Structure of high-risk papillomavirus type 31 E6 oncogenic protein and characterization of E6/E6AP/p53 complex formation

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1 **Title:** Structure of high-risk papillomavirus type 31 E6 oncogenic protein and
2 characterization of E6/E6AP/p53 complex formation

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7 **Running Title** (54 characters): HPV 31E6 structure and complex characterization

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20 experimental design and data analysis/interpretation– MC, GG, AB, ACS, JL, FS, TI,
21 GT, CS; Fluorescence Polarization – MC, GG; crystallography – IS, MC, ACS, AM,
22 GG; MST – DIF, CS; GPCA – MC, MM

23 **Abbreviations**

24	E6AP	E3 ubiquitin ligase E6-associated protein
25	GPCA	<i>Gaussia princeps</i> protein complementation assay
26	His ₆	Hexa-histidine
27	HPV	Human papillomavirus
28	MBP	Maltose binding protein
29	OD ₆₀₀	Optical density at 600 nm
30	TCEP	Tris(2-carboxyethyl)phosphine

31

32 **Abstract (173/250 words)**

33 The degradation of p53 is a hallmark of “high-risk” HPV types of the alpha genus and
34 HPV-related carcinogenicity. The oncoprotein E6 forms a ternary complex with the
35 E3 ubiquitin ligase E6-associated protein (E6AP) and tumor suppressor protein p53
36 targeting p53 for ubiquitination. The extent of p53 degradation by different E6
37 proteins varies greatly, even for the closely-related HPV 16 and 31. 16E6 and 31E6
38 display high sequence identity (~67 %). We report here for the first time the structure
39 of HPV31 E6 bound to the LxxLL motif of E6AP. 16E6 and 31E6 are structurally very
40 similar in agreement with the high sequence conservation. Both E6 proteins bind
41 E6AP and degrade p53. However, the binding affinities of 31E6 to E6AP and p53,
42 respectively, are reduced 2-fold and 5.4-fold as compared to 16E6. The affinity of E6-
43 E6AP-p53 ternary complex formation parallels the efficacy of the subsequent
44 reaction, namely degradation of p53. Therefore, closely-related E6 proteins

45 addressing the same cellular targets may still diverge in their binding efficiencies,
46 possibly explaining their different phenotypic or pathologic impact.

47 **Importance** (150/150 words)

48 Variations of carcinogenicity of Human papillomaviruses are related to variations of
49 the E6 and E7 interactome. While different HPV species and genera are known to
50 target distinct host proteins, the fine differences between E6 and E7 of closely-related
51 HPV types, supposed to target the same cellular protein pools, remain to be
52 addressed. We compare the oncogenic E6 proteins of the closely related “high-risk”
53 HPV types 31 and 16 with regard to their structure and their efficiency of ternary
54 complex formation with their cellular targets p53 and E6AP, which results in p53
55 degradation. We solved the crystal structure of 31E6 bound to the E6AP LxxLL-motif.
56 16E6 and 31E6 structures are highly similar but a few sequence variations lead to
57 different protein contacts within the ternary complex and, as quantified here, an
58 overall lower binding affinity of 31E6 compared with 16E6. These results align with
59 the observed lower p53-degradation potential of 31E6.

60

61 Introduction

62 Human papillomaviruses (HPV) comprise over 200 types. In accordance to their
63 sequence alignment of the major capsid protein L1 they are divided in 5 genera –
64 alpha, beta, gamma, mu and nu. HPVs infect skin squamous epithelial cells (alpha
65 and beta genus) and mucosal epithelial cells (alpha genus) in humans. An infection
66 can either be asymptomatic or result in benign tumors or cancer. Cancer
67 development occurs only in the rare cases of persistent infection and failure of viral
68 clearance. However, so called “high-risk” HPVs are classified as most carcinogenic
69 by the International Agency for Research on Cancer (IARC) (1, 2) because they are
70 highly associated with cancer of cervix (100.0 %), oropharynx (30.8 %), vulvar
71 (24.9 %), vagina (78.0 %), penis (50.0 %), and anus (88.0 %) (3). The carcinogenic
72 potential varies between the different HPV types. All “high-risk” types belong to the
73 alpha genus, but to four different species: alpha-5 (HPV 51), alpha-6 (HPV 56),
74 alpha-7 (HPV 18, 39, 45, 59 and 68) and alpha-9 (HPV 16, 31, 33, 35, 52, 58).
75 HPV 16 is associated with ~ 50 %, HPV 18 with 20 % whereas the HPV 31, 33, 35,
76 39, 45, 51, 52, 56, 58, 59 and 68 together are associated with ~ 30 % of cervical
77 cancer (4-6).

78 Two viral oncoproteins E6 and E7, which are always expressed in HPV associated
79 cancers (7), are responsible for the immortalization of human keratinocytes, the
80 target cells of HPV (8). Current models suggest that protein-protein-interactions of
81 both viral oncoproteins with their cellular targets contribute to carcinogenicity. Most
82 E7 proteins share the capacity to bind to retinoblastoma (Rb) family members as well
83 as phosphatases PTPN14 and PTPN21 (9-13), independently of the carcinogenic
84 risk level of HPVs they belong to. In contrast, E6 proteins do not share any universal
85 target cell protein conserved for all HPVs. However, binding to the E3 ubiquitin ligase

86 E6-associated protein (E6AP), the tumor suppressor protein p53 and PDZ domain
87 proteins are key interactions that are specifically displayed only for E6 proteins of
88 “high-risk” alpha mucosal HPVs. The simultaneous recruitment of E6AP and p53 by
89 E6 results in the degradation of p53 (14, 15).

90 E6 is highly conserved among papillomaviruses and consists of two zinc-binding
91 domains (figure 1). An E6-based phylogenic classification depicts similar
92 relationships for E6 proteins within the alpha genus as the common L1-based
93 classification. Within the alpha-9 group HPV 16, 35 and 31 are the closest related
94 HPVs. Generally, E6 proteins bind to accessible LxxLL-motifs of various cellular
95 target proteins. The LxxLL-binding profile of different E6 proteins varies within the
96 alpha genus (16).

97 HPV 16 is the HPV type with highest carcinogenic risk. It has been shown previously,
98 that 16E6 forms a ternary complex with the E6AP-LxxLL motif and the p53 core
99 domain (17). Once formed, the E6/E6AP/p53 complex mediates ubiquitination and
100 subsequent proteasomal degradation of p53 (18). Among all cellular interaction
101 partners of E6, this ternary complex is the best characterized interaction in structure
102 and function for 16E6. Studies on binding parameters of the ternary complex
103 depicted binding affinities of the 16E6-LxxLL(E6AP) dimer to p53 in a μ M-range (17)
104 and defined crucial amino acids for complex formation and subsequent p53
105 degradation (table 2 and 3). Figure 1 shows a sequence alignment of E6 proteins of
106 the alpha-9 genus and HPV 18 (alpha-7) as the second most prevalent “high-risk”
107 type. Amino acids of 16E6, which significantly contribute to LxxLL(E6AP) or p53
108 binding are not completely conserved among the alpha-9 HPVs. In table 2 and 3
109 these amino acids are compared regarding their conservation between 16E6 and
110 31E6. Despite the high sequence homology of 16E6 and 31E6 of 67 % identity

111 (ClustalO (19)) both binding sites show sequence variations, which can potentially
112 interfere with target binding, the formation of the ternary complex and subsequent
113 p53 degradation. Additionally, quantitative assays using a luciferase-p53 fusion
114 protein as substrate for E6 proteins revealed that HPV 16 E6 is more active in
115 initiating p53 degradation than HPV 31 E6 (20). These data suggest that besides
116 qualitative also quantitative differences among E6 and E7 protein interactions may
117 explain the different carcinogenic behavior.

118 HPV 16 and 31 are closely related, belong to the same genus and species and show
119 consistence in phylogeny and pathology. However, it is not completely understood,
120 why HPV 16 is far more carcinogenic than other “high-risk” alpha-9 HPV types. In this
121 work, the crystal structure of 31E6 was solved by X-ray crystallography. The binding
122 properties of 31E6 to p53 and LxxLL(E6AP) were analyzed quantitatively in
123 comparison to 16E6. Both HPV types represent “high-risk” types of alpha-9 genus
124 infecting the mucosal keratinocytes but reveal a different p53 degradation potential in
125 cell-based assays (2, 6, 20). Presumably, the degradation of p53 is greatly related to
126 carcinogenicity and variations in p53 degradation could be one factor in this process.
127 Hence, our structural and quantitative analysis bridges sequence, structure and
128 function together and further, suggests explanations regarding the different p53
129 degradation potential of two very closely related HPV E6 proteins both targeting the
130 same cellular targets E6AP and p53.

131 Results

132 *Structural analysis of 31E6*

133 Recombinant E6 proteins are notoriously prone to solubility issues (reviewed in (21)).
134 To overcome this problem, we fused a crystallization-prone mutant of the bacterial
135 maltose-binding protein (MBP) to the N-terminus of the HPV 31 E6 protein and the
136 E6-binding LxxLL sequence of E6AP (figure 5) to the C-terminus of the HPV 31 E6
137 protein. The resulting MBP-31E6-LxxLL(E6AP) triple fusion protein was purified as a
138 soluble monomer and yielded crystals that diffracted until 2.8 Å. The structure was
139 solved by molecular replacement using the known structures of MBP and HPV 16 E6
140 as a template.

141 The overall domain organization and structure is very similar (figure 3A) to the
142 published 16E6 structures. Two zinc ions are present in the structure, each
143 coordinated by four cysteine residues of the highly conserved CxxC motifs. Notably,
144 the amino acid R102 is conserved by sequence and structure. This residue is
145 important, because it bridges the E6N and E6C domain via two hydrogen bonds to
146 the backbone carbonyls of the E6N and contributes to LxxLL-motif binding in both E6
147 structures.

148 The structure of 16E6 was previously solved as a heterodimer 16E6/MBP-
149 (LxxLL)E6AP complex (Protein Data Bank (PDB) 4GIZ, (22)) and as a heterotrimer
150 (16E6/MBP-LxxLL(E6AP)/p53core (PDB 4XR8 (17)). An alignment of these
151 structures with the heterodimeric structure of 31E6/LxxLL(E6AP) based on the
152 LxxLL(E6AP)-peptide shows a mostly unperturbed E6N domain. Remarkably, the
153 structure of the ternary complex 16E6/MBP-LxxLL(E6AP)/p53core contains two
154 conformers in the asymmetric unit, trimer A and B, which differ in the position of the

155 E6C domain. 16E6C of trimer A superimposes well with E6C of the 16E6/MBP-
156 (LxxLL)E6AP dimeric complex. In comparison, the E6C domain of trimer B is
157 distorted. Notably, the crystal structure of 31E6-LxxLL(E6AP) only contains one
158 conformer. Here, the 31E6C domain aligns well with the 16E6/MBP-
159 LxxLL(E6AP)/p53core trimer B conformation. The differences of the E6C domain
160 positions become obvious by comparing the root-mean-square deviation (RMSD) of
161 the corresponding amino acid C α positions in the different 16E6 protein structures
162 related to the structural data of 31E6-LxxLL(E6AP) (figure 3B) as an indicator of
163 protein backbone alignment. This shows that the E6C domain deviates up to 5.5 Å
164 among the E6 proteins, whereas the E6N shows more similar RMSD values and, that
165 31E6-LxxLL(E6AP) aligns best with trimer B of 16E6/LxxLL(E6AP)/p53core. From
166 now on the obtained structure of 31E6-LxxLL(E6AP) will be compared with 16E6 of
167 the 16E6/LxxLL(E6AP)/p53core trimer B ternary complex as presented in PDB 4XR8
168 (16).

169 *31E6 interacts specifically with E6AP and LxxLL(E6AP)-peptides*

170 The interaction of E6 with the LxxLL-motif of the cellular E6AP is a requirement for
171 the formation of the ternary complex E6/E6AP/p53 followed by proteasomal
172 degradation of p53.

173 We examined the interaction of the oncoprotein E6 from HPV 31 with the host cell
174 protein E6AP in comparison to HPV 16 E6 as previously published (23). The
175 interaction was tested *in cellulo* using the qualitative *Gaussia princeps* protein
176 complementation assay (GPCA) (24, 25), where proteins are expressed and assayed
177 in a mammalian cellular environment. The GPCA signal of 31E6 (normalized
178 luciferase ratio (NLR) = 55 ± 9) is decreased by 35 % compared to 16E6 (NLR = $84 \pm$
179 5) (figure 2G) clearly indicating, that 31E6 shows a reduced interaction with E6AP *in*

180 *cellulo*. We used 16E6 L50E as a negative control ($NLR = 7 \pm 1$), which was
181 previously shown not to interact with E6AP. The expression of all three tested E6
182 constructs was verified by western analysis (figure 2H).

183 Further, we used a fluorescence anisotropy assay to quantify the binding affinities of
184 16E6 and 31E6 to the LxxLL-motif of E6AP (figure 2). To eliminate the fluorophore-
185 induced effects on the measured affinities, we used a fluorescein-labeled
186 LxxLL(E6AP)-peptide as a tracer to monitor the binding of an unlabeled
187 LxxLL(E6AP)-peptide. We observed a low-micromolar affinity between the unlabeled
188 LxxLL-peptide of E6AP and 16E6 ($K_d = 7.8 \pm 0.4 \mu M$) and a 2-fold weaker affinity
189 with 31E6 ($K_d = 13.6 \pm 0.9 \mu M$).

190 Even though the general architecture of 16E6 is maintained in 31E6, the structural
191 conformation of R10, which is conserved by sequence in 16E6 and 31E6 (figure 3), is
192 different. This is accompanied by a different orientation of the LxxLL(E6AP)-peptide
193 at its C-terminal part. In 16E6 (PDB 4XR8, figure 3 D) R10 forms a hydrogen bond
194 with carbonyl oxygen of G⁶ of the LxxLL(E6AP)-peptide (trimer A) or a salt bridge
195 with E⁷ or E⁸ of the LxxLL(E6AP)-peptide (trimer B) (The numbering of the
196 LxxLL(E6AP)-peptide is: [E⁻⁶S⁻⁵S⁻⁴E⁻³L⁻²T⁻¹L¹Q²E³L⁴L⁵G⁶E⁷E⁸R⁹]). In 31E6, R10 adopts a
197 conformation which does not contribute to peptide binding (figure 3C). Further, some
198 amino acids of the LxxLL(E6AP) binding pocket of 31E6 diverge from 16E6 (figure 1
199 and table 2). The difference in position 78 (H in 16E6 and W in 31E6) retains the
200 polar nitrogen atom (N3 of the imidazole ring compared to the indol nitrogen) but the
201 water-bridged hydrogen bond as in 16E6/LxxLL(E6AP) (PDB 4GIZ) or direct
202 interaction as in 16E6/LxxLL(E6AP)/p53core (PDB 4XR8) is not retained. Another
203 important difference between 31E6 and 16E6 is position 129. 16E6 R129 contacts
204 two amino acids of the LxxLL(E6AP) peptide (trimer B, figure 3D). This interaction

205 was revealed by molecular dynamics simulation (22) based on the dimeric
206 16E6/LxxLL(E6AP) (PDB 4GIZ) structure and was later directly found in the crystal
207 structure of the ternary 16E6/LxxLL(E6AP)/p53core complex (PDB 4XR8). 31E6
208 G129 is not able to establish these sidechain interactions (hydrogen bond and salt
209 bridge). 16E6 C51 contributes to LxxLL(E6AP) binding via a hydrogen bond between
210 the C51 amine and the LxxLL(E6AP) backbone carbonyl. C51 is not conserved
211 among the alpha-9 HPV group (figure 1). 31E6 T51 contributes to LxxLL(E6AP)
212 binding via a hydrogen bond between its sidechain hydroxyl group and the
213 LxxLL(E6AP) backbone carbonyl. In order to analyze, if these minor amino acid
214 variations impact LxxLL(E6AP) binding, a 16E6 mutant analogous to 31E6 (16E6-
215 C51T/H78W/R129G) was tested by fluorescence anisotropy (figure 2) expecting a
216 decreased binding to the LxxLL(E6AP)-peptide. Indeed, the binding decreased
217 dramatically with a non-detectable interaction in competitive fluorescence anisotropy.

218 Taken together, minor amino acid variations together with structural differences
219 between 16E6 and 31E6 accomplish an impaired binding of 31E6 to the
220 LxxLL(E6AP)-peptide.

221 *31E6-LxxLL(E6AP) interaction with p53*

222 The ternary complex 31E6-LxxLL(E6AP) + p53core was modeled based on the
223 published 16E6/LxxLL(E6AP)/p53core structure (PDB 4XR8, (17)) and the herein
224 presented structure of 31E6-LxxLL(E6AP) by superimposing 31E6 onto 16E6 of
225 trimer B. Compared to 16E6 the p53 interface of 31E6 shows amino acids essentially
226 contributing to p53 binding of which some are not conserved (figure 1 and table 3).
227 Above all, 16E6 E18 is not conserved in 31E6 and other alpha-9 HPV. The mutant
228 16E6 E18A has been described previously to reduce E6AP pulldown and p53
229 degradation efficiency by more than 50 % (17, 26). Our structural data clearly show,

230 that in 31E6 A18 the interaction with p53 would be disfavored because the loss of the
231 salt bridge between 16E6 E18 and p53 K101 is not compensated in 31E6 (figure 3A).
232 A second difference, which is unique to 31E6 in the alpha-9 genus, is a double amino
233 acid variation of I23/Y43 in 16E6 and Y23/L43 in 31E6. This double amino acid
234 variation seems to maintain an efficient hydrophobic packing at the E6/p53 interface
235 in 31E6. In 16E6 I23/Y43 are important for binding of the α 3 helix of p53 (amino acid
236 277-292) by hydrophobic interaction. Presumably, L289 of this helix is still capable to
237 bind to the hydrophobic pocket in 31E6 (figure 3G). Remarkably, the hydroxyl groups
238 of the tyrosines Y43 in 16E6 and Y23 in 31E6 are almost at the same position,
239 indicating, that the association to the carbonyl oxygen of K292 of p53 is retained in
240 31E6. The amino acids Q6 and Q14 of 16E6, which contribute to p53 binding (17),
241 are neither conserved in 31E6 nor in the alpha-9 HPV genus (figure 1). Q6
242 contributes backbone-backbone interactions which are likely impaired in 31E6 A6.
243 Q14 of 16E6 is responsible for sidechain-sidechain interactions with T102 and N268
244 of p53. Potentially, E14 of 31E6 would still interact.

245 To complement the analysis of the ternary complex E6/E6AP/p53, we analyzed the
246 interaction of the MBP-31E6-LxxLL(E6AP) construct, which was used for
247 crystallization, to the p53 core domain quantitatively using microscale thermophoresis
248 (MST). The direct fusion of the LxxLL-motif to the E6 protein mimics E6/E6AP
249 complex formation, which is required for p53 binding. As a comparison, an analogous
250 MBP-16E6-LxxLL(E6AP) construct was used. The affinity of the MBP-31E6-
251 LxxLL(E6AP) construct to the p53 core domain ($K_d = 91.7 \pm 1.26 \mu\text{M}$) was 5-fold lower
252 compared with the analogous MBP-16E6-LxxLL construct ($K_d = 18.1 \pm 2.47 \mu\text{M}$)
253 (figure 4). The binding of 31E6 to the p53 core domain appears to be reduced, which
254 agrees with the structural differences observed between 16E6 and 31E6 and the

255 sequence variations (table 3). Since it was previously shown, that the 16E6 E18A
256 mutant binds to and degrades p53 less efficiently (17, 26), we designed a 31E6 A18E
257 mutant analogous to 16E6 expecting an increased binding to the p53 core domain.
258 Here the binding increased slightly to $66.6 \pm 20.7 \mu\text{M}$, indicating that the E18
259 contributes to the binding of the p53 core domain but that additional protein features
260 account for p53 core domain binding.

261

262 Discussion

263 All “high-risk” HPV types inactivate the tumor suppressor protein p53 via E6AP-
264 dependent proteasomal degradation, which promotes cell immortalization. However,
265 they differ in their ability to degrade p53, what may affect carcinogenic potential. E6
266 recruits E6AP and p53 to form the ternary complex E6/E6AP/p53, which is required
267 for p53 degradation. The inactivation of p53 via proteasomal degradation is based on
268 the formation of the ternary complex E6/E6AP/p53 (17). Differences in the assembly
269 of this complex can alter the p53 degradation efficiency. The scope of this work was
270 to characterize the ternary complex of two very closely related alpha-9 “high-risk”
271 HPV types 16 and 31 in order to investigate whether (I) phylogenetic similarity results
272 in structural conservation and (II) the binding of E6 proteins to the same cellular
273 targets differs structurally and quantitatively between 16E6 and 31E6.

274 As expected, the overall structure of 31E6 resembles that of 16E6: two zinc-binding
275 domains E6N and E6C forming a binding cleft for LxxLL-motifs (figure 3). Due to the
276 high sequence conservation, especially of the zinc-binding motifs of the HPV E6
277 proteins this can very likely be claimed also for other HPV E6 proteins. The sequence
278 alignment of HPV alpha-9 E6 (figure 1 and table 2) shows, that the LxxLL-motif
279 binding site of E6 is highly conserved. Amino acid 16E6L50 in the hydrophobic
280 LxxLL-motif binding pocket, which abolishes LxxLL(E6AP) binding if mutated (27,
281 28), is conserved between 16E6 and 31E6, and within all alpha species. Additionally,
282 mutations of 16E6 R102 and R131 to alanine largely impair E6AP interaction. These
283 amino acids are also conserved in alpha-9 HPV types and contribute to LxxLL(E6AP)
284 binding by polar interactions. However, we found that the affinity of 31E6 to the
285 LxxLL(E6AP)-peptide is 2-fold lower than of 16E6 to the same peptide. Structural
286 comparison of 16E6/LxxLL(E6AP) and 31E6-LxxLL(E6AP) showed a slightly shifted

287 E6C domain. Indeed, the two different conformations of the E6C domain can be
288 related to the heterogeneous dynamic behavior of the E6C domain, which was
289 previously reported in NMR solution studies performed on various E6C domains (29-
290 31). The E6C domain is one building block of the LxxLL(E6AP) binding cleft.
291 Subsequently, flexibility of the E6C domain can be one reason for different binding
292 affinities to the LxxLL(E6AP)-peptide. Moreover, sequence differences between 16E6
293 and 31E6 as described in table 2, participating in LxxLL(E6AP) binding, result in less
294 protein contacts in 31E6. 16E6, if mutated to the 31E6 analogous amino acids
295 (C51T, H78W, R129G), resulted in a tremendously reduced binding affinity to the
296 LxxLL(E6AP)-peptide. These amino acids are not conserved in the alpha-9 genus at
297 all (Figure 1). As a conclusion, minor amino acid variations are another possibility of
298 the lower affinity of 31E6 to LxxLL(E6AP).

299 Notably, 16E6 mutants showing impaired binding to LxxLL(E6AP) also showed less
300 efficient p53 degradation (22, 28). As neither E6 nor E6AP alone are able to interact
301 directly with p53 (32-35), the binding to p53 requires the formation of E6/E6AP
302 complex. The binding of E6 to the LxxLL(E6AP)-peptide is sufficient to recruit the
303 core domain of p53 (17). However, it was recently reported, that additional binding
304 sites at the N-terminal region of E6AP are necessary to stimulate the ubiquitin-ligase
305 activity of E6AP by 16E6 (28). Here, we focused on one interaction site of E6 and
306 E6AP, the LxxLL(E6AP) motif, necessary for p53 binding, but not sufficient for p53
307 degradation. The structure and binding affinities of the functional complex in terms of
308 p53 ubiquitination are still elusive.

309 The binding of E6AP to E6 is required prior to binding of p53 to E6. In order to
310 investigate the binding of p53 to E6, we mimicked an "p53-ready" E6 by fusing the
311 LxxLL(E6AP)-peptide to the C-term of E6 (Figure 5). In this proximity, the

312 LxxLL(E6AP)-peptide is bound to E6 and therefore, the measured binding of the p53
313 core domain is presumably independent from the required binding of LxxLL(E6AP).
314 Of course, in the cellular environment the sequential binding of E6AP and p53 to E6
315 finally determines p53 degradation.

316 Some amino acids (D44, F47, D49), crucial for p53 core domain interaction in the
317 16E6/LxxLL/p53 complex are conserved within 16E6 and 31E6, suggesting that
318 31E6 can bind to p53, table 3).

319 However, the 31E6 binding site shows striking amino acid differences compared to
320 16E6. Of these amino acid variations, it was shown, that 16E6 mutants Q6A and
321 Q14A (not conserved in alpha HPV) bind to E6AP and degrade p53 similarly to
322 wildtype 16E6 *in cellulo* (17) indicating that variations at these positions have minor
323 influence on p53 binding and degradation *in cellulo*. In contrast the 16E6 E18A
324 mutant showed 75 % lower binding to p53 and decrease in p53 degradation
325 efficiency (17). Strikingly, in 31E6 this position is an alanine residue (A18). Indeed,
326 the mutation of A18 to E18 in 31E6 resulted in an increased affinity to p53 core
327 domain. This position may have an influence on p53 binding affinity and subsequent
328 degradation and accordingly it is subject to variation across alpha species, where it is
329 not conserved, neither in “high-risk”, nor in “low-risk” HPV types. The gain of affinity
330 of the 31E6A18E mutant is rather low, indicating that additional variations between
331 16E6 and 31E6 influence the binding to the p53 core domain, like the observed shift
332 in the E6C domain or other sequence variation. For example, our structural analysis
333 indicates that the sequence variation 16E6Y43 and 31E6L43 is compensated by
334 16E6I23 and 31E6Y23, retaining the hydrophobic pocket for p53 binding. However,
335 slight variations in the p53 binding pocket can also lead to different affinities. Overall,

336 the sequence differences and structural analysis parallels the obtained 5.4-fold lower
337 K_d for 31E6-LxxLL(E6AP) binding to the p53 core domain.

338 The formation of the ternary complex is presumably stronger for 16E6, because it
339 shows higher affinities to both, LxxLL(E6AP) and the p53 core domain. Our affinity
340 analysis strongly agrees with the previously reported >2-fold less efficient
341 degradation of p53 in HPV 31 E6 transfected cells compared to 16E6 transfected
342 cells, even though 31E6 shows an almost 3-fold higher cellular level than 16E6 in
343 these experiments (20).

344 HPV 18 is the second most prevalent HPV type associated with cervical cancer and
345 belongs to the alpha-7 HPV species. The intracellular level of 18E6 resembles 16E6,
346 but it shows almost 2-fold less efficient p53 degradation (20). Overall, 18E6 shares
347 less sequence identity (~57 %) with 16E6 compared to 31E6 with 16E6 (~66 %).
348 Slight structural differences, e.g. the position of E6C domain are not predictable but
349 can change the binding to the p53 core domain and E6AP. 18E6 does possess all
350 crucial amino acids, necessary for p53 core domain binding, only 16D44 is found as
351 the homologous amino acid E in 18E6. On the other hand, LxxLL(E6AP) binding
352 16R131, which shows ~ 50 % reduced binding to LxxLL(E6AP) if mutated to A, is not
353 conserved in 18E6 (18E6 H131, see figure 1). These variations can potentially
354 influence the efficiency of ternary complex formation and subsequent p53
355 degradation. "Low-risk" HPV types already show a much lower sequence identity to
356 16E6 (e.g. 11E6 36 %) and already possess amino acid differences, which neglect
357 binding to p53 (e.g. 11E6 has no conserved 16E6 E18, D44, F47, D49 crucial for p53
358 core domain binding). Consequently, "low-risk" HPV types are inactive in E6AP-
359 dependent p53 degradation at all (20).

360 It is important to note, that apart from defined crucial amino acids in 16E6 (table 2
361 and 3) the individual subset of minor sequence differences and the flexibility of the
362 E6C domain position, influence E6 structure and the binding to E6AP and p53. These
363 sequence variations increase with decreasing phylogenetic relation of E6 proteins.
364 Their impact on the E6 structure and binding affinities is not predictable. As a
365 conclusion, binding affinities certainly vary between the E6 proteins but must be
366 analyzed individually.

367 Further, it must be pointed out, that the alpha-9 “high-risk” HPV types 52 and 58 E6
368 proteins show higher p53 degradation efficiencies *in cellulo* than 16E6 despite similar
369 intracellular protein levels of E6 proteins (20), but possess a lower carcinogenic
370 potential. Here, p53 degradation potential does not correlate with the cancerogenic
371 risk (20, 36). The physiological context likely represents a more complex situation.
372 Apart from p53 degradation, many other factors contribute to viral persistence and
373 HPV-associated cancer which further differ among different HPV genus, species and
374 types. Carcinogenicity of HPV may be influenced by many parameters, including the
375 entire viral interactome of E1 to E7, transcription regulation, half-life of proteins,
376 deregulation of posttranslational modifications (37-39); all playing a role in DNA-
377 damage response (reviewed in (40-42)), persistence and immune response ((43) and
378 reviewed in (44, 45)), E6-mediated degradation of other cell proliferation regulatory
379 proteins, e.g. NHERF1 (27) and still elusive factors. Notably, human keratinocytes
380 can be immortalized by 16E7 alone (46). Co-expression with 16E6 increases the
381 immortalization rate (47), highlighting the important concomitant role of E7 in HPV-
382 associated carcinogenesis. Nonetheless, the inactivation of p53 remains a very
383 important process with respect to cell immortalization. A 16E6 mutant deficient in p53

384 interaction showed tremendously decreased potency of cell immortalization though
385 being co-expressed with 16E7 (22).

386 Further, the multiple interactions of E6 play important roles in cell immortalization
387 (48). E6 proteins can bind to various LxxLL-motifs of other cellular targets, e.g.
388 hTERT (49) and IRF3 (50, 51). Additionally, E6 PDZ-binding motifs (PBM) differ even
389 within HPV species (reviewed in (52)). The last 4 amino acids of the PBM of 31E6
390 (ETQV) differ only slightly from 16E6 (ETQL). However, it was shown previously, that
391 HPV 18 E6 (ETQV) has a different PDZ-binding profile compared with 16E6 (55).
392 Assumingly the PDZ-binding profile of 31E6 is also different from 16E6. It was
393 shown, that an interaction of E6 with the PDZ-containing protein MAGI-1 results in
394 degradation of MAGI-1, for carcinogenic as well as non-carcinogenic types (36, 53) of
395 alpha papillomaviruses in a very similar efficiency. The authors draw the conclusion
396 that MAGI1 degradation alone cannot result in carcinogenesis but in concert with p53
397 degradation and hTERT (54). In conclusion, different PDZ-binding partners can also
398 influence the carcinogenic potential of E6.

399 The interactome of both oncoproteins, E6 and E7, facilitates cell transformation. The
400 p53 degradation potential is one important factor in carcinogenesis especially for
401 “high-risk” types. Semi-quantitative analysis revealed a link between carcinogenicity
402 and p53 degradation (20) for some HPV types, as HPV 16 and 31. Further, HPV 16
403 is associated with ~ 50 % cervical cancers, in contrast HPV 31 is only associated
404 with 3-8 % (56, 57). This difference is even more significant in HPV positive tumors of
405 the oropharynx, where HPV 16 accounts for 93 % and HPV 31 together with 12 other
406 HPV types for 4 % of these cancers (58). In this context it is interesting, that 16E6
407 and 31E6 share the same structural fold but 31E6 forms the ternary complex
408 E6/E6AP/p53 less efficiently. Consequently, the E6-mediated proteasomal

409 degradation of p53 can be impaired. In principle, these findings are likely conferrable
410 to the alpha-9 species and beyond, and are not limited to the proteins of ternary
411 complex, analyzed here. In summary, additionally to the diverse interactions of E6
412 with different interaction partners (qualitative differences like 16E6 binds to E6AP and
413 8E6 binds to MAML1 (16, 59, 60)), divergence of E6 proteins could also be explained
414 by different affinities of very closely related proteins to the same cellular targets
415 (quantitative differences like 16E6 and 31E6 bind to LxxLL(E6AP) and p53 with
416 different affinities).

417

418 **Materials and Methods**

419 **Recombinant Protein Production and Purification**

420 The E6 proteins possess cysteine mutations to decrease oxidation and
421 oligomerization. An overview of used E6 constructs is given in figure 5. Proteins are
422 fused N-terminally to maltose-binding protein (MBP) to increase solubility and C-
423 terminally to LxxLL(E6AP)-peptides in pETxM1 plasmids. The proteins were
424 produced in BL21(DE3) at 20 °C after addition of 100 µM ZnCl₂ and induction with
425 1 mM IPTG overnight at OD₆₀₀ ~0.8. MBP-31E6 was expressed in TB-medium
426 (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 5 g/L NaCl, 0.017 M KH₂PO₄,
427 0.072 M K₂HPO₄), MBP-16E6 and MBP-31E6-LxxLL(E6AP) in LB-medium (10 g/L
428 tryptone, 10 g/L NaCl, 5 g/L yeast extract).

429 Proteins were lysed in 50 mM Tris-HCl pH 8 at 8 °C, 400 mM NaCl, 5 % (w/v
430 glycerol, 2 mM Tris(2-carboxyethyl)phosphine (TCEP) using a micro-fluidizer or
431 French press. Lysate was cleared by centrifugation (1 h, 100 000 x g, 4°C) and
432 applied to an equilibrated affinity column (self-packed amylose column, 30 mL
433 amylose resin (New England Biolabs) for MBP-16E6, MBP-16E6-LxxLL(E6AP),
434 MBP-31E6A18E-LxxLL(E6AP), MBP-16E6mut and MBP-31E6-LxxLL(E6AP)) or
435 MBPTrap (GE Healthcare) 3x 5 mL in a row for MBP-31E6. Proteins were eluted with
436 lysis buffer containing 10 mM maltose. Elution fractions were pooled and centrifuged
437 overnight at 100 000 x g to sediment agglomerates. The supernatant was applied to
438 a S200 XK16/60 or XK26/60 column (GE healthcare) pre-equilibrated with 50 mM
439 Tris-HCl pH 8 at 8 °C, 400 mM NaCl, 2 mM TCEP. Fractions containing monomeric
440 protein were pooled, if needed concentrated (>45 µM for FP, >60 mg/mL for Xtal,
441 >20 µM for Microscale Thermophoresis (MST)) and stored at -80 °C.

442 The p53 core domain construct is produced as a His₆-MBP-p53core following the
443 protocol previously described (61). After MBP-affinity chromatography (MBPTrap (GE
444 Healthcare) 3x 5 mL in a row) the N-terminal His₆-MBP-fusion was cleaved by His₆-
445 TEV-Protease followed by Heparin column (GE healthcare, 5 mL) to separate
446 cleaved MBP and TEV-protease from p53core and size exclusion chromatography
447 (Superdex S200 XK 16/60, GE healthcare) using the same buffers as for E6.

448 **Fluorescence Anisotropy**

449 Fluorescence anisotropy (FA) was measured with a PHERAstar (BMG Labtech,
450 Offenburg, Germany) microplate reader by using 485 ± 20 nm and 528 ± 20 nm
451 band-pass filters. In direct measurements, a dilution series of the MBP-E6 protein
452 was prepared in 96 well plates (96 well skirted PCR plate, 4ti-0740, 4titude, Wotton,
453 UK) in a 20 mM HEPES pH 7.5 buffer that contained 150 mM NaCl, 0.5 mM TCEP,
454 0.005 % Tween 20 and 50 nM fluorescently labeled E6AP-peptide (fE6AP, FI-ttds-
455 PESSSELTLQELLGEER). The volume of the dilution series was 40 μ L, which was
456 later divided into three technical replicates of 10 μ L during transferring to 384 well
457 micro-plates (low binding microplate, 384 well, E18063G5, Greiner Bio-One,
458 Kremsmünster, Austria). In total, the anisotropy of the probe was measured at 8
459 different protein concentrations (whereas one contained no protein and corresponded
460 to the free peptide). In competitive FA measurements, the same buffer was
461 supplemented with the E6 protein and fluorescently labeled LxxLL(E6AP)-peptide to
462 achieve a complex formation of 60-80 %, at concentrations based on the titration of
463 direct binding. Then, this mixture was used for creating a dilution series of the non-
464 fluorescent competitor (i.e. the biotinylated LxxLL-peptide of E6AP, Biotin-ttds-
465 PESSSELTLQELLGEER) and the measurement was carried out identically as in the
466 direct experiment. Analyses of FA experiments were carried out in ProFit (62).

467 **GPCA**

468 GPCA was performed as previously described (23, 25).

469 HEK-293T Pasteur cells were reverse-transfected in white 96-well plates at a
470 concentration of 4.2×10^5 cells per well using PEI MAX (Polysciences) with 100 ng
471 of pSPICA-N2-E6 and 100 ng of pSPICA-N1-target protein E6AP Δ 1-290. These
472 plasmids encode E6 and E6AP fused to the split fragments G1 or G2 (figure 5) of the
473 Gaussia luciferase (G), which complement the enzyme upon interaction of E6 and
474 E6AP. At 48 h post-transfection, cells were washed with 50 μ L of PBS and lysed with
475 40 μ L of Renilla Lysis Buffer (Promega, Madison, WI, USA, E2820) for 30 min. Split
476 *Gaussia princeps* luciferase enzymatic activity was measured using a Berthold
477 Centro LB960 luminometer by injecting 50 μ L of luciferase substrate reagent
478 (Promega, E2820) per well and counting luminescence for 10 s. Results were
479 expressed as a x-fold change of signal normalized over the sum of controls, specified
480 herein as normalized luciferase ration (NLR). For a given protein pair A/B: $NLR =$
481 $(G1-A + G2-B) / [(G1-A + G2) + (G1 + G2-B)]$ as described in (24).

482 **Crystallization and structure refinement**

483 The MBP-31E6-GSSGSGSGSGSAAA-LxxLL(E6AP) fusion protein was cloned
484 into pETxM1, purified as described, concentrated to 30 mg/mL in 50 mM Tris pH 8.0,
485 150 mM NaCl, 2 mM TCEP, and crystallized. Crystals were obtained at 20 °C in
486 200 mM tri-lithium citrate, 200 mM ammonium citrate tribasic pH 7.0, 20% (w/v)
487 polyethylene glycol 3350. All crystals were flash-cooled in a cryo-protectant solution
488 containing 25% (w/v) glycerol in crystallization buffer and stored in liquid nitrogen
489 before data collection. X-ray diffraction data were collected at the Synchrotron Swiss
490 Light Source (SLS) (Switzerland) on the X06DA (PXIII) beamline and processed with

the program XDS (63). The crystal structure was solved by molecular replacement with a high resolution MBP structure (PDB ID: 5H7Q, (64)) and 16E6 (PDB ID: 4XR8, (17)) using Phaser (65) and structure refinement was carried out with PHENIX (66). TLS refinement was applied during the refinement. Coot was used for model building (67). The crystallographic parameters and the statistics of data collection and refinement are shown in Table 1. The refined model and the structure factor amplitudes have been deposited in the PDB with the accession code 6SLM. Figures were prepared using PyMOL 2.3.3. RMSD (root-mean-square deviation) per residue was calculated on superpositioned structures by calling the rms_cur function in PyMOL on every pair of corresponding C α atoms using a custom Python script. Rms_cur returns the rms difference of the selection (here C α distance) without performing any superpositioning. The structure of 31E6 was used as the reference. RMSD (here C α distance) was plotted against the residue position. Superposition was based on the ligand (LxxLL-peptide). Trimer B of the ternary complex in PDB 4XR8 (17) was used for comparison.

Microscale Thermophoresis

The affinity of MBP-E6-LxxLL(E6AP) to p53 was determined by the K_d using Microscale Thermophoresis (MST), a small-scale method based on the principle of thermophoresis (68-70). The purified proteins MBP-31E6-LxxLL(E6AP), MBP-31E6A18E-LxxLL(E6AP) and MBP-16E6-LxxLL(E6AP) were labeled at lysine residues with the Monolith NTTM Protein labeling Kit RED-NHS first generation (NanoTemper Technologies GmbH, Munich, Germany) following the user manual instructions. Note, that (I) here the 16E6 does not carry the F47R mutation, because this mutation diminishes p53 interaction (22) and (II) p53 core domain is used, because it was previously shown, that only this region binds to E6 (17, 61).

516 In order to ensure a sufficient fluorescence signal, to prevent adsorption to the
517 capillary walls and to exclude auto fluorescence of the measurement buffer (20 mM
518 HEPES pH 6.8 at room temperature, 200 mM NaCl, 0.5 mM TCEP, 0.05 % Tween
519 20), a pre-test was performed according to the manufacturer manual and evaluated
520 positive. Binding was measured by incubating 200 nM of the labeled MBP-16E6-
521 LxxLL(E6AP) or MBP-31E6-LxxLL(E6AP) with a 2-fold serial dilution of p53 core
522 domain, starting with 87.5 μ M or 600 μ M, respectively. Samples at higher ligand
523 concentrations showed aggregation. The sample preparation was performed as
524 recommended by MO.Control software (NanoTemper Technologies GmbH, Munich,
525 Germany). The measurements were performed at 25 °C using 5 % excitation power
526 for MBP-16E6-LxxLL(E6AP) or 20 % excitation power for MBP-31E6-LxxLL(E6AP)
527 and MBP-31E6A18E-LxxLL(E6AP). Standard treated capillaries (Monolith NT.115
528 Capillary) were used. Thermophoresis was measured using the Monolith NT.115
529 (NanoTemper Technologies GmbH, Munich, Germany) and analyzed by MO.Affinity
530 analysis software (NanoTemper Technologies GmbH, Munich, Germany). Samples
531 leading to heterogenous fluorescence intensity were neglected. The K_d was
532 calculated using the MO.Affinity analysis software (K_d -model) only fixing the template
533 concentrations and implying fluorescence signals at 19 to 20 s of thermophoresis for
534 all samples.

535 **Data availability**

536 The structural data of the MBP-31E6-LxxLL(E6AP) are deposited in PDB with the
537 accession code 6SLM.

538

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556

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- 768

769 **Figure legends**

770 **Figure 1 - Multiple sequence alignment of E6 proteins sequences of alpha 9**
771 **HPV types and alpha 7 HPV type 18:** Except HPV 67 all α -9 HPV types are
772 classified as “high-risk”. After HPV16, HPV 18 (α -7) is the second most “high-risk”
773 HPV type. E6 proteins are ordered based on the phylogenetic relation of E6
774 (depicted left). Residue positions identified in the 16E6 ternary complex structure to
775 be responsible for 16E6/p53 interaction, for 16E6/ LxxLL(E6AP) motif interaction or
776 for both, p53 and LxxLL(E6AP), are indicated below as \$, # or &, respectively. E6
777 domain organization and structural features based on the published structure of 16E6
778 (PDB 4rxn) are shown above, illustrating the two zinc-finger domains E6N and E6C,
779 and the C-terminal PDZ Binding motif (PBM). The CxxC motifs (zinc binding) are
780 depicted below.

781 ClustalX coloring scheme in Jalview, sequences from PAVE database, aligned using
782 T-Coffee.

783

784 **Figure 2 - Interaction of E6 proteins with E6AP:** Purified E6 proteins show binding
785 to a fluorescein-labeled LxxLL peptide of E6AP (fE6AP) in fluorescence anisotropy
786 measurements in direct (A, C, E) and competitive (B, D, F; competition with
787 unlabeled peptide) measurements. Concluding from the competitive measurements,
788 31E6 has a 2-fold lower affinity to the E6AP peptide than 16E6. The 31E6 analogous
789 triple mutant of 16E6 (16E6_mut, C51T/H78W/R129G) shows a largely decreased
790 binding to LxxLL(E6AP). (G) GPCA analyses the interaction of proteins by
791 complementation of Gaussia-princeps split fragments *in cellulo*. 31E6 shows only
792 55% of the normalized luciferase ratio (NLR) compared with 16E6 indicating a lower
793 interaction of 31E6 with E6AP protein. 16E6 L50E is a negative control. The
794 expression of the E6 proteins was verified by Western blot analysis (H) using an
795 antibody, which detects the luciferase split fusion (G2) of the E6 protein. Actin serves
796 as a loading control.

797

798 **Figure 3 - A comparison of protein structures of 16E6 and 31E6 with the LxxLL**
799 **(E6AP) peptide:** (A) A superposition of the E6 structures of 31E6/LxxLL(E6AP) in
800 cyan, 16E6/LxxLL(E6AP)/p53 Trimer B in grey and 16E6/LxxLL(E6AP) in green
801 shows that all E6 proteins adopt a similar overall structural fold. (B) The root-mean-
802 square deviation (RMSD) of the C α -atoms of the 16E6 protein structures in relation to
803 31E6, as an indicator of protein backbone alignment, shows that the E6C domain for
804 the dimeric 16E6/LxxLL(E6AP) and ternary complex 16E6/LxxLL(E6AP)/p53 trimer A
805 deviates most from 31E6-LxxLL(E6AP), whereas the 16E6 ternary complex trimer B
806 shows very low deviation to the 31E6-LxxLL(E6AP) structure. This indicates, that
807 31E6-LxxLL(E6AP) adopts the conformation of 16E6/LxxLL(E6AP)/p53 trimer B.
808 (C+D) Polar interactions between E6 proteins and the LxxLL(E6AP) peptide. MBP

809 and p53 molecules are omitted in the representation for clarity. Most E6 interacting
810 amino acids are conserved (Figure 1, table 2) but the C-terminus of the LxxLL(E6AP)
811 peptide (top) is differently organized accompanied by a loss of interaction in 31E6
812 with the R10 conformers and R102 compared to 16E6. (F) The 31E6/LxxLL(E6AP)
813 structure was superimposed onto the ternary complex structure of
814 16E6/LxxLL(E6AP)/p53core trimer B based on the LxxLL peptide as in (A). Key
815 amino acid differences are shown in (E) where the interaction of 16E6 residues Q14
816 and E18 to p53 residues N268, T102 and K101 will properly not be supported by
817 31E6 E14 and A18. The hydrophobic packing of the p53 core domain C-terminus is
818 very similar in the model even though different residues can be found in 16E6 (I23,
819 Y43) and 31E6 (Y23, L43) which complement each other on the structural level.

820

821 **Figure 4 - Interaction of 31E6 and 16E6 with p53:** p53 core domain was titrated
822 from 87.5 μ M or 600 μ M against fluorescently labeled 200 nM MBP-16E6-
823 LxxLL(E6AP), MBP-31E6-LxxLL(E6AP) or MBP-31E6 A18E-LxxLL(E6AP) in two-fold
824 serial dilutions ($n=3$), at 25 $^{\circ}$ C measured by microscale thermophoresis (MST). Data
825 analysis was performed using the manufacturers MO.affinity analysis software. The
826 fraction of formed complex was calculated at 19-20 s of thermophoresis and plotted
827 against ligand concentration. Data points showing heterogenous fluorescence
828 intensity were neglected. Concomitant fitting applying the softwares K_d -model yielded
829 a K_d of 18.1 ± 2.47 μ M for 16E6-p53core and a 5-times higher K_d of 91.7 ± 1.26 μ M for
830 31E6-p53core. Mutating A18 in 31E6 to the 16E6 analogous E18 increased the
831 affinity to the p53 core domain slightly to a $K_d = 66.6 \pm 20.7$ μ M. The standard error of
832 regression (S) is 5.5 for MBP-31E6-LxxLL(E6AP), 3.8 for MBP-31E6A18E-
833 LxxLL(E6AP) and 5.0 for MBP-16E6-LxxLL(E6AP).

834

835 **Figure 5: Representation of the used constructs and mutants with respect to**
836 **their application in Fluorescence anisotropy, Microscale thermophoresis**
837 **(MST), GPCA and crystallization:** 31E6 has two, 16E6 has four surface exposed
838 cysteines, which were mutated to alanines (lines). The dashed line in 16E6 indicates
839 the F47R mutation, which decreases E6 oligomerization. Since this mutation
840 abolishes p53 interaction, it was not applied for p53 interaction studies using MST.
841 Fusion tags (MBP or luciferase fragments), linkers, proteins of interest and fused
842 peptide ligands are colored in orange or cyan, blue, lily and magenta, respectively.

843

844 **Table 1 data collection and refinement statistics (highest-resolution shell is**
 845 **shown in parentheses)**

	MBP-31E6-LxxLL(E6AP)
Wavelength	1.0
Resolution range (Å)	49.21 - 2.8 (2.9 - 2.8)
Space group	P 61 2 2
Unit cell (Å°)	113.64 113.64 185.97 90 90 120
Total reflections	350689 (33992)
Unique reflections	18044 (1756)
Multiplicity	19.4 (19.3)
Completeness (%)	99.34 (98.82)
Mean I/sigma (I)	14.71 (1.60)
R-meas	0.2318 (2.025)
CC1/2	0.998 (0.599)
R-work	0.2308 (0.3296)
R-free	0.2698 (0.3355)
Number of non-hydrogen atoms	4281
macromolecules	4213
ligands	37
solvent	31
Protein residues	539
RMS (bonds)	0.003
RMS (angles)	0.62
Ramachandran favored (%)	96.07
Ramachandran allowed (%)	3.93
Ramachandran outliers (%)	0
Rotamer outliers (%)	2.53
Clashscore	6.76
Average B-factor	85.21
macromolecules	85.56
ligands	67.19
solvent	59.1

846

847 **Table 2 conservation of amino acids of 16E6 which participate in LxxLL(E6AP)**
 848 **binding (22, 28) – variations between 16E6 and 31E6 are highlighted in**
 849 **grey/bold, * mutations show a significant decrease in binding**

16E6	R10	K11	L50*	C51	R55	S74	R77	H78	R129	R102*	R131*
31E6	R10	K11	L50*	T51	R55	S74	R77	W78	G129	R102	R131

850

851 **Table 3 conservation of amino acids of 16E6 important for p53 binding (17),**
852 **variations between 16E6 and 31E6 are highlighted in grey/bold, * mutations**
853 **show a significant decrease in binding**

16E6	Q6	E7	R8	Q14	E18*	Y43	D44*	F47*	D49*	L100	P112
31E6	A6	E7	R8	E14	A18	L43	D44*	F47*	D49*	L100	P112

854

855

A Superposition 31E6, 16E6 and 16E6 with LxxLL (E6AP)

B

C 31E6 / LxxLL (E6AP)

D 16E6 / LxxLL (E6AP)/p53

E

F

G

A Constructs for Fluorescence Anisotropy

B Constructs for MST

C Constructs for GPCA

D Construct for Crystallization