

1 **Rotavirus NSP1 localizes in the nucleus to disrupt PML nuclear bodies during infection**

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3 **Running title:** Rotavirus NSP1 disrupts PML nuclear bodies

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19

20 **ABSTRACT**

21           The rotavirus nonstructural protein 1 (NSP1) antagonizes interferon (IFN) induction in  
22 infected host cells. The primary function of NSP1 is thought to be degradation of interferon  
23 regulatory factors (IRFs) and beta-transducin repeat-containing protein ( $\beta$ -TrCP) in the  
24 cytoplasm to inhibit IFN induction. Here, we report that NSP1 localizes to the cytoplasm and  
25 nucleus and disrupts promyelocytic (PML) nuclear bodies (NB) in the nucleus during infection.  
26 Nuclear localization of NSP1 did not require an intact C terminus, suggesting NSP1 has a novel  
27 function in the nucleus independent of degradation of IRFs or  $\beta$ -TrCP. NSP1 expression either  
28 led to a reduction in PML NB number or a change in PML NB morphology from sphere-shaped  
29 foci to oblong-shaped structures, depending on the virus strain. Additionally, infection was not  
30 affected when cells lack PML NB, suggesting that rotavirus does not require PML for replication  
31 in highly permissive cell types. PML was not essential for nuclear localization of NSP1, but PML  
32 was required for NSP1 nuclear focus formation. PML NBs play an important role in many  
33 cellular functions that include IFN induction and host stress responses. This is the first report  
34 that rotavirus, a cytoplasmically replicating virus, encodes a viral protein that localizes to the  
35 nucleus during infection, and may suggest a new function of NSP1 in the nucleus.

36

37 **IMPORTANCE**

38           Rotavirus causes severe gastroenteritis in young children and leads to over 200,000  
39 deaths per year. Rotavirus is a cytoplasmically replicating virus, and must find ways to avoid or  
40 actively inhibit host antiviral responses to efficiently replicate. The nonstructural protein NSP1 is  
41 known to inhibit IFN induction by promoting degradation of host proteins in the cytoplasm of  
42 infected cells. Here, we demonstrate that NSP1 also localizes to the nucleus of infected cells,  
43 specifically to PML NB. NSP1 causes a disruption of PML NB, which may serve as an additional  
44 mechanism of IFN inhibition or interfere with other nuclear processes to promote viral

45 replication. A detailed exploration of the manipulation of nuclear processes in cells infected with  
46 cytoplasmically replicating viruses will lead to new insights into viral evasion of host responses.

47

## 48 INTRODUCTION

49 Rotaviruses are double-stranded RNA viruses containing a segmented genome  
50 packaged inside a multi-layered, non-enveloped viral particle (1). Most steps of the rotavirus  
51 replication cycle take place in the cytoplasm of infected cells in replication centers known as  
52 viroplasms, and final assembly takes place via a process of budding through the endoplasmic  
53 reticulum (ER) (2). Although the virus relies on its host to replicate efficiently, it must also find  
54 ways to avoid host antiviral responses. As with many viruses, rotaviruses target several different  
55 steps of the type I interferon (IFN) response in order to prevent the expression or activity of host  
56 antiviral proteins. For instance, the viral protein VP3 functions within the innermost layer of the  
57 viral particle as the capping enzyme, but also works within the infected cells to cleave 2'-5'-  
58 oligoadenylates to inhibit the activation of ribonuclease L (RNaseL) (3, 4).

59 Rotavirus also encodes the nonstructural protein NSP1 to inhibit the production of type I  
60 IFN, which in turn is thought to limit the expression of antiviral IFN-stimulated genes (ISGs) (5-  
61 10). Depending on the virus strain, NSP1 induces proteasomal degradation of IFN regulatory  
62 factors (IRFs) or  $\beta$ -TrCP, a protein component of a host cullin-RING E3 ubiquitin ligase complex  
63 that activates NF- $\kappa$ B (5, 8, 11, 12). Other cellular targets of NSP1 have also been identified, all  
64 of which appear to be targeted for degradation by the proteasome (13-16). NSP1 has been  
65 shown to localize diffusely throughout the cytoplasm of infected cells, but appears to be  
66 excluded from the viroplasms (17-19). Recent studies using screening approaches to identify  
67 host proteins that associate with NSP1 have found evidence for nuclear proteins in NSP1 pull  
68 down samples, but much of this data not been validated or investigated in detail (20, 21).

69 In this study, we demonstrate that rotavirus NSP1 localization is not restricted to the  
70 cytoplasm of infected or transfected cells; NSP1 also localizes to the nucleus, which still occurs  
71 in the absence of an intact C-terminal domain. Some rotavirus strains, such as SA11-4F, were  
72 found diffusely distributed at low levels in the nucleus of infected cells, while other strains, such  
73 as OSU, formed distinct nuclear foci. The rotavirus strains that formed nuclear foci were found

74 to colocalize with promyelocytic (PML) nuclear bodies (NB). NSP1 nuclear localization led to  
75 changes in the morphology or a reduction in the number of PML NB, depending on the virus  
76 strain. In the absence of PML, virus titers were unchanged, but fewer infected cells contained  
77 NSP1 nuclear foci, suggesting the irregularly shaped foci are attributed to the presence of PML.  
78 Together, the data suggest that NSP1 can localize to the nucleus to disrupt PML NB. This is the  
79 first report of a rotavirus protein found to localize to the nucleus, an unexpected finding given  
80 that the virus replicates in the cytoplasm of infected cells.

81

## 82 **RESULTS**

83 **NSP1 localizes to the nucleus and the cytoplasm of infected cells.** We previously  
84 examined host proteins that associate with NSP1 by performing pull down assays followed by  
85 mass spectrometry (20). The NSP1 protein from SA11-4F and OSU strains of rotavirus were  
86 found to associate with proteins associated with the nuclear pore, including exportin-1 (CRM1),  
87 nuclear pore complex protein NUP205, and NUP93. To determine if NSP1 localizes to the  
88 nucleus of infected cells, HT29 cells were mock infected or infected with SA11-4F or OSU  
89 rotavirus at a MOI of 5 for 8 h, which is when peak expression of NSP1 is expected to occur  
90 during infection (22). Cells were harvested and fractionated into whole cell, cytosolic and  
91 nuclear fractions, followed by SDS-PAGE. Immunoblotting for SA11-4F NSP1 and OSU NSP1  
92 demonstrated that the NSP1 proteins were localized to both cytosolic and nuclear fractions of  
93 infected cells (Fig. 1A). Nuclear fractions did not contain GAPDH, indicating that they were clear  
94 from cytosolic contamination. To ensure efficient separation of the endoplasmic reticulum (ER)  
95 from the nuclear membrane, calnexin was included as a control. To demonstrate that nuclear  
96 localization was not generalizable to other viral proteins, the viroplasm scaffolding protein NSP2  
97 was detected only in the cytoplasmic fraction.

98 To gain more detailed information about the nuclear distribution of NSP1 in infected  
99 cells, MA104 cells were mock infected or infected with SA11-4F or OSU rotavirus at a MOI of 5

100 for 8 h, followed by immunostaining and confocal microscopy (Fig. 1B). Lamin A/C was used to  
101 visualize the boundaries of the nuclear membrane. In cells infected with SA11-4F, NSP1  
102 appeared to be diffuse throughout the cytoplasm and nucleus. However, the nuclear distribution  
103 differed in cells infected with OSU, where NSP1 was found to form distinct foci within the  
104 nucleus. Together, these data show that NSP1 from SA11-4F and OSU strains of rotavirus is  
105 not restricted to the cytoplasm of infected cells, but also localizes to the nucleus.

106 **OSU-like and UK-like NSP1 proteins form foci within the nucleus.** To investigate the  
107 nuclear distribution of NSP1 proteins from other strains of rotavirus, the localization of tagged  
108 NSP1 proteins from different rotaviruses was examined in transfected cells. A transfection  
109 approach was used because antibodies are not available to all NSP1 proteins, and those  
110 antibodies that are available tend to not cross-react with NSP1 from different rotavirus isolates  
111 (8, 23). 293T cells were transfected with plasmids expressing Halo-tagged NSP1 from SA11-4F,  
112 OSU, WI61, UK, and DS-1 rotaviruses and harvested at 48 h p.t. Cells were then fractionated  
113 into whole cell, cytosolic and nuclear fractions, followed by SDS-PAGE and immunoblotting.  
114 Halo-tagged NSP1 from all strains tested was found to localize to the nuclear fraction of  
115 transfected cells (Fig. 2A). The levels of SA11-4F and DS-1 NSP1 proteins in the nucleus were  
116 consistently lower than what was observed for OSU, WI61, and UK NSP1 proteins. Although  
117 low levels of the HaloTag alone were detectable in the nucleus due to its small size, the levels of  
118 Halo-NSP1 proteins were notably higher in the nuclear fraction of transfected cells.

119 To determine if the subcellular distribution of Halo-tagged NSP1 proteins was similar to  
120 that observed in infected cells (Fig. 1B), MA104 cells were transfected with plasmids expressing  
121 the same Halo-tagged NSP1s for 48 h, as well as two C-terminally truncated NSP1 proteins,  
122 SA11-5S and OSU  $\Delta$ C13, followed by immunostaining and confocal microscopy (Fig. 2B).  
123 Lamin A/C was used to visualize the boundaries of the nuclear membrane. In cells transfected  
124 with Halo-NSP1 from SA11-4F rotavirus, the NSP1 protein was diffusely localized throughout  
125 the cytoplasm and nucleus, similar to what was observed in infected cells. The SA11-5S NSP1

126 contains a 17 amino acid C-terminal truncation, but is otherwise identical to SA11-4F NSP1;  
127 cells transfected with SA11-5S NSP1 also contained NSP1 in the nucleus in a diffusely  
128 distributed pattern. In cells transfected with Halo-NSP1 from OSU, WI61, UK, and DS-1  
129 rotaviruses, the NSP1 protein was diffusely localized throughout the cytoplasm, but distinct  
130 NSP1 foci formed in the nucleus. The removal of 13 amino acids from the OSU NSP1 (OSU  
131  $\Delta$ C13) or WI61 NSP1 (WI61  $\Delta$ C13) did not have an impact on the ability of NSP1 to localize and  
132 form distinct foci in the nucleus of transfected cells. The levels of nuclear Halo-NSP1 from  
133 SA11-4F and DS-1 in immunostained cells appeared to be rather low (Fig. 2B), as was reflected  
134 in the fractionation assay (Fig. 2A). Together, these data show that NSP1 from several different  
135 strains of rotavirus localizes to both the cytoplasm and nucleus, and that in most cases,  
136 excepting the SA11-4F and SA11-5S NSP1, distinct nuclear foci are formed by NSP1.

137 **OSU NSP1 forms foci in the nucleus at early times post-infection.** To determine at  
138 what time post-infection NSP1 begins to accumulate in the nucleus of infected cells, MA104  
139 cells were mock infected or infected with SA11-4F or OSU rotavirus and harvested at 2 h  
140 intervals for 12 h total. Fixed cells were then immunostained with antibodies specific to SA11-4F  
141 NSP1 or OSU NSP1 (8). In mock-infected cells stained with the SA11-4F NSP1 antibody, no  
142 signal was detected, suggesting that the SA11-4F NSP1 did not cross-react with any cellular  
143 proteins (Fig. 3A). In SA11-4F-infected cells, NSP1 was first observed in the cytoplasm and the  
144 nucleus at 6 h p.i., and continued to accumulate through 12 h p.i. SA11-4F NSP1 was diffusely  
145 distributed in the nucleus, and did not form distinct foci during the course of infection. At late  
146 times post-infection (10 and 12 h p.i.), the cytoplasmic NSP1 appeared to be excluded from the  
147 viroplasms, as has previously been observed (17). In mock-infected cells stained with the OSU  
148 NSP1 antibody, a low-level background signal was detected, indicating that the OSU NSP1  
149 antibody cross-reacted with a cellular protein (Fig. 3B). However, the accumulation of OSU  
150 NSP1 over the background staining was evident by 4 h p.i., and levels of OSU NSP1 continued  
151 to increase through 12 h p.i. Notably, the OSU NSP1 nuclear foci appeared by 6 h p.i.,

152 suggesting their formation was not due to over-expression of NSP1 protein at late times post-  
153 infection.

154 **OSU NSP1 nuclear foci colocalize with PML nuclear bodies during infection.** To  
155 determine the nature of the NSP1 nuclear structures in OSU-infected cells, HaCaT cells were  
156 mock infected or infected with SA11-4F or OSU rotavirus and fixed at 8 h p.i.. HaCaT cells are a  
157 human keratinocyte cell line that is highly amenable to immunofluorescence microscopy. Cells  
158 were co-stained with an antibody to NSP1 and markers of nuclear gems (gemin 2), nuclear  
159 speckles (SC35, also known as the serine/arginine-rich splicing factor SRSF2), or promyelocytic  
160 leukemia (PML) nuclear bodies (NB). A colocalization histogram was generated for imaged cells  
161 to determine if the signals from NSP1 and the subnuclear structures occurred in the same  
162 space. In mock-infected cells, gemin 2 appeared as one or two sphere-shaped foci in the  
163 nucleus, and no cross-reactivity with the SA11-4F NSP1 antibody was observed (Fig. 4A). In  
164 cells infected with SA11-4F or OSU rotavirus, no colocalization was apparent between nuclear  
165 NSP1 from either virus and the gemin 2 protein. Additionally, there appeared to be no major  
166 changes in the morphology or distribution of nuclear gems in rotavirus infected cells. Nuclear  
167 speckles were stained with an antibody to SC35, which appeared as irregularly distributed areas  
168 throughout the nucleus of mock-infected cells (Fig. 4B). In cells infected with SA11-4F or OSU  
169 rotavirus, no colocalization was apparent between nuclear NSP1 from either virus and SC35,  
170 nor was there a change in the morphology and distribution of nuclear speckles in infected cells.

171 Interestingly, in OSU-infected cells the NSP1 foci localized in the nucleus were found to  
172 colocalize with PML NB (Fig. 4C). Visual inspection of immunofluorescence images showed  
173 overlap between the signal from the OSU NSP1 and the PML antibody, and the OSU NSP1 and  
174 PML signal peaks corresponded with one another in the histogram profile. There also appeared  
175 to be changes in the morphology of PML NB during infection with OSU when compared to PML  
176 NB in mock-infected cells, where PML staining appeared as sphere-shaped foci throughout the  
177 nucleus. Colocalization was not observed between SA11-4F NSP1 and PML in infected cells,



178 likely because of the diffuse distribution pattern of SA11-4F NSP1 in the nucleus. Similar results  
179 were observed in infected MA104 cells and MA104 cells transfected with plasmids encoding  
180 Halo-tagged NSP1 (data not shown). Together, the data indicate that OSU NSP1 nuclear foci  
181 colocalize with PML NB in infected cells.

182 **Changes in the number and area of PML NB in rotavirus infected cells.** To follow up  
183 on the observation that the morphology of PML NB appeared to be altered in OSU-infected  
184 HaCaT cells, measurements of the number and area of PML NB present in the nucleus of  
185 rotavirus infected cells were made in comparison to uninfected cells. MA104 cells were mock  
186 infected or infected with SA11-4F, SA11-5S, or OSU rotaviruses and fixed at 8 h p.i. The SA11-  
187 5S rotavirus is identical to the SA11-4F virus strain except for a rearrangement in the NSP1  
188 coding gene that results in a small C-terminal deletion in the NSP1 protein; this truncated NSP1  
189 protein is no longer able to bind to IRF3 and target it for degradation (5, 11). Because of this C-  
190 terminal deletion, the SA11-5S NSP1 protein is no longer detected by available NSP1  
191 antibodies, thus the cells were stained with an antibody to the viral protein VP6 to detect  
192 infected cells, and an antibody to PML to detect the PML NB (Fig. 5A). The number of PML NB  
193 in 54 cells was counted in maximum projection of z-stack images of each condition, and the  
194 area of 100 PML NB was measured for quantification. In mock-infected cells, PML NB appeared  
195 sphere-shaped in the nucleus (Fig. 5A). Mock-infected cells on average contained  
196 approximately 12 PML NB per nucleus with an area of about  $0.21 \mu\text{m}^2$  (Fig. 5B, 5C).

197 In SA11-4F-infected cells, the PML NB retained their round morphology (Fig. 5A), but  
198 there was a notable decrease in the number of PML NB per nucleus, to about half of the  
199 number found in mock-infected cells (Fig. 5B). The decrease in the number of PML NB in SA11-  
200 4F-infected cells was not accompanied by an increase in the area of PML NB (Fig. 5C). When  
201 PML protein was examined by immunoblot there was not a decrease in overall PML protein  
202 levels (data not shown), suggesting the possibility that SA11-like NSP1s may be causing a  
203 dispersal of PML NB, but are not inducing degradation of PML. PML NB in SA11-5S-infected

204 cells retained their sphere-shaped morphology (Fig. 5A) and showed no change in number or  
205 area when compared to mock (Fig. 5B, 5C). In OSU-infected cells, PML NB took on an oblong  
206 appearance, suggesting a change in their morphology (Fig. 5A). This change was reflected by  
207 the near doubling of the average diameter of PML NB in OSU-infected cells to  $0.56 \mu\text{m}^2$  (Fig.  
208 5C). However, the number of PML NB in OSU-infected cells was similar to mock-infected cells  
209 (Fig. 5B), which suggests that the alteration in size and morphology was not due to fusion of  
210 existing PML NB.

211 **Changes to PML NB do not consistently group with SA11-4F-like or OSU-like**  
212 **NSP1s.** NSP1 proteins can be generally grouped into SA11-like, based on their ability to induce  
213 IRF degradation, and OSU-like, based on their ability to induce  $\beta$ -TrCP degradation (24). To  
214 determine if changes to PML NB generally grouped with SA11-4F-like or OSU-like NSP1s,  
215 MA104 cells were infected with a panel of monoreassortant viruses that contain distinct NSP1  
216 genes in the same genetic background of the SA11-L2 parental virus. The SNF and SRF  
217 viruses encode NSP1 proteins derived from K9 and RRV rotaviruses, respectively, and given  
218 their ability to induce IRF degradation are considered to have SA11-4F-like NSP1s (8, 20, 24).  
219 The SDF, SKF, and SOF viruses encode NSP1 proteins derived from DS-1, KU, and OSU  
220 rotaviruses, respectively, and are considered to have OSU-like NSP1s based on their ability to  
221 induce  $\beta$ -TrCP degradation (20, 24, 25). At 8 h p.i., cells were fixed and immunostained for the  
222 viral protein VP6 and PML, and nuclei were counterstained with DAPI.

223 The SA11-L2 virus is related to SA11-4F (the NSP1 proteins are identical), and as  
224 expected PML NB appeared morphologically similar in SA11-L2- and SA11-4F-infected cells  
225 (Fig. 6A). In addition, the number of PML NB in SA11-L2-infected cells was about half of that  
226 observed in mock-infected cells (Fig. 6B). Cells infected with the SA11-like viruses SNF and  
227 SRF also contained approximately 6 PML NB per cell, half of the number found in mock-infected  
228 cells. Like infection with OSU, the OSU-like viruses SOF and SKF resulted in somewhat larger  
229 and more oblong PML NB (Fig. 6A). While the OSU- and SOF-infected cells had approximately

230 the same number of PML NB as mock-infected cells, there were nearly two-times the number of  
231 PML NB found in SKF-infected cells (Fig. 6B). Unexpectedly, the OSU-like virus SDF caused a  
232 reduction in the number of PML NB, which appeared morphologically similar to PML NB in cells  
233 infected with SA11-like viruses (Fig. 6B, 6A).

234 **OSU NSP1 nuclear foci no longer form in PML-deficient cells.** To determine if PML  
235 is necessary for the formation of NSP1 foci in the nucleus of OSU-infected cells, HaCaT cells  
236 were transduced with lentivirus to knock down all isoforms of the PML protein by shRNA to  
237 create a stable cell line deficient in PML (shPML) (26). Previously published studies have shown  
238 no off-target effects using this same PML shRNA (26-30). HaCaT cells were also transduced  
239 with a lentivirus expressing a scrambled shRNA (shNEG) as a control (31). The loss of PML  
240 protein in shPML HaCaT cells was demonstrated by immunoblot, and quantification of PML  
241 protein levels consistently showed greater than 90% reduction (Fig. 7A). The loss of PML NB in  
242 shPML HaCaT cells was also evident by immunofluorescence microscopy (Fig. 7B). Next, the  
243 shPML and shNEG HaCaT cells were mock infected or infected with the OSU strain of rotavirus  
244 for 8 h followed by immunostaining for NSP1 and PML. In shNEG HaCaTs, the OSU NSP1  
245 protein was diffusely distributed throughout the cytoplasm and found in distinct foci that  
246 colocalized with PML NB as had been observed in other cell types (Fig. 7C). In the shPML  
247 HaCaTs, the OSU NSP1 nuclear foci no longer formed in the absence of the PML protein, but  
248 some OSU NSP1 was still diffusely distributed in the nucleus. These results suggest that NSP1  
249 nuclear localization is not dependent on the presence of PML, but that the formation of OSU  
250 NSP1 nuclear foci relies on the formation of PML NB.

251 To determine if the loss of PML affected viral titer, shNEG and shPML cells were mock  
252 infected or infected with SA11-4F or OSU rotavirus at a MOI of 5 for 8 h. Cells were lysed by  
253 multiple freeze-thaw cycles, and then viral titers were determined by plaque assay on MA104  
254 cells. In shNEG HaCaT cells, the average titer of SA11-4F rotavirus was approximately  $5.0 \times$   
255  $10^4$  PFU/ml, which was unchanged in shPML HaCaT cells (Fig. 7D). The average titer of OSU

256 rotavirus was slightly higher at approximately  $2.0 \times 10^5$  PFU/ml in shNEG and shPML cells, but  
257 there was no measurable difference in OSU replication in the absence of PML protein. The  
258 importance of the association of OSU NSP1 with PML NB might not be adequately determined  
259 in highly permissive cell lines such as HaCaT and MA104, as it has previously been shown that  
260 rotaviruses expressing defective NSP1 proteins can replicate to similarly high titers as their wild-  
261 type parental counterparts in most cell lines (32-35).

262

## 263 **DISCUSSION**

264 For DNA viruses that replicate in the nucleus of infected cells, there are a number of  
265 well-studied examples of viral genomes and proteins localizing to PML NB in infected cells (36).  
266 Some viruses with RNA genomes that replicate in the nucleus of infected cells have been  
267 shown to manipulate other types of nuclear bodies to enhance viral replication. There is now a  
268 growing appreciation for the manipulation of nuclear processes by cytoplasmically replicating  
269 viruses (37-40). Here we report that in addition to its known cytoplasmic localization, the  
270 rotavirus IFN antagonist protein NSP1 is also found in the nucleus of infected cells. Some  
271 rotavirus isolates, including OSU, WI61, and UK, formed punctate structures within the nucleus  
272 (Fig. 2). It was determined that the punctate NSP1 structures co-localized with PML NB (Fig. 4).  
273 Localization to the nucleus or to PML NB was not dependent on the C terminus of NSP1, which  
274 contains the substrate-binding domain that targets certain host proteins for proteasomal  
275 degradation (Fig. 2).

276 Previously published studies have concluded that NSP1 localizes to the cytoplasm of  
277 infected cells, based on immunofluorescence imaging (17-19). Re-examination of images from  
278 these studies indicates that NSP1 from SA11-5N, UK, UK variant brvA, and RRV did localize to  
279 the nucleus, sometimes as punctate spots, in addition to its cytoplasmic localization. Although  
280 the nuclear staining may have previously been dismissed as background, our use of subcellular  
281 fractionation confirms that NSP1 does indeed localize to the nucleus (Fig. 2). Given that the UK

282 variant brvA expresses only the first 258 amino acids of the NSP1 protein due to the insertion of  
283 a premature stop codon, it is possible that the region responsible for nuclear localization or  
284 association with PML NBs is found in the N-terminal half of NSP1.

285 NSP1 has mainly been studied for its role in inhibition of the IFN- $\beta$  response (reviewed in  
286 41, 42) and host tropism (43, 44). SA11-4F-like rotaviruses have been shown to target IRFs for  
287 proteasomal degradation, whereas OSU-like rotaviruses have been shown to target  $\beta$ -TrCP for  
288 degradation (42). IRF or  $\beta$ -TrCP degradation prevents the activation of transcription factors that  
289 translocate to the nucleus, resulting in an inhibition of the type I IFN response. Given that the  
290 pathway of IFN induction begins in the cytoplasm and proceeds through a well-orchestrated  
291 series of signaling events, it is logical to predict that NSP1 is found only in the cytoplasm in  
292 order to induce IRF or  $\beta$ -TrCP degradation. However, IRFs and  $\beta$ -TrCP also localize to the  
293 nucleus; therefore, the role of NSP1 in the nucleus could potentially be to promote degradation  
294 of IRFs or  $\beta$ -TrCP in that cellular compartment. Transient expression of NSP1 proteins with  
295 small C-terminal truncations demonstrated that localization to the nucleus and PML NB  
296 occurred in the absence of the substrate binding domain, suggesting that the function of NSP1  
297 in the nucleus is not related to degradation of IRFs or  $\beta$ -TrCP (Fig. 2).

298 PML NB have roles in a variety of cellular activities such as DNA damage and repair  
299 (45), apoptosis (46), and the IFN response (47-49). IFN- $\beta$  induction leads to an increase in the  
300 number and size of PML NB (50, 51). Our data demonstrated that OSU infection caused an  
301 increase in the area but not the number of PML NB when compared to mock-infected cells (Fig.  
302 5). Previous studies have shown that OSU NSP1 is able to inhibit IFN- $\beta$  induction, although  
303 perhaps not as efficiently as the SA11-4F NSP1, which may account for the increase in PML NB  
304 area, but the reason for the size increase requires additional experimentation (8, 12). PML NB  
305 morphology is similarly altered in some DNA virus infections; for example, adenovirus E4 ORF3  
306 causes PML NB to transform from sphere-shaped foci into track-like structures (52), and BK  
307 virus infection increases the size and decreases the number of PML NB to promote viral

308 infection (53). Infection with other rotaviruses that express OSU-like NSP1 proteins (SDF, SOF,  
309 and SKF) did not consistently change the number of PML NB per cell; SDF infection caused a  
310 significant reduction, whereas SKF infection caused a significant increase in the number of PML  
311 NB (Fig. 6). While grouping NSP1 proteins into SA11-4F-like and OSU-like based on their target  
312 for degradation is convenient, it may not fully capture the spectrum of NSP1 activities in infected  
313 cells. Infection with other SA11-4F-like rotaviruses (SA11-L2, SNF, and SRF) resulted in a  
314 substantial reduction in the number of PML NB per cell when compared to mock, but the reason  
315 for such a reduction requires further exploration.

316 Many proteins other than PML reside in PML NB, and most often their localization is  
317 transient. Proteins that localize to PML NBs typically contain a SUMO-interacting motif (SIM)  
318 that is necessary for recruitment to sumoylated PML (54). SIM interactions with SUMO may  
319 favor the retention of proteins in PML NBs. At this time it is unknown if NSP1 associates with  
320 sumoylated PML or other NB-associated proteins, but studies are underway to identify a  
321 possible SIM motif in NSP1. It is thought that PML, global SUMO conjugation, and SUMO-  
322 dependent ubiquitination are tightly connected (55). NSP1 contains a conserved RING domain  
323 that bears similarity to other E3 ubiquitin ligases, and it is possible that the RING domain is  
324 essential for PML NB localization (23).

325 One of the challenges of studying NSP1 in highly permissive cell culture systems is the  
326 difficulty in ascertaining if the activities of NSP1 are essential to promote rotavirus replication,  
327 due to the fact that the NSP1 protein can be truncated or eliminated and the virus continues to  
328 replicate to high titers (17, 32-35). Although SA11-4F and OSU rotaviruses replicated to similar  
329 titers in cells lacking PML (Fig. 7D), the data is not sufficient to conclude that NSP1 localization  
330 to PML NB has no role in the viral life cycle. The development of a cell culture model that shows  
331 restricted replication in the absence of NSP1 is needed to better test the importance of NSP1  
332 interactions with host proteins.

333 While this is the first report of a rotavirus nonstructural protein localizing to the nucleus of  
334 infected cells, NSP1 is not the first rotavirus protein shown to manipulate nuclear processes.  
335 The NSP3 protein expressed by rotaviruses, which binds to the eukaryotic translation initiation  
336 factor 4G and the 3' consensus sequence of viral transcripts to alter translation, has been  
337 shown to disrupt nuclear-cytoplasmic transport of poly(A)-binding protein (PABP) (56). NSP3  
338 appears to cause nuclear accumulation of poly(A)-containing mRNAs, thus preventing host  
339 mRNAs from reaching the cytoplasm to be translated (57). Other cytoplasmically replicating  
340 dsRNA viruses have also been shown to express proteins that localize to the nucleus. The  
341 reovirus  $\mu$ 2 protein, which interferes with the type I IFN response in a strain-specific manner (58,  
342 59), has been shown to localize to nuclear speckles and alter host cell splicing (40). Orbiviruses  
343 including bluetongue virus (BTV) and African horse sickness virus express a nonstructural  
344 protein NS4 that has been shown to localize to the cytoplasm and nucleus of infected cells (60-  
345 62). BTV NS4, which also has been shown to antagonize the IFN response, is found in the  
346 nucleolus of infected cells but it does not appear to influence mRNA splicing or translation (63).  
347 Although each of these viruses and rotaviruses are members of the *Reoviridae* family, the  
348 structural and non-structural components vary widely. Though the mechanisms by which these  
349 viruses alter nuclear events to promote viral replication may vary, it is becoming clear that  
350 cytoplasmically replicating viruses utilize their limited coding space to modify the host and  
351 create a favorable replication environment.

352

## 353 **MATERIALS AND METHODS**

354 **Cells and media.** Human 293T cells were cultured in high glucose Dulbecco's MEM  
355 (DMEM; Corning) supplemented with 5% fetal bovine serum (FBS) and 1% MEM non-essential  
356 amino acids (NEAA; HyClone). Human HT29 cells were cultured in high glucose DMEM  
357 supplemented with 10% FBS. Simian MA104 cells were cultured in Medium 199 (M199;  
358 Corning) supplemented with 5% FBS. Human HaCaT cells were cultured in low glucose

359 Dulbecco's MEM (DMEM; Corning) supplemented with 5% FBS. All cells were cultured at 37°C  
360 and 5% CO<sub>2</sub>.

361 **Viruses and infection.** The rotavirus strains SA11-4F, SA11-5S (33), SA11-L2 (64),  
362 SDF, SKF, SNF (32), SOF, SRF (65), and OSU were propagated and quantified in MA104 cells  
363 as previously described (66). Viruses were activated by incubation with 10 µg/mL of trypsin for  
364 30 min prior to infection of MA104 cells or 5 µg/mL of trypsin for 60 min prior to infection of  
365 HT29 or HaCaT cells. Cells were washed three times with serum-free medium and then  
366 inoculated with trypsin-activated rotavirus at a multiplicity of infection (MOI) of 5. At 1 hour post-  
367 infection (p.i.), cells were washed once with serum-free DMEM followed by addition of complete  
368 medium and incubation at 37°C for the specified time. Virus titers from infected HaCaT cell  
369 lysates were determined by plaque assay on MA104 cells as previously described (66).

370 **Antibodies.** The following commercial antibodies and dilutions were used for  
371 immunofluorescence staining: rabbit polyclonal antibody to HaloTag (Promega; G9281; 1:500  
372 dilution), mouse monoclonal antibody to lamin A/C (Cell Signaling; #4777; 1:150 dilution),  
373 peptide affinity purified rabbit polyclonal antisera to PML (Bethyl Laboratories; A301-167A;  
374 1:500 dilution), mouse monoclonal antibody to SC-35 (Abcam; ab11826; 1:2,000 dilution),  
375 mouse monoclonal antibody to Gemin 2 (Abcam; ab6084; 1:250 dilution), and affinity purified  
376 rabbit polyclonal antibody to fibrillarin (CST Cat# cs-2639; 1:400 dilution). Affinity purified rabbit  
377 polyclonal antisera to NSP1 from SA11-4F and OSU were used at 1:500 dilution as previously  
378 described (8, 20). Polyclonal guinea pig antiserum to VP6 was used at 1:2,000 dilution as  
379 previously described (20). Secondary antibodies used for immunostaining were: AlexaFluor 488  
380 goat anti-rabbit (Life Technologies; A-11034; 1:1,000 dilution), AlexaFluor 594 goat anti-mouse  
381 (Life Technologies; A-11032; 1:1,000 dilution), and AlexaFluor 594 goat anti-guinea pig (Life  
382 Technologies; A-11076; 1:1,000 dilution).

383 The following commercial antibodies and dilutions were used for immunoblotting: rabbit  
384 polyclonal antibody to HaloTag (Promega; G9281; 1:1,000 dilution), mouse monoclonal



385 antibody to lamin A/C (Cell Signalling; #4777; 1:2,000 dilution), mouse monoclonal antibody to  
386 glyceraldehyde-3-phosphoate dehydrogenase (GAPDH) (Santa Cruz; sc-32233; 1:1,000  
387 dilution), rabbit peptide affinity purified polyclonal antisera to PML (Bethyl Laboratories; A301-  
388 167A; 1:500 dilution), and affinity purified rabbit polyclonal antibody to calnexin (Cell Signaling;  
389 #2433; 1:2,000 dilution). Affinity purified rabbit polyclonal antisera to NSP1 from SA11-4F and  
390 OSU were used at 1:1,000 dilution as previously described (8, 20). Polyclonal antiserum to  
391 NSP2 from the simian SA11 strain of rotavirus was produced by Pacific Immunology  
392 Corporation (Ramona, CA). A peptide corresponding to amino acids 286 to 299 of SA11-5N  
393 NSP2 (C-KRLLFQKMKPEKNP) was conjugated to the carrier protein keyhole limpet  
394 hemocyanin. The peptide was used to immunize New Zealand White rabbits. NSP2-specific  
395 antiserum was collected and affinity purified using the immunizing peptide, which tested  
396 negative for cross-reactivity with other rotavirus proteins. Secondary antibodies used for  
397 immunoblotting were: IRDye 680RD goat anti-mouse (LiCor; 926-68070; 1:15,000 dilution),  
398 IRDye 800CW goat anti-rabbit (LiCor; 926-32211; 1:15,000 dilution), IRDye 680RD goat anti-  
399 rabbit (LiCor; 926-68073; 1:15,000 dilution), and IRDye 800CW goat anti-mouse (LiCor; 926-  
400 32210; 1:15,000 dilution).

401 **Plasmids and transfections.** The following plasmids were used in transfection  
402 experiments: HaloTag vector (Promega; pHTN), pHTN-NSP1 (SA11-4F), pHTN-NSP1 (OSU),  
403 pHTN-NSP1 (WI61), pHTN-NSP1 (UK) or pHTN-NSP1 (DS-1) (20). To generate C-terminal  
404 truncations in the NSP1 proteins expressed from these plasmids, outward PCR amplification  
405 was used with each pHTN-NSP1 plasmid as a template and suitable primers. All plasmid  
406 sequences were verified by sequencing. For subcellular fractionation experiments,  $3.0 \times 10^6$   
407 293T cells were plated in 60-mm dishes, and transfected 24 h later with 2.5  $\mu\text{g}$  of plasmid DNA  
408 with PolyJet (SignaGen, Rockville, MD). For immunofluorescence experiments,  $5.0 \times 10^5$   
409 MA104 cells were plated in 6-well plates, and transfected the following day with 1.0  $\mu\text{g}$  of  
410 plasmid DNA with PolyJet reagent. All transfection assays were analyzed at 48 h p.t.

411           **Subcellular fractionation.** Cells were scraped into 1.5 mL cold phosphate buffered  
412 saline (PBS) and resuspended by vortexing. To generate whole cell lysates (WCL), 500  $\mu$ L of  
413 cell suspension was centrifuged at  $9.6 \times g$  for 2 min to pellet cells. The supernatant was  
414 removed and the pellet was frozen at  $-80^{\circ}\text{C}$  for at least 30 min. Cell pellets were lysed in 100  
415  $\mu$ L of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 150 mM 1M Tris [pH 8.0],  
416 0.5% sodium deoxycholate, 1.0% IGEPAL CA-630, 0.1% SDS, 1 $\times$  protease inhibitor cocktail).  
417 Samples were incubated on ice for 20 min, then centrifuged at  $18.8 \times g$  for 3 min to pellet  
418 insoluble material. WCL was mixed with an equal part of 2 $\times$  tricine sample buffer (100 mM 1M  
419 Tris-Cl [pH 6.8], 25% glycerol, 2% SDS, 0.02% bromophenol blue, 5% beta-mercaptoethanol)  
420 then boiled at  $100^{\circ}\text{C}$  for 5 min.

421           To generate the cytosolic fraction (CF) and nuclear fraction (NF), 1000  $\mu$ L of cell  
422 suspension was centrifuged at  $0.6 \times g$  for 5 min to pellet cells. The supernatant was removed  
423 and pellet was resuspended in 150  $\mu$ L of reticulocyte standard buffer (RSB) (10 mM Tris [pH  
424 7.5], 16 mM NaCl, 3 mM  $\text{MgCl}_2$ ) containing 0.1 M sucrose, 1% IGEPAL CA-630, 0.5% sodium  
425 deoxycholate and 1 $\times$  protease inhibitor cocktail. Samples were incubated on ice for 15 min, then  
426 centrifuged at  $0.9 \times g$  for 5 min to separate the CF and NF. The CF was mixed with an equal  
427 part of 2 $\times$  tricine sample buffer and boiled at  $100^{\circ}\text{C}$  for 5 min. The nuclear pellet was washed  
428 three times in 1 mL of PBS and stored at  $-80^{\circ}\text{C}$  for at least 30 min. Nuclear pellets were lysed  
429 in 100  $\mu$ L of RIPA buffer, incubated on ice for 20 min, and centrifuged at  $9.6 \times g$  for 2 min to  
430 pellet insoluble material. The NF was mixed with an equal part of 2 $\times$  tricine sample buffer and  
431 boiled at  $100^{\circ}\text{C}$  for 5 min. Samples were stored at  $-80^{\circ}\text{C}$  for long-term storage or used  
432 immediately for SDS-PAGE. All centrifugation steps were performed at  $+4^{\circ}\text{C}$ .

433           **SDS-PAGE and immunoblotting.** Proteins were resolved by SDS-PAGE in 10% tris-  
434 tricine gels and transferred to a nitrocellulose membrane (Li-Cor). Membranes were blocked in  
435 Odyssey TBS blocking buffer (Li-Cor) and then incubated with primary antibody in Odyssey  
436 blocking buffer containing 0.1% Tween 20. Membranes were washed three times with Tris

437 buffered saline (50 mM Tris [pH 7.5], 150 mM NaCl) containing 0.1% Tween 20 (TBS-T).  
438 Secondary antibodies conjugated to IRDye680 or IRDye800 (Li-Cor) were added to TBS-T  
439 containing 1% nonfat dry milk. Membranes were washed three times with TBS-T and imaged  
440 using the Odyssey CLx infrared imaging system (Li-Cor) at a 5.5 intensity for each channel.

441 **Immunofluorescence staining and microscopy.** Infected or transfected cells plated  
442 on glass coverslips were fixed by incubating with 11% formaldehyde in PBS at room  
443 temperature for 10 min. Fixed cells were permeabilized and blocked in PBS containing 0.05%  
444 Triton X-100 and 5% BSA at room temperature for 45 min. Permeabilized cells were incubated  
445 with primary antibody diluted in PBS containing 0.05% Triton X-100 and 1% BSA for 1 h at  
446 37°C. Coverslips were washed four times in PBS at room temperature. Coverslips were then  
447 incubated with secondary antibody diluted in PBS containing 0.05% Triton X-100 and 1% BSA  
448 for 1 h at 37°C, followed by washing three times in PBS at room temperature. Coverslips were  
449 stained with 300 nM 4',6-diamidino-2-phenylindole nucleic acid stain (DAPI; Invitrogen) in PBS  
450 for 5 min followed by washing three times in PBS at room temperature. Coverslips were rinsed  
451 briefly in sterile water and allowed to dry for 1 h prior to mounting to slides with ProLong Gold  
452 Antifade Reagent (ThermoFisher). All immunofluorescence images were captured using a Nikon  
453 SIM-E & A1R confocal microscope with an SR Apo TIRF 100x/1.49 NA oil objective lens.  
454 Images were processed uniformly with Adobe Photoshop software. The number and area of  
455 PML NB were quantified in maximum projection of z-stack images. The area of PML NB was  
456 calculated by manually drawing a polygonal structure around the edges of PML NB chosen at  
457 random using the Nikon NIS Elements software.

458 **Lentivirus transduction.** Lentiviruses expressing anti-PML and anti control shRNAs  
459 were a kind gift from Dr. Roger Everett, MRC University of Glasgow Centre for Virus Research  
460 (26). The anti-PML shRNA coding strand sequence was 5'-AGATGCAGCTGTATCCAAG-3',  
461 which lies in conserved exon 4. HaCaT cells were transduced with lentiviruses expressing  
462 shRNAs directed against a scrambled control (shNEG) or PML (shPML) by adding 1 mL of

463 transduction media (low glucose DMEM, polybrene (5-8 ug/mL) and lentivirus) every 3 hours  
464 until the final volume was 3 mL. Cells were incubated for an additional 24 hours at 37°C prior to  
465 washing the transduction media and beginning selection with 10 ug/mL puromycin.

466 **Statistical analysis.** For quantification of PML NB, the number per cell was counted in  
467 54 cells per infection condition in a maximum intensity projection of confocal images. Counts  
468 were repeated in three independent experiments. One-way ANOVA analysis was performed  
469 using the mean number of PML NB per cell for each biological replicate and comparing the  
470 sample to the mean of the mock-infected control. For quantification of PML NB area, 100 PML  
471 NB were measured by manually drawing a polygonal shape around the edges randomly chosen  
472 PML bodies per infection condition in a maximum intensity projection of confocal images.  
473 Measurements were repeated in three independent experiments. One-way ANOVA analysis  
474 was performed using the mean area of PML NB per cell for each biological replicate and  
475 comparing the sample to the mean of the mock-infected control. Nikon NIS elements software  
476 was used for counting and area measurements. GraphPad Prism 8 was used for statistical  
477 analysis.

478

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491

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- 678

679 **FIGURE LEGENDS**

680

681 **FIG 1. NSP1 localizes to the cytoplasm and nucleus of infected cells.** (A) HT29 cells were  
682 mock infected or infected with SA11-4F or OSU rotavirus (MOI = 5) for 8 h. Whole cell lysates,  
683 cytosolic fractions, and nuclear fractions were resolved by SDS-PAGE and analyzed by  
684 immunoblotting for SA11-4F NSP1, OSU NSP1, SA11-4F NSP2, calnexin (loading control to  
685 exclude ER contamination), GAPDH (loading control for cytosolic extract), or lamin A/C (loading  
686 control for nuclear extract). Blots were imaged using the Odyssey infrared imaging system  
687 (LiCor). (B) MA104 cells were mock infected or infected with SA11-4F or OSU rotavirus (MOI =  
688 5) for 8 h. Cells were fixed and stained with  $\alpha$ -SA11-4F or  $\alpha$ -OSU NSP1 (green) and  $\alpha$ -lamin  
689 A/C (red) antibodies followed by secondary staining with AlexaFluor488 goat  $\alpha$ -rabbit and  
690 AlexaFluor594 goat  $\alpha$ -mouse. Lower panels are zoomed in images of cells in white box in upper  
691 panels.

692

693 **FIG 2. NSP1 localizes to the cytoplasm and nucleus of transfected cells.** (A) 293T cells  
694 were transfected with a plasmid encoding Halo-tagged NSP1 from SA11-4F, OSU, WI61, UK, or  
695 DS-1 rotavirus strains, or with empty Halo tag vector, for 48 h. Whole cell lysates, cytosolic  
696 fractions, and nuclear fractions were resolved by SDS-PAGE and analyzed by immunoblotting  
697 for the Halo tag, GAPDH (loading control for cytosolic fraction), or lamin A/C (loading control for  
698 nuclear fraction). Blots were imaged using the Odyssey infrared imaging system (LiCor). (B)  
699 MA104 cells were transfected with a plasmid encoding Halo-tagged NSP1 from SA11-4F, OSU,  
700 WI61, UK, or DS-1 rotavirus strains, small C-terminal NSP1 deletions of SA11-4F (SA11-5S),  
701 OSU (OSU  $\Delta$ C13), WI61 (WI61  $\Delta$ C13), or with empty Halo tag vector, for 48 h. Cells were fixed  
702 and stained with  $\alpha$ -Halo tag (green) and  $\alpha$ -lamin A/C (red) antibodies followed by secondary  
703 staining with AlexaFluor488 goat  $\alpha$ -rabbit and AlexaFluor594 goat  $\alpha$ -mouse.

704

705 **FIG 3. Time course of NSP1 nuclear accumulation in infected cells.** MA104 cells were mock  
706 infected or infected with SA11-4F or OSU rotavirus (MOI = 5) for 2, 4, 6, 8, 10 or 12 h. Cells  
707 were fixed and stained for **(A)**  $\alpha$ -SA11-4F NSP1 (green), or **(B)**  $\alpha$ -OSU NSP1 (green), and  $\alpha$ -  
708 lamin A/C (red) antibodies followed by secondary staining with AlexaFluor488 goat  $\alpha$ -rabbit and  
709 AlexaFluor594 goat  $\alpha$ -mouse. A zoomed in image at 6 h p.i. is included for SA11-4F and OSU  
710 infected cells, which are surrounded by a white box in upper panels.

711  
712 **FIG 4. Intranuclear OSU NSP1 localizes to PML nuclear bodies.** HaCaT cells were mock-,  
713 SA11-4F-, or OSU-infected (MOI = 5) for 8 h. Cells were fixed and stained with  $\alpha$ -SA11-4F or  $\alpha$ -  
714 OSU NSP1 (green) antibodies and **(A)**  $\alpha$ -Gemin2 (red), **(B)**  $\alpha$ -SC35 (red), or **(C)**  $\alpha$ -PML (red)  
715 antibodies. Nuclei were counterstained with DAPI (blue). Histograms display measured  
716 fluorescence signal intensity (x100) along the arrow in the image panels.

717  
718 **FIG 5. PML body morphology, quantity, and size in rotavirus-infected cells. (A)** MA104  
719 cells were mock infected or infected with SA11-4F, SA11-5S, or OSU rotavirus (MOI = 5) for 8  
720 h. Cells were fixed and stained with  $\alpha$ -VP6 (green) and  $\alpha$ -PML (red) antibodies followed by  
721 secondary staining with AlexaFluor488 goat  $\alpha$ -guinea pig and AlexaFluor594 goat  $\alpha$ -mouse.  
722 Nuclei were counterstained with DAPI (blue). Lower panels are zoomed in images of cells in  
723 white box in upper panels. **(B)** The number of PML nuclear bodies (NB) per cell was quantified  
724 in 54 cells per experiment for each infection condition. Each point represents the number of  
725 PML NB per cell. Lines represent the mean  $\pm$  standard deviation. Graph shows a single  
726 representative experiment (n = 3). One-way ANOVA was performed using the mean for each  
727 biological replicate and comparing the sample to the the mock-infected control. The number of  
728 PML NB per cell was statistically lower than mock in SA11-4F-infected cells ( $p < 0.05$ ), but not  
729 significantly different in SA11-5S- or OSU-infected cells. **(C)** The area of 100 PML NB was  
730 measured for each infection condition. Each point represents the area of a single PML NB. Line

731 represents mean area  $\pm$  standard deviation. Graph shows a single representative experiment (n  
732 = 3). One-way ANOVA was performed using the mean for each biological replicate and  
733 comparing the sample to the the mock-infected control. The area of PML NB per cell was  
734 statistically higher than mock in OSU-infected cells ( $p < 0.005$ ), but not significantly different in  
735 SA11-4F- or SA11-5S-infected cells.

736

737 **FIG 6. PML body morphology and quantity does not segregate into clear SA11-like or**  
738 **OSU-like groups. (A)** MA104 cells were mock infected or infected with rotavirus strains as  
739 labeled (MOI = 5) for 8 h. Cells were fixed and stained with  $\alpha$ -VP6 (red) and  $\alpha$ -PML (green)  
740 antibodies followed by secondary staining with AlexaFluor594 goat  $\alpha$ -guinea pig and  
741 AlexaFluor488 goat  $\alpha$ -mouse. Nuclei were counterstained with DAPI (blue), but omitted for  
742 clarity in some images. **(B)** The number of PML nuclear bodies (NB) per cell was quantified in  
743 54 cells per experiment for each infection condition. Each point represents the number of PML  
744 NB per cell. Lines represent the mean  $\pm$  standard deviation. Graph shows a single  
745 representative experiment (n = 3). One-way ANOVA was performed using the mean for each  
746 biological replicate and comparing the sample to the mock-infected control. The number of PML  
747 NB per cell was statistically lower than mock in SA11-4F, SA11-L2, SNF, SRF, and SDF  
748 infected cells. The number of PML NB per cell was not statistically different than mock in OSU  
749 or SOF infected cells, but was statistically higher in SKF infected cells.

750

751 **FIG 7. OSU NSP1 nuclear foci no longer form in PML-deficient cells. (A)** HaCaT cells were  
752 transduced with shNEG (scrambled control) or shPML (PML knockdown) lentiviruses. Whole  
753 cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting for PML or GAPDH  
754 (loading control). Blots were imaged using the Odyssey infrared imaging system (LiCor). **(B)**  
755 HaCaT cells were fixed and stained for PML (green) followed by secondary staining with  
756 AlexaFluor594 goat anti-mouse. Nuclei were counterstained with DAPI (blue). **(C)** shNEG and

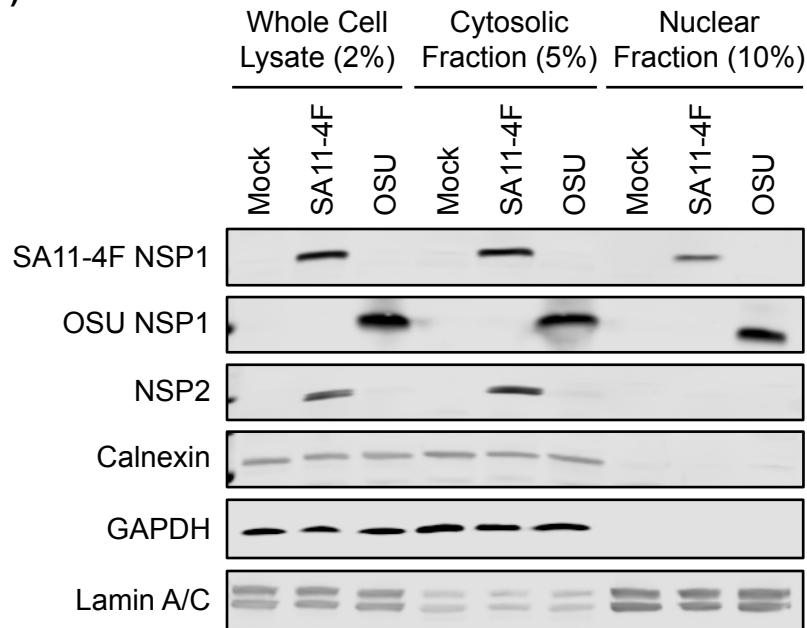
757 shPML HaCaT cells were infected with the OSU strain of rotavirus (MOI = 5) for 8 h. Cells were  
758 fixed and stained with  $\alpha$ -OSU NSP1 (green) and  $\alpha$ -PML (red) antibodies followed by secondary  
759 staining with AlexaFluor488 goat  $\alpha$ -rabbit and AlexaFluor594 goat  $\alpha$ -mouse. Nuclei were  
760 counterstained with DAPI (blue). (D) shNEG and shPML HaCaT cells were infected with the  
761 SA11-4F and OSU strains of rotavirus (MOI = 5) for 8 h. Cells were lysed by freeze-thaw and  
762 titered on MA104 cells.

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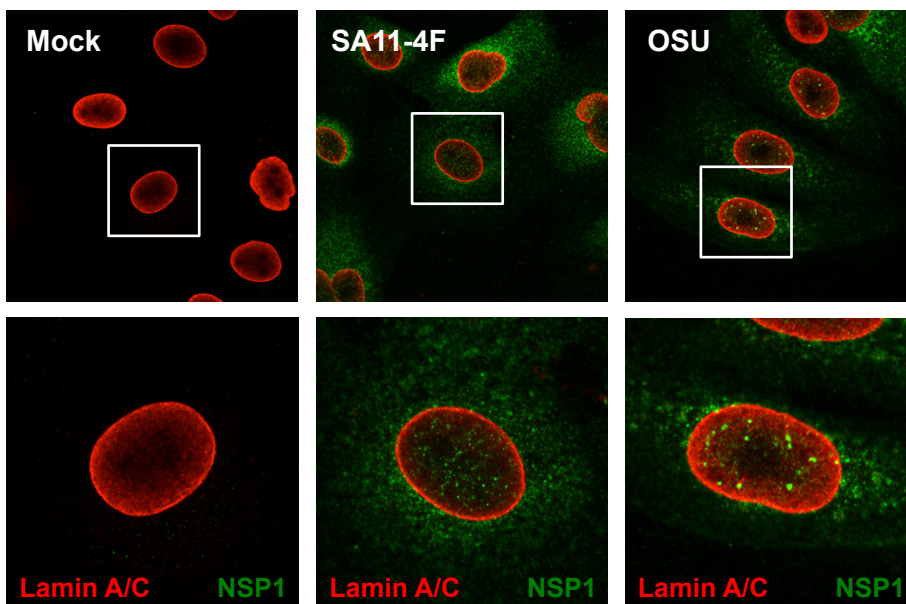
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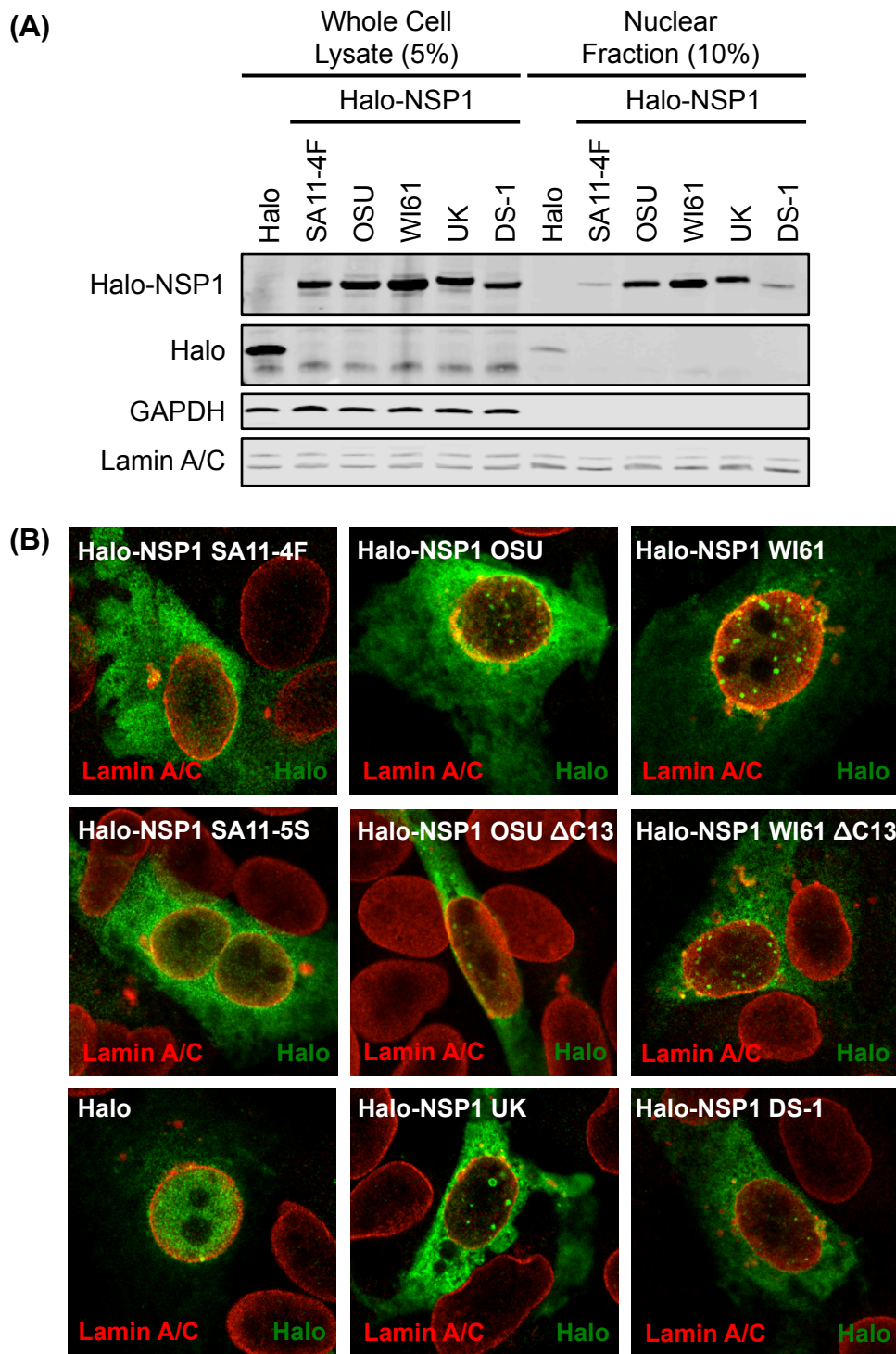
(A)



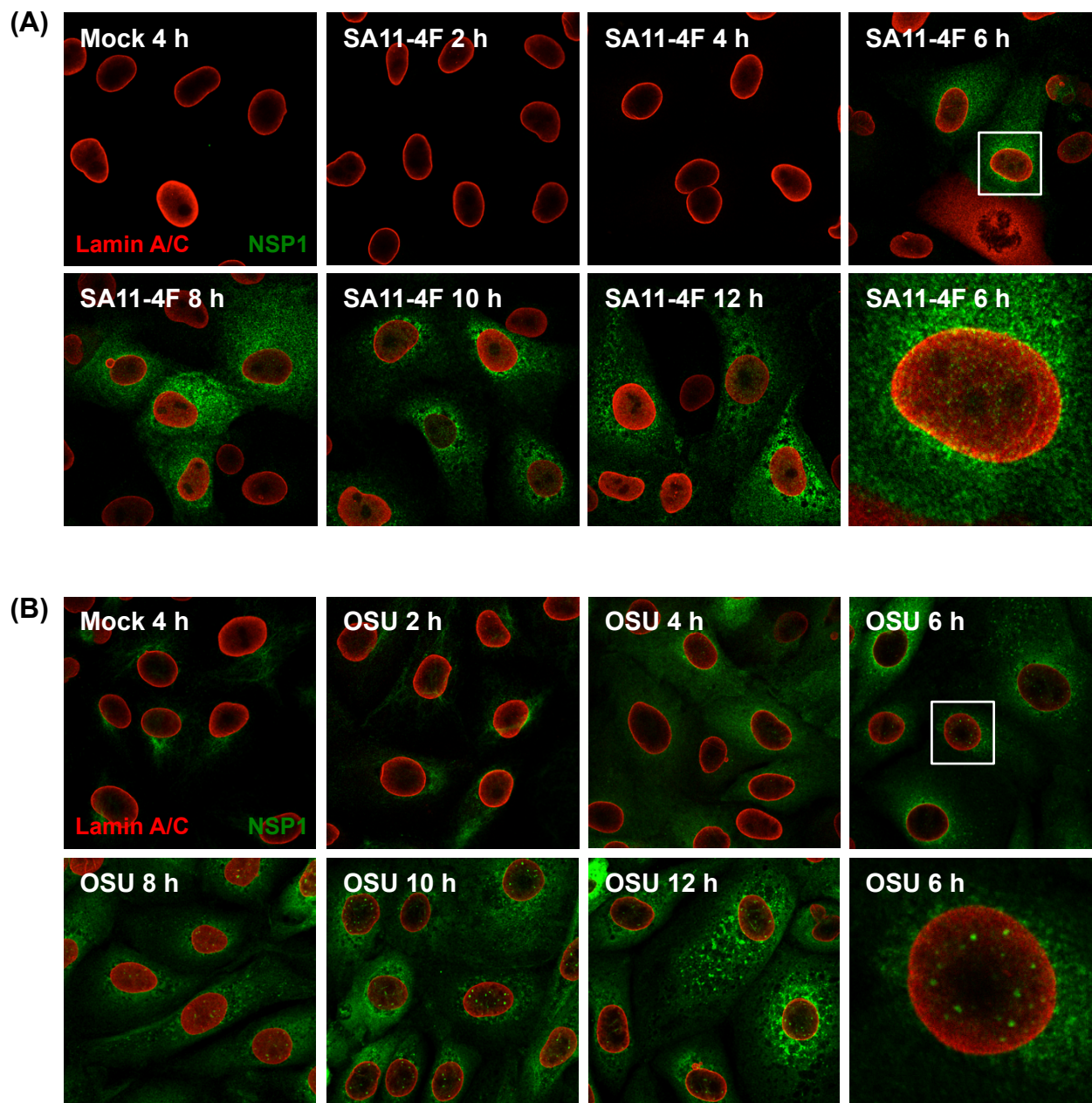
(B)



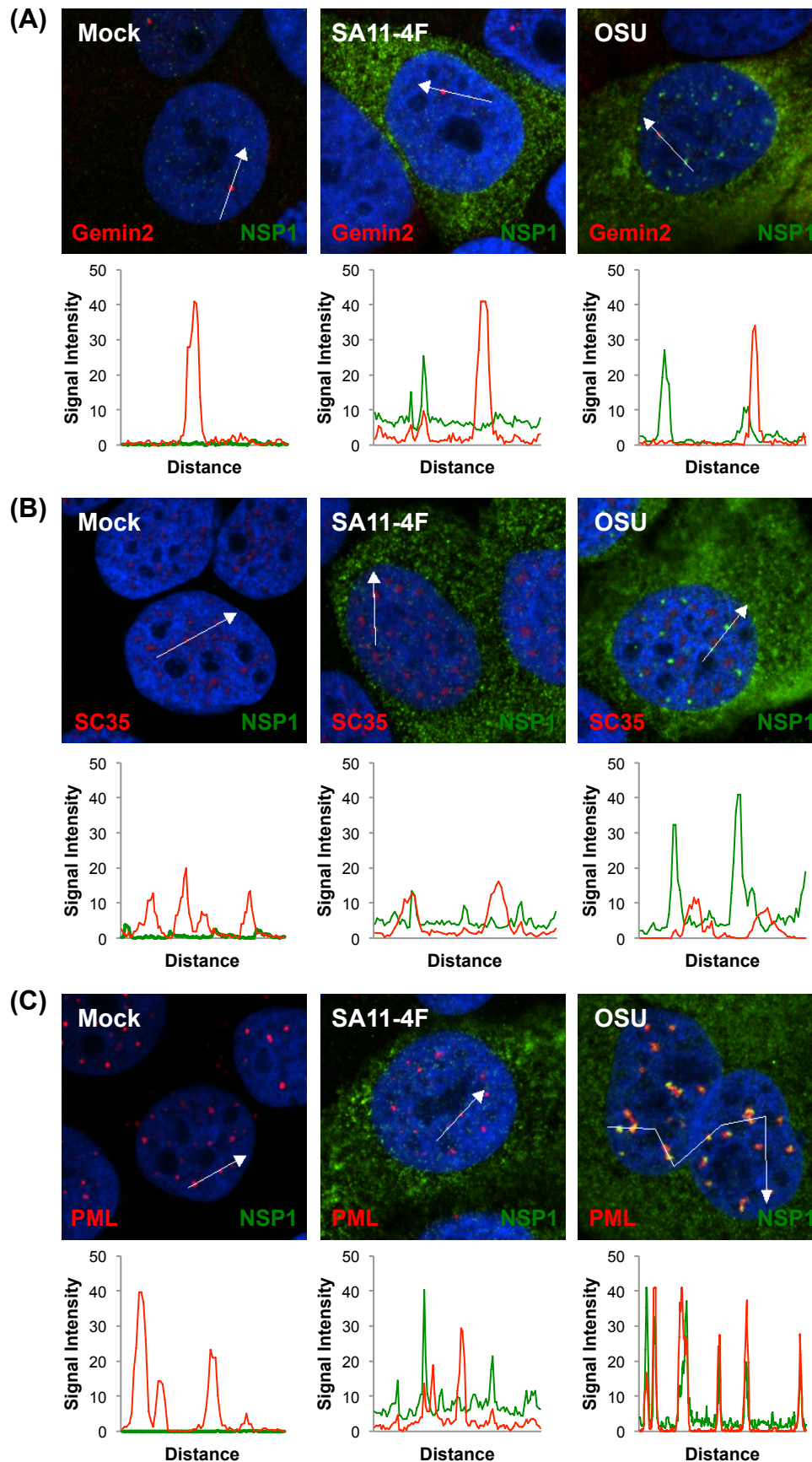
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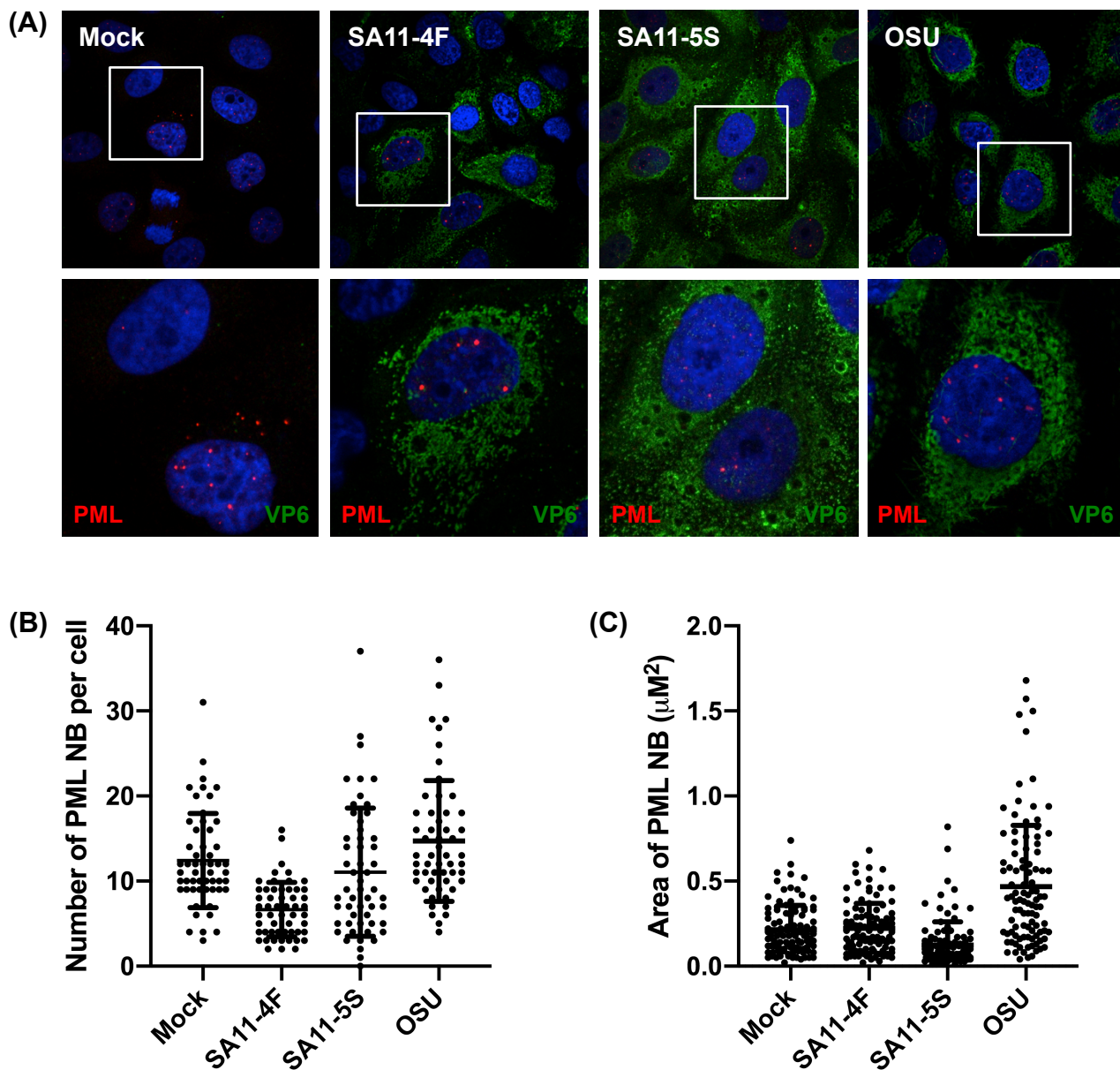
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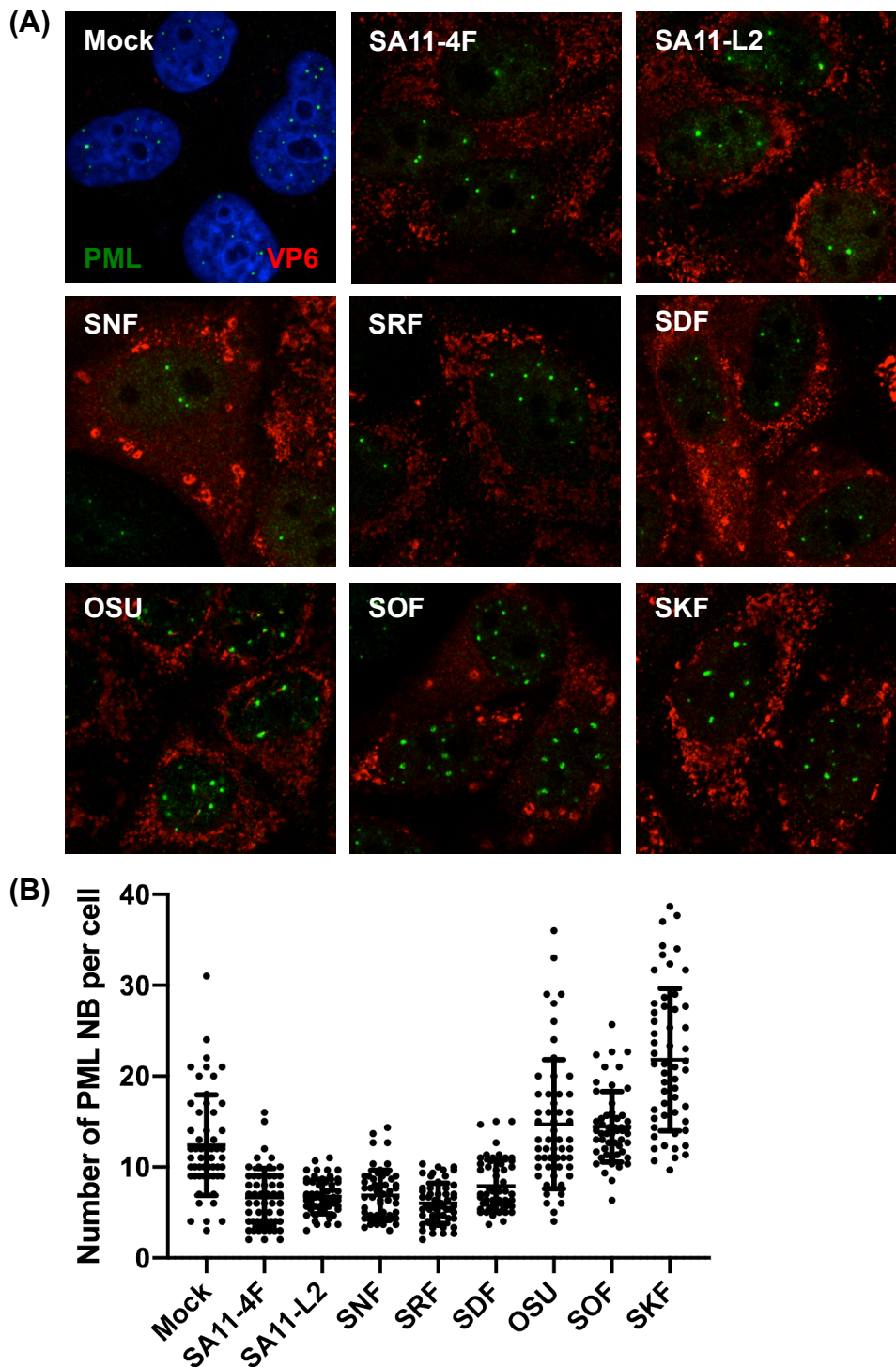
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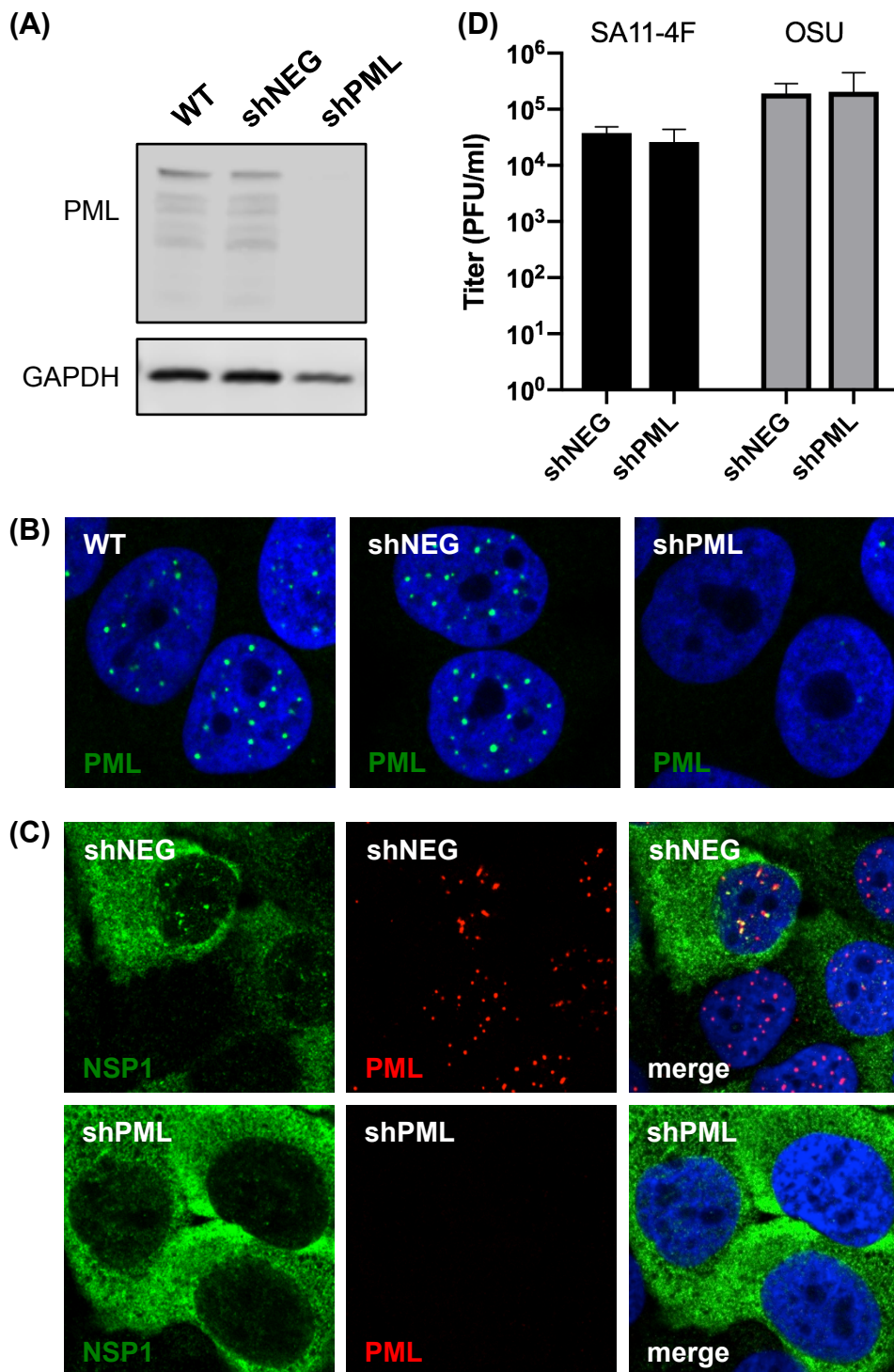
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**FIG 6. PML body morphology and quantity does not segregate into clear SA11-like or OSU-like groups.** (A) MA104 cells were mock infected or infected with rotavirus strains as labeled (MOI = 5) for 8 h. Cells were fixed and stained with  $\alpha$ -VP6 (red) and  $\alpha$ -PML (green) antibodies followed by secondary staining with AlexaFluor594 goat  $\alpha$ -guinea pig and AlexaFluor488 goat  $\alpha$ -mouse. Nuclei were counterstained with DAPI (blue), but omitted for clarity in some images. (B) The number of PML nuclear bodies (NB) per cell was quantified in 54 cells per experiment for each infection condition. Each point represents the number of PML NB per cell. Lines represent the mean  $\pm$  standard deviation. Graph shows a single representative experiment (n = 3). One-way ANOVA was performed using the mean for each biological replicate and comparing the sample to the mock-infected control. The number of PML NB per cell was statistically lower than mock in SA11-4F, SA11-L2, SNF, SRF, and SDF infected cells. The number of PML NB per cell was not statistically different than mock in OSU or SOF infected cells, but was statistically higher in SKF infected cells.



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