JVI Journals.ASM.org

The Transcription Factors TBX2 and TBX3 Interact with Human Papillomavirus 16 (HPV16) L2 and Repress the Long Control Region of HPVs

Marc A. Schneider,[a] Konstanze D. Scheffer,[a] Timo Bund,[a,d] Fatima Boukhallouk,[a] Carsten Lambert,[a] Cristina Cotarelo,[b] Gert O. Pflugfelder,[c] Luise Florin,[a] Gilles A. Spoden[a]

Department of Microbiology and Hygiene, University Medical Center of Johannes Gutenberg University Mainz, Mainz, Germany[a]; Department of Pathology, University Medical Center of Johannes Gutenberg University Mainz, Mainz, Germany[b]; Institute of Genetics, Johannes Gutenberg University Mainz, Mainz, Germany[c]; Max Planck Institute for Polymer Research, Mainz, Germany[d]

The minor capsid protein L2 of human papillomaviruses (HPVs) has multiple functions during the viral life cycle. Although L2 is required for effective invasion and morphogenesis, only a few cellular interaction partners are known so far. Using yeast two-hybrid screening, we identified the transcription factor TBX2 as a novel interaction partner of HPV type 16 (HPV16) L2. Coimmunoprecipitations and immunofluorescence analyses confirmed the L2-TBX2 interaction and revealed that L2 also interacts with TBX3, another member of the T-box family. Transcription of the early genes during HPV infection is under the control of an upstream enhancer and early promoter region, the long control region (LCR). In promoter-reporter gene assays, we observed that TBX2 and TBX3 repress transcription from the LCR and that this effect is enhanced by L2. Repression of the HPV LCR by TBX2/3 seems to be a conserved mechanism, as it was also observed with the LCRs of different HPV types. Finally, interaction of TBX2 with the LCR was detected by chromatin immunoprecipitation, and we found a strong colocalization of L2 and TBX2 in HPV16-positive cervical intraepithelial neoplasia (CIN) I-II tissue sections. These results suggest that TBX2/3 might play a role in the regulation of HPV gene expression during the viral life cycle.

Human papillomaviruses (HPVs) are small, nonenveloped viruses that infect stratified squamous epithelia of the skin or mucous membranes, inducing a variety of hyperproliferative lesions (1). The mucosotropic HPVs are classified as either low-risk or high-risk types. Lesions resulting from high-risk HPV infections (e.g., HPV type 16 [HPV16], HPV18, and HPV31) can progress to cervical and other cancers (2, 3). The icosahedral capsid of HPVs consists of 360 copies of the major capsid protein L1 (4, 5) and up to 72 copies of the minor capsid protein L2 (6). During HPV infection, L2 is required for the egress of the viral genome from endosomes (7) and for the dynein-mediated transport of the viral genome along microtubules to the nucleus (8–10). In the final steps of viral entry, L2 chaperones the viral genome to nuclear domain 10 (ND10), where initial transcription of the viral genes takes place (11). Transcription of the early viral proteins E6 and E7, representing the transforming oncogenes in high-risk HPVs, is controlled by an upstream enhancer sequence, the long control region (LCR) (also called the upstream regulatory region [URR]), which comprises the early promoter. A complex array of cellular and viral factors binds to the LCR and regulates expression of the early viral genes (12–21). The composition of the factors binding and regulating the LCR is dependent on differentiation of the host keratinocytes (22, 23). In the granular cell layers, the activation of the late promoter drives the expression of the capsid proteins L1 and L2 (24–26). Besides its importance in viral entry, L2 plays a major role during virus morphogenesis. L2 reorganizes ND10 protein composition and recruits L1 into these subnuclear structures, promoting assembly of papillomaviruses (27, 28). In addition, L2 might be required for efficient DNA packaging into HPV virions (29–31).

T-box proteins are transcription factors with a highly conserved DNA-binding domain, the T box, which recognizes a palindromic DNA sequence or the corresponding half-site (32, 33). The family of T-box genes contains 16 members in humans, which are classified into five subgroups, T, TBR1, TBX1, TBX2, and TBX6 (34). T-box proteins play important roles in metazoan development (35). TBX2 and TBX3 are members of the TBX2 subfamily and act as transcriptional repressors (36, 37). Both were first reported to be involved in embryonic development. TBX2 especially influences the development of the heart, limbs, and mammary glands (38–40). Haploinsufficiency of TBX3 results in ulnar-mammary syndrome (41). Recent studies suggest that TBX2 and TBX3 are also associated with cancer development. Overexpression of TBX2 and TBX3 has been found in various cancers, including breast, pancreas, melanoma, ovarian, liver, lung, and cervix cancers (reviewed in reference 42). TBX2 and TBX3 can both function as immortalizing genes, enabling cells to circumvent senescence (43, 44) by repressing $p14^{ARF}$/$p19^{ARF}$ (44, 45) and $p21^{CIP1}$ (46). Other genes repressed by TBX2/3 are the TRP-1, Connexin 43, NDRG1, and E-cadherin genes (36, 47–49). A recent study identified further potential targets regulated by TBX2 and showed that interaction of TBX2 with the retinoblastoma protein Rb1 enhances TBX2 DNA binding and transcriptional repression (50).

In this study, we identified the transcription factors TBX2 and

Received 17 July 2012 Accepted 28 January 2013
Published ahead of print 6 February 2013
Address correspondence to Luise Florin, lflorin@uni-mainz.de, or Gilles A. Spoden, gilles.spoden@gmx.net.

doi:10.1128/JVI.01803-12

TBX3 as novel cellular interaction partners of the HPV16 capsid protein L2. In addition, promoter-reporter gene assays showed that TBX2 and TBX3 repress the LCRs of different HPV types and that this effect is enhanced by L2. The results suggest that TBX2 and/or TBX3 is involved in the control of HPV transcription.

MATERIALS AND METHODS

Plasmids. The pT-REx-DEST30/TBX2-3×FLAG and pT-REx-DEST30/TBX3-3×FLAG expression vectors were constructed by A. Legler (unpublished data). FLAG-tagged expression vectors for the murine Tbx2 wild type (wt) and the DNA-deficient binding mutant Tbx2 R122/123E (49) were provided by Colin R. Goding, Oxford, United Kingdom. To construct a yellow fluorescent protein (YFP)-L2 expression vector, L2 was amplified from codon-optimized HPV16 L2 (51) using the forward primer 5′-AAAGGATTCACCATGAGGCACAAGAGG-3′ and the reverse primer 5′-AAAGGATTCGGCGGCCAGGCTCAC-3′. The DNA fragment was inserted into the BamHI site of pEYFP-C1 (Clontech). $L2_{1-380}$ was amplified with the forward primer 5′-AAAGAATTCATGGACCCCG ACTTCCTGGAC-3′ and the reverse primer 5′-TTTCTCGAGTCAGGT GGTGCTGGTGTCGG-3′ and inserted into the EcoRI-XhoI site of pcDNA3.1(+) (Invitrogen). pdEYFP-C1amp/TBX2 was obtained from imaGenes. The promoter reporter plasmids pGL3-P_{97} (19), pGL3 HPV16 LCR (12), pGL4.20 HPV11 LCR (Addgene plasmid 22858), and pGL4.20 HPV18 LCR (Addgene plasmid 22859) (52) were provided by Iwao Kukimoto, Tokyo, Japan; Lucia Pirisi, Columbia; and Peter M. Howley, Boston, MA. pGL4.20 HPV16 LCR was cloned by isolating the HPV16 LCR (12) with HindIII, blunting it with Klenow, and ligating it into the pGL4.20 SacI-BglII site (Klenow blunted). To monitor transfection efficiencies, the pSV-β-galactosidase control vector (Promega) was used. Cloning details and primer sequences for generation of the LCR deletion mutant reporter vectors are available on request.

Antibodies. LexA-L2 280 to 473 bait expressed in yeast strain L40 was detected with monoclonal anti-LexA antibody (sc-7544; Santa Cruz Biotechnology) and hemagglutinin (HA)-tagged TBX2 prey with monoclonal anti-HA antibody (16B12; Covance). For detection of YFP and YFP fusion proteins after Western blotting, the monoclonal antibody Jl-8 (Clontech) was used. FLAG-tagged proteins were stained with polyclonal anti-FLAG antibody (Rockland) in immunofluorescence assays and with monoclonal anti-FLAG (M2; Sigma-Aldrich) in immunoblots. L2 was stained with mouse monoclonal L2-1 antibody (53), and detection of β-actin as an input control in Western blots was done with monoclonal anti-β-actin antibody (AC-15; Sigma-Aldrich). Promyelocytic leukemia protein (PML) bodies were detected with monoclonal mouse anti-PML (PG-M3; Santa Cruz). Immunofluorescence staining of TBX2 in tissue sections was done with polyclonal rabbit anti-TBX2 antibody (HPA008586; Sigma-Aldrich), and L1 was detected with a polyclonal anti-L1 antiserum (K75) (54). HPV18 E6 Western blots on polyvinylidene difluoride (PVDF) were probed with a monoclonal mouse anti-HPV18 E6 antibody (sc-365089; Santa Cruz Biotechnology) and Western blots of endogenous TBX2 with a polyclonal goat anti-TBX2 antibody (sc-17880). Highly cross-absorbed Alexa Fluor-conjugated secondary antibodies for immunofluorescence staining were purchased from Invitrogen.

Yeast two-hybrid screening. Yeast two-hybrid screening with L2 (amino acids 280 to 473) as bait and with a cDNA library (pJG4-5) derived from human serum-starved WI-38 fibroblasts (55) was performed with the Hybrid Hunter two-hybrid system (Invitrogen) and has been described previously (10).

Cell culture and transfections. The human cervical carcinoma cell line HeLa was purchased from the German Resource Centre for Biological Material (DSMZ). HaCaT cells (human non-virus-transformed keratinocytes) were obtained from Cell Lines Services (CLS), Eppelheim, Germany. The human cervical carcinoma cell lines SiHa and CaSki were kindly provided by W. Zwerschke, Institute for Biomedical Aging Research, Innsbruck, Austria, and the human melanoma cell line WM266-4 was provided by M. Eisenhut, German Cancer Research Center (DKFZ), Heidelberg, Germany. The cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% Glutamax I (Invitrogen), 1% modified Eagle's medium, nonessential amino acids, and antibiotics. Normal human epidermal keratinocytes (NHEK) were purchased from PromoCell and cultivated according to the manufacturer's instructions. JetPEI DNA Transfection Reagent (Polyplus Transfection) was used for transfections according to the manufacturer's instructions.

Immunofluorescence microscopy. Cells were grown and transfected on coverslips; 24 h after transfection, the cells were washed and fixed with methanol (−20°C; 10 min). The fixed cells were washed three times with phosphate-buffered saline (PBS) and blocked for 30 min with PBS, 1% bovine serum albumin (BSA). The coverslips were incubated for 1 h at 37°C with the indicated primary antibodies. After washing with PBS, the coverslips were again blocked for 10 min with PBS-BSA and subsequently incubated at 37°C with Alexa-conjugated species-specific secondary antibodies (Invitrogen) for 45 min. The DNA was stained with Hoechst 33342 (Sigma) and is shown in blue. The coverslips were washed with PBS-BSA and PBS and mounted onto slides using Fluoprep mounting medium (bioMérieux). Images were acquired using a Zeiss Axiovert 200 M microscope equipped with a Plan-Apochromat 1006 (1.4 numerical aperture [NA]) and a Zeiss Axiocam digital camera. Axiovision software 4.7 was used for merging images. Images were deconvoluted using the software supplied by Zeiss (Axiovision 4.7). Tiffs were assembled into figures using Photoshop CS (Adobe).

Immunostaining of tissue slices. HPV16-positive paraffin-embedded cervix tissue slices were deparaffinized with Xylol two times for 20 min each time and rehydrated in decreasing concentrations of ethanol (90, 70, and 50%) for 10 min each. The slices were rinsed with water and boiled 3 times for 30 s in 10 mM citric buffer (pH 6.0). After that, the slices were washed with PBS for 15 min and blocked with PBS-BSA (1%) for 1 h. The staining was performed as described above, except that the first antibody was incubated overnight at 4°C. Images were acquired using a Zeiss Axiovert 200 M microscope equipped with a Plan-Apochromat 1006 (1.4 NA) and a Zeiss Axiocam digital camera. Axiovision software 4.7 was used for merging pictures. Colocalization of L2 and TBX2 was analyzed with the LSM 710 (Carl Zeiss, Jena, Germany) and the LSM software ZEN 2008.

Coimmunoprecipitation assays. HeLa cells (9×10^5 cells) were seeded on 60-mm dishes and transfected for 24 h with the indicated expression vectors. Immunoprecipitations of YFP and YFP-L2 were performed with a μMACS anti-green fluorescent protein (GFP)-tagged Protein Isolation Kit (MACS molecular) according to the manufacturer's instructions. For immunoprecipitation of FLAG-tagged proteins, transfected cells were washed with PBS and lysed in interaction buffer containing 150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), and protease inhibitor mixture (Halt protease inhibitor cocktail; EDTA free; Thermo Scientific). Lysates were treated 3 time for 20 s each time with an ultrasonifier (30% duty cycle; output control, 30%; Branson Sonifier 250) and incubated for 30 min at 4°C on a rotating wheel. Lysates were centrifuged for 10 min at 10,000 × *g* and 4°C. The lysates were precleared with 50 μl protein A/G agarose (Santa Cruz Biotechnology) and then incubated with 1 μl mouse monoclonal anti-FLAG antibody (Sigma) for 1 h at 4°C on a rotating wheel and for another hour after addition of 50 μl protein A/G agarose. The agarose was washed with wash buffer containing 500 mM NaCl, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), and protease inhibitor mixture. The precipitates were boiled in 2.5× SDS sample buffer and processed for Western blotting.

Luciferase reporter assays. Cells were seeded on 12-well plates and transfected with a total of 1.7 μg of plasmid DNA/well, consisting of 0.5 μg luciferase reporter vector together with one of the following combinations: 1 μg of control vector pT-REx-DEST30, 0.5 μg TBX2/3 plus 0.5 μg pT-REx-DEST30, or 0.5 μg TBX2/3 plus 0.5 μg L2. In serial-dilution assays, decreasing amounts of expression vector were replaced with control vector. To monitor transfection efficiency, 0.2 μg pSV-β-Gal (Pro-

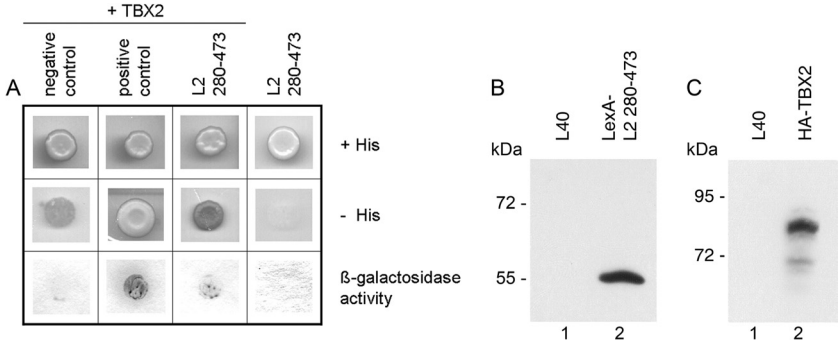

FIG 1 Identification of TBX2 as an HPV16 L2-interacting protein. (A) Yeast strain L40 expressing a negative control (LexA-lamin), a positive control (LexA-Fos2), or the bait LexA-L2 280 to 473 was transformed with the prey construct B42-HA-TBX2 (+ TBX2) or with empty prey vector pJG4-5 (right column). The plates were incubated for 4 days at 30°C. Transformants were tested for the prototrophic marker histidine (− His) and β-galactosidase activity indicating bait-prey interaction. (B) Expression of the bait LexA-L2 280 to 473 (lane 2) in the yeast strain L40 was shown by Western blotting using monoclonal anti-LexA antibody. As a control, lysate of untransformed L40 yeast cells was used in lane 1. (C) Expression of the B42-HA-TBX2 prey fusion protein in L40 yeast cells was shown by Western blotting (lane 2) using anti-HA antibody. Untransformed yeast cells served as a control (lane 1).

mega) or pcDNA3/β-Gal was added to each assay mixture. After 24 h, the cells were lysed with Cell Culture Lysis Reagent (Promega), and the relative luciferase activity was measured with a luciferase assay system (Promega) according to the manufacturer's instructions using the luminometer Lumat LB 9507 (Berthold Technologies). Lysates were additionally used to determine β-galactosidase activity with β-Gal-Juice Plus (pjk) as a measure of transfection efficiency. Luciferase activity values were normalized to the β-galactosidase activity for HaCaT cells and NHEK. pSV-β-Gal and pcDNA3/β-Gal were strongly regulated by TBX2/3 in HeLa cells; therefore, luciferase activity was not normalized to β-galactosidase activity in HeLa cells. However, as normalization did not affect the values in HaCaT cells and NHEK and HeLa transfections were done in parallel with HaCaT cells and NHEK, using the same transfection master mixes, we conclude that the values in HeLa cells are valid. Each experiment was done at least in triplicate and repeated at least three times. Statistical significances ($P < 0.05$) were calculated with the *t* test (two-tailed; paired).

ChIP. For chromatin immunoprecipitation (ChIP), HeLa cells were seeded on 15-cm dishes (1.6×10^6 cells/dish) and transfected for 48 h with pT-REx-DEST30 or pT-REx-DEST30/TBX2-3×FLAG. Chromatin preparation and immunoprecipitation were performed with the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling) according to the manufacturer's instructions. Lysates were precleared with protein G magnetic beads (preadsorbed with salmon sperm DNA [Clontech]) for 1 h at 4°C. Part (3%) of the precleared lysates was stored as an input control, and the rest was processed by overnight incubation at 4°C with anti-FLAG (M2; Sigma-Aldrich) or control mouse IgG. Protein G magnetic beads (preadsorbed with salmon sperm DNA) were added, and incubation was continued for 2 h at 4°C. After washing, elution, cross-link reversal, and proteinase K digestion, the precipitated DNA fragments were purified and used as templates for PCR amplification to detect coprecipitation of the HPV18 LCR with TBX2. The forward primer 5′-CTTGTACAACTACTTTCATGTCC-3′ and reverse primer 5′-GCGCCATAGTATTGTGGTGTG-3′ were used to amplify the 246-bp 3′-terminal fragment from nucleotide (nt) 7735 to nt 124 of the HPV18 genome. PCR products were separated by 2% agarose gel electrophoresis. Immunoprecipitation of the HPV16 LCR with endogenous TBX2 was done with SiHa cells in a manner similar to that described for HeLa cells. Endogenous TBX2 of SiHa cells was precipitated with goat anti-TBX2 antibody (Santa Cruz Biotechnology), and the coprecipitated HPV16 LCR was amplified with forward (5′-CCTGTTTTCCTGACCTGCAC-3′) and reverse (5′-GTGACATTTAGTTGGCCTTAGAAG-3′) primers, giving rise to a 220-bp DNA fragment (HPV16 LCR nt 7561 to 7781).

Fluorescence-assisted cell sorting. HeLa cells were transfected for 48 h with YFP or YFP-TBX2, trypsinized, and resuspended in PBS-0.1% FCS-1 mM EDTA. Sorting of transfected cells was done with a BD FACSVantage SE at the FACS and Array Core Facility (FacsLab) of the University Medical Center Mainz. Sorted cells were counted, and equal amounts of cells were processed by Western blotting.

RESULTS

TBX2 binds to HPV16 L2 in a yeast two-hybrid screen. To identify new cellular interaction partners of HPV16 L2, yeast two-hybrid screening was performed. As the full-length L2 exposed transactivating activity and induced expression of the reporter genes in the yeast two-hybrid system (data not shown), we used a C-terminal fragment of L2 (amino acids 280 to 473) fused to the LexA-binding domain as bait. For screening of interaction partners, bait-expressing yeast cells were transformed with a prey cDNA library. Transformants with high expression of the reporter genes were selected, prey plasmids were isolated, and prey cDNAs were identified by sequencing. One of the isolated plasmids contained the full-length cDNA of the transcription factor TBX2. Interaction of L2 and TBX2 in yeast was reproduced by retransforming the corresponding bait and prey vectors into the L40 yeast strain and monitoring expression of reporter genes (Fig. 1A). Yeast cells expressing the L2 bait together with the TBX2 prey were able to grow on histidine-deficient selection agar and expressed β-galactosidase, confirming the L2-TBX2 interaction. Combinations of L2 bait with negative-control prey or TBX2 prey with negative-control bait showed neither growth on selection agar nor expression of β-galactosidase. Expression of the L2 bait (Fig. 1B) and of the TBX2 prey (Fig. 1C) in L40 yeast was controlled by Western blotting.

L2 interacts with TBX2 and TBX3 *in vitro*. Given the high similarity of TBX2 and TBX3, both belonging to the TBX2 subgroup of the T-box family, the subsequent analyses were performed for TBX2 and TBX3. To confirm the interaction of HPV16 L2 with TBX2 and to analyze whether L2 interacts with TBX3, coimmunoprecipitation studies were done. FLAG-tagged TBX2 and TBX3 were coexpressed with YFP-L2 or YFP (control) in HeLa cells. Cell lysates were prepared, and YFP, as well as YFP-L2, was immunoprecipitated. Precipitates and a minor fraction of the cell lysates, as a control for protein expression, were processed by Western blotting with GFP- and FLAG-specific antibodies (Fig. 2A and B). FLAG-specific immunoblotting showed that TBX2 and TBX3 were specifically coprecipitated with YFP-L2

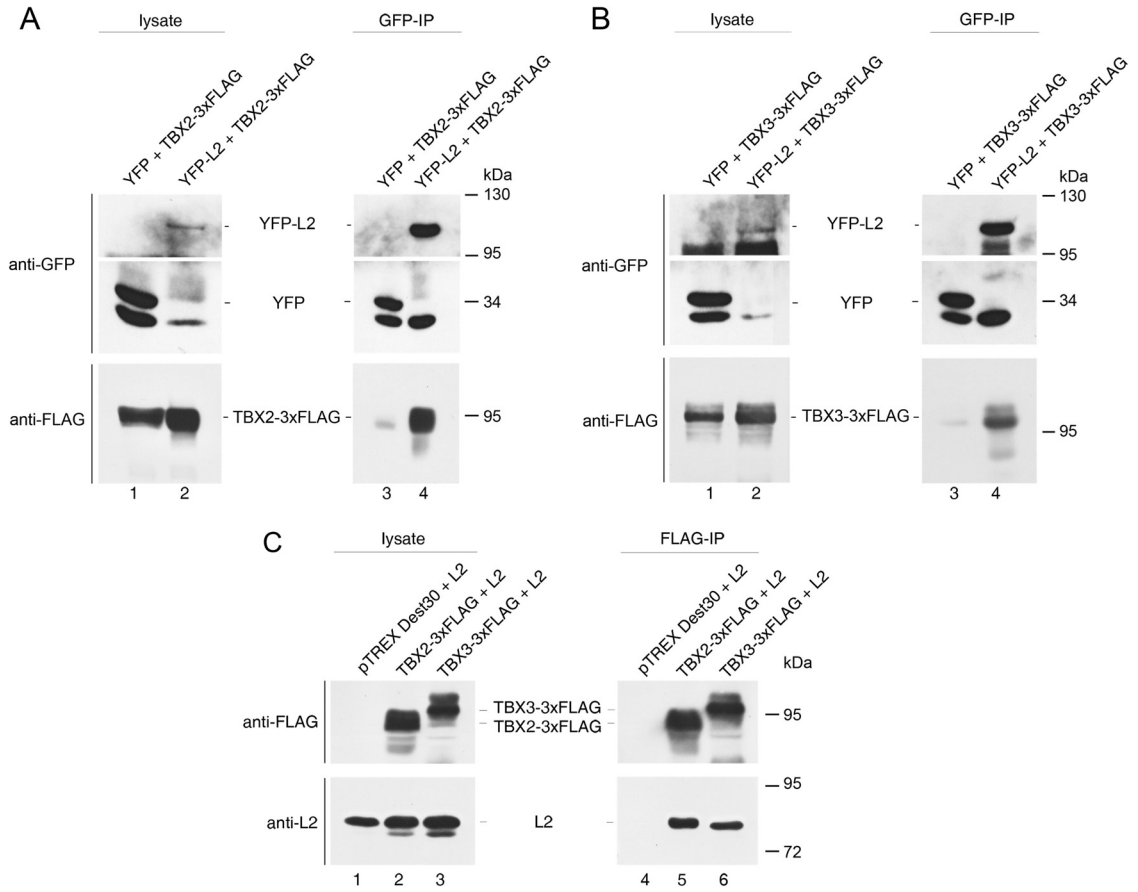

FIG 2 L2 interacts with TBX2 and TBX3 in HeLa cells. Lysates were prepared from HeLa cells transiently cotransfected with YFP or YFP-L2 and TBX2-3×FLAG (A), YFP or YFP-L2 and TBX3-3×FLAG (B), or pT-Rex-Dest30 and L2 or TBX2/3-3×FLAG and L2 (C), as indicated. Protein expression was verified by Western blotting of the lysates with the specified antibodies (A, B, and C, left). The lysates were subjected to immunoprecipitation (IP) with antibodies specific for GFP or FLAG, as indicated. The precipitated proteins were detected by Western blotting using anti-L2, anti-GFP, and anti-FLAG antibodies (A, B, and C, right).

(Fig. 2A and B, lanes 4), but not with the YFP control precipitation (Fig. 2A and B, lanes 3). For the inverse coimmunoprecipitation assay, L2 was coexpressed with FLAG-tagged TBX2 or TBX3 or with an empty control vector in HeLa cells (Fig. 2C, left). TBX2 and TBX3 were precipitated with a FLAG-specific antibody (Fig. 2C, right). Precipitates and expression controls were processed by Western blotting with L2- and FLAG-specific antibodies. L2 was specifically coprecipitated with TBX2 and TBX3 (Fig. 2C, right).

L2 colocalizes with TBX2 and TBX3 in the cell nucleus. To verify the interaction of HPV16 L2 with TBX2/3 in mammalian cells, the distribution of the proteins was analyzed by immunofluorescence deconvolution microscopy in HeLa cells. As HeLa cells do not express endogenous TBX2/3 (36) (see Fig. 4A), we transiently transfected FLAG-tagged TBX2 and TBX3, alone or together with L2, and stained the proteins with FLAG- and L2-specific antibodies (Fig. 3A). Overexpressed TBX2 and TBX3 were predominantly localized in the nucleus independently of L2. Overexpressed L2 that accumulated in ND10 (Fig. 3B), as described previously (11, 27, 28), colocalized only poorly or not at all with TBX2 or TBX3 (Fig. 3A, rows 2 and 4). However, in about 30% of double-transfected cells, L2 was not confined to ND10 but showed extensive colocalization with TBX2 or TBX3 (Fig. 3A, rows 1 and 3). To corroborate the TBX2/L2 colocalization, we analyzed the cellular distribution of L2 in cell lines expressing TBX2 endogenously. It has been shown previously that the human melanoma cell line WM266-4 expresses TBX2 (56) (Fig. 4A and B). We detected in Western blots that TBX2 is also expressed in the human cervical carcinoma cell line SiHa, but to a lesser degree than in WM266-4 cells (Fig. 4A and B). In both cell lines, SiHa and WM266-4, we observed colocalization of ectopically expressed L2 with endogenous TBX2 by immunofluorescence microscopy (Fig. 4B).

To further validate the finding that HPV16 L2 is able to colocalize with TBX2, even in the context of a natural infection, we analyzed the *in vivo* expression patterns of L2 and TBX2 by laser scanning microscopy (LSM) in HPV16-infected cervix tissue. The expression of the early and late genes in HPV-infected tissue is tightly regulated during epithelial cell differentiation (22). In early neoplasia stages, the L1 and L2 late genes are expressed in the granular epidermis. Therefore, we typed cervical intraepithelial neoplasia (CIN) I-II tissue slices for HPV16 infection by PCR (data not shown) and for expression of late viral genes by staining with L1- and L2-specific antibodies (Fig. 4D). HPV16-positive slices were costained for TBX2 and L2 and analyzed by laser scanning microscopy (Fig. 4C). TBX2 was easily detectable in cell nuclei of the upper cell layers (Fig. 4C, left). In cells of the upper layers coexpressing TBX2 and L2 (Fig. 4D, right), strong colocalization of L2 and TBX2 in nuclei was observed, and L2 staining

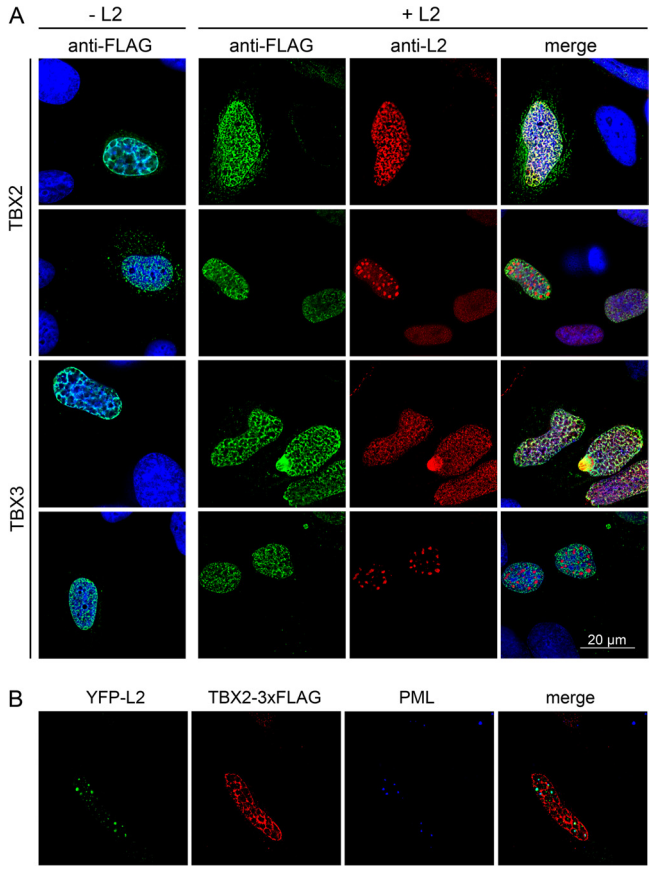

FIG 3 L2 colocalizes with TBX2 and TBX3 in the cell nucleus, but not in ND10. (A) TBX2-3×FLAG and TBX3-3×FLAG were either expressed alone (−L2) or coexpressed with HPV16 L2 (+L2) in HeLa cells for 24 h. The cells were fixed and permeabilized with methanol. Transiently expressed TBX2 or TBX3 was detected with rabbit polyclonal anti-FLAG antibody (green), and L2 was stained with mouse monoclonal anti-L2 antibody (red). DNA was stained with Hoechst and is shown in blue. (B) YFP-L2 was coexpressed with TBX2-3×FLAG in HeLa cells. TBX2 was stained with rabbit polyclonal anti-FLAG antibody (red) and endogenous PML with a specific mouse monoclonal antibody (blue).

showed no PML-like distribution, as previously described (57, 58). These data suggest that HPV16 L2 and TBX2/3 also interact *in vivo* in the context of a naturally occurring HPV16 infection.

TBX2 and TBX3 repress the long control region of HPV16. Since TBX2 and TBX3 are transcription factors, we were interested to see whether the two proteins regulate HPV gene expression. Expression of the HPV early genes E6 and E7 is under the control of the noncoding LCR, which comprises the early promoter. To monitor possible effects of TBX2/3 on HPV early-gene expression, we performed promoter-reporter gene assays with pGL3-P_{97} (19), a reporter vector with luciferase under the control of the HPV16 LCR. The vector was cotransfected with either a control plasmid or a TBX2 or TBX3 expression plasmid into HeLa and HaCaT cells and primary NHEK. Twenty-four hours posttransfection, the cells were lysed, and the relative luciferase quantity was assessed. TBX2 and TBX3 significantly repressed the LCR activity to 18 to 44% or 35 to 66% compared to controls, depending on the cell type (Fig. 5). Interestingly, inhibition of LCR was even more pronounced when TBX2 or TBX3 was coexpressed with L2, while expression of L2 alone had divergent effects on the LCR depending on the cell type. In HeLa cells, the LCR was strongly activated by L2, while it was weakly repressed in HaCaT cells and NHEK. Expression of TBX2, TBX3, and L2 was controlled by immunoblotting (Fig. 5). These results were reproduced with a second reporter vector, pGL3 HPV16 LCR (59), and also with the cell lines MCF-7, COS-7, and 293TT (data not shown). In concentration series of TBX2 or TBX3 cotransfected with pGL3-P_{97}, we observed that small amounts of TBX2 repressed the LCR significantly in HeLa and HaCaT cells while larger amounts of TBX3 were required for a similarly efficient repression of LCR activity (Fig. 6). It must be mentioned that all LCR activity assays were directly normalized by a β-Gal transfection control vector, except for the assays in HeLa cells. Indeed, we observed a strong regulation of transfection control vectors by TBX2/3 in HeLa cells. While expression under the control of a simian virus 40 (SV40) promoter (pSV-β-Gal) was repressed by TBX2/3, expression from a cytomegalovirus (CMV)-driven vector (pcDNA3/β-Gal) was strongly enhanced by TBX2/3 in HeLa cells (up to 14-fold) (data not shown). TBX2/3 might act in *cis* on the viral promoters of control vectors, but it is also conceivable that it acts in *trans*. As we show below (see Fig. 10), expression of the early genes of the integrated HPV18 genome in HeLa is highly regulated by TBX2. It has been previously observed that some viral promoters, among them the SV40 promoter, are regulated by HPV E6 (60). Hence, regulation of HPV18 early genes in HeLa cells by TBX2 might successively affect the promoter activity of control vectors. The mechanism underlying the regulation of SV40 and CMV promoters by TBX2 in HeLa cells is beyond the scope of this study. In order to control/normalize the transfection efficiencies of LCR assays in HeLa cells, transfections were done in parallel in HaCaT and HeLa cells from the same transfection master mixes, and LCR activity in HeLa cells was monitored by transfection efficiencies in HaCaT cells.

The L2 C terminus binds to TBX2 and enhances LCR repression. Using a deletion mutant of L2, we were able to confine the L2-TBX2 interaction domain to the C terminus of L2. HeLa cells were cotransfected with TBX2-3×FLAG and L2 or $L2_{1\text{-}380}$ and subsequently processed by FLAG-specific immunoprecipitations. In contrast to full-length L2, the C-terminal deletion mutant $L2_{1\text{-}380}$ was not coprecipitated by TBX2 (Fig. 7A, right). This suggests that the domain required for binding to TBX2 is located within the stretch of 93 amino acids at the 3′ end of L2. This is also consistent with our findings in the yeast two-hybrid assay, where TBX2 bound to an L2 bait comprising amino acids 280 to 473 of L2 (Fig. 1A). As we had already observed that L2 enhanced the repressive activity of TBX2 on the LCR (Fig. 5), we analyzed whether this effect is abolished with $L2_{1\text{-}380}$, which did not interact with TBX2. The pGL3-P_{97} vector was cotransfected with TBX2, L2, or $L2_{1\text{-}380}$, alone or in combination, into HaCaT cells, and luciferase was measured 24 h after transfection (Fig. 7A and C, left). Both L2 and $L2_{1\text{-}380}$ exercised a moderate but significant repressive effect on the LCR. The strong repression of LCR by TBX2 was even significantly enhanced by L2, but not by $L2_{1\text{-}380}$. This suggests that the cumulative repression of the LCR mediated by the coexpression of TBX2 and L2 is dependent on the TBX2-L2 interaction, as it is not observed with the TBX2 binding-deficient mutant $L2_{1\text{-}380}$.

A DNA-binding-deficient murine Tbx2 is impaired in repressing the LCR. The arginines in positions 122 and 123 of TBX2 are essential for DNA binding (61, 62). We analyzed the effects of murine wt Tbx2 and DNA-binding-deficient Tbx2 R122/123E

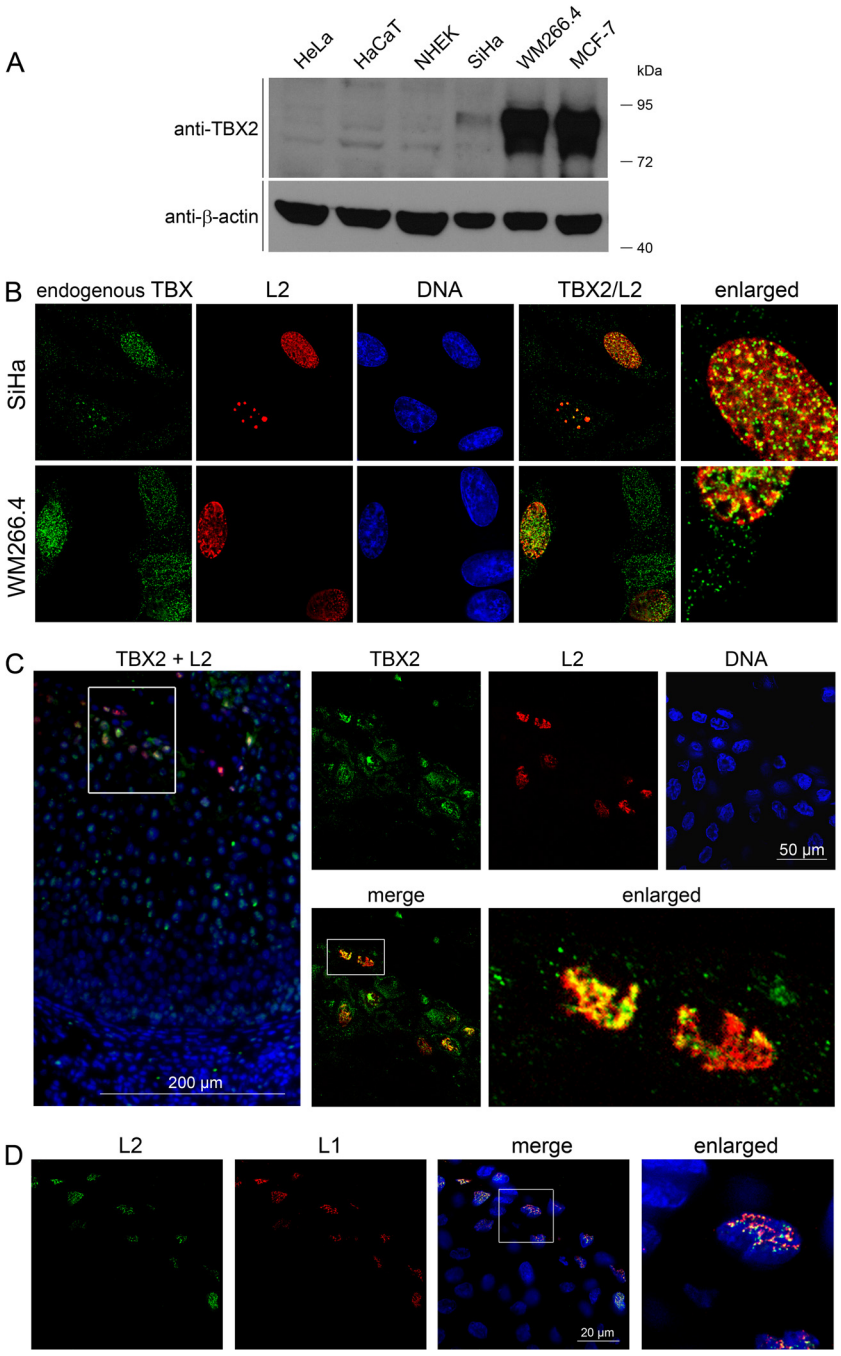

FIG 4 Colocalization of HPV16 L2 with endogenous TBX2. (A) Expression of TBX2 in different cell lines was controlled by Western blotting. Cells were lysed in SDS sample buffer and subsequently processed by SDS-PAGE and immunoblotted with a goat anti-TBX2 antibody and a mouse anti-β-actin antibody. (B) SiHa and WM266-4 cells were transfected with L2 for 24 h and processed by immunofluorescence staining. L2 was detected with a monoclonal mouse anti-L2 antibody (red), and endogenous TBX2 was detected with a polyclonal rabbit anti-TBX2 antibody (green). The DNA of nuclei was stained with Hoechst 33342 (blue). (C) HPV16-positive, paraffin-embedded cervical intraepithelial neoplasia tissue was deparaffinized, rehydrated, and stained with rabbit polyclonal anti-TBX2 antibody (green) and with mouse monoclonal anti-L2 antibody (L2-1) (red). DNA was stained with TO-PRO-3 (blue). (D) As in panel C, L1 was stained with rabbit polyclonal anti-L1 antibody (K75) (red) and L2 was detected with mouse monoclonal anti-L2 antibody (L2-1) (green). DNA was stained with Hoechst (blue). The boxed areas are shown enlarged on the right.

(49) on the transcriptional activity of the LCR in promoter-reporter gene assays (Fig. 7B, left, and C, right). The pGL3-P_{97} vector was cotransfected with a control or different TBX2 constructs, alone or in combination with L2, into HaCaT cells, and luciferase was measured 24 h after transfection. Similar to human TBX2, the murine Tbx2 repressed LCR activity significantly (Fig. 7B, left), and again, this repression was enhanced by coexpression of L2. In contrast, the DNA-binding-deficient Tbx2 R122/123E did not repress LCR activity on its own. However, coexpression of Tbx2 R122/123E with L2 led to a repression significantly stronger than

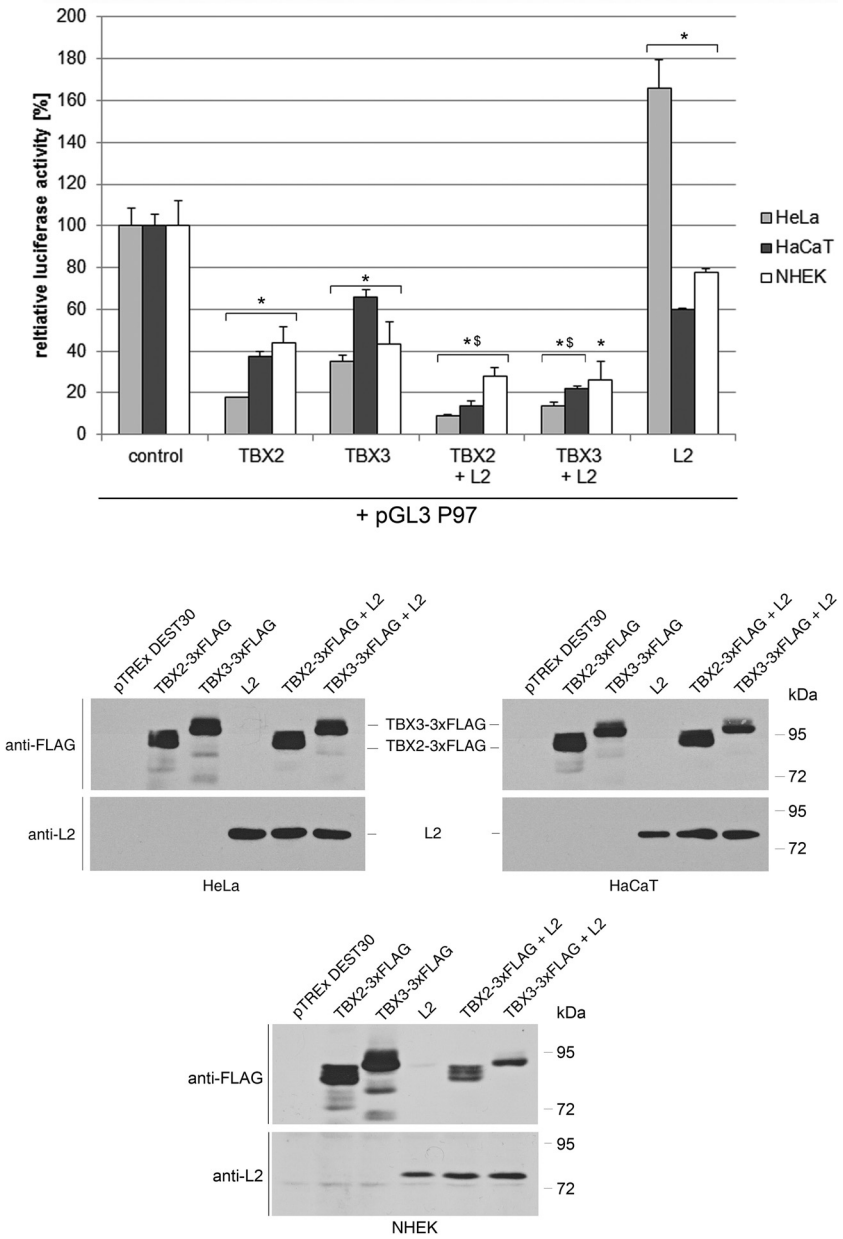

FIG 5 TBX2 and TBX3 repress the long control region of HPV16. HeLa and HaCaT cells and NHEK were transiently transfected for 24 h with 0.5 μg luciferase reporter vector containing the HPV16 long control region as a promoter (pGL3-P_{97}), together with 1 μg control vector or a combination of 0.5 μg TBX2/3 vector with 0.5 μg control vector, with 0.5 μg L2 vector, or, finally, with 0.5 μg L2 combined with 0.5 μg control vector. β-Galactosidase reporter plasmid (0.2 μg) was cotransfected to control transfection efficiency. Cells were lysed, and luciferase activity and β-galactosidase activity were determined. The relative luciferase quantity with control vector pT-Rex-DEST30 was set to 100%. Four individual experiments are represented as means and standard deviations (SD). *, $P < 0.05$ compared to the control; $, $P < 0.05$ compared to TBX2 or TBX3 without L2. Lysates of luciferase reporter assays were processed by Western blotting. FLAG-tagged TBX2 and TBX3 were detected with monoclonal anti-FLAG antibody and L2 with a monoclonal anti-L2 antibody (L2-1).

that by L2 alone. This suggests that Tbx2 represses the HPV16 LCR by direct interaction but is also able to enhance the L2-mediated LCR repression, probably through recruitment of Tbx2 to the LCR by L2.

Using immunofluorescence microscopy, we analyzed the cellular distribution of the murine Tbx2 constructs with regard to HPV16 L2 (Fig. 7D). Both Tbx2 wt and Tbx2 R122/123E showed the same nuclear localization patterns as human TBX2 when expressed alone (Fig. 7D, left). Wild-type Tbx2 also behaved like human TBX2 when coexpressed with L2, and only a small amount of colocalization of Tbx2 with L2 in ND10 was observed; however, increased colocalization took place when L2 did not accumulate in ND10 (Fig. 7D, rows 1 and 2). In contrast, DNA-binding-deficient Tbx2 R122/123E also nearly completely colocalized with L2 in ND10 (Fig. 7D, row 3), suggesting that Tbx2 incapable of direct DNA binding is recruited into ND10 by L2. Interaction of L2 with the Tbx2 R122/123E mutant was also observed in coimmunoprecipitations (Fig. 7B, right).

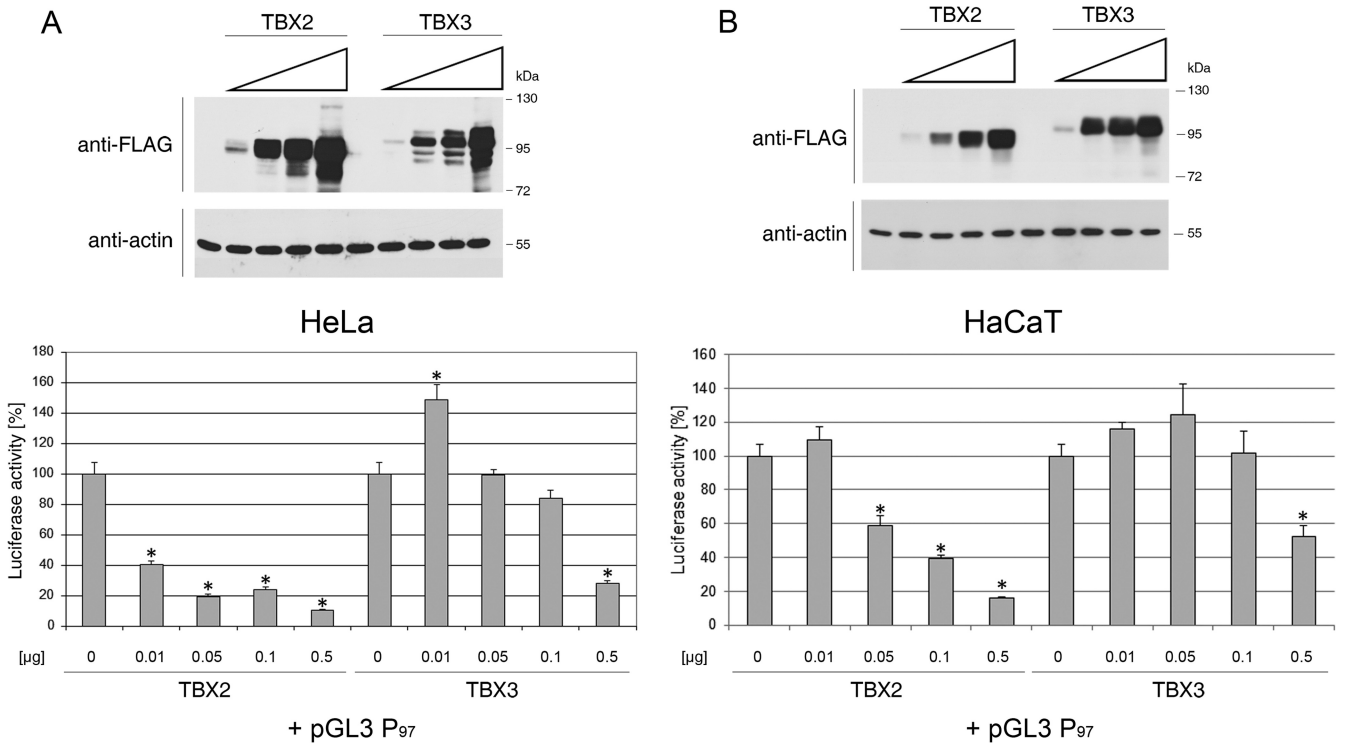

FIG 6 TBX2 and TBX3 dose-dependent regulation of LCR. HeLa and HaCaT cells were transiently transfected for 24 h with 0.5 μg luciferase reporter vector containing the HPV16 LCR. The cells were cotransfected with increasing amounts of TBX2 or TBX3. The cells were lysed to quantify luciferase activity and to monitor TBX2 and TBX3 expression by immunoblotting. β-Actin was detected as a loading control. The relative luciferase activity of pGL3-P_{97} with control vector pT-REx-DEST30 was set to 100%. Three individual experiments are represented as means and SD. *, $P < 0.05$.

The major element required for repression by TBX2 is located between nt 7450 and 7760 of the HPV16 LCR. In order to confine the LCR regulated by TBX2, a series of 5′ deletion sequences of the HPV16 LCR were generated and ligated into the pGL3 reporter gene vector upstream of luciferase. The activity of the LCR deletion mutants with or without TBX2 was analyzed in HaCaT cells (normalized by β-Gal) (Fig. 8). The data indicate that the LCR sequence between nt 7564 and 7756 comprises the element required for repression by TBX2. A DNA fragment (LCR 7450 to 7760) encompassing the repression-relevant region 7564 to 7756 but lacking the HPV16 LCR promoter fortuitously exhibited intrinsic promoter activity in the context of pGL3-basic and was repressed by TBX2 to an extent similar to that of the other constructs containing this region (data not shown). This confirmed the presence of a TBX2-responsive element in the 7450-to-7760 interval.

TBX2 and TBX3 also repress the LCRs of other HPV types. In order to check whether repression of the HPV16 LCR by TBX2 and TBX3 might be a conserved mechanism, we analyzed the effects of TBX2 and TBX3 on the LCRs of other HPV types. Promoter-reporter gene vectors containing the LCR of HPV type 11, 16, or 18 were cotransfected with a control, TBX2, or TBX3 vector into different cell types, and luciferase was measured after 24 h as a readout of LCR activity. In all three cell lines, HeLa, HaCaT, and NHEK, TBX2 and TBX3 significantly inhibited the LCRs of all three HPV types (Fig. 9). Inhibition of the three LCRs was stronger with TBX2 than with TBX3. Repression of the HPV LCR by TBX2/3 seems to be a general mechanism, as TBX2 and TBX3 significantly inhibited the LCRs of high-risk (22, 63) and low-risk (14) HPV types.

TBX2 represses the integrated HPV18 LCR of HeLa cells. After showing that TBX2 represses the LCRs of different HPV types, we analyzed whether TBX2 might also affect the LCR activity and early gene expression of integrated HPV genomes. HeLa cells are transformed by the stable integration of HPV18 genomes and the constitutive expression of the HPV18 early genes E6 and E7. To check the effect of TBX2 on the integrated HPV18 LCR, HeLa cells were transfected for 48 h with TBX2, and the expression of HPV18 E6 was monitored by Western blotting (Fig. 10A). Expression of TBX2 alone and, to a greater extent, coexpression of TBX2 with L2 significantly reduced E6 levels in HeLa cells compared to control transfections. In contrast, L2 expression alone did not significantly affect E6 expression levels. To account for transfection efficiencies, the effect of TBX2 on E6 levels was analyzed in 100% transfected HeLa cells by means of a fluorescence-activated cell sorting (FACS) approach. HeLa cells were transfected with YFP as a control or with YFP-TBX2 for 48 h, positive cells were isolated by FACS, and subsequently, an equal number of sorted cells were subjected to E6 Western blotting (Fig. 10B). E6 was strongly reduced in HeLa cells expressing YFP-TBX2 compared to YFP-transfected cells, most probably resulting from LCR inhibition by TBX2.

TBX2 interacts with the LCR. To investigate whether TBX2 interacts with the LCR, we performed ChIP assays in HeLa cells that had stably integrated the HPV18 genome (with the corresponding LCR) (64). HeLa cells were transfected with FLAG-

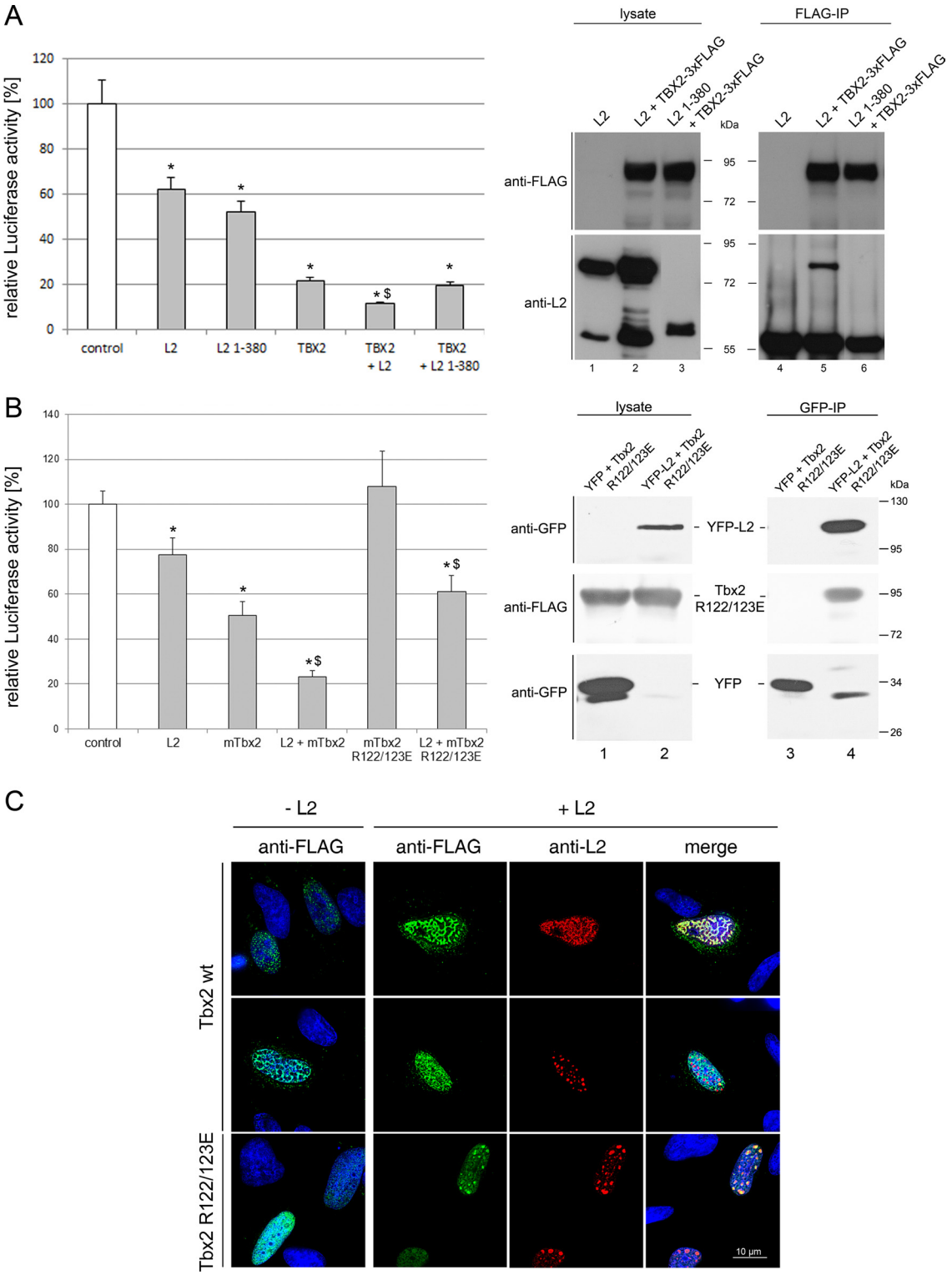

FIG 7 The L2 C terminus is required for TBX2 interaction and cumulative LCR repression. DNA-binding-deficient Tbx2 R122/123E does not repress the LCR but still interacts and colocalizes with L2. (A) (Left) HaCaT cells were transiently transfected for 24 h with the LCR-luciferase vector pGL3-P_{97}, together with the indicated control and/or expression vectors encoding L2, $L2_{1-380}$, or TBX2. The cells were lysed, and the luciferase activity was measured. The relative luciferase quantity with control vector pT-Rex-DEST30 was set to 100%. Three individual experiments are represented as means and SD. *, $P < 0.05$ compared to the control. $, $P < 0.05$ compared to TBX2 alone. (Right) Lysates for immunoprecipitation were prepared from HeLa cells transiently cotransfected as indicated. Protein expression was verified by Western blotting of the lysates with the specified antibodies. The lysates were subjected to immunoprecipitation with anti-FLAG antibody. The precipitated proteins were detected by Western blotting using anti-L2 and anti-FLAG antibodies. (B) (Left) As in panel A, left, with the indicated control and/or expression vectors encoding Tbx2, Tbx2 R122/123E, or L2. *, $P < 0.05$ compared to the control. $, $P < 0.05$ compared to L2 alone. (Right) As in panel A, right, with indicated antibodies. (C) For immunofluorescence analyses, HeLa cells were either transiently transfected with expression vectors for FLAG-tagged murine Tbx2 and Tbx2 R122/123E alone (column 1) or together with HPV16 L2 (columns 2 to 4). After 24 h, the cells were fixed and permeabilized with methanol. Transiently expressed murine Tbx2 or the mutant Tbx2 R122/123E was detected with rabbit polyclonal anti-FLAG antibody (green), and L2 was stained with mouse monoclonal anti-L2 antibody (red). DNA was stained with Hoechst and is shown in blue.

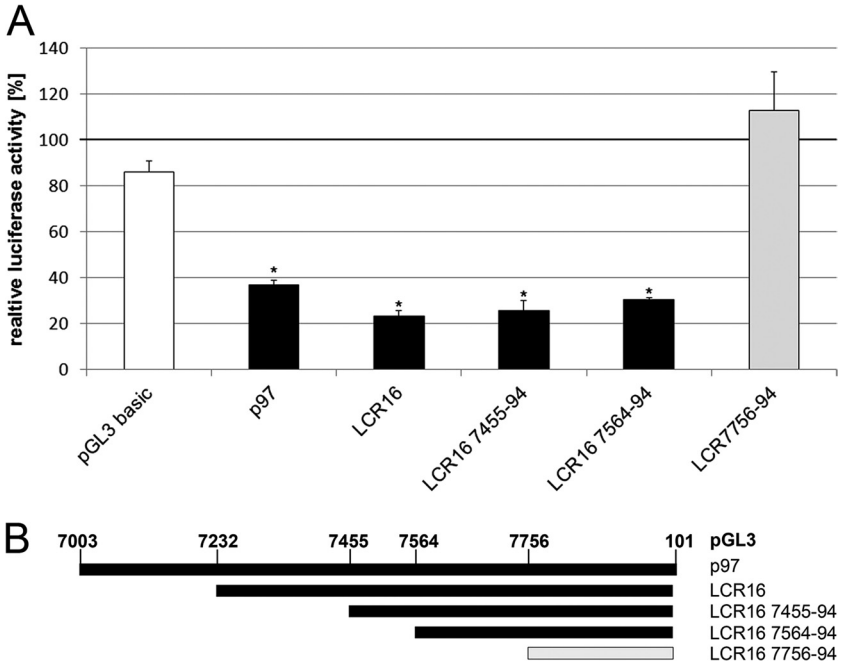

FIG 8 Mapping of the TBX2-responsive element within the HPV16 LCR. (A) The empty pGL3 reporter vector (pGL3-basic) or vector containing different sequences of the HPV16 LCR (B) was cotransfected with the control vector pT-Rex-DEST30 or with TBX2 and pSV-β-Gal into HaCaT cells. Twenty-four hours posttransfection, cells were lysed, and luciferase activity was measured and normalized with β-Gal. The relative luciferase activity of transfections with the control vector was set to 100% (thick line). The bars represent the relative luciferase activities of the corresponding transfections with TBX2. The results of three individual experiments are represented as means and SD. *, $P < 0.05$ compared to the control.

tagged TBX2 or a control vector. Forty-eight hours later, the cells were cross-linked with formaldehyde and lysed. Immunoprecipitations of DNA-protein complexes were done using either mouse anti-FLAG antibody or control mouse IgG. In the precipitates, a DNA fragment covering nucleotides 7735 to 124 of the HPV18 genome (a 247-bp 3′ segment of the HPV18 LCR) was amplified by PCR and analyzed by agarose gel electrophoresis (Fig. 11A). In cells transfected with TBX2, the DNA fragment was detectable in the precipitate with specific FLAG antibody (Fig. 11A, lane 5), but not with control IgG (lane 4). In precipitates with specific FLAG antibody in control-transfected cells, the DNA fragment was also not detectable (Fig. 11A, lane 2). This indicates that TBX2 specifically interacts with the HPV18 LCR. To analyze whether L2 impacts the ability of TBX2 to bind to the LCR, HeLa cells were transfected with FLAG-tagged TBX2 alone or in combination with L2 and subsequently subjected to ChIP with anti-FLAG antibody. Coexpression of L2 strongly enhanced the interaction of TBX2 with the LCR (Fig. 11B). This suggests that L2 enhances or stabilizes the interaction of TBX2 with the LCR, resulting in the increased inhibition of transcription from the LCR that we observed previously. In addition to ectopically expressed TBX2 in HeLa cells, we performed ChIP in SiHa cells with endogenously expressed TBX2 and an integrated HPV16 LCR. As shown in Fig. 11C, the HPV16 LCR from SiHa cells was specifically immunoprecipitated with endogenous TBX2. Coprecipitation of HPV16 LCR by TBX2 was again more efficient in the presence of L2.

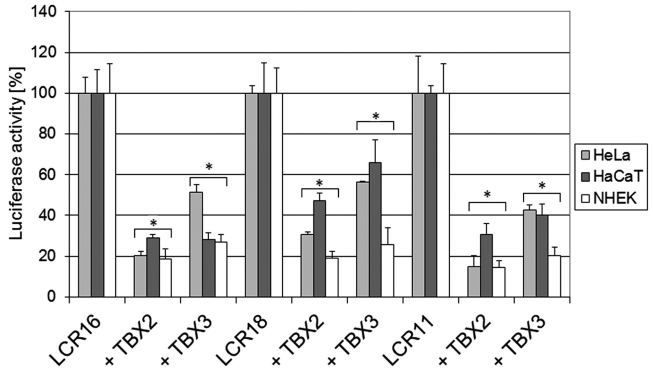

FIG 9 TBX2 and TBX3 also repress the LCRs of other HPV types. HeLa and HaCaT cells and NHEK were transiently transfected with pGL4.20 vector containing either the HPV11, -16, or -18 LCR in combination with control vector or TBX2 or TBX3 expression plasmid. The pSV-β-Gal control plasmid was cotransfected to monitor transfection efficiencies. Cells were lysed, and luciferase and β-galactosidase activities were determined. The relative luciferase quantity of the HPV16, HPV18, or HPV11 LCR constructs with control vector pT-REx-DEST30 was set to 100%. Three individual experiments are represented as means and SD. *, $P < 0.05$.

DISCUSSION

The minor capsid protein L2 of HPVs is functionally required during viral invasion and early steps of infection (7, 10, 11) and also plays a major role in virus morphogenesis at the end of viral replication (27). L2 is probably even involved in regulation of viral gene expression, as it inhibits the transcriptional activation function of E2 (65, 66) and is able to promote viral gene expression (67). Hence, screening for interaction partners of L2 constitutes an obvious strategy to identify new cellular proteins involved in the viral life cycle. In this study, using the yeast two-hybrid system,

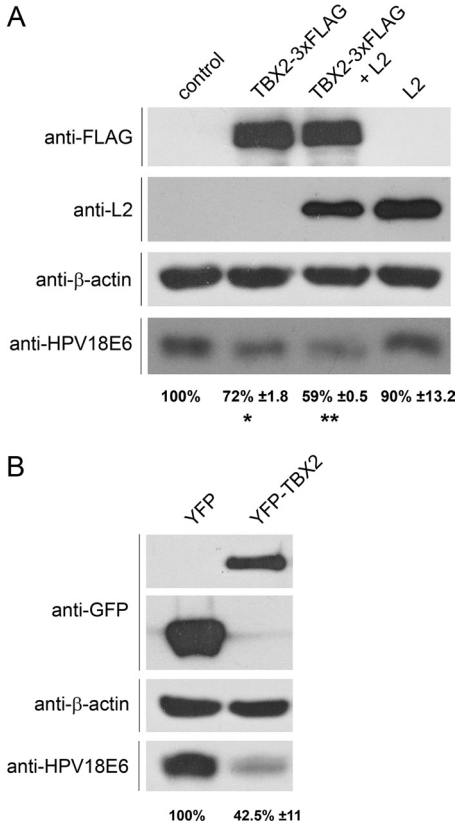

FIG 10 Repression of HeLa-integrated HPV18 LCR by TBX2. (A) HeLa cells were transfected with control, TBX2, TBX2+L2, or L2 vector for 48 h and processed by Western blotting. Expression of TBX2 and L2 was monitored with a mouse monoclonal anti-FLAG or anti-L2 antibody, respectively. β-Actin served as a loading control and was probed with a mouse monoclonal anti-β-actin antibody. HPV18 E6 was detected with a mouse monoclonal anti-HPV18 E6 antibody, and relative band intensities were quantified densitometrically. The E6 expression level of control transfection was set to 100%. Data from two individual experiments are represented as means ± SD. *, $P < 0.05$ compared to the control; **, $P < 0.05$ compared to TBX2 without L2. (B) HeLa cells were transfected with YFP-TBX2 or YFP, as a control, for 48 h. YFP- or YFP-TBX2-positive cells were isolated by FACS and processed by Western blotting. YFP and YFP-TBX2 expression was shown by means of a mouse monoclonal anti-GFP antibody, and β-actin was probed as a loading control. HPV18 E6 was detected and quantified densitometrically as a measure of LCR activity. The E6 level of YFP (control) transfection was set to 100%. Shown are mean values ± SD of two individual experiments.

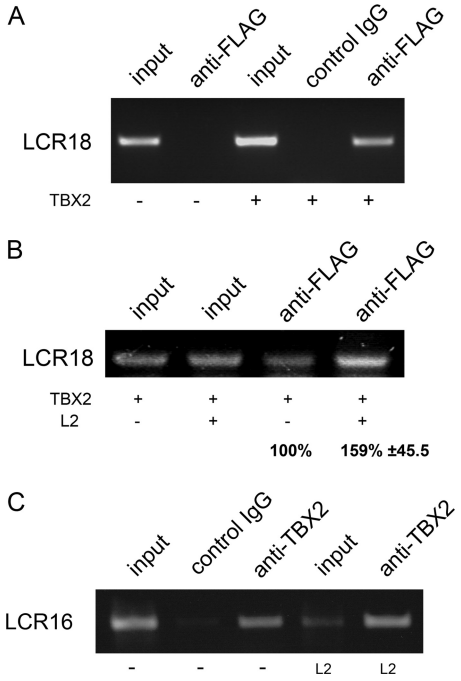

FIG 11 TBX2 interacts with the HPV18 and HPV16 LCRs. (A) For chromatin immunoprecipitation, HeLa cells were transfected for 48 h with control plasmid or TBX2-3×FLAG expression vector. Cells were lysed, and the chromatin was prepared. Chromatin-TBX2 complexes were precipitated with anti-FLAG antibody or normal mouse IgG as a control. Control ChIP with anti-FLAG antibody was also performed with the lysate of control transfected cells. DNA was purified from precipitates as a template for PCR amplification of an nt 7735 to nt 124 LCR fragment. Part of the total chromatin for ChIP was used as input. (B) Effect of L2 on LCR precipitation by TBX2. HeLa cells were transfected with TBX2-3×FLAG together with a control vector or an L2 vector for 48 h and processed by ChIP. Precipitates were quantified densitometrically, and ChIP of TBX2 with the control vector was set to 100%. Shown are mean values ± SD from 3 individual assays. (C) For HPV16 LCR ChIP, SiHa cells were transfected for 48 h with control plasmid or L2 expression vector. Cells were lysed, and the chromatin was prepared. Chromatin-TBX2 complexes were precipitated with anti-TBX2 antibody or normal goat IgG as a control. DNA was purified from precipitates as a template for PCR amplification. Part of the total chromatin for ChIP was used as input.

we found the transcription factor TBX2 to be a novel interaction partner of HPV16 L2. The interaction was confirmed in coimmunoprecipitation assays, and we observed that L2 also binds TBX3, a highly TBX2-related member of the TBX2 subfamily of T-box genes. Several other interaction partners of L2 have been described. The transcription factor Daxx binds to L2, as well as the chaperone protein Hsc70 and the dynein light chains DYNLT1/3 (10, 28, 68). A previous two-hybrid screen with HPV L2 (69) identified several interacting proteins, among them the transcription factor PATZ (70). Although the functions of the L2 interaction partners in the viral life cycle have not yet been defined for all candidates, they all have in common the fact that in cell culture, their interaction with L2 mainly takes place in ND10 of cellular nuclei. In contrast, we found colocalization of ectopically expressed L2 and TBX2/3 in the nuclei of HeLa cells only when L2 was not accumulated in ND10. This is in concordance with a previous study that did not detect localization of TBX2 in ND10 bodies (71) and with the nuclear distribution of L2 in HPV-infected cervix tissue. Indeed, in previous analyses of naturally HPV-infected cells of CIN lesions, L2 showed a punctate distribution and colocalization with Sp100 in only a few cells of the intermediate layers, where L2 was weakly expressed (28). In the stratified cells of the upper layers, L2 distribution in nuclei was diffuse (28) and no longer ND10-like (58). In the tissue stainings of the present study, it was in these cells that we detected TBX2 colocalized with L2. On the other hand, in SiHa cells, an HPV16-transformed cell line expressing endogenous TBX2, we detected colocalization of TBX2 with ectopic L2 also in defined nuclear speckles, probably ND10. This would support a more recent study that has shown that TBX2 is able to interact with PML (72). In addition, a DNA-binding-deficient mutant of Tbx2, Tbx2 R122/123E, was found to colocalize nearly completely with L2 in ND10 of several tested cell lines, in contrast to wild-type Tbx2, which behaved like ectopic human TBX2. It seems that wild-type Tbx2

and TBX2 are not recruited into ND10 by L2 when they are strongly engaged with chromatin. It is conceivable that the localization of the L2-TBX2 interaction, either in ND10 or not, is dependent on the cellular background or is tightly regulated during cell cycle progression. Further investigations are required to unravel the conditions and factors influencing the L2-TBX2 localization and the possible functional consequences.

The minor capsid protein L2 is associated with the viral genome at different stages during the HPV life cycle and regulates viral transcription by binding E2, as stated above. As TBX2 and TBX3 are transcription factors, we were interested in whether they might also have a regulatory effect on the level of HPV gene expression, especially in conjunction with L2. To address this question, we performed luciferase reporter assays with the LCR of HPV16 in the presence and absence of TBX2/3. Expression from the HPV16 LCR was significantly repressed by TBX2 and TBX3 in HeLa and HaCaT cells and NHEK (and other cell lines) (data not shown). LCR transcription repression was significantly enhanced when TBX2 or TBX3 was coexpressed with HPV16 L2, indicating that the TBX2/3-L2 interaction has functional relevance. This was confirmed with the C-terminally truncated, TBX2-binding-deficient $L2_{1-380}$, which, in contrast to L2, did not enhance LCR repression by TBX2. The T-box protein activity and specificity are regulated by protein-protein interactions (42). For example, the interaction of TBX2 with pRb affects TBX2 DNA binding and transcriptional repression (50). Similarly, we observed in ChIP assays that the specific interaction of TBX2 with the HPV LCR is enhanced or stabilized by L2, probably contributing to the increased repression of the LCR by TBX2-L2. All members of the T-box family identified to date have been shown to bind to the core sequence A/GGTGTGA, referred to as the T element (34). The HPV16 LCR does not contain a canonical T element; however, TBX2 and TBX3 were shown to also bind to diverged variants of this motif (61). The DNA-binding-deficient Tbx2 R122/123E mutant, in contrast to the wild-type Tbx2, did not repress the HPV16 LCR. This suggests that TBX2/3 represses the LCR by binding to a noncanonical sequence. In addition, the moderate repression of the LCR by L2 in HaCaT cells was significantly enhanced by the DNA-binding-deficient Tbx2 R122/123E mutant. As L2 interacts with HPV DNA (29, 73), it is conceivable that Tbx2 R122/123E was recruited to the LCR by L2 and exerted a repressive effect on the LCR. This again supports our hypothesis of the functional relevance of the TBX2-L2 interaction. Using deletion mutants in reporter assays, we were able to confine the TBX2-responsive element to a 196-nt sequence within the HPV16 LCR. Repression of the LCR by TBX2/3 might be the result of binding competition with other transcription factors of the LCR. Further studies, for instance, electrophoretic shift assays, are required to determine the LCR core sequence interacting with TBX2.

Repression of the HPV LCR by TBX2/3 seems to be a conserved mechanism, as we were able to reproduce it with the LCRs of three different HPV types, HPV11, -16, and -18. In addition, ectopically expressed TBX2 inhibited the LCR of the integrated HPV18 genome in HeLa cells, as we observed reduced expression levels of the viral E6 protein in TBX2-transfected HeLa cells. A number of studies have explored different approaches (peptide aptamers, RNA interference [RNAi], and E2 expression) to inhibit E6 and E7 function in HPV-transformed cell lines, with different outcomes ranging from growth inhibition through senescence to apoptosis (63, 74–80). Interestingly, inhibition of E6, and probably also E7, expression by TBX2 in HeLa cells did not lead to a significant increase in apoptosis, as tested by propidium iodide and annexin V staining in flow cytometry (data not shown); this might result from compensation by TBX2, which is able to disrupt apoptosis and circumvent senescence. Also, TBX2 might directly replace E6 and E7 functions, as, for instance, both TBX2 and E7 repress p21 (46, 81).

Altogether, our studies revealed two new cellular interaction partners of HPV16 L2. Both have the capacity to influence L2 localization in the cellular nucleus. Further, they repress expression from the HPV LCR, and this effect is enhanced in the presence of L2, indicating a functional relevance of the interaction. In the upper cell layers of infected tissues, TBX2 might constitute a crucial factor to inhibit transcription of viral genes and to initiate a transition to viral morphogenesis/assembly during productive infection. Consistent with this, it has been reported that in productive papillomavirus infection, and also in low-grade squamous intraepithelial lesions, the presence of E7 and of surrogate markers of E7 is absent or diminished in the upper cell layers compared to the basal and suprabasal layers (22, 82–84), probably resulting from reduced P97 activity. In line with this, it has been shown that in differentiated SiHa cells, mimicking the upper tissue cell layers where we detected TBX2 and L2, P97 activity is inhibited (85). This could be the result of an increase in TBX2 levels during differentiation of SiHa cells, similar to our observation that TBX2 is accumulated in the differentiated upper cell layers. Whether TBX2 is supported by L2 in repressing the LCR, as our data suggest, remains to be clarified, as a previous study in raft cultures suggested that the amount of virus production is independent of L2 (86). Future studies in the organotypic raft culture system should therefore shed light on the role of TBX2 alone or in combination with L2 in the viral life cycle. Additionally, a combined regulatory effect of TBX2 and L1 on virus production is conceivable and should be analyzed, as we recently observed that TBX2 and L1 colocalize in cellular nuclei. Furthermore, whether TBX2 or TBX3 also affects viral genome replication and whether other members of the T-box family regulate viral genome transcription/replication must be analyzed.

ACKNOWLEDGMENTS

This work was supported by a grant (SFB490/D2) from the German Research Foundation to L.F. and C.L. and a grant (MAIFOR 2010) from the University Medical Center Mainz to G.A.S.

We thank Iwao Kukimoto, Tokyo, Japan; Lucia Pirisi, Columbia; and Peter M. Howley, Boston, MA, for generously providing plasmid DNA constructs and Colin R. Goding, Oxford, United Kingdom, for vectors and helpful discussions. We thank Teodora Nikolova and Julia Lenz, Mainz, Germany, for assistance with LSM and FACS, respectively. We are grateful to Matthias Klein, Ursula Kurzik-Dumke, Arno Schad, and Matthias Husmann, Mainz, Germany, for advice on methods and helpful discussions and to Anne Legler, Mainz, Germany, for technical support.

REFERENCES

1. **Howley PM, Lowy DR.** 2007. Papillomaviruses, p 2299–2354. *In* Knipe DM, Howley PM (ed), Fields virology, 5th ed. Lippincott, Williams & Wilkins, Philadelphia, PA.
2. **Laimins LA.** 1993. The biology of human papillomaviruses: from warts to cancer. Infect. Agents Dis. 2:74–86.
3. **zur Hausen H.** 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat. Rev. Cancer. 2:342–350.
4. **Baker TS, Newcomb WW, Olson NH, Cowsert LM, Olson C, Brown JC.** 1991. Structures of bovine and human papillomaviruses. Analysis by cryo-

electron microscopy and three-dimensional image reconstruction. Biophys. J. **60**:1445–1456.
5. **Modis Y, Trus BL, Harrison SC.** 2002. Atomic model of the papillomavirus capsid. EMBO J. **21**:4754–4762.
6. **Buck CB, Cheng N, Thompson CD, Lowy DR, Steven AC, Schiller JT, Trus BL.** 2008. Arrangement of L2 within the papillomavirus capsid. J. Virol. **82**:5190–5197.
7. **Kamper N, Day PM, Nowak T, Selinka HC, Florin L, Bolscher J, Hilbig L, Schiller JT, Sapp M.** 2006. A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. J. Virol. **80**:759–768.
8. **Florin L, Becker KA, Lambert C, Nowak T, Sapp C, Strand D, Streeck RE, Sapp M.** 2006. Identification of a dynein interacting domain in the papillomavirus minor capsid protein l2. J. Virol. **80**:6691–6696.
9. **Florin L, Sapp M, Spoden GA.** 2012. Host-cell factors involved in papillomavirus entry. Med. Microbiol. Immunol. **201**:437–448.
10. **Schneider MA, Spoden GA, Florin L, Lambert C.** 2011. Identification of the dynein light chains required for human papillomavirus infection. Cell Microbiol. **13**:32–46.
11. **Day PM, Baker CC, Lowy DR, Schiller JT.** 2004. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. Proc. Natl. Acad. Sci. U. S. A. **101**:14252–14257.
12. **Baldwin A, Hypes MK, Pirisi L, Creek KE.** 2007. NFI is an essential positive transcription factor for human papillomavirus type 16 early gene expression. Open Virol. J. **1**:33–38.
13. **Chong T, Apt D, Gloss B, Isa M, Bernard HU.** 1991. The enhancer of human papillomavirus type 16: binding sites for the ubiquitous transcription factors oct-1, NFA, TEF-2, NF1, and AP-1 participate in epithelial cell-specific transcription. J. Virol. **65**:5933–5943.
14. **Chong T, Chan WK, Bernard HU.** 1990. Transcriptional activation of human papillomavirus 16 by nuclear factor I, AP1, steroid receptors and a possibly novel transcription factor, PVF: a model for the composition of genital papillomavirus enhancers. Nucleic Acids Res. **18**:465–470.
15. **Cripe TP, Haugen TH, Turk JP, Tabatabai F, Schmid PG, III, Durst M, Gissmann L, Roman A, Turek LP.** 1987. Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. EMBO J. **6**:3745–3753.
16. **Dollard SC, Broker TR, Chow LT.** 1993. Regulation of the human papillomavirus type 11 E6 promoter by viral and host transcription factors in primary human keratinocytes. J. Virol. **67**:1721–1726.
17. **Garcia-Carranca A, Thierry F, Yaniv M.** 1988. Interplay of viral and cellular proteins along the long control region of human papillomavirus type 18. J. Virol. **62**:4321–4330.
18. **Ishiji T, Lace MJ, Parkkinen S, Anderson RD, Haugen TH, Cripe TP, Xiao JH, Davidson I, Chambon P, Turek LP.** 1992. Transcriptional enhancer factor (TEF)-1 and its cell-specific co-activator activate human papillomavirus-16 E6 and E7 oncogene transcription in keratinocytes and cervical carcinoma cells. EMBO J. **11**:2271–2281.
19. **Kukimoto I, Takeuchi T, Kanda T.** 2006. CCAAT/enhancer binding protein beta binds to and activates the P670 promoter of human papillomavirus type 16. Virology **346**:98–107.
20. **Kyo S, Inoue M, Nishio Y, Nakanishi K, Akira S, Inoue H, Yutsudo M, Tanizawa O, Hakura A.** 1993. NF-IL6 represses early gene expression of human papillomavirus type 16 through binding to the noncoding region. J. Virol. **67**:1058–1066.
21. **Thierry F, Spyrou G, Yaniv M, Howley P.** 1992. Two AP1 sites binding JunB are essential for human papillomavirus type 18 transcription in keratinocytes. J. Virol. **66**:3740–3748.
22. **Doorbar J.** 2006. Molecular biology of human papillomavirus infection and cervical cancer. Clin. Sci. **110**:525–541.
23. **Lee C, Laimins LA.** 2007. The differentiation-dependent life cycle of human papillomaviruses in keratinocytes, p 45–67. *In* Garcea RL, DiMaio D (ed), The papillomaviruses. Springer Science and Business Media, New York, NY.
24. **del Mar Pena LM, Laimins LA.** 2001. Differentiation-dependent chromatin rearrangement coincides with activation of human papillomavirus type 31 late gene expression. J. Virol. **75**:10005–10013.
25. **Grassmann K, Rapp B, Maschek H, Petry KU, Iftner T.** 1996. Identification of a differentiation-inducible promoter in the E7 open reading frame of human papillomavirus type 16 (HPV-16) in raft cultures of a new cell line containing high copy numbers of episomal HPV-16 DNA. J. Virol. **70**:2339–2349.
26. **Hummel M, Hudson JB, Laimins LA.** 1992. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J. Virol. **66**:6070–6080.
27. **Day PM, Roden RB, Lowy DR, Schiller JT.** 1998. The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. J. Virol. **72**:142–150.
28. **Florin L, Schafer F, Sotlar K, Streeck RE, Sapp M.** 2002. Reorganization of nuclear domain 10 induced by papillomavirus capsid protein L2. Virology **295**:97–107.
29. **Okun MM, Day PM, Greenstone HL, Booy FP, Lowy DR, Schiller JT, Roden RB.** 2001. L1 interaction domains of papillomavirus L2 necessary for viral genome encapsidation. J. Virol. **75**:4332–4342.
30. **Stauffer Y, Raj K, Masternak K, Beard P.** 1998. Infectious human papillomavirus type 18 pseudovirions. J. Mol. Biol. **283**:529–536.
31. **Zhao KN, Sun XY, Frazer IH, Zhou J.** 1998. DNA packaging by L1 and L2 capsid proteins of bovine papillomavirus type 1. Virology **243**:482–491.
32. **Kispert A, Hermann BG.** 1993. The Brachyury gene encodes a novel DNA binding protein. EMBO J. **12**:4898–4899.
33. **Tada M, Smith JC.** 2001. T-targets: clues to understanding the functions of T-box proteins. Dev. Growth Differ. **43**:1–11.
34. **Wilson V, Conlon FL.** 2002. The T-box family. Genome Biol. **3**: REVIEWS3008. doi:10.1186/gb-2002-3-6-reviews3008.
35. **Smith J.** 1999. T-box genes: what they do and how they do it. Trends Genet. **15**:154–158.
36. **Carreira S, Dexter TJ, Yavuzer U, Easty DJ, Goding CR.** 1998. Brachyury-related transcription factor Tbx2 and repression of the melanocyte-specific TRP-1 promoter. Mol. Cell. Biol. **18**:5099–5108.
37. **He M, Wen L, Campbell CE, Wu JY, Rao Y.** 1999. Transcription repression by Xenopus ET and its human ortholog TBX3, a gene involved in ulnar-mammary syndrome. Proc. Natl. Acad. Sci. U. S. A. **96**:10212–10217.
38. **King M, Arnold JS, Shanske A, Morrow BE.** 2006. T-genes and limb bud development. Am. J. Med. Genet. A **140**:1407–1413.
39. **Plageman TF, Jr, Yutzey KE.** 2005. T-box genes and heart development: putting the "T" in heart. Dev. Dyn. **232**:11–20.
40. **Rowley M, Grothey E, Couch FJ.** 2004. The role of Tbx2 and Tbx3 in mammary development and tumorigenesis. J. Mammary Gland Biol. Neoplasia **9**:109–118.
41. **Bamshad M, Lin RC, Law DJ, Watkins WC, Krakowiak PA, Moore ME, Franceschini P, Lala R, Holmes LB, Gebuhr TC, Bruneau BG, Schinzel A, Seidman JG, Seidman CE, Jorde LB.** 1997. Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. Nat. Genet. **16**:311–315.
42. **Lu J, Li XP, Dong Q, Kung HF, He ML.** 2010. TBX2 and TBX3: the special value for anticancer drug targets. Biochim. Biophys. Acta **1806**:268–274.
43. **Carlson H, Ota S, Campbell CE, Hurlin PJ.** 2001. A dominant repression domain in Tbx3 mediates transcriptional repression and cell immortalization: relevance to mutations in Tbx3 that cause ulnar-mammary syndrome. Hum. Mol. Genet. **10**:2403–2413.
44. **Jacobs JJ, Keblusek P, Robanus-Maandag E, Kristel P, Lingbeek M, Nederlof PM, van Welsem T, van de Vijver MJ, Koh EY, Daley GQ, van Lohuizen M.** 2000. Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. Nat. Genet. **26**:291–299.
45. **Yarosh W, Barrientos T, Esmailpour T, Lin L, Carpenter PM, Osann K, Anton-Culver H, Huang T.** 2008. TBX3 is overexpressed in breast cancer and represses p14 ARF by interacting with histone deacetylases. Cancer Res. **68**:693–699.
46. **Prince S, Carreira S, Vance KW, Abrahams A, Goding CR.** 2004. Tbx2 directly represses the expression of the p21(WAF1) cyclin-dependent kinase inhibitor. Cancer Res. **64**:1669–1674.
47. **Chen JR, Chatterjee B, Meyer R, Yu JC, Borke JL, Isales CM, Kirby ML, Lo CW, Bollag RJ.** 2004. Tbx2 represses expression of Connexin43 in osteoblastic-like cells. Calcif. Tissue Int. **74**:561–573.
48. **Redmond KL, Crawford NT, Farmer H, D'Costa ZC, O'Brien GJ, Buckley NE, Kennedy RD, Johnston PG, Harkin DP, Mullan PB.** 2010. T-box 2 represses NDRG1 through an EGR1-dependent mechanism to drive the proliferation of breast cancer cells. Oncogene **29**:3252–3262.
49. **Rodriguez M, Aladowicz E, Lanfrancone L, Goding CR.** 2008. Tbx3

represses E-cadherin expression and enhances melanoma invasiveness. Cancer Res. **68**:7872–7881.
50. **Vance KW, Shaw HM, Rodriguez M, Ott S, Goding CR.** 2010. The retinoblastoma protein modulates Tbx2 functional specificity. Mol. Biol. Cell **21**:2770–2779.
51. **Leder C, Kleinschmidt JA, Wiethe C, Muller M.** 2001. Enhancement of capsid gene expression: preparing the human papillomavirus type 16 major structural gene L1 for DNA vaccination purposes. J. Virol. **75**:9201–9209.
52. **Schweiger MR, Ottinger M, You J, Howley PM.** 2007. Brd4-independent transcriptional repression function of the papillomavirus e2 proteins. J. Virol. **81**:9612–9622.
53. **Volpers C, Sapp M, Snijders PJ, Walboomers JM, Streeck RE.** 1995. Conformational and linear epitopes on virus-like particles of human papillomavirus type 33 identified by monoclonal antibodies to the minor capsid protein L2. J. Gen. Virol. **76**:2661–2667.
54. **Rommel O, Dillner J, Fligge C, Bergsdorf C, Wang X, Selinka HC, Sapp M.** 2005. Heparan sulfate proteoglycans interact exclusively with conformationally intact HPV L1 assemblies: basis for a virus-like particle ELISA. J. Med. Virol. **75**:114–121.
55. **Gyuris J, Golemis E, Chertkov H, Brent R.** 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell **75**:791–803.
56. **Vance KW, Carreira S, Brosch G, Goding CR.** 2005. Tbx2 is overexpressed and plays an important role in maintaining proliferation and suppression of senescence in melanomas. Cancer Res. **65**:2260–2268.
57. **Florin L, Sapp C, Streeck RE, Sapp M.** 2002. Assembly and translocation of papillomavirus capsid proteins. J. Virol. **76**:10009–10014.
58. **Lin Z, Yemelyanova AV, Gambhira R, Jagu S, Meyers C, Kirnbauer R, Ronnett BM, Gravitt PE, Roden RB.** 2009. Expression pattern and subcellular localization of human papillomavirus minor capsid protein L2. Am. J. Pathol. **174**:136–143.
59. **Baldwin A, Pirisi L, Creek KE.** 2004. NFI-Ski interactions mediate transforming growth factor beta modulation of human papillomavirus type 16 early gene expression. J. Virol. **78**:3953–3964.
60. **Kang YH, Kang M-J, Paik SG, Park SN, Yoon D-Y.** 2003. Repression of HPV E6-activated RSV promoter activity by anti-cancer agents. Antiviral Res. **58**:65–71.
61. **Lingbeek ME, Jacobs JJ, van Lohuizen M.** 2002. The T-box repressors TBX2 and TBX3 specifically regulate the tumor suppressor gene p14ARF via a variant T-site in the initiator. J. Biol. Chem. **277**:26120–26127.
62. **Sinha S, Abraham S, Gronostajski RM, Campbell CE.** 2000. Differential DNA binding and transcription modulation by three T-box proteins, T, TBX1 and TBX2. Gene **258**:15–29.
63. **Desaintes C, Demeret C, Goyat S, Yaniv M, Thierry F.** 1997. Expression of the papillomavirus E2 protein in HeLa cells leads to apoptosis. EMBO J. **16**:504–514.
64. **Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, zur Hausen H.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature **314**:111–114.
65. **Heino P, Zhou J, Lambert PF.** 2000. Interaction of the papillomavirus transcription/replication factor, E2, and the viral capsid protein, L2. Virology **276**:304–314.
66. **Okoye A, Cordano P, Taylor ER, Morgan IM, Everett R, Campo MS.** 2005. Human papillomavirus 16 L2 inhibits the transcriptional activation function, but not the DNA replication function, of HPV-16 E2. Virus Res. **108**:1–14.
67. **Schreiner S, Martinez R, Groitl P, Rayne F, Vaillant R, Wimmer P, Bossis G, Sternsdorf T, Marcinowski L, Ruzsics Z, Dobner T, Wodrich H.** 2012. Transcriptional activation of the adenoviral genome is mediated by capsid protein VI. PLoS Pathog. **8**:e1002549. doi:10.1371/journal.ppat.1002549.
68. **Florin L, Becker KA, Sapp C, Lambert C, Sirma H, Muller M, Streeck RE, Sapp M.** 2004. Nuclear translocation of papillomavirus minor capsid protein L2 requires Hsc70. J. Virol. **78**:5546–5553.
69. **Gornemann J, Hofmann TG, Will H, Muller M.** 2002. Interaction of human papillomavirus type 16 L2 with cellular proteins: identification of novel nuclear body-associated proteins. Virology **303**:69–78.
70. **Fedele M, Benvenuto G, Pero R, Majello B, Battista S, Lembo F, Vollono E, Day PM, Santoro M, Lania L, Bruni CB, Fusco A, Chiariotti L.** 2000. A novel member of the BTB/POZ family, PATZ, associates with the RNF4 RING finger protein and acts as a transcriptional repressor. J. Biol. Chem. **275**:7894–7901.
71. **Bilican B, Goding CR.** 2006. Cell cycle regulation of the T-box transcription factor tbx2. Exp. Cell Res. **312**:2358–2366.
72. **Martin N, Benhamed M, Nacerddine K, Demarque MD, van Lohuizen M, Dejean A, Bischof O.** 2012. Physical and functional interaction between PML and TBX2 in the establishment of cellular senescence. EMBO J. **31**:95–109.
73. **Zhou J, Sun XY, Louis K, Frazer IH.** 1994. Interaction of human papillomavirus (HPV) type 16 capsid proteins with HPV DNA requires an intact L2 N-terminal sequence. J. Virol. **68**:619–625.
74. **Dowhanick JJ, McBride AA, Howley PM.** 1995. Suppression of cellular proliferation by the papillomavirus E2 protein. J. Virol. **69**:7791–7799.
75. **Goodwin EC, Yang E, Lee CJ, Lee HW, DiMaio D, Hwang ES.** 2000. Rapid induction of senescence in human cervical carcinoma cells. Proc. Natl. Acad. Sci. U. S. A. **97**:10978–10983.
76. **Hall AH, Alexander KA.** 2003. RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. J. Virol. **77**:6066–6069.
77. **Lea JS, Sunaga N, Sato M, Kalahasti G, Miller DS, Minna JD, Muller CY.** 2007. Silencing of HPV 18 oncoproteins With RNA interference causes growth inhibition of cervical cancer cells. Reprod. Sci. **14**:20–28.
78. **Nauenburg S, Zwerschke W, Jansen-Durr P.** 2001. Induction of apoptosis in cervical carcinoma cells by peptide aptamers that bind to the HPV-16 E7 oncoprotein. FASEB J. **15**:592–594.
79. **Nishimura A, Ono T, Ishimoto A, Dowhanick JJ, Frizzell MA, Howley PM, Sakai H.** 2000. Mechanisms of human papillomavirus E2-mediated repression of viral oncogene expression and cervical cancer cell growth inhibition. J. Virol. **74**:3752–3760.
80. **Wells SI, Francis DA, Karpova AY, Dowhanick JJ, Benson JD, Howley PM.** 2000. Papillomavirus E2 induces senescence in HPV-positive cells via pRB- and p21(CIP)-dependent pathways. EMBO J. **19**:5762–5771.
81. **Morandell D, Kaiser A, Herold S, Rostek U, Lechner S, Mitterberger MC, Jansen-Durr P, Eilers M, Zwerschke W.** 2012. The human papillomavirus type 16 E7 oncoprotein targets Myc-interacting zinc-finger protein-1. Virology **422**:242–253.
82. **Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, Stanley MA.** 2012. The biology and life-cycle of human papillomaviruses. Vaccine **30**(Suppl. 5):F55–F70.
83. **Isaacson Wechsler E, Wang Q, Roberts I, Pagliarulo E, Jackson D, Untersperger C, Coleman N, Griffin H, Doorbar J.** 2012. Reconstruction of human papillomavirus type 16-mediated early-stage neoplasia implicates E6/E7 deregulation and the loss of contact inhibition in neoplastic progression. J. Virol. **86**:6358–6364.
84. **Middleton K, Peh W, Southern S, Griffin H, Sotlar K, Nakahara T, El-Sherif A, Morris L, Seth R, Hibma M, Jenkins D, Lambert P, Coleman N, Doorbar J.** 2003. Organization of human papillomavirus productive cycle during neoplastic progression provides a basis for selection of diagnostic markers. J. Virol. **77**:10186–10201.
85. **Hansen CN, Nielsen L, Norrild B.** 2010. Activities of E7 promoters in the human papillomavirus type 16 genome during cell differentiation. Virus Res. **150**:34–42.
86. **Holmgren SC, Patterson NA, Ozbun MA, Lambert PF.** 2005. The minor capsid protein L2 contributes to two steps in the human papillomavirus type 31 life cycle. J. Virol. **79**:3938–3948.