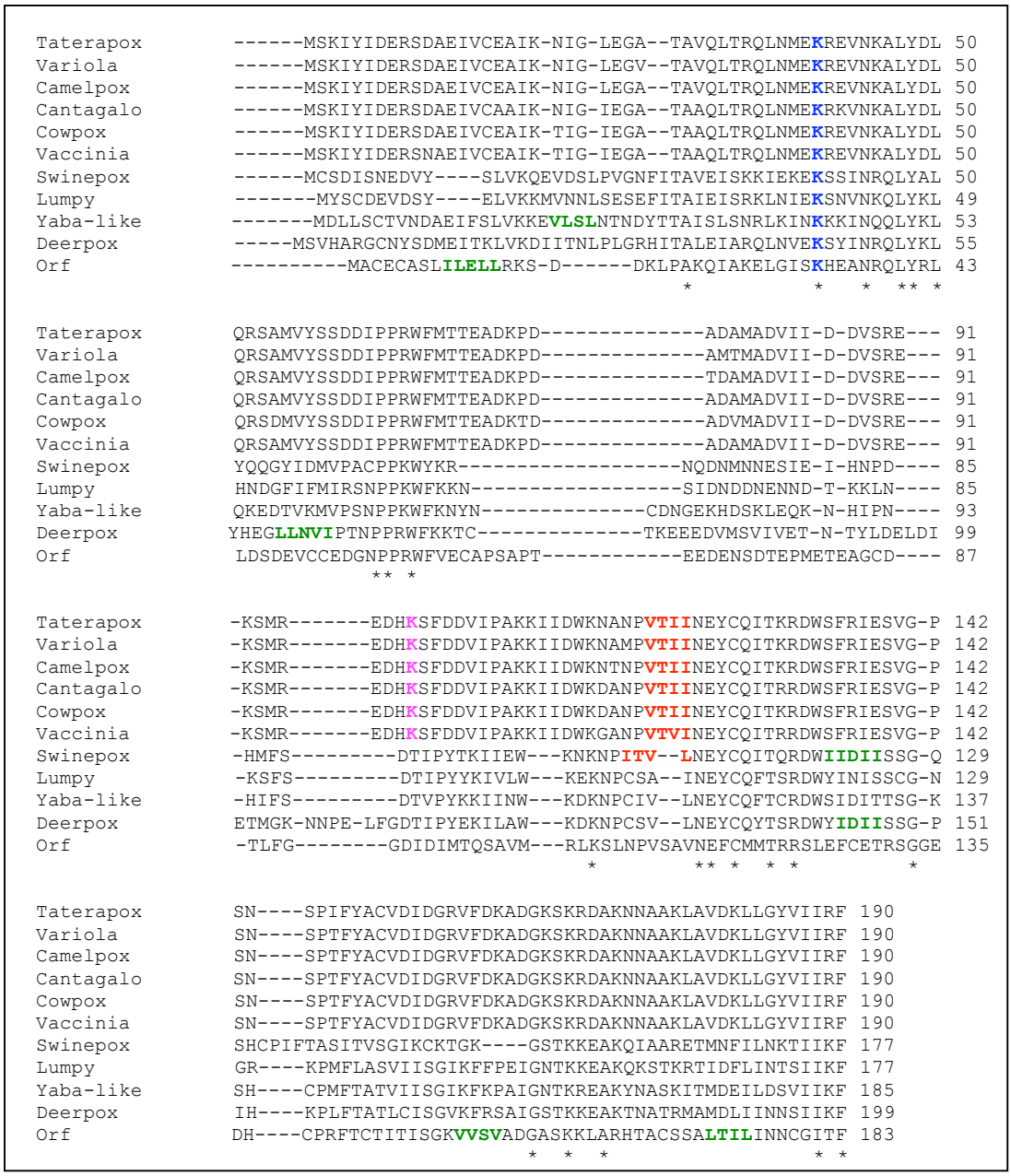
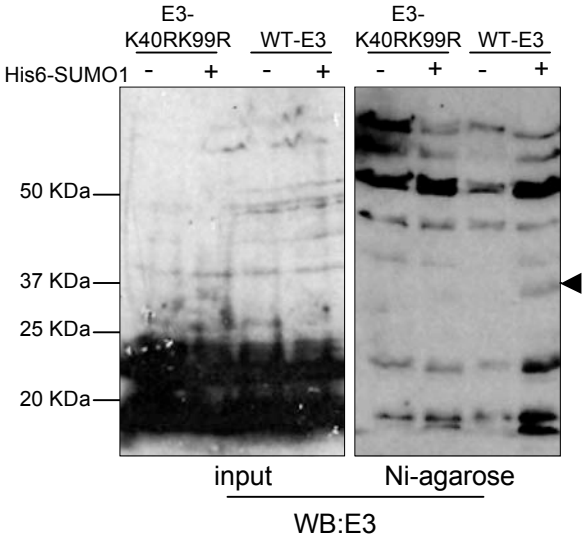
Supplementary information. González-Santamaría et al., 2011

Supplementary Table 1

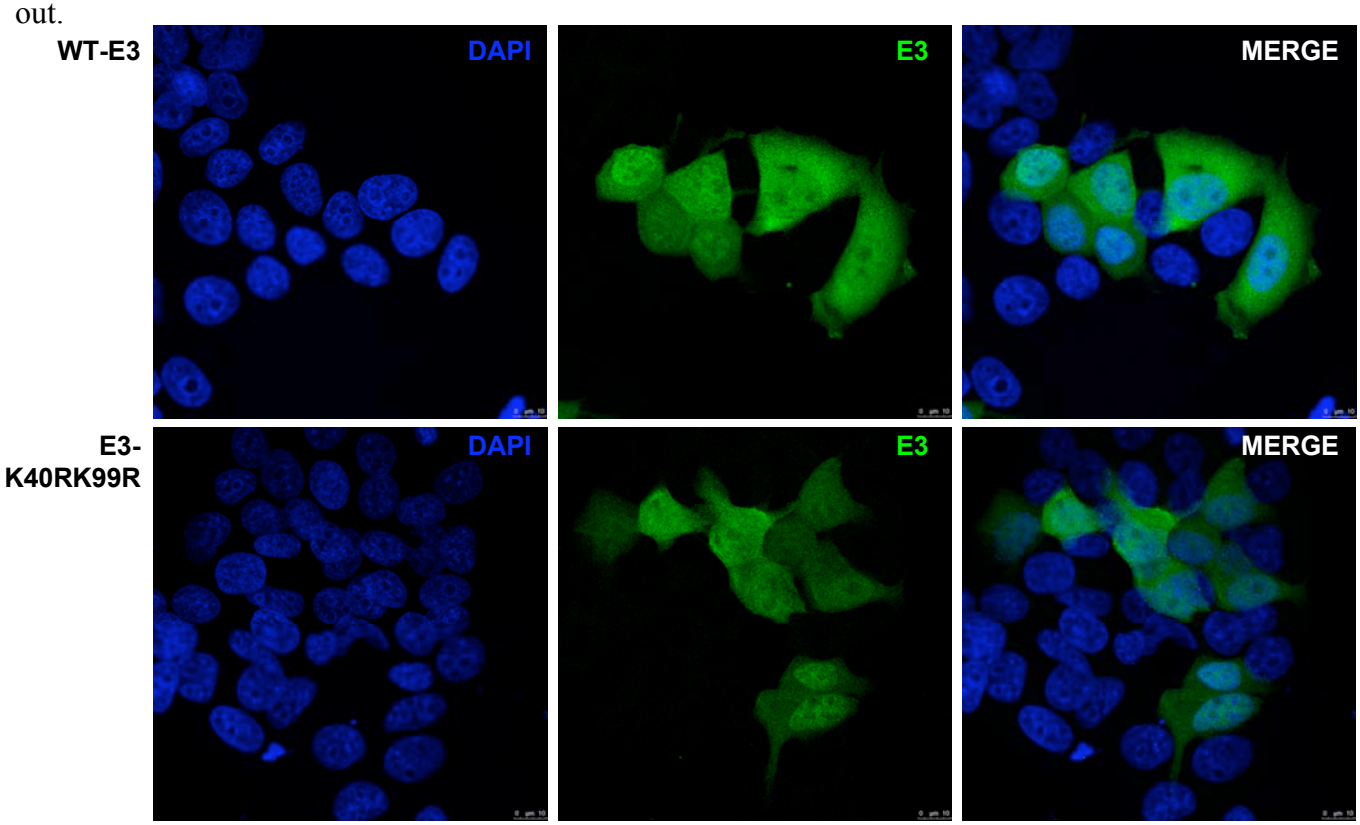
Oligonucleotides for mutagenesis
E3L-K40R-F-5'-CAACTTAATATGGAGAGGCGAGAAGTTAATAAAGC-3'
E3L-K40R-R-5'-GCTTTATTAACTTCTCGCCTCTCCATATTAAGTTG-3'
E3L-K99R-F-5'-GAGAGAGGATCATAGGTCTTTTGATGATGTTATTCCG-3'
E3L-K99R-R-5'-CGGAATAACATCATCAAAAGACCTATGATCCTCTCTC-3'
E3L-119ATAA122-F-5'-GGTGCTAACCCTGCCACCGCTGCTAATGAGTACTGCC-3'
E3L-119ATAA122-R-5'-GGCAGTACTCATTAGCAGCGGTGGCAGGGTTAGCACC-3'



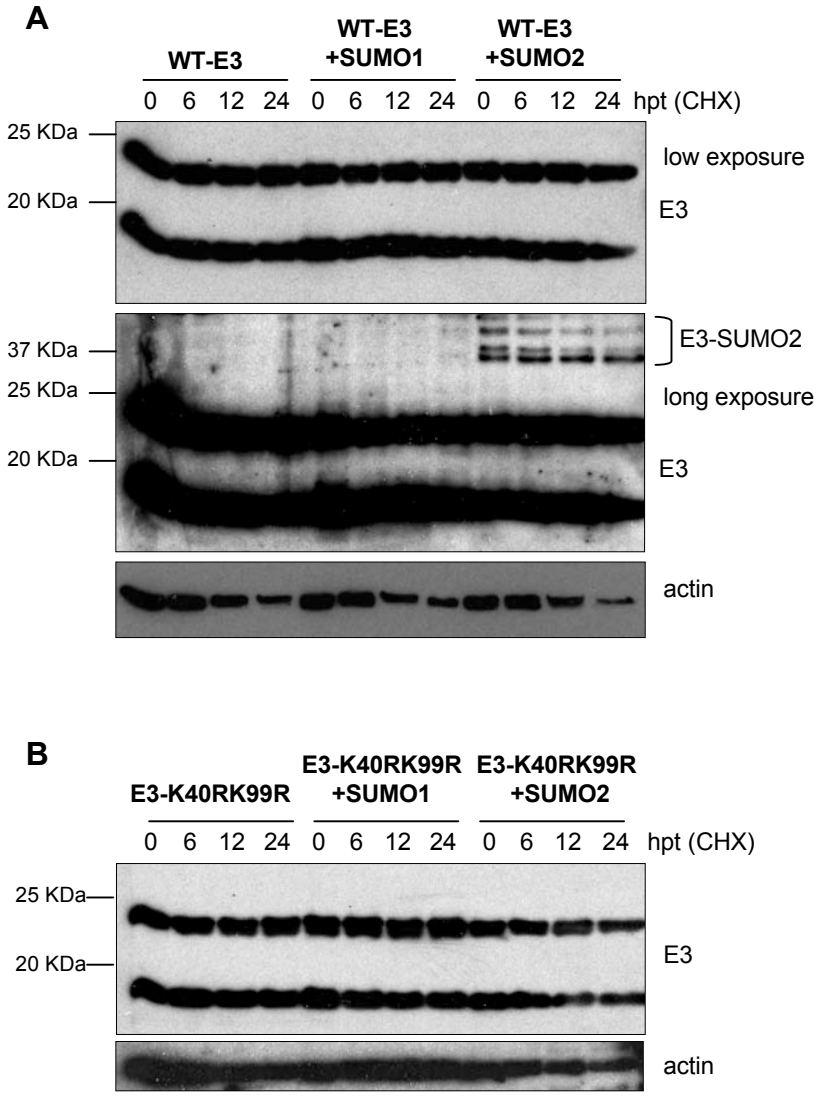
Supplementary Figure 1. Aligment using the Clustal X program of E3 proteins from different poxvirus. Conserved lysine residues in the consensus sequence for SUMOylation ψKxE in different poxvirus are shown in blue font. Conserved lysine residues in a non-consensus SUMOylation sequence in some poxvirus are shown in pink font. Conserved putative SIM domains in some poxvirus are shown in red font. Other putative SIM domains are shown in green font.



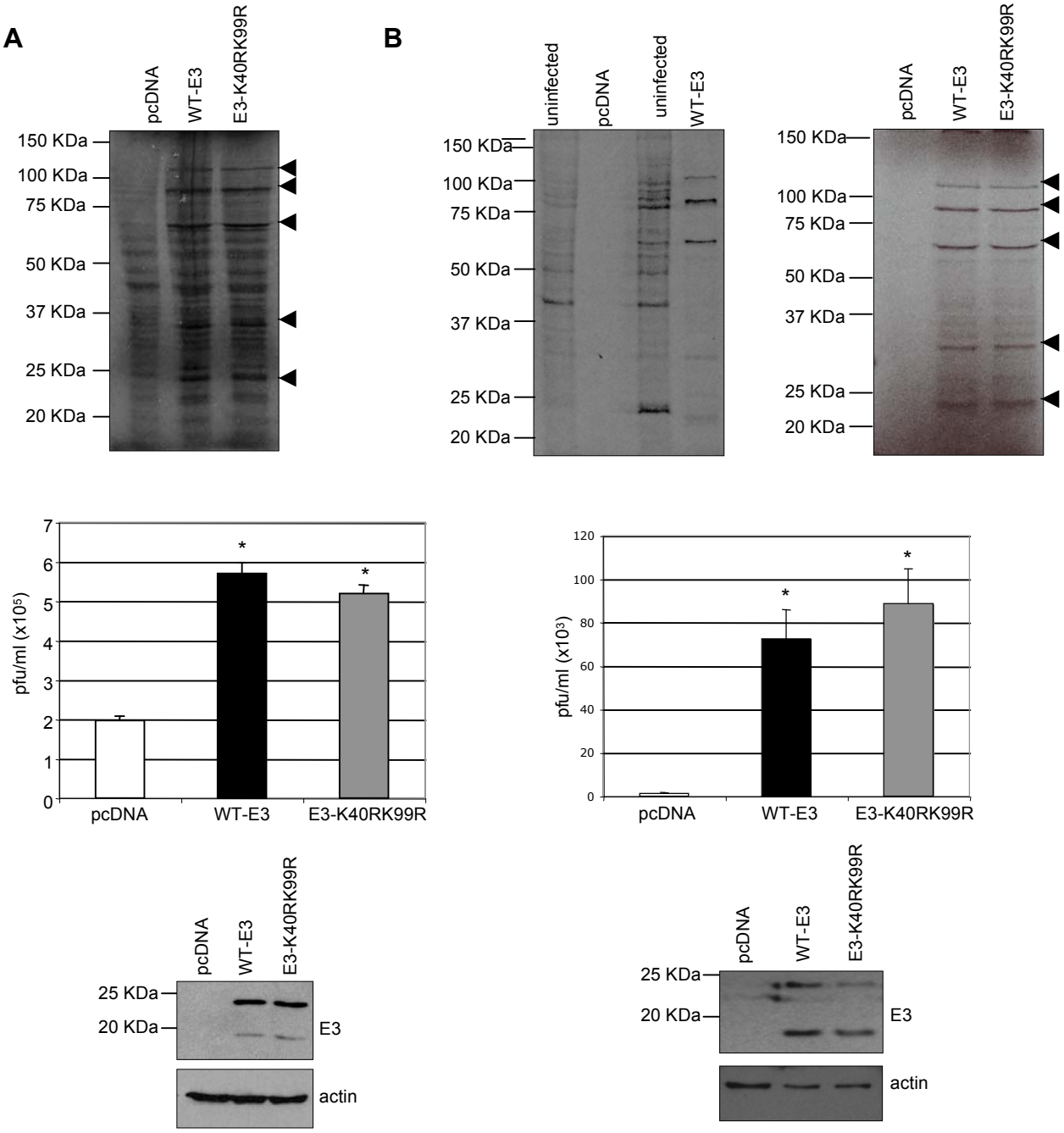
Supplementary Figure 2. Total extracts or Histidine-Tagged purified proteins were prepared from HEK-293 cells co-transfected with pCINEO-E3-WT or pCINEO-E3-K40RK99R together with pcDNA or pcDNA-Ubc9 and pcDNA-His6-SUMO1, and immunoblot analysis with an anti-E3 antibody was carried out.



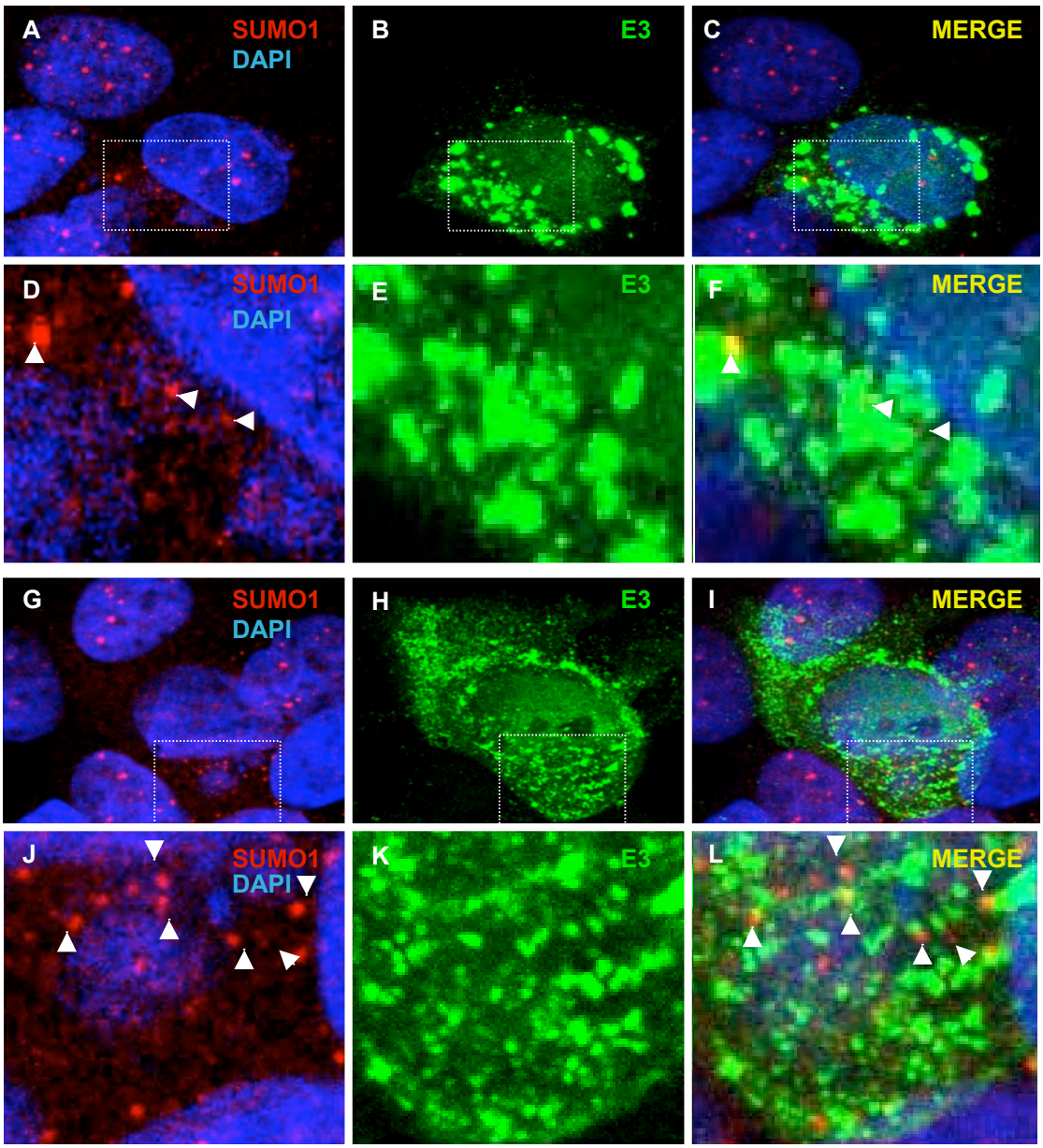
Supplementary Figure 3. Subcellular localization of WT-E3 or the E3-K40RK99R mutant in MCF-7 cells. MCF-7 cells were transfected with pCINEO-E3-WT or pCINEO-E3-K40RK99R. At 48 h after transfection, the cells were fixed and stained with DAPI and an anti-E3 antibody and analyzed by confocal microscopy.



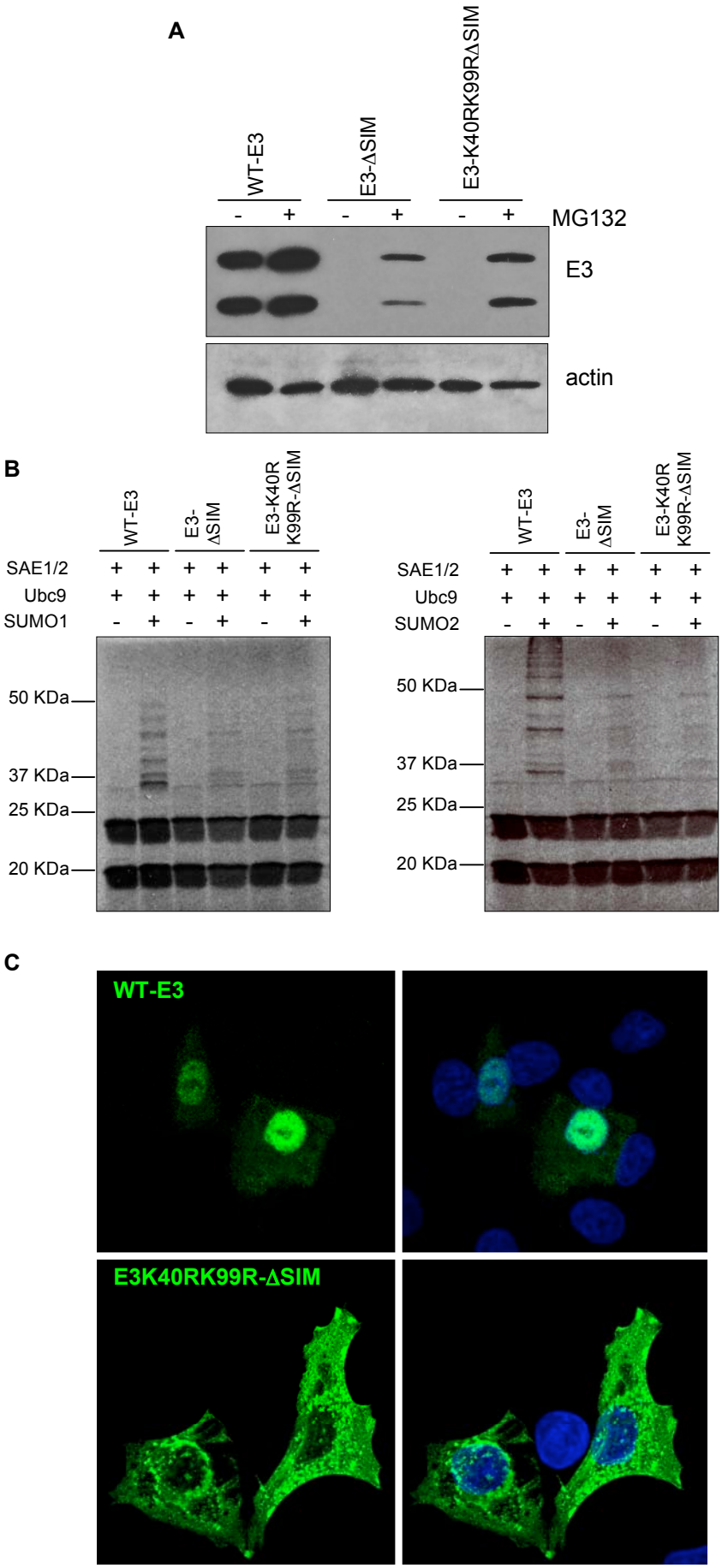
Supplementary Figure 4. E3 protein stability is unaffected by SUMOylation. A, MCF-7 cells were transfected with pCINEO-E3-WT together with pcDNA, Ubc9 and SUMO1 or Ubc9 and SUMO2, and treated with cycloheximide (CHX) for the indicated times to block protein synthesis. After CHX treatment, cells were lysed and proteins were subjected to Western-blotting with an anti-E3 antibody (upper panel A shows low exposure time and lower panel A shows long exposure time). B, MCF-7 cells were transfected with pCINEO-E3-K40RK99R together with pcDNA, Ubc9 and SUMO1 or Ubc9 and SUMO2, treated with CHX for the indicated times and analyzed as above described.


A
pcDNA
WT-E3
E3-K40RK99R
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
B
uninfected
pcDNA
uninfected
WT-E3
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
pcDNA
WT-E3
E3-K40RK99R
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
C
pfu/ml (x10⁵)
pcDNA
WT-E3
E3-K40RK99R
pfu/ml (x10³)
pcDNA
WT-E3
E3-K40RK99R
pcDNA
WT-E3
E3-K40RK99R
25 KDa
20 KDa
E3
actin
pcDNA
WT-E3
E3-K40RK99R
25 KDa
20 KDa
E3
actin

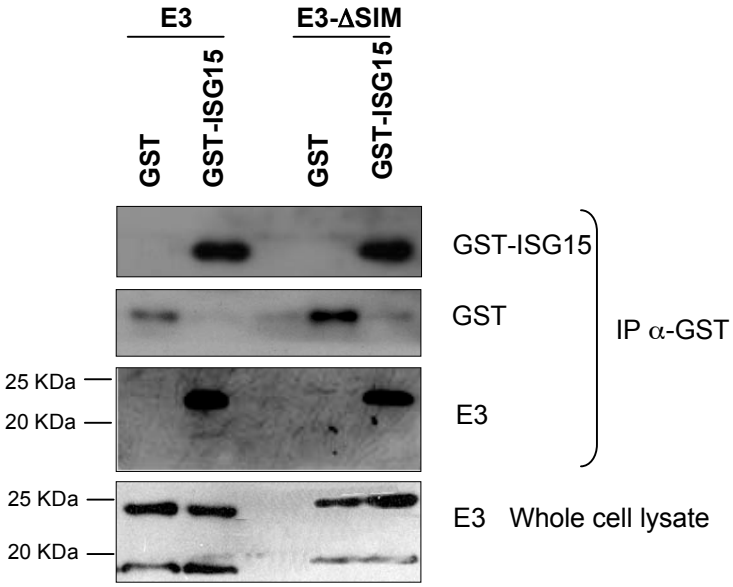
Supplementary Figure 5. A, Cultures of HeLa cells were transfected with pcDNA, pCINEO-E3-WT or pCINEO-E3-K40RK99R. At 48 h after transfection, cells were infected with VACV-ΔE3L (7.5 PFU per cell) for 16 h. Then, cultures were labelled with ^{35}S-methionine for 30 min and newly synthesized proteins were identified by 12% SDS-polyacrylamide gel electrophoresis and autoradiography. B, Cultures of HEK-293 cells were transfected with pcDNA or E3-WT and at 48 h after transfection, cells were infected with VACV-ΔE3L (7.5 PFU per cell) for 16 h or left uninfected. Then, newly synthesized proteins were labelled with ^{35}S-methionine for 30 min and identified by 12% SDS-polyacrylamide gel electrophoresis and autoradiography (left panel). HEK-293 cells were transfected with pcDNA, WT-E3 or E3-K40RK99R, infected with VACV-ΔE3L for 16 h and newly synthesized proteins were labelled with ^{35}S-methionine and analyzed as above described (right panel). C, Cultures of HeLa cells (left panel) or HEK-293 cells (right panel) were transfected with pcDNA, pCINEO-E3-WT or pCINEO-E3-K40RK99R and then infected with VACV-ΔE3L (7.5 PFU per cell). Infected cells were harvested after 24 h and virus yields were determined by plaque titration on BHK-21 cells. The results are the means of triplicate determinations +/- standard deviations for one experiment. *, $P<0.05$, compared with pcDNA transfected cells, Student's test. Similar results were obtained at least in three different experiments. The lower panel represents the expression levels of E3L gene transfected in each experiment.



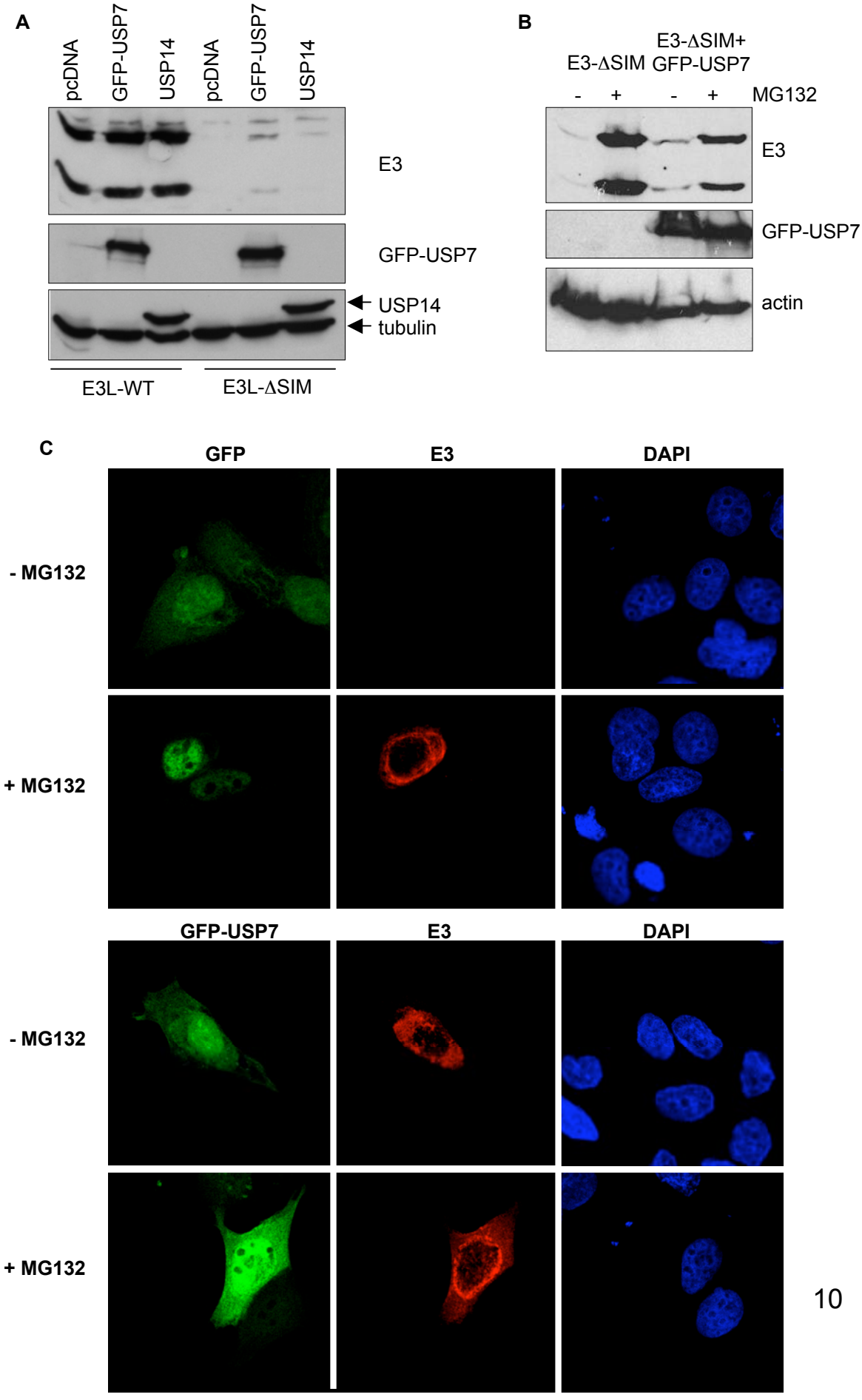
Supplementary Figure 6. E3-K40RK99R colocalizes with SUMO1 in the viral factories. MCF-7 cells were transfected with pCNIEO-E3-WT (A-F) or the pCINEO-E3-K40RK99R mutant (G-L) and then infected with VACV-ΔE3L for 6 h. Then, cells were fixed and labeled with DAPI, anti-SUMO1 and anti-E3 antibodies. The images were analyzed by confocal microscopy. The lower panels (D, E, F) and (J, K, L) show a higher magnification of the inserts shown in the upper panel (A, B, C) and (G, H, I), respectively.


A
WT-E3
E3-ΔSIM
E3-K40RK99RΔSIM
MG132
E3
actin
B
WT-E3
E3-ΔSIM
E3-K40R K99R-ΔSIM
SAE1/2
Ubc9
SUMO1
SUMO2
50 KDa
37 KDa
25 KDa
20 KDa
C
WT-E3
E3K40RK99R-ΔSIM

Supplementary Figure 7. The integrity of the SIM domain in E3 is required for its stability, SUMOylation and nuclear localization. A, HEK-293 cells were transfected with WT-E3, E3-ΔSIM or E3-K40RK99R-ΔSIM expression plasmids and treated or not with MG132 for 24 h. Analysis of the protein extracts by Western-blot with anti-E3 antibody was then carried out. B, [^{35}S]methionine-labelled WT-E3, E3-ΔSIM or E3-K40RK99R-ΔSIM proteins were used as substrates in an *in vitro* SUMOylation assay in the presence of SUMO1 (left panel) or SUMO2 (right panel). The reaction products were visualized by autoradiography. C, MCF-7 cells were transfected with WT-E3 or E3-K40RK99R-ΔSIM expression plasmids and treated with MG132 for 24 h. Cells were then stained with DAPI and an anti-E3 antibody, and the localization of E3 was detected by confocal microscopy.



Supplementary Figure 8. SIM is not required for the interaction between E3 and ISG15. HEK-293 cells were co-transfected with WT-E3 or E3-ΔSIM together with GST or GST-ISG15 expression plasmids and treated with MG132 for 24 h. Cells were washed and then lysed in RIPA buffer containing proteinase inhibitor cocktail. After a brief sonication, the lysates were cleared by centrifugation. A 50% protein A-sepharose slurry, which has been incubated with the anti-GST antibody at 4^{0}C overnight, was added to the cleared lysates and incubated at 4^{0}C for 2 h. Afterwards, the Sepharose beads were collected and washed five times in RIPA buffer. The precipitated protein complexes were recovered, boiled in SDS sample buffer and analyzed by Western-blotting using an anti-E3 antibody.


A
pcDNA
GFP-USP7
USP14
pcDNA
GFP-USP7
USP14
E3
GFP-USP7
USP14
tubulin
E3L-WT
E3L-ΔSIM
B
E3-ΔSIM
E3-ΔSIM+
GFP-USP7
- + - +
MG132
E3
GFP-USP7
actin
C
GFP
E3
DAPI
- MG132
+ MG132
GFP-USP7
E3
DAPI
- MG132
+ MG132

Supplementary Figure 9. Overexpression of the ubiquitin specific protease 7 (USP7/HAUSP) partially rescues E3-ΔSIM from degradation without altering its subcellular localization. A, HEK-293 cells were co-transfected with WT-E3 or E3-ΔSIM together with the indicated plasmids and at 48 h after transfection, analysis of the protein extracts by Western-blot with an anti-E3 antibody was carried out. B, HEK-293 cells were transfected with E3-ΔSIM in the presence or absence of GFP-USP7 and treated or not with MG132 for 24 h. Then, analysis of the protein extracts by Western-blot with an anti-E3 antibody was carried out. C, MCF-7 cells were co-transfected with E3-ΔSIM and GFP or GFP-USP7 and treated with MG132 for 24 h. Cells were then stained with DAPI and an anti-E3 antibody, and the localization of E3-ΔSIM was detected by confocal microscopy.