1 **Legend for Supplemental Material**

2 Methods text. Detailed methods used in these studies.

3 Table S1. List of oligonucleotides used in these studies.

4 Table S2. List of antibodies used in these studies.

5 Supplemental Figure Legends for Supplemental Figures 1-6.

6 **Figure S1. HPV16.L2F efficiently infects cells.**

7 HeLa cells were infected with HPV16.L2HA and HPV16.L2F (normalized to the same
8 number of encapsidated GFP plasmids), and the fraction of infected cells was
9 determined 48 hours post-infection by flow cytometry for GFP fluorescence.

10

11 **Figure S2. Visualization of HPV16.L2F during entry.**

12 **(a)** HeLa-sen2 cells were infected with HPV16.L2HA or HPV16.L2F at MOI of 10. 16
13 hours later, cells were immunostained with anti-HA antibody for HPV16.L2HA-infected
14 cells or anti-FLAG antibody for HPV16.L2F-infected cells (both green). Nuclei were
15 stained with DAPI (blue). Cells were visualized by fluorescence confocal microscopy,
16 and a single confocal slice is shown in each panel. **(b)** HPV16.L2F was preincubated
17 with 100μg/ml heparin for one hour at 37°C or left untreated. HeLa-Sen2 cells were then
18 mock-infected or infected with these viruses at MOI of 20. After 16 hours, cells were
19 immunostained with anti-FLAG antibody (green). Nuclei were stained with DAPI (blue).
20 Cells were visualized by fluorescence confocal microscopy, and a single confocal slice
21 is shown in each panel.

22

23 **Fig. S3. γ-secretase is not required for HPV internalization or capsid disassembly.**
24 **(a)** HeLa-Sen2 cells were treated with 250nM XXI or left untreated. One hour later, cells
25 were mock-infected (in the absence of XXI treatment) or infected at MOI of 20 with
26 HPV16.L2F PsV for 16 hours and then stained with anti-L1 polyclonal antibody (green).
27 Nuclei were stained with DAPI (blue). Cells were visualized by fluorescence confocal
28 microscopy, and a single confocal slice is shown in each panel. **(b)** HeLa-Sen2 cells
29 were transfected with control siRNA or siRNA targeting APH1A. 48 hours later, cells
30 were mock-infected or infected at MOI of 20 with HPV16.L2HA PsV. After 16 hours,
31 cells were stained with anti-33L1-7 antibody (green) and DAPI (blue). Cells were
32 visualized as in panel a.
33
34 **Figure S4. γ-secretase is required for L2 entry into the Golgi in HaCaT cells**.
35 HaCaT cells were treated with XXI or left untreated, and then mock-infected (without
36 XXI treatment) or infected with HPV16.L2F at MOI of 100. At 8 and 16 hours post-
37 infection, the cells were incubated with anti-FLAG and an antibody recognizing EEA1;
38 and at 16 hours post-infection, cells were incubated with anti-FLAG and anti-
39 TGN46. Cells were then processed for PLA, and proximity of L2 with the indicated
40 marker was visualized in green by fluorescence microscopy. Nuclei were stained with
41 DAPI (blue). A single confocal slice is shown in each panel.
42
43 **Figure S5. γ-secretase is required for Golgi localization of L2 during infection.**
44 HeLa-Sen2 cells were treated with inhibitor XXI or left untreated, and then mock-
45 infected (without XXI treatment) or infected with HPV16.L2F PsV at MOI of 100 for eight

46 or 16 hours. The cells were then incubated with anti-FLAG and an antibody recognizing
47 the Golgi marker GM130. Cells were processed for PLA, and proximity of L2 with
48 GM130 was visualized in green by fluorescence confocal microscopy. Nuclei were
49 stained with DAPI (blue). A single confocal slice is shown in each panel. These data
50 are quantified in Fig. 5b.

51

52 **Figure S6. Localization of HPV16 L1 in the Golgi requires γ-secretase activity.**
53 HeLa-Sen2 cells were treated with inhibitor XXI or left untreated, and then mock-
54 infected (without XXI treatment) or infected with HPV16.L2F at MOI of 100. 16 hours
55 later, the cells were incubated with 33L1-7 antibody and anti-TGN46. Cells were
56 processed for PLA, and proximity of L1 with TGN46 was visualized in green by
57 fluorescence confocal microscopy. Nuclei were stained with DAPI (blue). A single
58 confocal slice is shown in each panel.

59

60

61

62 **Supplemental Methods**

63 **Cell culture, plasmids and virus**

64 HeLa-S3 cells (hereafter HeLa cells) and human ectocervical keratinocytes (catalogue
65 numbers CCL2.2 and CRL-2614, respectively) were purchased from the American Type
66 Culture Collection (ATCC). HeLa-Sen2 cells are a cloned strain of HeLa cells, which
67 infects efficiently with SV40 and is suitable for immunofluorescence experiments (1).
68 293TT cells were obtained from Christopher Buck (NIH). All cells except 2614
69 keratinocytes were cultured in Dulbecco's MEM (DMEM) supplemented with 10% fetal
70 bovine serum (FBS), 10mM L-glutamine, 10mM HEPES pH 7.3 and Pen/Strep. 2614
71 cells were cultured in keratinocyte serum-free media (Life Technologies, Carlsbad, CA)
72 supplemented with bovine pituitary extract, recombinant human epidermal growth factor
73 and 400μM calcium chloride.

74 HPV pseudovirus production plasmids and reporter plasmids, including
75 p16sheLL, p5sheLL, p18sheLL and pCIneo-GFP, were gifts from Christopher Buck
76 (NIH). p16LlwCHA, which is used to generate HPV16 containing an HA tag at the C-
77 terminus of L2 (hereafter HPV16.L2HA), was a gift from Patricia Day (NIH). pCAG-
78 HcRed was purchased from Addgene (plasmid 11152, Cambridge, MA). The plasmid
79 encoding a secreted Gaussia luciferase gene, pMCS-Gaussia Luc, was purchased from
80 Thermo Scientific (Fremont, CA). p16sheLL.L2F used for producing HPV16.L2F PsV
81 was constructed from p16sheLL, and p16sheLL.L2FTHA used for producing
82 HPV16.L2FTHA was constructed from p16sheLL.L2F. In both cases, we used Phusion
83 HF DNA Polymerase from New England Biolabs (Beverly, MA) and standard cloning
84 procedures. Details of plasmid construction are available from the authors on request.

85 HPV PsV were produced, subjected to a maturation step and purified by iodixanol
86 (OptiPrep) density gradient centrifugation as described (2). Raft culture-derived HPV16
87 was the gift of Craig Meyers (Hershey Medical Center) (3,4) and was handled in
88 accordance with institutional biosafety requirements. Adenovirus type 5 expressing GFP
89 (Ad5-GFP) was purchased from Vector Biolabs (Philadelphia, PA), and SV40 was
90 produced and titered in CV1 cells as previously described (5). Virus preparations were
91 aliquoted and stored at −80°C.
92
93 **Antibodies and chemicals**
94 The anti-L1 rabbit polyclonal serum and 33L1-7 mouse antibody were gifts of Patricia
95 Day (NIH) and Martin Sapp (Louisiana State University), respectively. Other antibodies
96 used are listed in Table S2. γ-secretase inhibitor XXI [Compound E (#565790)] and furin
97 inhibitor I (#344930) were purchased from Millipore (Billerica, MA). Retro-2 was
98 obtained from Chembridge (Cambridge, MA). Heparin was purchased from Sigma.
99 siRNAs against APH1A, furin and Vps29, and the control scrambled RISC-free siRNA
100 were purchased from Dharmacon (Lafayette, CO). The sequences of these siRNAs are
101 listed in Table S1.
102
103 **Genome quantification**
104 Purified HPV16.L2HA and HPV16.L2F PsV were pretreated with DNAse kit (Promega,
105 Madison, WI) to remove any free DNA associated with capsids. After inactivating the
106 DNAase at 75°C for 30 min, the capsids were digested with 50 µg/ml proteinase K
107 (Roche, Indianapolis, IN) for one hour at 37°C, and DNA was isolated with a PCR

purification kit (Qiagen). Encapsidated GFP reporter plasmid was then quantified by qPCR using primers for GFP; a 10-fold serial dilution of purified pCIneo-GFP was used to generate a standard curve.

Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was isolated using the RNeasy kit (Qiagen, Germantown, MD) following the manufacturer's instructions. RNA was reverse transcribed into cDNA by iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). The amplification reactions were carried out by using single-color real-time PCR detection system (Bio-Rad) and the specific primers listed in Table S1. Actin was used as the internal control. All experiments were done in triplicate. To measure authentic HPV infection, HeLa cells were infected with 5 µL raft-derived HPV16 or PsV in the presence and absence of 250nM XXI, and RNA was isolated at 48 hours post-infection.

Flow cytometry

To measure infectivity of HPV16 PsV, untreated cells or cells pretreated for one hour with inhibitors or transfected 48 hours earlier with siRNAs were incubated with PsV at MOI of 0.5 (as determined by flow cytometry for reporter protein expression in untreated HeLa cells) or an equivalent number of packaged reporter plasmids. 48 hours post-infection, cells were harvested by trypsinization and fixed in 4% Formalde-Fresh solution. The fraction of cells expressing the reporter protein was assayed by flow cytometry for reporter gene expression on a BD Biosciences FACSCalibur flow cytometer. To measure the disassembly of HPV capsids during infection, HeLa cells

131 were infected with HPV16.L2HA or HPV16.L2F at MOI of 20 at 4°C for 2 hours to
132 synchronize infection. Cells were washed to remove unbound viruses and shifted to
133 37°C to initiate infection. Samples were fixed in ice-cold methanol at indicated time
134 points post-infection. The samples were stained with 33L1-7, Roche HA antibody, or
135 anti-FLAG at 1:100 dilution and incubated with corresponding AlexaFluor secondary
136 antibodies. Fluorescence intensity was assayed on a Becton-Dickinson FACSCalibur
137 flow cytometer.
138

139 **Neutralization assay**

140 Wild-type HPV16 PsV and HPV16.L2F both containing the GFP reporter plasmid were
141 incubated with Sigma anti-FLAG antibody (final concentration 10 μg/ml) in DMEM-10 for
142 60 min at 37°C. HeLa cells plated 24 hours earlier at 10^5 cells per well in six-well plates
143 were infected at MOI of one. 48 hours post-infection, cells were harvested with trypsin,
144 fixed with 4% formaldehyde (Formalde-Fresh, Fisher Scientific), and GFP-positive cells
145 were counted on a BD Biosciences FACSCalibur flow cytometer. All results were
146 normalized to corresponding mock-treated sample.
147

148 **Thrombin cleavage**

149 Wild-type HPV16 PsV and HPV16.L2FTHA (~10^6 infectious units) were mixed with 10
150 NIH units of thrombin protease (GE Healthcare) in physiological buffered saline (PBS)
151 or with PBS alone and incubated at 37°C overnight. A portion of the HPV16.L2FTHA
152 samples was subjected to 7.5% SDS-polyacrylamide gel electrophoresis and
153 immunoblotting with anti-FLAG (M2 from Sigma, 0.2 μg/ml), anti-HA (C29F4 from Cell

154 Signalling, dilution 1:1000), and anti-L1 (from BD Biosciences, 0.5 μg/ml) antibodies.
155 The samples were also tested for infectivity. HeLa cells plated 24 hours earlier at 10^5
156 cells per well in six-well plates were infected at MOI of one with thrombin-treated and
157 control PsV. 48 hours post-infection, cells were harvested with trypsin, fixed with
158 Formalde-Fresh (Fisher Scientific), and subjected to flow cytometry to count GFP-
159 positive cells.
160

161 **Internalization assay and Immunoblotting**

162 HeLa cells were transfected with Lipofectamine RNAiMAX reagent (Life Technologies,
163 Carlsbad, CA) according to manufacturer's instructions with siRNAs targeting APH1A or
164 furin, or with control siRNA 48 hours prior to infection, or pretreated for one hour at 37ºC
165 with 1μM furin inhibitor I, 250nM compound XXI, or 10nM bafilomycin A. Alternatively,
166 HPV16.L2F was pre-incubated with 100μg/ml heparin for one hour at 37°C. Cells were
167 infected at MOI of 10 with HPV16.L2F for two hours at 4°C, and washed and shifted to
168 37°C for six hours. The samples were harvested and treated with 100μg/ml proteinase
169 K to remove non-internalized virus particles. Proteinase K was then inactivated with
170 1μM phenylmethylsulfonyl fluoride, and internalized viruses were detected by western
171 blotting using rabbit anti-L1 antibody or anti-actin.
172

173 **Immunofluorescence microscopy and PLA**

174 50,000 HeLa-sen2 cells grown on glass coverslips in a 24-well plate overnight were
175 infected with HPV16.L2HA or HPV16.L2F at MOI of 20. At various times post-infection,
176 samples were fixed with 4% Formalde-Fresh, permeabilized with 1% saponin for one

177 hour at room temperature and processed for immunofluorescence with the following
178 antibodies: Sigma anti-FLAG mouse antibody (1:1000), Cell Signaling anti-HA rabbit
179 antibody (1:500), anti-L1 rabbit polyclonal serum (1:5000), and anti-33L1-7 (1:500)
180 mouse antibody. The cells were then stained with the corresponding AlexaFluor-
181 conjugated secondary antibodies diluted at 1:100 in blocking buffer. Nuclei were stained
182 by incubation with 5µg/ml DAPI for 10 min. Images were acquired with a ZEISS Axiovert
183 200 inverted fluorescent microscope using filters specific for the DAPI (blue),
184 AlexaFluor-488 (green), or AlexaFluor-568 (red) fluorochromes and processed with
185 ImageJ.

186 For the proximity ligation assay, 50,000 HeLa-sen2 cells grown overnight on
187 glass coverslips in 24-well plate were transfected with siRNA targeting APH1A or
188 control scrambled siRNA 48 hours prior to infection or pretreated with 250nM XXI for
189 one hour at 37°C. The cells were infected with HPV16.L2F at MOI of 50-100, fixed with
190 4% Formalde-Fresh at various times post-infection, and permeabilized with 1% saponin
191 for one hour at room temperature. The cells were stained with pairs of antibodies, one
192 recognizing the FLAG or the 33L1-7 epitope for capsid proteins L2 and L1, respectively,
193 and the other recognizing EEA1, TGN46, GM130, BiP or PDI. The antibodies were used
194 at the following dilutions: Sigma anti-FLAG mouse antibody (1:1000), Cell Signaling
195 anti-FLAG rabbit antibody (1:500), anti-33L1-7 mouse antibody (1:500), Cell Signaling
196 anti-EEA1 rabbit antibody (1:100), anti-TGN46 rabbit antibody (1:200), anti-GM130
197 mouse antibody (1:1000), anti-BiP rabbit antibody (1:1000) and anti-PDI rabbit antibody
198 (1:200). PLA was performed with Duolink reagents from Olink Biosciences (Uppsala,
199 Sweden) as described (6). Briefly, samples were incubated with a pair of suitable PLA

200 probes diluted 1:5 in blocking buffer in a humidified chamber for one hour and
201 processed for ligation for 30 min at 37°C. The cells were then amplified with fluorescent
202 substrates for 100 min at 37°C. Nuclei were stained with DAPI as above, and images
203 were acquired as described above. Approximately 100 nuclei were imaged in each
204 sample. The images were processed with ImageJ and quantitatively analyzed with
205 BolbFinder software to measure total fluorescence intensity in each sample. The
206 average fluorescence intensity per cell in each sample was normalized to control
207 samples as indicated in each experiment. All the experiments were done independently
208 three times with similar results, and one representative experiment is shown.
209
210 **Visualization of viral DNA**
211 To generate PsV containing EdU-substituted DNA (7), 293TT cells at 50% confluency in
212 15cm dishes were transfected with p16sheLL.L2F and pMCS-Gaussia Luc. 24 hours
213 later, medium was replaced with medium containing 10μM 5-ethynyl-2'-deoxyuridine
214 (EdU). At 72 hours post-transfection, cells were collected and PsV were purified.
215 HeLa cells were infected with EdU-labeled HPV16.L2F at MOI of 100. Eighteen
216 hours post-infection, cells were fixed with 4% FormaldeFresh for 15 min at room
217 temperature, permeabilized with 0.5% TritonX100 in PBS for 20 min at room
218 temperature, and processed for EdU visualization according to manufacturer's
219 instructions (EdU Imaging Kit from Invitrogen [kit C10339]). In brief, Click-iT reaction
220 cocktail (Click-iT reaction buffer, $CuSO_4$, AlexaFluor 594 azide and reaction buffer
221 additive) was added to permeabilized cells for 30 min at room temperature in the dark.
222 Cells were washed three times with blocking buffer and stained with anti-TGN46 and

223 DAPI as above, imaged with the Axiovert 200 microscope, and analyzed by imageJ.

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