Nuclear domain 10 components upregulated via interferon during human cytomegalovirus infection potently regulate viral infection

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Abstract

Human cytomegalovirus (HCMV) is a ubiquitous betaherpesvirus that causes life-threatening disease in immunocompromised and immunonaïve individuals. Type I interferons (IFNs) are crucial molecules in the innate immune response to HCMV and are also known to upregulate several components of the interchromosomal multiprotein aggregates collectively referred to as nuclear domain 10 (ND10). In the context of herpesvirus infection, ND10 components are known to restrict gene expression. This raises the question as to whether key ND10 components (PML, Sp100 and hDaxx) act as antiviral IFN-stimulated genes (ISGs) during HCMV infection. In this study, analysis of ND10 component transcription during HCMV infection demonstrated that PML and Sp100 were significantly upregulated whilst hDaxx expression remained unchanged. In cells engineered to block the production of, or response to, type I IFNs, upregulation of PML and Sp100 was not detected during HCMV infection. Furthermore, pre-treatment with an IFN-β neutralizing antibody inhibited upregulation of PML and Sp100 during both infection and treatment with HCMV-infected cell supernatant. The significance of ND10 components functioning as anti-viral ISGs during HCMV infection was determined through knockdown of PML, Sp100 and hDaxx. ND10 knockdown cells were significantly more permissive to HCMV infection, as previously described but, in contrast to control cells, could support HCMV plaque formation following IFN-β pre-treatment. This ability of HCMV to overcome the potentially anti-viral effects of IFN-β in ND10 expression deficient cells provides evidence that ND10 component upregulation is a key mediator of the anti-viral activity of IFN-β.

INTRODUCTION

Human cytomegalovirus (HCMV) is endemic in both developed and developing countries [1, 2]. However, despite its high prevalence the level of HCMV awareness is extremely low [3–5]. This may be attributable to its generally asymptomatic presentation, ability to persist in a state of latency with highly restricted gene expression [6] or the potential for reactivation without clinically detectable virus shedding [7, 8]. The true pathogenic capability of HCMV is made obvious when primary infection or reactivation from latency occurs in immunonaïve, immunocompromised or immunosuppressed individuals [9–14]. In these populations, HCMV infection can be life-threatening, an eventuality which makes fully understanding the mechanisms by which a healthy immune system can combat HCMV essential.

A set of cytokines that play a crucial role in the innate immune response to HCMV are IFNs. Pre-treatment of cells with either type I (IFNα and IFNβ) or type II IFNs (IFNγ) inhibits HCMV replication [15–17] and abrogation of the type I IFN response facilitates significantly enhanced viral replication and spread [18]. This phenomenon can be at least partially attributed to the induction of an anti-viral state in bystander cells by IFNs and their associated autocrine and paracrine acting IFN-stimulated genes (ISGs) [19, 20].

In the context of herpesviruses such as HCMV, a set of intrinsically expressed, inter-chromosomal, multiprotein...
aggregates, collectively referred to as nuclear domain 10 (ND10), are well known to have direct anti-viral activity [21–34]. These structures were first observed as nuclear speckles in biliary cirrhosis patients [35] and are also known as promyelocytic leukemia (PML) bodies as the PML protein is key to their formation and function [36]. SUMOylated PML recruits two other fundamental ND10 proteins, speckled protein of 100 kDa (Sp100) and human death domain associated protein 6 (hDaxx) [37–39]. Whilst these three proteins form the core of PML bodies there are more than 80 proteins with which they transiently associate [40]. This wide variety of interactions allows ND10 to play a role in a range of endogenous regulatory processes [41]. Many of the proteins associated with ND10 have chromatin remodelling functions capable of repressing transcription [42–47]. During HCMV infection, PML bodies associate closely with the viral genome and create a condensed chromatin environment around the major immediate early (IE) promoter of HCMV, resulting in the inhibition of IE gene transcription and subsequent productive viral infection [34]. Overexpression of the major ND10 components (PML, Sp100 and hDaxx) limits HCMV infection [34, 48] whilst knockdown enhances it [29, 30, 49–51]. In an attempt to evade the anti-viral action of ND10s HCMV-encoded proteins such as IE1 and pp71 are capable of dispersing and disabling them [32, 33, 52–58].

Type I IFNs play a major role in the innate immune response to HCMV as evidenced by the fact the abrogation of the type I IFN response significantly enhances HCMV replication [18]. In human cells, type I IFNs have been shown to upregulate both Sp100 and PML [59–62] whilst the data concerning the ability of hDaxx to be induced by type I IFNs [58, 63] are contrasting. In the current study, we sought to determine whether or not the ability of the IFN response to combat HCMV infection is dependent upon ND10.

**RESULTS**

**ND10 components PML and Sp100 are upregulated following HCMV infection independent of de novo viral gene expression**

Total RNA was harvested from primary human fibroblasts (HFs) at 2, 6 and 24 h post infection (h p.i.) with the low passage Merlin strain of HCMV in a viable or UV-irradiated form, whilst mock treatment was performed in parallel. It is an established principle that the damage caused by UV irradiation renders HCMV virions incapable of de novo gene expression whilst still enabling binding and entry [64–67]. The relative mRNA levels of ND10 components PML, Sp100 and hDaxx were then determined by qRT-PCR. This analysis revealed upregulation of PML and Sp100 by both HCMV and UV-HCMV. The upregulation of PML transcription by HCMV infection became statistically significant at 6 h p.i. with both viable HCMV and UV-HCMV and was maintained at 24 h p.i. (Fig. 1a). Sp100 mRNA upregulation was significant at 2 h p.i. with UV-HCMV and by 6 h p.i. was significant for both UV-HCMV infection and viable HCMV (Fig. 1b). Although not statistically significant at 24 h p.i., there was a trend towards upregulation of Sp100 at this time point (Fig. 1b). In contrast to PML and Sp100, hDaxx was not significantly upregulated following exposure to either viable HCMV or UV-HCMV at any time point post-infection (Fig. 1c).

The upregulation of PML and Sp100 mRNA following exposure of cells to UV-HCMV indicated that the mechanism of this upregulation was independent of de novo viral gene expression. Type I IFNs are key mediators of the innate response to HCMV whose secretion is upregulated independently of HCMV gene transcription [68–70] and are known to upregulate PML and Sp100 [59, 62]. Thus, to determine whether a secreted factor may be involved in the observed upregulation of PML and Sp100 transcription, HFs were treated for 6 h with supernatants from mock-, HCMV- or UV-HCMV-infected HFs that had been passed through a 0.1 μm filter to ensure they were free of any infectious virions. The absence of virions in filtered supernatants was confirmed by plaque assay (data not shown). The 6 h time point was chosen for RNA extraction, and subsequent qRT-PCR analysis, as this was the time of maximal PML and Sp100 transcript upregulation following infection (Fig. 1a, b). PML transcript levels were elevated significantly following incubation with supernatant from cells exposed to either HCMV or UV-HCMV (Fig. 1d). Sp100 mRNA levels increased similarly in response to incubation of HFs with either HCMV or UV-HCMV supernatant (Fig. 1e). There was no significant change in hDaxx mRNA levels in HFs incubated with HCMV or UV-HCMV supernatant (Fig. 1f). Together these data demonstrated differential regulation of ND10 components following HCMV infection, with PML and Sp100 upregulated by soluble factor(s) and hDaxx transcription unchanged.

**ND10 component upregulation following HCMV infection does not occur in cells engineered to block the response to, or production of, IFN**

To determine whether the soluble factor upregulating PML and Sp100 transcripts post-exposure to HCMV might be IFN, two previously described cell lines with an abrogated IFN response were utilized [71]. HFs expressing the nPro protein of bovine viral diarrhea virus, which targets IFN regulatory factor 3 (IRF3) for proteasomal degradation [72], are unable to produce IFN, whilst HFs expressing the STAT1 degrading V protein from parainfluenza virus 5 [73] cannot respond to IFN [71]. V/HFs and nPro/HFs infect with the same efficiency as the parental HFs by plaque assay [18]. Parental HFs, nPro/HFs and V/HFs were mock-, HCMV- or UV-HCMV-infected and RNA was extracted at 6 h p.i. Analysis of relative transcript levels by qRT-PCR demonstrated that PML was significantly upregulated in HCMV and UV-HCMV infected parental HFs, but not in nPro/HFs or V/HFs (Fig. 2a). Sp100 expression followed the same trend as PML, with significant transcript upregulation in parental HFs infected with either HCMV or UV-
HCMV, but not in infected nPro/HFs or V/HFs (Fig. 2b). As in parental HFs, levels of hDaxx expression were not significantly altered with HCMV or UV-HCMV infection of nPro/HFs or V/HFs (Fig. 2c). When nPro/HFs or V/HFs were treated with supernatant from infected parental HFs, upregulation of PML and Sp100 was significant and comparable to that observed in parental HFs (Fig. 2d, e). This finding is consistent with the ability of IRFs deficient cells to upregulate ISG expression in response to type I IFNs [71]. The levels of hDaxx transcription did not change significantly following infected cell supernatant treatment of nPro/HFs, V5/HFs or parental HFs (Fig. 2f). These data demonstrate that upregulation of PML and Sp100 transcription during HCMV infection is mediated by a soluble factor whose action is dependent upon the capacity to respond to IFN.

**Treatment with IFN-β upregulates ND10 components**

In HFs the IFN produced most abundantly in response to HCMV infection is IFN-β [69, 70, 74, 75]. Whilst it is well established that PML and Sp100 are induced by type I IFNs [59–62], we felt that the contrasting data regarding hDaxx upregulation [58, 63] warranted a brief investigation of the regulation of PML, Sp100 and hDaxx in our system to aid the interpretation of subsequent experiments. Primary HFs were incubated with 100U of recombinant IFN-β in the presence of an IFN-β neutralizing antibody or rabbit IgG (R IgG) isotype control. Six hours after IFN-β treatment, RNA was extracted and the relative transcript levels of PML, Sp100 and hDaxx were determined by qRT-PCR. Fold changes in mRNA levels compared to the mock infection at each time point (set to 1) are depicted. Error bars indicate the SEM statistical significance was determined using Student’s two-tailed t-test, with n=3, *P<0.05, **P<0.01. (d-f) Primary HFs were treated for 6 h with supernatant taken from mock-, HCMV- or UV-HCMV-infected primary HFs. The RNA extracted was converted to cDNA then analysed by qRT-PCR to determine the relative levels of PML, Sp100 and hDaxx transcripts (normalized to the housekeeping gene GAPDH). Individual bars depict the average fold change in the transcript level compared to the mock infection for each cell type (set to 1). Error bars indicate the SEM and statistical significance was calculated using Student’s two-tailed t-test, n=3, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

**IFN-β neutralization inhibits ND10 component upregulation following HCMV infection**

To determine whether IFN-β was the soluble factor responsible for upregulation of PML and Sp100 in response to HCMV, an IFN-β neutralizing antibody was used to treat HFs before infection with HCMV, or incubation with filtered supernatant from HCMV-infected HFs.
RNA was extracted 6 h post treatment and qRT-PCR was performed for PML, Sp100 and hDaxx. An isotype control antibody (R IgG) was included in all experiments. The upregulation of PML and Sp100 mRNA in infected HFs was abrogated in the presence of an IFN-β neutralizing antibody (Fig. 3d, e). Levels of hDaxx expression were unchanged following HCMV infection, whether or not an anti-IFN-β antibody was present (Fig. 3f). The upregulation of PML and Sp100 mRNA expression by uninfected HFs incubated with infected cell supernatant was also abrogated in the presence of an anti-IFN-β antibody (Fig. 3g, h). As before, hDaxx mRNA levels remained unchanged, with or without an IFN-β neutralizing antibody (Fig. 3i). These results demonstrate that the upregulation of PML and Sp100 transcripts in HCMV infection is dependent upon IFN-β.

### ND10 knockdown impairs the ability of IFNβ to inhibit HCMV infection

The importance of ND10 component upregulation in the anti-HCMV immune response was assessed by knockdown of PML, Sp100 and hDaxx. A lentiviral vector whose transfer plasmid (shDPS) coded for three short hairpin RNAs (shRNAs) under the control of three separate promoters designed to knockdown expressions of PML, Sp100 and hDaxx (shDPS) was generated in parallel with a lentivirus designed to express a non-specific control shRNA (shNEG) [26]. Primary HFs were transduced with these lentiviruses to generate shDPS HFs and shNEG HFs. The efficiency of knockdown was determined by immunofluorescent staining using antibodies specific for PML, Sp100 or hDaxx. In shNEG HFs (Fig. 4a) staining for PML, hDaxx and Sp100 remained readily detectable in shDPS HFs exposed to IFN-β. In contrast, IE1-positive cells were infected at an m.o.i. of 3 with HCMV or UV-irradiated HCMV, and mock infection performed in parallel. 6 h p.i. RNA was extracted, converted to cDNA and the relative levels of PML, Sp100 and hDaxx transcripts (normalized to the housekeeping gene GAPDH) were calculated. Individual bars represent the average fold change in the transcript level compared to the mock infection for each cell type (set to 1). Error bars indicate the SEM and statistical significance was determined using Student’s two-tailed t-test. n=3, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

HCMV strain Merlin infection of these ND10 component knockdown HFs yielded more cells positive for the HCMV IE gene product IE1 than infection of the parental HFs and control shNEG HFs (Fig. 4c), which is consistent with the outcome of infection in similarly knocked down cells with HCMV strain AD169 [26]. This experiment was then undertaken in the presence of IFN-β to determine whether the anti-viral effect of this cytokine was altered when the expression of ND10 components were inhibited. When treated with IFN-β, IE1-specific staining was almost completely absent in parental HFs and shNEG HFs (Fig. 4d), consistent with the ability of IFN-β to restrict HCMV infection [18]. In contrast, IE1-positive cells remained readily detectable in shDPS HFs exposed to IFN-
(Fig. 4d). Quantification of IE1-positive cells demonstrated that there were significantly more IE1-positive cells in the shDPS HFs than in either of the control cell lines (HFs and shNEG HFs) with or without IFN-β pre-treatment (Fig. 4e). These results demonstrate that knockdown of ND10 components renders cells less susceptible to the anti-viral effects of IFN-β.

To further examine this apparent increase in permissivity to HCMV due to reduced inhibitory capacity of IFN-β in shDPS HFs, a plaque assay was performed to determine the number of infectious centres. The ability of HCMV to form plaques in ND10 knockdown shDPS HFs was assessed by infecting cells at low multiplicity (100 p.f.u./1.5 x 10^5 cells) followed by culture under a 2% Avicel overlay for 10 days (to inhibit secondary virus spread). In the absence of exogenous IFN-β, the number of plaques in the infected shDPS HFs was significantly higher than in the parental HFs or control shNEG HFs (Fig. 4f), which is consistent with the increased number of IE1-positive shDPS HFs (Fig. 4c). Importantly, whilst pre-treatment with IFN-β was able to effectively block HCMV infection in parental HFs and the control shNEG HFs (no plaques observed) this inhibitory capacity was overcome when ND10 components were knocked down, as plaques were readily observed in shDPS HFs (Fig. 4f). These data show that HFs with stable combined knockdown of ND10 components are more permissive to HCMV infection, and that the ability of IFN-β to inhibit HCMV infection is abrogated when ND10 components are depleted.

To determine whether PML, Sp100 and hDaxx played distinct roles in the anti-viral IFN-β response to HCMV, primary HFs were transduced with lentiviruses engineered to knockdown either PML, hDaxx or Sp100 [26, 76]. This resulted in the creation of three separate single knockdown cell lines: shPML HFs, shSp100 HFs and shDaxx HFs. Efficient knockdown was confirmed by immunofluorescent

| Fig. 3. The role of IFN-β in the regulation of ND10 transcription by HFs following HCMV infection and infected cell supernatant treatment. (a–c) Primary HFs were treated with 100U of recombinant IFN-β, infected with intact HCMV at an m.o.i. of 3 (d–f) or treated with supernatant from HCMV infected HFs (g–i) in the presence of 100 neutralization units of anti-IFN-β antibodies or an equivalent concentration of the appropriate isotype control. Mock treatments were performed in parallel. Six hours after treatment RNA was extracted, converted to cDNA and the relative transcript levels of PML, Sp100 and hDaxx were determined (normalized to levels of the housekeeping gene transcript GAPDH). The average fold changes relative to mock treatment (set to 1) are depicted. Error bars represent SEM and statistical significance was calculated using Student’s two-tailed t-test. n=3, *P<0.05. |
Fig. 4. Determining the contribution of ND10 component upregulation to the inhibitory ability of IFN during HCMV infection. (a, b) Primary HFs were transduced with 10 µl ml⁻¹ of lentivirus expressing either (a) the shNEG non-specific scrambled shRNA (control), or (b) shDPS shRNAs targeting PML, Sp100 and hDaxx transcripts for degradation. At 72 h post transduction, cells were fixed and stained for PML, Sp100, hDaxx, and DAPI. (c) Percentage of total cells IE1+ was determined for each condition. (d) Average Plaque Count was also assessed. Isotype controls were included for each condition. 

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staining (data not shown). In the absence of IFN-β pre-treatment, HCMV strain Merlin infected these three cell lines with similar efficiency (Fig. 4g). However, with IFN-β pre-treatment, there were significantly more IE1+ cells in the infected shPML HFs and shSp100 HFs than the shNEG control HFs (Fig. 4g). Additionally, the percentage of IE1+ cells was significantly greater in the shPML HFs and shSp100 HFs compared to both the shSp100 HFs and shDaxx HFs (Fig. 4g). Overall these results indicate that PML, the key structural component of ND10s, contributes the most, of the three ND10 components, to both the shSp100 HFs and shDaxx HFs compared to the shSp100 HFs and shDaxx HFs (Fig. 4g). Overall these results indicate that PML, the key structural component of ND10s, contributes the most of the three ND10 proteins tested, to the IFN-β-dependent anti-viral activity of ND10 domains. Sp100 exerted a small, but still statistically significant impact whilst hDaxx appeared to play little role in the anti-viral, ND10 mediated, IFN-β response (Fig. 4g). This lack of hDaxx knockdown impact is consistent with the results shown in Fig. 3(c) which demonstrate an inability of hDaxx to be regulated by IFN-β.

**DISCUSSION**

It is an established principle that IFN-β is capable of limiting HCMV replication [15, 17, 18] and this study has revealed that ND10 component expression regulation by IFN-β significantly influences the ability of HCMV to form plaques and express immediate early genes. Enhancement of both of these measures of productive infection had been previously observed following HCMV infection of ND10 component knockdown cells [26]. However, the way in which ND10 component knockdown impairs the anti-viral ability of IFN-β during HCMV infection has not previously been described. Given the hundreds of ISGs that can potentially mediate the anti-viral activity of type I IFNs [19], it is remarkable that the ability of IFN-β to inhibit HCMV gene expression and plaque formation is so markedly depleted following knockdown of ND10 components. However, it is a result supported by the finding that PML nuclear bodies are required for type I IFN-dependent repression of foreign DNA expression [77] and a recent study implicating type I IFNs in the regulation of ND10 components during murine cytomegalovirus infection [78].

HCMV is known to actively combat ND10 formation, a fact which makes their apparent significance to the anti-viral type I IFN response even more surprising. Although, despite the destruction of ND10s by HCMV, it has been demonstrated that during infection a transient increase in the number of ND10s is observed in some cells [52]. We propose that these are the uninfected bystander cells in which an anti-viral state, manifested as increased ND10 formation, has been induced by type I IFN and that these cells are evidently a powerful tool utilized by this anti-HCMV type I IFN response. It is also important to note that whilst ND10 component knockdown can significantly overcome the inhibitory ability of IFN-β, the levels of infection were still not comparable to those in the absence of IFN-β, and this is because there are many other IFN-mediated anti-viral effects that can combat HCMV infection [64, 70, 75, 79–83].

The significant role of PML and Sp100 in the anti-viral IFN response is an observation supported by findings that both are transcriptionally upregulated post-infection with HCMV laboratory strain AD169 [83], a result reproduced in our study using the low passage HCMV strain Merlin. In addition, when the ability of IFN to upregulate PML, Sp100 and hDaxx was tested in our experimental system, directly and independently of infection, through treatment with 100U of IFN-β, transcript levels of PML and Sp100 both increased significantly. Furthermore, when an IFN-β neutralizing antibody was applied during HCMV infection, the virus-mediated upregulation of PML and Sp100 was significantly impaired. The links these results draw between IFN and ND10 component upregulation during HCMV infection are consistent with evidence that PML and Sp100 can be upregulated by IFN-β [59–62]. They also give credit to the idea that the existence of a positive feedback loop between IFN production and PML expression [84–86] may contribute to the significance of ND10 component upregulation to the anti-viral type I IFN response to HCMV. Contrastingly, our investigation of hDaxx transcript regulation during HCMV infection and in the presence or absence of IFN-β, did not reveal any significant changes in expression. This is in line with findings that hDaxx is not IFN-inducible in human cells [58]. Taken together, the data collected on PML, Sp100 and hDaxx regulation by IFN-β in this study fit with the significant differences in the ability of IFN-β to inhibit HCMV infection observed in the respective single knockdown cell lines, with PML and Sp100...
knockdown significantly reducing the anti-viral capacity of IFN-β, and not hDaxx. Interestingly, outside of the context of IFN-β, studies have shown that PML, Sp100 or hDaxx all play independent and important roles in inhibiting HCMV infection [26, 30].

Previous studies have examined regulation of PML, Sp100 and hDaxx protein levels during infection with the HCMV laboratory strains Ad169 and Towne [28, 50]. Tavalai et al. [28] demonstrated an upregulation of PML protein levels supporting the data described here [49]. On the other hand, Sp100 protein levels have been shown to decrease following high m.o.i. infection with either Towne or AD169 [28, 87]. However, these same studies demonstrate that during infection with either an IE1 deletion mutant HCMV or UV-HCMV, Sp100 protein levels are upregulated [28, 87]. This is consistent with an infection-mediated upregulation of Sp100 that can be masked by an IE1-dependent targeting of Sp100 by HCMV. Taking the results of our study together with those described here, we propose that IFN-induced expression of Sp100 is controlled post transcriptionally via IE1 in infected cells to facilitate efficient viral gene expression and replication whilst in uninfected bystander cells elevated Sp100 levels can persist.

Tavalai et al. [28] also indicated that hDaxx protein levels were upregulated by HCMV infection but only at later time points post-infection (>48 h p.i.). As we did not examine such time points in our study it is possible that hDaxx could be upregulated transcriptionally at late times post-infection in an IFN-independent manner or additional post transcriptional mechanisms exist to modulate hDaxx protein levels post-infection.

In addition to playing an anti-viral role in HCMV infection, ND10s are also known to act in such a manner during infection with other herpesviruses including varicella zoster virus, Herpes simplex virus type 1, Kaposi’s sarcoma-associated herpes virus and Epstein–Barr virus [21–26]. It is possible that the action of ND10s during these infections is also directly related to the IFN response generated.

Through demonstrating the importance of ND10s to the anti-viral capability of IFN this investigation has characterized an essential mechanism by which the innate and intrinsic immune responses coordinate to combat HCMV infection in healthy individuals with IFN inducing and utilizing ND10 upregulation to inhibit viral gene expression and spread.

**METHODS**

**Cell culture, viral infection and treatment of cells with conditioned supernatants**

HFF-1 primary human foreskin fibroblasts (HFs) and HEK293T cells sourced from the ATCC were grown at 37 °C and 5% CO₂ in DMEM media supplemented with 10% FCS and penicillin streptomycin (100 units ml⁻¹).

The low passage clinical isolate Merlin (used in all HCMV infections in this study) was generated from a bacterial artificial chromosome BAC pAL1111 as described previously [88]. Virus stocks were generated from the supernatant of infected HFs. Supernatant was collected when all HFs in infection demonstrating the importance of ND10ted flask displayed cytopathic effect. Supernatants were spun at 845 g for 10 mins to pellet cell debris before a second centrifugation at 12,000 r.p.m. in an ultracentrifuge for 2 h to pellet the virus (Thermo Scientific A-621 6 Fixed-Angle Rotor, Thermo Scientific Sorvall WX+ ultracentrifuge). Concentrated virus pellets were resuspended in fresh supplemented DMEM and stored at −80 °C.

UV irradiation of virus was performed by applying 720 mJ cm⁻² of UV using a CL-1000 Ultraviolet Crosslinker. To confirm successful inactivation of virus, UV-irradiated virus was added to fresh monolayers of fibroblasts and the development of cytopathic effect was not observed. Cell free viable or UV-irradiated virus was applied to cultures for 90 min before being washed off, this point was taken as time zero of the infection. To assay infection though plaque formation 1.5×10⁵ cells were infected in six-well plates and cultured under a 2% Avicel:EMEM (1:1) overlay for 10 days at 37 °C, 5% CO₂. Plaques were counted with the use of a Motic AE2000 inverted light microscope (20×).

For treatment of uninfected HFs with supernatants from infected HF supernatants were taken at 24 h.p.i. from mock-, HCMV- and HCMV-UV-infected primary HFs and stored at −80 °C. Before use in treatment supernatants were filtered (0.1µm) to ensure they contained no infectious virions (approximately 230nm in diameter [89]). Supernatants were diluted 1:1 with cell culture media before being applied to uninfected cells.

IFN-β neutralization was achieved by pre-treating cells and the treatments to be applied to them (virus, infected cell supernatant or recombinant IFN-β) for 1 h with 100 neutralization units of anti-IFN-β (Merck Millipore).

**Lentivirus generation**

pLKO-shNEG, pLKO-shPML, pLKO-ShSp100, pLKO-shD and pLKO-shDPS containing NEG control shRNA, anti-PML shRNA, anti-Sp100 shRNA, anti-hDaxx shRNA and anti-PML/Sp100/hDaxx shRNA respectively have been described previously [26, 76]. The envelope and packaging plasmids used were pMD2G and psPAX2 respectively. All plasmids were grown up in Stbl2 C. elegans and extracted using the NucleoBond Xtra Midi Plus Plasmid DNA purification kit (Macherey-Nagel) and associated protocol, obtained online. Plasmid transfections with the respective shRNA constructs, envelope (pMD2G) and packaging plasmid (psPAX2) into HEK293T cells were performed using the FuGENE HD transfection reagent (Promega). Supernatants were harvested at 48 h post transfection, filtered through a 0.45 µm filter before being added to low passage HFs. Transduced cells were selected with 1 µg ml⁻¹ puromycin (Sigma).
qRT-PCR

Total RNA was extracted from HFs using an innuPREP RNA minikit (Analytik-Jena) prior to cDNA synthesis using the AffinityScript cDNA synthesis kit (Agilent Technologies). For PCR, reaction mixtures were created using Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies) and relative levels of mRNA expression recorded by qRT-PCR (StepOne-Plus Real Time PCR machine) at 50°C for 2 mins followed by 10 mins at 95°C for denaturation then 50 amplification cycles of 15s at 95°C and 45s at 60°C, finally melt curve data were generated through 1 min at 95°C, 30s at 50°C, 30s at 95°C. Test gene mRNA levels were normalized to mRNA levels of the following: GAPDH-F, TCACCAGGGCTGTTTTGAA; GAPDH-R 5'- AGTTTGGAGAAGGCGT-3'; PML-F 5'- ATCACCCAGGGAAAAATGTC-3'; PML-R 5'- GCT TTTGATGGAGAAGGCGT-3'; hDaxx-F 5'- GCA GAAGCCCTATGCTCTCTC-3'; hDaxx-R 5'- GATGTTGCA GAACCTCGCGG-3'; SP100-F 5'- AAAGTTGAG TGGCAAGCCCAAG-3'; SP100-R 5'- CTCTAAGGGCTCA TCAAGTGCAGT-3'.

Immunofluorescence

Cells seeded onto coverslips (5 × 10⁴ cells coverslip⁻¹), following treatment/infection were washed with PBS and fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X (in PBS) before being stained with the appropriate primary antibody: anti-hDaxx (Upstate), anti-PML (Santa Cruz Biotechnology), anti-SP100 (Santa Cruz Biotechnology), anti-IE1 (Merck Millipore), or isotype control. Primary antibodies were detected by staining with the appropriate secondary antibody: anti-mouse IgG-AF549 (Invitrogen) and anti-rabbit IgG-AF488 (Invitrogen) and coverslips were counterstained with slowfide DAPI (Thermo Fisher Scientific) before imaging on a wide-field fluorescent light Zeiss deconvolution microscope.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Abbreviations

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