

## E6AP Ubiquitin Ligase Mediates Ubiquitin-Dependent Degradation of Peroxiredoxin 1

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### ABSTRACT

E6-associated protein (E6AP) is a cellular ubiquitin protein ligase that mediates ubiquitylation and degradation of tumor suppressor p53 in conjunction with the high-risk human papillomavirus E6 protein. We previously reported that E6AP targets annexin A1 protein for ubiquitin-dependent proteasomal degradation. To gain a better understanding of the physiological function of E6AP, we have been seeking to identify novel substrates of E6AP. Here, we identified peroxiredoxin 1 (Prx1) as a novel E6AP-binding protein using a tandem affinity purification procedure coupled with mass spectrometry. Prx1 is a 25-kDa member of the Prx family, a ubiquitous family of antioxidant peroxidases that regulate many cellular processes through intracellular oxidative signal transduction pathways. Immunoprecipitation analysis showed that E6AP binds Prx1 *in vivo*. Pull-down experiments showed that E6AP binds Prx1 *in vitro*. Ectopic expression of E6AP enhanced the degradation of Prx1 *in vivo*. *In vivo* and *in vitro* ubiquitylation assays revealed that E6AP promoted polyubiquitylation of Prx1. RNAi-mediated downregulation of endogenous E6AP increased the level of endogenous Prx1 protein. Taken together, our data suggest that E6AP mediates the ubiquitin-dependent proteasomal degradation of Prx1. Our findings raise a possibility that E6AP may play a role in regulating Prx1-dependent intracellular oxidative signal transduction pathways. *J. Cell. Biochem.* 111: 676–685, 2010. © 2010 Wiley-Liss, Inc.

**KEY WORDS:** E6AP; Prx1; UBIQUITIN; DEGRADATION

E6-associated protein (E6AP) is the prototype of a family of ubiquitin ligases called HECT domain ubiquitin ligases, all of which contain a domain homologous to the E6AP carboxyl terminus [Huibregtse et al., 1995]. E6AP was initially identified as the cellular factor that stimulates ubiquitin-dependent degradation of the tumor suppressor p53 in conjunction with the E6 protein of cervical cancer-associated human papillomavirus (HPV) types 16 and 18

[Huibregtse et al., 1993; Scheffner et al., 1994]. The E6–E6AP complex functions as an E3 ubiquitin ligase in the ubiquitylation of p53 [Scheffner et al., 1993]. Known substrates of the E6–E6AP complex include the tumor suppressor p53 [Scheffner et al., 1993], the PDZ domain-containing protein Scribble [Nakagawa and Huibregtse, 2000], and NFX1-91, a transcriptional repressor of the gene encoding hTERT [Gewin et al., 2004]. The ability of E6 to

Abbreviations: E6AP, E6-associated protein; Prx, peroxiredoxin; HPV, human papillomavirus; MS, mass spectrometry; MAbs, monoclonal antibody; PAB, polyclonal antibody; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; CHX, cycloheximide.

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utilize E6AP to target p53 and other cellular proteins for degradation contributes to its oncogenic functions [Matentzoglou and Scheffner, 2008]. Interestingly, E6AP is not involved in the ubiquitylation of p53 in the absence of E6 [Talis et al., 1998].

In an attempt to understand the physiological function of E6AP, several potential E6-independent substrates for E6AP have been identified, such as HHR23A and HHR23B (the human orthologs of *Saccharomyces cerevisiae* Rad23) [Kumar et al., 1999], Blk (a member of the Src family kinases) [Oda et al., 1999], Mcm7 (which is involved in DNA replication) [Kuhne and Banks, 1998], trihydrophobin 1 [Yang et al., 2007], and AIB1 (a steroid receptor coactivator) [Mani et al., 2006]. We previously reported that E6AP mediates ubiquitylation and degradation of annexin A1 in a Ca<sup>2+</sup>-dependent manner [Shimoji et al., 2009].

Some patients with Angelman syndrome, a severe neurological disorder linked to E6AP, have mutations within the catalytic cleft that have been shown to reduce E6AP ubiquitin ligase activity [Kishino et al., 1997; Matsuura et al., 1997; Cooper et al., 2004]. Despite the significant progress in the study of Angelman syndrome-associated E6AP mutations, none of the identified E6AP substrates have been directly linked to the disorder. Much research is still needed to fully understand the functional links between lack of E6AP expression and clinical manifestations of Angelman syndrome [Dan, 2009]. We previously reported that E6AP mediates ubiquitin-dependent proteasomal degradation of hepatitis C virus (HCV) core protein, thereby affecting the production of HCV particles [Shirakura et al., 2007; Suzuki et al., 2009]. It is becoming increasingly clear that E6AP plays important roles in human diseases, such as cervical cancer, Angelman syndrome, and hepatitis C [Scheffner et al., 1993; Kishino et al., 1997; Shirakura et al., 2007].

In this study, we attempted to identify the novel functions of E6AP. We screened for potential binding partners for E6AP. A tandem affinity purification procedure coupled with mass spectrometry analysis identified peroxiredoxin 1 (Prx1) as a novel binding partner for E6AP. We provide evidence suggesting that E6AP mediates the ubiquitin-dependent proteasomal degradation of Prx1.

## MATERIALS AND METHODS

### CELL CULTURE AND TRANSFECTION

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10% (v/v) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) at 37°C in a 5% CO<sub>2</sub> incubator. HEK293T cells were transfected with plasmid DNA using TransIT-LT1 (Mirus, Madison, WI).

### PLASMIDS AND RECOMBINANT BACULOVIRUSES

To make a fusion protein consisting of hexahistidine (His<sub>6</sub>)-tag fused to the N-terminus of Prx1 in *Escherichia coli*, pET17b-Prx1 [Kang et al., 1998] was digested with *Nde*I and *Bam*HI, and a Prx1 fragment was subcloned into the *Nde*I-Bpu1120I site of pET19b, resulting in pET19b-Prx1. The expression plasmid pET19b-Prx2 was constructed similarly. The plasmids, pET17b-Prx1 and pET17b-Prx2,

were kind gifts from Dr. S.G. Rhee, Ewha Women's University, Korea.

To express the Prx1 protein as a FLAG-tagged fusion protein in mammalian cells, pCAG-FLAG-Prx1 was constructed as follows. The DNA fragment of Prx1 was amplified from pET17b-Prx1 by polymerase chain reaction (PCR) using two oligonucleotides, 5'-GCGGCCGCCACCACCATGGACTACAAAGACGATGACGATAAAGG-AGGCGGCGGATCCATGTCTTCAGGAAATGC-3' and 5'-AGATCTTCACTTCTGCTTGGAG-3'. To express FLAG-tagged Prx2 protein in mammalian cells, the DNA fragment of Prx2 was amplified from pET17b-Prx2 by PCR using two oligonucleotides, 5'-GCGGCCGCCACCACCATGGACTACAAAGACGATGACGATAAAGGAGGCGGCGGATCCATGGCCTCCGGTAACGC-3' and 5'-AGATCTCTAATTGTG-TTTGGAG-3'. The amplified PCR fragments were subcloned into pGEM T-Easy (Promega, Madison, WI) and verified by sequencing. Then the Prx1 and Prx2 gene fragments were digested with *Not*I and *Bgl*III, and ligated into the *Not*I-*Bgl*III site of pCAG-MCS2 [Shirakura et al., 2007]. The MEF-tag cassette (containing Myc-tag, the tobacco etch virus protease cleavage site, and FLAG-tag) was fused to the N-terminus of the cDNA encoding E6AP [Ichimura et al., 2005; Shirakura et al., 2007]. MEF-tagged E6AP and MEF-tagged E6AP C-A were subcloned into pcDNA3, pCAGGS, and pVL1392. pCAG-HA-E6AP, pCAG-HA-E6AP C-A, and pCAG-HA-Nedd4 were described previously [Shirakura et al., 2007; Shimoji et al., 2009]. The ubiquitin expression plasmids, pRK5-HA-Ubiquitin wild type (WT), pRK5-HA-Ubiquitin-K48R, and pRK5-HA-Ubiquitin-K48 [Lim et al., 2005], were provided by Dr. T. Dawson (Johns Hopkins University, MD).

### ANTIBODIES

The mouse monoclonal antibodies (MAbs) used in this study were anti-hemagglutinin (HA) MAb (12CA5; Roche, Mannheim, Germany), anti-HA MAb (16B12; Covance, Princeton, NJ), anti-FLAG M2 mouse MAb (Sigma-Aldrich), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) MAb (Chemicon, Temecula, CA), anti-E6AP MAb (E6AP-330; Sigma-Aldrich), and anti-polyhistidine (His-1) MAb (Sigma-Aldrich). The c-Myc tagged protein mild purification kit (MBL) was used for immunoprecipitation. The polyclonal antibodies (PABs) used in this study were anti-HA rabbit PAb (Y-11; Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG rabbit PAb (F7425; Sigma-Aldrich), anti-Prx1 rabbit PAb (ab16805-100) (Abcam, Cambridge, Oxford), and anti-GST goat PAb (Amersham, Buckinghamshire, UK).

### EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

*E. coli* BL21 (DE3) cells were transformed with plasmids expressing His<sub>6</sub>-tagged protein and grown at 37°C. Expression of the fusion protein was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25°C for 4 h. Bacteria were harvested, suspended in lysis buffer [50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 5 mM Imidazole, 0.1% Triton X-100, protease inhibitor cocktail (Complete EDTA-free; Roche)], and sonicated on ice. His<sub>6</sub>-tagged proteins were purified on Ni-NTA beads (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The MEF-E6AP and MEF-E6AP C-A were purified on anti-FLAG M2 agarose beads (Sigma-Aldrich) as described previously [Shirakura et al., 2007].

## PURIFICATION OF E6AP-BINDING PROTEINS BY MEF PURIFICATION PROCEDURE

HEK293T cells were transfected with the plasmid expressing MEF-E6AP C-A by the calcium phosphate precipitation method, and the E6AP-binding proteins were recovered following the procedure described previously [Ichimura et al., 2005]. The inactive form of E6AP was expressed to inhibit ubiquitin-dependent degradation of potential substrates. Bound proteins were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining. The stained bands were excised and digested in the gel with lysylendoprotease-C (Lys-C), and the resulting peptide mixtures were analyzed using a direct nanoflow liquid chromatography-tandem mass spectrometry (MS/MS) system, equipped with an electrospray interface reversed-phase column, a nanoflow gradient device, a high-resolution Q-time of flight hybrid mass spectrometer (Q-TOF2; Micromass, Manchester, UK), and an automated data analysis system [Natsume et al., 2002; Shirakura et al., 2007]. All the MS/MS spectra were searched against the nonredundant protein sequence database maintained at the National Center for Biotechnology Information using the Mascot program (Matrix Science, London, UK) to identify proteins. The MS/MS signal assignments were also confirmed manually.

## Ni-NTA PULL-DOWN ASSAY

For Ni-NTA pull-down assays, purified MEF-E6AP was incubated with His<sub>6</sub>-Prx proteins immobilized on Ni-NTA agarose beads (Qiagen) in 1 ml of the binding buffer [50 mM Tris-HCl (pH 7.5), 10% glycerol, 1% Triton X-100, 150 mM NaCl, 5 μM ZnCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM EGTA, protease inhibitor cocktail (Complete EDTA-free)] at 4°C for 30 min. The beads were washed four times with wash buffer [50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 50 mM Imidazole, 0.1% Triton X-100, protease inhibitor cocktail (Complete EDTA-free)], and the pull-down complexes were separated by SDS-PAGE on 12.5% polyacrylamide gels and analyzed by immunoblotting with anti-FLAG MAb and anti-polyhistidine (His-1) MAb.

## IMMUNOFLUORESCENCE MICROSCOPY

Cells were transfected with pCAG-HA-E6AP C-A and pCAG-FLAG-Prx1 using TransIT-LT1 according to the manufacturer's instructions. Transfected cells grown on collagen-coated coverslips were washed with PBS, fixed with 4% paraformaldehyde for 30 min at 4°C, and permeabilized with PBS containing 2% FCS and 0.3% Triton X-100. Cells were incubated with anti-HA mouse MAb and anti-FLAG rabbit PAb as primary antibodies, washed, and incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) and Alexa Fluor 555 goat anti-rabbit IgG (Molecular Probes) as secondary antibodies. Then, the cells were washed with PBS, mounted on glass slides, and examined with a BZ-8000 microscope (Keyence).

## siRNA TRANSFECTION

HEK293T cells ( $3 \times 10^5$  cells in a six-well plate) were transfected with 40 pmol of either E6AP-specific small interfering RNA (siRNA; Sigma-Aldrich), or scramble negative-control siRNA duplexes

(Sigma-Aldrich) using HiPerFect transfection reagent (Qiagen) following the manufacturer's instructions. The E6AP-siRNA target sequences were as follows:

siE6AP-1 (sense), 5'-GGGUCUACACCAGAUUGCUTT-3'; scramble negative control (siCont-1, sense), 5'-UUGCGGGUCUAAUCCACC-GATT-3' [Shirakura et al., 2007].

## IN VIVO UBIQUITYLATION ASSAY

In vivo ubiquitylation assays were performed essentially as described previously [Shirakura et al., 2007]. Where indicated, cells were treated with 25 μM MG132 (Calbiochem, La Jolla, CA) or with dimethylsulfoxide (DMSO; control) for 30 min prior to collection. FLAG-Prx1 was immunoprecipitated with anti-FLAG MAb. Immunoprecipitates were analyzed by immunoblotting, using either anti-HA PAb or anti-FLAG MAb to detect ubiquitylated Prx1.

## IN VITRO UBIQUITYLATION ASSAY

In vitro ubiquitylation assays were performed essentially as described previously [Shirakura et al., 2007]. For in vitro ubiquitylation of Prx1, purified His<sub>6</sub>-Prx1 was used as a substrate. Assays were done in 40-μl volumes containing 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM ATP, 8 μg of bovine ubiquitin (Sigma-Aldrich), 0.1 mM DTT, 200 ng of mouse E1, 200 ng of E2 (UbcH7), and 0.5 μg of MEF-E6AP. The reaction mixtures were incubated at 37°C for 120 min followed by immunoprecipitation with anti-Prx1 PAb. The samples were analyzed by immunoblotting with anti-Ub MAb.

## RESULTS

### IDENTIFICATION OF Prx1 AS A BINDING PARTNER FOR E6AP

To identify novel substrates for E6AP, we screened for E6AP-binding proteins using a tandem affinity purification procedure with a tandem tag (known as MEF-tag) [Ichimura et al., 2005; Shirakura et al., 2007]. Seven proteins were reproducibly detected from lysed cells transfected with MEF-E6AP C-A (Fig. 1A, lane 2), but none were recovered from lysed control cells transfected with empty vector alone (Fig. 1A, lane 1). To identify the proteins, silver-stained bands were excised from the gel, digested with Lys-C, and analyzed using a direct nanoflow liquid chromatography-MS/MS system. One of these bands, migrating at 25 kDa (Fig. 1A, lane 2, No. 7), was identified as Prx1 based on two independent MS spectra (Fig. 1B,C). To confirm the proteomic identification of Prx1, HEK293T cells were transfected with MEF-E6AP C-A plasmid or empty plasmid. The cells were lysed and immunoprecipitated with anti-Myc MAb or control IgG. Endogenous Prx1 was co-immunoprecipitated with anti-Myc MAb, suggesting that E6AP binds endogenous Prx1 (Fig. 1D, lane 4).

### IN VIVO INTERACTION BETWEEN Prx1 AND E6AP

To determine whether E6AP specifically interacts with Prx1, HA-E6AP plasmid was introduced into HEK293T cells together with either FLAG-Prx1 plasmid or FLAG-Prx2 plasmid. Prx1 and Prx2 share 77.4% sequence identity at the protein level. Cells were lysed and immunoprecipitated with anti-HA MAb, anti-FLAG MAb, or

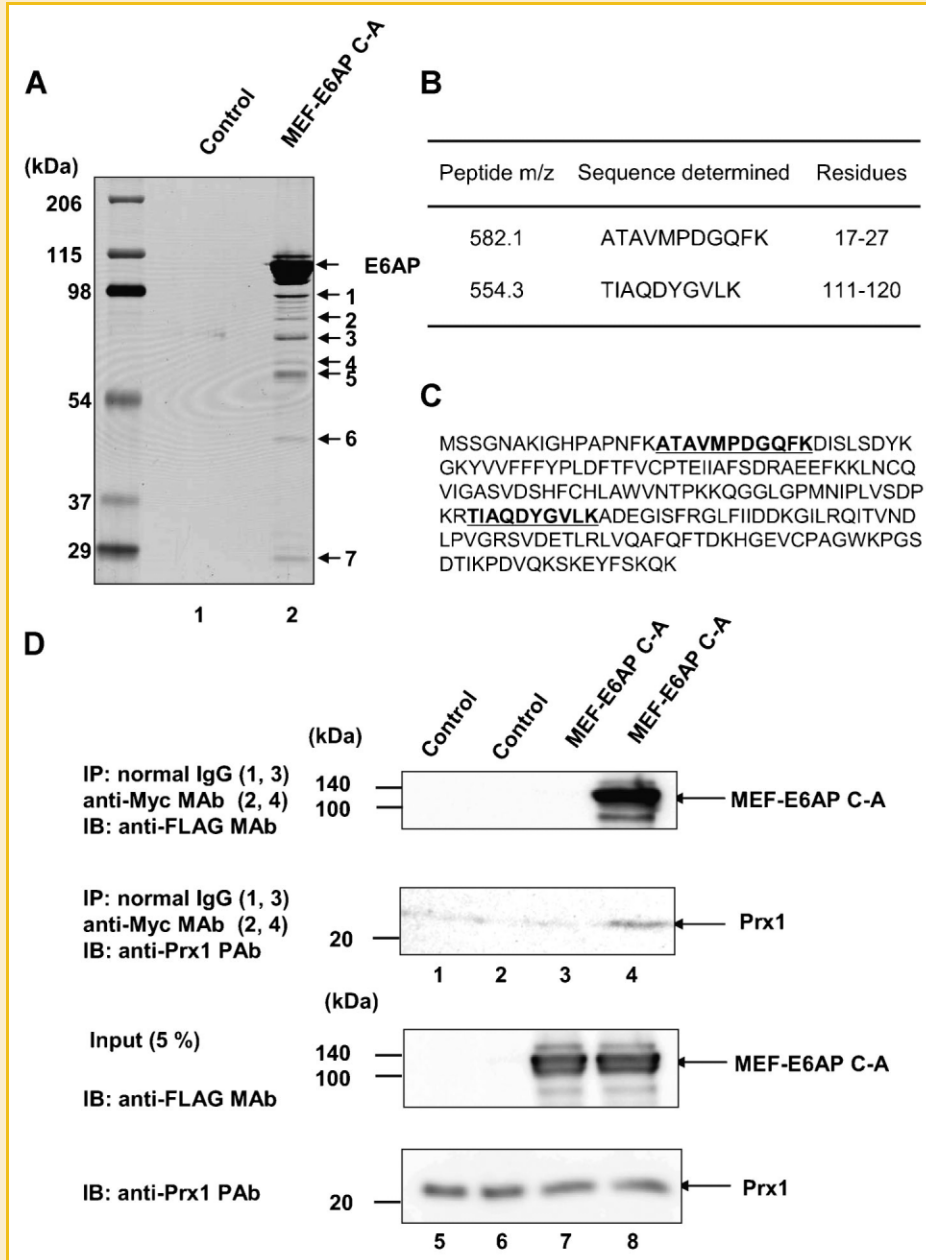


Fig. 1. Identification of Prx1 as a binding partner for E6AP. A: Prx1 interacts with E6AP in vivo. HEK293T cells were transfected with pcDNA3-MEF-E6AP C-A or empty plasmid, incubated for 48 h, and then harvested. The expressed MEF-E6AP C-A and binding proteins were recovered using the MEF purification procedure. Proteins bound to the MEF-E6AP C-A immobilized on anti-FLAG beads were dissociated with FLAG peptides, resolved by 7.5% SDS-PAGE, and visualized by silver staining. Control experiments were performed using HEK293T cells transfected with vector alone. Bound proteins were detected by SDS-PAGE and silver staining. Molecular weight markers are indicated as well as the position of p25 (No. 7), which likely corresponds to Prx1. B: Peptide masses were identified by tandem mass spectrometry. The protein was Prx1 (GenBank accession No. BC021683). C: Corresponding amino acids of Prx1 (peptides in bold print). D: HEK293T cells were co-transfected with MEF-E6AP C-A plasmid. Control experiments were performed using HEK293T cells transfected with vector alone. Cell lysates were immunoprecipitated with anti-Myc MAb or normal mouse IgG (lanes 1–4), eluted with c-Myc tag peptide. Eluates were analyzed by immunoblotting with anti-FLAG MAb or anti-Prx1 PAb. The input samples were separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG MAb or anti-Prx1 PAb (lanes 5–8). The positions of Prx1 and MEF-E6AP C-A are indicated by arrows. IB, immunoblot; IP, immunoprecipitation.

normal IgG (Fig. 2A, lanes 1–6). FLAG-Prx1 but not FLAG-Prx2 was co-immunoprecipitated with anti-HA MAb (Fig. 2A, lower panel, lanes 1 and 2). Conversely, HA-E6AP was co-immunoprecipitated with FLAG-Prx1 but not FLAG-Prx2 using anti-FLAG MAb (Fig. 2A, upper panel, lanes 3 and 4). These results suggest that E6AP specifically interacts with Prx1.

To determine whether Prx1 and E6AP co-localize in the cells, immunofluorescence microscopy analysis was performed in HEK293T cells. There was no staining without primary antibodies (data not shown). The immunofluorescence study showed that E6AP and Prx1 mainly localize in the cytoplasm and that E6AP and Prx1 co-localize in the cytoplasm (Fig. 2B, Merge).

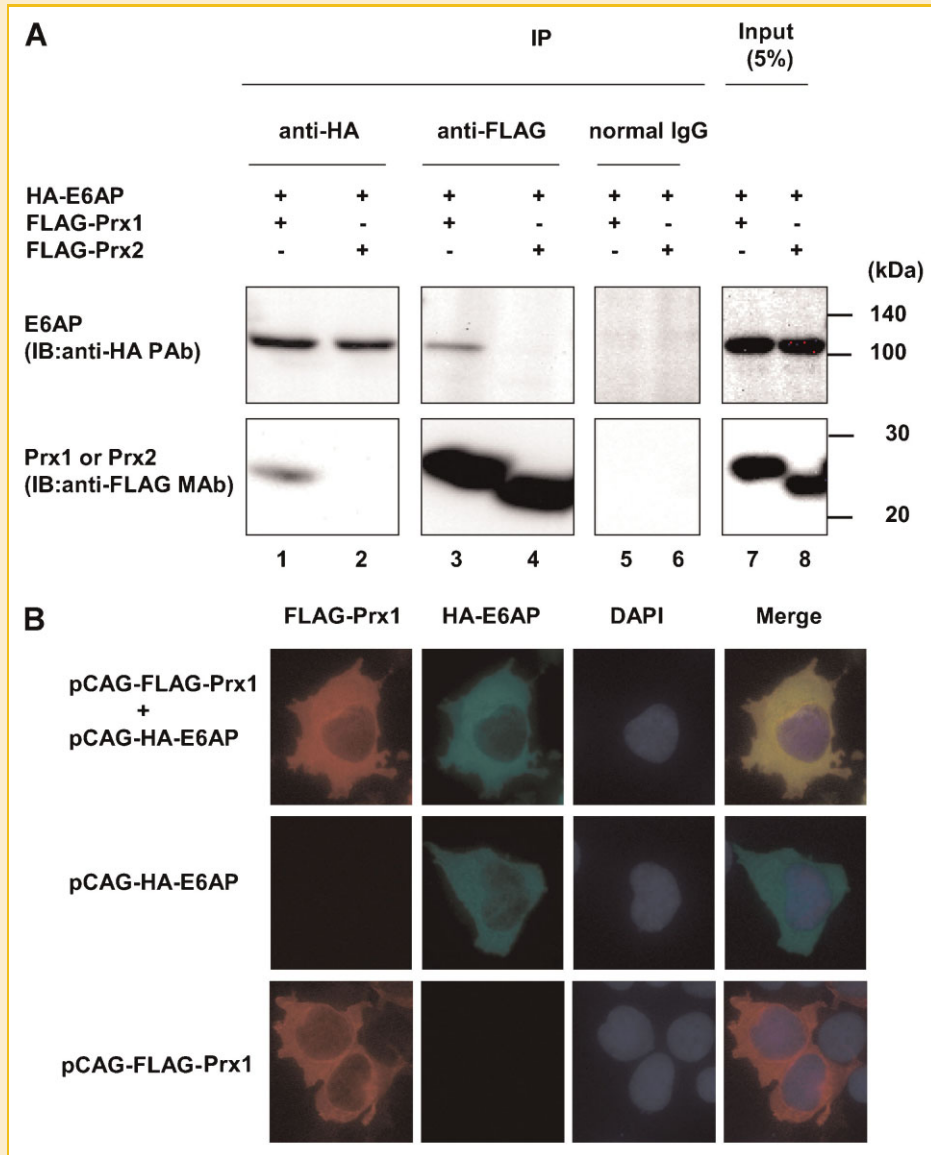


Fig. 2. In vivo interaction between Prx1 and E6AP. A: HEK293T cells were co-transfected with pCAG-HA-E6AP together with either pCAG-FLAG-Prx1 or pCAG-FLAG-Prx2. Cell lysates were immunoprecipitated with anti-HA mouse MAb, anti-FLAG mouse MAb, or normal mouse IgG and analyzed by immunoblotting with anti-HA PAb or anti-FLAG MAb. B: HEK293T cells were transfected with either HA-E6AP plasmid or FLAG-Prx1 plasmid, grown on coverslips, fixed, and processed for double-label immunofluorescence for HA-E6AP or FLAG-Prx1. All the samples were examined with a BZ-8000 microscope (Keyence).

### IN VITRO INTERACTION BETWEEN Prx1 AND E6AP

To determine whether E6AP interacts with Prx1 in vitro, purified recombinant MEF-E6AP, MEF-annexin A1 expressed in insect cells using a baculovirus system and purified recombinant His<sub>6</sub>-Prx1 and His<sub>6</sub>-Prx2 expressed in *E. coli* were used. The His<sub>6</sub>-tagged Prx proteins were mixed with either MEF-E6AP or MEF-annexin A1, incubated, pulled down with Ni-NTA agarose, and analyzed by immunoblotting with anti-FLAG MAb (Fig. 3, upper panel) or anti-polyhistidine MAb (Fig. 3, middle panel). MEF-annexin A1 served as a negative control to confirm that MEF-tag does not bind Prx1. MEF-E6AP was pulled down with Prx1, but not with Prx2 (Fig. 3, lanes 1 and 3), whereas annexin A1 was not pulled down with either Prx1 or Prx2 (Fig. 3, lanes 2 and 4). These

results suggest that E6AP directly and specifically binds Prx1 in vitro.

### E6AP DECREASES STEADY-STATE LEVELS OF Prx1 IN HEK293T CELLS

One of the characteristic features of HECT domain ubiquitin ligases is their direct association with their substrates. Thus, we hypothesized that E6AP would function as an E3 ubiquitin ligase for Prx1. We assessed the effects of E6AP on the steady-state levels of Prx1 in HEK293T cells. FLAG-Prx1 together with HA-tagged E6AP, catalytically inactive E6AP, E6AP C-A, or Nedd4 (another HECT domain ubiquitin ligase) was introduced into HEK293T cells, and the levels of Prx1 were examined by immunoblotting. The steady-state

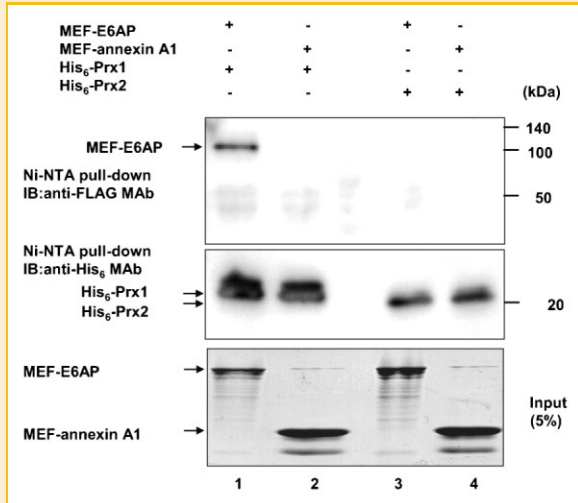


Fig. 3. In vitro interaction between Prx1 and E6AP. Purified MEF-E6AP, MEF-Annexin A1, His<sub>6</sub>-Prx1, and His<sub>6</sub>-Prx2 were used to examine the interaction between E6AP and Prxs. Purified MEF-E6AP was mixed with either His<sub>6</sub>-Prx1 or His<sub>6</sub>-Prx2, incubated, and pulled down with Ni-NTA agarose. MEF-Annexin A1 served as a negative control. The bound proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG MAb (upper panel) or anti-His<sub>6</sub> MAb (middle panel). The input MEF-E6AP and MEF-annexin A1 were separated by SDS-PAGE and stained with Coomassie brilliant blue (lower panel).

levels of Prx1 decreased with an increase in the amount of E6AP plasmid (Fig. 4A lanes 1–3, Fig. 4B). However, neither E6AP C-A nor Nedd4 decreased the steady-state levels of Prx1 (Fig. 4A lanes 4–6 and 7–9, Fig. 4B), indicating that E6AP specifically decreases Prx1.

To determine if endogenous E6AP is critical for the degradation of endogenous Prx1 in the cells, the expression of E6AP was knocked down by siRNA and the expression of Prx1 and E6AP was analyzed by immunoblotting. Transfection of siE6AP into HEK293T cells resulted in a decrease in E6AP levels by 97% (Fig. 4C, upper panel, lane 2). Knock-down of endogenous E6AP resulted in accumulation of endogenous Prx1 (Fig. 4C, lane 2, middle panel), suggesting that endogenous E6AP plays a role in the proteolysis of endogenous Prx1.

To further investigate if the E6AP-induced reduction of Prx1 is dependent on the proteasome, we examined the effects of the proteasome inhibitor clasto-lactacystin and the lysosomal enzyme inhibitors, E-64d and Pepstatin A, on the level of Prx1. Clasto-lactacystin was used to examine if Prx1 gets stabilized after the treatment, because it has an irreversible inhibitory effect on proteasome. HEK293T cells were transfected with pCAG-FLAG-Prx1 plus either empty vector or pCAG-HA-E6AP. Overexpression of E6AP resulted in a remarkable reduction of the Prx1 (Fig. 4D, lane 2, middle panel), whereas the Prx1 protein level was increased after treatment with clasto-lactacystin (Fig. 4D, lane 4, middle panel). In contrast, the Prx1 protein level was unchanged after treatment with E-64d plus Pepstatin A (Fig. 4D, lane 6, middle panel). These results indicate that E6AP-induced reduction of Prx1 is proteasome-dependent.

## E6AP-DEPENDENT POLYUBIQUITYLATION OF Prx1 IN VIVO

To determine whether E6AP can induce ubiquitylation of Prx1 in cells, we performed in vivo ubiquitylation assays. HEK293T cells were transfected with FLAG-Prx1 plasmid and either E6AP or Nedd4 plasmid, together with a plasmid encoding HA-tagged ubiquitin to facilitate the detection of ubiquitylated Prx1. Cell lysates were immunoprecipitated with anti-FLAG MAb and immunoblotted with anti-HA PAb to detect ubiquitylated Prx1 protein. No ubiquitin signal was detected in the cells co-transfected with empty plasmid or Nedd4 plasmid (Fig. 5A, lanes 4 and 6). In contrast, co-expression of E6AP led to readily detectable polyubiquitylated forms of the Prx1 as a smear of higher-molecular weight bands (Fig. 5A, left panel, lane 5). Immunoblot analysis with anti-FLAG PAb confirmed that FLAG-Prx1 was immunoprecipitated and that higher-molecular weight bands conjugated with HA-ubiquitin were indeed polyubiquitylated forms of the FLAG-Prx1 (Fig. 5A, right panel, lane 5). These results suggest that E6AP enhances polyubiquitylation of Prx1 in vivo.

To further investigate if E6AP is involved in K48-linked ubiquitylation of Prx1, we performed in vivo ubiquitylation assay using HA-tagged K48R dominant negative ubiquitin mutant and K48 only ubiquitin mutant expression plasmids. HEK293T cells were transfected with FLAG-Prx1 plasmid and E6AP plasmid, together with a plasmid encoding HA-ubiquitin WT, HA-K48R ubiquitin, or HA-K48 ubiquitin to facilitate the detection of ubiquitylated Prx1. Ubiquitin signal was detected in the cells transfected with either HA-ubiquitin WT or HA-K48 ubiquitin plasmid (Fig. 5B, lane 1 or 3), whereas no ubiquitin signal was detected in the cells transfected with HA-K48R ubiquitin (Fig. 5B, lane 2), suggesting that E6AP enhances K48-linked polyubiquitylation of Prx1. These results are consistent with the notion that E6AP is involved in proteasomal degradation of Prx1.

## E6AP MEDIATES POLYUBIQUITYLATION OF Prx1 IN VITRO

To reconstitute the E6AP-mediated polyubiquitylation of Prx1 in vitro, we performed an in vitro ubiquitylation assay of the Prx1 using purified MEF-E6AP and His<sub>6</sub>-Prx1 as described above. When the in vitro ubiquitylation reaction was carried out in the presence of MEF-E6AP C-A, no ubiquitylation signal was detected (Fig. 5C, lanes 3). However, inclusion of purified MEF-E6AP in the reaction mixture resulted in ubiquitylation of His<sub>6</sub>-Prx1 (Fig. 5C, lane 4). No signal was detected when His<sub>6</sub>-Prx1 was not included in the reaction mixture (Fig. 5C, lane 2). From these results, we concluded that E6AP mediates polyubiquitylation of Prx1.

## DISCUSSION

In this study, we identified Prx1 as a novel E6AP-binding protein using a tandem affinity purification procedure coupled with mass spectrometry. Overexpression of E6AP enhances proteasomal degradation of Prx1, and siRNA-mediated knockdown of endogenous E6AP results in accumulation of endogenous Prx1. E6AP enhances the polyubiquitylation of Prx1 in vivo and in vitro. We conclude that E6AP mediates ubiquitin-dependent degradation of

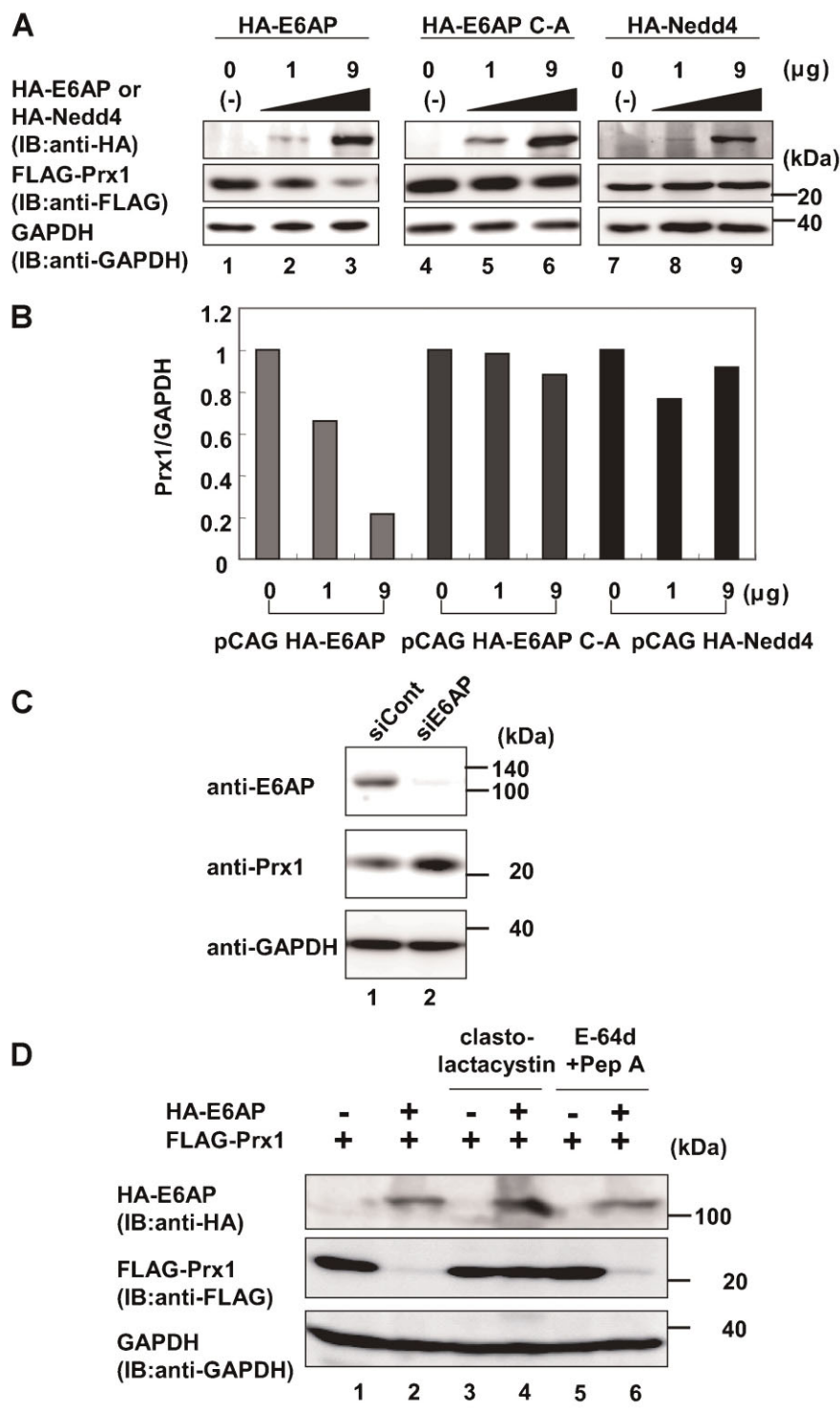


Fig. 4. E6AP decreases the steady-state levels of Prx1 protein in HEK293T cells. A: HEK293T cells ( $5 \times 10^5$  cells/six-well plate) were transfected with 0.5  $\mu$ g pCAG-FLAG-Prx1 plus empty vector and 1 or 9  $\mu$ g of pCAG-HA-E6AP, pCAG-HA-E6AP C-A, or pCAG-HA-Nedd4. At 48 h posttransfection, equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA MAb (top panel), anti-FLAG MAb (middle panel), and anti-GAPDH MAb (bottom panel). The results shown are representative of three independent experiments. B: Quantitation of the data shown in panel A. The intensities of the gel bands were quantitated using the ImageJ 1.43 program. The level of GAPDH served as a loading control. C: Knockdown of endogenous E6AP by siRNA resulted in the accumulation of endogenous Prx1 in HEK293T cells. HEK293T cells ( $3 \times 10^5$  cells/six-well plate) were transfected with 40 pmol of E6AP-specific duplex siRNA (or a scramble negative control). The cells were harvested at 96 h after siRNA transfection. D: HEK293T cells ( $5 \times 10^5$  cells/six-well plate) were transfected with 0.5  $\mu$ g of pCAG-FLAG-Prx1 plus 9  $\mu$ g of empty vector or pCAG-HA-E6AP. At 36 h after transfection, the cells were treated with DMSO control (lanes 1 and 2), 30  $\mu$ M clasto-lactacystin (lanes 3 and 4), or 40  $\mu$ M E-64d plus 20  $\mu$ M Pepstatin A (lanes 5 and 6). Cells were collected at 12 h after treatment with the inhibitors. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA MAb (upper panel), anti-FLAG MAb (middle panel), or anti-GAPDH MAb (lower panel).

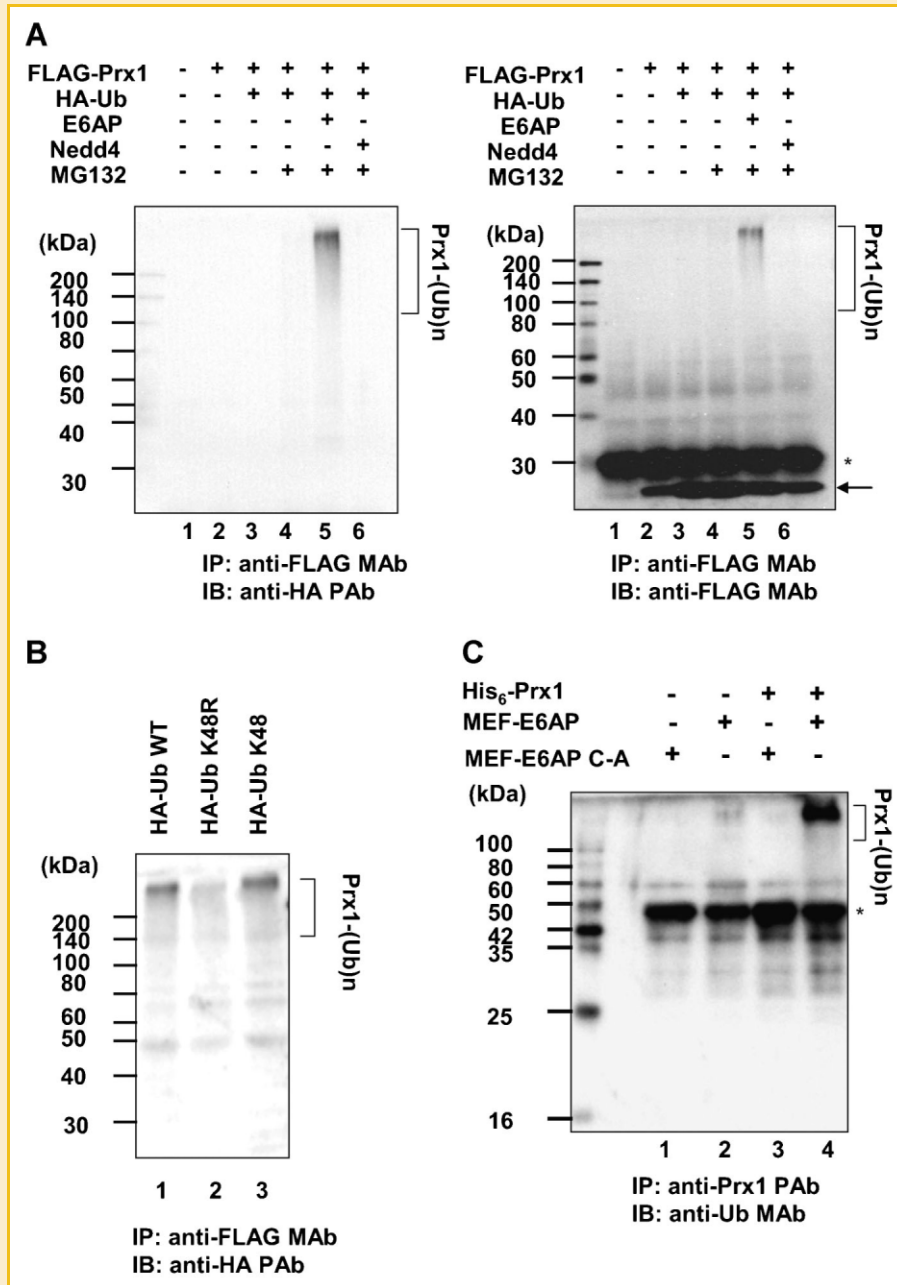


Fig. 5. E6AP mediates ubiquitylation of Prx1 in vivo and in vitro. A: HEK293T cells ( $2 \times 10^6$  cells/10-cm dish) were transfected with 1  $\mu$ g of pCAG-FLAG-Prx1 together with 2  $\mu$ g of plasmid encoding E6AP or Nedd4 as indicated. Each transfection also included 2  $\mu$ g of plasmid encoding HA-ubiquitin. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb (left panel) or anti-FLAG MAb (right panel). Arrow indicates FLAG-Prx1. Asterisk indicates immunoglobulin light chain. Ubiquitylated species of FLAG-Prx1 are marked by brackets. B: HEK293T cells ( $2 \times 10^6$  cells/10-cm dish) were transfected with 1  $\mu$ g of pCAG-FLAG-Prx1 together with 2  $\mu$ g of plasmid encoding E6AP plasmid. Each transfection also included 2  $\mu$ g of plasmid encoding HA-Ub WT, HA-Ub K48R, or HA-Ub K48 as indicated. At 36 h after transfection, the cells were treated with 25  $\mu$ M MG132 and cultured for 12 h. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb. Ubiquitylated species of FLAG-Prx1 are marked by brackets. C: In vitro ubiquitylation of Prx1 by recombinant E6AP. For in vitro ubiquitylation of Prx1 protein, purified His<sub>6</sub>-Prx1 was used as a substrate. Assays were done in 40- $\mu$ l volumes containing each component as indicated. The reaction mixture is described in Materials and Methods Section. The reaction was carried out at 37°C for 120 min followed by immunoprecipitation with anti-Prx1 PAb and analysis by immunoblotting with anti-Ub MAb. Ubiquitylated species of His<sub>6</sub>-Prx1 are marked by brackets. Asterisk indicates immunoglobulin heavy chain.

Prx1. Our results suggest that E6AP is involved in the regulation of Prx1 activity through the ubiquitin-proteasome pathway.

Prx1 is a 25-kDa member of the Prx family, a ubiquitous family of antioxidant peroxidases that regulate many cellular processes

through intracellular oxidative signal transduction pathways. More than 50 members of the Prx family have been identified in a wide variety of organisms ranging from prokaryotes to mammals. Prxs are widely expressed hydrogen peroxide scavenger proteins best



known for their role in detoxifying reactive oxygen species, DNA damage, and cancer, but they also act in cellular signaling and as molecular chaperones [Jang et al., 2004; Hall et al., 2009]. Mammalian cells express six isoforms of Prx (Prx1–6), which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) [Rhee et al., 2005]. Prx1 is a 2-Cys thiol reductase that forms a component of cellular antioxidant and thermal stress defense mechanisms through its ability to metabolize H<sub>2</sub>O<sub>2</sub>, and its properties as a molecular chaperone [Wood et al., 2003a,b; Jang et al., 2004]. Furthermore, Prx1 controls neuronal differentiation by a thiol-redox-dependent cascade [Yan et al., 2009].

The peroxidase activity of Prx1 is regulated by phosphorylation, which is mediated by cyclin-dependent kinases [Chang et al., 2002]. Phosphorylation at Thr90 by several Cdk, including Cdc2, results in inhibition of its peroxidase activity. Another known regulatory mechanism is cysteine sulfinic acid formation [Woo et al., 2003]. The active-site cysteine of Prx1 is selectively reduced to cysteine sulfinic acid during catalysis, which leads to inactivation of peroxidase activity. Reversing the inactivation of Prx1 was previously identified as a mechanism for its regulation. The sulfinic form of Prx1, produced during the exposure of cells to H<sub>2</sub>O<sub>2</sub>, is rapidly reduced to the catalytic active thiol form [Woo et al., 2003]. We propose here a novel regulatory mechanism of Prx1. Our results show for the first time that the E6AP-mediated ubiquitin-proteasome pathway is a mechanism for irreversibly attenuating the activity of Prx1. Our data suggest that E6AP specifically targets Prx1 for ubiquitin-dependent proteasomal degradation. Prx1 and Prx2 share 77.4% sequence identity at the protein level. However, our data showed that E6AP does not interact with Prx2 in vivo and in vitro, suggesting a specific interaction between E6AP and Prx1.

Angelman syndrome is a neurologic disorder characterized by developmental delay, severe intellectual disability, motor impairment, absent speech, happy demeanor, and epilepsy, and is attributed to an absence of UBE3A/E6AP gene expression that may be caused by various abnormalities of chromosome 15. Although the genetic link between UBE3A/E6AP and Angelman syndrome was identified more than 13 years ago [Kishino et al., 1997; Matsuura et al., 1997], the underlying pathophysiology is poorly understood. Ubiquitin-mediated proteolysis may be important in a number of processes of neuronal development, including synaptogenesis and mechanisms of long-term memory. Recent findings in animal models of Angelman syndrome have demonstrated altered dendritic spine formation as well as both synaptic and nonsynaptic influences in various brain regions, including hippocampus and cerebellar cortex [Dan, 2009]. Yan et al. [2009] provided several lines of evidence suggesting that the requirement for Prx1 in motor neuron differentiation stems from a previously uncharacterized enzymatic function that is distinct from its molecular chaperone or H<sub>2</sub>O<sub>2</sub> metabolic activities. It may be intriguing to investigate the functional link between lack of UBE3A/E6AP expression and stability of Prx1 with regard to the pathogenesis of Angelman syndrome.

The expression of Prx1 is induced by oxidative stress including that from the exposure to O<sup>2</sup>, Fe<sup>3+</sup>, or 2-mercaptoethanol. In addition to H<sub>2</sub>O<sub>2</sub>, other chemicals such as phorbol ester and okadaic acid have also been shown to induce the expression of Prx1

[Immenschuh et al., 2002; Wijayanti et al., 2008]. Increased expression of Prx1, in turn, contributes to greater resistance to oxidative stress. Many studies have indicated that aberrant expression of Prx1 was found in various kinds of cancers, such as thyroid tumors, oral cancers, lung cancers, and esophageal carcinoma [Yanagawa et al., 1999; Yanagawa et al., 2000; Chang et al., 2001; Qi et al., 2005]. As the hypoxic and unstable oxygenation microenvironment of a tumor is one of the key factors influencing tumor growth and progression, the induction of Prx1 expression might be an adaptive response of the cancer cells [Zhang et al., 2009]. Although the molecular mechanism responsible for the abnormal elevation of Prx1 is still unclear, it may be interesting to investigate the gene polymorphisms of E6AP and the stability of Prx1 in cancer cells.

There are several modes of substrate recognition in the ubiquitin-proteasome system. Recognition can be made via several mechanisms, such as (1) NH<sub>2</sub>-terminal residues (the N-end rule pathway), (2) allosteric activation, (3) recognition of phosphorylated substrate, (4) phosphorylation of E3, (5) phosphorylation of both the ligase and its substrate, (6) recognition in trans via an ancillary protein, (7) abnormal/mutated/misfolded proteins, and (8) recognition via hydroxylated protein [Glickman and Ciechanover, 2002]. It is known that E6AP uses several mechanisms for substrate recognition. E6AP recognizes p53 in conjunction with the HPV16 E6 protein [Scheffner et al., 1993; Talis et al., 1998]. E6AP also recognizes the tyrosine-phosphorylated form of Blk [Oda et al., 1999] and the Ca<sup>2+</sup>-binding form of annexin A1 [Shimoji et al., 2009]. Further studies will be needed to elucidate the mode of Prx1 recognition by E6AP.

In conclusion, we demonstrated that E6AP mediates the ubiquitin-dependent degradation of Prx1. Future efforts will focus on understanding the role of the E6AP-mediated proteolysis of Prx1 in the defense against oxidative stress and thermal stress as well as the ubiquitylation signal of Prx1. Insights into the physiological function of E6AP will be gained by investigating the effects of various oxidative stresses on the stability and functional control of Prx1.

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