Recruitment of a SAP18-HDAC1 Complex into HIV-1 Virions and Its Requirement for Viral Replication

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Abstract

HIV-1 integrase (IN) is a virally encoded protein required for integration of viral cDNA into host chromosomes. INI1/hSNF5 is a component of the SWI/SNF complex that interacts with HIV-1 IN, is selectively incorporated into HIV-1 (but not other retroviral) virions, and modulates multiple steps, including particle production and infectivity. To gain further insight into the role of INI1 in HIV-1 replication, we screened for INI1-interacting proteins using the yeast two-hybrid system. We found that SAP18 (Sin3a associated protein 18 kD), a component of the Sin3a-HDAC1 complex, directly binds to INI1 in yeast, in vitro and in vivo. Interestingly, we found that IN also binds to SAP18 in vitro and in vivo. SAP18 and components of a Sin3A-HDAC1 complex were specifically incorporated into HIV-1 (but not SIV and HTLV-1) virions in an HIV-1 IN–dependent manner. Using a fluorescence-based assay, we found that HIV-1 (but not SIV) virion preparations harbour significant deacetylase activity, indicating the specific recruitment of catalytically active HDAC into the virions. To determine the requirement of virion-associated HDAC1 to HIV-1 replication, an inactive, transdominant negative mutant of HDAC1 (HDAC1H141A) was utilized. Incorporation of HDAC1H141A decreased the virion-associated histone deacetylase activity. Furthermore, incorporation of HDAC1H141A decreased the infectivity of HIV-1 (but not SIV) virions. The block in infectivity due to virion-associated HDAC1H141A occurred specifically at the early reverse transcription stage, while entry of the virions was unaffected. RNA-interference mediated knock-down of HDAC1 in producer cells resulted in decreased virion-associated HDAC1 activity and a reduction in infectivity of these virions. These studies indicate that HIV-1 IN and INI1/hSNF5 bind SAP18 and selectively recruit components of Sin3a-HDAC1 complex into HIV-1 virions. Furthermore, HIV-1 virion-associated HDAC1 is required for efficient early post-entry events, indicating a novel role for HDAC1 during HIV-1 replication.

Introduction

HIV-1 replication is characterized by a dynamic interplay between the host and the virus [1]. While host restriction factors inhibit HIV-1 replication at various steps, HIV-1 actively engages cellular proteins for its own replication and for subversion of antiviral effects. Several host factors that either restrict or promote HIV-1 replication have been identified in recent years. A high-throughput screening of siRNA mediated knock-down of cellular proteins identified several hundred host proteins that played activating or inhibitory roles in HIV-1 replication highlighting the importance of studying host-virus interaction during HIV-1 replication [2]. While the high through-put screen allows the identification of proteins whose loss of function is not lethal to cells, it is likely that many host factors that are both essential for cellular function and play a role in HIV-1 replication were not identified in such a screen. Several host factors including INI1/hSNF5 and LEDGF, both of which directly bind to HIV-1 integrase (IN), a virally encoded protein required for insertion viral cDNA into host chromosomal DNA, have been identified using the yeast two hybrid system [3,4]. LEDGF appears to be involved in tethering integrase to transcriptionally active regions [5]. However, INI1/hSNF5 appears to play dual roles in HIV-1 replication in that while INI1/hSNF5 present in the producer cells appears to be required for HIV-1 replication, INI1/hSNF5 present in the target cells may be inhibitory. Our previous studies have indicated that ectopic (cytoplasmic) expression of a dominant negative mutant of INI1, harboring the minimal IN-binding domain, inhibits HIV-1 p24 release in a manner dependent on IN-INI1 interactions [6]. INI1/hSNF5 is specifically incorporated into HIV-1 virions and


Editor: Thomas J. Hope, Northwestern University, United States of America

Received April 22, 2008; Accepted May 4, 2009; Published June 5, 2009

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Funding: This work was supported by the National Institutes of Health grant R01 AI30051-01, and by an Irma T. Hirschl scholar award to GVK. MS, JC, and SM wish to acknowledge Institutional AIDS training grant T32-AID07501. This work was supported in part by the Center for AIDS Research at the Albert Einstein College of Medicine and Montefiore Medical Center funded by the National Institutes of Health (NIH AI-51519). This project was funded in part by NIH under contract N01-CO-12400 to DO. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Author Summary

The interaction between the host and HIV-1 virus is a dynamic process. While some host cellular proteins mount antiviral response, HIV-1 utilizes other host proteins for its propagation. It is crucial to understand the role of host proteins in HIV-1 replication for successful development of strategies to combat AIDS. INI1/hSNF5 is a cellular protein that directly binds to an HIV-1 protein, integrase (IN). It is selectively encapsidated into HIV-1 virions and is required for the subsequent viral replication. However, how INI1 mediates its effects is not completely understood. Here we report a novel finding that INI1 as well as IN directly binds to SAP18 (Sin3a-associated protein of 18 kD), a component of the cellular Sin3a/HDAC (histone deacetylase) complex. The HDAC complexes are multiprotein epigenetic modifiers known to affect cellular transcription. In this report we find that along with INI1, SAP18 and the components of the Sin3a+HDAC1 complex are specifically recruited into HIV-1 virions. In addition, we find that virion-associated deacetylase activity is required for efficient reverse transcription in target cells. These studies indicate that HIV-1 IN protein may recruit HDAC1 complex into the virions for utilizing them in the subsequent stages of replication. Our findings provide new insight into the roles of cellular HDAC1 complex in HIV-1 replication and provide a novel paradigm to understand host-virus dynamic interaction.

Here we report that INI1 and IN directly associate with SAP18, a component of the Sin3a-HDAC1 complex. We report a surprising finding that components of Sin3a-HDAC1, but not the core components of the SWI/SNF complex are specifically incorporated into HIV-1 virions. Furthermore, we found that incorporation of a dominant negative mutant of HDAC1 decreases the HDAC1 activity associated with the HIV-1 virions and that this decrease in HDAC1 activity is correlated to a decrease in infectivity of these virions. Finally, we demonstrate that HDAC1 activity is required for modulating a post-entry step at or before reverse transcription during HIV-1 replication. These results indicate an unanticipated role of IN and INI1 in recruiting the HDAC1 complex, independent of SWI/SNF complex, into HIV-1 virions and provide new insights into the role of INI1/hSNF5 and Sin3a-HDAC1 complex in HIV-1 replication.

Results

INI1 directly interacts with SAP18, a component of the Sin3a-HDAC1 complex

Since INI1/hSNF5 is specifically incorporated into HIV-1 virions [6,7], we tested to determine if other core components of SWI/SNF complex such as BRG1, BAF155, and BAF170, were present in the virus particles. HIV-1 virions were purified by density gradient centrifugation to separate the microvesicular fraction and the virions were subjected to subtilisin treatment to remove any cellular proteins non-specifically associated with the virions [17]. These purified, subtilisin-treated virions were subjected to immunoblot analysis using antibodies to components of SWI/SNF complex. Protein extracts from (i) CHO (INI1−/−; rhabdoid cells) that do not express INI1 but express all other components of the SWI/SNF complex; and (ii) 293T cells that express endogenous levels of INI1 and all the components of the SWI/SNF complex, were used as controls in these experiments. The same blot was subjected to sequential immunoblot analysis using several different antibodies, to allow the identification of multiple proteins in the same virion preparation. This analysis revealed the presence of INI1, consistent with the previous reports [7]. However, other components of the SWI/SNF complex including BRG1, BRM, BAF155, and BAF170 were not detected, despite the presence of these components in the control 293T cells used as producer cells (Figure 1A and Figure S1). Preliminary density gradient analysis of total nuclear extracts demonstrated that INI1 indeed is present in several high molecular weight fractions, including those devoid of BRG1 (data not shown), suggesting the existence of other unidentified INI1-associated complexes in the cell.

To determine if IN11 could associate with proteins other than SWI/SNF complex, we carried out a yeast two-hybrid analysis where LexADB (DNA Binding domain)-fusion of IN11 was used as bait to screen a HL60 cDNA library fused to GAL4AC (activation domain) [18]. One of the positive clones (#13029-49.1) that specifically interacted with IN11 but not with controls such as LexADB, LexADB-IN, LexaDB-cMYC, LexaDB-Laminin and LexaDB-cMYC (Figure 1B) was identified as a fragment of SAP18 (aa 45–153). Both cMYC and IN are known IN11 interacting proteins and Laminin is a negative control [18].

IN and INI1/SNF5 directly interact with SAP18 in vitro

To determine if the interaction of SAP18 with IN11 is direct, we carried out a GST pull down assay using purified components of GST (glutathione-S-transferase)-SAP18 and 6His(hexa-histidine)-INI1/hSNF5 fusion proteins, as well as IN. We found IN11 interacts with GST-SAP18 but not GST (Figure 1C, lanes 3 and 4). Interestingly, IN also interacted with GST-SAP18 but not with
GST-SAP18 were incubated with purified IN or INI1 or both and bound SAP18, INI1 and IN. Glutathione sepharose beads bound to GST alone or domain. (C) GST pull-down assay to demonstrate interaction between interaction; and ‘’ = no interaction, LexADB = LexA DNA binding domain. (A) Purified and concentrated HIV-1MN virions were immunoprecipitated with either α-HA, α-HDAC1, control α-6His, or no antibody. The results indicated that α-HA and α-HDAC1 antibodies were able to co-immunoprecipitate HA-INI1/hSNF5 and HDAC1 respectively (Figure 2B, lanes 3, 5, 9, and 11), while the control antibodies did not (Figure 2B, lanes 4, 6, 10, and 12). Additionally, α-HA antibodies co-immunoprecipitated BRG1, consistent with INI1 being part of the SWI/SNF complex (Figure 2B, lanes 3 and 9). We probed the above co-immunoprecipitates with antibodies against CHD3, an exclusive component of NuRD complex. While α-HDAC1 antibody co-immunoprecipitated CHD3, α-HA antibody did not (Figure 2B, lanes 5 and 11). Furthermore, another component of NuRD complex, MTA1 was also not pulled down by HA-INI1 in the same immunoprecipitates (data not shown). These results suggested that INI1/hSNF5 specifically associates with the Sin3a-HDAC1 complex.

To further establish that components of Sin3a-HDAC1 complex associate with INI1, we carried out co-immunoprecipitation experiments in the presence and absence of HIV-1 vectors, and a plasmid expressing FLAG-INI1. Co-immunoprecipitation experiments with α-FLAG antibodies indicated an association of SAP18 and HDAC1 with INI1 (Figure 2C, lane 5). Interestingly, association of these components and Sin3A was enriched when cells were cotransfected with HIV-1 vector (Figure 2C, compare lanes 5 and 7). This enrichment was not due to an increase in the level of these components in the presence of HIV-1 vector, as the input control from cells expressing Flag-INI1 with or without co-transfection of HIV-1 vectors indicated identical levels of expression (Figure 2C, lanes 1 and 2, in the presence and absence of HIV-1). Furthermore, immunoprecipitation with α-FLAG antibodies resulted in similar amounts of FLAG-INI1 both in the presence and absence of viral vectors (Figure 2C, compare lanes 5 and 7). These results suggested that association of SAP18, HDAC1 and Sin3A is preferentially increased in the presence of HIV-1, despite the presence of similar levels of these proteins (Figure 2C, compare lanes 5 to 7). This is consistent with the idea that HIV-1 IN also binds to SAP18. Since both IN and INI1 bind to SAP18, it is possible that association of INI1 with SAP18-Sin3a-HDAC1 complex is increased upon HIV-1 infection. In fact, we have found that co-expression of IN with INI1 increases the ability of INI1 to co-immunoprecipitate HDAC1, consistent with our hypothesis (Figure S2). Furthermore, we found that co-expression of GFP-IN with FL-SAP18 in INI1−/− MON

IN and INI1/hSNF5 co-immunoprecipitate with SAP18 and associate with components of Sin3a-HDAC1 complex in vivo

To further corroborate the in vitro finding, we carried out a series of co-immunoprecipitation assays. First, we found that antibodies to SAP18 could co-immunoprecipitate endogenous INI1, indicating that the two endogenous proteins interact with each other in vivo (Figure 2A).

HDAC1 is associated with several complexes, such as Sin3a, NuRD and CoREST [19]. Both Sin3a and SAP18 are components of Sin3a-HDAC1 complex but not that of NuRD and CoREST complexes. On the other hand, MTA1 and CHD3 are exclusive components of NuRD complex but not that of Sin3a complex [19]. To determine if INI1 can specifically associate with the Sin3a-HDAC1 complex, we carried out immunoprecipitation studies to determine the ability of INI1 to pull down HDAC1 and CHD3. MON (INI1−/− rhabdoid cells) and HeLa cells were transfected with a plasmid expressing HA-INI1/hSNF5, and total proteins were immunoprecipitated with either α-HA, α-HDAC1, control α-6His, or no antibody. The results indicated that α-HA and α-HDAC1 antibodies were able to co-immunoprecipitate HA-INI1/hSNF5 and HDAC1 respectively (Figure 2B, lanes 3, 5, 9, and 11), while the control antibodies did not (Figure 2B, lanes 4, 6, 10, and 12). Additionally, α-HA antibodies co-immunoprecipitated BRG1, consistent with INI1 being part of the SWI/SNF complex (Figure 2B, lanes 3 and 9). We probed the above co-immunoprecipitates with antibodies against CHD3, an exclusive component of NuRD complex. While α-HDAC1 antibody co-immunoprecipitated CHD3, α-HA antibody did not (Figure 2B, lanes 5 and 11). Furthermore, another component of NuRD complex, MTA1 was also not pulled down by HA-INI1 in the same immunoprecipitates (data not shown). These results suggested that INI1/hSNF5 specifically associates with the Sin3a-HDAC1 complex.

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GST-SAP18 suggesting that the three proteins have the potential to form ternary complex (Figure 1C, lanes 5 and 6).
Figure 2. INI1 associates with components of Sin3a-HDAC1 complex in vivo. (A) Interaction between endogenous INI1 and SAP18. Cell lysates from confluent HeLa cells were subjected to co-immunoprecipitation analysis using α-SAP18 antibodies. Rabbit IgG was used as a control. (B) Immunoprecipitation of HA-INI1 with HDAC1 in vivo. Total proteins from MON (INI1−/−) and HeLa cells, transfected with HA-INI1, were subjected to co-immunoprecipitation analyses using α-HA and α-HDAC1 antibodies. No antibodies (beads) and α-6His antibodies were used as negative controls. The immunoblots were sequentially probed with α-HA, α-HDAC1, α-BRG1, and α-CHD3 antibodies. (C) Association of INI1/hSNF5 with components of SIN3a/HDAC1 complex in the presence and absence of HIV-1 viral proteins. 293T cells were co-transfected with HIV-1 viral vectors or an empty vector and plasmid expressing either FLAG-INI1 or HA-INI1. Immunoprecipitation was performed using α-FLAG antibodies. Immunoblots were sequentially probed with α-IN1, α-SAP18, α-HDAC1, α-IN2A, α-BRG1 and α-HDAC3 antibodies as indicated. (Note: A weak Sin3a band is visible upon longer exposure in lane 7. The BRG1 band in the lane 6 is due to spill over from lane 5). (D–G) In vivo association of HA-INI1, GFP-IN, and FLAG-SAP18. 293T cells were transfected with vectors expressing FL-SAP18 and HA-INI1 (D), HA-INI1 and YFP-IN (E), FL-SAP18 and YFP-IN (F), or FLAG-SAP18, HA-INI1, and YFP-IN (G). Immunoprecipitations were carried out using α-FLAG (D), α-HA (E), α-GFP (F), or α-HA (G) antibodies. Bound proteins and input controls were immunoblotted with α-HA, α-FLAG, or α-IN antibodies. In all panels, WB = Western blot analysis.

doi:10.1371/journal.ppat.1000463.g002
cells results in weak association of IN with SAP18 in the absence of INI1 (Figure S3). As a control, we demonstrate that BRG1, a known protein associated with INI1, co-immunoprecipitates equally well in the presence and absence of HIV-1 (Figure 2C, compare lanes 5 and 7).

There are at least four classes of histone deacetylase (HDAC) enzymes, grouped according to their homology to yeast histone deacetylases. Class I HDACs (HDAC1, 2, 3 and 8) are homologous to yeast Rpd3 and are localized in the nucleus, with the exception of HDAC3, which localizes to both nucleus and the cytoplasm [20]. Among these, HDAC1 and HDAC2 are part of the Sin3A-HDAC1 complex. To further test the specificity of association of various HDACs with INI1, we probed the INI1 co-immunoprecipitates with HDAC3, a class I HDAC, not present in the Sin3a-HDAC1 complex. Our results indicated that HDAC3 was not co-immunoprecipitated by FLAG-INI1 in the presence or absence of HIV-1 (Figure 2C, lanes 5 and 7). Furthermore, the specificity of α-FLAG antibodies to pull down FLAG-INI1 was indicated by the fact that no co-immunoprecipitation of components of Sin3a-HDAC1 complex was obtained by α-FLAG antibodies in cells expressing HA-INI1 (Figure 2C, lane 9). These results together indicate a selective association of INI1 with the components of SIN3a-HDAC1, and enhancement of this association in the presence of HIV-1 proteins.

To further confirm that IN, INI1 and SAP18 form a complex in vivo, we carried out co-immunoprecipitation studies using cells transiently transfected with combinations of either YFP-IN and HA-INI1, HA-INI1 and FLAG-SAP18, YFP-IN and FLAG-SAP18 or all the three proteins simultaneously (Figure 2D–G). The antibodies to FLAG, HA, or GFP (as they are cross reactive with YFP) were used for the co-immunoprecipitation experiment. We found that α-FLAG antibodies could co-immunoprecipitate FLAG-SAP18 and HA-INI1 (Figure 2D), α-HA antibodies could co-immunoprecipitate HA-INI1 and YFP-IN (Figure 2E), and α-GFP antibodies could co-immunoprecipitate YFP-IN and FLAG-SAP18 (Figure 2F), confirming the interaction of pairs of these proteins, in vivo. Finally, we found that α-HA antibodies could simultaneously co-immunoprecipitate HA-INI1, FLAG-SAP18 and YFP-IN consistent with the idea that these proteins form a ternary complex in vivo (Figure 2G). To further establish the stringency of our immunoprecipitation reactions, we subjected the α-GFP immunoprecipitates from the cells transfected with YFP-IN to silver staining. We found that only a select set of protein bands specific to α-GFP lane was observed, indicating the stringency of immunoprecipitation reactions (Figure S4).

Components of the Sin3a-HDAC1 complex are specifically incorporated into HIV-1 but not SIVmac particles in an IN-dependent manner

Since INI1 is specifically incorporated into HIV-1 virions and the other components of SWI/SNF complex are not, we examined the possibility that INI1-associated components of SIN3a-HDAC1 complex could be recruited to HIV-1 virions. HIV-1 virions were purified, treated with subtilisin and subjected to sequential immunoblot analysis using antibodies to components of SWI/SNF complex and that of Sin3a-HDAC1 complex. The results of these analyses indicated that while INI1/sSNF5, SAP18, SAP30, and HDAC1 were incorporated into virions, the components of the SWI/SNF complex were not (Figure 3A, lanes 1 and 2). Previously, we had reported that INI1 is not incorporated into other lentiviral or retroviral particles including SIV-1, HTLV-1, and MuLV [7]. Therefore, we examined purified and subtilisin treated preparations of SIV and HTLV-1 virions for the presence of components of Sin3a-HDAC1 complex. We found that while INI1/hSNF5, SAP18, SAP30, and HDAC1 were clearly present in HIV-1 virions, they were absent from SIVmac and HTLV-1 virions (Figure 3A, lanes 3–6). Furthermore, BRG1 and BAF155, the two core components of the SWI/SNF complex were absent from all virion preparations (Figure 3A, lanes 1–6). These results indicate that INI1 and its associated components of Sin3a-HDAC1 complex are specifically incorporated into HIV-1 virions.

![Image](58x24 to 76x41)

Figure 3. Specific IN-dependent incorporation of the INI1-associated components of SIN3A/HDAC1 complex into HIV-1 virions. (A) Incorporation of components of SIN3A/HDAC1 complex into primate retroviral particles such as HIV-1MN, SIVmac and HTLV-1. Immunoblot of purified subtilisin-treated virions (~125 μg each lane), sequentially probed using α-IN1, α-BRG1, α-BAF155, α-SAP18, α-SAP30, and α-HDAC1 antibodies. (B) IN protein is required for incorporation of INI1/SNF5 and associated components into HIV-1 virions. Purified and subtilisin-treated, wild type, and ΔIN (lacking IN) virion particles were sequentially immunoblotted using α-IN1, α-SAP18, α-SIN3A, α-VSVG, and α-p24 antibodies. Note that IN1, SAP18, and SIN3a are absent in virion treated with subtilisin (lane 4).

doi:10.1371/journal.ppat.1000463.g003
To determine if the IN-IN1 interaction mediates specific recruitment of the Sin3a-HDAC1 complex, we generated virions lacking IN protein. Immunoblot analysis of virions lacking IN indicated that while wild type virions contained significant levels of INI1, SAP18, and SIN3a, none of these proteins were present in the subtilisin-treated virus particles lacking IN (Figure 3B, compare lanes 4 with 7). Thus, IN1 and its associated Sin3a-HDAC1 complex are specifically recruited into HIV-1 virions in a manner dependent on IN. This is consistent with the observation that IN binds to both IN1 and SAP18 in vitro (Figure 3B).

HDAC1 activity is associated with HIV-1 virions

Recent findings have established a complex interplay between host cellular proteins including HDACs, histone acetyl transferase (HAT), SWI/SNF complex and HIV-1 Tat during HIV-1 LTR transcription [21–23]. It has been established that nucleosome organization at the LTR and proviral transcription is modulated by acetylation and deacetylation of histones [24]. While these histone modifications, occurring at the LTR promoter site, have been well established, our results indicate an association of the Sin3a-HDAC1 complex within purified HIV-1 virions. To our knowledge, specific recruitment of HDAC proteins to virions has not been documented before, either for HIV-1 or any other virus. Therefore, we decided to further investigate specifically the role of virion-associated HDAC1 in HIV-1 replication.

We first examined for the presence of deacetylase activity in HIV-1 virion preparations to ensure that the Sin3a-HDAC1 complex is catalytically active. A fluorescence-based assay was used to detect the presence of deacetylase activity in different preparations of virions including HIV-1R3B and three-plasmid based HIV-1 vectors, using histones as substrates (Figure 4A and B). The results indicated the presence of HDAC activity that was above background and directly proportional to the amount of virus assayed. This activity was sensitive to 4 μM TSA (trichostatin A), a non-specific inhibitor of deacetylase activity (Figure 4A and B). The HDAC activity present in mock varied from batch to batch (perhaps due to the presence of cellular HDACs). Therefore, the HDAC activity of each virus preparation was normalized by subtracting mock activity. Furthermore, to eliminate the contaminating cellular HDAC proteins, we treated both virion and mock preparations with subtilisin and found that virion preparations have subtilisin-resistant HDAC1 activity, while mock preparations...

**Figure 4.** HDAC activity is associated with HIV-1 virions. Graphic representation of TSA-sensitive HDAC activity associated with HIV-1 virions, determined using a fluorimetric analysis. (A,B) HDAC activity in HIV-1R3B (A) and three plasmid-based HIV-1 [3pV, (B)] viral preparations. (C,D) Effect of incorporation of a catalytically inactive dominant negative mutant, HDAC1H141A on HDAC activity associated with HIV-1 3pV (C) and HIV-1R3B (D) virions. The bar graphs represent mean of three to four independent experiments +/- SEM.
do not, indicating that HDAC1 activity is incorporated specifically within the virion cores (Figure S5). It should be noted that deacetylase activities of different virion preparations varied considerably depending on the type of virus, the HDAC1 activity kit and sample preparation. The three-plasmid-based vectors demonstrated highest activity (200–3000 units/ng p24) as compared to R3B (20–400 units/ng p24). We also attempted to determine the HDAC1 activity associated with HIV-1 virions produced in IN1 to MON cells, however, these results were inconclusive due to low virus yield and consequent high background (data not shown).

The fluorescence based deacetylase activity assay is relatively non-specific and will not distinguish between different HDACs. Based on our co-immunoprecipitation studies, IN1 appears to specifically associate with the Sin3a-HDAC1 complex. To investigate if deacetylase activity associated with HIV-1 virions is due to the recruitment of HDAC1, we employed an HDAC1 mutant containing a single amino acid substitution in the catalytic site (HDAC1H141A). This mutation renders HDAC1 protein catalytically inactive without impairing its structure, stability or association with other components of the Sin3a-HDAC1 complex [25]. Three-plasmid based HIV-1 virions were produced either in the presence of empty vector or in the presence of a vector expressing Flag-HDAC1H141A. The virions produced were then normalized for p24 and were assessed for the presence of deacetylase activity using a fluorescence-based assay, as above.

The results of these analyses indicated that while virions produced in the presence of empty vectors harboured significant HDAC activity above the background, the virions (normalized for p24) produced in the presence of HDAC1H141A demonstrated 3–10 fold statistically significant decrease ($p = 0.0013$) in HDAC activity compared to the controls (Figure 4C and Table S1). These results established that the deacetylase activity associated with HIV-1 virions is mainly contributed by HDAC1 protein and that the active HDAC1 and the associated proteins are selectively recruited into HIV-1 virions. We further assessed the activity of full-length molecular clones HIV-1K53 in the presence or absence of HDAC1H141A after normalizing the virions for p24. We found that there is a statistically significant decrease ($p = 0.004$, albeit to a lesser degree, in activity associated with HIV-1K53 produced in the presence of HDAC1H141A (Figure 4D).

Requirement of HDAC1 for early events of HIV-1 replication

To determine the functional significance of virion-associated HDAC1 for HIV-1 replication, we tested the effect of expressing a catalytically inactive mutant, HDAC1H141A, that acts as a dominant negative mutant, on HIV-1 p24 release and infectivity. Pharmacological agents such as TSA are not specific to HDAC1 and would inhibit all Class I and Class II HDACs. The effect of HDAC1H141A is specific and it impairs neither the structure nor the stability of the protein, nor the association of the mutant protein with other components of the Sin3a-HDAC1 complex [25]. Therefore, by expressing this mutant in the producer cells, we could incorporate it into the virions and selectively test the requirement of virion-associated HDAC1. We produced HIV-1 virions in the presence of HDAC1H141A or the wild type HDAC1 as control, by co-expressing these proteins in the producer cells along with the viral vectors. Immunoblot analysis of normalized amounts of purified virions produced from these cells revealed specific incorporation of equivalent amounts of FLAG-tagged HDAC1 or HDAC1H141A proteins in the virions (Figure 5A, lanes 6 and 7). Furthermore, re-probing the same blot with antibodies specific to IN and p24 indicated identical amounts of these proteins, suggesting that incorporation of HDAC1 mutant does not impair the level of these viral proteins (Figure 5A).

To assess the effect of HDAC1H141A expression on p24 release, virions were collected from cells expressing empty vector, HDAC1 or HDAC1H141A and subjected to p24 analysis. We observed a significant increase in viral p24 release when FLAG-HDAC1H141A was expressed in the producer cells ($p = 0.0027$, Figure 6A; Table 1). There was a slight increase in p24 release when wild type HDAC1 was expressed as compared to empty vector, but the difference was much less ($p = 0.0948$). We next assessed the infectivity of virions produced in the presence of either FLAG-HDAC1 or FLAG-HDAC1H141A for their ability to infect target cells, after normalizing for p24 levels. We found that virions produced in the presence of FLAG-HDAC1H141A were significantly reduced in their infectivity (over 3–10 fold reduction; $p = 0.0005$ and 0.0062 for 10 ng and 5 ng p24 of virus, respectively) compared to virions produced in the presence of empty vector, given the identical amount of p24 (Figure 5C and Table 2). There was a slight decrease in the infectivity of virions produced in the presence of wild type HDAC1, but the difference was much less compared to virions produced in the presence of FLAG-HDAC1H141A (Figure 5C). Since these virions were produced in cells containing endogenous wild type HDAC1, it is likely that we were unable to completely eliminate encapsidation of this protein into virions. This is reflected by the presence of significant residual HDAC1 activity within virions harbouring mutant FLAG-HDAC1H141A (Figure 4D). Overall, these results indicated that virion-associated HDAC1 activity is required for efficient infectivity of HIV-1.

HDAC1 activity does not modulate SIV replication

IN1 interacts with HIV-1 but not SIV IN [7]. Furthermore, IN1 and the components of Sin3a-HDAC1 complex are incorporated into HIV-1 but not SIVmac virions (Figure 3A). To further confirm the functional specificity of the Sin3a-HDAC1 complex to HIV-1, we tested the effect of FLAG-HDAC1H141A on the production and infectivity of a VSVG pseudotyped SIV-based vector, carrying a GFP reporter gene [26]. SIV virions were produced in the presence and absence of FLAG-HDAC1H141A in 293T cells. We observed that expression of FLAG-HDAC1H141A did not significantly increase SIV particle production as compared to empty vector control ($p = 0.5287$, Figure 6A). Contrary to the effect on HIV-1, presence of FLAG-HDAC1H141A did not significantly affect the infectivity of SIV virions ($p = 0.1748$, Figure 6B). To further confirm that HDAC1 is not incorporated into SIV, we carried out a fluorescent-based deacetylase activity assay as described above using SIV and HIV-1, produced in the presence or absence of FLAG-HDAC1H141A. We found that SIV virions produced in the presence or absence of FLAG-HDAC1H141A exhibited deacetylase activity similar to that of the mock control ($p = 0.5916$, Figure 6C). Unlike SIV, HIV-1 virions exhibited higher deacetylase activity compared to mock control (Figure 6C). Furthermore, presence of FLAG-HDAC1H141A decreased the HDAC1 activity in HIV-1 virions ($p = 0.0265$, Figure 6C). These results establish that HDAC1 activity is specifically associated with HIV-1 but not SIV, consistent with the selective incorporation of the Sin3a-HDAC1 complex into HIV-1.

HIV-1 virion-associated HDAC1 activity is required for a post-entry step during or before early reverse transcription

The results of the above experiments strongly suggest that virion-associated HDAC1 is required for early events of HIV-1 replication, which include virus entry, uncoating, reverse tran-
scription, nuclear translocation and integration. To rule out the possibility that incorporation of HDAC1 mutant may affect general processing or incorporation of viral proteins, the purified viral preparations were subjected to immunoblot analysis using anti-HIV-1 serum. The results indicated that the general processing of viral proteins was not affected in the presence of FLAG-HDAC1H141A (Figure 7A). To determine if the incorporation of FLAG-HDAC1H141A blocks virus entry, we carried out a FRET-based entry assay, using Vpr-fused β-lactamase (BLAM) [27]. In this assay, entry of HIV-1 leads to cleavage of a substrate by virion-associated β-lactamase, which can be quantitated by observing changes in FRET. Cells successfully infected with HIV-1 emit a blue FRET whereas cells without viral entry remain green. We produced HIV-1 virions in the presence and absence of FLAG-HDAC1H141A, simultaneously incorporating Vpr-BLAM. Normalized amounts of virions containing Vpr-BLAM were then used to carry out the entry assay. We quantitated blue cells and determined the ratio of blue cells (successful HIV-1 entry) to blue cells expressing HDAC1H141A.

Figure 5. Virus-associated HDAC1 activity is required for HIV-1 replication in target cells. (A) Immunoblot analysis of virions produced in the presence of either an empty vector or vectors expressing FLAG-HDAC1 or FLAG-HDAC1H141A, using indicated antibodies. (B) Effect of expression of FLAG-HDAC1 or FLAG-HDAC1H141A vectors on the production of HIV-1 virus particles. The bars represent average p24 values obtained from three independent experiments (+/- SEM). (C) Effect of incorporation of HDAC1H141A on the infectivity of HIV-1 virions. Graphic representation of the % GFP positive (infected) cells obtained when 293T cells were exposed to three plasmid based HIV-1 harbouring either FLAG-HDAC1 or FLAG-HDAC1H141A (average of 3 independent experiments +/- SEM).

doi:10.1371/journal.ppat.1000463.g005
plus green cells (total number of cells). We found that virions containing FLAG-HDAC1H141A exhibited the same level of entry compared to that of the empty vector control, indicating that FLAG-HDAC1H141A does not affect HIV-1 entry (Figure 7B).

We next examined if replication of virions harbouring FLAG-HDAC1H141A is blocked at a post entry step such as uncoating, early or late reverse transcription. A quantitative-real-time PCR (Q-PCR) analysis was carried out using DNA isolated from cells infected with equal amounts of virions normalized by p24 ELISA.

**SEM = standard mean of error.**

doi:10.1371/journal.ppat.1000463.t001

Table 1. Effect of co-transfection of HDAC1 and HDAC1H141A on p24 release.

<table>
<thead>
<tr>
<th>Viral DNA (10 ng)</th>
<th>Plasmid co-transfected</th>
<th>Mean p24 ng/ml*</th>
<th>+/− SEM**</th>
</tr>
</thead>
<tbody>
<tr>
<td>3pV</td>
<td>pCDNA</td>
<td>29509</td>
<td>9299</td>
</tr>
<tr>
<td>3pV</td>
<td>pHDAC1</td>
<td>134460</td>
<td>8802</td>
</tr>
<tr>
<td>3pV</td>
<td>pHDAC1H141A</td>
<td>279801</td>
<td>63706</td>
</tr>
</tbody>
</table>

*Indicates mean of six different experiments done in triplicates. The average of each experiment was computed to obtain mean value.

**SEM = standard mean of error.**

doi:10.1371/journal.ppat.1000463.t002

Discussion

Our results provide a novel paradigm for the role of INI1 and components of the Sin3a-HDAC1 complex in HIV-1 replication. Interaction of the SWI/SNF complex with components of the HDAC1 complex has been previously reported [14,28]. Our results demonstrate that INI1/hSNF5 and IN directly interacts with SAP18, a component of Sin3a-HDAC1 complex. Furthermore, for the first time, we find that components of the Sin3a-HDAC1 complex are selectively recruited into HIV-1 virions. It is also interesting to note that core components of the SWI/SNF complex such as BRG1, BRM, BAF155 and BAF170 are absent from HIV-1. To our knowledge, this is the first report where association of INI1 with components of an HDAC1 complex has been demonstrated in the absence of the SWI/SNF complex. We believe that further analysis of this association will lead to uncovering novel biological functions of INI1 and the Sin3a-HDAC1 complex.

The recruitment of the Sin3a-HDAC1 complex appears to be specific to HIV-1 and dependent on IN. However, more experiments are needed to determine if IN alone can recruit the complex into virions and if INI1 is required for this function. Furthermore, we have demonstrated that HIV-1 virions harbour deacetylase activity, which is reduced when a catalytically inactive mutant, HDAC1H141A, is incorporated into virions. To our knowledge, this is the first report where association of INI1 with components of an HDAC1 complex has been demonstrated in the absence of the SWI/SNF complex. We believe that further analysis of this association will lead to uncovering novel biological functions of INI1 and the Sin3a-HDAC1 complex.

Table 2. Effect of co-transfection of HDAC1 and HDAC1H141A in the producer cells on viral infectivity.

<table>
<thead>
<tr>
<th>Viral preparation</th>
<th>Amount of virus used (p24)</th>
<th>% cells infected (mean)*</th>
<th>+/− SEM**</th>
</tr>
</thead>
<tbody>
<tr>
<td>3pV+pCDNA</td>
<td>10 ng</td>
<td>13.07</td>
<td>1.413</td>
</tr>
<tr>
<td>3pV+pHDAC1</td>
<td>10 ng</td>
<td>10.51</td>
<td>1.116</td>
</tr>
<tr>
<td>3pV+pHDAC1H141A</td>
<td>10 ng</td>
<td>4.871</td>
<td>0.6632</td>
</tr>
<tr>
<td>3pV+pCDNA</td>
<td>5 ng</td>
<td>7.216</td>
<td>1.116</td>
</tr>
<tr>
<td>3pV+pHDAC1</td>
<td>5 ng</td>
<td>5.569</td>
<td>0.8977</td>
</tr>
<tr>
<td>3pV+pHDAC1H141A</td>
<td>5 ng</td>
<td>2.414</td>
<td>0.3753</td>
</tr>
</tbody>
</table>

*Indicates mean of six different experiments done in triplicates. The average from each experiment was computed to obtain mean value.

**SEM = standard mean of error.**

doi:10.1371/journal.ppat.1000463.t001
producer cells also resulted in decrease of virion-associated HDAC1 activity and reduction in infectivity of those particles, consistent with the results of the HDAC1H141A mutant.

We surmise that the increase in particle production observed when HDAC1H141A is expressed is due to the derepression of viral and/or cellular transcription. We found that intracellular p24 was indeed increased when HDAC1H141A mutant was expressed (data not shown), consistent with this idea. The decrease in infectivity of HIV-1 virions harbouring HDAC1H141A is directly correlated to a decrease in virion-associated deacetylase activity. While the HIV-1-associated deacetylase activity is not required for entry into target cells, it appears to be required for early reverse transcription. These results suggest that virion-associated Sin3a-HDAC1 complex is required for either efficient reverse transcription or uncoating after entry. It is interesting to note that virions produced in INI1−/− cells are defective for reverse transcription,

Figure 6. Effect of HDAC1H141A on SIV replication. (A) Effect of expression of FLAG-HDAC1H141A vectors on the production of SIV-1 particles. The bars represent average p27 values obtained from three independent experiments (+/−SEM). (B) Effect of incorporation of HDAC1H141A on the infectivity of SIV virions. Graphic representation of the % GFP positive (infected) cells obtained upon infection of 293T cells with SIV vector produced in the presence or absence of pFLAG-HDAC1H141A (average of 3 independent experiments +/−SEM). (C) Comparative analysis of HDAC1 activity associated with HIV-1 and SIV virions in the presence and absence of HDAC1H141A. Equal amounts of SIV (in p27 ng) and HIV-1 (in p24 ng) were subjected to HDAC activity assay along with the mock-transfected culture supernatants. Note: the HDAC activity detected in SIV is similar to that of mock control, and HIV-1 harbours higher levels of HDAC activity compared to that of SIV and mock.

doi:10.1371/journal.ppat.1000463.g006
and that INI1 is associated with the HIV-1 reverse transcription complex [29,30]. Furthermore, many laboratories including ours, have demonstrated that IN directly interacts with RT and that mutants of IN affect RT function [31–34]. These observations raise the possibility that one mechanism by which IN influences RT function is via the recruitment of the Sin3a-HDAC1 complex through its direct interaction with SAP18 and INI1. Future experiments to address the relationship between binding of IN to INI1 and SAP18, and its influence on RT function, are likely to shed new light on the dynamic and functional interaction between HIV-1 RT, IN and IN-associated complexes.

Our results also parallel an earlier report where, it was demonstrated that cellular Sin3a protein was required for retrotransposition of Schistosaccharomyces pombe transposon, Tf1. In the Tf1 system, Sin3a appears to be required for a post reverse transcription event and mutations in Sin3a block a step before nuclear entry [35]. This genetic study indicated that the Sin3A-HDAC1 complex is required for a step other than viral transcription in retroviral/retrotransposon replication. However, in the case of HIV-1, we have found that the Sin3A-HDAC1 complex is required for early reverse transcription. Future experiments are required to uncover the exact mechanism by which virion-associated HDAC1 modulates post entry events of HIV-1.

INI1 appears to play a complex role in HIV-1 replication due to its multitude of effects. Studies using INI1 dominant negative
that while knocking-down INI1 in the producer cells has no effect, knocking-down INI1 in the target cells increases infection of the virus, indicating that it is an anti-viral protein [12]. In this report, virions produced in the INI1 knock-down cells were not analyzed for the presence of INI1 and hence the data is not interpretable. Furthermore, it is possible that INI1 was not completely knocked-down in the producer cells and that INI1 in the target cells plays different roles to INI1 in the producer cells.

Studies on siRNA-mediated knock-down of INI1 is complicated due to the fact that INI1 is a tumour suppressor and it is required for the survival of many cell types. Complete knock down of INI1 results in flat cell formation and apoptosis in HeLa cells [36]. Furthermore, other studies demonstrated that: (i) homozygous deletions of INI1 causes embryonic lethality at the peri-implantation stage; (ii) embryonic cells lacking INI1 undergo apoptosis; and (iii) conditional knock-out of INI1 results in massive apoptosis [37–41]. These studies highlight the fact that knocking-down INI1 may cause cell lethality. Inefficient transient knock-down of INI1 can be achieved in cells, but may not be suitable for assessment of the effect of its loss, as it has been documented for LEDGF that even a small amount of the protein is sufficient to provide the desired function during HIV-1 replication [42]. In addition to playing a role in late events during p24 release, INI1 binds Tat and is required for Tat-mediated LTR transcription [8–11]. Because of these reasons, it is important to first to segregate multiple effects of INI1 on cellular and viral functions to fully comprehend its effect on HIV-1 replication.

Based on our current study, we hypothesize that while the virion-associated INI1-Sin3a-HDAC1 complex may affect early reverse transcription, cellular INI1 associated with the SWI/SNF complex may have antiviral effects in target cells. It is possible that deacetylation of a substrate (an acetylated viral or cellular protein) by virion-associated HDAC1, is required to mediate efficient early reverse transcription of HIV-1. The acetylated viral or cellular protein may block early post-entry events and deacetylation may be necessary to overcome this block. Alternatively, a viral or cellular protein required for an early event may need deacetylation to become active. While histones are classic substrates that are modified by acetylation and deacetylation, an increasing list of non-histone proteins that are similarly modified have been identified [43]. Possible candidate viral proteins include IN, although requirement of acetylation for IN activity is controversial [44,45]. Alternatively, other viral proteins or unidentified cellular proteins could be substrates for deacetylation by the virion-associated Sin3a-HDAC1 complex.

While our studies investigate the virion encapsidation and functional significance of Sin3A-HDAC1 complex in HIV-1 replication, a recent report illustrated the association of NURD, another HDAC1 complex, with the HCMV viral protein UL38. In this case, association of UL38 with the Sin3a-HDAC1 complex also serves to inhibit host stress response [46]. Whether association of IN, INI1 with the SAP18-HDAC1 complex also serves to inhibit host stress response remains to be determined. We have recently found that INI1 is involved in inducing an interferon response in rhabdoid tumour cells [47]. By binding to INI1 and SAP18, HIV-1 IN may inhibit anti-viral interferon response during infection. Future experiments to determine such interference of the host innate immune response (due to the binding of viral and cellular proteins such as IN, IN1, and the SAP18-HDAC1 complex), are likely to shed novel insight into complex host-virus interactions. Furthermore, we believe that strategies involving disruption of INI1-SAP18-HDAC1 interactions or selective targeting of virion-associated HDAC complexes may lead to novel methods to inhibit HIV-1 replication.

Figure 8. siRNA knockdown of HDAC1 in virus producer cells results in decreased infectivity of HIV-1 in target cells. Analysis of the effect of HDAC1 knockdown on viral particle production and subsequent infectivity of HIV-1: (A) Immunoblot analysis of 293T cells exposed to either siHDAC1 or siControl. (B) Effect of HDAC1 knockdown on viral particle production. Viral supernatants from cells co-transfected with either siHDAC1 or siControl along with HIV-1aq were subjected to p24 analysis. Bars represent average p24 ng/ml values obtained from three independent experiments. (C) Analysis of infectivity of normalized amounts of HIV-1aq virus produced in cells transfected with either siControl or siHDAC1 using GHOST reporter cells. Bars represent the average fold change in infectious units/ng p24 from three independent experiments. doi:10.1371/journal.ppat.1000463.g008

mutants as well as p24 release in INI1(−/−) cells indicated that INI1 is required for HIV-1 replication [6,29]. Furthermore, particle produced in INI1−/− MON cells were defective for infection and were blocked at the stage of reverse transcription [29]. These latter results are consistent with the current report in that virion-associated HDAC1 activity is required for efficient early events of HIV-1. On the contrary, Maroun et al reported...
Virion-Associated HDAC1 in HIV-1 Replication

Materials and Methods

Plasmids

pGEX-SAP18 expressing GST-SAP18 was generated by cloning into pGEX3X-PL, a BamHI-EcoRI fragment generated by PCR amplification of a full length SAP18 EST clone (IMAGE 364760, GenBank accession number AA025356) using primers 5'-CTGACGGAAAAATGAAATTCAC-3' and 5'-GGCCGTAAGAGATCCTGGCCGGTTC-3'. A plasmid encoding IN1-FLAG fusion protein, pBABEpuro-IN1-FLAG was generated as described [48]. The pBABEpuro-FLAG control plasmid was generated from pBABEpuro-IN1-FLAG by deleting IN1 fragment. The gag-pol expression vector containing IN deletion, pCmvVar8.2AIN was generated as follows. First, an intermediate vector (pSP72-Cla-Sal) carrying a ClaI-SalI fragment of HIV-1 R3B virus was generated and subjected to directed mutagenesis using the primers 5'...GCTTCTCAATTACTGC-TagTCCTCAGTGGTCTTG...3' and 5'...CTTGAAGAGCTGAAGC-TAGACAGTAATTGGAGAGC...3' to generate stop codon at the beginning of IN (pSP72-deltaIN). The Satt-Bspl fragment containing IN mutation was isolated from pSP72-deltaIN and cloned into the pCmvVarR8.2 vector to generate pCmvVar8.2AIN. The pCDNA3.1-HDAC1 and pCDNA3.1-HDAC1H141A plasmids were gifts from Dr. Schreiber (Harvard Medical School). Three-plasmid HIV-1-based vectors were obtained from Dr. Trono and the SIV-based lentiviral system (pCAG-SIVgprre, pCAG4-RTR-plasmid) HIV-1-based vectors were obtained from Dr. Trono and Nienhuis (St. Jude Children’s Research Hospital). These vectors were pseudotyped by VSV G (expressed from pMDG-VSV G).

Virus stocks (5 ng p24) produced in 293T cells were treated with 50 μl buffer G (dpBS containing 0.1% IGEPAL, 1 mM DTT, 2 mg/ml BSA, 1 mM PMSF and 1 μg/ml each of pepstatin, aprotinin and leupeptin). Lysates were pre-cleared by incubating with 30 μl of protein A sepharose and washed 6 times with buffer G without BSA, separated by SDS-PAGE and subjected to immunoblot analysis. Co-immunoprecipitations of FLAG fusion proteins were carried out using FLAG Immunoprecipitation Kit (Sigma cat # A2220) and Western blot analysis was carried out using antibody for HA-INI1, HA-HRP (Sigma H6533), monoclonal α-GFP (Sigma G6539) and for Western blot analysis

Protein–protein interactions

SAP18 as an interacting partner for IN1 was isolated by using LEXADB-IN1 as a bait and screening HL60 cDNA library in yeast two hybrid system as described [18]. For GST pull down assay to analyze direct interaction between GST-SAP18 and His-IN1 and His-IN, glutathione sepharose 4B beads bound to 5 μg of either GST or GST-SAP18 were prepared as described [19] and incubated with 2 μg of either Ni-NTA purified His-IN or Ni-NTA and hydroxylapatite purified His-IN1 in buffer containing 20 mM HEPES-KOH (pH 6.8), 200 mM NaCl, 0.1 mM EDTA, 2-5 mM DTT, 0.1% IGEPAL, 100 μg/ml ethidium bromide and protease inhibitors and treated with 10 U of DNaseI. Following incubation, beads were washed 3–5x with buffer containing 20 mM HEPES-KOH (pH 6.8), 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 100 μg/ml ethidium bromide, 0.5% IGEPAL and 1 mM PMSF. Bound proteins were resolved by SDS-PAGE and analyzed by Western blot using indicated antibodies.

Co-immunoprecipitation analyses

(i) Co-immunoprecipitation of IN1 with components of HDAC1 complex. HeLa and MON cells were collected 40 hours after transfection and sonicated in 500 μl buffer G (dpBS containing 0.1% IGEPAL, 1 mM DTT, 2 mg/ml BSA, 1 mM PMSF and 1 μg/ml each of pepstatin, aprotinin and leupeptin). Lysates were pre-cleared by incubating with 30 μl of protein A sepharose and incubated overnight at 4°C. Bound proteins were washed 4 times with buffer G without BSA, separated by SDS-PAGE and subjected to immunoblot analysis. Co-immunoprecipitations of FLAG fusion proteins were carried out using FLAG Immunoprecipitation Kit (Sigma cat # A2220) and Western blot analysis was carried out using affinity purified rabbit polyclonal α-SAP18 antibody as per manufacturer’s recommendations.

(ii) Co-immunoprecipitation of endogenous proteins. Confluent HeLa cells were lysed in co-IP buffer containing 20 mM HEPES-KOH (pH 7.9), 150 mM NaCl, 5 mM MgCl2, 5 mM CaCl2, 0.1 mM EDTA, 1 mM DTT, 1% Triton-X, treated with 0.033 U/μl micrococcal nuclease and immunoprecipitated overnight with 1.6 μg of α-SAP18 antibody (ABC-Am ab31748-25) or rabbit IgG. Complexes were pulled down with Protein A agarose and washed 3 times with wash buffer containing 20 mM HEPES-KOH (pH 7.9), 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1% Triton-X. Complexes were resolved by SDS-PAGE and Western blot analysis was carried out using affinity purified rabbit polyclonal α-SAP18 antibody.

(iii) Co-immunoprecipitation of IN-IN1-SAP18 in vivo. For analysis of complex formation between HA-IN1, FLAG-SAP18 and YFP-IN, 293T cells were transfected with the plasmids expressing these proteins. 48 hrs post-transfection, cells were lysed in co-IP buffer as above and treated with 0.033 U/μl micrococcal nuclease and immunoprecipitated overnight with 2 μg of indicated antibodies or control IgG. Complexes were pulled down with Protein A agarose and washed 3–5x with wash buffer as above. Complexes were resolved by SDS-PAGE and Western blot analysis carried out using indicated antibodies. The antibodies used for immunoprecipitation were rabbit polyclonal α-HA (Santa Cruz sc805), monoclonal α-FLAG (Sigma F3165), monoclonal α-GFP (Sigma G6539) and for Western blot analysis were α-HA-HRP (Sigma H5633), α-FLAG-HRP (Sigma A6592) and monoclonal α-IN antibodies.

Quantitative real-time PCR (qPCR)

Virus stocks (5 ng p24) produced in 293T cells were treated with 50 U ml-1 with DNase (Roche) for 60 min at 37°C, and used to infect 2.0×10⁵ 293T cells in 6-well plates. After 2 hours of infection, the cells were washed with PBS, and incubated with fresh DMEM. Genomic DNA was isolated at various time points.
using DNeasy kit (Qiagen). Early HIV-1 reverse transcripts were quantified using primers eRT2, eRT2 and the ERT2 probe; and late reverse transcripts were quantified using primers MH531 and MH532 respectively, as described [49] using Taqman method. Reactions were analyzed in triplicates using the ABI Prism 7700 (Applied Biosystems).

Quantitation of HDAC activity in HIV-1/SIV virions

Virion-associated HDAC activity was measured using HDAC Fluorometric Activity Assay kit (Upstate #17-356) as per manufacturer’s recommendations. The HDAC Activity assays were measured using a Victor 2 multi-well plate reader (Perkin Elmer) with an excitation and emission wavelength set to 355 and 460, respectively. The lamp energy was set to 3948 with a measurement time of 0.1 sec and an emission aperture set to normal. For determining the HDAC activity of HIV or SIV based vectors in the presence or absence of HDAC1 or HDAC1H141A mutant, equal amount of virion preparations (by p24 ELISA) were subjected to HDAC1 activity assay. Mock-transfected culture supernatants were clarified in a manner similar to viral preparations and were used to determine the background level activities. HDAC activities detected in the mock samples were subtracted from the control and test samples, except in Figure 6C.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com. All data points (including outliers) were included in the analysis for significance and paired comparisons were carried out using t-statistic (two tailed) where equal variance in data between categories was assumed.

Gene symbols and nomenclature used in the manuscript have been provided in the Table S2.

Supporting Information

Figure S1 Immunoblot analysis of virions. Purified, concentrated, and subtilisin-treated HIV-1 mm virions were immunoblotted with various antibodies. The same blot was sequentially probed with α-IN1 (A), α-BRG1 (B), α-BAF170 (C), α-BAF155 (D), α-gp41 (E), and α-gp24 (F) antibodies, as indicated. Total protein lysates from MON [IN1−/− rhabdoid cells] and 293T [IN1+/+] were used as controls.

Found at: doi:10.1371/journal.ppat.1000463.s001 (0.24 MB PDF)

Figure S2 IN enhances the formation of IN1 with HDAC1, 293T cells were transfected with FLAG-IN1 and either pCDNA or YFP-IN plasmids and subjected to immunoprecipitation with either IgG or anti-FLAG monoclonal antibody agarose, as indicated. Immunoprecipitated complexes were analyzed by anti-FLAG monoclonal, anti-GFP polyclonal, and anti-HDAC1 polyclonal antibodies. Input lysates showing equal loading for both YFP-IN and FLAG-SAP18 plasmids and subjected to immunoprecipitation using either mouse IgG or anti-FLAG monoclonal antibody agarose. Immunoprecipitated complexes were analyzed using western blots with both anti-GFP polyclonal and anti-FLAG monoclonal antibodies. Input lysates showing equal loading for both YFP-IN and FLAG-SAP18 are shown.

Found at: doi:10.1371/journal.ppat.1000463.s002 (0.14 MB PDF)

Figure S3 Interaction of IN with Sap18 in [IN1−/−] MON cells. MON cells were transfected with YFP-IN and FLAG-SAP18 plasmids and subjected to immunoprecipitation using either mouse IgG or anti-FLAG monoclonal antibody agarose. Immunoprecipitated complexes were analyzed using western blots with both anti-GFP polyclonal and anti-FLAG monoclonal antibodies. Input lysates showing equal loading for both YFP-IN and FLAG-SAP18 are shown.

Found at: doi:10.1371/journal.ppat.1000463.s003 (0.15 MB PDF)

Figure S4 Silver stain analysis of immunoprecipitations to determine the specificity of interaction. Silver stain analysis of immunoprecipitations to determine the specificity of interaction. 293T cells were transfected with YFP-IN plasmid and subjected to immunoprecipitation using either mouse IgG or anti-GFP monoclonal antibody. Immunoprecipitated complexes were analyzed by SDS-PAGE followed by silver staining. Position of YFP-IN, IgG heavy (Hc) and light (Lc) chains are indicated with an arrow. Polypeptides that are specifically immunoprecipitated with anti-GFP antibodies are indicated with asterisk.

Found at: doi:10.1371/journal.ppat.1000463.s004 (0.55 MB PDF)

Figure S5 Presence of subtilisin-resistant HDAC1 activity in the HIV-1 virus. Graphic representation of HDAC activity associated with HIV-1 virions, determined using a fluorimetric analysis. 3pV = three plasmid based vectors, and mock = culture supernatant of mock transfected cells. HIV-1 (3pV) and mock were treated with subtilisin and subjected to HDAC activity assay. Note the presence of subtilisin-resistant activity in HIV-1 (3pV), indicating that this activity is present within the virions.

Found at: doi:10.1371/journal.ppat.1000463.s005 (0.12 MB PDF)

Figure S6 Reduction in HDAC activity within the virions correlates with a reduction in infectivity of virus. Three different representative experiments are indicated to illustrate the correlation of virion-associated HDAC1 activity to infectivity. Viral supernatants collected from producer cells transfected with siHDAC1 or siControl from Experiments I-III were normalized for p24 and subjected to HDAC activity (A) and infectivity (B) assays. Bars represent the fold change in HDAC activity/ng p24 (A) and Infectivity, i.u./ng p24 when compared with virus produced in the presence of siControl (B).

Found at: doi:10.1371/journal.ppat.1000463.s006 (0.27 MB PDF)

Table S1 HDAC activity of virus produced in the presence of HDAC1H141A (activity/ng p24). 293T cells were co-transfected with three plasmid-based vectors along with either empty vector or vector expressing HDAC1H141A, and virions produced from these cells were used in HDAC activity assays. The results represent HDAC activity units minus background/ng p24.

Found at: doi:10.1371/journal.ppat.1000463.s007 (0.05 MB DOC)

Table S2 Gene symbols.

Found at: doi:10.1371/journal.ppat.1000463.s008 (0.03 MB DOC)

Acknowledgements

We thank Drs. V. Prasad and M. Kielian at AECOM for critically reading the manuscript and S. Goff at Columbia University College of Physicians and Surgeons, F. Kashanchi at The George Washington University for useful discussions. We thank Drs. W. Wang at the National Institutes of Health, S. Schreiber and A. Engelman at Dana-Farber Cancer Institute, D. Helland at the University of Bergen, P. Bieniasz at Aaron Diamond AIDS Research Center (ADARC), and A. Nienhuis at St. Jude Children's Research Hospital for reagents.

Author Contributions

Conceived and designed the experiments: MS GVK. Performed the experiments: MS JC SRD SM XW KPD XS GSWC. Analyzed the data: MS JC SM KPD GVK. Contributed reagents/materials/analysis tools: DEO GVK. Wrote the paper: MS JC GVK.
References


