1. *The authors first demonstrate a physical interaction between c-Abl and E6AP (Figure 1). Describe the various experiments used to demonstrate the interaction. Identify and explain the importance of the different controls used in each case. Note, MEFs (Mouse Embryonic Fibroblasts) are commonly derived from genetically modified mice (e.g. from the c-Abl KO (knockout) mice used here) and provide a convenient means by which to study the function of targeted genes in cultured cells. 25 marks.*

   Physical interaction between c-Abl and E6AP. Overall, the interaction is demonstrated by extensive immunoprecipitation and immunoblotting, where E6AP are tagged with HA- or myc- and appropriate antibodies are employed.

   a. 1st experiment:
      The interaction is first demonstrated *in vivo* in HEK293 cells. Three types of plasmid combinations are transfected:
         i. c-Abl-only
         ii. HA-E6AP-only
         iii. c-Abl and HA-E6AP
         iv. (Here the absolute control is missing, where no c-Abl or HA-E6AP is transfected.)
   b. The grown cells are then harvested and processed with three IP-IB combinations to measure abundance of different species:
         i. IP: anti-Abl + IB: anti-HA, this measures abundance of c-Abl-E6AP complex
         ii. IP: anti-Abl + IB: anti-Abl, this measures abundance of total c-Abl.
         iii. IP: n/a + IB: anti-HA, this measures abundance of total E6AP, it is not clear why IP with anti-HA is omitted.
   c. Conceptually speaking, it's only the amount of c-Abl-E6AP complex of real interest. However, total c-Abl and total E6AP are also measured to demonstrate that transfections are successful and antibodies are indeed specific to their epitopes.
   d. In conclusion, c-Abl-E6AP complex is only present in doubly transfected HEK293 cells, thus evidencing a physical interaction between c-Abl and E6AP.

   a. 2nd experiment:
      The interaction is then demonstrated *in vivo* using mouse embryonic fibroblasts (MEF). Three types of MEF are employed:
         i. E6AP-wt
         ii. E6AP-KO
         iii. E6AP-KO/myc
   b. A preliminary quality test is conducted to confirm the abundance of E6AP amount follows expectation in each strain. Here the HSP60 abundance is employed as a control to confirm successful extraction of cellular protein. These experiments are immunoblotted with anti-E6AP and anti-HSP60.
   c. Interaction between myc-E6AP and c-Abl is then demonstrated. Here E6AP-wt strain is not included in the IP-IB process for unknown

reason, which otherwise serves a useful control. The extraction is processed under 2 IP-IB combinations:

   i. IP:anti-myc + IB:anti-Abl, this serve to measure abundance of myc-Abl. myc-Abl supposedly reflects abundance of myc-E6AP-Abl, however the authors did not rule out existence of other myc-tagged protein complexed with c-Abl.
   ii. IP:anti-myc + IB:anti-E6AP, this serve to measure abundance of myc-E6AP. Again this is an indirect evidence for E6AP-Abl interaction.

d. Overall, myc-Abl and myc-E6AP are only detected in E6AP-KO/myc strain. This evidence a myc-E6AP-c-Abl complex. However, this line of evidence is indirect, due to possible (though very unlikely) other myc-tagged protein in the strain. The E6AP-KO strain clearly does not contain any myc-tagged E6AP or c-Abl, but it's unclear whether it contains any other myc-tagged proteins.
e. To rule out other myc-tagged protein, a doubly IP process might be considered, ie. IP:anti-myc + IP:anti-E6AP, followed by IB:anti-Abl, this should directly confirm the existence of myc-E6AP-c-Abl complex.

a. 3rd experiment:
   The interaction is further demonstrated *in vitro* using bacterially derived GST-E6AP (GST for glutathione-S-transferase ) and HEK293-derived c-Abl. Moreover, 4 variants of c-Abl are employed to identify importance of different c-Abl sections in interacting with E6AP, they are:

   i. wt
   ii. KD (kinase defective, a.k.a. K290H mutant)
   iii. delta-proline
   iv. negative control (no c-Abl is transfected)

b. The experiment involves incubation of HEK293 extraction with GST(control) or GST-E6AP, followed by

   i. IP:glutathione/anti-GST + IB: anti-c-Abl, serve to measure GST-c-Abl.
   ii. IP:n/a + IB: anti-c-Abl, serve to measure total c-Abl
   iii. IP: glutathione/anti-GST + IB: anti-E6AP (supplementary), serve to measure GST-E6AP amount
   iv. IP: n/a + IB: anti-E6AP (supplementary), serve to measure total E6AP. (Confusingly, total E6AP appear to give less colouring than GST-E6AP, possibly because E6AP epitope is masked in raw mixture )
   v.

c. Negative control confirms that native HEK293 only contains very little amount of c-Abl compared to transfected strains. GST controls confirm that the GST itself does not interact with c-Abl thus ruling out spurious interaction and confirm the detected interaction is between E6AP-c-Abl.
d. Pull-down: glutathione/anti-GST + IB:anti-Abl serves to measure abundance of GST-E6AP-c-Abl. Based on these controls, GST-E6AP-c-Abl faithfully reflects the interaction between E6AP-c-Abl, thus warranting the result to evidence that E6AP interacts directly with wt-c-Abl, with KD-c-Abl and with delta-proline-c-Abl, *in vitro.*

e. This experiment is not strictly *in vitro*, since the HEK293 extraction is not purified for E6AP prior to co-incubation. It therefore does not rule out a HEK293-origin proxy relaying the interaction between E6AP and c-Abl.
f. Moreover, additional experiments using c-Abl-GFP constructs help establish that SH3 domain on c-Abl is important to interact with E6AP. However, the labelling of the corresponding figure (figure S4) is too poor to allow any critical evaluation (the status of Myc-E6AP is missing for the last 6 lanes). Thus no comment is made here concerning this claim.

2. *In order to demonstrate that c-Abl phosphorylates E6AP, the authors present data from an experiment in which the phosphorylation status of E6AP was measured following its purification from either wild type (wt) or c-Abl KO MEFs. Describe why additional experiments using purified proteins and a kinase-defective mutant of c-Abl were also required to prove E6AP is a target of c-Abl. 25 marks.*

   Knowing c-Abl is a tyrosine kinase, the author go on to demonstrate E6AP does not only interact with c-Abl, but also get phosphorylated by c-Abl. Here the phosphorylation is detected using anti-pY (phosphotyrosine), combined with various pull-down assays.

   1st Experiment:

   a. c-Abl-wt and c-Abl-KO MEF are employed to measure *in vivo* phosphorylation of E6AP by c-Abl. The extraction is processed under:
      i. IP:anti-E6AP + IB:anti-pY, serves to measure abundance of pY-E6AP (a.k.a. tyrosine-phosphorylated form of E6AP).
      ii. IP: anti-E6AP + IB: anti-E6AP, serves to measure abundance of E6AP. (Intriguingly, multiple bands show up, indicating E6AP is native in different complexes)
      iii. Overall, the experiment demonstrate the pY level on E6AP-complex is c-Abl-dependent. However, the exact position of pY remains unclear (maybe on a complexed protein), and the *in vivo* nature of measurement does not distinguish between direct phosphorylation and indirect phosphorylation.

   2nd Experiment

   a. *In vitro* phosphorylation of GST-E6AP by HEK293-derived c-Abl is measured. Both proteins are purified by appropriate IP (anti-GST and anti-Abl) prior to co-incubation Conclusion is drawn from comparing c-Abl-wt and c-Abl-KD(control). Enzyme-only and substrate-only mixtures are also employed as negative controls.
   b. The incubated mixture is subject to following processes:
      i. IB: anti-pY
      ii. IB: anti-GST
      iii. IB: anti-Abl
   c. Based on these controls, pY-level faithfully reflects amount of pY-E6AP. c-Abl-origin pY is ruled out since no pY is detected in c-Abl-only mixture added. However it would be preferential to include GST as a negative control for substrate to rule out a GST-origin pY.

Abolishment of pY-level with KD variant also strongly supports c-Abl as the major kinase giving rise to the detected phosphorylation. Overall, this evidence E6AP as a direct substrate of c-Abl.

Since *in vivo* situation is complicated, it's always preferential to carry out *in vitro* assay between 2 proteins to rule out indirect effects and to characterise the detail of the interaction.

*2.*

3. *The authors use mass spectrometry to identify a major c-Abl target site on E6AP. Explain how identifying a specific phosphorylation site enabled them to propose and test their model that phosphorylation by c-Abl affects the catalytic activity of E6AP. Comment on the inferences drawn from examining conservation of the site and surrounding sequence across species and in HECT domains from different E3 ligases. 35 marks.*

   Mass Spectrometry:The author identify Y636 as a major phosphorylation site of E6AP by c-Abl.

   a. Background knowledge: c-Abl kinase activity protects p53 from E6AP-dependent ubiquitin-mediated degradation.
   b. The fact that Y636 is within the HECT region that provide E3-ligase activity, together with the background knowledge, warrants a hypothesis where c-Abl controls p53 degradation with E6AP's E3-ligase activity as a proxy.
   c. With this information, they can generate E6AP mutant that mimic either a constitutively phosphorylated form or a constitutively-non-phosphorylated form. The former is achieved by introducing a negatively charged residue at the position of Y636. The latter is achieved by removing the -OH group on the tyrosine in a Y to F replacement. Such mutants are particularly convenient since it allows experimenter to identify the impact of enzymatic modification without actually using the enzyme, but by comparing wild-type and mutant-type instead.
      (Such mutagenesis, however, does suffer from side-effects where introduction of mutation damage the general protein structure and gives rise to spurious establishment or abolishment of interactions. )
   d. However Y636F mutant is still detected by anti-pY after co-incubation with c-Abl. This implies Y636 is not the only phosphorylation site. Thus more evidence are required to establish a connection between Y636 and regulation of p53 by c-Abl.
   e. Conservation of Y636 and its flanking sequence:
      i. Y636 and its flanking sequence is well-conserved in mammalian. The conservation up to Drosophila is acceptable, but the orthologue in *C. elegans* appear to be only weakly related.
         Therefore there is enough evidence to infer a similar role of E6AP in mammalians and in Drosophila but not in *C. elegans.* These orthologues however only evidence the conservation of E6AP as a whole and do not give information about functional importance of conserved tyrosine.

ii. In paralogous HECT E3 ligase, the flanking sequence appear to be weakly-conserved, though the tyrosine residue is completely conserved in the 6 paralogues.
iii. Given the common knowledge that only functional/structural residues are heavily conserved between the homologues, it is reasonable to suggest a conserved role of Y636 across the species. However the fact that NEDD4 has consecutive tyrosine residues near the conserved position raise the question about how could upstream enzyme identify the right residue to phosphorylate.
iv. On the other hand, given the common knowledge that kinase recognises phosphorylation site via its flanking short motifs, it's questionable whether the diverse flanking sequence can be recognised by the c-Abl kinase similarly. One could argue that additional conservation hot-spots exist near the conserved tyrosine, but an alignment of 6 E3-ligase does not give adequate/confident information about the conservation of the motif.
v. Overall, there is weak evidence to suggest a conserved role of Y636. The evidence can be reinforced with a bigger alignment or with high-throughput tests of phosphorylation status of the corresponding tyrosine residues.

f. With E3-ligase activity as a readout, multiple experiments are conducted to test the hypothesis:

1st Experiment:

a. E3-ligase activity on p53 is measured. Three pharmacological conditions are employed to vary the *in vivo* c-Abl activity (Imatinib 1uM and Imatinib 5uM, c-Abl inhibitor) and proteasome activity(MG132 100uM, proteasome inhibitor). HSP60 is employed as a neutral control for protein content to confirm the observed impact is specific to p53. The extraction is subject to IB:anti-p53 or IB:anti-HSP60.
b. The authors claimed that inhibition of c-Abl with Imatinib enhanced degradation of p53. This is poorly supported by the pale difference in colouring between control set and Imatinib set. MG132, in contrast, convincingly rescue the degradation of p53 specifically (not rescuing HSP60).

2nd Experiment:

a. A *in vitro* sequential enzymatic assay is performed, namely combining phosphorylation assay of GST-E6AP (variants) by c-Abl (variants) and a ubiquitination assay of hHR23 by phosphorylated GST-E6AP mixture, where E1,E2 ubiquitins are supplied. The amounts and sizes of radiolabelled hHR23 serve as readout for E3-ligase activity. Comparison between c-Abl-neg, c-Abl-wt and c-Abl-KD evidenced that active signalling by c-Abl-wt inhibit the ubiquitination activity of GST-E6AP-wt, whereas GST-E6AP-Y636F's E3-ligase activity is actually elevated if interacted with c-Abl-wt or c-Abl-KD. The latter effect is not explained by the current model and likely attribute to other phosphorylation sites on E6AP.

3rd Experiment:

a. E3-ligase activity of non-phosphorylated E6AP, E6AP-Y636F and E6AP-Y636D are compared. This confirm E6AP-Y636F largely preserve its E3-ligase activity (with moderate loss) and is justified as a functional phosphorylation-resistant mutant (though not perfect). In contrast, E6AP-Y636D shows a 40% reduction in its E3-ligase activity. These results strongly support that Y636 is involved in controlling E3-ligase activity.

Other Experiments:

a. The author then went on to test and compare E3-ligase activity using different substrate for readout (E6AP,p53,hHR23), under a unified protocol, and demonstrate that the choice of substrate indeed has an effect on the measurement of E3-ligase activity.

Overall, identification of Y636 as the major phosphorylation site by mass-spectrometry gives hint that the readout of the regulatory event is E3-ligase activity and warrant further mutagenesis and enzymatic assay to indirectly demonstrate the effect of phosphorylation with this specific readout.

*4. Knowledge of the E6AP crystal structure allowed the authors to hypothesize and test the mechanism by which Y636 phosphorylation may decrease ubiquitin ligase activity. Comment on how these experiments gave insight into how E6AP may behave differently in the absence and presence of HPVE6 but also describe the limitations of using mutagenesis of specific amino acids to infer their functional importance. 15 marks.*

Use of Crystal structure: Crystal structure of E6AP contain a trimer of E6AP complexed with a UbcH7(E2). Close inspection indicate that Y636 lies on-test the hypothesis that multimeric complex is important for E3-ligase activity. This is done by re-interpreting E3-ligase activity of Y636F, Y636D, and testing E544A and doubly mutant Y636D/E544R.

b. Y636F has slightly reduced E3-ligase activity. This agree with the multimeric model
c. Y636D has a dramatically reduced E3-ligase activity, agreeing with the multimeric model.
d. E544A has a slightly reduced activity, but does not inhibit self-ubiquitination, contrast to the dramatic activity reduction in Y636F. This implies E544A and Y636F might contribute to the E3-ligase activity independently as well as collectively (via multimeric effect).
e. Y636D/E544R show dramatic reduction in E3-ligase activity similar to those of Y636D. a.k.a. E544R is unable to rescue the phenotype of Y636D, disagreeing with the multimeric model.
f. Out of the three ubiquitination readouts, ubiquitination of p53 is measured with HPVE6 present, while ubiquitination of hHR23 and self-ubiquitination are measured without HPVE6.
g. The author claim that E544A mutant retains activity in self-ubquitination but not in hHR23 ubiquitination, arguing that the

hydrogen bond is important with and without HPVE6. However, the reduction in activity is very moderate. And the evidence does not support an important role of hydrogen bonding with HPVE6 present (since only p53 is tested with HPVE6 present)

h. The presented experimental data, however, does exhibit a robust trend regarding p53 ubiquitination, that is, Y636F and E544A do not result in any reduction in activity compared to E6AP-wt. In contrast, in readouts without HPVE6, activity is more or less reduced in Y636F and E544A mutants. This suggest in the presence of HPVE6, the E3-ligase activity is more robust to minor perturbation, thus HPVE6 serves to stabilise the functional form of E6AP.
i. Overall, the result supports the idea that multimeric E6AP form is important for HPVE6-independent ubiquitination (p53 ubiquitination) but dispensable for HPVE6-independent ubiquitination (hHR23 and E6AP itself)
j. Furthermore, the use of Y636F and E544A does not interrupt the intermolecular hydrogen bond specifically, but might as well introduce general structural malfunction. Thus one could not tell whether the observed effect is due to structural malfunction or interrupted hydrogen bond.  Particularly, Y636D/E544R does not rescue the phenotype, but it’s unclear whether Y636 and E544 act independently or Y636D/E544R introduced too much structural constraint. Thus mutagenesis is useful to perform structural perturbation, but also risking perturb in the unwanted aspect.
k. Y636F/E544A might be a useful double mutant to test whether effects of Y636F and E544A are additive. But again, effect of double mutant are hard to predict.