Supporting Information

Zaaroor-Regev et al. 10.1073/pnas.1003108107

SI Text

Materials and Methods. ***Preparation and fractionation of crude reticulocyte lysate.*** Reticulocytosis was induced in rabbits and lysates were prepared as described in ref. S1. The lysate was fractionated over DEAE cellulose (Whatman) into unabsorbed material (Fraction I) and high salt eluate (Fraction II). Fraction II (~600 mg) was further fractionated by ammonium sulfate into proteins that precipitate at 0–45% and >45%. Most of the Ring1B conjugating activity was found in the 0–45% fraction that was further resolved (~200 mg) on a MonoQ column using a linear salt gradient of 0–0.6 M KCl. The fractions containing the ligating activity were subjected to hydroxyapatite chromatography. Proteins were eluted using a linear gradient of 10–700 mM KPi pH 7.0. The active fractions were applied to a gel filtration Superdex 200 column. Elution was carried out in a buffer containing 20 mM Tris · HCl, pH7.2, 150 mM NaCl, and 1 mM DTT. The E3 activity was eluted in a discrete peak corresponding to an apparent native molecular size of ~280–410 kDa, and was applied to a MonoP column. Elution was preformed with a linear pH gradient of 6.3–4.0. The active fractions were subjected to mass spectrometrical analysis. All the chromatographical columns were purchased from GE Healthcare except for the hydroxyapatite column (BioRad).

In vitro translation, expression, and purification of recombinant proteins. cDNAs were translated in vitro from the pCS2+ vector in the presence of [^{35}S]methionine (GE Healthcare) using wheat germ coupled transcription–translation extract and SP6 RNA polymerase (Promega). Rosetta™(DE3) pLysS *Escherichia coli* cells (Novagen) were used for bacterial expression, and His-Ring1B and His-Bmi1 were purified as previously described (S2). For the induction of His- or GST-E6-AP, transformed bacteria were grown to 0.6 o.d., transferred to 42 °C for 30 min, cooled to 16 °C, and induced with IPTG (0.5 mM) for 48 h. Untagged ubiquitin mutants were purified as previously described (S3). E6 purification was described elsewhere (S4).

Cell lines and transfection. All cell lines were grown at 37 °C in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (all were from Biological Industries). Transient transfections were carried out using the jetPEI™ transfection reagent according to the manufacturer's (Polyplus Transfection) instructions, and cells were analyzed 24–72 h after transfection. siRNAs were transfected using Lipofectamine-2000 according to the manufacturer's (Invitrogen) instructions. H1299 cells in which E6-AP was stably silenced were maintained in the presence of 4 μg/mL puromycin. Frozen pellets of HeLa cells for nucleosome purification were from the National Cell Culture Center (BioVest International, Inc). For experiments monitoring stability of proteins, cycloheximide (100 μg/mL) was added for the indicated time periods.

Acid extraction of histones. Cells (1×10^7) were collected and washed twice in ice-cold PBS. The pellet was flash frozen in liquid nitrogen and quickly thawed in PBS containing protease inhibitors and N-ethylmaleimide (10 mM). Cells were further incubated for 10 min on ice and centrifuged for 10 min at 1,500 rpm. The pellet was then resuspended in H_2SO_4 (0.2 M) and incubated on ice for 30 min. The mixture was then centrifuged at 14,000 rpm for 10 min, the pH of the supernatant was adjusted to 7 with NaOH, and the mixture was dialyzed against 100 mM Tris · HCl pH 7.6.

Analysis of Ring1B and ubiquitinated histone H2A in E6-AP-deficient mice. E6-AP-deficient mice have been described previously (S5) and were kindly provided by Arthur L. Beaudet (Baylor College of Medicine, Houston) and Ygal Haupt (Hebrew University, Jerusalem). PCR-based genotyping of tail DNA was performed using the following primers: P1 (genomic forward): CTCTGAGTTGTTAGATGCACCTT; P2 (reverse): TGACAAGTATGGCTCAAGG; P3 [Hypoxanthine-guanine phosphoribosyltransferase forward]: GCATCGCATTGTGTGAGTAGG. For analysis of Ring1B expression by Western blot and immunohistochemistry (IHC), and for IHC of ubiquitinated histone H2A, tissues from E6-AP-deficient mice and appropriate background matched controls were harvested at 7–8 weeks of age. Western blot analysis was performed using tissue homogenates that were prepared in p300 buffer (20 mM NaH_2PO_4, 250 mM NaCl, 30 mM $Na_4P_2O_7$, 0.1% NP40, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors, pH 7.0). For IHC, tissues were fixed in 4% buffered formalin, embedded in paraffin and sectioned (5 μm). Antigen retrieval for both Ring1B and ubiquitinated histone H2A was performed in 100 mM glycine buffer pH 9 in a pressure cooker at 115 °C for 3 min. After 5 min treatment in 3% H_2O_2, slides were incubated (at 4 °C overnight) with antibodies against Ring1B or ubiquitinated histone H2A, diluted 1:400 and 1:50, respectively [in CAS-block™ (Invitrogen)]. Slides were washed three times with Optimax (Biogenex), followed by immunoperoxidase staining (Biocare Medical MACH 2 Polymer detection). All animal experiments were approved by the Hebrew University-Hadassah medical school animal studies committee.

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