Imaging analysis of nuclear antiviral factors through direct detection of incoming adenovirus genome complexes

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A B S T R A C T

Recent studies involving several viral systems have highlighted the importance of cellular intrinsic de- fense mechanisms through nuclear antiviral proteins that restrict viral propagation. These factors include among others components of PML nuclear bodies, the nuclear DNA sensor IFI16, and a potential re- striction factor PHF13/SPOC1. For several nuclear replicating DNA viruses, it was shown that these factors sense and target viral genomes immediately upon nuclear import. In contrast to the anticipated view, we recently found that incoming adenoviral genomes are not targeted by PML nuclear bodies. Here we further explored cellular responses against adenoviral infection by focusing on specific conditions as well as additional nuclear antiviral factors. In line with our previous findings, we show that neither interferon treatment nor the use of specific isoforms of PML nuclear body components results in co-localization between incoming adenoviral genomes and the subnuclear domains. Furthermore, our imaging ana- lyses indicated that neither IFI16 nor PHF13/SPOC1 are likely to target incoming adenoviral genomes. Thus our findings suggest that incoming adenoviral genomes may be able to escape from a large repertoire of nuclear antiviral mechanisms, providing a rationale for the efficient initiation of lytic replication cycle.

1. Introduction

Host cells have developed intrinsic mechanism(s) against invading viruses, which viruses try to evade on their part by evolving countermeasures against the cellular antiviral responses [1,2]. Cellular intrinsic defense mechanisms include the sensing and suppression of incoming viral genomes [2,3]. In this context, the interplay between cells and viruses has been best studied with herpes simplex virus type-1 (HSV-1) [3]. It has been shown that upon HSV-1 infection, incoming viral genomes are immediately targeted by PML nuclear bodies (PML-NBs) and/or its components [3]. Everett and co-workers have extensively studied the mecha- nisms of how PML-NBs respond against HSV-1 infection and demonstrated that ICP0, an E3 ubiquitin ligase encoded by the immediate-early gene of HSV-1, is a key factor to counteract the PML-NB-mediated repression [4]. It was shown that ICP0 induces degradation of PML, a central component of PML-NBs [5], and that the replication-defective phenotype of ICP0-null viruses can be rescued by depletion of PML, as well as of other PML-NB compo- nents [6e8]. Another important antiviral factor is IFI16, a nuclear DNA sensor that can induce the IRF3 and NF-kB signaling pathways [9]. Recent studies have revealed that IFI16 is immediately recruited onto incoming HSV-1 genomes for repression [10,11], but this process can be inhibited by ICP0 [10,12]. Intriguingly, it has been reported that IFI16 knockdown reduces the recruitment of PML-NB components onto HSV-1 genomes [10], suggesting a potential link between the two mechanisms.

In contrast to the non-chromatinized herpesviral genome, the adenovirus (Ad) genome is a linear double-stranded DNA forming a chromatin-like complex with viral basic core proteins inside the virion [13]. Core protein VII is the major, most abundant genome- bound factor, and the association with the viral genome has been thought to last at least during the first hours of infection even after nuclear import, as demonstrated by biochemical and microscopic analyses [14e17]. Ad genomes, like their herpesviral counterparts [2], are transported to the nuclear pore complex (NPC) still associated with the viral capsid, likely shielding it from

cellular recognition [18]. At the NPC, the genome separates from the capsid and is imported into the nucleus where it can be targeted by cellular factors [18]. It has been shown that the host nuclear protein TAF-I/SET binds to Ad genomes through the interaction with protein VII upon nuclear import of the genomes [15,17]. Thus, protein VII and TAF-I can be used as surrogate markers to visualize the localization of incoming Ad genome complexes in cells [17]. Recently we have conducted detailed imaging analyses and reported that unlike HSV-1, incoming Ad genome complexes neither target nor are targeted by PML-NBs and/or its components up to 4 hpi (hours post infection) [19]. This suggests that cellular responses against nuclear replicating DNA viruses may differ between viral systems. However, as PML-NB formation and/or expression of its components is enhanced by interferon (IFN) treatments [20], IFN may augment antiviral properties of PML-NBs against incoming Ad genome complexes. In addition, given the reported isoform-specific roles of PML-NB components in virus control [21], it remains possible that a certain isoform(s) of the components might play a distinct role against (or in favor of) Ad infection. Indeed, it was reported that the PML-NB resident factor Sp100 is regulated in an isoform-specific manner during Ad infection, suggesting that Sp100A promotes viral transcription while the other isoforms Sp100B, C, and HMG seem to play an opposite role [22,23]. In contrast to PML-NBs, no study has yet directly analyzed if IFI16 targets incoming Ad genome complexes. The cellular chromatin protein PHF13/SPOC1 is another factor that has been described recently to possess potential antiviral functions against Ad infection [24]. It was proposed that PHF13/SPOC1 targets incoming Ad genome complexes for repression, but genome-associated protein VII may protect the viral genome from the PHF13/SPOC1-mediated antiviral response by binding to PHF13/SPOC1 [24]. However, formal evidence for the targeting of incoming Ad genomes by PHF13/SPOC1 is currently missing.

To extend our understanding of how the host cell responds against Ad infection, here we used our previously developed imaging approaches for the visualization of individual incoming Ad chromatin complexes in living cells and fixed materials [17] to examine how the different potential antiviral factors behave upon infection. In particular we tested IFN treatments and the use of specific isoforms of Sp100, but failed to observe co-localization between PML-NB components and incoming Ad genome complexes, as was observed in the previous study [19]. Furthermore, our imaging analyses indicated no specific changes in the localization of IFI16 and PHF13/SPOC1 upon Ad infection. Taken together, our findings suggest that incoming Ad genome complexes may be able to escape from and/or fail to be recognized by several nuclear antiviral factors/mechanisms, which target herpesviruses such as HSV-1.

2. Materials and methods

2.1. Cells and viruses

U2OS (ATCC #HTB-96) cells were maintained in DMEM Glutamax (Life Technologies) supplemented with 10% of fetal calf serum (FCS). Recombinant replication-competent human adenovirus type 5 (Ad5) and replication-deficient E1-deleted GFP-expressing Ad5 vector (Ad5-GFP) were amplified and purified as described previously [25,26]. The transfection of plasmids was done using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. For the interferon a (IFNa) treatment, cells were incubated in the presence of 1000 units/mL IFNa (PBL Assay Science) for 18 h before infection.

2.2. Antibodies

Antibodies used in this study are as follows: rat anti-protein VII [14], mouse anti-protein VII [17], mouse anti-IFI16 (Abcam, ab55328), and mouse anti-PML (sc-966, Santa Cruz Biotechnology), and rat monoclonal anti-PHF13/SPOC1 (6F6) [27] antibodies. Rabbit anti-Sp100 antibody was generously provided by T. Sternsdorf (Research Institute Children's Cancer Center Hamburg).

2.3. Plasmids

The expression vectors for EGFP-tagged and mCherry-tagged TAF-Ib (pEGFP-C1-TAF-Ib and pCHA-puro-FLAG-mCherry-TAF-Ib) are described elsewhere [17,19]. The expression vectors for mCherry-tagged IFI16 and PHF13/SPOC1 (pcDNA3-IFI16-mCherry and pcDNA3-PHF13-mCherry) were obtained from MGC Montpellier Genomic Collections (Institut de Genetique Moleculaire de Montpellier). The expression vectors for EYFP-tagged Sp100A and Sp100B (pEYFP-C1-Sp100A and pEYFP-C3-Sp100B) were kindly provided by S. M. Janicki (Wistar Institute) [23].

2.4. Immunofluorescence and live-cell imaging analysis

Indirect immunofluorescence (IF) and live-cell imaging analyses were performed as described previously [17,19,28]. For pre-extractions, cells were first incubated with Transport buffer containing 0.5% Triton X-100 [17] and then fixed with 4% paraformaldehyde. IF samples were analyzed by a Leica SP5 confocal microscope. Confocal stacks were taken every 0.3 mm, and images were processed using ImageJ and presented as maximum intensity projections. For live-cell imaging, cells were seeded in ibidi m-slide VI$^{0.4}$ (Ibidi), and images were acquired using a Leica spinning-disk microscopy system (x100 objective) equipped with an incubation chamber at 37 °C. Frames were taken every 3 s for each color channel and assembled into movies using MetaMorph software.

3. Results and discussion

3.1. IFN treatments or the use of specific Sp100 isoforms do not enable PML-NBs to target incoming Ad genome complexes

IFN is well known to enhance the expression of PML-NB components [20], possibly enforcing the antiviral role of the domains. This led us to test the possibility that IFN treatments may permit PML-NBs to target incoming Ad genome complexes. U2OS cells were first pre-treated with IFNa (or not) and then infected with Ad5, followed by IF analyses at 3 hpi using antibodies against PML and protein VII (Fig. 1A and B). As expected, the IFNa treatment enhanced the expression of PML as shown by an increased nuclear signal with anti-PML antibody (Fig. 1A). Increased expression of PML was also confirmed by Western blotting (Sup Fig. S1A). The results showed that irrespective of the IFNa treatment and despite the increase of PML expression, at 3 hpi no co-localization with protein VII foci was observed (Fig. 1B), consistent with a recent report using HDF-TERT cells [29]. This result suggests that PML-NBs do not target incoming Ad genomes even if stimulated through IFN treatments and/or upon increased expression of its components.

Next we tested if Sp100 isoforms exhibit distinct behaviors during immediate early phases of infection, as they were suggested to undergo isoform-specific regulations to regulate Ad gene expression [22]. First, we performed IF analyses using the specific antibody against Sp100 to investigate the localization of all isoforms of the endogenous protein at 3 hpi (Fig. 1C). Consistent with

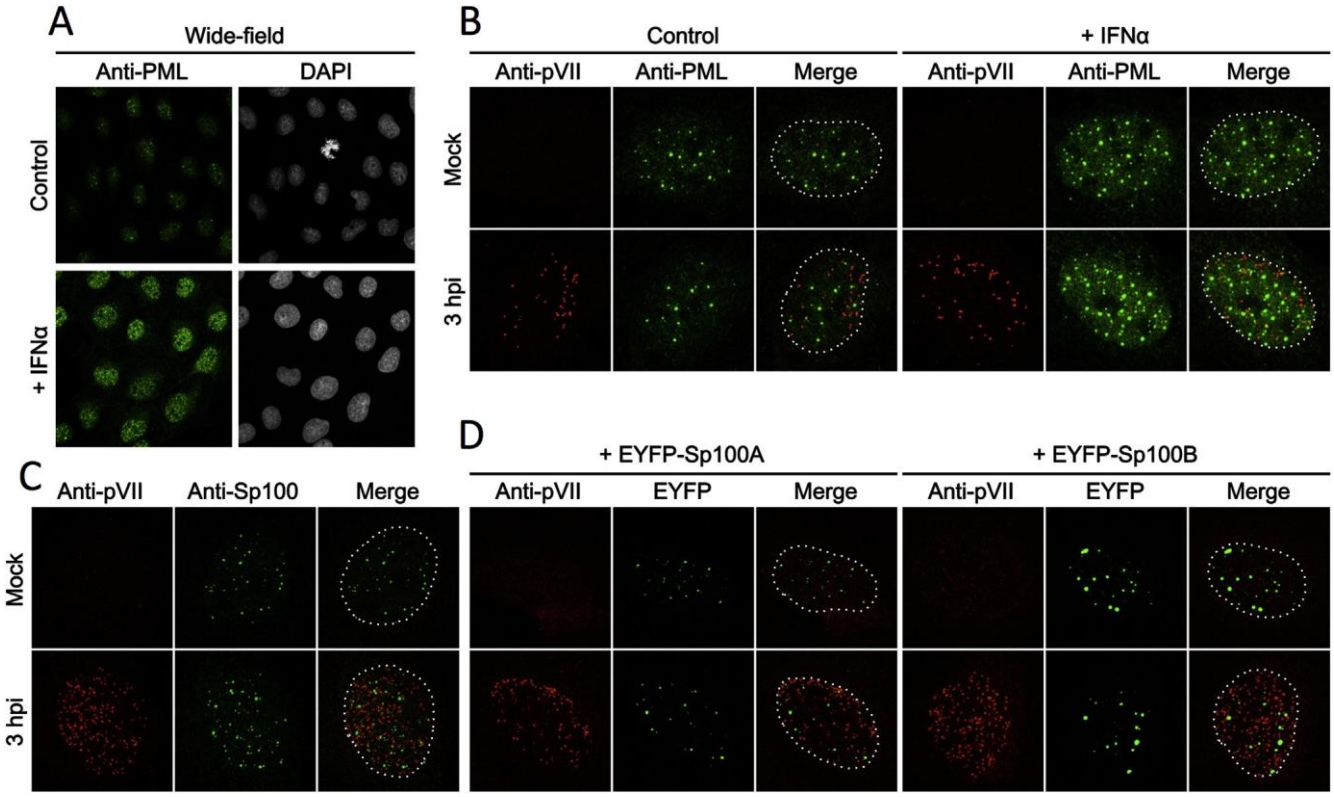

Fig. 1. Localization of PML-NB factors upon Ad infection. (A) IF analyses with IFNa-treated cells. U2OS cells were cultivated in the absence (Control, upper panels) or presence of 1000 units/mL IFNa (þIFNa, lower panels) for 18 h. Cells were then subjected to IF analyses against anti-PML antibody (green, left column). DAPI staining is shown in the right column. (B) IF analyses with infected cells. Control cells (left panels) or IFNa-treated cells (right panels) were either mock-infected (upper panels) or infected with Ad5 (lower panels) and at 3 hpi subjected to IF analyses using antibodies against protein VII (red, first and fourth columns) and PML (green, second and fifth columns). Merged images are shown in the third and sixth columns together with dashed lines indicating the shapes of the nuclei shown by DAPI staining (not shown). (C) IF analyses using anti-Sp100 antibody. U2OS cells were either mock-infected or infected with Ad5 and at 3 hpi subjected to IF analyses using antibodies against protein VII (red, first column) and Sp100 (green, second column). (D) IF analyses with EYFP-tagged Sp100 isoforms. U2OS cells were first transfected with the expression vectors for either EYFP-Sp100A (left panels, green, second column) or EYFP-Sp100B (right panels, fifth column), and at 24 hpt (hours post-transfection) IF analyses were performed as described above using anti-protein VII antibody (red, first and fourth columns). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the previous study [19], we did not observe specific co-localization between incoming Ad genome complexes and endogenous Sp100. Next we carried out IF analyses using EYFP-tagged Sp100A and B as representatives for potentially positive and negative isoforms in transcriptional regulations, respectively [22,23] (Fig. 1D). Cells were transfected with the expression vectors for each isoform individually and then infected with Ad5 for IF analyses. Again we found no co-localization between Ad genome complexes and Sp100 isoforms. To exclude transient recruitment of the isoforms, we next performed live-cell imaging using fluorescently labeled TAF-I, a marker for incoming Ad genomes in living cells [17]. The analysis using mCherry-tagged TAF-I and EYFP-tagged Sp100 isoforms confirmed the absence of co-localization also in living cells (Sup Fig. S1B and C, and Sup Movies S1 and S2). Thus, these results indicate that neither Sp100A nor B engages in a specific response against incoming Ad genome complexes, similar to what we observed with other PML-NB components [19]. Therefore, the reported differential regulation of both isoforms may occur in later phases of infection [22] and does not represent a nuclear response against the incoming genomes *per se*.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.078.

3.2. *IFI16 does not target incoming Ad genome complexes*

Next we investigated the localization of IFI16 in Ad-infected cells (Fig. 2). We first carried out IF analyses using anti-IFI16 antibody to analyze the localization of the endogenous protein (Fig. 2A). IFI16 showed a diffuse distribution throughout the nucleoplasm in uninfected cells, and we did not observe any changes in its localization upon Ad infection up to 3 hpi, the time when immediate early gene expression has started (Fig. 2A). Similar results were obtained when we analyzed instead exogenously expressed mCherry-tagged IFI16 (Fig. 2B). A recent report demonstrated the dynamic interactions of IFI16 with incoming HSV-1 genomes in living cells [11]. To examine if IFI16 may display similar behavior in living Ad-infected cells, live-cell imaging was performed using EGFP-tagged TAF-I and mCherry-tagged IFI16 (Fig. 2C, Sup Movie S3). As reported previously, EGFP-TAF-I showed infection-specific puncta in living cells, indicating the localization of incoming Ad genome complexes [17] (Fig. 2C, upper panels, arrowheads). In contrast, we did not observe any transient accumulation or recruitment of IFI16-mCherry onto viral genomes under the same conditions (Fig. 2C, lower panels, arrowheads). Taken together, our IF and live-cell imaging analyses indicate that IFI16 does not target incoming Ad genome complexes, unlike reported for HSV-1 [10,11].

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.078.

Recent biochemical studies on IFI16 have suggested that cellular nucleosome structures may inhibit the DNA recognition by IFI16 [30,31]. Incoming HSV-1 genomes are possibly non-chromatinized immediately after nuclear import, while Ad genomes form

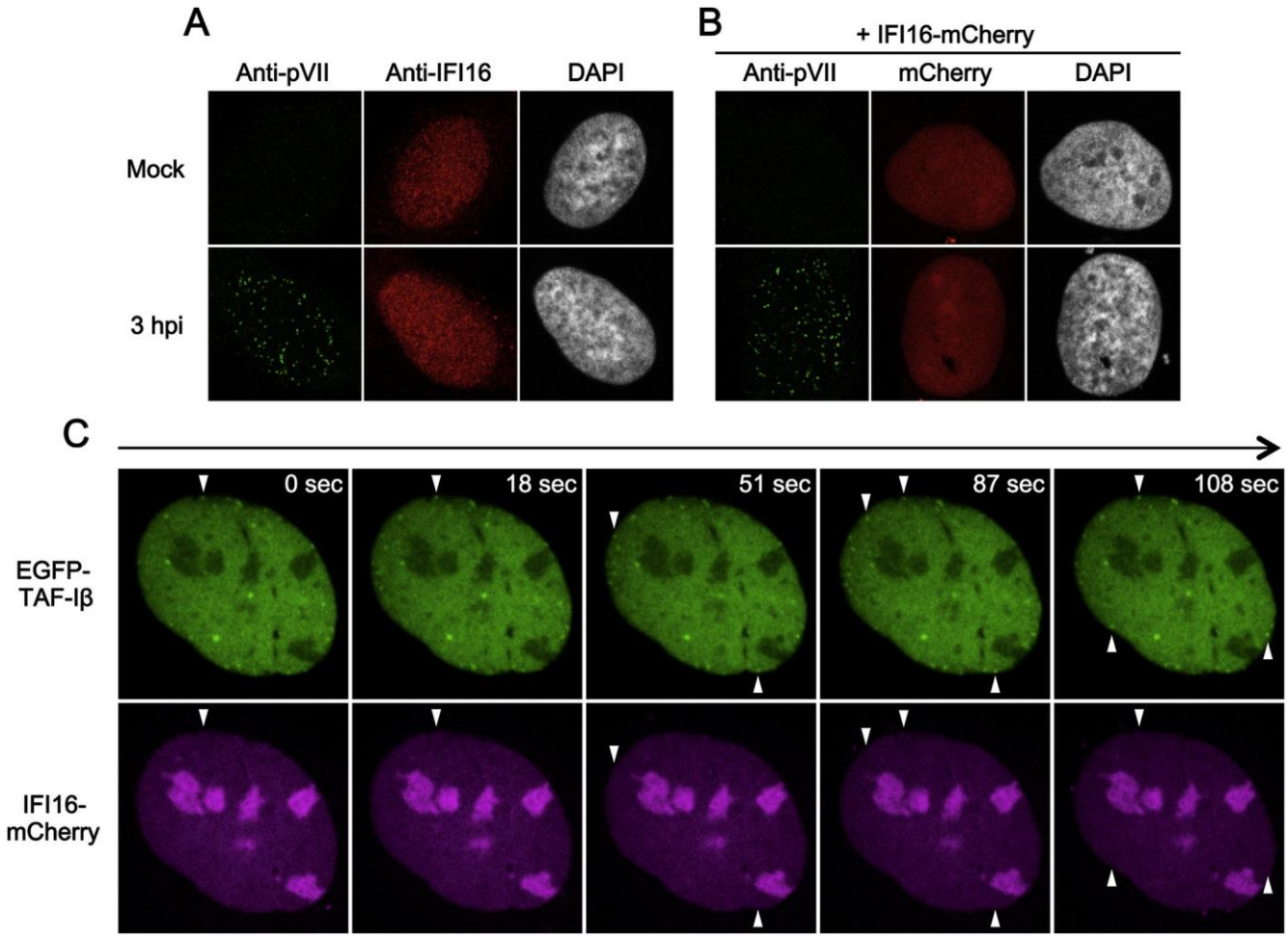

Fig. 2. Localization of the nuclear DNA sensor IFI16 upon Ad infection. (A) IF analyses using anti-IFI16 antibody. U2OS cells were either mock-infected or infected with Ad5 and at 3 hpi subjected to IF analyses using antibodies against protein VII (green, first column) and IFI16 (red, second column). DAPI staining is shown in the third column (gray). (B) IF analyses with mCherry-tagged IFI16. U2OS cells were first transfected with the expression vector for IFI16-mCherry (red, second column), and at 24 hpt IF analyses were performed as described above using anti-protein VII antibody (green, first column). (C) Live-cell imaging using mCherry-tagged IFI16. U2OS cells were transiently transfected with the expression vectors for EGFP-TAF-Iβ (green, upper panles) and IFI16-mCherry (magenta, lower panels), infected with Ad5-GFP, and subjected to imaging around 3 hpi. Frames were taken every 3 s for 2 min, and snapshots from the movies are shown. Arrowheads indicate TAF-I puncta at the nuclear periphery. Full movies are provided as Supplementary Movie S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chromatin-like structures with protein VII. Therefore, we would argue that viral genome structures such as chromatinization with protein VII in the case of Ad genomes may determine how host antiviral factors respond, although this possibility needs to be addressed in future studies. As mentioned above, a potential interplay between PML-NBs and IFI16 has been reported [10]. Furthermore, the recent work of Knipe and co-workers suggested that IFI16 is linked to the cGAS-STING pathway, a major cytoplasmic mechanism for detecting foreign DNA [32], during HSV-1 infection [33]. In contrast to HSV-1, our findings in this and previous studies suggest that neither PML-NBs nor IFI16 appear to target incoming Ad genome complexes. This study thus would be in line with a previous report showing that depletion of IFI16 does not augment transduction levels during Ad5-GFP infection [12]. Intriguingly, it has been reported that although Ad infection induces the cGAS-STING and its downstream pathways, knockdown of cGAS or STING does not promote viral propagation [34], suggesting that the activation of the cGAS-STING cascade is unable to protect cells from Ad infection. Thus, Ad may have evolved unknown mechanism(s) to escape from several antiviral responses that work efficiently to target HSV-1.

3.3. PHF13/SPOC1 neither targets incoming Ad genomes nor binds to genome-associated protein VII

Finally we examined the localization of PHF13/SPOC1 upon Ad infection to directly test the previously proposed role of the host protein against incoming Ad genomes [24] (Fig. 3). Although PHF13/SPOC1 had been proposed to target incoming Ad genomes [24], we did not find co-localization between protein VII foci and endogenous (Fig. 3A) as well as mCherry-tagged PHF13/SPOC1 (Fig. 3B). Furthermore, PHF13/SPOC1-mCherry, like IFI16-mCherry, did not show any form of co-localization with EGFP-TAF-I puncta in living Ad-infected cells (Fig. 3C, arrowheads, and Sup Movie S4). Since the previous report indicated the potential interaction between protein VII and PHF13/SPOC1, we also examined the localization of PHF13/SPOC1 together with that of TAF-I using pre-extraction treatment (Fig. 3D). When infected cells are pre-extracted with a detergent-containing buffer before fixation, EGFP-tagged TAF-I shows small dots that are well overlapping with protein VII foci in fixed cells, reflecting the interaction between TAF-I and genome-bound protein VII [17]. Here we observed that when co-expressed in non-infected cells, both EGFP-TAF-I and PHF13/SPOC1-mCherry showed a diffuse nuclear localization

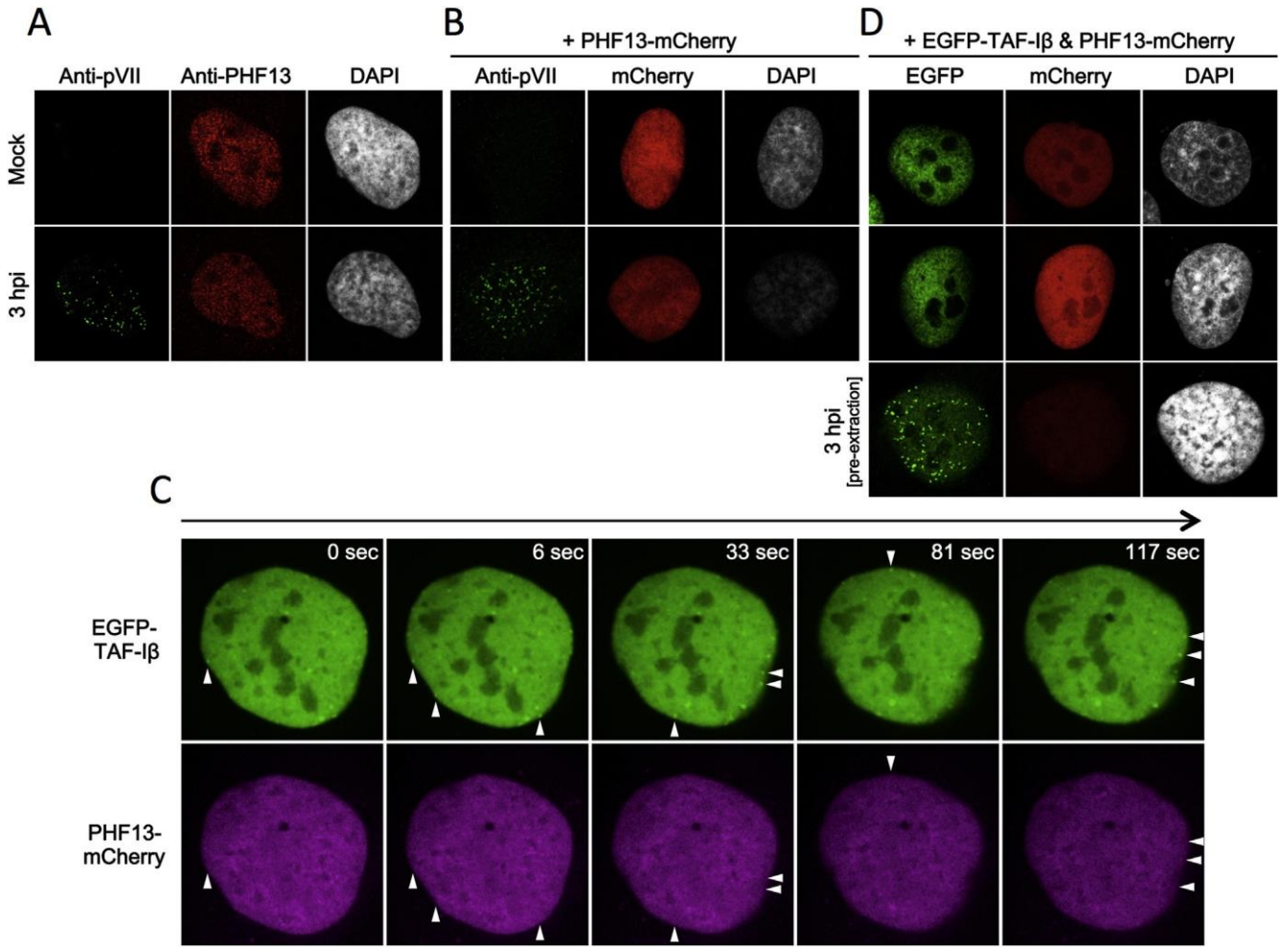

Fig. 3. Localization of the potential antiviral factor PHF13/SPOC1 upon Ad infection. (A) IF analyses using anti-PHF13/SPOC1 antibody. U2OS cells were either mock-infected or infected with Ad5 and at 3 hpi subjected to IF analyses using antibodies against protein VII (green, first column) and PHF13/SPOC1 (red, second column). DAPI staining is shown in the third column. (B) IF analyses with mCherry-tagged PHF13/SPOC1. U2OS cells were first transfected with the expression vector for PHF13/SPOC1-mCherry (red, second column), and at 24 hpt IF analyses were performed as described above using anti-protein VII antibody (green, first column). (C) Live-cell imaging using mCherry-tagged PHF13/SPOC1. U2OS cells were transiently transfected with the expression vectors for EGFP-TAF-Iβ (green, upper panels) and PHF13/SPOC1-mCherry (magenta, lower panels), infected with Ad5-GFP, and subjected to imaging around 3 hpi. Frames were taken every 3 s for 2 min, and snapshots from the movies are shown. Arrowheads indicate TAF-I puncta at the nuclear periphery. Full movies are provided as Supplementary Movie S4. (D) Subcellular localization of fluorescently labeled proteins after pre-extraction treatment. U2OS cells were transiently transfected with the expression vectors for EGFP-TAF-Iβ (green, first column) and PHF13/SPOC1-mCherry (red, second column) and at 24 hpt were either mock-infected or infected with Ad5. At 3 hpi, cells were either immediately fixed (first and second rows) or pre-extracted with a buffer containing 0.5% Triton X-100 and then fixed (third row, pre-extraction) and analyzed by microscopy. DAPI staining is shown in the third column. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

without major changes upon Ad infection (Fig. 3D, Mock and 3 hpi), consistent with our previous results [17]. When infected cells were pre-extracted, EGFP-TAF-I showed puncta as reported previously [17], but PHF13/SPOC1-mCherry did not (Fig. 3D, pre-extraction). Taken together, these results suggest that PHF13/SPOC1 neither targets incoming Ad genome complexes directly nor binds to genome-associated protein VII upon entry.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.078.

In the previous report, it was shown using chromatin immunoprecipitation (ChIP) assays that PHF13/SPOC1 binds to Ad genomes [24]. However, the ChIP assays were carried out at 24 hpi, where multiple rounds of viral DNA replication have taken place. Thus, the results cannot distinguish if PHF13/SPOC1 binds to incoming Ad genomes in early phases or newly replicated viral DNA in late phases of infection. Indeed, the authors also showed using IF analyses that PHF13/SPOC1 is recruited into Ad DNA replication centers late in infection [24]. Therefore, we suggest that the antiviral effect of PHF13/SPOC1 may target replicating or replicated, but not incoming, viral genomes in later phases of infection. Furthermore, the observation that PHF13/SPOC1 is downregulated and/or degraded in other viral infection systems including HSV-1 [24] is as compatible with an indirect as with a direct role of the protein against genomes of those viruses. PHF13/SPOC1 knockdown experiments in the previous study, which enhanced Ad propagation [24], would also be compatible with an indirect antiviral effect of PHF13/SPOC1 on Ad infection. Thus further studies on other viral systems are clearly needed to prove if PHF13/SPOC1 has a general antiviral property associated with targeting viral genomes.

In summary, in this study we used direct visualization in fixed and living cells to investigate how several (potential) nuclear antiviral factors respond to incoming Ad genome complexes. Our analysis showed that neither IFN-stimulated PML-NBs nor specific Sp100 isoforms nor IFI16 target incoming Ad genome complexes, thus showing a clear difference between the cellular response

towards invading HSV-1 and Ad genomes. Furthermore, our findings argue against the previously proposed antiviral model for PHF13/SPOC1 upon Ad infection. Taken together, our studies suggest a diversity of cellular responses against viral infection, which should be further investigated using other viral systems and which may help elucidating why some viruses efficiently enter lytic replication while others respond with latency.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.078.

Transparency document

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