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TXNIP interacts with hEcd to increase p53 stability and activity

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ABSTRACT

The p53 protein plays a central role in cell cycle arrest and apoptosis in response to diverse stress stimuli. Human ecdysoneless (hEcd) is known for its role in stabilizing the p53 protein level and increasing p53-mediated transcription. Here, we report that thioredoxin interacting protein (TXNIP), a member of the tumor suppressor family, interacts with hEcd and decreases MDM2-mediated p53 ubiquitination, leading to p53 stabilization and an increase in p53 activity. The ectopic overexpression of both TXNIP and Ecd increased actinomycin D-mediated cell death in MCF-7 cells, whereas knockdown of TXNIP and Ecd decreased cell death. These results show that TXNIP is a new regulator of the Ecd-MDM2-p53 loop.

1. Introduction

Ecd (ecdysoneless) is required for normal embryogenesis, adult eclosion, and oocyte development [1–3]. Ecd has been implicated in the regulation of ecdysteroid synthesis in *Drosophila melanogaster* [4,5]. Ecd loss disrupts production of the steroid hormone ecdysone and causes abnormal lymph gland cell division and defective nerve terminal development [6,7]. The human Ecd ortholog, known as hGST1 (human suppressor of glucocorticoid receptor (GCR) 2), is highly expressed in muscle and the heart and rescues the growth defect induced by the *gcr2* mutation, in which glycolytic genes transcription is dysregulated [8]. In a recent study, hEcd was identified as a novel p53-interacting protein [9]. hEcd interacts with murine double minute-2 (Mdm2) and stabilizes p53 by inhibiting the Mdm2-mediated degradation of p53 [9]. Additionally, Ecd-null mouse embryonic fibroblasts show a delay in G_1-S cell cycle progression [10]. Ecd acts in the cell cycle via the Rb-E2F pathway by directly binding to Rb and inhibiting Rb-E2F binding, thereby inducing E2F target gene expression and cell cycle progression [10]. Ecd expression is highly expressed in breast cancer tissue compared with normal breast tissue [11]. Ecd shuttles between the nucleus and the cytoplasm, and its strong cytoplasmic localization depends on active CRM1-mediated nuclear export [12]. In the nucleus, Ecd interacts with p300 to increase the transactivation activity [12].

TXNIP, also known as vitamin D3-upregulated protein 1 (VDUP1) and thioredoxin (TRX)-binding protein-2, is involved in many cellular activities. It inhibits tumor cell growth by regulating cell cycle progression [13]. The nuclear localization of TXNIP with importin α_1 (Rch1) leads to growth suppressive activity in MCF-7 cells [14]. TXNIP forms a transcriptional repressor complex through interacting with promyelocytic leukemia zinc-finger, Franconi anemia zinc-finger, and histone deacetylase 1, which are transcriptional corepressors [13]. TXNIP is also dominantly expressed during the apoptotic process of various cells. TXNIP overexpression alone is not sufficient to induce apoptosis, and additional stimuli, such as genotoxic agents, are required to induce cell apoptosis [15].

We used a yeast two-hybrid screening strategy to identify novel binding partners of TXNIP. We identified hEcd in a HeLa cDNA library and investigated the functional significance of interaction between TXNIP and hEcd.

2. Materials and methods

2.1. Cell lines

Human HeLa, 293T, MCF-7 and HCT 116 cells were used in this study. The cells were grown at 37 °C in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS, L-glutamate, penicillin, and streptomycin in a humidified 95% air and 5% CO_2 incubator.

2.2. Yeast two-hybrid screen

A yeast two-hybrid screen was performed according to the methods of Gyuris et al. [16]. Full-length human TXNIP (391

Abbreviations: hEcd, human ecdysoneless; Mdm2, murine double minute-2; TRX, thioredoxin; TXNIP, thioredoxin interacting protein.

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aa: NM_006472) was cloned into the EcoRI/PstI sites of the pGBKL vector. Human HeLa cDNA library inserts were cloned as SalI/NotI fragments into pPC86. pGBKL-TXNIP was used as a bait and the HeLa cDNA library was used for the yeast two-hybrid screen.

2.3. Precipitation of GST-fusion proteins and co-immunoprecipitation assay

HeLa and 293T cells were lysed at 4 °C in lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 10% glycerol, 20 mM HEPES (pH 7.2), and a 1 × protease inhibitor mixture (Calbiochem). GST fusion protein pull-down assays were performed through the incubation of 500 μl of lysate with glutathione-Sepharose 4B (GE Healthcare) for 12 h at 4 °C. The co-immunoprecipitation of endogenous TXNIP with endogenous Ecd was analyzed in HeLa cell lysates. Protein G-agarose beads (Roche) were preincubated with rabbit anti-TXNIP antiserum, mouse anti-Ecd serum or control preimmune serum at 4 °C for 30 min. The mixtures were then incubated with HeLa cell lysates for an additional 5 h. GST complexes and immunocomplexes were washed five times with lysis buffer. The bound proteins were resuspended in SDS sample buffer, subjected to SDS–PAGE, and detected by autoradiography.

2.4. p53-dependent luciferase reporter assay

To analyze p53 activity, cells were split into 12-well plates. On the next day, the cells were transiently cotransfected with the indicated expression vectors with the p53-Luc reporter plasmid and pRL-CMV (Promega), a Renilla-derived luciferase reporter plasmid for transfection efficiency control, using Lipofectamine (Invitrogen) reagents. Following the indicated treatments, the cells were lysed and subjected to a luciferase assay.

2.5. Flow cytometric analysis

H1299 and MCF-7 cells (4×10^5) were seeded into 60 mm plates. After 24 h of incubation, the cells were cotransfected with expression plasmids as indicated in the figure legends and incubated for an additional 16 h in the presence or absence of 10 nM actinomycin. The cells were harvested by trypsinization, washed with 1 × PBS and resuspended in 50 μl of 1 × PBS. Cell death was

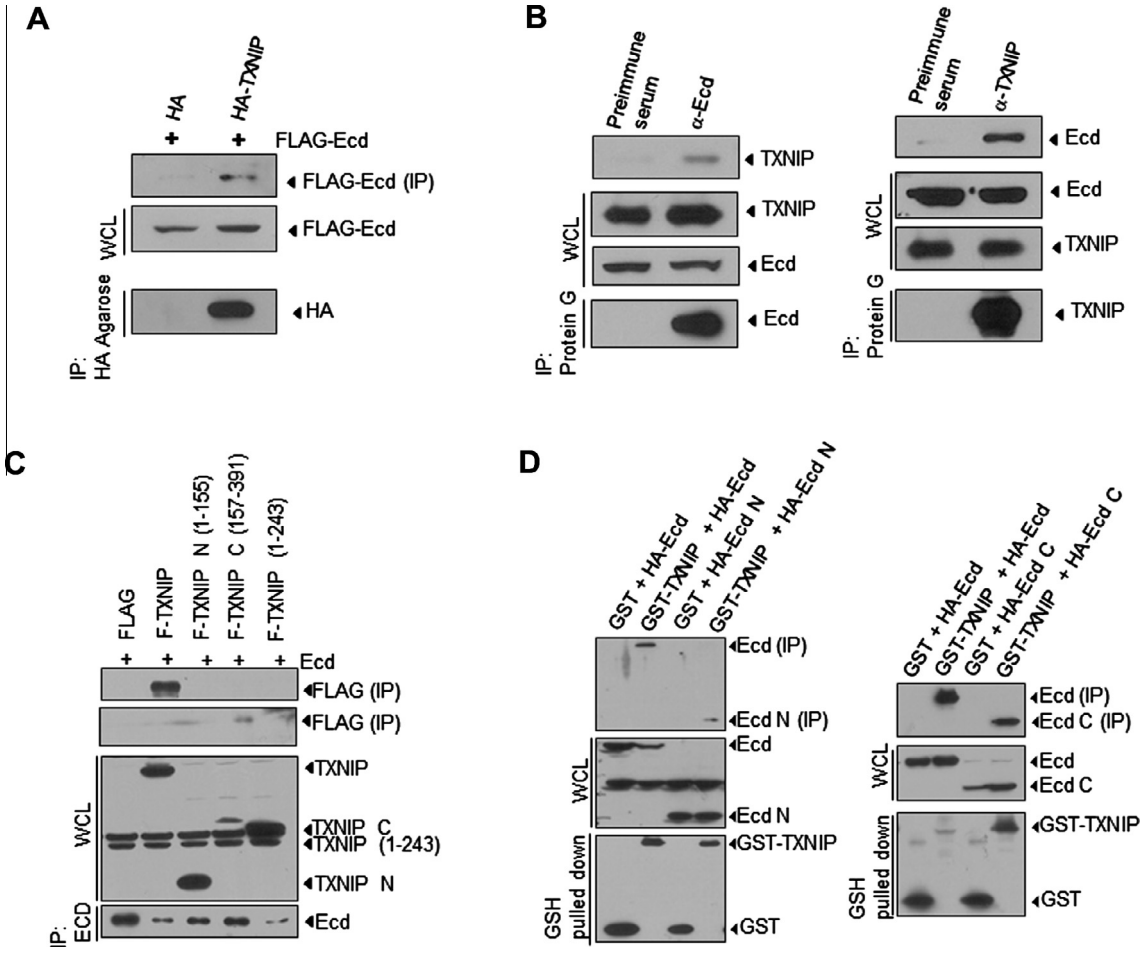

Fig. 1. Interaction between TXNIP and hEcd *in vitro* and *in vivo*. (A) Co-immunoprecipitation of HA-tagged TXNIP and FLAG-tagged hEcd in HEK 293T cells. Immobilized proteins on HA-agarose beads were visualized by immunoblotting with anti-FLAG antibody. (B) Endogenous TXNIP-hEcd complexes were immunoprecipitated from HeLa cell lysates with anti-hEcd (left) or anti-TXNIP (right) antibodies, and the complexes were detected by immunoblotting using anti-TXNIP (left) or anti-hEcd (right) antibodies. (C) Binding of TXNIP or its deletion mutants to hEcd. Co-immunoprecipitation assays were performed using lysates from HEK 293T cells transfected with combinations of pFLAG alone, pFLAG-TXNIP or pFLAG-TXNIP deletion mutants together with the hEcd expression vector. hEcd coprecipitated with vectors containing residues 157–391 of TXNIP and full-length TXNIP. (D) The same cells were transfected with GST-TXNIP and HA-hEcd deletion constructs and precipitated with GSH agarose. GST-TXNIP coprecipitated with vectors containing the COOH-terminal region of hEcd or full-length hEcd.

assessed by flow cytometry following staining with FITC-Annexin V and PI.

3. Results

3.1. Binding of TXNIP to hEcd

Previous studies have suggested that TXNIP has diverse effects on cell proliferation, apoptosis, and tumorigenesis through interactions with many cellular components. To identify novel functions of TXNIP in cellular signaling, we performed a yeast-two hybrid screen. An hEcd clone and multiple TRX cDNA clones were identified in a human HeLa cDNA library and confirmed by the DNA sequence analysis (Supplementary Fig. S1). To confirm the physical interaction between TXNIP and hEcd, co-immunoprecipitation assays were carried out. Expression vectors encoding FLAG-tagged full-length of hEcd were cotransfected into 293T cells in the presence of an expression vector encoding either HA alone or the HA-TXNIP fusion protein. Consistent with the data from the yeast two-hybrid assay, FLAG-hEcd coprecipitated with HA-TXNIP (Fig. 1A). In addition, endogenous TXNIP also bound to endogenous hEcd in HeLa cells (Fig. 1B, left). In reverse order, the specific co-immunoprecipitation of endogenous hEcd was detected through Western blotting with anti-hEcd when anti-TXNIP antibody was used for the immunoprecipitation of endogenous TXNIP (Fig. 1B, right). To further analyze the binding region of TXNIP to hEcd, analyzed the binding of FLAG-tagged deletion constructs of TXNIP to full-length hEcd. hEcd bound to amino acid residues 244–391 of TXNIP (Fig. 1C). Additionally, the C-terminal region of hEcd bound strongly to TXNIP (Fig. 1D). Overall, the results of the protein–protein interaction assays and the yeast two-hybrid screen indicate that TXNIP binds specifically to hEcd.

3.2. TXNIP and Ecd reduce Mdm2-p53 association and Mdm2-dependent p53 degradation

The p53 is a stress-inducible transcription factor that regulates many genes involved in cell cycle arrest, apoptosis, and differentiation. A recent study showed that hEcd stabilizes p53 and that the NH_2-terminal region of hEcd interacts with p53 [9]. We investigated the potential relationship between VDUP and p53. A GST pulldown assay showed that GST-p53 coprecipitated with FLAG-TXNIP, and the binding was also detected under Ecd overexpression (Fig. 2A). p53 stability is primarily regulated by the p53-Mdm2 interaction. Mdm2 contains a p53-binding domain and promotes the ubiquitination and proteasomal degradation of p53 [17,18].

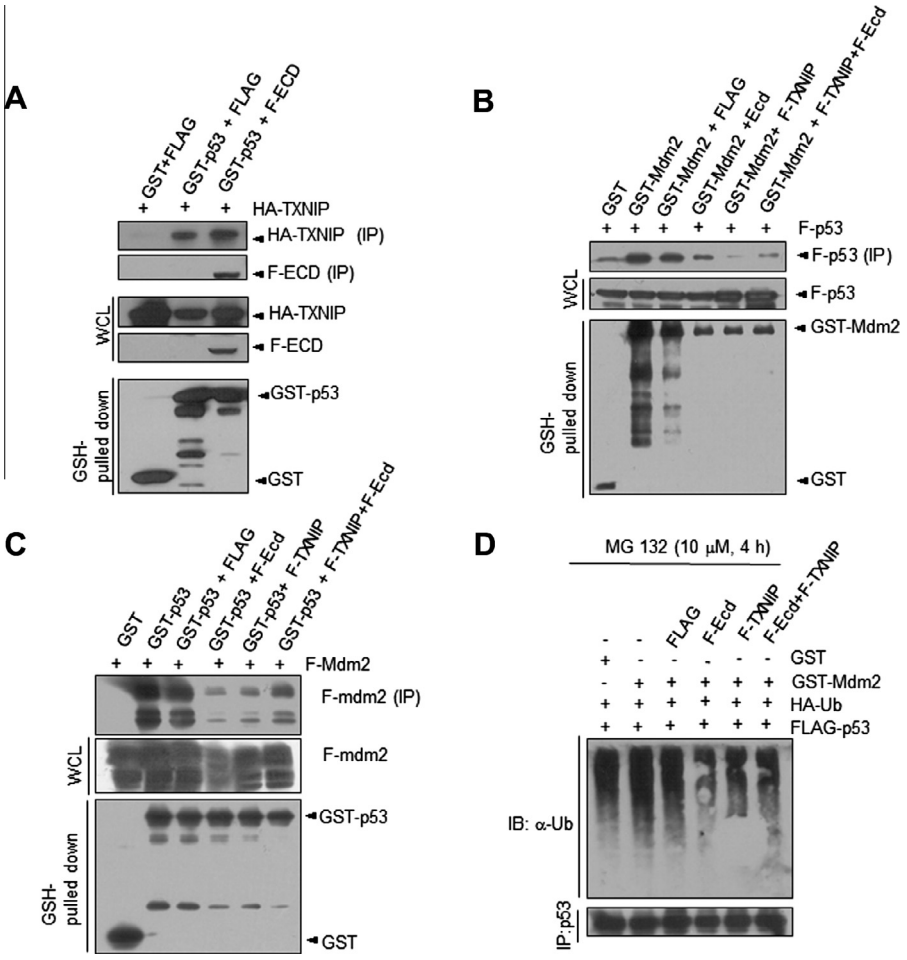

Fig. 2. TXNIP and hEcd reduce p53-Mdm2 association and Mdm2-dependent p53 degradation. (A) HEK 293T cells were transiently cotransfected with either pFLAG or pFLAG-hEcd vector together with pHA-TXNIP and pGST-p53. Lysates were precipitated with GSH agarose and analyzed by Western blotting for HA-TXNIP and FLAG-hEcd. (B) HEK 293T cells were transiently cotransfected with plasmids expressing GST-Mdm2, FLAG-p53, hEcd, or TXNIP as indicated. Cell extracts were subjected to GSH agarose binding. Immobilized proteins on GSH-agarose were visualized by Western blotting using anti-FLAG or anti-GST antibodies. (C) Cells were transfected with the plasmids GST-p53, FLAG-Mdm2, hEcd, or TXNIP as indicated. Lysates were precipitated with GSH agarose and analyzed by Western blotting for FLAG-Mdm2 and GST-p53. (D) HEK 293T cells were cotransfected as indicated and treated for 4 h in the presence of the proteasome inhibitor MG132 (10 μM). Ubiquitinated p53 proteins were immunoprecipitated by with anti-p53 antibody and blotted with anti-Ub antibody.

We examined whether TXNIP was involved in the association between Mdm2 and p53. An expression vector encoding FLAG-tagged p53 was cotransfected into 293T cells in the presence of an expression vector encoding either GST alone or the GST-Mdm2 fusion protein. Expectedly, GST-Mdm2 coprecipitated with FLAG-p53 (Fig. 2B). The ectopic overexpression of TXNIP or hEcd decreased the binding of Mdm2 to p53 (Fig. 2B). TXNIP and hEcd co-overexpression also inhibited Mdm2 binding to p53 at a similar level (Fig. 2B). In reverse order, GST-p53 also coprecipitated with FLAG-tagged Mdm2 (Fig. 2C) and the p53-Mdm2 binding was also decreased by TXNIP or hEcd overexpression (Fig. 2C). The ubiquitin–proteasome pathway plays a major role in the degradation of proteins involved in the cell cycle, differentiation, and cell death. Because Mdm2 mediates the ubiquitin-dependent proteolysis of p53, we examined whether TXNIP or hEcd inhibited Mdm2-mediated p53 ubiquitination. We transfected p53, Mdm2, hEcd, TXNIP, or both TXNIP and hEcd into 293T cells together with a plasmid encoding HA-tagged ubiquitin, as indicated in Fig. 2D. Mdm2 overexpression promoted p53 ubiquitination (Fig. 2D). The ectopic overexpression of TXNIP or hEcd effectively reduced p53 ubiquitination by Mdm2 (Fig. 2D). These results suggest that TXNIP and hEcd increase p53 stability by suppressing the binding activity of Mdm2 to p53.

3.3. TXNIP increases p53-dependent luciferase activity

Based on the observation that both TXNIP and hEcd increase p53 stability, we examined whether ectopically overexpressed TXNIP could modulate p53-responsive luciferase activity. The p53 pathway can activated by low-dose actinomycin D (ActD) [19]. The p53 activation induced by ActD (10 nM) was markedly increased by both TXNIP and hEcd overexpression in HeLa cells (Fig. 3A). TXNIP increased ActD-induced and overexpressed p53-mediated p53 luciferase activity in a dose-dependent manner in HeLa cells transfected with a fixed amount of hEcd (Fig. 3B). In addition, cotransfection with TXNIP and hEcd increased p53-dependent luciferase activity in p53-positive human breast cancer MCF-7 cells (Fig. 3C). Similarly, TXNIP overexpression increased ActD-induced and overexpressed p53-mediated p53 luciferase activity in MCF-7 cells transfected with a fixed amount of hEcd (Fig. 3D). Together, these results indicate that the combination of TXNIP and hEcd increases p53 activity in HeLa and MCF-7 cells.

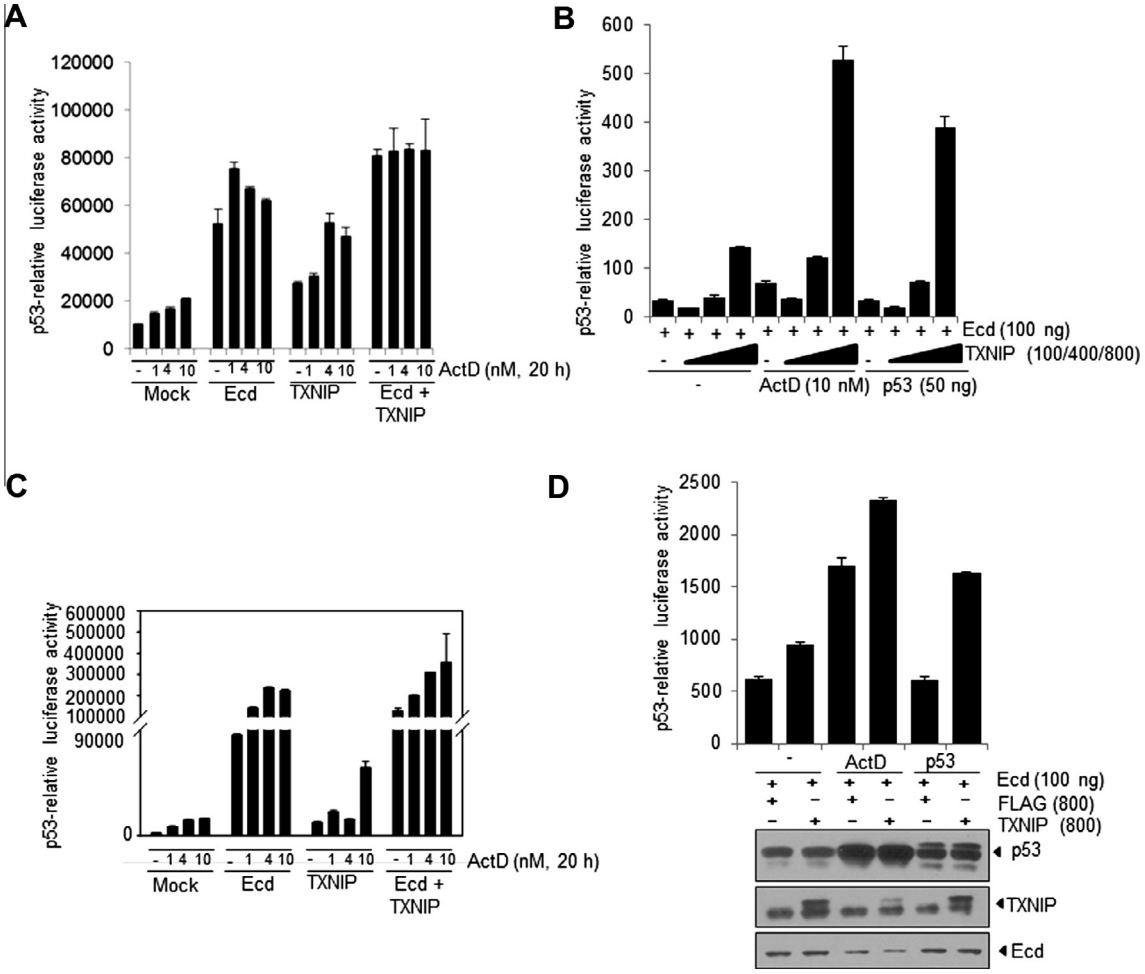

Fig. 3. TXNIP and hEcd enhances p53-associated luciferase activity. (A) HeLa cells were transiently cotransfected with the indicated plasmids together with the p53 reporter plasmid and pRL-CMV. At 8 h posttransfection, the cells were treated with ActD (0, 1, 4, or 10 nM) for 20 h. p53-induced luciferase activities were measured after normalizing for transfection efficiency against Renilla luciferase activity. (B) HeLa cells were transfected with a fixed amount of hEcd vector and increasing amounts of TXNIP together with the p53 reporter plasmid and pRL-CMV with or without the p53 (50 ng/ml) expression vector. The cells were treated with or without 10 nM ActD for 20 h and analyzed for luciferase activity. (C) MCF-7 cells were transfected with Mock, Ecd, or TXNIP together with the p53 reporter plasmid and pRL-CMV. The cells were treated with or without ActD (1.4, or 10 nM) for 20 h and analyzed for luciferase activity. (D) MCF-7 cells were transfected with the indicated amounts of hEcd or TXNIP vectors together with the p53 reporter plasmid and pRL-CMV with or without the p53 (50 ng/ml) expression vector. At 8 h posttransfection, the cells were treated with or without 10 nM ActD for 20 h and analyzed for luciferase activity. TXNIP, Ecd, and p53 protein levels were visualized with Western blotting (*bottom*).

3.4. TXNIP and hEcd increase cells death in response to ActD treatment

Cell death through apoptosis plays a crucial role in physiological and pathological cellular regulation [20]. The metabolic inhibitor ActD is a potent inducer of p53 and increases the sensitivity of many cells to apoptosis in response to death receptor stimulation [19,21]. We investigated whether treatment with low-dose ActD induced cell death in p53-negative H1299 cells and p53-positive MCF-7 cells. Treatment with ActD (10 nM) for 72 h induced significant cell death in p53-positive MCF-7 cells but not in H1299 cells (Fig. 4A). However, treatment with 30 nM ActD induced significant H1299 cell death similar to MCF-7 cell death (Fig. 4A). The ectopic co-overexpression of both TXNIP and hEcd markedly increased cell death in MCF-7 cells at 48 h after 10 nM ActD treatment but did not increase H1299 cell death (Fig. 4B). The p53 protein levels in response to ActD treatment for 20 h were higher in MCF-7 cells in which TXNIP or Ecd was overexpressed than those in control cells (Supplementary Fig. S2A). Next, we determined whether small interfering RNA corresponding to both TXNIP and Ecd could decrease cell death. The co-transfection of si-TXNIP and si-Ecd into MCF-7 cells inhibited p53 induction by ActD treatment and decreased cell death (Fig. 4C and Supplementary Fig. S2B). Taken together, these results indicate that TXNIP and hEcd cooperatively induce p53-positive MCF-7 cell death.

4. Discussion

Recently, Ecd was reported to function as a novel promoter of mammalian cell cycle progression and play an important role in human pancreatic cancer cell growth and Her2/neu-overexpressing breast cancer [10,11,22]. In contrast, TXNIP is a candidate tumor suppressor protein [13,23,24]. Its expression is up-regulated in cell cycle-arrested cells and down-regulated in human breast, lung, and colon tumor tissues and various tumor cell lines [25]. For that reason, TXNIP and Ecd may have opposite effects on cell growth and tumor promotion. However, we show here that TXNIP and Ecd may act cooperatively in regulating p53 stability and p53 activity. It is hypothesized that the physiological roles of TXNIP and Ecd may differ substantially from in p53-dependent and p53-independent cell survival/growth. However, the detailed mechanisms are unknown.

TXNIP plays multiple roles in the regulation of cell growth, apoptosis, and differentiation [26]. To identify novel functions of TXNIP, we searched for novel TXNIP-interacting proteins using a yeast two-hybrid strategy. We identified hEcd as a binding partner

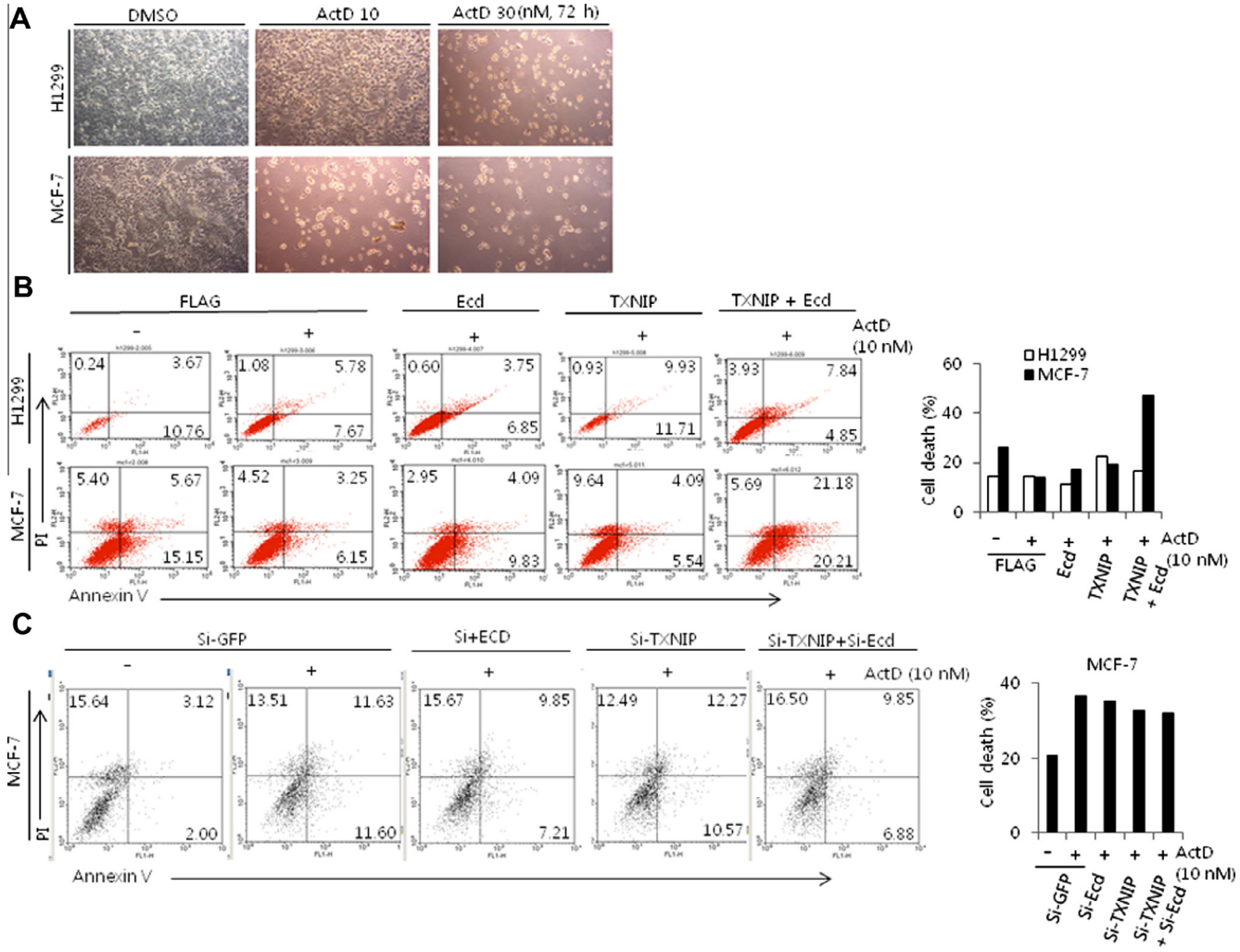

Fig. 4. TXNIP and Ecd increase cell death in response to ActD treatment of MCF-7 cells. (A) H1299 and MCF-7 cells were left untreated or treated with 10 nM ActD or 30 nM ActD. Cell morphologies were observed with phase-contrast microscopy. (B) H1299 and MCF-7 cells were transfected with the plasmids FLAG, Ecd, or TXNIP. After 6 h, cells were untreated or treated with 10 nM ActD for 48 h. (C) H1299 and MCF-7 cells were transfected with the plasmids Si-GFP, Si-Ecd, or Si-TXNIP. After 6 h, the cells were left untreated or treated with 10 nM ActD for 48 h. Cell death was assessed by flow cytometry following staining with FITC-Annexin V and PI. The percentage of cell death (FITC-Annexin V positive or PI positive) is shown.

of TXNIP. We showed that TXNIP physically interacts with hEcd *in vitro* and *in vivo*. TXNIP and hEcd co-overexpression increases p53 stability and promotes cell death in response to ActD treatment of MCF-7 cells. Conversely, silencing VDUP and Ecd using siRNA decreased MCF-7 cell death. Together, these data suggest that TXNIP and hEcd cooperatively lead to p53-positive MCF-7 cell death in response to ActD treatment.

Collectively, our results show that TXNIP binds to Ecd, and both play cooperative roles in p53 stability and p53 activity. In addition, TXNIP and Ecd increased ActD-medicated cell death of MCF-7 cells ($p53^{+/+}$) but not in H1299 cells ($p53^{-/-}$). These observations may provide an understanding of the mechanism of p53-induced apoptosis and suggest the potential for new strategies for p53-based cancer therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.036.

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