post-inoculation with the virus, total DNA was isolated and subjected to qPCR with the primers specific for positive strand cDNA. Results are shown as mean of three independent experiments±SEM; asterisk indicates $p \leq 0.05$. (F) Jurkat cells infected as in C were harvested 48 h post-inoculation, total DNA was isolated and subjected to a two-step *Alu*-based nested PCR. Results are shown as mean of three independent experiments±SEM; asterisk indicates $p \leq 0.05$. (G) The cores of NL4-3 virions from pCMV-MCM5 transfected and empty vector transfected HEK293T cells were p24 normalized and subjected to Endogenous Reverse Transcription (ERT) assay. DNA was purified and analyzed by qRT-PCR with primer set specific for strong-stop viral DNA. Results are shown as mean of three independent measurements±SEM.

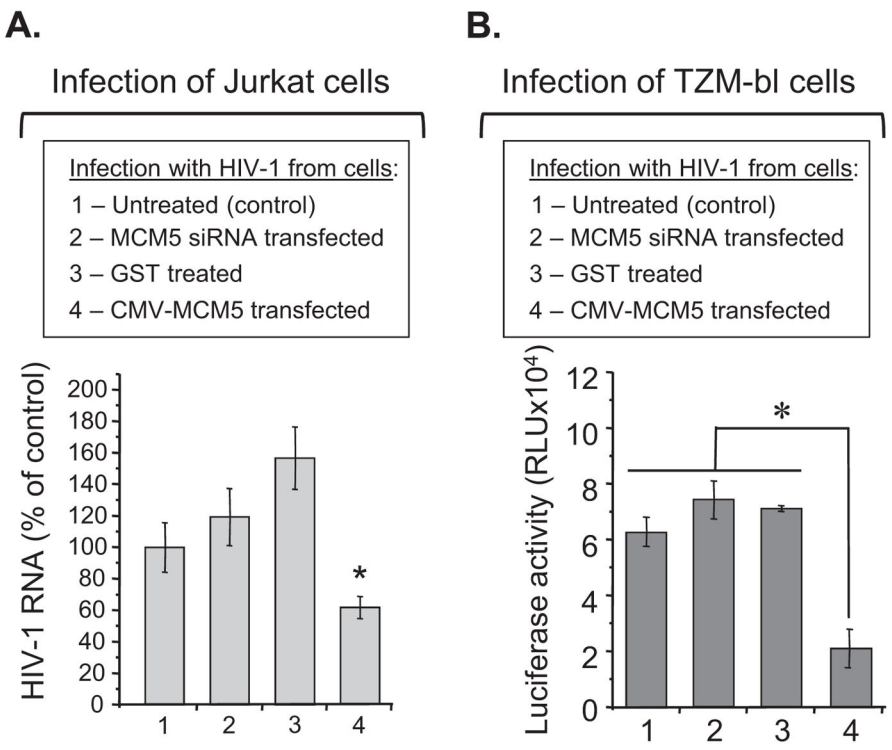

Fig. 4.

Increased incorporation of MCM5, but not its deficiency in HIV-1 virions, leads to decreased HIV-1 viral replication in the infected cells. (A) The purified and p24 normalized NL4-3 virions from MCM5 siRNA-transfected, genistein-treated, or pCMV-MCM5-transfected HEK293T cells were used for infection of Jurkat cells. The cells were harvested at 48 h post-inoculation and subjected to RT-PCR analysis with the primers specific for HIV-1 Gag. Results are shown as mean of three independent measurements±SEM. Asterisk indicates $p<0.05$. (B) The TZM-bl cells were infected with the same virus suspensions as in A, incubated for 72 h, lysed and subjected to analysis of luciferase activity. Results are shown as mean of three independent measurements±SEM; asterisk indicates $p<0.01$.

Table 1

MS/MS detection of MCM2–7 complex subunits in the cores of virions derived from different infected cell lines.

Cores of virions from	MCM complex subunits											
	MCM2		MCM3		MCM4		MCM5		MCM6		MCM7	
	Prot. score[a]	# of pept.[b]	Prot. score	# of pept.	Prot. score	# of pept.	Prot. score	# of pept.	Prot. score	# of pept.	Prot. score	# of pept.
Sup-T1, CEM_{NKR} ($CD4^+$ T cells)	5.07	4	2.11	2	2.21	3	4.01	3	7.62	4	7.18	2
THP-1 (monocytes)			+[c]		2.51	4	10.08	3	5.69	3		
HEK293T (epithelial)			+				7.13	3			3.60	2

[a] Log_{10} transorfmation of the protein identification confidence calculated from peptides shared with no other protein (proteotypic peptides): $-Log_{10}[1-(\%Confidence/100)]$.

[b] The number of proteotypic peptides detected by LC-MS/MS analysis

[c] Protein earlier detected only by Western blot (Santos et al., 2012).