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Host-cell dependent role of phosphorylated keratin 8 during influenza A/NWS/33 virus (H1N1) infection in mammalian cells

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# Virus Research

## Host-cell dependent role of phosphorylated keratin 8 during influenza A/NWS/33 virus (H1N1) infection in mammalian cells --Manuscript Draft--

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<b>Abstract:</b>	<p>In this study, we investigated the involvement of keratin 8 during human influenza A/NWS/33 virus (H1N1) infection in semi-permissive rhesus monkey-kidney (LLC-MK2) and permissive human type II alveolar epithelial (A549) cells. In A549 cells, keratin 8 showed major expression and phosphorylation levels.</p> <p>Influenza A/NWS/33 virus was able to subvert keratin 8 structural organization at late stages of infection in both cell models, promoting keratin 8 phosphorylation in A549 cells at early phases of infection. Accordingly, partial colocalizations of the viral nucleoprotein with keratin 8 and its phosphorylated form were assessed by confocal microscopy at early stages of infection in A549 cells. The employment of chemical activators of phosphorylation resulted in structural changes as well as increased phosphorylation of keratin 8 in both cell models, favoring the influenza A/NWS/33 virus's replicative efficiency in A549 but not in LLC-MK2 cells.</p> <p>In A549 and human larynx epidermoid carcinoma (HEp-2) cells inoculated with respiratory secretions from pediatric patients positive for, respectively, influenza A virus or respiratory syncytial virus, the keratin 8 phosphorylation level had increased only in the case of influenza A virus infection.</p> <p>The results obtained suggest that in A549 cells the influenza virus is able to induce keratin 8 phosphorylation thereby enhancing its replicative efficiency.</p>
<b>Suggested Reviewers:</b>	<p>Joanna Rzeszowska-Wolny joanna.rzeszowska@polsl.pl Her scientific interest concerns the information stored in living systems: regulation of the expression of genetic information, signal transduction, mechanisms induced by DNA damage and repair.</p> <p>Akira Ono akiraono@umich.edu His scientific interest is focused on mechanisms regulating assembly of viral components and effects of host factors</p> <p>simone giannecchini simone.giannecchini@unifi.it His scientific interest is focused on host cell-virus interaction</p> <p>Maria Antonia De Francesco maria.defrancesco@unibs.it Her scientific interest is focused on the molecular characterization of different viruses</p>

## **Highlights**

In A549 cells keratin 8 shows major expression and phosphorylation levels

Influenza A/NWS/33 virus promotes keratin 8 phosphorylation in A549 cells

Activators of phosphorylation favour the replication of influenza A/NWS/33 virus

Influenza A virus enhances its replication in A549 cells by phosphorylation

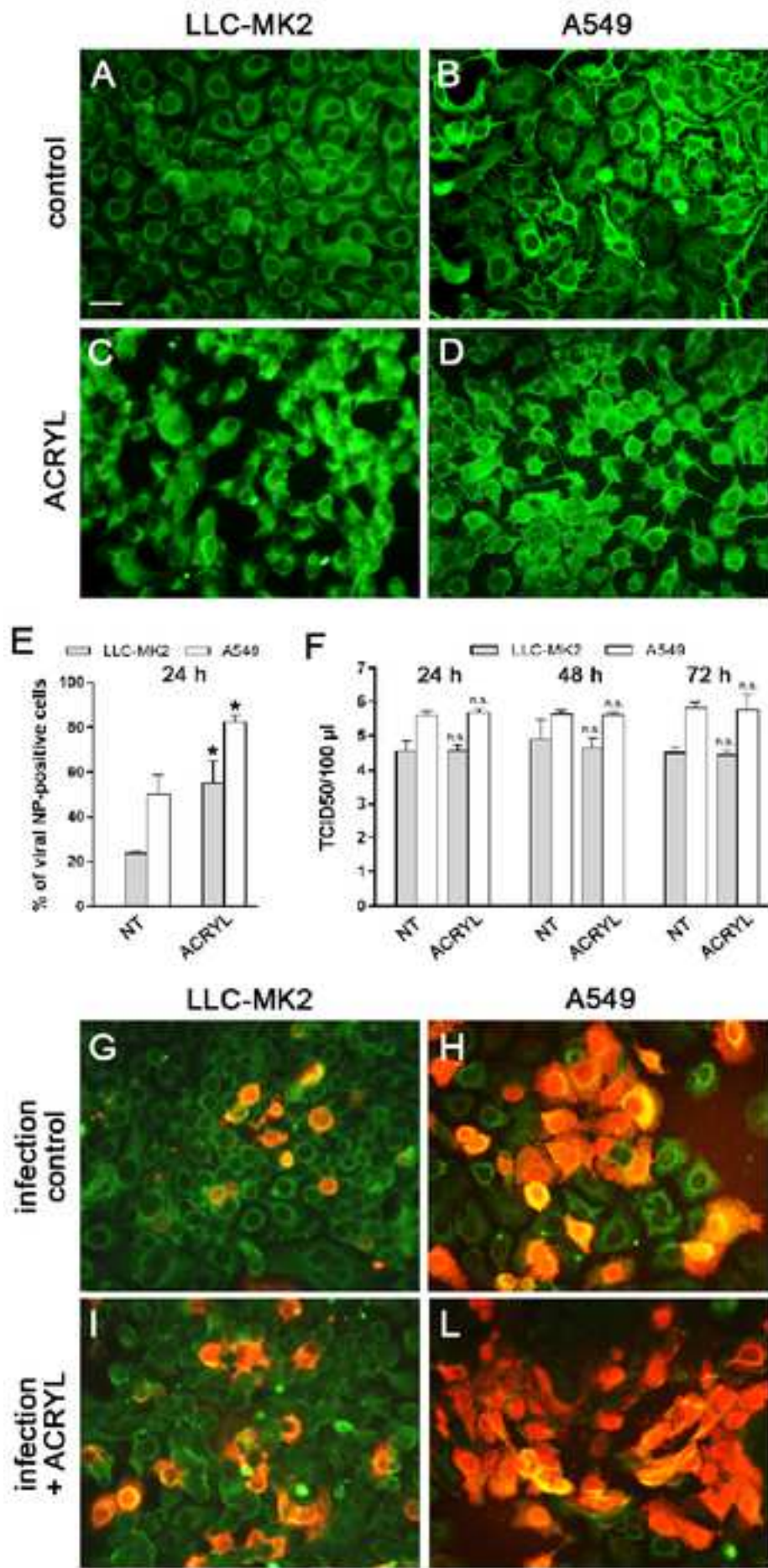


FIG.1

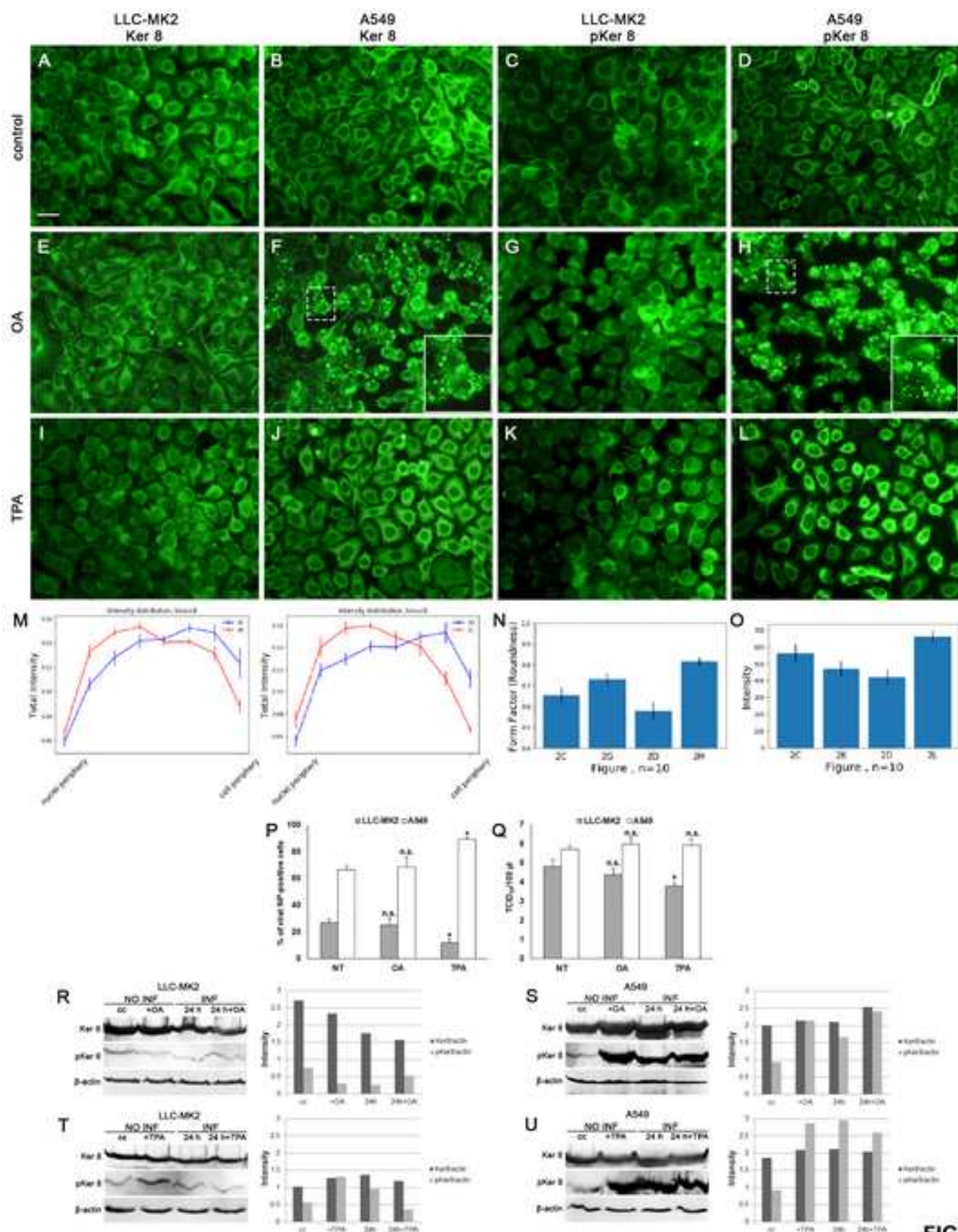


FIG.2

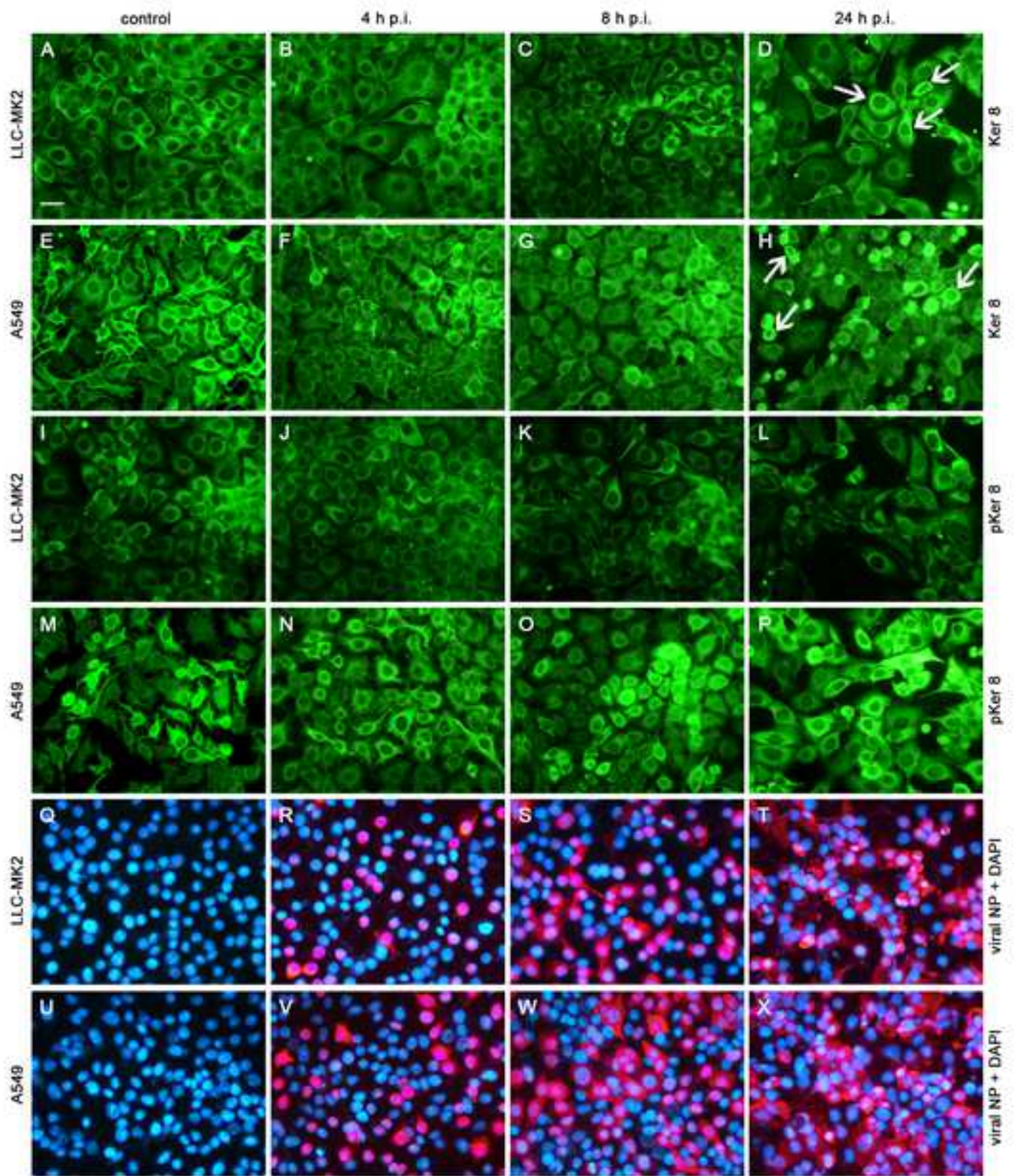


FIG.3

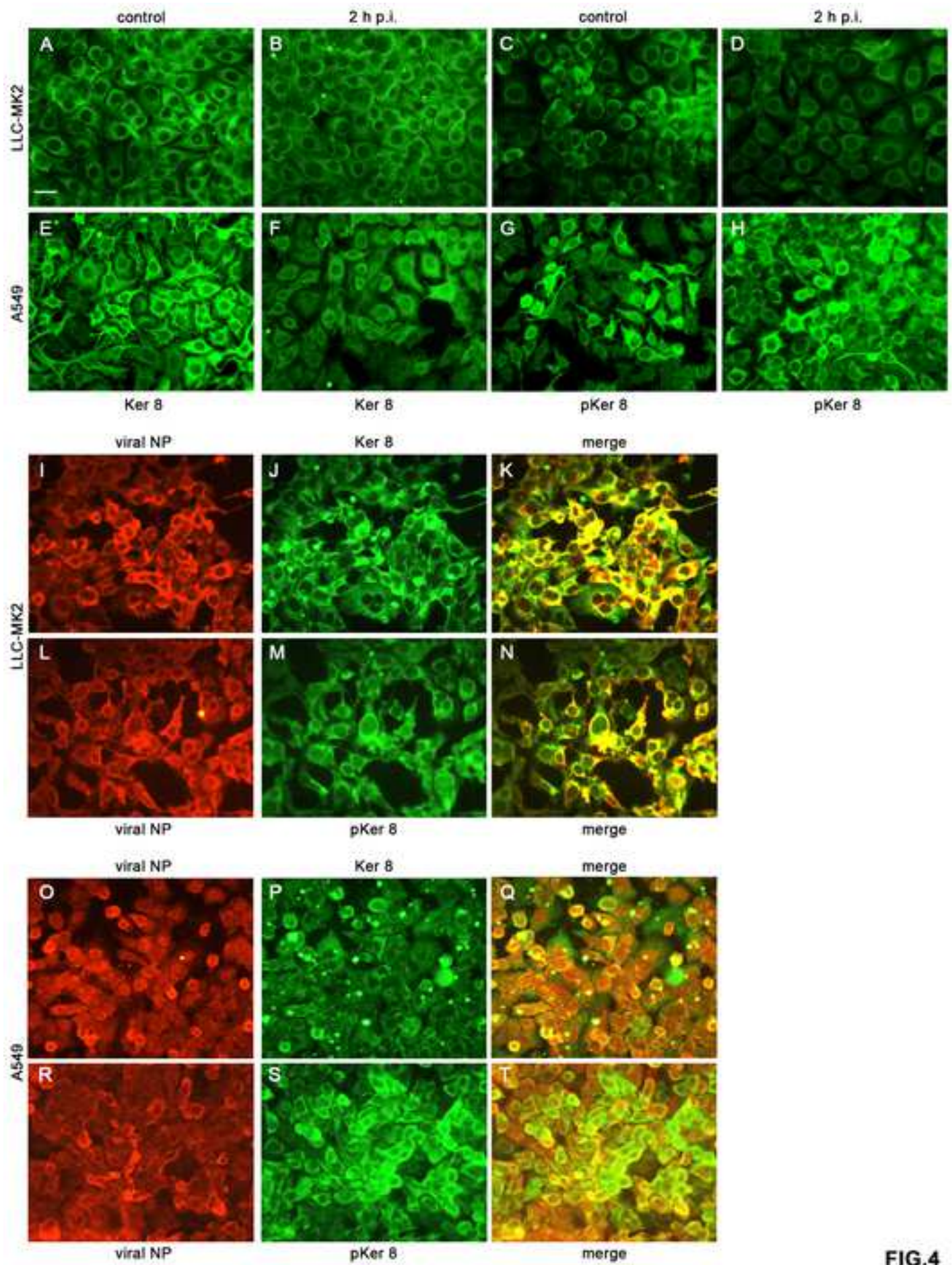
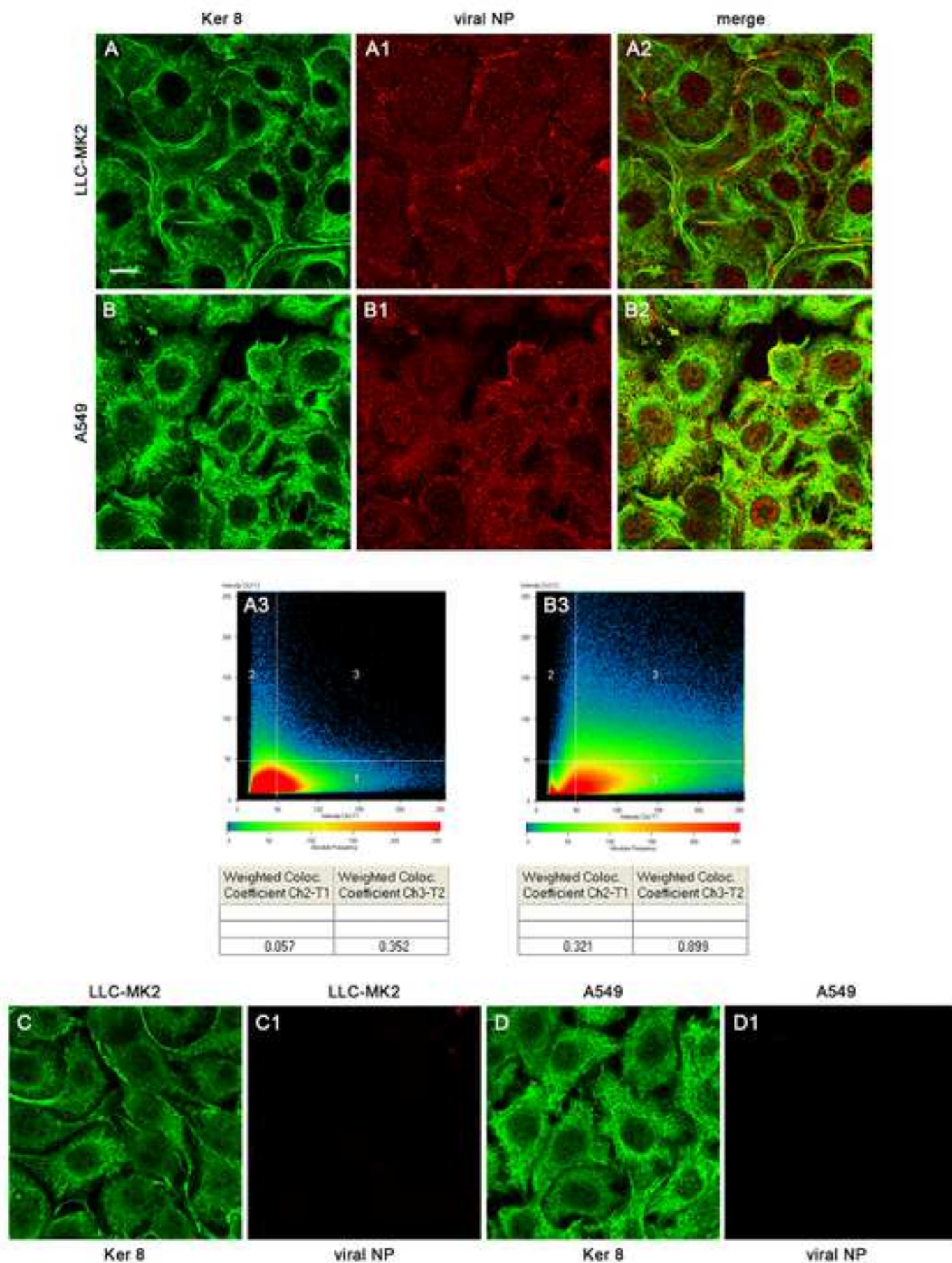


FIG.4

**FIG.5**

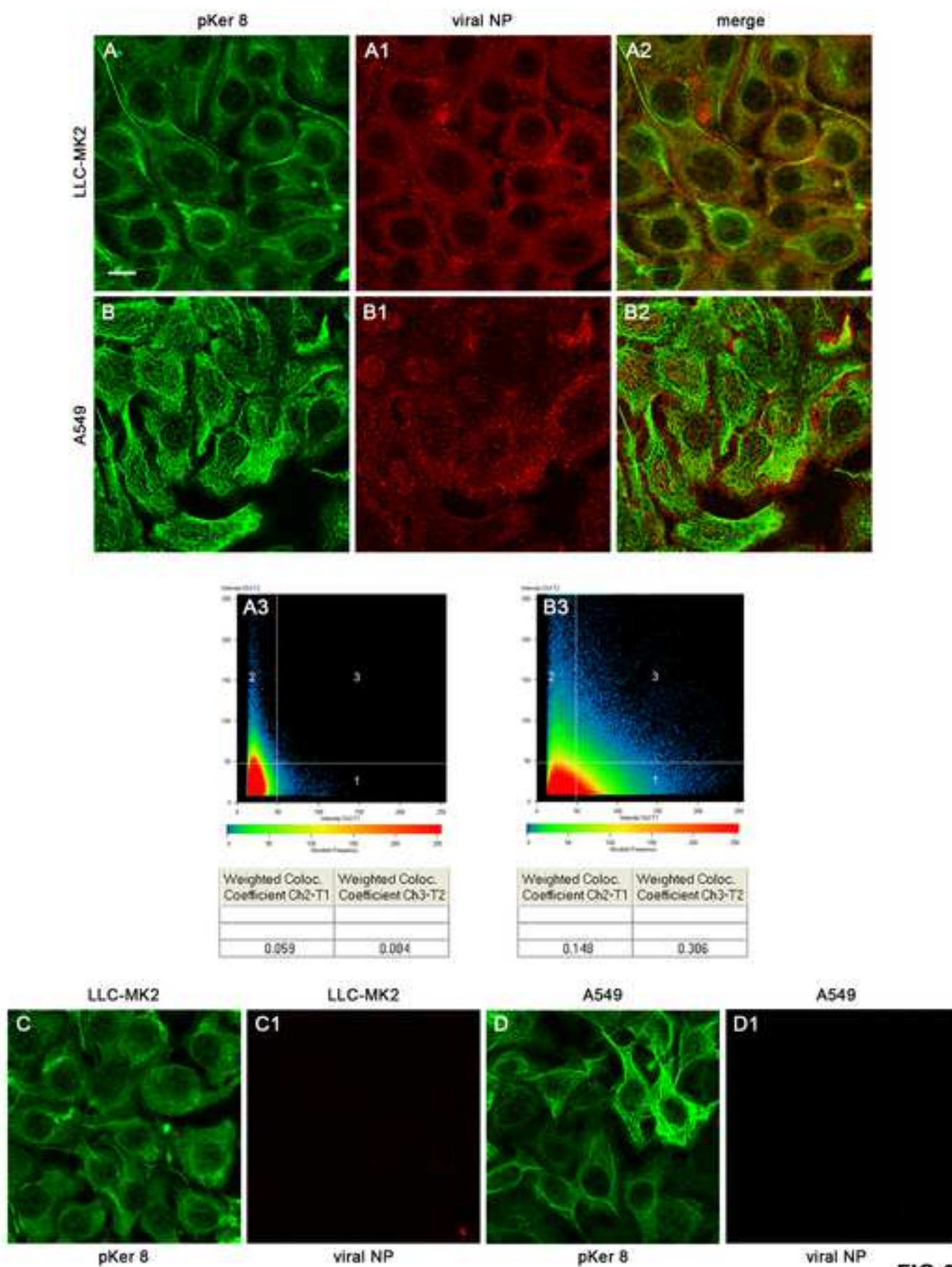
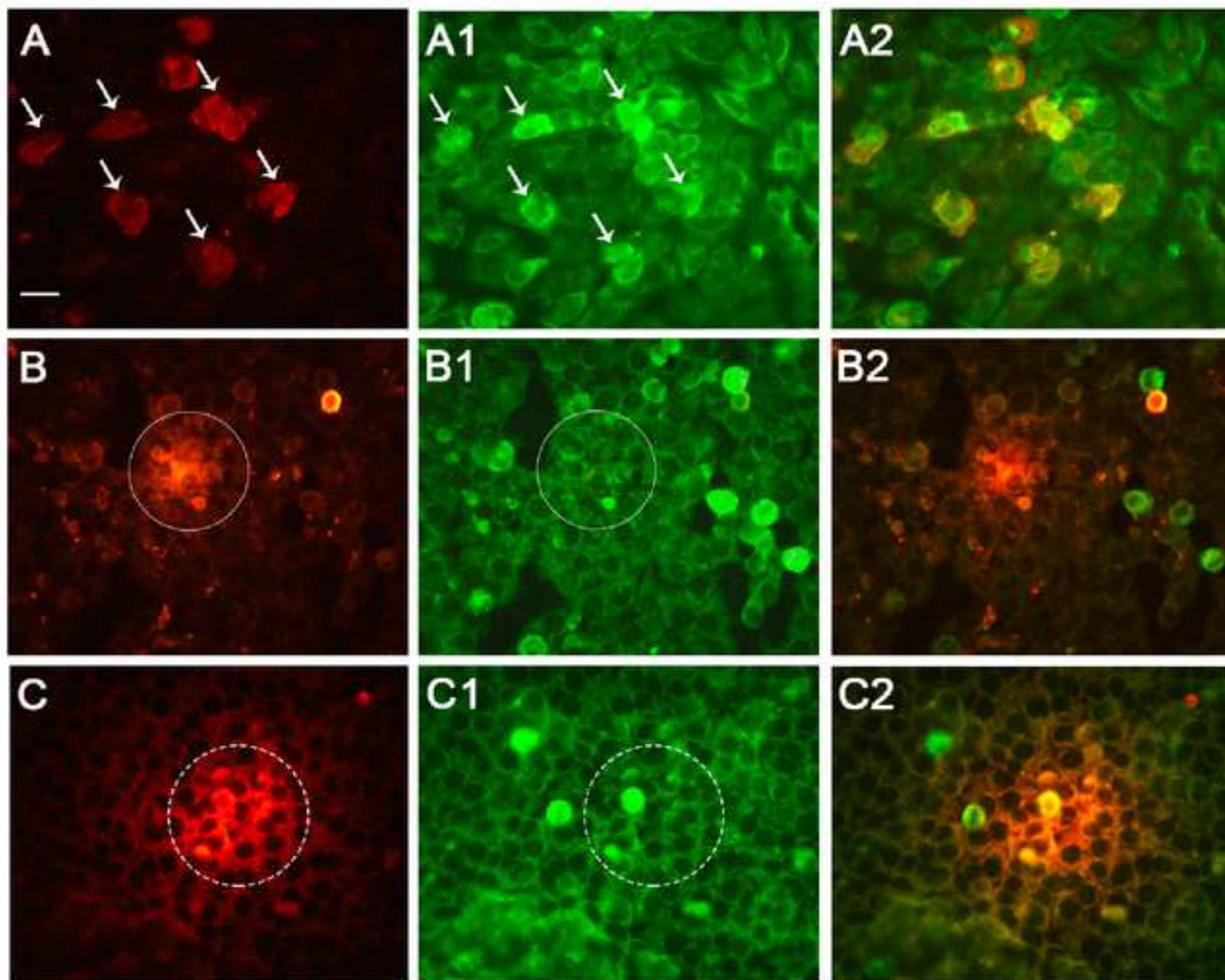


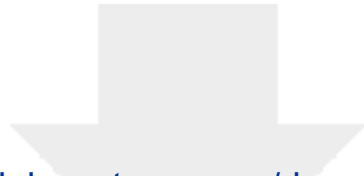
FIG.6

**FIG.7**

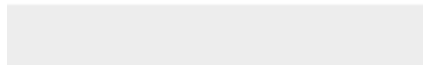
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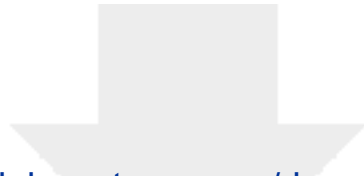
**Flora De Conto**: conceptualization, validation, investigation, funding acquisition, writing original draft, writing review and editing, resources; **Francesca Conversano**: investigation, writing original draft, visualization; **Sergey V. Razin**: investigation, writing review and editing; **Silvana Belletti**: investigation, visualization; **Maria Cristina Arcangeletti**: investigation, data curation; **Carlo Chezzi**: writing review and editing, supervision, validation; **Adriana Calderaro**: writing review and editing, supervision, validation.



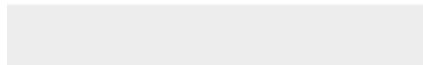
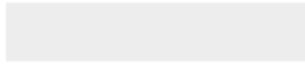


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**Supplementary Material**  
SUPPLEMENTARY FIG1.tif





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**Supplementary Material**  
SUPPLEMENTARY FIG2.tif



1 **Host-cell dependent role of phosphorylated keratin 8 during influenza A/NWS/33 virus (H1N1) infection**  
2 **in mammalian cells**

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## 1 **ABSTRACT**

2

3 In this study, we investigated the involvement of keratin 8 during human influenza A/NWS/33 virus (H1N1)  
4 infection in semi-permissive rhesus monkey-kidney (LLC-MK2) and permissive human type II alveolar  
5 epithelial (A549) cells. In A549 cells, keratin 8 showed major expression and phosphorylation levels.  
6 Influenza A/NWS/33 virus was able to subvert keratin 8 structural organization at late stages of infection in  
7 both cell models, promoting keratin 8 phosphorylation in A549 cells at early phases of infection. Accordingly,  
8 partial colocalizations of the viral nucleoprotein with keratin 8 and its phosphorylated form were assessed by  
9 confocal microscopy at early stages of infection in A549 cells. The employment of chemical activators of  
10 phosphorylation resulted in structural changes as well as increased phosphorylation of keratin 8 in both cell  
11 models, favoring the influenza A/NWS/33 virus's replicative efficiency in A549 but not in LLC-MK2 cells.  
12 In A549 and human larynx epidermoid carcinoma (HEp-2) cells inoculated with respiratory secretions from  
13 pediatric patients positive for, respectively, influenza A virus or respiratory syncytial virus, the keratin 8  
14 phosphorylation level had increased only in the case of influenza A virus infection.  
15 The results obtained suggest that in A549 cells the influenza virus is able to induce keratin 8 phosphorylation  
16 thereby enhancing its replicative efficiency.

1 **Keywords:** virus-host interaction; keratin 8; phosphorylation; intermediate filaments; Influenza A virus.

2

3 **Abbreviations:** A549 – human type II alveolar epithelial; ACRYL – acrylamide; BSA – bovine serum

4 albumin; DAPI – 4',6-diamidino-2-phenylindole dihydrochloride; IF – intermediate filament; IIF – indirect

5 immunofluorescence; HEp-2 human larynx epidermoid carcinoma; Ker 8 – keratin 8; LLC-MK2 – rhesus

6 monkey-kidney; MDCK – Madin-Darby canine-kidney; MOI – multiplicity of infection; NP –nucleoprotein;

7 n.s. – not statistically significant; NWS/33 virus – human influenza A/NWS/33 virus; OA – okadaic acid; PBS

8 – phosphate-buffered saline; PFU – plaque forming units; p.i. – post-infection; pKer 8 – phosphorylated keratin

9 8 on serine 432; PKC – protein kinase C; PTM – post-translational modification; RSV – respiratory syncytial

10 virus; TCID<sub>50</sub> – fifty % tissue culture infectious dose; TPA – 12-O-tetradecanoylphorbol-13-acetate; WB –

11 Western blotting.

## 1 **1. Introduction**

2

3 Influenza viruses depend on specific cellular components/functions to perform their replication. A widespread  
4 understanding of the influenza virus-host cell interface could highlight suitable targets for innovative antiviral  
5 drugs, improving the treatment and prevention of influenza.

6 Concerning the involvement of the cell cytoskeleton, we previously demonstrated that stable microfilaments  
7 and microtubules constitute a restriction factor for human influenza A/NWS/33 virus (NWS/33 virus) infection  
8 (Arcangeletti et al., 2008; De Conto et al., 2018, 2015, 2012, 2011). Importantly, although previous studies  
9 mainly described the regulatory role of intermediate filaments (IFs) during virus infection through chemical  
10 perturbation or small interfering RNA (siRNA)-mediated depletion of specific IFs components (Arcangeletti  
11 et al., 1997; Hertel, 2011; Matsuda et al., 2005; Miller and Hertel, 2009; Shoeman et al., 2001; Sripada and  
12 Dayaraj, 2010; Wu and Pantè, 2016), the knowledge about the involvement of the IFs post-translational  
13 modifications remains limited (Stefanovic et al., 2005; Chiou et al., 2012; McIntosh et al., 2010).

14 IFs are formed by members of a very large family of cell-specific proteins sharing both sequence homology  
15 and structural features (Busch et al., 2012). Among IF proteins, keratins are the most abundantly expressed in  
16 epithelial cells (Hyder et al., 2008). IF proteins are regulated by several post-translational modifications  
17 (PTMs), which mainly appear under specific conditions, such as stress-response, cell migration, mitosis, and  
18 apoptosis (Hyder et al., 2008; Omary et al., 2006; Sawant and Leube, 2017; Snider and Omary, 2016). Among  
19 the PTMs, phosphorylation has been found to be involved in the control of the assembly/disassembly of IFs  
20 (Hyder et al., 2008; Izawa and Inagaki, 2006; Omary et al., 2006; Sihag et al., 2007). Specifically, keratin 8  
21 (Ker 8) phosphorylation induces the reorganization of keratin filaments, promoting the migration of epithelial  
22 tumor cells (Busch et al., 2012) and modulating the stretch response of keratin in epithelial cells (Fois et al.,  
23 2013).

24 This study aimed to investigate the role of Ker 8 and its phosphorylated state during NWS/33 virus infection  
25 in LLC-MK2 and A549 mammalian cells, showing differing levels of permissiveness. To this aim, experiments  
26 were carried out by using chemical treatments able to modulate the structural organization and phosphorylation  
27 level of Ker 8.

1 Here we describe that Ker 8 phosphorylation modulates virus infection in a virus- and cell-dependent way.  
2 More specifically, Ker 8 phosphorylation augmented in A549 but not in LLC-MK2 cells during influenza A  
3 virus infection, favoring the virus replication cycle. Conversely, the parallel analysis of respiratory syncytial  
4 virus (RSV) infection in HEp-2 cells assessed unchanged levels of Ker 8 phosphorylation.

## 1 **2. Materials and methods**

2

### 3 *2.1. Cell lines*

4 A549 (TCL 101), LLC-MK2 (BS CL 57), HEp-2 (BS TCL 23), and Madin-Darby canine-kidney (MDCK, BS  
5 CL 64) cells were from the Lombardy and Emilia Romagna Experimental Zootechnic Institute (*IZSLER*)  
6 (Brescia, Italy). Cells were cultured in Ham's F-12 Nutrient Mixture (A549) and Earle's Modified Eagle's  
7 Medium (LLC-MK2, HEp-2, and MDCK), containing 2 mM L-glutamine, 10% fetal bovine serum and  
8 antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were kept at 37°C in a humidified  
9 atmosphere with 5% CO<sub>2</sub>. Culture reagents were from EuroClone.

10

### 11 *2.2. Viral infection*

12 Human influenza A/NWS/33 virus (H1N1; ATCC VR 219) was propagated as previously detailed  
13 (Arcangeletti et al., 2008). LLC-MK2 and A549 cells grown to confluence in shell-vials or 6-well plates were  
14 infected at a multiplicity of infection (MOI) of 0.1, 2, and 10 plaque-forming units (PFU)/cell to either better  
15 evidence the host cell-dependent susceptibility to virus infection or better visualize the virus staining by  
16 confocal microscopy analyses. After adsorption for 75 min at 4°C, the viral inoculum was removed, and cells  
17 were washed twice with serum-free Earle's Modified Eagle's Medium, before incubation for the time indicated.

18

### 19 *2.3. Infection of A549 and HEp-2 cells with respiratory samples from patients with acute respiratory disease*

20 A total of 10 nasal and throat swabs from pediatric patients with acute respiratory disease observed at the  
21 University Hospital of Parma (Northern Italy) were stored at 4°C in viral transport medium until submitted to  
22 the Unit of Virology. Laboratory diagnosis of respiratory infections was performed upon medical request. The  
23 patients' identity and medical information were rigorously protected and remained anonymous throughout the  
24 study. Upon rapid culture method carried out as previously described (De Conto et al., 2019), the samples found  
25 positive for either influenza A virus (5 samples) or RSV (5 samples), were, respectively, inoculated in A549 and  
26 HEp-2 cell monolayers. At 24 or 48 h post-infection (p.i.), IIF analysis of phosphorylated Ker 8 on serine 432  
27 (pKer 8) together with influenza A virus nucleoprotein (NP) and RSV fusion protein was carried out.

28

#### 1 2.4. *Antibodies*

2 For IIF assays, mouse monoclonal anti-influenza A virus NP (1:30; Argene/BioMérieux) and anti-RSV fusion  
3 protein (1:30; Argene/BioMérieux) antibodies were used. Additionally, rabbit polyclonal anti-Ker 8 (1:100;  
4 Thermo Fisher Scientific) and anti-pKer 8 (1:100; Thermo Fisher Scientific) antibodies were employed. Bound  
5 antibodies were detected by Alexa Fluor 568 goat anti-mouse IgG (1:470; Molecular Probes) and fluorescein  
6 isothiocyanate-conjugated donkey anti-rabbit IgG (1:70; Li StarFish) antibodies. Lastly, 4',6-diamidino-2-  
7 phenylindole dihydrochloride (DAPI; 2.5 µg/ml; Sigma-Aldrich) was employed during the IIF assays to detect  
8 nuclear chromatin.

9 For Western blotting (WB) assays, mouse monoclonal anti-beta-actin IgG (1:400; Santa Cruz Biotechnology),  
10 rabbit polyclonal anti-Ker 8 (1:3,000; Thermo Fisher Scientific), and anti-pKer 8 (1:500; Thermo Fisher  
11 Scientific) antibodies were employed. Bound antibodies were detected by anti-mouse (1:5,000; Sigma-  
12 Aldrich) and anti-rabbit (1:5,000; Sigma-Aldrich) IgG alkaline phosphatase-conjugated antibodies.

13

#### 14 2.5. *Drug treatment*

15 Cells grown in shell-vials or 6-well plates were treated with different chemical modulators of IFs.  
16 Depolymerization of the IFs was obtained using acrylamide (ACRYL; 5 mM) treatment. To induce Ker 8  
17 phosphorylation, the cells were treated with either phosphatase inhibitor okadaic acid (OA; 0.05 µg/ml) or  
18 protein kinase C (PKC) activator 12-O-tetradecanoylphorbol-13-acetate (TPA; LLC-MK2: 25 nM; A549: 50  
19 nM). ACRYL was from Bio-Rad, while OA and TPA were from Sigma-Aldrich.

20

#### 21 2.6. *Indirect immunofluorescence assay*

22 After performing the experimental infection and/or drug treatments, LLC-MK2 and A549 cell monolayers  
23 were fixed and permeabilized in methanol for 5 min at -20°C. The cells were then washed with phosphate-  
24 buffered saline (PBS, pH 7.4; 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl), blocked with  
25 1% bovine serum albumin (BSA; PAA Laboratories GmbH) in PBS, and incubated with primary antibodies  
26 diluted in 0.2% BSA in PBS for 1 h at 37°C. After washes with PBS, the cells were incubated with the  
27 secondary antibodies diluted in 0.2% BSA in PBS for 45 min at 37°C. For negative controls, the primary  
28 antibodies were replaced by 0.2% BSA in PBS. The cells were mounted in buffered glycerol solution

1 (Argene/BioMérieux) and analyzed through an epifluorescence microscope (DMLB Leica). For each cell  
2 monolayer, ten randomly selected microscopic fields were acquired using a DC 300F camera (Leica) and then  
3 the most representative images were selected. For viral NP fluorescence quantization, ten randomly selected  
4 fields per cell monolayer were analyzed, and viral NP-positive cells expressed as mean percentage values of  
5 the total cell number per field, estimated by chromatin staining with DAPI. IIF assays were performed twice  
6 in two replicate shell-vials for each experimental condition.

7

### 8 *2.7. Fifty percent tissue culture infectious dose (TCID<sub>50</sub>) assay*

9 Viral yields from culture supernatants were assessed in MDCK cells using a TCID<sub>50</sub> assay, as previously  
10 described (De Conto et al., 2012).

11

### 12 *2.8. Western blotting assay*

13 Cell lysates were collected as previously described (Arcangeletti et al., 2008). Briefly, aliquots containing 30  
14 µg of proteins were loaded onto sodium dodecyl sulphate-13% polyacrylamide gel electrophoresis. The  
15 resolved proteins were transferred onto nitrocellulose blotting membranes (Bio-Rad). Subsequently, WB  
16 assays were performed according to the experimental protocol previously described (De Conto et al., 2018)  
17 and carried out twice with cell lysates collected from two replicate wells for each experimental condition.

18 For the assay of phosphorylation specificity, the nitrocellulose blotting membrane was first incubated for 30  
19 min in phosphatase buffer (0.2 mM EDTA, 0.1 M TrisHCl, pH = 8.5) in the presence or absence of alkaline  
20 phosphatase from calf intestine (20 units/ml, ThermoFisher Scientific), according to Maya and Oren (2000).  
21 The membrane was then rinsed twice in distilled water, before the WB assay.

22

### 23 *2.9. Confocal microscopy*

24 Confocal microscopy was carried out as previously described (De Conto et al., 2018), using a Zeiss LSM 510  
25 Meta inverted confocal microscope with a 63 x 1.4 NA oil immersion objective. For analysis of colocalization,  
26 a scatter diagram of the number of red pixels against the number of green pixels was generated from each  
27 merged image. Negative controls were examined in parallel to assess the specificity of the signals and absence  
28 of any background. No mutual cross talking of the red and green signals was detectable. The weighted

1 colocalization coefficients represent the sum of intensity of colocalizing pixels in red and green channels, as  
2 compared to the overall sum of pixel intensity above threshold. The weighted coefficient could be 0 (no  
3 colocalization) or 1 (all pixels colocalized). Bright pixels contributed more signal than faint pixels. Profile  
4 display mode was used for the intensity profile graphs.

5

#### 6 *2.10. Trypan blue exclusion assay*

7 To determine the cell vitality, the trypan blue exclusion assay was carried out accordingly to Strober (2015).  
8 Briefly, the cells grown in 6-well plates were first subjected to different chemical treatments and then  
9 trypsinized. The cells were collected in PBS, before centrifugation at 2,500 x *g* for 5 min. The cell pellet was  
10 resuspended in 0.5 ml of PBS, before addition of 0.5 ml of 0.4% trypan blue in PBS (1:1 dilution). The mixture  
11 was incubated for 3 min at room temperature. The viable cells were counted within 5 minutes with a Burker  
12 counting chamber through the exclusion of trypan blue.

13

#### 14 *2.11. Quantitative image analysis*

15 Cells images were analyzed using the CellProfiler software (version 3.1.5). Boundary of 10 cells per  
16 experiment were manually identified and nuclei region was excluded. To analyze distribution of intensity of  
17 immunostaining of proteins, each cell was divided onto 8 equal size shells. For each shell, the fraction of total  
18 staining (FractAtD) was calculated. To analyze the overall intensity of immunostaining an Integrated Intensity  
19 parameter was calculated for each cell.

20 Western blot images were analyzed by using the Fiji software (version 2.0.0-rc69/1.52p).

21

#### 22 *2.12. Statistical analysis*

23 Statistical analysis was performed using GraphPad Prism software. A two-tailed Student's t-test was used to  
24 analyze differences between untreated and drug-treated as well as uninfected and infected LLC-MK2 and A549  
25 cells. *P* values < 0.05 were considered statistically significant.

26

### 1 3. RESULTS

2

#### 3 3.1. Depolymerization of IFs modulates influenza A/NWS/33 virus replication in LLC-MK2 and A549 cells

4 In the first set of experiments, we analyzed the effects of IF depolymerization on influenza virus infection in  
5 LLC-MK2 and A549 cells. Preliminary experiments (data not shown) were carried out to assess the  
6 concentration and the minimum treatment-time with ACRYL (*i.e.* 4 h) able to induce well discernible IFs  
7 depolymerization. Then the ACRYL treatment was maintained twice as long (*i.e.* 8 h), verifying that no further  
8 morphological changes of IFs as well as toxic effects occurred (Supplementary Table 1).

9 The cells either left untreated or treated with ACRYL for 8 h, were subjected to IIF analysis to detect Ker 8  
10 (Figs. 1A-D). The results in untreated cells showed the radial distribution of Ker 8 filaments from the  
11 perinuclear region to the cell periphery, with a more pronounced network of filaments in A549 cells (Fig. 1B  
12 vs. 1A). Treatment with ACRYL stimulated the depolymerization of Ker 8 in both models, with more overt  
13 outcomes in LLC-MK2 cells (Fig. 1C vs. 1D).

14 Then, cells which were either left untreated or pre-treated for 4 h with ACRYL to induce IFs depolymerization  
15 were infected with NWS/33 virus (MOI = 0.1 PFU/cell, 24 h) in the absence or presence of the drug for 4 h  
16 p.i. for a total 8 h treatment-time (Figs. 1E and 1G-L). The cells were then subjected to double IIF staining to  
17 detect viral NP in comparison with Ker 8 (Figs. 1E, and 1G-L). The infection rate was monitored by evaluating  
18 viral NP-positive cells (Fig. 1E). The results showed that the depolymerization of the IFs had caused an  
19 increase in the portion of viral NP-positive cells in both cell models (Fig. 1E; Fig. 1I vs. 1G, and Fig. 1L vs.  
20 1H).

21 Next, we checked using a TCID<sub>50</sub> assay whether the above-mentioned drug treatment had interfered with  
22 emergence of the viral progeny (Fig. 1F). Surprisingly, the titers of infectious viruses assessed in culture  
23 supernatants of LLC-MK2 and A549 cells treated with ACRYL at 24 h p.i., as described above, were similar  
24 to those of supernatants from untreated cells. Therefore, additional experiments were carried out in order to  
25 determine if longer times of virus infection (*i.e.* 48 and 72 h p.i.) at the above-described experimental  
26 conditions would have allowed the emergence of greater viral yields. The results assessed almost univariate  
27 viral yields over several infectious cycles (Fig. 1F).

28

### 1 3.2. Chemical activation of Ker 8 phosphorylation favors influenza A/NWS/33 virus infection in A549 cells

2 Considering the fact that depolymerization of Ker 8 may well be stimulated by Ker 8 phosphorylation (Moch  
3 et al., 2013; Sivaramakrishnan et al., 2009), we next focused on the consequences of chemical modulation of  
4 Ker 8 phosphorylation. To this aim, we used both the phosphatase inhibitor OA and the protein kinase activator  
5 TPA, showing a correlation with Ker 8 phosphorylation (Cadrin et al., 1992; Lee et al., 2014; Tao et al., 2016).  
6 Preliminary experiments (data not shown) evidenced that Ker 8 phosphorylation is induced by a minimum  
7 treatment-time with, respectively, OA for 2 h and TPA for 40 min at the concentrations reported in the Methods  
8 section. By temporally doubling these treatment times, no further modifications of Ker 8 nor cytotoxic effects  
9 were observed (Supplementary Table 1).

10 To this end, LLC-MK2 and A549 cells were either left untreated or treated with OA (for 4 h) or TPA (for 1 h  
11 and 20 min), before IIF assays to detect both Ker 8 and pKer8 (Figs. 2A-L).

12 The treatment with OA resulted in the clustering of pKer 8 filaments in the perinuclear area and cell rounding  
13 in both models (Figs. 2G, 2H, and 2N). This effect was rather moderate in LLC-MK2 cells but very significant  
14 in A549 cells, where the OA had induced the collapse of the pKer 8 network into dense granules, as evidenced  
15 by their strong fluorescence (see inset in Fig. 2H).

16 The treatment with TPA had caused the perinuclear ramming of pKer 8 (Figs. 2K and 2L), and raised the Ker  
17 8 phosphorylation, especially in A549 cells (Fig. 2L, and 2O). In particular, the quantitative analysis (Fig. 2M)  
18 of the immunofluorescence signal associated to pKer8 in LLC-MK2 and A549 cells (Fig. 2C vs. 2K and Fig.  
19 2D vs. 2L) well evidenced its redistribution toward the cell nuclei upon TPA treatment.

20 The parallel analysis of Ker 8 expression and localization after the above-mentioned treatments in both cell  
21 models (Figs. 2E, 2F, 2I, and 2J) pointed up effects that involve the entire network of Ker 8 filaments and not  
22 only its phosphorylated component.

23 The quantitative analyses of the immunofluorescence signal associated to Ker 8 and pKer 8 shown in the  
24 Supplementary Fig. 1 were in general consistent with the findings above reported.

25 In parallel, the specificity of the anti-pKer 8 antibodies was evaluated by immunoblotting assays  
26 (Supplementary Fig. 2). To this aim, the nitrocellulose membranes were either untreated or pretreated with  
27 alkaline phosphatase before incubation with anti-pKer 8 antibodies, as detailed in the Methods section. The  
28 results demonstrate the specificity of the above-mentioned antibodies, because the treatment with alkaline

1 phosphatase almost completely eliminated their immunoreactivity (Supplementary Fig. 2 AP pretreatment vs.  
2 untreated control).

3 Having observed that the drug treatments had affected pKer 8 distribution in different ways in the models  
4 studied, we were interested to discover whether and how this influences virus infection. To this end, LLC-  
5 MK2 and A549 cells were either left untreated or pre-treated with OA (for 2 h) and TPA (for 40 min) to induce  
6 IFs modifications, and then subjected to NWS/33 virus infection (MOI = 0.1 PFU/cell, 24 h) in the absence or  
7 presence of OA for 2 h p.i. and TPA for 40 min p.i. for a total treatment-time of 4 h with OA and 1 h 20 min  
8 with TPA, before IIF staining of viral NP. The results (Fig. 2P) showed that the portion of viral NP-positive  
9 cells had diminished to some extent in LLC-MK2 cells treated with OA, while in A549 cells it had slightly  
10 increased. In contrast, treatment with TPA had decreased the portion of viral NP-positive LLC-MK2 cells and  
11 increased that of A549 cells (Fig. 2P).

12 We next checked, using TCID<sub>50</sub> assays, whether the above-mentioned treatments had influenced the emergence  
13 of the viral progeny. Figure 2Q shows that the yield of the infectious virus was in general consistent with the  
14 results of the IIF assays.

15 We then analyzed by WB how the levels of Ker 8 and pKer 8 had been affected by treatments with the above-  
16 described drugs and virus infection (Figs. 2R-U). In LLC-MK2 cells, the treatments with OA (Fig. 2R) and  
17 TPA (Fig. 2T) slightly affected Ker 8 levels compared to the untreated cells. NWS/33 virus infection (MOI =  
18 2 PFU/cell, 24 h) carried out in the absence or presence of OA had slightly decreased the levels of Ker 8 (Figs.  
19 2R), which remained almost univariate in the absence or presence of TPA (Fig. 2T).

20 In A549 cells, the exposure to both OA and TPA had not significantly changed Ker 8 expression compared to  
21 the untreated cells (Figs. 2S and 2U). NWS/33 virus infection carried out alone or in the presence of OA and  
22 TPA had slightly increased the Ker 8 level (Figs. 2S and 2U).

23 pKer 8 was scarcely expressed in both models (Figs. 2R-U). In LLC-MK2 cells, no relevant changes in pKer  
24 8 levels were seen as a result of either the drug treatments or viral infection (Fig. 2R and 2T). Conversely, in  
25 A549 cells, the levels of pKer 8 had increased after drug treatments and viral infection with more relevant  
26 effects in case of TPA treatment (Figs. 2S and 2U).

27

28

### 1 3.3. *Influenza A/NWS/33 virus infection differentially modifies Ker 8 network in LLC-MK2 and A549 cells*

2 We then analyzed the expression and phosphorylation state of Ker 8 during NWS/33 virus infection. To this  
3 end, LLC-MK2 and A549 cells were either uninfected or infected with NWS/33 virus (MOI = 2 PFU/cell,  
4 from 2 to 24 h of infection), before IIF staining carried out at different time intervals of virus infection to detect  
5 both Ker 8 and pKer 8 (Fig. 3, and Fig. 4).

6 At late times of NWS/33 virus infection, we observed changes in Ker 8 distribution and expression as a likely  
7 consequence of the cytopathic effect. More specifically, at 24 h p.i. we observed the collapse of the Ker 8  
8 network around the nucleus of a large number of cells (Arrows in Figs. 3D and 3H).

9 In LLC-MK2 cells, the level of pKer 8 had remained almost unchanged throughout the 24 h of infection (Figs.  
10 3J, 3K, 3L vs. 3I, and Fig. 4D vs. 4C), while in A549 cells an increase in pKer 8 level starting from 2 h p.i.  
11 (Fig. 4H vs. 4G) until 24 h p.i. was evident (Figs. 3N, 3O, and 3P vs. 3M). In parallel, NWS/33 virus infection  
12 was monitored by IIF staining of viral NP in LLC-MK2 (Figs. 3R, 3S, and 3T) and A549 (Figs. 3V, 3W, and  
13 3X) cells. At 4 h p.i. an initial viral NP nuclear accumulation was observable in infected-LLC-MK2 cells with  
14 a mean of  $46.8 \pm 2.7\%$  of the cell monolayer (Fig. 3R), while in the most part of infected-A549 cells ( $53.3 \pm$   
15  $6.2\%$ ) viral NP was present in both the cytoplasm and nucleus (Fig. 3V). The portion of viral NP present in the  
16 cytoplasm progressively increased from 8 (LLC-MK2:  $63.2 \pm 2.5\%$ ; A549:  $78.5 \pm 4.4\%$ ) to 24 h p.i. (LLC-  
17 MK2:  $92 \pm 2.7\%$ ; A549:  $96.4 \pm 3.7\%$ ) in both models, involving almost all of the cell monolayers. The  
18 intracellular distribution of viral NP in relation to either Ker 8 or pKer 8 at 24 h p.i. was assessed in the studied  
19 models by double immunostaining experiments (Fig. 4I-T).

20

### 21 3.4. *Viral NP partially colocalizes with Ker 8 and pKer 8 during the early stages of influenza A/NWS/33 virus* 22 *infection in A549 cells*

23 In order to analyze the subcellular distribution of Ker 8 and pKer 8 in comparison with viral NP, LLC-MK2  
24 and A549 cells were infected for, respectively, 1 h and 30 min and 1 h with NWS/33 virus (MOI = 10 PFU/cell),  
25 followed by confocal microscopy analysis (Figs. 5 and 6). The subcellular distribution of Ker 8 and viral NP  
26 evidenced a partial colocalization in A549, while in LLC-MK2 cells very weak signals of colocalization were  
27 detected (Figs. 5B2 and 5B3 vs. Figs. 5A2 and 5A3). Controls were in parallel carried out in uninfected A549  
28 and LLC-MK2 cells. To this aim, the cells were immunostained for the detection of Ker 8 (5C and 5D) or pKer

1 8 (6C and 6D) in association with viral NP (5C1, 5D1, 6C1, and 6D1) in order to assess the absence of  
2 background signals in the red channel.

3 Moreover, faint signals of colocalization of pKer 8 with viral NP were evidenced in A549, but not in LLC-  
4 MK2 cells (Figs. 6B2 and 6B3 vs. Figs. 6A2 and 6A3).

5

### 6 *3.5. Influenza A virus but not RSV infection enhances Ker 8 phosphorylation in human epithelial cells*

7 In order to deepen the results previously described, we next investigated the phosphorylation level of Ker 8 in  
8 human epithelial cells inoculated with samples from the upper respiratory tract of pediatric patients with acute  
9 respiratory disease found positive for either influenza A virus or RSV, as described in the Methods section  
10 (Fig. 7). Upon 24 (Fig. 7A-B2) or 48 h p.i. (Fig. 7C-C2), A549 (Fig. 7A-A2) and HEp-2 (Fig. 7 B-C2) cell  
11 monolayers were subjected to IIF assays in order to detect pKer 8 (Fig. 7A1, B1, and C1) in comparison with  
12 influenza A virus NP (Fig. 7A) or RSV fusion protein (Fig. 7B, and C).

13 The results showed an increased pKer 8 level in influenza A virus-positive cells (pointed arrows in Fig. 7A1).  
14 Conversely, pKer 8 had remained almost unchanged in RSV-positive cells, also at late times of infection (circle  
15 in Fig. 7B1, and dashed circle in Fig. 7C1).

## 1 4. DISCUSSION

2

3 Influenza is one of the main public health problems worldwide (Belser et al., 2011; Ryan et al., 2018).  
4 Productive infection strictly depends on the influenza virus's ability to co-opt the biosynthetic apparatus of the  
5 host cell. We ourselves have previously assessed various cytoskeletal restriction factors against NWS/33 virus  
6 infection (Arcangeletti et al., 2008; De Conto et al., 2018, 2015, 2012, 2011).

7 The objective of this study was to evaluate the involvement of Ker 8 during NWS/33 virus infection in LLC-  
8 MK2 (semi-permissive) and A549 (permissive) mammalian cells in order to ascertain if this cytoskeletal  
9 component represents a restriction factor. To this end, we analyzed Ker 8 phosphorylation, which has been  
10 associated with structural reorganization of IFs (Lee et al., 2014) and keratin's ability to polymerize (Liao and  
11 Omary, 1996; Omary et al., 1998; Ridge et al., 2005; Strnad et al., 2001; Strnad et al., 2002).

12 Depolymerization of IFs with acrylamide (Eckert, 1985) showed more evident outcomes in LLC-MK2 than in  
13 A549 cells, where Ker 8 and its phosphorylated form showed a higher level and specific distribution. IF  
14 depolymerization during the early stages of NWS/33 virus infection had augmented the portion of viral NP,  
15 suggesting that a dynamic arrangement of IFs may favor the viral replication cycle. However, it had not resulted  
16 in a consistent increase in the titers of the viral progeny, as assessed also at very late times of infection. In  
17 agreement with previous observations (Bhattacharya et al., 2007; Kanlaya et al., 2010), it is likely that IF  
18 depolymerization fosters the biosynthetic activities required for viral replication, affecting the late phases of  
19 the infection. Nonetheless, the time frame(s) and mechanism(s) required to completely restore the IF  
20 physiological structure after acrylamide removal remain not fully understood (Arcangeletti et al., 1997). This  
21 aspect could account for the fact that partially depolymerized IFs linger on in the late stages of the infection,  
22 affecting the emergence of viral progeny. Additionally, we showed that in LLC-MK2 cells infected with avian  
23 influenza A/FPV/Ulster 73 (H7N1) virus, the IF depolymerization hinders the release of the viral progeny  
24 (Arcangeletti et al., 1997). Similarly, the occurrence of IF modifications during infection with Argentinian  
25 Mammarenavirus (Cordo and Candurra, 2003), Foot-and-mouth disease virus (Gladue et al., 2013), Dengue  
26 virus (Kanlaya et al., 2010), and Cytomegalovirus (Miller and Hertel, 2009) lead to a decreased viral  
27 replication efficiency.

1 NWS/33 virus had been able to induce the onset of Ker 8 structural modifications at late stages of infection.  
2 Importantly, in A549 cells, the Ker 8 dynamism had been enhanced at early stages of infection through  
3 phosphorylation (Fig. 3). Therefore, it may be assumed that in LLC-MK2 cells there are specific regulatory  
4 factors that can counteract virus-induced phosphorylation. Nonetheless, the Ker 8 phosphorylation level  
5 assessed in LLC-MK2 cells under physiological conditions was lower compared to the A549 cells.  
6 It has been proposed that Ker 8 might be phosphorylated by protein kinases stimulated by mitogens (Omary,  
7 2009; Omary et al., 2006). Influenza virus activates protein kinases to promote both the transport of viral  
8 ribonucleoprotein complexes and the release of viral progeny (Kujime et al., 2000; Pleschka et al., 2001).  
9 Specifically, NWS/33 virus triggers the protein kinase levels at early stages of infection (Arora and Gasse,  
10 1998; Kunzelmann et al., 2000), easing its entry into the host cell (Root et al., 2000). Notably, protein kinase  
11 activation favors virus entry through endocytosis (Constantinescu et al., 1991; Toker, 1998). We ourselves had  
12 reported previously that NWS/33 virus entry into LLC-MK2 cells occurs mainly by macropinocytosis (De  
13 Conto et al., 2011). Therefore, increased phosphorylation levels may be relevant for NWS/33 virus entry in  
14 A549, but not in LLC-MK2 cells.  
15 Importantly, high phosphorylation levels have been correlated with IF disassembly (Cadrin et al., 1992) and  
16 increased keratin solubility (Omary et al., 1998). In addition, phosphorylation affects the reorganization of Ker  
17 8 in the perinuclear area (Beil et al., 2003), correlates with disease progression (Toivola et al., 2004; Zatloukal  
18 et al., 2004), and exerts regulatory effects on keratin organization and its interaction with other molecules  
19 (Coulombe and Omary, 2002). Importantly, Rotavirus and Hepatitis B and C virus infections increase Ker 8  
20 phosphorylation (Liao et al., 1995; Shi et al., 2010; Toivola et al., 2004).  
21 The aforesaid evidence accounts for the fact that the IF system is affected by the regulatory activity of several  
22 cellular factors and viruses can contribute to its modulation. Interestingly, human rhinovirus serotype 2 virus  
23 is able to stimulate the cleavage of Ker 8, causing IF changes which facilitate the virus egress (Seipelt et al.,  
24 2000).  
25 In this study we found that OA, an inhibitor of protein phosphatases (Sitprija and Sitprija, 2019), had strongly  
26 affected the IF network in A549 cells in accordance with previous data (Kasahara et al., 1993; Ku et al., 2002;  
27 Strnad et al., 2002; Yatsunami et al., 1993). Contrarily, mild effects were observed in LLC-MK2 cells. These  
28 conflicting results might be related to the presence of different phosphatases levels, which are cell model-

1 dependent (Duchesne et al., 2003; Ingebritsen and Cohen, 1983). Moreover, treatment with TPA, a PKC  
2 activator (Castagna et al., 1982; Chen et al., 2013), had induced a stronger accumulation of pKer 8 in the  
3 perinuclear region and, in general, an increased level of pKer 8, especially in A549 cells, as reported by other  
4 Authors (Cadrin et al., 1992; Lee et al., 2014). Of note, the above-mentioned drug treatments had augmented  
5 the replicative efficiency of NWS/33 virus in A549 cells.

6 Although both OA and TPA were able to modulate the phosphorylated state of Ker 8, especially in A549 cells,  
7 cautious conclusions should be drawn, since such drugs could also have effects on other cellular proteins.  
8 Further studies are needed in order to better dissect the multifaceted relationship between the influenza virus  
9 and cellular phosphorylation pathways.

10 The obtained findings explain the complexity of the mechanisms regulating keratin phosphorylation, which  
11 involve several factors, such as epidermal growth factor, phosphatase inhibitors and B4 leukotriene (Kasahara  
12 et al., 1993; Ku and Omary, 1997; Sivaramakrishnan et al., 2009), and show a differential expression depending  
13 on the cellular model and its condition.

14 In A549 cells, NWS/33 virus infection had increased the pKer 8 level. Other Authors have shown increased  
15 K10 levels in A549 cells infected with avian influenza A (H9N2) virus (Yu et al., 2016), while conflicting data  
16 have revealed a reduction in K14 upon infection with papillomavirus (Bowden et al., 1992). These observations  
17 highlight the uniqueness of the interaction occurring between different viruses and specific IF components.

18 Lastly, the increased Ker 8 phosphorylation observed in human epithelial cells inoculated with respiratory  
19 samples from paediatric patients positive for influenza A virus, but not for RSV, supports the supposition that  
20 this is a cell- and virus-dependent mechanism.

21 In conclusion, this study has developed new knowledge on the role of IFs during influenza virus infection,  
22 showing that in permissive A549 cells, but not in semi-permissive LLC-MK2 cells, NWS/33 virus is able to  
23 activate Ker 8 phosphorylation, which enhances its replicative efficiency. In this regard, it has to be considered  
24 that in LLC-MK2 cells Ker 8 is poorly expressed and organized, and, therefore, does not represent a  
25 cytoskeletal restriction factor of an extent comparable to those previously detected (De Conto et al., 2018,  
26 2015, 2012).

27 Overall, the use of two cell models having different permissivity for NWS/33 virus infection allowed to  
28 highlight the uniqueness of the interaction occurring between influenza virus and Ker 8 and showed how

- 1 specific cellular factors are crucial in modulating the virus's replicative efficiency. The nature of these factors
- 2 and the high complex mechanisms that regulate their functions calls for further investigation.
- 3

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6

## 7 **Compliance with Ethical Standards**

8 This article does not include any studies with human participants or animals performed by any of the authors.  
9 Laboratory diagnosis for respiratory infections was performed according to the medical order; consequently,  
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13

## 14 **Conflict of interest**

15 The authors declare that they have no conflict of interest.

16

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## 1 **Figure legends**

2

3 **Fig. 1. IF depolymerization during A/NWS/33 virus infection in LLC-MK2 and A549 cells enhances the**  
4 **portion of viral NP but not the viral yields.** LLC-MK2 (A, C) and A549 (B, D) cells were either left untreated  
5 (A, B) or treated with ACRYL (5 mM) (C, D) for 8 h. The cells were then subjected to IIF assays to detect Ker  
6 8 (A-D). Images were collected with a conventional fluorescence microscope (magnification x500). (E) The  
7 graph shows the percentage of viral NP-positive LLC-MK2 and A549 cells evaluated by IIF in untreated cells  
8 (NT) and in cells pre-treated with ACRYL (5 mM) for 4 h, before inoculation with A/NWS/33 virus (MOI =  
9 0.1 PFU/cell, 24 h) in the absence (NT) or presence (ACRYL) of the drug for 4 h p.i. The cells were then  
10 immunostained with anti-NP antibodies and the number of positive cells in relation to the total cell population  
11 was expressed as a percentage. (F) Viral yields were evaluated by TCID<sub>50</sub> assays in MDCK cells from  
12 supernatants of LLC-MK2 and A549 cells at, respectively, 24, 48, and 72 h p.i. Values represent the mean of  
13 two independent experiments. Error bars in graphs correspond to standard deviations. \*  $P < 0.05$ ; not  
14 statistically significant (n.s.)  $P > 0.05$ . (G-L) Merged images showing the overlap (orange/yellow) between  
15 viral NP (red) and Ker 8 (green) in LLC-MK2 and A549 cells evaluated by IIF. (G, H) Untreated cells and (I,  
16 L) cells pre-treated with ACRYL (5 mM) for 4 h, before inoculation with A/NWS/33 virus (MOI = 0.1  
17 PFU/cell, 24 h) in the absence (G, H) or presence (I, L) of the drug for 4 h p.i. Scale bar = 20  $\mu\text{m}$ .

18

19 **Fig. 2. Ker 8 phosphorylation activation by chemical treatments enhances A/NWS/33 virus infection**  
20 **efficiency in A549 cells.** LLC-MK2 (A, C, E, G, I, K) and A549 (B, D, F, H, J, L) cells were untreated (A-D)  
21 or treated with either OA (0.05  $\mu\text{g}/\text{ml}$ ) (E- H) for 4 h or TPA (LLC-MK2: 25 nM; A549: 50 nM) (I- L) for 1 h  
22 and 20 min. The cells were then subjected to IIF assays to detect Ker 8 (A, B, E, F, I, J) and pKer 8 (C, D, G,  
23 H, K, L). Images were collected using a conventional fluorescence microscope (magnification x500). Scale bar  
24 = 20  $\mu\text{m}$ . (F, H) The insets show a higher magnification (x2000) of the Ker 8 and pKer 8 patterns of the cell  
25 included within the dotted area. (M, O) Quantitative analysis with the CellProfiler software of the  
26 immunofluorescence signal of pKer 8 of the images related to the TPA treatment in A549 and LLC-MK2 cells.  
27 Boundary of 10 cells per experiment were manually identified and the nuclei region was excluded. (M) To  
28 analyze the distribution of the intensity of the immunostaining of pKer 8, each cell was divided onto 8 equal

1 size shells. For each shell, the fraction of total staining (FractAtD) was calculated. To analyze the overall  
2 intensity of immunostaining an Integrated Intensity parameter was calculated for each cell. (N) Bar histogram  
3 related to the analysis of cell shape upon OA treatment in LLC-MK2 and A549 cells. (P) The graph shows the  
4 percentage of viral NP-positive LLC-MK2 and A549 cells evaluated by IIF in untreated cells (NT) or in cells  
5 pre-treated with OA for 2 h and TPA for 40 min, before inoculation with A/NWS/33 virus (MOI = 0.1 PFU/cell,  
6 24 h) in the absence (NT) or presence of OA for 2 h p.i. and TPA for 40 min p.i. The cells were then  
7 immunostained with anti-NP antibodies and the number of positive cells in relation to the total cell population  
8 was expressed as a percentage. (Q) Viral yields were evaluated by TCID<sub>50</sub> assays in MDCK cells from  
9 supernatants of LLC-MK2 and A549 cells. Values represent the mean of two independent experiments. Error  
10 bars in graphs correspond to standard deviations. \*  $P < 0.05$ ; n.s.  $P > 0.05$ . (R-U) The level of Ker 8 and pKer  
11 8 was evaluated by WB assays in uninfected (NO INF) LLC-MK2 (R, T) and A549 (S, U) cells either left  
12 untreated (cc) or treated with OA (+OA) for 4 h and TPA (+TPA) for 1 h and 20 min. In parallel, Ker 8 and  
13 pKer 8 levels were evaluated in infected (INF) LLC-MK2 (R, T) and A549 (S, U) cells either left untreated  
14 (24 h) or pre-treated with OA (24 h+OA) for 2 h and TPA (24 h+TPA) for 40 min, before inoculation with  
15 A/NWS/33 virus (MOI = 2 PFU/cell, 24 h) in the absence (24 h) or presence of OA (24 h+OA) for 2 h p.i. and  
16 TPA (24 h+TPA) for 40 min p.i. (R-U) The presence of beta-actin was checked in parallel as a protein loading  
17 control. Quantitation of protein band intensity relative to beta-actin control are shown in the bar histograms to  
18 the right of each WB assay.

19

20 **Fig. 3. A/NWS/33 virus modifies Ker 8 organization in LLC-MK2 and A549 cells late in infection, while**  
21 **it enhances Ker 8 phosphorylation in A549 cells at early stages of its replication cycle.** LLC-MK2 and  
22 A549 cells were either uninfected (A, E, I, M, Q, U) or infected with A/NWS/33 virus at an MOI of 2 PFU/cell  
23 for, respectively, 2 h (see Fig. 4), 4 h (B, F, J, N, R, V), 8 h (C, G, K, O, S, W), and 24 h (D, H, L, P, T, X). The  
24 cells were then subjected to IIF assays to detect Ker 8 (A-H), pKer 8 (I-P) and a merge (LLC-MK2: Q-T;  
25 A549: U-X) between viral NP (red) and nuclear chromatin staining with DAPI (blue). Merged images show  
26 the overlap (pink) between viral NP and nuclei. Images were collected with a conventional fluorescence  
27 microscope (magnification x500). Scale bar = 20  $\mu$ m.

28

1 **Fig. 4. Analysis of the effects of A/NWS/33 virus infection on the expression and phosphorylation levels**  
2 **of Ker 8 at 2 and 24 h p.i. in LLC-MK2 and A549 cells.** LLC-MK2 and A549 cells were either uninfected  
3 (A, E, C, G) or infected with A/NWS/33 virus at an MOI of 2 PFU/cell for, respectively, 2 h (B, F, D, H) and  
4 24 h (I-T). The cells were then subjected to IIF assays to detect Ker 8 (A, B, E, F, J, P), pKer 8 (C, D, G, H,  
5 M, S), viral NP (I, L, O, R), and a merge (K, N, Q, T). Merged images show the overlap (orange/yellow)  
6 between viral NP (red) and either Ker 8 (green) or pKer 8 (green). Images were collected with a conventional  
7 fluorescence microscope (magnification x500). Scale bar = 20  $\mu$ m.

8

9 **Fig. 5. At early phases of A/NWS/33 virus infection viral NP partially co-localizes with Ker 8 in A549**  
10 **cells.** LLC-MK2 and A549 cells were infected with A/NWS/33 virus at an MOI of 10 PFU/cell for 1 h and 30  
11 min (A, A1, A2) and 1 h (B, B1, B2), respectively. The cells were then stained with IIF to detect Ker 8 (A, B)  
12 and viral NP (A1, B1), before confocal microscopy analysis. The colocalizing areas (orange/yellow) (A2, B2)  
13 are indicated by superposition of the two signals; no colocalizing areas have the original colors (green, Ker 8;  
14 red, viral NP). (A3, B3) scatter diagrams generated from the merged images A2 and B2, respectively, outlining  
15 the pixel-to-pixel correlation between the red and green channels. The Zeiss LSM 510 meta software was used  
16 to measure the weighted colocalization coefficients (green channel: Ch2-T1; red channel: Ch3-T2). In parallel,  
17 uninfected LLC-MK2 and A549 cells (C-D1) were stained with IIF for the detection of Ker 8 (C, D) and viral  
18 NP (C1, D1) to assess the absence of background signals in the red channel. Scale bar = 10  $\mu$ m.

19

20 **Fig. 6. Co-localization signals between viral NP and pKer 8 are detected at early phases of A/NWS/33**  
21 **virus infection in A549 cells.** LLC-MK2 and A549 cells were infected with A/NWS/33 virus at an MOI of 10  
22 PFU/cell for 1 h and 30 min (A, A1, A2) and 1 h (B, B1, B2), respectively. The cells were then stained with  
23 IIF to detect pKer 8 (A, B) and viral NP (A1, B1), before confocal microscopy analysis. The colocalizing areas  
24 (orange/yellow) (A2, B2) are indicated by superposition of the two signals; no colocalizing areas have the  
25 original colors (green, pKer 8; red, viral NP). (A3, B3) scatter diagrams generated from the merged images A2  
26 and B2, respectively, outlining the pixel-to-pixel correlation between the red and green channels. The Zeiss  
27 LSM 510 meta software was used to measure the weighted colocalization coefficients (green channel: Ch2-  
28 T1; red channel: Ch3-T2). In parallel, uninfected LLC-MK2 and A549 cells (C-D1) were stained with IIF to

1 for the detection of pKer8 (C, D) and viral NP (C1, D1) to assess the absence of background signals in the red  
2 channel. Scale bar = 10  $\mu$ m.

3

4 **Fig. 7. Ker 8 iperphosphorylation is a cell- and virus-dependent mechanism observable for influenza A**  
5 **virus infection in A549 cells but not for RSV infection in HEp-2 cells.** Human respiratory secretions positive  
6 for either influenza A virus or RSV were inoculated in A549 (A-A2) and HEp-2 (B-C2) cell monolayers. At  
7 24 (A-B2) or 48 h p.i. (C-C2) the cells were stained by IIF with anti-pKer 8 (A1, B1, C1), anti-influenza A  
8 virus NP (A) and anti-RSV fusion protein (B, C) antibodies. Images representing the results obtained from 10  
9 samples (5 positive for influenza A virus, and 5 positive for RSV) were collected using a conventional  
10 fluorescence microscope (magnification x500). (A2, B2, C2) Merged images showing the overlap  
11 (orange/yellow) between viral NP (red) and pKer 8 (green) in A549 cells (A2) or between RSV fusion protein  
12 (red) and pKer 8 (green) in HEp-2 cells (B2, C2) evaluated by IIF. Arrows (A, A1) and circles (B, B1, C, C1)  
13 highlight infected cells. Scale bar = 20  $\mu$ m.

14

15 **Supplementary Fig. 1. Quantitative analysis of the immunofluorescence signal associated to Ker 8 and**  
16 **pKer8 upon treatment with the phosphorylation modulators OA and TPA in LLC-MK2 and A549 cells.**

17 (A-F) The IIF images related to Ker 8 and pKer 8 immunostainings in LLC-MK2 and A549 cells shown in  
18 Fig. 2A-L were analyzed with the CellProfiler software. Boundary of 10 cells per experiment were manually  
19 identified and the nuclei region was excluded. (A, C, D, F) To analyze the distribution of the intensity of the  
20 immunostainings, each cell was divided onto 8 equal size shells. For each shell, the fraction of total staining  
21 (FractAtD) was calculated. To analyze the overall intensity of immunostaining an Integrated Intensity  
22 parameter was calculated for each cell. (B, E) Bar histograms showing the analysis of the cell shape.

23

24 **Supplementary Fig. 2. Analysis of phosphorylation specificity by Western blotting using alkaline**  
25 **phosphatase nitrocellulose membrane treatment.** Cell lysates of LLC-MK2 and A549 cells were loaded  
26 onto sodium dodecyl sulphate-13% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose  
27 blotting membranes. The membranes were then incubated for 30 min in phosphatase buffer, in the presence

- 1 (AP pretreatment) or absence (untreated control) of alkaline phosphatase (20 units/ml). Subsequently, WB
- 2 assays were performed for the detection of pker 8 and beta-actin.
- 3

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: