

2 **Identification of an arginine-rich motif in human papillomavirus type 1 E1[^]E4**
4 **protein necessary for E4 mediated inhibition of cellular DNA synthesis *in vitro* and in**
6 **cells**

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Abstract

Productive infections by human papillomaviruses (HPVs) are restricted to non-dividing, differentiated keratinocytes. HPV early proteins E6 and E7 deregulate cell cycle progression and activate the host cell DNA replication machinery in these cells, changes essential for virus synthesis. Productive virus replication is accompanied by abundant expression of the HPV E4 protein. Expression of HPV1 E4 in cells is known to activate cell cycle checkpoints, inhibiting G2-to-M transition of the cell cycle and also suppressing entry of cells into S phase. We report here that the HPV1 E4 protein, in the presence of a soluble form of the replication-licensing factor (RLF) Cdc6, inhibits initiation of cellular DNA replication in a mammalian cell-free DNA replication system. Chromatin-binding studies show that E4 blocks replication initiation *in vitro* by preventing loading of RLFs Mcm2 and Mcm7 onto chromatin. HPV1 E4 mediated replication inhibition *in vitro* and suppression of entry of HPV1 E4 expressing cells into S phase are both abrogated upon alanine replacement of arginine 45 in the full-length E4 protein (E1^{E4}), implying that these two HPV1 E4 functions are linked. We hypothesize that HPV1 E4 inhibits competing host cell DNA synthesis in replication-activated suprabasal keratinocytes by suppressing licensing of cellular replication origins, thus modifying the phenotype of the infected cell in favour of viral genome amplification.

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Introduction

Human papillomaviruses (HPVs) are a large group (> 100 types) of small DNA viruses that replicate in keratinocytes of squamous epithelia. HPV infections produce hyperproliferative warts that are in most instances benign. A small subset of HPV types however form lesions on the skin, and on the oropharyngeal and anogenital tract mucosa, that have a significant risk of malignant transformation. The most common cancer attributable to infection with the high-risk HPV types is cancer of the uterine cervix (35). Despite the differences in pathogenesis between virus types, their life cycles are similar (10), beginning with infection of keratinocytes within the basal cell compartment of squamous epithelia. Here the HPV genome is replicated as a low copy (between 50-100 copies per cell) episome in synchrony with the replication of the host cell genome, a process that requires HPV E1 and E2 functions. HPV early proteins E6 and E7 act to expand the population of HPV infected keratinocytes once they migrate up from the basal layer, by stimulating cell cycle entry and cell survival. The virus then utilizes the host cell's replication machinery that has been activated in these cells to amplify the HPV DNA to many thousands of copies per cell during the vegetative stage of the life cycle. Finally, the capsid proteins L1 and L2 are produced and new progeny are assembled in the most superficial cells prior to their release from the highly differentiated squames.

A major protein produced during the HPV life cycle is the E4 protein. It is expressed as an E1^{E4} fusion protein from spliced transcripts formed between the N-terminus of the E1 open-reading frame (ORF) and almost the complete ORF of E4 (21). The precise function of E4 has not been defined, but loss of expression of the full-length E1^{E4} polypeptide has a severe adverse effect on viral genome amplification of HPV types 16, 18 and 31 genomes following introduction of mutant genomes unable to support E1^{E4} expression into keratinocytes and subsequent induction of cellular differentiation (20, 38, 39). Failure to complete the vegetative stage of the virus life cycle is also the outcome of loss of E1^{E4} expression in rabbit papillomas induced by a mutant cottontail rabbit papillomavirus genome (22). These studies suggest that E4 function is necessary for efficient vegetative replication of the virus, a hypothesis supported by coincidence between onset of viral

genome amplification and induction of high-level E4 production in natural papillomavirus
90 infections (23).

Examination of E4 activity in epithelial cell cultures has revealed diverse biological
92 actions that perhaps imply a multifunctional role for this viral protein in the virus life
cycle. These include disruption of ND10 body organization that might be required for
94 viral DNA replication, either by organization of viral replication centres or by inactivation
of an host anti-viral response mediated through the nuclear ND10 body (8, 29). A potent
96 G2 arrest function is a conserved function of E4 proteins between HPV types with
dissimilar tropism and it is thought that division-arrest of infected cells might be necessary
98 to support efficient viral DNA amplification (5, 13, 19). E4 inclusion bodies found in the
cytoplasm of cells of HPV1 skin warts contain a kinase SRPK1, a binding partner of
100 E1^{E4} proteins, that is associated with regulating the function of splicing factors (1).
Sequestration of SRPK1 by E4 could be an HPV mechanism to regulate expression of viral
102 late transcripts at the late stages of the replication cycle (1). Late in the infectious cycle,
the E4 protein may also act to diminish the integrity of the keratinocyte by disrupting the
104 keratin cytoskeleton and cornified envelope formation, and inducing apoptosis through
alteration of mitochondria function, to facilitate egress of the newly formed HPV virions
106 (3, 6, 24, 26).

Execution of multiple functions might be assisted by conversion of the E4 protein into
108 multiple forms, brought about by a combination of sequential N-terminal proteolysis of the
E1^{E4} polypeptide (7, 25) and by phosphorylation (9, 21). Indeed, a study of the
110 interaction between E4 and cell growth revealed an interesting relationship between
modification of the HPV1 E4 protein and dysregulation of the cell cycle (13). During the
112 HPV1 infectious cycle N-terminal sequences are removed from the full-length 17-kDa
E1^{E4} polypeptide to produce smaller E4 species of 16-, 11- and 10-kDa that
114 progressively replace the full-length protein as the replication cycle proceeds (7, 25).
Expression of the 17-kDa E1^{E4} protein in the presence of a protein mimicking the 16-
116 kDa polypeptide in epithelial cells inhibits G2-to-M transition of the cell cycle and, in a

population of cells, prohibition of entry into S phase is also observed (13). The negative
 118 effect on S phase entry however, was not apparent in cells expressing the individual forms
 of E4, although expression of the truncated 16-kDa protein alone was sufficient to block
 120 cell division (13). Further analysis revealed that HPV1 E4 employed two distinct
 mechanisms to inhibit G2-to-M transition, the first, mediated by the combined expression
 122 of 17- and 16-kDa proteins, was found to be dependent on maintenance of high levels of
 the Wee1 kinase to inhibit cdk1 activity, and the second, mediated by the 16-kDa protein,
 124 was associated with insufficient production of cyclin B1 to enable the cells to transverse
 G2 to M (13, 14). Employment of two distinct mechanisms to inhibit cell division
 126 suggests that the G2 arrest function of HPV1 E4 is important in the HPV life cycle. In this
 study we investigated how HPV1 E4 inhibits progression of cells into S phase and show
 128 that HPV1 E4 affects a key step in the cellular DNA replication process.

Materials and methods

130 HPV1 E4 expression plasmids

Construction of plasmids based on pcDNA3.1 that deliver expression of the 17-kDa full-
 132 length HPV1 E1^ΔE4 (E4-17K) and an N-terminal truncation (E4-16K) equivalent to the
 16-kDa E4 polypeptide have been described previously (29). A set of previously described
 134 deletions within the full-length E1^ΔE4 coding sequence (25) were excised and inserted into
 the BamHI restriction site of pcDNA3.1 (Invitrogen, Carlsbad, CA). The sequence
 136 integrity of plasmid inserts was verified by bi-directional DNA sequencing. Substitutions
 E4R45A, E4R47A and E4R48A are described elsewhere (1).

138 Expression of recombinant proteins

HPV1 E4 and *Xenopus laevis* Cdc6 (His₆-XeCdc6) proteins were purified from Sf9 insect
 140 cells following infection with appropriate recombinant baculoviruses, as previously
 described (28, 31). Histidine-tagged human geminin (His₆-hsGeminin) was expressed in
 142 *Escherichia coli* and purified as described (36). For expression of histidine-tagged HPV1
 E4 protein in bacteria, the E1^ΔE4 wild type and mutant coding sequences were inserted
 144 into the BamHI – EcoRI cloning site of the expression vector pRSET-C (Invitrogen) and

expressed in *E. coli* strain BL21 (DE3) pLysS (Novagen, Madison, WI, USA). The
146 recombinant protein was purified by IMAC using NiCl₂-charged HiTRAP chelating
columns (GE Healthcare Europe GmbH, Munich, Germany). Non-specifically bound
148 proteins were removed with 4 column volumes (c.v.) of 10% elution buffer (30 mM Tris-
Cl pH 8, 300 mM imidazole, 30 mM NaCl, 0.1 mM PMSF) and 8 c.v. of 20% elution
150 buffer. The E4 proteins were eluted with 2 c.v. of 100% elution buffer and desalted into
20 mM Tris-Cl pH 8, 50 mM NaCl.

152 ***In vitro* cellular DNA replication assays**

Nuclei and cytosolic extracts were prepared from synchronized NIH3T3 and HeLa S3 cells
154 and supplemented as described previously (11, 15, 31, 32). *In vitro* DNA replication
assays were performed as described (15, 31) (Fig. 1A). Briefly, reactions contained 30 µl
156 of cytosolic extracts (250-300 µg of protein), 10 µl of premix buffer (160 mM K-HEPES
pH 7.8, 28 mM MgCl₂, 12 mM ATP, 0.4 mM of GTP, CTP, UTP, dATP, dGTP and dCTP,
158 1 µM biotin-16-dUTP, 2 mM dithiothreitol, 160 mM creatine phosphate, 20 µg
phosphocreatine kinase), 1 x 10⁵ nuclei and, where indicated, up to 10 µl of recombinant
160 protein(s). His₆-XeCdc6 protein in 20 mM HEPES pH 7.4, 150 mM NaCl, 5% glycerol, 1
mM DTT was added to *in vitro* replication reactions at a final concentration of 0.65 µM,
162 baculovirus-expressed HPV1 E4 proteins in 10 mM phosphate buffer pH 7.4, 0.1 mM DTT
at 3 µM, bacterially-expressed His₆-E4 proteins in 40 mM Tris-HCl pH 7.6, 30 mM NaCl,
164 and His₆-hsGeminin protein in 50 mM Na-phosphate at 4 µM. Equal volumes of
appropriate buffers were added to control reactions. All components of the replication
166 reactions were incubated together on ice for 15 min prior to the addition of S phase cytosol
and incubation for 3 h at 37°C. For analysis of *in vitro* DNA synthesis reactions by
168 confocal microscopy, reactions were stopped by diluting with 500 µl of phosphate-
buffered saline (PBS) and nuclei fixed for 5 min in 4% paraformaldehyde. After fixation,
170 nuclei were spun through a 30% sucrose/PBS cushion onto poly-L-lysine coated
coverslips. All subsequent washing and staining steps were carried out in PBS, 0.2%

172 Triton X-100, 0.04% SDS. Coverslips were washed, stained for incorporated biotin-16-
dUTP with fluorescein-linked streptavidin (1:100 dilution, Amersham) and for DNA with
174 propidium iodide/RNase A (both at 50 ng/ml), washed again, and mounted in Vectorshield.
Confocal fluorescence microscopy of random fields of nuclei was performed on a Leica
176 TCS DMRE confocal microscope and the number of nuclei incorporating biotin-16-dUTP
in vitro and non-replicating nuclei were counted. Routinely 800-1000 nuclei were scored
178 blind by a single individual for each reaction and quantitated as percentages of the total
number of nuclei that synthesized DNA *in vitro*. More than one preparation of nuclei was
180 assayed, in triplicate, for each set of experiments and analysis performed by two
individuals. Statistical analysis of data from multiple independent experiments was
182 performed by single factor analysis of variance (ANOVA).

Chromatin-binding assay

184 *In vitro* DNA replication assays were set up as described above. After 3 h incubation,
nuclei were pelleted by low speed (1300xg) centrifugation and used in chromatin-binding
186 reactions performed as described previously (12). Samples were immunoblotted with
antibodies against Mcm2 (BD Biosciences, #610701), Mcm7 (Neomarkers, Lab Vision,
188 Suffolk, UK, #MS-862-P), Cdc6 (Santa Cruz, CA, USA, #9964), HPV1 E4 (MAb 4.37,
(7)) and Histone H1 (Santa Cruz, CA, #10806). Analysis of protein bands using
190 densitometry was determined using ImageJ (<http://rsb.info.nih.gov/ij>). Chromatin-binding
reactions were repeated at least twice in separate *in vitro* DNA replication assays.

Cell transfection and cell cycle analysis

Cos-1 cells were transfected with the appropriate combinations of HPV1 E4 expression
194 plasmids, or the pcDNA3.1 empty vector as a control plasmid, as described previously
(13). At 48 h post-transfection, the cells were incubated with 5-bromodeoxyuridine
196 (BrdU) at a final concentration of 33 μ M for 2 h. Cells were then fixed, incubated with an
anti-BrdU antibody conjugated to fluorescein isothiocyanate, labelled with propidium

198 iodide and analysed by dual-parameter flow cytometry as described (13). Statistical
analysis of data derived from multiple independent experiments was performed by
200 ANOVA. Expression of E4 proteins was confirmed by immunoblot analysis using an
HPV1 E4 monoclonal antibody (MAb 4.37).

202

Results

HPV1 E4 inhibits cellular DNA replication *in vitro*

204 We sought to establish whether the negative effect of HPV1 E4 upon S phase entry
reflected an interaction between the viral protein and the process of cellular DNA synthesis
206 itself. To do this, we took advantage of an established cell-free DNA replication system
that supports efficient cellular DNA synthesis under somatic cell cycle control (31) (Fig.
208 1A). Previous characterisation of this system revealed that nuclei prepared from quiescent
(G0) NIH3T3 fibroblasts cannot initiate DNA synthesis in S phase cytosolic extracts of
210 HeLa cells, whilst G1 nuclei become competent to initiate DNA replication in the S phase
extracts when prepared 16 to 18 hours after release from G0 (31).

212 NIH3T3 G1 nuclei were combined with HeLa S phase cytosol and incubated for 3 h in the
presence of an ATP generation system and nucleotides (NTPs and dNTPs) including
214 biotin-labelled dUTP as a marker to enable detection of DNA synthesis by confocal
microscopy (Fig. 1A). We observed that 19.3% of G1 nuclei were capable of DNA
216 synthesis in the presence of S phase cytosol in comparison to only 2.2% following
incubation of the G1 nuclei in a physiological buffer (Buffer A) that supports elongation
218 but not initiation of DNA replication (Fig. 1C). The small proportion of replication
competent nuclei observed in Buffer A represent contaminating S phase nuclei present in
220 the G1 nuclear preparation that continue DNA synthesis at replication forks established *in*
vivo prior to their isolation (31). Thus, 17.1% of G1 nuclei undergo true replication
222 initiation in the presence of S phase cytosol. To confirm that our preparations of G1 nuclei
and S phase cytosol respond to exogenous factors, we first tested the response to
224 recombinant preparations of the replication-licensing factor (RLF) Cdc6 (His₆-XeCdc6)
and geminin (His₆-*hs*Geminin), a known cellular repressor of replication licensing (18).

226 The percentage of replicating nuclei increased from 19.3% to 23% in reactions containing
His₆-XeCdc6 (Fig. 1C). Since Cdc6 is known to be rate-limiting for replication
228 competence after release from G0 (31), the small but consistent increase in replicating
nuclei in the presence of His₆-XeCdc6 indicates a low number of G1 nuclei are responsive
230 to this RLF. In contrast, in the presence of His₆-hsGeminin there was a marked and
significant decrease (7.2%) in the percentage of replication competent nuclei (Fig. 1C).

232 To investigate if HPV1 E4 might interfere with cellular DNA synthesis, the viral protein
was expressed in Sf9 insect cells using a recombinant baculovirus and the purified protein
234 (Fig. 1B, WT38) titrated into *in vitro* replication reactions (Fig. 1C). Whilst we observed
no significant effect of E4 on the percentage of nuclei synthesizing DNA in co-incubations
236 of G1 nuclei and S phase cytosol (19.7%), when E4 was added to reactions that also
contain exogenous Cdc6 (His₆-XeCdc6), the percentage of replicating nuclei decreased
238 significantly from 23% to 10.6%, indicating that 54% of the replication-competent nuclei
failed to initiate DNA synthesis (Fig. 1C). Notably, the scale of E4-induced replication
240 inhibition was comparable to the inhibitory effect (67%) of His₆-hsGeminin (Fig. 1C).

The E4 protein added to the *in vitro* replication assays contained the full-length E1^ΔE4
242 protein (17-kDa) plus small quantities of truncated polypeptides (16-, 11-, and 10-kDa)
(Fig. 1B, WT38). Interestingly, addition of recombinant E4 protein that contains the
244 truncated proteins, but no full-length E1^ΔE4 polypeptide (Fig. 1B, WT43) to the cell-free
replication assay did not inhibit DNA synthesis in the G1 nuclei either in the absence (data
246 not shown) or presence of exogenous Cdc6 (Fig. 1C, ΔE4). This observation suggests that
E4-induced inhibition of cellular DNA synthesis *in vitro* requires the presence of the full
248 length E1^ΔE4 protein.

To validate the specificity of our findings, identical replication reactions to those described
250 above, but using a separate preparation of baculovirus-expressed E4 protein containing a
similar profile of E4 species to WT38, and G1 nuclei prepared from human WI38 diploid
252 fibroblasts, achieved a similar level of replication inhibition (55%; data not shown).

HPV1 E4 does not arrest ongoing cellular DNA synthesis *in vitro*

254 Unlike G1 nuclei, nuclei isolated from cells in the S phase of the cell cycle contain active
 replication forks and are thus competent for DNA synthesis in the absence of cytosolic S
 256 phase extracts (31). Addition of geminin, an inhibitor of origin licensing, to *in vitro*
 replication reactions containing NIH3T3 S phase nuclei as the source of template failed to
 258 affect ongoing DNA synthesis (Fig. 1D) consistent with previous reports (18, 33, 40).
 Significantly, cellular DNA elongation was also not affected by the addition of
 260 recombinant E4 protein (WT38) to S phase nuclei, either in the absence or presence of
 His₆-XeCdc6 (Fig. 1D). The data from the *in vitro* replication assay (Fig. 1) indicate that
 262 in the presence of exogenous Cdc6, HPV1 E4 inhibits initiation of DNA synthesis, but
 fails to arrest ongoing DNA synthesis.

HPV1 E4 blocks recruitment of replication licensing proteins onto chromatin *in vitro*

264 Initiation of cellular DNA replication is achieved by the ordered assembly of pre-
 replicative complexes (pre-RCs) at origins of replication (34). During late mitosis (M) and
 266 early G1 phase, RLFs Cdc6 and Cdt1, by interacting with the origin recognition complex,
 load the putative DNA replicative helicase Mcm2-7 onto chromatin to form pre-RCs. In
 268 the subsequent S phase, DNA replication is initiated at these “licensed” origins by the
 concerted action of cyclin dependent kinases and Cdc7-Dbf4. To investigate whether
 270 HPV1 E4 might inhibit replication initiation by blocking assembly of pre-RCs onto
 272 chromatin, we resolved chromatin-bound protein fractions prepared from G1 and S phase
 nuclei taken through *in vitro* replication reactions by gel electrophoresis and probed for
 274 RLFs Cdc6, Mcm2 and Mcm7 (Fig. 2). Histone H1 levels were used as a loading control.
 Chromatin prepared from G1 nuclei incubated with S phase cytosol showed a two-fold
 276 increase in the binding of endogenous Cdc6 and MCM factors compared to the elongation
 control reaction (Buffer A) (Fig. 2A). Addition of recombinant Cdc6 to replication
 278 reactions led to a 1.5-fold increase in the total amount of chromatin-bound Cdc6 and
 further increased levels of chromatin-bound MCMs (Fig. 2A), correlating with the
 280 observed small increase in the percentage of replication-competent nuclei (Fig. 1C). In
 contrast, geminin inhibited origin licensing by blocking loading of Mcm2 onto chromatin

282 (Fig. 2A), indicating that increased levels of chromatin-bound RLFs in nuclei taken
 through the replication assay are a result of genuine pre-RC assembly *in vitro*. Addition of
 284 HPV1 E4 protein (WT38) with His₆-XeCdc6 to the replication reactions was associated
 with a 3.5-fold and 2-fold decrease in chromatin-bound Mcm2 and Mcm7 proteins
 286 respectively, compared to reactions containing His₆-XeCdc6 alone (Fig. 2A). Notably, the
 reduced levels of chromatin-bound MCM proteins were close to base levels measured in
 288 G1 nuclei incubated with Buffer A (Fig. 2A). The reduction of chromatin-bound MCM
 proteins by the HPV1 protein correlated with its ability to inhibit replication, as Mcm2 and
 290 Mcm7 levels were not affected by addition of E4 in the absence of exogenous Cdc6, or by
 the addition of E4 protein lacking full-length E1^ΔE4 protein (ΔE4) to reactions containing
 292 His₆-XeCdc6 (Fig. 2A). There was no evidence that E4 became bound to chromatin, either
 in the presence or absence of exogenous Cdc6 (see Fig. 4b).

294 Chromatin-binding studies on S phase nuclei taken through replication elongation assays
 show that neither addition of geminin nor E4 together with His₆-XeCdc6 affected the
 296 chromatin-binding status of Mcm2 and Mcm7 (Fig. 2B).

Together, the *in vitro* replication and chromatin-binding data indicate that inhibition of
 298 initiation of cellular DNA synthesis by HPV1 E4 in the presence of His₆-XeCdc6 correlates
 with reduced MCM loading onto chromatin.

300 **Repression of S phase entry by HPV1 E4 in epithelial cells is dependent on an arginine-rich motif in the E1^ΔE4 protein**

302 To determine whether there is a relationship between the negative effect of HPV1 E4 on
 cell proliferation (13) and E4's ability to inhibit cellular DNA replication *in vitro*, we first
 304 identified the HPV1 E1^ΔE4 sequences required for suppression of S phase entry in
 epithelial cells. Since the negative effect of HPV1 E1^ΔE4 on S phase entry was dependent
 306 on the presence of a truncated 16-kDa E4 protein (13), HPV1 E1^ΔE4 (E4-17K) expression
 plasmids containing small deletions that cover the majority of the E1^ΔE4 sequence, were
 308 individually co-transfected with the plasmid expressing the truncated protein (E4-16K)
 into Cos epithelial cells, and cellular DNA synthesis monitored by BrdU incorporation. In

310 keeping with our previous findings (13), transient expression of the full-length E1^ΔE4
 312 protein together with E4-16K reduced S phase BrdU incorporation by nearly 2-fold, in
 comparison to cells expressing the individual proteins, or control cells (Table 1). Of the
 E1^ΔE4 deletion plasmids tested, only one, containing a deletion of residues 44 to 48
 314 (GRPRR), did not inhibit S phase entry following co-transfection with E4-16K (Table 1).
 The G2 arrest function of this mutant E1^ΔE4 protein however remained intact (data not
 316 shown).

The contribution of individual amino acids within the ⁴⁴GRPRR⁴⁸ sequence to E1^ΔE4
 318 function was examined by substituting the arginine residues at positions 45, 47 and 48 by
 alanine residues (E4R45A, E4R47A, E4R48A). Following co-transfection of these mutant
 320 E1^ΔE4 plasmids with the E4-16K expression plasmid into Cos cells, BrdU incorporation
 revealed that inhibition of entry into S phase was sensitive to alanine substitution of Arg45,
 322 but not mutation of Arg47 or Arg48 (Fig. 3A). Loss of inhibitory action was not due to
 any change in the stability of the E4R45A E1^ΔE4 protein in epithelial cells (Fig. 3B), and
 324 all three mutants promoted G2 arrest to levels comparable with E4-17/16K expressing cells
 (data not shown).

326 **The arginine-rich motif in E1^ΔE4 is necessary for inhibition of cellular DNA synthesis initiation *in vitro***

328 Next, we wanted to determine whether the ⁴⁴GRPRR⁴⁸ sequence E1^ΔE4 was also involved
 in inhibition of cellular origin licensing in the *in vitro* replication assay. In this instance,
 330 the recombinant HPV1 E4 proteins were expressed and purified from bacteria. To verify
 that a bacterial form of the E4 protein encodes an inhibitory function, the wild-type protein
 332 was titrated into the *in vitro* replication reactions. Consistent with the data using
 baculovirus recombinant E4 protein, we observed no significant effect of E4 on the
 334 percentage of nuclei synthesizing DNA following addition of the bacterial E4 protein to
 co-incubations of NIH3T3 G1 nuclei and HeLa S phase cytosol (24%, Fig. 4A). However
 336 when E4 was added to reactions that also contain exogenous Cdc6 (His₆-XeCdc6), the
 percentage of replicating nuclei decreased significantly from 29% to 13.8%, indicating that

338 in the presence of a bacterial derived HPV1 E4 protein nearly 53% of the replication-
competent nuclei failed to initiate DNA synthesis (Fig. 4A). Inhibition of cellular
340 replication initiation was however abrogated upon the addition of the E4 protein containing
deletion of ⁴⁴GRPRR⁴⁸ to the *in vitro* reactions containing His₆-XeCdc6 (26.5%, Fig. 4A),
342 and alanine substitution of arginine 45 (E4R45A) within this motif was sufficient to relieve
the inhibitory effect of the HPV protein (23.2%, Fig. 4A).

344 Our analysis of pre-RC assembly in the *in vitro* replication assay had indicated that HPV1
E4 protein inhibited loading of MCM onto chromatin (Fig. 2). Therefore we investigated
346 whether failure to inhibit replication initiation by the mutant E1^ΔE4 proteins correlated
with efficient assembly of pre-RCs onto chromatin. Chromatin-bound protein fractions
348 prepared from G1 and S phase nuclei taken through *in vitro* replication reactions were
probed for RLFs Cdc6, and MCM proteins 2 and 7 (Fig. 4B). Chromatin prepared from
350 G1 nuclei incubated with S phase cytosol in the presence of His₆-XeCdc6 and the wild-type
protein derived from bacteria showed a 51% and 41% decrease in chromatin-bound Mcm2
352 and Mcm7 proteins respectively, compared to the level of these proteins in the reaction
containing His₆-XeCdc6 alone (Fig. 4B). The reduced levels of chromatin-bound MCM
354 proteins in the presence of the bacterial preparation of E4 were similar to the decrease
observed with the baculovirus-recombinant E4 protein (Fig. 2). However, in the presence
356 of mutant E1^ΔE4 proteins E4^Δ44-48 and E4R45A both Mcm2 and Mcm7 were efficiently
recruited to chromatin (Fig. 4B).

358 Together, our data suggest that HPV1 E4 inhibits initiation of cellular DNA replication *in*
vitro by blocking MCM loading onto chromatin and that this is dependent on an arginine-
360 rich motif within the full-length form of the viral protein.

Discussion

362 Using a cell-free cellular DNA replication assay, we have shown that HPV1 E4 protein is a
potent inhibitor of cellular replication licensing; a novel function for this protein.
364 Chromatin binding studies indicate that E4 blocks replication initiation *in vitro* by
preventing loading of licensing factors Mcm2 and Mcm7 onto chromatin. The functional

366 effect of E4 mimics the cellular repressor of replication licensing geminin (18), but while
geminin interacts with the RLF Cdt1 to inhibit assembly of MCMs into pre-RCs, the
368 mechanism by which E4 blocks MCM loading appears different to that of the cellular
repressor. Unlike geminin, inhibition of licensing *in vitro* by E4, requires addition of an
370 exogenous supply of soluble Cdc6. The requirement for exogenous Cdc6 for E4 mediated
inhibition of DNA replication may be explained by the differential regulation of Cdc6 in
372 normal proliferating cells, where Cdc6 is found in both the soluble and chromatin-bound
fractions (30) and during the G₀-S transition, where Cdc6 is synthesised *de novo* and
374 immediately recruited to replication origins (12). In the *in vitro* replication assay used in
this study, NIH3T3 G₁ nuclei are prepared during release from density-dependent growth
376 arrest (G₀) and therefore contain only chromatin-bound Cdc6 protein (31). How HPV1 E4
and the soluble form of the licensing factor Cdc6 function together to inhibit replication is
378 not yet understood, but they are sufficient to block MCM recruitment to origins even in the
presence of chromatin bound Cdc6. One possibility is that E4 is able to complex with
380 MCM factors in a soluble Cdc6-dependent manner. We have evidence of an association
between a GST-HPV1 E1^ΔE4 fusion protein and an epitope-tagged form of Mcm7
382 expressed in cell lysates containing soluble Cdc6 (I. Bell and Sally Roberts, preliminary
data). Association between E1^ΔE4 and this MCM factor however, might not be a complete
384 description of the mechanism of E4-mediated replication inhibition since ΔE4, the form of
HPV1 E4 defective in inhibiting cellular DNA synthesis, can form an association with
386 Mcm7 (I. Ashmole and S. Roberts, unpublished data). It is feasible that further
modification, for example a phosphorylation event, is necessary to achieve an “active”
388 inhibitory complex and this might be dependent on N-terminal sequences specific to the
full-length E1^ΔE4 polypeptide. Whatever the underlying mechanism, inhibition of
390 initiation of cellular DNA replication *in vitro* and suppression of entry of epithelial cells
into S phase are functions both dependent on arginine 45 in the HPV1 E1^ΔE4 polypeptide,
392 suggesting that these two functions are linked and hence implies that E4 can block cellular
DNA synthesis in the presence of endogenous soluble Cdc6.

394 Our studies have shown that E4-induced inhibition of *in vitro* replication initiation is
dependent on a full-length E1^{E4} molecule. It is possible that the smaller forms of E4 that
396 exist in the purified preparations of HPV1 E4 protein used in this study may contribute to
this E1^{E4} function. Indeed HPV1 E4 expression studies show that co-expression of full-
398 length and truncated forms of HPV1 E4 act to repress cell proliferation, whilst expression
of the full-length form alone did not (13). Complex formation between the different E4
400 polypeptides (14) might be one explanation, either the complex inhibiting S phase entry
directly or, upon formation, depleting free full-length protein to a level that it is then active
402 with regards to blocking cell proliferation. This latter explanation might well explain why
there is no block in cell proliferation in cells expressing the E1^{E4} protein alone even
404 though a small amount of the truncated E4 species does accumulate in these cells.

We do not know at this stage of our investigations whether this novel HPV1 E4 function is
406 conserved between the different phylogenetic types. Arginine 45 lies in a region of HPV1
E4 that is rich in basic amino acids and indeed similar (but not identical) regions are to be
408 found in E4 proteins of types with a dissimilar tropism to HPV1, such as HPV16 and 18
that have preference for epithelia of the oral and anogenital tracts (1). The basic region of
410 HPV16 E4 forms part of the G2 arrest domain (5), but in HPV1 E4 is not a required
element of the G2 arrest function, and nor do these regions contribute to the interaction
412 with the keratin cytoskeleton (25, 27). An association between HPV1 E4 and the SR
protein kinase SRPK1 is dependent on arginine 45 (1), although other sequences required
414 to maintain this interaction do not contribute to inhibition of cell proliferation, suggesting
that this E4 binding partner is unlikely to be involved in the underlying mechanism of
416 replication inhibition by HPV1 E4. Therefore, either arginine 45 mediates an association
to a novel E4-binding protein, or dictates a specific cellular localization, necessary for
418 replication inhibition.

Host cell DNA synthesis is blocked during Epstein-Barr virus (EBV) lytic infection cycle
420 during which there is high level amplification of the EBV genome (17). EBV inactivates
MCM helicase function by phosphorylation of MCM proteins and this might be sufficient

422 to block cellular DNA synthesis in lytic infected cells (16). Infection by another DNA
virus, cytomegalovirus (CMV) also abrogates cellular replication licensing by inhibiting
424 chromatin loading of MCM proteins (2, 37). Even though the underlying mechanism of
repression of cellular DNA replication by EBV and CMV were not identified, taken
426 together with our study, it implies that unrelated DNA viruses may have evolved similar
strategies to selectively inhibit host cell DNA synthesis. This function could prove
428 advantageous to viruses that depend on the host cell for the supply of essential replication
enzymes and nucleotides for viral DNA synthesis. Papillomaviruses have three phases of
430 replication; establishment and maintenance of the genome in basal cells is followed by
vegetative genome amplification in cells that have migrated up from the basal layer and
432 differentiated (10). Because keratinocyte differentiation normally correlates with exit from
the cell cycle, the virus induces S phase gene activity in these cells, and eventually they
434 initiate vegetative viral genome replication, whereby the viral genome is amplified to high
copy number (4). Notably, the switch to genome amplification is associated with induction
436 of E4 protein (23). Furthermore, more recently, it has been shown that this switch also
correlates with suppression of cellular DNA synthesis in replication-activated HPV16-
438 containing keratinocytes (20). We therefore hypothesize that in these cells, E4 acts to
preserve the supply of essential host replication factors by inhibiting licensing of cellular
440 origins of replication and thus repress competing cellular DNA synthesis. Combined with
action on G2-to-M transition of the cell cycle (5, 13, 19), E4 could be a key player in
442 ensuring successful replication of the virus. Indeed, loss of expression of the full-length
E1^ΔE4 protein is associated with an abrogation of efficient vegetative genome replication
444 in systems that recapitulate the productive replication life cycles of HPV16, 18 and 31 and
cottontail papillomavirus (20, 22, 38, 39).

446 Viral factors such as E4 could provide powerful molecular tools that can be utilized to
dissect the molecular mechanisms regulating initiation of eukaryotic DNA replication.
448 Furthermore, because the origin licensing machinery has been proposed as a novel
attractive target for anti-cancer therapy, the design of E4-based mimetic compounds could
450 provide novel non genotoxic agents.

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Figure legends

590 **Figure 1. HPV1 E4 inhibits initiation of cellular DNA synthesis in an *in vitro***
replication assay. (A) Cell-free cellular DNA replication system. Nuclei (N)
 592 prepared from G1 phase NIH3T3 fibroblasts, synchronized by release from
 quiescence (G0), initiate a single round of semi-conservative DNA replication in
 594 cytosolic extracts (SC) from S phase HeLa cells following incubation in buffer A
 (BA) that support elongation, nucleotides (dNTPs, NTPs) and an ATP regeneration
 596 system (CP, CK). Nuclei are stained with propidium iodide to reveal DNA (red)
 and with fluorescein-streptavidin (green) to detect biotin-16-dUTP incorporation
 598 resulting from *in vitro* DNA synthesis. **(B)** Coomassie-stained SDS-PAGE gel of
 baculovirus recombinant HPV1 E4 proteins. Lane 1, molecular weight standards
 600 (12.3-, 17.2-, 30-, 42.7-, 66-, 76-kDa); lanes 2 and 3, purified HPV1 E4 proteins
 WT38 and WT43 containing variable levels of full-length E1^{E4} (17-kDa) and
 602 processed (16- and 11-kDa) species. **(C)** NIH3T3 G1 nuclei were incubated in
 cytosolic extracts from S phase HeLa cells, which induce initiation in competent
 604 nuclei, or in elongation buffer (Buffer A) which only supports elongation DNA
 synthesis in nuclei that are already in S phase. Addition of E4 (but not Δ E4) and
 606 Cdc6 to co-incubations inhibits DNA synthesis at a level comparable to the effect
 of geminin. Results are expressed as the percentage of nuclei replicating (mean \pm
 608 standard deviation), and asterisks indicate a significance of >99.99% in the
 decrease of replicating nuclei compared to control replication assays. **(D)** Addition
 610 of E4 and Cdc6 to co-incubations of NIH3T3 S phase nuclei and HeLa S phase
 cytosol had no effect on replication potential. Similarly, addition of geminin did
 612 not affect ongoing DNA synthesis. Data analyzed as described in C.

614 **Figure 2. HPV1 E4 suppresses recruitment of MCM proteins onto chromatin *in vitro*.**
 Immunoblots of chromatin-bound protein fractions prepared from NIH3T3 G1
 616 phase **(A)** and S phase **(B)** nuclei taken through *in vitro* replication assays. The
 densities of protein bands were determined and shown in the histograms relative to

618 those measured in nuclei incubated in S phase cytosol (SC), after normalization
 620 against histone H1 loading,

620

**Figure 3. Inhibition of S phase entry by HPV1 E4 in epithelial cells is dependent on
 622 an arginine residue within the E1^ΔE4 protein.** Cos-1 epithelial cells were
 624 transfected with expression plasmids and pulse-labelled with BrdU, and the
 626 percentage of BrdU-positive S phase cells counted. (A) Co-expression of full-
 628 length E1^ΔE4 with the truncated E4 protein E4-16K inhibits S phase entry
 compared to expression of the polypeptides alone. Alanine replacement of arginine
 45 (R45A), but not of arginines 47 (R47A) or 48 (R48A), is sufficient to relieve the
 inhibitory effect upon S phase progression (mean ± standard deviation). The
 double and single asterisk(s) indicates a significance of 99.99% and 99.98%
 630 respectively, in the decrease in the percentage of BrdU-positive cells compared to
 Cos cells transfected with empty vector. The data shown was collected from seven
 632 independent experiments. The two-dimensional BrdU-PI profiles of cells
 expressing E4-16K, E1^ΔE4+E4-16K, R45A+E4-16K and R47A+E4-16K are as
 634 shown. (B) Immunoblot of protein extracts showing E4 protein expression in Cos-
 1 cells. Migration of full-length (E1^ΔE4) and truncated (E4-16K) polypeptides are
 636 as indicated.

**Figure 4. The arginine-rich motif in E1^ΔE4 is necessary for inhibition of cellular DNA
 638 synthesis initiation *in vitro*.** (A) Addition of bacterial recombinant wildtype
 640 HPV1 E4 protein (WTE4) and Cdc6 to co-incubations of NIH3T3 G1 nuclei and
 HeLa S phase cytosol inhibits cellular DNA synthesis in replication competent
 642 nuclei. E4-mediated inhibition is relieved following addition of mutant E4 proteins
 containing either a deletion of residues 44 to 48 (E4 Δ 44-48) or a single alanine
 644 replacement of arginine 45 (E4R45A), together with Cdc6. Data from three
 independent experiments are given as the mean ± standard deviation, and a single

646 asterisk indicates a >99.99% significance in the decrease of replicating nuclei
compared to control replication assay containing exogenous Cdc6. **(B)** Immunoblot
648 of chromatin-bound protein fractions prepared from NIH3T3 G1 nuclei taken
through in vitro replication reactions containing wild type and mutant E4 proteins.
650 The histogram shows the densities of the protein bands, after normalization against
histone H1 loading, relative to those in nuclei incubated in S phase cytosol (SC).

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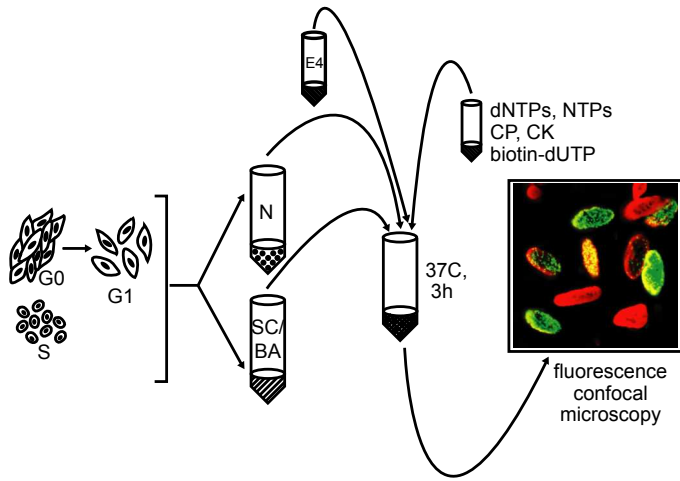
674 TABLE 1. Percentage of BrdU positive S phase Cos-1 epithelial cells following
 676 transfection with wild type and mutant HPV1 E4 expression plasmids^a

678	HPV1 E4	% of BrdU-positive S phase cells
	E4-17K	15.2 ± 1.8
	E4-16K	14.4 ± 1.5
	E4-17/16K	7.9 ± 0.3
	E4-17KΔ2-5/16K	9.0 ± 1.5
680	E4-17KΔ10-14/16K	8.1 ± 1.7
	E4-17KΔ21-24/16K	8.8 ± 1.7
	E4-17KΔ24-27/16K	6.0 ± 1.8
	E4-17KΔ27-30/16K	9.7 ± 1.5
	E4-17KΔ32-33/16K	8.8 ± 1.6
682	E4-17KΔ44-48/16K	16.2 ± 1.6
	E4-17KΔ49-53/16K	7.6 ± 0.9
	E4-17KΔ57-60/16K	6.3 ± 1.1
	E4-17KΔ110-115/16K	8.2 ± 0.6
	E4-17KΔ61-125/16K	10.0 ± 1.2
684	pcDNA	16.6 ± 2.1

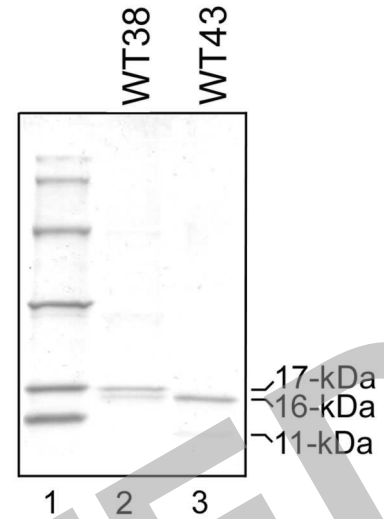
686 ^aData taken from four independent experiments and results are shown as the means
 ± standard deviations.

Figure 1

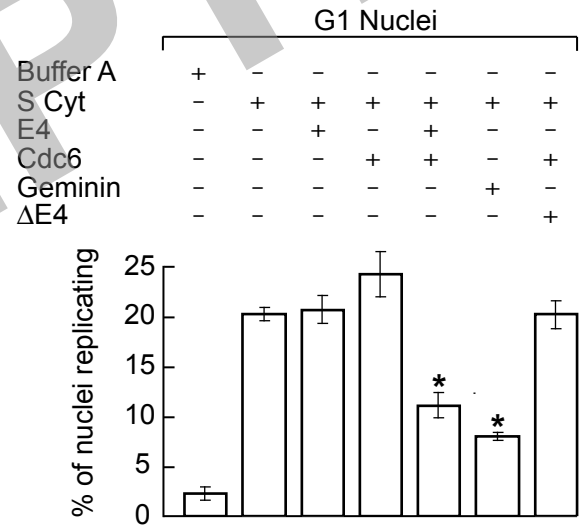
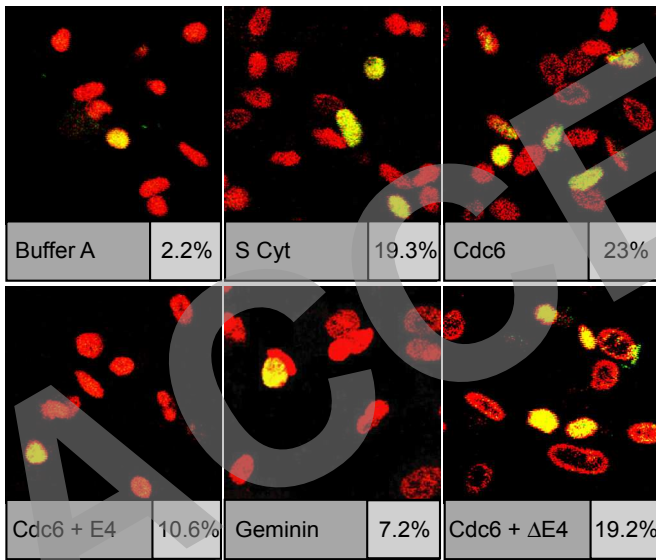
a



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c



d

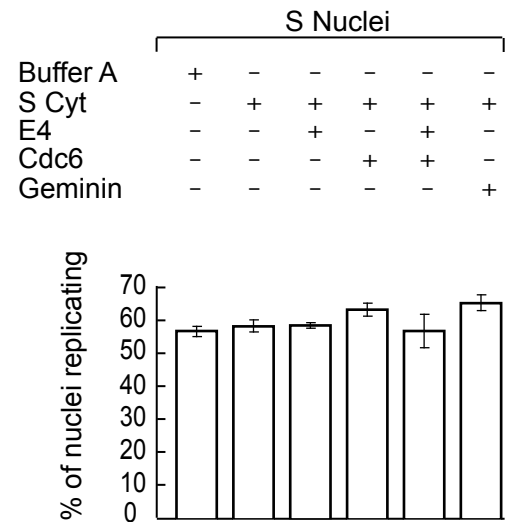
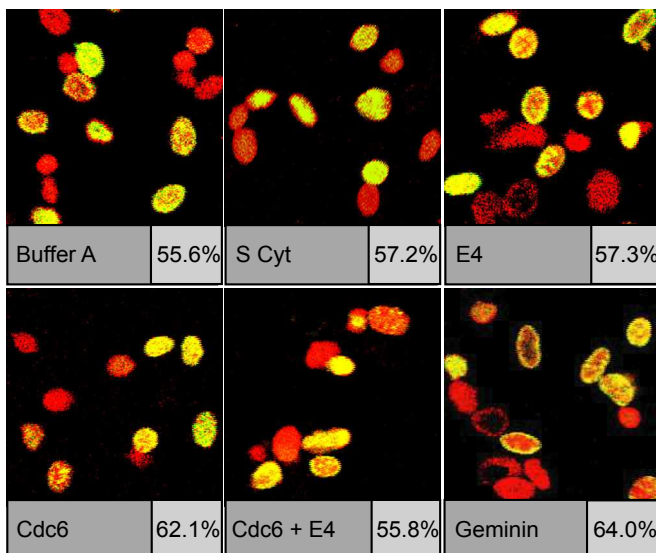
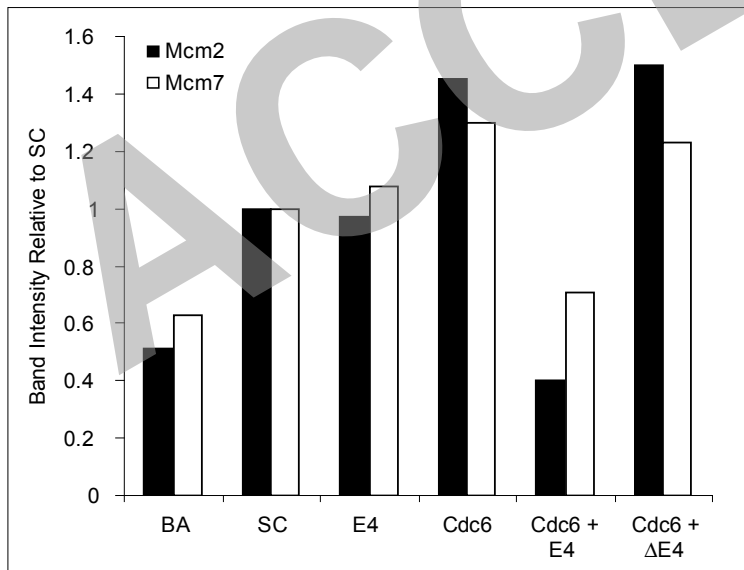
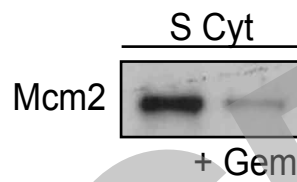
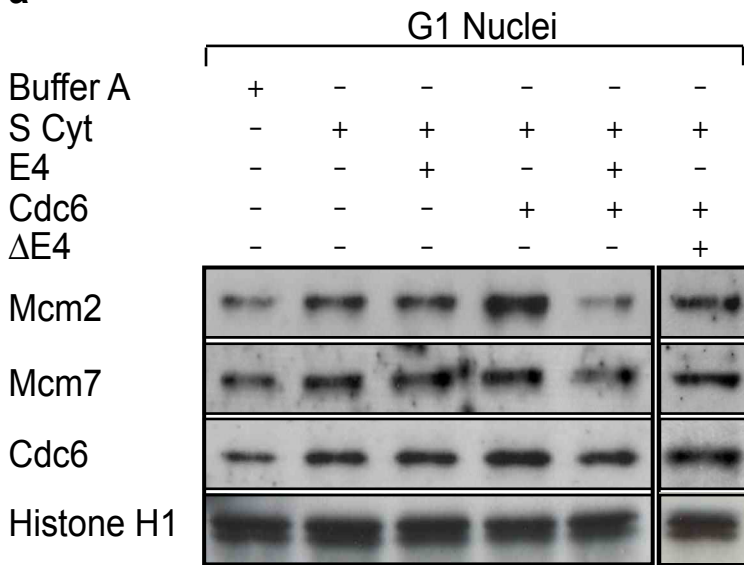


Figure 2

a



b

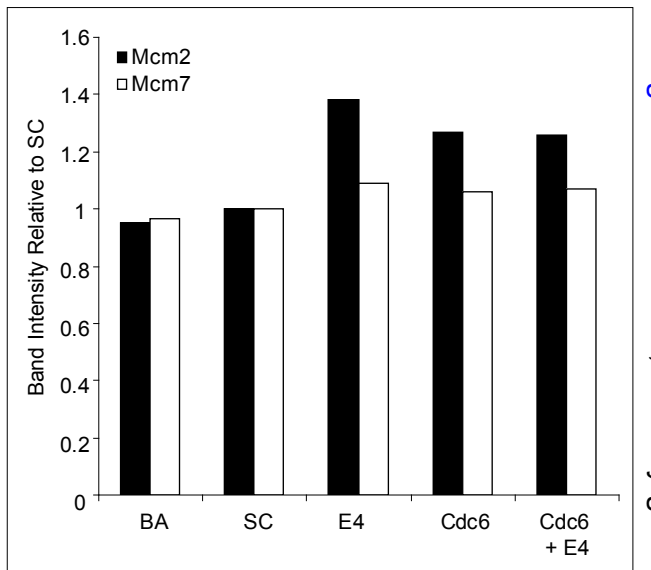
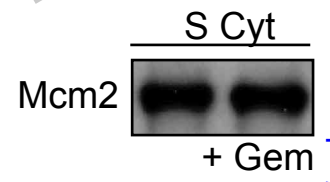
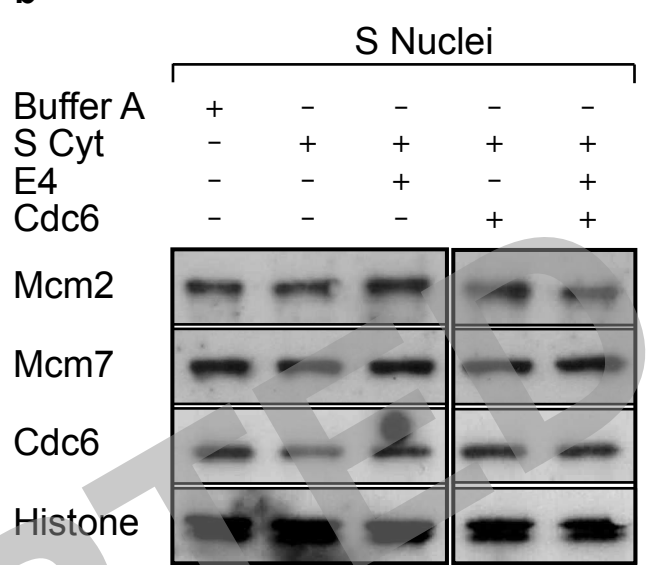
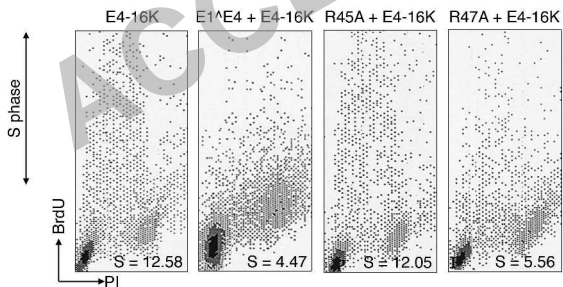
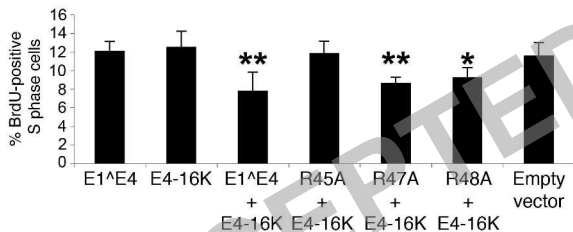


Figure 3

A



B

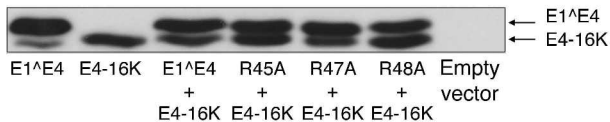
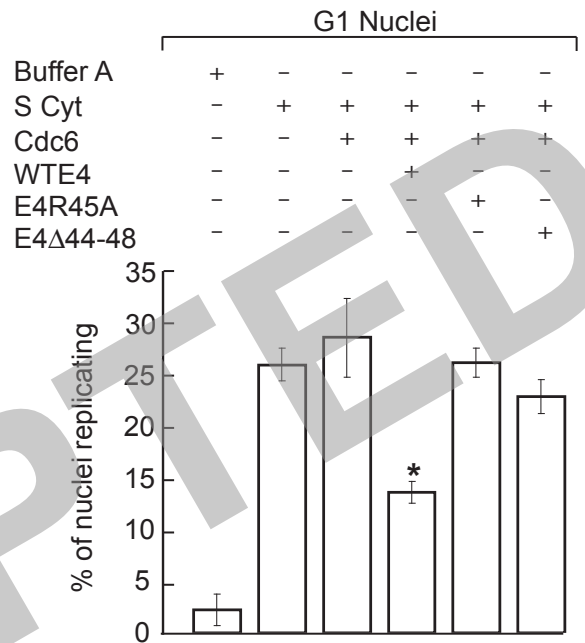
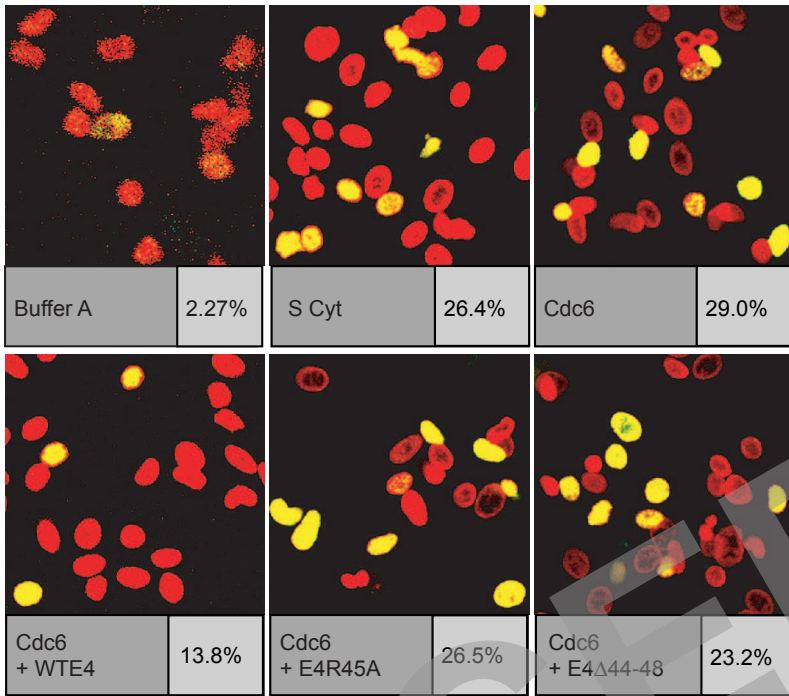


Figure 4

a



b

