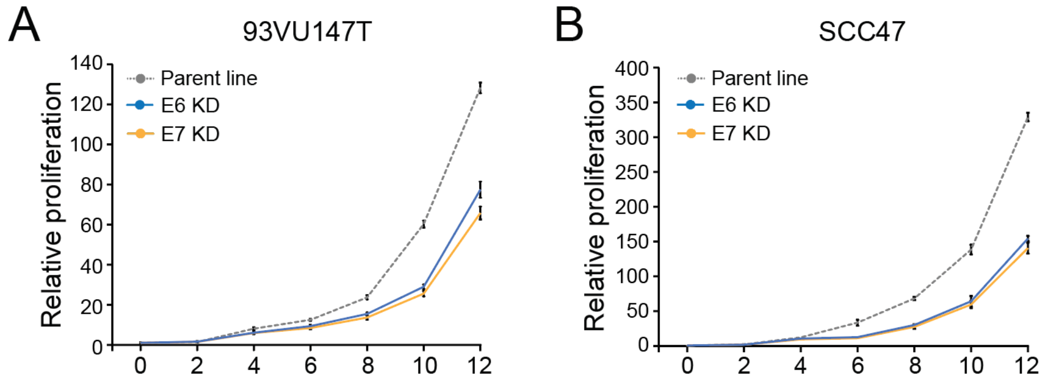
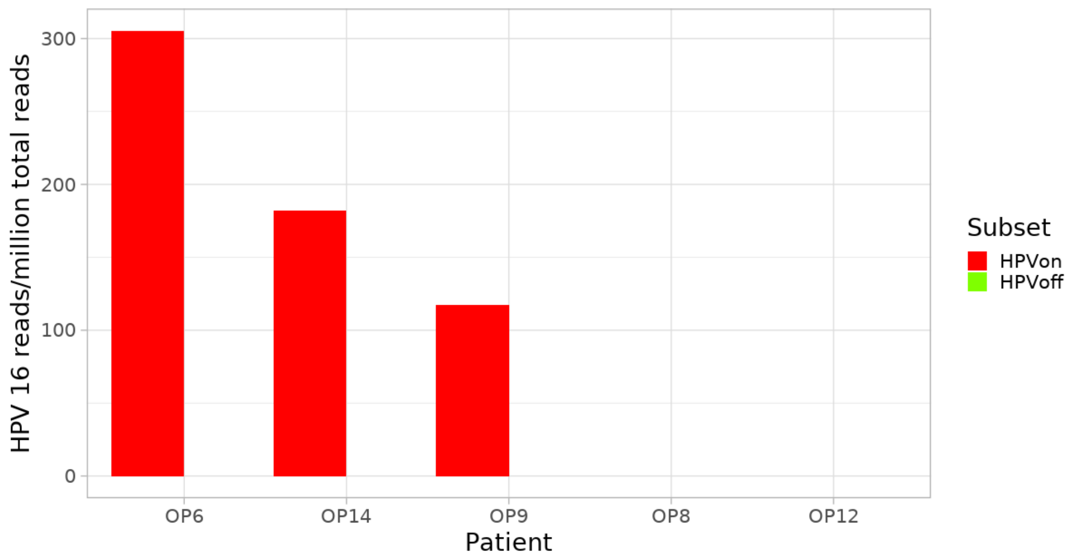
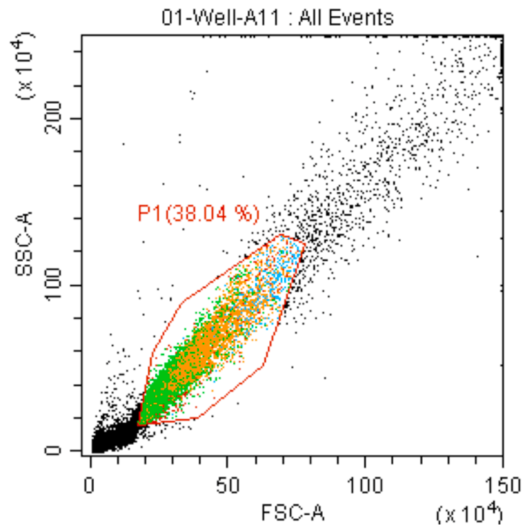


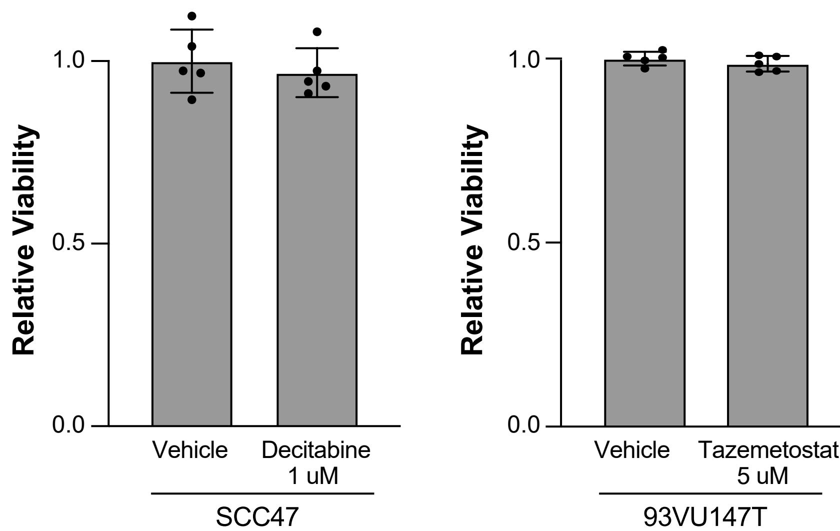
Supplementary Figure 1. Proliferation is reduced in E6 and E7 knockdown lines compared to control 93VU147T or SCC47 lines.



Supplementary Figure 2. HPV16 reads detected by HPV-EM in five patients. HPVon and HPVoff cells were analyzed separately for OP6, OP9 and OP14 (HPV-positive tumors) as well as OP8 (p16 positive, HPV negative) and OP12 (p16/HPV negative). No HPV reads were detected among HPVoff cells using this method in OP6, OP9, or OP14 and similarly HPV reads were not detects in OP8 or OP12.



Supplementary Figure 3. Gating for flow cytometry analyses of cell cycle phase (see Extended Data 7J).



Supplementary Figure 4. Bar plot shows relative viability by Cell Titer Glo following treatment with decitabine in SCC47 (*left*) or tazemetostat in 93VU147T (*right*) (n=3).

Supplementary Note 1: Additional methods relating to tissue staining and proliferation measurements

Immunocytochemistry

Cells were fixed in freshly prepared 4% paraformaldehyde for 20 minutes at room temperature, washed with PBS and subsequently blocked and permeabilized with 0.1% Triton X in 10% goat serum containing PBS in room temperature for 1 hour. Cells were then probed with primary antibodies, Ki-67 1:500 dilution (D2H0 rabbit mAb, Cell Signaling), E6 1:100 dilution (mouse anti-virus, clone C1P5, Invitrogen), E7 1:100 dilution (mouse anti-virus, clone TVG701Y, Invitrogen) diluted in 10% goat-serum PBS and incubated overnight at 4 degree Celsius. Cells were washed with PBS and then probed with secondary antibody, goat anti-rabbit IgG (H+L), Fab2 Alexa Fluor 594, goat anti-mouse IgG (H+L), or Fab2 Alexa Fluor 488 (Cell Signaling) at 1:400 dilution in 2% goat serum containing PBS for 1 hour at room temperature followed by PBS washes and mounted with DAPI (Fluoroshield, Sigma). Imaging was completed using Fluorescence Eclipse Ti2 Inverted microscope (Nikon).

Molecular Fluorescent *In Situ Hybridization* (MFISH)

Viral RNA ISH (RNAScope) and DNA ISH (DNAScope) were performed using RNAscope 2.5 HD Reagent Kit Red assay combined with Immunohistochemistry (Advanced Cell Diagnostics) according to manufacturer's instructions. Briefly, slides were baked in dry air oven for one hour at 60°C, deparaffinized (Xylene for five minutes twice followed by 100% ethanol for two minutes twice), hydrogen peroxide was applied for 10 minutes at room temperature, and co-detection target retrieval was done using Steamer (BELLA) for twenty minutes and washed with PBS-T. Tissue slides were then incubated overnight with p16-INK4a antibody (LSBio) in HybEz Slide Rack in the Humidity Control Tray with damp humidifying paper and incubated overnight at 4°C. The next day, post-primary fixation was done by washing slides with PBS-T and submerging slides in 10% NBF for 30 minutes at room temperature. Slides are washed with PBS-T and Protease Plus was added to each slide for 30 minutes at 40°C then washed with distilled water. RNAscope antisense probes were utilized to target RNA of specified viral genes, while DNAScope sense probes were utilized targeting DNA of specified viral genes[48]. Selected probes were warmed at 40°C and hybridized with specific oligonucleotide probes for 2 hours at 40°C in HybEZ Humidifying System. Details of antibodies, probes, and sequences are in **Supplementary Table 11**. RNA/DNA was then serial amplified and stained with Fast Red solution. Slides were blocked with co-detection blocker for 15 minutes at 40°C and washed in PBS-T. Secondary Alexa Flour 488 antibody (Abcam) was applied for one hour at room temperature in the dark. Finally, slides were washed with PBS-T and counter stained with DAPI (Sigma) and mounted with ProLong Gold Antifade Reagent (Invitrogen). RNAScope was optimized with a PPIB probe as a positive control, while a DapB probe and no secondary antibody served as negative controls. All slides were imaged on the EVOS M5000 Imaging System (Invitrogen) for scoring.

Quantification was performed with CellProfiler using the ISH pipeline by Erben et al[49]. Adjustments were made to accommodate cell size as well as green versus red staining. Dot staining was identified based on intensity and distinct pixel ranges for DAPI (nucleus, 20-50 pixels), green (p16, 10-30 pixels), and red (E6 or E7, 3-12 pixels). Cell size was identified using a 5-pixel radius from nucleus and images were overlaid to count dots per cell. A positive stain scoring of p16 was determined as greater than 1, while a negative

stain score was determined as less than 1. Red (E6 and E7) RNA ISH signal was detected within individual cells and scored using ACD scoring bins. Bin scoring ranged from < 1 dot/cell designated as bin 0, 1–3 dots/cell designated as bin 1, 4–9 dots/cell designated as bin 2, 10–15 dots/cell designated as bin 3, and > 15 dots/cell designated as bin 4. Stain scoring remained the same for all genes of interest with positive staining determined as >0 dots/cell and negative staining as 0 dots/cell per ACD guidelines. DNA ISH signal was detected and scored in a similar fashion. RNAse treatment was used to confirm that signal was specific to DNA. All RNA and DNA ISH images were reviewed with a dedicated head and neck pathologist, who confirmed the heterogeneous pattern of HPV RNA expression compared to a more homogeneous pattern of HPV DNA detected.

Dual-Stain Immunohistochemistry

FFPE blocks of the patient tumors were sectioned onto slides at 4 µm. Slides were baked at 60 degrees for 30 minutes followed by deparaffinization with xylene and graded ethanol. Diva Decloaker (Biocare Medical) was used for heat mediated antigen retrieval for all stains. Blocking was performed with Dako Dual Endogenous Enzyme Block (5 minutes). HPV TYPE 16/18 E6 Mouse Monoclonal antibody (1:50 dilution, Thermofisher, cat# MA1-46057) was applied first and incubated for 30 minutes. Secondary antibody incubation was performed with the Dako EnVision+ Dual Link System-HRP for 30 minutes, followed by DAB staining for 5 minutes. Blocking with Dako Dual Endogenous Enzyme Block was then repeated for 5 minutes. Staining with P16-INK4A polyclonal antibody (1:75 dilution, Thermofisher, cat# 10883-1-AP) was then performed with a 30 minute incubation time. Dako Powervision Poly-AP was used for secondary antibody staining (30 minutes), followed by incubation with AP Red substrate for 5 minutes. Sections were then mounted with a coverslip with Glycergel (Dako).

Cell Proliferation

CellTitre-Glo (CTG) proliferation assays (Promega) were completed according to manufacturer protocols. Briefly, 2000 cells were seeded per 96 wells in technical replicates of 5. Cells were lysed on day 0 (one hour after seeding of cells), 1, 3, 5, 7, and 9 for HPV single clones and day 0, day 2, day 4, day 6, day 8, day 10 and day 12 for E6 and E7 HNSCC knockdown cell lines by addition of the CTG reagent followed by measurement of luminescence using the Biotek Cytation 5 (BioTek, Winooski, VT). Background luminescence was removed. Luminescence values were adjusting based on 2µM Adenosine triphosphate (ATP) luminescence measured on the same plate for each day.

References

1. Deleage, C. *et al.* Defining HIV and SIV Reservoirs in Lymphoid Tissues. *Pathog. Immun.* **1**, 68–106 (2016).

2. Erben, L., He, M.-X., Laeremans, A., Park, E. & Buonanno, A. A Novel Ultrasensitive In Situ Hybridization Approach to Detect Short Sequences and Splice Variants with Cellular Resolution. *Mol. Neurobiol.* **55**, 6169–6181 (2018).