High-risk HPV16E6 stimulates hADA3 degradation by enhancing its SUMOylation

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Despite significant research, our understanding of the molecular mechanisms of Human Papilloma Virus (HPV) induced cancers remains incomplete. Majority of invasive cervical cancers are caused by high-risk HPV 16 and 18. Two potent HPV oncoproteins, E6 and E7, promote human malignancies by disrupting the activities of key regulators of cell proliferation and apoptosis. Recent investigations have identified hADA3, a transcriptional coactivator protein as a target of high-risk HPV16E6. However, the mechanism of degradation of hADA3 by E6 and its contribution in HPV induced carcinogenesis is poorly understood. Here, we showed that E6-mediated proteolysis of hADA3 is responsible for maintaining low levels of hADA3 in HPV-positive cervical cancer cell lines. We demonstrate that HPV16E6 targets hADA3 for ubiquitin-mediated degradation via E6AP ubiquitin ligase. We also show that hADA3 undergoes accelerated SUMOylation in the presence of HPV16E6. Our data represent the first evidence that hADA3 is posttranslationally modified by SUMOylation, which makes it unstable and establishes a link between SUMOylation and E6-mediated ubiquitination of hADA3. Furthermore, depletion of Ubc9 prevented rapid degradation of hADA3 in E6 expressing cervical cancer cells and overexpression of hADA3 resulted in suppression of proliferation and migration abilities of SiHa cells. Overall, this study underscores the importance of posttranslational modifications in HPV16E6-mediated downregulation of hADA3 thereby unveiling a novel mechanism by which HPV induces oncogenesis.

Introduction

Cervical cancer is a major public health problem. This life-threatening disease is primarily caused by certain types of high-risk Human Papilloma Virus (HPV) that can transform the cells in the lining of the cervix giving rise to cancerous lesions (1–4). Cervical cancer continues to be one of the common malignancies affecting women in spite of the availability of improved diagnostic and screening techniques and vaccines. Therefore, understanding the molecular pathway that contributes to HPV oncogenesis constitutes an important area of research. HPV oncogenicity is contributed by two major multifunctional oncoproteins E6 and E7, which are extensively expressed in all HPV induced carcinomas (4). E6 and E7 cause human malignancies by abrogating the activities of key cell cycle regulators and tumor suppressors (5–10). Past investigations have revealed numerous cellular proteins that interact with these oncoproteins thereby allowing uncontrolled proliferation of the infected cells (1,2,4,7,9,11,12).

The present study focuses on hADA3 (human alteration/deficiency in activation3), a previously identified cellular target of HPV16E6 (13). The transcriptional coactivator hADA3 plays a key role in DNA damage induced acetylation of p53 leading to stabilization and augmentation of its transcriptional activity (14–16). Human ADA3 has been implicated to play important role in diverse physiological processes including embryonic development, cell cycle progression (17), regulation of nuclear receptor functions (18,19), chromatin remodeling by virtue of its association with HAT complexes like PCAF (20), p300 (21), STAGA (22), GCN5 (23) and in senescence (15). Given the importance of hADA3 in normal cellular physiology, we speculate that functional inactivation of hADA3 by HPV16E6 may help in causing cellular transformation; however, the mechanism of hADA3 regulation remains unclear. The purpose of the current study was to gain insight into the molecular mechanism of HPV6-mediated degradation of hADA3 as a crucial part of the oncogenic machinery.

In the present investigation, we report that HPV16E6 destabilizes hADA3 by promoting it’s ubiquitination via E6AP ubiquitin ligase. We provide novel evidence showing that E6-mediated destabilization of hADA3 is induced by SUMO modification. Taken together, the current investigation demonstrates that E6 driven degradation of SUMOylated hADA3 is a novel mechanism for HPV-mediated inactivation of hADA3, which may play a key role in HPV16E6 related oncogenic transformation.

Materials and methods

Antibodies and plasmid constructs

Monoclonal antibodies against the epitope tag Flag were purchased from Sigma (St. Louis, MO.), while antibodies against β-actin, ubiquitin, GFP, HPV16 E6, Ubc9, p53 and E6AP were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Myc, anti-V5 and anti-SUMO2/3 were purchased from Invitrogen (Carlsbad, CA). Plasmids expressing Myc-HPV16E6, Flag-hADA3 and EGFP-hADA3 were generous gifts from Vimla Band (UNMC, USA) described in (3,14). Expression constructs of HA-SUMO1, 2 and 3 were kindly provided by Ron Hay (University of Dundee, UK), Myc-Ubc9 and Myc-Ubc9DN were kind gifts from William T. Beck (UCI, USA), Myc-PIAS1 was a generous gift by Ed Schmidt (Montana State University, USA), while Flag-SENP1 was gifted by Michael J. Matunis (John Hopkins University, USA). V5-E6AP and HA-CS33A were generous gifts from Niraj Ranjan Jana (National Brain Research Center, India). Plasmids expressing HA-Ub and Flag-Ub were kindly provided by Pradip Raychaudhuri (UCI, USA) and pSGS-Flag E6 was a kind gift from Srilata Bagchi (UCI, USA).

Cell culture, transfection and western blotting

HEK293T, C33A, HeLa and SiHa cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA) whereas Caski was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL of penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA) whereas Caski was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL of penicillin and 100 ng/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator with 5% CO2 atmosphere at 37°C. For overexpression experiments, HEK293T cells were transfected by calcium phosphate method (24), whereas Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was employed for transfection in rest of the cells as per manufacturer’s instructions. The medium was changed after 16 h of transfection and the cells were harvested 2 h posttransfection in lysis buffer containing 50 mM Tris-HCl, 400 mM NaCl, 0.2% Nonidet P-40, 10% glycerol and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Equal amount of whole cell lysates were processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting with appropriate antibodies.

Coimmunoprecipitation analyses

Cell extracts were prepared in lysis buffer containing 150 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 2 mM PMSF (phenylmethylsulfonyl fluoride), 2 mM NaF, 1 mM Na3VO4, and protease inhibitor cocktail. Lysates were precleared with 10 µl Protein-G bead slurry (50%) at 4°C with constant rocking for 1 h. For immunoprecipitation, 1.5 µg precleared extract was gently rocked (with 1 µg of antibody for 4 h at 4°C). Thereafter, 40 µl of Protein-G bead suspension was added to the above mixture and allowed torock for an additional 1 h at 4°C. The beads were washed 3–4 times with the lysis buffer and the bound proteins were eluted with SDS-PAGE sample dye.

Abbreviations: CHX, cycloheximide; hADA3: human alteration/deficiency in activation3; HEK293T, human embryonic kidney 293T cells; HPV, human papilloma virus, NEM, N-ethylmaleimide; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; shRNA, Short hairpin RNA; EGFP, enhanced green fluorescent protein; SUMO, small ubiquitin-like modifier.

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Beads were spun down and the clear supernatant was resolved by SDS-PAGE and subjected to immunodetection with appropriate antibodies.

**Analyses of protein turnover**

Cells were transfected with the desired expression vectors and 16 h posttransfection, they were trypsinized, pooled and resuspended equally in four petri plates for each set. After growing for another 16 h, cells were treated with 100 µg/ml cycloheximide (CHX: Sigma, St Louis, MO), harvested at the indicated time intervals and equal amounts of the whole cell lysates were subjected to Western blot analysis. Densitometric analyses of scanned images were carried out using Multi Gauge V3.0 (Fujifilms, Tokyo, Japan) software.

**In vivo ubiquitination assay**

HEK293T or C33A cells were transfected with vectors expressing Flag-hADA3, HA-UB and Myc-E6 as indicated in the figures using calcium phosphate method. After 36 h of transfection, cells were treated with 20 µM MG132 (Sigma, St Louis, MO) for 5 h. Cell lysates were prepared with lysis buffer containing 150 mM NaCl, 1% NP-40, 1 mM EDTA, 40 mM HEPES (pH 7.0), 5 µM N-ethylmaleimide (NEM from Sigma, St Louis, MO), protease inhibitor cocktail, 2 mM PMSF, 2 mM NaF and 1 mM Na3VO4. Lysates were precleared with 15 µl Protein G-beads for 1 h at 4°C. Thereafter, immunoprecipitation against Flag epitope was performed by incubating 1.5 mg of precleared supernatants with 1.5 µg antibody for overnight at 4°C. The unbound proteins were removed by washing the beads extensively with modified RIPA buffer (25) containing 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.05% SDS, 1 mM EDTA, 40 mM HEPES (pH 7.0), 5 µM NEM and 2 mM PMSF. Protein complexes bound to beads were resolved by SDS-PAGE and detection was carried out using appropriate antibodies.

**In vivo SUMOylation Assay**

HEK293T or C33A cells were transfected with Flag-hADA3, HA-SUMO1, 2 and 3 or Flag-hADA3, Myc-Ubc9 and Myc-PIAS1 or Flag-hADA3 and Myc-E6 expression vectors followed by MG132 (20 µM) treatment for 5 h. Subsequently, cell lysates were prepared with lysis buffer containing 150 mM NaCl, 1% NP-40, 1 mM EDTA, 40 mM HEPES (pH 7.0), 5 µM NEM, protease inhibitor cocktail, 2 mM PMSF, 2 mM NaF and 1 mM Na3VO4. For immunoprecipitation, 1.5 mg protein was incubated with anti-Flag antibody and protein-antibody complex was pull down with Protein-G-Sepharose beads. Dilution of cell lysate and washing of beads were accomplished with modified RIPA buffer under denaturing condition. Immunocomplexes were resolved by SDS-PAGE and detection was carried out by Western blot analyses with anti-HA, anti-Flag, anti-SUMO and anti-Myc antibodies.

**Generation of stable cells**

The pSUPER-retro vector system was used for expression of shRNA in mammalian cells. The hADA3 and Ubc9 specific RNAi sequences (sense, 5'-GGC CAG CCA UCA CAA ATT-3' and antisense, 5'-UUG AUU GUG AUG GCU GCT CTC-3') cloned in shRNA constructs are described elsewhere (14,27). SiHA cells were transduced with either shADA3 or shUbc9 retroviral supernatants followed by selection in media containing puromycin (0.5 µg/ml) for 10 days. The knockdown was verified by assessing the expression of endogenous hADA3 and Ubc9 using Western blot.

**Wound healing assay**

For wound healing assay, stable cells were seeded at a density of 1x10^5 cells in 24 well plates and allowed to grow up to full confluence in a monolayer. A single linear scratch of approximately 5 mm was introduced in each well using a sterile micro-tip. The debris was washed with PBS and replaced with fresh growth medium containing appropriate drug. Wound closure was monitored by capturing images at different time intervals after scratching. Three independent experiments were carried out in duplicate.

**Soft agar assay**

For transformation and anchorage independent colony formation ability, soft agar assay was performed. Cells (4 x 10^4) were suspended in a medium containing 0.4% agarose and then poured onto 60 mm culture dish coated with 0.8% agarose. The top agar surface was layered with complete medium every third day and cells were allowed to grow for 20 days. Colonies larger than 0.1 mm in soft agar were stained with 0.1% crystal violet for counting and capturing images.

**Results**

**HPV16E6 downregulates hADA3 protein expression by promoting its ubiquitination in cervical cancer cells**

HPV16E6 has been shown to destabilize hADA3 in overexpression system (13,15). To extend these findings in physiological setting, hADA3 protein levels in whole cell lysate of HPV-negative C33A cell line was compared with those of the HPV-positive cervical cancer cell lines like HeLa, SiHa and Caski by immunoblotting. Notably, HPV-positive cells exhibited lower levels of hADA3 compared to the HPV-negative cells (Figure 1A). This result supports the notion that HPV16E6 targets endogenous hADA3 for degradation. Further, to examine the role of proteasome in this process, cervical cancer cells (C33A, HeLa, SiHa and Caski) were treated with MG132, a potent inhibitor of 26S proteasome complex before assessing the level of hADA3 protein. As demonstrated in Figure 1A, inhibition of proteasome activity by MG132 prevented the E6-mediated degradation of hADA3, resulting in accumulation of endogenous hADA3. To investigate whether the decrease in hADA3 level in E6 expressing cells correlates to an alteration in protein turnover, HPV16E6 was ectopically expressed in non-HPV cervical cancer cell line C33A and the half-life of the cellular hADA3 protein was assessed after a CHX block. As indicated in Figure 1B, overexpression of Myc-E6 resulted in rapid turnover of endogenous hADA3 thereby reducing its half-life from 6.7 to 2.7 h (Figure 1C). These results demonstrate that E6 induces degradation of hADA3 via proteasomal-mediated pathway.

Since degradation of proteins via proteasome pathway generally involves ubiquitination, we speculated that HPV16E6 may promote ubiquitination of hADA3 to stimulate its proteolysis. To investigate this possibility, we performed an in vivo ubiquitination assay in which HEK293T cells were transfected with plasmids expressing Flag-hADA3 and HA-ubiquitin in the presence and absence of Myc-E6. As shown in Figure 1D, an enhanced ubiquitination signal of hADA3 was observed in presence of HPV16E6. Identical result was obtained in HPV-negative cervical cancer cell line C33A as well upon overexpression of HPV16E6 (Figure 1E). Blot for Figure 1E was probed with anti-ubiquitin antibody which showed enriched endogenous ubiquitination of hADA3 in presence of HPV16E6. These results support the conclusion that HPV16E6 enhances ubiquitination of hADA3.

E6AP has been shown to facilitate E6-mediated destabilization of numerous cellular proteins via proteasome pathway including a recent report for hADA3 (5,28–31). However, their physical interaction was not assessed before. To this end, we overexpressed Flag-hADA3, V5-E6AP and Flag-E6 in HEK293T cells followed by MG132 treatment and immunoprecipitation with anti-Flag antibody. As shown in Figure 1F, E6AP was found to coimmunoprecipitate with Flag-hADA3, revealing an interaction between E6AP and hADA3 even in the absence of HPV16E6. As expected, no E6AP signal was detected in the control immunoprecipitates of cells transfected with hADA3 alone, whereas a band for E6AP protein was clearly detected in anti-Flag immunoprecipitates of cells co-transfected with hADA3 and E6AP with or without E6. Further, in vivo ubiquitination experiment demonstrated that hADA3 is ubiquitinated in presence of E6AP and the levels of ubiquitinated hADA3 was found to be higher upon HPV16E6 cotransfection (Figure 1G). Altogether, these observations provided supporting evidence for a specific interaction between hADA3 and E6AP and that E6 contributes to enhanced hADA3 ubiquitination through E6AP.

**hADA3 is SUMOylated in vivo**

Next, we investigated whether SUMOylation could play a role in regulating the level of cellular hADA3 and subsequent influence of E6 in this process. For this purpose, the SUMOylation status of hADA3 in vivo was evaluated. Flag-hADA3 was overexpressed in the absence or presence of HA tagged SUMO1, 2 and 3 constructs in HEK293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody under denaturing condition. Western blot analysis with anti-HA antibody (Figure 2A) revealed increased appearance of high molecular weight hADA3 bands in presence of SUMO overexpression indicating that hADA3 was covalently modified by SUMO. However, intense laddering was observed in presence of SUMO2 and SUMO3 (as compared to SUMO1) indicating higher preference for these forms of substrates. The presence of multiple bands also implies that hADA3 could be SUMOylated at multiple sites regardless of SUMO isoforms.
Fig. 1. HPV16E6 stimulates E6AP-mediated ubiquitination of hADA3. (A) Effect of MG132 on the endogenous levels of hADA3 in cervical cancer cells. C33A, HeLa, SiHa and Caski cell lines were either mock treated with DMSO or with MG132 for 5h. Equal amount of whole cell extracts were then subjected to SDS-PAGE followed by immunoblotting with hADA3 and β-actin antibodies. (B) Half-life of hADA3 in presence of HPV16E6. HPV negative cervical cancer cell line C33A was transfected either with empty vector or with vector expressing Myc-E6. After 36h of transfection, cells were treated with CHX and harvested at the indicated time intervals. Equal amounts of cell lysates were analyzed by Western blotting using anti-hADA3, anti-Myc and anti-β-actin antibodies. (C) The intensity of hADA3 bands were quantified by densitometric analysis and a graph was plotted between hADA3 and time of CHX addition. (D) In vivo ubiquitination of Flag-hADA3 in presence of 16E6. HEK293T cells were transfected with either Flag-hADA3 or HA-Ub alone or in combination with HPV16 E6. Transfected cells were treated with MG132 for 5h followed by immunoprecipitation with Flag-antibody in modified RIPA buffer. Level of ubiquitination was checked using anti-HA-antibody. Equal amounts of cell lysates were subjected to SDS-PAGE to check expression of all transfected constructs by Western blotting using antibodies against Flag and Myc epitopes. Similarly, in vivo ubiquitination was carried out in C33A cell line (E) transfected with Flag-hADA3 in presence and absence of E6. Cells were subjected to MG132 treatment followed by immunoprecipitation with anti-Flag antibody and immunoblotting with antibodies against ubiquitin, Flag and Myc. (F) In vivo association between E6AP and hADA3. HEK293T cells were transfected with Flag-hADA3 and HA-Ub in combination with either E6AP alone or E6AP and E6. After 36h of transfection, cells were incubated with the proteasome inhibitor MG132 for 5h and whole cell lysates were immunoprecipitated with anti-Flag antibody followed by immunoblotting with anti-HA antibody. All results were replicated in at least three independent experiments and a representative picture has been shown.
Fig. 2. SUMOylation of hADA3. (A) In vivo SUMOylation of hADA3. HEK293T cells were transfected with either Flag-hADA3 alone or in combination with HA-SUMO1, 2 or 3. After treating the cells with MG132, immunoprecipitation was carried out using anti-Flag antibody followed by immunoblotting with anti-HA antibody. (B) Effect of MG132 on in vivo SUMOylation of hADA3. SUMOylation was carried out (with HA-SUMO2) as described above in presence or absence of MG132. (C and D) Association of hADA3 with Ubc9 and PIAS1. HEK293T cells were co-transfected with either (C) Myc-Ubc9 or (D) Myc-PIAS1 alone or together with Flag-hADA3. Cells were lysed and equal amounts of protein were subjected to immunoprecipitation with anti-Flag antibody followed by SDS-PAGE and immunoblotting with anti-Ubc9, anti-Myc and anti-Flag antibodies. Band marked with (*) indicates the signal due to IgG in the control IP lanes of (C) and (D). (E and F) SUMOylation of Flag-hADA3 in presence of Ubc9 and PIAS1. HEK293T cells were transfected with either Flag-hADA3 alone or in combination with Myc-Ubc9 and Myc-PIAS1. After treating the cells with MG132 for 5h, immunoprecipitation was carried out with anti-Flag antibody followed by immunoblotting with mixture of anti-SUMO1 and SUMO2 antibodies (E). The blot was stripped and probed with anti-Flag and anti-Myc antibodies (F). Each experiment was replicated at least three times and a representative picture has been shown in the figure.
and that they accumulate in presence of proteasome inhibitor (shown in Figure 2B) suggesting that these forms are prone to proteasomal degradation.

Ubc9 and PIAS are two key components of the SUMOylation machinery (32–34). Therefore, our next aim was to assess the interaction of hADA3 with Ubc9 and PIAS1 in vivo by coimmunoprecipitation experiments. HEK293T cells were transfected with Flag-hADA3 and Myc-Ubc9 constructs. Transfected cell lysates were immunoprecipitated with anti-Flag antibody and coimmunoprecipitated Ubc9 was detected by Western blot analysis. Our result shown in Figure 2C clearly demonstrates the coimmunoprecipitation of Myc-Ubc9 with Flag-hADA3 indicating that these proteins do interact upon overexpression. Consistent with this finding, immunoprecipitation experiment with PIAS1 overexpression revealed interaction of hADA3 with PIAS1 as well (Figure 2D). Comigration of IgG with Flag-hADA3 has been indicated by (*) in Figure 2C and D. Collectively, these data suggest that, hADA3 is a specific SUMO substrate and it physically interacts with the major components of SUMOylation machinery.

To further define the role of Ubc9 and PIAS1 in SUMOylation of hADA3 in vivo, we examined the effect of ectopically expressed Myc-Ubc9 and Myc-PIAS1 on the SUMOylation status of Flag-hADA3. Transfected cell lysates were immunoprecipitated with anti-Flag antibody in modified RIPA buffer followed by detection of SUMO, hADA3 and Ubc9. Immunoblotting with anti-SUMO1 and anti-SUMO2 (Figure 2E) and anti-Flag (Figure 2F) antibodies (after blot stripping) confirmed that overexpression of Ubc9 and PIAS1 augmented the intensity of SUMOylated hADA3 bands supporting the conclusion that Ubc9 and PIAS1 act as E2 SUMO conjugating and E3 SUMO ligating enzyme respectively for hADA3. Figure 2F also verified that the slower migrating bands seen after overexpression of Ubc9 and PIAS1 are indeed the higher molecular weight species of Flag-hADA3. Taken together, these findings clearly demonstrate that hADA3 is a substrate for SUMOylation.

SUMOylation destabilizes hADA3 through SUMO-dependent ubiquitination

Considering the suggested role of SUMOylation in destabilization of hADA3 as above, we examined the turnover rate of hADA3 in presence of ectopically expressed Ubc9 and PIAS1. Results of half-life assay (As shown in Figure 3A and C), demonstrated that overexpression of Ubc9 and PIAS1 accelerated the decay of hADA3 compared to control cells with a sharp decline in hADA3 half-life from approximately 5 to 2 h. To further substantiate these results, we examined the turnover of hADA3 in cells overexpressing the de-SUMOylating enzyme SENP1 (35). As expected, overexpression of SENP1 resulted in extension of hADA3 half-life from 5 to 7 h (Figure 3B and C). Taken together, these results suggest that SUMOylation leads to destabilization of hADA3 protein and de-SUMOylation by SENP1 reverses this effect (Figure 3C).

Above data demonstrated that SUMO modified hADA3 was destined for proteasome-mediated degradation. Moreover, SUMO modification was shown to promote degradation of several proteins (36–38). This prompted us to investigate whether there exists a link between SUMOylation and ubiquitination of hADA3. To examine this, an in vivo ubiquitination assay of hADA3 was performed in the absence or presence of overexpressed SUMO. Immunoprecipitation of Flag-hADA3 from transfected HEK293T cell extracts were examined by Western blot analysis using anti-ubiquitin antibodies. As shown in Figure 3D, enhanced ubiquitination of hADA3 was observed in cells overexpressing SUMO2 indicating that SUMOylation facilitates ubiquitination of hADA3. In order to confirm this observation further, in vivo SUMOylation of hADA3 was performed in the presence of overexpressed E6AP and a dominant negative mutant of the same (HA-C833A). As seen in Figure 3E, the results showed a significant accumulation of slower migrating SUMO conjugated forms of hADA3 in the presence of E6APDN (HA-C833A) even without MG132 treatment. This evokes the thought that SUMOylation may be important for subsequent ubiquitination and degradation of hADA3 in cervical cancer cell lines. Altogether, these data suggest that SUMO conjugated hADA3 is a potential target for ubiquitin-mediated degradation.

**E6 induces hADA3 SUMOylation and targets SUMOylated hADA3 for degradation**

To investigate the effect of E6 in SUMOylation of hADA3, an in vivo SUMOylation assay was performed in C33A cells after transfecting with Flag-hADA3 along with empty vector or in combination with Myc-E6. When anti-Flag immunoprecipitates were probed with anti-SUMO1+2 antibodies (Figure 4A), elevated basal levels of SUMOylated hADA3 was observed in E6 expressing cells. This clearly suggests a role of HPV16E6 in stimulation of hADA3 SUMOylation in cervical cancer cells. Moreover, SENP1 overexpression was found to reduce the level of ubiquitinated hADA3 (Figure 4B) indicating a direct correlation between SUMO conjugation and ubiquitination of hADA3. To further investigate if E6 is involved in the disposal of SUMO conjugated hADA3 through ubiquitination and also to address the critical role of Ub9 on hADA3 stability, the effect of Ub9DN on hADA3 protein levels was examined in the presence of E6. As shown in Figure 4C (in HEK293T) and 4D (in C33A), inhibition of SUMOylation machinery by overexpression of Ub9DN abolished the E6-mediated degradation of hADA3 resulting in significant accumulation of hADA3 protein. Further, to gain insight into the E6-mediated degradation of SUMO-hADA3, the decay rate of EGFP-hADA3 was examined in SiHa cells overexpressing shUb9. As expected, depletion of Ub9 in SiHa cells led to extension of hADA3 half-life from approximately 5.5 to 7.5 h as shown in Figure 4E and quantitated in Figure 4F. A statistical analysis showed that there is a statistically significant difference between the half-lives of EGFP-ADA3 in control and Ub9 depleted cells (P=0.0082). These results support the hypothesis that HPV16E6 targets hADA3 for ubiquitin-mediated degradation by enhancing it’s SUMOylation.

**E6-mediated destruction of hADA3 contributes to malignant progression of HPV transformed cervical cancer cells**

To investigate the functional consequences of E6-mediated destabilization of hADA3 in HPV positive cervical cancer cell lines, hADA3 depleted SiHa cells were generated (Figure 5A) and the cell transformation properties were examined. First, a soft agar assay was performed to test the ability of these cells to grow in an anchorage independent manner. Results (Figure 5B and C) revealed that cells deficient in hADA3 formed increased number of colonies (by 1.4-fold) compared to the control cells. This signifies that hADA3 suppresses the transformation ability of cells. Next, in vitro wound closure assay was used to assess the migration potential of these cells. The results showed that the cells expressing shADA3 exhibited increased migratory property leading to faster closure of the wounded area (Figure 5D). Depletion of hADA3 enhanced migration of SiHa cells by two fold compared to the control and it took less time to cover the wound in comparison to the control cells. These findings clearly indicated that level of hADA3 is crucial for growth and migration of cervical cancer cells. To avoid SUMOylation-mediated destabilization of hADA3, we designed another experiment wherein hADA3 is overexpressed in Ubc9 depleted SiHa cells so as to ensure sustained overexpression of hADA3. These cells were then probed for their transformation and migration abilities. There was a two fold reduction in the colony formation in shUb9 cells overexpressing EGFP-hADA3, as revealed by a soft agar assay (Figure 5F and G). Furthermore, the wound closure rate of these cells overexpressing hADA3 was also delayed (by 2.4-fold at 48 h) compared to the control cells as shown in Figure 5H. These observations suggest that HPV16E6 targets SUMO-hADA3 thereby reducing the levels of hADA3 in cervical cancer cells with significant impact on their proliferative potential. This effect is reversed by restoration of hADA3 levels. On the basis of these findings, we conclude that hADA3 overexpression suppresses the transformation phenotype and thus may reduce the tumorigenicity of cervical cancer cells.
Discussion

Several studies have identified cellular proteins that interact with HPV16E6 (5–7,9–11,28–30), one among which is hADA3, a core component of HAT complex (13). While previous reports have established that HPV16E6 inactivates hADA3 function (13,18,19), the molecular details of such inactivation still remains unclear. The present investigation attempts to outline the underlying mechanism. The first clue came from our initial observation of the steady state levels of endogenous hADA3 in cervical cancer cells; levels of the transcriptional co-activator negatively correlated with HPV status (Figure 1A). This is consistent with the earlier observation...
that ectopically expressed HPV16E6 reduces the turnover of overexpressed hADA3 protein (13) supporting the hypothesis that E6 targets hADA3 for degradation. We further extended this investigation by demonstrating that this process of degradation is proteasome dependent since proteasome inhibition resulted in rapid accumulation of hADA3 in E6 expressing cervical cancer cells. This might explain the reason for weak detection of ubiquitinated hADA3 bands in the absence of proteasome inhibitor.

Fig. 4. E6 accelerates hADA3 degradation via SUMOylation. (A) E6 enhances hADA3 SUMOylation. C33A cells were transfected with Flag-hADA3 in absence and presence of Myc-E6. After 5 h incubation with MG132, cell lysates were prepared and immunoprecipitation was performed using anti-Flag antibody. Immunocomplexes were analyzed for SUMO conjugates by Western blotting with anti-SUMO 1 and 2 antibodies. (B) Overexpression of Flag-SENP1 downregulates the ubiquitination of hADA3. HEK293T cells were transfected with the indicated plasmids. After MG132 treatment, cell lysates were prepared. Transfected hADA3 was immunoprecipitated with anti-Flag antibody followed by immunoblotting with anti-Flag antibody. (C and D) Rescue of hADA3 by overexpression of Myc-Ubc9DN. (C) Flag-hADA3 was overexpressed in HEK293T cells in presence of Myc-E6 with or without Myc-Ubc9DN. Equal amount of cell lysates were subjected to Western blotting with the indicated antibodies. Similarly, (D) C33A cells were transfected with either E6 alone or in combination with Myc-Ubc9DN and the whole cell lysates were immunoblotted with the indicated antibodies. (E) Half-life of EGFP-hADA3 in Ubc9 depleted cells. SiHa cells with stable expression of control vector or shUbc9 construct were overexpressed with EGFP-hADA3. Decay of hADA3 protein was followed as described in materials and method section. Immunoblot was analyzed with antibodies against EGFP, Ubc9 and β-Actin. (F) The intensity of EGFP-hADA3 bands were quantified using densitometric analysis and a graph was plotted between hADA3 and time of CHX addition. Solid line indicates the relative level of EGFP-hADA3 in control cells whereas dotted line represents EGFP-hADA3 levels in Ubc9 depleted cells. Results are plotted as standard error of mean with P value < 0.01 between the two groups. All the results are shown from one representative of at least three independent experiments.
Fig. 5. hADA3 suppresses the transformation phenotypes of cervical cancer cells. Depletion of hADA3 expression stimulates cell proliferation, migration and anchorage independent growth in cervical cancer cells. (A) Western blot analysis of SiHa stable cells expressing control vector or shADA3 with anti-hADA3 antibody. (B) Soft agar assay was performed with stable SiHa cells expressing pSUPER retro puro vector and shADA3 construct. Cells were suspended in 0.4% agarose and plated on 0.8% bottom agar layer. The appearance of colonies was analyzed after 20 days. Experiments were replicated thrice and representative picture is shown. (C) Colonies were manually counted after staining with crystal violet. The number of colonies in the plot represents the mean number from three plates. Results are given as standard error of mean (**P < 0.05). (D) A scratch assay was performed as indicated in experimental procedures and migration was recorded at 0, 24, 48 and 72h. Results shown are representative of triplicate experiments. (E) Western blot analysis of SiHa stable cells expressing control vector or shUbce9 with anti-Ubc9 antibody. (F) Soft agar assay was performed with the indicated SiHa stable cells as described above. Colonies were stained using crystal violet at the end of 20 days. Experiments were replicated thrice and representative picture is shown. (G) The plot shows the quantification (mean colony scores obtained from three plates) of the above experiment. Results are given as standard error of mean (*P < 0.05). (H) A wound healing assay was performed with SiHa cells expressing shUbc9 with EGFP alone or shUbc9 with EGFP-hADA3 as indicated in the figure according to experimental procedures. Migration was recorded at 0, 24 and 48h. Results shown are representative of triplicate experiments.
E6AP is known to participate in degradation of several E6 targets (5,28–31,39). A recent finding also implicated its involvement in downregulation of hADA3 (31). Our study shows that E6AP associates with hADA3 and promotes its degradation. Though their physical interaction was found to be independent of E6, presence of E6 accelerated hADA3 turnover. Moreover, our unpublished laboratory observations suggest that hADA3 levels are normally regulated by E6AP (data not shown). Hence, our investigation identified a novel mechanism of cellular regulation of hADA3. However, significance of this observation under physiological conditions still remains to be elucidated.

The present study provides the first evidence that the coactivator hADA3 is posttranslationally modified and regulated by SUMOylation. We also show that components of SUMO pathway i.e. Ube9 and PIAS1 are novel interactors of hADA3. Our results indicate that hADA3 SUMOylation results in protein destabilization, suggesting that SUMO modification can affect hADA3 half-life perhaps by augmenting the ubiquitination process. We speculate that attachment of SUMO moiety brings in topological changes in the hADA3 protein. As a result, some of the hidden ubiquitination sites may get exposed thereby making the protein susceptible to degradation. Additionally, Ube9 knockdown experiments, where we showed that disruption of SUMOylation machinery stabilizes hADA3 (Figure 4F), reinforced our argument in favor of a role of SUMOylation in hADA3 degradation. Thus, the discovery of hADA3 SUMOylation revealed an important aspect of gene regulation at the posttranscriptional level and added a new dimension to our understanding of hADA3 stability. These findings open up exciting perspectives in the study of this new posttranslational modifications of hADA3 as well (40). Our results showed that ablation of SUMOylation machinery by overexpression of Ubc9DN resulted in accumulation of hADA3 even in absence of MG132. This finding suggested that SUMOylation may be important for subsequent ubiquitination and degradation of hADA3 in cervical cancer cell lines. Furthermore, accumulation of SUMOylated hADA3 in the presence of E6APDN also indicated that SUMOylation directs ubiquitination. Taken together, these results suggest that HPV16E6 targets SUMOylated hADA3 for E6AP-mediated ubiquitination and proteasome degradation. We also found that ectopic expression of Ubc9DN markedly suppresses HPV16E6 dependent degradation of hADA3. This shows that disruption of cellular SUMOylation machinery blocks ubiquitination of hADA3. Therefore, our experimental evidences strongly favor the idea that HPV16E6 induces SUMO-dependent ubiquitination and degradation of hADA3.

Current study also implies a multitude of intriguing possibilities regarding SUMOylation of hADA3. For instance, SUMOylation can alter hADA3 activity by tinkering its association with other proteins like HATs or HDACs, transcription factors or modulate its localization thereby influencing its function as a coactivator. Cancer associated properties of hADA3 have been highlighted by several recent reports. However, contribution of hADA3 in development and progression of cancer is not well understood. Thus, SUMOylation-mediated regulation of hADA3 may have important consequences not only in the context of normal cellular physiology but also in malignancy and disease. While the effects of SUMOylation on hADA3 function are yet to be identified, it is likely that enhanced SUMOylation of hADA3 by HPV16E6, apart from being critical for viral oncogenic function, may also facilitate viral survival and reproduction (41).

The molecular events underlying hADA3 controlled transforming activity of HPV 16E6 in cervical cancer cells have been highlighted in this study. Results indicated that loss of hADA3 contributes to the malignant phenotype of cervical cancer cells. However, when expression of hADA3 was restored in cervical cancer cells (Figure 5G–H), it was found to negatively regulate cell proliferation, migration and anchorage independent growth, implicating a potential role of hADA3 in the inhibition of tumorigenesis. This may reflect a unique feature of hADA3 protein associated with HPV pathogenesis. Since loss of hADA3 has also been shown to inhibit cell proliferation in earlier studies (17,23), the results may suggest that levels of hADA3 is critical for normal cell growth and both up and down regulation of hADA3 may perturb cellular proliferation.

Thus, our findings provide important insights into the hADA3 biology and its potential contribution to the tumorigenic process. HPV16E6-mediated enhanced SUMOylation appears to represent another mechanism by which hADA3 is deregulated. A better understanding in this regard may aid in devising highly selective therapeutic strategies for growth suppression of HPV-mediated cancers.

Funding
Department of Biotechnology (DBT), Department of Science and Technology, R&D grants from University of Delhi and UGC (under SAP Programme), Government of India, to A.N. Fellowships were provided by Council of Scientific and Industrial Research to V.C. and DBT to R.J. and N.J.

Acknowledgements
We thank Dr Vimla Band, UNMC, Nebraska, USA, for valuable discussion and suggestions. We also thank Dr Suman Kundra, Dept. of Biochemistry, UDSC, India, for critical reading of our manuscript and constructive comments.

Conflict of Interest Statement: None declared.

References
34. Everett, R. D. et al. (2006) PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. J. Virol., 80, 7995–8005.

Received February 18, 2014; revised April 9, 2014; accepted April 27, 2014