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Identification of an arginine-rich motif in human papillomavirus type 1 E1^E4 protein necessary for E4 mediated inhibition of cellular DNA synthesis *in vitro* and in 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.

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 infections (23).

Examination of E4 activity in epithelial cell cultures has revealed diverse biological 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 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 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 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 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 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 keratin cytoskeleton and cornified envelope formation, and inducing apoptosis through alteration of mitochondria function, to facilitate egress of the newly formed HPV virions (3, 6, 24, 26).

Execution of multiple functions might be assisted by conversion of the E4 protein into 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 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 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 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-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 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 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 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, 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 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 that HPV1 E4 affects a key step in the cellular DNA replication process.

Materials and methods

HPV1 E4 expression plasmids

Construction of plasmids based on pcDNA3.1 that deliver expression of the 17-kDa full-length HPV1 E1^E4 (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 deletions within the full-length E1^E4 coding sequence (25) were excised and inserted into the BamHI restriction site of pcDNA3.1 (Invitrogen, Carlsbad, CA). The sequence integrity of plasmid inserts was verified by bi-directional DNA sequencing. Substitutions E4R45A, E4R47A and E4R48A are described elsewhere (1).

Expression of recombinant proteins

HPV1 E4 and *Xenopus laevis* Cdc6 (His_6-*Xe*Cdc6) proteins were purified from Sf9 insect cells following infection with appropriate recombinant baculoviruses, as previously described (28, 31). Histidine-tagged human geminin (His_6-*hs*Geminin) was expressed in *Escherichia coli* and purified as described (36). For expression of histidine-tagged HPV1 E4 protein in bacteria, the E1^E4 wild type and mutant coding sequences were inserted 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 recombinant protein was purified by IMAC using $NiCl_2$-charged HiTRAP chelating columns (GE Healthcare Europe GmbH, Munich, Germany). Non-specifically bound 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 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.

In vitro cellular DNA replication assays

Nuclei and cytosolic extracts were prepared from synchronized NIH3T3 and HeLa S3 cells 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 of cytosolic extracts (250-300 µg of protein), 10 µl of premix buffer (160 mM K-HEPES pH 7.8, 28 mM $MgCl_2$, 12 mM ATP, 0.4 mM of GTP, CTP, UTP, dATP, dGTP and dCTP, 1 µM biotin-16-dUTP, 2 mM dithiothreitol, 160 mM creatine phosphate, 20 µg phosphocreatine kinase), 1 x 10^5 nuclei and, where indicated, up to 10 µl of recombinant protein(s). His_6-*Xe*Cdc6 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, baculovirus-expressed HPV1 E4 proteins in 10 mM phosphate buffer pH 7.4, 0.1 mM DTT at 3 µM, bacterially-expressed His_6-E4 proteins in 40 mM Tris-HCl pH 7.6, 30 mM NaCl, and His_6-*hs*Geminin protein in 50 mM Na-phosphate at 4 µM. Equal volumes of appropriate buffers were added to control reactions. All components of the replication 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 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, 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%

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 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 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 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 assayed, in triplicate, for each set of experiments and analysis performed by two individuals. Statistical analysis of data from multiple independent experiments was performed by single factor analysis of variance (ANOVA).

Chromatin-binding assay

In vitro DNA replication assays were set up as described above. After 3 h incubation, nuclei were pelleted by low speed (1300x*g*) centrifugation and used in chromatin-binding reactions performed as described previously (12). Samples were immunoblotted with antibodies against Mcm2 (BD Biosciences, #610701), Mcm7 (Neomarkers, Lab Vision, 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 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 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 (BrdU) at a final concentration of 33 μM for 2 h. Cells were then fixed, incubated with an anti-BrdU antibody conjugated to fluorescein isothiocyanate, labelled with propidium

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

Results

HPV1 E4 inhibits cellular DNA replication *in vitro*

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 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. 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 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).

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 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 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 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 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 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 recombinant preparations of the replication-licensing factor (RLF) Cdc6 (His_6-*Xe*Cdc6) and geminin (His_6-*hs*Geminin), a known cellular repressor of replication licensing (18).

The percentage of replicating nuclei increased from 19.3% to 23% in reactions containing His_6-*Xe*Cdc6 (Fig. 1C). Since Cdc6 is known to be rate-limiting for replication competence after release from G0 (31), the small but consistent increase in replicating nuclei in the presence of His_6-*Xe*Cdc6 indicates a low number of G1 nuclei are responsive to this RLF. In contrast, in the presence of His_6-*hs*Geminin there was a marked and significant decrease (7.2%) in the percentage of replication competent nuclei (Fig. 1C).

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 (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 of G1 nuclei and S phase cytosol (19.7%), when E4 was added to reactions that also contain exogenous Cdc6 (His_6-*Xe*Cdc6), the percentage of replicating nuclei decreased 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 inhibition was comparable to the inhibitory effect (67%) of His_6-*hs*Geminin (Fig. 1C).

The E4 protein added to the *in vitro* replication assays contained the full-length E1^E4 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 truncated proteins, but no full-length E1^E4 polypeptide (Fig. 1B, WT43) to the cell-free replication assay did not inhibit DNA synthesis in the G1 nuclei either in the absence (data 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 length E1^E4 protein.

To validate the specificity of our findings, identical replication reactions to those described 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 fibroblasts, achieved a similar level of replication inhibition (55%; data not shown).

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

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 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 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 recombinant E4 protein (WT38) to S phase nuclei, either in the absence or presence of His_6-*Xe*Cdc6 (Fig. 1D). The data from the *in vitro* replication assay (Fig. 1) indicate that 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*

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 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 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 HPV1 E4 might inhibit replication initiation by blocking assembly of pre-RCs onto 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 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 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 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 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

(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 HPV1 E4 protein (WT38) with His_6-*Xe*Cdc6 to the replication reactions was associated with a 3.5-fold and 2-fold decrease in chromatin-bound Mcm2 and Mcm7 proteins respectively, compared to reactions containing His_6-*Xe*Cdc6 alone (Fig. 2A). Notably, the reduced levels of chromatin-bound MCM proteins were close to base levels measured in 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 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^E4 protein (ΔE4) to reactions containing His_6-*Xe*Cdc6 (Fig. 2A). There was no evidence that E4 became bound to chromatin, either in the presence or absence of exogenous Cdc6 (see Fig. 4b).

Chromatin-binding studies on S phase nuclei taken through replication elongation assays show that neither addition of geminin nor E4 together with His_6-*Xe*Cdc6 affected the chromatin-binding status of Mcm2 and Mcm7 (Fig. 2B).

Together, the *in vitro* replication and chromatin-binding data indicate that inhibition of initiation of cellular DNA synthesis by HPV1 E4 in the presence of His_6-*Xe*Cdc6 correlates with reduced MCM loading onto chromatin.

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

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 identified the HPV1 E1^E4 sequences required for suppression of S phase entry in epithelial cells. Since the negative effect of HPV1 E1^E4 on S phase entry was dependent on the presence of a truncated 16-kDa E4 protein (13), HPV1 E1^E4 (E4-17K) expression plasmids containing small deletions that cover the majority of the E1^E4 sequence, were 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

keeping with our previous findings (13), transient expression of the full-length E1^E4 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^E4 deletion plasmids tested, only one, containing a deletion of residues 44 to 48 (GRPRR), did not inhibit S phase entry following co-transfection with E4-16K (Table 1). The G2 arrest function of this mutant E1^E4 protein however remained intact (data not shown).

The contribution of individual amino acids within the ^{44}GRPRR^{48} sequence to E1^E4 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 E1^E4 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, 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^E4 protein in epithelial cells (Fig. 3B), and all three mutants promoted G2 arrest to levels comparable with E4-17/16K expressing cells (data not shown).

The arginine-rich motif in E1^E4 is necessary for inhibition of cellular DNA synthesis initiation *in vitro*

Next, we wanted to determine whether the ^{44}GRPRR^{48} sequence E1^E4 was also involved in inhibition of cellular origin licensing in the *in vitro* replication assay. In this instance, 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 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 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 when E4 was added to reactions that also contain exogenous Cdc6 (His_6-*Xe*Cdc6), the percentage of replicating nuclei decreased significantly from 29% to 13.8%, indicating that

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 replication initiation was however abrogated upon the addition of the E4 protein containing deletion of ^{44}GRPRR^{48} to the *in vitro* reactions containing His$_6$-*Xe*Cdc6 (26.5%, Fig. 4A), 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).

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 whether failure to inhibit replication initiation by the mutant E1^E4 proteins correlated with efficient assembly of pre-RCs onto chromatin. Chromatin-bound protein fractions 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 G1 nuclei incubated with S phase cytosol in the presence of His$_6$-*Xe*Cdc6 and the wild-type protein derived from bacteria showed a 51% and 41% decrease in chromatin-bound Mcm2 and Mcm7 proteins respectively, compared to the level of these proteins in the reaction containing His$_6$-*Xe*Cdc6 alone (Fig. 4B). The reduced levels of chromatin-bound MCM 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 of mutant E1^E4 proteins E4Δ44-48 and E4R45A both Mcm2 and Mcm7 were efficiently recruited to chromatin (Fig. 4B).

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-rich motif within the full-length form of the viral protein.

Discussion

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. Chromatin binding studies indicate that E4 blocks replication initiation *in vitro* by preventing loading of licensing factors Mcm2 and Mcm7 onto chromatin. The functional

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 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 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 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 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 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 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 MCM factors in a soluble Cdc6-dependent manner. We have evidence of an association between a GST-HPV1 E1^E4 fusion protein and an epitope-tagged form of Mcm7 expressed in cell lysates containing soluble Cdc6 (I. Bell and Sally Roberts, preliminary data). Association between E1^E4 and this MCM factor however, might not be a complete 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 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" inhibitory complex and this might be dependent on N-terminal sequences specific to the full-length E1^E4 polypeptide. Whatever the underlying mechanism, inhibition of 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^E4 polypeptide, suggesting that these two functions are linked and hence implies that E4 can block cellular DNA synthesis in the presence of endogenous soluble Cdc6.

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 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-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 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 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 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 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 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 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 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 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 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 replication inhibition.

Host cell DNA synthesis is blocked during Epstein-Barr virus (EBV) lytic infection cycle 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

to block cellular DNA synthesis in lytic infected cells (16). Infection by another DNA virus, cytomegalovirus (CMV) also abrogates cellular replication licensing by inhibiting 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 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 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 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 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 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 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-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 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 ensuring successful replication of the virus. Indeed, loss of expression of the full-length E1^E4 protein is associated with an abrogation of efficient vegetative genome replication in systems that recapitulate the productive replication life cycles of HPV16, 18 and 31 and cottontail papillomavirus (20, 22, 38, 39).

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. 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 provide novel non genotoxic agents.

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Figure legends

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) prepared from G1 phase NIH3T3 fibroblasts, synchronized by release from quiescence (G0), initiate a single round of semi-conservative DNA replication in cytosolic extracts (SC) from S phase HeLa cells following incubation in buffer A (BA) that support elongation, nucleotides (dNTPs, NTPs) and an ATP regeneration system (CP, CK). Nuclei are stained with propidium iodide to reveal DNA (red) and with fluorescein-streptavidin (green) to detect biotin-16-dUTP incorporation resulting from *in vitro* DNA synthesis. (**B**) Coomassie-stained SDS-PAGE gel of baculovirus recombinant HPV1 E4 proteins. Lane 1, molecular weight standards (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 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 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 ΔE4) and 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 ± standard deviation), and asterisks indicate a significance of >99.99% in the decrease of replicating nuclei compared to control replication assays. (**D**) Addition 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 not affect ongoing DNA synthesis. Data analyzed as described in C.

Figure 2. HPV1 E4 suppresses recruitment of MCM proteins onto chromatin *in vitro.* Immunoblots of chromatin-bound protein fractions prepared from NIH3T3 G1 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 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^E4 protein.** Cos-1 epithelial cells were transfected with expression plasmids and pulse-labelled with BrdU, and the 624 percentage of BrdU-positive S phase cells counted. (**A**) Co-expression of full-length E1^E4 with the truncated E4 protein E4-16K inhibits S phase entry 626 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 628 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^E4+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^E4) and truncated (E4-16K) polypeptides are 636 as indicated.

638 **Figure 4. The arginine-rich motif in E1^E4 is necessary for inhibition of cellular DNA 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 (E4Δ44-48) 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

asterisk indicates a >99.99% significance in the decrease of replicating nuclei compared to control replication assay containing exogenous Cdc6. (**B**) Immunoblot of chromatin-bound protein fractions prepared from NIH3T3 G1 nuclei taken through in vitro replication reactions containing wild type and mutant E4 proteins. 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).

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

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
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
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
pcDNA	16.6 ± 2.1

[a]Data taken from four independent experiments and results are shown as the means ± standard deviations.

Figure 1

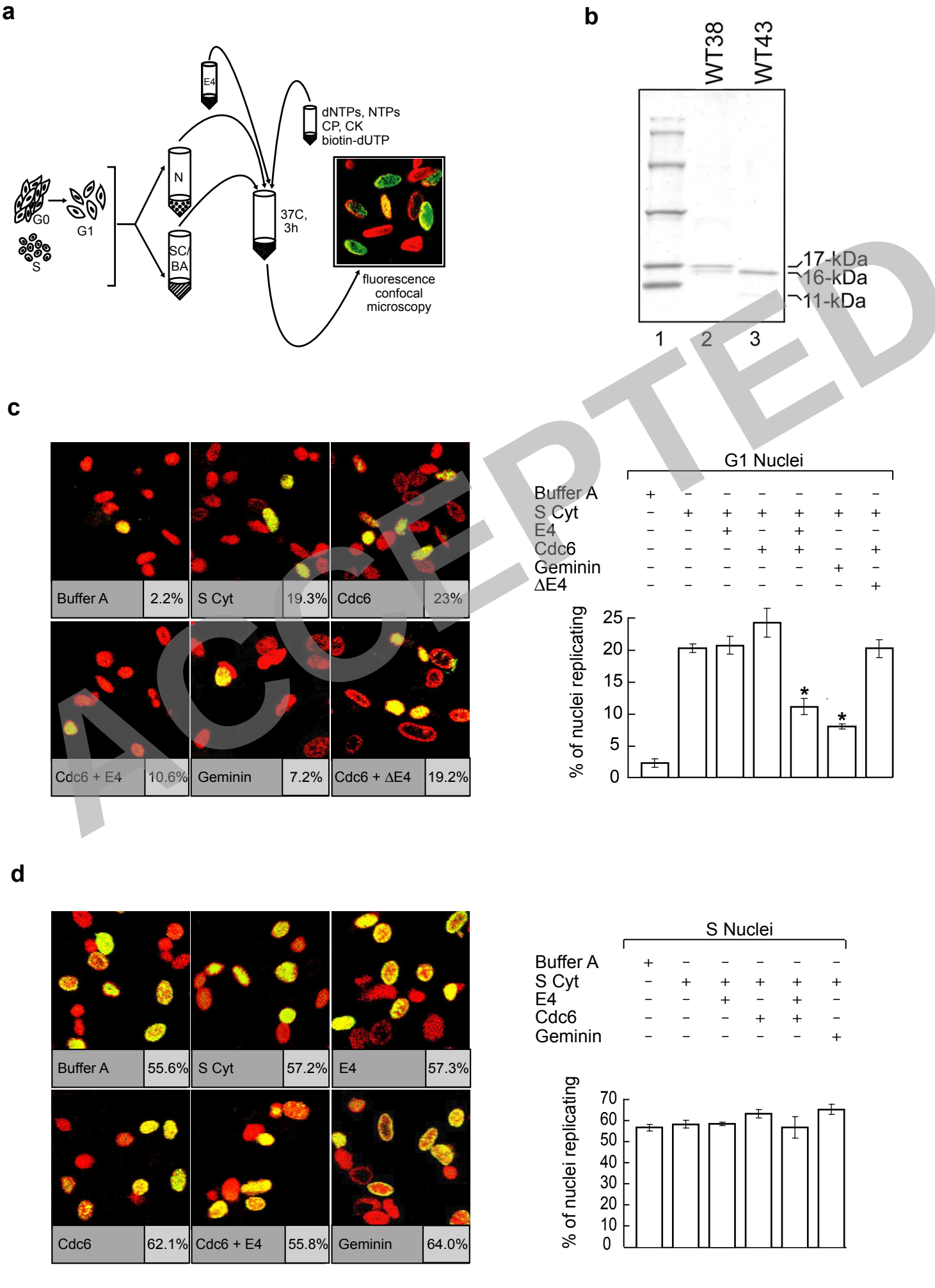


Figure 2

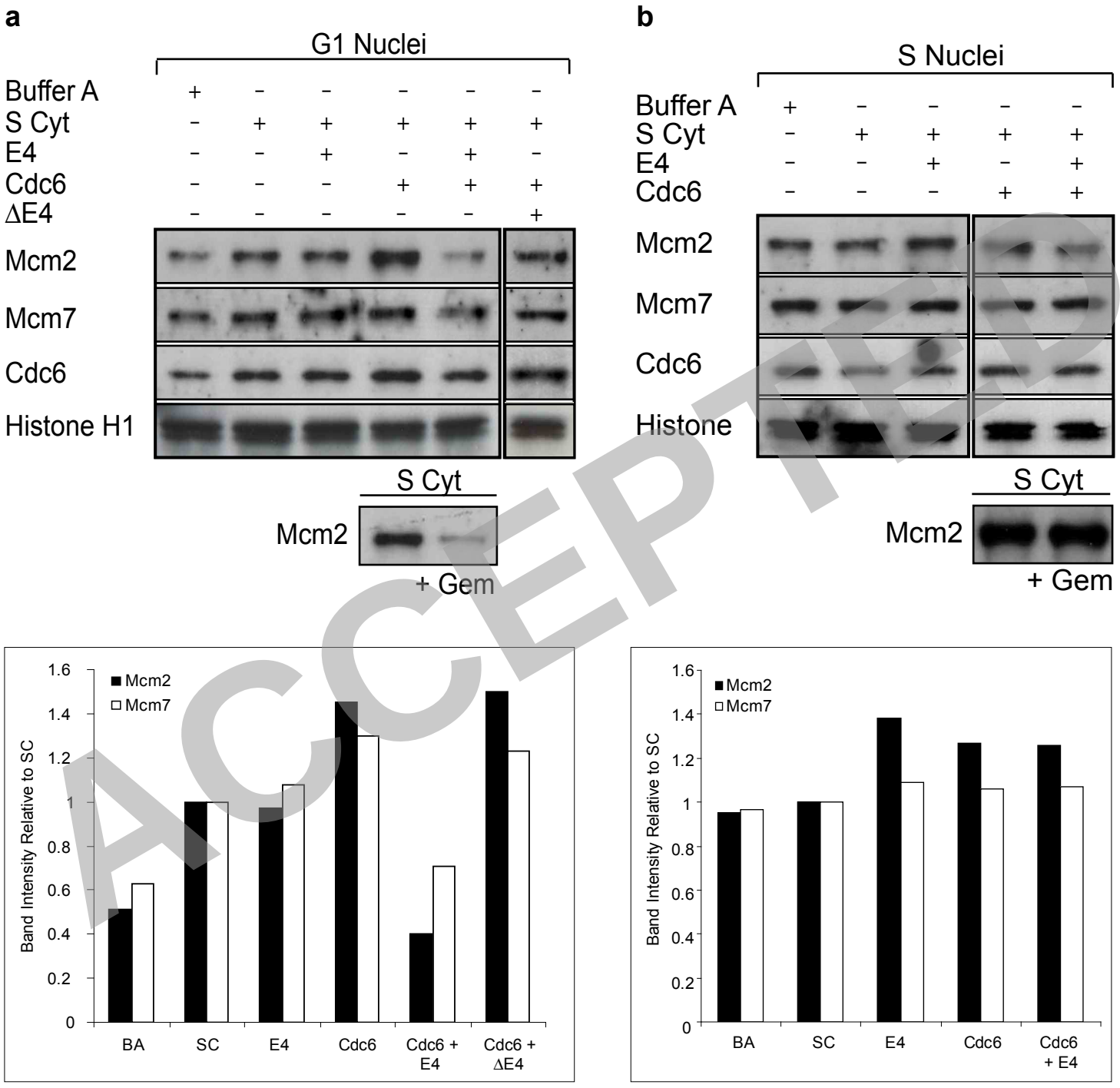


Figure 3

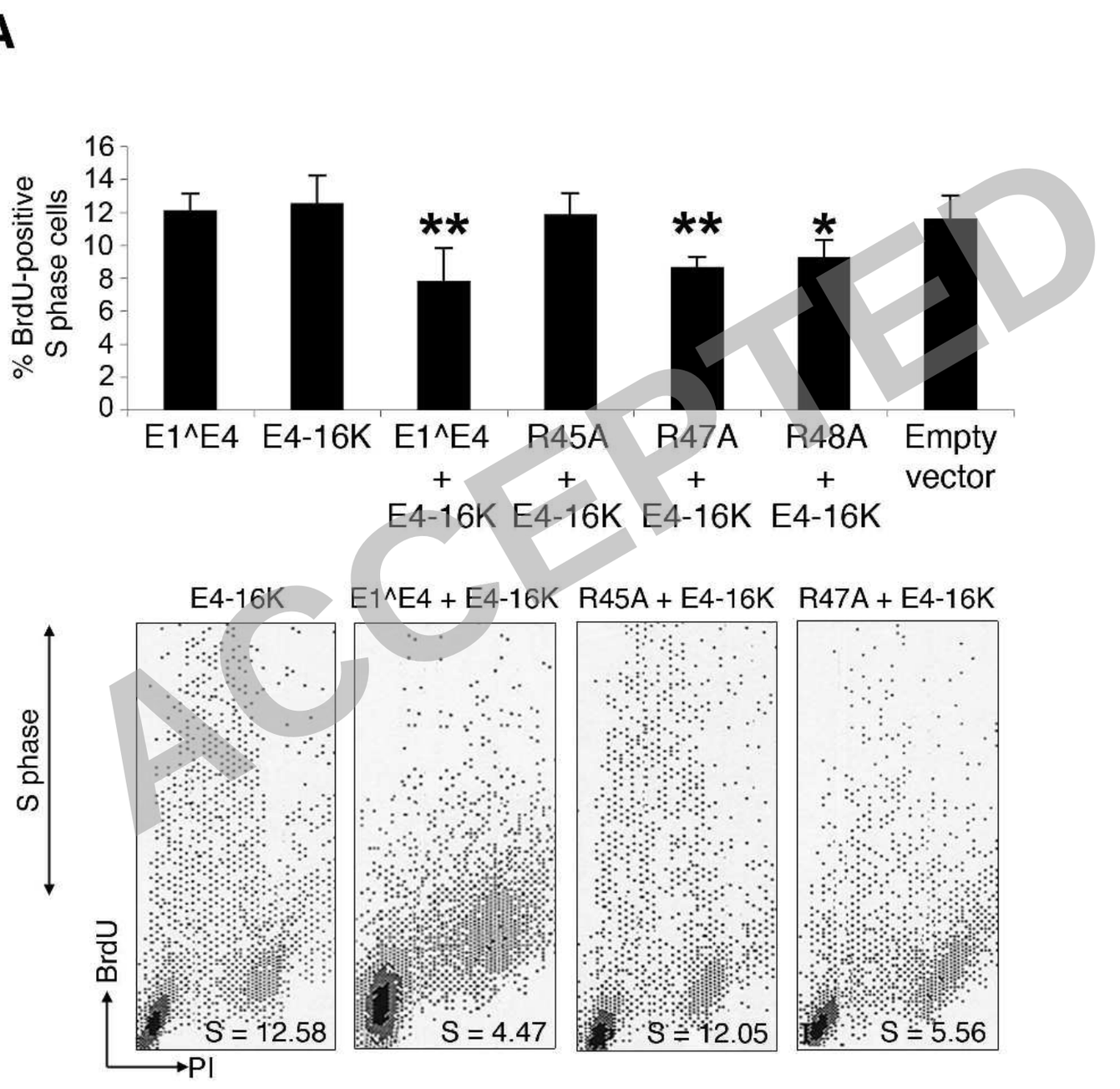
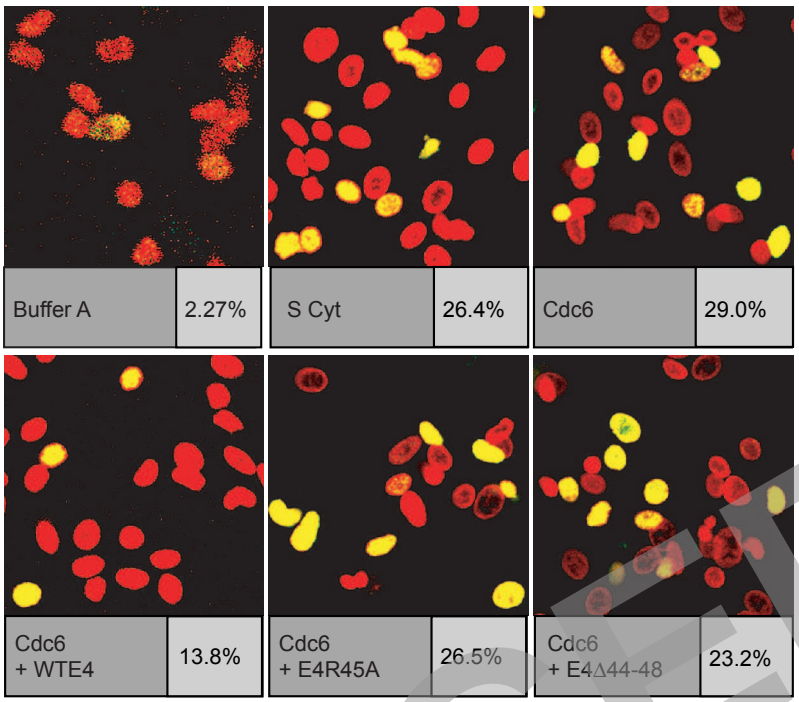
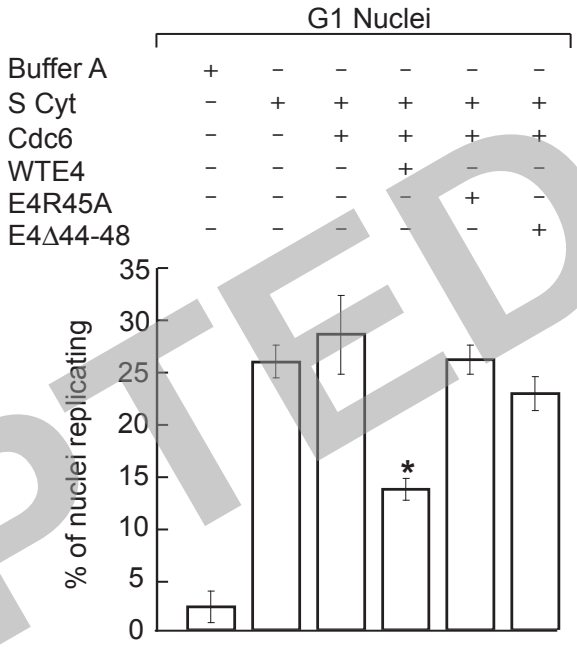


Figure 4

a





b