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RESEARCH PAPER

CBP80/20-dependent translation initiation factor (CTIF) inhibits HIV-1 Gag synthesis by targeting the function of the viral protein Rev

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ABSTRACT

Translation initiation of the human immunodeficiency virus type-1 (HIV-1) full-length RNA has been shown to occur through cap-dependent and IRES-driven mechanisms. Previous studies suggested that the nuclear cap-binding complex (CBC) rather than eIF4E drives cap-dependent translation of the full-length RNA and we have recently reported that the CBC subunit CBP80 supports the function of the viral protein Rev during nuclear export and translation of this viral transcript. Ribosome recruitment during CBC-dependent translation of cellular mRNAs relies on the activity CBP80/20 translation initiation factor (CTIF), which bridges CBP80 and the 40S ribosomal subunit through interactions with eIF3g. Here, we report that CTIF inhibits HIV-1 and HIV-2 Gag synthesis from the full-length RNA. Our results indicate that CTIF associates with HIV-1 Rev through its N-terminal domain and is recruited onto the full-length RNA ribonucleoprotein complex in order to interfere with Gag synthesis. We also demonstrate that CTIF induces the cytoplasmic accumulation of Rev impeding the association of the viral protein with CBP80. We finally show that Rev interferes with the association of CTIF with CBP80 indicating that CTIF and Rev compete for the CBC subunit.

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Introduction

Ribosome recruitment onto the HIV-1 full-length RNA has been shown to occur through cap-dependent and cap-independent mechanisms [1–4]. Initial studies showed that the highly structured 5′-untranslated region (5′-UTR) present within the full-length RNA was inhibitory for translation in cell-free translation systems suggesting that cap-dependent ribosome scanning was not an efficient mechanism of translation initiation operating in HIV-1 transcripts [5–7]. In agreement with this idea, several groups have reported that the 5′-UTR of the HIV-1 full-length RNA harbors a cell cycle-dependent internal ribosome entry site [8–12]. However, more recent reports including ours have shown that cap-dependent ribosome scanning operates on the full-length RNA in a mechanism supported by the host DEAD-box RNA helicase DDX3 but independent of the cap-binding protein eIF4E [13–17]. In this sense, HIV-1 full-length RNA translation was also proposed to occur in a nuclear cap-binding complex (CBC)-dependent fashion during the virally induced arrest of the cell cycle [18]. Consistent with these observations, we have recently reported that the CBC subunit CBP80 (NCBP1) promotes nuclear export and translation of the HIV-1 full-length RNA by supporting the functions of the viral protein Rev on these processes [19].

The CBC is recruited onto the nascent transcript early during transcription and has been involved in every step of mRNA metabolism from transcription to splicing, nuclear export, translation and decay [20]. Two different CBCs have been described so far in mammalian well) were transfected with, the canonical CBC composed by CBP20 (NCBP2) and CBP80 and a recently described CBC in which CBP80 interacts with the newly discovered cap-binding protein NCBP3 [21,22]. CBC-dependent translation has mostly been associated to the mRNA quality control mechanism known as nonsense-mediated mRNA decay (NMD), which triggers the degradation of mRNA harboring premature termination codons [23–25].

Translation initiation driven by the CBC relies on the CBP20/80 translation initiation factor (CTIF), which binds to the CBP80 subunit and interacts with eIF3 to promote 40S small ribosomal subunit recruitment [26,27]. CTIF also recruits the DEAD-box RNA helicase eIF4AIII, which drives the unwinding of RNA structures on the 5′-UTR of mRNA translated through a CBC-dependent mechanism [28]. More recently, it was shown that CTIF links translation and autophagy by driving misfolded polypeptides to aggresomes [29]. Here, we report that CTIF inhibits HIV-1 and HIV-2 Gag synthesis. We also present evidence showing that CTIF interacts with HIV-1 Rev through its CBP80-binding N-terminal

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domain and is incorporated into the full-length RNA ribonucleoprotein complex most probably at the 5′-UTR. We also demonstrate that CTIF induces the cytoplasmic accumulation of Rev avoiding its massive nuclear localization. Finally, we show that CTIF interferes with the association between the viral protein Rev and CBP80, and in concordance with this, Rev interferes with the association between CTIF and CBP80.

Together, our data provide evidence for a novel inhibition of HIV-1 and HIV-2 replication by CTIF, which is exerted at the level of Gag synthesis and targets the function of human lentiviral Rev proteins.

Materials and methods

DNA constructs

The pNL4.3-ΔEnv, pNL4.3R, pNL4.3R-ΔRev, pNL4.3-EGFP, pNL4.3R-CMV-ΔU3/U5, pROD10R and pNCAC proviral vectors were previously described [19,30,31]. The pcDNA3-Flag-CTIF, pcDNA3-Flag-CTIF-N-ter (1–305), pcDNA3-Flag -CTIF-C-ter (306–598) and pCMV-myc-CBP80, pcDNA-d2EGFP, pcDNA3-Renilla, pCIneo-Renilla, pCMV-myc-CBP80, pcDNA-V5-CBP80 and pEGFP-Rev were previously described [19,26,27,31,32].

Cell culture and DNA transfection

HeLa and HEK293T well) were transfected with were maintained in DMEM (Gibco) supplemented with 10% FBS (Pan-Biotech) at 37°C and a 5% CO_2 atmosphere. The Jurkat clone E6-1 was maintained in RPMI 1640 (Gibco) supplemented with 10% FBS (Pan-Biotech) and antibiotics (Sigma-Aldrich) at 37°C and a 5% CO_2 atmosphere. Cells were transfected using linear PEI ~25,000 Da (Polysciences) as described previously [19,31].

Analysis of Renilla and firefly luciferase activities

Renilla luciferase activity was determined using the Renilla Reporter Assay System (Promega) in a GloMax® 96 microplate luminometer (Promega).

Western blot

Cell extracts were subjected to 10% SDS-PAGE and transferred to an Amersham Hybond™-P membrane (GE Healthcare). Membranes were incubated with an anti-HIV-1 p24 monoclonal antibody diluted to 1/1000 (NIH AIDS Reagents Catalog number #3537) [33–35], a rabbit anti-CTIF antibody (ThermoFisher Scientific) diluted to 1/250, a mouse anti-GAPDH antibody (Santa Cruz Biotechnologies) diluted to 1/5000, a rabbit anti-MLV CA (kindly provided by Dr. Gloria Arriagada, UNAB, Chile) diluted to 1/1000, a rabbit anti-Flag antibody (Sigma-Aldrich) diluted to 1/1000, a mouse anti-F protein of hRSV (Santa Cruz Biotechnologies) diluted to 1/500, a mouse anti-puromycin antibody (Milipore, clone 12D10) diluted to 1/1000 and anti-actin antibody (Santa Cruz Biotechnologies) diluted to 1/750. Upon incubation with the corresponding HRP-conjugated secondary antibody (Jackson ImmunoResearch) diluted to 1/5000, membranes were analyzed with the Pierce® ECL substrate or the SuperSignal™ West femto (ThermoFisher Scientific) using a Mini HD9 Western blot Imaging System (UVItec).

Generation of CTIF knockdown cell lines

Lentiviral particles carrying an anti-CTIF shRNA were produced in HEK293T well) were transfected with by transfecting a commercially available pLKO.1 vector containing the shRNA sequence targeting the 3′-UTR of the CTIF mRNA (Sigma-Aldrich) together with the pCMV-VSV-G (kindly provided by Dr. Philippe Mangeot, INSERM, France) and psPax2 (Addgene #12260) vectors. Supernatants were collected at 48 hours post transfection (hpt), cleared through a 0.22 µm filter and used to transduce HeLa well) were transfected with. The medium containing lentiviral particles was replaced by fresh DMEM containing puromycin (1 µg/mL) at 48 hours post-transduction and well) were transfected with were selected for 10 days at 37°C and 5% CO_2. CTIF knockdown was evaluated by Western blot as mentioned above. Lentiviral particles carrying a scramble shRNA were used in parallel as a control. The sequences of CTIF and scramble shRNAs are in Supplementary Table 1.

Pseudotyped virus production and infection assays

For the analysis of endogenous CTIF in infected CD4 + T-well) were transfected with (Jurkat E1), HEK293T cells were co-transfected with the pNL4.3-ΔEnv provirus and pCMV-VSV-G and cell supernatants were collected at 72 hour post-transfection, cleared through a 0.22 µm filter and used to infect Jurkat cells mixing in a proportion 1:1 volume of virus:volume of cells. Infected cells were recovered at 8, 12, 20, 24, 48, 72, 96 hours post-infection and washed with 300 µl of PBS and pelleted at 500 x g for 5 min at 4°C. Cells were resuspended in 100 µl of ice cooled lysis Buffer I (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 7.5 and protease inhibitor [Roche]), vortexed for 5 seconds, incubated for 30 min at 4°C under agitation and centrifuged at 12,000 rpm for 20 min at 4°C. In order to check viral production under CTIF overexpression, HEK293T cells were co-transfected with the pNL4.3-EGFP provirus and pCMV-VSV-G together with pcDNA-d2EGFP or pcDNA3-Flag-CTIF. Cells supernatants collection and infection was performed as described above. At 48 hours post-infection, cells were washed with 300 µl of 1x PBS and pelleted at 500 x g for 5 min at 4°C. Cells were resuspended in 100 µl of ice-cold lysis Buffer II (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA and protease inhibitors [Roche]), vortexed for 5 seconds, incubated for 30 min at 4°C under agitation and centrifuged at 12,000 rpm for 20 min at 4°C. For both experiments the supernatant containing the whole cellular lysate was recovered and 30 µg of total protein were used for Western blot analysis. For hRSV production HEp-2 cells were infected with 500 µl of the virus and supernatants were recovered when an 80% of cytopathic effect was observed. Supernatants were cleared by

centrifugation and stored at −80°C. For quantification of hRSV we perform a $TCID_{50}$ in the MA104 cell line as previously described [36].

Surface sensing of translation (SUnSET) assay

Global proteins synthesis under control or CTIF overexpression was analyzed using the puromycin incorporation into new peptide synthesis as previously described [37]. Briefly, HeLa cells were transfected with pcDNA-d2EGFP or pcDNA3-Flag-CTIF and treated with 10 µg/ml of puromycin for 10 min at 37°C and 5% CO_2 at 24 hpt. Cells were washed with 1x PBS twice and lysed with lysis Buffer II (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA and EDTA-free protease inhibitors cocktail [Roche]). As a control of protein synthesis inhibition, HeLa cells were treated with 0.5 mM of sodium arsenite (Sigma-Aldrich) for 45 min prior to puromycin treatment. 20 µg of total proteins were resolved by SDS-PAGE and analyzed by Western blot analysis.

RNA extraction and RT-qPCR

Cytoplasmic RNA extraction and RT-qPCR from cytoplasmic RNA were performed as we have previously described [19,31]. Briefly, cells were washed intensively with 1x PBS, recovered with 1x PBS-EDTA 10 mM and lysed for 1–2 min at room temperature with 200 µl of RNLa buffer (10 mM Tris–HCl pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5% NP40 and 2 mM of vanadyl-ribonucleoside complex (VRC) [New England Biolabs]). Cell lysates were centrifuged at 2100 x g for 5 min at 4°C and supernatant containing the cytoplasmic fraction were recovered and RNA extraction was carried out by adding 1 ml of TRIzol™ (Thermo Fisher) as indicated by the manufacturer. Cytoplasmic RNAs (1 µg) were reverse-transcribed using the High Capacity cDNA reverse transcription (Life Technologies). For quantitative PCR, a 20-µl reaction mix was prepared with 5 µl of template cDNAs (previously diluted to 1/10), 10 µl of Brilliant II SYBR® Green QPCR master mix (Agilent), 0.2 µM of sense and antisense primers and subjected to amplification using the Agilent AriaMx™ Real Time PCR system (Agilent). The GAPDH housekeeping gene was amplified in parallel to serve as a control reference. Relative copy numbers of HIV-1 full-length RNA was compared to GAPDH using $x^{-\Delta Ct}$ (where x corresponds to the experimentally calculated amplification efficiency of each primer couple). Sequences of the primers used and the experimentally calculated amplification efficiency of the primers are presented in Supplementary Table 2.

RNA immunoprecipitation

6 $x10^6$ HEK293 cells were seeded in a 150 mm dish and co-transfected with 10 µg pNL4.3-ΔEnv, 10 µg pcDNA3-Flag-CTIF and 3 µg pEGFP-Rev or pEGFP as a control. At 24 hours post-transfection, cells were washed twice with PBS 1X, centrifuged at 500 g for 5 minutes and resuspended in 10 mL PBS 1X/formaldehyde 0.1%. Cells were cross-linked for 15 minutes at room temperature and then 1.25 mL of quenching solution (2 M Glycine, 25 mM Tris-HCl pH7.5) was added and incubated for 5 minutes. Cells were centrifuged and the pellet was resuspended in 1 mL Hypotonic Lysis Buffer (20 mM Tris-HCl pH 7.5, 15 mM NaCl, 10 mM EDTA, 0.5% Nonidet P40, 0.1% Triton X-100, 2 mM VRC and protease inhibitors) and incubated for 10 minutes on ice. The cell lysate was centrifuged at 13.000 x g for 10 minutes at 4°C to remove cell membranes and NaCl concentration was adjusted to 150 mM. A fraction of the cell lysate (200 µL) was stored at −20°C as input for further analysis.

Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich) were washed twice with Hypotonic Lysis Buffer and added to the cell lysate. The mixture was incubated with rotation for 2 hours at 4°C and beads were then washed three times with 1 mL of Wash Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Nonidet P40 and 2 mM VRC). Beads were resuspended in 100 µL of Wash Buffer and 25 µL were recovered for Western blot analysis. Then, 1 µL of proteinase K (NEB) was added and incubated at 52°C for 1 hour followed an incubation at 95°C for 5 minutes.

Total RNA extraction was performed using the TRIzol reagent and RNA was precipitated overnight at −20°C with 100% ethanol, 20 µg glycogen and 10 mM magnesium chloride. Then, the RNA was pelleted at 12.000 x g for 40 minutes at 4°C, washed with 70% ethanol and treated with TURBO-DNAse (Invitrogen) following manufacturer instructions.

Total RNA was quantified and 300 ng were subjected to reverse transcription and qPCR using specific primers for HIV-1 full-length mRNA and GAPDH as described above.

RNA fluorescent *in situ* hybridization, immunofluorescence and confocal microscopy

The specific probes of 100–200 bp against the HIV-1 full-length RNA were generated by in vitro transcription of the pBSK-Gag/Pol vector using digoxin-11-UTP (Roche) and subsequent fragmentation using the RNA fragmentation buffer as previously described [19]. RNA FISH analyses were performed as we have recently described [19]. Briefly, HeLa cells were cultured in a 12-well plate with coverslips and transfected with 1 µg of pNL4.3-ΔEnv or 1 µg of the corresponding expression vector as indicated in each figure. At 24 hpt, cells were washed twice with 1x PBS and fixed for 10 min at room temperature with 4% paraformaldehyde. Cells were subsequently permeabilized for 10 min at room temperature with 0.2% Triton X-100 and hybridized overnight at 37°C in 200 µl of hybridization mix (10% w/v dextran sulfate, 2 mM VRC, 0.02% w/v RNase-free BSA, 50% v/v formamide, 1.5 µg/µL of tRNA and 0.6 ng/µL of 11-digoxigenin-UTP probes) in a humid chamber. Cells were washed with 0.2x SSC/50% v/v formamide during 30 min at 50°C and then incubated three times with antibody dilution buffer (2x SSC, 8% v/v formamide, 2 mM vanadyl-ribonucleoside complex, 0.02% w/v RNase-free BSA). Mouse anti-digoxigenin (Roche) and rabbit anti-Flag (Sigma Aldrich) primary antibodies diluted to 1/100 in antibody dilution buffer were added for 2 h at room temperature. After three washes with antibody dilution buffer, cells were incubated for 90 min at room

temperature with anti-mouse Alexa 488 (Invitrogen) and anti-rabbit Alexa 594 antibodies (Invitrogen) diluted at 1/500. Cells were washed three times in wash buffer (2x SSC, 8% v/v formamide, 2 mM vanadyl-ribonucleoside complex), twice with 1x PBS, incubated with DAPI (0.3 µg/mL in 1x PBS) (Life Technologies) for 1 min at room temperature, washed three times with 1x PBS, three times with water and mounted with Fluoromount™ Aquous Mounting Medium (Sigma-Aldrich).

For immunofluorescence, HeLa cells transfected with 1 µg of the corresponding expression vector were washed, fixed and permeabilized as in RNA FISH protocol. Subsequently, cells were incubated with blocking reagent (BioRad) for 30 min at room temperature and incubated with primary antibody mouse anti-Flag (Sigma-Aldrich), rabbit anti-myc (Sigma-Aldrich) or rabbit anti-CTIF (ThermoFisher Scientific) diluted 1/100 for 1 h at room temperature. After 3 washes with 1x PBS cells were incubated with anti-mouse Alexa 647 and/or anti-rabbit Alexa 594 secondary antibodies (Molecular Probes) diluted at 1/500 for 1 h at room temperature. Cells were washed three times 1x PBS, incubated with DAPI (0.3 µg/mL in 1x PBS) (Life Technologies) for 1 min at room temperature, washed three times with 1x PBS, three times with water and mounted with Fluoromount™ Aquous Mounting Medium (Sigma-Aldrich).

Images were obtained with a TCS SP8 Confocal Microscope (Leica Microsystems) or an LSM 800 Confocal Microscope (Zeiss) and processed using FIJI/ImageJ (NIH).

Proximity ligation assay (PLA)

The PLA was carried out using the DUOLINK II *In Situ* kit (Sigma-Aldrich) and the PLA probe anti-mouse minus and the PLA probe anti-rabbit plus (Sigma-Aldrich) as we have previously described [19]. Briefly, PFA-fixed HeLa cells were pre-incubated with blocking reagent (BioRad) for 30 min at room temperature. Primary antibodies anti-protein or -tag of interest were diluted in 40 µl of blocking reagent and incubated at 37°C for 1 h. Samples were washed three times with 1x PBS for 5 min each and secondary antibodies (DUOLINK anti-rabbit PLA plus probe and DUOLINK anti-mouse PLA-minus probe) were added and incubated at 37°C for 1 h. Ligation and amplification reactions were performed following the same protocol described in [38]. Samples were incubated with DAPI (0.3 µg/mL in 1x PBS) (Life Technologies) for 1 min at room temperature, washed three times with 1x PBS, three times with water and mounted with Fluoromount™ Aquous Mounting Medium (Sigma Aldrich). For immunofluorescence combined with the PLA, the cells were washed twice with 1x PBS and secondary antibody Alexa anti-rabbit 647 (Invitrogen) was added to the coverslip after the rolling circle amplification. Secondary antibody was incubated 15 minutes at room temperature and washed twice with 1x PBS. Finally, nuclei were stained with DAPI as mentioned above. Images were obtained and processed as described above.

In situ hybridization coupled to PLA (ISH-PLA)

The ISH-PLA was carried out as a combination of the RNA FISH and the PLA as we previously described [19]. Briefly, PFA-fixed HeLa cells growing on coverslips were permeabilized for 10 min at RT with 0.2% Triton X-100 and hybridized overnight at 37°C in 200 µl of hybridization mix (10% w/v dextran sulphate, 2 mM vanadyl–ribonucleoside complex, 0.02% w/v RNase-free bovine serum albumin, 50% v/v formamide, 1.5 µg/µL of tRNA and 0.6 ng/µL of 11-digoxigenin-UTP probes) in a humid chamber. Cells were washed with 0.2x SSC/50% v/v formamide during 30 min at 50°C and incubated with blocking reagent for 30 min at room temperature. Cells were then incubated three times with antibody dilution buffer (2x SSC, 8% v/v formamide, 2 mM vanadyl–ribonucleoside complex and 0.02% w/v RNase-free bovine serum albumin). Primary antibodies, mouse anti-digoxigenin and rabbit anti-Flag diluted to 1/100 and 1/200, respectively, in antibody dilution buffer were added for 2 h at room temperature. After three washes with antibody dilution buffer and two washes with 1x PBS for 5 min each, the secondary antibodies (DUOLINK anti-rabbit PLA-plus probe, DUOLINK anti-mouse PLA minus probe) were added and incubated at 37°C for 1 h. The ligation and amplification reactions were performed following the same protocol described above. Thereafter, coverslips were incubated with a solution of DAPI (0.3 µg/mL in 1x PBS) (Life Technologies) for 1 min at room temperature, washed three times with 1x PBS, three times with water and mounted with Fluoromount™ Aquous Mounting Medium (Sigma-Aldrich). Images were obtained and processed as described above.

Conservation analysis

HIV-1 sequences (879 sequences) were selected from the HIV-1 database from the Los Alamos National Laboratory (www.hiv.lanl.gov/content/index). Sequence alignment was performed using MAFFT [39] and the sequence logo was generated with WebLogo 3.6.0 [40,41].

Results

CTIF inhibits human lentiviral Gag synthesis during viral replication

We have recently reported that CBP80 promotes Gag synthesis from the HIV-1 full-length RNA in association with the viral protein Rev [19]. Since CTIF bridges CBP80 and the 40S ribosomal subunit by interacting with eIF3 during CBC-dependent translation [26,27], we sought whether CTIF was involved in Gag synthesis from the HIV-1 full-length RNA. We first analyzed the expression of endogenous CTIF in T-cells infected with VSV-G-pseudotyped HIV-1 and consistently observed that the presence of the virus induced an increase in the levels of the cellular protein from 8 to 24 hours post-infection to then decrease at later time points (Fig. 1A). Of note, we detected endogenous CTIF as a double band in Jurkat cells while it was detected as a single band in other cell lines including H9, CEM, THP1, HeLa and

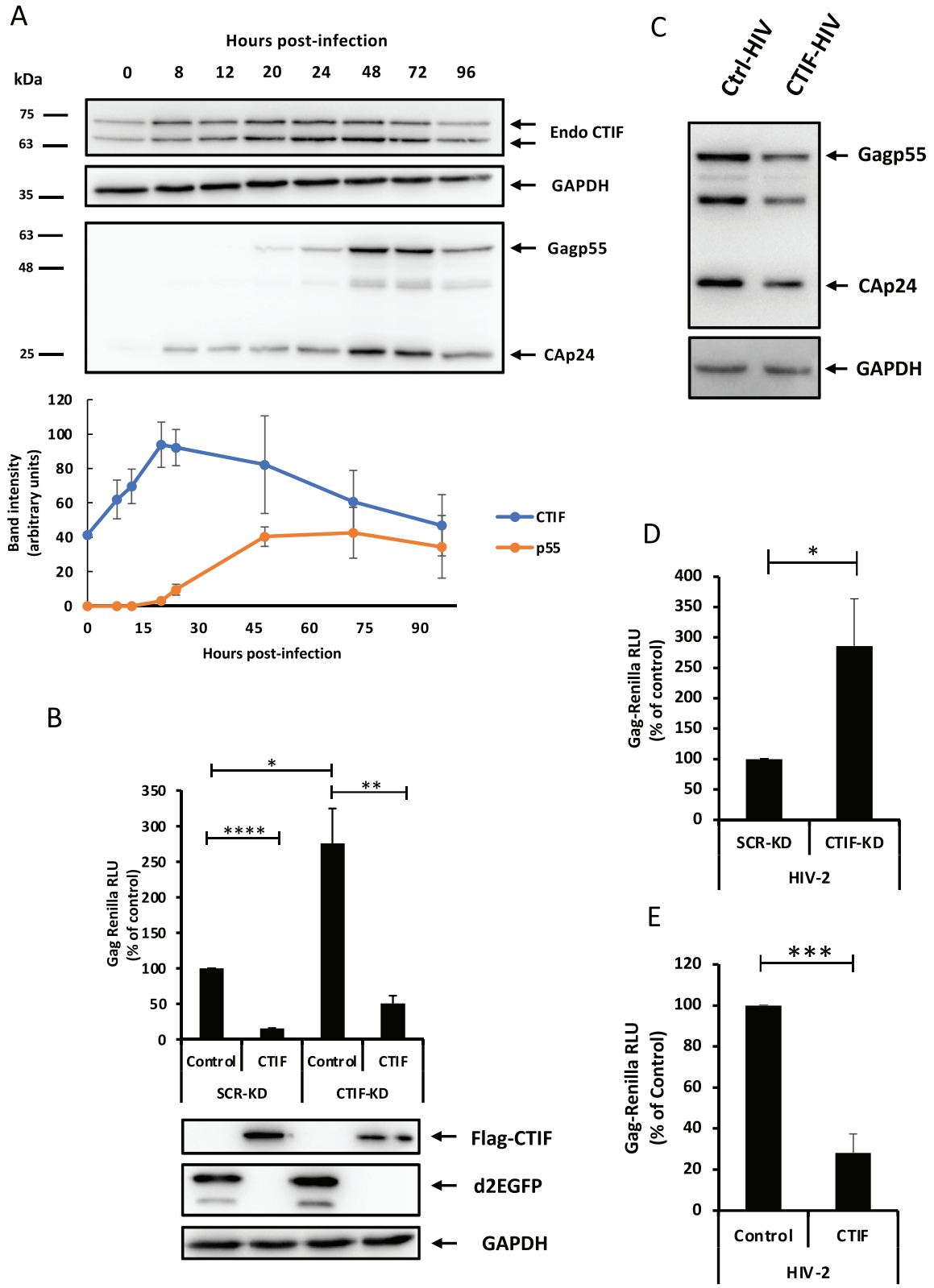

Figure 1. CTIF inhibits HIV-1 and HIV-2 Gag synthesis.

A) Jurkat cells were infected with VSV G-pseudotyped HIV-1 and cell extracts prepared at 0, 8, 12, 20, 24, 48, 72 and 96 hours post-infection were used to detect Gag (and its processing intermediates) and CTIF by Western blot. GAPDH was used as loading control. Band intensity for CTIF and Gag (pr55) from three independent experiments were quantified, normalized to GAPDH and plotted (n = 3, ± SEM). B) SCR-KD or CTIF-KD cells ($1x10^5$ cells/well) were transfected with 1 µg of pcDNA-d2EGFP (used as a control) or pcDNA3-Flag-CTIF together with 0.3 µg of pNL4.3R as described in materials and methods. Gag-Renilla activity was determined at 24 hpt. Results were normalized to the control (arbitrary set to 100%) and correspond to the mean ± SD of three independent experiments (*P < 0.05; **P < 0.01 and ****P < 0.0001, t-test). Cells extracts were used to verify Flag-CTIF and d2EGFP expression by Western blot. GAPDH was used as a loading control.C) Jurkat cells were infected with the VSV G-pseudotyped HIV-1 produced under control and Flag-CTIF expression and cellular extracts prepared at 48 hours post-infection were used to detect Gag and its processing intermediates. GAPDH was used as loading control. This image corresponds to a representative Western blot from three independent experiments.D) SCR-KD or CTIF-KD cells ($1x10^5$ cells/well) were transfected with 0.3 µg of the HIV-2 pROD10R reporter proviral DNA as described in materials and

HEK293T (data not shown). A search at the INTERFEROME database [42] indicates that CTIF is not expressed from an interferon stimulated gene (data not shown) and thus, an additional regulatory mechanism must regulate CTIF protein levels during HIV-1 infection in Jurkat cells. It is noteworthy that we consistently observed that the drop in the levels of CTIF during the course of infection in Jurkat cells was correlated with the accumulation of the Gag polyprotein being more evident from 24 to 72 hpi (Fig. 1A), which further prompted us to investigate the impact of CTIF on Gag synthesis.

In order to study the involvement of CTIF on Gag synthesis and separate any potential effect of the cellular protein in the early steps of the replication cycle, we used transfection of a reporter proviral DNA in the permissive HeLa cell model as we have previously reported [14,16,19,31]. We used a validated shRNA targeting the 3′-UTR of the CTIF mRNA and generated a stable cell line (CTIF-KD cells) with reduced levels of the endogenous protein (Supplementary Fig. 1A). Interestingly and despite a partial knockdown of endogenous CTIF in these cells, we observed a 2.5 to 3-fold increase in HIV-1 Gag synthesis in CTIF-KD cells compared to the cell line expressing the scramble shRNA (Fig. 1B, compare 'Control' in SCR-KD and CTIF-KD cells). Interestingly, the stimulation in Gag synthesis observed in CTIF-KD cells was completely lost when Flag-CTIF (lacking the sequence targeted by the shRNA in its coding mRNA) was ectopically expressed and this reduction in Gag synthesis was even more evident when Flag-CTIF was expressed in SCR-KD cells (Fig. 1B, compare 'CTIF' in SCR-KD and CTIF-KD cells) thus, indicating that CTIF inhibits Gag synthesis from the HIV-1 full-length RNA. Of note, Flag-CTIF expression has no effect on the Renilla luciferase expressed from a reporter DNA suggesting that increased levels of CTIF may not impact on the Renilla reporter gene (Supplementary Fig. 1B). Analysis of global protein synthesis in control and Flag-CTIF expressing cells by SUnSET revealed that Flag-CTIF is not a general inhibitor of translation (Supplementary Fig. 1C). In order to investigate whether the negative role of CTIF on Gag synthesis impacts on viral production, we produced VSV-G-pseudotyped HIV-1 in HEK293T cells expressing Flag-CTIF or d2EGFP as control (CTIF-HIV and Ctrl-HIV, respectively) (Supplementary Fig. 1D). At 72 hours post-transfection, we recovered the supernatants and used them to infect Jurkat cells for 48 hours (Fig. 1C). We observed that Jurkat cells infected with CTIF-HIV produced less Gag when compared to Ctrl-HIV indicating that expression of Flag-CTIF in the producer cells and the subsequent decrease in Gag synthesis impairs viral particle production (Fig. 1C). We then evaluated whether the effect of CTIF was conserved in the related lentivirus HIV-2 and observed that similar to HIV-1, Gag synthesis from the HIV-2 full-length RNA was stimulated by near 3-fold in CTIF-KD cells (Fig. 1D). In agreement with this observation, HIV-2 Gag synthesis was strongly impaired in cells expressing Flag-CTIF (Fig. 1E). We also evaluated the effect of Flag-CTIF in the distant gammaretrovirus Murine Leukemia Virus (MLV) but no effect Gag levels was observed (Supplementary Fig. 1E). As an additional control, we analyzed whether Flag-CTIF expression impacts protein synthesis of the non-related human respiratory syncytial virus (hRSV), but we were not able to observe any effect (Supplementary Fig. 1F) indicating a specific negative effect of CTIF on Gag synthesis at least for the human lentiviruses HIV-1 and HIV-2.

CTIF inhibits Gag synthesis in a Rev-dependent manner

While MLV uses the Tap/NXF1-mediated nuclear export pathway for the exit of its full-length RNA that will be used as the substrate for Gag synthesis [43–45], nuclear export of the HIV-1 and HIV-2 full-length RNA relies on the viral protein Rev, which bridges the viral transcript with the karyopherin CRM1 [46]. Thus, we speculated that CTIF interferes with human lentiviral Gag synthesis by targeting the functions of the viral protein Rev and decided to use HIV-1 as a model to characterize the molecular mechanism at play. For this, we first analyzed whether Rev and CTIF interact in intact cells using the proximity ligation assay (PLA) and observed that EGFP-Rev form complexes with both endogenous and Flag-tagged CTIF (Fig. 2A, Supplementary Figs. 2A and 2B). Consistent with the localization of endogenous CTIF at the cytoplasmic face of the nuclear envelope with a minor presence in the nucleus (Supplementary Fig. 2A) [26], we observed most CTIF-Rev PLA dots localizing within or around the DAPI signal (Fig.2A). Since Flag-CTIF accumulated both around the nucleus and in the cytoplasm, we observed CTIF-Rev dots at these subcellular locations indicating that CTIF may impact on Rev localization (Supplementary Fig. 2B and see below).

Having determined that CTIF associates with Rev, we then sought to determine whether Rev was involved in the inhibitory activity of CTIF on Gag synthesis by using wild type and ΔRev HIV-1 reporter proviruses. Consistent with the critical role of Rev in Gag synthesis, we observed that Gag-Renilla synthesis from the ΔRev reporter provirus was strongly reduced but not abolished in the absence of Rev (Supplementary Fig. 2C). Compared to the wild type provirus, we observed that the low level of Gag synthesis from the ΔRev provirus was not as much affected as the wild type provirus both in CTIF-KD cells or in Flag-CTIF expressing cells suggesting that CTIF requires the presence of Rev to fully exert its negative effect on Gag synthesis (Fig. 2B and Supplementary Fig. 2D). Since CTIF is located at the cytoplasmic face of the nuclear pore complex and recruited to the mRNP upon nuclear export [26,27], we reasoned that CTIF

methods. Gag-Renilla activity was determined at 24 hpt. Results were normalized to the control (arbitrary set to 100%) and correspond to the mean ± SD of three independent experiments (*P < 0.05, t-test).E) HeLa cells (1x10^5 cells/well) were transfected with 1 µg of pcDNA-d2EGFP (used as control) or pcDNA3-Flag-CTIF together with 0.3 µg of pROD10R as described in materials and methods and Gag-Renilla activity was determined at 24 hpt. Results were normalized to the control (arbitrary set to 100%) and correspond to the mean ± SD of three independent experiments (***P < 0.001; t-test). In parallel, cells extracts were used to detect Flag-CTIF by Western blot. GAPDH was used as a loading control.

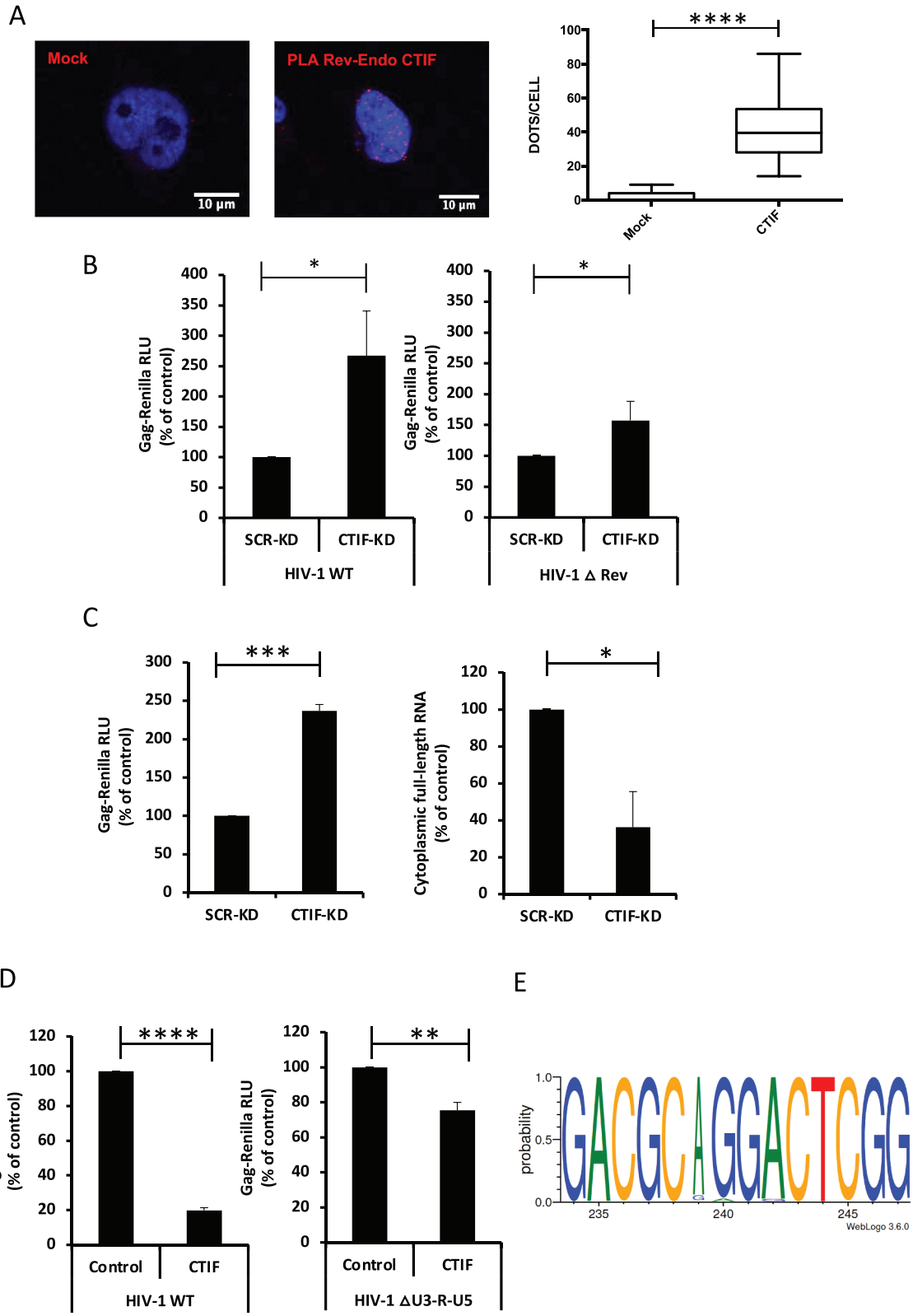

Figure 2. CTIF acts on Rev function.

A) HeLa cells (8x10^4 cells/well) transfected with 1 µg pEGFP-Rev were subjected to the proximity ligation assay using a rabbit anti-CTIF antibody diluted 1/100 and a mouse anti-GFP antibody diluted 1/100 together with the Duolink® *in situ* kit as described in materials and methods. Mock corresponds to non-transfected cells. The PLA dots are shown in red and DAPI in blue. Scale bar 10 µm. Quantification of dots per cell in Mock-CTIF (n = 34 cells) and Rev-CTIF (n = 33 cells) are presented (****P < 0.0001, Mann–Whitney test).B) SCR-KD and CTIF-K cells (1x10^5 cells/well) were transfected with 0.3 µg of pNL4.3R or pNL4.3R-ΔRev as described in materials and methods and Renilla luciferase activity was determined at 24 hpt. Results were normalized to the control (arbitrary set to 100%) and correspond to the mean ± SD of three independent experiments (*P < 0.05, t-test).C) SCR-KD or CTIF-KD cells(1x10^5 cells/well) were transfected with 0.3 µg of pNL4.3 R as described in materials and methods. Gag-Renilla activity (left panel) and cytoplasmic full-length RNA levels (right panel) were determined at 24 hpt. Results were normalized to the control (arbitrary set to 100%) and correspond to the mean ± SD of three independent experiments (***P < 0.001, *P < 0.05, t-test).D) HeLa cells (1x10^5 cells/well) were transfected with 1 µg of pcDNA-d2EGFP(used as a control) or pcDNA-Flag-CTIF together with 0.3 µg pNL4.3 R (left panel) or pNL4.3 R-CMV-ΔU3/U5 (right panel) as described in materials and methods. Results were normalized to the control (arbitrary set to 100%) and correspond to the mean ± SD of three independent experiments (**P < 0.01, ****P < 0.0001, t-test t-test).E) Conservation analysis of the AGGA loop (nucleotides 234 to 247 in NL4.3) from the 5′-UTR of 879 HIV-1 isolates.

may interfere with the cytoplasmic functions of Rev. Interestingly, we observed that increased levels of Gag synthesis in CTIF-KD cells were accompanied by a significant reduction in the cytoplasmic levels of the full-length RNA suggesting either that endogenous CTIF is required for the cytoplasmic accumulation of the full-length RNA but may interfere with ribosome recruitment or that enhanced translation of the full-length RNA is accompanied by a faster turnover (Fig. 2C and see discussion). However, we observed that despite a strong inhibition of Gag synthesis under expression of Flag-CTIF, the cytoplasmic levels of the full-length RNA were minimally affected (Supplementary Fig. 2E). The discrepancies in the effect on the cytoplasmic full-length RNA levels observed under CTIF KD (decrease) and overexpression (no changes) must be related to the role that endogenous CTIF is already exerting on the viral RNA. It should be mentioned that any contamination of our cytoplasmic fractions with nuclear RNA was discarded as previously described (data not show) [19].

In addition to the Rev response element (RRE) present within the region of the *env* gene of the full-length RNA previously shown as critical for Rev-mediated nuclear export [47], the HIV-1 Rev protein was also shown to bind the AGGA loop present at the base of SL1 within the 5′-UTR in order to promote protein synthesis [48,49]. Thus, in order to obtain information of any involvement of the 5′-UTR we determined the impact of Flag-CTIF expression on Gag synthesis using the pNL4.3R-CMV-ΔU3/U5, a reporter proviral DNA that lacks the ΔU3/R/U5 region and most of the 5′-UTR (including the previously described Rev binding site) and has the CMV IE promoter instead [31]. Interestingly, we observed that the CMV-ΔU3/U5 provirus was not as much affected as the wild type provirus by Flag-CTIF expression indicating that the negative effect of CTIF on Gag synthesis are mostly exerted at the level of the 5′-UTR (Fig. 2D). Of note, Gag synthesis from the pNL4.3 R-CMV-ΔU3/U5 provirus was not stimulated by CBP80 overexpression suggesting that CTIF and CBP80 may have a common target during Gag synthesis that involves the 5′-UTR of the full-length RNA (Supplementary Fig. 2F and see below). Conservation analysis of the Rev binding site in the AGGA loop [48] revealed a high conservation amongst HIV-1 isolates suggesting an important role of this Rev binding site on viral replication (Fig. 2E).

Taking together, these data suggest that CTIF interacts with Rev and targets protein synthesis from the full-length RNA in a 5′-UTR-dependent manner.

CTIF inhibits Gag synthesis through its N-terminal CBP80-binding domain

CTIF is composed of two major domains, the N-terminal region (aa 1–305) that contains the CBP80-binding domain and the C-terminal region (aa 306–598), which harbors a MIF4G domain [26] (Fig. 3A, upper scheme). Thus, we took advantage of the inhibitory effect on Gag synthesis observed upon expression of Flag-CTIF to investigate whether the inhibitory activity was contained in one of these two domains. Interestingly, we observed that expression of the N-terminal domain alone (Flag-CTIF-N-ter) was able to inhibit the synthesis of HIV-1 Gag in a similar way to the full-length protein (Fig. 3A). The C-terminal domain (Flag-CTIF-C-ter) was unable to interfere with Gag synthesis indicating that the N-terminal CBP80-binding domain contains the inhibitory activity of CTIF. In agreement with data presented above, we observed that the N-terminal domain, but not the C-terminal domain, interacts with EGFP-Rev at levels similar to those obtained with the full-length protein as evidenced by PLA (Fig. 3B). Of note, the lack of PLA dots was not due to defects in the expression of Flag-CTIF-C-ter as evidenced by immunofluorescence staining performed in parallel (Supplementary Fig. 3A). It should be mentioned that we consistently observed that the interaction between EGFP-Rev and Flag-CTIF (or the N-terminal domain) mainly occurs in the cytoplasm, which is consistent with the subcellular localization of the proteins (Supplementary Fig. 3A and see below).

We then wanted to evaluate whether CTIF was recruited to the full-length RNA during viral replication and to determine the involvement of Rev in this process. For this, we performed RNA immunoprecipitation of Flag-CTIF, which was co-expressed with the pNL4.3 or pNL4.3-ΔRev proviral DNA (Fig. 3C). Interestingly, we observed a decrease in the association between CTIF and the full-length RNA in the absence of Rev, which was restored upon expression of EGFP-Rev in *trans* suggesting that CTIF is mostly recruited to the full-length RNA ribonucleoprotein complex in a Rev-dependent manner (Fig. 3C). We also observed that EGFP-Rev was immunoprecipitated with Flag-CTIF confirming the interaction between both proteins (Fig. 3C). We then used *in situ* hybridization coupled to the proximity ligation assay (ISH-PLA) in order to evaluate the involvement of the domains of CTIF in its recruitment to the full-length RNA ribonucleoprotein complex during viral replication. In agreement with their ability to interact with Rev, we observed that both full-length CTIF and the N-terminal domain form complexes with the full-length RNA (Fig. 3D and Supplementary Fig. 3B). Of note, ISH-PLA dots between CTIF and the full-length RNA were observed around the nucleus indicating that CTIF may reach the viral RNP upon or during nuclear export. Consistent with its inability to interact with Rev and to interfere with Gag synthesis, we observed that the C-terminal domain of CTIF was not recruited to the full-length RNA (Fig. 3D and Supplementary Fig. 3B). It should be mentioned that neither full-length CTIF nor the isolated domains altered the subcellular localization of the full-length RNA as judged by RNA FISH and confocal microscopy analyses performed in parallel to the ISH-PLA (Supplementary Fig. 3C).

Taking together, these data suggest that CTIF interacts with Rev through its N-terminal domain and this interaction allow the incorporation of CTIF to the full-length RNA ribonucleoprotein complex leading to the inhibition of Gag synthesis.

CTIF induces the cytoplasmic accumulation of Rev

From the data presented above, it seems that CTIF and Rev mainly interact in the cytoplasm despite most of the viral protein is normally found in the cell nucleus and concentrated at the nucleolus. Thus, we sought to determine whether CTIF

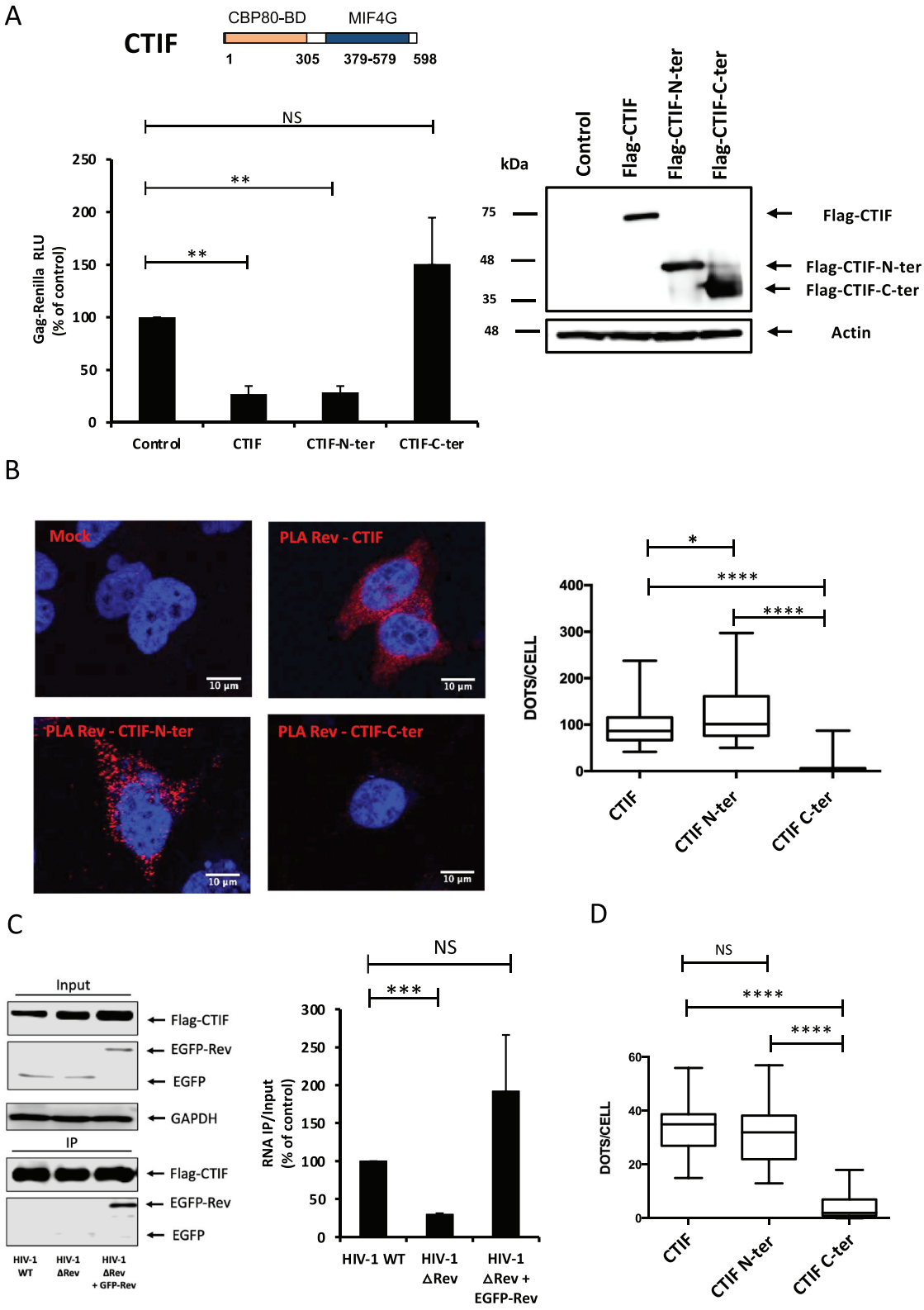

Figure 3. CTIF inhibits Gag synthesis through its N-terminal domain.

A) Upper panel: Scheme of CTIF showing its N-terminal CBP80-binding domain and its C-terminal MIF4G domain. Numbers indicate amino acid positions. Bottom panel: HeLa cells ($1x10^5$ cells/well) were transfected with 1 µg of pcDNA-d2EGFP (used as a control), pcDNA3-Flag-CTIF, pcDNA3-Flag-CTIF-N-ter or pcDNA-Flag-CTIF -C-ter together with 0.3 µg of pNL4.3R as described in materials and methods. Gag-Renilla activity was determined at 24 hpt. Results were normalized to the control (arbitrary set to 100%) and correspond to the mean ± SD of three independent experiments (**P < 0.01; NS = non-significant, t-test). Cells extracts were used to detect Flag-CTIF by Western blot. Actin was used as a loading control.B) HeLa cells ($8x10^4$ cells/well) transfected with 1 µg of pEGFP-Rev together with 1 µg of pcDNA3-Flag-CTIF, pcDNA3-Flag-CTIF-N-ter or pcDNA-Flag-CTIF-C-ter were subjected to the proximity ligation assay using a rabbit anti-Flag antibody diluted 1/200 and a mouse anti-GFP antibody diluted 1/100 together with the Duolink□ *in situ* kit as described in materials and methods. Mock corresponds to non-transfected cells. The PLA signal is shown in red and DAPI in blue. Scale bar 10 µm. Quantification of dots per cell in Rev-CTIF (n = 29 cells), Rev-CTIF-N-ter (n = 43 cells) and Rev-CTIF-C-ter (n = 43 cells) are presented (****P < 0.0001, NS; non-significant, Mann–Whitney test).C) HeLa cells ($6x10^6$ cells/plate) were transfected with 10 µg of

has an impact on the subcellular localization of Rev. For this, we performed PLA under the same conditions described above but performed immunofluorescences staining in parallel and proceed to analyze the localization of Rev (Fig. 3B and Supplementary Fig. 3A). As expected, EGFP-Rev signal was mainly present in the nucleus/nucleolus of the cells under control conditions as evidenced by confocal microscopy (Fig. 4A). However, we observed a significant increase in the cytoplasmic signal of EGFP-Rev when either full-length CTIF or the N-terminal domain were co-expressed together with the viral protein (Fig. 4A). Of note, both EGFP-Rev and Flag-CTIF were localized diffuse along the cytoplasm as evidenced by phase-contrast microscopy performed in parallel. Quantification of the subcellular localization of EGFP-Rev indicates that under control conditions near 90% of the cells exhibited an exclusive nuclear localization of EGFP-Rev (Fig. 4B). In contrast, we observed that 70–80% of the cells presented a cytoplasmic/nuclear localization of EGFP-Rev when full-length CTIF or the N-terminal domain were co-expressed further confirming that the N-terminal domain of CTIF harbors the functional role. (Fig. 4B). Consistent with its inability to interact with Rev and interfere with Gag synthesis, the C-terminal domain of CTIF did not impact on the subcellular localization of Rev (Fig.s 4A and 4B). We then combined the PLA strategy with immunofluorescence staining in order to analyze whether changes in Rev localization induced by CTIF were associated to the interaction between both proteins (Fig. 4C). As observed, cells expressing EGFP-Rev and Flag-CTIF in the cytoplasm also present interaction dots at the same subcellular location. Similar results were obtained with Flag-CTIF-N-ter but not with the Flag-CTIF-C-ter further indicating that the association of Rev with the N-terminal CBP80-binding domain of CTIF might sequester the viral protein in the cytoplasm.

CTIF interferes with the interaction between Rev and CBP80

We recently reported that Rev associates with CBP80 in order to promote nuclear export and translation of the full-length RNA [19]. Moreover, from data obtained with the pNL4.3R-CMV-ΔU3/U5 it seems that CTIF and CBP80 have the same target during HIV-1 Gag synthesis. Thus, we finally wanted to investigate whether the inhibitory activity of full-length CTIF on Rev function was related to the interaction between the viral protein and the CBC subunit. For this, we employed the PLA strategy in order to analyze and quantify the interaction between EGFP-Rev and myc-CBP80 in the presence or absence of Flag-CTIF. Interestingly, quantification of our PLA experiments showed that expression of CTIF strongly interferes with the interaction between Rev and CBP80 (Fig. 5A). Immunofluorescence staining performed in parallel show the correct expression of all components indicating that differences are not due to defects in expression (Supplementary Fig. 4A). We also analyzed whether Rev was able to interfere with the interaction between Flag-CTIF and V5-CBP80 and observed that EGFP-Rev expression indeed interferes with the association between these cellular proteins (Fig. 5B). Immunofluorescence staining performed in parallel show the correct expression of all components analyzed (Supplementary Fig. 4B).

Taken together, these data suggest that CTIF interferes with the association between Rev and CBP80, which results in the inhibition of Gag synthesis.

Discussion

Compared to protein synthesis from a canonical mammalian mRNA, Gag synthesis from the HIV-1 full-length RNA should not be as efficient as it is. This is because the viral transcript possesses several features known to be incompatible with efficient mRNA nuclear export and translation in mammalian cells. First, since the full-length RNA is unspliced but contains functional splice sites, it needs to subvert the cellular splicing machinery in order to accumulate in the nucleus of infected cells. Second, the lack of intron removal avoids the splicing-dependent recruitment of nuclear host factors such as Aly/REF or the exon junction complex that license nuclear export and thus, the full-length RNA is not assembled into a canonical mRNP that exits the nucleus associated to Tap/NXF1 and is directed to the translational machinery in the cytoplasm. Third, the full-length RNA harbors a highly structured 5′-UTR expected to interfere with the classical cap-dependent ribosomal scanning mechanism of translation initiation. Despite all these functional constraints, the HIV-1 full-length RNA reaches the cytoplasm efficiently and is highly specialized in ribosome recruitment producing huge amounts of Gag protein during viral replication. The viral protein Rev orchestrates the post-transcriptional regulation of the full-length RNA by acting as a multifunctional bridge between host factors and the viral transcript. By binding to the Rev response element (RRE) present at the 3′-UTR of the full-length RNA through an RNA binding domain and the host karyopherin CRM1 through its nuclear export signal (NES), Rev ensures the cytoplasmic accumulation of its RNA targets. Rev has also been involved in ribosome recruitment of the full-length RNA through the binding to a highly conserved AGGA loop present at the SL1 within the 5′-UTR although the mechanisms at play are poorly understood. Recent work including ours reported that Rev interacts with the CBC subunit CBP80. In our study, we demonstrated that CBP80 cooperates with Rev during nuclear export and translation of the full-length RNA. We also showed that the full-length RNA remains associated with CBP80 in the cytoplasm

pNL4.3 together with 10 µg pcDNA3-Flag-CTIF and 3 µg of pEGFP or pEGFP-Rev as indicated. At 24 hpt, cell extracts were used for RNA immunoprecipitation as described in materials and methods. Results are expressed as the IP/input ratio normalized to the pNL4.3 condition (arbitrary set at 100%). Results correspond to the average ± SEM of three independent experiments. (***P < 0.001; NS = non-significant, t-test). Cells extracts were used to detect Flag-CTIF, EGFP and EGFP-Rev by Western blot.D) HeLa cells ($8x10^4$ cells/well) transfected with 0.3 µg of pNL4.3 together with 1 µg pcDNA3-Flag-CTIF, pcDNA3-Flag-CTIF-N-ter or pcDNA-Flag-CTIF-C-ter were subjected to ISH-PLA using a rabbit anti-Flag antibody diluted 1/200 and a mouse anti-digoxigenin antibody diluted 1/100 together with the Duolink *in situ* kit as described in materials and methods. Mock corresponds to non-transfected cells. Quantification of dots per cell in full-length RNA-CTIF (n = 36 cells), full-length RNA-CTIF-N-ter (n = 30 cells) and full-length RNA-CTIF-C-ter (n = 27 cells) are presented (****P < 0.0001, NS; non-significant, Mann–Whitney test).

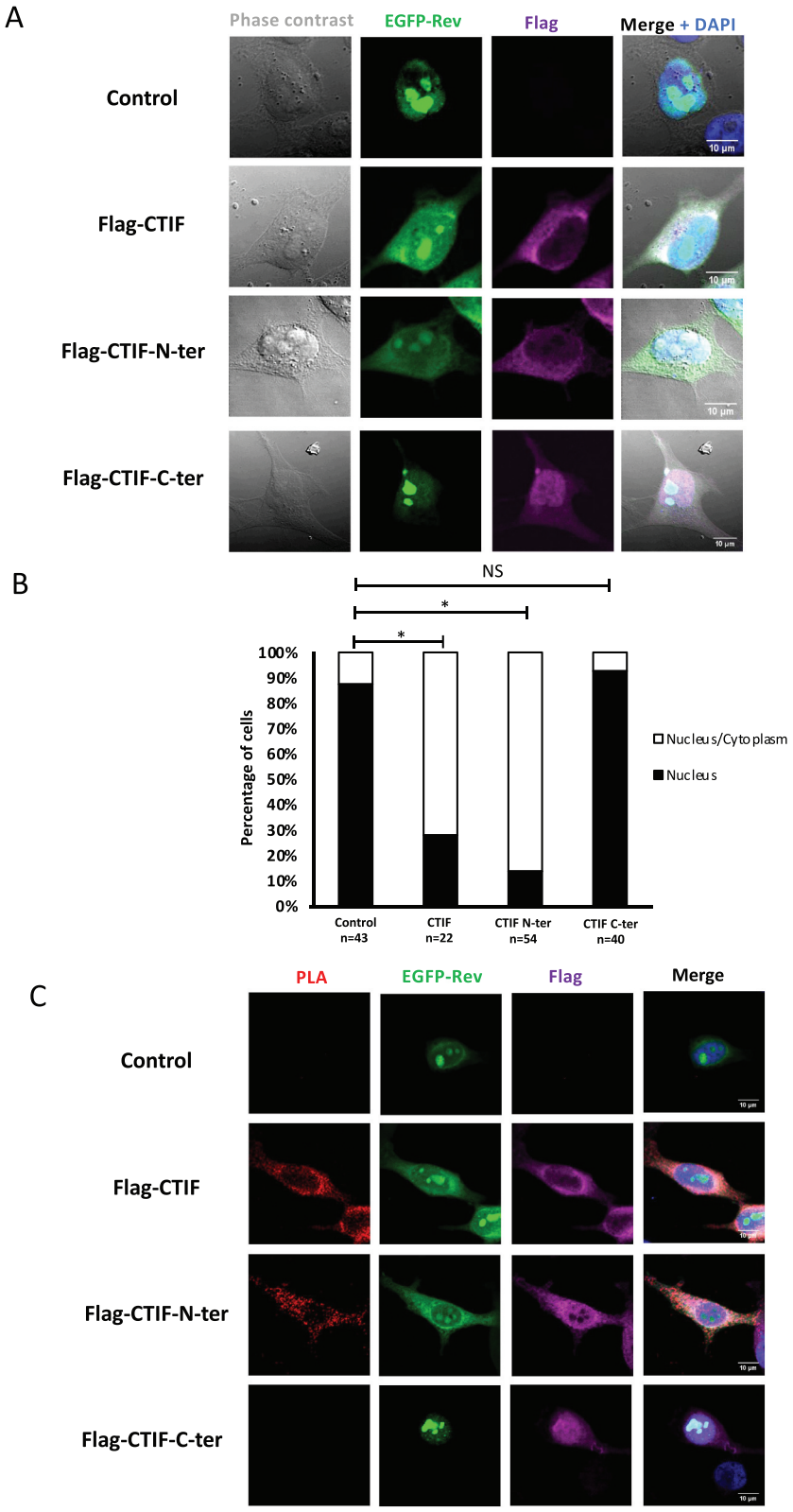

Figure 4. CTIF modifies Rev localization within the cell.

A) HeLa cells (8x10^4 cells/well) transfected with 1 µg pEGFP-Rev together with 1 µg of pcDNA3-Flag-CTIF, pcDNA3-Flag-CTIF-N-ter, pcDNA3-Flag-CTIF-C-ter or pcDNA-β-globin (used as a control) were subjected to immunofluorescence assay using a rabbit anti-Flag antibody. Phase contrast signal is shown in grey, EGFP-Rev signal in green, Flag-tag signal in magenta and DAPI in blue. Scale bar 10 µm.B) Quantification of the sub-cellular localization of EGFP-Rev in conditions presented in A) (*P < 0.05; NS; non-significant, t-test).C) HeLa cells (8x10^4 cells/well) transfected with 1 µg of pEGFP-Rev together with 1 µg of pcDNA3-Flag-CTIF, pcDNA3-Flag-CTIF -N-ter or pcDNA3-Flag-CTIF-C-ter were subjected to the proximity ligation assay using a rabbit anti-Flag antibody and a mouse anti-GFP antibody and the Duolink *in situ* kit. After the rolling circle amplification of the proximity ligation assay, cells were incubated with Alexa anti-rabbit 647 secondary antibody as described in materials and methods. Mock corresponds to non-transfected cells. The PLA signal is shown in red, EGFP-Rev signal in green, Flag signal in magenta and DAPI in blue. Scale bar 10 µm.

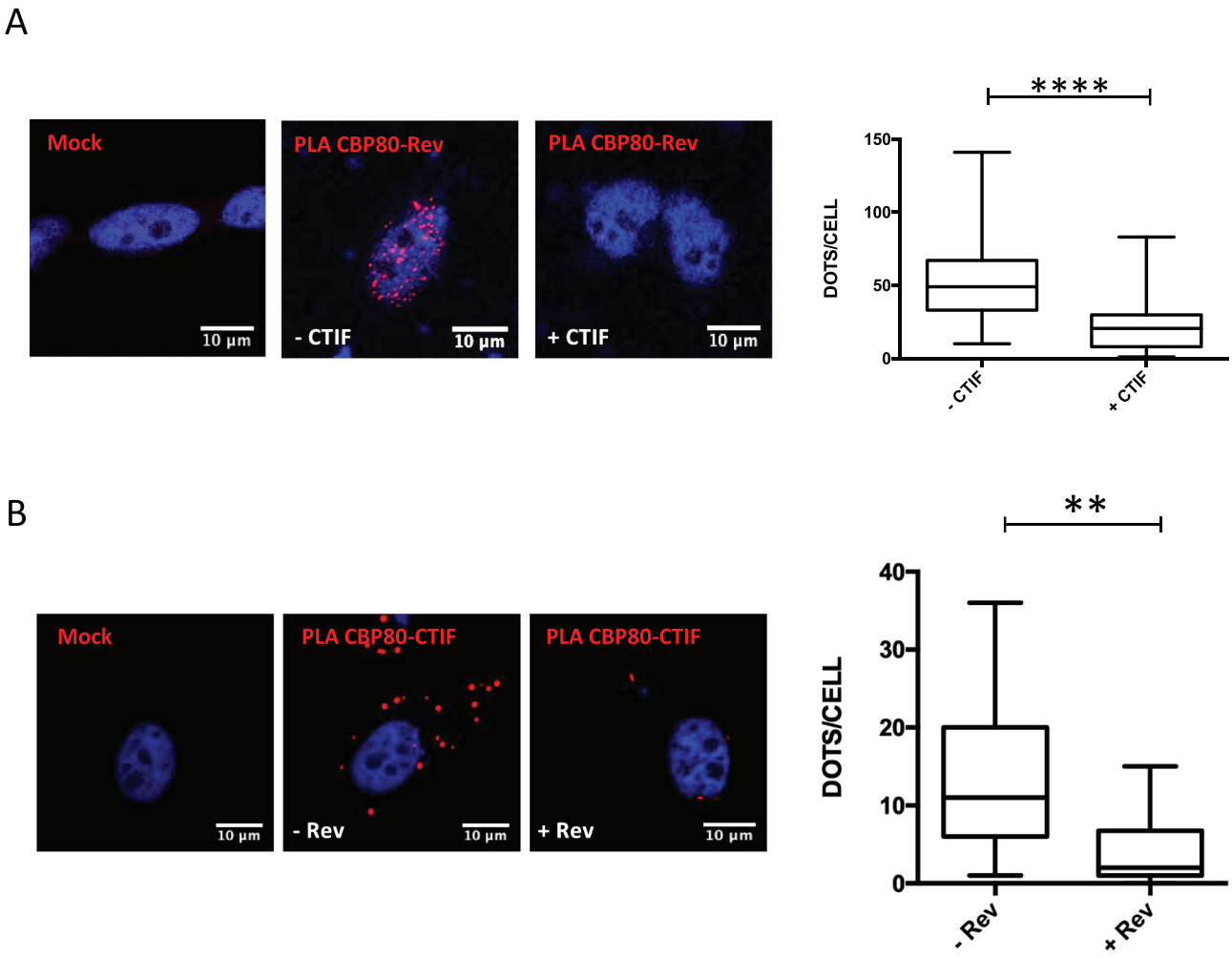

Figure 5. And interferes with the Rev-CBP80 interaction.

A) HeLa cells (8x10^4 cells/well) transfected with pEGFP-Rev and pCMV-myc-CBP80 together with pcDNA β-globin (- CTIF condition) or pcDNA3-Flag-CTIF (+ CTIF condition) were analyzed for the Rev-CBP80 interaction by PLA using a rabbit anti-GFP antibody diluted 1/100 and a mouse anti-Myc antibody (Sigma-Aldrich) diluted 1/100 together with and the Duolink *in situ* kit as described in materials and methods. Mock corresponds to non-transfected cells. The PLA signal is shown in red and DAPI in blue. Scale bar 10 µm. Quantification of dots per cell in – CTIF (n = 43 cells) and + CTIF (n = 37 cells) is presented (****: P < 0.0001, Mann–Whitney test).B) HeLa cells (8x10^4 cells/well) transfected with pcDNA3-Flag-CTIF and pcDNA-V5-CBP80 together with pEGFP (- Rev condition) or pEGFP-Rev (+ Rev condition) were analyzed for the CTIF-CBP80 interaction by PLA using a rabbit anti-Flag antibody diluted 1/200 and a mouse anti-V5 antibody (Santa Cruz) diluted 1/100 together with the Duolink *in situ* kit as described in materials and methods. Mock corresponds to non-transfected cells. The PLA signal is shown in red and DAPI in blue. Scale bar 10 µm. Quantification of dots per cell in – Rev (n = 46 cells) and + Rev (n = 40 cells) is presented (**: P < 0.01, Mann–Whitney test).

and that Rev promotes this association. Since we reported that Rev favored the association of a complex also containing the DEAD-box RNA helicase eIF4AI but no other translation initiation factors such as eIF4GI or eIF3g, we decided to further investigate the composition of this unusual viral mRNP. We focused on CTIF since it is the translation initiation factor that scaffolds the CBC-bound mRNP and the 40S ribosomal subunit through interactions with CBP80 and eIF3g. However, we observed that CTIF associates with Rev and is incorporated into the full-length RNA ribonucleoprotein complex in order to interfere with Gag synthesis in a 5´-UTR-dependent manner. Interestingly, we observed that CTIF binds Rev and interferes with the assembly of the Rev-CBP80 complex, which is required for proper Gag synthesis from the full-length RNA. In addition, Rev impedes the association of CTIF with CBP80 suggesting that CTIF and Rev may compete for CBP80 binding. Thus, from our data it is tempting to speculate that CTIF may bind Rev displacing CBP80 from the Rev/full-length RNA nuclear export complex. This viral mRNP complex containing CTIF instead CBP80 is not efficient in recruiting ribosomes, which results in low levels of Gag synthesis.

The full-length RNA is also used as the viral genome incorporated into nascent virions and a recent report showed that only half of the full-length RNA present in the cytoplasm is translated at a given time and Gag only packages non-translating full-length RNA [50]. Thus, it would be of interest to evaluate whether the CTIF-bound full-length RNA, which is not competent for translation, is addressed for packaging. The role of CTIF in the cytoplasmic abundance and/or turnover of the full-length RNA (as evidenced in our CTIF-KD cells) also requires further investigation. Indeed, despite the full-length RNA of Retroviruses contains long 3`-UTR typical of a class of NMD substrates, they escape this RNA decay pathway [51]. In the case of HIV-1, we have previously reported that the NMD factor UPF1 rather promotes HIV-1

full-length RNA nuclear export by forming a complex with Rev, CRM1 and DDX3 [52]. The interplay between the HIV-1 full-length RNA and cytoplasmic RNA decay factors is a poorly explored field that warrants further exploration.

Considering the different origins of HIV-1 and HIV-2, the conserved inhibition of Gag synthesis observed in both human lentiviruses and the high conservation of CTIF between human and non-human primates (data not shown), it is tempting to speculate that the inhibitory effect of CTIF on Gag synthesis must be conserved at least in primate lentiviruses. Whether CTIF also inhibits the functions of Rev-like proteins from other retroviruses such as Human T-cell lymphotropic virus (HTLV) Rex or mouse mammary tumor virus (MMTV) Rem, amongst others, needs further exploration [53,54].

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Authors contributions

FGdG and RSR designed the experiments, analyzed the data and wrote the manuscript. JC and FVE contributed with the experimental design and with data analysis. FGdG, SRB CPM, BRA, PA, AGA, DTA, AOA, MLA performed the experiments. CRF performed the bioinformatic analysis. All the authors read and approved the final version of the manuscript.

Availability material

Plasmids generated in our laboratory are available upon request

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