The Human papillomavirus type 16 E7 oncoprotein induces a transcriptional repressor complex on the Toll-like receptor 9 promoter

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Human papillomavirus type 16 (HPV16) and other oncogenic viruses have been reported to deregulate immunity by suppressing the function of the double-stranded DNA innate sensor TLR9. However, the mechanisms leading to these events remain to be elucidated. We show that infection of human epithelial cells with HPV16 promotes the formation of an inhibitory transcriptional complex containing NF-κBp50–p65 and ERα induced by the E7 oncoprotein. The E7-mediated transcriptional complex also recruited the histone demethylase JARID1B and histone deacetylase HDAC1. The entire complex bound to a specific region on the TLR9 promoter, which resulted in decreased methylation and acetylation of histones upstream of the TLR9 transcriptional start site. The involvement of NF-κB and ERα in the TLR9 down-regulation by HPV16 E7 was fully confirmed in cervical tissues from human patients. Importantly, we present evidence that the HPV16-induced TLR9 down-regulation affects the interferon response which negatively regulates viral infection. Our studies highlight a novel HPV16-mediated mechanism that combines epigenetic and transcriptional events to suppress a key innate immune sensor.

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Abbreviations used: ChIP, chromatin immunoprecipation; EBV, Epstein-Barr virus; EMSA, electromobility shift assay; ERE, estrogen response element; HBV, hepatitis B virus; HCV, hepatitis C virus; HDAC, histone deacetylase; HPV, human papillomavirus; pDC, plasmacytoid DC; PRR, pathogen recognition receptor; RIG-I, retinoic acid-inducible gene I; TSA, Trichostatin A.

Approximately 26% of all human cancers worldwide are associated with infectious agents, among which 80% are viruses (Bouvard et al., 2009). Hepatitis C virus (HCV), hepatitis B virus (HBV), Human T-lymphotropic virus type I (HTLV-1), Epstein-Barr virus (EBV), Kaposi sarcoma-associated virus (KSHV), and the mucosal high-risk (HR) human papillomavirus (HPV) types have been clearly implicated in different types of human cancers. In particular, HPV is the etiological factor of cervical cancer and is responsible for ∼20% of all human cancers linked to infection. More than 500,000 new cervical cancer cases and 275,000 deaths are reported each year worldwide (Tay, 2012). In addition, HR HPV types are responsible for a proportion of oropharyngeal cancers (Ryerson et al., 2008; Marur et al., 2010) that appear to have steadily increased in the last two decades in the USA and Europe (Ryerson et al., 2008; Marur et al., 2010). Similarly to several oncogenic viruses, HR HPV types are able to alter the immune surveillance and cellular homeostasis, mainly deregulating the cellular gene expression and promoting epigenetic changes (Kim et al., 2003; Zhang et al., 2004; Rincon-Orozco et al., 2009; Moore and Chang, 2010; Nelson, 2011). The initial outcome is the

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increase of cellular proliferation that favors viral replication and persistence. This event is mainly mediated by the HR HPV oncoproteins E6 and E7, which have the properties to target many cellular proteins. In particular, E6 and E7 have the properties to bind and degrade the tumor suppressor gene products p53 and pRb, respectively. As a consequence, the transcription of several cell cycle check point tumor suppressors, such as p21, FAS, DEK, and B-MyB, is prevented (Tommasino et al., 2003; Malanchi et al., 2004; Rampias et al., 2009). In addition, the HR HPV types are able to deregulate several immune-related pathways to guarantee the persistence of the infection.

Innate immunity is the first line of host defense against infections. The cellular innate immune response is mediated by both hematopoietic and nonhematopoietic cells that express pathogen recognition receptors (PRRs). Viral nucleic acids are sensed by different PRR families, which include: cytosolic retinoic acid–inducible gene I (RIG-I)–like helicases for viral RNA; cytosolic DNA sensors such as DNA-dependent activation of IFN (DAI), Absent in Melanoma 2 (AIM2), and others; and several members of the TLR family (Coban et al., 2005; Adams et al., 2008; Fernandes-Alnemri et al., 2009; Gondois-Rey et al., 2009; Rathinam et al., 2010). TLR9 was one of the first innate immune receptors to be identified (Hemmi et al., 2000). Historically, in humans TLR9 was reported to be expressed in plasmacytoid DCs (pDCs) and B cells. However, recent studies have shown that it is also present in nonimmune cells such as endothelial and epithelial cells (Lebre et al., 2007; Pivarcsi et al., 2007; Morizane et al., 2012). Upon recognition of unmethylated double-stranded DNA CpG motifs present in the genome of viruses such as EBV, HSV, and HPV (Lund et al., 2003; Hasan et al., 2007a; Fiola et al., 2010; Zauner et al., 2010), TLR9 initiates a signaling cascade that leads to the production of type I IFN and proinflammatory cytokines (Sepulveda et al., 2009; Sasai et al., 2010; Avalos and Ploegh, 2011; Ewald et al., 2011), the release of which activates host immune defenses against infection.

Despite the efficiency of innate immune response, HR HPV types have developed strategies to persist in the host. Indeed, HR HPV16 and 18 are able to inhibit the transcription of proinflammatory chemokines and cytokines, such as CCL5 and IL-1β (Karim et al., 2011). In addition, the IFN inducible antiviral genes IFIT1 and MX1, proapoptotic genes (TRAIL and XAF1), and PRRs (TLR3, RIG-I, and MDA5) are also inhibited by HPV16, 18, and 31 (Reiser et al., 2011). We and others have observed that HPV16, EBV, and HBV impair the expression and function of the innate immune receptor TLR9 (Hasan et al., 2007a; Fathallah et al., 2010; Hirsch et al., 2010; Vincent et al., 2011). TLR9 expression is severely suppressed in the cervical epithelium of women with HPV16-positive cervical lesions compared with that of women that are healthy or with regressing infection (Hasan et al., 2007a; Daud et al., 2011). The viral oncoproteins E6 and E7 have been linked to the HPV16-mediated TLR9 transcriptional down-regulation (Hasan et al., 2007a). However, the mechanisms involved in this event remain to be elucidated.

Here, we have elucidated the mechanism of HR HPV16 E7 mediated down-regulation of TLR9. This viral oncoprotein induces the formation of transcriptional inhibitory complexes, including NF-κBp50–p65, ERα, and chromatin modifying enzymes, at the TLR9 promoter and induce epigenetic changes. Thus, HPV16 employs a unique mechanism to turn down TLR9 transcription, expression, and function, which is an essential event required for oncoviral mediated carcinogenesis.

RESULTS

Infection of human epithelial cells with HPV16 down-regulates TLR9 expression and function in an E6/E7-dependent manner

To understand how TLR9 expression is regulated by HPV16 we generated quasi-virions (16QsV) that closely resemble the natural virus (Flores et al., 1999; Pyeon et al., 2005). Infection of C33A cells (an HPV-negative cervical epithelial cell line) with 16QsV for 8 h led to reduced TLR9 mRNA levels with a further decrease observed at 24 h (Fig. 1 A). The effect seen at 24 h after infection was dependent on viral genome replication, as UV-treated 16QsV did not suppress TLR9 levels (Fig. 1 A, right). In contrast, CpG 2006 and HSV-2, which strongly activate the TLR9–NF-κB axis, transiently suppressed TLR9, with restoration of mRNA and protein levels 24 h after stimulation (Fig. 1 A). Addition of TNF, pseudo-virions (late proteins L1 and L2 encapsidated GFP expression plasmid, abbreviated as PV), and GpC controls did not down-regulate TLR9 levels (Fig. 1 A). Thus, TLR9 expression was severely suppressed by infection with 16QsV. 16QsV infection and replication was controlled by qPCR using specific primers for the early genes E1 and E7 (Fig. 1 B, left and middle). The number of added 16QsV viral genome equivalents inversely correlated with TLR9 mRNA levels (Fig. 1 B, right). We previously reported that TLR9 down-regulation was associated with E6 and E7 expression in primary keratinocytes as well as in cervical cancer–derived cell lines (Hasan et al., 2007a). Accordingly, C33A cells infected with 16QsV for 24 h in the presence of a siRNA against HPV16E6E7 restored TLR9 mRNA levels and promoter activity (Fig. 1, C and D). We next investigated the biological consequence of TLR9 suppression by HPV16. Human keratinocytes are strong producers of proinflammatory cytokines such as IL-6, IL-8, and MIP3α (Debenedictis et al., 2001; Hudak et al., 2002; Ito et al., 2003; Metz et al., 2008; Bangert et al., 2011; Kaplan et al., 2012). We have previously shown that HPV16 E6E7 prevented secretion of MIP3α and IL-8 when cells were stimulated as a result of the loss of TLR9 expression (Hasan et al., 2007a). We next evaluated whether infection with 16QsV also suppressed TLR9 functional signaling. C33A cells were infected with 16QsV for 36 h, washed, and stimulated with a CpG motif from HPV16 genome (Hasan et al., 2007a) or CpG 2006. We observed that 16QsV infection hindered TLR9 function, as CpG 2006 and CpG motifs from HPV16 did not lead to the secretion of IL-8, IL-6, and MIP3α (unpublished data). TLR9 is also a strong inducer of type I IFN, the release of which activates host immune defenses against viral spread

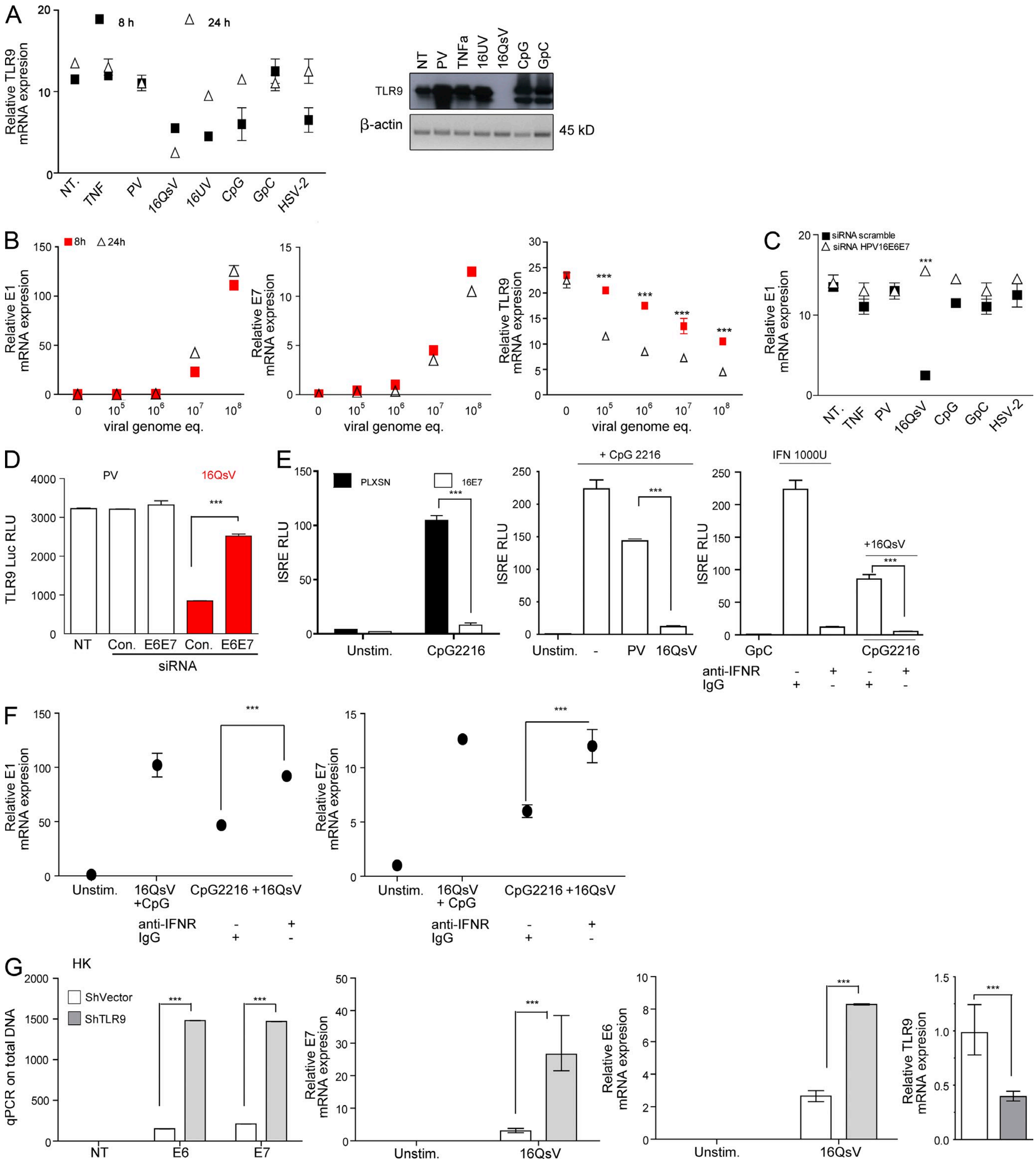

Figure 1. TLR9 expression is suppressed 24 h after infection by native 16QsV. (A, left) C33A cells were not treated (NT) or treated for 8 or 24 h with TNF, PV, 16QsV (at 10^7 viral concentrations genome equivalents), 16UV (rendered replication incompetent using UV), CpG 2006, GpC 2006, or infected with HSV-2. TLR9 mRNA levels were determined by qPCR. Shown are data from four independent experiments performed in triplicate. Error bars indicate SEM. (A, right) TLR9 protein was examined by immunoblotting in C33A cells. Cells were harvested after 24 h treatment with PV, TNF, 16UV, 16QsV CpG 2006, and GpC control. (B) C33A cells were treated with increasing viral concentrations genome equivalents (v.g.e.; as measured by qPCR on the viral DNA of infected cells) of 16QsV for 8 or 24 h). E1, E7 (left and middle), and TLR9 mRNA levels (right) were determined for their relative expression by qPCR. Shown are data from five independent experiments performed in duplicate with ***, P < 0.0001, based on an unpaired Student's *t* test. (C) C33A cells were infected as indicated for 24 h. siRNA against HPV16E6E7 was transfected 24 h after stimulation and TLR9 mRNA levels were determined by

(Sepulveda et al., 2009; Sasai et al., 2010; Avalos and Ploegh, 2011; Ewald et al., 2011). We tested the ability of HK transduced with HPV16E6E7 or with empty vector (PLXSN) to produce type I IFN upon TLR9 stimulation with CpG 2216. HPV16E6E7 expression severely impaired the ability of TLR9 to produce type I IFN compared with cells transduced with the vector alone (Fig. 1 E, left). The same block in IFN production was observed in C33A cells infected with 16QsV before CpG stimulation (Fig. 1 E, middle) and correlated to the loss TLR9 mRNA levels. No effect on IFN production was observed when PV was used as a control Fig. 1 E, middle). Addition of CpG 2216 24 h before 16QsV infection permitted type I IFN activation of the ISRE minimal promoter that was abrogated in the presence of an antibody against the type I IFN receptor (anti-IFNR; Fig. 1 E, right). Most importantly, prestimulation of TLR9 with CpG 2216 before 16QsV infection significantly decreased the expression of the viral early genes which was blocked in the presence of anti-IFNR (Fig. 1 F). To demonstrate that this event was linked to TLR9 down-regulation and not to the alteration of IFN signaling (Ronco et al., 1998), we tested whether 16QsV blocks the type I IFN production signaling pathway of RIG-I. Indeed, ectopic levels of RIG-I–stimulated cells infected with 16QsV did not affect type I IFN bioactivity (unpublished data). To gain more insights on the biological significance of HPV-induced TLR9 down-regulation, we silenced its expression in HK by using a short hairpin RNA (Fig. 1 G, left). Subsequently, these cells were infected with 16QsV and viral load was determined. Cells expressing TLR9 shRNA had a higher copy number of HPV16 genome in comparison with mock cells (Fig. 1 G, middle). Accordingly, HPV16 viral transcription was increased in cells with reduced TLR9 expression (Fig. 1 G, right). Collectively, these data show that infection with 16QsV of human epithelial cells inhibited TLR9 expression and signaling in an E6- and/or E7-dependent manner and that TLR9 plays an essential role in limiting HPV16 life cycle.

HPV16 down-regulation is dependent on NF-κB signaling

NF-κB signaling was shown to regulate TLR9 (Takeshita et al., 2004) and we reported that deletion of putative NF-κB sites in the TLR9 promoter restored its transcriptional activity in the presence of HPV16 E6 and E7 (Hasan et al., 2007a). We next determined whether TLR9 down-regulation induced by 16QsV is mediated by the NF-κB pathway. C33A were transiently transfected with the TLR9 promoter/luciferase reporter gene and treated with siRNA for IKKα or IKKβ (Fig. 2 A, right), two cytoplasmic kinases that promote the nuclear translocation of active NF-κB transcriptional factor (Häcker and Karin, 2006). Cells were then exposed for 24 h to 16QsV or TNF. In the presence of IKKα or IKKβ siRNA, TLR9 promoter activity and mRNA levels were rescued compared with cells treated with scramble siRNA (Fig. 2 A). Interestingly, TNF, a known activator of the NF-κB pathway, was unable to inhibit TLR9 transcription (Fig. 2 A). Ectopic expression of a dominant-negative MyD88 mutant did not restore TLR9 transcription or protein levels in cells infected with native 16QsV, indicating that a MyD88–NF-κB pathway was not involved in this phenomenon (unpublished data). In contrast, the suppression of TLR9 expression by UV-treated 16QsV or HSV2, which both contain CpG elements (Hasan et al., 2007a), was prevented in the presence of a dominant-negative MyD88 mutant.

A 1-h treatment with a chemical inhibitor of IKKβ (Bay 11) also restored TLR9 mRNA and protein levels in all cervical cancer–derived cell lines (Fig. 2 B). TLR9 expression upon Bay 11 treatment correlated with loss of NF-κBp65 nuclear localization (Fig. 2 C). In addition, gene silencing of IKKα, IKKβ, or NF-κBp65 in SiHa cells by siRNA resulted in the recovery of TLR9 expression, as measured by luciferase activity or by the endogenous TLR9 levels (unpublished data). Thus, TLR9 transcriptional inhibition depends on the activation of NF-κB signaling after infection with 16QsV.

We next characterized which HPV16 oncoprotein was responsible for NF-κB-dependent-TLR9 down-regulation. Human primary keratinocytes (HK) were transduced with E6 and/or E7 and the pLXSN vector control. Immunoblotting showed that several positive regulators of the canonical NF-κB signaling, i.e., IKKβ, p50, and p65, were activated by E7, and to a lesser extent by E6. Stimulation of the canonical NF-κB pathway leads to IKK complex activation, which

qPCR. Shown are data from four independent experiments performed in triplicate and error bars indicate SEM with ***, P < 0.0001, based on an unpaired Student's *t* test. (D) C33A cells were transfected with the TLR9 luciferase promoter and, 24 h later, treated with 16 or control PV. siRNA against HPV16E6E7 was transfected 24 h after infection and TLR9 luciferase levels were measured 18 h later. Shown are data from four independent experiments performed in triplicate, and error bars indicate SEM with ***, P < 0.0001, based on an unpaired Student's *t* test. (E, left) HK transduced with the pLXSN vector control or HPV16E7 were stimulated for 24 h with TLR9 agonist CpG ODN 2216 (type A IFN inducer) and the IFN bioassay was performed. (E, middle) C33A cells were unstimulated or infected with 16QsV or PV for 36 h, and cells were washed and then treated with CpG 2216 for 24 h. Supernatants were collected and the type I IFN bioassay was performed. (E, right) C33A cells were stimulated with GpC or CpG 2216 for 24 h and then infected with 16QsV for 36 h. Supernatants were harvested and treated with anti-IFNR or IgG control and the type I IFN bioassay was performed as described. (F) C33A cells were unstimulated and infected with 16QsV for 36 h + CpG 2216 stimulation for 24 h or vice versa ± anti-IFNR or IgG control. Cells were harvested for RNA extraction and qRT-PCR was performed for E1 (left) or E7 (right) relative gene expression. Shown are data from four independent experiments performed in triplicate, and error bars indicate SEM with ***, P < 0.0001, based on an unpaired Student's *t* test. (G) HK cells were transfected for a total of 48 h with shTLR9 and shControl (scramble sequence). 20 h after transfection, HK cells were infected for 24 h with 16QsV at a viral load of 10^7 v.g.e. Viral load was measured by qPCR using E6- and E7-specific primers. HPV16 E6 and E7 transcripts were also measured by qPCR and TLR9 transcripts were assessed by qPCR after 24 h transfection with shRNA. Data are representative of six independent experiments performed in triplicate. Shown are the mean ± SEM with ***, P < 0.0001, based on an unpaired Student's *t* test.

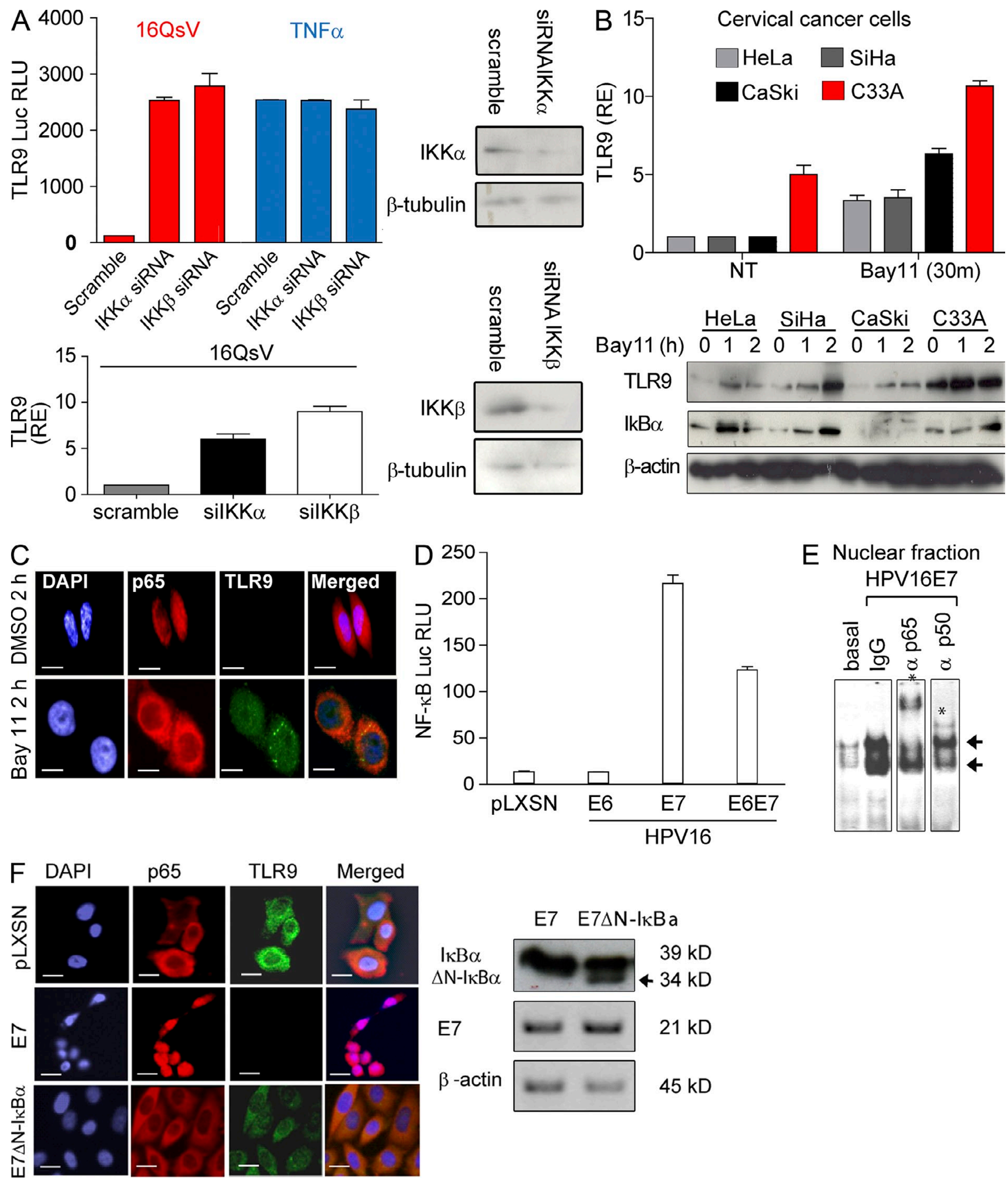

Figure 2. HPV16E7 activates the NF-κB pathway that leads to the suppression of TLR9. (A, top) C33A cells were treated with siRNA for IKKα or β for 16 h, and then transfected with TLR9 luciferase promoter. After 24 h, cells were exposed to the indicated treatments and harvested 24 h later to measure activity (left). IKKα or β levels as determined by immunoblotting (right) were analyzed. (A, bottom) C33A cells were treated with siRNA for IKKα or β for 24 h and TLR9 mRNA levels were measured by qPCR. Shown are data from six independent experiments performed in triplicate and error bars indicate SEM. (B) SiHa (HPV16+), HeLa (HPV18+), CaSki (HPV16+), and C33A control cells were treated with the IKKβ inhibitor Bay11. At the indicated times, mRNA and protein expression of TLR9 as well as protein levels of IκBα was determined by immunoblotting. Shown are data from seven independent experiments performed in triplicate and error bars indicate SEM. RE, mRNA relative expression. (C) SiHa cells were treated with Bay11 for the indicated time and immunofluorescence was performed to determine NF-κBp65 cellular localization (red) and TLR9 expression (green). Nuclear staining was controlled using DAPI. Shown are data from one out of four examined fields and one out of three independent experiments. Bars, 10 µm. (D) Human female primary keratinocytes (HK) were transduced with the indicated retroviruses and transfected with the NF-κB reporter gene construct. At 48 h after

subsequently phosphorylates and induces the ubiquitination/degradation of IκBα promoting the nuclear translocation of NF-κB. We noted that HPV16E7, but not E6, induced the phosphorylation and degradation of IκBα (unpublished data). To further corroborate the role of E7 on TLR9 suppression in model of natural HPV16 infection, SiHa cells were treated with a siRNA for HPV16E7 for 48 h. We observed an increase in TLR9 transcripts when E7 levels were suppressed. In addition, C33A cells infected with 16QsV gained the ability to produce IL-8 via TLR9 stimulation only when E7 expression was inhibited by a siRNA (unpublished data). We then transiently expressed the NF-κB minimal promoter linked to luciferase. 24 h after transfection, cells were lysed and luminescence was measured. We observed that the oncoprotein E7, but not E6, was able to induce NF-κB activity (Fig. 2 D). Accordingly, HPV16 E7-transduced primary keratinocytes displayed NF-κB binding to a consensus cis element as shown by electromobility shift assay (EMSA; Fig. 2 E). Antibodies against p65 and p50 (Fig. 2 E), but not the NF-κB family members RelB, c-rel, or p52 (not depicted), induced a supershift.

We inhibited the NF-κB canonical pathway by expressing a nondegradable deletion mutant of IκBα (ΔN-IκBα) that lacks the first 36 amino acids at the N terminus containing the IKK-phosphorylated amino acid. ΔN-IκBα expression in HPV16 E7 HK led to cytoplasmic sequestration of NF-κBp65 and restoration of TLR9 levels without affecting the expression of the viral genes (Fig. 2 F). Thus, E7 down-regulates TLR9 via the activation of the canonical IκBα release of NF-κBp65.

HPV16E7 recruits an inhibitory NF-κBp50–p65 complex to a novel cis element on the TLR9 promoter

The observation that NF-κB is required for TLR9 suppression by HPV16E7 prompted us to determine which sites of the TLR9 promoter are involved in this HPV16-induced event. Takeshita et al. (2004) previously described an NF-κB site that is important for the regulation of TLR9 upon its engagement by CpG 2006 (hereafter referred to as Site D). Using genomatix and tescan programs, we identified three additional NF-κB cis elements in the TLR9 promoter that we termed A, B, and C (Fig. 3 A). To determine which site was involved in TLR9 down-regulation by HPV16E7, the TLR9 promoter luciferase construct was mutated individually for the sites A, B, C, and D and then transiently expressed into primary human keratinocytes transduced with HPV16E7 or the empty vector (pLXSN). We observed that mutation of site B, and not A, C, or D, restored TLR9 promoter activity in the presence of HPV16E7 (Fig. 3 B). Similar results were obtained using HK from 10 different donors transduced with HPV16 E7 recombinant retrovirus and subsequently transfected with wild-type or mutant TLR9 promoter cloned in front of the luciferase reporter (unpublished data). We next determined which NF-κB family members bound to the site B region on the TLR9 promoter in the presence of HPV16E7. Chromatin immunoprecipitation (ChIP) for NF-κBp65, p50, RELB, c-rel, and p52 was performed in HK transduced with HPV16E7 or stimulated with TNF or CpG 2006 (for 4 and 24 h). We observed that both NF-κBp65 and p50 forms were recruited to site B (Fig. 3 C) and not RelB, p52, or c-rel (not depicted). In addition, reChIP assays revealed that NF-κBp50 and p65 bound as heterodimers on site B, whereas no NF-κB complexes were found associated with sites A, C, or D (Fig. 3 C). In untreated cells an NF-κBp50 and p65 complex was constitutively recruited to site D where it most likely activates TLR9 transcription because high levels of TLR9 are detected in these cells (unpublished data). We corroborated our findings by infecting human epithelial cells with 16QsV, which also induced the suppressive NF-κBp50–p65 complex to site B on the TLR9 promoter (Fig. 3 D). In addition, HPV16E7 gene silencing by siRNA blocked NF-κBp50–p65 binding to site B (Fig. 3 D). No NF-κBp50–p65 recruitment at site B was observed in cells treated with TNF (Fig. 3 D).

NF-κBp50–p65 heterodimers are typically associated with active gene transcription (Häcker and Karin, 2006). The finding that a NF-κBp50–p65 heterodimer acts as a gene suppressor in HPV16E7-infected cells led us to examine whether HPV16E7 was recruited to site B along with NF-κB. To test this hypothesis, we transduced HK with HPV16E7HA or an empty vector (pBABE-HA) and performed ChIP analysis using anti-HA antibody. No HPV16E7-HA recruitment was observed, whereas NF-κBp50 and p65 were still found located at site B (Fig. 3 E). Together, these data show that HPV16E7 induced the recruitment of an inhibitory NF-κBp50–p65 complex at site B of TLR9 promoter without being recruited itself to that site.

ERα cooperates with NF-κB complex to suppress TLR9

As our ChIP amplification for site B is comprised of the 200 bp region around the cis element, we next evaluated whether the HPV16E7-induced inhibition of TLR9 transcription was solely mediated on a NF-κB cis element found on site B. We generated an artificial promoter by cloning the site B cis element in front to a minimal promoter (pTAL) linked to the luciferase

transfection, luciferase activity was determined. Data are from one assay representative of seven independent experiments; shown are mean ± SEM from triplicate values in three. (E) EMSA was performed on HPV16E7-transduced HK using the NF-κB EMSA kit (Panomics) according to the manufacturer's instructions. For the supershift analyses, nuclear extracts were incubated with NF-κBp50 or p65 or IgG control. The arrow indicates NF-κB complexes, and the asterisk indicates a supershift. Shown are data from one out of four independent experiments that gave identical results. (F, left) HKs were retrovirally transduced with HPV16E7, empty vector (pLXSN), or HPV16E7ΔN-IκBα and were harvested at the doubling population 7 and stained by immunofluorescence for TLR9 and NF-κBp65. Either Alexa Fluor 488 nm (green) or 594 nm (red) was used as secondary antibodies. Nuclear staining was controlled using DAPI. Shown are data from one out of six examined fields and one out of three independent experiments. Bars, 10 µm. (F, right) Western blot for ΔN-IκBα (marked with an arrow), E7, and β-actin. Shown are data from one out of five independent experiments that gave identical results.

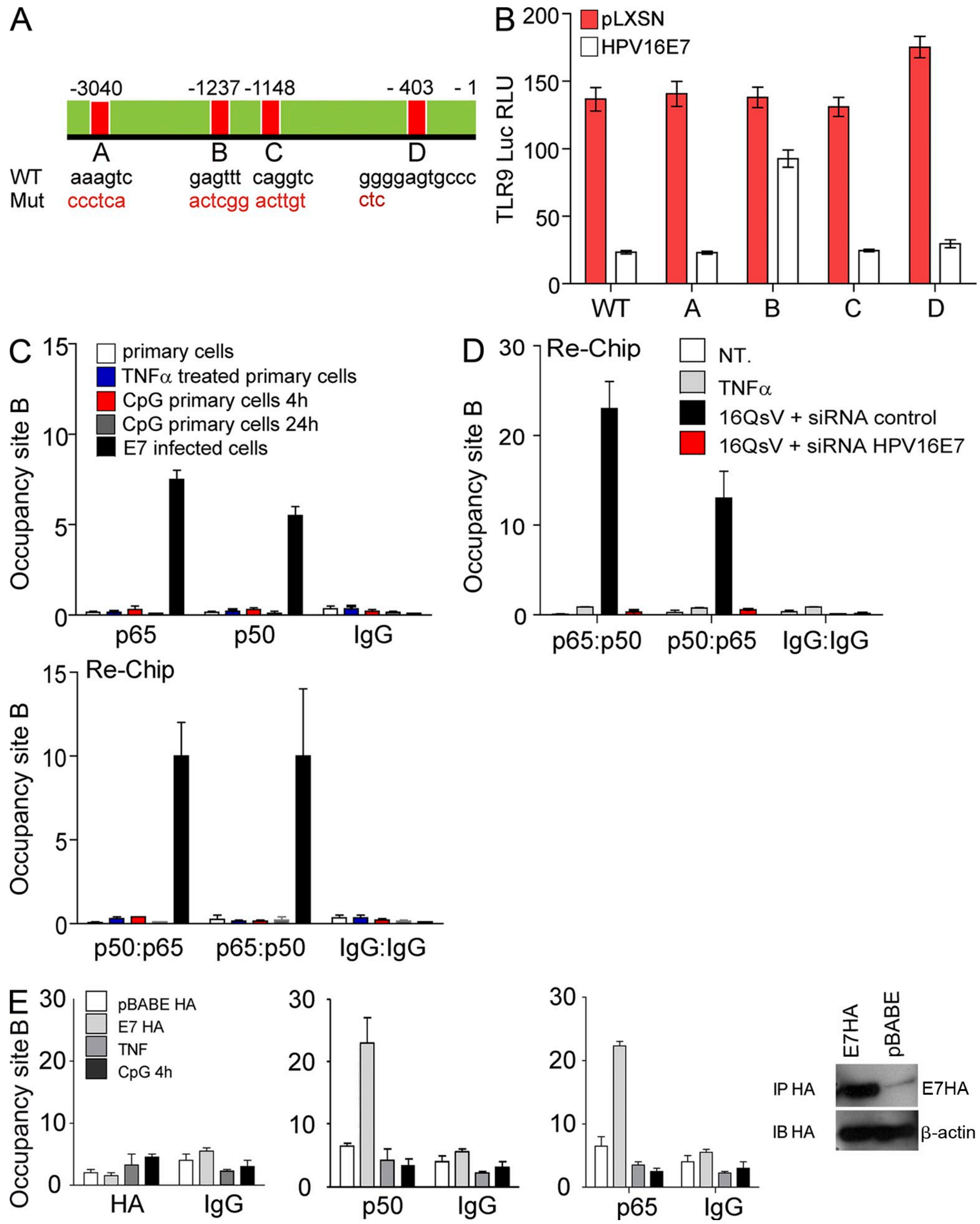

Figure 3. TLR9 promoter regulation by HPV16E7 depends on the locality of the NF-κB complex at a specific cis site. (A) Predicted NF-κB binding sites (A, B, C, and D) in the TLR9 promoter. Sequence mutations were made at site A, B, C, and D to inactivate the binding sites. Top sequences are the native form; bottom sequences indicate the mutations made. (B) HKs were transduced with HPV16E7 or empty vector. Transduced cells were then transfected with TLR9 promoter luciferase expression vectors containing either a wt promoter sequence or sequences with the mutation in the A, B, C, or D site indicated in A. 40 h after transfection, luciferase activity was measured. Shown are data from six independent experiments performed in triplicate and error bars indicate SEM. (C) The binding of NF-κB complexes to the TLR9 promoter in HK was determined by ChIP and ReChIP assays. Sheared chromatin from HK transduced with HPV16E7 or stimulated with TNF or CpG 2006 (4 and 24 h) was immunoprecipitated with antibodies to NF-κBp50 or p65. Site B on the TLR9 promoter was amplified by qPCR to determine the specific binding of transcription factors bound to DNA. Immunoprecipitated DNA and input DNA was amplified with gene-specific and β-globin (Hbb) primers by qPCR, using input DNA to generate a standard curve. ChIP data are represented as % input (gene-specific)/% input(β-globin) = occupancy site B. Shown are data from six independent experiments performed in triplicate and error bars indicate SEM. (D) Re-ChIP analysis was performed for p50–p65 NF-κB complexes using C33A cells first infected with 16QsV for 36 h. Cells were then transfected with siRNA for HPV16E7 and harvested 24 h later. Shown are data from five independent experiments performed in triplicate and error bars indicate SEM. (E) E7 does not bind to the NF-kB cis element site B. ChIP was performed using human epithelial cells transfected with pbabe HA, pbabe HPV16E7-HA, or stimulated with CpG (4 h) or TNF (8 h). ChIP was performed using HA, NF-κBp50 or p65, or an IgG control antibody to examine occupancy of site B. Shown are data from six independent experiments performed in triplicate, and error bars indicate SEM. Shown is an immunoblot to control the ChIP for HA-E7.

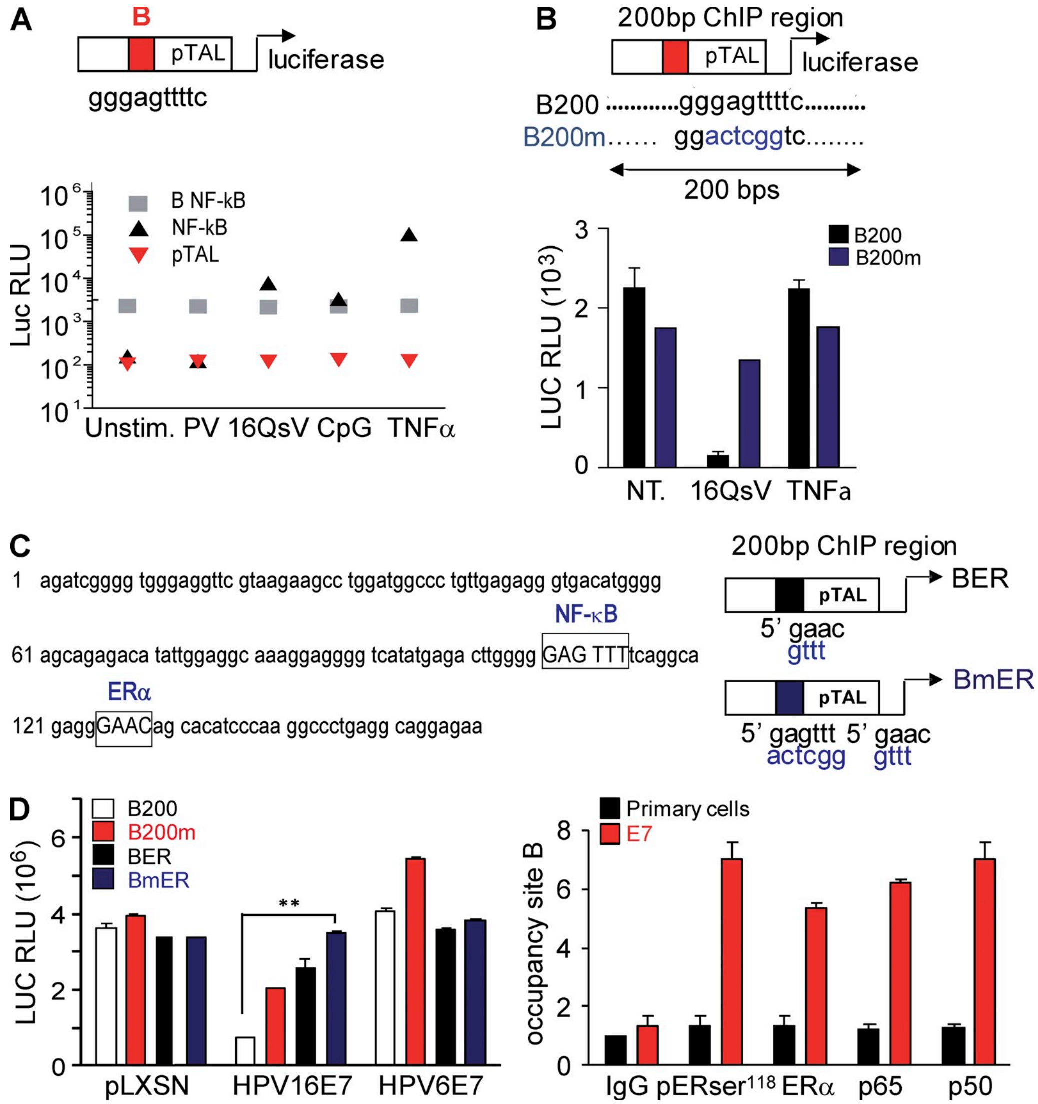

Figure 4. The negative regulatory effect of HPV16 on the TLR9 promoter requires NF-κB and ERα binding to site B flanking sequences in human epithelial cells and HK. (A, top) Diagram illustrates the minimal B site promoter. (A, bottom) Human epithelial cervical cells were transfected with the NF-κB luciferase consensus site, the B minimal promoter or the control vector pTAL. 24 h after transfection 16QsV, control PV (native), CpG 2006, or TNF were added. Cells were harvested and luciferase activity was measured after 24 h. (B, top) Diagram depicting constructs including a 200 bp sequence of the TLR9 promoter containing the NF-κB cis element on site B WT (B200) or mutated (Bm). (B, bottom) C33A cells were transfected with B200 or B200m. 24 h after transfection, 16QsV or TNF were added. Cells were harvested and luciferase activity was measured after 24 h (right). (C, left) The 200 bp sequence around site B on the TLR9 promoter with mapped NF-κB and ERα cis sites. (C, right) Site B was mutated at the cis ERα site (BER) and double mutation for NF-κB and ERα cis elements (BmER). (D, left) HK cells transduced with pLXSN, HPV16E7, or HPV6E7 were transfected with B200, B200m, BER, or BmER. 24 h after transfection luciferase activity was measured. (D, right) ChIP analyses of ERα phosphorylated and total forms and NF-κBp65 or p50 binding to site B on the TLR9 promoter in HK transduced ± HPV16E7. Data are representative of the mean of five or more independent experiments performed in triplicate, and error bars indicate SEM. **, $P < 0.001$ based on an unpaired Student's *t* test.

gene (termed B NF-κB; Fig. 4 A). C33A were transfected with the B NF-κB, control pTAL, or the NF-κB consensus minimal promoter linked to luciferase. Surprisingly, 16QsV exerted no effect on the minimal B NF-κB, although it was able to activate the consensus NF-κB promoter (Fig. 4 A). As controls, the same transfected cells were exposed to PV or TNF. PV did not affect the activity of any of the promoters, whereas TNF activated the NF-κB consensus promoter, but not site B NF-κB. These data suggest that additional cis elements are required for the HPV16 inhibitory TLR9 transcriptional activity. To evaluate this hypothesis, we cloned a 200 bp fragment containing the site B element in the pTAL vector (B200; Fig. 4 B). Transfection of B200 into C33A promoted the transcription of the luciferase gene that was repressed in the presence of 16QsV, but not by TNF (Fig. 4 B). Mutations of the NF-κB site on B200 (Bm) restored luciferase activity (Fig. 4 B). Together,

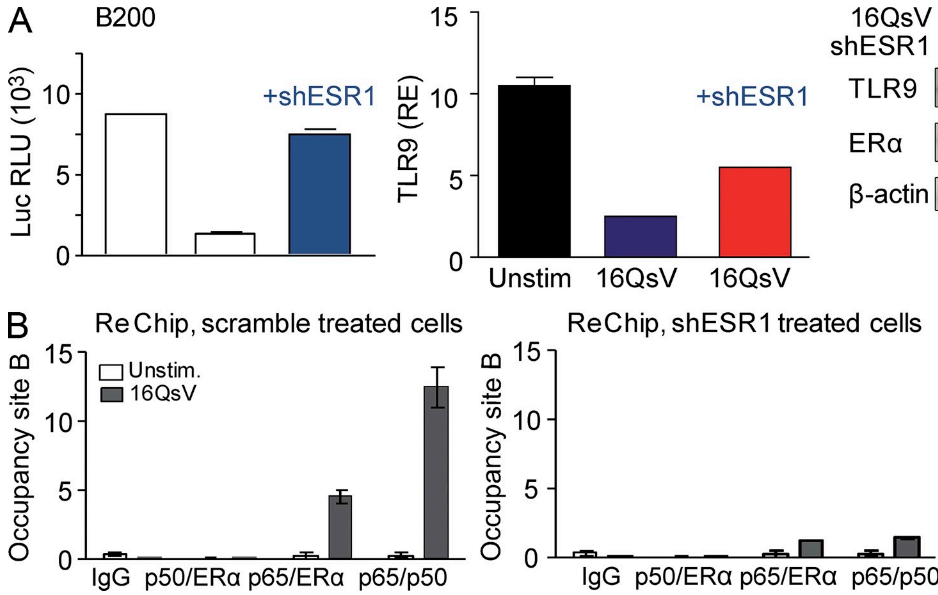

Figure 5. ERα complexes with NF-κBp65 to suppress TLR9 transcription by 16QsV. (A, left) C33A cells were transfected with B200 and, 24 h later, shESR1 was introduced. 12 h after shESR1 introduction, cells were infected with 16QsV. Cells were harvested and luciferase activity was measured after 24 h. (A, right) qPCR and immunoblot analysis for TLR9 and ERα in the human epithelial cervical cells treated with shESR1. (B) C33A cells were transfected with a shRNA scramble for ERα (left) or with an shRNA for ERα (shESR1; right) for 24 h. Cells were then infected for 12 h with 16QsV and analyzed by ReChIP for NF-κBp50–ERα or NF-κBp65–ERα or NF-κBp65–p50 occupancy on site B of the TLR9 promoter. Data are representative of the mean of five or more independent experiments performed in triplicate; graphs show the mean ± SEM.

these data indicate that other cis elements in the proximity of the predicted NF-κB cis element may be required to suppress the TLR9 promoter by HPV16. We identified within the 200 bp region a putative estrogen response element (ERE; Fig. 4 C). To determine whether the ERE site was involved in HPV16E7-mediated TLR9 down-regulation, we generated mutants of B200 promoter, in which ERE cis element was mutagenized alone (BER) or together with the NF-κB cis element at site B (BmER; Fig. 4 C). Mutagenesis of ERE significantly alleviated the E7-induced inhibition of luciferase activity (Fig. 4 D). This phenomenon was even more evident in the case of BmER (Fig. 4 D, left). In agreement with previous data (Hasan et al., 2007a) E7 from low-risk HPV type 6 did not have any effect on the regulation of the B200, BER, or BmER promoters (Fig. 4 D, left). ChIP experiments in HK using antibodies against estrogen α (ERα) or its phosphorylated form at serine 118 confirmed that ERα bound to the ERE element on the TLR9 promoter in the presence of HPV16E7 (Fig. 4 D, right). In addition, silencing of ERα expression by short hairpin construct against ERα (shESR1) restored the luciferase activity of the B200 bp promoter in 16QsV-infected cells as well as the endogenous levels of TLR9 mRNA and protein (Fig. 5 A). Similarly, exposure of the cells to melatonin, an inhibitor of ERα, blocked HPV16E7 or 16QsV ability to suppress the B200 promoter (unpublished data). Finally, reChIP experiments revealed that in 16QsV-infected cells the recruitment of a p50–p65 complex to site B was inhibited when ERα expression was down-regulated by shESR1 (Fig. 5 B). In summary, our data show that ERα is recruited with the p50–p65 NF-κB complex to site B resulting in transcriptional repression of TLR9.

HPV16 E6E7 induces epigenetic changes in TLR9 promoter

Gene expression is regulated by DNA binding transcriptional factors and by chromatin modifications (Weng et al., 2012). We therefore analyzed the chromatin organization within TLR9 promoter in mock or 16QsV-infected cells by monitoring Histone 4 acetylation (AceH4) and trimethylation of histone H3 at lysine 4 (H3K4me3), which are events associated with transcriptionally active chromatin (Foster et al., 2007). AceH4 and H3K4me3 were observed at the region surrounding site B on TLR9 promoter in untreated C33A cells (Fig. 6 A, left). A similar situation was detected 6 h after infection of C33A cells with 16QsV, but not 8 or 36 h after infection, in which the AceH4 and H3K4me3 near site B were strongly decreased (Fig. 6 A, left). Silencing of HPV16 E7 by siRNA in 16QsV-C33A cells restored AceH4 and H3K4me3 association at site B (Fig. 6 A, right). Loss of AceH4 and H3K4me3 was not only limited to the TLR9 promoter region around site B but also occurred in the chromatin down-stream of site B until the transcription start site of the *TLR9* promoter (Fig. 6 B). These histone modifications coincided with the recruitment to the same regions of histone deacetylases (HDACs) 1–3 (Fig. 6 C). Previous studies have shown that p65 can form a complex with HDAC3 (Xu et al., 2007). However, ChIP/reChIP experiments in cells infected with 16QsV showed that HDAC3 recruited to the site B region was not directly associated with

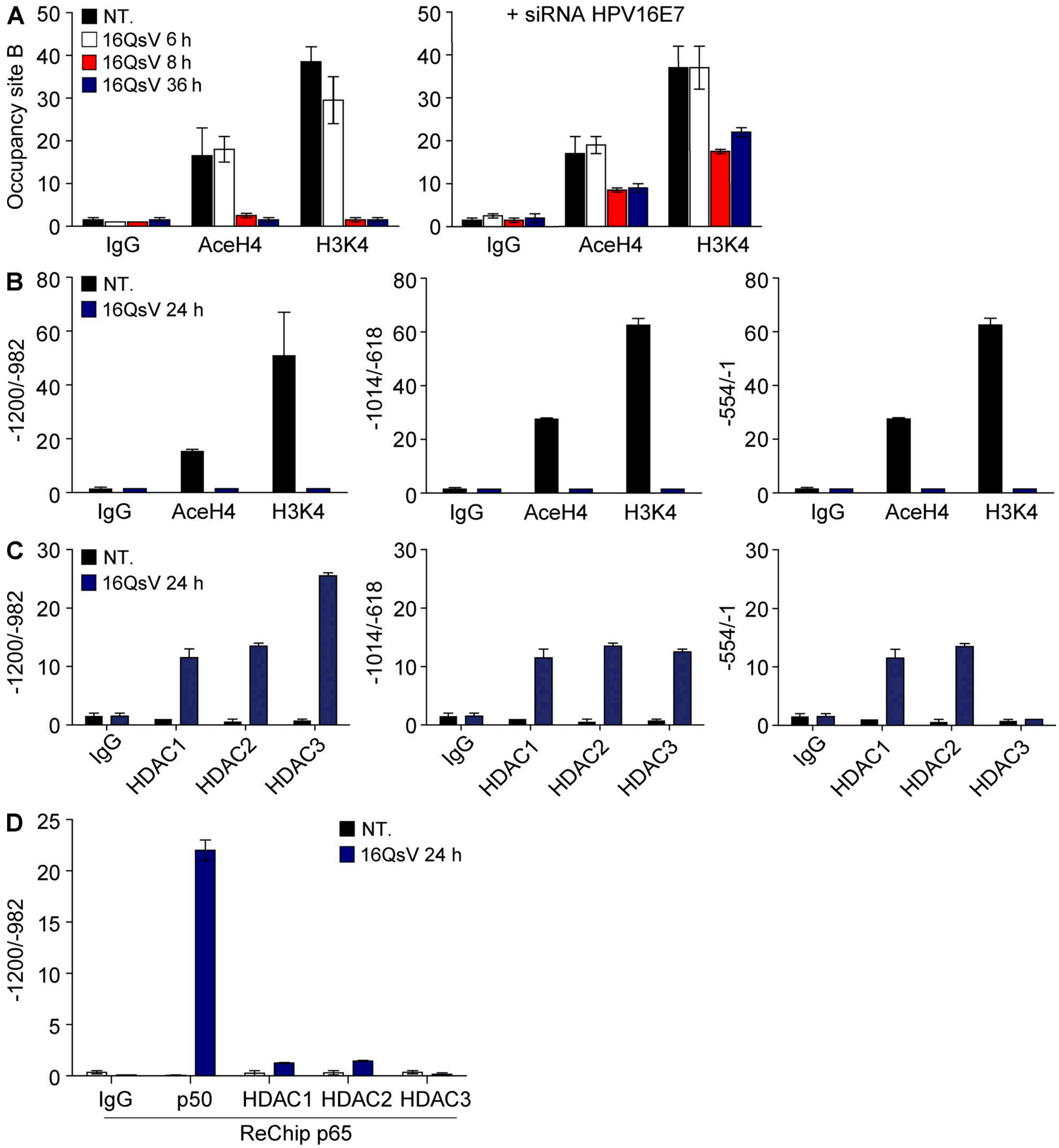

Figure 6. 16QsV induces closing of the chromatin structure on the TLR9 promoter from site B until the transcription start site. (A, left) ChIP using anti AceH4 or H3K4me3 histone antibodies was performed for site B using C33A cells infected with 16QsV for 6, 8, and 36 h. (A, right) C33A cells were treated as in A (left) except that 1 h after incubation with 16QsV, cells were treated with siRNA against HPV16E7. (B) Chromatin from C33A cells that had been incubated ± 16QsV for 24 h was analyzed by ChIP for AceH4 or H3K4me3 histones interacting with chromatin DNA along the TLR9 promoter. AceH4 or H3K4me3 binding to histones associated DNA upstream of site B on the TLR9 promoter were amplified by qPCR along the regions −1200 until −1. (C) HDAC1-3 binding to histone associated DNA of upstream of site B on the TLR9 promoter were amplified by qPCR along the regions −1200 until −1. (D) ReChIP for NF-κBp65–p50 or NF-κBp65–HDAC1-3 was performed on C33A cells treated with 16QsV for 36 h. Data are representative of three or more independent experiments; graphs shown are mean ± SEM from triplicate values.

the p65 (Fig. 6 D). We therefore evaluated whether ERα was responsible for the recruitment of HDACs to TLR9 promoter in cells infected with 16QsV. Re-ChIP experiments using a specific pERα (ser 118) antibody revealed that p65 and HDAC1 were recruited in proximity to site B on the TLR9 chromatin in 16QsV-infected C33A cells (Fig. 7 A). In addition, immunoprecipitation experiments with an ERα antibody revealed that ERα coprecipitated with NF-κBp65 and/or HDAC1 in chromatin fractions extracted from 16QsV-infected C33A cells, whereas only a weak association of ERα in chromatin fractions from untreated C33A cells was observed (Fig. 7 B). In agreement with these data, down-regulation of ERα expression by shRNA restored AceH4 at site B on the TLR9 promoter in 16QsV-infected cells (Fig. 7 C). Furthermore, inhibition of HDAC1 by Trichostatin A (TSA) restored AceH4 and reduced HDAC1 recruitment to site B in the presence of 16QsV (unpublished data) which coincided with an increase of TLR9 mRNA and protein levels. We also observed that 16QsV infection decreases H3K4me3 at site B (Fig. 6 A). Gene silencing of ERα in the same cells also restored H3K4 trimethylation near site B, indicating that ERα is also involved in H3K4 demethylation on the TLR9 promoter (Fig. 7 D). We next wanted to determine which demethylase was involved. A new class of demethylase enzyme, called JARID1B, has been shown to be highly expressed in ERα-positive breast cancer cells and tumors (Dey et al., 2008; Kim et al., 2010; Catchpole et al., 2011; Nijwening et al., 2011). JARID1B interacts with ERα and catalyzes the removal of methyl groups from lysine 4 of histone H3. We observed that JARID1B protein levels were elevated in HPV16E7 HK in comparison with mock-infected cells (Fig. 7 E, top). We hypothesized that JARID1B recruitment via ERα was responsible for the loss of H3K4me3 on the TLR9 promoter at site B. Indeed, blocking ERα expression in HPV16E7 HK decreased JARID1B and increased H3K4 levels in chromatin fractions (Fig. 7 E, bottom). Re-ChIP experiments in 16QsV-infected C33A cells showed that JARID1B was recruited in association with $ER\alpha^{ser118}$ at site B on the TLR9 promoter (Fig. 7 F). Other histone demethylases, such as LSD1 or RBP2, were not recruited to the TLR9 promoter (unpublished data). Abrogating ERα expression reduced JARID1B recruitment to site B on the TLR9 promoter (Fig. 7 G). Therefore, HPV16 induces ERα to recruit HDAC1 and JARID1B histone modification enzymes as well as NF-κBp50–p65 to prevent TLR9 transcription. We also confirmed that NF-κBp65, p50, HDAC1, and JARID1B all immunoprecipitated with ERα using chromatin fractions from 16QsV-infected C33A cells (Fig. 7 H). However, the formation of the different complexes were dependent on the integrity of the DNA because none of the subunits were immunoprecipitated after DNase I treatment of the chromatin (Fig. 7 H). To determine whether the ERα and NF-κB complex are independently or dependently recruited to TLR9 promoter, we performed oligo pulldown experiments using biotinylated DNA probes which contain a region of the TLR9 promoter encompassing both ERE and the NF-κB cis elements (B), intact ERE with a mutated NF-κB cis element (Bm), or vice versa (BER; Fig. 7 I). We observed that the intact site B probe sequence from TLR9 promoter (B) precipitated ERα, NF-κBp65, p50, HDAC1, and JARID1B. However, mutation of NF-κB cis element (Bm) resulted in the loss of binding of p50 and p65, without significantly affecting the recruitment of ERα, HDAC1, and JARID1B (Fig. 7 I). An opposite scenario was observed when the ERE was mutated (Fig. 7 I). Thus, the two repressive complexes appear to be independently recruited to TLR9 promoter. However, mutation of either ERE or NF-κB cis elements strongly affected the ERα, and NF-κBp65 interaction. In summary, these data show that HPV16 promoted the formation of a repressive chromatin modification complex that negatively regulates TLR9 gene expression.

NF-κBp65 and ERα are involved in the regulation of TLR9 expression in HPV16-positive cervical cancers

To corroborate our findings in cervical cancer samples, we examined by immunohistochemical analysis the expression and cellular localization of NF-κBp65 and ERα in normal cervical tissues ($n = 8$) and HPV16-positive cancers ($n = 8$; Fig. 8). Examination of normal cervical tissue revealed high expression of TLR9 in the basal (B) and suprabasal (S) layers (Fig. 8 A), which was lost in tumor samples (Fig. 8 B). In contrast, NF-κBp65 nuclear staining was increased in tumors compared with normal tissue (Fig. 8, A and B). Only a subtle difference in ERα nuclear levels was observed in the tumor and normal tissues (Fig. 8, A and B). However, according to the ChIP data shown in Fig. 5, the NF-κBp65/ERα nuclear colocalization was significantly increased in tumor samples in comparison to healthy tissues (Fig. 8 C, $P < 0.0001$, unpaired Student's *t* test). To evaluate whether these two cellular proteins interact in cervical cancer cells, we performed in vivo immunoprecipitation using the DUOLINK technology, which determines the localization in tissues of two proteins in the proximity of <40 nm. With this technique, protein–protein interaction or proximity is revealed by the appearance of distinct bright dots when tissue is analyzed with a confocal microscope. Using specific NF-κBp65 and ERα antibodies, no or mainly cytoplasmic red dots (interacting NF-κBp65/ERα) were detected in the three normal cervical tissues examined (Fig. 8, D and E). In contrast, cancer specimens displayed high levels of NF-κBp65–ERα interactions which were mostly located perinuclearly or in the nucleus of the cells. *Ortho* slicer movement (total of 30 Z stack slices of 0.3 µm) allowed us to consolidate that the red staining could be seen penetrating through the nucleus of the cell (Fig. 8 D, bottom). ChIP experiments using tissue from normal cervical tissue or HPV16-positive cancer tissue revealed that NF-κBp50–p65 and ERα were recruited to the TLR9 promoter only in cancer cells (Fig. 8 F). The interaction between ERα and p65 showed by the DUOLINK assay was also confirmed in cervical cancer cell lines as well as in primary keratinocytes expressing HPV16 E6 and E7 (Fig. 8 G and not depicted). In addition, nuclear NF-κBp65–ERα interactions were lost in the presence of a siRNA for NF-κBp65 or a shRNA against ERα (Fig. 8, H and I; and not depicted). In summary, the analysis of human specimens fully confirmed the data obtained in in vitro experimental models that showed the involvement of ERα and NF-κBp65 complex in transcriptional down-regulation of TLR9 gene.

DISCUSSION

The characterization of HPV mechanisms in deregulating the immune surveillance is extremely important to fully understand

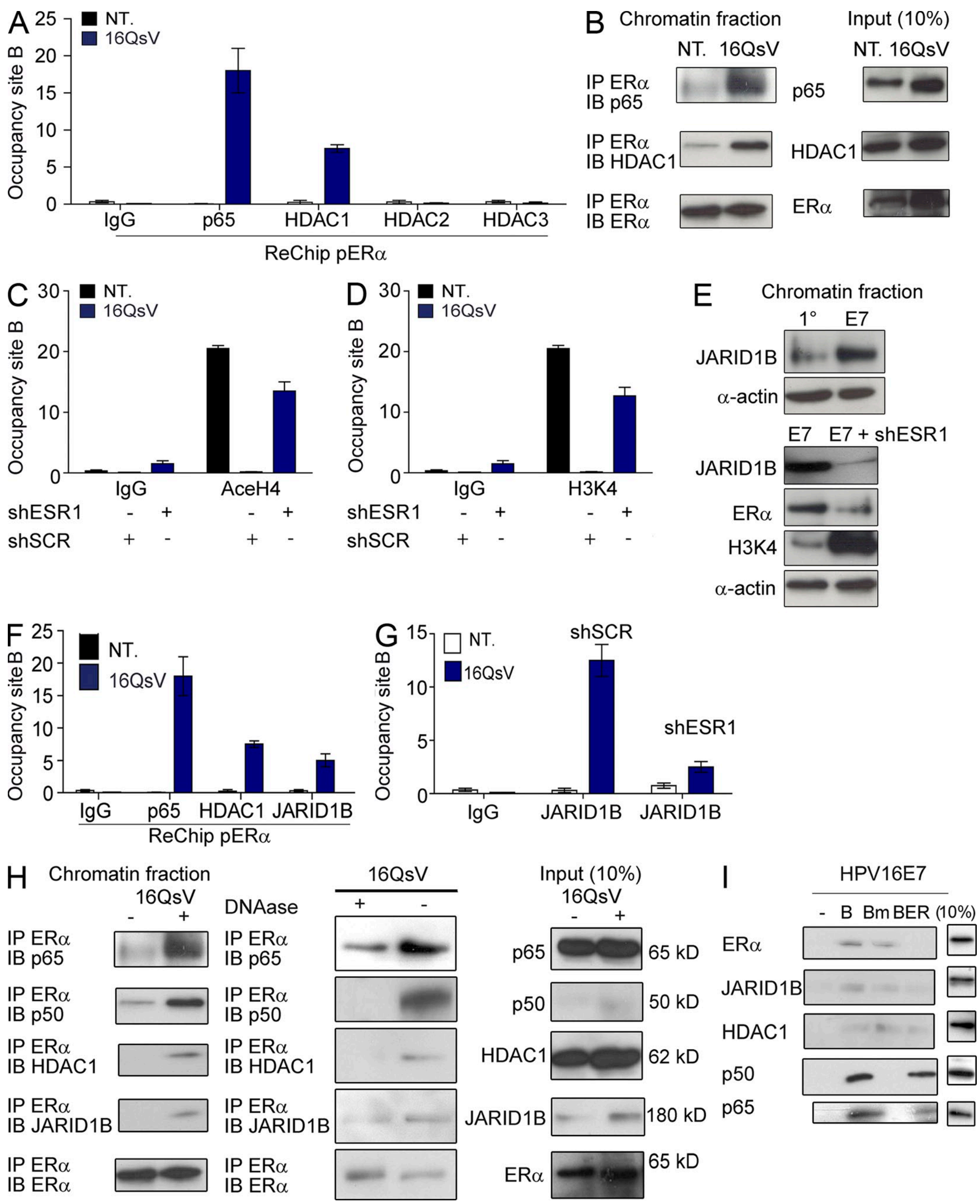

Figure 7. Recruitment of epigenetic demethylating and deacetylating enzymes by ERα to site B on the TLR9 promoter in 16QsV human epithelial cells. (A) ReChIP for pERα–p65 or HDAC1-3 was performed on C33A cells treated with 16QsV for 24 h. (B, left) Immunoprecipitation for ERα interactions with NF-κBp65 or HDAC1 was performed on chromatin fraction of C33A infected for 36 h with 16QsV. (B, right) Input controls. (C) ChIP using anti AceH4 histone antibodies was performed for site B on C33A cells infected with 16QsV for 24 h ± shESR1. (D) ChIP using anti H3K4me3 histone antibodies was performed for site B on C33A cells infected with 16QsV for 24 h ± shESR1. (E) Chromatin fraction Western blot analysis of JARID1B, ERα, or H3K4me3 expression in pLXSN or HPV16E7 ± shESR1-transduced HK. (F) ReChIP for pERα–p65 or pERα/HDAC1 or pERα/JARID1B was performed on C33A cells treated with 16QsV for 36 h. (G) ChIP using anti-JARID1B antibody was performed for site B on C33A cells infected with 16QsV for 24 h ± shScramble control sequence (shSCR) or shESR1. (H, left) Immunoprecipitation for ERα interactions with NF-κBp65, p50, JARID1B, or HDAC1 was performed on chromatin fractions of C33A infected for 36 h with 16QsV ± DNase I treatment. (H, right) 10% of input loaded protein. (I, left) Oligo pulldown assay for site B, Bm, and BER using protein lysates from pLXSN or HPV16E7-transduced cells. Bound proteins were assessed by immunoblotting for NF-κBp65, p50, JARID1B, HDAC1, or ERα. (I, right) Input controls (10%). Data are representative of three or more independent experiments; graphs shown are the mean ± SEM from triplicate values.

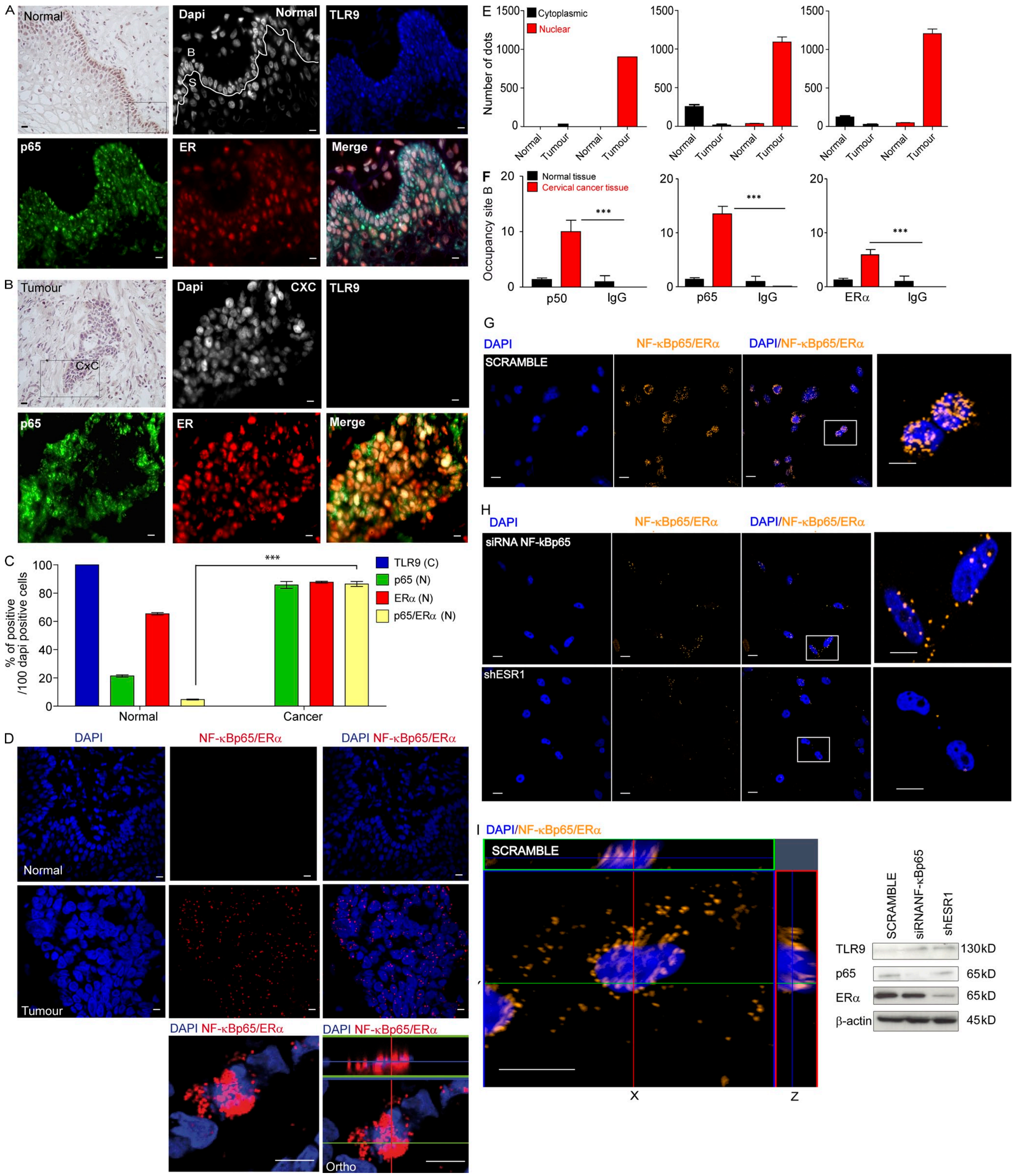

Figure 8. HPV16-positive cervical cancer lesions contain NF-κBp65–ERα nuclear complexes which bind to site B on the TLR9 promoter. Histology and immunofluorescence (IF) of normal cervical issue (A) and HPV16+ cervical cancer biopsies (B). Nuclear (white), TLR9 (blue), ERα (red), or NF-κBp65 (green). The box in the histology staining indicates which part of the slide was examined by IF. Normal (HPV−) and a neoplastic biopsy (HPV16+) from one representative patient out of eight tested with similar results is shown. Bars, 10 µm. (C) Histograms representing the cellular distribution for TLR9, ERα, and NF-κBp65 in normal (HPV−) and cancer cervical tissue (HPV16 positive by Apex screening). For each stained biopsy, six fields were examined and cytoplasmic or nuclear staining was counted manually and the percentage scored out of 100 cells. Data are representative of three

the events involved in the establishment of cervical diseases. In this study, we found that the oncovirus HPV16 activates a unique NF-κBp50–p65 and ERα inhibitory complex that suppresses TLR9 transcription and function. This event resulted in an inhibition of IFN production, which appears to negatively influence the HPV viral life cycle. We showed that the oncoprotein HPV16E7 activates the NF-κB canonical pathway, leading to the formation of a suppressive NF-κBp50–p65 complex, which binds a specific NF-κB element (site B) of TLR9 promoter. Gene silencing, chemical inhibitor, and ectopic mutant levels of NF-κB regulators alleviate the E7-mediated inhibition of TLR9 expression. Similarly, mutation of NF-κB site B prevented E7 to inhibit TLR9 promoter activities. Interestingly, TNF, a strong NF-κB signaling activator, did not lead to TLR9 down-regulation. TLR9 engagement by HSV2, CpG oligos, or UV-inactivated 16QsV resulted in a temporary TLR9 down-regulation, which was mediated by MyD88 and was not dependent on NF-κBp50–p65 binding to site B, indicating that one of the other identified NF-κB sites (A, C, and/or D) may be required. Indeed, overexpression of MyD88DN efficiently abolished the TLR9 transcriptional repression after TLR9 engagement. In contrast, it did not affect the E7-TLR9 transcriptional abrogation. These data suggest that activation of NF-κB signaling by different means, i.e., E7 expression, TNF treatment, and TLR9 engagement, led to the formation of distinct NF-κB complexes which may bind to exclusive sites in the TLR9 promoter. Furthermore, in untreated TLR9-expressing cells the NF-κBp50–p65 was isolated at site D on the TLR9 promoter, suggesting that in this context the NF-κBp50–p65 complex was transcriptionally active. To our knowledge, this is the first description of NF-κBp50–p65 complex mediating differential regulation of a target gene depending on the binding site in its promoter. Generation of artificial minimal promoter comprising only the NF-κB cis element B (Fig. 4 A) revealed that additional elements were required to fully repress TLR9 promoter activity by E7. We identified, in close proximity to the NF-κB cis element at site B, an ERE, which we found was essential for HPV16E7 to turn down TLR9 transcription.

The transcription factor ERα is a member of the nuclear receptor family which translocates to the nucleus upon binding to the sex hormone estradiol (Gibson and Saunders, 2012). Epidemiological studies showed that estrogen is a risk factor for both breast and HPV-mediated cervical carcinogenesis (Brake and Lambert, 2005; Chung et al., 2008, 2010; Shai et al., 2008; Chung and Lambert, 2009). In addition, experiments with transgenic mice expressing HPV16 E6 and E7 in the basal layers of the epithelia demonstrated that the estrogen cooperated with the viral oncoproteins in promoting cervical cancers (Chung et al., 2010). Our ChIP experiments confirmed that a phosphorylated form of ERα (S118) was recruited to the TLR9 promoter. However, no phosphorylation of the other amino acid residues known to be linked to ERα activation was observed (unpublished data). It has been previously reported that CDK7 is involved in phosphorylation of ser118 (Joel et al., 1998), but it is not yet known whether this kinase is activated in HPV16-positive cells. In addition, blocking ERα expression or function in cells infected with 16QsV prevented NF-κBp50–p65 recruitment to site B, thus restoring TLR9 expression. Immunoprecipitation of chromatin fractions from 16QsV-infected cells revealed the presence of ERα–p65 complexes, a complex which has been previously reported to act as an inhibitor for estrogen-regulated genes (Feldman et al., 2007). More importantly, the ERα–p65 interaction was confirmed in HPV16-positive cervical cancer cell lines and tissues by DUOLINK assays, whereas this complex was not present in the nucleus of normal tissues. In addition, ChIP experiments using chromatin fractions prepared from normal cervical or cancer epithelia showed that ERα and p65 were recruited to TLR9 promoter only in cervical cancer cells. We have examined the role of ERα signaling on TLR9 expression in normal HK by addition of its ligand estradiol 17β; indeed, we observed that TLR9 mRNA levels were increased (unpublished data). These data are consistent with the nuclear expression of ERα seen in the normal cervical in which TLR9 expression is observed (Fig. 8). Based on our data, we hypothesize that ERα signaling favors cervical cancer development, in part by promoting an efficient and permanent inhibition of TLR9 expression only in HPV16-infected

independent experiments performed on 16 biopsies (eight normal and eight neoplastic); graphs shown are the mean ± SEM. ***, $P < 0.0001$. (D) Duolink analysis of NF-κBp65–ERα in normal versus cervical cancer tissue. DAPI marks the nucleus and the red dots represent NF-κBp65–ERα proximity ligation <40 nm distance. Shown are data from one out of five independent experiments that gave identical results. Represented here is one out of six fields examined for each section. Bars, 10 µm. (E) NF-κBp65 and ERα proximity interaction locality were estimated by counting the number of red dots manually and using the Duolink Image tool (Olink, Biosciences) present in three field sections on three normal versus three cervical cancer tissues. Data are representative of three independent experiments performed on six biopsies (normal and neoplastic); graphs shown are the mean ± SEM. (F) ChIP analysis was performed on normal cervical tissue and cervical cancer biopsies for NF-κBp65, p50, or ERα binding to site B on the TLR9 promoter (represented one experiment out of three ChIP, and qPCR for occupancy on site B was performed in duplicate three times on three normal vs. four cervical cancer tissues). Shown are data from six independent experiments performed in triplicate, and error bars indicate SEM. ***, $P < 0.0001$ based on an unpaired Student's *t* test. (G) Duolink analysis of NF-κBp65–ERα in the cervical cancer cell line CaSki. DAPI marks the nucleus and the orange dots represent NF-κBp65–ERα proximity ligation <40 nm distance. Shown are data from one out of three independent experiments that gave identical results in which one of five fields were examined. Bars, 20 µm. (H) As in G, however cells were treated with a siRNA sequence for NF-κBp65 or small hairpin for ERα (as described in Fig. 5). Shown are data from one out of five independent experiments that gave identical results. Bars, 20 µm. (I, left) 63× zoom × 2 and ortho Z slices (3.3 µM) show nuclear penetration of the NF-κBp65 and ERα proximity ligation of <40 nm in scramble short hairpin for ERα-treated cells. Shown are data from one out of five fields examined and one out of five independent experiments that gave identical results. Bar, 20 µm. (I, right) Immunoblot demonstrating the efficiency of siRNA knockdown of NF-κBp65 and shESR1. Shown are data from one out three immunoblots performed.

cells. Most importantly, we report that ERα was associated with two chromatin modification enzymes, the histone demethylase JARID1B and deacetylase HDAC1. This complex inhibited histone methylation (H3K4me3) and acetylation (AceH4) at site B and, consequently, downstream toward the transcriptional start site on the TLR9 promoter. There are an increasing number of reports highlighting the importance of deacetylases and demethylases in innate immune gene regulation. Several deacetylase enzymes, such as HDAC1, HDAC8, and HDAC6, influence IFN-β gene expression with opposing effects (Nusinzon and Horvath, 2006). Although HDAC1 and HDAC8 repress IFN-β expression, HDAC6 acts as a coactivator essential for enhancer activity. Virus replication is enhanced in HDAC6-depleted cells, demonstrating that HDAC6 is an essential component of innate antiviral immunity (Nusinzon and Horvath, 2006). We demonstrated that blocking HDAC1 with the use of TSA restored TLR9 expression. Interestingly, Lin et al. (2009) treated cervical cancer cell xenografts with TSA and retarded tumor growth significantly. These data indicate the use of HDAC inhibitors in cervical cancer therapy (Takai et al., 2011). JARID1B (also known as PLU-1) has been shown to demethylate H3K4me3 and binds to ERα in breast cancer tumors (Scibetta et al., 2007; Catchpole et al., 2011). We show for the first time that JARID1B levels are increased and bind to ERα, which prevented H3K4me3 of TLR9 in the chromatin fraction of HPV16-infected cells. Independently of ERα, JARID1B has been shown to bind to another demethylating enzyme, LSD1, and repress the transcription of CCL14, an epithelial derived chemokine known to reduce the angiogenic and metastatic potential of breast cancer cells in vivo (Pedersen and Helin, 2010). These data address the role of deacetylases and demethylases separately. Previous work by Feldman et al. (2007) showed that ERα and the p65 colocalized on DNA which was an essential interaction that was inhibitory for ER transcriptional activity. Our work further analyzed the ability of ERα to collectively bring deacetylase and demethylase enzymes along with a NF-κBp50–p65 complex to silence the transcription of a key innate sensor. HPV16 aims to suppress TLR9 as a means to avoid consequent recognition and/or prevents prestimulation of TLR9 by the microflora in the cervix, which may be protective against HPV infection. Gillet et al. (2011) reported an association between alteration of the vaginal flora and HPV infection, suggesting that commensal bacterial subspecies could be protective against HPV infection (such as lactobacilli in the microflora). This suppression of TLR9 could prevent an efficient innate response against HPV and facilitate the establishment of a chronic infection, which is considered a necessary condition for cervical and other virus-induced cancers. The importance of our findings have been corroborated in clinical studies showing that clearance of HPV16 infection in women correlated to increased TLR9 expression in the epithelium (as well as other TLRs; Yu et al., 2010; Daud et al., 2011; DeCarlo et al., 2012).

TLR9 down-regulation in HPV-induced carcinogenesis is underlined by the fact that it has been shown that specific TLR9 polymorphisms in women were associated with an increased risk of cervical cancer development (Roszak et al., 2012) and that other oncoviruses, such as EBV, HCV, and HBV, share the property to persistently repress TLR9 expression although with distinct mechanism (Hasan et al., 2007a; Fathallah et al., 2010; Vincent et al., 2011). More specifically, we have recently shown that the oncoprotein LMP1 from EBV down-regulates the transcription of TLR9 in human B cells via activation of NF-κB (Fathallah et al., 2010). HBV and HCV particles can block the ability of pDC to produce IFN-α in response to TLR9, but not TLR7 agonists (Daud and Scott, 2008; Xu et al., 2009; Hirsch et al., 2010; Daud et al., 2011; van Gent et al., 2011; Woltman et al., 2011). In addition, reports have shown that a strong dysfunction of tumor-associated pDCs in their capacity to produce type I IFN in response to CpG-A (TLR9 agonist), but not to TLR7 ligands, was observed in human primary breast and ovarian tumors (Hirsch et al., 2010). These data indicate that TLR9 function is suppressed in viral and nonviral–associated cancers via a unique mechanism targeting TLR9 but not other TLRs which share immune signaling pathways. More work in the field of TLR9 regulation is thus required to understand how a variety of cancers affect differently the same innate immune receptor.

In summary, our work demonstrates that the oncovirus HPV16 induces a transcriptional repressive complex that suppresses TLR9 expression. This suggests that TLR9 may play a tumor-suppressive role in cervical cancers, perhaps by inducing type-I IFN and proinflammatory responses, which are known to induce cell cycle arrest, apoptosis, and death of viral infected cells. Thus, interfering with the regulation of TLR9 with synthetic transcriptional agonists that target ERα levels may provide a novel therapeutic strategy for cervical cancers.

MATERIALS AND METHODS

Cell culture procedures. NIH3T3, Phoenix, HEK 293T, HEK 293TT (for virus production), and cervical cancer–derived cell lines HeLa, SiHa, C33A, and CaSki were maintained as previously described (Hasan et al., 2007a). Primary human female skin keratinocytes (HK) were grown as previously described (Hasan et al., 2007a; Mansour et al., 2007). High-titer retroviral supernatants (>5 × 10^6 IU/ml) were generated as previously described (Hasan et al., 2007a; Mansour et al., 2007). The 16QsV and PV production, infection, and viral genome expression quantification of HPV16 have been performed as previously described (Buck et al., 2005).

Construct information. The retroviral pBabe-puro encoding HPV16 and 6 E6 and or E7 have been previously described (Hasan et al., 2007a). The constructs pLXSN-HPV16 and HPV6 E6/E7 were a gift from D. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA). The plasmids used for HPV16 structural genes and control PV production, the target HPV16 genome, and GFP (for PV control) were kindly donated from the laboratories of Martin Muller and Angel Alonso (DKFZ, Germany). The NF-κB reporter plasmid was obtained from BD. The TLR9 promoter luciferase construct has been previously described (Takeshita et al., 2004). TLR9 mutated promoters were generated using the Quikchange site-directed mutagenesis kit (Stratagene). NF-κB minimal promoters were cloned into pTAL-LUC vector (BD). Minimal promoters for sites B200 and B200m were amplified from the TLR9 promoter and mutated site B promoter, respectively, and cloned into the pTAL-LUC vector. The human RIG-I plasmid was donated to us from the laboratory of T. Taniguchi (Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Tokyo, Japan). The ΔN-IκBα, which lacks the sequence that codes for the first N-terminal 36 amino acids (pBabe-puro-ΔN-IκBα), was generated by introduction of the PCR-amplified DNA fragment from pcDNA3-Flag-IκBα (obtained from T. Gilmore, Boston University, Boston, MA, and E. Kieff, Harvard Medical School, Boston, MA). The MyD88DN has been previously described (Hasan et al., 2004). Small hairpin RNA lentiviral constructs for TLR9 (shTLR9) and control were provided by the Procan Axe II CLARA platform. shERα was provided by D. Picard (Geneva University, Geneva, Switzerland) siRNA for IKKα or IKKβ was purchased from Ambion, and the sequence used was previously published (Accardi et al., 2011). siRNA for HPV16E6E7 or E7 was purchased from Dharmacon and previously published (Tang et al., 2006; McCloskey et al., 2010). The ISRE Luciferase minimal promoter was purchased from Stratagene.

Stimuli and inhibitors. TNF (210-TA) was purchased from R&D systems. HSV-2 was provided by the laboratory of A. Iwasaki (Yale Medical School, New Haven, CT). BAY 11–7082 (EMD Millipore) and melatonin (Sigma-Aldrich), TLR9 CpG 2006 and 2216 ODN, and negative GpC ODN controls, as well as RIG-I 5′ppp dsRNA were used between 3 and 10 μM (InvivoGen). TSA was provided by the laboratory of Z. Herceg (IARC, Lyon, France). The anti-IFNR and IgG control were purchased from PBL and used as previously described (Hasan et al., 2007b).

Immunofluorescence. Keratinocytes were treated as previously described (Hasan et al., 2007a). Biopsies of normal and cancer cervical tissues were taken from patients, snap frozen with liquid nitrogen, and stored at −80°C until required (obtained from M. Sideri, Istituto Europeo di Oncologia, Milano, Italia). Approval was obtained according to local ethic committees of the Istituto Europeo di Oncologia, Italy. Sections of 5-μm thickness were cut and either stained for immunofluorescence using the TSA system (PerkinElmer). Cells or tissues were washed, the coverslips were mounted onto slides using a 1/10 dilution of 4′,6′-diamidino-2-phenylindole (nuclear stain; Invitrogen) in fluoromount (Southern Biotechnology Associates), and protein expression was detected by direct fluorescence microscopy. Photographs were taken at 40× magnification. Microscopes and software used for immunofluorescence imaging were performed using the NIKON Eclipse TI-NIS-Elements AR 3.10, Axioplan 2 epifluorescence microscope (Carl Zeiss). Data presented are representative images from at least four independent experiments in which >90% of the cells showed similar staining patterns.

Proximity ligation duolink assay. Histological tumor sections or spotted cervical cancer cell lines were fixed for 30 min with cold 4% PFA. Blocking step was performed with Duolink Blocking solution according to manufacturer's instructions. Slides were incubated overnight at 4°C with primary antibodies directed against NF-κBp65 (0.15 μg/ml; Cell Signaling Technology) or ERα (1.5 μg/ml; Cell Signaling Technology) and then with the appropriate DNA-linked secondary antibodies. Duolink II Detection Orange Reagents were subsequently used according to the manufacturer's instructions (Eurogentec). Slides were analyzed using an inverted confocal microscope LSM710 (Leica).

ELISA. To measure secreted cytokines, C33A cells were seeded at 4 × 10^4 cells/96 wells in 200 μl of growth medium. The next day the cells were infected with 16QsV for 24 h. Cells were washed with PBS, and CpG or GpC 2006 was added. 24 h later, supernatants were collected for analysis of

Table 1. Oligo sequences used in this study

Primers	Forward (5′–3′)	Reverse (5′–3′)
TLR9	CGTCTTGAAGGCCTGGTGTTGA	CTGGAAGGCCTTGGTTTTAGTGA
β2Microglobulin	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
HPV16E1	CATAGAGATGCAGTACAGG	CTCACCCCGTATAACTC
HPV16E6	CCCACAGGAGCGACCCAGAAAGTT	CCCATCTCTATATACTATGCATAAATCCC
HPV16E7	As previously described (Mansour et al., 2007)	
NF-κB for ChIP		
Site A	TGGGTCTGTACCTGTGTGTGCA	TTCATTCCCTCCATCCACCTC
Site B	TGGATGGCCCTGTTGAGAGGG	TAGCCCCTGGGCATTCTCCTG
Site C	CTGGAGAGCACTCAGGGGAAC	GTCACACTAGGTCCCTCCTC
Site D	AGGCCCTGCAGAACTCTGGAG	TCAGGCAGAGAGCAGGGAGA
Cloning		
Site B	CCGCTAGCAGATCTGGGGTGGGAGGTTT	CTCGAGCCCCTGCTTGCAGTGATCGTG
Mutations TLR9 promoter of NF-κB sites		
Site A	NFAF: AAGGGACTCTGGGCCCTCATCAGGCTTG	NFAR: CAAGCCTGATGAGGGCCCAGAGTCCCTT
Site B	NFBF: GAGACTTGGGGACTCGGTCAGGCAGAGGGA	NFBR: TCCCTCTGCCTGACCGAGTCCCCAAGTCTC
Site C	NFCF: ACA/GCG/GGT/GGA/CTT/GTC/CAT/AGG/GCC/TT	NFCR: AAGGCCCTATGGACAAGTCCACCCGCTGT
Site D	As previously described (Fathallah et al.)	
ERα site biotinylated[a]	TCAGGCAGAGGTTTCAGCACATC	GATGTGCTGAAACCTCTGCCTGA
NF-κB minimal promoter sites, annealing primers	CCGCTAGCGAGTTTCTCGAGCC	GGCGATCGCTCAAAGAGCTCGG
siRNA		
HPV16E6E7	UCCAUAUGCUGUAUGUGAU	
HPV16E7	GCACACACGUAGACAUUCG	
IKKα	GCAGGCUCUUUCAGGGACA	
IKKβ	GGUGGAAGAGGUGGUGAGC	
NF-κB	As previously described (Hasan et al., 2005)	
Scramble	CGAAUGUCUACGUGUGUGC	

[a]Other biotin-labeled oligo probes were generated using site B forward WT or mutated biotinylated primers and respective nonbiotinylated reverse primers. Probe DNA was amplified using the TLR9 promoter plasmid as a template.

IL-8, MIP3α, or IL-6 secretion using Quantikine ELISA kits (R&D Systems) as previously described (Hasan et al., 2007a).

Immunoblotting, immunoprecipitation, and EMSA. Biochemical analysis of harvested cells was performed as described previously (Hasan et al., 2007a). To obtain cytoplasmic and nuclear extracts, cells were harvested and lysed as previously described (Gonda et al., 1996). Chromatin fractions were performed as previously described (Méndez and Stillman, 2000), omitting nuclease treatment. Where mentioned, DNase I (Fermentas) was added to chromatin fractions. 20 µg of protein extracts (determined by the Bradford assay; Bio-Rad Laboratories) were used for immunoblotting. EMSA and supershift assays were performed using the NF-κB EMSA kit (Panomics). For each binding reaction, 5 µg of nuclear extracts was used. Proteins or protein–DNA complexes were detected using ECL (GE Healthcare). Immunoprecipitations were performed as previously described (Hasan et al., 2005).

ChIP assay. ChIP assays were performed using the Shearing Optimization kit and the OneDay ChIP kit (Diagenode). For C33A cells or primary keratinocytes, cell sonication cycles last 15 s with 5 s on and 2 s off at 20% of amplitude and were repeated four times. For tissue, immunoprecipitation was performed over night on a rotating wheel at 4°C. 2.5 µl/reaction of DNA solution was used for qPCR. The primers used to amplify TLR9 promoter regions are listed above. ReChip was performed using the diagenode protocol one day ChIP kit up until step 49 and then after using the procedure from the Epigenome Network of Excellence website. ChIP on tissue was performed according to the protocol from Epigenome Network of Excellence for tissue preparation, after the Red ChIP kit from diagenode was used to prepare chromatin and the 1-d ChIP kit for the immunoprecipitation. Immunoprecipitation was performed overnight on a rotating wheel at 4°C. 2.5 µl/reaction of DNA solution was used for qPCR.

Chromatin fractions. Chromatin fractions were prepared as above, omitting micrococcal nuclease treatment.

Oligo pulldown. Oligo pulldown was performed as previously described (López-Rovira et al., 2002) with nuclear extracts as stated in the figure legend and oligo probes as listed in Table 1.

Transfections and luciferase assay. Cells were transiently transfected with the luciferase constructs or sh vectors using FuGene (Roche) as described previously (Hasan et al., 2007a). Each experiment was repeated three times in triplicate; results generally deviated by <10% of the mean value. SiRNA were transfected as previously described (Hasan et al., 2005).

Type I IFN bioassay. Supernatants were harvested, UV inactivated, and placed onto transfected HEK293T cells that express the IFN-β–inducible cis element ISRE-linked to the luciferase gene. 24 h after stimulation with supernatants, cells were harvested and luciferase activity was measured as previously described (Hasan et al., 2005).

Genotyping. Tumor samples were genotyped using multiplex PCR with HPV type-specific primers for amplification of viral DNA and array primer extension for typing (Hasan et al., 2007a).

RT-qPCR. Total RNA was extracted from cells using the RNeasy Mini kit (QIAGEN and Machery Nagel). cDNA was synthesized with the First strand cDNA synthesis kit (MBI, Fermentas). The Mx3000P real-time PCR system (Stratagene) was used to perform qPCR with Mesa green qPCR MasterMix plus (Eurogentec). Primer sequences are enclosed in Table 1.

Statistical analysis. GraphPad (version 5) was used to calculate unpaired and paired p-values.

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REFERENCES

Accardi, R., M. Scalise, T. Gheit, I. Hussain, J. Yue, C. Carreira, A. Collino, C. Indiveri, L. Gissmann, B.S. Sylla, and M. Tommasino. 2011. IkappaB kinase beta promotes cell survival by antagonizing p53 functions through DeltaNp73alpha phosphorylation and stabilization. *Mol. Cell. Biol.* 31:2210–2226. http://dx.doi.org/10.1128/MCB.00964-10

Adams, S., D.W. O'Neill, D. Nonaka, E. Hardin, L. Chiriboga, K. Siu, C.M. Cruz, A. Angiulli, F. Angiulli, E. Ritter, et al. 2008. Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. *J. Immunol.* 181:776–784.

Avalos, A.M., and H.L. Ploegh. 2011. Competition by inhibitory oligonucleotides prevents binding of CpG to C-terminal TLR9. *Eur. J. Immunol.* 41:2820–2827. http://dx.doi.org/10.1002/eji.201141563

Bangert, C., P.M. Brunner, and G. Stingl. 2011. Immune functions of the skin. *Clin. Dermatol.* 29:360–376. http://dx.doi.org/10.1016/j.clindermatol.2011.01.006

Bouvard, V., R. Baan, K. Straif, Y. Grosse, B. Secretan, F. El Ghissassi, L. Benbrahim-Tallaa, N. Guha, C. Freeman, L. Galichet, and V. Cogliano; WHO International Agency for Research on Cancer Monograph Working Group. 2009. A review of human carcinogens—Part B: biological agents. *Lancet Oncol.* 10:321–322. http://dx.doi.org/10.1016/S1470-2045(09)70096-8

Brake, T., and P.F. Lambert. 2005. Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse model. *Proc. Natl. Acad. Sci. USA.* 102: 2490–2495. http://dx.doi.org/10.1073/pnas.0409883102

Buck, C.B., D.V. Pastrana, D.R. Lowy, and J.T. Schiller. 2005. Generation of HPV pseudovirions using transfection and their use in neutralization assays. *Methods Mol. Med.* 119:445–462.

Catchpole, S., B. Spencer-Dene, D. Hall, S. Santangelo, I. Rosewell, M. Guenatri, R. Beatson, A.G. Scibetta, J.M. Burchell, and J. Taylor-Papadimitriou. 2011. PLU-1/JARID1B/KDM5B is required for embryonic survival and contributes to cell proliferation in the mammary gland and in ER+ breast cancer cells. *Int. J. Oncol.* 38:1267–1277.

Chung, S.H., and P.F. Lambert. 2009. Prevention and treatment of cervical cancer in mice using estrogen receptor antagonists. *Proc. Natl. Acad. Sci. USA.* 106:19467–19472. http://dx.doi.org/10.1073/pnas.0911436106

Chung, S.H., K. Wiedmeyer, A. Shai, K.S. Korach, and P.F. Lambert. 2008. Requirement for estrogen receptor alpha in a mouse model for human papillomavirus-associated cervical cancer. *Cancer Res.* 68:9928–9934. http://dx.doi.org/10.1158/0008-5472.CAN-08-2051

Chung, S.H., S. Franceschi, and P.F. Lambert. 2010. Estrogen and ERalpha: culprits in cervical cancer? *Trends Endocrinol. Metab.* 21:504–511. http://dx.doi.org/10.1016/j.tem.2010.03.005

Coban, C., K.J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M. Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, et al. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* 201:19–25. http://dx.doi.org/10.1084/jem.20041836

Daud, I.I., and M.E. Scott. 2008. Validation of reference genes in cervical cell samples from human papillomavirus-infected and -uninfected women for quantitative reverse transcription-PCR assays. *Clin. Vaccine Immunol.* 15:1369–1373. http://dx.doi.org/10.1128/CVI.00074-08

Daud, I.I., M.E. Scott, Y. Ma, S. Shiboski, S. Farhat, and A.B. Moscicki. 2011. Association between toll-like receptor expression and human papillomavirus type 16 persistence. *Int. J. Cancer.* 128:879–886. http://dx.doi.org/10.1002/ijc.25400

Debenedictis, C., S. Joubeh, G. Zhang, M. Barria, and R.F. Ghohestani. 2001. Immune functions of the skin. *Clin. Dermatol.* 19:573–585. http://dx.doi.org/10.1016/S0738-081X(00)00173-5

DeCarlo, C.A., B. Rosa, R. Jackson, S. Niccoli, N.G. Escott, and I. Zehbe. 2012. Toll-like receptor transcriptome in the HPV-positive cervical cancer microenvironment. *Clin. Dev. Immunol.* 2012:785825. http://dx.doi.org/10.1155/2012/785825

Dey, B.K., L. Stalker, A. Schnerch, M. Bhatia, J. Taylor-Papidimitriou, and C. Wynder. 2008. The histone demethylase KDM5b/JARID1b plays a role in cell fate decisions by blocking terminal differentiation. *Mol. Cell. Biol.* 28:5312–5327. http://dx.doi.org/10.1128/MCB.00128-08

Ewald, S.E., A. Engel, J. Lee, M. Wang, M. Bogyo, and G.M. Barton. 2011. Nucleic acid recognition by Toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase. *J. Exp. Med.* 208:643–651. http://dx.doi.org/10.1084/jem.20100682

Fathallah, I., P. Parroche, H. Gruffat, C. Zannetti, H. Johansson, J. Yue, E. Manet, M. Tommasino, B.S. Sylla, and U.A. Hasan. 2010. EBV latent membrane protein 1 is a negative regulator of TLR9. *J. Immunol.* 185:6439–6447. http://dx.doi.org/10.4049/jimmunol.0903459

Feldman, I., G.M. Feldman, C. Mobarak, J.C. Dunkelberg, and K.K. Leslie. 2007. Identification of proteins within the nuclear factor-kappa B transcriptional complex including estrogen receptor-alpha. *Am. J. Obstet. Gynecol.* 196:394: e1–e11, discussion :394: e11–e13. http://dx.doi.org/10.1016/j.ajog.2006.12.033

Fernandes-Alnemri, T., J.W. Yu, P. Datta, J. Wu, and E.S. Alnemri. 2009. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature.* 458:509–513. http://dx.doi.org/10.1038/nature07710

Fiola, S., D. Gosselin, K. Takada, and J. Gosselin. 2010. TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells. *J. Immunol.* 185:3620–3631. http://dx.doi.org/10.4049/jimmunol.0903736

Flores, E.R., B.L. Allen-Hoffmann, D. Lee, C.A. Sattler, and P.F. Lambert. 1999. Establishment of the human papillomavirus type 16 (HPV-16) life cycle in an immortalized human foreskin keratinocyte cell line. *Virology.* 262:344–354. http://dx.doi.org/10.1006/viro.1999.9868

Foster, S.L., D.C. Hargreaves, and R. Medzhitov. 2007. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature.* 447:972–978.

Gibson, D.A., and P.T. Saunders. 2012. Estrogen dependent signaling in reproductive tissues - a role for estrogen receptors and estrogen related receptors. *Mol. Cell. Endocrinol.* 348:361–372. http://dx.doi.org/10.1016/j.mce.2011.09.026

Gillet, E., J.F. Meys, H. Verstraelen, C. Bosire, P. De Sutter, M. Temmerman, and D.V. Broeck. 2011. Bacterial vaginosis is associated with uterine cervical human papillomavirus infection: a meta-analysis. *BMC Infect. Dis.* 11:10. http://dx.doi.org/10.1186/1471-2334-11-10

Gonda, T.J., D. Favier, P. Ferrao, E.M. Macmillan, R. Simpson, and F. Tavner. 1996. The c-myb negative regulatory domain. *Curr. Top. Microbiol. Immunol.* 211:99–109.

Gondois-Rey, F., C. Dental, P. Halfon, T.F. Baumert, D. Olive, and I. Hirsch. 2009. Hepatitis C virus is a weak inducer of interferon alpha in plasmacytoid dendritic cells in comparison with influenza and human herpesvirus type-1. *PLoS ONE.* 4:e4319. http://dx.doi.org/10.1371/journal.pone.0004319

Häcker, H., and M. Karin. 2006. Regulation and function of IKK and IKK-related kinases. *Sci. STKE.* 2006:re13. http://dx.doi.org/10.1126/stke.3572006re13

Hasan, U.A., S. Dollet, and J. Vlach. 2004. Differential induction of gene promoter constructs by constitutively active human TLRs. *Biochem. Biophys. Res. Commun.* 321:124–131. http://dx.doi.org/10.1016/j.bbrc.2004.06.134

Hasan, U., C. Chaffois, C. Gaillard, V. Saulnier, E. Merck, S. Tancredi, C. Guiet, F. Brière, J. Vlach, S. Lebecque, et al. 2005. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J. Immunol.* 174:2942–2950.

Hasan, U.A., E. Bates, F. Takeshita, A. Biliato, R. Accardi, V. Bouvard, M. Mansour, I. Vincent, L. Gissmann, T. Iftner, et al. 2007a. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. *J. Immunol.* 178:3186–3197.

Hasan, U.A., C. Caux, I. Perrot, A.C. Doffin, C. Menetrier-Caux, G. Trinchieri, M. Tommasino, and J. Vlach. 2007b. Cell proliferation and survival induced by Toll-like receptors is antagonized by type I IFNs. *Proc. Natl. Acad. Sci. USA.* 104:8047–8052. http://dx.doi.org/10.1073/pnas.0700664104

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature.* 408:740–745. http://dx.doi.org/10.1038/35047123

Hirsch, I., C. Caux, U. Hasan, N. Bendriss-Vermare, and D. Olive. 2010. Impaired Toll-like receptor 7 and 9 signaling: from chronic viral infections to cancer. *Trends Immunol.* 31:391–397. http://dx.doi.org/10.1016/j.it.2010.07.004

Hudak, S., M. Hagen, Y. Liu, D. Catron, E. Oldham, L.M. McEvoy, and E.P. Bowman. 2002. Immune surveillance and effector functions of CCR10(+) skin homing T cells. *J. Immunol.* 169:1189–1196.

Ito, T., N. Seo, H. Yagita, K. Tsujimura, M. Takigawa, and Y. Tokura. 2003. Alterations of immune functions in barrier disrupted skin by UVB irradiation. *J. Dermatol. Sci.* 33:151–159. http://dx.doi.org/10.1016/S0923-1811(03)00177-4

Joel, P.B., J. Smith, T.W. Sturgill, T.L. Fisher, J. Blenis, and D.A. Lannigan. 1998. pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol. Cell. Biol.* 18:1978–1984.

Kaplan, D.H., B.Z. Igyártó, and A.A. Gaspari. 2012. Early immune events in the induction of allergic contact dermatitis. *Nat. Rev. Immunol.* 12:114–124.

Karim, R., C. Meyers, C. Backendorf, K. Ludigs, R. Offringa, G.J. van Ommen, C.J. Melief, S.H. van der Burg, and J.M. Boer. 2011. Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes. *PLoS ONE.* 6:e17848. http://dx.doi.org/10.1371/journal.pone.0017848

Kim, J., S. Shin, M. Subramaniam, E. Bruinsma, T.D. Kim, J.R. Hawse, T.C. Spelsberg, and R. Janknecht. 2010. Histone demethylase JARID1B/KDM5B is a corepressor of TIEG1/KLF10. *Biochem. Biophys. Res. Commun.* 401:412–416. http://dx.doi.org/10.1016/j.bbrc.2010.09.068

Kim, K., P.A. Garner-Hamrick, C. Fisher, D. Lee, and P.F. Lambert. 2003. Methylation patterns of papillomavirus DNA, its influence on E2 function, and implications in viral infection. *J. Virol.* 77:12450–12459. http://dx.doi.org/10.1128/JVI.77.23.12450-12459.2003

Lebre, M.C., A.M. van der Aar, L. van Baarsen, T.M. van Capel, J.H. Schuitemaker, M.L. Kapsenberg, and E.C. de Jong. 2007. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J. Invest. Dermatol.* 127:331–341. http://dx.doi.org/10.1038/sj.jid.5700530

Lin, Z., M. Bazzaro, M.C. Wang, K.C. Chan, S. Peng, and R.B. Roden. 2009. Combination of proteasome and HDAC inhibitors for uterine cervical cancer treatment. *Clin. Cancer Res.* 15:570–577. http://dx.doi.org/10.1158/1078-0432.CCR-08-1813

López-Rovira, T., E. Chalaux, J. Massagué, J.L. Rosa, and F. Ventura. 2002. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J. Biol. Chem.* 277:3176–3185. http://dx.doi.org/10.1074/jbc.M106826200

Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9–mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* 198:513–520. http://dx.doi.org/10.1084/jem.20030162

Malanchi, I., R. Accardi, F. Diehl, A. Smet, E. Androphy, J. Hoheisel, and M. Tommasino. 2004. Human papillomavirus type 16 E6 promotes retinoblastoma protein phosphorylation and cell cycle progression. *J. Virol.* 78:13769–13778.http://dx.doi.org/10.1128/JVI.78.24.13769-13778.2004

Mansour, M., M. Touka, U. Hasan, A. Bellopede, A. Smet, R. Accardi, A.S. Gabet, B.S. Sylla, and M. Tommasino. 2007. E7 properties of mucosal human papillomavirus types 26, 53 and 66 correlate with their intermediate risk for cervical cancer development. *Virology.* 367:1–9. http://dx.doi.org/10.1016/j.virol.2007.05.005

Marur, S., G. D'Souza, W.H. Westra, and A.A. Forastiere. 2010. HPV-associated head and neck cancer: a virus-related cancer epidemic. *Lancet Oncol.* 11:781–789. http://dx.doi.org/10.1016/S1470-2045(10)70017-6

McCloskey, R., C. Menges, A. Friedman, D. Patel, and D.J. McCance. 2010. Human papillomavirus type 16 E6/E7 upregulation of nucleophosmin is important for proliferation and inhibition of differentiation. *J. Virol.* 84:5131–5139. http://dx.doi.org/10.1128/JVI.01965-09

Méndez, J., and B. Stillman. 2000. Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Mol. Cell. Biol.* 20:8602–8612. http://dx.doi.org/10.1128/MCB.20.22.8602-8612.2000

Metz, M., F. Siebenhaar, and M. Maurer. 2008. Mast cell functions in the innate skin immune system. *Immunobiology*. 213:251–260. http://dx.doi.org/10.1016/j.imbio.2007.10.017

Moore, P.S., and Y. Chang. 2010. Why do viruses cause cancer? Highlights of the first century of human tumour virology. *Nat. Rev. Cancer*. 10: 878–889. http://dx.doi.org/10.1038/nrc2961

Morizane, S., K. Yamasaki, B. Mühleisen, P.F. Kotol, M. Murakami, Y. Aoyama, K. Iwatsuki, T. Hata, and R.L. Gallo. 2012. Cathelicidin antimicrobial peptide LL-37 in psoriasis enables keratinocyte reactivity against TLR9 ligands. *J. Invest. Dermatol.* 132:135–143. http://dx.doi.org/10.1038/jid.2011.259

Nelson, H.S. 2011. Some highlights of the first century of immunotherapy. *Ann. Allergy Asthma Immunol.* 107:417–421. http://dx.doi.org/10.1016/j.anai.2011.05.013

Nijwening, J.H., E.J. Geutjes, R. Bernards, and R.L. Beijersbergen. 2011. The histone demethylase Jarid1b (Kdm5b) is a novel component of the Rb pathway and associates with E2f-target genes in MEFs during senescence. *PLoS ONE*. 6:e25235. http://dx.doi.org/10.1371/journal.pone.0025235

Nusinzon, I., and C.M. Horvath. 2006. Positive and negative regulation of the innate antiviral response and beta interferon gene expression by deacetylation. *Mol. Cell. Biol.* 26:3106–3113. http://dx.doi.org/10.1128/MCB.26.8.3106-3113.2006

Pedersen, M.T., and K. Helin. 2010. Histone demethylases in development and disease. *Trends Cell Biol.* 20:662–671. http://dx.doi.org/10.1016/j.tcb.2010.08.011

Pivarcsi, A., A. Müller, A. Hippe, J. Rieker, A. van Lierop, M. Steinhoff, S. Seeliger, R. Kubitza, U. Pippirs, S. Meller, et al. 2007. Tumor immune escape by the loss of homeostatic chemokine expression. *Proc. Natl. Acad. Sci. USA*. 104:19055–19060. http://dx.doi.org/10.1073/pnas.0705673104

Pyeon, D., P.F. Lambert, and P. Ahlquist. 2005. Production of infectious human papillomavirus independently of viral replication and epithelial cell differentiation. *Proc. Natl. Acad. Sci. USA*. 102:9311–9316. http://dx.doi.org/10.1073/pnas.0504020102

Rampias, T., C. Sasaki, P. Weinberger, and A. Psyrri. 2009. E6 and e7 gene silencing and transformed phenotype of human papillomavirus 16-positive oropharyngeal cancer cells. *J. Natl. Cancer Inst.* 101:412–423. http://dx.doi.org/10.1093/jnci/djp017

Rathinam, V.A., Z. Jiang, S.N. Waggoner, S. Sharma, L.E. Cole, L. Waggoner, S.K. Vanaja, B.G. Monks, S. Ganesan, E. Latz, et al. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat. Immunol.* 11:395–402. http://dx.doi.org/10.1038/ni.1864

Reiser, J., J. Hurst, M. Voges, P. Krauss, P. Münch, T. Iftner, and F. Stubenrauch. 2011. High-risk human papillomaviruses repress constitutive kappa interferon transcription via E6 to prevent pathogen recognition receptor and antiviral-gene expression. *J. Virol.* 85:11372–11380. http://dx.doi.org/10.1128/JVI.05279-11

Rincon-Orozco, B., G. Halec, S. Rosenberger, D. Muschik, I. Nindl, A. Bachmann, T.M. Ritter, B. Dondog, R. Ly, F.X. Bosch, et al. 2009. Epigenetic silencing of interferon-kappa in human papillomavirus type 16-positive cells. *Cancer Res.* 69:8718–8725. http://dx.doi.org/10.1158/0008-5472.CAN-09-0550

Ronco, L.V., A.Y. Karpova, M. Vidal, and P.M. Howley. 1998. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes Dev.* 12:2061–2072. http://dx.doi.org/10.1101/gad.12.13.2061

Roszak, A., M. Lianeri, A. Sowińska, and P.P. Jagodziński. 2012. Involvement of Toll-like Receptor 9 polymorphism in cervical cancer development. *Mol. Biol. Rep.* 39:8425–8430. http://dx.doi.org/10.1007/s11033-012-1695-8

Ryerson, A.B., E.S. Peters, S.S. Coughlin, V.W. Chen, M.L. Gillison, M.E. Reichman, X. Wu, A.K. Chaturvedi, and K. Kawaoka. 2008. Burden of potentially human papillomavirus-associated cancers of the oropharynx and oral cavity in the US, 1998-2003. *Cancer*. 113:2901–2909. http://dx.doi.org/10.1002/cncr.23745

Sasai, M., M.M. Linehan, and A. Iwasaki. 2010. Bifurcation of Toll-like receptor 9 signaling by adaptor protein 3. *Science*. 329:1530–1534. http://dx.doi.org/10.1126/science.1187029

Scibetta, A.G., S. Santangelo, J. Coleman, D. Hall, T. Chaplin, J. Copier, S. Catchpole, J. Burchell, and J. Taylor-Papadimitriou. 2007. Functional analysis of the transcription repressor PLU-1/JARID1B. *Mol. Cell. Biol.* 27:7220–7235. http://dx.doi.org/10.1128/MCB.00274-07

Sepulveda, F.E., S. Maschalidi, R. Colisson, L. Heslop, C. Ghirelli, E. Sakka, A.M. Lennon-Duménil, S. Amigorena, L. Cabanie, and B. Manoury. 2009. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. *Immunity*. 31:737–748. http://dx.doi.org/10.1016/j.immuni.2009.09.013

Shai, A., H.C. Pitot, and P.F. Lambert. 2008. p53 Loss synergizes with estrogen and papillomaviral oncogenes to induce cervical and breast cancers. *Cancer Res.* 68:2622–2631. http://dx.doi.org/10.1158/0008-5472.CAN-07-5266

Takai, N., N. Kira, T. Ishii, M. Nishida, K. Nasu, and H. Narahara. 2011. Novel chemotherapy using histone deacetylase inhibitors in cervical cancer. *Asian Pac. J. Cancer Prev*. 12:575–580.

Takeshita, F., K. Suzuki, S. Sasaki, N. Ishii, D.M. Klinman, and K.J. Ishii. 2004. Transcriptional regulation of the human TLR9 gene. *J. Immunol.* 173:2552–2561.

Tang, S., M. Tao, J.P. McCoy Jr., and Z.M. Zheng. 2006. Short-term induction and long-term suppression of HPV16 oncogene silencing by RNA interference in cervical cancer cells. *Oncogene*. 25:2094–2104. http://dx.doi.org/10.1038/sj.onc.1209244

Tay, S.K. 2012. Cervical cancer in the human papillomavirus vaccination era. *Curr. Opin. Obstet. Gynecol.* 24:3–7. http://dx.doi.org/10.1097/GCO.0b013e32834daed9

Tommasino, M., R. Accardi, S. Caldeira, W. Dong, I. Malanchi, A. Smet, and I. Zehbe. 2003. The role of TP53 in Cervical carcinogenesis. *Hum. Mutat.* 21:307–312. http://dx.doi.org/10.1002/humu.10178

van Gent, M., B.D. Griffin, E.G. Berkhoff, D. van Leeuwen, I.G. Boer, M. Buisson, F.C. Hartgers, W.P. Burmeister, E.J. Wiertz, and M.E. Ressing. 2011. EBV lytic-phase protein BGLF5 contributes to TLR9 downregulation during productive infection. *J. Immunol.* 186:1694–1702. http://dx.doi.org/10.4049/jimmunol.0903120

Vincent, I.E., C. Zannetti, J. Lucifora, H. Norder, U. Protzer, P. Hainaut, F. Zoulim, M. Tommasino, C. Trépo, U. Hasan, and I. Chemin. 2011. Hepatitis B virus impairs TLR9 expression and function in plasmacytoid dendritic cells. *PLoS ONE*. 6:e26315. http://dx.doi.org/10.1371/journal.pone.0026315

Weng, N.P., Y. Araki, and K. Subedi. 2012. The molecular basis of the memory T cell response: differential gene expression and its epigenetic regulation. *Nat. Rev. Immunol.* 12:306–315. http://dx.doi.org/10.1038/nri3173

Woltman, A.M., M.L. Op den Brouw, P.J. Biesta, C.C. Shi, and H.L. Janssen. 2011. Hepatitis B virus lacks immune activating capacity, but actively inhibits plasmacytoid dendritic cell function. *PLoS ONE*. 6: e15324. http://dx.doi.org/10.1371/journal.pone.0015324

Xu, W.S., R.B. Parmigiani, and P.A. Marks. 2007. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene*. 26:5541–5552. http://dx.doi.org/10.1038/sj.onc.1210620

Xu, Y., Y. Hu, B. Shi, X. Zhang, J. Wang, Z. Zhang, F. Shen, Q. Zhang, S. Sun, and Z. Yuan. 2009. HBsAg inhibits TLR9-mediated activation and IFN-alpha production in plasmacytoid dendritic cells. *Mol. Immunol.* 46:2640–2646. http://dx.doi.org/10.1016/j.molimm.2009.04.031

Yu, L., L. Wang, M. Li, J. Zhong, Z. Wang, and S. Chen. 2010. Expression of toll-like receptor 4 is down-regulated during progression of cervical neoplasia. *Cancer Immunol. Immunother.* 59:1021–1028. http://dx.doi.org/10.1007/s00262-010-0825-1

Zauner, L., G.T. Melroe, J.A. Sigrist, M.P. Rechsteiner, M. Dorner, M. Arnold, C. Berger, M. Bernasconi, B.W. Schaefer, R.F. Speck, and D. Nadal. 2010. TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. *Oncogene*. 29:4588–4598. http://dx.doi.org/10.1038/onc.2010.203

Zhang, B., R.N. Laribee, M.J. Klemsz, and A. Roman. 2004. Human papillomavirus type 16 E7 protein increases acetylation of histone H3 in human foreskin keratinocytes. *Virology*. 329:189–198. http://dx.doi.org/10.1016/j.virol.2004.08.009