Supporting Information

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SI Results and Discussion

In vitro ubiquitination experiments are generally used to provide evidence that a given protein can serve as ubiquitination substrate for a particular E3 ligase. Thus, we performed in vitro ubiquitination experiments by incubating in vitro-translated, radiolabeled activity-regulated cytoskeleton-associated protein (Arc) with recombinant ubiquitin-activating enzyme E1 and the ubiquitin-conjugating enzymes (Ubc)H5b and/or UbcH7 in the absence or presence of ubiquitin and E6 associated protein (E6AP) (for further details, see *SI Materials and Methods*). In parallel, ubiquitination assays with really interesting new gene 1b (Ring1b)-I53S (1), tumor suppressor protein p53 (2), and the N-terminally truncated form of HECT domain and RCC1-like domain-containing protein 2 (HERC2_tr) (see the legend to Fig. 2) as substrates were performed. As shown in Fig. S2*A*, all four proteins were ubiquitinated by E6AP in a dose-dependent manner, although with somewhat different efficiencies.

At first glance, the observations that E6AP is capable of targeting Arc for ubiquitination in vitro but not within cells (Fig. 2*D*) may seem to be contradictory. However, this apparent paradox is readily explained by the notion that in contrast to cell-based assays, the substrate specificity of in vitro ubiquitination systems appears to be rather limited. Firstly, E6AP was originally isolated as a protein that in complex with human papillomavirus E6 targets p53 for ubiquitination and degradation (3–5), whereas E6AP alone (i.e., in the absence of E6) does not bind to p53 and, consequently, does not play a role in p53 turnover in cells (6–9). Nonetheless, E6AP is capable of ubiquitinating p53 in vitro in the absence of E6 (2) (Fig. S2*A*). Secondly, similar to the results obtained with E6AP, Arc, Ring1b, HERC2_tr, and p53 are ubiquitinated in vitro in the presence of the human ortholog of murine double minute 2 (Mdm2) (Hdm2), an E3 ligase that is not related to E6AP (Fig. S2*B*). However, with the exception of p53 (10), none of the proteins studied is known to interact with Hdm2 or to serve as substrates for Hdm2 within cells in coexpression experiments. Finally, all of the proteins studied are either known to be degraded by the ubiquitin-proteasome system (p53, Arc, Ring1b) and/or are components of it (Ring1b, HERC2) (10–13). Thus, these proteins are likely to contain amino acid sequences or regions that serve as ubiquitination signals and can be recognized in vitro by E3 ligases, which are not involved in ubiquitination/degradation of the respective proteins within cells (in other words, these signals apparently suffice to be recognized by different E3 ligases in vitro but not in a cellular environment). In conclusion, we propose that similar to members of the protein kinase family (e.g., refs. 14–17), at least some E3 ligases are promiscuous in vitro with respect to their substrate specificity, and, thus, results obtained in in vitro ubiquitination experiments have to be interpreted with caution.

SI Materials and Methods

Cell Lines and Plasmids. H1299 cells and HEK293T cells were grown in DMEM supplemented with 10% (vol/vol) FBS. SH-SY5Y cells were grown in DMEM/F12 (1:1) plus GlutaMAX supplemented with 10% (vol/vol) FBS.

The bacterial expression constructs for the GST fusion proteins of full-length human homolog of Rad23 (HHR23)A and Arc and of various deletion mutants of HHR23A, Arc, and Sacsin (Fig. 1 and Fig. S1) were generated by PCR-based approaches (further details will be provided upon request). Expression constructs (in vitro translation, transient transfection experiments) encoding HA-tagged wild-type E6AP [isoform 1 (18); note that although all experiments shown were performed with isoform 1, similar results were obtained with isoforms 2 and 3 in Arc-degradation experiments and luciferase reporter assays], the HA-tagged catalytically inactive mutant E6AP-C820A (substitution of Cys-820 by Ala), the catalytically inactive mutant Ring1b-I53S, human p53, and His-tagged ubiquitin were described previously (1, 19). Expression constructs (transient transfection experiments) encoding HA-tagged wild-type E6AP with a C-terminal Flag tag, wild-type E6AP with an N-terminal (GFP-E6AP) or C-terminal (E6AP-GFP) GFP extension, HA-tagged Arc, HA-tagged HERC2_tr (amino acids 2958–4834 of HERC2), and HA-tagged estrogen receptor (ER)α, as well as dehydrofolate reductase (DHFR)–HA–ubiquitin fusion proteins of HA-Arc, HA-Ring1b-I53S, and HA-HERC2_tr were generated by PCR-based approaches (further details will be provided upon request). The ER-responsive reporter construct (3×ERE TATA Luc) was obtained from Addgene (20).

Transfection and Antibodies. For transient expression, cells were transfected with the respective constructs by lipofection (Lipofectamine 2000; Invitrogen) according to the manufacturer's instructions. Protein extracts were prepared 24 h after transfection as described (19). Levels of the various HA-tagged proteins were determined by Western blot analysis using the mouse monoclonal HA.11 (Hiss Diagnostics). Where indicated, Arc, Ring1b-I53S, E6AP, and tubulin were detected by an anti-Arc mouse monoclonal antibody (612602; BD Transduction Laboratories), an anti-Ring1b antibody (MBL International), a mouse monoclonal antibody against E6AP (7), and an anti-tubulin mouse monoclonal antibody (Abcam).

To generate cell lines, in which E6AP expression is stably suppressed by RNA interference, HEK293T cells were transfected with pMSCV-Hygro-NA8 (Clontech) and SH-SY5Y cells were transfected with pcDNA4TO-NA8-IRES-Puro by lipofection (Lipofectamine 2000; Invitrogen) and nucleofection (Cell Line Nucleofector Kit V; Amaxa), respectively. Both constructs express an shRNA directed against nucleotides 300–318 of the E6AP mRNA [numbering according to E6AP isoform 1, with nucleotide 1 referring to A of the start codon (18)]. Cells stably containing the respective expression construct were selected by resistance to hygromycin (Sigma) and puromycin (Sigma), respectively. After establishing respective cell lines, protein extracts were prepared, and E6AP levels were determined by Western blot using an anti-E6AP mouse monoclonal antibody (7).

Coprecipitation Assays. In vitro coprecipitation experiments using GST fusion proteins were performed as described previously (4). Briefly, 500 ng of baculovirus expressed E6AP were incubated with 1 μg of bacterially expressed GST or GST fusion proteins as indicated (Fig. 1). After 4 h, bound proteins were purified by glutathione affinity chromatography and electrophoresed in 8% SDS/polyacrylamide gels, and E6AP was detected by Western blot analysis.

In Vitro Ubiquitination. For in vitro ubiquitination experiments, the ubiquitin-activating enzyme E1 and E6AP (isoform 1) were expressed in the baculovirus system, and UbcH5b and UbcH7 were expressed in *Escherichia coli* BL21 by using the pET expression system as described (21). For in vitro ubiquitination, 1 μL of rabbit reticulocyte lysate-translated ^{35}S-labeled substrate (Arc, HERC2_tr, p53, Ring1b-I53S) was incubated with 50 ng of E1, 50 ng of UbcH5b or UbcH7, increasing amounts (100–400 ng) of baculovirus-expressed E6AP or Hdm2, and 20 μg of ubiquitin

(Sigma) in 40-μL volumes. In addition, reactions contained 25 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 2 mM ATP, and 4 mM $MgCl_2$. After incubation at 25 °C (E6AP) or 35 °C (Hdm2) for 2 h, total reaction mixtures were electrophoresed in 10% SDS/polyacrylamide gels, and ^{35}S-labeled proteins were detected by fluorography.

Luciferase Reporter Assays. Transfections of H1299 or H1299-shE6AP [stable knockdown of E6AP expression (19)] cells were performed in six-well plates. One well was transfected with 1 μg of the ER reporter construct and expression constructs encoding β-galactosidase (400 ng), HA-ERα (50 ng), and E6AP or E6AP-C820A (1.5 μg) as indicated (Fig. 3 *A* and *B*). Twenty-four hours after transfection, cells were harvested and lysed on ice in 120 μL of 1× Luciferase Cell-Culture Lysis Reagent (Promega). After 30 min, samples were centrifuged for 15 min at 4 °C, and the supernatant used to determine both luciferase activity and β-galactosidase activity to adjust for transfection efficiency. Furthermore, each transfection was performed in duplicate. Luciferase assays were performed using a Luciferase Assay System kit (Promega) and analyzed on a Wallac 1420 multilabel counter (PerkinElmer).

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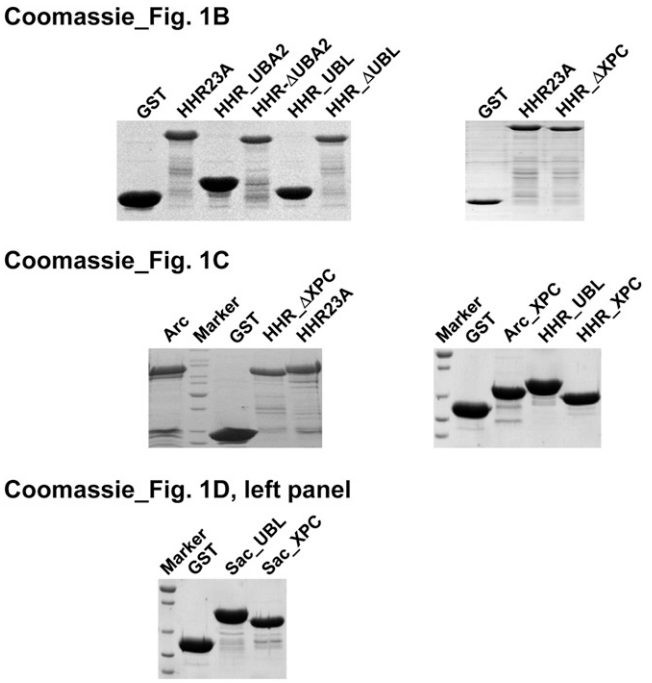

Fig. S1. Coomassie stain of the various GST fusion proteins used in pull-down assays with E6AP. The various proteins (for details, see Fig. 1) were bacterially expressed as GST fusion proteins and purified by affinity chromatography using glutathione–Sepharose. Protein amounts were adjusted, and the various fusion proteins were used in GST pull-down assays (Fig. 1). (*Top*) Coomassie from Fig. 1*B*. (*Middle Left*) Coomassie from Fig. 1*C*, *left*. Marker: 25, 30, 40, 50, 60, 70, 85, 100, and 120 kDa (from bottom to top). (*Middle Right*) Coomassie from Fig. 1*C*, *right*. Marker: 20, 25, 30, 40, and 50 kDa (from bottom to top). (*Bottom*) Coomassie from Fig. 1*D*, *left*. Marker: 20, 25, 30, 40, and 50 kDa (from bottom to top).

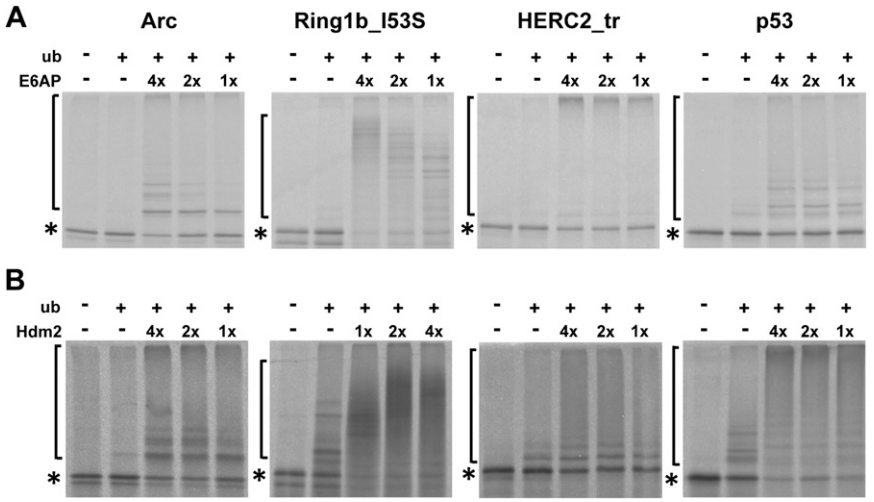

Fig. S2. E6AP is a promiscuous ubiquitin–protein ligase in vitro. In vitro-translated radiolabeled Arc, p53, Ring1b-I53S, or HERC2_tr were incubated in the absence or presence of ubiquitin (ub) with increasing amounts of baculovirus-expressed E6AP (*A*) or bacterially expressed GST fusion protein of Hdm2 (*B*) under standard ubiquitination conditions (*SI Materials and Methods*). After 120 min, reactions were stopped and reaction products analyzed by SDS/PAGE followed by fluorography. Running positions of the nonmodified form and of the ubiquitinated forms of the various substrates are indicated by asterisks and brackets, respectively. Ring1b-I53S was used as substrate rather than wild-type Ring1b, because, as a result of the mutation, Ring1b-I53S is severely compromised in its function as E3 ligase and cannot ubiquitinate itself (1). Thus, ubiquitination of Ring1b-I53S by E6AP can be easily monitored. HERC2_tr represents an N-terminal truncation mutant of HERC2 comprising amino acid residues 2958–4834 and was used because it binds to E6AP and represents an efficient substrate for E6AP.

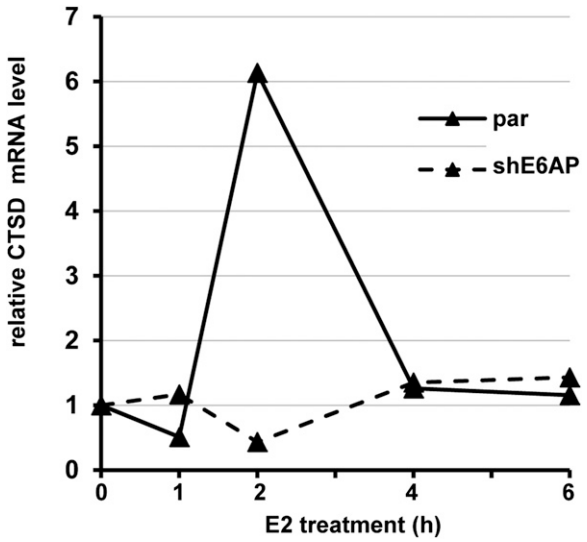

Fig. S3. Knockdown of E6AP expression levels interferes with estradiol-mediated transcription of the Cathepsin D (*CTSD*) gene. SH-SY5Y cells (par) and SH-SY5Y cells, in which endogenous E6AP expression is stably down-regulated by RNA interference (shE6AP), were treated with 10 nM estradiol (E2). Cells were harvested at the times indicated and used to isolate total RNA, followed by cDNA synthesis and determination of relative levels of CTSD mRNA by real-time PCR. Simultaneous determination of Actin mRNA levels served as reference. The ratio between CTSD mRNA and Actin mRNA at time 0 was set to 1.