Dynamics of the ternary complex formed by c-Myc interactor JPO2, transcriptional co-activator LEDGF/p75 and chromatin*

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Background: The function of transcription factors can be investigated by monitoring their movement inside the nucleus of cells with biophysical techniques.

Results: A model for interaction of JPO2 with chromatin in absence and presence of its interaction partner LEDGF/p75 is proposed.

Conclusion: JPO2 adopts the dynamics of LEDGF/p75.

Significance: Our approach is generic for the study of transcription factor dynamics.

ABSTRACT

Lens epithelium-derived growth factor (LEDGF/p75) is a transcriptional co-activator involved in targeting human immunodeficiency virus (HIV) integration and the development of MLL fusion-mediated acute leukemia. A previous study revealed that LEDGF/p75 dynamically scans the chromatin, and upon interaction with HIV-1 integrase (IN), their complex is locked on chromatin. At present, it is not known whether LEDGF/p75-mediated chromatin locking is typical for interacting proteins. Here, we employed continuous photobleaching, fluorescence correlation and cross-correlation spectroscopy to investigate in vivo chromatin binding of JPO2, a LEDGF/p75- and c-Myc-interacting protein involved in transcriptional regulation. In the absence of LEDGF/p75, JPO2 performs chromatin scanning inherent to transcription factors. However, while the dynamics of JPO2 chromatin binding are decelerated upon interaction with LEDGF/p75, very strong locking of their complex onto chromatin is absent. Similar results were obtained with the domesticated transposase pogZ, another cellular interaction partner of LEDGF/p75. We furthermore show that diffusive JPO2 can oligomerize, that JPO2 and LEDGF/p75 interact directly and specifically through the specific interaction domain (SID) of JPO2 and the C-terminal domain of LEDGF/p75 comprising the integrase binding domain (IBD), and that modulation of JPO2 dynamics requires a functional PWWP domain in LEDGF/p75. Our results suggest that the dynamics of the LEDGF/p75-chromatin interaction depend on the specific partner and that strong chromatin locking is not a property of all LEDGF/p75 binding proteins.

Lens epithelium-derived growth factor (LEDGF/p75) and its shorter p52 splice variant were identified by co-purification with the transcriptional co-activator PC4, suggesting a role...
Dynamics of the JPO2-LEDGF/p75-chromatin complex in transcriptional regulation (1). Based on sequence similarity, LEDGF/p75 is a member of the hepatoma-derived growth factor related protein (HRP) family [reviewed in (2)]. HRPs are characterized by a conserved N-terminal PWWP domain, a 90-135-amino acid module found in a variety of nuclear proteins (3,4). The PWWP domain of LEDGF/p75 recognizes H3K36me3 marks (5,6) and cooperates with AT-hooks and the nuclear localization signal (NLS) to interact with DNA/chromatin as has been shown in vitro (7) and in cells (8). Next to the PWWP domain, LEDGF/p75 contains a conserved integrase binding domain (IBD) absent in the p52 splice variant (9). This domain binds lentiviral integrases, as well as several other cellular proteins such as JPO2, pogZ, MLL-menin and Cdc7-ASK (10-16). Through its interaction with MLL-menin, LEDGF/p75 is implicated in embryonic development and MLL-fusion driven forms of leukemia (15-17). In addition, LEDGF/p75 has also been linked to solid cancers (18,19), and is involved in stress response and cell survival, as its expression is induced by a wide range of stress stimuli (18,20). A vast amount of LEDGF/p75 research was focused on its role during HIV replication. Through its interaction with HIV-1 integrase (IN), LEDGF/p75 locks the latter onto chromatin (21) and tethers the HIV pre-integration complex (PIC) containing IN to chromatin, targeting integration to the body of active genes (22-25). This preference is abolished upon knockdown or knockout of LEDGF/p75 (24-27). The role of LEDGF/p75 as a molecular tether of IN is further supported by the finding that the HIV-1 integration pattern is reminiscent of the LEDGF/p75 chromatin interaction profile (28).

The LEDGF/p75 interactor JPO2 (CDCA7L, RAM2, R1) was first identified as a c-Myc transcription factor interacting protein that complements c-Myc transforming activity (29) and shows anti-apoptotic activity (30). JPO2 consists of 454 amino acids and contains a PEST domain, a putative leucine zipper (LZ), a putative nuclear localization signal (NLS) and a zinc-binding RING-finger-like domain (Fig. 1A). Through interaction with the androgen (AR) and retinoic acid receptor (RAR), JPO2 is implicated in the regulation of prostate specific genes (31). A complex interplay at the MAO A gene promoter between Sp1, JPO2, the AR and glucocorticoid receptor has been reported (30,32). Low levels of JPO2 increase MAO A expression, a finding that is correlated to suicide victims suffering from major depression disorder (33,34). In addition, transcriptional activity of the MAO B gene is also regulated by JPO2 through the interplay with Sp1 and E2F-associated phosphoprotein (35), that upon SUMOylation binds JPO2 at a SUMO interaction motif in its N-terminal part (36). All these data are indicative of a role of JPO2 in transcriptional regulation. Independently, JPO2 was identified as a cellular binding partner of LEDGF/p75 (11,14). In these studies, the stability and association of JPO2 with chromatin were shown to depend on the presence of LEDGF/p75. The p75-binding domain in JPO2, also referred to as the specific interaction domain (SID), was mapped between residues 62-94 (11,14). Like IN, JPO2 interacts with LEDGF/p75 via the IBD (11,14). In this regard it was shown that binding was mutually exclusive although the IBD residues engaged by JPO2 and IN were not identical (11,14).

Fluorescence fluctuation spectroscopy methods such as continuous photobleaching (CP) and fluorescence correlation spectroscopy (FCS) were originally developed to measure diffusion coefficients (37,38), but can also be used to study dynamic chromatin interactions in cells (39). In addition, fluorescence cross-correlation spectroscopy (FCCS) can be used to specifically study protein-protein interactions in cells (40). Using CP and FCS, we could show that the dynamic chromatin scanning/hopping mode of LEDGF/p75 inside live HeLa cell nuclei is mediated in part by its PWWP domain (21). Additionally, when IN was overexpressed in these cells, LEDGF/p75 was shown to lock the LEDGF/p75-integrase complex onto the chromatin via its PWWP domain (75-fold increase in affinity). Since IN has a low intrinsic affinity for chromatin, chromatin locking cannot solely be explained by the presence of IN (21). Moreover, although this mechanism may be a crucial step for HIV integration targeting, it is not known whether it is a general mechanism upon binding of cellular and/or viral proteins to the IBD of LEDGF/p75. Here we explored the dynamic behavior of JPO2 inside living cells in the absence and presence of LEDGF/p75 using CP and FCS and investigated in detail its interaction with LEDGF/p75 by FCCS.
EXPERIMENTAL PROCEDURES

**Plasmids** - Plasmids for expression of eGFP (-fusion) proteins in eukaryotic cells were based on the peGFP-C1 backbone (Clontech Laboratories, Inc., Mountain View, CA). The plasmid for the expression of eGFP-JPO2 has been described elsewhere (pEGFP-JPO2) (11). The expression plasmid for eGFP-JPO298-454 was constructed by inserting the coding sequence into the BamHI and XhoI digested peGFP-C1 constructs downstream of and in frame with the eGFP coding sequence. The JPO298-454 coding sequence was amplified from pEGFP-JPO2 with the following primers: 5'-GCGGGATCCGAAGTAATGGTGTTGAG and 5'-ACCTCGAGTCAATTGTCTTCTACCAGC. The plasmid encoding RNAi-resistant mRFP-LEDGF/p75 for expression in HeLa p75KD cells was created by amplifying the ‘LEDGF BC’ gene (41,42) by PCR with 5'-GGCGAATTCAACTCGCGATTTCAAACCTGG and 5'-GAATTCGGATCCCTAGTTATCTAGTGTAGAAT primers. The amplicon was subsequently ligated in the pmRFP-C1 plasmid (21) after digestion with EcoRI/BamHI. The plasmid for expression of mRFP-LEDGF/p75k56D/R74D,BC was created from pmRFP-LEDGF/p75BC by site-directed mutagenesis as described elsewhere (21). A plasmid for eukaryotic expression of mRFP1-labeled pogZ 1117-1410 (mRFP-ΔNpogZ) has been described elsewhere (10).

**Cell lines** - HeLa cells were obtained from the NIH Reagent program and were cultivated at 5% CO$_2$ and 37°C in a humidified atmosphere in high-glucose Dulbecco’s Modified Eagle Medium (Life Technologies Europe B.V., Ghent, Belgium), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich gmbh, Taufkirchen, Germany) and 50 µg/mL Gentamycin (Life Technologies). Monoclonal HeLa cells with a strong (> 97%) knockdown of endogenous LEDGF/p75 (HeLa_p75KD) were cultivated as described previously (42).

**Transfections** - Transient expression was achieved via cationic lipid-mediated plasmid DNA transfection (Lipofectamine2000, Life Technologies). One day before transfection, 0.5-1×10$^6$ cells were seeded per well in a #1 Lab-Tek Chambered Coverglass (VWR International, Leuven, Belgium). Cells were transfected at 50-70% cell confluency. Plasmid DNA (0.5 µg) and Lipofectamine2000 (0.5-1 µL/0.5 µg DNA) were diluted in 50 µL OptiMEM (Life Technologies) per well and incubated for 30 minutes at RT. Subsequently, 350 µL pre-warmed growth medium was added, the cell medium was replaced with the transfection mixture and cells were incubated for at least 12 hours prior to the microscopy experiments.

**Western blot** - Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on NuPage 10% Bis-Tris gels (Life Technologies), followed by electroblotting onto polyvinylidene difluoride membranes. LEDGF/p75 was detected using a polyclonal rabbit derived antibody (A300-847A, Bethyl Laboratories Inc., Montgomery, TX, USA) and eGFP was detected using an in-house polyclonal anti-GFP rabbit derived antibody. Detection was performed with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibodies (Dako Belgium nv/sa, Heverlee, Belgium) using an Amersham ECL+ chemiluminescence kit (GE Healthcare Europe GmbH, Diegem, Belgium).

**Spectroscopy** - Continuous photobleaching (CP) (43), fluorescence correlation (FCS) (38) and cross-correlation (FCCS) spectroscopy (40) experiments were performed on a commercial microscope (LSM510/ConfoCor2, Carl Zeiss, Jena, Germany) as previously described (21). The enhanced green fluorescent protein (eGFP) and monomeric red fluorescent protein 1 (mRFP) (44) were used as fluorescent markers.

For CP analysis, the fluorescence intensity $F(t)$ per cell was normalized to $F(0)$ after which different cells were averaged. This average was subsequently fitted with (45):

$$F(t) = y_0 + (1 - y_0) \left[ \frac{F_1 \exp(-k_1 t)}{(1 - F_1 \exp(-k_2 t))} \right],$$  
Eq. 1

where $y_0$ is the offset, $F_1$ and $k_1$ are the immobile/slow fraction and corresponding bleaching constant, respectively, and $k_2$ is the decay constant representing photobleaching due to the finite size of the nuclear compartment. The percentage photobleaching after 20 seconds, $F_{20s}$, was used to compare different proteins.
For FCS analysis, the experimental autocorrelation functions (ACF) from different cells were globally fitted in Igor Pro 5 (Wavemetrics, Portland, OR) with (46,47):

$$G(r) = \frac{1}{N} \prod_{i=1}^{n} \left[ 1 + F_i \left( 1 - F_i \right)^{-1} \exp(-\tau / \tau_i) \right] \cdot \sum_{i=1}^{n} \left[ F_i \left( 1 + 4D_i \tau \right)^{-1} \left( 1 + 4D_i S^{-2} \tau \right)^{1/2} \right],$$

Eq. 2

where $\tau$ is the correlation lag time, $N$ the average number of particles in the confocal volume, $m$ the total number of fast blinking processes of the fluorescent protein, $m = 0$ for cross-correlation, $F_i$ and $\tau_i$ the fraction and relaxation time blinking process $j$, $n$ the total number of diffusing species, $F_i$ and $D_i$ the fraction and diffusion coefficient of species $i$, $\sum_{i=1}^{n} F_i = 1$ and $S$ the ratio of axial ($\omega_z$) to lateral ($\omega_r$) waist of the confocal volume. All fitting parameters except the intermediate relaxation time ($500 \mu$s) of eGFP blinking (21,48) were optimized during the fitting process. In the figures, ACFs from different cells were normalized on $G(0)$ and subsequently averaged.

The molar concentration $c$ of the fraction measured with FCS was calculated from $N$ with:

$$c = \frac{N}{N_A 2 \pi \omega_z^2 \omega_r},$$

Eq. 3

where $N_A$ is Avogadro’s number. For comparing the dynamics of different proteins, the two-component diffusion parameters were converted into a species-weighted $D$:

$$\overline{D} = F_1 D_1 + F_2 D_2.$$

Eq. 4

A species-weighted $\overline{D}$ performs well in all types of kinetic chromatin binding regimes and allows a direct comparison with previously determined values of $D$ for other proteins (21). For FCCS analysis, all three correlation functions (ACF$_{\text{green}}$, ACF$_{\text{red}}$ and CCF) were fitted with Eq. 2 and for those measurements with equimolar expression of eGFP- and mRFP-labeled protein (i.e. $0.75 < N_{\text{green}} / N_{\text{red}} < 1.25$) the CCF was normalized by $G(0)_{\text{ACF,green}}$. In this case, the normalized CCF amplitude is directly proportional (but not equal to) the concentration of green-red complexes. To compare different measurements, the relative cross-correlation was calculated (49,50):

$$CC_{\text{rel}} = \frac{G_{\text{CCF}}(0)}{G_{\text{ACF,green}}(0)},$$

Eq. 5

where $G_{\text{ACF,green}}(0)$ is the diffusion amplitude of the ACF calculated with photons from the eGFP detection channel, and $G_{\text{CCF}}(0)$ is the amplitude of the CCF. Due to bleed through of eGFP in the detection channel of mRFP, a non-zero CCF amplitude and thus $CC_{\text{rel}}$ is observed even for non-interacting species.

**FLIM-FRET** - Fluorescence lifetime imaging microscopy based Förster resonance energy transfer (FLIM-FRET) was performed on a two-photon excitation laser scanning microscope (Leica TCS SP5, Leica Microsystems CMS GmbH, Mannheim, Germany + MaiTai-HP Ti:Sapphire, Newport Spectra-Physics) equipped with a LSM upgrade kit for performing time resolved measurements (Picoquant GmbH, Berlin, Germany). eGFP was excited at 1.5% (~5 mW) of the 880-nm pulsed laser light through a HCX-PLAPO-63x NA1.2 water objective (Leica) and emission was captured through a 535/50 bandpass filter on single-photon avalanche diodes. The fluorescence decay was measured with time-correlated single-photon counting and was calculated from all cytosolic or nuclear pixels. Analysis was performed by mono- or bi-exponential convolution fitting. The instrument response function was determined with malachite green.

**RESULTS**

*Dynamic chromatin interaction of JPO2* - To study the interaction between JPO2 and chromatin, experiments were performed in HeLa cells with a stable 97% knockdown of LEDGF/p75 (HeLap75KD) (42). JPO2 was N-terminally labeled with eGFP (eGFP-JPO2) (Fig. 1A). Previous studies reported that the cellular distribution of JPO2 with different N-terminal fluorescent tags (eGFP, HcRed, mRFP1) or MBP is reminiscent of that of wild-type JPO2. In addition, these fusions do not disturb the interaction with LEDGF/p75 (11,14). eGFP-JPO2 expressed as a full-length protein inside HeLa cells, as verified by western
blotting (Fig. 1B). In HeLa p75KD cells, eGFP-JPO2 localized heterogeneous in the nucleus, but did not interact with chromosomes in mitotic cells (Fig. 1C). At high expression levels eGFP-JPO2 partially localized in the cytoplasm (data not shown), as has been observed before (8). Next, we studied the dynamics of eGFP-JPO2 inside the nuclei of HeLa p75KD cells by recording fluorescence time traces and analyzing these with CP and FCS methods. With the CP method, the time-dependence of photobleaching of a fluorescently labeled molecule (in our case eGFP - JPO2) is analyzed to quantify its mobility. In the case of a chromatin-binding protein inside the nucleus, diffusion is slowed down, or sometimes even halted, rendering the CP method suitable for quantifying chromatin interactions in cells. As a relative measure for chromatin binding, we used the relative amount of fluorescence photobleached after 20 seconds, \( F_{20s\text{-}eGFP-JPO2} \). After 20 seconds, a near-steady state is reached, during which remaining signal fluctuations can be analyzed with the FCS method. With FCS, a temporal autocorrelation function (ACF) of the fluorescence signal is calculated. This ACF carries information on the absolute protein concentration, \( c \), and the diffusion coefficient, \( D \). In the case of dynamic chromatin interactions, an effective \( D \) is usually measured in the nucleus, that is much lower than expected for free Brownian diffusion.

Compared to eGFP that is freely diffusing in the nucleus of HeLa p75KD cells, eGFP-JPO2 exhibited significantly more photobleaching (\( p < 0.01 \)) as measured with CP (\( F_{20s\text{-}eGFP} = 7\pm1\% \) vs. \( F_{20s\text{-}eGFP-JPO2} = 16\pm7\% \)) suggesting chromatin interaction, and strongly reduced overall dynamics as measured with FCS (\( D_{eGFP} = 33.0\pm3.5 \, \mu m^2/s \) and \( D_{eGFP-JPO2} = 1\pm0.4 \, \mu m^2/s \) for) in cells with comparable expression levels (Fig. 1D-E and Table 1). Noteworthy, eGFP-JPO2 displayed lower photobleaching and faster diffusion as compared to eGFP-tagged LEDGF/p75 (\( F_{20s\text{-}eGFP-JPO2} = 39\pm3\% \) and \( D_{eGFP-JPO2} = 0.5\pm0.1 \, \mu m^2/s \)) (Fig. 1D-E). The larger standard deviation for \( F_{20s\text{-}eGFP-JPO2} \) and for \( D \) for eGFP-JPO2 relative to eGFP-LEDGF/p75 (Table 1), may be due to variable, low amounts of endogenous LEDGF/p75 still present in the HeLa p75KD cells. Taken together, these results show that JPO2 can interact dynamically with chromatin in the absence of LEDGF/p75, albeit with a lower affinity for chromatin than LEDGF/p75 itself.

**JPO2 is decelerated by LEDGF/p75** - To analyze the LEDGF/p75-JPO2 interaction, LEDGF/p75 was fused to the red fluorescent protein mRFP1 (mRFP-LEDGF/p75) (Fig. 2A). Expression of mRFP-LEDGF/p75 was confirmed by Western blot (Fig. 2B) and confocal microscopy (Fig. 2C). As seen for endogenous LEDGF/p75 (51), mRFP-LEDGF/p75 displayed a typical dense fine speckled distribution and interacted with mitotic chromatin. The protein exhibited very slow dynamics in HeLa p75KD cells as measured with FCS (Fig. 2D), but the obtained diffusion coefficient was slightly increased (Table 1) as compared to the one previously reported for eGFP-LEDGF/p75 (21). The mRFP1 is known to have an intrinsically low photostability (44), causing increased photobleaching during FCS experiments, which in turn leads to artificially increased diffusion coefficient (52), explaining this observation.

Next, we probed direct intracellular complex formation of mRFP-LEDGF/p75 and eGFP-JPO2. In HeLa p75KD cells co-expressing both proteins, colocalization was observed throughout the nucleus in interphase cells, as has been shown before (11,14), and on chromosomes in mitotic cells (Fig. 2E). In cells with equimolar expression of both proteins, we performed FCCS experiments. With the FCCS method, a temporal cross-correlation function (CCF) of the fluorescence signals coming from the eGFP and mRFP fluorophores is calculated. This CCF carries information on the absolute binding affinity. To quantify FCCS experiments, the \( C_{rel} \) value is typically calculated (Eq. 5). Diffusion of two (differentially labeled) proteins as part of the same complex results in increased \( C_{rel} \) values with respect to a negative control. In cells containing equimolar expression levels of eGFP-JPO2 and mRFP-LEDGF/p75, the \( C_{rel} \) was indeed significantly higher (\( C_{rel} = 0.32\pm0.10 \)) than in the control cells (eGFP-JPO2 + mRFP; \( C_{rel} = 0.17\pm0.15 \), \( p < 0.01 \)), proving that both proteins are indeed engaged in a mobile protein-protein interaction complex in cell nuclei. This now allows studying the impact of LEDGF/p75 on the dynamics of JPO2 with continuous photobleaching and FCS.

In the presence of mRFP-LEDGF/p75, photobleaching (\( F_{20s\text{-}eGFP-JPO2} \) increased...
significantly from 16±7% to 39±6% (p < 0.01) while the diffusion coefficient decreased two-fold ($D_{\text{eGFP-JPO2}} = 1.0±0.4 \ \mu m^2/s$ and $D_{\text{eGFP-JPO2+mRFP-LEDGF/p75}} = 0.6±0.1; p < 0.01$) (Fig. 2G,H and Table 1), supporting reduced overall mobility of eGFP-JPO2. Strikingly, the $F_{20s}$ and $D$ values of the complex approached that of LEDGF/p75 only, suggesting LEDGF/p75 determines the mobility of the LEDGF/p75-JPO2 complex (Table 1). Importantly, given the pronounced blinking and lower photostability of the mRFP1 fluorophore under the experimental conditions used here for FCS (Fig. 2D), it is more correct to use the values measured for fusions to the same fluorescent protein, in this case eGFP, when comparing the dynamics ($F_{20s}$) of JPO2 and LEDGF/p75 (Table 1, compare values for eGFP-LEDGF/p75 with those for eGFP-JPO2 + mRFP-LEDGF/p75). The two-fold decrease in the diffusion coefficient stands in stark contrast to the ~75-fold decrease we observed earlier for the LEDGF/p75-IN complex (Table 1) suggesting a different mechanism of chromatin interaction (21). To confirm that the co-diffusing JPO2-LEDGF/p75 complexes result from directly and specifically interacting proteins, we made a JPO2 deletion mutant lacking amino acids 1-97 (comprising the SID, residues 62-94) (eGFP-JPO298-454) (Fig. 1A). Expression was verified with Western blot (Fig. 3A). In HeLa p75KD cells this protein still localized in the nucleus, but did not interact with mitotic chromosomes (Fig. 3B). As expected, the interaction defective eGFP-JPO2 mutant did not co-localize with mRFP-LEDGF/p75 (Fig. 3C). We next performed measurements in cells with similar expression levels for eGFP-JPO298-454 as for the wild-type protein (Table 1, c). Interestingly, compared to eGFP-JPO2 the deletion mutant displayed slower dynamics as measured with CP ($F_{20s}$,eGFP-JPO2 = 16±7% and $F_{20s}$,eGFP-JPO298-454 = 32±7%) (Table 1). Possibly, deletion of residues 1-97 might increase the affinity for chromatin or other unknown cellular proteins. FCS measurements with eGFP-JPO298-454, on the other hand, were indistinguishable from those of wild-type JPO2 (Table 1), suggesting that the first 97 amino acids of JPO2 are not necessary for dynamic chromatin interactions of JPO2. A domain in JPO2 mediating dynamic chromatin scanning would thus lie outside of residues 1-97. Finally, as expected, mRFP-LEDGF/p75 co-expression did alter neither the $F_{20s}$ nor the $D$ of eGFP-JPO298-454 (Fig. 3D-E) and no significant cross-correlation was observed in cells after equimolar expression of both proteins (Fig. 3F). Taken together, we showed that colocalizing mRFP-LEDGF/p75 and eGFP-JPO2 constitute a mobile interacting protein complex and that LEDGF/p75 dictates the mobility of this complex. We proved the specificity of the observed interaction by deleting the LEDGF/p75 binding domain of JPO2, which resulted in a loss of the effect of mRFP-LEDGF/p75 on the dynamics of eGFP-JPO2.

**Insights in the JPO2-LEDGF/p75 chromatin tethering mechanism** - Previous experiments have shown that the LEDGF/p75-HIV-1 IN complex is locked onto the chromatin and that this requires the presence of PWWP chromatin interacting domain of LEDGF/p75 (Fig. 2A) (21). Inhibition of the PWWP-chromatin interaction by specific mutations in the PWWP domain (K56D/R74D) resulted in a mobile, nuclear IN-LEDGF/p75 complex. Here, we investigated whether the mobility of the LEDGF/p75-JPO2 complex is also dependent on the PWWP domain. Therefore, we compared the dynamics of eGFP-JPO2 in the presence of mRFP-LEDGF/p75 or the PWWP-chromatin interaction mutant mRFP-LEDGF/p75K56D/R74D. Expression of mRFP-LEDGF/p75K56D/R74D was confirmed by Western blot (Fig. 4A). In HeLa p75KD cells, the protein lacked the typical dense fine speckled distribution of LEDGF/p75, i.e. the protein was dispersed equally over the nucleus and not able to interact with mitotic chromatin (Fig. 4B), as has been shown before for eGFP-LEDGF/p75K56D/R74D (21). This mutant LEDGF/p75 still co-localized with eGFP-JPO2 in interphase cells, suggesting their interaction is preserved (Fig. 4C). As expected, in cells co-expressing mRFP-LEDGF/p75K56D/R74D and eGFP-JPO2, significantly less photobleaching of eGFP-JPO2 occurred (p < 0.01) and the dynamics of eGFP-JPO2 as measured with FCS were also increased (p = 0.017), as compared to co-expression with wild-type mRFP-LEDGF/p75 (Fig. 4D,E and Table 1). FCCS analysis confirmed that a direct interaction between JPO2 and LEDGF/p75K56D/R74D was still possible (p < 0.01) (Fig. 4F).

Finally, we corroborated that JPO2 interacts with the C-terminal domain of LEDGF/p75. To
this end, we performed FCCS experiments in cells co-expressing eGFP-JPO2 and either mRFP-LEDGF/p75326-530 or mRFP-p52 (Fig. 2A). While the former LEDGF/p75 deletion mutant comprises the IBD (residues 347–429), the latter is the alternative splice variant of the ledgf gene mRNA product, sharing its first 325 residues with those of LEDGF/p75. We only observed positive cross-correlation in cells expressing eGFP-JPO2 and mRFP-LEDGF/p75326-530 (Fig. 4F), confirming that, indeed, JPO2 interacts with the C-terminal part of LEDGF/p75 in cells.

In summary, our results show that the deceleration of the dynamics of the LEDGF/p75-JPO2 complex relative to JPO2 is due to chromatin interactions of the LEDGF/p75-PWWP domain and that JPO2 interacts with the C-terminal part of LEDGF/p75 comprising the IBD.

Oligomerization of JPO2 (and IN) – To get more insight into the structure of diffusive nuclear JPO2, we tested whether JPO2 is able to form oligomers in cells with FLIM-FRET. With this method, protein-protein interactions can be determined in a sensitive manner. Practically, the average fluorescence lifetime, \( \tau_f \), of the binding partner carrying the more blue-shifted fluorophore (the FRET donor, in our case eGFP) is quantified in the absence or presence of the binding partner carrying the more red-shifted fluorophore (the FRET acceptor, in our case mRFP1). If the binding partners interact and the two fluorophores are within 10 nm distance, Förster-type energy transfer will cause the \( \tau_f \) of the donor to be lowered (quenched). As a control experiment, we measured a direct fusion of donor and acceptor inside HeLa cells (mRFP-eGFP) (Table 2). Indeed, relative to freely diffusing eGFP (\( \tau_f = 2.43\pm0.02 \text{ ns} \)), this protein exhibited a strongly quenched eGFP \( \tau_f \) (\( \tau_f = 1.95\pm0.05 \text{ ns} \)). Next, we performed FLIM-FRET in the nucleus of HeLa\(_{p75KD}\) cells co-expressing eGFP-JPO2 and mRFP-JPO2. In these cells, the eGFP fluorophore was again significantly quenched (\( \tau_f = 2.02\pm0.08 \text{ ns} \)) relative to eGFP-JPO2 expressed alone (\( \tau_f = 2.17\pm0.06 \text{ ns} \)) or co-expressed eGFP-JPO2 and mRFP1 (\( \tau_f = 2.18\pm0.06 \text{ ns} \)) (Table 2). This strongly suggests the JPO2 protein is present (at least) as a dimer in the nucleosol.

As a comparative study, we also performed FLIM-FRET in cells co-expressing eGFP- and mRFP-tagged IN. From these experiments, it was clear that also IN is present as a stable oligomer in the nucleus of HeLa\(_{p75KD}\) cells (Table 2), which had been shown before in vitro (53).

Taken together, FLIM-FRET experiments showed that both JPO2, as well as IN, are present as oligomers in the nucleus of HeLa\(_{p75KD}\) cells.

Mobility and interaction of pogZ-LEDGF/p75 – To verify whether other cellular interaction partners of LEDGF/p75 possessed a similar dynamical signature, as did JPO2, we performed similar experiments with the pogZ domesticated transposase (10). Like JPO2 and IN, pogZ interacts with LEDGF/p75 via its IBD domain. Since the full-length pogZ protein (1410 residues) is known to exhibit very low expression in HeLa cells, we performed these experiments employing an N-terminal truncation mutant of pogZ labeled with the mRFP1 fluorescent protein (mRFP-\( \Delta \)NpogZ). This mutant retains the pogZ DDE domain and its ability to interact with LEDGF/p75 (10). In HeLa\(_{p75KD}\) cells, this protein exhibited a pan-cellular distribution, with a slight enrichment in the cytosol (Fig. 5A). In the nucleus, mRFP-\( \Delta \)NpogZ diffused slower than expected for free nuclear diffusion (\( \overline{D} = 2.1\pm0.6 \mu m^2/s \), Table 1) (Fig. 5B). Such mobility is indicative for interactions within the nuclear compartment in the absence of significant amounts of endogenous LEDGF/p75. Upon co-expression of eGFP-LEDGF/p75, mRFP-\( \Delta \)NpogZ relocated almost completely to the nucleus (Fig. 5C), and its dynamics lowered to a \( \overline{D} \) of 1.2\( \pm \)0.5 \( \mu m^2/s \) (Fig. 5B and Table 1). Thus, similar to what we observed for JPO2 (Fig. 2H and Table 1), the dynamics of pogZ approached those of LEDGF/p75 (mRFP-LEDGF/p75, \( \overline{D} = 0.9\pm0.3 \mu m^2/s \), Table 1), when the latter is co-expressed. To investigate whether pogZ and LEDGF/p75 engaged in a mobile complex, we performed a cross-correlation analysis. Indeed, significant cross-correlation could be observed (Fig. 5D), and after analysis a \( CC_{rel} \) of 0.28\( \pm \)0.11 (n = 12 cells) could be determined. Taken together, like JPO2, pogZ exhibits slow nuclear dynamics independent of LEDGF/p75, and forms a two-fold slower complex with LEDGF/p75 when the latter is present.

DISCUSSION
JPO2 exhibits dynamics typical for a chromatin binding protein - When expressed at low, nanomolar concentrations in HeLa-p75KD cells, eGFP-JPO2 localized in the nucleus. Four explanations are possible: (i) active nuclear import, (ii) high intrinsic affinity for chromatin, (iii) chromatin tethering by one or more proteins or (iv) chromatin tethering by remaining endogenous LEDGF/p75. Active nuclear import is plausible since JPO2 possesses a predicted NLS (Fig. 1A) (32). Once translocated, JPO2 could remain localized in the nucleus due to its intrinsic affinity for chromatin. In agreement with this, the protein does contain different domains that would allow direct interactions with DNA, such as a leucine zipper and a RING-finger-like (CXXC), Zn-binding domain (Fig. 1A). In addition, the protein is known to interact with different cellular chromatin interacting proteins that could tether it to chromatin. In this work, we found that even in absence of one of its natural binding partners, LEDGF/p75, JPO2 exhibits remarkably slow dynamics (Fig. 1D,E). Its diffusion coefficient ($D = 1.0\pm0.4 \mu m^2/s$) was about 30-fold slower than the eGFP control for free nuclear diffusion ($D = 33.0\pm3.5 \mu m^2/s$). Given that for free Brownian motion, the diffusion coefficient of a molecule is proportional to $M_t^{-1/3}$, and given the $M_t$ of eGFP-JPO2 ($M_t = 78$ kDa), and of eGFP ($M_t = 27$ kDa), this 30-fold difference cannot solely be explained by a difference in molecular mass (Fig. 1E, dashed curve). A similar dynamic behavior has been observed before with similar methods for other chromatin-interacting proteins such as (but not limited to) the nucleosomal binding protein HMG-17, the pre-mRNA splicing factor SF2/ASF, the rRNA processing protein fibrillarin (54), the RNA polymerase II (subunit B1), the Heterochromatin Protein 1β, the Histone H1 (21,55), the Sec combs reduced (Scr) peptides transcriptional regulator (56), and finally, LEDGF/p75 (Fig. 2D). With respect to the latter, we have recently shown with a combination of fluorescence recovery after photobleaching, FCS, FCCS and tunable-focus FCS, that the slow apparent dynamics of LEDGF/p75 are due to fast-kinetic interactions with chromatin (21).

More generally, in recent years, sensitive and high time-resolution fluorescence methods such as FCS, fluorescence recovery after photobleaching and single particle tracking have allowed detailed investigations of the (spatio-)temporal organization of proteins belonging to different dynamical classes. These classes range from the quasi-static nuclear structures such as (but are not limited to) core histones, heterochromatin and the nuclear lamina (57), to the highly dynamic class of transcription factors and related proteins (54,58-62). Especially with respect to the latter class, proteins nearly always exhibit slow ($D \sim 0.1-5 \mu m^2/s$) apparent free (i.e. Brownian-like) diffusion. When analyzed in more detail, however, these dynamics can often be attributed to frequent (high on-rate, high off-rate) interactions with either chromatin and/or with other slow chromatin interacting proteins (21,55,63-69). Such dynamical regime is likely true for JPO2 (as well as pogZ), and is illustrated in Fig. 6A: JPO2 interacts dynamically with chromatin through its known chromatin interacting domains, and dynamically moves about in the nuclear compartment by chromatin scanning and/or hopping. Finally, although its intracellular interaction with LEDGF/p75 and chromatin seems unaffected by the presence of an N-terminal tag [Fig. 1, Fig. 2 and (11,14)], it needs to be emphasized that the hydrodynamical properties observed and quantified in this work, are those of eGFP-labeled JPO2, and are therefore not necessarily or completely representative for endogenous JPO2.

Relative cross-correlation – Previously, we have measured the relative cross-correlation, $CC_{rel}$, for the IN-LEDGF/p75 interaction with the same method. Because of the high affinity of this complex for the nearly immobile chromatin, and since FCCS only allows quantification of interactions of mobile complexes, different strategies had to be pursued first to render the complex mobile in cells, either by re-targeting to the cytosol (70,71), or by targeting away from chromatin in the nucleus (21). In those experiments, a $CC_{rel}$ of 0.47-0.60 was observed, which is significantly higher than that observed here for the JPO2-LEDGF/p75 ($CC_{rel} = 0.32\pm0.10$) and pogZ-LEDGF/p75 ($CC_{rel} = 0.28\pm0.11$) interaction. Noteworthy, the $CC_{rel}$ for JPO2-LEDGF/p75 did not scale significantly with the eGFP-JPO2 concentration (data not shown), which suggests that the measured $CC_{rel}$ value is not biased by endogenous JPO2. Two properties might explain this difference in $CC_{rel}$ between IN and
cellular partners of LEDGF/p75: binding affinity and binding stoichiometry.

A lower affinity of the cellular binding partners for LEDGF/p75 would lead to a lower $CC_{rel}$ value (72,73), which would be in line with previous results from in vitro AlphaScreen experiments on JPO2, pogZ and IN (10,11). Furthermore, a lower binding affinity would render the formation of a significant population of near-immobile complex improbable, which is in line with our observations (Fig. 2, Fig. 5 and Table 1).

On the other hand, a lower binding stoichiometry would also lead to a lower $CC_{rel}$ value (74). With FLIM-FRET, we observed di- or oligomers of JPO2, as well as of IN (Table 2), suggesting a lower stoichiometry of the JPO2-LEDGF/p75 complex is not the reason for the lower $CC_{rel}$. At the moment of this writing, LEDGF/p75 is known to interact with the SID of JPO2, but no evidence exists that it would favors binding to a JPO2 dimer, or even enhance JPO2 oligomerization. For the IN-LEDGF/p75 complex on the other hand, it is known that LEDGF/p75 requires an IN dimer for interacting with the latter (75), an IN dimer already contains two LEDGF/p75 binding sites (75), and LEDGF/p75 even stimulates IN tetramerization in vitro (76). The latter has also been hypothesized from in vivo experiments (21). Future experiments might clarify whether the observed lower $CC_{rel}$ for JPO2-LEDGF/p75 is related to a different stoichiometry within the complex.

**Trailing of cellular cargo versus locking of IN by LEDGF/p75 -** When mRFP-LEDGF/p75 and eGFP-JPO2 were co-expressed in HeLa-p75KD cells, no dramatic increase of photobleaching (as measured with CP) or decrease in the dynamics (as measured with FCS) of eGFP-JPO2 was observed (Fig. 2G,H). The small, but significant decrease of the diffusion coefficient of eGFP-JPO2 in the presence of LEDGF/p75 (from 1.0±0.4 $\mu m^2/s$ to 0.6±0.1 $\mu m^2/s$) was overcome by mutating the PWPP domain of LEDGF/p75 or by mutating the SID of JPO2 (Fig. 3 and 4). Furthermore, in cells co-expressing wild-type JPO2 and LEDGF/p75, we clearly observed their interaction in the nucleolus (Fig. 2F). These experiments thus suggest that LEDGF/p75 provides a specific and dynamic link between JPO2 and the chromatin, as illustrated in Fig. 6B. For pogZ, another cellular interaction partner of LEDGF/p75, a similar dynamic link model is likely true. This is a fundamentally different observation than the ‘chromatin locking’ observed previously for the IN-LEDGF/p75 complex (21). Indeed, for the LEDGF/p75-IN interaction, the highest concentration of interacting protein was found associated with chromatin, not freely diffusing in the nucleosol. Chromatin locking, the process by which LEDGF/p75 attaches IBD-bound IN to chromatin by its PWPP domain, resulted in an IN-LEDGF/p75 complex with severely reduced dynamics ($D = 0.006±0.001 \mu m^2/s$) and only by mutating the PWPP domain, the complex was targeted away from the near-immobile chromatin (77) and a high $CC_{rel}$ was observed. This locking did not seem to be related to the intrinsic affinity of IN for chromatin, since IN has been shown to exhibit fast nucleosomal dynamics in cells with a transient LEDGF/p75 knockdown ($\bar{D} = 15.2 \mu m^2/s$) (78). Since the diffusion coefficient for the IN-LEDGF/p75 complex ($D = 0.006±0.001 \mu m^2/s$) is ~2 orders-of-magnitude slower than what we observed for the JPO2-LEDGF/p75 complex ($\bar{D} = 0.6±0.1 \mu m^2/s$) and pogZ-LEDGF/p75 complex ($\bar{D} = 1.2±0.5 \mu m^2/s$), chromatin locking thus does not seem to be a general mechanism by which LEDGF/p75 tethers protein cargo to chromatin. The absence of an increased stoichiometry of the JPO2-LEDGF/p75 interaction would be in line with the observed absence of chromatin locking, since, in the absence of any allosteric effects, locking would likely only be observed when JPO2-LEDGF/p75 complexes contain an extraordinarily high avidity for chromatin binding, e.g. multiple LEDGF/p75 molecules working in concert for binding the complex to chromatin.

**Absolute affinity and stoichiometry by fluctuation analysis? -** With respect to the FCCS method, currently available FCCS analysis models would allow calculating both binding affinity and stoichiometry directly from experimental data (72-74). However, for technical reasons such as (but not limited to) spectral crosstalk and photobleaching, this was not possible with our implementation of FCCS (dual-color continuous-wave excitation in combination with fluorescent proteins). More recent fluctuation methods, however, do render such quantitative analyses...
possible. For example, fluctuation imaging rather than point-analysis limits photobleaching (79-81), single- instead of dual-color excitation FCCS reduces analysis complexity (82), pulsed interleaved excitation (PIE) avoids false-positive cross-correlation due to spectral crosstalk (e.g. observed for the negative control in Fig. 2F) (83,84), and the recently developed combination of PIE and fluctuation imaging both reduces photobleaching and avoids crosstalk (85,86). Applied to LEDGF/p75 and its cellular interaction partners, such methods would possibly allow a direct measurement of stoichiometry and binding affinity, or even allow quantification of intracellular competition between different IBD-binding cargoes in a three- or multicolor approach.

CONCLUSION

We have shown that JPO2, by itself, can interact directly and dynamically with chromatin. In the presence of its natural binding partner LEDGF/p75, JPO2 is tugged along chromatin by PWWP-mediated chromatin-scanning LEDGF/p75. Studying the dynamics clearly showed that the intracellular mechanism of chromatin tethering of JPO2 by LEDGF/p75 is different than that of IN; whereas IN is locked onto chromatin, JPO2 simply adapts to the diffusion mode of LEDGF/p75, and is being passively transported along the chromatin. We showed that JPO2 as well as IN can oligomerize in cells, although the lower $CC_{rel}$ suggests a lower binding affinity and/or stoichiometry of the JPO2-LEDGF/p75 complex than that of IN-LEDGF/p75. Our findings implicate LEDGF/p75 in the biological function of JPO2 inside cells. More generally, the high affinity of IN for LEDGF/p75 and the locking mechanism would be consistent with a pathogen (HIV-1) overruling the cellular interaction partners of LEDGF/p75. Future experiments should consider interactions of LEDGF/p75 with other cellular binding partners such as Cdc7-ASK and/or MLL-menin, or should be aimed at studying intracellular competition between different IBD-binding cargoes. Such investigations would provide a more complete insight in the dynamic function of LEDGF/p75 as a molecular tether inside cell nuclei.
REFERENCES


Dynamics of the JPO2-LEDGF/p75-chromatin complex


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Author contributions - JH and JdR conceived and designed the research. JH and BvH performed experiments and data analysis. EvS and DD provided assistance with FLIM-FRET. JH and JdR wrote the paper. All authors carefully edited the manuscript.
### Table 1 – Analysis of JPO2–chromatin interaction in living cells in the absence and presence of LEDGF/p75.

<table>
<thead>
<tr>
<th>protein 1</th>
<th>protein 2</th>
<th># cells</th>
<th>$c$±s.d. [µM]</th>
<th>$F_{20s}$±s.d. [%]</th>
<th>$\overline{D}$±s.d. [µm²/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP-JPO2</td>
<td></td>
<td>13</td>
<td>0.60±0.25</td>
<td>16±7</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td>mRFP-LEDGF/p75</td>
<td></td>
<td>10</td>
<td>0.40±0.19</td>
<td>33±9</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>eGFP-LEDGF/p75</td>
<td></td>
<td>39±3</td>
<td></td>
<td></td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>eGFP-LEDGF/p75</td>
<td>mRFP-IN</td>
<td></td>
<td></td>
<td></td>
<td>0.006±0.001</td>
</tr>
<tr>
<td>eGFP</td>
<td>mRFP-LEDGF/p75</td>
<td>8</td>
<td>0.81±0.41</td>
<td>39±6f</td>
<td>0.6±0.1f</td>
</tr>
<tr>
<td>eGFP-JPO2</td>
<td>mRFP-LEDGF/p75</td>
<td>68</td>
<td>0.69±0.33</td>
<td>23±7</td>
<td>0.8±0.3</td>
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<tr>
<td>eGFP-JPO2_{98-454}</td>
<td>mRFP-LEDGF/p75</td>
<td>48</td>
<td>0.77±0.31</td>
<td>32±7</td>
<td>0.8±0.4</td>
</tr>
<tr>
<td>eGFP-JPO2_{98-454}</td>
<td>mRFP-LEDGF/p75</td>
<td>6</td>
<td>0.55±0.20</td>
<td>31±11</td>
<td>1.1±0.1</td>
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<tr>
<td>mRFP-ΔNpogZ</td>
<td></td>
<td>19</td>
<td>0.29±0.10</td>
<td></td>
<td>2.1±0.6c</td>
</tr>
<tr>
<td>mRFP-ΔNpogZ</td>
<td></td>
<td>19</td>
<td>0.31±0.22</td>
<td></td>
<td>1.2±0.5c,g</td>
</tr>
</tbody>
</table>

$F_{20s}$ is the percentage of photobleaching after 20 seconds, calculated by fitting CP data with Eq. 1; $\overline{D}$ is the species-weighted diffusion coefficient calculated from fitting FCS data of protein 1 with Eq. 2; cMRFp is particularly prone to photobleaching and blinking, resulting in a slight overestimation of $D$; dFrom reference (21); eMeasured with spot fluorescence recovery after photobleaching (sFRAP) and ~75-fold smaller than the corresponding value measured for eGFP-LEDGF/p75 ($D=0.41$ µm²/s); f$< 0.01$ from an unpaired Student’s t-test with cells expressing only eGFP-JPO2. g$< 0.01$ from an unpaired Student’s t-test with cells expressing only mRFP-ΔNpogZ.

### Table 2 – Analysis of JPO2 and IN oligomerization in living cells with FLIM-FRET.

<table>
<thead>
<tr>
<th>protein 1</th>
<th>protein 2</th>
<th># cells</th>
<th>$\tau$±s.d. [ns]</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP</td>
<td></td>
<td>11</td>
<td>2.43±0.02</td>
</tr>
<tr>
<td>eGFP</td>
<td>mRFP</td>
<td>10</td>
<td>1.95±0.05</td>
</tr>
<tr>
<td>eGFP</td>
<td>JPO2</td>
<td>10</td>
<td>2.17±0.06</td>
</tr>
<tr>
<td>eGFP</td>
<td>mRFP</td>
<td>5</td>
<td>2.18±0.06</td>
</tr>
<tr>
<td>eGFP</td>
<td>mRFP-JPO2</td>
<td>15</td>
<td>2.02±0.08</td>
</tr>
<tr>
<td>eGFP</td>
<td>IN</td>
<td>40</td>
<td>2.37±0.05</td>
</tr>
<tr>
<td>eGFP</td>
<td>mRFP</td>
<td>20</td>
<td>2.35±0.04</td>
</tr>
<tr>
<td>eGFP</td>
<td>mRFP-IN</td>
<td>42</td>
<td>2.19±0.02</td>
</tr>
</tbody>
</table>

$\tau$ is the fluorescence lifetime of eGFP, which is lowered (quenched) if the donor (eGFP) and acceptor (mRFP) fluorescent proteins are within 10 nm distance, i.e. when a protein complex containing both labels is present. b$< 0.01$ from an unpaired Student’s t-test with cells expressing only the FRET donor.
FIGURE LEGENDS

Fig. 1 – JPO2 interacts dynamically with chromatin. (A) Schematic representation of JPO2 with indication of the known/predicted chromatin and protein–protein interaction domains. Abbreviations are explained in the Introduction section. (B) Western blot of HeLa cells expressing eGFP-JPO2, 24 hours post-transfection. The eGFP-JPO2 protein was detected using an eGFP antibody. Non-transfected cells were used as a negative control. (C–E) Confocal imaging, CP and FCS analysis of HeLa p75KD cells expressing eGFP-JPO2. (C) Both interphase (top) and mitotic (bottom) cells were imaged after staining for DNA with DAPI. (D) CP curves were fitted with Eq. 1 (solid line). Error bars show the standard deviation after normalization. (E) Average FCS autocorrelation functions were fitted with Eq. 2 (solid line). Error bars represent the standard deviation on the normalized data after fitting. (D–E) CP and FCS data in HeLa p75KD cells expressing eGFP or eGFP-LEDGF/p75 are shown for comparison (21). Parameters after fitting are given in Table 1.

Fig. 2 – Dynamic chromatin interaction of JPO2 is enhanced by LEDGF/p75. (A) Schematic representation of LEDGF/p75 with indication of the known chromatin and protein–protein interaction domains. Abbreviations are explained in the introduction section. (B) Western blot of HeLa cells expressing mRFP-LEDGF/p75, 24 hours post-transfection. Endogenous LEDGF/p75 (L) and mRFP-LEDGF/p75 (mL) were detected using a LEDGF/p75 specific Ab. The minor band below the mRFP-LEDGF/p75 band is due to partial intrachain cleavage of the mRFP-moiety (87). Non-transfected cells were used as a negative control. (C–D) Confocal imaging and FCS analysis of HeLa p75KD cells expressing mRFP-LEDGF/p75. (C) Both interphase (top) and mitotic (bottom) cells were imaged after staining for DNA with DAPI. (D) FCS analysis of eGFP- and mRFP-tagged LEDGF/p75. Average autocorrelation functions and fit with Eq. 2 (solid line). Error bars represent the standard deviation on the normalized data after fitting. Parameters after fitting are depicted in Table 1. The differences between the eGFP- and mRFP-LEDGF/p75 autocorrelation functions in the 10-1000-µs time scale are due to the strong blinking contribution in the mRFP1 autocorrelation function (52). (E–H) Confocal imaging, CP, FCS and FCCS analysis of HeLa p75KD cells co-expressing eGFP-JPO2 and mRFP-LEDGF/p75. (E) Both interphase (top) and mitotic (bottom) cells were imaged after staining for DNA with DAPI. (F) Normalized average FCCS cross-correlation function from cells with equimolar expression of eGFP- and mRFP-labeled proteins. Error bars represent standard error of mean. (G) CP curves were fitted with Eq. 1 (solid line). Error bars show the standard deviation after normalization. (H) Average FCS autocorrelation functions were fitted with Eq. 2 (solid line). Error bars represent the standard deviation on the normalized data after fitting. (G–H) CP and FCS data in HeLa p75KD cells expressing eGFP-LEDGF/p75 (21) and/or eGFP-JPO2 (Fig. 1D–E) are shown for comparison. Parameters after fitting are depicted in Table 1.

Fig. 3 – Fluorescently tagged JPO2 and LEDGF/p75 interact in vivo through the SID domain of JPO2. (A) Western blot of HeLa cells expressing eGFP-JPO2 (WT) or the eGFP-JPO2 mutant lacking the first 97 amino acids of JPO2 (98-454). The eGFP-JPO2 protein was detected using an eGFP antibody. (B–F) Confocal imaging, CP, FCS and FCCS analysis of HeLa p75KD cells expressing eGFP-JPO298-454 or eGFP-JPO298-454+mRFP-LEDGF/p75. (B) Both interphase (top) and mitotic (bottom) cells were imaged after staining for DNA with DAPI. (C) CP curves were fitted with Eq. 1 (solid line). Error bars show the standard deviation after normalization. (D) Average FCS autocorrelation functions were fitted with Eq. 2 (solid line). Error bars represent the standard deviation on the normalized data after fitting. Parameters after fitting are given in Table 1. (F) Boxplot of the $C_{rel}$ values measured in cells with equimolar expression of eGFP- and mRFP-labeled proteins. Whiskers represent the 5th and 95th percentile. The * indicates a statistically significant difference (p < 0.01 from an unpaired Student’s t-test. FCCS data from Fig. 2F is shown for comparison.

Fig. 4 – LEDGF/p75 trails JPO2 along chromatin via its PWWP domain. (A) Western blot of HeLa cells expressing mRFP-LEDGF/p75 (WT) or the mRFP-LEDGF/p75 mutant lacking the first 97 amino acids of JPO2 (98-454). The mRFP-LEDGF/p75 protein was detected using a LEDGF/p75 specific Ab. (B–F) Confocal imaging, CP, FCS and FCCS analysis of HeLa p75KD cells expressing eGFP-JPO298-454 or eGFP-JPO298-454+mRFP-LEDGF/p75. (B) Both interphase (top) and mitotic (bottom) cells were imaged after staining for DNA with DAPI. (C) CP curves were fitted with Eq. 1 (solid line). Error bars show the standard deviation after normalization. (D) Average FCS autocorrelation functions were fitted with Eq. 2 (solid line). Error bars represent the standard deviation on the normalized data after fitting. (D–E) CP and FCS data in HeLa p75KD cells expressing eGFP-LEDGF/p75 (21) and/or eGFP-JPO2 (Fig. 1D–E) are shown for comparison.
were detected using a LEDGF/p75 specific Ab. (B-F) Confocal imaging, CP, FCS and FCCS analysis of HeLa p75KD cells expressing mRFP-LEDGF/p75K56D/R74D or eGFP-JPO2 + mRFP-LEDGF/p75K56D/R74D. (B-C) Both interphase (top) and mitotic (bottom) cells were imaged after staining for DNA with DAPI. (D) CP curves were fitted with Eq. 1 (solid line). Error bars show the standard deviation after normalization. (E) Average FCS autocorrelation functions were fitted with Eq. 2 (solid line). Error bars represent the standard deviation on the normalized data after fitting. (D-E) CP and FCS data in HeLa p75KD cells expressing eGFP-JPO2 (Fig. 1D) or eGFP-JPO2 + mRFP-LEDGF/p75 (Fig. 2G-H) are shown for comparison. Parameters after fitting are given in Table 1. (F) Boxplot of the \( CC_{rel} \) values from different cells (# cells > 6) with equimolar expression of eGFP- and mRFP-labeled proteins. Whiskers represent the 5\(^{th}\) and 95\(^{th}\) percentile. The * indicates a statistically significant difference (p < 0.01 from an unpaired Student’s t-test). FCCS data from Fig. 2F is shown for comparison.

**Fig. 5** – Ternary complex analysis of pogZ, LEDGF/p75 and chromatin. (A, C) Confocal imaging. Scale bars are 10 µm. (B) FCS and (D, E) FCCS analysis of HeLa p75KD cells expressing mRFP-ΔNpogZ or mRFP-ΔNpogZ+eGFP-LEDGF/p75. (B) Average FCS autocorrelation functions were fitted with Eq. 2 (solid line). Error bars represent the standard deviation on the normalized data after fitting. (D) Normalized average FCCS cross-correlation function from cells with equimolar expression of eGFP- and mRFP-labeled proteins. Error bars represent standard error of mean. (E) Boxplot of the \( CC_{rel} \) values measured in cells with equimolar expression of eGFP- and mRFP-labeled proteins. Whiskers represent the 5\(^{th}\) and 95\(^{th}\) percentile. The * indicates a statistically significant difference (p < 0.01 from an unpaired Student’s t-test).

**Fig. 6** – Model of LEDGF/p75 trailing JPO2 along the chromatin. (A) In the absence of LEDGF/p75, JPO2 dynamically scans the chromatin, presumably with its leucine zipper (LZ) and RING-like domains, and either by direct or indirect contacts with chromatin. (B) LEDGF/p75 trails JPO2 along chromatin. Through its IBD domain, LEDGF/p75 interacts specifically with the SID domain of JPO2. Since the dynamics of LEDGF/p75 are slower than those of JPO2, the latter adopts the diffusion mode of the former. eGFP and mRFP tags are illustrated in green and red, respectively.
Fig. 5

A mRFP-ΔNpogZ

B 2.0

G(τ)

0.0

0.5

1.0

1.5

2.0

0.01 1 10 100

lag time τ [ms]

mRFP-ΔNpogZ

mRFP-ΔNpogZ (eGFP-LEDGF/p75)

C mRFP-ΔNpogZ eGFP-p75 merge

D 0.50

G_{eGFP} (τ)/G_{AC, green} (τ)

0.00 0.25 0.50

0.01 1 10 100

lag time τ [ms]

mRFP-ΔNpogZ + eGFP

mRFP-ΔNpogZ + eGFP-LEDGF/p75

E

C_r

p75: no (eGFP)

WT WT

Fig. 6

A chromatin fiber

B chromatin fiber
Dynamics of the Ternary Complex Formed by c-Myc Interactor JPO2, Transcriptional Co-Activator LEDGF/p75 and Chromatin
Jelle Hendrix, Bart van Heertum, Els Vanstreels, Dirk Daelemans and Jan De Rijck

J. Biol. Chem. published online March 14, 2014

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