

1           **Role of PML-Nuclear Bodies in Human Herpesvirus 6A and 6B**

2   **Genome Integration.**

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## 17 **Abstract**

18 Human herpesviruses 6A and 6B (HHV-6A/B) are two betaherpesviruses that readily  
19 integrate their genomes into the telomeres of human chromosomes. To date, the cellular  
20 or viral proteins that facilitate HHV-6A/B integration remain elusive. In the present study,  
21 we demonstrate that the immediate early protein 1 (IE1) of HHV-6A/B colocalizes with  
22 telomeres during infection. Moreover, IE1 associates with PML-NBs, a nuclear complex  
23 that regulates multiples cellular mechanism including DNA repair and antiviral responses.  
24 Furthermore, we could demonstrate that IE1 targets all PML isoforms and that both  
25 proteins colocalize at telomeres. To determine the role of PML in HHV-6A/B integration,  
26 we generated PML knockout cell lines using CRISPR/Cas9. Intriguingly, in the absence of  
27 PML, the IE1 protein could still localize to telomeres albeit less frequently. More  
28 importantly, HHV-6A/B integration was impaired in the absence of PML, indicating that  
29 it plays a role in the integration process. Taken together, we identified the first cellular  
30 protein that aids in the integration of HHV-6A/B and shed light on this targeted integration  
31 mechanism.

32

## 33 **Author summary**

34 Human herpesviruses type 6A and 6B are relatively common viruses whose infections can  
35 be life threatening in patients with a compromised immune system. A rather unique feature  
36 of these viruses is their ability to integrate their genome in human chromosomes.  
37 Integration takes place is a specialized region of the chromosomes known as telomeres, a  
38 region that controls cellular lifespan. To date, the mechanisms leading to HHV-6A and  
39 HHV-6B integration remain elusive. Our laboratory has identified that the IE1 protein of

40 HHV-6A and HHV-6B target the telomeres. Moreover, we have shown that IE1 associates  
41 with a cellular protein, PML, that is responsible for the regulation of important cellular  
42 mechanisms such as the life span of cells and DNA repair. Hence, we studied the role of  
43 PML in HHV-6 integration. Our study demonstrates that in absence of PML, the HHV-6A  
44 and HHV-6B integrate 50-70% less frequently. Thus, our study unveils the first cellular  
45 protein involved in HHV-6A and HHV-6 chromosomal integration.

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47

## 48 **Introduction**

49 Human herpesviruses type 6A and 6B (HHV-6A/B) are members of the *betaherpesvirinae*  
50 that were isolated in the 1980's. In 2013, the International Committee on Taxonomy of  
51 Viruses recognized HHV-6A and HHV-6B as distinct viral species (1). HHV-6B is known  
52 as the etiologic agent of *exanthem subitum*, a childhood disease whose symptoms include,  
53 fever, occasional skin rash and respiratory distress (2). HHV-6A is much less characterized  
54 than HHV-6B. Considering that many HHV-6A/B proteins share 90-95% homology, the  
55 symptoms of primary HHV-6A infections are likely lessened in part due to cross-protective  
56 immunity developed against HHV-6B. Upon primary infection, HHV-6A/B establish  
57 latency like all herpesviruses. During latency, most herpesviruses maintain their genome  
58 in a circularized form (episome). The viral episomes are generally tethered to the human  
59 chromosomes, ensuring transmission to daughter cells following cell division (3), (4).  
60 However, to date, the presence of HHV-6A/B episomes during latency have yet to been  
61 demonstrated.

62

63 Despite the fact that no episomes of HHV-6A/B have been reported, both viruses can  
64 reactivate and cause secondary infections. In healthy subjects, HHV-6B reactivation is  
65 mostly subclinical and controlled by the immune system. However, in  
66 immunocompromised individuals, HHV-6B reactivation can be problematic and  
67 occasionally life-threatening (5),(6),(7). In case of HHV-6A, several reports have  
68 associated the virus with neurodegenerative diseases such as multiple sclerosis and more  
69 recently with Alzheimer's disease (8), (9), (10), (11). In spite of their pathological  
70 differences, both HHV-6A and HHV6B can readily integrate their genomes into host

71 chromosomes (12), (13), (14). HHV-6A/B integration can take place in various  
72 chromosomes but invariably occurs within the telomeric region (12), (13), (14). In 1993,  
73 Luppi *et al* reported three cases of individuals with telomeric integration of HHV-6A/B  
74 (13). In 1999, Daibata *et al* subsequently demonstrated that chromosomally-integrated  
75 HHV-6 can be inherited (12). Subjects with inherited chromosomally-integrated HHV-  
76 6A/B (iciHHV-6A/B) have at least one (occasionally 2 or 3) copy of the viral genome  
77 present in every somatic cells, with the viral genome transmitted to 50% to their children  
78 (15), (16). Viral integration into telomeres could be an alternative latency mechanism for  
79 HHV-6A/B. In support, the integrated HHV-6A/B genomes are generally intact and  
80 conserved without any gross rearrangements or mutations (17). Furthermore, integrated  
81 HHV-6A/B genomes can express genes and lead to complete viral reactivation  
82 (18)(19),(20),(7). Reactivation of HHV-6A/B can be life threatening for  
83 immunocompromised hosts.

84

85 Telomeres are non-coding (TTAGGG)<sub>n</sub> hexanucleotides present at the chromosome  
86 termini and contain a single-stranded 3' extension of 30-500 G rich nucleotides. They  
87 protect chromosomes against the loss of genetic information, which would result in  
88 premature cell senescence and prevent the recognition of chromosome ends by the DNA  
89 damage response (DDR) machinery. The telomere end forms a t-loop (22),(23) that is  
90 maintained by a complex of 6 proteins, the shelterin proteins (24), (25) that protect the  
91 chromosomes against DNA damage response. The HHV-6A/B genome is about 160  
92 kilobase pairs (kbp) in length and contains a unique region (U) with close to 100 open  
93 reading frames (26), (27), (28). This U region is flanked by identical direct repeat regions

94 (DR<sub>L</sub> and DR<sub>R</sub>) of 8-9 kbp that contain telomere sequences identical to human telomeres  
95 at both ends (27), (29). Wallaschek and al. recently demonstrated that these telomeric  
96 sequences facilitate integration of HHV-6A into host telomeres (30). This indicated that  
97 integration is likely mediated by homologous DNA recombination events. To date, no viral  
98 or cellular proteins have been identified that are involved in HHV-6A/B integration.

99

100 An interesting candidate involved in viral integration is the immediate-early protein 1 (IE1)  
101 of HHV-6A/B, which can be transcribed without *de novo* protein synthesis (31). The IE  
102 proteins of herpesviruses regulate early genes and plays an important role in the initiation  
103 of lytic virus replication. Moreover, they establish a favorable environment by  
104 manipulating PML-Nuclear bodies (PML-NBs), which are part of the cellular antiviral  
105 defense (32), (33), (34). In the context of an infection, PML-NBs have been shown to  
106 repress replication of various viruses with its components SP-100 and DAXX. PML-NBs  
107 are found mostly in the nucleus and contain large quantities of the PML protein (35), (36).  
108 Some viruses have developed ways to overcome this antiviral mechanism by degrading or  
109 manipulating PML-NBs. For example, herpes simplex virus 1 (HSV-1) encodes the E3  
110 ligase ICP0 that conjugates ubiquitin to PML and induces its degradation (37), (38). Human  
111 cytomegalovirus (hCMV) IE1 de-SUMOylates PML-NBs resulting in PML redistribution  
112 (39). In contrast, HHV-6A/B infection does induce dispersal of PML-NBs but reduces and  
113 increases their size (32), (33), (40). Intriguingly, HHV-6B IE1 has been shown to  
114 colocalize with PML during infection (32), (33) however, the role of this PML-IE1  
115 interaction remains unknown.

116

117 Considering that 1) PML is located at telomeres, 2) PML-NBs associate with proteins  
118 involved in homologous recombination and 3) viral integration occurs at telomeres, we  
119 hypothesize that PML likely plays a role in HHV-6A/B chromosomal integration. We  
120 addressed this hypothesis and could demonstrate that HHV-6A/B IE1 not only localizes  
121 with PML, but also the host telomeres. In addition, we could demonstrate that PML indeed  
122 plays a role in HHV-6A/B integration.

123

## 124 **Materials and methods**

### 125 **Cell lines and viruses**

126 HeLa cells with long telomeres (Hela LT) (41) and HEK293T (ATCC, Manassas, VA,  
127 USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning Cellgro,  
128 Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher  
129 Scientific, Waltham, MA, USA), nonessential amino acids (Corning Cellgro), HEPES,  
130 sodium pyruvate (Wisent Inc., St-Bruno, Québec, Canada), and 5 µg/ml plasmocin  
131 (Invitrogen, San Diego, CA, USA). U2OS (osteosarcoma) cells (ATCC) were cultured in  
132 the same medium but supplement with 10% of Nu serum (Corning Cellgro) instead of  
133 FBS.

134

### 135 **Plasmids**

136 Expression vectors for HHV-6A IE1 (pCDNA4/TO-IE1A) and HHV-6B IE1  
137 (pCDNA4/TO-IE1B) control vector (pCDNA4/TO) were described previously (42).  
138 Plasmids expressing PML isoforms were kindly provided by Jin-Hyun Ahn (43). To  
139 generate a PML-I lentiviral vector, the PML-1 gene was PCR amplified with *attB1* and

140 *attB2* sites added to the forward and reverse primer, respectively. The PCR amplicon was  
141 recombined into pDonor221 vector followed by a second recombination into pLenti  
142 CMV Hygro DEST vector, a kind gift from Eric Campeau and Paul Kaufman (Addgene  
143 plasmid # 17454) (44). The PML Double Nickase Plasmids (h2) (sc-400145-NIC-2) were  
144 bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

145

#### 146 **Immunofluorescence (IFA)**

147 Coverslips were incubated for 30 minutes in blocking solution (1 mg/ml BSA; 3% goat  
148 serum; 0.1% Triton X-100; 1 mM EDTA pH 8.0, in phosphate-buffered saline (PBS)).  
149 After blocking, coverslips were incubated for 1 hour in primary antibody diluted in  
150 blocking solution. Coverslips were washed with PBS, three times for five minutes.  
151 Coverslips were incubated for 30 minutes with secondary antibody diluted in blocking  
152 solution. Coverslips were washed with PBS, three times for five minutes. When the IFA  
153 was done, coverslips were air dried at room temperature and mounted with *SlowFade* Gold  
154 Antifade reagent containing DAPI (Invitrogen, Eugene, Oregon USA).

155

#### 156 **Immunofluorescence conjugated to *in situ* hybridization (IF-FISH)**

157 Cells on coverslips were stained as for IFA. Once IFA was completed, cells were fixed for  
158 2 minutes at room temperature with 1% paraformaldehyde in PBS. Coverslips were washed  
159 two times for five minutes with PBS. Cells were dehydrated with 5 minutes each  
160 consecutive ethanol baths (70%, 95%, 100%). Once dried, coverslips were placed upside  
161 down on a drop of hybridizing solution (70% formamide; 0.5% blocking reagent; 10 mM  
162 Tris-HCl pH 7.2; 1/1000 Cy3 or Cy5-TelC PNA probe). Samples were denatured for 10



163 minutes at 80°C on a heated block. Coverslips were incubated over night at 4°C in the dark  
164 and washed two times for 15 minutes in washing solution (70% formamide; 10 mM Tris-  
165 HCl pH 7.2). Coverslips were washed 3 time for 5 minutes with PBS and were air dried,  
166 slow fade was added and coverslips were sealed.

167

### 168 **Transfection assays**

169 U2OS cells were seeded at  $2 \times 10^5$  cells/well in a 6-well plate containing glass coverslips  
170 in 2 mL of medium. Cells were transfected 24 hours post-seeding with pCDNA4/TO,  
171 pCDNA4/TO-IE1A, pCDNA4/TO-IE1B expression vector using the *TransIT-LT1*  
172 Transfection Reagent (Mirus Bio LLC, Madison, WI, USA). After 48 hours of transfection,  
173 cells were washed 3 times with PBS and fixed in 2% of paraformaldehyde and used for  
174 immunofluorescence (IFA) assay. HeLa LT cells were seeded at  $1 \times 10^5$  cells/well in a 6-  
175 well plate containing glass coverslips in 2 mL of medium. Cells were transfected 24 hours  
176 post-seeding with pCDNA4/TO, pCDNA4/TO-IE1A, pCDNA4/TO-IE1B expression  
177 vector using Lipofectamine 2000 (Thermo Fischer Scientific). After 48 hours of  
178 transfection, cells were fixed in 2% paraformaldehyde and used for IFA.

179

### 180 **Infection assays**

181 U2OS cells were seeded at  $2 \times 10^5$  cells/well in a 6-well plate containing glass coverslips  
182 in 2 mL of medium. Cells were infected 24 hours post-seeding with U1102 (HHV-6A) and  
183 Z29 (HHV-6B). After 48 hours post-infection, cells were washed 3 times with PBS and  
184 fixed in 2% of paraformaldehyde and used for immunofluorescence (IFA) assay.

185

## 186 **Generation of PML Knockout cell line**

187 HeLa LT and U2OS cells were transfected with CRISPR-Cas9 vector targeting PML as  
188 described. After 48 hours, cells were selected with 1 µg/mL of puromycin. Selected cells  
189 were harvested, counted and seeded at a density of 1 cell per well in three 96-well flat-  
190 bottom plates. After 10 to 14 days, wells containing only a single clone were identified.  
191 Clones were propagated for an additional 3 weeks and transferred into wells of a 12-well  
192 plate. Clones were screened by PCR, sequenced and analyzed by IFA for PML expression.  
193 PML negative clones were expanded and kept frozen until used.

194

## 195 **HHV-6A/B integration assays**

196 Integration assays were performed as described previously (45). Briefly, ten thousand  
197 cells per well (U2OS PML WT, U2OS PML<sup>-/-</sup> #1, U2OS PML<sup>-/-</sup> #2, HeLa LT PML  
198 WT, HeLa LT PML<sup>-/-</sup> #1, HeLa LT PML<sup>-/-</sup> #2) were seeded in 48-well plates. The next  
199 day, the cells were infected with U1102 or Z29 at a multiplicity of infection (MOI) of 1  
200 followed by overnight incubation at 37°C. Cells were washed 3X with 1X PBS to remove  
201 unabsorbed virions prior to the addition of fresh culture medium. Upon infection, cells  
202 were passaged for 4 weeks and analyzed by droplet digital PCR (ddPCR). For this, DNA  
203 was isolated using the QiaAMP blood extraction kit as described by the manufacturer  
204 (Qiagen Inc., Toronto, ON, Canada).

205

## 206 **qPCR**

207 qPCR was performed as described previously by Gravel et al. (45). Briefly, DNA was  
208 extracted using QiaAMP blood extraction kit as described by the manufacturer (Qiagen

209 Inc.) and analyzed using primers and probes against *U65-66* (HHV-6A/B) and *RPP30*  
210 (reference gene). Data was normalized against the corresponding genome copies of the  
211 cellular *RPP30* protein.

212

### 213 **Quantification of HHV-6A/B integration by droplet digital PCR (ddPCR)**

214 The HHV-6A/B copy number per cell was determined by ddPCR as previously described  
215 by Sedlak et al. (46).

216

### 217 **Statistical analysis**

218 Unpaired t-test with Welch correction was used to compare the number of PML-NBs at  
219 telomeres in IE1 expressing and control cells. It was also used to compare the number of  
220 IE1 at telomeres in PML<sup>+/+</sup> and PML<sup>-/-</sup> cells. Chi-square analysis was used to compare  
221 integration frequency between PML<sup>+/+</sup> and PML<sup>-/-</sup> cell lines.

222

## 223 **Results**

### 224 **IE1A/B localize at the site of integration, the telomeres.**

225 Upon cell entry, HHV-6A/B can either actively replicate or establish latency. This  
226 decision is often influenced by the permissivity of the target cells. We have previously  
227 shown that U2OS and Hela cells are semi-permissive to infection,  
228 as the HHV-6A/B initiates replication in only a minority of cells despite considerable  
229 expression of IE and E proteins (45), (47). Both cell lines have been extensively used  
230 to assess HHV-6A/B integration (48), (49), (50). To determine if IE proteins might  
231 contribute to HHV-6A/B integration, we first determined whether they localize to sites

232 of integration, the telomeres. U2OS cells were infected with HHV-6A (U1102) or  
233 HHV-6B (Z29) for 2 days and analyzed for IE1 expression by confocal microscopy.  
234 IE1 was detected as distinct nuclear foci upon infection (Fig 1A), with a proportion of  
235 IE1 localizing with telomeres (yellow asterisks). Quantification of Z stacks revealed  
236 that 20.4% and 26.38% of the IE1A/B foci (red) localize with telomeres (Fig 1B).  
237 To assess if IE1A or IE1B localize with cellular or viral telomeres, we transfected U2OS  
238 cells with IE1A/B expression vectors and analyzed IE1 localization in the absence of  
239 viral genomes. Ectopically-expressed IE1A and IE1B localized with cellular telomeres  
240 to the same extent as during infection (Fig 2A and B), indicating that cellular telomeres  
241 were targeted by these viral proteins.

242

### 243 **Both IE1A and IE1B colocalize with PML**

244 We previously demonstrated that IE1 of HHV-6B associates with PML-NBs during  
245 productive T cell infection (31). We next determined IE1A and IE1B colocalization with  
246 PML-NBs would also be observed in semi-permissive cells. IE1A and IE1B expression  
247 vectors were transfected in U2OS cells and their localization was assessed by IFA. IE1  
248 from both viruses efficiently associated with PML (Fig 3).  
249 PML is actually not a single protein but a mixture of seven different isoforms, whereby  
250 the first six isoforms (I to VI) are nuclear proteins (51). To determine if IE1  
251 preferentially colocalizes with certain PML isoforms, we co-transfected PML negative  
252 (PML<sup>-/-</sup>) cells with individual expression plasmids for the six nuclear PML isoforms and  
253 IE1 expression vectors. Western blotting confirmed that all six PML isoforms are  
254 efficiently expressed upon transfection of PML<sup>-/-</sup> cells (Fig 4A). IE1B colocalized with

255 all PML isoform tested. Similarly, IE1A localized with all 6 PML isoforms (data not  
256 shown).

257

### 258 **Presence of IE1A/B affects the number of PML-NBs present at cellular telomeres**

259 U2OS cells do not express telomerase and elongate their telomeres via alternative  
260 lengthening of telomere mechanisms (ALT) (52), (41), (53). In ALT cells, a significant  
261 proportion (75%) of PML-NBs localize at telomeres and are referred to as ALT-associated  
262 PML-NBs (APBs) (Fig 5) (54). In HeLa LT cells that rely mostly on the telomerase complex  
263 for telomere elongation the number of APBs was much lower (Fig 5).

264 Since IE1A/B colocalize with PML-NBs, we next assessed whether IE1's presence might  
265 affect PML-NBs localization at telomeres. U2OS and HeLa LT cells were transfected with  
266 IE1A/B expression vectors, and the proportion of PML-NBs localizing to telomeres was  
267 determined by IF-FISH (Fig 6A and B). The frequency of PML-NBs located at the host  
268 telomeres was reduced by 58% (25.8+/- 22.53) in U2OS cells upon expression of IE1A  
269 compared to the empty vector control (63.35+/- 16.97) (Fig 6C). A comparable reduction  
270 in APBs of 50% was also observed in U2OS cells expressing IE1B. We confirmed this  
271 observation in HeLa LT cells, where the PML-NBs localizing at telomeres was reduced by  
272 64% and 61% upon expression of IE1A and IE1B respectively. Similar results were  
273 obtained in U2OS and HeLa LT cells infected with HHV-6A/B (data not shown).

274

### 275 **The absence of PML does not affect the presence of IE1A/B at telomeres.**

276 Considering that IE1A/B colocalize with PML-NBs and that a significant proportion of  
277 PML-NBs are located at host telomeres, we next determined if PML contributes to the

278 localization of IE1A/B's to the telomeres. PML knockout (KO) U2OS and HeLa LT cell  
279 were generated using the CRISPR-Cas9 system (Fig 7). Deletion of a part of exon 1 (Fig  
280 7A) resulted in a pre-mature STOP codon resulting in a short truncated PML protein (Fig  
281 7B). Abrogation of PML expression was confirmed in U2OS (Fig 7C) and HeLa LT (Fig  
282 7D) cells by IFA.

283 Following transfection of IE1A/B expression vectors in WT and PML<sup>-/-</sup> U2OS cells, we  
284 observed that IE1A/B localized at telomeres despite PML's absence, albeit at a slightly  
285 lower frequency (Fig 8A and B). There was however an increased proportion of cells in  
286 which IE1A/B were not present at telomeres. As shown in Fig 8C, the number of U2OS  
287 PML<sup>-/-</sup> nuclei with no IE1A/B at telomeres was significantly increased relative to WT  
288 nuclei (\*\*p<0.0001).

289

### 290 **PML is required for efficient HHV-6A/B chromosomal integration.**

291 Considering that a proportion of PML-NBs localize at telomeres and that PML plays a role  
292 in DNA repair by homologous recombination, we investigated if PML plays a role in HHV-  
293 6A/B integration into telomeres. PML knockout and control cells lines were infected with  
294 HHV-6A or HHV-6B and integration frequency assessed after four weeks post infection  
295 by droplet digital PCR as described (48). The absence of PML was confirmed at the  
296 beginning (T0) and the end (T28) of the experiment by IFA for U2OS (Fig 9A) and HeLa  
297 LT cells (Fig 10A). ddPCR revealed that HHV-6A and -6B integration was significantly  
298 reduced in U2OS cells in the absence of PML by 64% and 50% respectively (Fig 9B and  
299 C). In HeLa LT cells, HHV-6A integration was reduced by approximately 50% in the  
300 absence of PML (Fig 10B). The reductions were even more pronounced for HHV-6B in

301 HeLa LT cells where the integration frequency was reduced by 73% and 90.6% in the two  
302 independent clones used in this study (Fig 10C). Taken together, our data demonstrates  
303 that integration occurs less efficient in the absence of PML in two standard models for  
304 HHV-6A/B integration.

305

## 306 **Discussion**

307 One key interest of our laboratory is to identify proteins that facilitate HHV-6A/B  
308 chromosomal integration. We previously demonstrated that the putative HHV-6A/B  
309 integrase U94 possesses DNA binding, helicase and exonuclease activity, suggesting that  
310 the protein could be involved in HHV-6A/B integration (55). However, recombinant  
311 HHV-6A lacking U94 integrated as efficient as WT virus, indicating that U94 is  
312 dispensable for integration of HHV-6A in vitro (50).

313

314 Another hypothesis is that HHV-6A/B chromosomal integration occurs with the help of  
315 telomerase, the enzyme responsible for telomere elongation (56). We have previously  
316 shown that telomerase is not essential for HHV-6A/B integration, as it occurs in both  
317 telomerase negative and positive cells (48), (49). However, in telomerase expressing cells,  
318 telomerase is likely important for the generation a neo-telomere at the end of DR<sub>L</sub>  
319 (reviewed in (57)). In support, blockade of telomerase activity by the G-quadruplex  
320 (guanine rich structure (G4) present in telomeres) stabilizing agent BRACO-19, negatively  
321 affects HHV-6A integration (49). Such effect is not observed in telomerase negative cells  
322 such as U2OS cells.

323

324 The fact that HHV-6A telomeric repeats are required for efficient integration (30) argues  
325 in favor of a homologous recombination (HR) events between cellular and viral telomeres.  
326 Cellular telomeres are protected by the shelterin complex that consists of 6 proteins:  
327 telomeric repeat binding factor 1 (TRF1), TRF2, protection of telomere 1 (POT1), telomere  
328 protection protein 1 (TPP1), TRF-interacting nuclear protein 2 (TIN2) and repressor  
329 activation protein 1 (RAP1) (58). The main function of the shelterin complex is to protect  
330 chromosome end from being recognized as damaged DNA. Of the 6 shelterin proteins,  
331 TRF2 is the key factor that blocks DNA repair proteins at telomeres by inhibiting the  
332 Ataxia-telangiectasia-mutated (ATM) pathway that senses double-stranded DNA breaks  
333 (59). In addition to the shelterin complex, other proteins can also localize to telomeres. In  
334 telomerase negative cells such as U2OS cells, telomeres are elongated by an Alternative  
335 Lengthening of Telomeres (ALT+) associated PML-Nuclear Bodies (APBs) mechanism  
336 (60),(54),(61),(62). These nuclear bodies primarily formed by the Promyelocytic Leukemia  
337 Protein (PML) itself that recruits hundreds of interacting partners at telomeres such as  
338 helicases implicated in G-quadruplex structure resolution like the bloom syndrome protein  
339 (BLM), the Werner Syndrome Protein (WRN) and other protein implicated in DNA  
340 recombination and repair (63), (54), (64), (65), (53). Osterwalds et al. have shown that in  
341 ALT<sup>+</sup> cells such as U2OS, PML-NBs (APBs) are frequently present at telomeres. We've  
342 confirmed this result (Fig 5). We also noticed that a significant proportion of PML-NBs  
343 also localize to telomeres of telomerase expressing cells such as HeLa LT cell (Fig 5), in  
344 agreement with Marchesini et al (63). Marchesini demonstrated that PML is essential for  
345 telomere maintenance in non-neoplastic cells, as cells undergo apoptosis in absence of  
346 PML after DNA damage at these sites (63). These findings support the role of PML in



347 DNA repair mechanism. Considering this, we hypothesized that PML could aid in HHV-  
348 6A/B integration.

349

350 In addition to their roles in telomere stability, PML-NBs have antiviral defense functions  
351 (36), (35). In contrast to many other viruses including HSV, CMV, EBV and HHV-8,  
352 HHV-6A/B infection does not lead to the dispersal of PML-NBs but rather to PML-NBs  
353 coalescence (38), (66), (67), (68), (33). Whether this affects antiviral functions of PML-  
354 NBs remains to be determined. We could demonstrate that the HHV-6A/B IE1 protein, a  
355 protein that play roles in innate immune evasion mechanisms (69), (42), is associated with  
356 PML-NBs (31). Here we also report that IE1A/B also associates with telomeres.  
357 Considering that PML also associates with telomeres, we hypothesized that IE1A/B  
358 localization to telomeres could be PML dependent. However, in PML<sup>-/-</sup> U2OS and HeLa  
359 LT cells, a significant proportion of IE1A/B remained associated with telomeres. However,  
360 the proportion of nuclei in which IE1A/B could not be detected at telomeres was largely  
361 increased in PML<sup>-/-</sup> cells. Thus, although not essential, PML does influence the  
362 localization of IE1A/B at telomeres. One possible explanation resides in the fact that  
363 IE1A/B are SUMOylated proteins and that PML itself and/or other PML-NB associated  
364 proteins contain SUMO interacting motif (SIM) could facilitate interactions at telomeres  
365 (31), (70). Moreover, IE1A/B also possess putative SIMs that can bind SUMOylated  
366 proteins present at telomeres, possibly explaining why IE1A/B can localize at telomeres in  
367 the absence of PML.

368

369 Finally, we tested if PML played a role in HHV-6A/B chromosomal integration. We used  
370 the CRISPR-Cas9 to abrogate PML expression (Fig 7). For each cell line used, two  
371 independent PML KO clones were assessed to ensure reproducibility and avoid potential  
372 off-target effects. In U2OS cells (Fig 9), HHV-6B integration was less frequent in PML<sup>-/-</sup>  
373 cells ( $p < 0.0001$ ). In HeLa LT cells (Fig 10), the same effect was observed for HHV-6A  
374 and HHV-6B. Of note, integration rates are higher in U2OS presumably because of higher  
375 constitutive DDR repair in the cells, supporting the hypothesis that DNA repair  
376 mechanisms are involved in the HHV-6A/B integration process (71). Globally, both cell  
377 lines studied suggest a role for PML in HHV-6A/B integration. However, since integration  
378 still occurred in PML KO cells indicates that PML contributes but is not essential for this  
379 process. The positive influence of PML on HHV-6A/B integration could be explained by  
380 a reduction of protein present at telomeres like those involve in the DDR. As mentioned  
381 above, TRF2 blocks DNA repair at double DNA strand breaks. Moreover, in PML-NBs at  
382 telomeres, TRF2 is SUMOylated by MMS21, resulting in a lower density of TRF2 on  
383 telomeres (72). These telomere regions can then be processed by other proteins and  
384 recombine with the HHV-6A/B telomeres.

385

386 In conclusion, we have demonstrated that HHV-6A/B IE1 proteins colocalize with all  
387 isoforms of PML and host telomeres. Abrogation of PML expression influences the  
388 presence of IE1 at telomeres and affects HHV-6A/B integration into host telomeres. To our  
389 knowledge, this is the first report of a cellular protein that is involved in HHV-6A/B  
390 integration.

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## 664 **Figures**

665

666 **Fig 1. Colocalization of IE1A/B at telomeres in infection.** U2OS cells were infected  
667 with U1102 (HHV-6A) and Z29 (HHV-6B). A) 48 hours post-infection, cells were fixed

668 with 2% paraformaldehyde and labeled for IF-FISH. IE1A/B was detected using anti-IE1-  
669 ALEXA-568 (red) labeled antibodies and telomeres were detected using a Cy5-labeled  
670 telomeric probe (Aqua). B) Percentage of IE1A/B foci localizing at telomeres in infected  
671 cells. P value was determined using an unpaired t-test with Welch correction. ns: p value  
672 is not significant.

673

674 **Fig 2. Colocalization of IE1A/B at telomeres in transfection.** (A) U2OS cells were  
675 transfected with pCDNA4TO (CTRL) and pCDNA4TO-IE1A/B expression vectors. Cells  
676 were analyzed by immunofluorescence (IFA) 48 hours post-transfection, using anti-IE1  
677 ALEXA-568-labeled (red) and anti-PML ALEXA-488-labeled antibodies (green). (B)  
678 Percentage of IE1A/B foci localizing at telomeres in transfected cells. P value was  
679 determined using an unpaired t-test with Welch correction. \*P<0.04.

680

681 **Fig 3. Ectopic IE1A/B colocalize with PML.** U2OS were transfected with IE1A/B  
682 expression vectors. 48 hours post-transfection, cells were analyzed by IFA using anti-PML  
683 ALEXA-488-labeled (green) and anti-IE1 ALEXA-568-labeled (red) antibodies.

684

685 **Fig 4. IE1A/B colocalize with all nuclear PML isoforms.** (A) HEK293T were  
686 transfected with PML isoforms expression vectors and analyzed by western blot using anti-  
687 Myc antibodies. U2OS PML<sup>-/-</sup> were co-transfected using pCDNA4TO-IE1B (B) vectors  
688 along with vectors expressing the various PML isoforms (I to VI). 48 hours post-  
689 transfection, cells were analyzed by IFA using anti-Myc ALEXA-488-labeled (green) and  
690 anti-IE1 ALEXA-568-labeled (red) antibodies.

691

692 **Fig 5. PML proteins colocalize at telomeres in U2OS and HeLa LT cells.** (A) U2OS  
693 cells (ALT+) and HeLa LT cells (telomerase +) were grown on coverslips and fixed with  
694 2% paraformaldehyde at sub confluence. Cells were analyzed by IF-FISH. PML proteins  
695 were detected using anti-PML ALEXA-488-labeled (green) antibodies and telomeres were  
696 detected using a Cy3-labeled telomeric probe (red). (B) The number of PML foci localizing  
697 at telomeres was calculated after analysis of U2OS (N=20) and HeLa LT (N=40) nuclei.

698

699 **Fig 6. IE1A/B colocalize with PML at telomeres.** U2OS (A) and HeLa LT (B) cells were  
700 transfected with pCDNA4/TO-IE1A or pCDNA4/TO-IE1B. 48 hours post-transfection,  
701 cells were fixed with 2% paraformaldehyde and analyzed by IF-FISH. IE1A/B were  
702 detected using anti-IE1-ALEXA-568-labeled antibodies (red), PML were detected using  
703 anti-PML ALEXA-488-labeled (green) antibodies and telomeres were labeled using a Cy5-  
704 labeled telomeric probe (Aqua). (C) Percentage of IE1A/B at telomeres in transfected  
705 U2OS (N=37) and HeLa LT (N=24) cells. P value was determined using an unpaired t-test  
706 with Welch correction. \*P<0.01; ns = p value is not significant. (D) Percentage of PML  
707 foci at telomeres in presence and in absence of ectopically expressed IE1A/B. CTRL:  
708 Empty vector. P value was determined using an unpaired t-test with Welch correction.  
709 \*\*\*p<0.0001

710

711 **Fig 7. Generation of PML Knockout cell lines.** U2OS and HeLa LT cells were  
712 transfected with expression vectors for Cas-9 nuclease and PML RNA guides. After  
713 puromycin selection, cells were seeded at a density of one cell/well to obtain unique clones.



714 (A) Each clone was screened by PCR with PML primers. Mutations were confirmed by  
715 sequencing the PCR amplicons. (B) Translation of the mutated sequence results into a  
716 truncated protein with three premature STOP codons. WT and PML<sup>-/-</sup> U2OS (C) and HeLa  
717 LT (D) cells were analyzed by IFA for PML expression using anti-PML ALEXA-488-  
718 labeled (green) antibodies.

719

720 **Fig 8. PML is dispensable for IE1A/B localization at telomeres** (A) U2OS<sup>-/-</sup> cells were  
721 transfected with pCDNA4TO-IE1A and pCDNA4TO-IE1B expression vectors. 48 hours  
722 post-transfection, cells were analyzed by IF-FISH for IE1A/B (red) and PML (green)  
723 expression using specific antibodies. Telomeres were detected using Cy5-labeled telomeric  
724 probe (Aqua). (B) Number of IE1A/B foci localizing at telomeres in the presence (N=37  
725 for IE1A PML<sup>+/+</sup>; N=24 for IE1B PML<sup>+/+</sup>) or in the absence of PML (N=46 for IE1A  
726 PML<sup>-/-</sup>; N=35 for IE1B PML<sup>-/-</sup>). P value was determined using an unpaired t-test with  
727 Welch correction. \*P<0.01. ns: p value is not significant. (C) Total number of IE1A/B that  
728 have no IE1A/B at telomeres was compared between PML<sup>+/+</sup> (N=37 for IE1A; N=24 for  
729 IE1B) and <sup>-/-</sup> cell lines (N=46 for IE1A; N=35 for IE1B). P value was determined using  
730 Chi-square analysis. \*\*\*P<0.0001

731

732 **Fig 9. HHV-6A/B integration in WT and PML<sup>-/-</sup> U2OS cells.** (A) PML expression in  
733 WT and PML<sup>-/-</sup> U2OS cells on day 0 and day 28 post-infection. WT and PML<sup>-/-</sup> U2OS  
734 cells were infected at a MOI of 1 with HHV-6A (B) and HHV-6B (C) and were cultured  
735 for a month. Cellular DNA was extracted, and integration frequency determined by ddPCR.  
736 Each integration assay was done three time for each cell lines (error bars). CTRL + :

737 iciHHV-6A/B donor DNA. P value was determined using Chi-square analysis. P value was  
738 determined using Chi-square analysis. \*\*\*P<0.0001; \*\*P<0.001

739

740 **Fig 10. HHV-6A/B integration in WT and PML<sup>-/-</sup> HeLa LT cells.** (A) PML expression  
741 in WT and PML<sup>-/-</sup> HeLa LT cells on day 0 and day 28 post-infection. WT and PML<sup>-/-</sup>  
742 HeLa LT cells were infected at a MOI of 1 with HHV-6A (B) and HHV-6B (C) and were  
743 cultured for a month. Cellular DNA was extracted, and integration frequency determined  
744 by ddPCR. Each integration assay was done three time for each cell lines (error bars).  
745 CTRL + : iciHHV-6A/B donor DNA. P value was determined using Chi-square analysis.  
746 \*\*\*P<0.0001; ns: p value is not significant.

747

## 748 **Supporting information**

749

750 **S1 Fig. PML KO does not create more DNA damages.** PML<sup>+/+</sup> and PML<sup>-/-</sup> cells from  
751 the integration assays at T28 were seeded on coverslips and fixed with 2% of  
752 paraformaldehyde. Cells were analyzed by IF-FISH for DNA damage protein 53BP1 (red)  
753 and PML (green) expression using specific antibodies. Telomeres and nuclei were detected  
754 using Cy5-labeled telomeric probe (Aqua). Number of 53BP1 foci per nuclei was counted  
755 for PML<sup>+/+</sup> U2OS cells (N=42), HeLa LT cells (N=37) and PML<sup>-/-</sup> U2OS cells (N=40)  
756 and HeLa LT cells (N=37). P value was determined using an unpaired t-test with Welch  
757 correction. ns: p value is not significant.

758

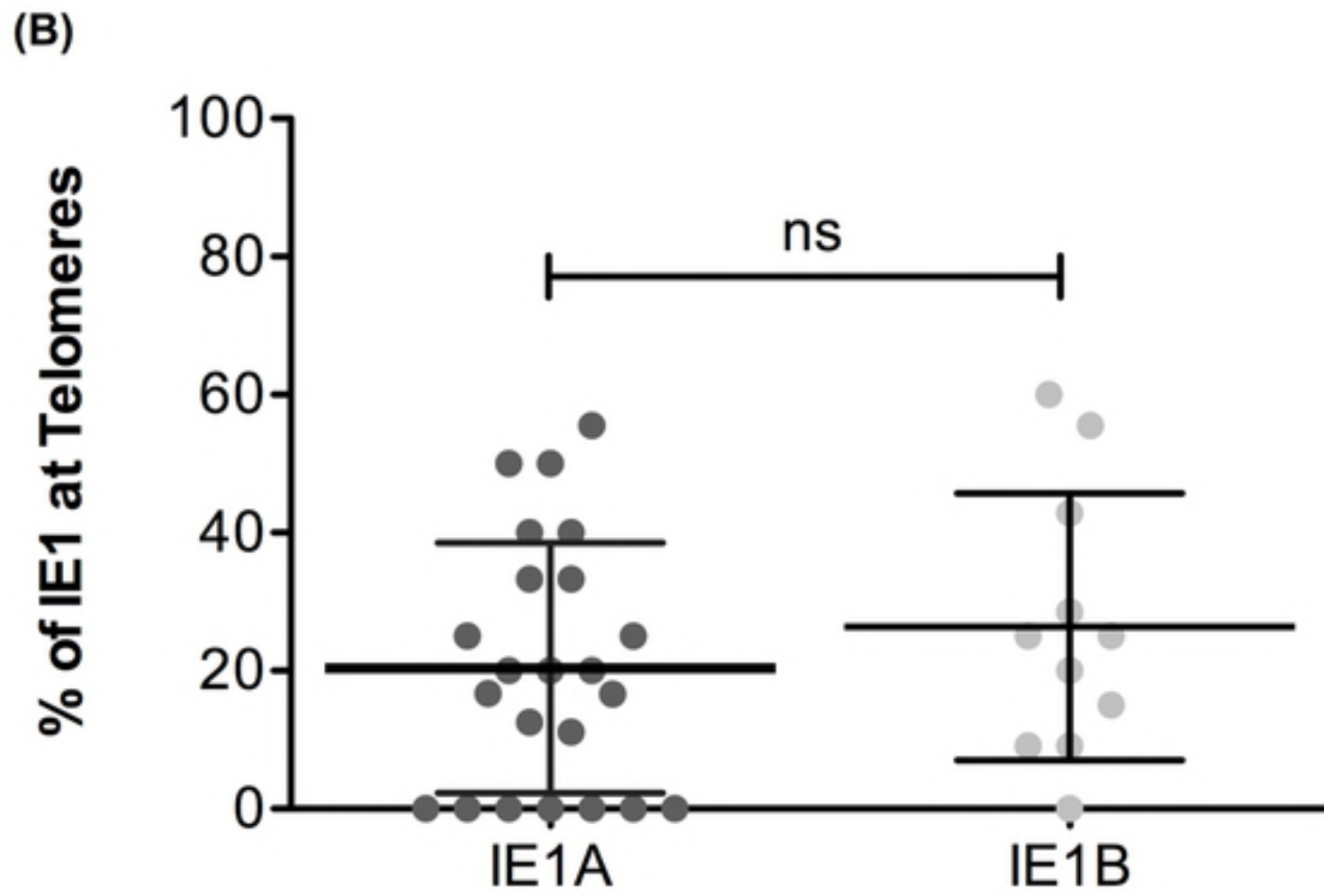
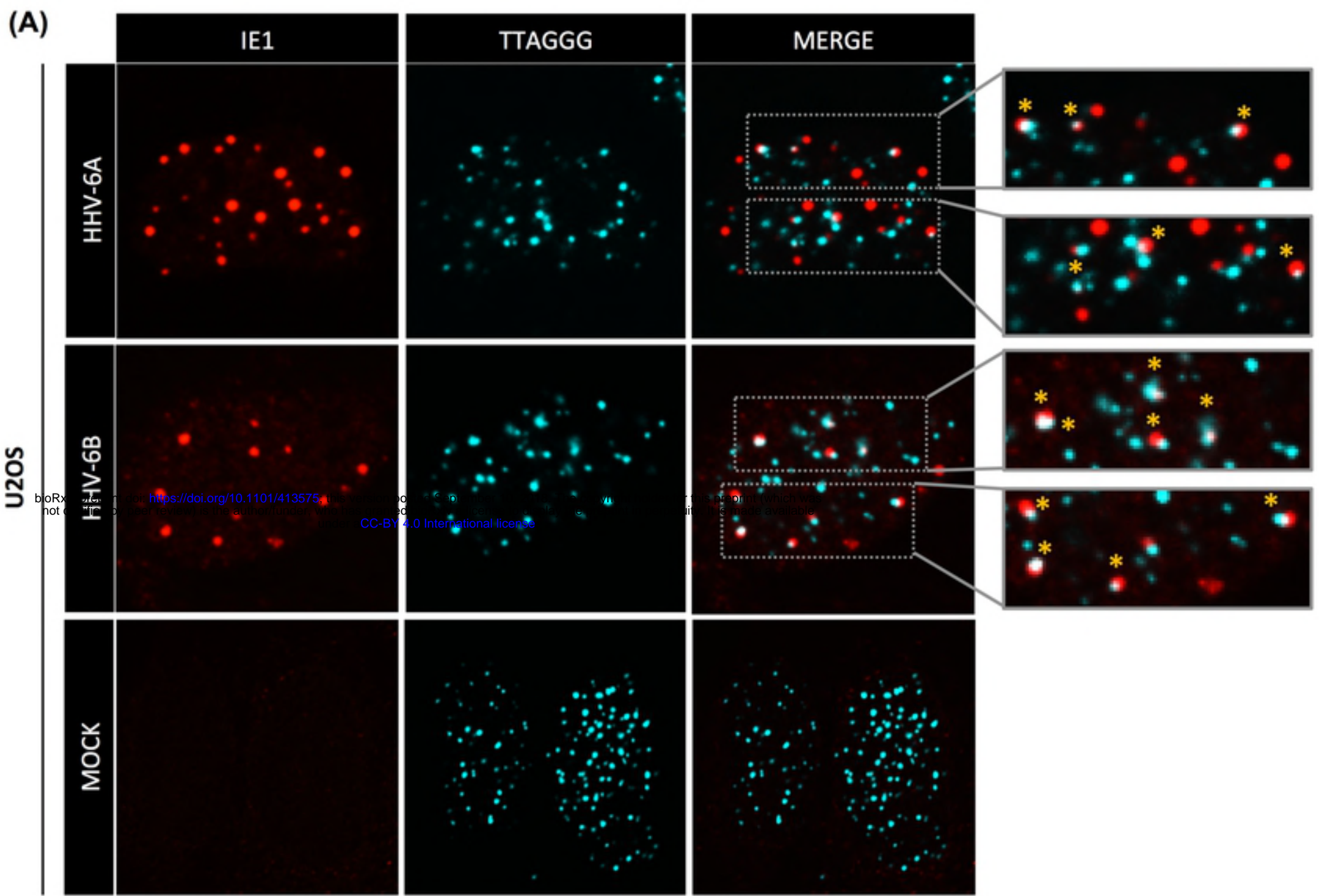
759 **S2. PML and IE1B localize in close proximity.** U2OS-Flp-In TREX cells were  
760 transfected with expression vectors containing FLAG-BirA-GFP and FLAG-BirA-IE1B  
761 and selected with hygromycin (250 $\mu$ g/ml) and blasticidin (50 $\mu$ g/ml). (B) Cells were seeded  
762 on coverslips and 24 hours later, 50nM of biotin was added to the medium for an additional  
763 24h before being fixed with paraformaldehyde 2%. IFA confirms BirA-GFP and BirA-  
764 IE1B expression (Flag) and biotinylation of proteins (Streptavidin-HRP). (C) Biotinylated  
765 proteins were immunoprecipitated with streptavidin magnetic beads followed by mass  
766 spectrometry.

767

768

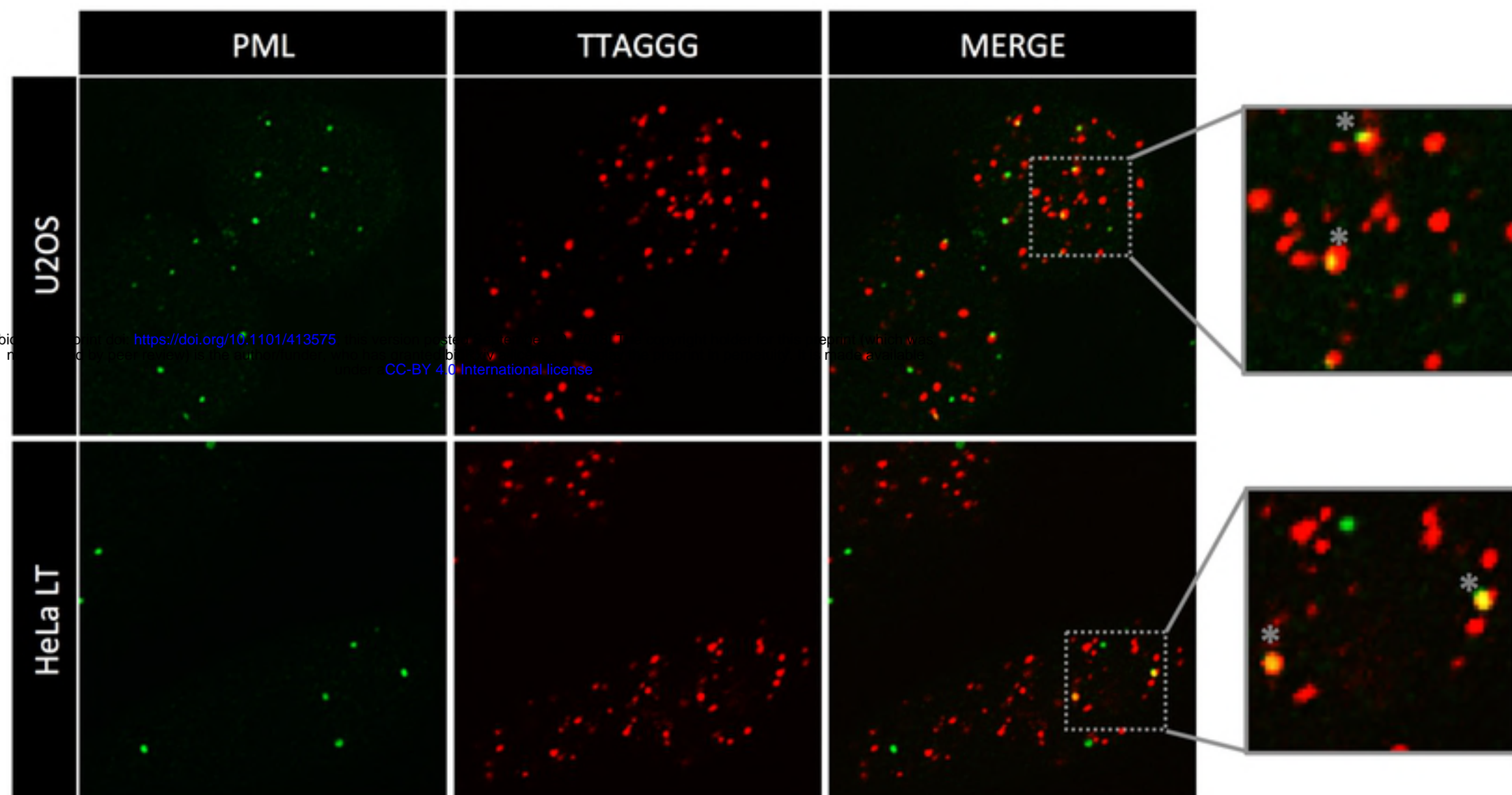
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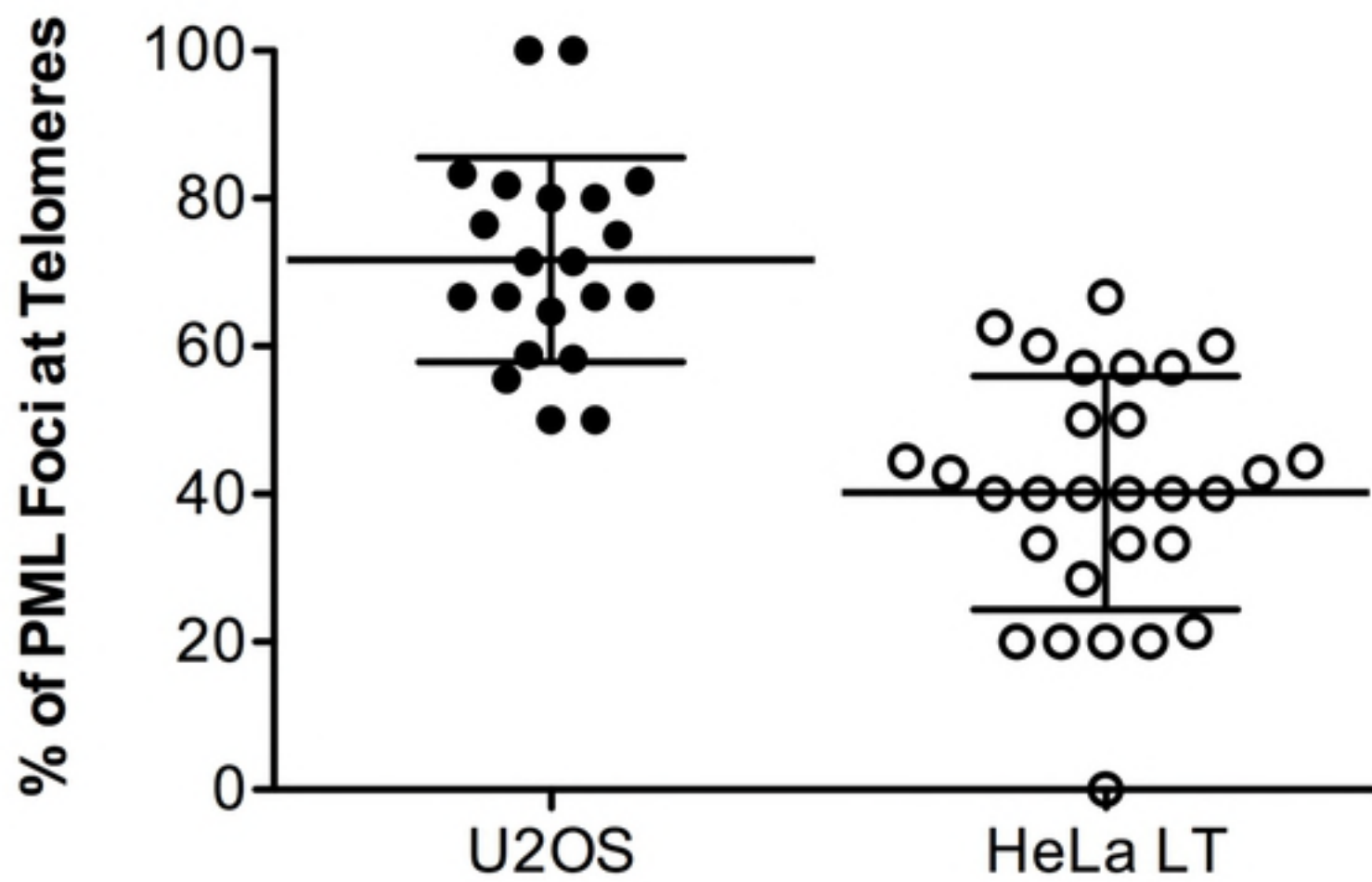


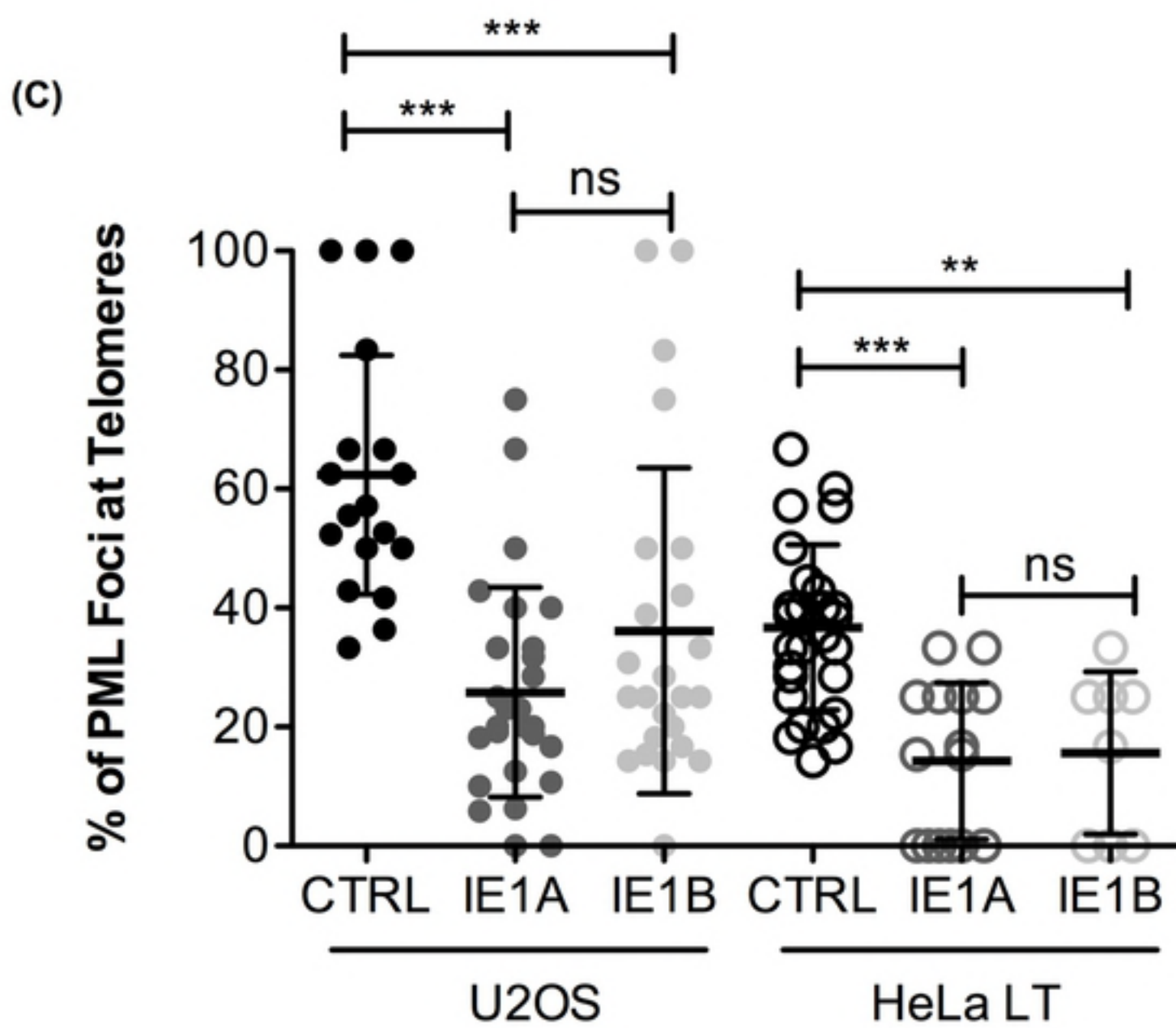
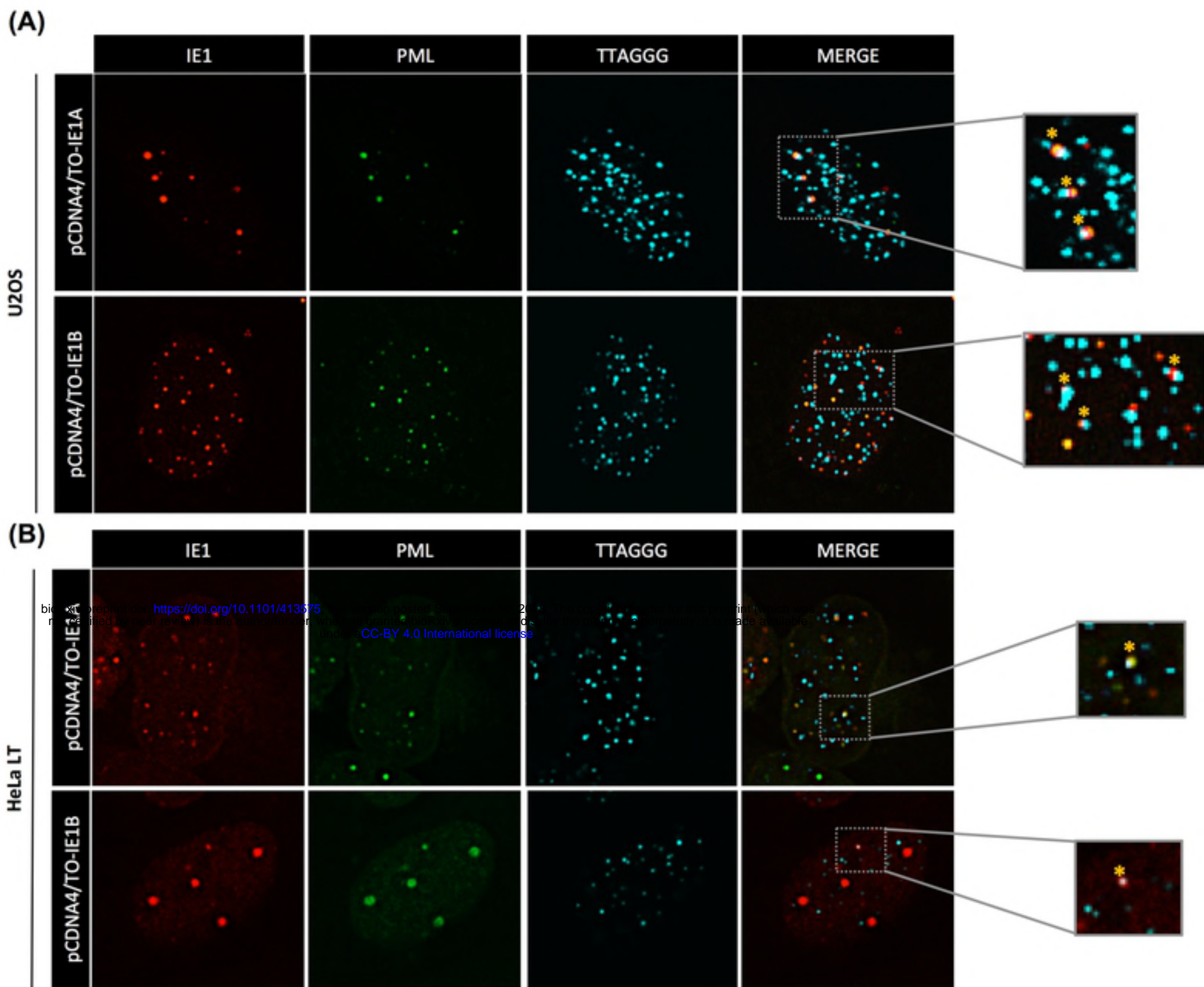


(A)



(B)





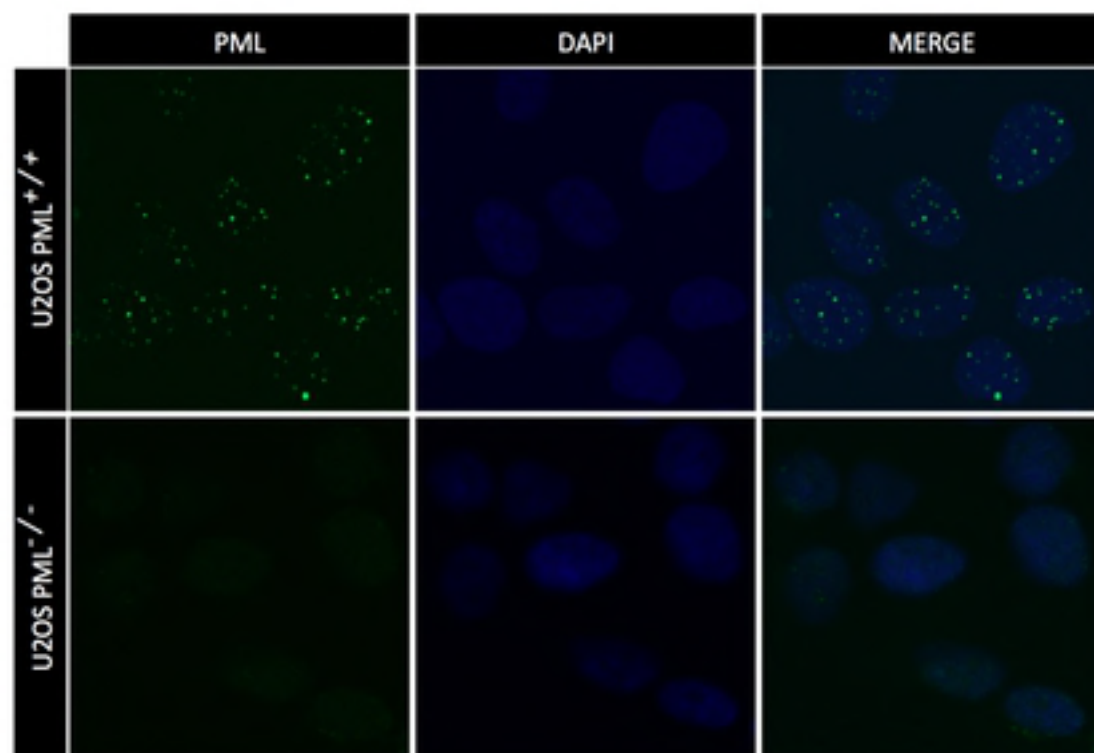
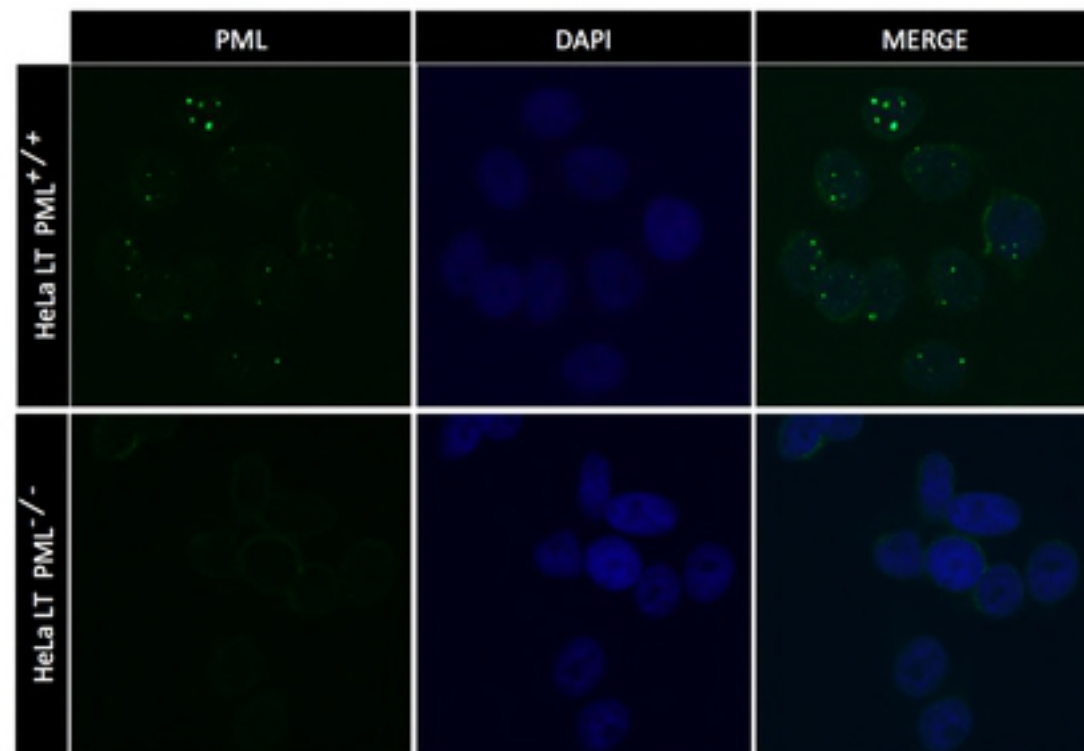
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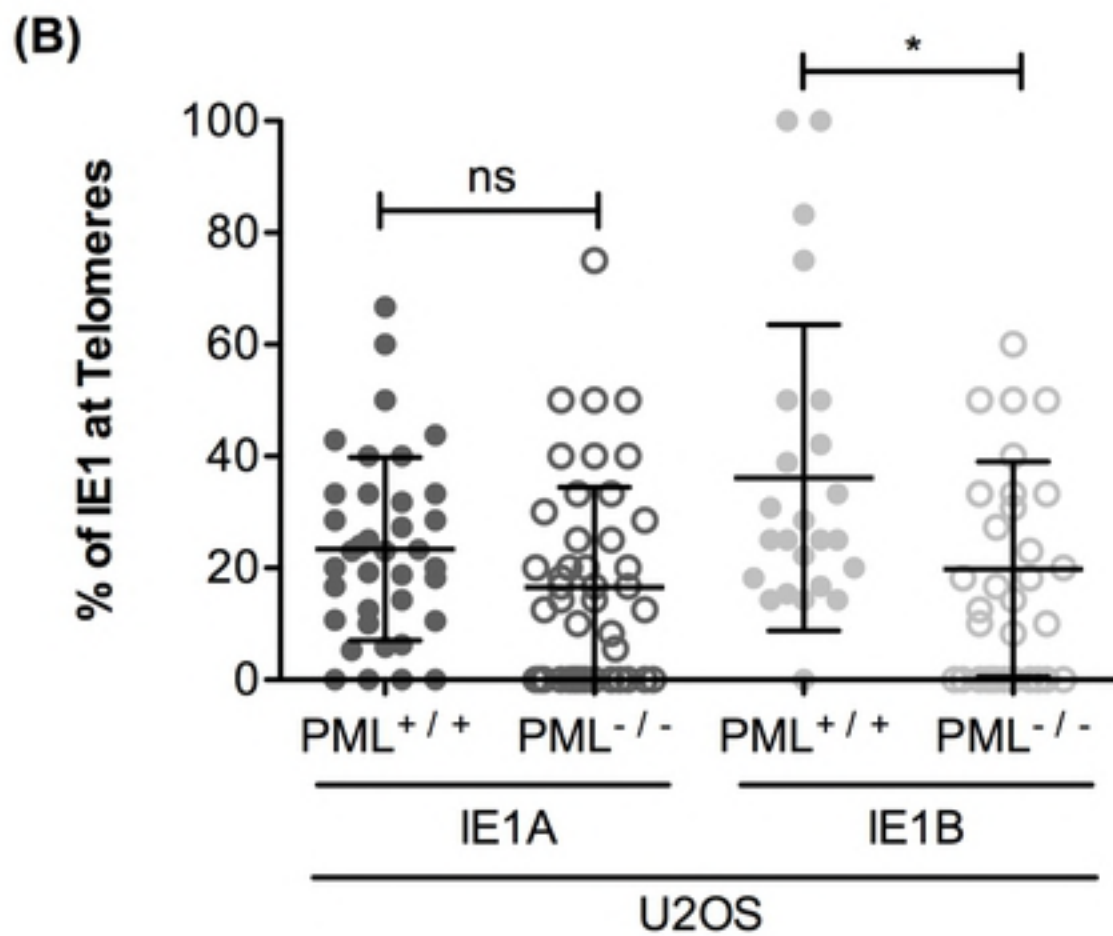
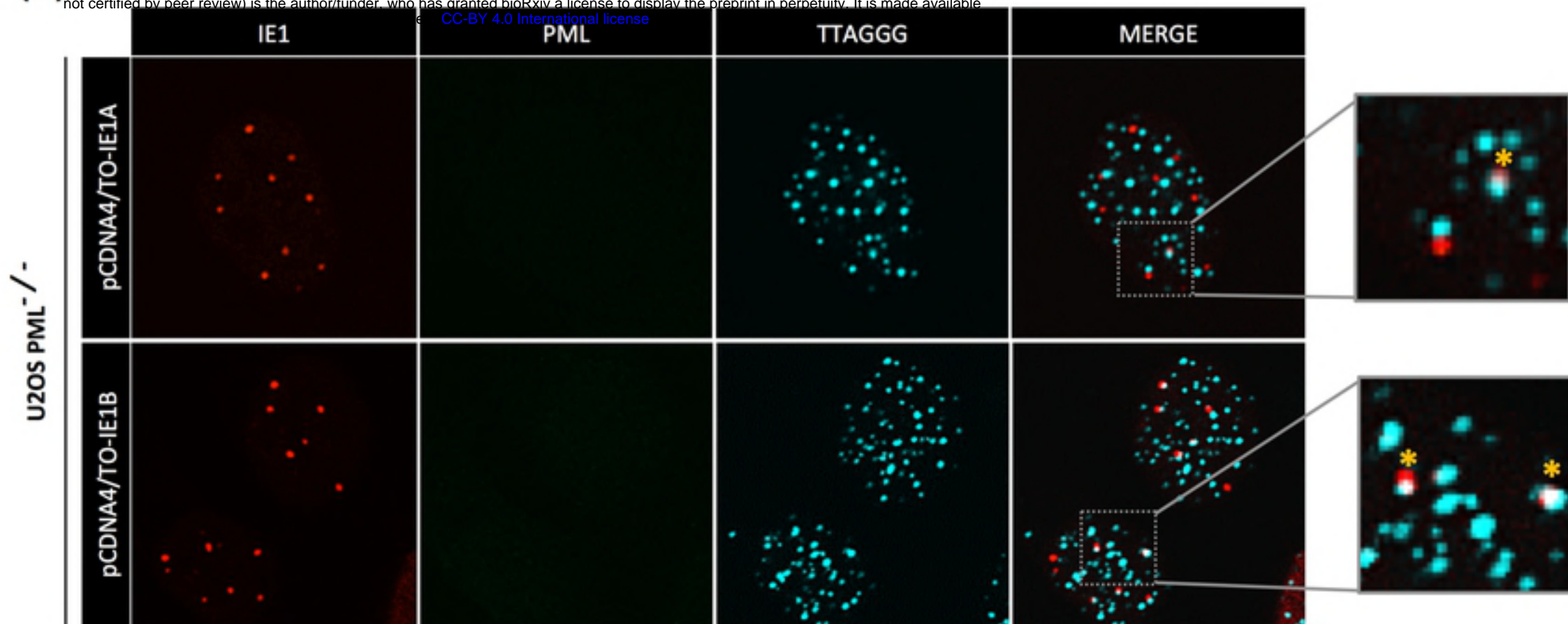
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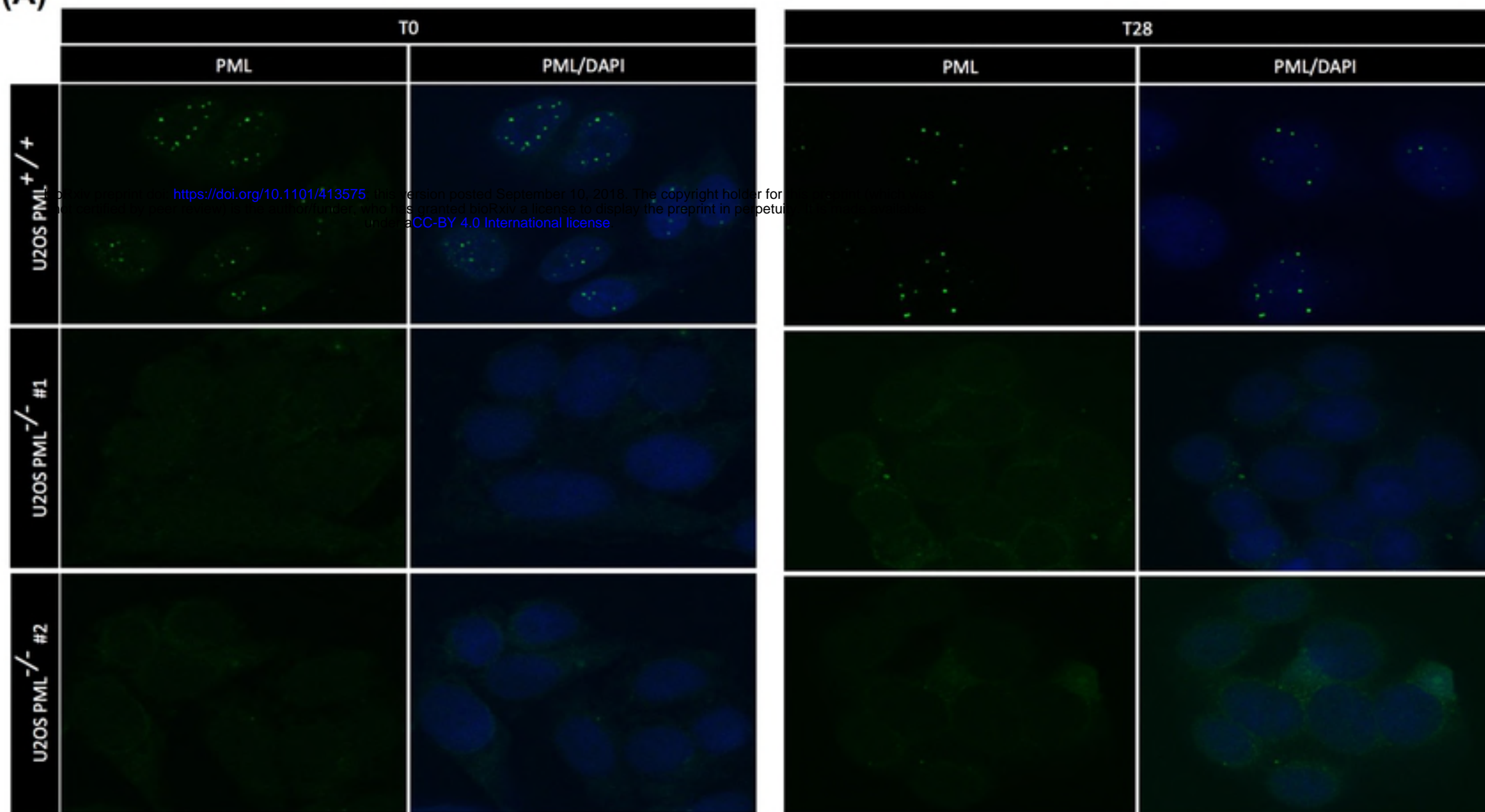
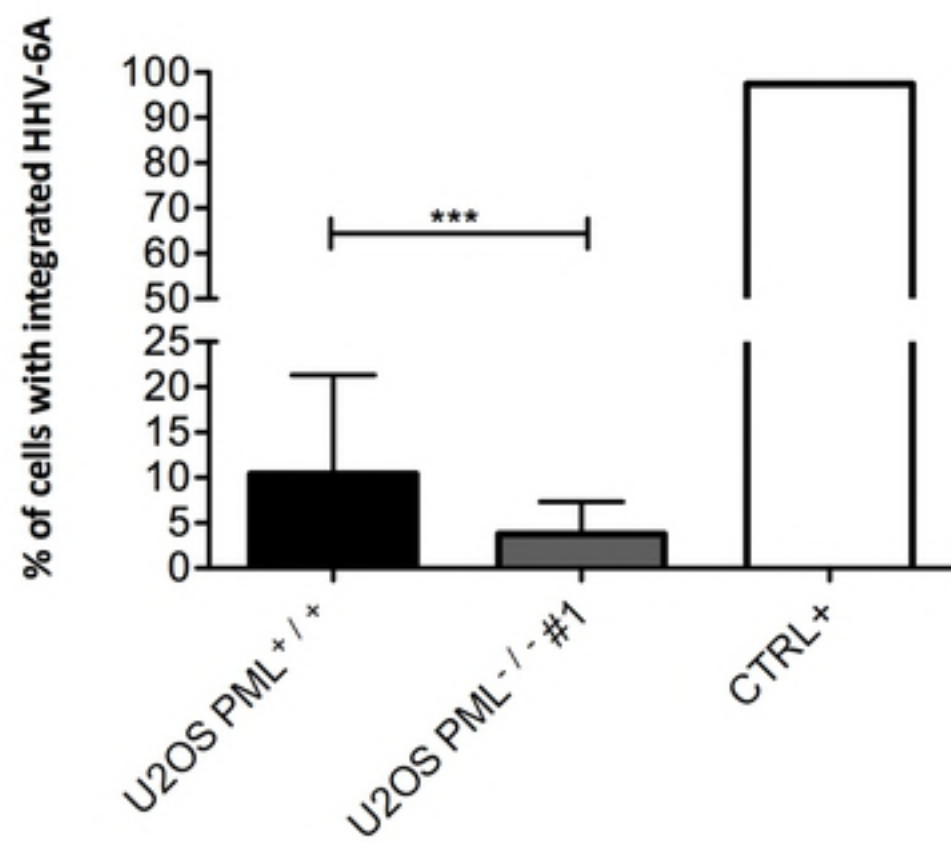
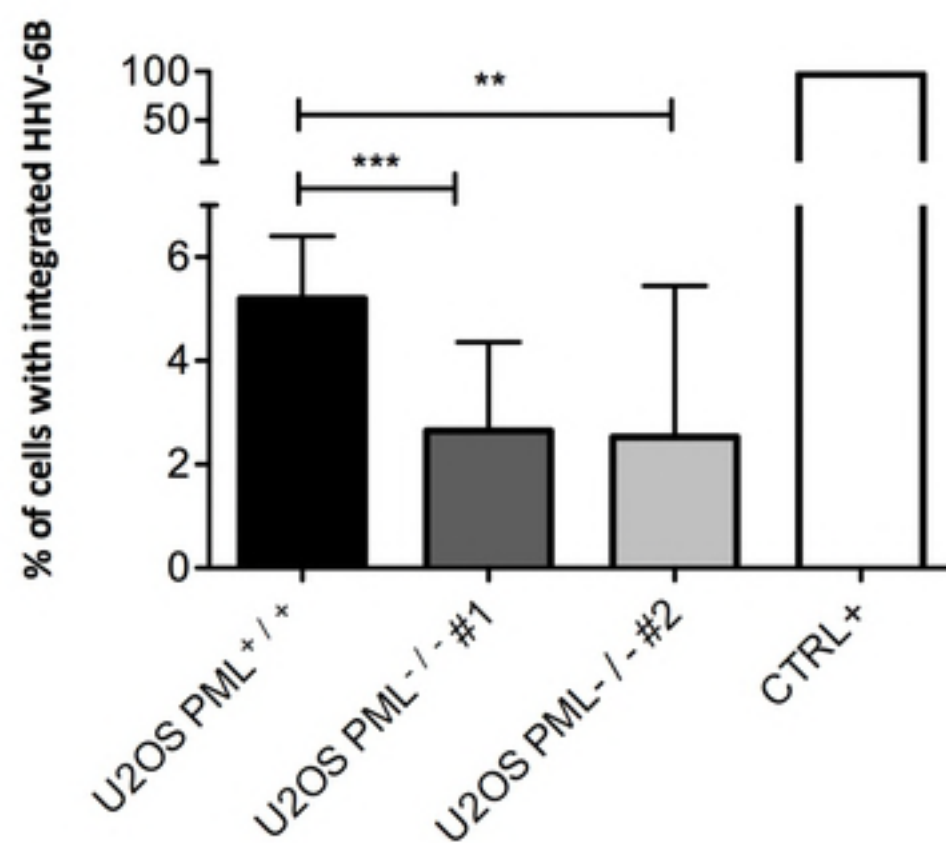
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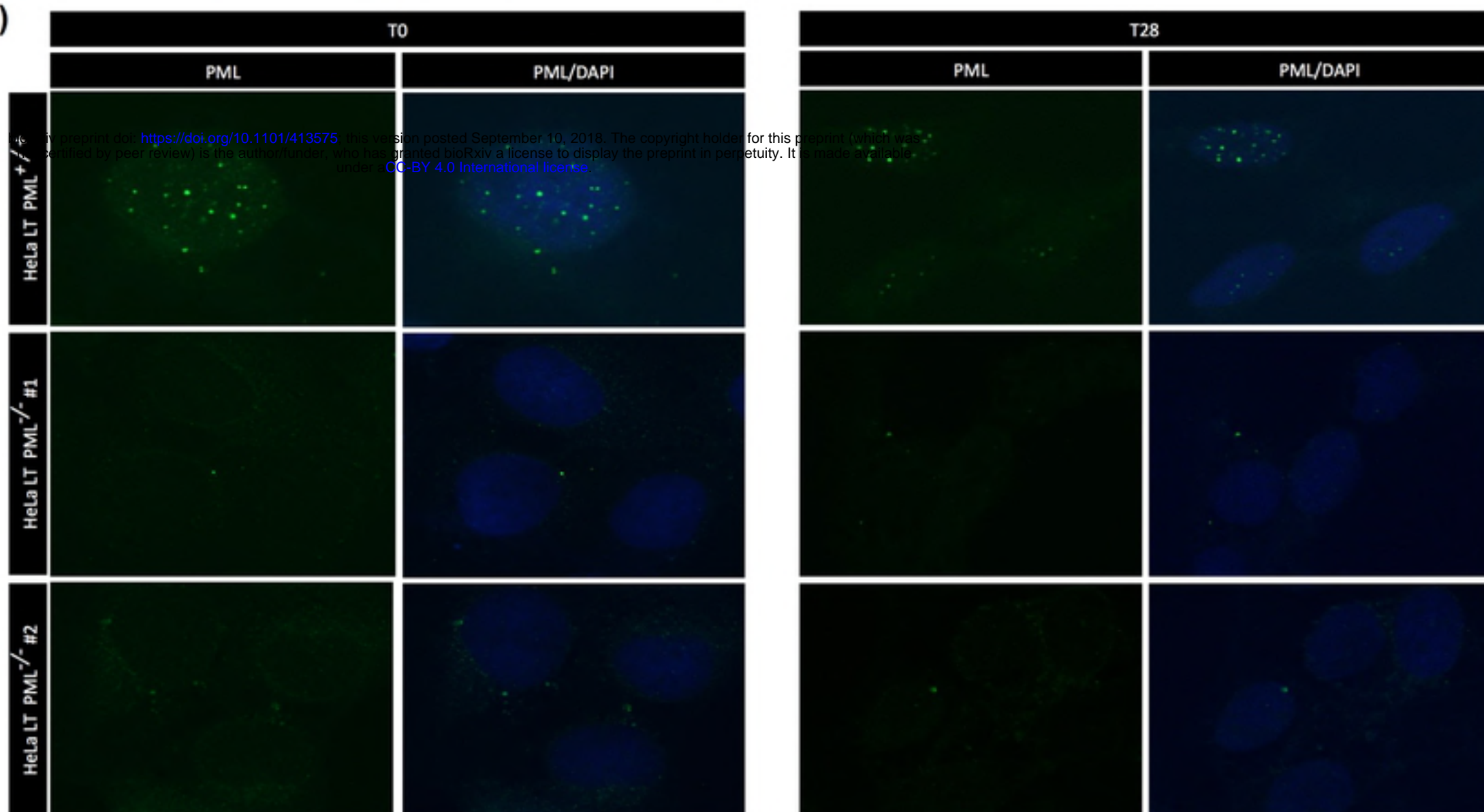
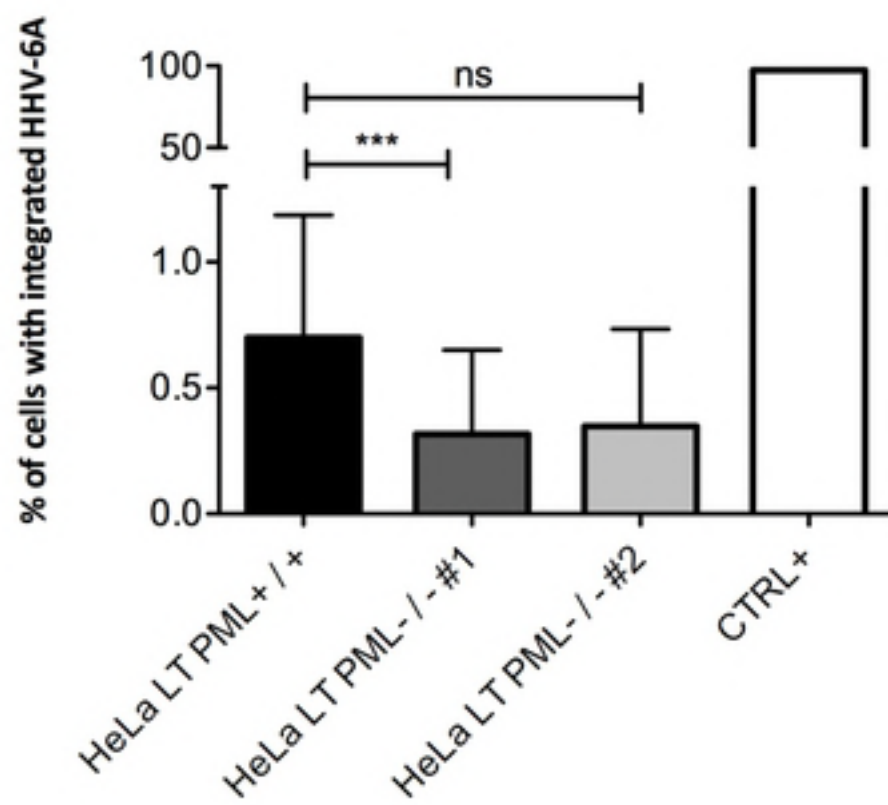
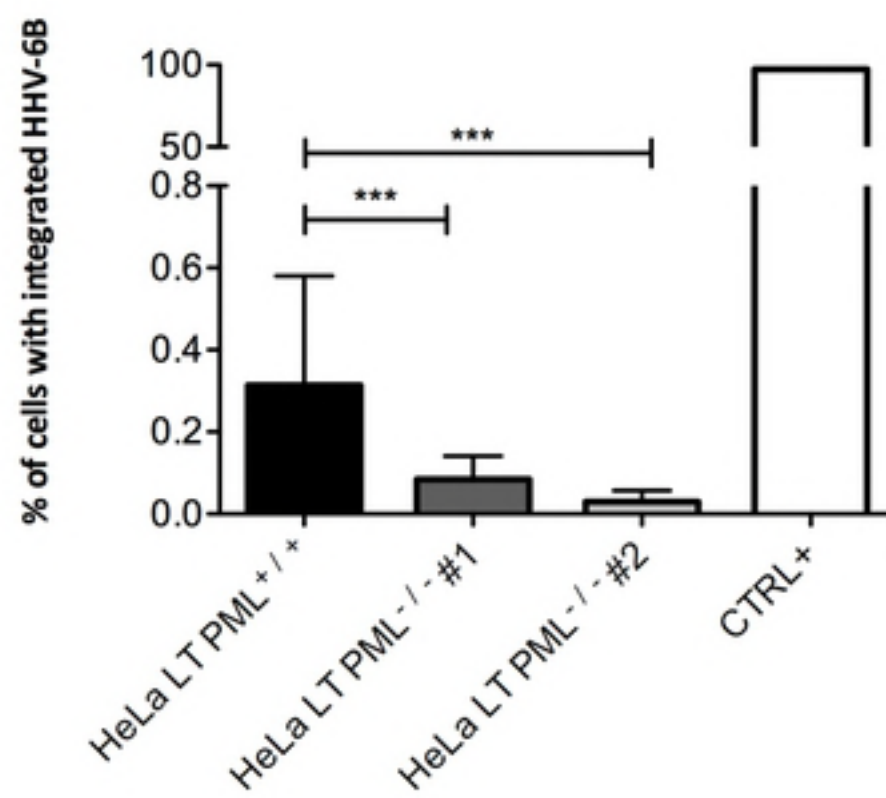
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**(C)****(D)**



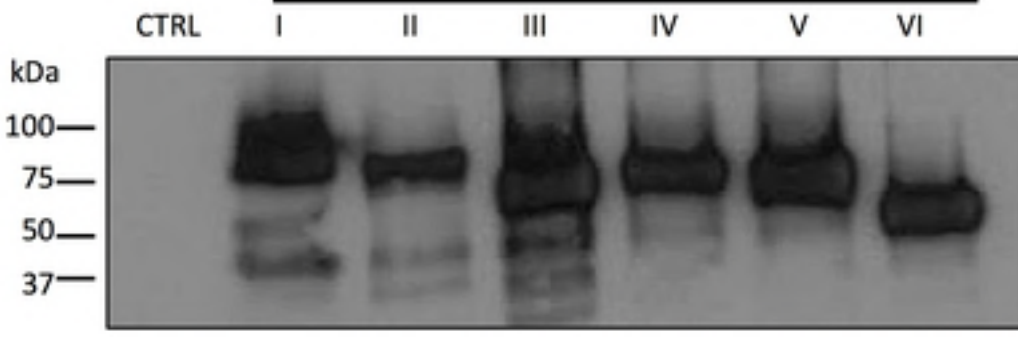


**(A)****(B)****(C)**

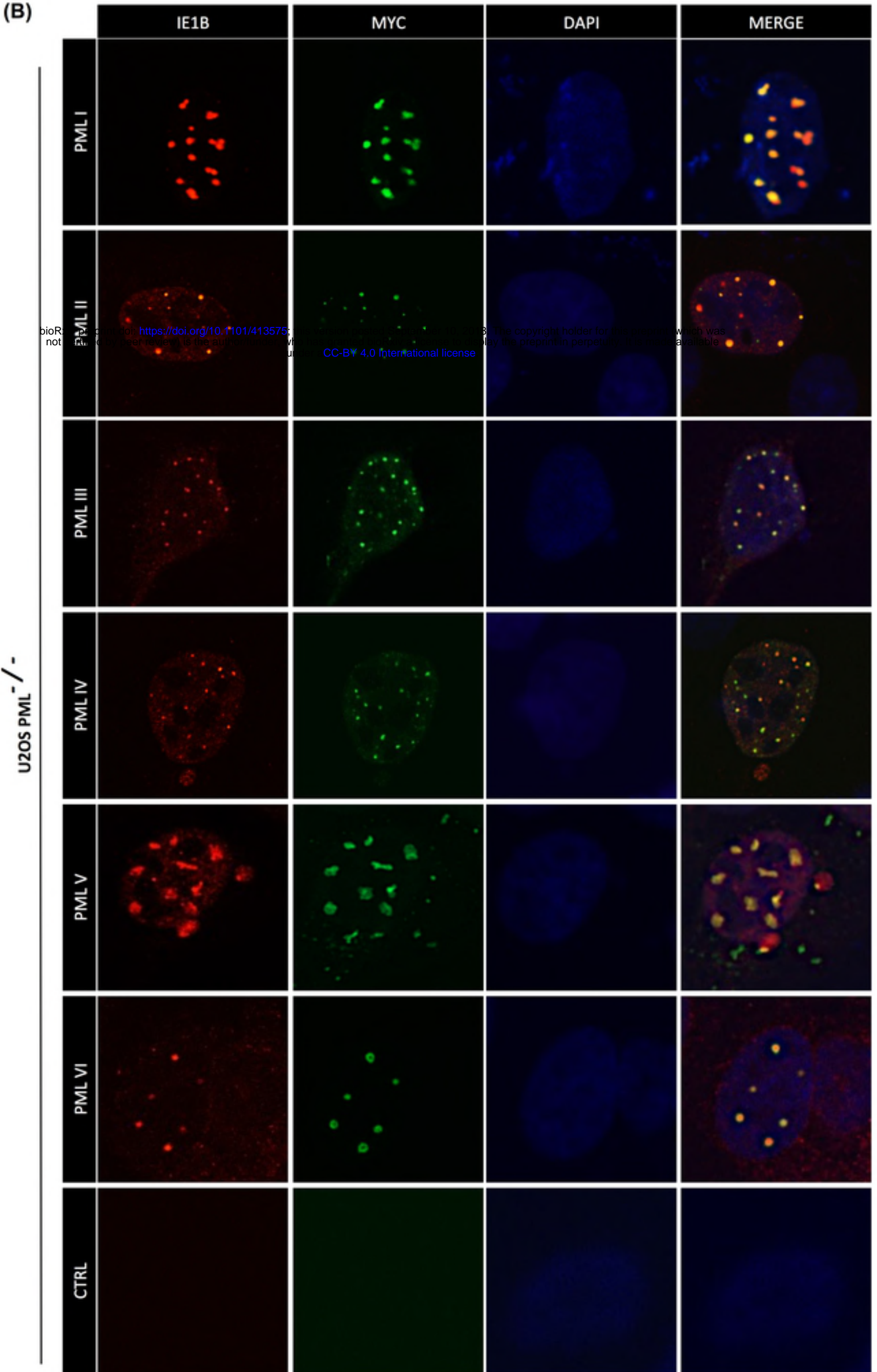
**(A)****(B)****(C)**

PML isoforms

(A)



(B)



U2OS

