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The human papillomavirus type 16 E7 oncoprotein targets Myc-interacting zinc-finger protein-1

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ABSTRACT

We demonstrate that HPV-16 E7 forms a complex with Miz-1. UV-induced expression of the CDK-inhibitor $p21^{Cip1}$ and subsequent cell cycle arrest depends upon endogenous Miz-1 in HPV-negative C33A cervical cancer cells containing mutated p53. Transient expression of E7 in C33A inhibits UV-induced expression of $p21^{Cip1}$ and overcomes Miz-1-induced G1-phase arrest. The C-terminal E7Δ79LEDLL83-mutant with reduced Miz-1-binding capacity was impaired in its capability to repress $p21^{Cip1}$ expression; whereas the pRB-binding-deficient E7C24G-mutant inhibited $p21^{Cip1}$ expression similar to wild-type E7. Using ChIP, we demonstrate that endogenous E7 is bound to the endogenous $p21^{Cip1}$ core-promoter in CaSki cells and RNAi-mediated knock down of Miz-1 abrogates E7-binding to the $p21^{Cip1}$ promoter. Co-expression of E7 with Miz-1 inhibited Miz-1-induced $p21^{Cip1}$ expression from the minimal-promoter via Miz-1 DNA-binding sites. Co-expression of E7Δ79LEDLL83 did not inhibit Miz-1-induced $p21^{Cip1}$ expression. E7C24G retained E7-wild-type capability to inhibit Miz-1-dependent transactivation. These findings suggest that HPV-16 E7 can repress Miz-1-induced $p21^{Cip1}$ gene expression.

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Introduction

High-risk human papillomaviruses (HPVs) are the major etiological agents for cervical cancer (zur Hausen, 2002). HPV-16 is the most prevalent high-risk genotype (Munoz et al., 2003). Co-expression of the early viral genes E6 and E7 is necessary and sufficient to immortalize primary human keratinocytes (Münger et al., 1989) and to maintain the transformed phenotype of cervical cancer cells (DeFilippis et al., 2003; Francis et al., 2000; Goodwin and DiMaio, 2000). E7 is the major HPV oncoprotein and its expression is sufficient to immortalize primary human epithelial cells at a low frequency (Halbert et al., 1991; Reznikoff et al., 1994; Wazer et al., 1995) and to induce cervical cancer in transgenic mice treated with estrogens (Riley et al., 2003). This is consistent with the strong increase of the HPV-16 E7 protein levels during cervical carcinogenesis and with high level expression of HPV-16 E7 in cervical cancers (Fiedler et al., 2004). According to the current model, E7 acts mechanistically through interaction with cellular regulatory protein complexes and alter, or neutralize, their normal functions (McLaughlin-Drubin and Munger, 2009; Pim and Banks, 2010). This leads to impaired cell cycle arrest responses and deregulation of pathways controlling cellular differentiation and apoptosis. Moreover, E7 elicits genomic instability and reprograms cellular metabolic pathways. The transforming activity of E7 was linked to its interaction with the retinoblastoma protein (pRB) and abrogation of the growth-suppressive function of pRB (Dyson et al., 1989), resulting in transcriptional activation of genes necessary for S-phase entry and progression (McLaughlin-Drubin and Munger, 2009). E7 mutants that are unable to bind pRB retain however transforming capacity (Funk et al., 1997), and E7 mutants with reduced transforming activity retain the ability to bind pRB (Massimi et al., 1997; Phelps et al., 1992). Moreover, E7 stimulates proliferation independently of its ability to associate with pRB (Caldeira et al., 2000). In keeping with these findings, critical roles for non-pRB targets of HPV-16 E7 have been demonstrated (Balsitis et al., 2006), including proteins involved in transcriptional activation and chromatin-remodelling (Bernat et al., 2003; Brehm et al., 1999; Prathapam et al., 2001). While these findings suggest that HPV-16 E7 has functions in transcriptional activation, little is known about a potential role of E7 in transcriptional repression.

Activation of the tumor suppressor p53 by DNA-damage induces cell cycle arrest mediated, at least in part, by transcriptional activation of the

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cyclin-dependent kinase inhibitor (cdki) gene p21^{Cip1} (Dulic et al., 1994). Growth arrest by induction of p53 in DNA-damaged keratinocytes is bypassed by HPV-16 E7 (Demers et al., 1994; Slebos et al., 1994) although E7 was found to stabilize p53 (Seavey et al., 1999). In keeping with studies showing that p21^{Cip1} gene expression is modulated by other signals independently of p53 (Macleod et al., 1995), these data suggest that E7 modifies pathways downstream or independently of p53 to allow cell division. However, the underlying mechanisms are not well understood. Immortalization of keratinocytes co-expressing HPV-16 E7 and oncogenic c-Myc is independent of p53 (Liu et al., 2007). C-Myc is a major transforming protein in human cancer with several functions such as inducing cell cycle progression (Bornkamm, 2009). C-Myc is a transcriptional regulator with the ability to activate some target genes while repressing others (Wanzel et al., 2003). The mechanisms by which c-Myc negatively regulates transcription are less well understood. According to the current model c-Myc represses transcription when tethered to promoters by transcriptional activators (Wanzel et al., 2003), as shown for Miz-1 (Myc-interacting zinc-finger protein-1), a transcription factor involved in transcriptional activation of the p15^{Ink4b} gene after TGF-β stimulation (Seoane et al., 2001; Staller et al., 2001) and the p21^{Cip1} gene in response to UV-irradiation induced DNA damage (Herold et al., 2002; Seoane et al., 2002). Miz-1 can bind directly to specific DNA sequences in the core promoter close to the start sites of the p21^{Cip1} gene and activate transcription (Herold et al., 2002; Wu et al., 2003). Miz-1-dependent transactivation can be repressed by a Miz-1-bound c-Myc through disruption of the interaction between Miz-1 and co-activators (Herold et al., 2002; Staller et al., 2001). The repression of p21^{Cip1} expression by c-Myc leads to enhanced cyclin E and cyclin A dependent kinase activity and hence cell-cycle progression of c-Myc-transformed cells despite DNA damage (Herold et al., 2002; Seoane et al., 2002).

In this study, we searched for novel cellular E7-binding proteins involved in cell cycle control. We found that HPV-16 E7 forms a complex with the anti-proliferative protein Miz-1. The transcription of p21^{Cip1} is regulated by Miz-1 as well as by p53 (Seoane et al., 2002). Moreover, cell cycle arrest induced by p53 in response to DNA damage can be bypassed by HPV-16 E7 (Demers et al., 1994; Slebos et al., 1994). For these reasons, we studied the effects of the E7/Miz-1 interaction on the regulation of p21^{Cip1} gene expression and cell cycle progression in response to UV-induced DNA damage in the HPV-negative C33A cervical cancer cells which harbor an inactive p53 protein mutated in the DNA binding domain (Crook et al., 1991; Scheffner et al., 1991).

Results

Interaction between HPV-16 E7 and Miz-1

We searched for novel cellular HPV-16 E7-binding proteins involved in cell cycle control in a yeast two-hybrid screen using the intact carboxyl-terminal domain of HPV-16 E7 as bait. In this screen we isolated cDNAs coding for carboxyl-terminal fragments of Myc-interacting zinc-finger protein-1 (Miz-1) alias zinc finger and BTB domain containing 17(ZBTB17) (Staller et al., 2001). A specific and strong interaction of Miz-1($_{aa331–803}$), encoding for the zinc-finger domains 2–13, with HPV-16 E7 but not with unrelated proteins was demonstrated in yeast using two distinct reporter genes, leu2::lexAo6-pLEU2 (Fig. 1A, left panels) and LexAo8-Gal1-lacZ::URA3 (Fig. 1A, right panels).

To determine whether both full length proteins can interact, purified GST-HPV-16 E7 was incubated with extracts from Miz-1 wild-type expressing cells. Miz-1 specifically bound to GST-HPV-16 E7 in this assay, whereas no binding was observed for GST alone (Fig. 1B, left panel), suggesting that both proteins interact *in vitro*. The E7 protein of the non-transforming low-risk HPV-11 bound with lower efficiency as HPV-16 E7 to Miz-1 in the GST pull down assays (Fig. 1B, left panel). To further map the binding site for Miz-1 in HPV-16 E7 a set of point and deletion mutants of E7 (Fig. 1C) was investigated for Miz-1-binding in the GST pull down assay. The conserved domain 2 (cd2) as well as the carboxyl-terminal domain (cd3) is essential for the transforming potential of HPV-16 E7 (Massimi et al., 1997; Phelps et al., 1992; Prathapam et al., 2001; Santer et al., 2007). These domains have been defined by inactivating mutations in cd2 (C24G) (Phelps et al., 1992) and cd3 (Δ79LEDLL83 (Massimi et al., 1997), Δ52YNIVT56 (Prathapam et al., 2001). Cd2 mutants are deficient in binding to pRB and some additional cellular proteins (McLaughlin-Drubin and Munger, 2009). The cd3 mutants are, for example, deficient in binding to IGF binding protein-3 (Santer et al., 2007) or the metabolic regulator pyruvate kinase M2 (Zwerschke et al., 1999). E7C24G bound Miz-1 with wild-type efficiency (Fig. 1B, right panel), suggesting that the interaction with Miz-1 is not a function of cd2. In contrast, the two C-terminal mutants Δ79LEDLL83 and Δ52YNIVT56 showed significantly reduced binding to Miz-1 (Fig. 1B, right panel). These data are in accordance with the fact that a C-terminal E7 fragment was used as the bait in the yeast two-hybrid screen. Moreover, these findings underline that E7 specifically interacts with Miz-1 and suggest that a region encompassing amino acids 52–83 within the α1-helix of the conserved E7 C-terminal zinc-binding fold (Ohlenschlager et al., 2006), but not the conserved domain 2, is necessary for the interaction with Miz-1.

To analyze whether E7/Miz-1 complexes exist in human cells, co-immunoprecipitation experiments were performed from lysates of U-2OS osteosarcoma cells transiently overexpressing HPV-16 E7 and Miz-1 (Fig. 2A). E7 was specifically co-precipitated with Miz-1 using anti-Miz-1 antibodies, but not with an unrelated isotypic control antibody (Fig. 2A). Antibodies against HPV-16 E7 precipitated E7 from U-2OS extracts and co-precipitation of Miz-1 was observed (Fig. 2A). Unrelated polyclonal control serum precipitated neither HPV-16 E7 nor Miz-1, underlining the specificity of the immunoprecipitations (Fig. 2A). The mouse monoclonal anti-Miz-1 antibody 10E2 recognizes more than one band of endogenous Miz-1 in human cancer cell extracts in western blotting experiments (Herkert et al., 2010; Herold et al., 2008; Wanzel et al., 2005). These bands represent most likely post-translational modified forms of Miz-1, such as differentially phosphorylated Miz-1 (Wanzel et al., 2005) and sumoylated Miz-1 (Herkert et al., 2010). We conducted a western blot experiment to analyze which forms of endogenous Miz-1 exist in cervical cancer cells (Fig. 2B). In CaSki cells the anti-Miz-1 antibodies recognized three major bands. Long exposure revealed however that additional forms of Miz-1 exist in low quantities (Fig. 2B). Similar results were found in HeLa and C33A cells although the abundance of the corresponding bands was somewhat different between the given cervical cancer cell lines (Fig. 2B). Comparison of endogenous Miz-1 and ectopically overexpressed Miz-1, especially in the long exposure, demonstrated the specificity of the endogenous Miz-1 bands (Fig. 2B). To determine whether the endogenous HPV-16 E7 and Miz-1 proteins can form a complex, CaSki cell lysates were immunoprecipitated with the anti HPV-16 E7 RabMab42-3 (Dreier et al., 2011), rabbit pre-immune serum served as a negative control (Fig. 2C). Immunoblotting showed that Miz-1 was specifically detected in anti-HPV-16 E7 immunoprecipitates, neither protein was present in the control immunoprecipitate (Fig. 2C). Interestingly, the anti-HPV-16 E7 RabMab42-3 immunoprecipitated only two Miz-1 bands (Fig. 2C). These findings suggest that HPV-16 E7 can form a complex with two forms of Miz-1 in living cervical cancer cells.

HPV-16 E7 represses Miz-1-induced expression of the endogenous p21^{Cip1} gene after UV irradiation in C33A cervical cancer cells

Like p53 (Dulic et al., 1994), Miz-1 can induce p21^{Cip1} gene expression and subsequent cell cycle arrest in response to UV irradiation in immortalized human keratinocytes (Herold et al., 2002).

Miz-1 acts directly via its DNA-binding sites at the p21^{Cip1} core promoter in a p53-independent manner (Herold et al., 2002; Seoane et al., 2002). We investigated whether E7 interferes with Miz-1-dependent transcriptional control of the p21^{Cip1} gene after DNA damage. To rule out any confounding effects by p53 the E7/Miz-1 interaction was studied in C33A cervical cancer cells which harbor an inactive p53 protein mutated in the DNA-binding domain (Crook et al., 1991; Scheffner et al., 1991). To analyze whether Miz-1 is essential for the induction of the p21^{Cip1} gene in response to UV irradiation in C33A cells, we used small interfering RNA duplexes (siRNAs) to knock down the expression of endogenous Miz-1. Depletion of the Miz-1 protein (Figs. 3A and C) resulted in a substantial abrogation of UV-induced expression of the endogenous p21^{Cip1} gene, relative to C33A cells transiently transfected with control siRNA, as shown in reduced p21^{Cip1} mRNA levels (Fig. 3B) as well as reduced p21^{Cip1} protein levels (Fig. 3C). These findings indicate that Miz-1 mediates UV-dependent induction of the p21^{Cip1} gene in the p53 negative C33A cells. To investigate the effect of HPV-16 E7 on UV-induced p21^{Cip1} gene expression in this system, C33A cells were transiently transfected with pX-HPV-16 E7 (Fig. 3C) and the endogenous p21^{Cip1} mRNA levels were measured after UV irradiation by quantitative RT-PCR analysis (Fig. 3B). Similar to the depletion of Miz-1, the expression of HPV-16 E7 resulted in a dramatic abrogation of UV-dependent induction of p21^{Cip1} gene expression. The transient expression of HPV-16 E7 resulted also in reduced p21^{Cip1} protein level in the UV-irradiated C33A cells (Fig. 3C). This suggests that HPV-16 E7 can abrogate UV-induced expression of endogenous p21^{Cip1} in a p53-independent manner.

From the Miz-1 western blot shown in Fig. 3C it seemed possible that the Miz-1 levels are somewhat reduced when E7 is expressed. Since this could be important for the underlying mechanism, we conducted western blot experiments to prove whether HPV-16 E7 has impact on the endogenous Miz-1 protein levels (Fig. 3D). We found that the Miz-1 protein levels did not change in E7 expressing cells. The Miz-1 protein levels were increased in CaSki cells and in the stable cell lines NHEK/HPV-16 E6 and NHEK/HPV-16 E7 relative to normal human epidermal keratinocytes (NHEK), suggesting that the Miz-1 levels are increased in immortalized and transformed cells. The Miz-1 protein levels were however similar in NHEK/HPV-16 E7 and in the NHEK/HPV-16 E6 control cells (Fig. 3D, left panel). Moreover, transient overexpression of HPV-16 E7 in experimental cell lines led not to strong changes in the Miz-1 levels (Fig. 3D, right panel and data not shown). The analysis of the impact of HPV-16 E7 on the Miz-1 levels warrants further studies.

Our findings that HPV-16 E7 blocks Miz-1-dependent transactivation of the p21^{Cip1} gene in UV-irradiated C33A cells raise the possibility that the ability of E7 to activate cell proliferation involves its ability to block Miz-1-induced p21^{Cip1} gene expression. To determine whether E7 can override UV-induced, Miz-1-dependent G1-cell cycle arrest in C33A cells, a transient transfection assay was established which allows monitoring of the ability of E7 to override G1-arrest. To this end, normal proliferating C33A cells were transiently transfected with expression vectors for E7, Miz-1 or empty vector. 24 h after UV irradiation, cell cycle distribution was analyzed among the surviving cell population using FACS analysis (Fig. 3E). In the C33A cells transiently overexpressing Miz-1, the number of G1-phase cells was considerably increased (Fig. 3E), whereas transient co-expression of E7 with Miz-1 abrogated Miz-1-induced G1-cell cycle arrest. This indicates that E7 can override Miz-1-mediated G1-cell cycle arrest induced after DNA damage.

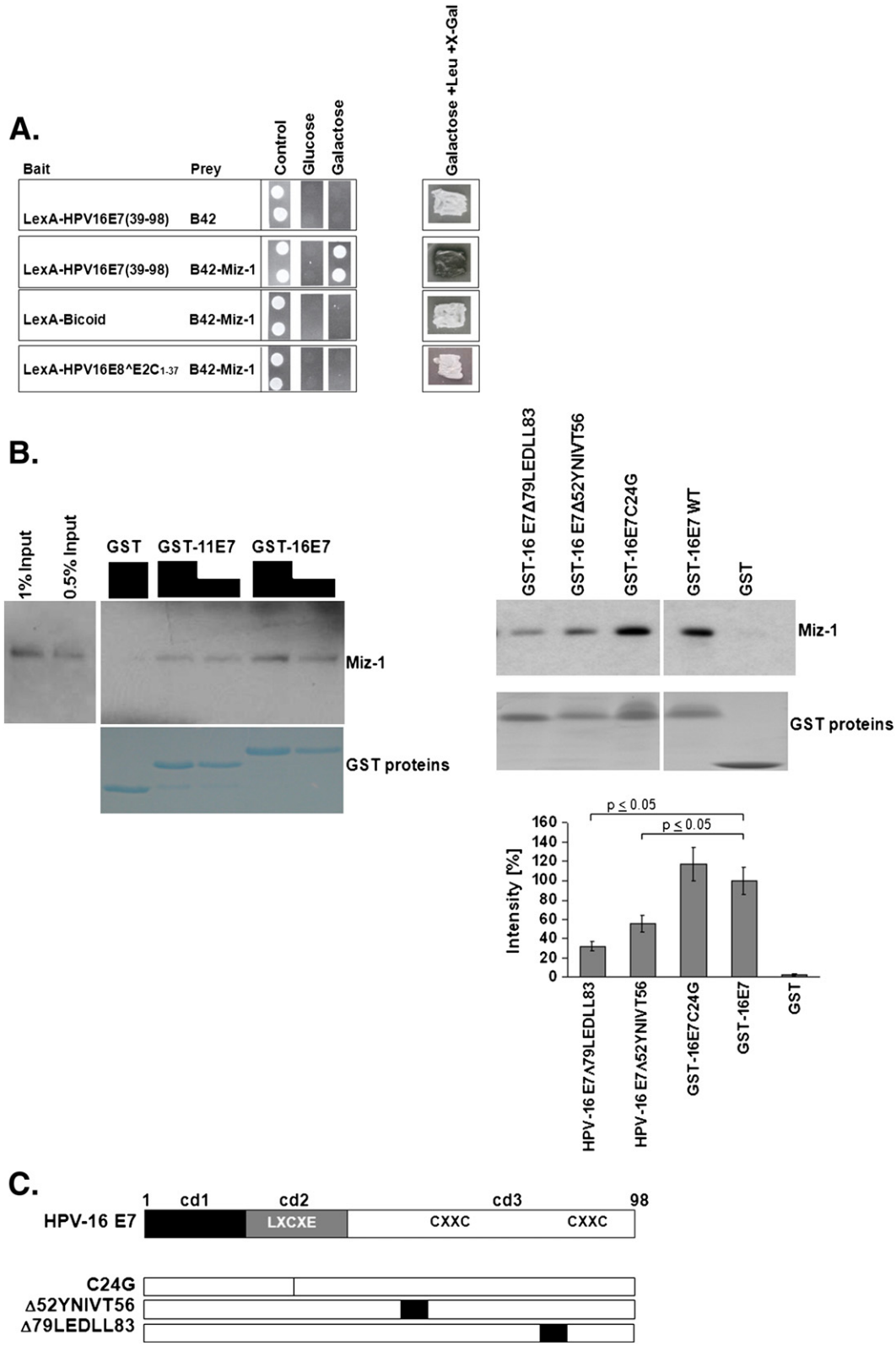
To further address the question whether E7-induced downregulation of p21^{Cip1} is Miz-1-dependent, we studied whether the two carboxyl-terminal HPV-16 E7 mutants, which have reduced Miz-1-binding capacity, are impaired in their ability to repress UV-induced expression of the endogenous p21^{Cip1} gene. C33A cells were transiently transfected either with pX-HPV-16 E7 wild-type, pX-HPV-16 E7Δ79LEDLL83, pX-HPV-16 E7Δ52YNIVT56, pX-HPV-16 E7C24G or pX, as indicated (Fig. 4A), and UV-irradiation induced p21^{Cip1} gene expression was studied by quantitative RT-PCR analysis (Fig. 4B). We found that the low efficiency Miz-1-binding HPV-16 E7Δ79LEDLL83 mutant significantly lost the ability to repress UV-irradiation induced expression of the endogenous p21^{Cip1} gene. This underlines the specificity of the repressive effect of HPV-16 E7 on Miz-1-dependent transactivation of the p21^{Cip1} gene and indicates that the 79LEDLL83 motif within the α1-helix in the C-terminal zinc-finger loop of HPV-16 E7 is necessary for repression of the p21^{Cip1} gene. The second C-terminal mutant, E7Δ52YNIVT56, showed a tendency to reduced repressing activity but expression was slightly lower than for wild-type E7. The cd2-mutant E7C24G repressed the p21^{Cip1} gene expression to a similar extent as wild-type E7 (Fig. 4B), suggesting that E7-induced repression of Miz-1-induced p21^{Cip1} gene expression is pRB-independent. These data further support the model that HPV-16 E7-induced downregulation of p21^{Cip1} is Miz-1-dependent. The data underline also that the HPV-16 E7/Miz-1 interaction contributes to the transforming potential of the virus, because the HPV-16 E7Δ79LEDLL83 mutation has reduced transforming activity (Massimi et al., 1997). To further address the question whether the E7/Miz1 interaction is important for cell transformation we analyzed whether the low-risk HPV-11 E7 protein has the ability to repress UV-induced expression of the endogenous p21^{Cip1} gene. HPV-11 E7 was transiently expressed in C33A cells (Fig. 4C), and UV-irradiation induced p21^{Cip1} gene expression measured by quantitative PCR analysis (Fig. 4B). We found that HPV-11 E7 could not repress UV-irradiation induced expression of the endogenous p21^{Cip1} gene. This underlines that the HPV-16 E7/Miz-1 interaction contributes to the transforming potential of the virus. Moreover, these data indicate that HPV-11 E7, although it binds to Miz-1 in the GST pull downs (Fig. 1), does not have the biochemical activity of HPV-16 E7 to repress UV-induced p21^{Cip1} transcription. Moreover, it was shown that low-risk

Fig. 1. Analysis of the HPV-16 E7/Miz-1 interaction. (A) Yeast strain EGY48/pSH1834 (Matα, his3, ura3, trp1, leu2::lexAo6-pLEU2/lexAo8-Gal1-lacZ::URA3) containing a LexA operator-LEU2 and a LexA operator-lacZ gene (Zwerschke et al., 1999) was used for both the LEU2 and β-galactosidase reporter gene assays. EGY48/pSH1834 derivatives expressing various LexA fusion proteins (Zwerschke et al., 1999) from pEG202::HIS3 vectors as indicated were transformed with the plasmid pB42-Miz-1-331-803::TRP1. pJG4-5::TRP1, expressing the unfused B42 transactivation domain (Zwerschke et al., 1999), was used as negative control. Transformants were selected for uracil, histidine and tryptophan prototrophy and grown in glucose minimal medium (Ura-, His-, Trp-). (Left panel) Yeast cells were dropped out onto each of three plates and incubated for 4 days at 30 °C under the following nutrient conditions: Control: Glucose minimal medium with leucine; all strains grow. Glucose: glucose minimal medium without leucine; selection for B42 fusion protein independent activation of the LexAo6-LEU2 reporter. Galactose: galactose minimal medium without leucine; selecting for B42 fusion protein-dependent activation of the LexAo6-pLEU2 gene. (Right panel) The yeast cells, containing the LexA operator-lacZ gene (LexAo8-GAL1-LacZ::URA3) on pSH1834 and expressing the various LexA fusion proteins and the B42-Miz-1331-803 prey or B42 as indicated, were streaked out onto Galactose + Leu + X-Gal plates and incubated for 4 days at 30 °C: Selecting for B42 fusion protein-dependent activation of the lexAo8-Gal1-lacZ::URA3 gene expressing the β-galactosidase enzyme, as indicated by the generation of the blue colour. (B) HPV E7 interacts with Miz-1 in GST pull down experiments. (Left panel) Purified GST, GST-HPV-11 E7 and GST-HPV-16 E7 fusion proteins immobilized on glutathione sepharose 4B beads were incubated with whole-cell extracts from C33A cells overexpressing the full length Miz-1 protein, as indicated. (Top) After washing, bound proteins were separated by SDS-PAGE and the amount of Miz-1 protein that was retained was determined by direct immunoblotting using a monoclonal anti-Miz-1 antibody. (Bottom) Input of the various GST fusion proteins was controlled by Coomassie staining. (Right panel) (Top) GST pull downs with various GST-HPV-16 E7 mutants are shown as indicated. (Bottom) The Miz-1 western blot and GST protein bands were scanned from low exposures and densitometrically analyzed for quantification (n = 3). The Miz-1 protein bound to HPV-16 E7 wild-type was set to 100%. The western blots shown are from one membrane which was cut after finishing the western blot procedure. (C) Structure of the HPV-16 E7 protein. Cd1, cd2, cd3 and the zinc-finger motifs (CXXC) are indicated. The mutants of E7 used in the assays are shown.

HPV-11 E7 does not bypass DNA damage induced G1 arrest (DeWeese et al., 1997). Together these data indicate that HPV-11 E7 can neither repress UV-induced p21^{Cip1} gene expression nor override UV-induced G1-cell cycle arrest. This suggests that the binding of HPV-11 E7 to Miz-1, as shown in our GST pull down experiments (Fig. 1), is not sufficient for the inactivation of Miz-1 induced G1 cell cycle arrest and UV-induced p21^{Cip1} transcription.

HPV-16 E7 binds to the p21^{Cip1} core promoter and represses Miz-1-induced expression from the Miz-1 binding sites

Having demonstrated that E7 forms a complex with Miz-1 and represses UV-induced p21^{Cip1} gene expression in a Miz-1-dependent manner, we aimed to further address the underlying mechanism. It is known that Miz-1 is bound to the consensus initiator sequence



(−40/+20) at the start site of transcription on the p21^{Cip1} promoter (Fig. 5A) and induces p21^{Cip1} gene expression in UV-irradiated cells (Herold et al., 2002). We tested in chromatin-immunoprecipitation (ChIP) experiments whether endogenous HPV-16 E7 is bound to the endogenous p21^{Cip1} core promoter in CaSki cervical cancer cells, first without UV irradiation (Fig. 5B, left panels). ChIP was performed using the rabbit monoclonal anti-HPV-16 E7 antibody (RabMab-42-3, Dreier et al., 2011), and the precipitates were monitored by RT-PCR for the content of the human p21^{Cip1} promoter DNA region from −46 to +156. Under these conditions the anti-HPV-16 E7 antibody specifically co-precipitated p21^{Cip1} promoter (−46/+156) DNA, as corroborated by sequencing. The anti-Miz-1 antibody and the anti-histone H3 antibody (positive control) also co-precipitated p21^{Cip1} promoter (−46/+156) DNA, while the negative control, rabbit pre-immune serum, did not (Fig. 5B, left panels). Next, we tested whether endogenous HPV-16 E7 and Miz-1 are also bound to the endogenous p21^{Cip1} promoter in UV-irradiated CaSki cells (Fig. 5B, right panels). Also under these conditions both the anti-HPV-16 E7 antibody and the anti-Miz-1 antibody specifically co-precipitated p21^{Cip1} promoter (−46/+156) DNA. Our protein-protein interaction studies suggest that HPV-16 E7 can form a complex with Miz-1 in CaSki cells (see below, Fig. 2). To further analyze whether Miz-1 is an anchor for HPV-16 E7 on the p21^{Cip1} core promoter, Miz-1 was knocked down in CaSki cells by RNAi (Fig. 5C) and ChIPs were conducted using the anti-HPV-16 E7 and anti-Miz-1 antibodies, respectively (Fig. 5D). As expected in this experiment the anti-Miz-1 antibodies did not co-precipitate p21^{Cip1} promoter DNA. The anti-HPV-16 E7 antibody did also not co-precipitate p21^{Cip1} promoter (−46/+156) DNA in the Miz-1 knock down cells, suggesting that the binding of HPV-16 E7 to the p21^{Cip1} promoter is dependent on Miz-1. Together, our data indicate that a HPV-16 E7/Miz-1 complex is bound to the p21^{Cip1} core promoter irrespective of UV irradiation. Moreover, the data suggest that HPV-16 E7 does not prevent Miz-1-binding to the promoter but rather blocks the ability of Miz-1 to transactivate.

To further investigate whether E7 represses p21^{Cip1} gene expression by interaction with Miz-1 on the p21^{Cip1}-core promoter, we analyzed whether E7 can inhibit Miz-1-dependent transactivation of the p21^{Cip1} core promoter (−93/+20) on pGL3p21min, which contains the Miz-1 consensus elements (Fig. 6A). The experiments were conducted in U-2OS cells which display intrinsic high transfection efficiency. U-2OS cells were transiently transfected with the pGL3p21min reporter plasmid, pUHDMiz-1 and increasing amounts of pxHPV16 E7 (Fig. 6B). Overexpressed Miz-1 activated transcription of the p21^{Cip1} minimal promoter (Fig. 6B). Co-expression of E7 caused a considerable abrogation of Miz-1-dependent transactivation of the p21^{Cip1} minimal promoter in a dose-dependent manner (Fig. 6B), suggesting that E7 can repress the transcriptional activity of Miz-1 on the p21^{Cip1} core promoter. To determine which domain in E7 is relevant for the repression of Miz-1-dependent induction of the p21^{Cip1} core promoter, E7 mutants were investigated in co-transfection experiments. All E7 proteins were well expressed in the U-2OS cells (Fig. 6C). While co-transfection of Miz-1 and the cd2-mutant E7C24G repressed transactivation by Miz-1 with activity similar to that of wild-type E7, the repressing activity of the two

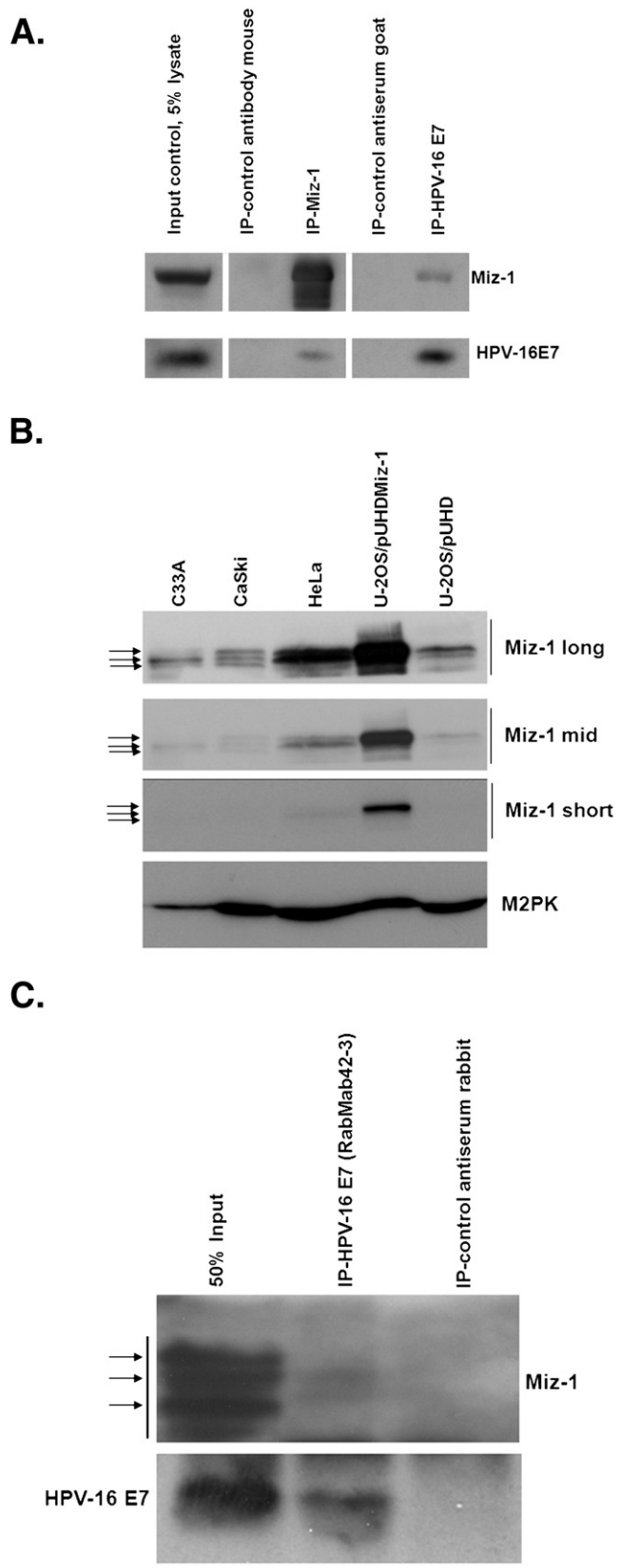


Fig. 2. Interaction of Miz-1 and HPV-16 E7 in cervical cancer cells. (A) HPV-16 E7 forms a complex with Miz-1 in U-2OS cells. U-2OS cells were co-transfected with pUHDMiz-1 and pXHPV-16 E7. 24 h later, cell lysates were prepared and subjected to immunoprecipitation using antibodies against HPV-16 E7, Miz-1 and isotypic control antibodies, as indicated. Cell lysate was used as input control. Precipitated proteins (Miz-1 and 16 E7) were separated on a 12.5% SDS-PAGE and detected by western blotting. (B) Detection of Miz-1 protein in cervical cancer cell lines. Cell lysates were prepared from C33A, CaSki, HeLa, U-2OS and U-2OS cells transiently transfected with pUHDMiz-1 (positive control). Cell lysates were separated on a 10% SDS-PAGE and Miz-1 protein was detected by western blotting using anti-Miz-1 10E2 antibodies. Three different exposures, long, mid and short, are shown. The Miz-1 bands are indicated by arrows. M2-PK served as input control for this experiment. (C) Interaction of endogenous HPV-16 E7 and Miz-1 in CaSki cells. CaSki cell lysates were prepared and subjected to immunoprecipitation using the anti-HPV-16 E7 RabMab42-3 and polyclonal rabbit control serum, as indicated. Cell lysate was used as input control. Precipitated proteins (Miz-1 and 16 E7) were separated on a SDS-PAGE and detected by western blotting using affinity-purified polyclonal rabbit anti-HPV-16 E7 antibodies and the mouse monoclonal anti-Miz-1 antibody 10E2. The Miz-1 bands are indicated by arrows.

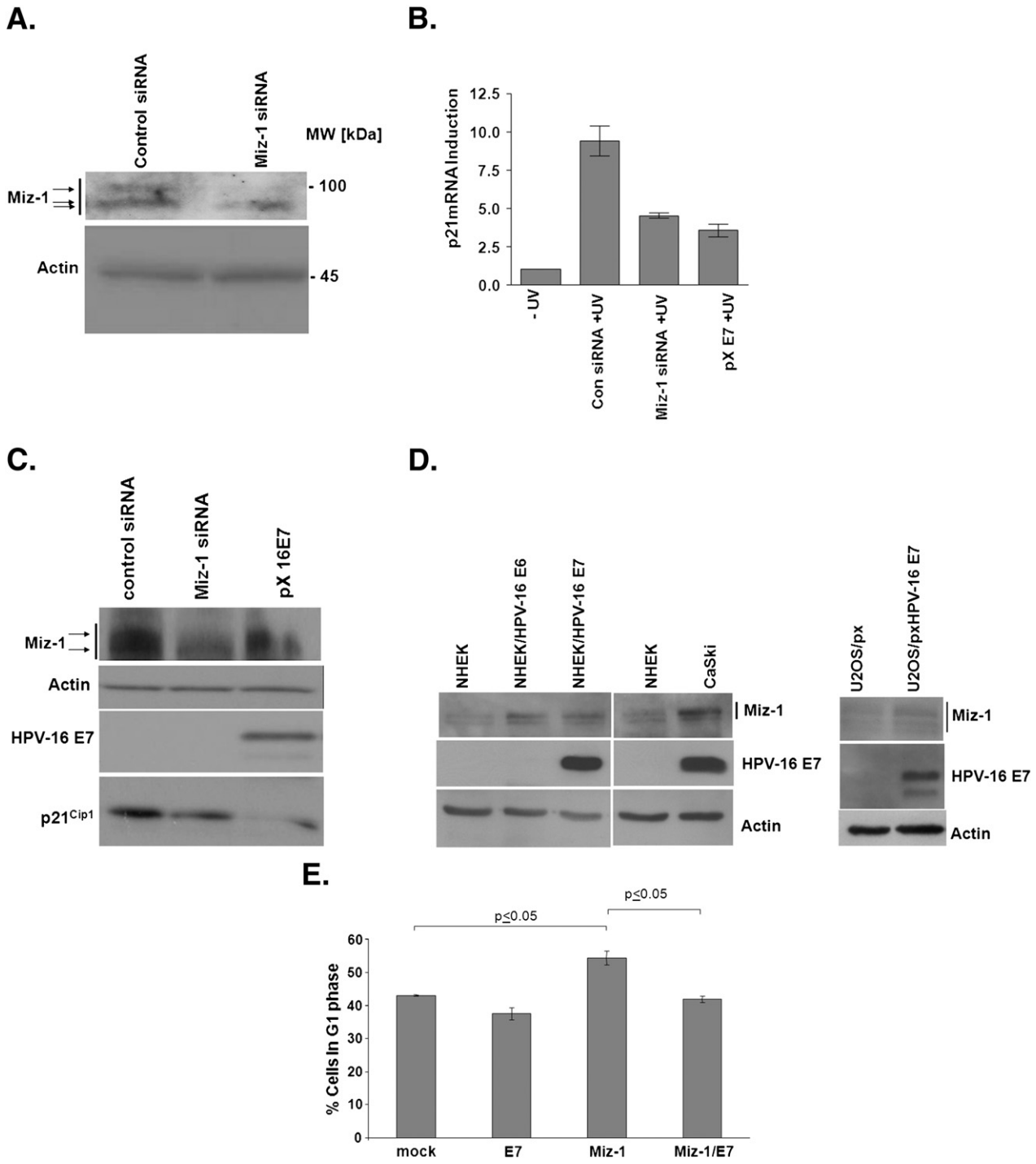


Fig. 3. Impact of siRNA-mediated knock down of endogenous Miz-1 and ectopic expression of HPV-16 E7, respectively, on UV-induced expression of $p21^{Cip1}$in C33A cells. (A) The siRNA oligonucleotide-mediated knock down of Miz-1 in C33A cells is shown (upper panel); siRNA oligonucleotide mixes were used for both Miz-1 and control siRNA (see ref. Herold et al., 2008). The Miz-1 bands are indicated by arrows. In the siRNA knock down the upper Miz-1 band is fully diminished and the faster migrating, more abundant, Miz-1 band is also reduced. Actin served as input control (lower panel). (B) UV-induced (25 J/m2) expression of the endogenous $p21^{Cip1}$ gene in C33A cells treated with control-siRNA, Miz-1-siRNA or transiently expressing HPV-16 E7, measured by quantitative RT-PCR. The values were normalized to β-actin. Values are the means ± standard deviation of at least 3 independent measurements. (C) Effects of the siRNA-mediated knock down of Miz-1 and of transient expression of HPV-16 E7 on the $p21^{Cip1}$ protein levels in UV-irradiated (25 J/m2) C33A cells. The corresponding cell lysates were run onto a SDS-PAGE and the protein level of HPV-16 E7, $p21^{Cip1}$, actin and Miz-1 are monitored by western blotting as indicated. The Miz-1 bands are indicated by arrows. (D) Detection of endogenous Miz-1 protein in different HPV-16 E7 expressing cells. Cell lysates were prepared from NHEK, NHEK/HPV-16 E6 (Blasko et al., 2000), NHEK/HPV-16 E7 (Mannhardt et al., 2000) and CaSki cells (left panel) and from U-2OS and U-2OS cells transiently transfected with pxHPV-16 E7 (right panel). Cell lysates were separated onto a SDS-PAGE and Miz-1 protein detected by western blotting using the anti-Miz-1 10E2 antibody. The expression of the HPV-16 E7 protein was also corroborated. Actin served as input control. (E) HPV-16 E7 overrides Miz-1 induced G1-cell cycle arrest in UV-irradiated C33A cells. C33A cells were transiently transfected with expression vectors for Miz-1, E7 or empty vector (mock) as indicated. Transfection efficiency of the C33A cells was over 90%. 24 h later the cells were irradiated with 25 J/m2 UV and another 24 h later the cells were propidium-iodide stained and the cell cycle distribution was determined by flow cytometry using a FACSCalibur. The percentage number of cells in G1-phase is shown. Values are the means ± standard deviation of three independent experiments.

carboxyl-terminal E7 mutants, Δ52YNIVT56 and Δ79LEDLL83, was significantly reduced (Fig. 6C). These results are consistent with the reduced binding efficiency of the carboxyl-terminal E7 mutants to Miz-1 (Fig. 1), reduced repression of Miz-1-dependent induction of the endogenous $p21^{Cip1}$ gene in C33A cells (Fig. 3) and the Miz-1-dependent binding of E7 to the $p21^{Cip1}$ core promoter in ChIP (Fig. 5D). In contrast, E7C24G, which fails to bind and inactivate pRB (Fiedler et al., 2004), was able to bind and repress Miz-1-induced $p21^{Cip1}$ gene expression with an efficiency similar to that of wild-type E7. These data suggest that the ability of E7 to form a complex with Miz-1 is consistent with the inhibition of Miz-1-driven transactivation of $p21^{Cip1}$ gene expression from the core promoter.

Discussion

The main finding of the present study is that HPV-16 E7 inhibits UV-induced $p21^{Cip1}$ gene expression in cervical cancer cells in a Miz-1-dependent manner. The ability to inhibit $p21^{Cip1}$ expression depends on E7 binding and repressing the activity of the transcription factor Miz-1 which is bound to the $p21^{Cip1}$ core promoter and activates $p21^{Cip1}$ gene expression. The ability of E7 to bind to Miz-1 and to repress Miz-1-dependent $p21^{Cip1}$ gene expression is significantly impaired by the C-terminal mutant E7Δ79LEDLL83 which exhibits reduced transforming activity (Massimi et al., 1997; Prathapam et al., 2001), suggesting that the ability of E7 to abrogate the function of Miz-1 contributes to its transforming capacity. This is underlined by our finding that HPV-11 E7 cannot repress UV-irradiation induced expression of the $p21^{Cip1}$ gene in C33A cells. Another E7 mutant, C24G, which is impaired in binding and inactivating the pRB family members (Phelps et al., 1992), can bind to Miz-1 and inhibit Miz-1-dependent $p21^{Cip1}$ expression similar to wild-type E7. This suggests that the interaction of E7 with pRB is not required for inactivation of Miz-1 and strengthens the current model that several distinct regulatory pathways must be subverted by oncogenic E7 to transform mammalian cells. This is in keeping with the results from numerous genetic, cell biological and biochemical studies (reviewed in ref. McLaughlin-Drubin and Munger, 2009; Pim and Banks, 2010; zur Hausen, 2002).

HPV-16 E7 can abrogate DNA-damage induced inhibition of DNA synthesis by various agents such as UV light, chemicals or ionizing radiation (Demers et al., 1994; Jones et al., 1999; Seavey et al., 1999; Slebos et al., 1994; Song et al., 1998). It is known that E7 can override p53-mediated G1-cell cycle arrest in response to DNA damage (Demers et al., 1994; Hickman et al., 1994); however, in cells expressing wild-type p53, p53 protein levels after DNA damage are not decreased but increased by E7 (Eichten et al., 2002; Funk et al., 1997; Jones et al., 1999; Seavey et al., 1999; Slebos et al., 1994; Song et al., 1998), reviewed in ref. (McLaughlin-Drubin and Munger, 2009). In fact, E7 has been shown to stabilize p53 (Seavey et al., 1999) but despite the high levels of p53 in E7 expressing cells the transcription of p53 target genes such as $p21^{Cip1}$ is not activated (Jones et al., 1999), and other studies showed that E7 represses transcriptional activation of reporter genes by p53 (Eichten et al., 2002; Massimi and Banks, 1997), reviewed in Ref. McLaughlin-Drubin and Munger, 2009). Thus the importance of the functional interaction between E7 and p53 (both proteins does not physically interact) for E7-induced

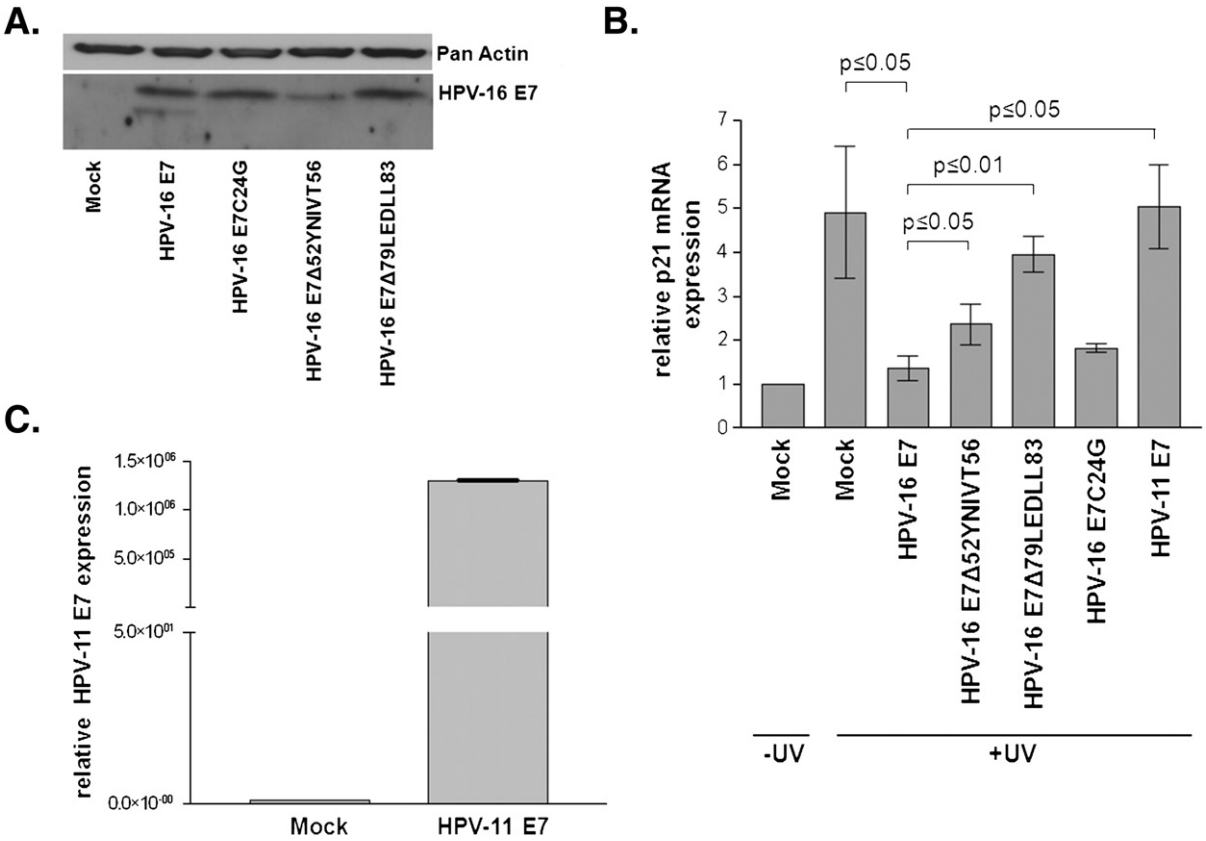


Fig. 4. HPV-16 E7 mediated repression of Miz-1-dependent $p21^{Cip1}$gene expression after UV irradiation is a function of the HPV-16 E7 carboxyl-terminal zinc-binding loop. (A) C33A cells were transiently transfected with either empty vector (mock) or expression vectors for HPV-16 E7 and HPV-16 E7 mutants, as indicated. The E7 protein levels were detected 24 h after transfection by western blotting. Pan-actin served as input control. (B) The transiently transfected C33A cells were irradiated with UV (25 J/m2); RNA samples were extracted before and 6 h after UV irradiation and subjected to quantitative PCR analysis to assess $p21^{Cip1}$ mRNA expression. The values were normalized to pan-actin. Values are the means ± standard deviation of 3 independent measurements. (C) HPV-11 E7 expression in C33A cells. C33A cells were transiently transfected with an HPV-11 E7 expression vector and with empty vector (mock). The expression of HPV-11 E7 was detected by q-RT-PCR. N = 6.

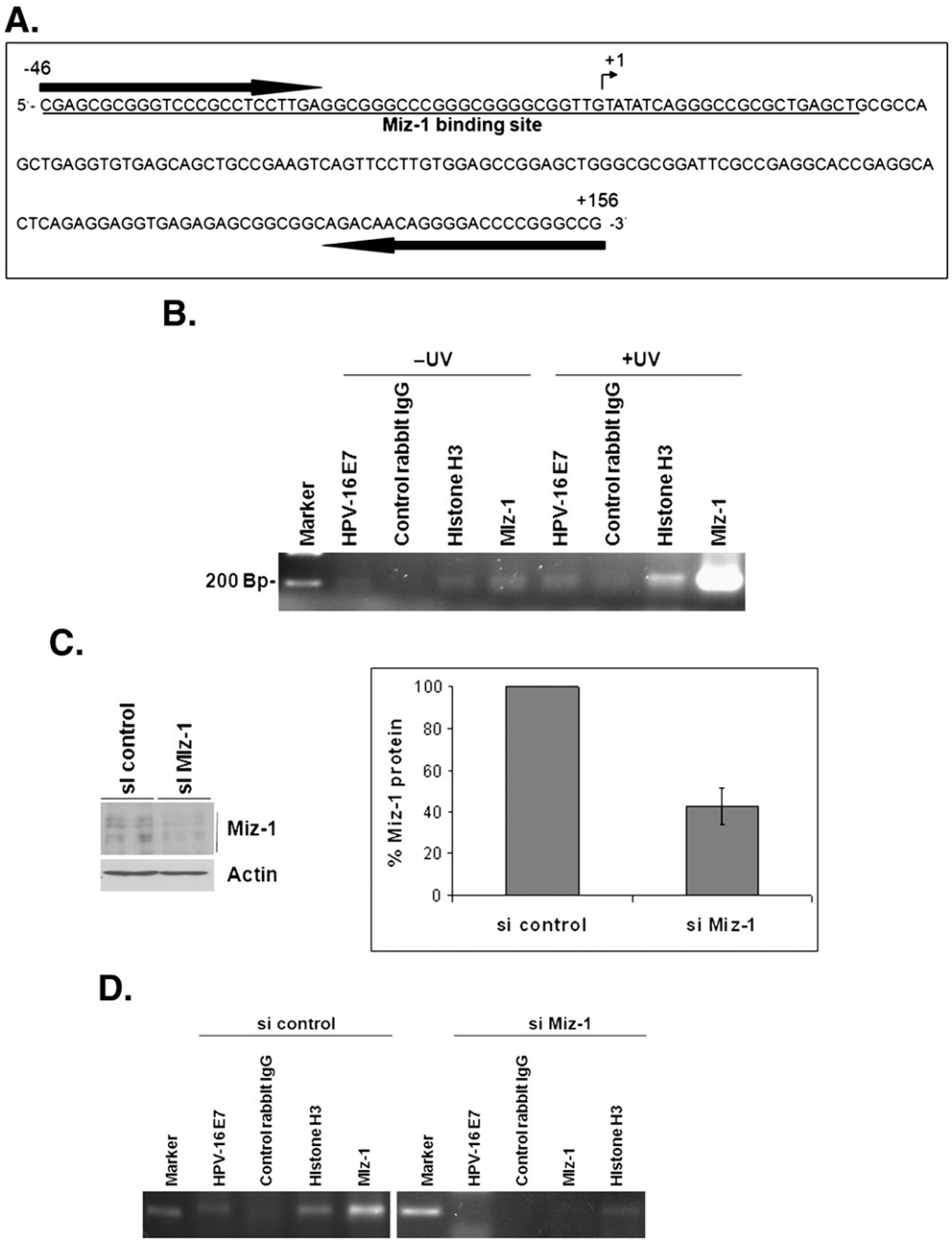


Fig. 5. HPV-16 E7 binds to the p21^{Cip1}core promoter. (A) Nucleotide sequence of the human p21^{Cip1} core promoter (−46/+156). The Miz-1 binding sites are underlined. The primers for the amplification of the p21^{Cip1} promoter region are indicated by arrows. (B) Chromatin immunoprecipitation (ChIP) of human p21^{Cip1} core promoter (−46/+156) DNA. Chromatin preparations from UV-treated and non-UV-treated CaSki cells were subjected to ChIP as indicated. The anti-HPV-16 E7 RabMab-42-3, anti-Miz-1 and anti-histone H3 antibodies co-immunoprecipitated human p21^{Cip1} core promoter (−46/+156) DNA. Unrelated rabbit immune serum failed to immunoprecipitate this DNA sequence. The amplified DNA bands were isolated from the agarose gel and the p21^{Cip1} core promoter (−46/+156) sequence verified by sequencing. The results are representative of three independent experiments. All antibodies failed to immunoprecipitate an unresponsive control. (C) SiRNA mediated knock down of Miz-1 in CaSki cells. (left panel) SiRNA oligonucleotide mixes were used for both Miz-1 and control siRNA (see ref. Herold et al., 2008). The efficiency of the siRNA-mediated Miz-1 knock down was analyzed by western blotting 72 h after transfection. Actin served as input control. (Right panel) The Miz-1 and actin western blot bands were scanned from low exposures and densitometrically analyzed for quantification (n=3). (D) ChIP of human p21^{Cip1} core promoter (−46/+156) DNA in Miz-1-depleted CaSki cells. Chromatin preparations from control siRNA and Miz-1 siRNA CaSki cells were subjected to ChIP as indicated. In control siRNA cells, the anti-HPV-16 E7 RabMab-42-3, anti-Miz-1 and anti-histone H3 antibodies co-immunoprecipitated human p21^{Cip1} core promoter (−46/+156) DNA. Unrelated rabbit immuneserum failed to immunoprecipitate this DNA sequence. In Miz-1 siRNA cells, the anti-HPV-16 E7 RabMab-42-3, anti-Miz-1 antibodies and unrelated rabbit immuneserum failed to co-immunoprecipitate human p21^{Cip1} core promoter (−46/+156) DNA. The amplified DNA bands were isolated from the agarose gel and the p21^{Cip1} core promoter (−46/+156) sequence was verified by sequencing. The results are representative of three independent experiments.

abrogation of DNA-damage induced inhibition of DNA-synthesis remains uncertain. In this study, we employed C33A cervical cancer cells which express an inactive p53 protein mutated in the DNA binding domain (p53R273C) (Crook et al., 1991; Scheffner et al., 1991), resulting in loss of DNA binding (Cho et al., 1994). This gave us the opportunity to analyse p53-independent p21^{Cip1} gene expression. We found that Miz-1 is necessary for UV-induced p21^{Cip1} expression and subsequent cell cycle arrest in the C33A cells. Moreover, we could show that E7 represses Miz-1-induced activation of p21^{Cip1} gene expression after UV irradiation in the C33A cells and this contributes to the abrogation of Miz-1-induced G1-cell cycle arrest. C33A cells responded with a moderate G1-cell cycle arrest to UV irradiation. This might be due to the mutated p53 as well as pRB alleles in this cell type (Crook et al., 1991; Scheffner et al., 1991; Zhu et al., 1993).

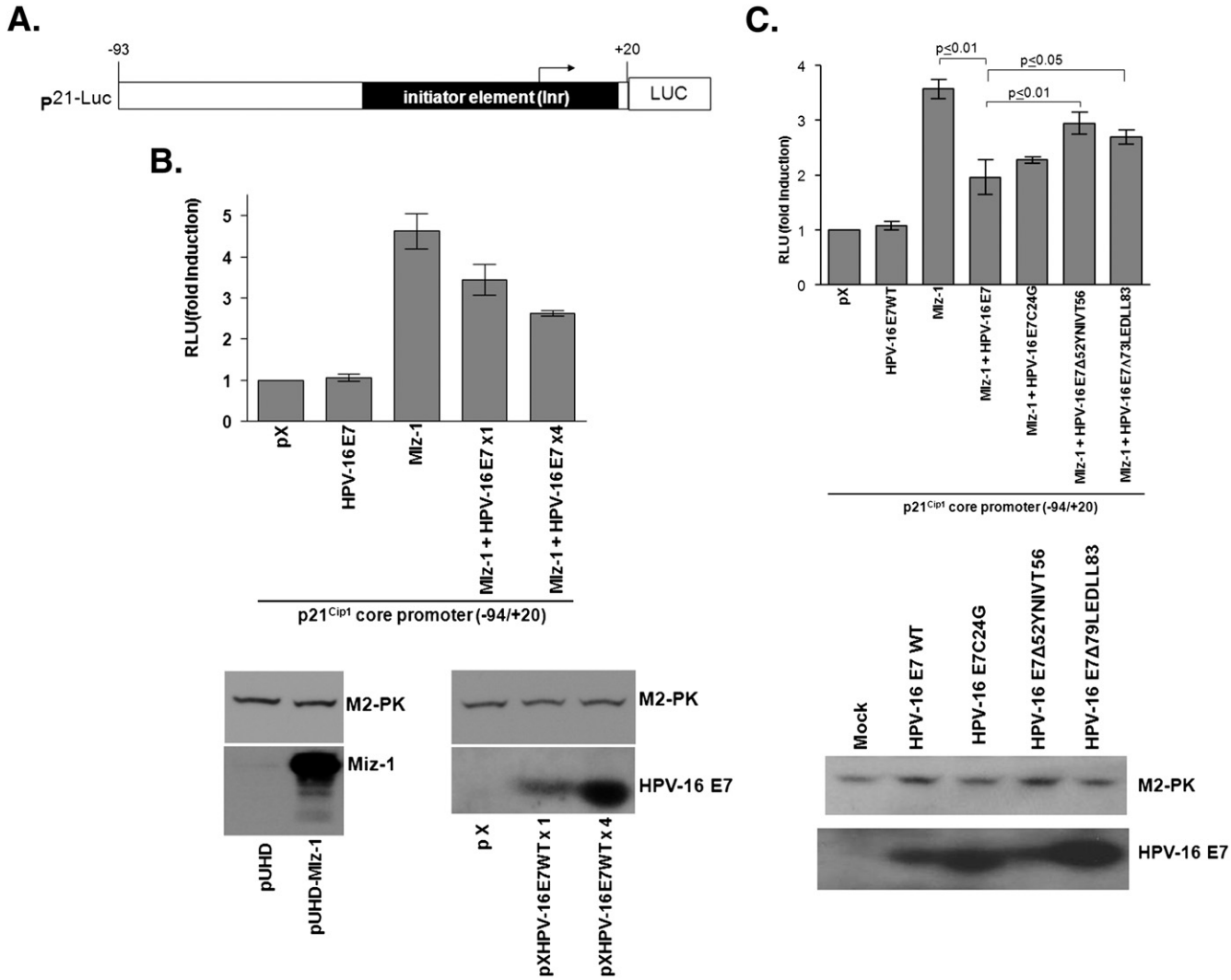


Fig. 6. HPV-16 E7 represses transactivation of the p21^{Cip1}core promoter by Miz-1. (A) Schematic representation of the p21^{Cip1}-core promoter (−93/+20)-luciferase reporter construct. Miz-1 binds to the initiator element (Inr) indicated as gray box. (B) (Upper panel) HPV-16 E7-mediated repression of Miz-1-dependent transactivation of the reporter pGL3-p21^{Cip1}-core promoter (−93/+20)-luciferase in U-2OS cells. U-2OS cells were transiently transfected by Effectene with the pGL3-p21^{Cip1}-core promoter (−93/+20)-luciferase reporter gene construct in the presence or absence of Miz-1 and increasing amounts of HPV-16 E7, as indicated. A cytomegalovirus-driven β-galactosidase (β-Gal) expression vector was co-transfected to control for transfection efficiency. The total amount of transfected DNA was always adjusted to 3 μg with empty vector pX. Luciferase activity was normalized to β-Gal activity. The experiments were repeated in triplicate. The results are representative of three independent experiments. Shown are means ± standard deviations (SD). (Lower panel) Detection of the expressed effectors. U-2OS cells were transiently transfected with empty vector (mock), expression vectors for Miz-1 and HPV-16 E7, as indicated and the Miz-1 and E7 protein levels were detected 24 h after transfection by western blotting. M2-PK is shown as a loading control. (C) (Upper panel) Repression of Miz-1-dependent transactivation of the reporter pGL3-p21^{Cip1}-core promoter (−93/+20)-luciferase by HPV-16 E7 and HPV-16 E7 mutants in U-2OS cells. U-2OS cells were transiently transfected by Effectene with the pGL3-p21^{Cip1}-core promoter (−93/+20)-luciferase reporter gene construct in the presence or absence of Miz-1, HPV-16 E7 and HPV-16 E7 mutants, as indicated. A cytomegalovirus-driven β-galactosidase (β-Gal) expression vector was co-transfected and luciferase activity was normalized to β-Gal activity. The total amount of transfected DNA was always adjusted to 3 μg with empty vector pX. The experiments were repeated in triplicates. The results are representative of three independent experiments. The values shown are means ± standard deviations (SD). (Lower panel) Detection of the transiently expressed, HPV-16 E7 and HPV-16 E7 mutants in U-2OS cells. The E7 protein levels were detected 24 h after transfection by western blotting. M2-PK is shown as a loading control.

Nevertheless, the data indicate that E7-mediated repression of Miz-1-induced p21^{Cip1} gene expression contributes to G1/S cell cycle progression.

Induced by UV irradiation Miz-1 binds to the p21^{Cip1} core promoter and transactivates expression of the p21^{Cip1} gene (Herold et al., 2002; Wu et al., 2003). Our results suggest a model in which E7 forms a complex with Miz-1 at the p21^{Cip1} core promoter in cervical cancer cells and specifically inhibits transactivation by Miz-1 which was activated by UV irradiation. This results in the inhibition of Miz-1-dependent expression of the p21^{Cip1} gene. A similar mechanism was shown for c-Myc, which can form a complex with Miz-1 at the core promoter of p21^{Cip1} and thereby repress p21^{Cip1} gene expression in response to DNA damage (Herold et al., 2002; Wu et al., 2003). In p53 wild-type cells this contributes to the inhibition of p21^{Cip1} induction by p53 from distal p53 response elements after exposure to DNA damage and facilitates p53-induced apoptosis by shifting the p53-response from anti-proliferative to pro-apoptotic (Seoane et al., 2002). In analogy, our results raise the possibility that E7-mediated repression of Miz-1-induced p21^{Cip1} gene expression could contribute to the inability of p53 to induce p21^{Cip1} gene expression in p53 wild-type cells (Jones et al., 1999). However, the role of E7 for p53-induced transactivation of the p21^{Cip1} promoter warrants further study.

What could be the reason for HPV-16 to evolve a strategy to inactivate the DNA-damage/Miz-1/p21^{Cip1} pathway by E7? Cells can respond to DNA damage by undergoing cell cycle arrest (Garner and Raj, 2008). In response to DNA damage signaling p53 is stabilized and induces the

expression of many target genes. Cell cycle arrest in G1 can be mediated by p53 through its induction of $p21^{Cip1}$. Like E7, E6, which induces proteolysis of p53, also abrogates DNA-damage-induced cell cycle arrest (Kessis et al., 1993). In fact, the p53 levels are quite low in HPV-positive cervical cancer cells and in cells expressing HPV E6 or HPV E6/E7 (reviewed in ref. McLaughlin-Drubin and Munger, 2009)). However, $p21^{Cip1}$ can also be induced through p53-independent pathways (Macleod et al., 1995). Thus, although p53-dependent antiproliferative pathways are inactivated by E6, antiproliferative signals that cannot be neutralized by E6 may affect the proliferation of HPV-16-infected cells and thereby viral replication. In this scenario, E7-driven inactivation of Miz-1 may add to inhibition of $p21^{Cip1}$ gene expression, and hence down modulation of the antiproliferative DNA-damage response of the infected host cells. In fact it was shown that E7 can abrogate the inhibition of DNA synthesis through both p53-dependent and p53-independent pathways (Jones et al., 1999; Song et al., 1998).

Transition from G1 to S phase is regulated by G1/S CDKs that phosphorylate and thereby inactivate pRB (Garner and Raj, 2008). E7 disrupts pRB/E2F complexes. This results in increased cyclin E and cyclin A levels and contributes to the induction of DNA synthesis. Thus the mechanisms by which E7 overrides DNA-damage induced inhibition of DNA synthesis may include the inactivation of pRB and deregulated expression of E2F transcriptional targets (Hickman et al., 1994). However, mutational analysis of E7 has shown that this activity is not solely responsible for cell transformation by E7 (reviewed in ref. McLaughlin-Drubin and Munger, 2009). E7 stimulates proliferation also independently of its ability to associate with pRB (Caldeira et al., 2000) and inactivation of pRB by E7 is not sufficient to overcome cell cycle arrest in human keratinocytes (Helt and Galloway, 2001). In keeping with these data, we showed in the present study that the E7 cd2 mutant C24G, which is deficient in binding to pRB, behaves as wild-type E7 in the functional interaction between E7 and Miz-1. Moreover, E7 contributes to abrogation of Miz-1-induced G1 cell cycle arrest in the C33A cells which contain mutated pRB alleles. This suggests that pRB-independent pathways contribute to HPV-16 E7-induced abrogation of DNA-damage induced G1/S cell cycle arrest in C33A cells. Thus repression of the expression of the cdki $p21^{Cip1}$ by E7 could be a strategy to overcome cyclin-E-Cdk2 and cyclin-A-Cdk2 inhibition and hence activation of additional targets which are necessary for cell cycle progression into S phase, such as the cyclin E-Cdk2 substrate NPAT (Zhao, 2004).

Several examples exist of tumor virus oncoproteins with diverse and partially redundant functions (reviewed in ref. McLaughlin-Drubin and Munger, 2008). The inactivation of the $p21^{Cip1}$ protein by physical interaction with E7 was previously shown as another mechanism contributing to the ability of E7 to override pathways inhibiting G1/S cell cycle transition after DNA damage (Funk et al., 1997) and in differentiating keratinocytes (Jones et al., 1997). However, it was also shown that high-risk HPV E7 cannot override a cell cycle arrest in HeLa cells induced by ectopic expression of $p21^{Cip1}$ (Niculescu et al., 1998). Moreover, studies in differentiated keratinocytes in warty lesions and in raft cultures suggested that $p21^{Cip1}$ is only detectable in post-mitotic E7-expressing cells but not in E7-expressing cells undergoing DNA synthesis (Banerjee et al., 2006; Noya et al., 2001). These data suggest that E7 inactivates $p21^{Cip1}$ by more than one mechanism. The data reported here suggest a novel mechanism contributing to E7-induced abrogation of DNA-damage induced $p21^{Cip1}$ expression by functional inactivation of Miz-1.

Materials and methods

Yeast two-hybrid interaction analysis

The Y2-HS was performed essentially as described (Zwerschke et al., 1999).

Plasmid construction

pUHDMiz-1 (Herold et al., 2002), the HPV-16 E7 and HPV-11 E7 expression vectors (Mannhardt et al., 2000; Massimi et al., 1997; Prathapam et al., 2001; Santer et al., 2007) are described. pGL3-$p21^{Cip1}$-promoter (−93/+20) containing the $p21^{Cip1}$ core promoter from −93 to +20 (Wu et al., 2003) was generated by the insertion of the following oligonucleotides into the Nhe/XhoI-site of pGL3 (Promega, Austria): p21minpGL3 1 F: 5′-ctagcgagcgcgggtcccgcctccttgaggcgggcccgggcggggcg gttgtatatca-3′; p21minpGL3 2 F: 5′-gggccgcgctgagctgcgccagctgaggtgtgag-cagctgccgaagtcagttcgttgtg-3′; p21minpGL3 1R: 5′-cagcgcggccctgatata-caaccgccccgcccgggcccgcctcaaggaggcgggacccgcgctcg-3′; p21minpGL3 2R: 5′-tcgagcacaaggaactgacttcggcagctgctcacacctcagctggcgcagct-3′

Cell culture and transfection

C33A cervical cancer cells and U-2OS osteosarcoma cells were cultured in DMEM plus 10% FCS (Santer et al., 2007). Cells were transiently transfected with 3 μg of various plasmids using Lipofectamine 2000 (Invitrogen, California). Transfection efficiency was controlled by co-transfecting pEGFP-C1 (Clontech, Germany) or pBB14 expressing membrane-bound GFP (Brideau et al., 1998). The transfection efficiency in both C33A and U-2OS cells was over 90%. Transfection efficiency varied less than 10% between individual experiments.

In vitro protein–protein interaction analysis

For GST pull-down assays, 10 ng/μl of GST and GST fusion proteins containing various mutants of E7 (Santer et al., 2007) were loaded on glutathione-Sepharose 4B beads. Total cell lysates from U-2OS cells overexpressing Miz-1 were incubated with GST-E7 fusion proteins and bound proteins were analyzed by western blotting using monoclonal anti-Miz-1 antibodies (Clone 10E2, ref. Herold et al., 2008).

Coimmunoprecipitation experiments

Co-IP in U-2OS cells: U-2OS cells cotransfected with pUHDMiz-1 and pX-HPV-16 E7 were harvested in lysis buffer (50 mM HEPES pH7, 150 mM (HPV-16 E7 IP) or 300 mM (Miz-1 IP) NaCl, 0.1% NP-40, 10 mM ß-glycerophosphate, 100 μM Na3VO4, 0.2 mM PMSF, 1 mM NaF, protease-inhibitor-mix no.1836170 (Roche, Germany). Total cell lysates were subjected to immunoprecipitation either with affinity-purified goat anti-HPV-16 E7 antibodies (Fiedler et al., 2005), mouse anti-Miz-1 antibodies (clone H-190, Santa Cruz), control mouse antibodies, or goat pre-immune serum. Beads were washed three times in lysis buffer and precipitated proteins were visualized by western blotting using affinity-purified polyclonal anti-HPV-16 E7 or mouse monoclonal anti–Miz-1 antibodies (clone 10E2; Wanzel et al., 2005; Herold et al., 2008; Herkert et al., 2010), respectively, essentially as described (Santer et al., 2007). Co-IP in CaSki cells: CaSki cells were harvested in lysis buffer (50 mM HEPES pH7, 150 mM NaCl, 0.1% NP-40, 10 mM ß-glycerophosphate, 100 μM Na3VO4, 0.2 mM PMSF, 1 mM NaF, protease-inhibitor-mix no.1836170 (Roche, Germany). Total CaSki cell lysates were subjected to immunoprecipitation either with the anti-HPV-16 E7 RabMab42-3 (Dreier et al., 2011) or rabbit pre-immune serum. Beads were washed three times in lysis buffer and precipitated proteins were visualized by western blotting using affinity-purified polyclonal anti-HPV-16 E7 or mouse monoclonal anti-Miz-1 antibodies (clone 10E2; Herkert et al., 2010; Herold et al., 2008; Wanzel et al., 2005), respectively. Note that the mouse monoclonal anti-Miz-1 antibody (clone 10E2) recognizes more than one band of Miz-1 in western blotting experiments (Herkert et al., 2010; Herold et al., 2008; Wanzel et al., 2005). These bands represent most likely post-translational modifications of Miz-1 (Herkert et al., 2010; Wanzel et al., 2005).

Cell cycle analysis

C33A cells transiently transfected (transfection efficiency >90 %) with pX, pX-HPV-16 E7 and/or pUHDMiz-1 were cultured in DMEM plus 10% FCS and 24 h later UV irradiated with 25 J/m2. 24 h later these cells were resuspended in Nicoletti buffer and the cell cycle profiles of PI-stained nuclei were measured by FACS analysis (FACSCalibur, Becton Dickenson, Austria). Cell cycle distribution was analyzed using ModFit software (Becton Dickenson, Austria).

SiRNA experiments

C33A cells were seeded in 6-well plates, maintained until 70% confluency and were then transfected with 100 nM siRNA oligonucleotide mixes for Miz-1 and control siRNA (Herold et al., 2008) using Lipofectamine 2000 (Invitrogen, UK). 70 h after transfection cells were subjected to UV irradiation (25 J/m2) with subsequent Quantitative RT-PCR (6–8 h after UV irradiation) or western blotting.

Luciferase reporter gene assays

U-2OS cells were seeded in 6-well plates and transiently transfected using the Lipofectamine 2000 (Invitrogen, California) with pGL3, pGL3-p21^{Cip1}-promoter (−93/+20) and expression vectors for Miz-1, HPV-16 E7 and mutants thereof. 24 h after transfection luciferase activity from at least 3 independent transfections was measured in an Anthos Lucy 1 photoluminometer (Anthos Labtec Instruments, Austria).

Quantitative RT-PCR experiments

RNA was purified using the RNeasy Kit (Quiagen, Germany). RNA was transcribed to cDNA using the First Strand cDNA Synthesis Kit (Fermentas, Ontario). Real-Time PCR was performed with iQ Sybr Green Supermix (Biorad, California) using a iCycler (Biorad, California). The final concentration of primers was 500 nM. Primer: β-actin forward LC 5′-GACGACATGGAGAAAATCTG-3′; β-actin reverse LC 5′-ACATGATCTGGGTCATCTTCT-3′; p21-LC1 5′-GGCGGCAGACCAGCATGACAGATT-3′; p21-LC2: 5′-GCAGGGGGCGGCCAGGGTAT-3′. HPV-11 E7 forward 5′-TGGACAAGGTGGACAAACAA-3′; HPV-11 E7 reverse 5′-TAGTGTGCCCAGCAAAAGCT-3′.

Western blot analysis

Western blot analysis was performed as described (Mück et al., 2010). Antibodies against Miz-1: IPs: clone H-190, Santa Cruz Biotechnology; Western blot: Miz-1 clone 10E2 (Herold et al., 2008), p21^{Cip1} (clone N20, Santa Cruz Biotechnology), Pan-actin (clone ACTN05 Labvision, California), p300 (BD Pharmingen), Myc (9E10, Santa Cruz Biotechnology). Anit-HPV-16 E7 antibodies: affinity-purified rabbit and goat anti-HPV-16 E7 antibodies (Fiedler et al., 2004, 2005), RabMab42-3 (Dreier et al., 2011).

UV-irradiation experiments

Cells were seeded in 6-well plates and transiently transfected with the corresponding expression vectors. 24 h later, cells were washed with PBS, covered with 25 μl/cm^2 PBS and irradiated with 25 J/m^2 UV in CL 1000 Cross linker from UVP, Inc (Upland, California). Cells were further processed 6–8 h after irradiation.

Chromatin immunoprecipitation (ChIP) assay

ChIP was conducted according to the SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling Technology, Inc, Danvers, MA, USA). For the immune-precipitation of chromatin complexes 18 μg precleared chromatin was incubated with sepharose beads and 5 μg of monoclonal rabbit anti-HPV-16 E7 antibodies (RabMab42-3) (Dreier et al., 2011), anti Miz-1 antibody (Clone C-19, Santa Cruz Biotechnology, Inc. Heidelberg, Germany), anti-Histone H3 RabMab (Clone D2B12, Cell Signaling Technology, Inc, Danvers, MA, USA) and unrelated rabbit immune-serum (Cell Signaling Technology, Inc, Danvers, MA, USA), respectively. To detect the sequence of the human p21^{Cip1}-core promoter (−46/+156) the samples were analyzed by RT-PCR using the forward primer 5′-AGC GCG GGT CCC GCC TCC TTG AG-3′and the reverse primer 5′-CGG CCC GGG GTC CCC TGT TGT CT-3′, essentially as described (Ceballos et al., 2005).

Statistical analysis

Statistical significances were calculated using the unpaired *t*-test (GraphPad PRISM version 3.03).

Acknowledgments

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The authors declare no conflict of interests.

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