SUPPORTING INFORMATION

A ubiquitin variant-based affinity approach selectively identifies substrates of the ubiquitin ligase E6AP in complex with HPV-11 E6 or HPV-16 E6

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Running title: Identification of substrates of the E6-E6AP ubiquitin ligase

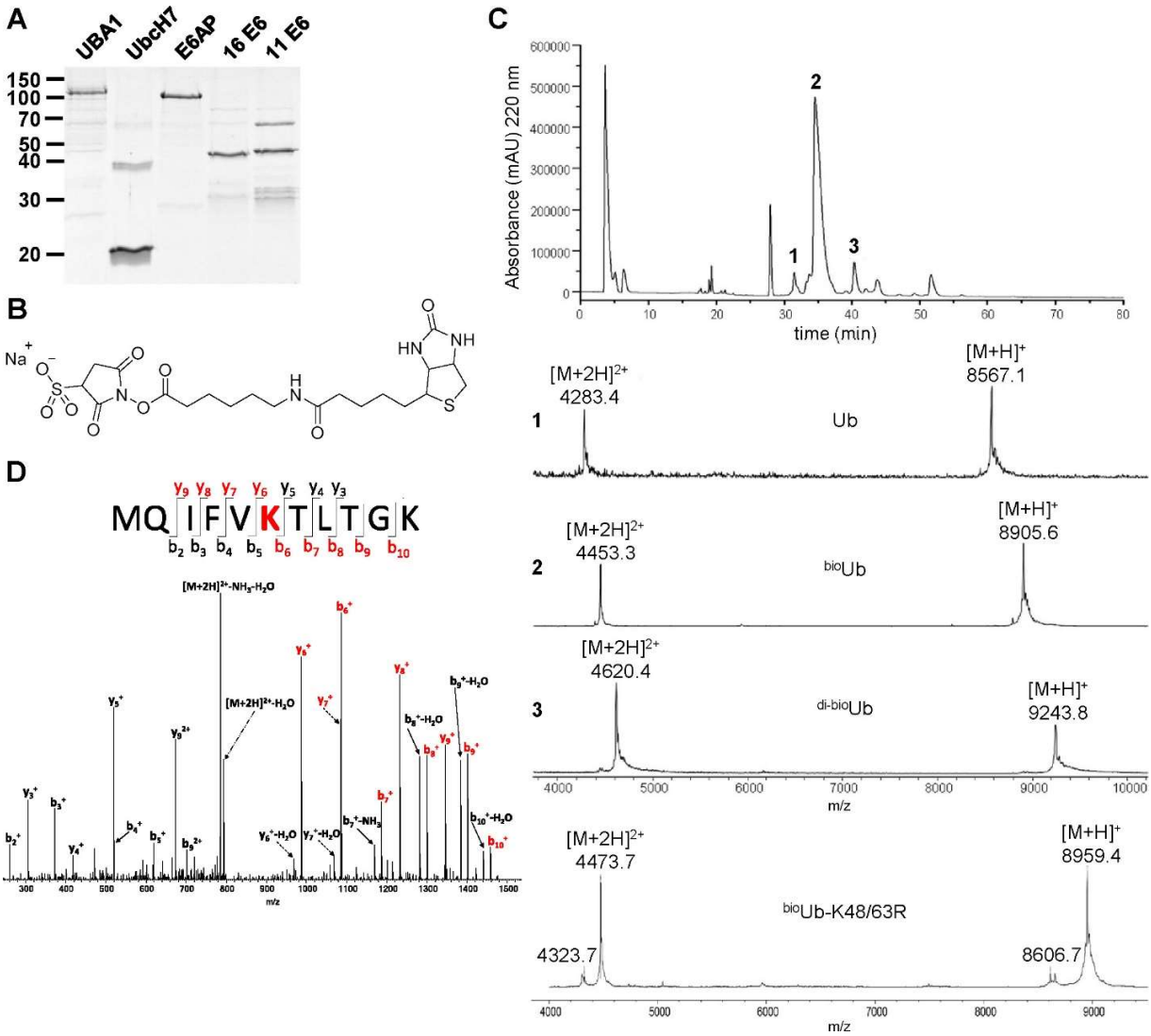

Fig. S1 *A*, The ubiquitin-activating enzyme UBA1 and E6AP (isoform 1 (15)) were expressed in the baculovirus system and purified as described (Experimental Procedures). The ubiquitin-conjugating enzyme UbcH7 and GST-fusion proteins of HPV-16 and HPV-11 E6 were bacterially expressed and purified as described (Experimental Procedures). Upon purification, concentrations of the respective protein preparations were determined and the proteins subjected to SDS-PAGE followed by Coomassie blue staining. Running positions of molecular mass markers are indicated. *B*, Structure of Sulfo-NHS-LC-biotin used for biotinylation of ubiquitin. *C*, HPLC chromatogram of ubiquitin (Ub) after reaction with Sulfo-NHS-LC-biotin, and MALDI-TOF spectra of peak 1, 2, and 3, as well as of mono-biotinylated Ub-K48/63R. *D*, LC-MS/MS analysis of mono-biotinylated ubiquitin.

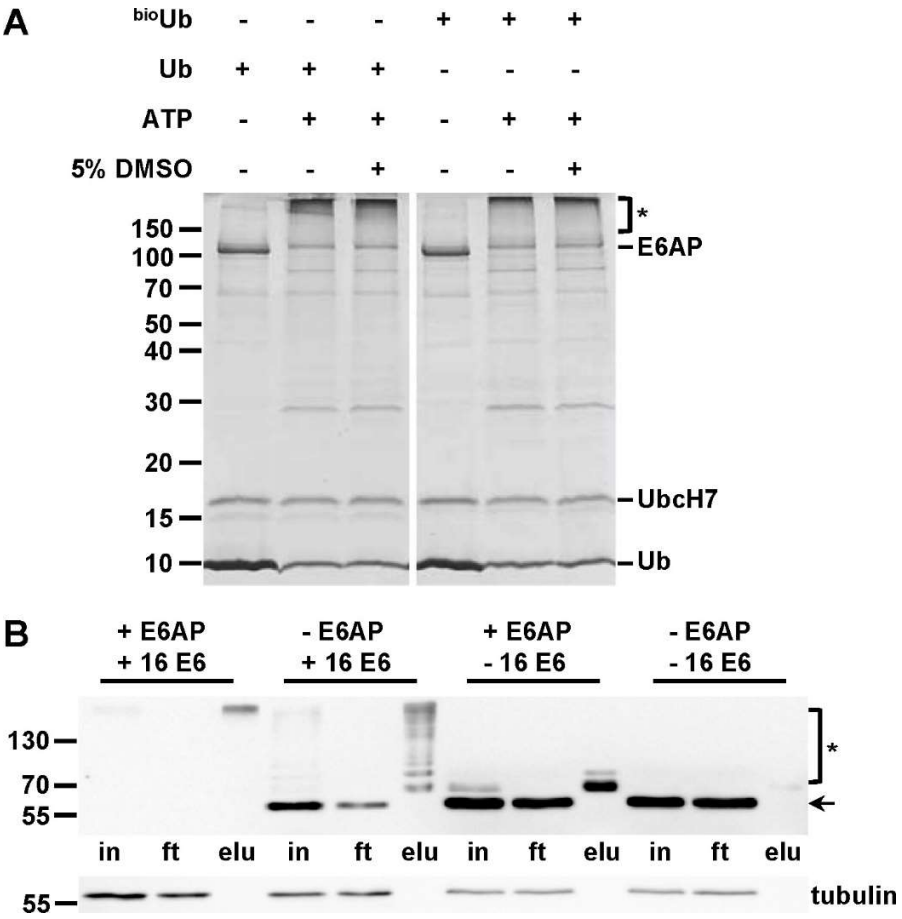

Fig. S2 *A*, Biotinylated ubiquitin (^{bio}Ub) is efficiently used by E6AP for auto-ubiquitination. E6AP was incubated with UBA1, UbcH7, and non-modified ubiquitin (Ub) or ^{bio}Ub under standard ubiquitination conditions (Experimental Procedures). After 90 min at 30 °C, reactions were stopped and analyzed by SDS-PAGE followed by Coomassie blue staining. Running positions of free ubiquitin and free ^{bio}Ub (Ub), non-modified E6AP, and poly-ubiquitinated E6AP (asterisk) are indicated. *B*, HEK293T cell extract was incubated with recombinant UBA1, UbcH7, and ^{bio}Ub in the absence and presence of recombinant E6AP and a GST fusion protein of HPV-16 E6. After 90 min at 30 °C, potentially ubiquitinated proteins were enriched via streptavidin-affinity purification and analyzed by Western blot analysis using an anti-p53 antibody or an anti-tubulin antibody as control. Note that HEK293T cells express endogenous E6AP at levels that are sufficient to facilitate E6-mediated ubiquitination of p53; however, the efficiency is improved by addition of recombinant E6AP, as witnessed by the decrease in levels of non-modified p53. Running positions of molecular mass markers, non-modified p53 (arrow), and ubiquitinated forms of p53 (asterisk) are indicated. in, cell extract used for affinity purification after ubiquitination reaction; ft, proteins not bound to streptavidin beads; elu, proteins bound to streptavidin beads.

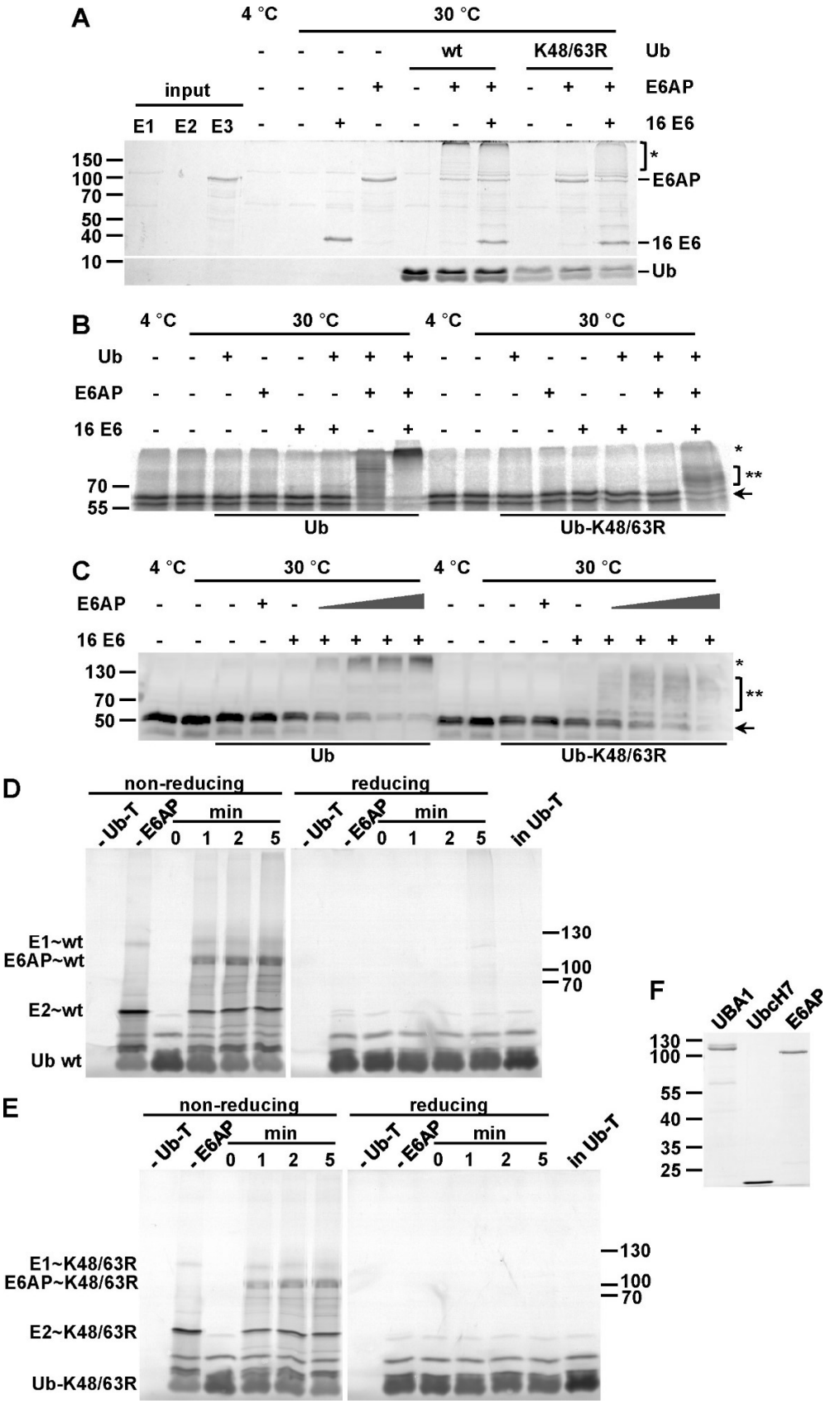
A
4 °C
30 °C
wt
K48/63R
Ub
input
E6AP
E1 E2 E3
16 E6
150
100
70
50
40
10
*
E6AP
16 E6
Ub
B
4 °C
30 °C
4 °C
30 °C
Ub
E6AP
16 E6
70
55
*
**
Ub
Ub-K48/63R
C
4 °C
30 °C
4 °C
30 °C
E6AP
16 E6
130
70
50
*
**
Ub
Ub-K48/63R
D
non-reducing
reducing
- Ub-T
- E6AP
min
0 1 2 5
in Ub-T
E1~wt
E6AP~wt
E2~wt
Ub wt
130
100
70
E
non-reducing
reducing
- Ub-T
- E6AP
min
0 1 2 5
in Ub-T
E1~K48/63R
E6AP~K48/63R
E2~K48/63R
Ub-K48/63R
130
100
70
F
UBA1
UbcH7
E6AP
130
100
55
40
35
25

Fig. S3 The ubiquitin variant Ub-K48/63R is poorly used by E6AP for ubiquitination in the absence of HPV-16 E6. *A*, E6AP auto-ubiquitination. Baculovirus-expressed E6AP (E3) was incubated with UBA1 (E1) and UbcH7 (E2) in the absence or presence of wild-type (wt) ubiquitin (Ub) or Ub-K48/63R and a GST fusion protein of HPV-16 E6. After 90 min at 30 °C, reaction mixtures were subjected to SDS-PAGE followed by Coomassie blue staining. Running positions of the various proteins and of molecular mass markers are indicated. Ubiquitinated forms of E6AP are indicated by an asterisk. *B*, E6AP-mediated ubiquitination of Ring1b-I53S, an E6-independent substrate of E6AP. *In vitro* translated, radiolabeled Ring1b-I53S (30) was incubated with E1 and UbcH7 in the absence and presence of wild-type ubiquitin (Ub) or the ubiquitin variant Ub-K48/63R, baculovirus-expressed E6AP, and a GST fusion protein of HPV-16 E6 as indicated. Upon 90 min at 30 °C, reactions were stopped and analyzed by SDS-PAGE followed by fluorography. Running positions of the non-modified form and poly-ubiquitinated forms of Ring1b-I53S are indicated by an arrow and an asterisk, respectively. **, presumably Ring1b-I53S mono-ubiquitinated at one or several lysine residues by Ub-K48/63R. *C*, E6-E6AP-mediated ubiquitination of p53. HEK293T cell extract was incubated with E1 and UbcH7 in the absence and presence of wild-type ubiquitin (Ub) or the ubiquitin variant Ub-K48/63R, increasing amounts of baculovirus-expressed E6AP, and a GST fusion protein of HPV-16 E6 as indicated. Upon 90 min at 30 °C, reactions were stopped and analyzed by Western blot analysis using an anti-p53 antibody. Running positions of the non-modified form and poly-ubiquitinated forms of p53 are indicated by an arrow and an asterisk, respectively. **, presumably p53 mono-ubiquitinated at one or several lysine residues by Ub-K48/63R. *D-E*, Thioester complex formation of E6AP with ubiquitin and Ub-K48/63R. Baculovirus-expressed E6AP was incubated with UBA1 (E1), UbcH7 (E2), and a mixture of TAMRA-labeled wild-type ubiquitin (Ub wt) with unlabeled ubiquitin (*D*) or Ub-K48/63R (K48/63R) with unlabeled Ub-K48/63R (*E*) (ratio 1:1) for the times indicated at 30 °C. Reactions were stopped by incubating the mixtures for 15 min at 30 °C in urea loading buffer (Experimental Procedures) in the absence (non-reducing) or presence (reducing) of 25 mM DTT. The reactions were separated on 7.5-15 % SDS-PA gradient gels at 4 °C and analyzed by fluorescence scan at 532 nm to monitor TAMRA-labeled ubiquitin and TAMRA-labeled Ub-K48/63R (Ub-T). Running positions of the respective ubiquitin thioester complexes (~), of free Ub-T (Ub wt or Ub-K48/63R), and of molecular mass markers are indicated. - Ub-T, reaction in the absence of TAMRA-labeled ubiquitin/Ub-K48/63R; - E6AP, reaction in the absence of E6AP; In Ub-T, input of TAMRA-labeled ubiquitin/Ub-K48/63R. *F*, Protein preparations used for E6AP-ubiquitin thioester complex formation as determined by SDS-PAGE and Coomassie blue staining.

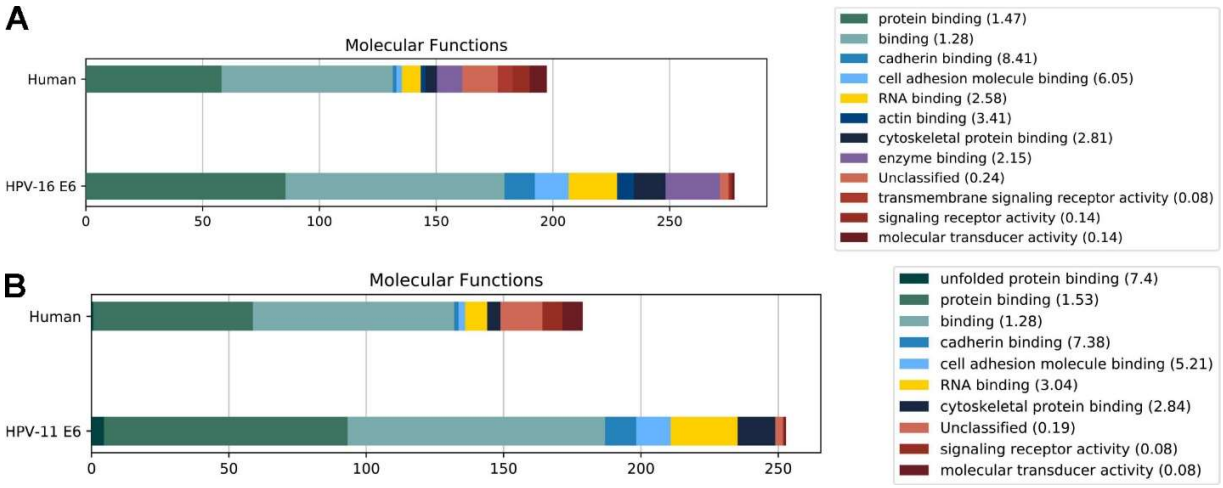

Fig. S4 PANTHER overrepresentation test of the GO annotation dataset 'molecular functions'. Shown is the human reference dataset with classes of molecular functions that are over- or under-represented for HPV-16 E6 and HPV-11 E6 compared to the human genome. Classes of molecular functions that are over-represented in HPV-16 E6/HPV-11 E6 are colored in green, blue, yellow and purple. Classes of molecular functions that are under-represented are colored in red. The 'fold enrichment' compared to the human genome as calculated by PANTHER is indicated in brackets for each class of molecular function. *A*, Classes of molecular functions that are over-or under-represented for HPV-16 E6 compared to the human genome in %. *B*, Classes of molecular functions that are over-or under-represented with HPV-11 E6 compared to the human genome in %. Note that most proteins map to more than one molecular function resulting in a total value >100 %.

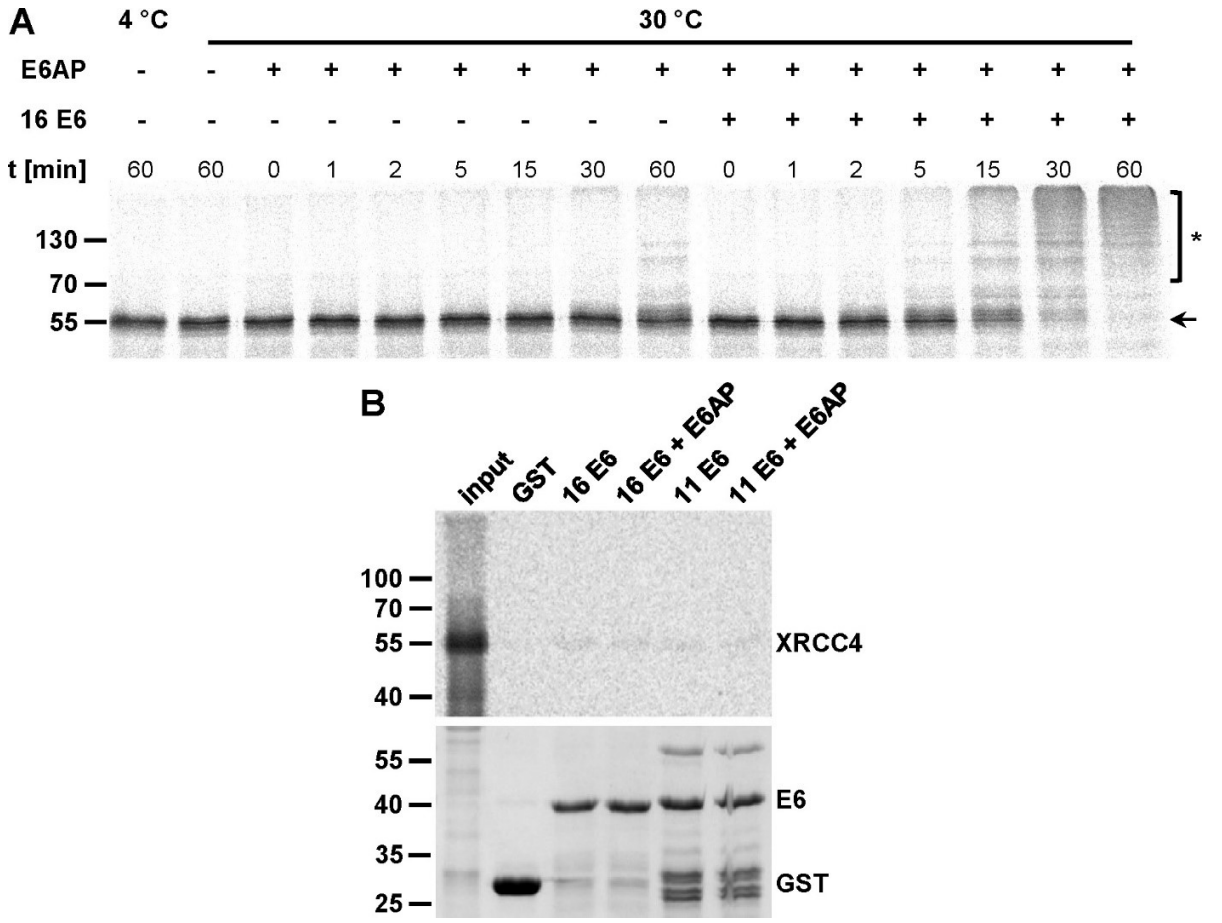

Fig. S5 *A*, Time course of XRCC4 ubiquitination. *In vitro* translated, radiolabeled XRCC4 was incubated at 30 °C with E1, UbcH7, and ubiquitin in the absence and presence of baculovirus-expressed E6AP and a GST fusion protein of HPV-16 E6. At the times indicated, reactions were stopped and XRCC4 ubiquitination analyzed by SDS-PAGE followed by fluorography. Running positions of molecular mass markers, the non-modified form (arrow) and ubiquitinated forms (asterisk) of XRCC4 are indicated. *B*, GST fusion proteins of HPV-16 E6 and HPV-11 E6 or GST alone bound to glutathione sepharose beads were mixed with *in vitro* translated, radiolabeled XRCC4 and baculovirus-expressed E6AP as indicated. Upper panel, upon incubation for 90 min at 4 °C, beads were washed and eluates analyzed by SDS-PAGE followed by fluorography. input, 10% of XRCC4 used in the binding reactions. Lower panel, amount of GST proteins used in the binding reactions as determined by SDS-PAGE and Coomassie blue staining.

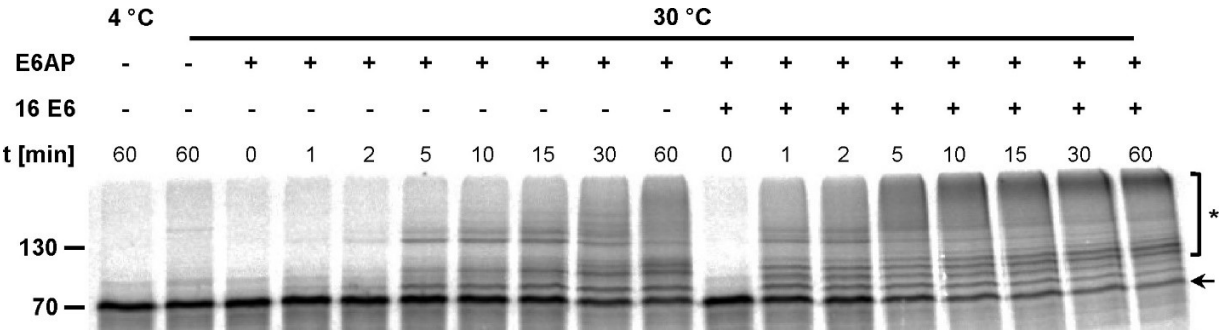

Fig. S6 Time course of OTUD5 ubiquitination. *In vitro* translated, radiolabeled OTUD5 was incubated at 30 °C with E1, UbcH7, and ubiquitin in the absence and presence of baculovirus-expressed E6AP and a GST fusion protein of HPV-16 E6. At the times indicated, reactions were stopped and OTUD5 ubiquitination analyzed by SDS-PAGE followed by fluorography. Running positions of molecular mass markers, the non-modified form (arrow) and ubiquitinated forms (asterisk) of OTUD5 are indicated.

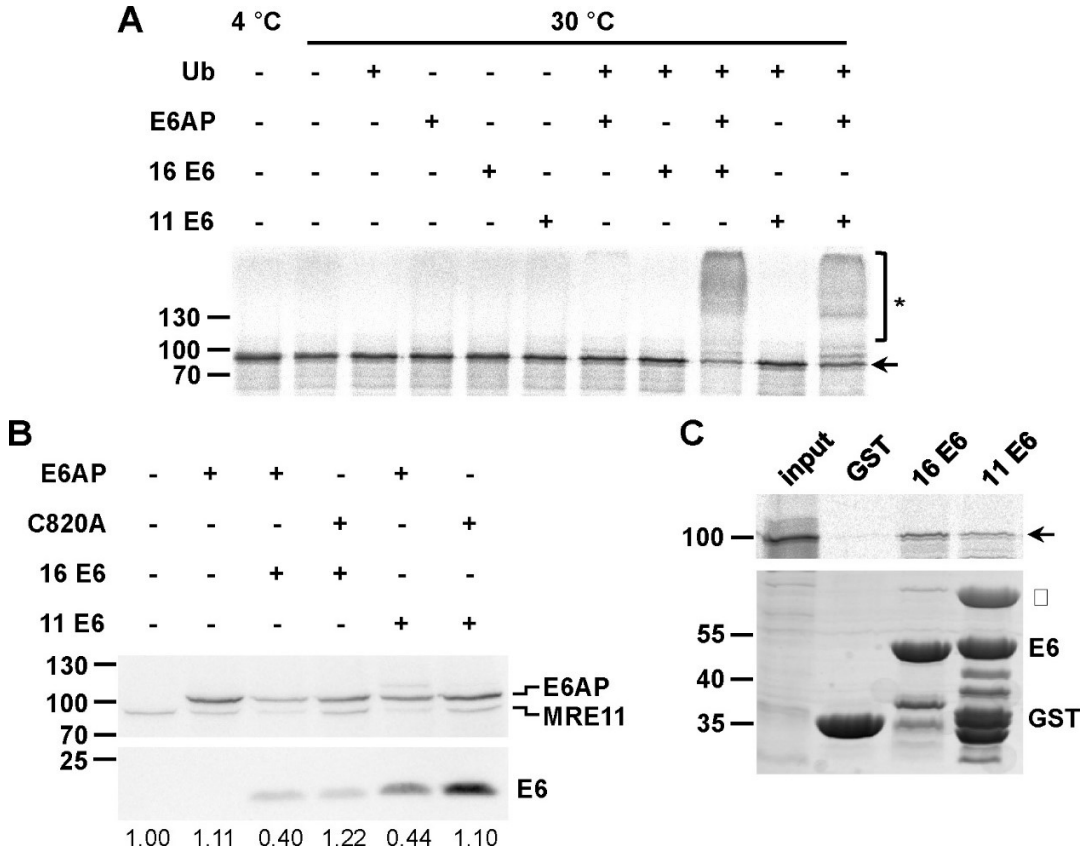

Fig. S7 MRE11 as potential substrate protein of the HPV E6 proteins. *A*, *In vitro* translated, radiolabeled MRE11 was incubated with E1 and UbcH7 in the absence and presence of ubiquitin (Ub), baculovirus-expressed E6AP, and GST fusion proteins of HPV-16 E6 or HPV-11 E6 as indicated. Upon 90 min at 30 °C, reactions were stopped and analyzed by SDS-PAGE followed by fluorography. Running positions of molecular mass markers, the non-modified form (arrow) and the ubiquitinated forms (asterisk) of MRE11 are indicated. *B*, H1299-K3 cells, in which endogenous E6AP expression is stably downregulated by RNA interference (11), were transfected with expression constructs encoding HA-tagged forms of MRE11, E6AP, a catalytically inactive E6AP mutant (C820A), HPV-16 E6, and HPV-11 E6 as indicated. 24 h after transfection, protein extracts were prepared and the levels of the various proteins determined by Western blot analysis using an anti-HA antibody. Note that prior to SDS-PAGE, the relative transfection efficiency of each transfection was determined (see Experimental Procedures) to adjust the amounts of the extracts used for analysis. Running positions of molecular mass markers and relative levels of HA-MRE11 are indicated. *C*, GST fusion proteins of HPV-16 E6 and HPV-11 E6 or GST alone bound to glutathione sepharose beads were mixed with *in vitro* translated, radiolabeled MRE11. Upper panel, upon incubation for 90 min at 4 °C, beads were washed and eluates analyzed by SDS-PAGE followed by fluorography. input, 10% of MRE11 used in the binding reactions. Lower panel, amount of GST proteins used in the binding reactions as determined by SDS-PAGE and Coomassie blue staining. The rectangle indicates the running position of a bacterial chaperonin (GroEL) that copurifies with the E6 proteins and levels of which differ between different E6 preparations.

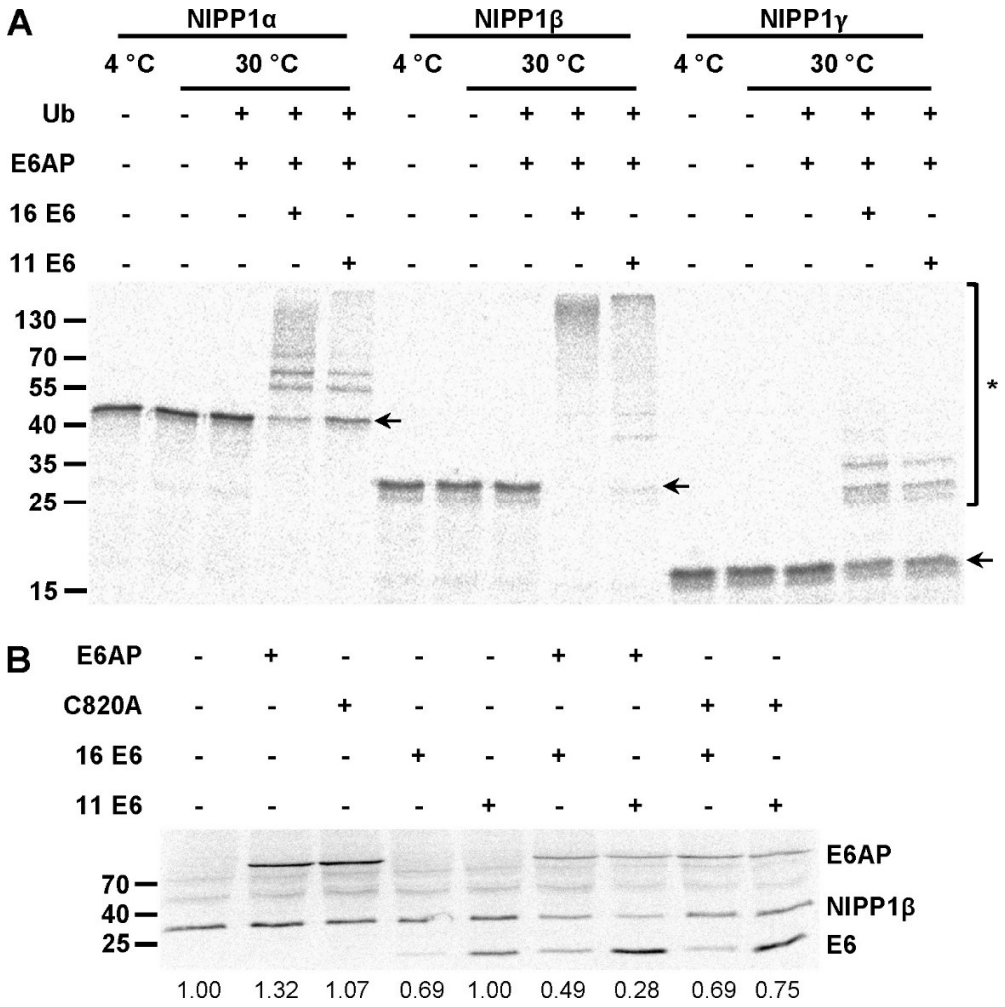

Fig. S8 NIPP1 as potential substrate protein of the HPV E6 proteins. The nuclear inhibitor of protein phosphatase 1 (NIPP1) is encoded by the *PPP1R8* gene which encodes several isoforms. Here, we studied the effect of the HPV E6 proteins on ubiquitination and degradation of the isoforms NIPP1α, NIPP1β, and NIPP1γ. NIPP1β and NIPP1γ differ from NIPP1α (351 amino acids) by the lack of the N-terminal 142 and 224 amino acids, respectively. *A*, *In vitro* translated, radiolabeled NIPP1α, NIPP1β, and NIPP1γ were incubated with E1 and UbcH7 in the absence and presence of ubiquitin (Ub), baculovirus-expressed E6AP, and GST fusion proteins of HPV-16 E6 or HPV-11 E6 as indicated. Upon 90 min at 30 °C, reactions were stopped and analyzed by SDS-PAGE followed by fluorography. Running positions of molecular mass markers, the non-modified form (arrow) and ubiquitinated forms (asterisk) of the NIPP1 isoforms are indicated. *B*, Since NIPP1β was the NIPP1 isoform that was most efficiently ubiquitinated by the HPV E6 proteins *in vitro*, we tested the effect of the HPV E6 proteins on NIPP1β levels in cotransfection assays in cells. H1299-K3 cells, in which endogenous E6AP expression is stably downregulated by RNA interference (11), were transfected with expression constructs encoding HA-tagged forms of NIPP1β, E6AP, a catalytically inactive E6AP mutant (C820A), HPV-16 E6, and HPV-11 E6 as indicated. 24 h after transfection, protein extracts were prepared and the levels of the various proteins determined by Western blot analysis using an anti-HA antibody. Prior to SDS-PAGE, the relative transfection efficiency of each transfection was determined (see Experimental Procedures) to adjust the amounts of the extracts used for analysis. Running positions of molecular mass markers and relative levels of HA-NIPP1β are indicated.

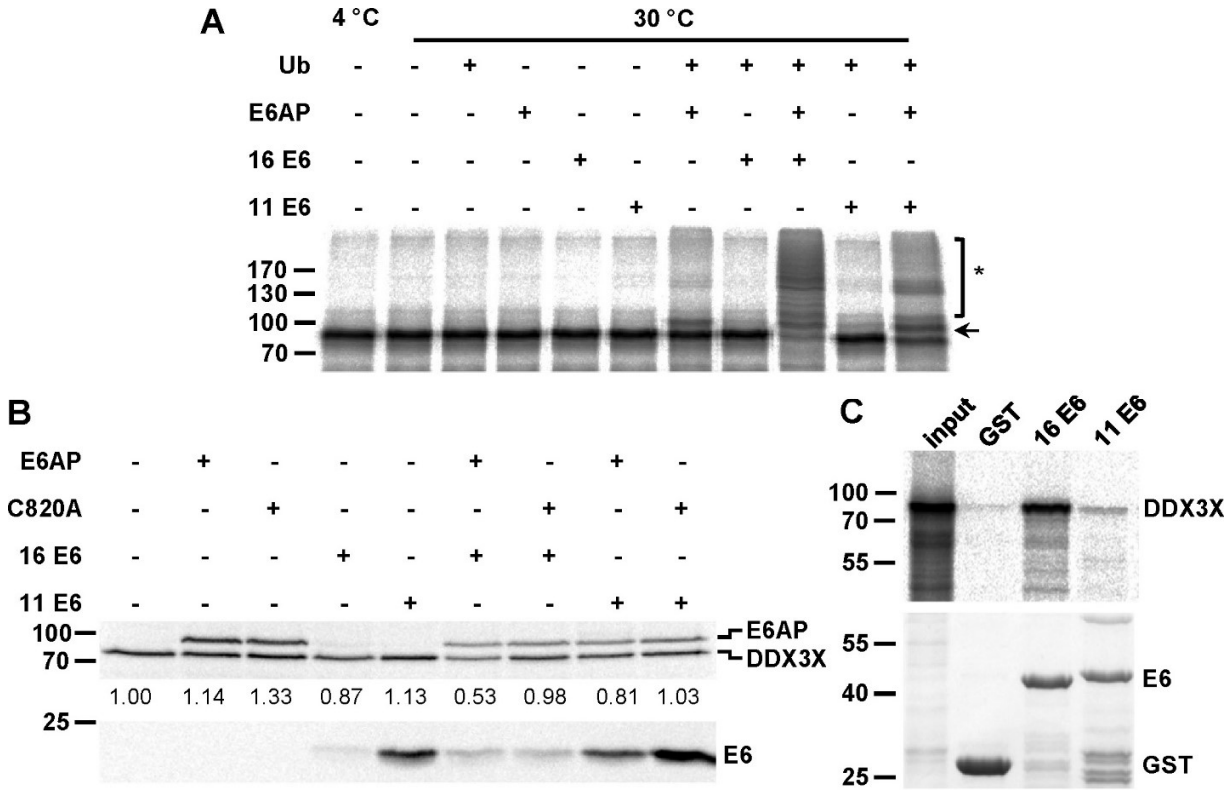

Fig. S9 DDX3X as potential substrate protein of the HPV-16 E6 protein. *A*, *In vitro* translated, radiolabeled DDX3X was incubated with E1 and UbcH7 in the absence and presence of ubiquitin (Ub), baculovirus-expressed E6AP, and GST fusion proteins of HPV-16 E6 or HPV-11 E6 as indicated. Upon 90 min at 30 °C, reactions were stopped and analyzed by SDS-PAGE followed by fluorography. Running positions of molecular mass markers, the non-modified form (arrow) and the ubiquitinated forms (asterisk) of DDX3X are indicated. *B*, H1299-K3 cells, in which endogenous E6AP expression is stably downregulated by RNA interference (11), were transfected with expression constructs encoding HA-tagged forms of DDX3X, E6AP, a catalytically inactive E6AP mutant (C820A), HPV-16 E6, and HPV-11 E6 as indicated. 24 h after transfection, protein extracts were prepared and the levels of the various proteins determined by Western blot analysis using an anti-HA antibody. Note that prior to SDS-PAGE, the relative transfection efficiency of each transfection was determined (see Experimental Procedures) to adjust the amounts of the extracts used for analysis. Running positions of molecular mass markers and relative levels of HA-DDX3X are indicated. *C*, GST fusion proteins of HPV-16 E6 and HPV-11 E6 or GST alone bound to glutathione sepharose beads were mixed with *in vitro* translated, radiolabeled DDX3X. Upper panel, upon incubation for 90 min at 4 °C, beads were washed and eluates analyzed by SDS-PAGE followed by fluorography. input, 10% of DDX3X used in the binding reactions. Lower panel, amount of GST proteins used in the binding reactions as determined by SDS-PAGE and Coomassie blue staining.