

Supplementary Materials

HPV16 induces formation of virus-p62-PML hybrid bodies to enable infection

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Supplementary Materials and Methods

Plasmids

Expression plasmid encoding LC3-GFP (pEGFP-C1/LC3-B full-length VK325) was a kind gift by Ivan Dikic (Institute of Biochemistry II, Goethe University Medical School, Frankfurt am Main, Germany).

Quantitative mass spectrometry

Protein digest preparation: Early endosomes were pelleted by ultracentrifugation (100.000 × g, 1 h, 4°C). Pelleted endosomes were solubilized in 25 mM ammonium bicarbonate containing 0.1 % RapiGest (Waters, Eschborn, Germany) (80°C, 15 min). Proteins were reduced by adding 5 mM DTT (45 min, 56 °C) and free cysteines alkylated with iodoacetamide (Sigma, Taufkirchen, Germany) (15 mM, 25°C, 1 h in dark). 0.2 µg porcine sequencing grade trypsin (Promega, Mannheim, Germany) were added and the samples were incubated overnight at 37°C. After digestion, RapiGest was hydrolyzed by adding 10 mM HCl (37°C, 10 min) and the resulting precipitate was removed by centrifugation (13.000 × g, 15 min, 4°C) and the supernatant was transferred into an autosampler vial for peptide analysis via LCMS.

UPLC configuration: Capillary liquid chromatography of tryptic peptides was performed with a Waters NanoAcuity UPLC system equipped with a 75 µm × 150 mm BEH C18 reversed phase column and a 2.6 µl PEEKSIL-sample loop (SGE, Darmstadt, Germany). The aqueous mobile phase (mobile phase A) was H2O (LC-MS Grade, Roth, Freiburg, Germany) with 0.1 % formic acid. The organic mobile phase (mobile phase B) was 0.1 % formic acid in acetonitrile (LC-MS grade, Roth). Samples (2.6 µl injection)

were loaded onto the column in direct injection mode with 3 % mobile phase B for 15 min at 400 nl/min, followed by an additional 10 min wash (3 % B) for 10 min at 300 nl/min. Peptides were eluted from the column with a gradient from 3-35 % mobile phase B over 90 min at 300 nl/min followed by a 20 min rinse of 80 % mobile phase B. The column was immediately re-equilibrated at initial conditions (3 % mobile phase B) for 20 min. [Glu1]fibrinopeptide was used as lockmass at 300 fmol/μl. Lockmass solution was delivered from the auxiliary pump of the NanoAcuity system at 400 nl/min to the reference sprayer of the NanoLockSprayTM source. Samples were analyzed in triplicate.

Mass spectrometer configuration: Mass spectrometry analysis of tryptic peptides was performed using a Waters Q-TOF Premier API system, operated in V-mode with typical resolving power of at least 10,000. All analyses were performed using positive mode ESI using a NanoLockSpray source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with a [Glu1]fibrinopeptide solution (300 fmol/μl) delivered through the reference sprayer of the NanoLockSpray source. Accurate mass LCMS data were collected in an alternating, low energy (MS) and elevated energy (MSE) mode of acquisition. The spectral acquisition time in each mode was 0.7 s with a 0.05-s interscan delay. In low energy MS mode, data were collected at constant collision energy of 3 eV. In MSE mode, collision energy was ramped from 16 to 36 eV during each 0.7 s data collection cycle. One cycle of MS and MSE data was acquired every 1.5 s. The radio frequency applied to the quadrupole mass analyzer was adjusted such that ions from m/z 300 to 1500 were efficiently transmitted, ensuring that any ions observed in the LC/MSE data less than m/z 300 were known to arise from dissociations in the collision cell.

Data processing and protein identification: The continuum LCMSE data were processed and searched using the IDENTITYE- Algorithm of ProteinLynx Global Server (PLGS) version 2.3. The resulting peptide and protein identifications were evaluated by the software using statistical models similar to those described by Skilling et al. [1]. Protein identifications were assigned by searching the UniProtKB/Swiss-Prot Protein Knowledgebase Release 52.3 for human proteins (12920 entries) supplemented with known possible contaminants and standard proteins (porcine trypsin, yeast enolase) using the precursor and fragmentation data afforded by the LCMS acquisition method as described before. The search parameter values for each precursor and associated fragment ions were set by the software using the measured mass error obtained from processing the raw continuum data. Peptide identifications were restricted to tryptic peptides with no more than one missed cleavage. Carbamidomethyl cysteine was set as fixed modification, and oxidized methionine, protein N-acetylation, and deamidation of asparagine and glutamine were searched as variable modifications. Database search was performed allowing a maximal mass deviation of 15 ppm for precursor ions and 30 ppm for fragment ions. For a valid protein identification, the following criteria had to be met: at least 2 peptides detected with together at least 7 fragments. All reported peptide identifications provided by

the IDENTITYE-algorithm are correct with >95% probability. The initial false positive rate for protein identification was set to 3 % based on search of a 5x randomized database, which was generated automatically using PLGS 2.3 by randomizing the primary amino acid sequence of each entry. Subsequently, by using a minimum replication rate of identification in two separate technical replicates as a filter, the false positive rate is further reduced to below 0.2%.

Western blot analysis of phosphorylated p62

For analysis of p62 phosphorylation, HeLa cells were seeded in a 12-well plate in DMEM medium with FCS (without antibiotics). On the next day, the cells were starved for 1 hour in DMEM without FCS prior to PsV addition. Afterwards, all tested samples were lysed at the same time in lysis buffer containing 5 mM Tris-HCl pH 7.4, 1 mM EGTA, 250 mM sucrose and 1% Triton X-100, and phosphatase inhibitor cocktail PhosSTOP (Roche, Penzberg, Germany). Samples underwent three freeze-thaw cycles (freezing at -80°C and thawing at 4°C), were transferred to the Eppis, centrifuged, and denatured at 95°C for 5 min in SDS sample buffer. Equal amounts of protein were loaded on an SDS-PAGE gel. The samples were electro-transferred onto a nitrocellulose membrane and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.01% Tween (TBST). After incubation with primary antibodies (in 5% BSA-TBST), proteins were detected using HRP-conjugated secondary antibodies. Detection was carried out using Western Lightning Plus ECL detection reagent (PerkinElmer, Waltham, MA). Signals were recorded with X-ray films for Western blot detection Super RX-N (Fujifilm, Duesseldorf, Germany).

Detection of L1-7 epitope by immunofluorescence

HeLa cells were grown on coverslips in a 12-well plate and transfected with siRNAs. 48 hours later cells were infected with HPV16 PsVs and incubated for 7 hours at 37 °C. Subsequently, the cells were fixed with methanol and processed for staining with mAb 33L1-7 (L1-7). This mAb recognizes a specific epitope located in the interior of the PsV capsid and is not accessible in intact virions [2]. The samples were analyzed by fluorescence microscopy using a Zeiss Axiovert 200M microscope and quantified by ImageJ software. Quantification of fluorescent pixels was performed with ImageJ software.

References

1. Skilling, J.; Denny, R.; Richardson, K.; Young, P.; McKenna, T.; Campuzano, I.; Ritchie, M. ProbSeq--a Fragmentation Model for Interpretation of Electrospray Tandem Mass Spectrometry Data. *Comparative and functional genomics* 2004, *5*, 61–68, doi:10.1002/cfg.370.
2. Spoden, G.; Freitag, K.; Husmann, M.; Boller, K.; Sapp, M.; Lambert, C.; Florin, L. Clathrin- and Caveolin-Independent Entry of Human Papillomavirus Type 16--Involvement of Tetraspanin-Enriched Microdomains (TEMs). *PLoS ONE* 2008, *3*, e3313, doi:10.1371/journal.pone.0003313.

Supplementary Figures

Figure S1: Quantitative Mass spectrometry detected enrichment of cellular proteins in endosomal fractions after HPV16 PsV incubation. The quantitative analysis of host-cell proteins of two individual LC-MS experiments is shown. Experiments were performed using HeLa cells. Three independent preparations at different timepoints (non-infected, 4h, 7h post infection) were analysed in five technical replicates in each experiment. Parts per million (ppm) values are calculated at the protein level. The amount of each protein is determined using the 3 "best ionizing" peptides, which are then compared to the total protein in the sample. The protein level values of the technical replicates (MW ppm) are highlighted in yellow; the data for HPV16 major capsid protein L1 are highlighted in red; for p62/sequestosome-1 in green and for Rab5a in gray.

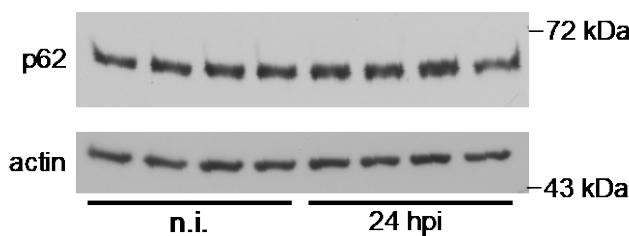


Figure S2. Representative p62 levels after HPV16 PsVs addition. HeLa cells were incubated with HPV16 PsVs for 24 hours (24 hpi) or left untreated (not infected, n.i.). P62 levels were analyzed by Western blotting using p62-specific mAb. β -actin was used as loading control.

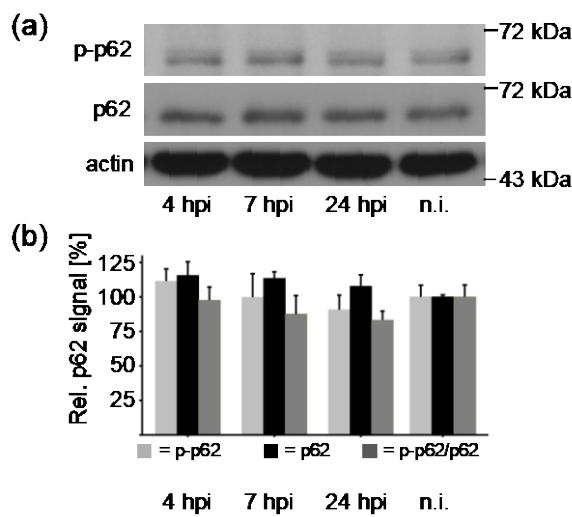


Figure S3. P62 phosphorylation is unaffected by HPV16 PsVs. (a) Western blotting showing p62 phosphorylation status of HeLa cells upon HPV16 PsVs addition. Cells were starved for 1 hour and subsequently exposed to PsVs for different time points. All samples were collected at the same time. For signal detection, the membrane was stained using p62-specific mAb (for total p62 protein detection) and phospho-p62-specific pAb (p-p62, for detection of phosphorylated p62 at Ser 349). β -actin was used as a loading control. (b) Quantification of the blots shown in (a) using ImageJ software. The amount of phosphorylated p62 protein is shown as a ratio of phosphorylated to total p62 form. The values are given as mean \pm SEM and the mean for not infected cells (n.i.) was set to 100%. Data (n =

4-9 out of three replicates) were analyzed for significant differences using unpaired *t*-test for total p62 ($p = 0.0176$, $p = 0.0017$ and $p = 0.1884$ for n.i. vs. 4 hpi, 7 hpi and 24 hpi, respectively), using unpaired *t*-test for phospho-p62 ($p = 0.4375$ and $p = 0.9878$ for n.i. vs. 4 hpi and 7 hpi, respectively) and Mann-Whitney test ($p = 0.3301$ for n.i. vs. 24 hpi), and using unpaired *t*-test for phospho-p62 per total p62 ($p = 0.8681$, $p = 0.4446$ and $p = 0.2509$ for n.i. vs. 4 hpi, 7 hpi and 24 hpi, respectively).

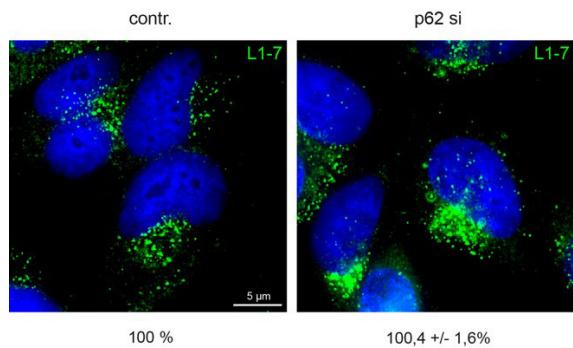


Figure S4. P62 knockdown has no effect on virus internalization. HeLa cells were transfected with control siRNA (contr.) or a p62 siRNA pool (p62 si) for 48 hours and infected with HPV16 PsVs for 7 hours. Cells were stained with L1-specific Ab (33L1-7) and nuclear stain solution (Hoechst 33342). Relative L1-7-positive pixels per DNA pixels were quantified using ImageJ software and the values are given as a mean \pm SEM. The mean for control siRNA-treated cells (contr.) was set to 100%.

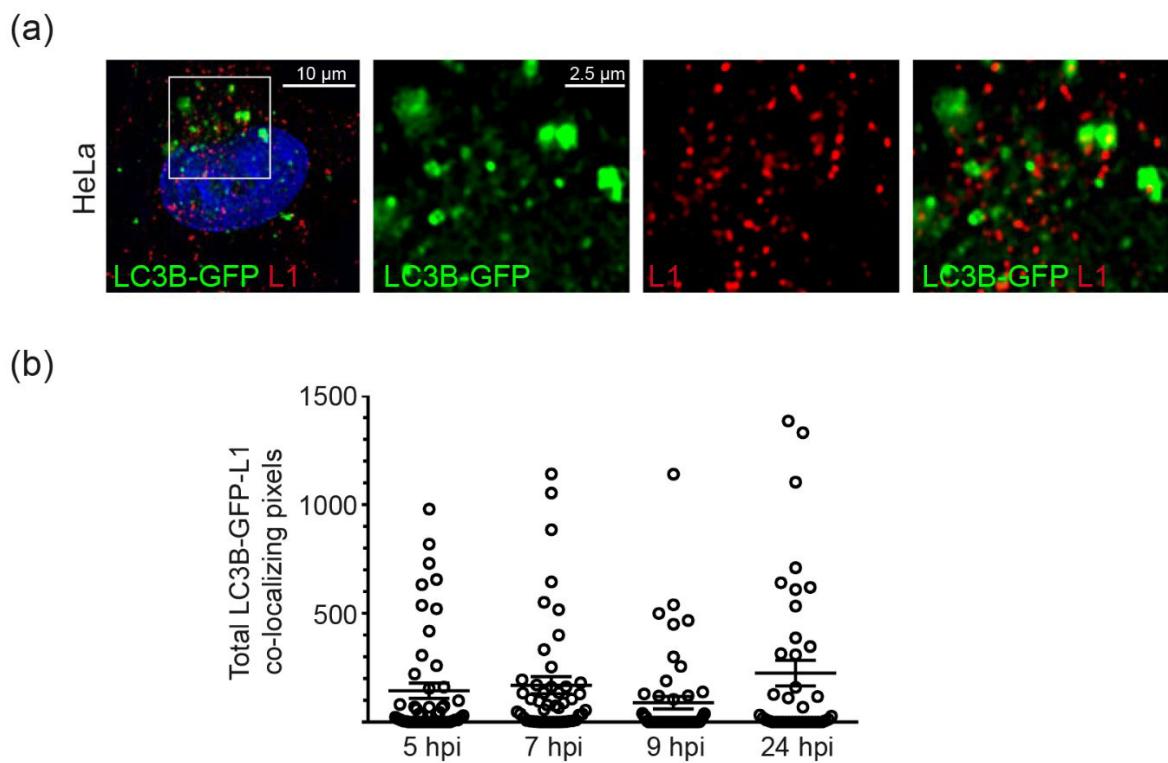


Figure S5. HPV16 major capsid protein L1 co-localizes stably over the time with autophagosome marker LC3B. **(a)** HeLa cells were transfected with LC3B-GFP expressing plasmid for 24 hours and infected with HPV16 PsVs for five, seven, nine or 24 hours (5h, 7h, 9h, or 24h). Cells were stained for HPV16 major capsid protein L1 by immunofluorescence using K75 antibody. **(a)** Representative pictures of LC3B-GFP and HPV16 L1 co-localization in HeLa (exposed to PsVs for 7 hours). LC3B-GFP is shown in green, L1 in red and nuclear DNA (stained by Hoechst) in blue. The box in the first panel outlines the following higher magnification images. **(b)** LC3B-GFP-L1 co-localization is not dependent on the duration of HPV16 infection in HeLa cells. Graphs show quantification of the total number of pixels representing LC3B-GFP-L1 co-localization after 5h, 7h, 9h, or 24 h of HPV16 PsVs. Data ($n = 40 - 52$ out of three replicates) are shown as scatter dot plot and the lines represent mean \pm SEM of the absolute numbers from three independent experiments. Significant differences were analyzed using ordinary one-way ANOVA ($p = 0.1252$).

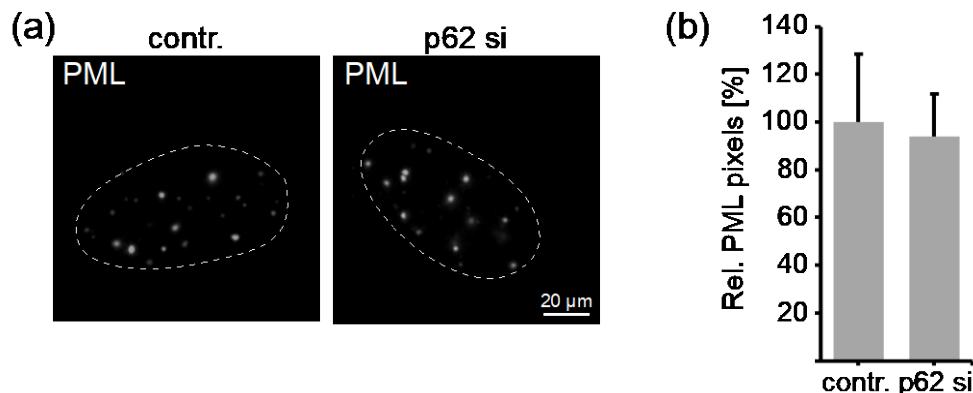


Figure S6. PML after p62 knockdown. **(a-b)** HeLa cells were transfected with control siRNA (contr.) or a p62 siRNA pool (p62 si) for 48 hours and infected with HPV16 PsVs for 24 hours. Cells were stained with PML-specific mAb and nuclear stain solution (Hoechst 33342). Representative pictures are shown in **(a)**. **(b)** Relative PML-positive pixels per DNA pixels were quantified using ImageJ software and are given as a mean \pm SEM. The mean for control siRNA-treated cells (contr.) was set to 100%. Data ($n = 57-60$ out of three replicates) were analyzed for significant differences using Mann-Whitney test: $p > 0.05$.