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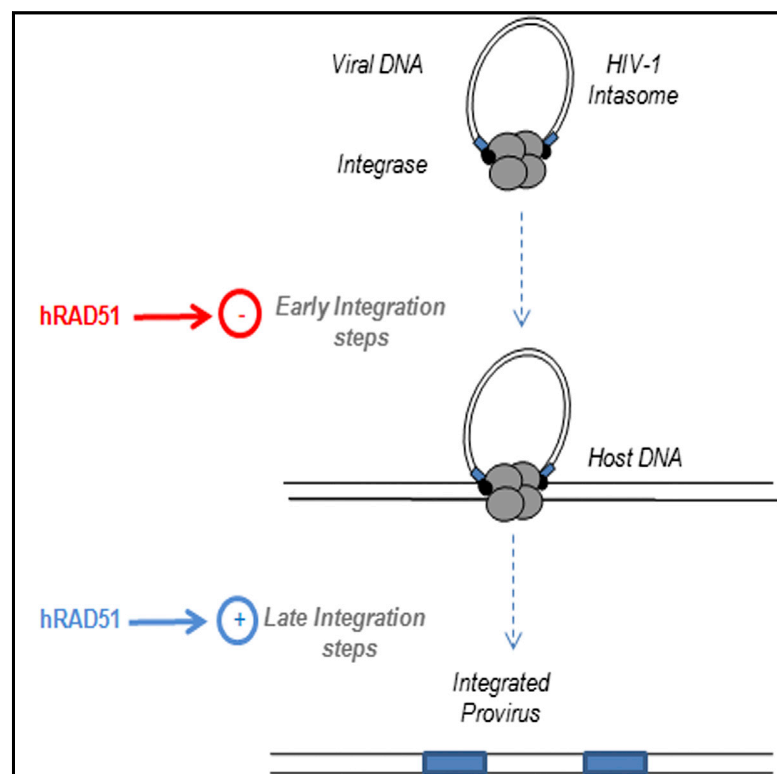
Dual and Opposite Effects of hRAD51 Chemical Modulation on HIV-1 Integration

Graphical Abstract



Highlights

- Recombinase activity of hRAD51 correlates with its ability to inhibit HIV-1 IN
- Chemical modulations of hRAD51 can have opposite effects on HIV-1 integration
- Optimal intracellular activity of hRAD51 is required for efficient HIV-1 replication
- Efficient HIV-1 integration depends on the cellular hRAD51 level

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In Brief

HIV-1 replication depends on the integration of the viral genome into the infected cell DNA. This step can be modulated by the hRAD51 DNA repair protein. Pharmacological strategies, employed by Thierry et al., establish a direct correlation between the stimulation of hRAD51 and the inhibition of HIV-1 integration, highlighting the multiple and opposite regulatory functions of the recombinase on this important replication step.

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Dual and Opposite Effects of hRAD51 Chemical Modulation on HIV-1 Integration

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SUMMARY

The cellular DNA repair hRAD51 protein has been shown to restrict HIV-1 integration both in vitro and in vivo. To investigate its regulatory functions, we performed a pharmacological analysis of the retroviral integration modulation by hRAD51. We found that, in vitro, chemical activation of hRAD51 stimulates its integration inhibitory properties, whereas inhibition of hRAD51 decreases the integration restriction, indicating that the modulation of HIV-1 integration depends on the hRAD51 recombinase activity. Cellular analyses demonstrated that cells exhibiting high hRAD51 levels prior to de novo infection are more resistant to integration. On the other hand, when hRAD51 was activated during integration, cells were more permissive. Altogether, these data establish the functional link between hRAD51 activity and HIV-1 integration. Our results highlight the multiple and opposite effects of the recombinase during integration and provide new insights into the cellular regulation of HIV-1 replication.

INTRODUCTION

Retroviral replication requires the integration of the viral cDNA into the host cell genome, a multistep process catalyzed by the intasome complex formed between the retroviral integrase (IN) and the viral DNA (Bowerman et al., 1989; Miller et al., 1997). After intasome binding to the host chromatin and insertion of the viral cDNA ends into the target DNA the post-integration repair (PIR) of the integration locus occurs. This step, probably catalyzed by host factors, leads to the stable insertion of the viral genome, the provirus (for reviews, see Hare et al., 2010; Grandgenett and Korolev, 2010; Cherepanov, 2010). We have previously shown that hRAD51, belonging to the homologous recombination DNA repair pathway (HR), could bind HIV-1 IN (Desfarges et al., 2006) and exert a negative effect on integration both in vitro and in vivo (Cosnefroy et al., 2012). Indeed, the stimulation of hRAD51 activity by the RAD stimulatory compound 1 (RS-1 [Jayathilaka et al., 2008]), inhibits HIV-1 integration, leading to a significant decrease of the viral replication (Cosnefroy et al., 2012). Moreover, hRAD51 has also been shown to activate the HIV-1 long terminal repeat (LTR) dependent transcription (Chipitsyna et al., 2006; Kaminski et al., 2014; Rom et al., 2010) and, thus, to stimulate viral genes expression. These data indicate that hRAD51 plays different roles during the retroviral replication cycle, which must be taken into account by clinical approaches. To better understand hRAD51 regulatory functions we performed a pharmacological analysis of the impact of hRAD51 chemical modulation on HIV-1 integration. Our results showed that hRAD51 stimulatory and inhibitory compounds induced multiple and opposite effects on this specific step of the retroviral replication cycle. These data indicate that efficient integration relies on equilibrium between pro- and anti-integration properties of hRAD51. This suggests that cellular pathways and/or treatments affecting this equilibrium could differently affect viral replication and reveals the complex regulatory functions of hRAD51 on integration.

RESULTS

Selection of hRAD51 Chemical Modulators Affecting Its HIV-1 IN Inhibition Ability

hRAD51 inhibits HIV-1 integration in vitro (Figure S1) and enhancement of the hRAD51/DNA active nucleofilaments formation by the RAD51 stimulatory compound 1, RS-1 (chemical structure shown in Figure 1A) improves the integration inhibition

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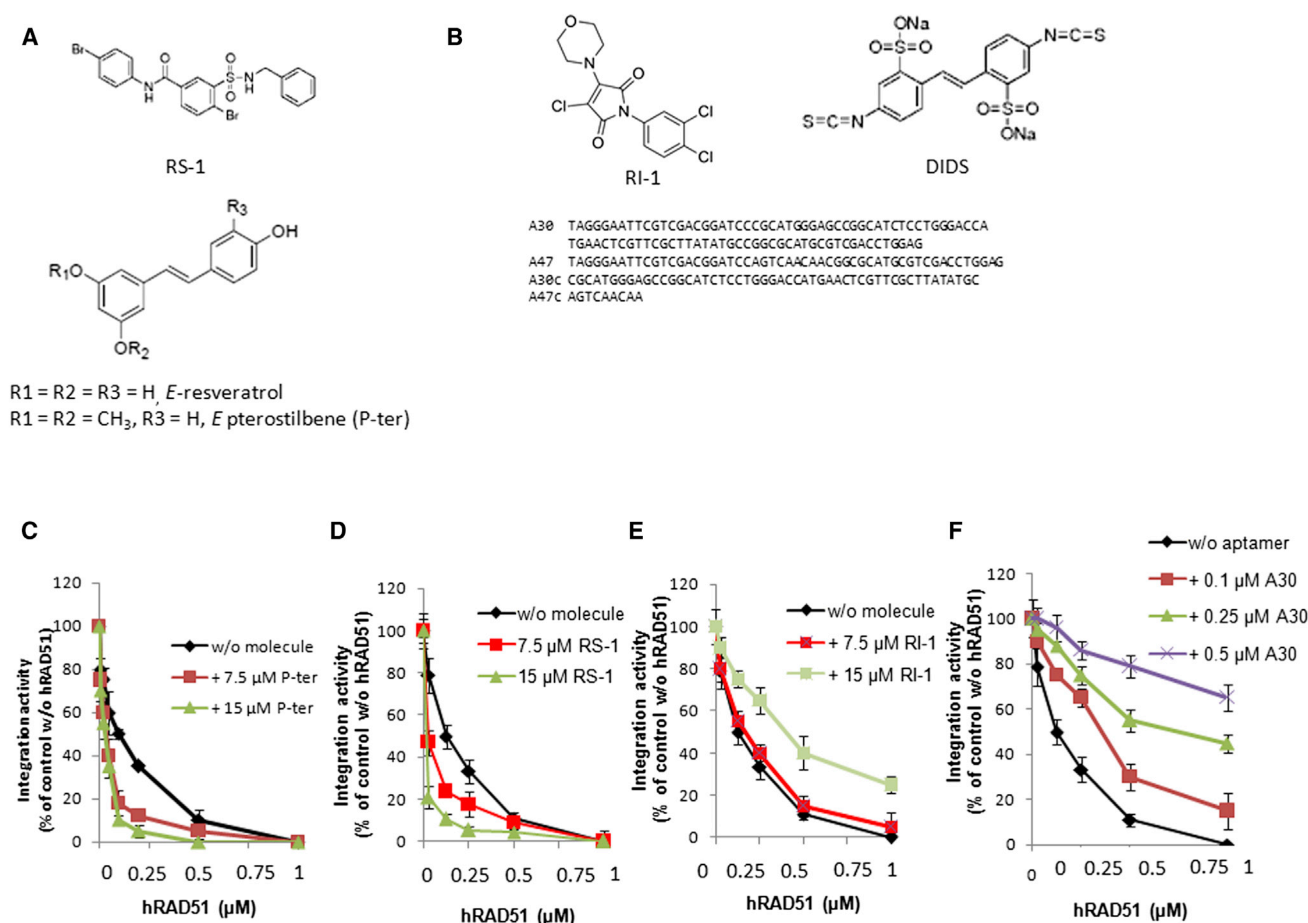

Figure 1. Effect of hRAD51 Modulators on HIV-1 Integration

The chemical structure of the stimulatory compounds RS-1 and P-ter (A) and the inhibitory compounds RI-1 and DIDS, as well as the sequence of the aptamers (B) are indicated. Increasing concentrations of wt-hRAD51 were added in a standard concerted integration assay in the presence of 100 μM ATP (without [w/o] molecule or aptamer), and with 7.5 or 15 μM RS-1 (C), P-ter (D), RI-1 (E), or 0.1, 0.25, or 0.5 μM of A30 (F). The data reported represent the mean values of at least three independent experiments ± SD (error bars). The activity in the absence of compounds, corresponding to the total amount of donor DNA integrated into the acceptor plasmid, as detected on agarose gel electrophoresis and shown in Figure S1, was normalized to 100%.

by the recombinase both in vitro and in vivo (Cosnefroy et al., 2012). These data highlighted the role of the nucleocomplex in the inhibition process. To better characterize the mechanism of inhibition, we searched for compounds capable of affecting the hRAD51/DNA binding properties and, thus, the formation of the active nucleofilament. Stilbenes, like disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS), having previously been shown to affect hRAD51 activity (Ishida et al., 2009), we tested natural stilbenes derivatives recently identified in our laboratory (Pflieger et al., 2013) in an hRAD51/DNA interaction assay. As reported in Figure S2, most of the 22 molecules tested were found to inhibit the hRAD51/DNA association, but only one, the *E*-pterostilbene (P-ter) (chemical structure shown in Figure 1A), stimulated binding. Given that hRAD51 stimulatory compounds are attractive candidates as antiviral agents (Cosnefroy et al., 2012), we first focused our interest on the stimulatory molecule. To verify its possible stimulation effect on hRAD51 strand exchange activity, P-ter was assayed in an in vitro recombination activity. As shown in Figure S3A, P-ter stimulates the recombinase activity at least as efficiently as RS-1, (EC_{50} [half maximal effective concentration] for P-ter = 19.2 ± 2.4 μM and EC_{50} for RS-1 = 25 ± 3 μM).

The hRAD51 stimulatory compounds were then tested on in vitro hRAD51-mediated inhibition of HIV-1 IN activity using the concerted integration assay described in Figure S1. As reported in Figure S4A and by Pflieger et al. (2013), P-ter was previously shown to inhibit HIV-1 IN activity with an IC_{50} (half maximal inhibitory concentration) = 47.5 ± 2.5 μM. Since P-ter showed hRAD51 stimulatory effect between 5 and 20 μM (see Figure S3) without significantly affecting HIV-1 IN activity, it was tested at concentrations up to 15 μM on hRAD51-mediated IN inhibition. As reported in Figure 1C, a strong improvement in integration restriction was observed in the presence of P-ter, as observed in the case of RS-1 (Figure 1D). This indicates that the stimulation of the hRAD51 activity not only by RS-1 but also by other stimulatory compounds such as P-ter can enhance the HIV-1 IN inhibition properties of the recombinase. To determine whether the active hRAD51 nucleofilament could serve as

a target for modulating hRAD51-mediated inhibition of HIV-1 IN, we next examined whether inhibition of the recombinase could also affect its integration restriction properties. Two hRAD51 chemical inhibitors, DIDS (Ishida et al., 2009) and RAD51 inhibitory compound 1 (RI-1) (Budke et al., 2012a, 2012b), and several DNA aptamers previously selected against hRAD51 (Martinez et al., 2010; Figure 1B), were tested. These molecules were assayed on the in vitro hRAD51 recombination activity shown in Figure S3A. Both RI-1 and DIDS displayed an inhibitory activity on the strand exchange catalyzed by hRAD51 (IC_{50} determined for RI-1 and DIDS were respectively 50 ± 5 μM and 90 ± 2.5 μM, Figure S3B). A47 and A30 aptamers (Martinez et al., 2010) were also found to strongly inhibit hRAD51 under these conditions (IC_{50} for A47 = 75 ± 4 nM and IC_{50} for A30 = 30 ± 2 nM), while their shortened control versions A47c and A30c did not (Figure S3C). Among the hRAD51 inhibitory molecules assayed, DIDS showed a significant IN inhibitory effect (IC_{50} = 5 ± 2 μM, Figure S4B) and, thus, was excluded from further analyses. We next compared the effect of RS-1 and P-ter with that of RI-1 and A30, which showed the best inhibitory effect, on hRAD51-mediated integration restriction. As reported in Figures 1E and F, good correlation was observed between hRAD51 activity and IN inhibition. Indeed, all the hRAD51 inhibitors induced a significant decrease in the hRAD51-mediated integration inhibition, which is in sharp contrast to the potentiation observed with the hRAD51 stimulatory compounds RS-1 and P-ter. No effect of molecules on the IN/hRAD51 interaction was detected (Figure S5), confirming that the modulation of hRAD51-mediated inhibition by the drugs was mainly due to their effect on the active hRAD51 nucleofilaments. This suggested that modulators of the nucleocomplex could be used as tools to explore its biological regulatory function in infected cells.

Opposite Effects of hRAD51 Chemical Modulation on HIV-1 Integration Step in 293T Cells

RS-1 has previously been shown to stimulate hRAD51 activity both in vitro and in vivo (Jayathilaka et al., 2008) and to inhibit HIV-1 replication in single- and multiple-round infection assays performed in different cell types, including primary peripheral blood mononuclear cells (PBMC) resting cells (Cosnefroy et al., 2012). To better characterize the mechanism of action of RS-1, especially at the integration step, we compared it with P-ter using a typical 293T single-round replication assay, in order to focus on the early steps of infection. Cytotoxicity measurement in cells treated with increasing concentrations of drug showed no significant effect on cells viability (cytotoxic concentration at which 50% cytotoxicity is observed [CC_{50}] > 250 μM, Figures S6A and S6B). We next tested the drugs for their effect on cellular hRAD51-mediated DNA repair activity using a typical cisplatin resistance assay in a non-toxic concentration range. As reported in Figures S7A and S7B, a 24-hr treatment of 293T cells with either RS-1 or P-ter induced an increase in the cisplatin resistance, as expected from the stimulation of the active hRAD51 nucleofilament. To determine the effect of the molecule on intracellular hRAD51 protein, we performed an immunolocalization analysis of the recombinase. As reported in Figure S7C, cells treated with either RS-1 or P-ter showed an increased number of hRAD51 nuclear foci compared with untreated cells. Quantification of the cytoplasmic and nuclear foci (Figure S7D) confirmed that treatment with the hRAD51 stimulatory compounds induced a nuclear relocalization of hRAD51, consistent with a stimulation of the formation of active nucleofilaments in the nuclear compartment. Since (1) hRAD51 activity could be altered during cell cycle and (2) the effect of the compounds on the viral replication might depend on the alternation of cell cycle, we analyzed the impact of drug treatments on the cellular cycle. As reported in Figure S7E, propidium iodide labeling of the cells showed no significant change in the cell cycle alternation of the treated versus untreated cells. This allowed further analyzes of drugs effects on early steps of retroviral replication.

As shown in Figure 2A, a 24-hr treatment of 293T cells with RS-1 prior to transduction with pNL4.3-based pRRLsin-PGK-eGFP-WPRE VSV-G pseudotyped viruses induced an inhibition of transduction efficiency. Quantification of the different viral DNA populations indicated that this phenotype was due to an inhibition of integration, as shown by an increase of the amount of unintegrated two-LTR DNA circles, and a decrease of the integrated DNA form, while the total DNA amount remained unchanged (Figure 2B). In contrast, treatments performed 5 hr after transduction induced an opposite phenotype, showing a stimulation of the viral replication correlated with an increased integration. To determine whether this dual effect was specific to the RS-1 molecule, we tested the newly selected P-ter. Assays performed on 293T cells transduced with the lentiviral vector produced results similar to those obtained with RS-1 (Figures 2C and 2D).

The RI-1 compound, exerting an opposite effect to RS-1 on hRAD51, was then tested. As reported in Figure S6C, no significant RI-1 toxicity was observed in a 1–50 μM range, although a slight decrease in cell viability could be observed at concentrations higher than 50 μM (CC_{50} = 200 ± 15 μM). A 24-hr pre-treatment of 293T cells with RI-1 was found to induce a decrease in both the cisplatin resistance (Figure S8A) and the nuclear foci formation (Figure S8B). These results confirmed that RI-1 could negatively modulate the hRAD51 DNA repair activity. The drug was next tested on early steps of retroviral replication. As reported in Figure 3A, a 24-hr pre-treatment led to an increase of the percentage of eGFP-positive 293T cells transduced with the lentiviral vector (EC_{50} = 50 ± 12 μM). Quantification of the viral DNA species indicated that the phenotype was due to a stimulation of integration, as shown by the significant increase in integrated DNA forms and the decrease of two-LTR circles, the global viral DNA amount remaining unchanged (Figure 3B). Strikingly, a 5-hr post-transduction treatment with RI-1 induced an opposite effect. Indeed, the viral replication was inhibited (EC_{50} = 50 ± 6 μM), with a decrease in the integrated DNA forms accompanied by slight increase of the two-LTR circles. In contrast, a 16-hr post-transduction treatment had no significant effect on replication.

These results demonstrated that the hRAD51 activity modulation can have opposite effects on integration depending on the chronology of the treatment. Especially, stimulation of the recombinase prior to transduction rendered the cells resistant to integration, whereas stimulation of hRAD51 after transduction had a positive effect on integration. Thus, depending on its catalytic status, hRAD51 can either positively or negatively influence the early steps of HIV-1 replication by acting at the integration step. In view of these data, one important conclusion is that cells

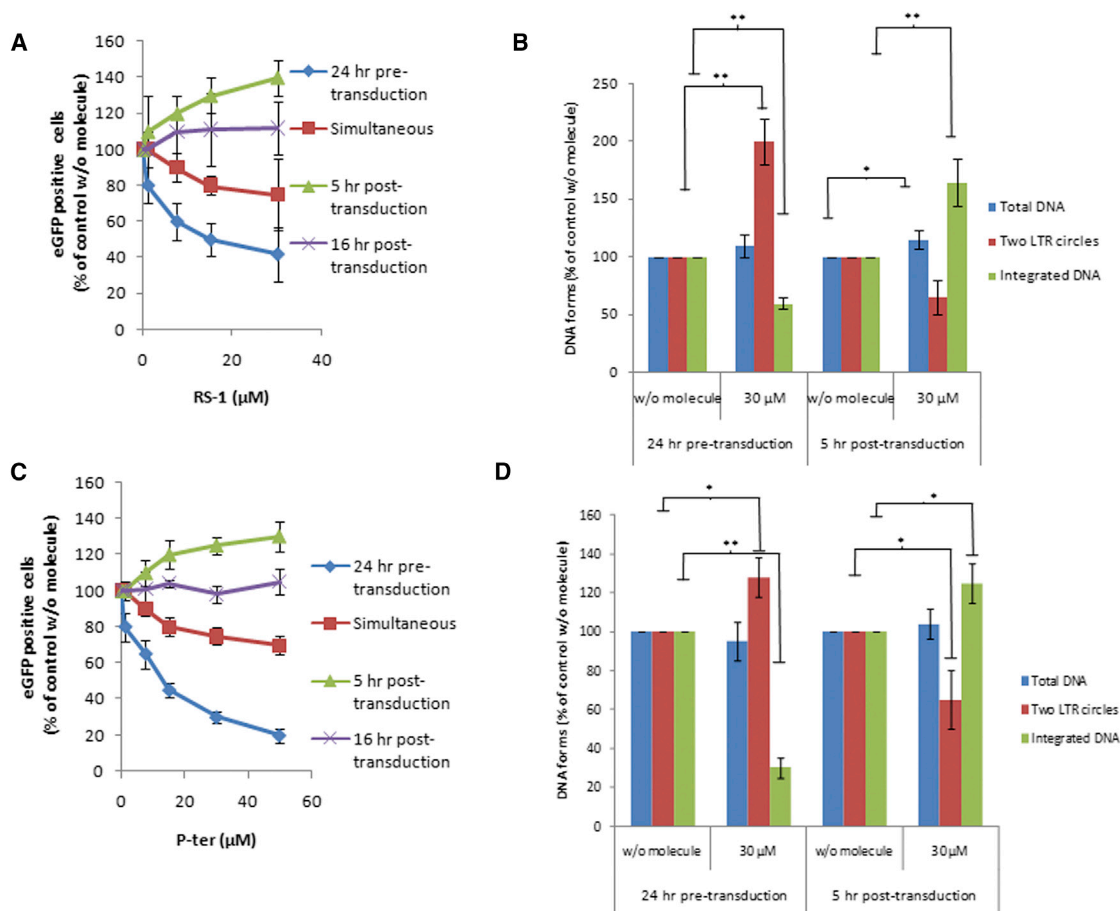

Figure 2. Effect of RS-1 and P-ter Treatment on Early Steps of HIV-1 Replication and Viral DNA Populations

Cells were treated for 24 hr prior to, concomitantly, or 5–16 hr after transduction with either RS-1 (A and B) or P-ter (C and D). The eGFP fluorescence was measured 10 days after transduction by flow cytometry. The percentage of eGFP-positive cells in the absence of compound was normalized to 100% (A and C). The amount of the total, integrated, and 2-LTR circles viral DNA forms was quantified as described in the Experimental Procedures at a fixed 30 µM concentration (effective and non-cytotoxic) of RI-1 or P-ter, under two distinct treatment conditions (early and late). The amount of each viral DNA species produced in the absence of compound was normalized to 100% (B and D).

Results are the mean of three independent experiments. The p values calculated using Student's t test are indicated as *$p < 0.05$, **$p < 0.005$. w/o, without.

with higher hRAD51 intracellular concentrations would be expected to be more resistant to HIV-1 infection. To verify this hypothesis, we studied the effect of the hRAD51 intracellular concentration on integration and viral replication.

Modulation of hRAD51 Expression Affects Both Cellular DNA Repair Activity and HIV-1 Integration

In order to increase the hRAD51 intracellular content, an hRAD51-FLAG tagged protein was overexpressed in 293T cells by transfection with the pcDNA-hRAD51 expression vector. Expression of the heterologous recombinase was checked by western blotting using an anti-FLAG antibody (Figure 4A). The global hRAD51 expression level was evaluated by western blotting using an anti-hRAD51 antibody, allowing detection of both the expressed and the endogenous hRAD51. Under our experimental conditions, we reached a 3- to 5-fold increase in hRAD51 expression compared with non-transfected cells or cells transfected with a BAP-FLAG control vector (Figure 4B). Immunolocalization experiments of the hRAD51-FLAG protein using an anti-FLAG antibody showed the formation of typical nuclear foci specific of the active DNA repair recombinase, while the BAP-FLAG control protein showed a more diffuse localization (Figure 4C). This was confirmed by measuring the hRAD51-mediated DNA repair activity through a cisplatin resistance assay, indicating a significant enhancement of the resistance to cisplatin of the cells overexpressing hRAD51-FLAG (Figure 4D). Altogether these data demonstrate that hRAD51-FLAG overexpression stimulates the intrinsic cellular DNA repair activity.

Early steps of HIV-1 replication were then analyzed in cells overexpressing hRAD51-FLAG by transduction with pRRLsin-PGK-eGFP-WPRE VSV-G pseudotyped viruses and measurement of the eGFP expression from the integrated gene. As reported in Figure 4E, hRAD51-FLAG overexpression induced a significant 40%–50% inhibition of HIV-1 replication, in contrast to BAP-FLAG. Under these conditions, no significant change in the total viral DNA amount was detected, while a strong decrease in the integrated DNA forms was observed in addition to an increase of the unintegrated two-LTR circles (Figure 4F). This

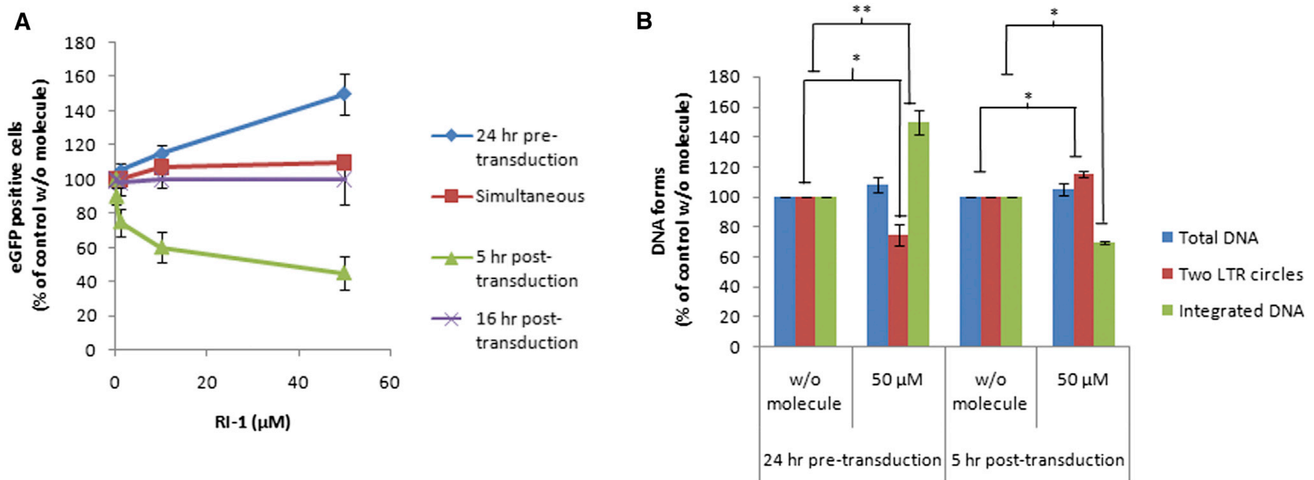

Figure 3. Effect of hRAD51 Inhibitory Compounds RI-1 on Early Steps of HIV-1 Replication

(A) Cells were treated for 24 hr prior to, concomitantly, or 5–16 hr after transduction. eGFP fluorescence was measured 10 days following transduction by flow cytometry. The percentage of GFP-positive cells obtained in the absence of compound was normalized to 100%.

(B) The effect of RI-1 on viral DNA production was measured by quantifying the total, integrated, and 2-LTR circles viral DNA forms at a fixed 50-μM concentration (non-cytotoxic and effective) of RI-1, under two treatment conditions. The proportion of the different DNA species obtained in the absence of compound was normalized to 100%.

Results are represented as the mean values calculated from three independent experiments. The p values are reported as $^{*}p < 0.05$, $^{**}p < 0.005$. w/o, without.

confirmed the effect of hRAD51 on the integration step following nuclear entry of the viral cDNA. To better characterize the relationship between the intracellular concentration of hRAD51 and HIV-1 integration efficiency, we tested whether decreasing the hRAD51 expression level could induce an opposite phenotype. For this purpose, a pharmacological approach was used to reduce hRAD51 expression. Imatinib (Figure 5A) was previously reported to decrease the hRAD51 protein levels and increase tumor cell radiosensitivity (Russell et al., 2003). In vitro control experiments showed that this compound did not affect HIV-1 IN and hRAD51 catalytic activities (Figure S9A), validating our approach. As reported in Figure S9B, treatment with imatinib concentrations above 10 μM led to a significant cellular toxicity (EC_{50} = 30 ± 8 μM). The effect of the drug on hRAD51 expression was thus evaluated using concentrations below 10 μM. Western blot quantifications of hRAD51 expression following imatinib treatment confirmed that the drug could induce an efficient 40%–50% decrease 10 hr after treatment, while the initial level of hRAD51 was recovered after 72 hr (Figure 5B). The decrease was also associated with a reduced hRAD51 DNA repair activity as measured by the quantification of cisplatin resistance (Figure 5C). Based on these data, HIV-1 replication was tested 24 hr after treatment. As reported in Figure 5D, imatinib treatment 24 hr before transduction of the cells led to an increase in eGFP expression associated with a typical enhancement of the integration efficiency (Figure 5E). These results demonstrate that an upregulation of hRAD51 expression both stimulates endogenous DNA repair and induces HIV-1 integration restriction.

hRAD51 Expression and Intracellular Localization Are Modulated during HIV-1 Early Steps of Replication

To determine whether hRAD51 expression could be modulated during the viral infection, we transduced 293T cells with pNL4.3-based pRRLsin-PGK-eGFP-WPRE VSV-G pseudotyped vectors and analyzed the hRAD51 protein content of cellular extracts obtained at different time points after transduction. As reported in Figure 6A, a decrease in the hRAD51 protein content was detected 4–12 hr after transduction and an increase of the protein level was observed 16–24 hr after transduction. In contrast, the hRAD51 protein amount was found unchanged in the non-transduced cells. Transcription activity of the RAD51 genes was then analyzed. We used the http://www.peachi.labtelenti.org web resource allowing the querying of cellular responses to infection in SupT1 T cells transduced by HIV-based vectors (Mohammadi et al., 2013). Genes encoding for RAD51 and paralogs were found highly downregulated during the early steps of infection (0–6 hr) and upregulated during the integration step (8–16 hr) in contrast to other RAD genes such as RAD18 or RAD54 (Figure S10). These data indicate that hRAD51 expression is modulated during the early steps of replication at the transcription level. We next analyzed the cellular behavior of the endogenous hRAD51 by immunofluorescence staining of transduced cells at different time points. As shown in Figure 6B, a modulation of the cellular localization could also be detected during the early stages of the replication. Indeed, while hRAD51 foci were found equally distributed in both cytoplasm and nuclear compartment during the 0–8 hr after transduction, a strong relocalization of the recombinase was detected during the 12–24 hr after transduction. No significant change was observed in the non-transduced cells (Figure S11). Consequently, all these data strongly suggest that viral infection modulates both hRAD51 expression and intracellular localization during the early stages of replication.

DISCUSSION

Retroviral integration introduces cuts in the host cell DNA that can be considered as potential mutagenic events by the cellular

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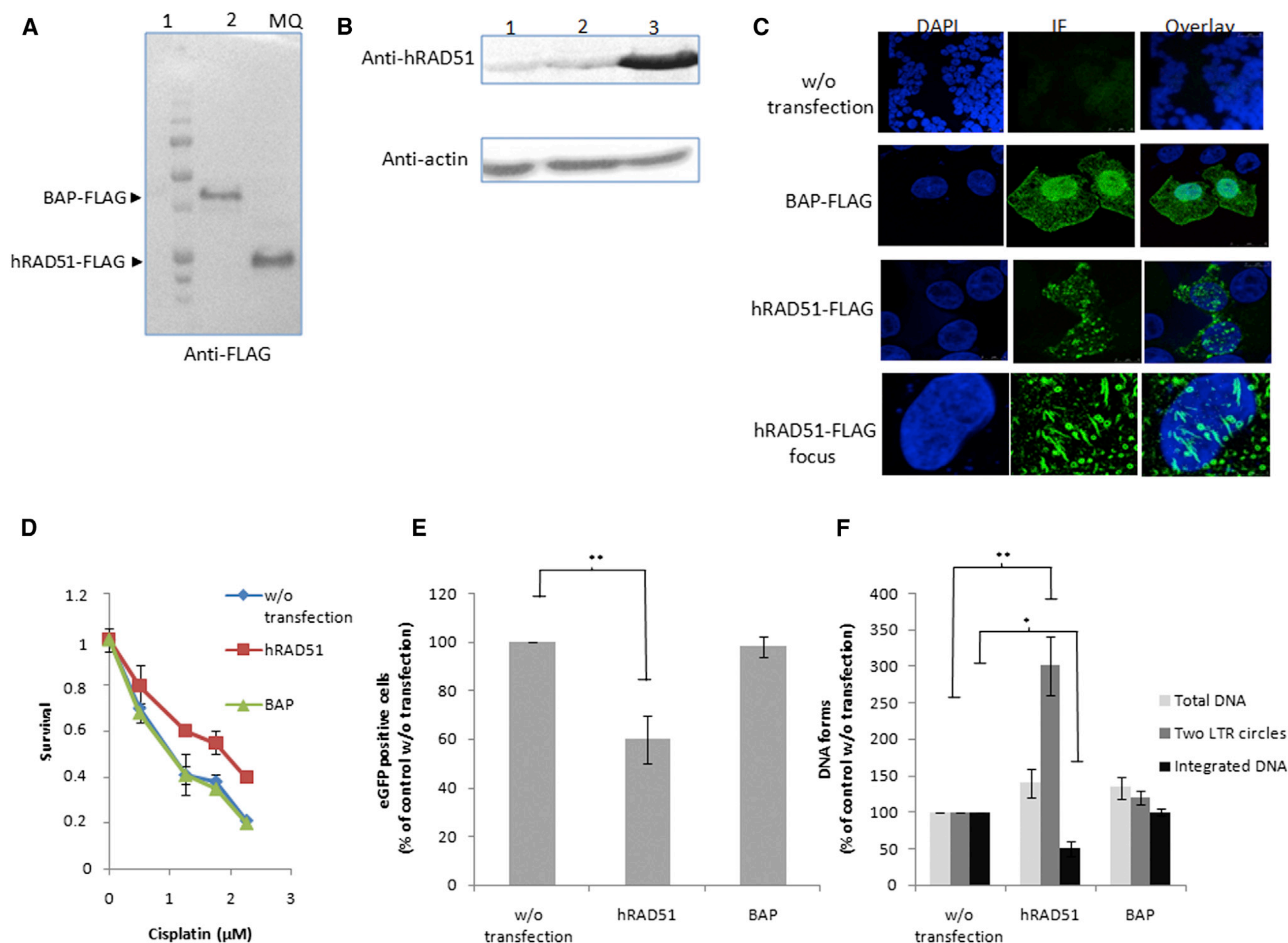

Figure 4. Effect of hRAD51 Overexpression on Endogenous DNA Repair and Early Steps of HIV-1 Replication and Viral DNA Productions

(A) The expression of hRAD51-FLAG or BAP-FLAG in 293T cells was checked 48 hr after transfection by western blotting using anti-FLAG antibodies (lane 1, protein extract from cells expressing hRAD51-FLAG; lane 2, protein extract from cells expressing BAP-FLAG).

(B) The global hRAD51 level was determined in cells transfected with the hRAD51-FLAG (hRAD51) and BAP-FLAG (BAP) expression vectors in parallel to untransfected control cells (without [w/o] transfection), by western blotting using anti-hRAD51 antibodies. The amounts of protein loaded were normalized to the endogenous actin protein revealed by western blotting using an anti-actin antibody.

(C) The cellular distribution of the overexpressed proteins was determined by immunolocalization using an anti-FLAG antibody.

(D) The hRAD51 activity was determined under each condition by a cisplatin resistance assay as described in the Experimental Procedures. The cells were transduced 48 hr after transfection with the hRAD51 or BAP expression plasmids.

(E) HIV-1 replication was evaluated from fluorescence measurement 10 days after transduction by flow cytometry. The percentage of untransfected eGFP-positive cells was normalized to 100%.

(F) The amount of total, integrated, and 2-LTR circles viral DNAs was measured by qPCR as described in the Experimental Procedures. The proportion of the different viral DNA species produced in untransfected control cells was normalized to 100%.

Results are represented as the mean values calculated from three independent experiments. The p values are shown as *$p < 0.05$, **$p < 0.005$.

DNA repair machinery. Furthermore, the incoming intasome containing blunt-ended viral DNA can also be recognized as a double-strand break (DSB) by the homologous repair (HR) pathway in the infected cells. This is supported by the formation of active hRAD51 nucleofilaments on viral DNA in presence of IN as detected in previous electron microscopy analyses (Rom et al., 2010). HR hRAD51 protein binds HIV-1 IN and restricts its activity both in vitro and in vivo through a DNA/IN dissociation process dependent on the formation of active hRAD51 nucleofilaments (Desfarges et al., 2006; Cosnefroy et al., 2012). The stimulation of hRAD51-mediated inhibition of HIV-1 integration, by using drugs like RS-1, for example, results in the suppression of viral replication in different cell types, including primary resting PBMCs (Cosnefroy et al., 2012). In addition, hRAD51 has been shown to stimulate the expression of proviral genes by enhancing LTR-dependent transcription (Kaminski et al., 2014; Rom et al., 2010). These data indicate that efficient HIV-1 infection relies on an optimal intracellular activity of hRAD51. Here, using a pharmacological approach allowing modulation of hRAD51 activity, we provide a comprehensive analysis of the mechanisms involved in the regulation of HIV-1 integration.

We first selected molecules capable of modulating the hRAD51-mediated inhibition of HIV-1 IN. Several previously described hRAD51 inhibitors such as RI-1 and A30 (Budke

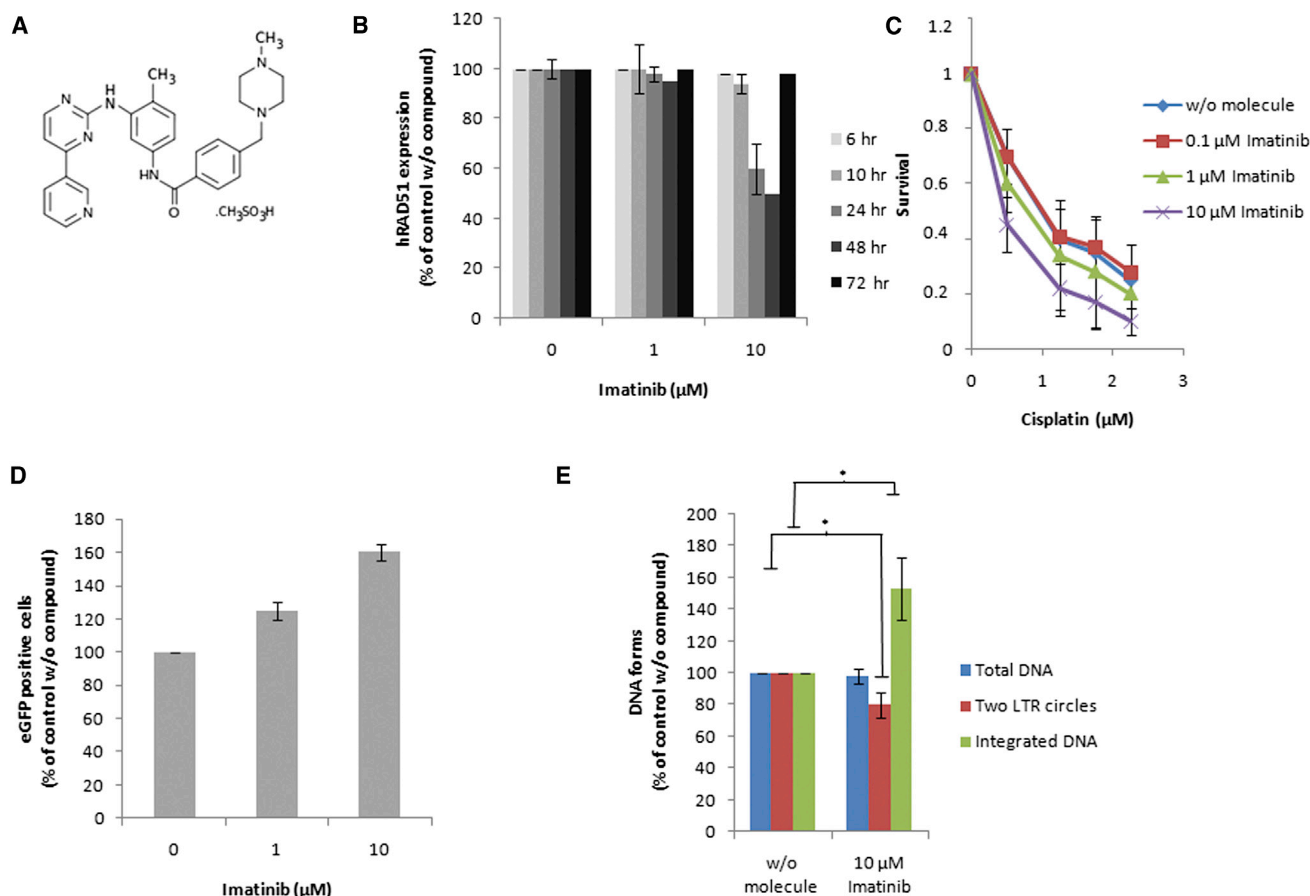

Figure 5. Effect of Imatinib Treatment on hRAD51 Expression Levels, Endogenous DNA Repair and Early Steps of HIV-1 Replication

(A) The chemical structure of imatinib.

(B) The total protein fraction was extracted 6–72 hr following treatment with imatinib. The hRAD51 protein levels were determined from western blot analyses using an anti-hRAD51 antibody. The amount of hRAD51 protein present in untreated cells was normalized to 100%.

(C) The ability of imatinib to affect cisplatin resistance was checked in a standard survival analysis performed after 24 hr of treatment with increasing concentrations of the compound. Survival was expressed as the ratio of absorbance at 492 nM (Synergy [BioTek] plate reader) of cisplatin-treated cells (pre-incubated with the compound or not) relative to untreated cells. Results represent the means of at least three independent experiments ± SD (error bars).

(D) The effect of imatinib on early steps of HIV-1 replication was analyzed following a 24-hr treatment of the cells before transduction with the lentiviral vector. eGFP fluorescence was measured 10 days after transduction by flow cytometry. The percentage of eGFP-positive cells in the absence of compound was normalized to 100%.

(E) The amount of total, integrated, and 2-LTR circles viral DNA forms was quantified at a fixed 10 μM concentration (effective and non-cytotoxic) of imatinib. The amount of each viral DNA species produced in the absence of compound was normalized to 100%. The results are presented as the mean of three independent experiments. $^{*}p < 0.05$.

et al., 2012b; Martinez et al., 2010) were found to alleviate the in vitro integration restriction properties of the recombinase. On the contrary, hRAD51 stimulatory compounds, like the previously reported RS-1 (Jayathilaka et al., 2008) and the newly selected *E*-pterostilbene purified from grape wine (Pflieger et al., 2013), promoted the hRAD51-induced restriction of HIV-1 integration. These results, summarized in Figure S12, show a strong correlation between the recombination activity of hRAD51 and its ability to inhibit IN. Furthermore, none of the drugs affected the IN/hRAD51 association. These data strongly suggest that integration restriction relies on the formation of active hRAD51 nucleofilaments. This nucleocomplex could, thus, constitute a valuable pharmacological target for strategies aiming to modulate HIV-1 replication by targeting the integration step. In addition, to provide new information about the hRAD51-induced integration restriction, these molecules could also serve as tools to further explore the role of hRAD51 in proviral LTR-driven gene expression.

The use of chemical modulators produced differential and opposite effects on both HIV-1 integration and replication depending on the time of treatment (data summarized in Figure S13). Early stimulation of hRAD51 (treatment at 24 hr before transduction) negatively impacted integration and replication, whereas late stimulation (i.e. 5 hr after transduction) had an opposite effect. Likewise, early inhibition of the hRAD51-mediated recombination induced a stimulation of integration and replication, in contrast to what was observed using a 5-hr post-transduction treatment, which led to a significant decrease

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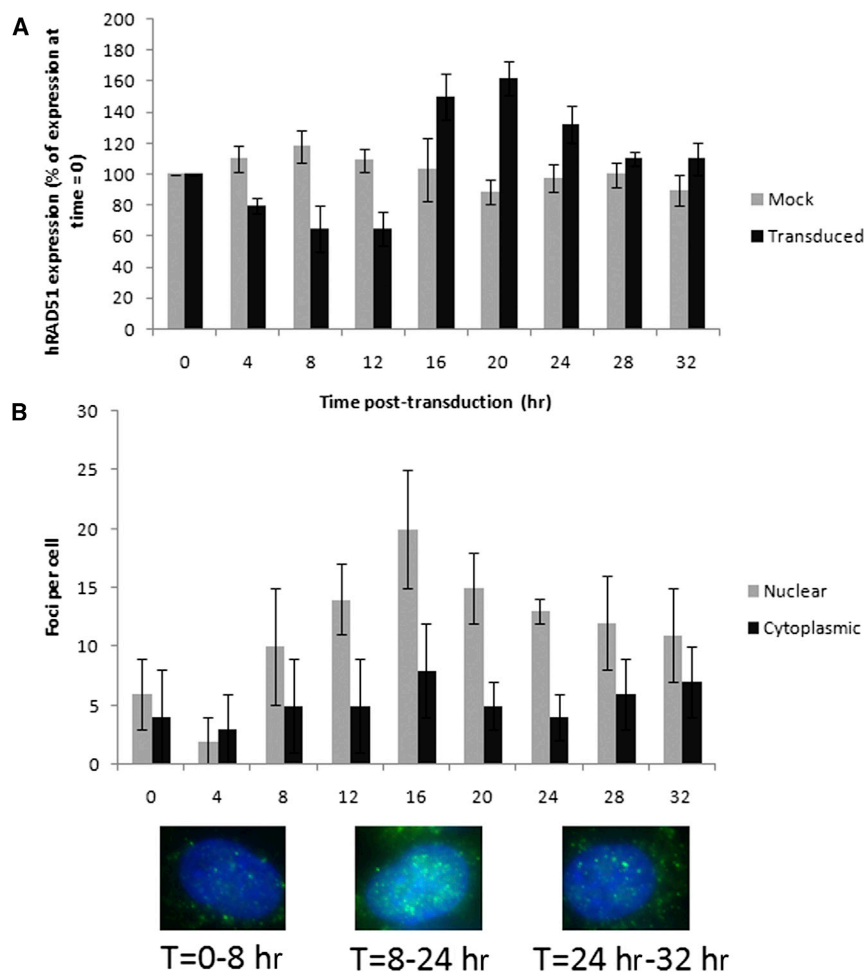

Figure 6. Expression and Intracellular Localization of hRAD51 during the Early Steps of HIV-1 Replication in 293T Cells

(A) Proteins were extracted at different time points (0–32 hr) after 293T cell transduction with pNL4.3-based pRRLsin-PGK-eGFP-WPRE VSV-G pseudotyped viruses and the hRAD51 expression level was analyzed by western blot. The amount of hRAD51 was normalized to the amount of actin detected by western blot. The quantity of hRAD51 detected at time point = 0 was then normalized to 100%. The results are represented as the mean of three independent experiments. Data obtained without transduction (mock) are also reported.

(B) The cellular distribution of hRAD51 was determined by immunolocalization at different time points after 297T cell transduction using an anti-hRAD51 antibody. Results are reported as the mean number ± SD of cytoplasmic and nuclear foci observed under each condition for at least 50 cells. A typical picture of hRAD51 intracellular localization is also provided for several time periods corresponding to the early and late steps of integration.

of the replication due to an integration defect. The integration window has been previously determined and is broadly admitted to take place between 6 and 20 hr after infection in T cell lines (Mohammadi et al., 2013; Manic et al., 2013). Since the expression of the reporter gene used in our lentiviral vectors is LTR independent, our data indicate that hRAD51 plays distinct and opposite functions in the regulation of HIV-1 integration, displaying an early restrictive effect and a late stimulatory effect (summary in Figure 7).

The integration restriction activity of hRAD51 is likely to be related to the previously reported IN/viral cDNA dissociation mechanism through the formation of the hRAD51/DNA nucleofilaments. Indeed, the incoming preintegration complexes (PICs) may be recognized early by hRAD51, thanks to (1) the affinity of the recombinase for both IN and the viral cDNA (Desfarges et al., 2006; Cosnefroy et al., 2012) and (2) the recognition of the blunt-ended retroviral genome as a DSB, as expected from its intrinsic DNA repair properties (Cosnefroy et al., 2012). This is also supported by our biochemical data showing that hRAD51 more efficiently targets the HIV-1 IN/DNA complex (see Figure S1B) and by the systematic correlations observed between HIV-1 integration restriction and hRAD51-mediated DNA repair activity, in vivo (see Figures S13 and S14). Moreover, under the same experimental conditions, integration inhibition was consistently found associated with an increase of the amount of two-LTR circles assumed to be formed in the nucleus. This indicates an effect downstream of the nuclear import of the PIC. hRAD51 targeting toward the incoming intasome in the nucleus could prevent the latter from reaching the integration locus. This hypothesis is also strongly supported by the treatment-dependent cellular localization of hRAD51, as stimulatory compounds were found to promote the nuclear translocation of the recombinase in contrast to hRAD51 inhibitors (see Figure S7C and S7D; Figure S8B).

The mechanisms of regulation brought into play in cells treated 5 hr after transduction remain to be fully determined. Nevertheless, the data obtained using stimulatory and inhibitory compounds strongly support that they target a pro-integrative function of the recombinase. Since hRAD51 is the main actor of the homologous DNA repair pathway, one relevant hypothesis may be that the recombinase acts at the step of PIR by promoting it. This relationship between retroviral integration and DNA repair has been previously proposed (Kilzer et al., 2003). The PIR and the involvement of hRAD51 during this process are currently under study in our laboratory. Especially, the possible role of the nucleofilament-mediated dissociation of HIV-1 IN following strand transfer, which has previously been shown to be required for efficient DNA repair of the integration locus (Yoder and Bushman, 2000; Brin et al., 2000), must be investigated. Indeed, this enhancement of the PIR by hRAD51 could be associated to the previously reported function of the recombinase in the LTR-driven transcription of the provirus (Chipitsyna et al., 2006; Kaminski et al., 2014; Rom et al., 2010) and/or to the chromatin remodeling properties of the recombinase (Dupaigne et al., 2008). Indeed, nucleosomal DNA remodeling at the

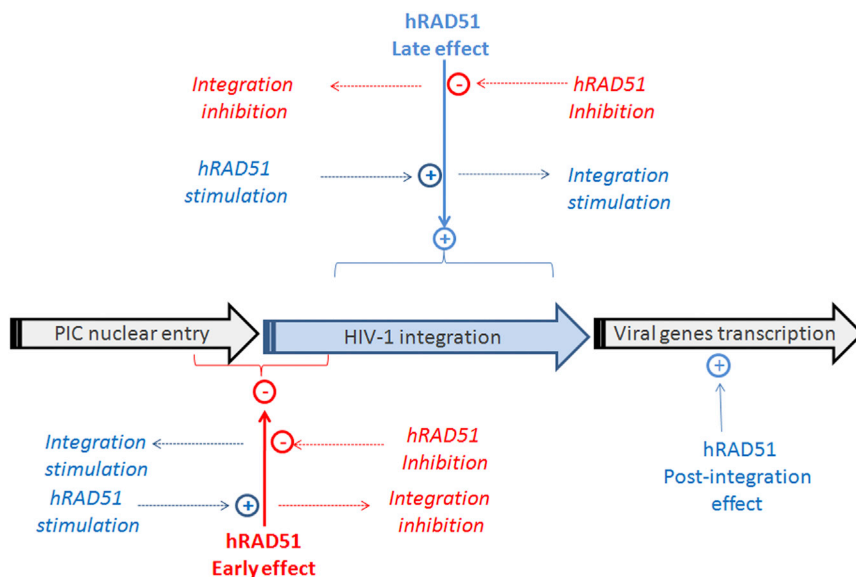

Figure 7. Influence of hRAD51 and Its Modulation on HIV-1 Integration

hRAD51 recombinase activity, namely the formation of the active nucleofilament, plays a negative regulatory role on the steps following PIC nuclear import and preceding strand transfer catalysis, and a positive regulatory role during the steps subsequent to integration, including DNA repair. Chemical modulators targeting these dual effects can exert opposite effects on HIV-1 integration, and hence on viral replication.

integration site has been proposed to be important for efficient integration (Lesbats et al., 2011a, 2011b). This is supported by the relocalization of endogenous hRAD51 protein during the integration step in the nuclear compartment where PIR must occur (Figure 6B). Biochemical data showing that hRAD51 can promote the in vitro DNA repair step catalyzed by human flap endonuclease 1 also support this hypothesis (data not shown). However, the role of hRAD51 during the DNA repair of the gapped intermediates remains to be fully investigated and, to this purpose, the chemical compounds reported in this work could constitute useful tools for further studies of this process.

Our data confirm that cellular DNA repair machineries can play multiple roles by restricting retroviral integration and/or directly participating in the stability of the integrated DNA. For an optimal viral replication equilibrium between these, opposite effect must be reached. Our results indicate that this can be accomplished by regulation of hRAD51 endogenous expression levels and localization during viral replication (cf. Figure 6). This suggests that the expression of the anti-integration property of hRAD51 during the early steps of replication (0–6 hr) can be overcome by the decrease of the protein level, and hRAD51 activity. Indeed, this is expected to limit the integration inhibition by the recombinase as observed during chemical treatments with imatinib decreasing the hRAD51 expression and promoting the integration (Figure 5). This would allow the virus to reach a suitable level of efficient integration for optimized replication. In addition, to highlight the biological regulatory functions of hRAD51 on HIV-1 integration, our data also provide new insights on the possible use of chemical compounds targeting hRAD51 as antiviral agents restricting HIV-1 replication by modulating the recombinase activity in infected cells. Interestingly, the drug concentrations used in our work are fully compatible with the concentrations used in cellular or clinical studies (Jayathilaka et al., 2008; Russell et al., 2003). However, our data indicate that further development toward antiviral compounds will be a difficult task due to the dual regulatory function of the recombinase described in this work, as well as the possible secondary effects due to an alteration of DNA repair processes in the infected cells. Nevertheless, we have previously shown that strategies aiming to stimulate hRAD51 activity in a multiple-round infection system, as in PBMCs, could be successful. Indeed, stimulation of the hRAD51-mediated DNA repair pathway prior to HIV-1 infection has shown a protective effect against viral replication without affecting the viability of the cells (Cosnefroy et al., 2012). Consequently, based on our results, we can speculate that early stimulation of hRAD51 activity (by overexpression or allosteric stimulation) would be efficient against viral replication and effort should be made toward this strategy. In addition, compounds targeting the hRAD51 DNA repair activity are potential anti-cancer drugs and some anti-cancer molecules, such as imatinib, which is already used in clinic, have an impact on hRAD51 expression. Importantly, these effects are observed using a micromolar range of drug amounts close to the plasmatic concentrations used in clinic. Consequently, another important conclusion to be drawn from our finding is that great care should be taken when treating HIV-1-infected patients with hRAD51 modulators, as a burst in viral replication may be expected to occur through LTR-dependent proviral transcription and/or under conditions favoring hRAD51 pro-integration functions.

SIGNIFICANCE

Regulation of HIV-1 integration is crucial for efficient viral replication and, thus, constitutes an attractive target for antiviral therapies. The hRAD51 DNA repair protein has been shown to modulate integration activity. A better understanding of the regulation properties of this recombinase is essential for therapeutic approaches targeting DNA repair. The work presented here establishes the link between hRAD51 activity and HIV-1 integration efficiency in infected cells. Indeed, the pharmacological analyses performed demonstrates that hRAD51 plays dual and opposite roles in the regulation of integration, indicating that efficient HIV replication depends on an optimal intracellular level of hRAD51. In addition, to provide new virus/host interactions, our data may have important implications in the development of antiviral strategies based on the modulation of the

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activity of the cellular DNA repair machinery. Indeed, the work presented here highlights the different pro- and anti-integration properties of hRAD51, which must be taken into consideration for future pharmacological development, and, importantly, shows how chemical modulation of the hRAD51 activity can induce opposite effects on viral replication.

EXPERIMENTAL PROCEDURES

Chemicals, Proteins, and Antibodies

RS-1 and RI-1 were previously selected from a 10,000-compound library (ChembridgeDIVERSet) screened for their capacity to affect the DNA binding properties of hRAD51 (Jayathilaka et al., 2008; Budke et al., 2012b). DIDS previously reported as an hRAD51 inhibitor (Ishida et al., 2009) was purchased from Sigma. P-ter was extracted from *Vitis vinifera* leaves (isolation and purification protocols described in Pflieger et al., 2013). Imatinib was purchased from Selleck Chemicals (Euromedex). Aptamers A30 and A47, previously selected as ligands of hRAD51 (Martinez et al., 2010), and their shortened versions A30c and A47c were purchased from MWG. Cisplatin was purchased from Sigma. Recombinant HIV-1 IN was expressed in yeast and purified using the IN_{Hybrid} method previously described (Lesbats et al., 2008). Wild-type recombinant hRAD51 was produced as described before (Chi et al., 2006). Monoclonal anti-FLAG and polyclonal anti-hRAD51 antibodies were purchased from Sigma.

In Vitro Enzymatic Assays

HIV-1 concerted integration reactions were performed as described previously (Lesbats et al., 2008) and in Figure S1. All IN activities were quantified by scanning the bands (half-site plus full-site integration products) following gel electrophoresis and autoradiography, using ImageJ software. hRAD51 activity was evaluated according to the strand exchange reaction previously reported (Takizawa et al., 2004) and adapted to our concerted integration conditions (see Figure S3). Strand exchange products were quantified following autoradiography of the gel, using ImageJ software. Measurements of the hRAD51 DNA binding activity was performed as previously described with slight modifications (Budke et al., 2012b) (see Figure S2).

Transfection Assay

293T cells were grown in DMEM Glutamax medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and 50 μg/ml gentamicin. The cells were seeded into 48-well plates 24 hr before transfection. Fifteen thousand cells were transfected with 1 μg of either p3X-FLAG-CMV-hRAD51 (hRAD51) expression vector or p3X-FLAG-CMV-7-BAP (BAP) expression control plasmid using lipofectamine 2000 (Invitrogen). DMEM containing 20% of FCS was added 4 hr after transfection. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 hr and then lysed with SDS loading buffer. Extracted proteins were separated on 12% SDS-PAGE and analyzed by western blotting.

Transduction of 293T Cells

293T cells were plated in 48-multiwell plates at 50,000 cells/well using 400 μl of DMEM (Invitrogen) containing 10% (v/v) FCS and 50 μg/ml of gentamycin (Invitrogen). Infection was assayed using pNL4.3-based pRRLsin-PGK-eGFP-WPRE VSV-G pseudotyped lentiviruses produced as described in Richard et al. (2001). After three washes with PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]), the cells were transduced with the lentiviral vector at an optimized MOI of 1, in a final volume of 400 μl (under these conditions, 25%–35% of the cells contained one integrated viral cDNA copy). After 10 days, the cells were washed twice with PBS, treated with trypsin for 5 min at room temperature, and centrifuged for 2 min at 2000 rpm. The pellet was washed twice with PBS and resuspended in 200 μl of PBS/200 mM EDTA. Fluorescence was quantified using 10,000 cells on a FACS Calibur (Beckton-Dickinson). Plots were analyzed using the FCS express v3.00.0103. Data are presented as the percentage of cells showing a significant level of fluorescence or as the fluorescence intensity calculated by the X-median fluorescence intensity of all cells.

Intracellular hRAD51 Immunolocalization

293T cells transfected with the p3X-FLAG-CMV-hRAD51 (hRAD51) or p3X-FLAG-CMV-7-BAP (BAP) expression vectors, or treated with one of our compounds, were washed three times with PBS and incubated for 10 min in fixation buffer containing 2% formaldehyde. After two washes, the cells were incubated for 5 min in permeabilization buffer (PBS supplemented with 0.4% saponin and 0.1% Triton X-100). The cells were washed with PBS supplemented with 0.1% saponin and incubated for 1 hr at room temperature in blocking buffer (PBS 0.1%, 1% BSA, 2% FCS). The cells were incubated with the primary antibody overnight at 4°C. After three washes with PBS, the secondary antibody coupled to Alexa 440 was added and the cells were further incubated for 1 hr at 37°C. DAPI was added at a final concentration of 1 μg/ml for 10 min at room temperature. The cells were then washed four times with PBS before fluorescence microscopy analysis. A similar procedure was used for hRAD51 intracellular localization analysis in 293T cells transduced with pRRLsin-PGK-eGFP-WPRE VSV-G pseudotyped lentiviruses.

Cytotoxicity and Cell Cycle Measurements

The cellular cytotoxicity of the compounds was determined by measuring the survival of cells treated with increasing concentrations of molecules for 2 days with a standard MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay (PromegaCellTiter 96 AQueous One Solution Cell Proliferation Assay). Analyses of the cell cycle were performed using a propidium iodide flow cytometry kit from Abcam following the manufacturer's instructions.

Quantification of Cisplatin Resistance

The effect of protein overexpression and chemical treatment on hRAD51-mediated homologous recombination was assessed using the standard cisplatin resistance assay described previously (Jayathilaka et al., 2008).

Quantification of HIV-1 DNA Species

The cells were harvested 8, 24, and 48 hr after infection by centrifugation, and aliquots of 2×10^6 to 6×10^6 cells were kept frozen at −80°C until analysis. Total DNA (including integrated and episomal HIV-1 DNA) was extracted using the QiAmp blood DNA mini kit (Qiagen) according to the manufacturer's instructions and eluted in 50 μl of elution buffer. Quantification of the viral DNA species was performed using the conditions and primers described in Brussel and Sonigo (2003). The total HIV-1 DNA was amplified by quantitative real-time PCR using a LightCycler instrument (Roche Diagnostics). Quantifications of total HIV-1 DNA, including 2-LTR circles and integrated HIV-1 cDNA, were performed by qPCR on a LightCycler instrument using the fit point method provided in the LightCycler quantification software, version 4.1 as previously described (Zamborlini et al., 2011). The copy numbers of 2-LTR circles and total viral DNA were determined with reference to a standard curve prepared by amplification of quantities ranging from 10×10^6 to 1×10^6 copies of plasmid comprising the HIV_{LAI} 2-LTR junction (Manic et al., 2013). The integrated HIV-1 cDNA copy number was determined with reference to a standard curve generated by the concomitant two-stage PCR amplification of serial dilutions of an integrated HIV-1 DNA standard from Hela-R7 Neocells (Munir et al., 2013). Cell equivalents were calculated according to amplification of the β-globin gene (two copies per diploid cell) with commercially available materials (Control Kit DNA; Roche Diagnostics). 2-LTR circles and total and integrated HIV-1 DNA levels were determined as copy numbers per 10^6 cells. Two-LTR circles and integrated cDNA were also expressed as a percentage of the total viral DNA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes fourteen figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.04.020.

AUTHORS CONTRIBUTIONS

T.S. designed and performed the cellular transduction experiments and viral DNA quantifications, discussed the data, drafted the manuscript; M.S.B.

designed and performed the in vitro experiments, discussed the data, drafted the manuscript; L.S. designed the in vitro experiments and discussed the data; E.T. performed the cellular transduction experiments and viral DNA quantifications; C.C. purified the recombinant IN proteins; S.C. carried out the mass spectrometry analyses of the IN/hRAD51 interaction (not shown), corrected the manuscript, and discussed the data; P.W.T. and J.M.M. purified the stilbene compounds and corrected the manuscript; A.C. performed the hRAD51 transcription study and analyzed the data. P.S. purified the recombinant hRAD51 protein, discussed the data, and corrected the manuscript; P.C. and B.B. performed the hRAD51/DNA binding assays, drafted, and corrected the manuscript; I.H. and J.H. carried out secondary experiments in animal models (not shown), discussed the data, and corrected the manuscript; M.L.A. discussed the data and corrected the manuscript; O.D. designed, performed, and discussed the viral DNA quantifications, corrected the manuscript; V.P. set up and coordinated the project, designed and performed the cellular and biochemical experiments, drafted the manuscript, and discussed the data.

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