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Premature transcription termination complex proteins PCF11 and WDR82 silence HIV-1 expression in latently infected cells

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Postintegration transcriptional silencing of HIV-1 leads to the establishment of a pool of latently infected cells. In these cells, mechanisms controlling RNA Polymerase II (RNAPII) pausing and premature transcription termination (PTT) remain to be explored. Here, we found that the cleavage and polyadenylation (CPA) factor PCF11 represses HIV-1 expression independently of the other subunits of the CPA complex or the polyadenylation signal located at the 5′ LTR. We show that PCF11 interacts with the RNAPII-binding protein WDR82. Knock-down of PCF11 or WDR82 reactivated HIV-1 expression in latently infected cells. To silence HIV-1 transcription, PCF11 and WDR82 are specifically recruited at the promoter-proximal region of the provirus in an interdependent manner. Codepletion of PCF11 and WDR82 indicated that they act on the same pathway to repress HIV expression. These findings reveal PCF11/WDR82 as a PTT complex silencing HIV-1 expression in latently infected cells.

HIV-1 | transcription | latency

Significance

One of the major challenges to cure HIV is to better characterize the molecular mechanisms repressing viral transcription in latently infected cells. The silencing of latent HIV-1 provirus is the result of multifactorial transcriptional blocks that lead to the accumulation of short attenuated viral RNAs. Here, we identify the key role of a novel mammalian nuclear complex composed of PCF11 and WDR82 in the attenuation of HIV-1 transcription. The transcription terminator WDR82 associates with PCF11 at a proximal RNAPII (RNA Polymerase II) elongation checkpoint on HIV-1 promoter to enforce premature transcription termination. This study unravels a new mechanism of viral silencing and could shed light on a widespread process of transcription attenuation at the cellular level.

Despite the successful use of antiretroviral therapy, HIV-1 infection cannot be eradicated as the virus persists in cellular reservoirs such as latently infected CD4+T cells. These reservoirs are established early during the course of infection and can persist for an extended period of time. HIV-1 latency is operationally defined as the persistence of cells harboring replication-competent integrated proviruses that are transcriptionally silent. A generally accepted model for the establishment of latency posits that HIV-1 infects activated CD4+T cells that could then revert to a quiescent memory state leading to a refractory environment for viral transcription. However, numerous studies have shown that HIV-1 latency also arises in activated T-cells, indicating that cellular mechanisms are taking place to silence viral transcription rapidly after integration and independently of the cellular activation state (1).

In the absence of the viral transactivator Tat, HIV-1 transcription is repressed at the promoter-proximal region where paused RNA polymerase II (RNAPII) is associated with short nascent transcripts that have been detected in cells from infected individuals showing suppression of viremia (2–5). We and others have identified several cellular factors enforcing RNAPII pause at the HIV-1 promoter, including negative elongation factor (NELF), Integrator, SPT6/IWS1, LEDGF/p75, PAF1C, and BRD4 (6–11). The release of RNAPII pause into productive elongation is triggered by the binding of Tat to the transactivation response element (TAR), the RNA stem loop present in 5′ of all HIV-1 transcripts, that mediates the recruitment of active P-TEFb (positive transcription elongation factor)-containing complex (12). However, if the paused RNAPII complex does not shift to productive elongation, it can instead undergo premature transcription termination (PTT). In metazoans, PTT is a widespread mechanism that occurs near the TSS (transcription start site) or within the gene body and negatively regulates gene expression (13). For HIV-1, it has been shown that promoter-linked PTT is mediated by the nuclear endoribonuclease complex Microprocessor, together with XRN2 and the RNA/DNA helicase Senataxin (SETX), and by nuclear RNA surveillance factors (14, 15).

In addition to the RNAPII promoter-proximal pausing, a subsequent early elongation checkpoint that involves promoter-proximal polyadenylation signal (PAS) recognition can take place at the first stable nucleosome for cellular genes (16). PAS motifs are present in transcribed pre-mRNAs of coding genes and direct the 3′-end cleavage and polyadenylation (CPA) reaction via their recognition by the CPA complex (17, 18). As the integrated HIV provirus contains a duplicated long terminal repeat (LTR) at each end of its genome, the PAS that regulates the 3′ end processing of viral mRNAs is also located in the 5′ LTR and must be occluded during active transcription by a mechanism requiring the U1 snRNP binding to the downstream major 5′ splice site (5′ss) D1 (19, 20). However, the role of the proximal PAS in repressing HIV transcription in latently infected cells remains to be explored. Previous studies have shown that depletion of the CPA complex

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subunit PCF11 reactivates HIV expression in latently infected cells, indicating that it contributes to the repression of HIV basal transcription (21, 22). Still, little is known about the mechanisms involved in this process.

In mammalian cells, PCF11 interacts with CLP1 to form the cleavage factor II (CFIIm) complex (23). Unlike other subunits, PCF11 is present at substoichiometric levels in purified CPA complex (17, 24). PCF11 is required for both transcription termination (22, 24–28) and CPA activities (23, 29). These two functions involve separate domains of the protein. PCF11 interacts directly with the C-terminal domain (CTD) of the RNAPII via its CTD interaction domain (30, 31). This interaction is capable of dismantling the RNAPII elongation complex and causes transcription termination in vitro (22, 25, 26). The CTD of PCF11 contains two zinc fingers that bind RNA in a nonspecific manner and contributes to the recognition of 3′ processing substrates (32, 33). PCF11 also modulates gene expression by regulating alternative cleavage and polyadenylation. Interestingly, PCF11 expression is autoregulated by a PAS-dependent premature termination of its own transcription (24, 28).

Amongst the factors regulating PTT, WDR82 was recently shown to enforce early termination of long noncoding RNAs (lncRNAs), eRNAs and PROMPTS in mammalian cells (34–37). WDR82 is a WD repeats protein found in both the SET1 H3K4 methyltransferase complex and the PNUTS protein phosphatase 1 complex and was shown to bind to the RNAPII CTD phosphorylated on Serine 5 (Ser5P) (34, 36). Swd2, the yeast homolog of WDR82, is a component of the RNA 3′-end processing and termination subcomplex associated with Pta1 (APT) that is involved in sn/snoRNA transcription and termination (38, 39). WDR82 was also found to interact with ZC3H4, the human orthologue of Suppressor of sable [Su(s)], a nuclear RNA-binding protein that attenuates transcription at protein-coding genes containing inserted transposable elements downstream of their promoter region (40, 41). In human cells, the WDR82/ZC3H4 complex is recruited to regions with high RNAPII levels and was found to repress lncRNAs characterized by inefficient splicing of their first exon (35–37, 42, 43).

In this study, we aimed at understanding the mechanisms by which PCF11 represses HIV-1 basal transcription. Our results reveal that PCF11 forms a complex with the transcription terminator WDR82. Loss of PCF11 and WDR82 activates HIV-1 expression in latently infected cells, independently of the CPA machinery. These two factors interdependently associate with the HIV-1 promoter-proximal region and repress HIV-1 transcription.

Results

PCF11 Regulates HIV-1 Basal Transcription in a CPA- and PAS-Independent Manner. To identify whether the CPA machinery and PAS motifs play a role in the repression of HIV transcription, we first used a HeLa cell line containing a unique integrated copy of the Luciferase (Luc) gene under the transcriptional control of HIV-1 5′LTR promoter (HIV-1 LTR-Luc) (Fig. 1*A*). In the absence of the viral transactivator Tat, the basal activity of the viral promoter in this cell line is low but reactivable upon various stimuli. Thus, we analyzed the role of components of the 3′ end processing machinery in the repression of the HIV-1 LTR activity. The CPA complex is composed of cleavage and polyadenylation specificity factor (CPSF), cleavage factors I and II (CFIm and CFIIm), and cleavage stimulation factor (CstF). CPSF contains 6 proteins and binds directly to the A(A/U)UAAA hexamer within the PAS via WDR33 and CPSF4 and the RNA cleavage is accomplished by the endonuclease CPSF3 at the cleavage site (CS) often identified by the CA dinucleotide. CFIIm, containing PCF11 and CLP1, interacts transiently with the CPA complex (Fig. 1*B*) (18). Interestingly, CPA factors including PCF11 are also detected on nascent transcripts near the TSS suggesting that the CPA complex is not only implicated in 3′-end termination but also in promoter-associated premature termination (13, 24, 44). To analyze the role of components of the 3′ end processing machinery in the repression of the HIV-1 LTR activity, we depleted subunits of CFIm (CPSF6), CPSF (CPSF2, CPSF3, CPSF4, WDR33 and FIP1), CFIIm (PCF11, CLP1), CstF (CSTF2) as well as the scaffold protein SYMP using specific siRNAs. We observed that knockdown of CPA subunits affected expression of the others (Fig. 1*C*). In particular, knockdown of CPSF subunits was associated with an increased expression of PCF11, reflecting an auto-inhibition mechanism involving premature CPA and termination of its own transcript (24, 28) (*SI Appendix*, Fig. S1*A*). Knockdown of PCF11 resulted in a robust and significant increase of abundance of LTR-driven mRNA, whereas loss of the other CFIIm subunit CLP1 had almost no effect (Fig. 1*D*). In addition, individual depletions of CPSF6, subunits of the CPSF complex, CSTF2 or SYMP had little to no effect on LTR-driven mRNA expression. Notably, simultaneous depletion of CPSF2, CPSF3, and CPSF6 or CPSF4, WDR33, and FIP1 showed no additional effect on the LTR-driven mRNA levels, suggesting that there is no redundancy or compensatory effect between CPA subunits in the control of HIV-1 basal transcription (*SI Appendix*, Fig. S1*B*). To assess whether the effect of PCF11 depletion on the activation of LTR-driven expression was PAS-dependent, we introduced a single-point mutation to the PAS hexamer sequence (AAUAAA->AAGAAA: PASmut) that abolishes its interaction with the CPSF complex (45). We also tested a second construct containing a mutation eliminating the downstream CS (CA->GA: CSmut) (Fig. 1*E*). Of note, the WT, PASmut, and CSmut LTR-Luc constructs are inserted at the same locus in a parental HeLa Flp-IN cell line to avoid integration site position effect. LTR-driven nascent transcripts from the WT and PASmut constructs were quantified by nuclear run-on assay (NRO). We detected high levels of short nascent transcripts extending beyond the PAS motif and showed that transcription is progressively attenuated along the 5′ LTR. Furthermore, mutation of the PAS hexamer does not affect HIV-1 nascent transcripts profile, suggesting that the 5′ LTR PAS has very little effect on RNAPII processivity (*SI Appendix*, Fig. S1*C*). Depletion of PCF11 leads to a similar reactivation of LTR-driven expression in all three cell lines, indicating that the PAS motif and the CS are not required for PCF11-mediated repression of the HIV-1 promoter (Fig. 1 *F* and *G*). Taken together, our results suggest that amongst the subunits of the 3′ end processing complex, only PCF11 plays a significant role in the repression of LTR-driven expression activity. This PCF11-mediated repression occurs for the most part independently of the PAS motif and the CPA activity.

PCF11 Interacts with WDR82 in an RNA-Independent Manner. Having shown that the effect of PCF11 depletion on LTR-driven expression is both CPA- and PAS-independent, we sought to determine whether PCF11 could interact with other factors involved in transcriptional termination. First, we performed glycerol gradient sedimentation of HEK293T cell nuclear extracts to determine whether PCF11 exists in one or several complexes. Western blotting analysis of the collected fractions shows that PCF11 and CLP1 peak in fractions of lower molecular weight than the peak of CPSF subunits (Fig. 2*A*). In addition, RNAPII was found in fractions of similar molecular weight to CPSF subunits. Interestingly, we observed that WDR82 displays a sedimentation profile similar to that of CFIIm (Fig. 2*A*). WDR82 is the human orthologue of the yeast Swd2, a subunit of the APT complex with phosphatase activity

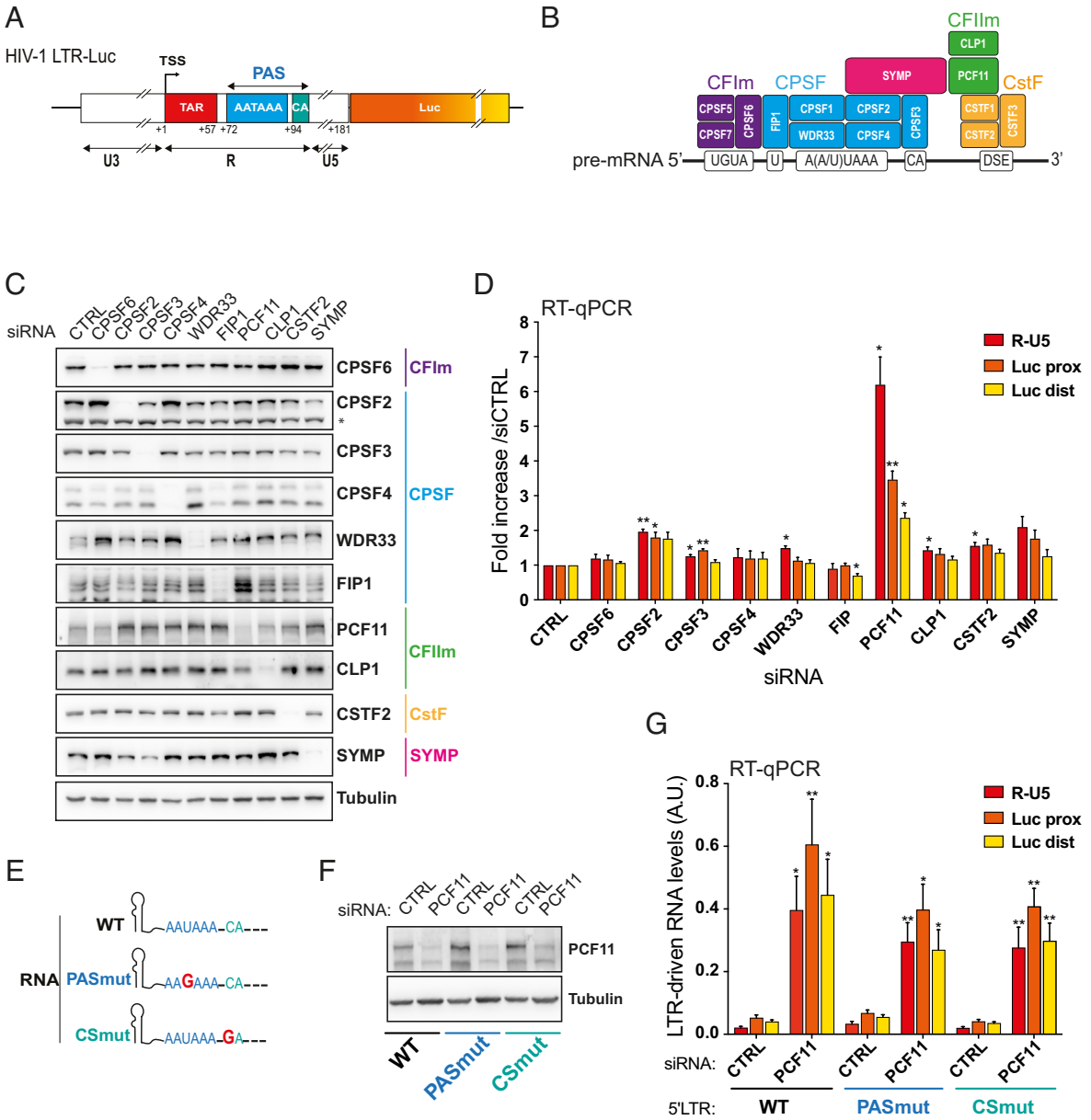

Fig. 1. PCF11 depletion stimulates HIV-1 LTR-driven RNA levels. (*A*) Schematic of the HIV-1 LTR Luc construct (HIV-1 LTR-Luc) integrated in the HeLa-LTR-Luc cell line, indicating TAR region, AATAAA hexamer, and CA dinucleotide site of cleavage from the 5′ PAS and the Luc coding region. Of note, HIV-1 mRNAs transcribed from the 5′ LTR are devoid of the UGUA sequence as the +1 TSS is positioned 3 nucleotides downstream of the corresponding TGTA motif. (*B*) Schematic of the mammalian 3′ end processing machinery, including CPA subcomplexes and *cis*-elements contributing to 3′ end processing. (*C*) HeLa-LTR-Luc cells were transfected with indicated siRNAs directed against CPA subunits or a control siRNA (CTRL). Cells were harvested at 72 h posttransfection and analyzed by western blotting using the indicated antibodies (* indicates nonspecific signal). (*D*) Cells described in (*C*) were analyzed for LTR-driven Luc RNA levels were measured by RT-qPCR upon each individual siRNA knockdown using 3 different pairs of primers amplifying the TAR-U5 region (R-U5), the 5′ proximal (Luc prox) region or the 3′ distal (Luc dist) region of the Luc gene. Results are presented as fold increase over siRNA control condition (siCTRL). Data represent mean ± SEM obtained from 3 independent experiments (n = 3). *P* values were calculated using student's one-sample *t* test. (*E*) Schematic of the LTR mutations introduced in the AATAAA hexamer (AATAAA->AAGAAA; PASmut) or CA dinucleotide site of cleavage (CA->GA; CSmut). Each of the HIV-1 LTR-Luc constructs (WT, PASmut and CSmut) was integrated at the same genomic location in the HeLa Flp-IN parental cell line, therefore avoiding position-effect differences of expression between the different HIV LTR-Luc constructs. (*F*) Western blotting showing the depletion of PCF11 in HeLa LTR-Luc cell lines 72 h after siRNA transfection. (*G*) LTR-driven Luc RNA levels were quantified by RT-qPCR after knockdown of PCF11 in HeLa LTR-Luc WT, PASmut, or CSmut. Data represent mean ± SEM obtained from 4 independent experiments (n = 4). *P* values were calculated using multiple unpaired *t* test.

that associates with the core CPF complex (38, 46). Because yeast Pcf11 was shown to interact directly with Swd2 in vitro (47, 48), we next investigated whether this interaction was conserved in human cells. We performed immunoprecipitation (IP) of endogenous PCF11 from the nuclear fraction of HEK293T cells and the presence of cofactors was assessed by western blotting. We found that PCF11 interacts robustly with its CFIIm cofactor CLP1, whereas association with CPSF subunits CPSF2, CPSF3, and WDR33 was weaker. In sharp contrast, we observed a strong enrichment for WDR82 with immunoprecipitated PCF11. The binding of PCF11 to CLP1 and WDR82 was not affected by RNAse A treatment, whereas its association with CPSF2, CPSF3, and WDR33 sharply decreased, indicating that PCF11 strongly associates with WDR82 through protein–protein interaction (Fig. 2*B*). Interestingly, IP of endogenous WDR82, revealed a robust association with CPSF2, CPSF3, CPSF4, and PCF11, indicating that WDR82 can also engage with the CPSF complex (Fig. 2*C*). To further characterize whether PCF11, WDR82, and the CPSFs can interact within the

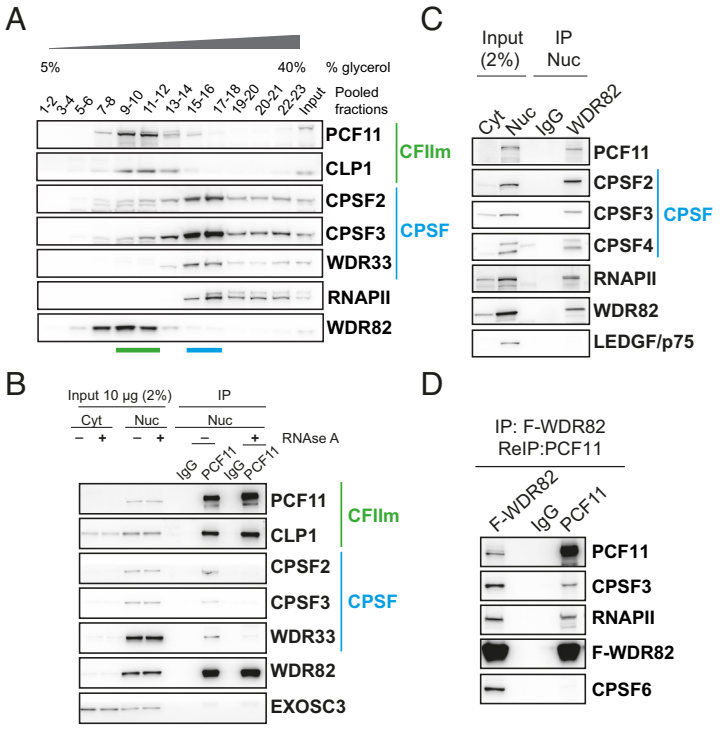

Fig. 2. PCF11 interacts with WDR82. (*A*) HEK293T cells nuclear extracts were separated by centrifugation through a 5 to 40% glycerol gradient. Fractions were collected, pooled 2 by 2 and analyzed by western blotting using the indicated antibodies. (*B*) HEK293T cells were first fractionated into cytoplasmic (Cyt) and nuclear extracts (Nuc). Nuclear extracts were further treated or not with RNAse A and subjected to PCF11 affinity IP. The anti-PCF11 immunoprecipitate was analyzed by western blotting using the indicated antibodies. (*C*) The HEK293T cells nuclear fraction treated with RNAse A was subjected to anti-WDR82 IP and analyzed by western blotting using the indicated antibodies. (*D*) Nuclear extracts from HEK293T cells transfected with a Flag-WDR82 (F-WDR82) expressing construct were subjected to anti-Flag IP then immunoprecipitated complexes were washed and eluted with an excess of Flag peptide and reimmunoprecipitated with an anti-PCF11 antibody. The resulting double immunoprecipitated complex was analyzed by western blotting using the indicated antibodies.

same complex, Flag-WDR82 was overexpressed in HEK293T cells, immunoprecipitated using an anti-Flag antibody and subjected to reciprocal IP using an anti-PCF11 antibody. Flag-WDR82 and CPSF3 were found to belong to the same complex, together with the RNAPII, whereas the CFIm subunit CPSF6 was not present (Fig. 2*D*). These results indicate that WDR82 and PCF11 associate within the same complex that also engages with CPSF subunits.

PCF11 and WDR82 Knockdown Increases HIV-1 Reactivation from Latently Infected Cells. To further address the role of PCF11, WDR82, and the CPA complex in HIV-1 latent provirus silencing, we first depleted CPSF (CPSF2, CPSF3, and FIP1) subunits, CFIIm (PCF11, CLP1) subunits, SYMP or WDR82 with specific shRNAs in the JLat A1 cell line containing a single integrated copy of LTR-Tat-GFP-LTR HIV minigenome (Fig. 3*A*) (49). We measured transcriptional reactivation from latency by monitoring the percentage of cells reexpressing GFP at day 6 and day 9 posttransduction (d.p.t.). Flow cytometry analysis revealed that depletion of CPSF subunits had no significant effect on basal HIV-1 expression. In contrast, we observed a strong reactivation of HIV-1 expression upon depletion of PCF11 and WDR82, and SYMP to a lesser extent (Fig. 3 *B* and *C*). Similar reactivation of HIV expression was observed when JLat A1 cells were transduced with a second shRNA targeting PCF11 (*SI Appendix*, Fig. S2*A*), or with a sgRNA against WDR82 using the CRISPR-Cas9 system (*SI Appendix*, Fig. S2*B*). We further confirmed that depletion of PCF11 and WDR82 significantly reactivates HIV expression in two other models of latency, JLat A2 cells that also contain a single integrated copy of the HIV minigenome, and JLat 10.6 cells that contain a nearly complete latent provirus (Fig. 3*E*) (49). Thus, we found that WDR82 and PCF11 are both involved in the repression of HIV-1 expression from different integration sites in latently infected cells.

PCF11 and WDR82 Are Required for HIV-1 Post-Integrative Transcriptional Silencing. To further assess the role of PCF11 and WDR82 on HIV latency in infected CD4+ cells, we used the dual-labeled HIV-GKO virus containing an LTR-driven GFP and expressing the mKO2 fluorescent protein under the constitutive EF1a promoter (50). Infection with HIV-GKO allows the quantification of both productively (GFP+ mKO2+) and latently (GFP– mKO2+) infected cells (Fig. 4*A*). Jurkat cells were first infected with HIV-GKO, then transduced with shRNAs targeting either PCF11 or WDR82 at 7 d post-infection, and analyzed by western blotting (Fig. 4*B*) and flow-cytometry 6 d posttransduction (Fig. 4 *C* and *D*). The total percentage of mKO2 expressing cells (sum of the percentages of GFP+ mKO2+ and GFP– mKO2+cells) upon PCF11 or WDR82 depletion was comparable to control conditions, indicating that their depletion does not impact the proportion of cells containing an integrated provirus (Fig. 4*C*). Amongst infected cells, PCF11 KD significantly increased the number of productive cells (48.5% GFP+ cells in shPCF11, compared to 29.0% for shCTRL), which correlated with a decrease in the proportion of latent cells. WDR82 KD also resulted in a significant increase of the percentage of productive cells (37.2% GFP+ cells in shWDR82) (Fig. 4*D*). To further quantify the proportion of latent cells reactivated after PCF11 or WDR82 KD, cells were infected with HIV-GKO for 4 d before sorting the latent population (GFP– mKO2+). These latently infected cells were then transduced with shRNA targeting PCF11 or WDR82 and HIV reactivation was monitored by measuring the percentage of GFP+ cells by FACS. Knocking down PCF11 or WDR82 lead to an increase of the percentage of GFP+ expressing cells (from 1.9% in shCTRL to 10.1 % and 4.8% in shPCF11 and shWDR82, respectively), indicating that both factors are involved in the maintenance of HIV-1 latency (Fig. 4 *E* and *F*). Of note, knocking down PCF11 in primary CD4+T cells resulted in high

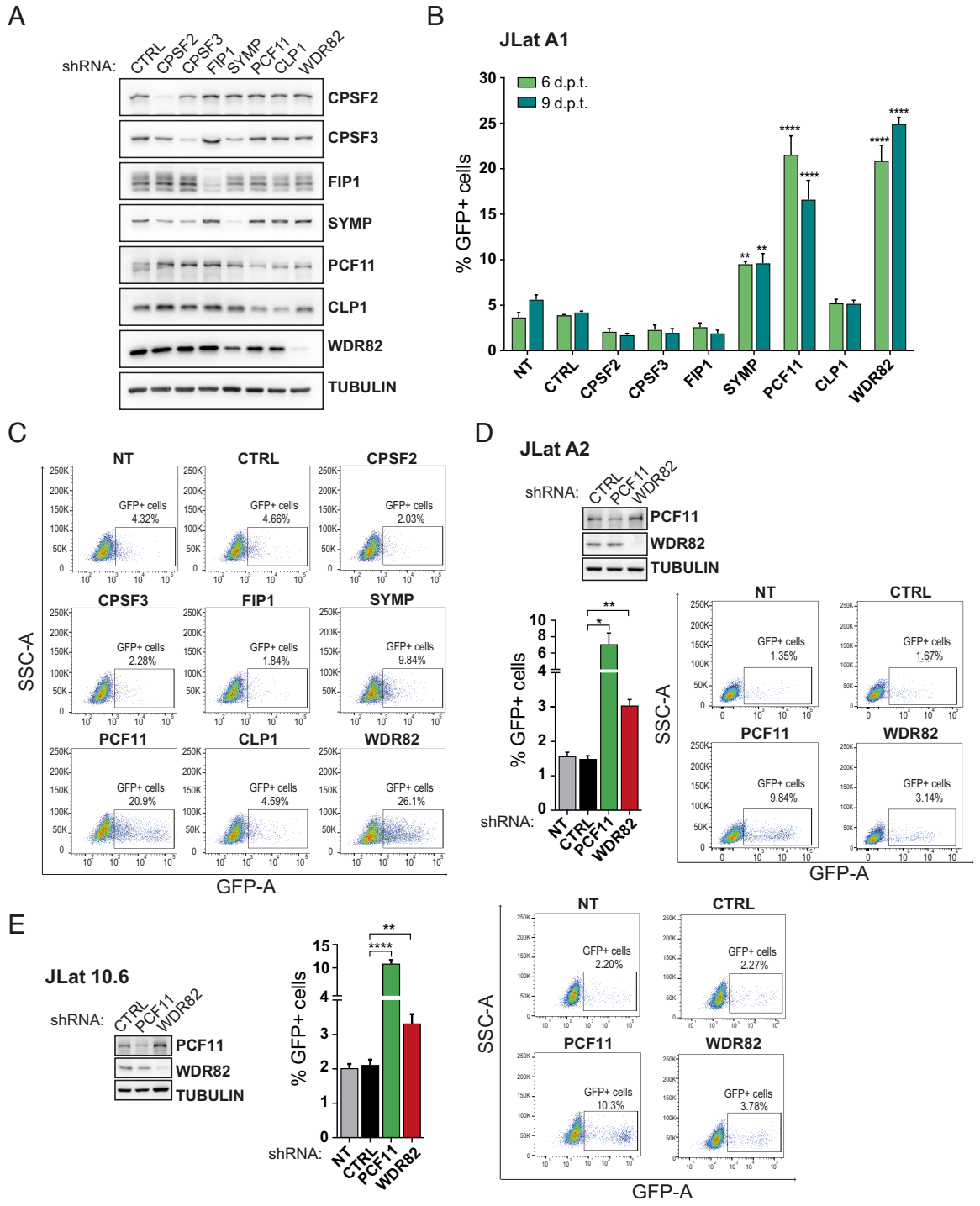

Fig. 3. Depletion of PCF11 and WDR82 reactivates latent HIV-1 expression. (*A*) JLat A1 cells were transduced with lentivectors expressing a shRNA targeting the indicated CPA subunits or a control (CTRL) shRNA. Cells were harvested at 6 d posttransduction and analyzed by western blotting using the indicated antibodies. (*B*) HIV-1 proviral reactivation was monitored in JLat A1 from (*A*) by flow cytometry analysis of GFP+ cells at day 6 and day 9 post transduction (d.p.t.) of the indicated shRNA or in nontransduced cells (NT). Data represent mean ± SEM obtained from 5 independent experiments (n = 5). *P* values were calculated using 2-way ANOVA test. (*C*) Representative FACS histograms and quantification of GFP+ cells at 6 d posttransduction. (*D*) Effect of PCF11 and WDR82 knockdown on HIV expression at 9 d posttransduction was measured by FACS quantification of GFP+ cells in JLat A2 cells containing a HIV minigenome. Data represent mean ± SEM obtained from 3 independent experiments (n = 3). (*E*) FACS quantification of GFP+ JLat 10.6 cells that contain a near full-length proviral genome harboring a GFP gene. Data represent mean ± SEM obtained from 6 independent experiments (n = 6). *P* values were calculated using unpaired *t* test.

levels of cell toxicity which prevented us from further studying its role in silencing HIV expression in this model. Thus, our results show that both PCF11 and WDR82 depletion reactivates latent proviruses expression from heterogeneous integration sites, suggesting that these 2 factors are involved in the maintenance of post-integration HIV-1 latency.

PCF11 and WDR82 Associate with the Silent Provirus and Repress Its Transcription. To assess whether PCF11 and WDR82 are directly implicated in the repression of the integrated latent provirus, we performed chromatin IP (ChIP) experiments in JLat A1 cells using specific primers (Fig. 5*A*). As expected, paused RNAPII was found to accumulate at the TSS and promoter-proximal (Prox) regions of

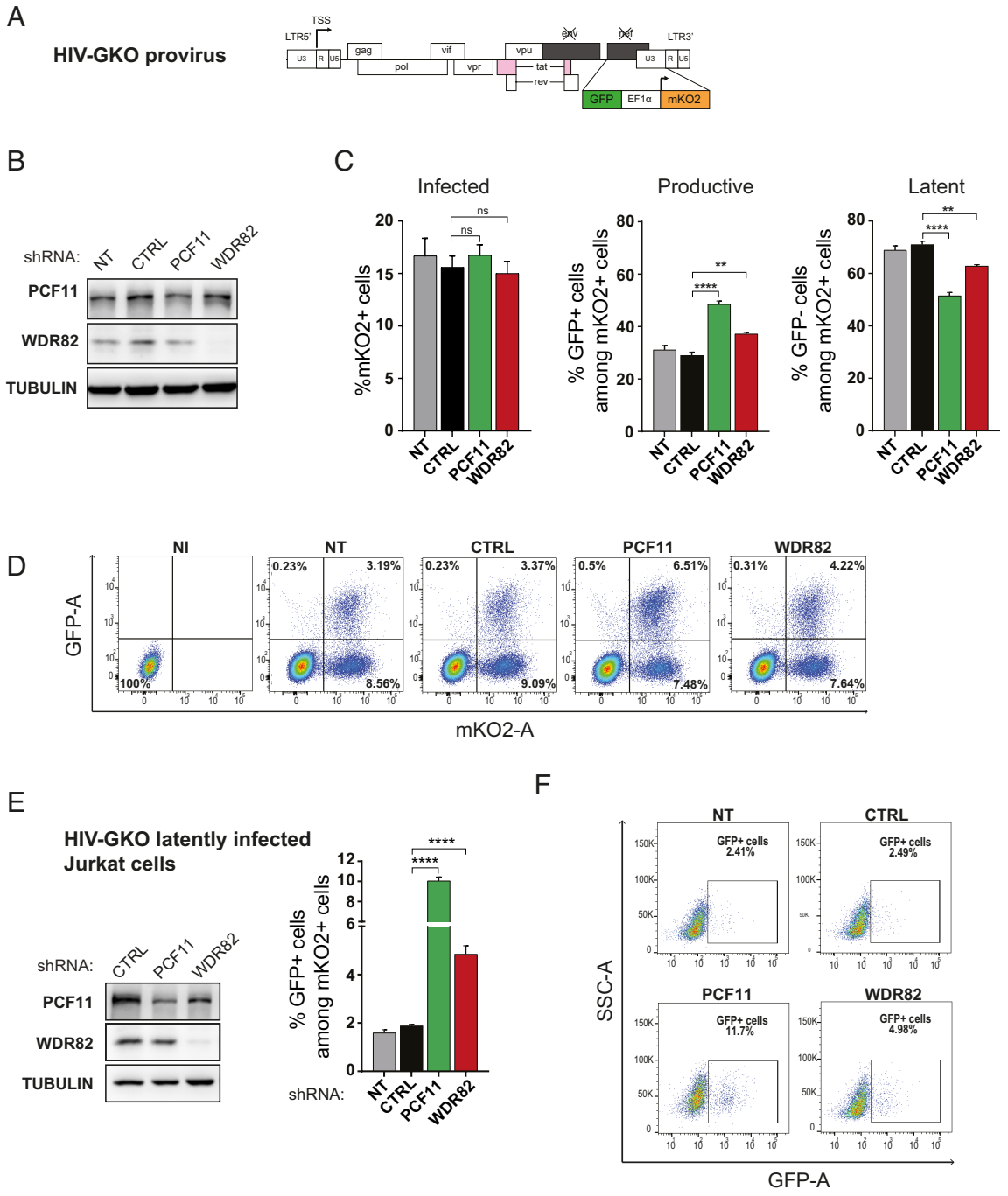

Fig. 4. PCF11 and WDR82 are involved in the maintenance of HIV-1 latency. (*A*) Schematic representation of HIV-GKO reporter virus. (*B*) Jurkat cells were infected with HIV-GKO and cultured for 7 d. Infected cells were then transduced with lentivectors expressing the indicated shRNAs, harvested at 6 d posttransduction and analyzed by western blotting using the indicated antibodies. (*C*) Cells from (*B*) were analyzed by FACS at 6 d posttransduction. Quantifications are shown as mean ± SEM obtained from 4 independent experiments (n = 4). *P* values were calculated using unpaired *t* test. (*D*) Representative FACS histograms and quantification of productively (GFP+ mKO2+) and latently (GFP− mKO2+) infected cells. (*E*) Jurkat cells were infected with HIV-GKO virus. At 4 d postinfection, the population of latently infected cells was sorted, maintained in culture for 6 wk to stabilize the latent population and transduced with lentivectors expressing shRNAs targeting PCF11 or WDR82, or a control shRNA (CTRL). The percentage of latent cells reactivated by the KD of PCF11 or WDR82 was quantified by FACS. Data represent mean ± SEM obtained from 4 independent experiments (n = 4). *P* values were calculated using unpaired *t* test. (*F*) Representative FACS histograms and quantification of reactivated cells (GFP+).

the HIV-1 promoter in latent cells (TSS and Prox, Fig. 5 *B*, *Upper*). PCF11 displayed a similar profile to the RNAPII while WDR82 specifically accumulated at the promoter-proximal region (Fig. 5 *B*, *Middle* and *Lower*). Thus, our results indicate that PCF11 and WDR82 are recruited at the promoter-proximal region in latent cells. Given that PCF11 and WDR82 were previously shown to regulate PTT at cellular genes and extragenic regions (24, 51), we performed 4-thiouridine (4sU) metabolic labelling of newly synthesized transcripts to determine the effect of their depletion on LTR-driven transcription in JLat A1 cells. Upon PCF11 and WDR82 depletion, we observed a robust increase in levels of nascent transcripts that was similar to the increase in levels of steady-state mRNAs, indicating that PCF11 and WDR82 act mainly as transcriptional repressors of the HIV-1 promoter (Fig. 5*C*).

We next analyzed the interdependence between PCF11 and WDR82 recruitment at the integrated latent provirus. Of note,

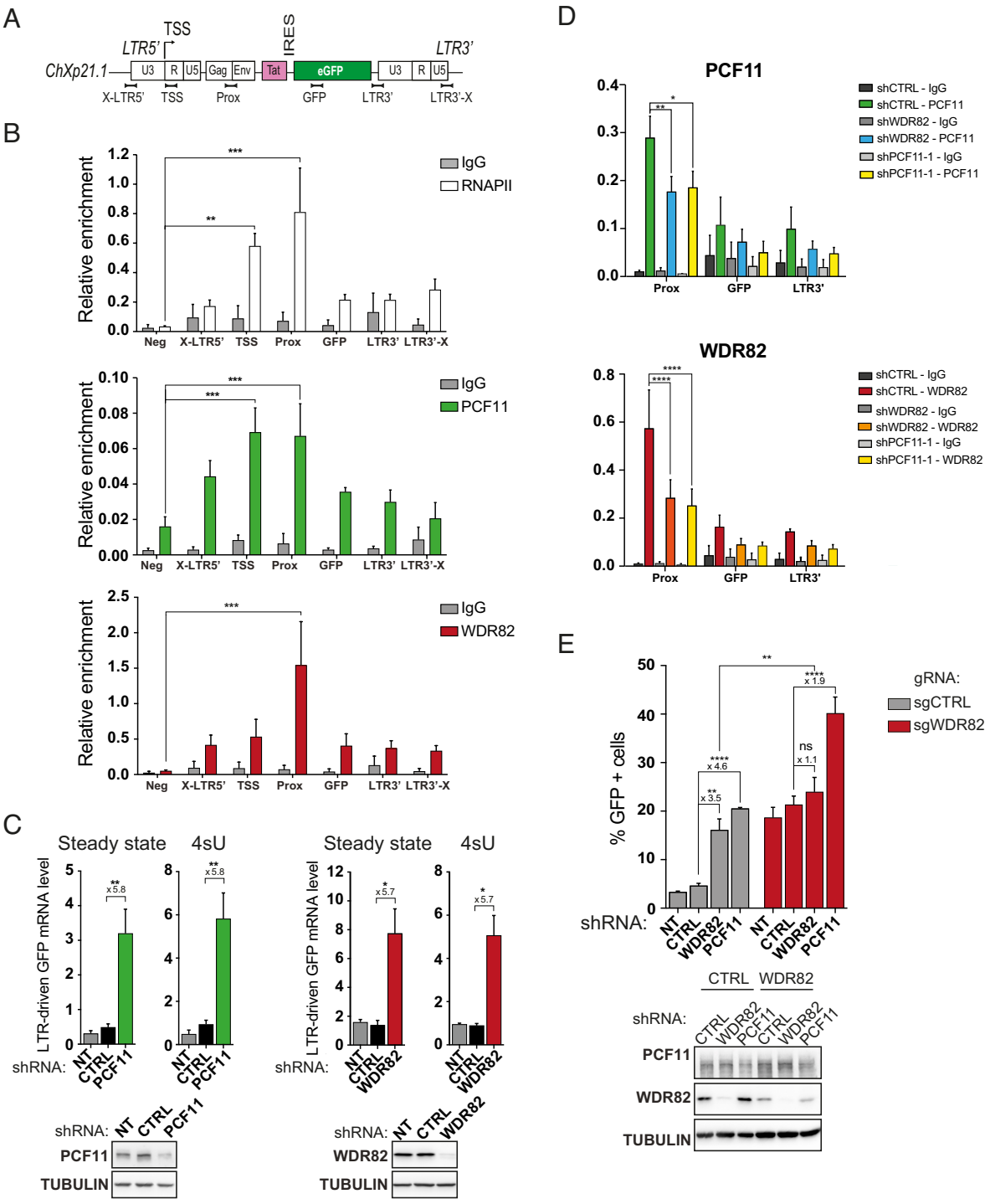

Fig. 5. PCF11 and WDR82 are required for transcriptional inhibition of HIV latent provirus. (*A*) Schema indicating the position of primers on the provirus in JLat A1 cells used for the ChIP-qPCR analysis. (*B*) RNAPII, WDR82, and PCF11 occupancy at the latent provirus in JLat A1 cells was measured by ChIP assay using indicated primers. Data represent mean ± SEM obtained from 4 independent experiments (n = 4). *P* values were calculated using 2-way ANOVA test with multiple comparisons. (*C*) Effect of PCF11 or WDR82 depletion on HIV transcription was monitored in JLat A1 cells by isolation and RT-qPCR quantification of newly transcribed HIV-1 RNA using 4-thiouridine (4sU) metabolic labelling. Steady-state levels of HIV-1 RNA were quantified by RT-qPCR on whole-cell RNA before 4sU isolation. Data represent mean ± SEM obtained from 3 or 4 independent experiments (WDR82, n = 3; PCF11, n = 4). *P* values were calculated using unpaired *t* test. (*D*) Impact of WDR82 or PCF11 depletion on their mutual recruitment on HIV-1 provirus was measured by ChIP assay in JLat A1 cells transduced for 6 d with a lentivector expressing control shRNA (shCTRL) or shRNA targeting PCF11 (shPCF11) or WDR82 (shWDR82). Data represent mean ± SEM obtained from 4 independent experiments (n = 4). *P* values were calculated using 2-way ANOVA test with multiple comparisons. (*E*) JLat A1 cells were transfected with sgCTRL or sgWDR82, as indicated, then transduced with shWDR82 or shPCF11 and the effect on HIV-1 reactivation was monitored by FACS 6 d after shRNA transduction. Data represent mean ± SEM obtained from 4 independent experiments (n = 4). *P* values were calculated using 2-way ANOVA test with multiple comparisons. shPCF11 fold increase over shCTRL in sgCTRL and sgWDR82 cells were compared using unpaired *t* test.

because the HIV-1-based lentivector shares the same LTR U5 sequence as the integrated HIV-1 minigenome, the TSS primers within this region cannot be used for the ChIP-qPCR assay. ChIP analysis showed that PCF11 and WDR82 occupancy significantly decreases at the promoter-proximal region upon the KD of each other, suggesting that both factors are required for the recruitment

of the PCF11/WDR82 complex (Fig. 5*D*). Next, WDR82 was KD in JLat A1 cells using WDR82-targeting sgRNA:Cas9 ribonucleotide complex. We show that partial loss of WDR82 specifically reduced PCF11 occupancy at the promoter-proximal region, without affecting its recruitment at the TSS. Remarkably, RNAPII recruitment to the promoter-proximal region was significantly increased while its level at the TSS was unaffected (*SI Appendix*, Fig. S3*A*). To further determine whether PCF11 and WDR82 are involved in the same pathway to repress HIV-1 expression or whether they act separately, we simultaneously knocked down the expression of both proteins in JLat A1 cells. In cells depleted for WDR82 expression, silencing PCF11 using shRNA increased HIV-1 expression by less than twofold compared to the control (Fig. 5*E*). This limited increase is below the level expected for an additive effect, suggesting that PCF11-mediated repression of HIV transcription requires, at least partially, the presence of WDR82. Taken together, these findings suggest that PCF11 and WDR82 cooperate at the promoter-proximal region downstream of the TSS to repress HIV-1 transcription in latently infected cells (*SI Appendix*, Fig. S3*B*).

Discussion

As for cellular promoters, RNAPII proximal pausing near TSS at the HIV 5′ LTR promoter is a rate-limiting regulatory checkpoint that can lead to PTT if paused RNAPII complexes do not shift to productive elongation and therefore contributes to HIV latency. Here, we present evidence that PCF11 and WDR82 interact at the promoter-proximal region downstream of the TSS on HIV-1 provirus to enforce transcriptional silencing in latently infected cells, independently of the CPA machinery. This model is based on the following observations: i) PCF11-mediated repression of HIV-1 transcription does not require the CPA complex nor the PAS motif present at the 5′ LTR. ii) PCF11 interacts with the termination factor WDR82. iii) PCF11 and WDR82 are involved in the repression of HIV-1 expression in different models of latently infected cells. iv) PCF11 and WDR82 control the maintenance of HIV latency during de novo infection in CD4+T lymphocytes. v) PCF11 and WDR82 associate with the promoter-proximal region of the latent provirus in an interdependent manner. vi) Knocking down PCF11 or WDR82 derepresses HIV-1 transcription in latently infected cells. vii) PCF11 and WDR82 act on the same pathway to silence HIV-1 expression.

Although termination factors have been reported to attenuate levels of promoter-associated RNAs via the recognition of cryptic PAS (16, 44, 52, 53), our results highlight a role for PCF11 in silencing transcription independently of other subunits of the CPA complex, further supporting a noncanonical role for PCF11 in regulating PTT at the HIV-1 promoter. In human cells, the binding profile of PCF11 at protein-coding genes shows an enrichment at both TSSs and 3′ends (24). This is consistent with observations showing that, in addition to its role in 3′ end transcription termination and CPA efficiency at protein coding genes, PCF11 is also involved in repressing TSS-associated noncoding transcription (24, 54). Interestingly, PCF11 also autoregulates its own expression by a premature termination mechanism. However, this effect requires PAS motifs within its first intron and is regulated by the CPA complex (24, 28). In sharp contrast, we found that repression of HIV-1 transcription by PCF11 involves a different mode of action as it is independent of the AATAAA poly(A) signal and CS.

Here, we show that PCF11 interacts with WDR82, a RNAPII CTD-binding factor shared by several protein complexes, including PNUTS, Restrictor, and SET1 H3K4 methyltransferases (34, 35, 37, 41, 55). In latently infected cells, PCF11 occupancy profile at the provirus indicates that it likely interacts with the promoter-proximal associated RNAPII complex that contains high levels of phosphorylated Ser5P CTD. However, as a subunit of the CPA complex that functions at 3′ end of genes, PCF11 was shown to bind preferentially to the CTD of the RNAPII phosphorylated on Serine 2 (Ser2P) that marks the latter stages of transcription elongation and termination (30, 31, 56). WDR82 is structured as a circular beta-propeller composed of seven WD40 repeats and binds directly to Ser5P-CTD (36, 57, 58). We propose that WDR82 directly participates in the recruitment of PCF11 to paused RNAPII at the HIV-1 promoter-proximal region. We observed that occupancy of PCF11 and WDR82 is co-dependent of the other suggesting that the interaction between PCF11 and WDR82 is important for their recruitment to the CTD of RNAPII at the promoter-proximal region. This might reflect previous observations showing that WDR82 alone binds poorly to the Ser5P-CTD RNAPII CTD, but this interaction is strongly enhanced when it is engaged within the SET1 or the ZWC complex (36, 57, 58).

Our results support previous observations showing that, in addition to a major pause site at the TAR region, RNAPII also accumulates at additional downstream sites on the latent HIV-1 LTR (7). Together with DSIF, NELF binds to the paused RNAPII and repressed HIV-1 transcription in latent cells (6–8). Part of this effect depends on the ability of NELF to facilitate PCF11 recruitment at the TSS on HIV-1 LTR and repress its expression (21). However, it has been shown that NELF does not affect RNAPII occupancy at additional downstream pause sites at the HIV-1 promoter, suggesting that the downstream transcriptional block does not depend on NELF (7). Similar to cellular genes, NELF function could regulate an early LTR TSS-associated step that is distinct from RNAPII pause–release mediated by P-TEFb (59). Instead, our work shows that depletion of WDR82 alleviates a PCF11-dependent transcription block downstream of the TSS, at the promoter-proximal region, suggesting a role at a step distinct from that regulated by NELF. Thus, our results indicate that PCF11 is recruited to the latent provirus by two distinct mechanisms: i) independently of WDR82 at the TSS, likely via its interaction with NELF as suggested by Natarajan et al. (21) and ii) via WDR82 at the promoter-proximal region (*SI Appendix*, Fig. S3*B*).

Interestingly, a direct in vitro interaction has been reported in yeast between Pcf11 and the WDR82 homolog Swd2 (47, 48). Swd2 is part of the APT subcomplex involved in the transcription termination of snoRNAs that are mostly encoded by independent transcription units in yeast (38, 39, 60–63). Mature snoRNAs are not polyadenylated and therefore do not require subunits of the core cleavage and polyadenylation factor (CPF) complex for their biogenesis. Instead, Pcf11 cooperates with both APT and the ncRNA Ndr1–Nab3–Sen1 (NNS) complex at 3′ ends of snoRNA to stimulate RNAPII termination (39, 62, 63). While the NNS complex is specific to yeast and not present in human cells, a role of PCF11/WDR82 in HIV-1 promoter-proximal transcription termination may share some similarity to the Pcf11/APT/NNS pathway involved in yeast snoRNAs termination (64).

Importantly, by using NRO approaches, we report that levels of short nascent transcripts that accumulate at the 5′ end of the viral gene progressively decrease along the 5′ LTR region, reflecting transcription attenuation rather than site-specific PAS-dependent cleavage. These results show that a mechanism of PTT takes place at the HIV promoter and occurs independently of the PAS, suggesting that these nascent viral RNAs are nonadenylated at their 3′ end. In addition, our study shows that PCF11 represses HIV expression at the transcriptional level and independently of other CPA subunits. However, we cannot rule out that other mechanisms

involving the cleavage of nascent transcripts could cooperate with PCF11/WDR82 to promote RNAPII termination at the HIV-1 promoter. The Microprocessor complex, composed of Drosha and DGCR8, cleaves HIV-1 TAR and recruits the 5′-3′ RNA exonuclease XRN2, the RNA/DNA helicase SETX, and the nuclear exosome subunit RRP6 to induce PTT at the 5′ LTR (14). In yeast, Pcf11 binding to Ser2P CTD-RNAPII facilitates the subsequent recruitment of SETX homolog Sen1 to the RNAPII. Sen1 then translocates along the nascent transcript and unwinds the RNA:DNA hybrid to promote RNAPII release (62). Similarly, under basal conditions, PCF11 could facilitate SETX occupancy at the HIV-1 promoter and further stimulate RNAPII removal from DNA to terminate transcription.

While PAS-dependent cleavage of nascent viral transcripts by the CPA complex is not required to repress HIV-1 transcription, our results show that WDR82/PCF11 can also interact with other CPA subunits. Recent studies have highlighted a role of WDR82 in transcription termination through its cooperation with nuclear exosome targeting complexes (43, 65). Interestingly, WDR82 was also found to cooperate with CPSF and human silencing hub complex to favor 3′-end transcription termination (66). As PCF11 is a master regulator of transcription termination, the identification of a novel complex containing PCF11 and WDR82 suggests an unsuspected relationship between subunits of the CPA pathway and WDR82-mediated premature termination. Further studies will determine whether PCF11/WDR82 could be involved in CPA-dependent termination at protein-coding and noncoding genes.

Materials and Methods

For detailed protocols on cell culture, transfection of small interfering RNAs in adherent cells, production and transduction of pseudotyped viruses, nucleofection of sgRNA:Cas9 and siRNA in nonadherent cells, glycerol gradient sedimentation, nuclear proteins IPs, Western blot analysis, total RNA extraction, reverse transcription and quantitative PCR analysis, NRO assay, 4sU labelling of newly synthesized RNAs, ChIP, flow cytometry analysis and cell sorting, and statistical analysis, see *SI Appendix, Materials and Methods*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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