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A novel HECT-type E3 ubiquitin ligase, NEDL2, stabilizes p73 and enhances its transcriptional activity

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Abstract

Expression of p73, a p53 family member regulating cell growth and apoptosis, is maintained at low levels in mammalian cells, and cellular activation of p73 is usually controlled at the protein level. However, the precise molecular mechanisms by which p73 stability is regulated are unclear. During the search for interacting molecules with the COOH-terminal proline-rich region of p73, we identified a novel NEDD4-related protein (termed as NEDL2) which contains a C2 domain at its NH_2-terminus, two WW domains, and a HECT domain at its COOH-terminus. As expected, NEDL2 catalyzed the ubiquitination of bacterial cellular proteins in vitro. Reciprocal co-immunoprecipitation experiments and in vitro pull-down assays revealed that NEDL2 bound to p73, which carries two putative PY motifs. p73 was efficiently ubiquitinated but stabilized in a NEDL2-dependent manner. Accordingly, p73 decayed at faster rates in the absence of NEDL2 than in its presence. Consistent with the NEDL2-mediated stabilization of p73, NEDL2 enhanced the p73-dependent transcriptional activation. Thus, our results suggest that NEDL2 activates the function of p73 by increasing its stability.

Keywords: NEDL2; E3 ubiquitin ligase; p53; p73; PY motif; WW domain

p73 has a considerable amino acid sequence homology with the tumor suppressor p53 [1]. Like p53, p73 binds to the p53-responsive elements found within the promoter regions of a variety of p53-target genes, and thereby induces cell cycle arrest and/or apoptosis in certain human cancerous cells [1–3]. In contrast to p53, p73 is expressed as multiple splicing isoforms arising from differential mRNA splicing [1,4–6]. These isoforms possess altered COOH-terminal structures with different transactivating potentials. The COOH-terminal extension outside the conserved oligomerization domain is unique to p73 and could be involved in the specific function of p73 [4,5]. Recently, Yang et al. [7] discovered the additional p73 variant (ΔNp73) which was generated by using an alternative promoter. ΔNp73 lacked the NH_2-terminal transactivation domain with dominant-negative behavior toward p73 as well as p53 and had an ability to induce malignant transformation [8,9]. Recently, we and others demonstrated that the increase of p73 directly induced ΔNp73 protein in mammalian cultured cells, suggesting that there exists a negative feedback regulation of p73 by its target ΔNp73 [10,11].

MDM2 is the major p53 E3 ubiquitin ligase which promotes the ubiquitination and subsequent degradation of p53 by the 26S proteasome [12–14]. DNA damage-induced phosphorylation of p53 at multiple sites including Ser-15 and Ser-20, prevents its association with MDM2 and results in its stabilization [15]. Recently, it has been shown that the intracellular levels of p73 are increased in the presence of the proteasomal inhibitor, suggesting that p73 stability is regulated by proteasomal degradation [16]. MDM2 also bound to the

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transactivation domain of p73 to repress its transcriptional activity, however, co-expression of MDM2 with p73 resulted in an increase in the level of p73 [17]. Thus, unlike p53, p73 stability may be regulated by another protein with E3 ubiquitin ligase activity. On the other hand, stabilization of p73 is crucial for its ability to induce cell cycle arrest and/or apoptosis. p73 is significantly stabilized in response to cisplatin treatment [18]. During the cisplatin-induced apoptosis, p73 was phosphorylated on Tyr-99 by non-receptor tyrosine kinase c-Abl. In addition, Steegenga et al. [19] described that p73 was stabilized upon adenovirus infection.

In the present study, we focused our attention on the COOH-terminal region of p73, which contains two putative PY motifs. By BLAST analysis, we identified a novel NEDD4-related E3 ubiquitin ligase NEDL2, and found that p73 and NEDL2 formed a complex via a p73 PY motif and the NEDL2 WW domain. p53, which lacks the PY motif did not bind to NEDL2. Overexpression of NEDL2 resulted in the ubiquitination of p73 but enhanced the steady-state level of p73 as well as the ability of p73 to transactivate the p53/p73-responsive promoters. Thus, the differential binding of NEDL2 to p53 family members might contribute to their functional divergence.

Materials and methods

Cell culture and transfection. COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies) and penicillin (100 IU/ml)/streptomycin (100 μg/ml). H1299 human large cell lung carcinoma cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin (100 IU/ml)/streptomycin (100 μg/ml). Cells were grown at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. Transient transfection was performed by LipofectAMINE Plus reagent according to the manufacturer's recommendations (Life Technologies).

Northern blot analysis. A human multiple tissue Northern blot (Clontech Laboratories), a nylon membrane on which 2 μg of poly(A)$^+$ RNA from various human normal tissues was blotted, was hybridized overnight at 65 °C in a solution containing 7.5% dextran sulfate, 1 M NaCl, 1% *N*-lauroyl sarcosine, 100 μg/ml of heat-denatured salmon sperm DNA, and the ^{32}P-labeled human *NEDL2* or *NEDD4* cDNA. The membrane was washed twice in 0.5× SSC/0.1% *N*-lauroyl sarcosine at 50 °C and exposed to an X-ray film at −70 °C.

Expression and purification of GST fusion proteins. Cultures of *Escherichia coli* containing the expression plasmids encoding glutathione *S*-transferase (GST) fusion proteins were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 h at 30 °C. Cells were then harvested by centrifugation, resuspended in NETN buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM EDTA), and lysed by sonication. The lysates were clarified by centrifugation at 10,000*g* for 30 min. GST fusion proteins were purified on a glutathione–Sepharose column. The integrity of the fusion proteins was assessed by SDS–polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. Protein concentration was determined using Bradford protein assay (Bio-Rad).

In vitro ubiquitination assay. Equal amount of purified GST-NEDL2 or GST-NEDD4 was mixed with bacterial lysates containing wheat E1, appropriate E2 (UbcH5c or UbcH7), and bovine ubiquitin (Sigma Chemical). After 2 h incubation at 30 °C, reactions were stopped by the addition of the SDS sample buffer (62.5 mM Tris–Cl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, and 0.01% bromophenol blue) and boiling. Reaction mixtures were then subjected to SDS–polyacrylamide gel electrophoresis and ubiquitinated proteins were visualized by immunoblotting using a monoclonal anti-ubiquitin antibody (1B3, Medical and Biological Laboratories).

Pull-down experiments. p73α or p73β was generated in vitro using a TNT Coupled Rabbit Reticulocyte Lysate System as directed by the manufacturer (Promega). In vitro-translated p73α or p73β was mixed with an equal amount of each GST fusion protein in NETN buffer and 1 mg/ml of bovine serum albumin (BSA). Following incubation in the presence of glutathione–Sepharose beads for 2 h at 4 °C with gentle shaking, the beads were washed three times with the binding buffer. The bound proteins were then eluted by boiling in the SDS sample buffer for 5 min, size fractionated on a 10% SDS–polyacrylamide gel, and immunoblotted with the antibody against p73 (Ab-4, NeoMarkers).

Production of polyclonal anti-NEDL2 antibody. The polyclonal anti-NEDL2 antibody was raised against a peptide "Cys" plus containing the amino acid sequences between positions 448 and 466 of human NEDL2 (448-RSSFPTDTRLNAMLHIDSD-466). The peptide and the polyclonal antibody were produced by Biologica. The specificity of the antibody was checked on its ability to immunoprecipitate NEDL2 expressed in COS7 cells and its ability to detect NEDL2 by Western blot analysis.

Immunoprecipitation and Western blot analysis. For immunoprecipitation experiments, COS7 cells were transfected with the appropriate expression plasmid. Forty-eight hours after transfection, cells were washed with phosphate-buffered saline (PBS), lysed in ice-cold EBC lysis buffer [50 mM Tris–Cl, pH 7.5, 120 mM NaCl, 0.5% (v/v) Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], and whole cell lysates were subjected to immunoprecipitation with the polyclonal anti-NEDL2, monoclonal anti-p53 (DO-1, Oncogene Research Products), monoclonal anti-HA (12CA5, Roche Molecular Biochemicals), or monoclonal anti-p73 (Ab-4, NeoMarkers) antibody. For Western blot analysis, whole cell lysates or immunoprecipitates were boiled in the SDS-sample buffer, subjected to SDS–polyacrylamide gel electrophoresis, and proteins were then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane filter (Immobilon-P, Millipore). The filter was blocked with Tris-buffered saline (TBS) containing 5% non-fat dried milk at room temperature for 1 h, and subsequently incubated for 1 h with the monoclonal anti-p73 (Ab-4, NeoMarkers), monoclonal anti-ubiquitin (1B3, Medical and Biological Laboratories), monoclonal anti-HA (12CA5, Roche Molecular Biochemicals), monoclonal anti-β-tubulin (5H1, PharMingen), monoclonal anti-FLAG (M2, Sigma Chemical), polyclonal anti-NEDL2, or polyclonal anti-actin ([20–33], Sigma Chemical) antibody, followed by an incubation with a goat anti-mouse or anti-rabbit IgG secondary antibody coupled to horseradish peroxidase. Protein signals were visualized with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Protein decay rate analysis. COS7 cells were transfected with the indicated combinations of the expression plasmids. Twenty-four hours after transfection, cycloheximide (ICN Pharmaceuticals) was added to the culture medium at a final concentration of 100 μg/ml and cells were harvested at the indicated time points. Whole cell lysates were extracted and subjected to immunoblot analysis with the anti-p73 antibody (Ab-4, NeoMarkers).

Reporter assay. For the luciferase assay, H1299 cells were grown in 24-well plates (5×10^4 cells/well), and transiently co-transfected with the expression plasmid for HA-p73α, p53/p73-responsive luciferase reporter construct containing $p21^{WAF1}$ or *MDM2* promoter, and pRL-TK (*Renilla* luciferase for internal control) together with or without the increasing amounts of *NEDL2* expression plasmid. At 48 h, the cells were collected, washed in ice-cold 1× PBS, and assayed for reporter gene activities.

Results

Identification and characterization of a novel E3 ubiquitin ligase, NEDL2

To find out interacting molecules with the COOH-terminal proline-rich region of p73, we decided to search for novel cellular proteins with the WW protein–protein interaction domains. The BLAST analysis of nucleotide and protein sequence databases identified one cDNA clone, *KIAA1301* (DDBJ/EMBL/GenBank Accession No., AB037722) [20]. *KIAA1301* encodes a deduced open reading frame of 1572 amino acids, which has a predicted molecular weight of 193 kDa, and contains an NH_2-terminal phospholipid/calcium-binding C2 domain, two WW domains, and a COOH-terminal HECT domain, with a significant structural similarity to NEDD4 E3 ubiquitin ligase (Figs. 1A and B). Accordingly, we named this gene product as NEDL2 (NEDD4-like ubiquitin protein ligase 2). Northern blot analysis of multiple human tissues with a *NEDL2* cDNA probe detected two mRNA species of roughly 9.5 and 15 kb, which were expressed predominantly in adult brain, lung, and heart (Fig. 1C), whereas *NEDD4* mRNA was abundantly expressed in adult skeletal muscle. As NEDL2 shared structural features with NEDD4, we asked whether NEDL2 had an E3 ubiquitin ligase activity in vitro. To this end, the HECT domain of NEDL2 fused to glutathione *S*-transferase [GST-NEDL2(1313–1572)] was generated and examined its ability to ubiquitinate bacterial cellular proteins. In these experiments, GST-NEDD4(52–777), which

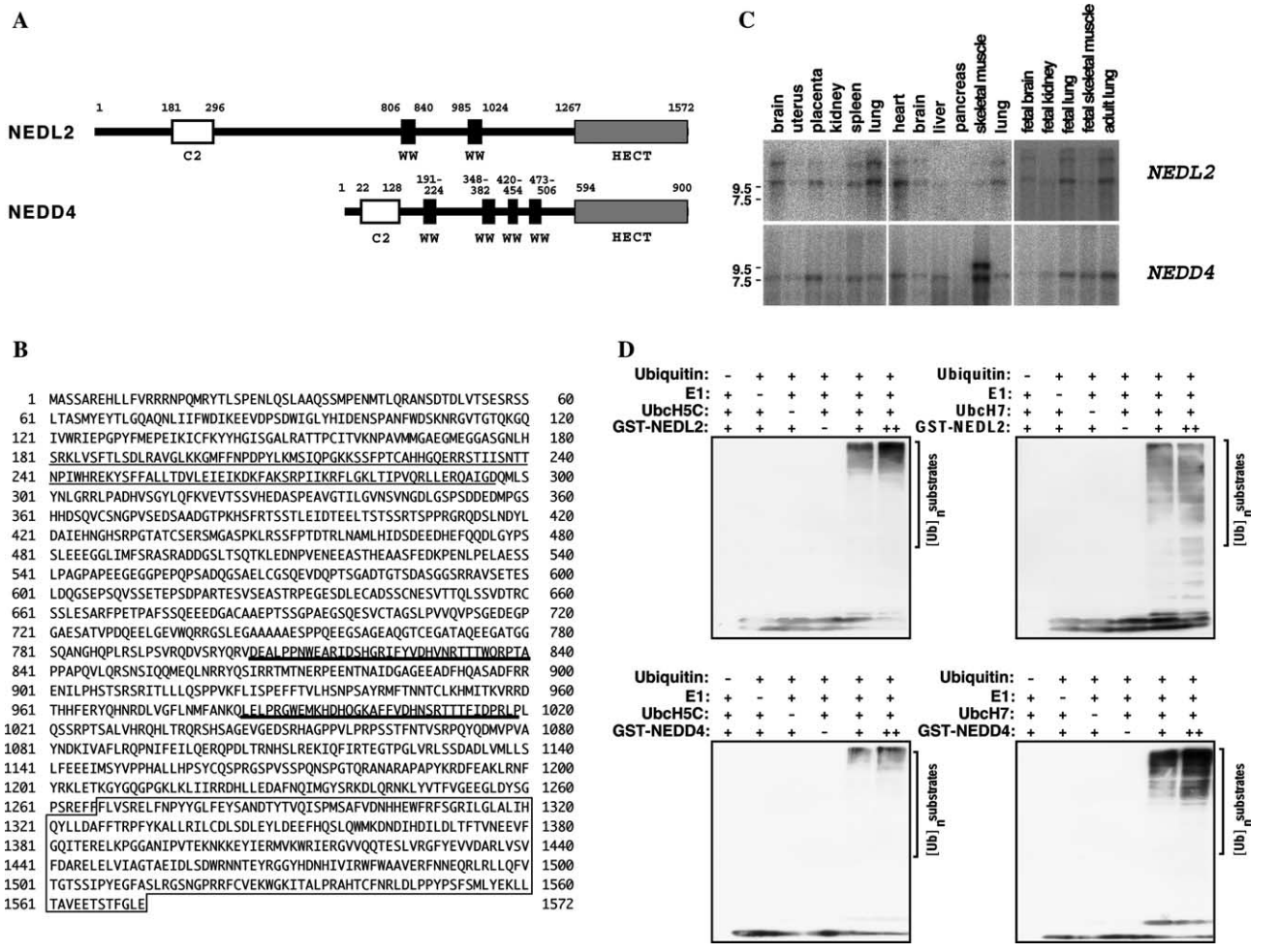

Fig. 1. *NEDL2* encodes an E3 ubiquitin ligase. (A) Schematic diagram of human NEDL2 and NEDD4. A lipid/Ca^{2+}-binding (C2) domain and WW protein interaction domains are indicated by open and closed boxes, respectively. A COOH-terminal catalytic HECT domain is denoted by gray box. Numbers indicate amino acid position. (B) Entire amino acid sequence of human NEDL2. The NH_2-terminal C2 domain and two WW domains are indicated by thin and thick lines, respectively. The box shows the COOH-terminal HECT domain. (C) Northern blot analysis of *NEDL2* mRNA expression in human tissues. The human multiple tissue Northern blot (Clontech) was hybridized with human *NEDL2* (upper panel) or with *NEDD4* (lower panel) cDNA probe. Molecular size markers are shown on the left. (D) E3 ubiquitin ligase activity of NEDL2 in vitro. Equal amounts of bacterial lysates were incubated with the indicated combinations of recombinant proteins in the absence or the presence of the increasing amounts of GST-NEDL2 (upper panels) or GST-NEDD4 (lower panels). The reaction mixtures were analyzed by immunoblotting with a monoclonal antibody against ubiquitin. The ubiquitinated bacterial cellular proteins are indicated by brackets.

encoded four WW domains and the COOH-terminal HECT domain of mouse NEDD4 [21], was used as a positive control. When the reaction mixtures were analyzed by immunoblotting with the anti-ubiquitin antibody, we observed the appearance of a smear of ubiquitinated bacterial cellular proteins in the presence of GST-NEDD4(52–777) or GST-NEDL2(1313–1572) under our experimental conditions (Fig. 1D), suggesting that the HECT domain of NEDL2 catalyzed ubiquitin ligation onto bacterial cellular proteins and that NEDL2 acted as an E3 ubiquitin ligase.

NEDL2 associates with p73

As described, p73α and p73β contain two putative PY motifs (405-PPSY-408 and 484-PPPY-487) which are not present in p53 (Fig. 2A). Interestingly, the interaction of p73 with Yes-associated protein (YAP) has been reported to be mediated by the region of p73 containing the PPPY motif and the WW domain of YAP [22]. To examine whether NEDL2 physically interacts with p73, COS7 cells were transiently transfected with the expression plasmids for hemagglutinin (HA)-tagged p73α and $(His)_6$-xpress-tagged NEDL2. The whole cell lysates were in turn immunoprecipitated with a polyclonal anti-NEDL2 antibody followed by immunoblotting with a monoclonal anti-p73 antibody. Control experiments revealed that equal amounts of proteins were expressed in each sample (Fig. 2B, lower panels). Immunoprecipitation of NEDL2 resulted in the co-precipitation of p73α (Fig. 2B, first panel). This observation was confirmed by the immunoprecipitation of p73α, which resulted in the co-precipitation of NEDL2 (Fig. 2B, second panel), suggesting that NEDL2 interacts with p73α in mammalian cultured cells. To identify the p73 region required for the interaction with NEDL2, we examined NEDL2 for its ability to bind to p73β or p73α (1–427) by co-immunoprecipitation experiments. As shown in Figs. 2C and D,

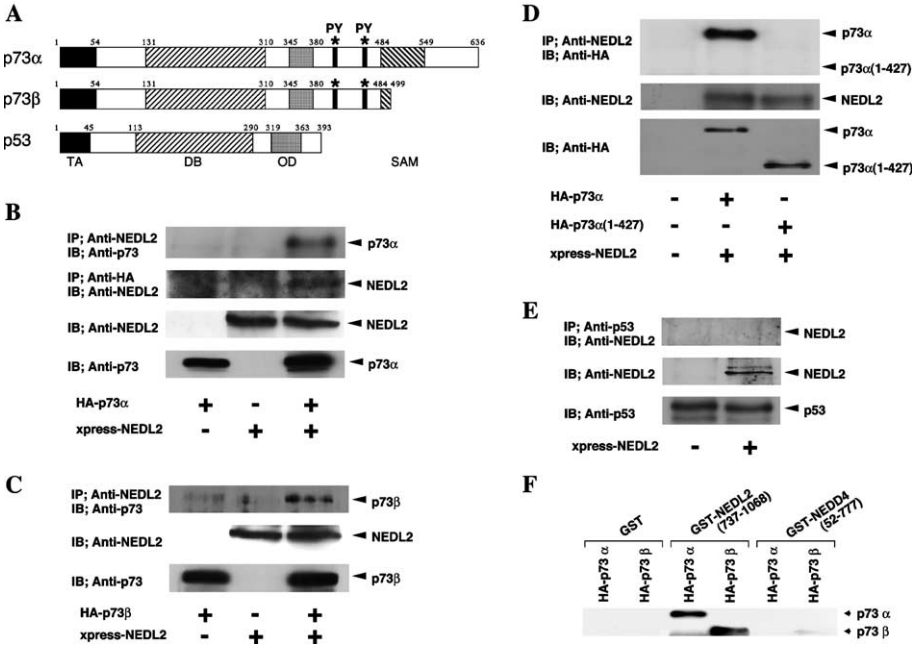

Fig. 2. Interaction of NEDL2 with p73. (A) Schematic representation of p73 and p53. TA, transactivation domain; DB, DNA-binding domain; OD, oligomerization domain; SAM, and sterile α motif domain. The possible PY motifs are indicated with asterisks. (B) Co-immunoprecipitation of p73α with NEDL2. COS7 cells were transfected with the indicated combinations of the expression plasmids. Whole cell lysates were immunoprecipitated (IP) with a polyclonal anti-NEDL2 (first panel) or with a monoclonal anti-HA (second panel) antibody, and precipitated proteins were analyzed by immunoblotting (IB) with the anti-p73 or with the anti-NEDL2 antibody, respectively. Lower panels show the immunoblotting with the anti-NEDL2 or with the anti-p73 antibody to monitor the expression level of the transfected constructs. (C) Interaction between p73β and NEDL2. Whole cell lysates prepared from COS7 cells transfected with the indicated combinations of the expression plasmids were immunoprecipitated with the anti-NEDL2 antibody followed by immunoblotting with the antibody against p73 (upper panel). Whole cell lysates were monitored on Western blot for expression of HA-p73β and NEDL2 (middle and lower panels, respectively). (D) Identification of the region of p73 required for the interaction with NEDL2. Whole cell lysates from COS7 cells transfected with the indicated combinations of the expression plasmids were immunoprecipitated with the anti-NEDL2 antibody, followed by immunoblotting with the monoclonal anti-HA antibody. Lower panels show the immunoblotting with the anti-NEDL2 or with the anti-HA antibody to monitor the expression level of exogenous NEDL2 or p73 derivatives, respectively. (E) Interaction between p53 and NEDL2. Immunoprecipitation assays were performed with whole cell lysates from COS7 cells transfected with the indicated expression plasmid. Whole cell lysates were immunoprecipitated with a monoclonal anti-p53 antibody and subjected to immunoblot analysis using the anti-NEDL2 antibody (upper panel). Whole cell lysates were monitored on Western blot for expression of these components (lower panels). (F) In vitro pull-down assay. COS7 cells were transiently transfected with the expression plasmid for HA-p73α or HA-p73β. Whole cell lysates were incubated with the indicated GST fusion proteins and then protein complexes were collected on the glutathione–Sepharose resin. Specifically bound p73α or p73β was detected by immunoblotting with the anti-p73 antibody.

p73β was co-immunoprecipitated with NEDL2, whereas p73α (1–427) which lacks the COOH-terminal PPPY motif, was undetectable in the anti-NEDL2 immunoprecipitates. In addition, p53 was not co-immunoprecipitated with NEDL2 (Fig. 4E). These results strongly suggested that the p73 region containing the PPPY motif was required for the interaction with NEDL2.

To test whether the WW domains of NEDL2 could be required for the interaction with p73, GST-NEDL2(737–1068) fusion protein which contained two WW domains was incubated with the in vitro-translated p73α or p73β in pull-down experiments. As shown in Fig. 2F, GST fusion protein containing NEDL2(737–1068) was associated with p73α and p73β, whereas GST or GST-NEDD4(52–777) was not, suggesting that the WW domains of NEDL2 are necessary for its interaction with p73, and these proteins interact directly.

NEDL2 stabilizes p73

We next asked whether or not NEDL2 ubiquitinates p73. COS7 cells were transiently co-transfected with the indicated combinations of the expression plasmids. Forty-eight hours after transfection, cells were treated with the proteasomal inhibitor MG-132 for 30 min and whole cell lysates were subjected to immunoprecipitation with the anti-p73 antibody followed by immunoblotting with the anti-ubiquitin antibody. As shown in the left panel of Fig. 3A, a high level of ubiquitinated p73α was detected in cells transfected with p73α and NEDL2. Similar results were also obtained by immunoblotting analysis (Fig. 3A, right panel). We then tested whether NEDL2 affected the stability of p73. To this end, COS7 cells were transiently co-transfected with the constant amount of the expression plasmid for HA-p73α, HA-p73β or FLAG-p53 together with or without the increasing amounts of the NEDL2 expression plasmid, and the levels of exogenously expressed HA-p73α, HA-p73β or FLAG-p53 were examined by immunoblotting. Unexpectedly, transient overexpression of NEDL2 resulted in an increase in the levels of HA-p73α as well as HA-p73β (Figs. 3B and C). On the other hand, the expression level of FLAG-p53 which was unable to interact with NEDL2, was not affected in the presence of NEDL2 under our experimental conditions (Fig. 3D). Similar results were also obtained in p53-deficient human large

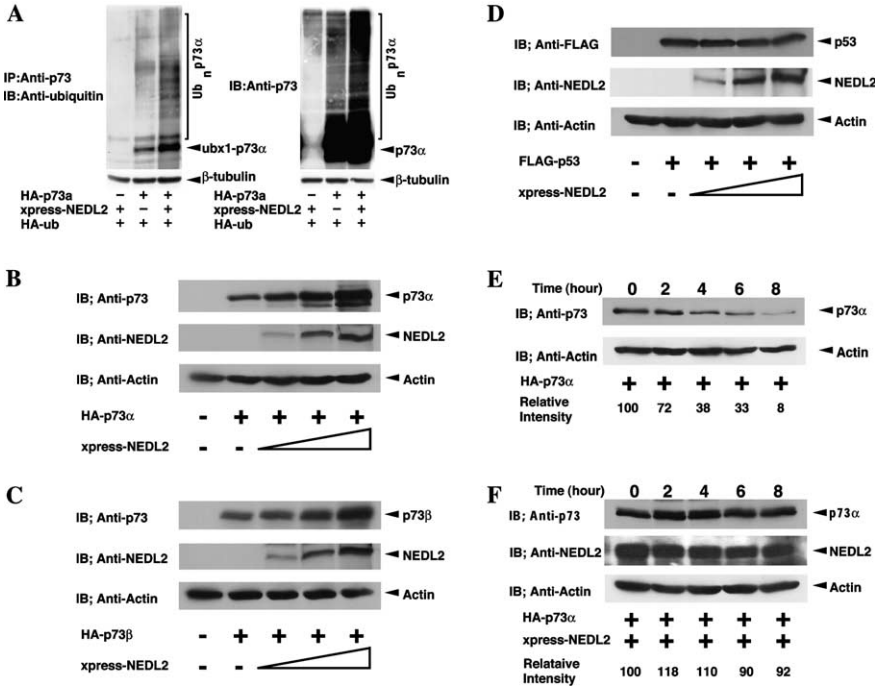

Fig. 3. NEDL2 stabilizes p73 in mammalian cultured cells. (A) NEDL2 ubiquitinates p73. COS7 cells were transfected with the indicated combinations of the expression plasmids. Forty-eight hours after transfection, cells were exposed to MG-132 (at a final concentration of 40 μM) for 30 min and subjected to immunoprecipitation (left panel) or immunoblotting (right panel) with the indicated antibodies. The expression level of β-tubulin is included as a loading control. (B,C) Overexpression of NEDL2 affects the steady-state level of p73α and p73β. COS7 cells were transiently transfected with the expression plasmid for HA-p73α (B) or HA-p73β (C) together with or without the increasing amounts of the NEDL2 expression plasmid. Whole cell lysates were analyzed by immunoblotting with the antibody against p73 (upper panels), NEDL2 (middle panels) or actin (lower panels). (D) NEDL2 does not affect the expression level of p53. Whole cell lysates prepared from COS7 cells transfected with the indicated combinations of the expression plasmids were subjected to immunoblotting with the indicated antibodies. (E,F) NEDL2 increases the half-life of p73α. COS7 cells were transiently transfected with the expression plasmid encoding HA-p73α in the absence (E) or in the presence of the *NEDL2* expression plasmid (F). Twenty-four hours after transfection, cycloheximide was added to the culture medium at a final concentration of 100 μg/ml and the cells were harvested at the indicated time points. Whole cell lysates were analyzed by immunoblotting with the anti-p73, the anti-NEDL2, or with the anti-actin antibody. Densitometry was used to quantify the amounts of p73α, which normalized to actin. The amounts of p73α are indicated as relative fold.

cell lung carcinoma H1299 cells (data not shown). We then examined the decay rate of p73 in the presence of NEDL2. COS7 cells were transiently co-transfected with the expression plasmid for HA-p73α together with or without the NEDL2 expression plasmid. Forty-eight hours after transfection, cells were treated with cycloheximide at a final concentration of 100 μg/ml. At the indicated time points, whole cell lysates were prepared and subjected to immunoblotting with the anti-p73 antibody. Consistent with the previous observations [23,24], the half-life of the exogenously expressed p73α was less than 4 h (Fig. 3E). Under our experimental conditions, p73α decayed at faster rates in the absence of NEDL2 than in its presence (Figs. 3E and F). Similarly, the half-life of p73β was prolonged in the presence of NEDL2 (data not shown). Thus, our results clearly indicated that NEDL2 had a stabilizing effect on p73.

NEDL2 enhances the p73-mediated transcriptional activation

To investigate the functional consequences of NEDL2 interaction with p73, we tested the ability of NEDL2 to affect the p73-mediated transcriptional activation. H1299 cells were transiently transfected with the expression plasmid for HA-p73α, a luciferase reporter construct containing the p53/p73-binding site from *p21*^{WAF1} or *MDM2* promoter in the presence or absence of the increasing amounts of the NEDL2 expression plasmid. As shown in Fig. 4A, NEDL2 enhanced the p73α-mediated transcriptional activation in a dose-dependent manner. In contrast, NEDL2 had a weak effect on the p53-dependent transactivation (Fig. 4B). These observations raised a possibility that NEDL2 stabilized p73 through direct protein–protein interactions, and thereby enhanced the ability of p73 to transactivate the *p21*^{WAF1} and *MDM2* promoter. Thus, NEDL2 may interact with p73 not only physically but also functionally.

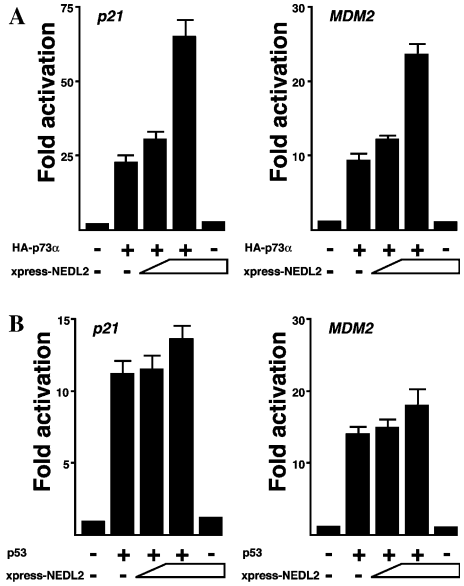

Fig. 4. NEDL2 enhances the p73-mediated transcriptional activation. p53-deficient H1299 cells were plated at a density of 5×10^4/well in 12-well plates and transiently transfected with the expression plasmid for HA-p73α (A) or p53 (B), pRL-TK, and a p53/p73-responsive luciferase reporter construct carrying *p21*^{WAF1} or *MDM2* promoter along with or without the increasing amounts of the *NEDL2* expression plasmid as indicated. Luciferase activity was quantified and normalized to internal *Renilla* reporter signal. Shown is the average of three independent experiments. Means of at least three independent experiments are shown with error bars indicating means ± standard deviations.

Discussion

In the present study, we identified a novel NEDD4-related E3 ubiquitin ligase NEDL2 and also showed that NEDL2 interacts with p73 but not with p53. Immunoprecipitation and in vitro pull-down analyses demonstrated that this interaction was mediated by the WW protein–protein interaction domains of NEDL2 and the region of p73 containing the PPPY motif. In contrast to NEDL2, the WW domains of NEDD4 did not have an ability to bind to p73. The previous reports suggest that the WW domains have distinct ligand specificities [25] and determine the substrate specificity of certain E3 ubiquitin protein ligases [26,27]. Our results also suggest that the WW domains of NEDD4 family members mediate specific and distinct protein–protein interaction.

Since the intracellular levels of p73 are increased in the presence of the proteasomal inhibitor, p73 stability is regulated in part by the protein degradation process through the ubiquitin–proteasome pathway [16], however, the relevant E3 ubiquitin ligase has not been identified. In contrast to p53, MDM2 which induces the ubiquitin-dependent degradation of p53, does not promote the degradation of p73 but in fact enhances its stability [17]. Recently, Ohtsuka et al. [24] have reported that cyclin G binds to p53 as well as p73, and negatively regulates their stability, however, the molecular mechanisms of the cyclin G-dependent down-regulation of p53 or p73 are unclear. Under our experimental conditions, NEDL2 with an E3 ubiquitin ligase activity in vitro has an ability to ubiquitinate p73 in cells, however, overexpression of NEDL2 increased the stability of exogenous p73. The intracellular level of p53 was not affected by NEDL2 without interacting with p53. Stabilization of p73 is crucial for its ability to induce cell cycle arrest and/or apoptosis. Recently, it has been shown that a certain polyubiquitination event does not serve to target substrate proteins for proteolytic degradation, suggesting that there exists a non-proteolytic regulatory function of ubiquitin [28,29]. Spence et al. [28] have found that ribosomal protein L28 is ligated to a chain of ubiquitin molecules joined through Lys-63 of ubiquitin and the heavily ubiquitinated ribosomes were active in translation. In addition, TRAF6-mediated

Lys-63-linked polyubiquitination is required for the activation of IKK [29]. On the other hand, it has been suggested that Lys-48-linked polyubiquitin chains might be critical for efficient recognition by 26S proteasome [30]. Although the chain structure of the polyubiquitinated p73 has not been determined, the NEDL2-mediated ubiquitination might play a regulatory role in p73 function.

Alternatively, it is possible that the direct interaction between p73 and NEDL2 might be sufficient to enhance the stability of p73 in the absence of ubiquitination. During the cisplatin-induced apoptosis, p73 is stabilized and activated in a c-Abl-dependent manner [18]. The PxxP motif (335-PAVP-338) of p73 is required for the interaction with c-Abl [31]. Recently, Ren et al. [32] have found that protein kinase Cδ catalytic fragment-mediated phosphorylation of p73 at Ser-289 is tightly associated with the accumulation as well as activation of p73. Considering that the DNA damage-induced phosphorylation of p53 prevents its association with MDM2 and results in its stabilization [15], it is likely that conformational alterations of p73 induced by its phosphorylation or its binding to NEDL2 might disrupt the interaction with certain cellular protein(s) required for the degradation of p73. Additionally, the binding of NEDL2 to p73 might mask site(s) of p73 targeted for degradation by the proteasome, and thereby p73 might accumulate to significantly higher level than that in the absence of NEDL2. To explore a possible role of the NEDL2-mediated ubiquitination in the regulation of p73 activity, we are generating a catalytic mutant form of NEDL2 in which the critical cysteine residue (at position 1540) within the HECT domain is replaced by alanine residue.

Consistent with the NEDL2-mediated stabilization of p73, p73-dependent transcriptional activity has been enhanced in the presence of NEDL2. Of note, Yes-associated protein (YAP) interacts with the PPPY motif of p73 but not with p53, resulting in the enhancement of the p73-mediated transcriptional activation [22]. We have reported previously that, in addition to the NH_2-terminal transactivation region, the COOH-terminal glutamine- and proline-rich domain of p73 including the PPPY motif (residues 382–491) has a transactivation function [33]. Like YAP, it is therefore likely that the differential binding of NEDL2 to p53 family members might contribute at least in part to their functional divergence.

In summary, we have found that p73 stability as well as its activity is significantly enhanced through the physical interaction with NEDL2, and this interaction may be mediated by the p73 region containing the PPPY motif and the WW domains of NEDL2. Although the precise molecular mechanisms that regulate the NEDL2-dependent upregulation of p73 have not yet been defined, identification of cellular components involved in this process should provide clue(s) to understand how p73 is regulated at the protein level.

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References

[1] M. Kaghad, H. Bonnet, A. Yang, L. Creancier, J.C. Biscan, A. Valent, A. Minty, P. Chalon, J.M. Lelias, X. Dumont, P. Ferrara, F. McKeon, D. Caput, Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers, Cell 90 (1997) 809–819.

[2] C.A. Jost, M.C. Marin, W.G. Kaelin, p73 is a human p53-related protein that can induce apoptosis, Nature 389 (1997) 191–194.

[3] L. Fang, S.W. Lee, S.A. Aaronson, Comparative analysis of p73 and p53 regulation and effector functions, J. Cell Biol. 147 (1999) 823–830.

[4] V. De Laurenzi, A. Costanzo, D. Barcaroli, A. Torrinoni, M. Falclco, M. Annicchiarico-Petruzzelli, M. Levrero, G. Melino, The new p73 splice variants, γ and δ, with different transcriptional activity, J. Exp. Med. 188 (1998) 1763–1768.

[5] Y. Ueda, M. Hijikata, S. Takagi, T. Chiba, K. Shimotohno, New p73 variants with altered C-terminal structures have varied transcriptional activities, Oncogene 18 (1999) 4993–4998.

[6] V. De Laurenzi, M.V. Catanl, A. Teminoni, M. Corazzari, G. Melino, A. Costanzo, M. Levrero, R.A. Knight, Additional complexity in p73: induction by mitogens in lymphoid cells and identification of two new splicing variants ε and ζ, Cell Death Differ. 6 (1999) 389–390.

[7] A. Yang, N. Walker, R. Bronson, M. Kaghad, M. Oosterwegel, J. Bonnin, C. Vagner, H. Bonnet, P. Dikkes, A. Sharpe, F. McKeon, D. Caput, p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours, Nature 404 (2000) 99–103.

[8] C.D. Pozniak, S. Radinovic, A. Yang, F. McKeon, D.R. Kaplan, F.D. Miller, An anti-apoptotic role for the p53 family member, p73, during developmental neuron death, Science 289 (2000) 304–306.

[9] T. Stiewe, S. Zimmermann, A. Frilling, H. Esche, B.M. Putzer, Transactivation-deficient ΔTA-p73 acts as an oncogene, Cancer Res. 62 (2002) 3598–3602.

[10] T.J. Grob, U. Novak, C. Maisse, D. Barcaroli, A.U. Luthi, F. Pirnia, B. Hugli, H.U. Graber, L.V. De, M.F. Fey, G. Melino, A. Tobler, Human ΔNp73 regulates a dominant negative feedback loop for TAp73 and p53, Cell Death Differ. 8 (2001) 1213–1223.

[11] T. Nakagawa, M. Takahashi, T. Ozaki, K. Watanabe, S. Todo, H. Mizuguchi, T. Hayakawa, A. Nakagawara, Autoinhibitory regulation of p73 by ΔNp73 to modulate cell survival and death through p73-specific target element within the Δ*Np73* promoter, Mol. Cell. Biol. 22 (2002) 2575–2585.

[12] Y. Haupt, R. Maya, A. Kazaz, M. Oren, Mdm2 promotes the rapid degradation of p53, Nature 387 (1997) 296–299.

[13] M.H.G. Kubbutat, S.N. Jones, K.H. Vousden, Regulation of p53 stability by Mdm2, Nature 387 (1997) 299–303.
[14] R. Honda, H. Tanaka, H. Yasuda, Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53, FEBS Lett. 420 (1997) 25–27.
[15] K.H. Vousden, Activation of the p53 tumor suppressor protein, Biochem. Biophys. Acta 1602 (2002) 47–59.
[16] E. Balint, S. Bates, K.H. Vousden, Mdm2 binds p73α without targeting degradation, Oncogene 18 (1999) 3923–3929.
[17] X. Zeng, L. Chen, C.A. Jost, R. Maya, D. Keller, X. Wang, W.G. Kaelin, M. Oren, J. Chen, H. Lu, MDM2 suppresses p73 function without promoting p73 degradation, Mol. Cell. Biol. 19 (1999) 3257–3266.
[18] J. Gong, A. Costanzo, H.Q. Yang, G. Melino, W.G. Kaelin, M. Levrero, J.Y.J. Wang, The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage, Nature 399 (1999) 806–809.
[19] W.T. Steegenga, A. Shvarts, N. Riteco, J.L. Bos, A.G. Jochemsen, Distinct regulation of p53 and p73 activity by adenovirus E1A, E1B, and E4orf6 proteins, Mol. Cell. Biol. 19 (1999) 3885–3894.
[20] T. Nagase, R. Kikuno, K.I. Ishikawa, O. Ohara, Prediction of the coding sequences of unidentified human genes. XVI. The complete sequences of 150 new cDNA clones from brain which code for large proteins in vitro, DNA Res. 7 (2000) 65–73.
[21] S. Hatakeyama, J.P. Jensen, A.M. Weissman, Subcellular localization and ubiquitin-conjugating enzyme (E2) interactions of mammalian HECT family ubiquitin protein ligases, J. Biol. Chem. 272 (1997) 15085–15092.
[22] S. Strano, E. Munarriz, M. Rossi, B. Cristofanelli, Y. Shaul, L. Castagnoli, A.J. Levine, A. Sacchi, G. Cesareni, M. Oren, G. Blandino, Physical interaction with Yes-associated protein enhances p73 transcriptional activity, J. Biol. Chem. 275 (2000) 29503–29512.
[23] C.-W. Lee, N.B. La Thangue, Promoter specificity and stability control of the p53-related protein p73, Oncogene 18 (1999) 4171–4181.
[24] T. Ohtsuka, H. Ryu, Y.A. Minamishima, A. Ryo, S.W. Lee, Modulation of p53 and p73 levels by cyclin G: implication of a negative feedback regulation, Oncogene 22 (2003) 1678–1687.
[25] G. Pirozzi, S.J. McConnell, A.J. Uveges, J.M. Carter, A.B. Sparks, B.K. Kay, D.M. Fowlkes, Identification of novel human WW domain-containing proteins by cloning of ligand targets, J. Biol. Chem. 272 (1997) 14611–14616.
[26] H. Zhu, P. Kavsak, S. Abdollah, J.L. Wrana, G.H. Thomsen, A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation, Nature 400 (1999) 687–693.
[27] L. Qiu, C. Joazeiro, N. Fang, H.-Y. Wang, C. Elly, Y. Altman, D. Fang, T. Hunter, Y.-C. Liu, Recognition and ubiquitination of Notch by Itch, a Hect-type E3 ubiquitin ligase, J. Biol. Chem. 275 (2000) 35734–35737.
[28] J. Spence, R.R. Gali, G. Dittmar, F. Sherman, M. Karin, D. Finley, Cell cycle regulated modification of the ribosome by a variant multiubiquitin chain, Cell 102 (2000) 67–76.
[29] L. Deng, C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, Z.J. Chen, Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain, Cell 103 (2000) 351–361.
[30] Q. Deveraux, V. Ustrell, C. Pickart, M. Rechsteiner, A 26S protease subunit that binds ubiquitin conjugates, J. Biol. Chem. 269 (1994) 7059–7061.
[31] R. Agami, G. Blandino, M. Oren, Y. Shaul, Interaction of c-Abl and p73α and their collaboration to induce apoptosis, Nature 399 (1999) 809–813.
[32] J. Ren, R. Datta, H. Shioya, Y. Li, E. Oki, V. Biedermann, A. Bharti, D. Kufe, p73β is regulated by protein kinase Cδ catalytic fragment generated in the apoptotic response to DNA damage, J. Biol. Chem. 277 (2002) 33758–33765.
[33] N. Takada, T. Ozaki, S. Ichimiya, S. Todo, A. Nakagawara, Identification of a transactivation activity in the COOH-terminal region of p73 which is impaired in the naturally occurring mutants found in human neuroblastomas, Cancer Res. 59 (1999) 2810–2814.