

Disruption of repressive p130–DREAM complexes by human papillomavirus 16 E6/E7 oncoproteins is required for cell-cycle progression in cervical cancer cells

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Human papillomaviruses (HPVs) with tropism for mucosal epithelia are the major aetiological factors in cervical cancer. Most cancers are associated with so-called high-risk HPV types, in particular HPV16, and constitutive expression of the HPV16 E6 and E7 oncoproteins is critical for malignant transformation in infected keratinocytes. E6 and E7 bind to and inactivate the cellular tumour suppressors p53 and Rb, respectively, thus delaying differentiation and inducing proliferation in suprabasal keratinocytes to enable HPV replication. One member of the Rb family, p130, appears to be a particularly important target for E7 in promoting S-phase entry. Recent evidence indicates that p130 regulates cell-cycle progression as part of a large protein complex termed DREAM. The composition of DREAM is cell cycle-regulated, associating with E2F4 and p130 in G0/G1 and with the B-myb transcription factor in S/G2. In this study, we addressed whether p130–DREAM is disrupted in HPV16-transformed cervical cancer cells and whether this is a critical function for E6/E7. We found that p130–DREAM was greatly diminished in HPV16-transformed cervical carcinoma cells (CaSki and SiHa) compared with control cell lines; however, when E6/E7 expression was targeted by specific small hairpin RNAs, p130–DREAM was reformed and the cell cycle was arrested. We further demonstrated that the profound G1 arrest in E7-depleted CaSki cells was dependent on p130–DREAM reformation by also targeting the expression of the DREAM component Lin-54 and p130. The results show that continued HPV16 E6/E7 expression is necessary in cervical cancer cells to prevent cell-cycle arrest by a repressive p130–DREAM complex.

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INTRODUCTION

Cervical cancer accounts for almost 12% of all cancers in women and represents the second most common cause of cancer death in females in the world. Almost all squamous-cell carcinomas and the vast majority of adenocarcinomas of the cervix are associated with infection by so-called high-risk human papillomavirus (HPV) types, most commonly HPV type 16 (reviewed by McLaughlin-Drubin & Münger, 2009). In contrast, low-risk HPV types that infect mucosal epithelium, for example HPV6, are generally associated with non-cancerous neoplasias such as condylomas. All HPV types infect replicating keratinocytes in the basal or parabasal layer of the epithelium, and the HPV life cycle is linked tightly to the differentiation state of these cells (reviewed by Doorbar, 2006). HPV replication requires that infected cells retain replicative capacity within the suprabasal

layer, and the expression of HPV E6 and E7 proteins is critical for this property. A common feature of carcinogenic progression of persistent high-risk HPV types is integration of part of the viral genome into the host chromosome, such that normal regulation of E6 and E7 is lost. Several studies have shown that the resultant overexpression of E6/E7 is critical for cervical cancer cell growth; for example, targeting the bicistronic HPV16 E6/E7 mRNA by RNA interference was found to result in G1 arrest and apoptosis (Jiang & Milner, 2002; Hall & Alexander, 2003; Tang *et al.*, 2006; Sima *et al.*, 2008; Yamato *et al.*, 2008).

E6 and E7 are small proteins of approximately 100 aa that appear to be derived from a common precursor, but have subsequently evolved distinct functions. In high-risk HPV types, the abilities of E6 and E7 to bind to and inactivate the functions of cellular p53 and Rb tumour-suppressor proteins, respectively, are critical to their functions as oncoproteins. In addition, E6 and E7 interact with many other cellular proteins that regulate cell survival and proliferation (reviewed by McLaughlin-Drubin & Münger,

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2009). Although it is generally accepted that E6/E7 proteins of high-risk HPV types target cellular tumour suppressors most effectively, high-risk and low-risk E7 proteins share the ability to target the Rb-related p130 protein for degradation (Zhang *et al.*, 2006). This property is significant, as other studies have shown that the capacity of different E7 types to target p130 correlates strongly with their ability to induce S phase in infected cells (Genovese *et al.*, 2008).

Recently, p130 and the related p107 protein have been found to be constituents of a transcriptionally repressive complex termed DREAM (or LINC). In this complex, p130 or p107 are associated with E2F4 or E2F5 and bind to the promoters of genes that regulate entry into S phase (Litovchick *et al.*, 2007; Pilkinton *et al.*, 2007; Schmit *et al.*, 2007; Knight *et al.*, 2009). Upon dissociation of p130/p107 and E2F4/5 from this complex at G1/S, the core DREAM proteins form an alternative complex with the B-myb transcription factor (Litovchick *et al.*, 2007; Pilkinton *et al.*, 2007; Schmit *et al.*, 2007; Knight *et al.*, 2009). B-myb–DREAM then plays an important role in promoting transcription of genes required for mitosis. The DREAM complex was originally discovered in *Drosophila melanogaster* (Korenjak *et al.*, 2004; Lewis *et al.*, 2004) and the protein components are conserved across many species including nematodes (Harrison *et al.*, 2006). The DREAM core consists of Lin-9, Lin-37, Lin-54, Lin-52 and RbAp48 in mammals (Fig. 1), the homologues of *Drosophila* Mip130, Mip40, Mip120, dLin-52 and Caf1p55, respectively.

In this study, we investigated whether expression of HPV16 E6/E7 in cervical cancer cells results in disruption of the

p130–DREAM and p107–DREAM complexes and conversely favours the formation of B-myb–DREAM. In addition, we have studied whether depletion of E6/E7 by RNA interference leads to reformation of p130–DREAM and whether this is required for the G1 arrest precipitated by E6/E7 depletion. Our results show that disruption of the p130–DREAM complex by HPV16 E6/E7 in CaSki cervical cancer cells is critical in order to induce cell-cycle progression from G1 to S phase.

RESULTS

DREAM complexes are disrupted in cervical cancer cell lines

To determine whether the overexpression of HPV16 E6/E7 in cervical cancer cell lines is associated with loss of specific DREAM complexes, four cell lines (T98G, C33A, SiHa and CaSki) were employed. T98G glioblastoma cells were used as a control, as the DREAM complexes have been well-characterized in this cell line (Schmit *et al.*, 2007). C33A is a cervical carcinoma cell line that does not carry any HPV genes, whereas SiHa and CaSki cells are HPV16-positive, with two and 600 copies, respectively, of the E6/E7 gene per cell. In these experiments, the core DREAM component (Fig. 1) was immunoprecipitated from nuclear lysates using Lin-9 antibodies, and the presence of p130, p107 or B-myb in the immunoprecipitates was determined by Western blotting. Input controls showed that expression of p130 is reduced in the HPV16-transformed cell lines (SiHa and CaSki) compared with the HPV-negative control T98G and C33A cell lines (Fig. 2). The reduction of p130 levels in SiHa and CaSki cells is presumably due to E7-mediated degradation (Zhang *et al.*, 2006). Furthermore, p130–DREAM complexes are most abundant in T98G cells (Fig. 2) and scarce in E7-expressing cells (in particular CaSki), and this presumably reflects both the reduced p130 levels and interference with binding of p130 to E2F4/5 by E7. Although the input control showed that p107 expression was abundant in C33A cells and could also be detected readily in SiHa cells (Fig. 2), p107 is at most a minor constituent of DREAM complexes in these cell lines and was undetectable in CaSki cells (Fig. 2). B-myb expression, as indicated by the input controls, was higher in SiHa and CaSki cells than in the control cell lines (Fig. 2); this is probably the result of deregulation of *B-myb* transcription by HPV16 E7 (Lam *et al.*, 1994). It is also apparent that B-myb was co-precipitated with Lin-9 in all cell lines, but the level of B-myb–DREAM was highest in E7-expressing cell lines (Fig. 2). This presumably results from a combination of increased B-myb expression and disruption of competing p130–DREAM complexes. The B-myb complex was also notably abundant in C33A cells, which may reflect the activity of cyclin–cdk complexes there. All blots were probed with Lin-9 antibody in this experiment as a control for the Lin-9 immunoprecipitation (Fig. 2). Although this showed similar immunoprecipitation efficiency in each cell

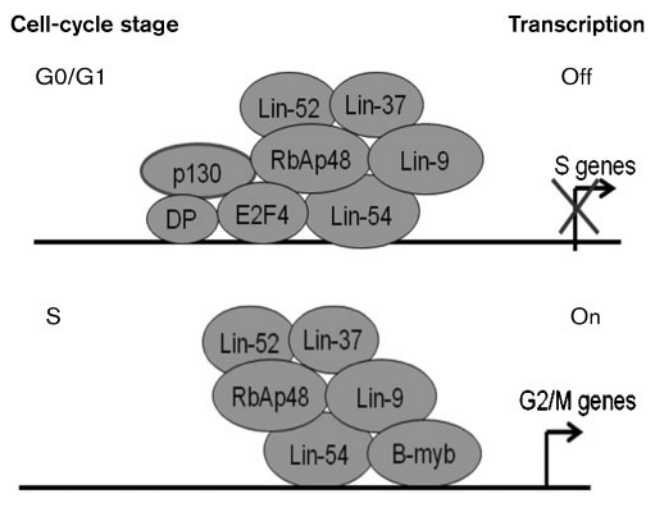


Fig. 1. Diagrammatic representation of the p130–DREAM and B-myb–DREAM complexes. The core DREAM constituents comprise Lin-52, Lin-37, Lin-9, Lin-54 and RbAp48. In quiescent cells (G0/G1), DREAM associates with E2F4–p130 to repress E2F-regulated genes. During cell-cycle entry, E2F4 and p130 dissociate and DREAM associates with B-myb to activate genes required for mitosis.

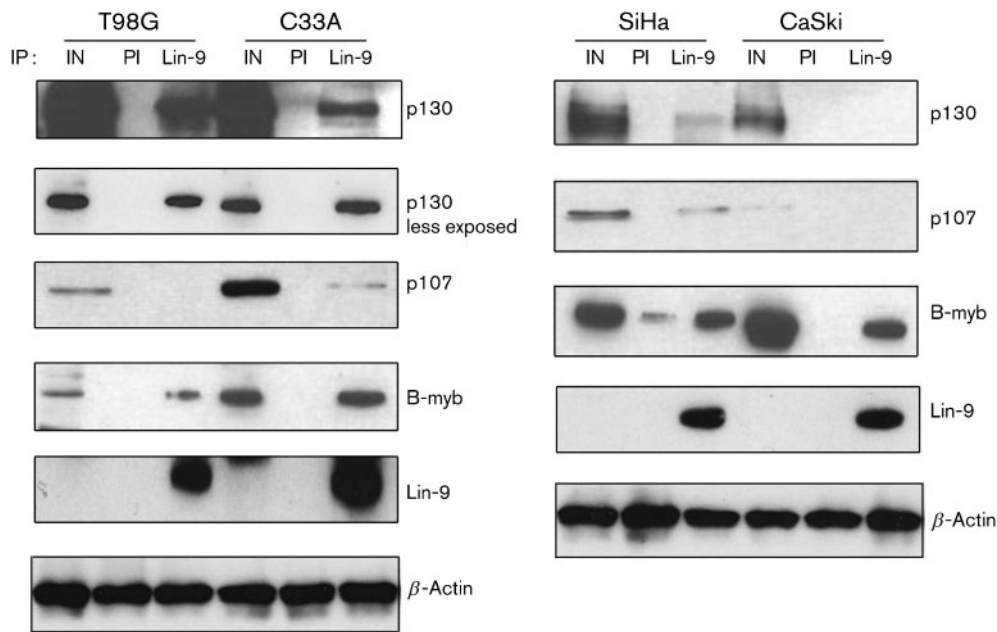


Fig. 2. Characterization of DREAM complexes in T98G, C33A, SiHa and CaSki cell lines. DREAM complexes were immunoprecipitated from nuclear lysates of the HPV-negative cell lines T98G and C33A and the HPV16-positive cell lines SiHa and CaSki with control pre-immune serum (PI) or Lin-9 antibody. Immunoprecipitates (IP) were blotted for p130, p107, B-myb and Lin-9 proteins. All protein samples were run in parallel and blots were exposed equally to film, except for the lesser exposure of the p130 blot of T98G and C33A lysates. The input control (IN) comprised 10% of the lysates used for immunoprecipitation. Ten per cent of the lysates remaining after immunoprecipitation was also run against an input control and probed with β -actin antibody as a check for loading.

line, Lin-9 could not be detected in the input control, similar to previous observations (Osterloh *et al.*, 2007).

Depletion of HPV16 E6/E7 results in cell-cycle arrest and reformation of the p130–DREAM complex

To confirm that disruption of pocket protein–DREAM complexes in CaSki and SiHa cells depended on HPV16 oncoproteins, we knocked down E6/E7 expression in SiHa and CaSki cells with lentivirus vectors (pLKO.1 puro) carrying small hairpin RNA (shRNA) genes directed to the E7 coding region of the bicistronic mRNA. The E7 shRNAs used in this study were selected from two published sequences shown to deplete HPV16 E7 expression effectively (Sima *et al.*, 2008; Rampias *et al.*, 2009) and were named 16E7A and 16E7B, respectively. Control cells were transduced with a lentivirus vector expressing a generic scrambled shRNA. After transduction of the packaged lentivirus vectors into SiHa and CaSki cells, the cells were selected with puromycin for 24 h and were harvested after a further 48 h. Quantitative PCR (qPCR) showed that E6/E7 mRNA expression was substantially reduced in CaSki and SiHa cells by RNA interference (Fig. 3a). Knockdown was more effective with 16E7B than with 16E7A shRNA, resulting in 98% depletion of the E6/E7 mRNA in SiHa cells and 87% in CaSki cells (Fig. 3a). As a further indication

of the relative efficacies of 16E7A and 16E7B shRNAs, we examined re-expression of p53 protein in CaSki cells that should result from depletion of E6 expression. This showed that p53 was induced more strongly by 16E7B shRNA, again demonstrating that this shRNA targeted the bicistronic E6/E7 mRNA more effectively than 16E7A shRNA. Analysis of cell-cycle status in SiHa and CaSki cells by propidium iodide staining and flow cytometry likewise showed a more profound effect with 16E7B shRNA than with 16E7A shRNA (Fig. 3c). In CaSki cells, knockdown caused an obvious G1 arrest, with 79.3 and 83.5% of cells in G1 after 16E7A and 16E7B shRNA transduction, respectively, compared with 61.6% in control cells (Fig. 3c). This G1 arrest was accompanied by reductions of S- and G2-phase cells. However, a subtly different effect was found in SiHa cells, where 16E7A and 16E7B shRNAs increased the proportion of cells with a 4n content, as well as reducing the proportion of S-phase cells (Fig. 3c). Such differences may reflect the influence of other underlying oncogenic mutations that have occurred in these cell lines during transformation *in vivo* and culture *in vitro*. As the effects of E6/E7 mRNA knockdown are more straightforward in CaSki cells, subsequent analyses concentrated on this cell line.

To investigate whether the impact of E6/E7 suppression on the CaSki cell cycle was reflected in effects on DREAM complexes, nuclear lysates from shRNA-transduced cells

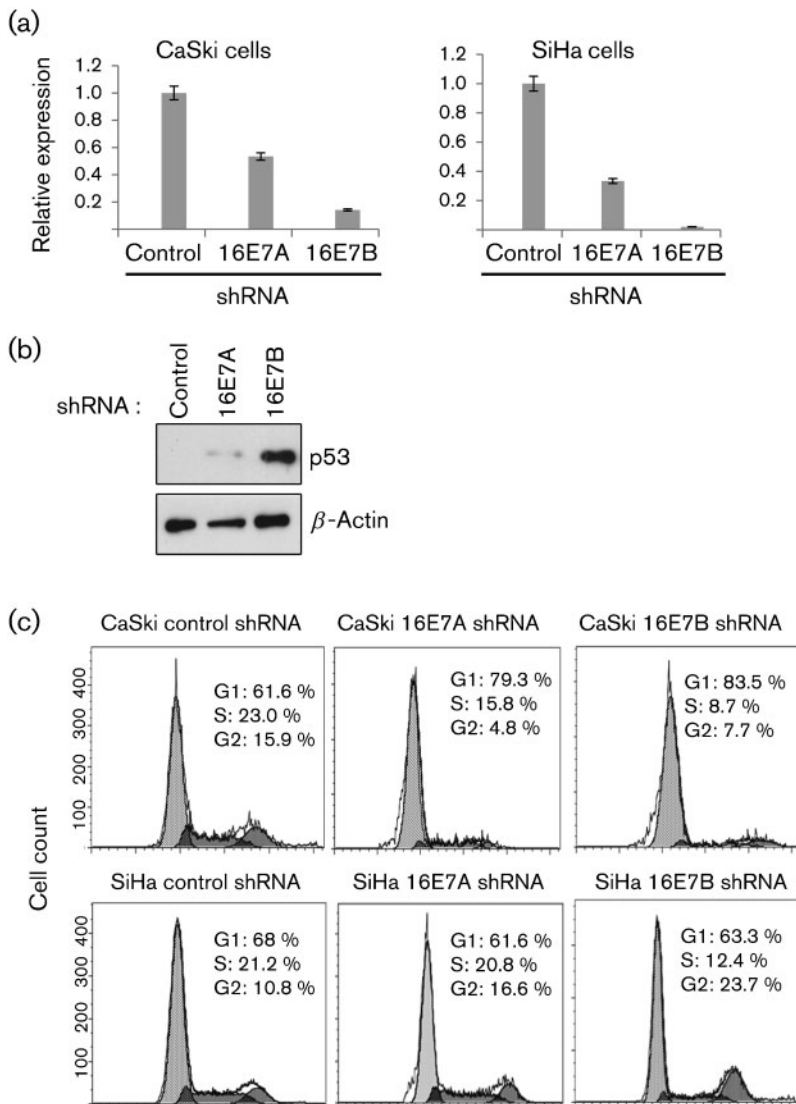


Fig. 3. Depletion of E6/E7 in CaSki and SiHa cells induces cell-cycle arrest. (a) qPCR analysis of E6/E7 RNA expression in CaSki and SiHa cells transduced with lentiviruses encoding a generic control shRNA or shRNAs targeting the E7 coding region (16E7A and 16E7B). Expression was normalized to levels in the control shRNA cells. (b) Western blot against p53 in lysates of CaSki cells transduced with lentiviruses encoding control, 16E7A or 16E7B shRNAs. β -Actin was used as a loading control. (c) Flow cytometry of propidium iodide-stained CaSki and SiHa cells transduced with control, 16E7A or 16E7B shRNA lentivirus vectors. The estimated percentages of cells in G1, S and G2/M phases are shown.

were immunoprecipitated with Lin-9 antibodies and Western-blotted against B-myb and p130. The results demonstrated that B-myb–DREAM was considerably reduced in abundance in 16E7A shRNA-transduced cells and virtually abolished upon 16E7B shRNA transduction (Fig. 4). In contrast, p130–DREAM was slightly increased in abundance in 16E7A shRNA-transduced cells and strongly increased upon 16E7B shRNA transduction (Fig. 4). Reformation of p130–DREAM in 16E7B shRNA-transduced cells probably accounts for the loss of *B-myb* gene expression evidenced by the input control (Fig. 4), as this complex is known to repress transcription through a promoter E2F-binding site (Lam & Watson, 1993; Litovchick *et al.*, 2007).

G1 arrest is dependent on DREAM complex reformation

To determine whether the profound G1 arrest precipitated by E6/E7 knockdown in CaSki cells is dependent on

repressive DREAM complex reformation, we depleted expression of one of the core DREAM complex components, Lin-54. A number of Lin-54 shRNA lentivirus vectors were first assessed by qPCR, and the most effective in depleting mRNA expression (data not shown) was used in subsequent analyses. As a further test for Lin-54 depletion, we immunoprecipitated DREAM complexes from CaSki cells transduced with the Lin-54 and control shRNA vectors and tested for co-immunoprecipitation of B-myb (Fig. 5a). This assay was performed as Lin-54 itself is difficult to detect by Western blotting, whereas B-myb is expressed at high levels in these cells. The results showed that Lin-54 expression was suppressed efficiently in CaSki cells compared with the control (Fig. 5a); the reduction in B-myb levels seen in the input control may be the result of instability seen previously when DREAM complexes are depleted (Pilkinton *et al.*, 2007). We also tested whether the formation of p130–DREAM induced by 16E7B shRNA transduction (Fig. 4) would be ablated by co-transduction of

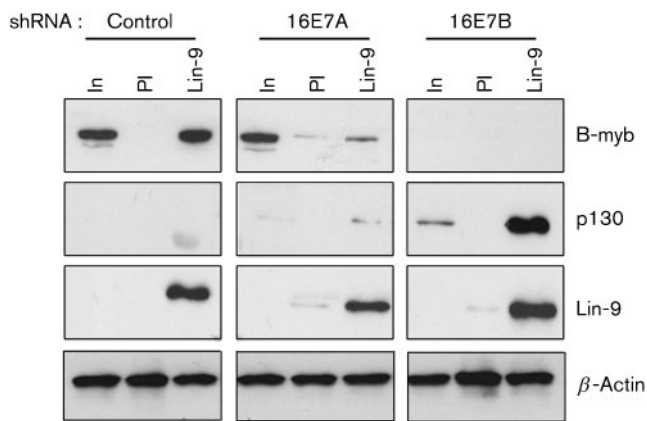


Fig. 4. Reformation of the p130–DREAM complex following E6/E7 knockdown. Nuclear lysates of CaSki cells transduced with lentiviruses encoding control, 16E7A or 16E7B shRNAs were immunoprecipitated with pre-immune serum (PI) or Lin-9 antibodies and blotted for B-myb, p130 and Lin-9. Protein samples were run on the same gel and probed with antibodies in parallel. The input controls (In) comprised 10% of the lysates used for immunoprecipitation. Ten per cent of the lysates remaining after immunoprecipitation was also run against an input control and probed with β -actin antibody as a check for loading.

Lin-54 shRNA; in this experiment, complexes were immunoprecipitated with Lin-9 antibody to ensure that Lin-54 suppression affects the formation of the core DREAM complex. We found that reformation of p130–DREAM upon targeting E6/E7 was abolished in cells co-transduced with Lin-54 shRNA (Fig. 5b). Subsequently, Lin-54 shRNA was co-transduced with 16E7B shRNA into CaSki cells and cells were harvested for cell-cycle analysis. This showed that, whilst the proportion of cells in G1 increased from 61.0 to 83.5% upon 16E7B shRNA transduction, this G1 arrest was prevented completely by co-transduction with Lin-54 shRNA (Fig. 5c). It is notable that, whilst there was a pronounced increase in the proportion of S-phase cells in 16E7B/Lin-54 shRNA-co-transduced cells compared with cells transduced with 16E7B shRNA alone (18.8 and 8.7%, respectively), there was still a reduction compared with cells transduced with the control shRNA (23% in S phase). It is likely that the co-transduced CaSki cells were arrested or delayed in G2/M as a consequence of B-myb–DREAM depletion (Knight *et al.*, 2009). Nonetheless, the results clearly demonstrate a requirement for Lin-54 and, by extension, the DREAM complex, for G1 arrest in CaSki cells depleted for E6/E7 expression.

To confirm that reformation of p130–DREAM is important for G1 arrest upon E6/E7 deletion, CaSki cells were transduced with 16E7B shRNA either alone or together with p130 shRNA. Flow cytometry showed that co-transduction of p130 shRNA strongly overcame the G1 arrest caused by 16E7B shRNA (Fig. 6b). In addition, qPCR analysis showed an increase in expression of B-myb and

cyclin A, which are transcriptionally repressed by p130–DREAM (Litovchick *et al.*, 2007), upon co-transduction of 16E7B shRNA-expressing cells with p130 shRNA (Fig. 6c). These results therefore confirm that continued expression of 16E6/E7 in CaSki cells is vital to overcome G1 arrest that would otherwise be imposed by p130–DREAM.

DISCUSSION

This study addressed whether repressive DREAM complexes are a significant target for HPV16 oncoproteins in cervical cancer cells. Our work showed that the repressive p130–DREAM complex was virtually abolished in CaSki cells, which express high levels of 16E6/E7, and was also much depleted in SiHa cells, which express lower levels of 16E6/E7. Depleting E6/E7 expression by RNA interference caused cell-cycle arrest in both CaSki and SiHa cells, as noted in previous studies (Tang *et al.*, 2006), and it was evident that cell-cycle arrest in CaSki cells depended on reformation of a repressive p130–DREAM complex. It can therefore be inferred that the continued proliferation of these HPV16-transformed cells is dependent upon disruption of p130–DREAM. Although both E6 and E7 are depleted by targeting the bicistronic mRNA with the shRNAs used, it is likely that the major effect on DREAM complexes, and thus the cell cycle, results from E7 depletion. HPV16 E7 is well-known to bind to all three members of the Rb/pocket protein family, preventing formation of transcriptionally repressive complexes by physically precluding interaction with E2F4/5 and additionally through degradation of pocket proteins. It cannot be excluded, however, that re-expression of p53 following E6 depletion could also impact on DREAM complexes. Under these conditions, p21^{Cip1} could inhibit cyclinE/A–cdk2 complexes, which would preclude both activating phosphorylation of B-myb (Saville & Watson, 1998) and inactivating phosphorylation of p130 (Cheng *et al.*, 2000).

It is very well-established that the association of different HPV types with cervical cancer correlates closely with the propensity of E7 to target inactivation and degradation of cellular Rb. More recently, it has been recognized that E7 proteins from both high- and low-risk HPV types share an ability to target p130 for degradation (Zhang *et al.*, 2006; Genovese *et al.*, 2008). Degradation of p130 by E7 appears to be important for driving quiescent cells into S phase and also reduces the expression of differentiated epithelial-cell markers (Zhang *et al.*, 2006; Genovese *et al.*, 2008). It can therefore be argued that p130 rather than Rb is the more significant cellular target of E7 during the normal replication cycle to establish conditions in suprabasal cells favouring HPV genome replication. This argument has been propounded in previous studies (Zhang *et al.*, 2006; Genovese *et al.*, 2008); however, it is notable in one of these publications that p130 levels in submerged cultures of CaSki and SiHa cells were found to be rather similar to those of the control primary human keratinocytes, suggesting that E7 had little impact on p130

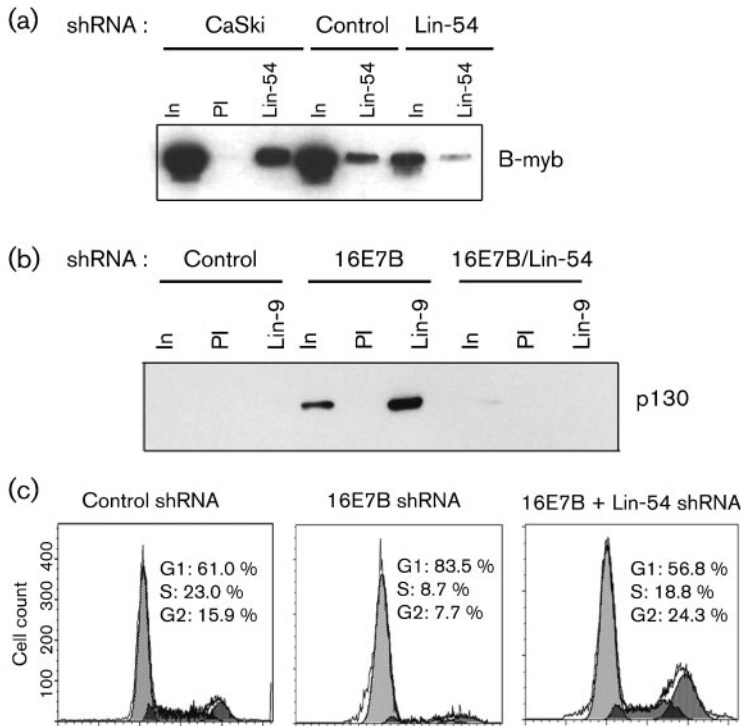


Fig. 5. G1 arrest upon E6/E7 depletion is dependent on Lin-54. (a) Nuclear lysates from untransduced CaSki cells (CaSki) or CaSki cells transduced with lentiviruses encoding control or Lin-54 shRNAs were immunoprecipitated with pre-immune serum (PI) or Lin-54 antibodies and blotted for B-myb. The input (In) control comprised 10% of the lysates used for immunoprecipitation. (b) Detection of p130 in immunoprecipitates of CaSki cells transduced with lentiviruses encoding control shRNA, 16E7B shRNA and a combination of 16E7B and Lin-54 shRNAs. Pre-immune serum (PI) and Lin-9 antibodies were used for immunoprecipitation and p130 was detected on Western blots. The input (In) control comprised 10% of the lysates used for immunoprecipitation. (c) Flow cytometry of propidium iodide-stained CaSki cells transduced with lentiviruses encoding control shRNA, 16E7B shRNA and a combination of 16E7B and Lin-54 shRNAs. The estimated percentages of cells in G1, S and G2/M phases are shown.

stability in these cervical cancer cells (Genovese *et al.*, 2008). We also detected p130 by Western blotting in SiHa and CaSki cells in our study, albeit at lower levels than in the control T98G and C33A cells. Importantly, our work showed a profound p130–DREAM deficit in cervical cancer cells compared with controls (Fig. 2). This was particularly evident with CaSki cells, and suggests that the p130 detected in this system is not part of the DREAM complex. The residual p130 protein may be unable to bind to E7, presumably reflecting the complex phosphorylation

events that regulate its activity (reviewed by Cobrinik, 2005), and is thus protected against degradation in CaSki cells. It is notable that a transcriptionally inactive p130–E2F4–cyclinE/A–cdk2 complex was observed in HeLa cells (Popov *et al.*, 2005); however, this complex was unable to revert to a repressive p130–E2F4 complex in this HPV18-transformed cervical cancer cell line. It is therefore plausible that E7 targets p130 specifically in the DREAM complex, thereby removing the barrier to entry into S phase.

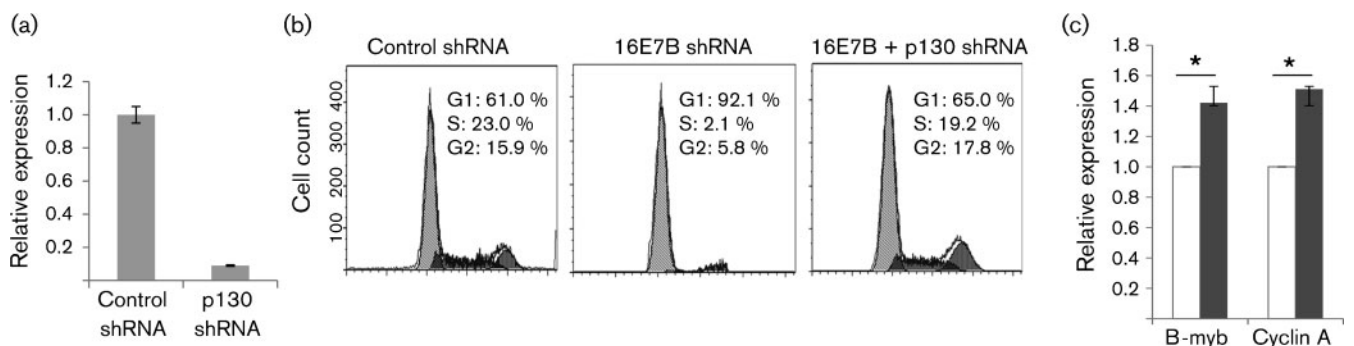


Fig. 6. G1 arrest upon E6/E7 depletion is dependent on p130–DREAM reformation. (a) qPCR to show that p130 mRNA expression is depleted in CaSki cells transduced with a lentivirus encoding p130 shRNA. (b) Flow cytometry of CaSki cells transduced with lentiviruses encoding control shRNA, 16E7B shRNA and a combination of 16E7B and p130 shRNAs. The estimated percentages of cells in G1, S and G2/M phases are shown. (c) qPCR analysis of B-myb and cyclin A RNA expression in CaSki cells co-transduced with 16E7B alone (empty bars) or a combination of 16E7B and p130 shRNAs (shaded bars). Differences in expression were found to be statistically significant by Student’s two-tailed *t*-test as indicated by asterisks, with $P < 0.045$ for B-myb and $P < 0.02$ for cyclin A.

HPV16 E6 and E7 proteins are known to interact with and regulate the activity of multiple cellular proteins implicated in cell proliferation, apoptosis and senescence (reviewed by Yugawa & Kiyono, 2009). Other studies have found that targeting E6/E7 expression by RNA interference induces apoptosis and/or senescence (Jiang & Milner, 2002; Hall & Alexander, 2003; Johung *et al.*, 2007; Sima *et al.*, 2008; Yamato *et al.*, 2008), in addition to effects on the cell cycle. We observed no obvious apoptosis in CaSki or SiHa cells during the course of our experiments, and there is no implication from our study that DREAM complexes regulate cell survival. Recent studies do suggest, however, that p130–DREAM is important for establishment of senescence, at least in the context of the RAS oncogene (Tschöp *et al.*, 2011). It will be interesting to determine whether the induction of senescence mediated by E7 repression, which has been reported to be initiated by a transcriptional cascade driven by the Rb family (Johung *et al.*, 2007), also depends upon reformation of p130–DREAM.

METHODS

Cell culture. Human glioblastoma (T98G), HPV-negative cervical cancer (C33A) and HPV16-positive cervical cancer (CaSki and SiHa) cell lines (ATCC) were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FCS, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Incubation was carried out at 37 °C in 10% CO₂ and 95% humidity.

Antibodies. The Lin-9 rabbit polyclonal antibody (Knight *et al.*, 2009) used for immunoprecipitation of DREAM complexes was purified by using a protein A–Sepharose column. The B-myb LX015.1 monoclonal antibody was described previously (Tavner *et al.*, 2007). Polyclonal Lin-9 antibody (ab46415) used in Western blotting was from Abcam; B-myb (sc-724), p107 (sc-318) and p130 (sc-317) antibodies were from Santa Cruz; β-actin (A-2066) antibody was from Sigma-Aldrich. Polyclonal Lin-54 antibody was a kind gift from Stefan Gaubatz (University of Würzburg, Germany).

Co-immunoprecipitation and Western blotting. Nuclear lysates were prepared by passing cells through a 25G syringe needle in buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM NaF, 1 mM DTT and Complete protease inhibitors; Roche) and collecting nuclei by brief microcentrifugation followed by incubation in 1 vol. buffer B (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 450 mM NaCl, 1 mM NaF, 25% glycerol, 1 mM DTT and Complete protease inhibitors; Roche). The lysate was cleared by microcentrifugation at 16 800 g for 10 min, then 300 µg was diluted with an equal volume of 20 mM HEPES pH 7.9 and mixed with 2 µg antibody overnight at 4 °C. Protein G–Sepharose beads were added and incubated for 1 h at 4 °C to collect immune complexes, which were washed four times with IP buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 0.5% Triton X-100] and eluted in SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and Western-blotted using standard techniques.

Lentiviral transduction. Lentiviral particles carrying shRNA genes were generated in the pLKO.1 vector following the supplier's instructions (Addgene). The four shRNAs used in this study were encoded by the following oligonucleotides inserted into *EcoRI* and *AgeI* sites of pLKO.1: 16E7A (forward, 5'-CCGGATGCATGGAGAT-ACACCTACACTCGAGTGTAGGTGTATCTCCATGCATTTTTTG-3', and reverse, 5'-AATTCAAAAATGCATGGAGATACACCTACACTC-

GAGTGTAGGTGTATCTCCATGCAT-3'); 16E7B (forward, 5'-CCG-GGGACAGAGCCATTACAATATCTCGAGATATTGTAATGGGC-TCTGTCCCTTTTG-3', and reverse, 5'-AATTCAAAAAGGACAGA-GCCATTACAATATCTCGAGATATTGTAATGGGCCTGTCC-3'); Lin-54 (forward, 5'-CCGGGGCTTCCATTCAATGGCATACTCGA-GTATGCCATTGAATGGAAGCCGTTTTTG-3', and reverse, 5'-AA-TTCAAAAACGGCTTCCATTCAATGGCATACTCGAGTATGCCA-TGAATGGAAGCCG-3'); and p130 (forward, 5'-CCGGATGGCCA-TGTTTTGGAAGCAACTCGAGTTGCTTCCAAAACATGGCCATTT-TTTG-3', and reverse, 5'-AATTCAAAAATGGCCATTTTTGGA-AGCAACTCGAGTTGCTTCCAAAACATGGCCAT-3'). SiHa and CaSki cells (1 × 10⁶) were plated in 10 cm dishes and incubated at 37 °C with 10% CO₂. The next day, the medium was changed to fresh medium containing 8 µg polybrene ml⁻¹. The lentiviral stock was added at 1 ml for each 10 cm dish and the cells were further incubated overnight. Following transduction, fresh medium supplemented with 2 µg puromycin ml⁻¹ was added 24 h post-transduction. Cells were harvested 48 h after puromycin selection for RNA and protein extractions.

Flow cytometry. Fluorescence-activated cell-sorting analysis was performed on ethanol-fixed cells stained with propidium iodide using a FACSCanto II analyser (BD) and analysed with FlowJo (Tree Star Inc.) software as described previously (Knight *et al.*, 2009). At least 10 000 cells were analysed.

RNA extraction and qPCR. Total RNA was extracted by using an RNeasy Mini kit (Qiagen) and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). qPCR was carried out using Absolute SYBR Green ROX (ABgene) and an ABI 7900HT sequence detector (Applied Biosystems). Relative quantification was calculated by normalizing against *ARP PO* gene primers (Knight *et al.*, 2009). Each PCR was performed on biological triplicates. Primer sequences and conditions were as described previously (Knight *et al.*, 2009).

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