

The Human Orthologue of *Drosophila* Ecdysoneless Protein Interacts with p53 and Regulates Its Function

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Abstract

Biochemical mechanisms that control the levels and function of key tumor suppressor proteins are of great interest as their alterations can lead to oncogenic transformation. Here, we identify the human orthologue of *Drosophila melanogaster* ecdysoneless (hEcd) as a novel p53-interacting protein. Overexpression of hEcd increases the levels of p53 and enhances p53 target gene transcription whereas hEcd knockdown has the opposite effects on p53 levels and target gene expression. Furthermore, hEcd interacts with murine double minute-2 and stabilizes p53 by inhibiting murine double minute-2-mediated degradation of p53. Thus, hEcd protein represents a novel regulator of p53 stability and function. Our studies also represent the first demonstration of a biochemical function for hEcd protein and raise the possibility that altered hEcd levels and/or function may contribute to oncogenesis. (Cancer Res 2006; 66(14): 7167-75)

Introduction

Normal cells are endowed with intrinsic checkpoints to prevent their oncogenic transformation. Indeed, tumor suppressor pathways in normal cells dominantly suppress oncogene-driven tumorigenesis (1), and oncogenic transformation invariably involves the inactivation of the tumor suppressor pathways (2). Not surprisingly, the mutations or deletions of the major tumor suppressors, such as p53 and Rb, together constitute the most frequent genetic alterations in human cancer (3-6). How tumor suppressor pathways prevent the emergence of oncogenically transformed cells has become a central theme in cancer cell biology.

In recent years, it has become clear that p53 plays an essential role in cellular response to DNA damage and other cellular stresses and prevents the emergence of cells with mutated genomes by inducing cell cycle arrest while facilitating DNA repair or facilitating apoptosis or senescence of cells if DNA damage is extensive. It has been shown the ability of p53 to transcriptionally activate genes that directly control the various cellular fates (5, 7, 8). Thus, elucidation of the components of the biochemical machinery that control p53 level and function is of substantial biological significance and these components may be involved in oncogenesis. For example, the ubiquitin ligase murine double minute-2 (Mdm2)

controls p53 levels by targeting it for ubiquitin-dependent degradation and increased Mdm2 levels are associated with high risk of cancers.

In this study, we report the identification of the human orthologue of *Drosophila melanogaster* ecdysoneless (hEcd; ref. 9) as a novel p53-interacting protein. Importantly, we show an interaction of hEcd with p53 and Mdm2, a physiologic role for hEcd in the stability and function of p53. The "ecdysoneless" (*ecd*) mutations in *D. melanogaster* have been used in numerous genetic analyses as the insect steroid hormone ecdysone is responsible for coordination of embryogenesis, larval molting, and metamorphosis (10). However, the *ecd* gene product was not known until recently. Gaziova et al. (9) have recently cloned *ecd* and found it to be expressed in both steroidogenic and nonsteroidogenic tissues and showed that the Ecd protein also plays a cell-autonomous role in development and oogenesis. However, the mechanistic basis of Ecd function remains unknown. Our study is the first report of a mechanistic basis for hEcd protein function with potential implications on the roles of this evolutionarily conserved protein family. Identification of hEcd as a novel p53 binding partner implicates hEcd as a novel participant in human cancer.

The human orthologue of Ecd was initially cloned in a screen of human genes that can rescue the growth defect in *Saccharomyces cerevisiae* mutants with a deletion of the growth control regulatory gene 2 (*GCR2*) gene, a transcriptional activator of the glycolytic gene expression, and was therefore designated as human suppressor of *GCR2* (hSGT1; ref. 11), its current database name [National Center for Biotechnology Information (NCBI) accession no. NM_007265; Unigene cluster Hs.446373 *Homo sapiens*]. However, hSGT1 has no structural relationship with yeast *GCR2* nor does a clear mammalian orthologue of *GCR2* (12) exist. Furthermore, an orthologue of hSGT1 (Ecd) is not discernible in the *S. cerevisiae* genome but highly conserved orthologues are found throughout higher eukaryotic organisms, including the fission yeast *Schizosaccharomyces pombe*.⁶ Given these findings and the fact that an unrelated and evolutionarily conserved group of SGT1 proteins (for suppressor of G2 allele of skp1; also known as SUGT1) already exists (13), we have adopted the nomenclature suggested by Gaziova et al. (9) and will refer to hSGT1 as human ecdysoneless (hEcd) to avoid any confusion that the continued use of the acronym hSGT1 is likely to create.

Materials and Methods

Yeast two-hybrid screen. Yeast two-hybrid screen of a human mammary epithelial cell library using the human papillomavirus (HPV)-16 E6 protein

Note: This work was initiated while Dr. Vimla Band's laboratory was at the New England Medical Center, Tufts University School of Medicine, Boston, MA.

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⁶ <http://www.sanger.ac.uk//Software/Pfam/data/jtml/full/PF07093.shtml.gz>

as a bait identified 28 specific E6-interacting clones (14). Homology analysis using “Gapped BLAST” search of the NCBI database revealed that one of these clones corresponded to residues 291 to 644 of hSGT1/Ecd (accession no. NM_007265; ref. 11).

Cell lines and culture conditions. 293T, H1299, WI-38, and mouse embryonic fibroblast (MEF) cell lines were grown in DMEM. U2OS, MCF-7, and Saos-2 cell lines were grown in α -MEM (Life Technologies, Rockville, MD). Both DMEM and α -MEM were supplemented with 10% FCS (HyClone, Logan, UT). The p53-negative breast cancer cell line 21PT was grown in DMEM supplemented with hydrocortisone and epithelial growth factor as previously described (15).

Antibodies. Anti-hEcd rabbit polyclonal antiserum was generated using purified GST-hEcd (amino acids 291-644) as an antigen by Animal Pharma (Healdsburg, CA). Other antibodies used were purchased commercially: α -tubulin, β -actin (Abcam); poly(ADP-ribose) polymerase (F-2), p53 (DO-1), p53 (Pab1801), p53 (Pab 240), p53 (FL-393), p21 (F-5), Bax (B-9) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); p53 (PAb 421), Mdm2 (Ab-1) (Calbiochem, San Diego, CA); p53 (Pab122; NeoMarkers, Fremont, CA); and FLAG M2 antibody and FLAG M2 horseradish peroxidase-conjugated antibody (Sigma, St. Louis, MO).

Immunoblotting. Cells were washed with PBS, lysed in 1× sample buffer [50 mmol/L Tris (pH 6.8); 2% SDS; 10% glycerol] lacking β -mercaptoethanol, and protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL). Lysates were analyzed immediately or frozen at -80°C . Before analysis, β -mercaptoethanol was added and samples were heated at 95°C for 5 minutes. Proteins were separated in denaturing polyacrylamide gels and transferred to polyvinylidene difluoride membranes, and the membranes were blocked with 1% bovine serum albumin in TBS/0.1% Tween 20, incubated with primary and secondary antibodies, and washed in TBS/0.1% Tween 20. Enhanced chemiluminescence signals were recorded using a light-sensitive film (NEN Life Science Products, Boston, MA).

Localization of hEcd by biochemical fractionation. To assess hEcd localization in subcellular fractions, U2OS cells were treated with 10 ng/mL of leptomycin B (Sigma) or 70% methanol (as control) and cells were harvested in 1× sample buffer. Nuclear and cytoplasmic fractions were prepared as described (16) and equal amounts of protein extracts were analyzed for hEcd levels by immunoblotting.

Plasmids, plasmid constructions, and hEcd RNAi oligonucleotides. GST-hEcd (amino acids 291-644) was constructed by subcloning the corresponding sequence derived from the yeast two-hybrid vector pGAD10 into pGEX-4T2 (Amersham-Pharmacia Biotech, Piscataway, NJ). Full-length hEcd was amplified from normal mammary gland cDNA (Clontech, Mountain View, CA) by using the Advantage cDNA amplification kit (Promega, Madison, WI) and the following primers: hEcd sense, GATCGGGCCGCCATGGACTACAAGGACGACGATGACAAGGAAGAA-ACCATGAAGCTTGC; hEcd antisense GCGGCAGCCGCAGAACGCTAAAT-GTGCAGGTTAAT. The resultant PCR product with an NH₂-terminal FLAG tag was cloned into pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, CA). Truncated versions of hEcd (amino acids 1-291, 292-492, Madison, WI and 493-644) were all FLAG tagged and constructed in pcDNA3.1 by PCR using full-length hEcd as template. Full-length hEcd protein fused to glutathione S-transferase (GST) was derived by subcloning hEcd cDNA from the pcDNA3.1-FLAG-hEcd construct into pGEX 4T-2 vector. Histidine-tagged hEcd was constructed by cloning the PCR-derived cDNA fragments into pEF6/His vector. Construction of GST-p53, pCR3.1-FLAG-p53, and pCMVp53 has previously been described (17). The luciferase reporters of Bax, cyclin G, Mdm2, and insulin-like growth factor binding protein 3 were provided by Dr. Carole Prives (Columbia University, New York, NY) and pG₁₃ reporter was provided by Dr. Wafik El-Diery (University of Pennsylvania, Philadelphia, PA; originally obtained from Dr. Bert Vogelstein, Johns Hopkins School of Medicine, Baltimore, MD). The RNA oligonucleotides used to generate 21-nucleotide small interfering RNA (siRNA) duplexes were synthesized by Dharmacon (Lafayette, CO) corresponding to the following human hEcd sequences: siRNA 1 (nucleotides 1,703-1,723), 5'-AAGGCCTAATGAGTCAGATTCDdT-3'; siRNA 2 (nucleotides 1,902-1,922), 5'-AAGAACCAAGUGGAACCUGUAdTdT-3'; and control

siRNA, 5'-AACCGAGUCUAUCCCUAUAdTdT-3'. pCMV-Mdm2 was constructed by cloning the PCR-derived cDNA fragments from pCMV-HA-Mdm2 provided by Dr. Hua Lu (Oregon Health & Science University, Portland, OR).

In vitro binding. The ³⁵S-labeled proteins were generated using wheat germ or rabbit reticulocyte lysate-based coupled *in vitro* transcription-translation system (Promega) and *in vitro* binding experiments with GST or GST fusion proteins were done as previously described (17).

Immunoprecipitation. Immunoprecipitations from cell lysates [lysis buffer contains 100 mmol/L Tris (pH 8.0), 100 mmol/L NaCl, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride] were done as described (14, 17) using protein A-Sepharose 4B and protein G-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ) as immunosorbents. Cell lysates were precleared with beads for 1 hour. Antibodies were added to the precleared cell lysates and incubated for 2 hours or overnight (indicated in each experiment). Beads were added and incubated for another 1 hour, then washed six times with immunoprecipitation lysis buffer. The immunoprecipitations or cell lysates were resolved on SDS-PAGE gels and immunoblotting was done as described above.

Turnover of p53. U2OS cells were transfected with indicated siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen). After 24 hours, cycloheximide (25 $\mu\text{g}/\text{mL}$) was added to cells, and lysates at different time periods were analyzed by immunoblotting. Densitometry was carried out on directly scanned images using ScionImage for Windows software (Scion Corp., Frederick, MD).

In vivo association of p53 and hEcd. 293T cells were transfected with pCR3.1-FLAG-p53 with or without pEF6/His-hEcd and cell lysates for immunoprecipitation were prepared as described above. His pulldown assay was done as previously described (17).

To assess the interaction of endogenous p53 and hEcd, U2OS cell lysates in immunoprecipitation lysis buffer were subjected to immunoprecipitation with a cocktail of anti-p53 antibodies (PAb421, PAb1801, PAb240, DO-1, and PAb122) or normal mouse immunoglobulin G (IgG; negative control), and associated hEcd was detected with anti-hEcd rabbit antibody.

Assessment of p53-dependent transactivation using luciferase reporters. 21PT cells (5×10^5 per 100-mm dish) were transfected with pCMV-p53 and pcDNA3.1-FLAG-hEcd together with the indicated luciferase reporters and SV40-Renilla luciferase reporter (pRL-SV40; transfection efficiency control). Luciferase activity was measured as previously described (17). Similar results were obtained with another cell line, Saos-2 (data not shown). For the assessment of p53-dependent luciferase reporter activity following hEcd knockdown, U2OS cells were transfected using Lipofectamine 2000 (Invitrogen) with control or hEcd siRNA or p53 RNAi (18) and Bax-luciferase reporter. Luciferase activity was measured as described above.

In vivo association of hEcd with Mdm2. H1299 cells (5×10^5 per 100-mm dish) were transfected with pcDNA 3.1-FLAG-hEcd (4 μg) and pCMV-Mdm2 (2.5 μg) using Lipofectamine 2000. Twenty hours after transfection, the cells were treated with MG132 (20 $\mu\text{mol}/\text{L}$) for 4 hours, lysed, and subjected to anti-hEcd immunoprecipitation followed by anti-Mdm2 immunoblotting.

Senescence experiments in WI-38 cells. WI-38 cells were retrovirally infected with pMSCVpuro vector or pMSCVpuro-FLAG-hEcd, selected in 1 $\mu\text{g}/\text{mL}$ of puromycin for 2 days, and then 2×10^4 cells were plated in 35-mm dishes for immunoblotting, [³H]thymidine incorporation, and senescence-associated β -gal (SA- β -gal) assays. These assays were done as previously described (19, 20).

Cell proliferation assay on expression of hEcd siRNA in p53-positive and p53-negative cells. Cells (1×10^5 ; two p53-positive cell lines, U2OS and MCF-7, and two p53-negative cell lines, H1299 and 21PT) were plated in six-well plates; after 24 hours, cells were transfected with 200 pmol siRNA duplex per well using Lipofectamine 2000. Twenty-four hours after transfection, equal numbers of cells were seeded in six-well plates for growth analyses. The cells were trypsinized and counted at the indicated time points. Cell lysates were analyzed at day 1 for protein expression by immunoblotting.

Results

Expression of hEcd protein in human cell lines. To examine the expression pattern of hEcd in human cells, we generated an anti-hEcd rabbit polyclonal antiserum using purified GST-hEcd (amino acids 291-644) as an immunogen. This antibody identified 73-kDa polypeptide in the whole-cell detergent extracts of all cell lines tested (Fig. 1A). When cell extracts were analyzed after biochemical fractionations, hEcd was detected in both nuclear and cytoplasmic fractions and the proportion of hEcd in the nuclear fraction increased on leptomycin B treatment (leptomycin B inhibits nuclear export; Fig. 1B). The relative purity of the subcellular fractions was shown by immunoblotting for nuclear poly(ADP-ribose) polymerase and cytoplasmic (α -tubulin) markers (Fig. 1B). Thus, hEcd seems to be localized in the nucleus as well as the cytoplasm.

hEcd interaction with p53. Given the fact that hEcd does not exhibit any known protein-protein interaction motifs, and the propensity of several HPVE6 binding proteins, such as E6AP, p300, and ADA3, to interact with p53, we examined if hEcd might interact with p53. GST-hEcd (Fig. 2A), but not GST, bound to 35 S-labeled p53 generated by *in vitro* translation in rabbit reticulocyte lysates. Conversely, 35 S-labeled hEcd generated by *in vitro* translation in rabbit reticulocyte (Fig. 2B) exhibited binding to GST-p53, but not to GST (control); GST-p53 binding to 35 S-labeled p53 (due to oligomerization; ref. 21) served as a positive control (Fig. 2B, bottom).

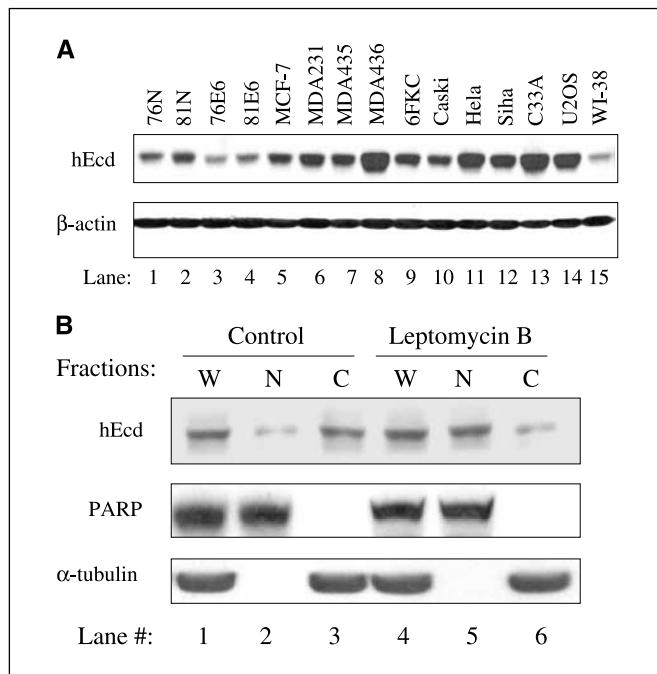


Figure 1. A, hEcd protein expression in cultured cells. Whole-cell lysates from normal (76N and 81N; lanes 1 and 2), immortal (76E6 and 81E6; lanes 3 and 4) mammary epithelial cells, mammary tumor (MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-436; lanes 5-8), normal foreskin keratinocyte (6FKC; lane 9), cervical cancer (Caski, HeLa, Siha, and C33A; lanes 10-13), osteosarcoma (U2OS; lane 14) cell lines, and lung fibroblast (WI-38; lane 15) cells were immunoblotted with indicated antibodies. B, biochemical localization of hEcd in mammalian cells. Nuclear and cytoplasmic extracts were prepared from control or leptomycin B-treated U2OS cells and 40- μ g aliquots of protein lysates were subjected to immunoblotting with the indicated antibodies. W, whole-cell lysate; N, nuclear extract; C, cytoplasmic extract.

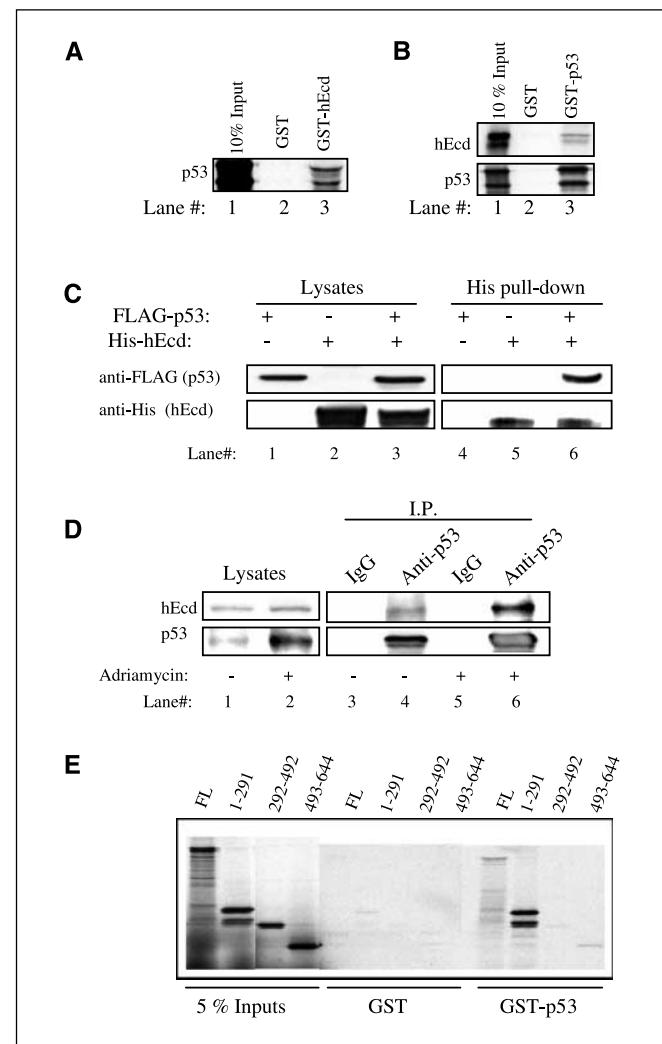


Figure 2. Interaction of hEcd with p53. A and B, *in vitro* binding of hEcd to p53. 35 S-labeled p53 (A and B) or hEcd (B) was generated by *in vitro* translation in rabbit reticulocyte lysates, incubated with indicated GST fusion proteins, and analyzed. C, association of overexpressed hEcd and p53 *in vivo*. 293T cells were transfected with 1 μ g of pCR3.1-FLAG-p53 and/or 8 μ g of pEF6/His-hEcd. Aliquots of cell lysates (400 μ g) were incubated with His-Bind resin to pull down transfected hEcd. Bead-bound hEcd-associated p53 was detected by anti-FLAG immunoblotting (top right, lanes 4-6) side by side with cell lysate immunoblotting (top left, lanes 1-3). Bottom, reprobing with anti-His antibody to detect transfected hEcd. D, association between endogenous hEcd and p53. Lysates were prepared from control or Adriamycin-treated U2OS cells and 800- μ g aliquots of lysate protein were subjected to immunoprecipitation with control mouse IgG or anti-p53 cocktail antibodies, followed by anti-hEcd and anti-p53 immunoblotting. E, *in vitro* binding of hEcd or its deletion mutants to GST-p53. 35 S-labeled hEcd or its deletion mutants were generated using rabbit reticulocyte lysates and equal aliquots were allowed to bind to 3 μ g of GST or GST-p53.

Following the results of the *in vitro* binding, we examined the association of hEcd and p53 *in vivo*. We cotransfected 293T cells with His-hEcd and FLAG-p53, affinity-isolated the His-hEcd protein on His-Bind resin, and assessed the presence of associated FLAG-p53 by anti-FLAG immunoblotting. A clear association between exogenously expressed p53 and hEcd was seen (Fig. 2C, lane 6). Next, we examined if endogenous hEcd and p53 also associate by doing anti-hEcd immunoblotting of anti-p53 immunoprecipitations from lysates of untreated or Adriamycin-treated U2OS cells. hEcd was detected in immunoprecipitations with anti-p53 (Fig. 2D, top, lane 4) but not with IgG (Fig. 2D, top, lane 3), and treatment with

Adriamycin, which expectedly increased the p53 levels, substantially increased the level of p53-associated hEcd (Fig. 2D, top, lane 6). Transfected FLAG-hEcd also associated with endogenous p53 in U2OS cells and this association increased on Adriamycin treatment (data not shown).

Specific binding of p53 to hEcd was assessed by doing *in vitro* binding using purified GST-p53 and ³⁵S-labeled hEcd NH₂-terminal, middle, and COOH-terminal fragments (described above). This *in vitro* binding shows that the NH₂-terminal region of hEcd interacts with p53 (Fig. 2E). These results strongly support the conclusion that hEcd and p53 associate both *in vitro* and *in vivo* in mammalian cells.

hEcd promotes the transcriptional activation function of p53. To assess if the hEcd-p53 association changes p53 function, we transfected p53-negative 21PT cells (15) with a fixed amount of p53 and increasing amounts of hEcd, together with a p53-responsive artificial luciferase reporter (pG13-luciferase; ref. 22) or luciferase linked to promoters of known p53 target genes. Transfection of p53, as expected, increased the reporter luciferase activity (Fig. 3). Significantly, cotransfection of hEcd dose-dependently enhanced the effect of p53 on all of the reporters tested, albeit to varying degrees (Fig. 3A-E). hEcd transfection without p53 had no effect on any of the reporters, indicating that

hEcd effects were p53 dependent (Fig. 3). Furthermore, no luciferase activity was seen when MG₁₅ luciferase (with a mutant p53-binding site; ref. 22) was cotransfected with p53 or p53 plus hEcd (data not shown). Notably, immunoblotting analysis of cell lysates used in luciferase assays showed that hEcd dose-dependently increased the levels of p53 protein (Fig. 3). Thus, hEcd-dependent increase in p53 levels promotes the function of p53 to transactivate target genes.

Next, we addressed if the endogenous hEcd was required for the p53-mediated transcriptional response. For this purpose, we introduced the p53 reporter Bax-luciferase into U2OS cells that were transiently transfected with control siRNA or two distinct hEcd siRNA oligonucleotides; immunoblotting confirmed a substantial decrease in hEcd protein levels in cells with both siRNAs (Fig. 3F). Cells with p53 RNAi (used as a control) had reduced levels of p53 (Fig. 3F). Indeed, reduced Bax-luciferase activity was seen in both hEcd siRNA transfectants and p53 siRNA-transfected cells when compared with the control siRNA (Fig. 3F). Furthermore, a substantial decrease in the protein levels of p53 target genes, *p21*, *Bax*, and *Mdm2*, was observed on knockdown of hEcd (Fig. 3G) under both untreated and Adriamycin-treated conditions, suggesting that hEcd controls p53 level under physiologic conditions. Next, we assessed the

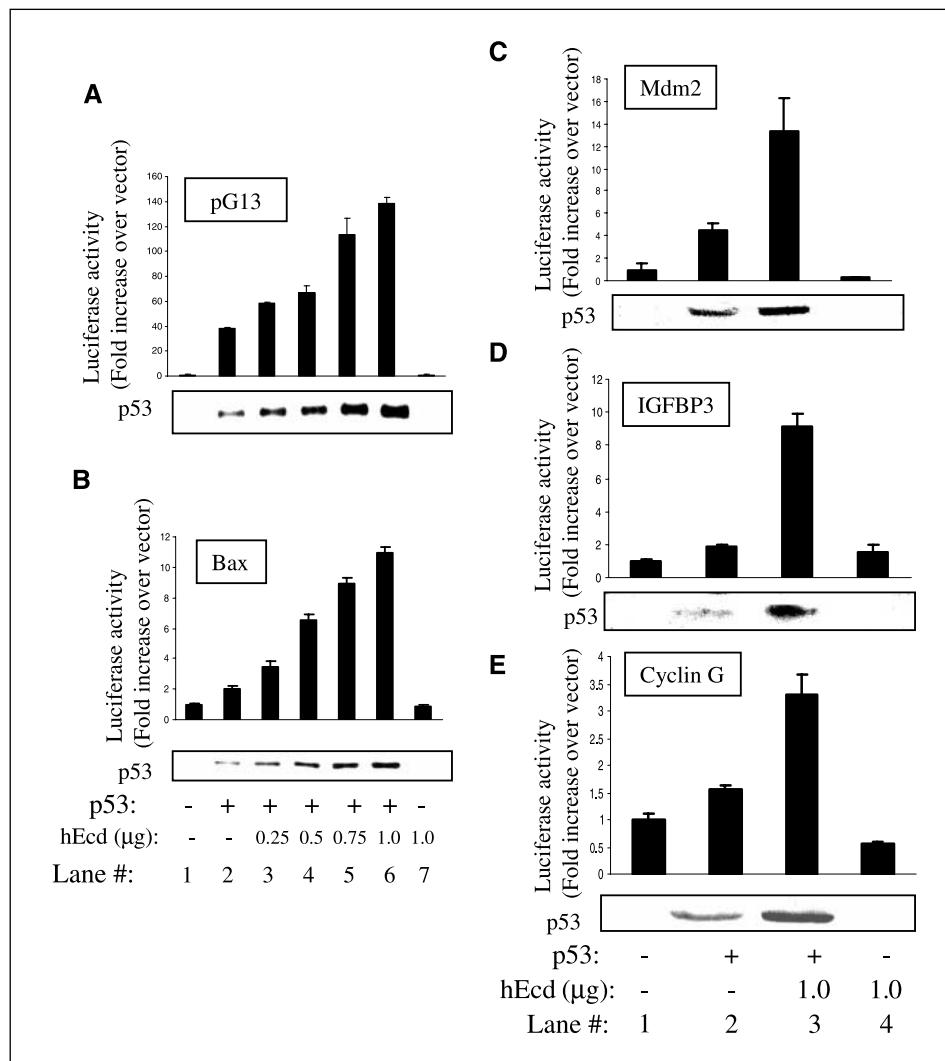


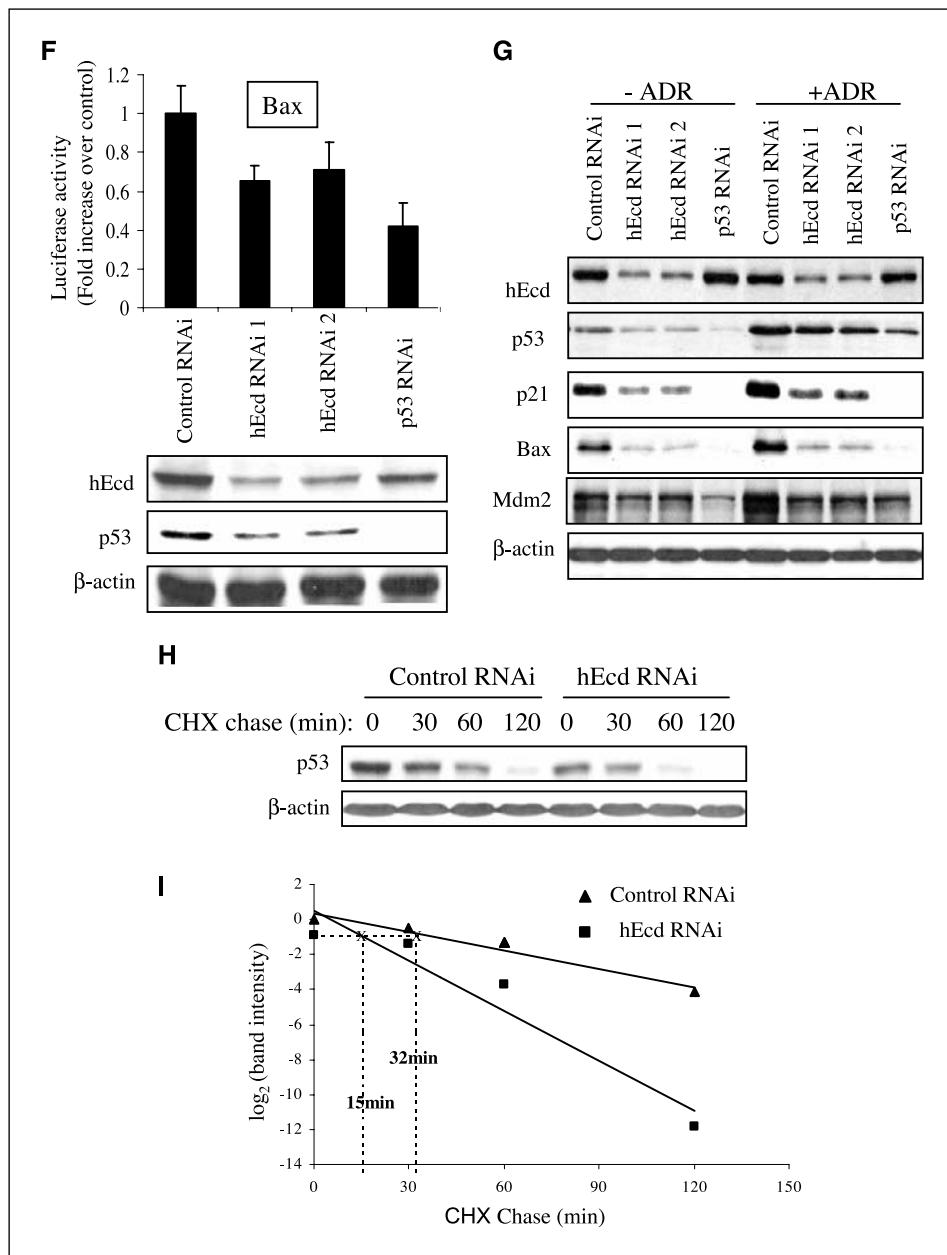
Figure 3. hEcd regulates p53 stability and transactivation. *A* to *E*, hEcd enhances p53-mediated transactivation by stabilizing p53 protein. 21PT cells were transfected with pCMV-p53 [10 ng (*A*); 25 ng (*B-E*)] and/or the indicated amounts of pcDNA3.1-FLAG-hEcd together with the indicated promoter-luciferase reporters [50 ng (*A*); 500 ng (*B-E*)] and pRL-SV40 (20 ng). The cells were lysed and the luciferase activity was measured. Results are shown as fold activity over vector alone after normalizing for transfection efficiency against *Renilla* luciferase activity. The lysates of cells used in luciferase assays were analyzed for p53 levels by immunoblotting (*bottom*).

half-life of the endogenous p53 after cycloheximide-induced block of new protein synthesis in cells expressing either control RNAi or hEcd siRNA. Immunoblotting analysis of cell lysates showed that the half-life of the endogenous p53 was reduced from ~30 minutes in control siRNA-treated cells to ~15 minutes in hEcd siRNA-treated cells (Fig. 3H and I). These results show that hEcd regulates the stability of p53 protein. Together, these analyses clearly show that hEcd interacts with p53 and increases p53-mediated transactivation by stabilizing the p53 protein. These results support the idea that hEcd plays a critical role in promoting the function of p53 as a transcription factor, which in turn mediates the p53-dependent functional effects of hEcd.

hEcd stabilizes p53 by negating Mdm2-dependent p53 degradation. As p53 turnover is regulated by a balance between its synthesis and Mdm2-dependent proteasomal degradation (23, 24), we wished to investigate if hEcd may counter Mdm2-

mediated p53 degradation. First, we examined the *in vivo* association between hEcd and Mdm2 in H1299, a p53-null cell line with low levels of Mdm2 and widely used for Mdm2 association experiments (25). H1299 cells were transfected with FLAG-tagged hEcd (to distinguish it from endogenous hEcd) and Mdm2 and cell lysates were prepared as such or after MG132 treatment. Anti-Mdm2 immunoblotting of anti-hEcd immunoprecipitations showed a clear association between hEcd and Mdm2 when both were cotransfected and cells were treated with MG132 (Fig. 4A, lane 8). No clear binding was noticed without MG132 treatment, probably due to the low levels of Mdm2 expression (data not shown). hEcd-Mdm2 association suggested that hEcd may stabilize p53 by inhibiting its Mdm2-mediated degradation. Notably, lack of stabilization of p53 was seen when hEcd was cotransfected with p53 into p53/Mdm2-null MEFs (ref. 26; Fig. 4B, lanes 1 and 2). Furthermore, cotransfection of hEcd together with p53 and Mdm2

Figure 3 Continued. F, knockdown of hEcd by siRNA reduces p53 protein levels and decreases p53-mediated transcriptional activation. U2OS cells in six-well plates were transfected with 200 pmol of the indicated siRNA duplexes together with 150 ng of Bax-luciferase reporter and 10 ng of pRL-SV40. Twenty-four hours posttransfection, lysates were analyzed for luciferase activity as in (A-E). Columns, mean of triplicates; bars, SD. Representative of three experiments. Fifty-microgram aliquots of lysate proteins from the same assay were immunoblotted with indicated antibodies. G, knockdown of hEcd by siRNA decreased the protein levels of p53 and its targets. U2OS cells in six-well plates were transfected with 200 pmol of the indicated siRNA duplexes. Twenty-four hours posttransfection, cells were treated with or without Adriamycin (0.5 µg/mL; Sigma) for 8 hours. Fifty-microgram aliquots of lysate proteins were immunoblotted with indicated antibodies. H and I, hEcd increases half-life of p53 protein. U2OS cells in six-well plates were transfected with 200 pmol of control or hEcd siRNA duplexes (hEcd RNAi). Twenty-four hours posttransfection, cycloheximide (25 µg/mL) was added and cell lysates were prepared at the indicated time periods. Fifty-microgram aliquots of lysate proteins were analyzed by immunoblotting. The p53 bands were quantified by densitometry against β-actin control using Scion Image software and plotted against time of cycloheximide treatment. Each decrease of 1 unit of log₂ (band intensity) is equivalent to one half-life (35).



in p53/Mdm2-null MEFs reduced the extent of Mdm2-mediated degradation of p53 (Fig. 4B, lanes 3 and 4). An equal amount of green fluorescent protein controls the transfection efficiency. These results showed that Mdm2 is required for hEcd-mediated stabilization of p53 protein.

Overexpression of hEcd leads to p53-dependent accelerated cellular senescence. Next, we examined the effects of hEcd overexpression on replicative senescence of WI-38 cells, a normal human lung-derived fibroblast cell line widely used for such studies (19). When retrovirally infected WI-38 cells were selected in puromycin for 48 hours and replated, maximal FLAG-hEcd expression was detected around day 6 postinfection (4 days postselection; Fig. 5A). Analysis of [³H]thymidine incorporation into nuclei at the peak of hEcd expression (day 6) revealed marked inhibition of cell proliferation in hEcd-overexpressing cells (Fig. 5B). Whereas control cells maintained their normal morphology, many of the hEcd-overexpressing cells became enlarged and attained a flat morphology, characteristic of senescence (Fig. 5C). Staining for SA- β -gal, a widely used histochemical marker of senescence (20), showed a nearly 3-fold increase in the proportion of senescent cells in hEcd-overexpressing cells compared with controls (Fig. 5D). These results indicated that hEcd overexpression accelerated the replicative senescence program in WI-38 cells.

Reprobing of blots of hEcd-overexpressing WI-38 cells showed an increase in p53 levels that coincided with peak hEcd overexpression (Fig. 5A); concomitantly, the levels of p53 target p21 were also elevated (Fig. 5A), suggesting that transcriptional activation of p53 may contribute to hEcd-induced senescence.

Consistent with this hypothesis, hEcd overexpression caused acceleration of senescence program in p53^{+/+}, but not in p53^{-/-}, MEFs (Fig. 5E and F) and in p53^{-/-} MEFs reconstituted with a temperature-sensitive p53 that is functional at 32°C and nonfunctional at 37°C (ref. 27; Fig. 5G). Together, these analyses provide strong evidence that hEcd overexpression activates a p53-dependent accelerated cellular senescence program.

Transient knockdown of endogenous hEcd expression increases cell proliferation in p53-positive but not in p53-negative cells. Next, we asked if hEcd knockdown can produce an opposite phenotype (i.e., enhance proliferation). Stable retrovirus-mediated knockdown of hEcd could not be used because both p53-positive and p53-null cells underwent growth inhibition and cell death, apparently due to a p53-independent role of hEcd in maintaining cell viability (data not shown). Therefore, we assessed cell proliferation after transient hEcd knockdown using double-stranded siRNA transfection in two p53-positive (U2OS and MCF-7) and two p53-negative (H1299 and 21PT) cell lines (poor transient transfection efficiency precluded the use of MEFs and WI-38). hEcd knockdown in U2OS and MCF-7 cells resulted in increased proliferation, similar to the effect of p53 knockdown (Fig. 6B and D) when assessed within 4 days posttransfection. These effects were p53 dependent as hEcd knockdown did not change the rate of proliferation of H1299 and 21PT cells (Fig. 6F and H). Notably, hEcd knockdown led to a substantial reduction in p53 levels in U2OS and MCF-7 cells (Fig. 6A and C). Taken together, these results strongly support a role for hEcd in the induction of p53-mediated cellular senescence.

Discussion

The p53 gene mutations remain the most frequent genetic alterations in human cancers. During various stress stimuli, the p53 protein is modified posttranslationally, and this leads to either cell cycle arrest, cellular senescence, or apoptosis, processes widely believed to prevent the emergence of cells with altered genome and increased propensity for oncogenic transformation (5, 28). Thus, a better understanding of how the levels of p53 protein and its functions are precisely regulated is of considerable significance.

Here, we report the identification and characterization of hEcd as a novel p53-interacting protein. Overexpression of hEcd promoted the accumulation of p53 protein. Conversely, RNAi knockdown studies showed that hEcd is a positive regulator of endogenous p53 protein levels. Furthermore, we show that hEcd interacts with p53 protein and enhances the p53-mediated transactivation and function. Altogether, these data provide strong support for the idea that hEcd is a novel regulator of p53.

During our analyses of the role of hEcd in p53-mediated transactivation, we consistently observed changes in p53 protein levels. We reasoned that the influence of hEcd on p53 levels could be indirect or a more direct action involving physical interaction with p53. A major cellular protein involved in the regulation of p53 stability is the ubiquitin ligase Mdm2. Our coimmunoprecipitation analyses established that hEcd interacts with p53 as well as Mdm2. More detailed studies to identify the structural determinants on hEcd, p53, and Mdm2 that mediate their interactions are required to definitely define the basis of these associations. Because Mdm2 and p53 are known to interact directly, hEcd, p53, and Mdm2 could form a trimeric complex, although competition of Mdm2 binding to p53 by hEcd remains an alternative similar to binding of p300 within Mdm2 binding region on p53 (29).

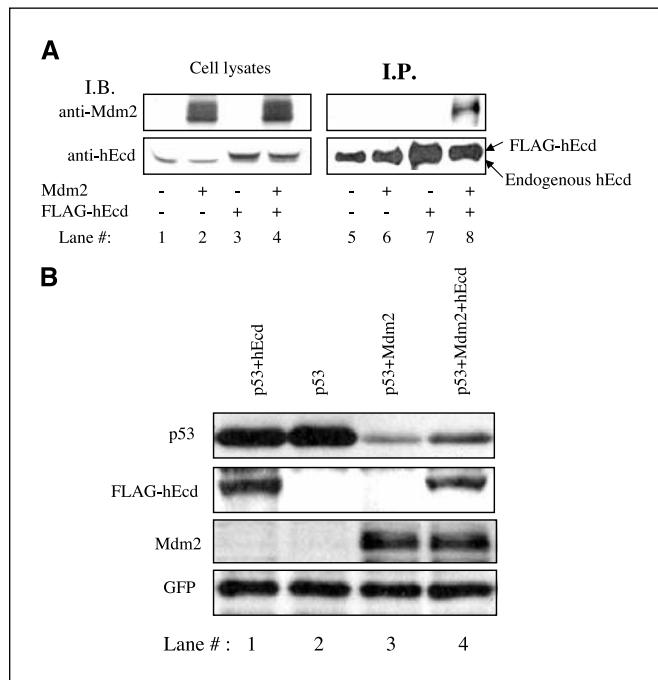
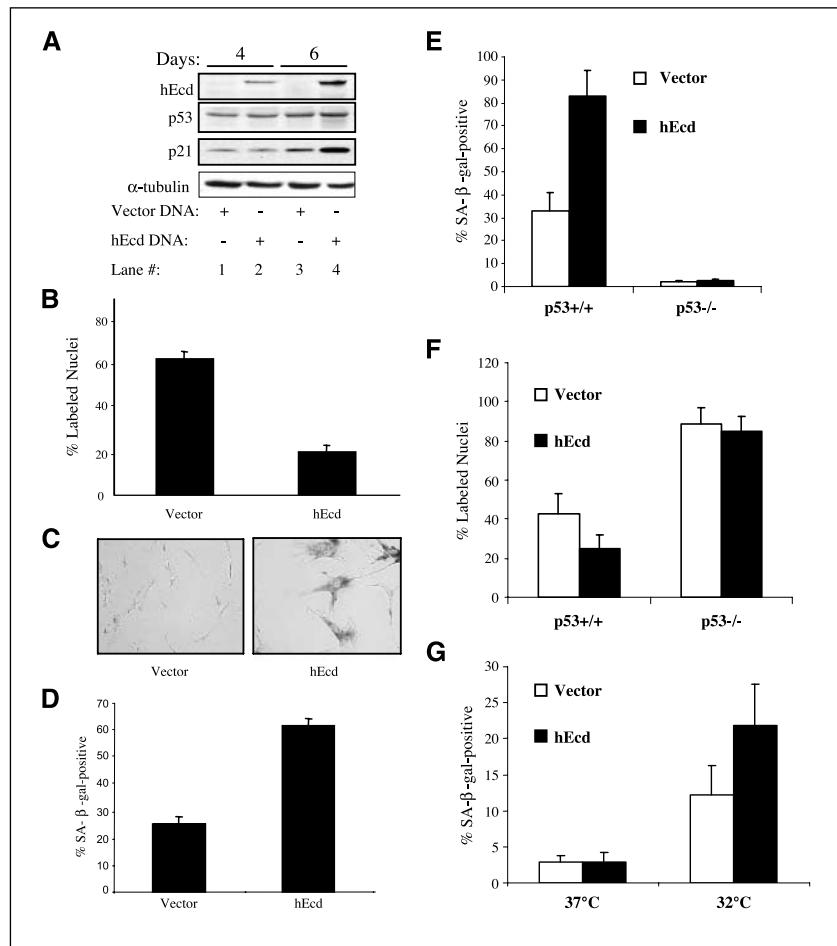


Figure 4. hEcd regulates p53 stability by associating with Mdm2 and inhibiting Mdm2-mediated p53 degradation. *A*, hEcd associates with Mdm2 *in vivo*. p53-null H1299 cells were transfected with pcDNA-FLAG-hEcd and/or pCMV-Mdm2, as indicated. Cells were treated with MG132 and lysates were immunoprecipitated using anti-hEcd antibody, followed by immunoblotting with indicated antibodies. *B*, hEcd overexpression inhibits Mdm2-dependent p53 degradation. p53/Mdm2-null MEFs were transfected with pcDNA-FLAG-hEcd, pCMV-Mdm2, pCMV-p53, and pEGFP vector in the indicated combinations using Lipofectamine 2000. Lysate proteins were subjected to immunoblotting using the indicated antibodies.

Figure 5. hEcd overexpression induces senescence in WI-38 cells. WI-38 cells were infected with retroviruses expressing FLAG-hEcd or empty vector, selected in puromycin for 2 days, and plated in 35-mm dishes for various analyses. *A*, aliquots (100 µg) of lysate protein were immunoblotted for hEcd (anti-FLAG), p53 (DO-1 monoclonal antibody), and p21 (F-5 antibody) proteins. *B*, the vector or hEcd transfected cells were analyzed for [³H]thymidine incorporation using autoradiography and enumeration of labeled nuclei at day 6 postinfection corresponding to peak hEcd expression. Two hundred cells were counted and the data are expressed as percentage of total cells with labeled nuclei. *C* and *D*, cells were stained for SA-β-gal at day 6 postinfection and photographed under phase-contrast optics at ×10 magnification. *C*, a representative field. *D*, quantification based on enumeration of SA-β-gal-positive cells in 200 cells counted. *E* to *G*, p53^{+/+} and p53^{-/-} MEFs (*E* and *F*) or p53-null MEFs reconstituted with a temperature-sensitive p53 mutant (*G*) were retrovirally infected as above, selected in puromycin for 2 days, and analyzed for senescence using SA-β-gal staining (*E* and *G*) and DNA synthesis (*F*) as above. *B*, *D*, *E*, *F*, and *G*, columns, mean of triplicates; bars, SD. Representative of three experiments.



Physical association of hEcd with p53 and Mdm2 suggested the possibility that hEcd controls p53 levels by antagonizing Mdm2-mediated p53 degradation. Indeed, overexpression of hEcd and p53 in p53/Mdm2-null MEFs did not stabilize p53 whereas reconstitution of Mdm2 resulted in hEcd-mediated p53 stabilization. How hEcd antagonizes the function of Mdm2 towards p53 remains to be determined. Aside from the physical competition mechanism alluded to above, hEcd may mask the sites of ubiquitination on p53 and/or facilitate the recruitment of deubiquitinating enzymes (30). Whereas future studies will address these important issues, our RNAi analyses clearly indicate that hEcd is a physiologic component of the biochemical networks that control the levels of p53. Whereas we have only assessed the role of Mdm2 in hEcd-mediated p53 stabilization, it remains possible that other recently identified E3 ligases of p53, such as COP1, pifh2, and ADP ribosylation factor-BP1/Mule (31–33), may also play a role in hEcd-mediated p53 stabilization.

As hEcd localizes in the nucleus as well as the cytosol, it was important to assess that hEcd-dependent stabilization of p53 correlates with an increase in the transcriptionally competent pool of p53. Indeed, our analyses of reporters incorporating consensus p53-binding sequences or natural target gene promoters directly established that hEcd regulates the levels of transcriptionally active pool of p53. As hEcd is also present in the cytoplasm, it will be of considerable future interest to determine if it might compete with PARC, a cytosolic protein that binds to and retains p53 in the cytoplasm and thereby antagonizes p53 function (16). At what step during the nuclear transport of p53 the hEcd protein associates with

it and what accessory proteins and/or posttranslational modifications promote this association will be critical questions for further analyses. It is important to note that hEcd does not contain a consensus nuclear localization signal, yet our leptomycin B experiments clearly indicate that this protein shuttles into and out of the nucleus. It remains possible that hEcd is imported into the nucleus by other nuclear proteins. Given its association with p53, its shuttling into the nucleus, and its ability to modulate p53-mediated transcriptional activation, it will be of interest to determine if the nuclear hEcd functions as a p53 coactivator like p300 and ADA3, two well-known p53 interacting proteins (17, 34).

Whereas hEcd interacts with p53 and we have seen increased association of p53 when cells are exposed to genotoxic stress (which increases p53 levels), we have not observed a detectable change in the levels of hEcd or any change in its gel mobility (which could suggest posttranslational modifications) on treatment of cells with DNA damaging agents (Adriamycin, actinomycin D, UV, etc.; Fig. 3G and data not shown). However, further studies are required to comprehensively investigate the possible role of hEcd in stress-related functions of p53. Alternatively, cytosolic and/or nuclear hEcd may interact with other partners to mediate p53-independent functions.

Notably, *Drosophila* Ecd protein was shown to have a cell-autonomous function in cell proliferation/survival in steroidogenic as well as nonsteroidogenic tissues. Further biochemical and genetic analyses of Ecd proteins are therefore likely to elucidate novel pathway of cell proliferation/survival control. Our analyses showing the role of hEcd as a regulator of stability of a

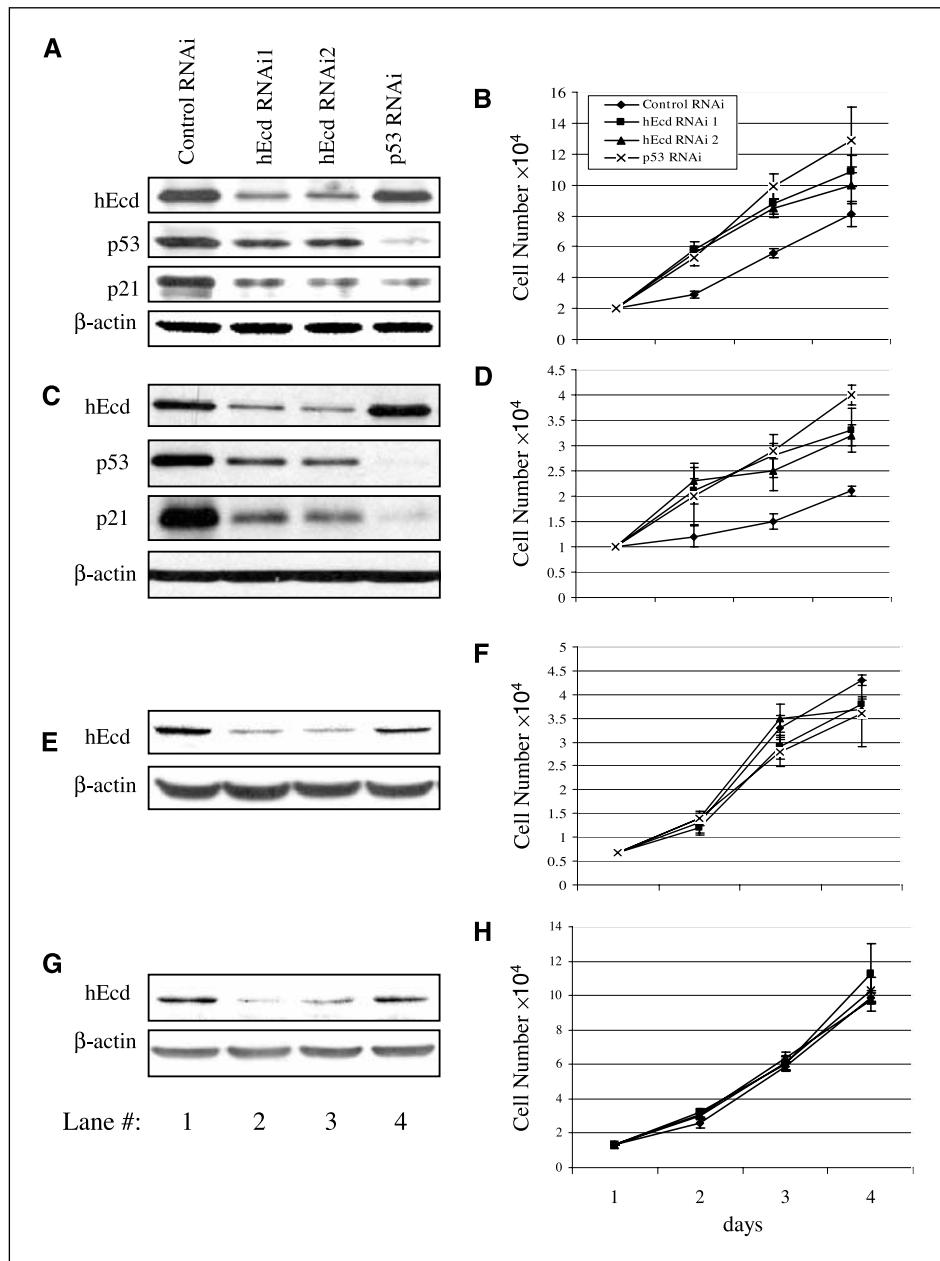


Figure 6. RNAi knockdown of endogenous hEcd expression enhances the proliferation of p53-positive but not of p53-null cells. p53-positive U2OS and MCF-7 (A–C) or p53-negative H1299 and 21PT (E–H) cells in six-well plates were transfected with indicated siRNA duplexes per well. A, C, E, and G, fifty-microgram aliquots of cell lysates were analyzed by immunoblotting with indicated antibodies after 24 hours. B, D, F, and H, parallel cultures of cells were plated in six-well plates, trypsinized at the indicated time points, and counted using Z1 Coulter Particle Counter (Beckman Coulter, Fullerton, CA). Points, mean of triplicates; bars, SD. Representative of three experiments.

transcriptional activator provide new insights into the possible biochemical basis for the function of hEcd protein.

In conclusion, we have identified the hEcd protein as a novel regulator of p53 stability and function. Future studies will address the further mechanisms by which hEcd regulates p53 and the biological roles of this interaction.

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Correction

hEcd, a p53 Regulator

In the article on hEcd, a p53 regulator, in the July 15, 2006 issue of *Cancer Research* (1), Jianming Chen should have been included as the second author.

1. Zhang Y, Chen J, Gurumurthy CB, Kim J, Bhat I, Gao Q, Dimri G, Lee SW, Band H, Band V. The human orthologue of *Drosophila* ecdysoneless protein interacts with p53 and regulates its function. *Cancer Res* 2006;66:7167–75.

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