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Research article

Ebola virus infection inversely correlates with the overall expression levels of promyelocytic leukaemia (PML) protein in cultured cells

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Abstract

Background: Ebola virus causes severe, often fatal hemorrhagic fever in humans. The mechanism of escape from cellular anti-viral mechanisms is not yet fully understood. The promyelocytic leukaemia (PML) associated nuclear body is part of the interferon inducible cellular defense system. Several RNA viruses have been found to interfere with the anti-viral function of the PML body. The possible interaction between Ebola virus and the PML bodies has not yet been explored.

Results: We found that two cell lines, Vero E6 and MCF7, support virus production at high and low levels respectively. The expression of viral proteins was visualized and quantified using high resolution immunofluorescence microscopy. Ebola encoded NP and VP35 accumulated in cytoplasmic inclusion bodies whereas VP40 was mainly membrane associated but it was also present diffusely in the cytoplasm as well as in the euchromatic areas of the nucleus. The anti-VP40 antibody also allowed the detection of extracellular virions. Interferon-alpha treatment decreased the production of all three viral proteins and delayed the development of cytopathic effects in both cell lines. Virus infection and interferon-alpha treatment induced high levels of PML protein expression in MCF7 but much less in Vero E6 cells. No disruption of PML bodies, a common phenomenon induced by a variety of different viruses, was observed.

Conclusion: We have established a simple fixation and immunofluorescence staining procedure that allows specific co-detection and precise sub-cellular localization of the PML nuclear bodies and the Ebola virus encoded proteins NP, VP35 and VP40 in formaldehyde treated cells. Interferon-alpha treatment delays virus production *in vitro*. Intact PML bodies may play an anti-viral role in Ebola infected cells.

Background

Ebola virus belongs to the family of negative strand RNA viruses (Mononegavirales) and together with the Marburg virus are the two known species of the *Filoviridae* family [1]. Electron microscopy of Ebola virions produced in cell culture have shown them to be pleomorphic, appearing as either 6-shaped, circular or as long filamentous (and sometimes branched) forms with a length up to 14,000 nm and a uniform diameter of 80 nm [2]. The 19 kb single-stranded RNA genome encodes seven viral proteins: the membrane associated (matrix) proteins VP24 and VP40, the viral glycoproteins, GP (that forms the 10 nm

long peplomers) and sGP (non-structural secreted form), the characteristic helical ribonucleocapsid that consists of the nucleoprotein (NP), VP30, VP35 and the L-protein that forms a RNA-dependent RNA polymerase [1].

Infection of humans and non-human primates by the Ebola virus often leads to severe disease, characterized by systemic hemorrhage, with mortality rates from around 50% up to nearly 90% [3]. The underlying pathogenic mechanism of the hemorrhagic fever is poorly understood. A major reason for the limited amount of information regarding these types of viruses is that the handling of these agents requires a biological safety level 4 (BSL4) containment laboratory.

The reason for the severe tissue damage and organ failure is still somewhat obscure. Both direct cytolysis by the virus and indirect pathology induced by immune-mediated responses have been suggested to play a role [4,5]. Several studies have suggested that the viral GP (the presumed attachment protein) is one of the major pathogenic factors [6,7].

It is not yet clear how the virus evades cellular anti-viral responses. Certain studies have indicated that a strong inflammatory response early in infection may be beneficial for the survival of the infected individual [8]. In a murine model system it was shown that the type I interferon (IFN) response plays a key role in the resistance of normal mice to sub-cutaneously inoculated mouse-adapted Ebola Zaire (EBO-Z) virus [9]. Successful filovirus therapy has been suggested to require active participation of the type I IFN response [9]. Emerging data suggests VP35 to function as a type I IFN antagonist [10].

Specific viral proteins are likely to contribute to the different aspects of the pathology. An important aspect for understanding the function of the different viral proteins is to characterize their precise sub-cellular localization and behaviour during the different phases of viral life cycle. High resolution localization of filovirus antigens is hampered by the difficulties associated with handling of BSL4 agents. Complex, computer supported imaging systems are not regular accessories of high containment level laboratories. To use microscope systems placed at a BSL2 laboratory requires complete inactivation of the virus e.g. prolonged fixation of the infected cells with formaldehyde. This fixation procedure however renders most Ebola antigens inaccessible for antibodies. Immunofluorescence staining of formaldehyde fixed cells requires epitope recovery by means of limited proteolysis [11] or permeabilization. Here we describe a simple fixation and immunofluorescence staining protocol that allows specific and sensitive detection of three viral proteins (NP, VP35 and VP40) using the corresponding monoclonal antibodies on infected cells that were fixed with formaldehyde for, or in excess of, 48 hours. A subsequent treatment with an acetone:methanol mixture served as permeabilization step. We also carried out double immunofluorescence staining of NP, VP35 and VP40 with promyelocytic leukaemia (PML) associated protein, a major component of the subnuclear organelle nuclear domain 10 (ND10; PML body).

The nuclear PML body was first identified as involved in the pathogenesis of acute promyelocytic leukaemia [12]. These distinct sub-nuclear structures are present in most mammalian nucleated cells and are part of an inducible intracellular defense mechanism [13]. The constitutive PML body expression is enhanced by type I and II IFN (alpha/beta and gamma) and in addition, the expression can be induced by viral infections, thereby displaying a defense mechanism to viruses [13]. Many viral infections results in the PML body disruption or delocalization. Various viral proteins from viruses such as lymphocytic choriomeningitis virus (LCMV) [14,15], human T-cell leukaemia virus type 1 (HTLV-I) [16], Rabies virus [17], Epstein-Barr virus (EBV) [18,19] and herpes simplex virus (HSV) [20] have been found to associate with PML bodies and interfere with its innate anti-viral function. Some viral proteins have been found to either physically associate with the PML body [13] or indirectly relocate its normal intra-nuclear positioning, thereby disrupting the normal PML body function. However, precise mechanisms for "disruption and delocalization" are not well understood.

To date, no data are published on the potential interaction between filovirus proteins and the PML nuclear bodies.

In this study, we report our observations concerning the sub-cellular localization of these proteins in two different cell lines (Vero E6 and MCF7) that show different sensitivity for Ebola infection. We have measured the levels of the viral proteins at different time points post-infection in IFN-alpha treated and non-treated cells using digital image quantification methods. We also correlated the expression of viral proteins with the expression of PML protein.

Results

Immunofluorescence detection of Ebola antigens

Staining of viral antigens outside a BSL4 laboratory requires complete inactivation of the virus. This was achieved by fixation of the infected cells with 4% buffered formaldehyde for 48 hours. To permeabilize the cells and recover the epitopes we treated the fixed cells with a 1:1 mixture of methanol and acetone for additional 12–24 hrs. Two different cell types, the human cell line MCF7 and the monkey-derived Vero E6 were used in this study. Both cell lines could be infected by the Ebola Zaire strain

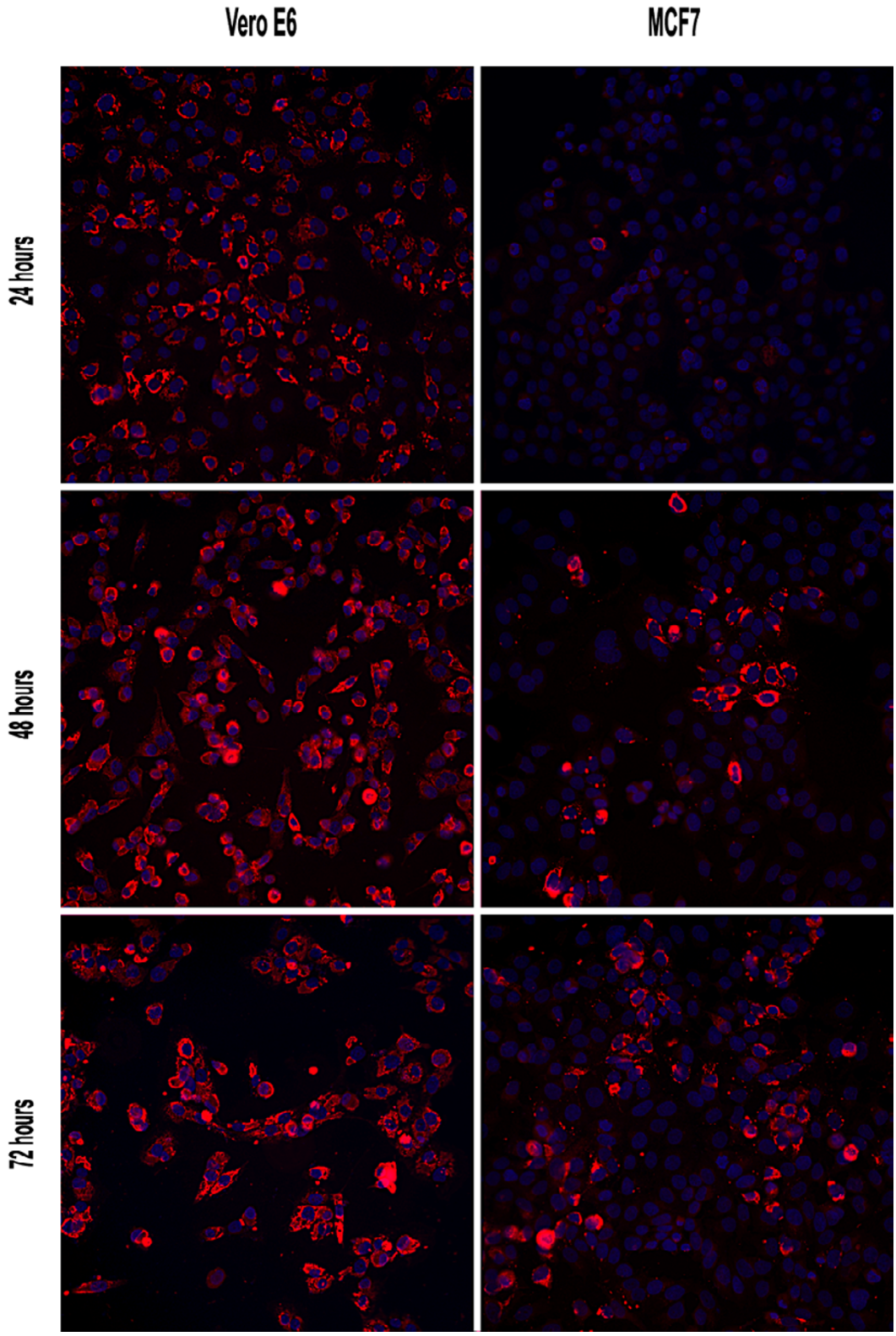

Figure 1
Ebola virus infectivity differs in MCF7 and Vero E6 cells. Vero E6 (left panel) and MCF7 (right panel) cells at 24, 48 and 72 hrs post-infection stained for Ebola virus nucleoprotein – NP (red). Cell nuclei are stained with Hoechst 33258 (blue).

(subtype Mayinga). Vero E6 cells were highly susceptible, as seen by viral antigen expression by immunofluorescence. Nearly all cells were positive already 24 hrs post-infection. In contrast, MCF7 cultures showed very few infected cells at 24 hrs post-infection. Even after 72 hours only around half of the cells contained viral antigens (Figure 1).

Sub-cellular localization of Ebola virus proteins

Nucleoprotein (NP)

All three antibodies directed against NP, VP35 and VP40 respectively gave a clear and distinct staining that was completely absent from the non-infected cells. The strongest and most easily recognizable pattern, which was also most clearly differing from existing cellular structures, was obtained with the anti-NP antibody. It detected well circumscribed cytoplasmic inclusions of variable size. The inclusions started as small round foci at the early phase of infection. They then grew bigger, started to form tubular structures with multiple branches and subsequently became confluent, often surrounding the nucleus in a ring-like fashion. High resolution, three dimensional reconstitution of immunostained cells, from a series of mathematically deblurred optical sections, showed that the inclusions were not anchored to the cell membrane or to the nucleus (Figure 2 top panel). At a late phase of infection the inclusions often turned into one or two nucleus size bodies (Figure 3 middle panel). Importantly, the presence of inclusions of often considerable size caused no visible distress to the cells in the first 24–48 hours. Cells with inclusions that occupied 1/3 to half of the cytoplasm had entered and successfully completed mitotic divisions (Figure 2 middle and bottom panels). Cytopathic effects were only prominent in the Vero E6 cultures. At 72 hours many of the Vero E6 cells rounded up and subsequently died containing huge cytoplasmic inclusions.

Viral protein 35 (VP35)

VP35 was primarily associated with distinct NP-like cytoplasmic inclusions. Unlike NP, however, VP35 was also distributed diffusely over the entire cytoplasm (Figure 3 bottom panel). No nuclear staining was detectable.

Viral protein 40 (VP40)

At an early stage of the infection VP40 showed fine granular, even distribution all over the cytoplasm. It was also present in the nucleus avoiding the nucleoli (Figure 4 top panel). At later stages of the infection VP40 became gradually associated with the cell membrane. It was also still present in the nuclei, mainly accumulating in the low DNA density regions corresponding to the euchromatic areas of the nucleus (Figure 3 top panel). In cells that started to round up VP40 was almost exclusively localized to the cell surface. VP40 has been shown to be a membrane-associated protein that induces formation of filamentous virus-like particles [2,21]. Consistent with these findings, we observed that VP40 was present in all the membrane structures and, importantly, also in numerous filamentous structures on the surface and in the vicinity of the infected cell. The filamentous particles had a uniform thickness, at the limit of the microscope resolution (<0.2 μm), but showed a great variability in length (0.4–10 μm) as shown in Figure 4 middle and bottom panel. The size and distribution of the particles and the known presence of VP40 in the virion membrane implies that the anti-VP40 antibody can detect Ebola virus particles and/or filamentous virus-like particles outside the cell. Superposition of VP40 staining on the phasecontrast images of the cells showed that virion release started before the appearance of detectable cytopathic effects (Figure 5).

Interferon alpha (IFN-alpha) treatment delays Ebola infection in vitro

In order to examine the possible anti-viral effects, cell cultures were pre-treated with IFN-alpha three hours before the time of the infection. The IFN-alpha concentration (200; 1,000 and 5,000 international units (IU)/ml) had been previously tested on the cell lines and 200 IU/ml was established to be the minimum concentration required for up regulation of PML expression (data not shown). This level also approximately corresponds to the *in vivo* concentrations achieved during anti-viral therapeutic treatment in patients. We observed that IFN-alpha treatment markedly decreased the Ebola virus protein production in both cell types. A pronounced effect was seen in Vero E6 cultures with a 2 to 4-fold reduction in the amount of viral proteins at 72 hrs post-infection (Figure 6). The expression levels were determined by quantifying the fluorescence signals of approximately 1,200 cells in three independent measurements on randomly selected fields. The total signal intensity was divided by the number of cells. The apparently paradoxal reduction of NP expression per cell in MCF7 cultures at 72 hrs as compared to 48 hrs (Figure 6 columns in the upper right graph) was a result of the proliferation of non-infected cells during the 3 days observation period. Plotting the total amount of NP in the culture showed an almost linear increase of NP expression in the non-treated cultures in contrast to IFN-alpha treated cells (Figure 6 lines in the upper right graph).

IFN-alpha treatment also delayed the appearance of cytopathic effects. In non-treated Vero E6 cultures the majority of infected cells eventually rounded up and detached from the substratum. IFN-alpha treatment not only reduced the size of NP inclusions but also inhibited the Ebola virus induced round up (Figure 7).

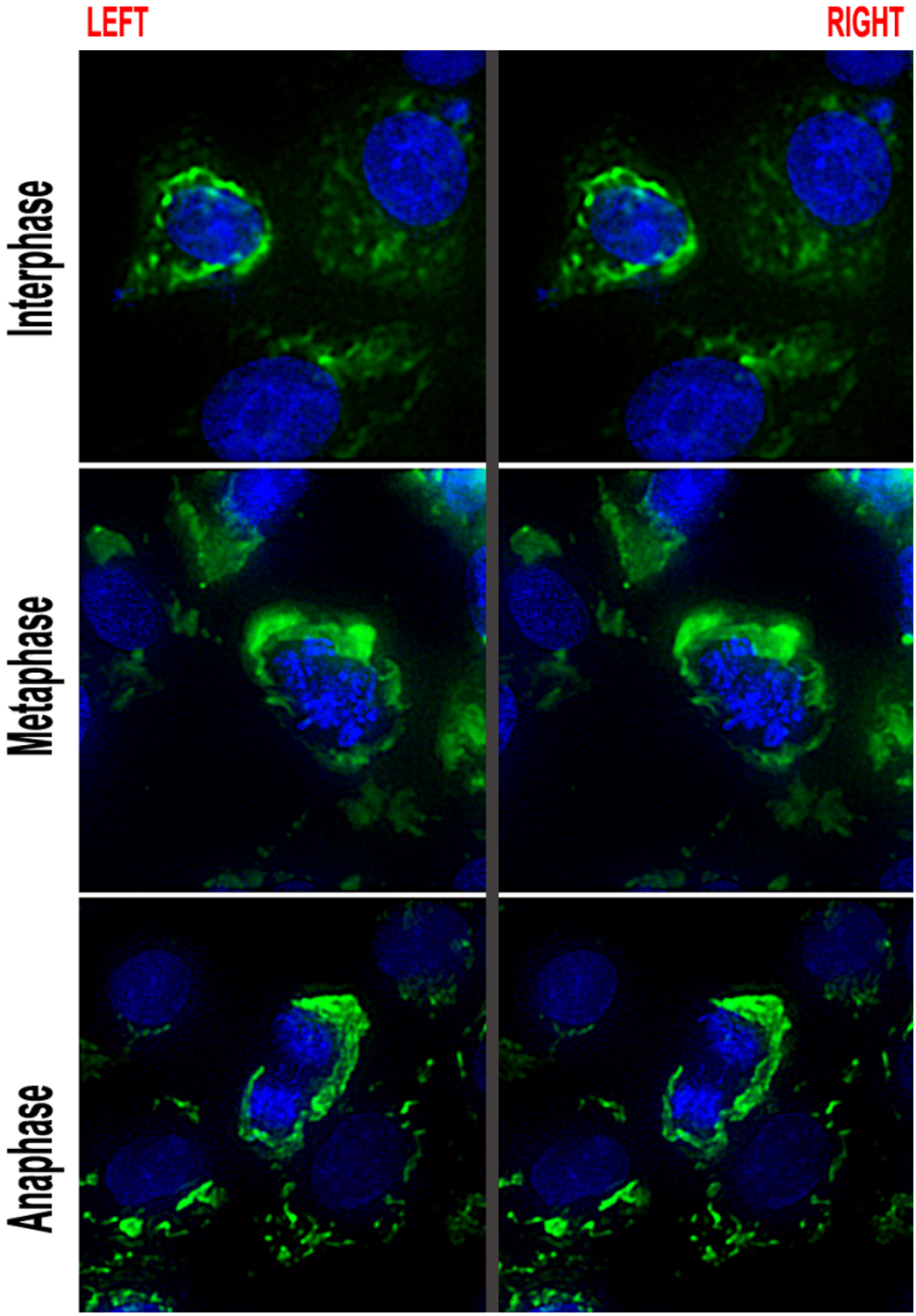

Figure 2
Ebola virus NP in Vero E6 cells at different phases of the cell cycle. Stereoprojected images of NP (green) that show the three dimensional distribution of the protein in cytoplasmic inclusions in interphase, metaphase and anaphase. The images were reconstituted from a series of fifteen, mathematically deblurred, optical sections 0.3 μm apart. Note that the presence of large inclusions does not interfere with the progression of the cell cycle. Interphase nuclei and chromosomes are stained blue. Pictures were taken 48 hrs post-infection.

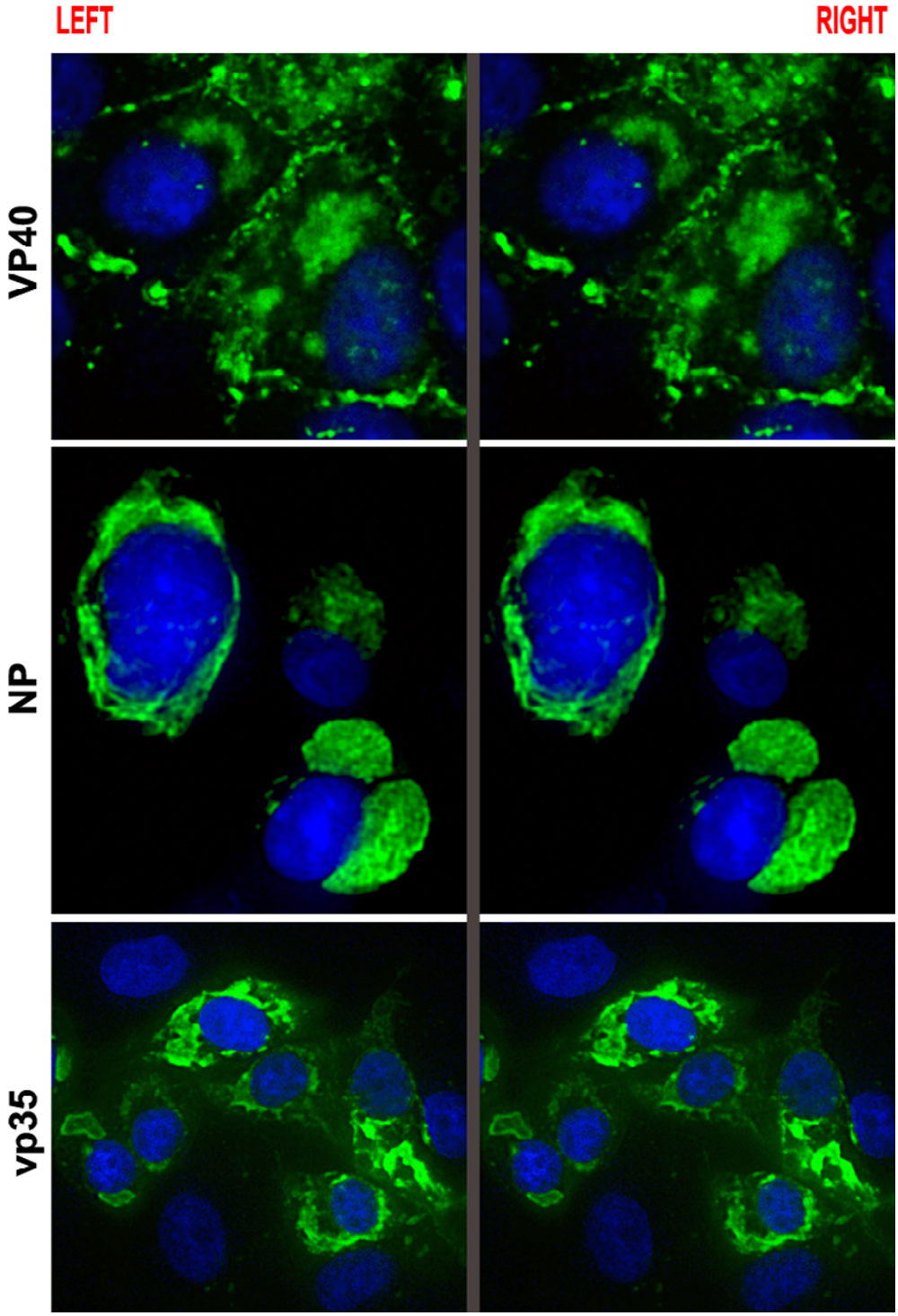

Figure 3
Comparison of the sub-cellular distribution of Ebola viral proteins VP40, NP and VP35. Three dimensional stereo-projected images of Vero E6 cells, 48 hours post-infection. DNA staining is blue. The upper panel shows that VP40 (green) is primarily membrane associated although it can be also present in the low DNA density regions of the nucleus or in diffuse areas of the cytoplasm. NP (green) on the other hand exclusively accumulates in well circumscribed cytoplasmic inclusions of various size, shape and confluency (middle panel). VP35 (green) shows similar distribution as NP with additional homogeneous staining all over the cytoplasm (bottom panel). Both NP and VP35 avoid the nuclei.

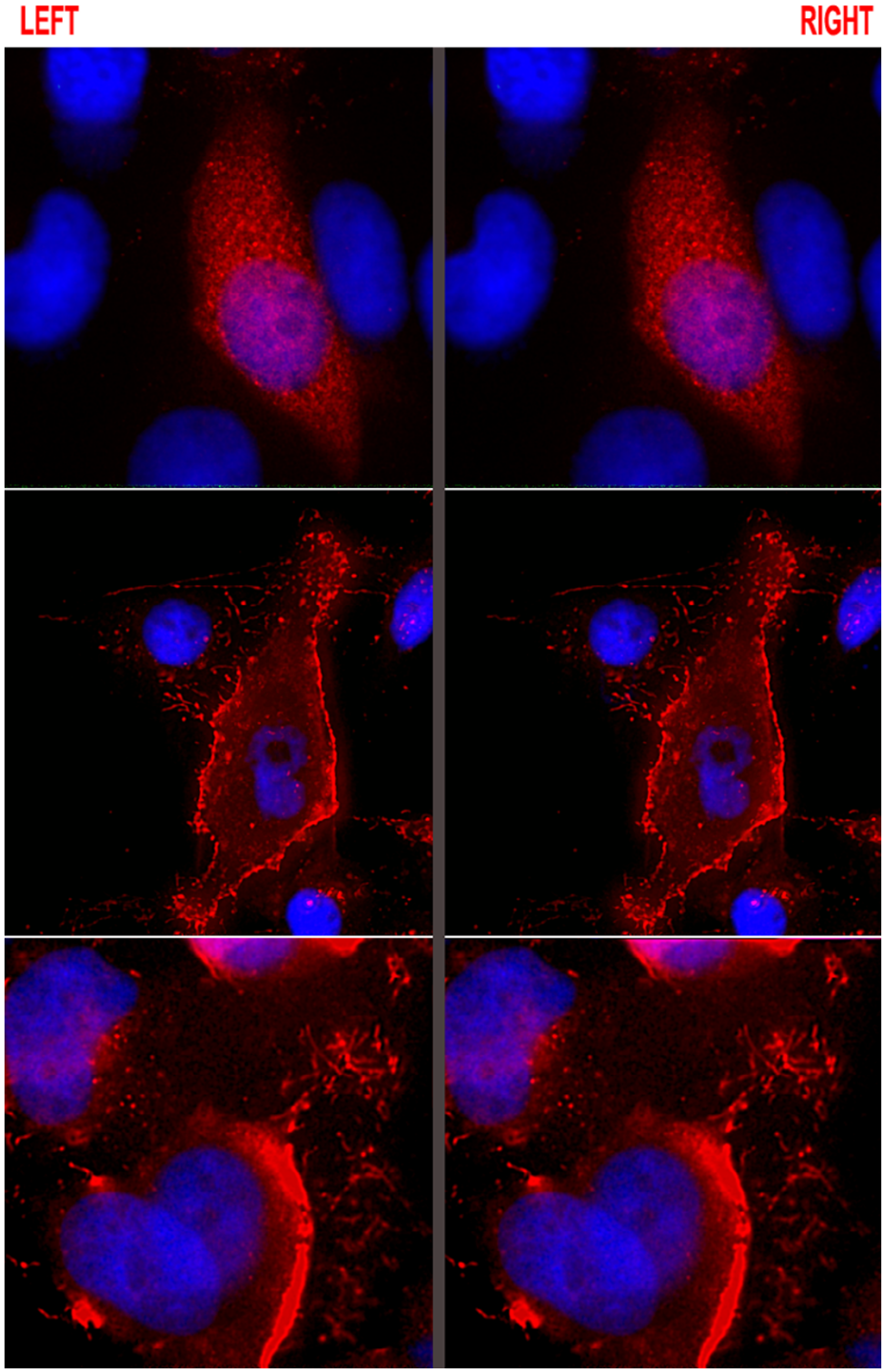

Figure 4
Ebola VP40 changes distribution during the course of infection. The stereoprojected images of MCF7 cells show that at the early phase of infection (top panel) VP40 is localized to homogeneously dispersed granules in the cytoplasm as well as in the nucleus. Later, at the initial phase of rounding up (middle panel), VP40 is almost exclusively membrane associated and it is present in extracellular filamentous structures as well. VP40 is also present in the virions that accumulate in large quantities around cells with pronounced cytopathic effects (bottom panel). DNA staining is blue.

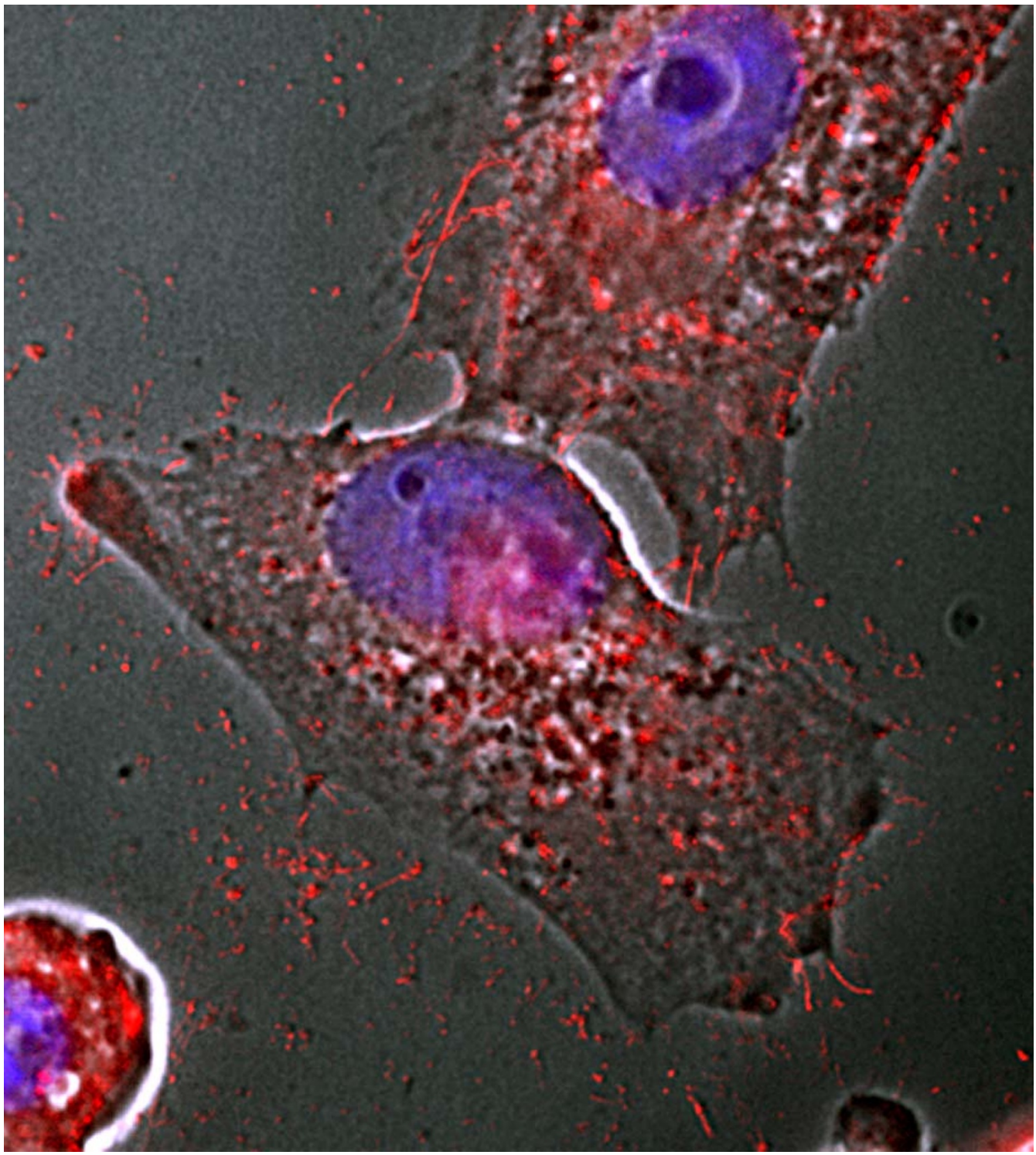

Figure 5
Release of filamentous Ebola virus particles from Vero E6 cells. VP40 staining (red) superimposed on a phasecontrast image of infected cells shows the appearance of extracellular virions even before the development of cytopathic effects. DNA staining is blue.

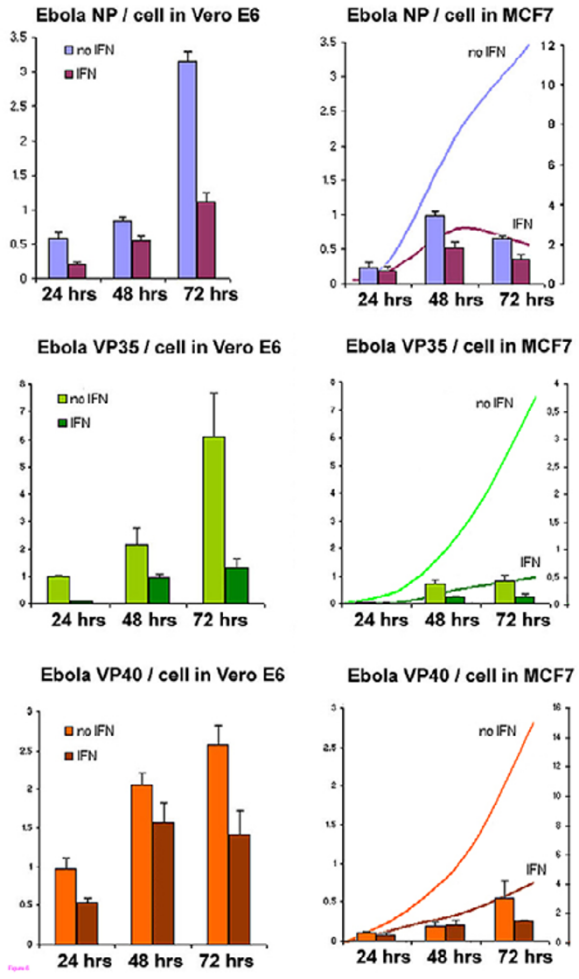

Figure 6

Interferon-alpha treatment inhibits the production of Ebola virus proteins. 200 IU/ml of IFN-alpha reduces the amount of NP, VP35 and VP40 both in the highly susceptible Vero E6 cells and in the less virus sensitive MCF7 cells. The Y axis shows the relative fluorescence intensity. The columns represent the mean fluorescence intensity *per cell*. Standard deviation was calculated from three independently captured random fields of immunostained cells. All the steps of IFN-alpha treatment, virus infection, fixation, immunofluorescence staining and image capturing were carried out in parallel for the two cell lines allowing the direct comparison of expression levels of the individual viral proteins between treated and non-treated as well as between Vero E6 and MCF7 cell lines. This is represented by the identical scale of the Y axis for the given proteins. Due to the unknown binding affinity of the antibodies the staining intensities for NP, VP35 and VP40 are not comparable with each other. To avoid confusion due to the presence of an expanding non-infected cell population in the MCF7 cultures we show not only the fluorescence intensity of staining *per cell* (columns of the upper right chart) but also the average fluorescence intensity for the entire field (lines – with a corresponding Y axis on the right).

PML expression in Ebola infected cultures

Ebola infection led to an increased expression of PML protein in MCF7 cultures whereas PML levels were not changed in mock-infected cells at any time point (data not shown). IFN-alpha treatment further increased PML protein expression. The elevation of PML levels was manifested in the increased size and staining intensity of the PML nuclear bodies. The increased PML expression corresponded to an overall decrease of Ebola virus protein expression. On the other hand Vero E6 cultures showed an overall low level of PML expression with small and less intensely stained nuclear bodies. Virus infection and IFN-alpha treatment was much less efficient in increasing PML expression in this cell type (Figure 8).

Double immunofluorescence staining of the three Ebola proteins (VP35, VP40 and NP in MCF7 and Vero E6 cells) in combination with PML showed that increased PML expression was associated mainly with the infected cells (as visualized in Figure 9 by VP35 and VP40 expression in MCF7 and NP expression in Vero E6). No disruption or dissolution of PML bodies was observed in the infected cells of either cultures.

Discussion

We could demonstrate that 4% formaldehyde fixation followed by acetone: methanol (1:1) treatment allowed the immunofluorescence detection of both the Ebola virus proteins NP, VP35, VP40 and the cellular PML protein in the virus infected cells. In fact, the fixation procedure was fully comparable with fixation in acetone alone (data not shown). This method provides an easier, and in routine laboratory procedure more reliable, alternative to the protocols of van der Groen [22] and Kurata [11] where the epitope recovery in formaldehyde fixed cells required controlled enzymatic treatment with trypsin or pronase. It gives a comparable result to paraformaldehyde fixation followed by Triton X-100 permeabilization [6].

Our staining data suggest that for routine detection of infectious virus or titration of virus stocks, immunofluorescence staining of NP protein (since staining of this protein gave rise to the most easily recognizable pattern, clearly differing from existing cellular structures) on formaldehyde fixed and subsequently methanol:acetone treated cells, is a useful tool.

The presence of NP-containing viral inclusions throughout the cell cycle indirectly suggests that Ebola virus does not induce an immediate re-programming of the cells by abruptly shutting down host protein synthesis but prepares for virus replication in a much more stealth-like fashion. The development of huge cytoplasmic inclusions without apparent cellular distress is in line with previous

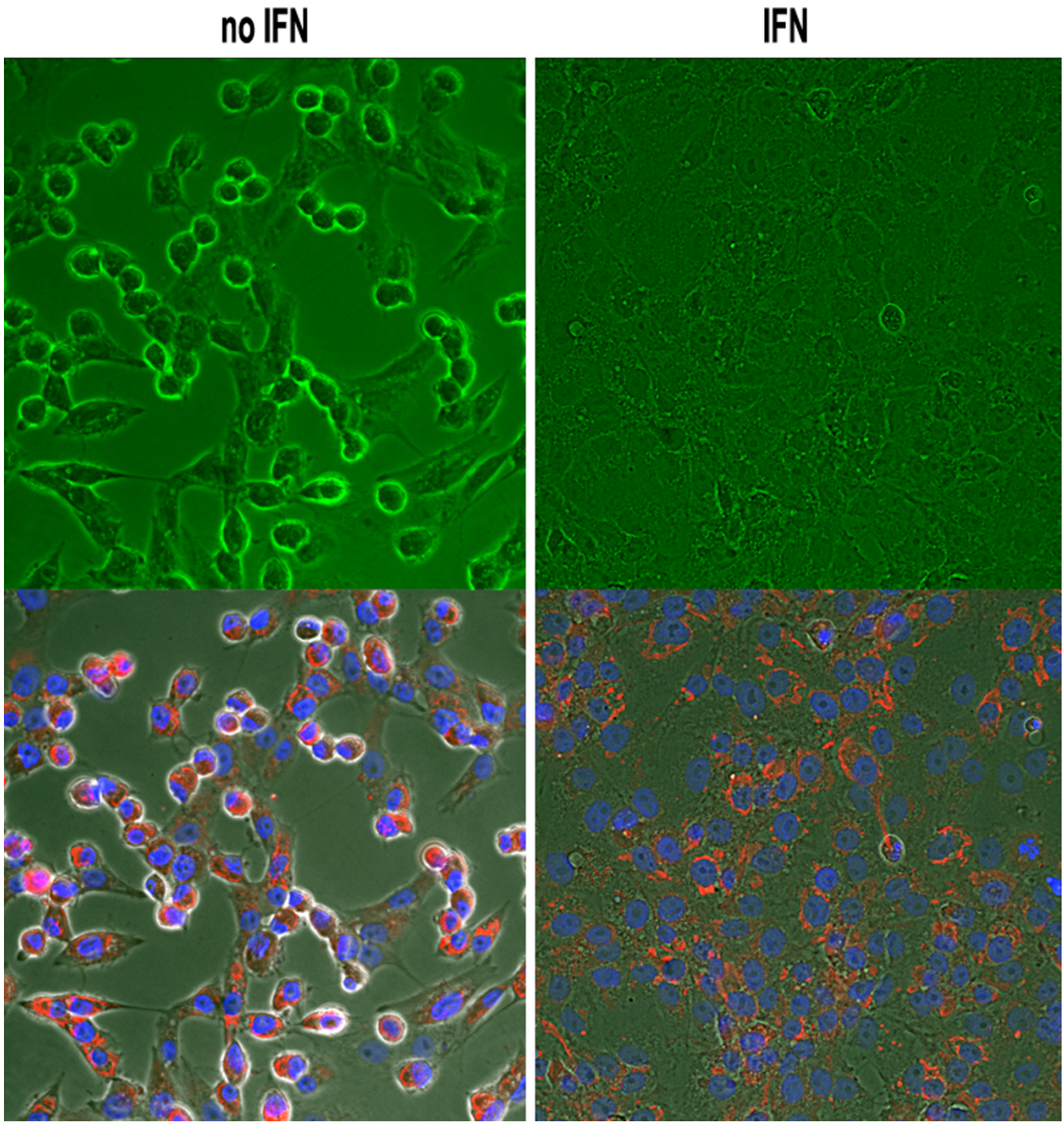

Figure 7
Interferon-alpha treatment decreases the Ebola virus induced cytopathic effects in Vero E6 cultures. Phasecontrast image of IFN-alpha treated and non-treated cells 72 hrs post-infection shows an efficient inhibition of rounding up (top right panel). Fluorescence staining for NP (red) and DNA (blue) is superimposed on the phase contrast image (bottom panel).

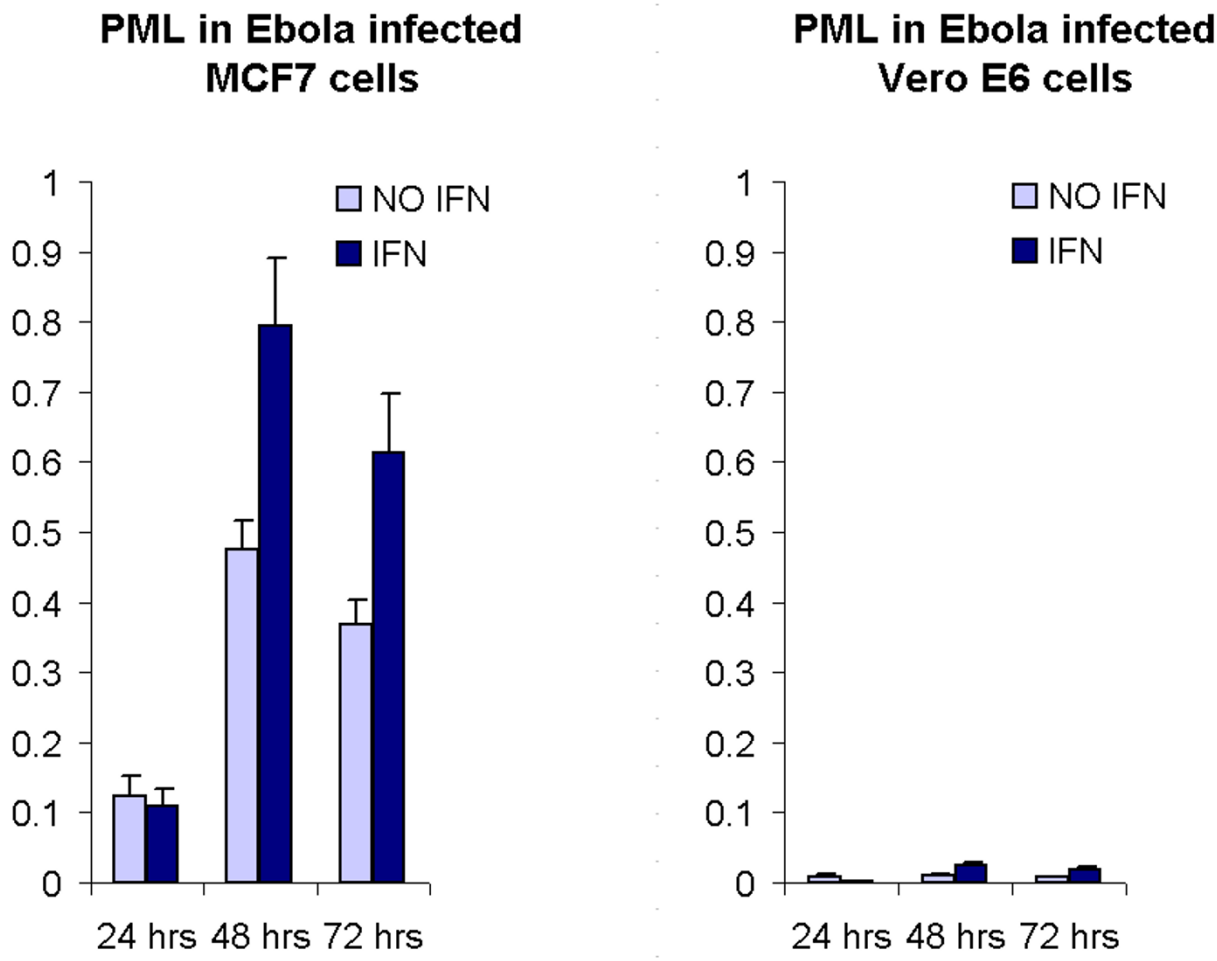

Figure 8
MCF7 and Vero E6 differs in the virus- and interferon treatment-induced PML expression levels. The Y axis shows the relative fluorescence intensity. The columns represent the mean fluorescence intensity of PML staining *per nucleus* in interferon-alpha treated cultures. Standard deviation is calculated from three independent capturings of random fields of immunostained cells.

observations that filoviruses induced cytopathic effects may be absent or require several days to manifest [23].

VP35 is an essential component of the Ebola nucleocapsid [24]. Here we show that VP35 accumulates in cytoplasmic inclusions that are morphologically indistinguishable from NP-containing viral inclusions. Ebola virus replicates in the cytoplasm through double stranded RNA intermediates and it appears that it has evolved a mechanism that interferes with dsRNA induced interferon response [25]. VP35 was shown to be able to rescue NS1 mutant influenza A virus replication arguing that it is a type I IFN antagonist. VP35 could also block dsRNA or virus-mediated induction of IFN responsive promoters [10]. Negative stranded RNA viruses often sequester their dsRNA intermediate into ribonucleoprotein complexes that form cytoplasmic inclusions. The presence of VP35 in the Ebola virus inclusions suggests that it intercepts the dsRNA induced response in a close spatial proximity to the viral RNA.

VP40 showed a completely different sub-cellular localization from NP and VP35. It was primarily associated with the cell membrane. Biochemical studies earlier showed that VP40 is a membrane binding protein that oligomerizes upon interaction with lipid membranes [26]. The ol-

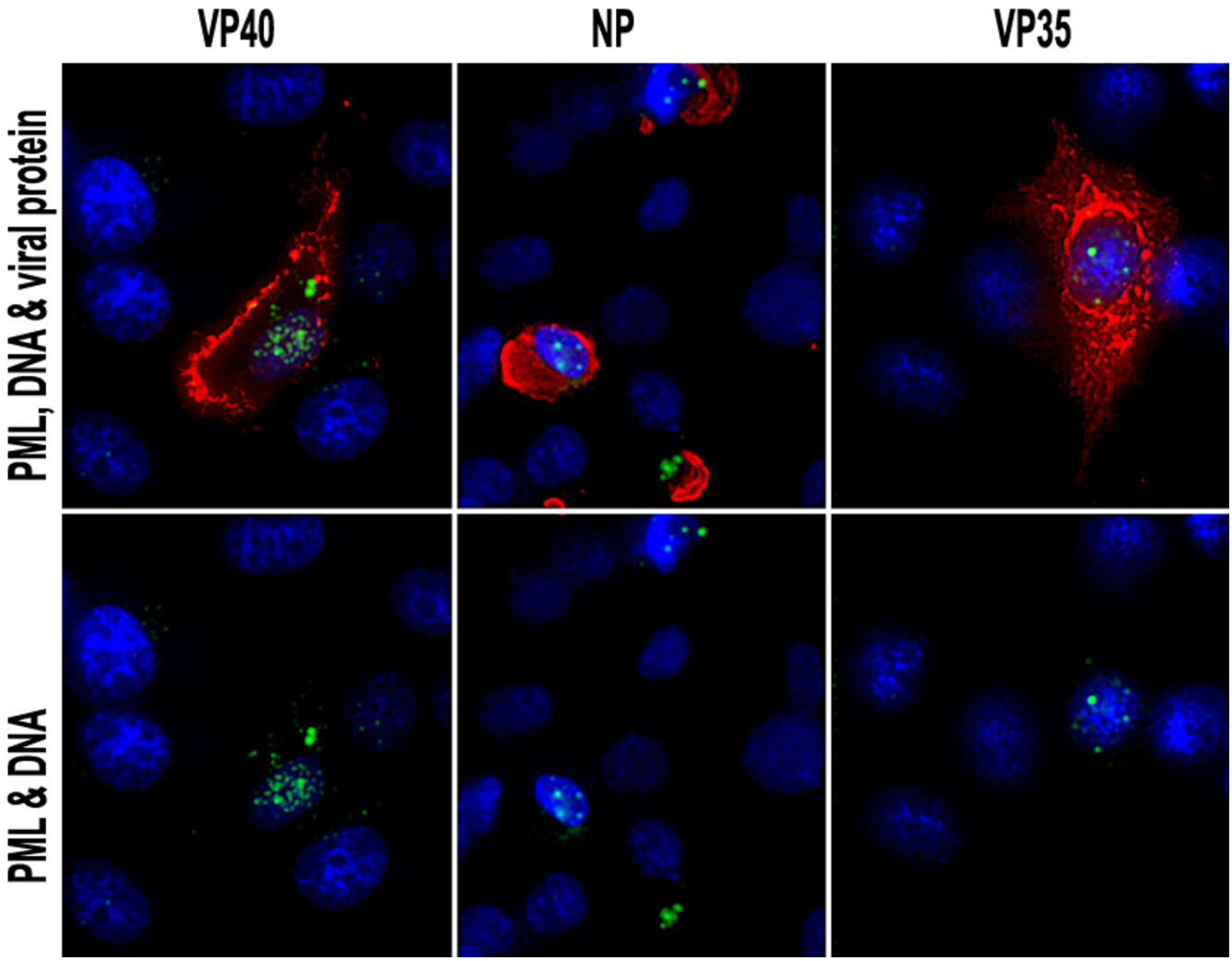

Figure 9
Ebola virus induces increased PML expression in the infected cells. Double immunofluorescence staining of VP40 and VP35 in MCF7 or NP in Vero E6 cells (red) with PML (green) 48 hrs post-infection. The pictures shows that infected cells express more PML proteins as compared to the non-infected neighbouring cells. DNA staining is blue. The intensity of the PML fluorescence signal is not directly comparable between Vero E6 (weak overall PML staining) and MCF7 cells (strong overall PML staining).

igomerization leads to self assembly into membrane-containing particles [27] and subsequent release into the cell culture medium [28]. Unlike the Marburg virus encoded VP40 that localized both to the viral inclusions as well as to clusters of intracellular membrane or to plasma membrane protrusions in infected macrophages [29], Ebola VP40 was primarily associated with the cell membrane. It also entered the nucleus, similarly to the Marburg encoded VP40, raising the possibility that VP40 may play additional, e.g. gene expression regulatory role beside the involvement in virus assembly and budding.

Importantly, the staining with anti-VP40 antibodies on formaldehyde fixed and acetone:methanol treated cells allowed the direct immunofluorescence detection of extracellular virions and/or filamentous virus-like particles. The size of most viruses is regularly below the resolution of the light microscope. Although the spatial resolution of the fluorescence microscope is constrained by the same physical laws as any other light microscopes, e.g. it does not allow the reliable separation of two points that are closer than 0.2 μm, it still allows the detection of light emitting particles on dark background that are far much

smaller, e.g. individual protein molecules. Ebola Zaire virions with average length of 1.3 ± 0.7 μm and occasional length up to 14 μm [2] are well within the limits of fluorescence microscopy. The possibility to carry out 3D reconstitution of fluorescence images picturing nascent virions in combination with staining for additional two or three viral and/or cellular proteins should provide ample amount of information about the molecular mechanism of the egress of Ebola virus.

IFN-alpha appears to play a crucial role in the anti-viral defense in Ebola virus infected experimental animals. Mice lacking the type I IFN receptor, but not wild type mice, died upon subcutaneous inoculation of the virus [9]. The adenosine analogue 3-Deazaneplanocin A was recently shown to protect from Ebola virus induced death of infected mice through massive induction of IFN-alpha [30].

We found that IFN-alpha treatment decreased Ebola virus protein production *in vitro* and inhibited the development of cytopathic effects. These data are in line with the findings that IFN-alpha could reduce infection 10 to 100-fold in cell cultures and also delay the death of infected monkeys [31]. One important anti-viral mechanism that is induced as part of the interferon response is the increased expression of the components of the subnuclear organelle ND10 or PML body. A variety of DNA and RNA viruses encode proteins that specifically interact with the PML bodies and regularly destroy them. Rhabdoviruses share several molecular features with filoviruses. The rabies virus encoded P phosphoprotein is a distant homologue of the Ebola VP35. It interacts with and reorganizes PML bodies [17]. Here we show that Ebola virus infection did not dissolve PML bodies. On the contrary, individual cells expressing viral proteins regularly showed increased expression of PML. Moreover, although Ebola virus interferes with the IFN response it could not inhibit the IFN-alpha mediated induction of PML. VP35, a possible candidate for interference with PML function, never entered the nucleus but remained exclusively cytoplasmic. Importantly, Vero E6 cells, that supported Ebola replication very efficiently, expressed low levels of PML and showed much smaller virus-dependent and IFN-mediated increase in PML expression than MCF7 cells that served as a poor host for the virus. Our data suggest that Ebola virus lacks effective anti-PML mechanism. The data are also consistent with the possibility that increased PML expression might hinder virus infection, similarly to what was previously demonstrated for vesicular stomatitis virus (VSV) [32,33], influenza virus [32], human foamy virus (HFV) [34] and lymphocytic choriomeningitis virus (LCMV) [33]. Our results lead to the testable hypothesis that introduction of constitutively expressed PML cDNA into Vero E6 cells should inhibit, and inhibitory RNA (RNAi) mediated downregulation of PML in MCF7 cells should promote, the propagation of the Ebola virus *in vitro*.

Conclusions

A modified fixation procedure allowed us to study the expression levels and sub-cellular localization of Ebola virus encoded proteins as well as the nuclear PML expression using advanced imaging techniques. NP and VP35 accumulated in cytoplasmic inclusion bodies whereas VP40 was mainly membrane associated. The anti-VP40 antibody stained extracellular virions and/or filamentous virus-like particles as well. We showed that the two cell lines studied supported virus propagation on different levels: Vero E6 – high, and MCF7 – low. IFN-alpha treatment delayed virus production *in vitro* in both lines. MCF7 but not Vero E6 showed efficient virus- and IFN-mediated induction of PML. We suggest that Ebola virus lacks an effective anti-PML mechanism and that intact PML bodies may play an anti-viral role in Ebola virus infected cells.

Methods

Cell lines and interferon-alpha treatment

The human epithelial breast carcinoma cell line MCF7 (ATCC no. HTB-22) and the African green monkey kidney cell line Vero E6 (originally named Vero C1008; ATCC no. CRL-1586) were grown on 8-well Lab-Tek II Chamber slides (Nunc, USA) in DMEM medium (high glucose) supplemented with 10% fetal calf serum (Life Technologies), penicillin/streptomycin, L-glutamine (Life Technologies) at 37°C and 5% CO_2. Cell cultures were grown to sub-confluence and thereafter used as target cells for the infections. Three hours pre-infection, selected cell cultures were treated with 200 IU/ml of interferon-alpha (Welferon; a generous gift from Dr. Norman Finter, GlaxoWelcome).

Infections

All work with infectious material was carried out at the BSL4 laboratory at the Center for Microbiological Preparedness at the Swedish Institute for Infectious Disease Control in Solna, Sweden. Infections were performed with Ebola Zaire subtype Mayinga (kindly provided by Dr Stephan Becker, Marburg, Germany). The virus stock was titrated on Vero E6 cells. For the experiments, the target cells were infected with 2.3 plaque-forming units (PFU) per cell (30,000 cells / well) in the total volume of 0.2 ml for 45 min at 37°C. The supernatant was then removed and 1 ml of fresh medium (D-MEM + 2% FCS) was added. Cultures were incubated further and harvested at 1, 2 and 3 days post-infection. At harvest, chambers were removed, slides were washed twice in PBS and subjected to fixation (and virus inactivation) in 4% formaldehyde (10% NBF) for 48 hr at +4°C.

Immunofluorescence staining

Infected cell cultures grown on 8-well Lab-Tek II Chamber slides (Nalge Nunc International, IL, USA) were analysed by indirect immunofluorescence 24 hrs, 48 hrs and 72 hrs post-infection. After formaldehyde fixation slides were quickly washed twice in PBS and permeabilized with ice-cold aceton:methanol (1:1) 12–24 hrs at -20°C. Slides were thereafter subjected to re-hydration in 2% FCS in PBS for at least 1.5 hrs at room temperature (RT) before incubated with primary anti-PML by using a rabbit-anti-PML polyclonal (sc-5621, Santa Cruz Biotechnology). Primary antibodies were diluted in blocking buffer (2% BSA / 0.2% Tween-20 / 10% glycerol / 0.05% NaN_3 in PBS) and incubated in a moist chamber for 60 min at RT. Cells were then washed five times with cold PBS before incubation with the secondary FITC-conjugated swine-anti-rabbit antibody (Dakopatts). The cells were then blocked (absorbed) for 30 min in normal mouse serum in PBS. Thereafter, Ebola viral proteins were stained by using the following monoclonal antibodies (mouse ascitic fluids from hybridoma cells generously provided by Dr Anthony Sanchez at the Centers for Disease Control, Atlanta, GA [2]): anti-EBOLA Zaire VP35 (BC03-BD09; EBO-Z-VP35); EBOLA Zaire Nucleoprotein (FB03-BE08; EBO-Z-NP) and EBOLA Zaire VP40 (BO04-BD07; EBO-Z-VP40). Ebola monoclonals were diluted 1:100 in blocking buffer (2% BSA / 0.2% Tween-20 / 10% glycerol / 0.05% NaN_3 in PBS) and incubated in a moist chamber for 60 min at RT. Cells were thereafter washed five times in cold PBS and a TRITC-conjugated rabbit-anti-mouse monoclonal antibody (Dakopatts) was added. Single staining of viral proteins was carried out using the FITC-conjugated secondary antibody. For DNA staining, Hoecht 33258 was used. Cover slips were mounted on the glass slides using 70% glycerol containing 2.5% DABCO anti-fading agent (Sigma).

Fluorescence microscopy and digital image analysis

Images were collected using a Leitz DM RB microscope, equipped with Leica PL Fluotar 100×, 40× and PL APO Ph 63× oil immersion objectives. Composite filter cubes were used for the FITC, TRITC and Hoechst 33258 fluorescence, respectively. The pictures were captured with a Hamamatsu dual mode cooled CCD camera (C4880), recorded and analyzed on a Pentium PC computer equipped with an AFG VISION*plus* – AT frame grabber board using Hipic4.0.4 (Hamamatsu), Image-Pro Plus (Media Cybernetics). Digital images were assembled using Adobe Photoshop software.

Measurement of protein expression levels was carried out on three randomly selected fields containing around 400 cells each. The slides were processed identically keeping the exposition time, and the upper and lower limits of computer LUT (look-up-table) constant. Fluorescence intensities were calculated for individual cells. Average and standard deviation values were calculated from the three independent measurements.

3D reconstituted images were collected using Zeiss Axiophot microscope equipped with ×63 oil Plan-Apochromat NA 1.4, and ×100 oil Plan-Neofluar NA 0.7–1.3 objectives, illuminated with Osram HBO 200 W mercury short arc lamp. A program, developed by us (RFH_ST_BIN), was used to produce images. This program also gives information about the depth distribution by creating a maximal-intensity projected image pair representing a 3D reconstitution of the original object as single or stereoscopic image. To remove out-of-focus blur, the program uses the nearest-neighbor deconvolution deblurring (NNDC) algorithm. Following excitation filters were used: single band UV exciter for Hoechst (84360), single band Blue exciter for FITC (84490), and single band green exciter for TRITC (84555). The images were captured with a PXL cooled camera (Photometrics, Munich, Germany) and analyzed using the Isee 5.1 graphical programming system (Inovision, Raleigh; NC) on Pentium PC computer with LINUX OS.

Author's contributions

Å. S. B. carried out the experiments and drafted the manuscript. L. S. helped with the digital image analysis and together with Å. S. B. conceived the study. F. E. participated in the design and coordination of the study. All authors read and approved the final manuscript.

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