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c-Abl Phosphorylates E6AP and Regulates Its E3 Ubiquitin Ligase Activity

Ai-Leen Chan,[†,‡,¶] Tamar Grossman,[§,¶] Valentina Zuckerman,[§] Dafne Campigli Di Giammartino,[§] Ofra Moshel,[§] Martin Scheffner,[∥] Brendon Monahan,[⊥] Pat Pilling,[⊥] Yong-Hui Jiang,[@] Sue Haupt,[†,‡,●] Ora Schueler-Furman,[#] and Ygal Haupt*[,†,‡,●,▽]

[†]Research Division, The Peter MacCallum Cancer Centre, St. Andrew's Place, East Melbourne 3002, Victoria, Australia

[‡]Sir Peter MacCallum Department of Oncology, University of Melbourne, Parkville 3010, Victoria, Australia

[§]The Hebrew University, Hadassah Medical School, Jerusalem, Israel

[∥]Department of Biology, Box M642, University of Konstanz, 78457 Konstanz, Germany

[⊥]CRC for Cancer Therapeutics, 343 Royal Parade, Parkville 3052, Victoria, Australia

[@]Division of Medical Genetics, Department of Pediatrics and Neurobiology, Duke University School of Medicine, Durham, North Carolina 27710, United States

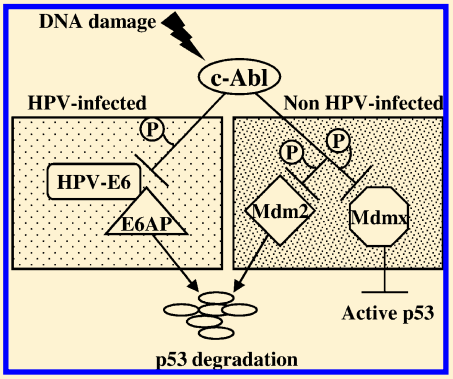
[#]Department of Microbiology and Molecular Genetics, Hebrew University, Hadassah Medical School, Jerusalem, Israel

[▽]Department of Biochemistry and Molecular Biology, Monash University, Clayton 3168, Victoria, Australia

[●]Department of Pathology, University of Melbourne, Melbourne 3010, Victoria, Australia

Supporting Information

ABSTRACT: In human papillomavirus (HPV)-infected cells, the p53 tumor suppressor is tightly regulated by the HPV-E6−E6AP complex, which promotes it for proteasomal degradation. We previously demonstrated that c-Abl tyrosine kinase protects p53 from HPV-E6−E6AP complex-mediated ubiquitination and degradation under stress conditions. However, the underlying mechanism was not defined. In this study, we explored the possibility that c-Abl targets E6AP and thereby protects p53. We demonstrated that c-Abl interacts with and phosphorylates E6AP. We determined that the E3 ligase activity of E6AP is impaired in response to phosphorylation by c-Abl. We mapped the phosphorylation site to tyrosine 636 within the HECT catalytic domain of E6AP, and using substitution mutants, we showed that this residue dictates the E3 ligase activity of E6AP, in a substrate-specific manner. On the basis of the crystal structure of the HECT domain of E6AP, we propose a model in which tyrosine 636 plays a regulatory role in the oligomerization of E6AP, which is a process implicated in its E3 ubiquitin ligase activity. Our results suggest that c-Abl protects p53 from HPV-E6−E6AP complex-mediated degradation by phosphorylating E6AP and impairing its E3 ligase activity, thus providing a molecular explanation for the stress-induced protection of p53 in HPV-infected cells.



Tight regulation of the p53 protein is critical for its proper function in healthy cells, to ensure appropriate responses and recovery from cellular stresses.[1] p53 regulation occurs predominantly at the level of protein stability, where Mdm2 and Mdmx (Mdm4) (Mdm proteins) are the major regulators. Mdm2 acts as the direct E3 ligase of p53, whereas Mdmx, which lacks E3 ligase activity, inhibits p53 transcriptional activity and cooperates with Mdm2 to promote p53 for proteasomal degradation.[2] Amplification of Mdm2 or Mdmx in human cancers such as breast, stomach, colon, and retinoblastomas often correlates with a wt status of p53, indicating that deregulating this pathway is sufficient to suppress p53 without the selection of direct mutations.[3,4] p53 ubiquitination by the E3 ligases Pirh2, COP1, and ARF-BP1 has also been shown under certain conditions.[2]

In contrast to normal cells, in HPV-infected cells, the regulated degradation of p53 is switched from Mdm2 to E6AP (HPV-E6-associated protein) in conjunction with viral E6.[5] E6AP is encoded by the *UBE3A* locus, which is mutated in Angelman syndrome, a human neurodevelopmental disorder[6] (reviewed in ref 7). E6AP is the prototype of the subfamily of E3 ligases that are characterized by a C-terminal HECT (homologous to the E6AP C-terminus) domain. Unlike the RING type of E3 ligases, the HECT E3 ligases bind ubiquitin covalently before transferring it to the substrate. In the absence of HPV-E6, E6AP has also been reported to affect p53 levels[8,9]

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and demonstrated to self-ubiquitinate[10] and regulate additional cellular targets (i.e., hHR23[11]).

In response to stress conditions, p53 is stabilized and activated through a process involving post-translational modifications of p53 and its inhibitors.[12] An important upstream activator of p53 is the c-Abl non-receptor tyrosine kinase.[13] c-Abl has been implicated in the cellular response to DNA damage[14,15] and plays an important role in the accumulation of p53 under stress conditions,[16] by neutralizing the inhibitory effects of Mdm2.[17−19] c-Abl phosphorylates Mdm2 and inhibits its E3 ligase activity.[18,19] Furthermore, c-Abl phosphorylates Mdmx and blocks its interaction with p53.[1] The phosphorylation of Mdm2 by c-Abl facilitates the Mdm2−Mdmx interaction and promotes the ubiquitination of Mdmx by Mdm2.[20]

We previously found that c-Abl also protects p53 from ubiquitination by the HPV-E6−E6AP complex, which in turns impairs the nuclear export of p53 and results in its accumulation.[17] In this study, we searched for the molecular explanation underlying the protection of p53 from the HPV-E6−E6AP complex, afforded by c-Abl. We found a physical and functional link between c-Abl and E6AP: c-Abl interacts with and phosphorylates E6AP. We have mapped this phosphorylation to a conserved tyrosine 636 (Y636) within the HECT domain. We showed that the phosphorylation status of E6AP residue 636, which is the target of c-Abl, regulates E6AP E3 ligase activity in a substrate-specific manner and modulates its interaction with c-Abl. Our results define E6AP as a novel target of c-Abl and provide a molecular explanation for the protection of p53 from inhibition by the HPV-E6−E6AP complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Assays. HEK293 and HeLa cells were grown in Dulbecco modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (FCS). MEFs were derived from E6AP null mice as recently described[21] and were grown in DMEM supplemented with 10% heat-inactivated FCS and supplements. Transfection and Western blot analysis were conducted as previously described.[22] wt and c-Abl null MEFs were treated with cisplatin (20 μM) for up to 3 h. HeLa cells were seeded overnight and treated with imatinib (1 and 5 μM) for 24 h, followed by 100 μM MG132 (Merck) for 4 h before being harvested for Western blot analysis.

Antibodies. The following antibodies were used: anti-c-Abl ABL-148 (Sigma), anti-Ha antibody HA.11 (Covance), anti-myc monoclonal antibody 9E10, anti-E6AP antibody E6AP-330 (Sigma), anti-phosphotyrosine 4G10 (Upstate Biotechnology), anti-GST polyclonal rabbit immunosera (purified on a GST column), anti-human p53 monoclonal antibodies PAb1801 and DO1, anti-GFP (Roche) and HRP-conjugated affinity-purified goat anti-mouse IgG and goat anti-rabbit IgG (Jackson Immuno Research Laboratories).

Plasmids. The following expression plasmids were used: human wt p53 (pRC-CMV-p53), mouse wt *c-abl* (pCMV-*c-abl*, type IV), mouse kinase-defective *c-abl* (pCMV-*c-ablK290H*, type IV), *c-abl* Δpro (pCDNA3-*c-abl*Δ793−1044, type IV), His-c-Abl (pCDNA3-*His-c-abl*, type IV), His-c-AblKD (pCDNA3-*His-c-ablK290H*, type IV),[19] HPV-16 E6 (pCB6 HPV16 E6) GST-HPV-16 E6, empty vectors (pCMV-Neo-Bam, pCDNA3), Ha-E6AP isoform 1, myc-tagged E6AP isoform 1, GFP (pEGFP-N1), GFP-c-Abl wt (pEGFP-N1-c-Abl wt), GFP-c-Abl ΔSH3 (pEGFP-N1-c-Abl ΔSH3), baculovirus expressing E6AP, myc-E6AP expressed in lentivirus,[23] PCMVΔR8.91 packaging construct, and PMD2.VSVG envelope construct. The following plasmids were used for *in vitro* transcription−translation (TNT) (Promega): human wt p53 (pRC-CMV-p53) and hHR23A. The following plasmids were used for the generation of expressed proteins: GST-E6AP (isoform 1), E2 UBCH5b in pPET3a, substitution mutants of E6AP (Y636F, Y636E, Y636D, E544A, and E544R) were generated on the background of GST-E6AP using site-directed mutagenesis on the relevant plasmid (Stratagene).

Retrovirus Generation and Infection. Retrovirus expressing E6AP wt was generated as previously described.[21]

Immunoblotting, Immunoprecipitation, Kinase Assay, and Ubiquitination Assay. Western blot analysis and the immunoprecipitation assay were performed as previously described.[1] *In vitro* and *in vivo* kinase assays were performed as previously described.[1,19] For bacterially expressed proteins, pGEX fusion plasmids were expressed in *Escherichia coli* BL21 (DE3)-pLysS (Bioline) bacteria. The expression of GST-E6, GST-E6AP wt, and mutant fusion proteins was induced when cells reached an OD_{600} of 0.6 with 0.1 mM IPTG at 37 °C for 3 h. GST fusion proteins were isolated from the bacterial extract on glutathione-Sepharose beads (Sigma). Purified proteins were stored at −20 °C. E6AP proteins subjected to ubiquitination assays were from the following sources: bacterially derived GST-E6AP on glutathione-Sepharose beads or baculovirus-derived E6AP as previously described.[24] GST-E6AP or E6AP, as indicated in each experiment, was added to *in vitro* translated, radioactively labeled substrate proteins (HHR23 and p53) synthesized in the TNT wheat germ system (Promega) and labeled with [^{35}S]methionine. In addition, reaction mixtures contained E1 (150 ng), UbcH5b (125 ng), 60 mM Tris-HCl (pH 7.6), dithiothreitol (1 mM), $MgCl_2$ (28 mM), ATP (2 mM), and ubiquitin (62.5 μg). Ubiquitination of p53 was conducted in the presence of GST-E6 (60 ng). The reaction was conducted in a total volume of 50 μL at the indicated temperature with agitation (1400 rpm). The reaction was stopped by the addition of protein sample buffer, and the mixture was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), followed by exposure of the dry gel to the Typhoon phosphorimager (GE).

Mass Spectrometric Analysis. Phosphorylated and non-phosphorylated E6AP were derived from a kinase assay using wt or a kinase-defective c-Abl and analyzed as previously described.[1] In essence, bacterially derived GST-E6AP was phosphorylated *in vitro* by c-Abl and subjected to mass spectrometric analysis using Qtof2 (Micromass) and nanospray attachment. Data analysis was conducted using the biolynx package (Micromass), and a database search was performed with Mascot (Matrix Science).

RESULTS

E6AP Interacts with c-Abl *in Vivo* and *in Vitro*. We previously demonstrated that c-Abl protects p53 from ubiquitination and degradation by the HPV-E6−E6AP complex.[17] To explore the mechanism of this protection, we examined the physical interaction between c-Abl and E6AP. To examine an interaction in cells, HEK293 cells were transfected with expression plasmids for Ha-tagged E6AP alone, or Ha-tagged E6AP together with c-Abl. HEK293 cells express low levels of c-Abl, which is wild type (wt) and functional (Figure 1A, II, lane 2[19]). Twenty-four hours post-transfection, cells

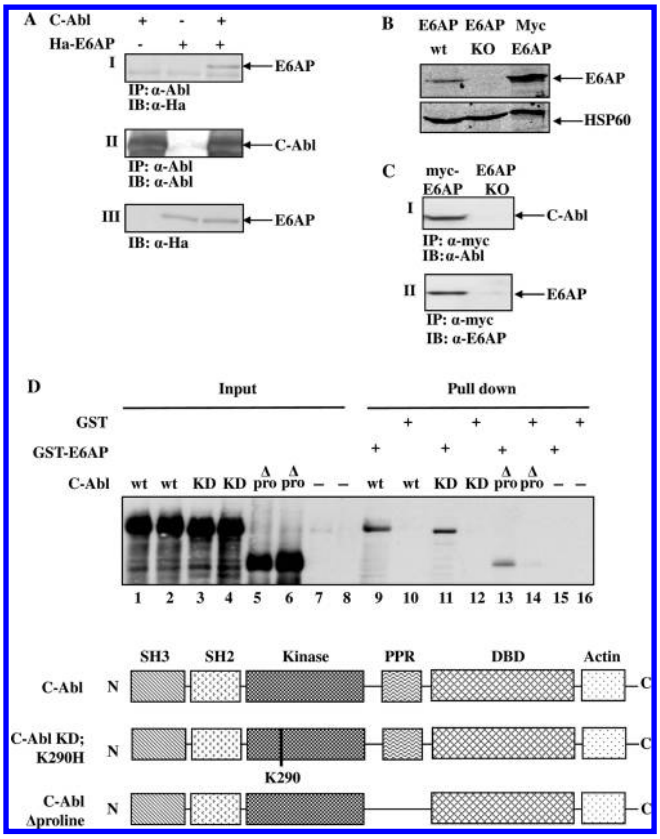

Figure 1. E6AP interacts with c-Abl *in vivo* and *in vitro*. (A) Interaction between E6AP and c-Abl in HEK293 cells. HEK293 cells were transfected with the indicated expression plasmids (7 μg each). Twenty-four hours post-transfection, cell extracts were subjected to immunoprecipitation using the anti-c-Abl antibody followed by Western blotting using anti-Ha (I). The expression levels of c-Abl in the transfected cells were monitored with the anti-c-Abl antibody (II), and that of Ha-tagged E6AP was monitored using the anti-Ha antibody (III) (in panel I, a band cross-reacting with anti-Ha in all extracts was deduced not to be E6AP). (B) E6AP expression levels in E6AP null MEFs reconstituted with myc-tagged E6AP. Cells were stably infected with myc-tagged E6AP-expressing viruses to achieve E6AP expression within physiological levels. Cells from E6AP wild-type MEFs, noninfected E6AP null MEFs, and E6AP null MEFs that were reconstituted with myc-E6AP were harvested, and extracts were subjected to immunoblotting with the anti-E6AP antibody and the anti-HSP60 antibody as a control for protein loading. (C) Interaction between E6AP and c-Abl in MEFs. E6AP null MEFs were stably infected with myc-E6AP-expressing viruses. Cell extracts from noninfected E6AP null MEFs and myc-E6AP-infected MEFs were subjected to immunoprecipitation using the anti-myc antibody, followed by Western blotting using the anti-c-Abl antibody (I). The expression levels of E6AP in the infected cells were monitored by the anti-E6AP antibody (II). (D) Interaction between E6AP and c-Abl *in vitro*. HEK293 cells were transfected with the indicated expression plasmids (10 μg each). Twenty-four hours post-transfection, cell extracts were subjected to a pull-down assay using bacterially derived GST-E6AP or GST (5 μg each) on glutathione beads followed by Western blotting using the anti-c-Abl antibody. A schematic representation of the c-Abl protein and the mutants used in this study is shown in the bottom panel. wt c-Abl contains an SH3 domain, an SH2 domain, tyrosine kinase (Y kinase), a polyproline region (PPR), a DNA binding domain (DBD), and an actin binding domain (Actin). The c-Abl kinase-defective (c-Abl KD) mutant contains a mutation at lysine 290, while c-AblΔproline lacks the proline rich region.

were harvested and the extracts were subjected to immunoprecipitation using the anti-c-Abl antibody, and bound E6AP was detected by immunoblotting using the anti-Ha antibody. As shown in Figure 1A, an interaction between E6AP and c-Abl was observed only in cells transfected with the two expression plasmids. To further validate the interaction between c-Abl and E6AP, MEFs from an E6AP KO mouse were employed. Cells were stably infected with myc-tagged E6AP-expressing viruses (Figure 1B). Cells from E6AP null MEFs either transduced to express myc-E6AP or not infected were harvested, and extracts were subjected to immunoprecipitation using the anti-myc antibody, followed by immunoblotting with the anti-c-Abl antibody. Endogenous c-Abl was coprecipitated efficiently with E6AP from myc-E6AP-expressing cells (Figure 1C, I, left lane), but not from E6AP KO MEFs (Figure 1C, I, right lane). These results provide support that the interaction between E6AP and c-Abl also occurs in non-transformed cells, and at physiological c-Abl levels.

Further, we examined the interaction between c-Abl and E6AP more directly. For this purpose, the binding between bacterially derived GST-E6AP and c-Abl derived from transfected HEK293 cells was measured. We also examined whether the kinase activity of c-Abl affected the interaction by comparing wt and a kinase-defective mutant of c-Abl (c-Abl KD; K290H). Because the proline rich region of c-Abl mediates its interaction with other proteins,[25−27] we also compared the interaction between wt and the c-Abl mutant lacking the proline rich region (c-AblΔproline). HEK293 cells were transfected with the relevant expression plasmids for wt or the c-Abl mutants. Twenty-four hours post-transfection, cells were harvested and the extracts were incubated with either GST-E6AP or GST only, followed by immunoblotting using the anti-c-Abl antibody. As shown in Figure 1D, kinase-defective and wt c-Abl interact with E6AP to similar degrees, suggesting that the kinase activity of c-Abl is not essential for its interaction with E6AP. c-Abl lacking the proline rich region also interacts with E6AP, albeit at a lower level, suggesting that although it is not essential, this region does contribute to this interaction. To further confirm this interaction, we have repeated the binding assay with untagged E6AP (Figure 5 of the Supporting Information). Similarly, we found that E6AP interacts with wt c-Abl, c-Abl KD, and c-AblΔpro. We have also explored the E6AP interaction between wt and the c-Abl mutant lacking the SH3 region (c-AblΔSH3) (Figure 4 of the Supporting Information). The wt and c-AblΔSH3 constructs are GFP fusion proteins that were transfected into HEK293 cells. Protein extracts were then subjected to immunoprecipitation using the anti-E6AP antibody, followed by immunoblotting with the anti-GFP antibody. As shown in Figure 4 of the Supporting Information, wt c-Abl was immunoprecipitated with E6AP (lane 3, marked with an asterisk) while the c-AblΔSH3 mutant did not (lane 4). These results suggest that the SH3 domain of c-Abl is required for the interaction with E6AP.

c-Abl Phosphorylates E6AP *in Vitro* and *in Vivo*. c-Abl is a tyrosine kinase, and its catalytic activity has been shown to contribute to the protection of p53 from its inhibitors.[1,17,19] We therefore asked whether c-Abl phosphorylates E6AP. For this purpose, we measured the ability of c-Abl to phosphorylate E6AP in cultured cells. E6AP phosphorylation was compared between wt and c-Abl KO MEFs (Figure 2). Lysates from both cell types were subjected to immunoprecipitation using the anti-E6AP antibody followed by immunoblotting using the anti-phosphotyrosine antibody. Furthermore, we measured the

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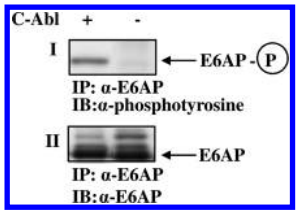

Figure 2. c-Abl-dependent tyrosine phosphorylation of E6AP *in vivo*. Cell extracts from wt and c-Abl null MEFs were subjected to immunoprecipitation using the anti-E6AP antibody (I) followed by Western blotting using the anti-phosphotyrosine antibody (II). The complete Western blot is shown in Figure 3 of the Supporting Information.

ability of c-Abl to phosphorylate E6AP in an *in vitro* kinase assay. wt c-Abl and a kinase-defective mutant were transfected into HEK293 cells, subsequently isolated by immunoprecipitation with the anti-c-Abl antibody, and then incubated with bacterially isolated GST-E6AP under kinase assay conditions. E6AP phosphorylation was detected with the phosphotyrosine antibody only in the presence of wt c-Abl (Figure 5A, I, lane 4) but not in the presence of the c-Abl kinase-defective mutant (Figure 5A, I, lane 5). This result strongly supports the tyrosine phosphorylation of E6AP in a c-Abl-dependent manner.

c-Abl Phosphorylates E6AP at Y636. The results described above encouraged us to identify the relevant tyrosine residue(s) within E6AP that is phosphorylated by c-Abl. The sequence of E6AP contains 35 tyrosine residues that are distributed throughout the protein. Mass spectrometry was used to identify the tyrosine residues that are phosphorylated specifically by c-Abl. Bacterially derived GST-E6AP was phosphorylated *in vitro* by c-Abl and subjected to mass spectrometric analyses. This analysis revealed that the peptide encompassing Y636 was phosphorylated (Figure 3A), demonstrating that Y636 of E6AP is a phosphorylation site for c-Abl *in vitro*. Tyrosine 636 lies within the HECT region, the catalytic E3 ligase domain of E6AP (Figure 3B). Tyrosine 636, in addition to the sequence surrounding it, is highly conserved within E6AP among mammals (human, bovine, rat, and mouse), as well as in *Drosophila* and *Caenorhabditis elegans* (Figure 3C). Tyrosine 636 is also conserved among six other HECT E3 ligases, including AIP4, WWP, and NEDD4 family

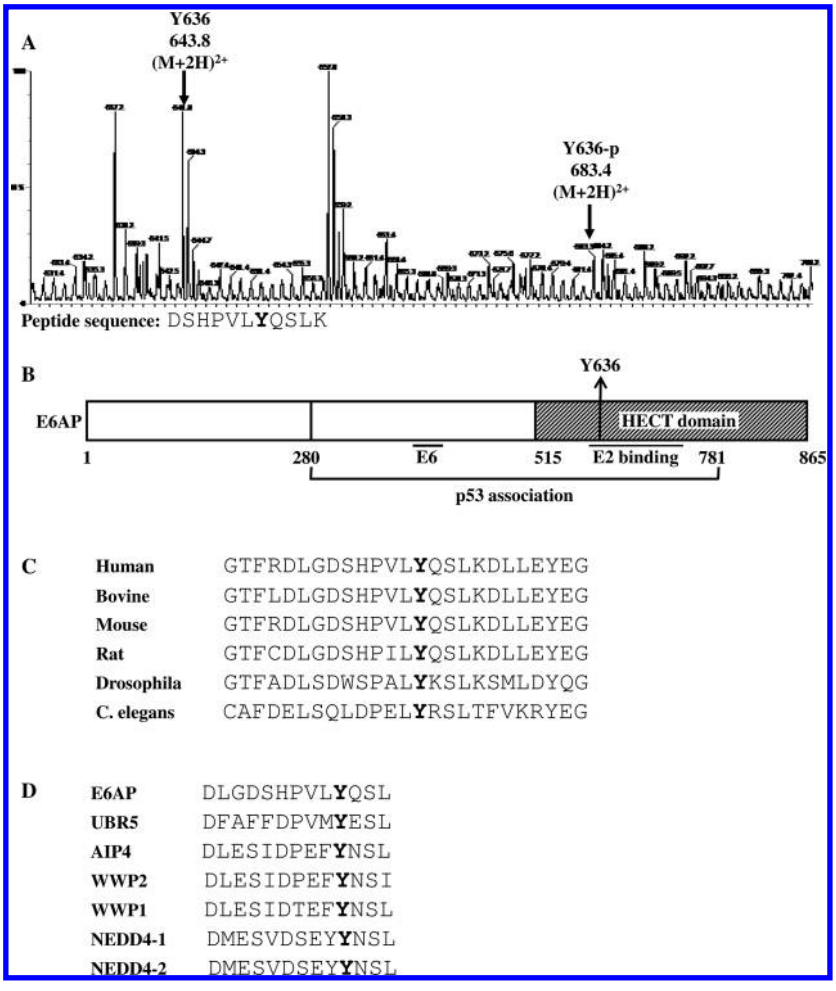

Figure 3. c-Abl phosphorylates E6AP at Y636. (A) Mapping the E6AP phosphorylation site by mass spectrometry. GST-E6AP was phosphorylated *in vitro* in the presence of c-Abl, separated via SDS−PAGE, excised from the gel, and subjected to mass spectrometric analysis using Qtof2 (Micromass). The peaks representing phosphorylated (Y636-p) and nonphosphorylated (Y636) residues are indicated. (B) Schematic representation of the E6AP protein comprising a p53 associating region, an E6 binding region, and the HECT domain.[29,38] Tyrosine 636 identified by mass spectrometry lies within the HECT domain. (C and D) Tyrosine 636 is conserved. Alignment of the E6AP amino acid sequences from human, bovine, mouse, rat, *Drosophila*, and *C. elegans* (C) and alignment of E6AP and the corresponding regions within the HECT domains of various E3 ligases (D) show that this tyrosine (Y636 in human E6AP, highlighted in bold) is completely conserved.

members (Figure 3D). Whether this conserved tyrosine in these HECT proteins is also phosphorylated by c-Abl is unknown. While the mass spectrometric analysis did not reveal additional major phosphorylation site(s), we have examined whether additional sites within E6AP are phosphorylated by c-Abl. For this purpose, tyrosine 636 has been replaced with phenylalanine (Y636F) via removal of a hydroxyl group and therefore prevention of phosphorylation of Y636. wt and Y636F mutant E6AP were then subjected to a c-Abl kinase reaction, which revealed that the Y636F mutant still undergoes c-Abl-dependent phosphorylation (Figure 5A, lane 7). This suggests that an additional phosphorylation site(s) exists within E6AP. We have focused on Y636 because it was identified as the major site of phosphorylation by mass spectrometry and it lies within the catalytic domain of E6AP, a highly conserved residue.

Mutation of E6AP Y636 Impairs Its E3 Ligase Activity. Because we found Y636 to be the major site of phosphorylation by c-Abl, we next examined the contribution of c-Abl-mediated phosphorylation of E6AP to its E3 ligase activity *in vitro*. For this purpose, we employed two approaches. First, we tested the effect of a known c-Abl inhibitor, imatinib, on the ability of E6AP to promote the degradation of p53, a well-established substrate of E6AP in HPV-infected cells. HeLa cells were treated with imatinib (1 and 5 μM for 28 h) or left untreated, and the effect on p53 expression was monitored by Western blot analysis. As shown in Figure 4, treatment of HeLa cells

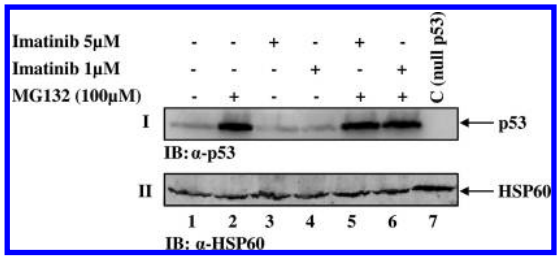

Figure 4. c-Abl inhibits the E3 ligase activity of E6AP. HeLa cells (HPV-infected cells) were treated with imatinib (1 and 5 μM), a c-Abl-specific inhibitor. After 24 h, the cells were treated with proteasome inhibitor MG132 (100 μM) for 4 h before being harvested. Cell extracts were subjected to immunoblotting using the anti-p53 antibody (I), and protein loading was monitored with anti-HSP60 (II). A control cell extract, H1299, was included as it is known to be null for p53.

with imatinib enhanced the degradation of p53 (Figure 4, lanes 3 and 4). This result supports our notion that c-Abl inhibits the E3 ligase activity of E6AP, at least toward p53.

In a second approach, we examined how phosphorylation of E6AP by c-Abl impacts the degradation of another well-established substrate, hHR23.[11] For this purpose, we performed sequential enzymatic assays. GST-E6AP was isolated from bacteria and incubated with a lysate expressing the wt or a catalytic mutant of c-Abl, under kinase assay conditions. Tyrosine-phosphorylated and nonphosphorylated GST-E6AP were then washed and added to a ubiquitination reconstitution assay containing E1, E2 (UbcH5b), and radioactively labeled hHR23. Following the ubiquitination assay, the samples were separated on a gel and subjected to phosphorimager analysis. The extent of ubiquitination of hHR23 induced by GST-E6AP was lower following incubation with wt c-Abl than with the kinase-defective mutant (Figure 5B, lanes 2 and 3 vs lane 1). These results suggest that tyrosine phosphorylation of E6AP by c-Abl impairs its E3 ligase activity, at least toward hHR23. In contrast, the E3 ligase activity of the E6AP Y636F (phosphorylation resistant) mutant was not affected following incubation with wt c-Abl (Figure 5A, lanes 5 and 6 vs lane 4).

Next, we attempted to address whether this effect of phosphorylation on the E3 ligase activity of E6AP is mediated through Y636. For this purpose, we compared the E3 ligase activity of wt E6AP with that of the E6AP Y636F (phosphorylation resistant) mutant over a range of E6AP doses using hHR23 as a substrate.[11] A comparison of E6AP activity over multiple doses of both proteins suggests that wt E6AP is at least 3 times more active than the E6AP Y636F mutant at low concentrations of the protein (Figure 5C). Further, the extent of hHR23 ubiquitination, as measured by the molecular weight size of the smear of hHR23 ubiquitin conjugates, was clearly higher following incubation of wt E6AP than with the equivalent amount of the E6AP Y636F mutant (Figure 5C, II, lane 4 vs lane 9). This finding of extensive E3 ligase efficacy of the Y636F mutant toward its substrate hHR23 suggests that phosphorylation prevention of this residue largely preserves its E3 ligase capacity (and some loss of activity may have resulted from slight E6AP structural malformation, caused by the introduction of phenylalanine). To further, more directly address the role of Y636 phosphorylation with respect to the E3 ligase ubiquitination activity of E6AP, we substituted Y636 with a negatively charged aspartate (Y636D) that mimics the negative charge that is introduced into the wt protein upon phosphorylation. The E3 ligase activity toward hHR23 of wt E6AP was then compared with those of Y636F (phosphorylation resistant) and Y636D (phosphorylation mimic) mutants in an *in vitro* ubiquitination assay. As shown in Figure 6A, the Y636F substitution led to a modest 15% reduction (Table 1), while for the Y636D mutant, activity was more pronounced with 40% reduction (Figure 6A, lanes 1, 2, and 4, respectively). This suggests that mimicking phosphorylation by aspartate had a marked negative effect on E6AP E3 ligase activity (consistent with c-Abl phophorylation modulating E6AP ligase activity, even in the absence of E6).

Further, as E6AP also mediates its own ubiquitination[10,28] it was of interest to define whether Y636 plays a role in this function. For this purpose, we compared wt E6AP and the substitution mutants for their self-ubiquitination efficiency in the absence of E6. In a manner similar to that used for the hHR23 substrate, the capacity of E6AP to self-ubiquitinate was reduced by 25% with the mutant Y636F and more markedly to 85% with the mutant Y636D (Figure 6C, lanes 2 and 4). These results demonstrate a critical role for Y636 in the regulation of E6AP self-ubiquitination in the absence of E6.

The capacity of E6AP residue Y636 to contribute to its E3 ligase competence toward p53 in the presence of HPV-E6 was subsequently determined. The E3 ligase activity of wt E6AP was compared with Y636F and Y636D mutants in an *in vitro* ubiquitination assay using p53 as a substrate in the presence of the HPV-E6 protein. As shown in Figure 6B, the Y636F substitution had no effect on p53 ubiquitination (lanes 1 and 2), while the Y636D substitution mutant lost ~50% of its activity (lanes 1 and 4). Together, these findings suggest that residue E6AP Y636 influences its E3 ligase capacity in a substrate-specific manner.

In Addition to Y636, E544 Is Also Important for Its E3 Ligase Activity. The crystal structure of the HECT domain of E6AP bound to the UbcH7 (E2) complex revealed that E6AP

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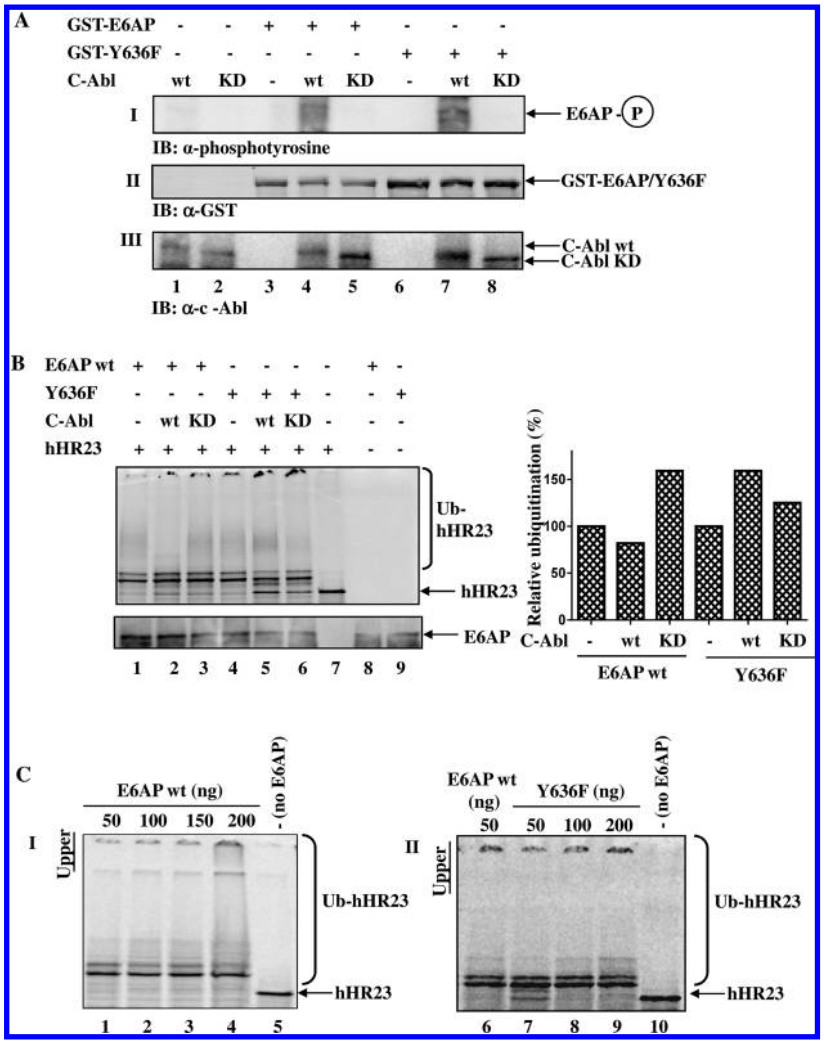

Figure 5. Tyrosine 636 plays an important role in the regulation of the E3 ligase activity of E6AP. (A) Phosphorylation of E6AP and Y636F by c-Abl *in vitro*. HEK293 cells were transfected with expression plasmids for either c-Abl wt (7 μg) or kinase-defective c-Abl (10 μg). Twenty-four hours post-transfection, cell extracts were subjected to immunoprecipitation using the anti-c-Abl antibody and incubated with bacterially purified GST-E6AP (3 μg) and GST-Y363F (3 μg) under kinase assay conditions. The incubation mix was subjected to Western blotting using the anti-phosphotyrosine antibody (I), and the amount of input GST-E6AP and c-Abl was monitored using anti-GST (II) anti-c-Abl (III) antibodies. (B) The level of E6AP-mediated ubiquitination of its substrate, hHR23, is markedly reduced upon phosphorylation by c-Abl. GST-E6AP and GST-Y636F were incubated with wt or kinase-defective c-Abl under kinase assay conditions. Following the incubations, GST-E6AP (both phosphorylated and nonphosphorylated) and GST-Y636F were subjected to an *in vitro* ubiquitination assay using *in vitro* translated radioactively labeled human hHR23 as a substrate. The reaction was conducted in the presence of E1 (150 ng), UbcH5b (125 ng), ubiquitin (62.5 μg), and ATP (2 mM) at 30 °C for 2 h. The reaction mixture was subjected to SDS−PAGE followed by phosphorimager analysis. The intensities of the indicated ubiquitinated bands were quantified and presented as a histogram relative to ubiquitination by either GST-E6AP or GST-Y636F (without the presence of c-Abl) (which were taken to be 100% activity). (C) The E3 ligase activity of wt E6AP is higher than that of E6AP Y636F. The *in vitro* reconstitution assay as described in panel A comparing wt with the Y636F mutant over different doses. As controls, hHR23 was incubated in the absence of E6AP (lanes 5 and 10). Wild-type E6AP and Y636F mutant proteins were not phosphorylated prior to the *in vitro* ubiquitination reconstitution assay.

crystallizes as a trimer[29] (Figure 2A of the Supporting Information). Intriguingly, Y636 was found at the interface between two E6AP monomers, where it interacts with a glutamate (E544) in a neighboring molecule, forming a strong hydrogen bond that can potentially contribute a significant part of the energy of the interaction between the monomers (Figure 2B of the Supporting Information). Phosphorylation of Y636 leads to electrostatic repulsion between the negative charges of phosphorylated Y636 (p-Y636) and E544, as well as to a steric clash with E544 [in particular its C atom (Figure 2C of the Supporting Information)], which disrupt the orientation between the two monomers, thus preventing the creation of the trimer.

The structure therefore suggests that the predicted interaction between Y636 and E544 is critical for the binding of E6AP molecules, whose interruption upon phosphorylation could affect the E3 ligase activity of E6AP. To evaluate the importance of the interaction between Y636 and E544 in the regulation of E6AP E3 ligase activity, we generated a mutant in which glutamate 544 was substituted with alanine (E544A), thereby reducing the potential interaction energy between the monomers in the trimer. A comparison between wt E6AP and the E544A mutant in an *in vitro* ubiquitination assay revealed a moderate effect toward hHR23 as a substrate (Figure 6A, lane 3), but no effect toward p53 in the presence of HPV-E6 (Figure 6B, lane 3) or self-ubiquitination (Figure 6C, lane 3). The

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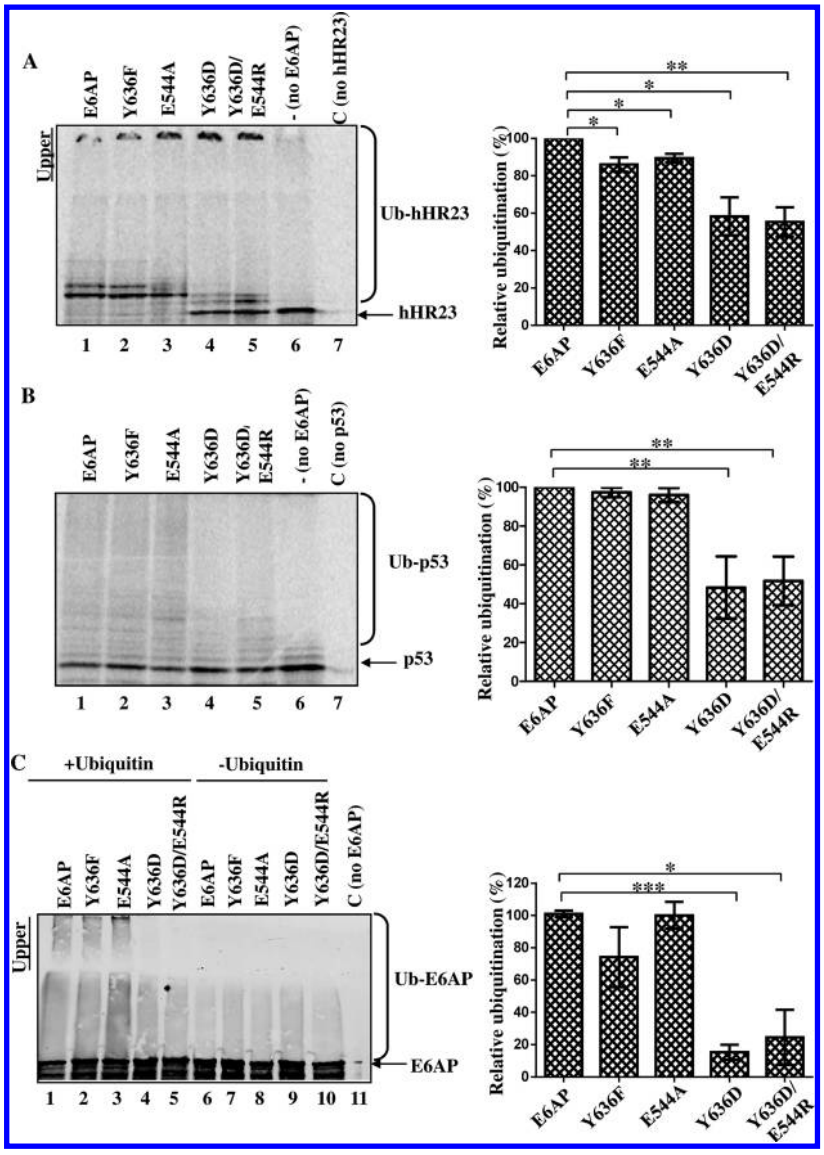

Figure 6. Effects of substitutions in E6AP on the ubiquitination of hHR23 (A) and p53 (B). Bacterially derived GST-E6AP mutants were incubated with E1 (150 ng), E2/UbcH5b (125 ng), and *in vitro* translated radioactively labeled hHR23 (A) and p53 (B) at 30 °C for 2 h. The mixture was then subjected to SDS–PAGE, followed by phosphorimager analysis. The intensities of the ubiquitinated bands were quantified and presented as a histogram relative to the level of ubiquitination by E6AP (lane 1, which was taken to be 100%). The histogram shows the means ± the standard error of the mean of three independent replicates. Two-tailed, unpaired *t* tests were used to determine if a significant difference existed between the E6AP mutants and wt E6AP. Results that were significant are denoted as follows: $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. The inputs of E6AP proteins used in panels A and B are represented in panel C. Incubation in the absence of ubiquitin followed by Western blotting using the anti-E6AP antibody was used to measure self-ubiquitination of E6AP (C, lanes 6–10). A control without any substrate (Figure 5A,B, lane 7) and without ubiquitin (Figure 5C, lane 11) is labeled as C. All the experiments were repeated at least three times.

effects of the E544A substitution toward the substrates are similar to those observed with the Y636F substitution mutant (Figure 6A–C, lanes 2). This suggests that in the absence of E6, in addition to Y636, E544 also contributes to E6AP activity in a substrate-specific manner and highlights the importance of the hydrogen bond between these two residues.

A pertinent question then followed: Is the interaction between Y636 in one monomer and E544 in a neighboring monomer important for E6AP function, or do both residues contribute independently to the activity of E6AP? To distinguish between these two options, we generated a compensatory double substitution mutant. The Y636D mutant introduces a repulsion between two adjacent negative charges (D636 and E544) and therefore prevents interaction between monomers. The compensatory E544R mutation introduces a positive charge that could potentially re-establish this interaction (Figure 2D of the Supporting Information). E6AP activity, as measured by ubiquitination of the substrate, showed no rescue of activity for this double mutant (Figure 6A,B, lane 5 vs lane 4) toward HHR23 and p53. This result indicates that both residues are important and can contribute independently to E6AP activity or, alternatively, that the Y636D substitution has additional effects on the E3 ligase activity of E6AP that are not compensated by the E544R mutation. Nonetheless, the lack of compensation does not rule out a contribution of the Y636–E544 interaction to the catalytic activity of E6AP. Intriguingly, in the context of E6AP self-ubiquitination, interference with this interaction, through the Y636F substitution, reduced this

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Table 1. Summary of the Effect of Substitutions in E6AP Studied Here on the E3 Ligase Activity of E6AP[a]

E6AP Residue 636 [Charge]	E6AP Residue 544 [Charge]	Deduced effect on E6AP multi-merization	% Ubiquitination of Substrate		
			E6AP	hHR23	p53
Y636 [no charge] (wt)	E544 [-] (wt)	interaction	100	100	100
636F [no charge] (prevents phosphorylation, prevents H-bond)	E544 [-] (wt)	weak interaction	74	86	100
Y636 [no charge] (wt)	544A [no charge] (prevents H-bond)	weak interaction	100	89	100
636D [-] (mimics phosphorylation)	E544 [-] (wt)	disrupted interaction	15	58	48
636D [-] (mimics phosphorylation)	544R [+]	disrupted Interaction	25	55	51

[a]The charges corresponding to the residues and the predicted E3 ligase activity are indicated. The values of mean % ubiquitination of p53 in the presence of HPV-E6 and hHR23 and self-ubiquitination of E6AP of E6AP substitution mutants are shown relative to the activity of wt E6AP (taken to be 100%). This table summarizes the results presented in Figure 6.

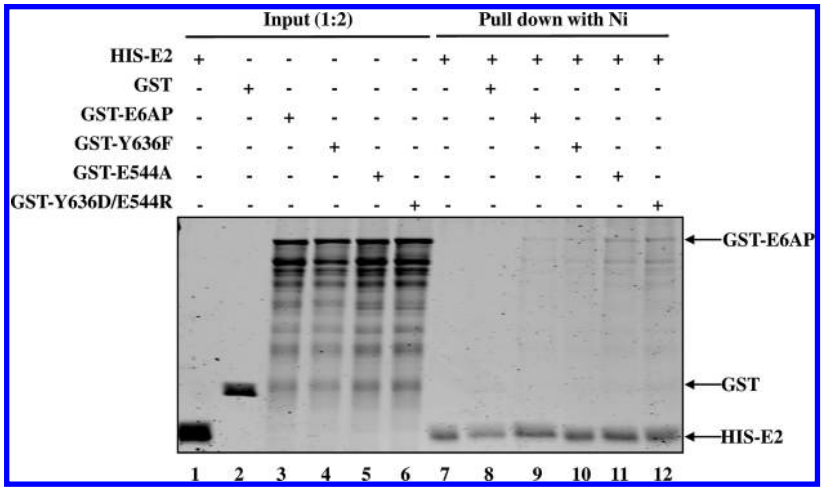

Figure 7. Interaction among UbcH7, wt E6AP, and E6AP mutants. His-tagged UbcH7 (E2), wt E6AP, and E6AP mutants were subjected to a pull-down assay followed by Coomassie staining of a protein blot.

capacity by 25% (Figure 6C, lane 2). Further, Y636D or the Y636D/E544R double mutant lost the majority of the activity (>70%) for self-ubiquitination (Figure 6C, lanes 4 and 5). These results demonstrate a critical role for Y636−E544 interaction in the regulation of E6AP self-ubiquitination.

Another possible impact of the phosphorylation at Y636 is on the interaction between E6AP and E2. Therefore, we have compared the interaction between UbcH7 (E2) and wt E6AP versus the phosphorylation mutants (Figure 7). His-tagged UbcH7 (E2), wt E6AP, and E6AP mutants were subjected to a pull-down assay followed by Coomassie staining of a protein gel. We found that UbcH7 pulled down wt and E6AP mutant proteins to an extent correlating with their input. This suggests that the phosphorylation of E6AP on Y636F or the interaction between Y636 and E544 does not directly impact the interaction with UbcH7 (E2).

DISCUSSION

The timely and efficient activation of p53 in response to DNA damage is essential for a proper cellular response to stress. This regulatory process requires the relief of p53 from inhibitory constraints, such as those imposed by the Mdm proteins, or by the HPV-E6−E6AP complex in HPV-infected cells (Figure 1 of the Supporting Information). This regulation is associated with extensive post-translational modifications of p53 and its negative regulators.[12,30−32] We previously found that c-Abl blocks the ubiquitination and degradation of p53 by the HPV-E6−E6AP complex.[17] In this study, we demonstrated that c-Abl interacts with E6AP (Figure 1) and phosphorylates it *in vitro* as well as *in vivo* (Figures 2 and 5). We mapped a single phosphorylation site to Y636 within the HECT domain of E6AP by mass spectrometry (Figure 3). Using a sequential kinase−ubiquitination enzymatic reaction, we found that phosphorylation of E6AP by c-Abl impairs its E3 ligase activity (Figure 5B). A substitution mutation of Y636 to D636 (Y636D), which mimics constitutive phosphorylation, or to F636 (Y636F), which prevents phosphorylation, supports a role for this residue in the regulation of E6AP E3 ligase activity toward p53 and hHR23 (Figure 6), albeit to a different extent. A role for c-Abl in the protection of p53 was previously described by us and others (reviewed in ref 16). c-Abl interacts with and phosphorylates Mdm2,[18,19] thereby protecting p53 from degradation. c-Abl also interacts with and phosphorylates Mdmx at the p53-binding domain, thereby relieving p53 from a transcriptional constraint.[1] Interestingly, the phosphorylation of

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Mdm2 by c-Abl promotes Mdm2−Mdmx interaction and promotes the ubiquitination of Mdmx.[20]

The crystal structure of the HECT domain of E6AP revealed a trimeric E6AP complex bound to a single E2 molecule (UbcH7).[29] Analysis (Figure 2 of the Supporting Information) of this structure revealed that Y636 is at the interface with E544 in a neighboring molecule forming a hydrogen bond, which may significantly contribute to the multimerization of E6AP. Substitution mutations of E6AP at Y636 (Y636F) or E544 (E544A), which prevent hydrogen bond formation, have been used to explore the role of this interaction in the E3 ligase activity of E6AP. We have also attempted to restore this interaction by introducing a positive charge on residue 544 through a substitution of glutamate with arginine. However, the Y636D/E544R double mutant did not regain E3 ligase activity, suggesting that either the opposing charges were insufficient to promote multimerization of E6AP or these residues make additional contributions to the catalytic activity of E6AP beyond the formation of multimers. One possibility is that phosphorylation of Y636 also interferes with E2 interaction, because it resides within the E2 binding site of E6AP.[29] Intriguingly, the inhibition of the Y636−E544 interaction by these substitution mutants impaired the E3 ligase activity of E6AP in a substrate-specific manner (Table 1). In the presence of HPV-E6, p53 ubiquitination was executed with complete efficiency when c-Abl phosphorylation of E6AP was prevented, through the Y636F substitution, as predicted. However, the prevention of E6AP intermolecular hydrogen bonding through the E544A substitution also resulted in efficient p53 ubiquitination. This suggests that monomeric E6AP can ubiquitinate p53 in the presence of HPV-E6. This finding is consistent with a previous report that E6AP can self-ubiquitinate as a monomer in the presence of the HPV-E6 protein.[10] Taken together, these data corroborate that multimerization is not essential for E6AP-mediated ubiquitination of p53 in the presence of HPV-E6. In contrast, in the absence of HPV-E6, Y636F exhibited some impaired activity toward hHR23 (15% inhibition) and inhibition of self-ubiquitination (25% inhibition) (Table 1). Further, our results raise the intriguing possibility that in the absence of HPV-E6, E6AP accumulation and consequent multimeric E6AP complex formation favor self-ubiquitination. In this model, E6AP expression levels would be regulated by the self-ubiquitination and fidelity of Y636 would be central. This model is consistent with previous findings demonstrating that in the absence of HPV-E6, ubiquitination of E6AP occurs largely in trans, which therefore requires its oligomerization.[28] Future studies will be required to demonstrate the oligomerization model. Our results support a regulatory role for Y636 in the self-regulation of cellular E6AP levels.

Given the high degree of conservation of Y636 among different species, including *Drosophila* and *C. elegans*, this residue may play an important and conserved regulatory role. Further, Y636 is also conserved among a number of HECT E3 ligases, including AIP4 and members of the WWP and NEDD4 families (Figure 3D). Whether the conserved Y636 also plays a regulatory role in other HECT enzymes is yet to be investigated. The role of multimerization in ubiquitination has recently been demonstrated for RNF4, where a hydrogen bond between Y193 and G159 bridges two monomers and is essential for ubiquitination. The ubiquitin is transferred from one monomer bound to E2 to a second monomer bound to the substrate.[33] Thus, multimerization may be a general mechanism of ubiquitination across different classes of E3 ligases.

In HPV-infected cells, the HPV-E6−E6AP complex regulates p53 stability.[5,34] Inhibition of the HPV-E6−E6AP complex is sufficient to restore p53 function and consequently the induction of growth inhibition. This has been demonstrated through a variety of approaches, including the antisense approach, downregulation via RNAi, a dominant negative mutant, or small molecule inhibitors.[35−40] Thus, restoration of p53 tumor suppression is an attractive approach to the treatment of HPV-related cervical carcinoma. Often, these approaches are combined with treatment using genotoxic agents, which are known to activate c-Abl.[14,15,41,42] Our results suggest that c-Abl-mediated phosphorylation of E6AP provides an important protection for p53 from the HPV-E6−E6AP complex.

■ ASSOCIATED CONTENT

Supporting Information

A schematic diagram of the role of c-Abl in the protection of p53 by phosphorylation of HPV-E6−E6AP (HPV-infected cells) and Mdm proteins (non-HPV-infected cells) (Figure 1), the structural basis of E6AP molecules and the importance of Y636 (Figure 2), phosphorylation of E6AP by c-Abl *in vivo* (Figure 3), and the interaction between E6AP and c-Abl (Figures 4 and 5). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Research Division, The Peter MacCallum Cancer Centre, St. Andrew's Place, East Melbourne 3002, Victoria, Australia. Telephone: 613-96565871. Fax: 613-96561411. E-mail: ygal.haupt@petermac.org.

Author Contributions

¶A.-L.C. and T.G. contributed equally to this work.

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Notes

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■ ABBREVIATIONS

HPV, human papillomavirus; E6AP, E6-associated protein; HECT, homologous to the E6AP C-terminus; MEF, murine embryonic fibroblast; KO, knockout; GST, glutathione *S*-transferase; wt, wild type; IP, immunoprecipitation; IB, immunoblot; Ub, ubiquitinated.

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