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Deregulated expression of E2F1 promotes proteolytic degradation of tumor suppressor p73 and inhibits its transcriptional activity

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ABSTRACT

The expression of tumor suppressor p73 is regulated at mRNA and protein levels. It has been shown that E2F1 acts as a transcriptional activator for *p73*. In this study, we have found that deregulated expression of E2F1 increases the mRNA level of *p73*, however, E2F1 promotes the degradation of p73. Immunoprecipitation experiments demonstrated that E2F1 forms a complex with p73 and inhibits the transcriptional activity of p73. Enforced expression of E2F1 induces degradation of p73 in a proteasome-independent manner. Additionally, the deletion analysis showed that E2F1(1–117) has an undetectable effect on p73, whereas E2F1(1–285) and E2F1(1–414) have an ability to promote degradation of p73 and inhibition of p73 transcriptional activity, suggesting that the region of E2F1 between amino acid residues 118 and 285 has a critical role in the regulation of p73. Taken together, our present study indicates that E2F1 has a dual role in the regulation of p73.

Introduction

p73 is one of tumor suppressor p53 family members of nuclear transcription factor, and transactivates its direct target genes implicated in the induction of cell cycle arrest and/or apoptotic cell death [1]. In a sharp contrast to *p53*, *p73* is rarely mutated in human tumor tissues [2], suggesting that p73 is not a classic Knudson-type tumor suppressor. However, recent studies demonstrated that p73-deficient mice develop spontaneous tumors, and their spectrum is quite different from that of *p53*-deficient mice [3], indicating that p73 act as a tumor suppressor. p73 is expressed as a variety of isoforms arising from alternative splicing events (pro-apoptotic TAp73) and alternative promoter usage (anti-apoptotic ΔNp73). TAp73 isoforms including p73α contain distinct COOH-terminal regions with different transcriptional as well as pro-apoptotic activity [4]. ΔNp73 which lacks NH_2-terminal transactivation domain, has an oncogenic potential and displays a dominant-negative behavior toward wild-type p53 as well as TAp73 [5,6]. *ΔNp73* is transactivated by wild-type p53 as well as TAp73, and thereby forming a negative auto-regulatory feedback loop [7–9].

E2F1 is a nuclear transcription factor and has an oncogenic and a pro-apoptotic property [10,11]. Previously, it has been shown that E2F1 acts as a transcriptional activator for TAp73 [12,13], suggesting that TAp73 contributes at least in part to E2F1-mediated apoptotic cell death. In the present study, we have found for the first time that the deregulated expression of E2F1 promotes a proteolytic degradation of p73 in a proteasome-independent manner, suggesting that the intracellular balance between the amounts of E2F1 and p73 plays a critical role in the regulation of cell fate determination.

Materials and methods

Cell lines and culture. Human cervical carcinoma-derived HeLa and human lung carcinoma-derived H1299 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/ml of penicillin and 100 μg/ml of streptomycin. Cells were grown at 37 °C in a humidified incubator with 5% CO_2.

Transfection. For transient transfection, cells were transfected with the indicated expression plasmids using FuGENE HD transfection reagent (Roche Applied Science) according to the manufacturer's instructions.

Colony formation assay. HeLa and H1299 cells were transfected with the indicated expression plasmids. Forty-eight hours after transfection, cells were transferred into the fresh medium containing G-418 (Sigma) at a final concentration of 800 μg/ml. Two weeks after the selection with G-418, drug-resistant colonies were fixed and stained with Giemsa's solution.

siRNA-mediated knockdown. HeLa cells were transiently transfected with control siRNA or siRNA against E2F1 (Dharmacon) by using LipofectAMINE RNAiMAX (Invitogen) according to the

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manufacturer's instructions. Forty-eight hours after transfection, total RNA was prepared and subjected to RT-PCR to ascertain the efficacy of siRNA transfection.

RT-PCR. Total RNA was extracted from the indicated cells using the RNeasy Mini Kit (Qiagen), and treated with DNase I (Qiagen) to eliminate genomic DNA. The quality of the extracted RNA was confirmed by electrophoresis on 1.2% denaturing agarose gels. Total RNA (5 μg) was reverse transcribed in the presence of random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. The resultant first-strand cDNA was amplified by PCR-based strategy to examine the expression levels of genes of interest. The oligonucleotide primers used in this study were as follows: human *E2F1*, 5′-GGTGAGGTCCCCAAAGTCAC-3′ (sense) and 5′-GCCACCATA GTGTCACCACC-3′ (antisense); human *p73α*, 5′-CCGGGAGAACTTTGAGATCC-3′ (sense) and 5′-AT CTTCAGGGCCCCCAGGTC-3′ (antisense); human *GAPDH*, 5′-ACCTGA CCTGCCGTCTAGAA-3′ (sense) and 5′-TCCA CCACCCTGTTGCTGTA-3′ (antisense). *GAPDH* was used as an internal control. PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Immunoblotting. Cells were lysed in a lysis buffer containing 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 62.5 mM Tris–HCl (pH 6.8). The protein concentration of cell lysates was determined by using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories) according to the manufacturer's instructions. Equal amounts of cell lysates (50 μg of protein) was separated by electrophoresis on SDS–polyacrylamide gels, and transferred onto the Immobilon-P membrane filters (Millipore). The membranes were incubated for 1 h at room temperature with monoclonal anti-p73 (Ab-4, Neomarkers), monoclonal anti-p53 (Oncogene Resesarch Products), polyclonal anti-E2F1 (C-20, Santa Cruz Biotechnology) or with polyclonal anti-Actin (20–33, Sigma) antibody followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) at room temperature for 1 h. Peroxidase activities were detected by ECL system (Amersham Biosciences) according to the manufacturer's instructions.

Immunoprecipitation. Cells were lysed in a lysis buffer containing 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EGTA, 50 mM β-mercaptoethanol ,1% Triton X-100 and a commercial protease inhibitor mixture (Sigma) for 30 min on ice, and subjected to a brief sonication for 10 s at 4 °C followed by centrifugation at 15,000 rpm at 4 °C for 10 min to remove insoluble materials. The protein concentrations were measured using the Bradford protein assay according to the manufacturer's instructions (Bio-Rad Laboratories). Whole cell lysates (1 mg) were precleared with 30 μl of protein G-Sepharose beads and used for immunoprecipitation with normal mouse serum (Invitrogen) or with anti-p73 antibody. After the addition of 30 μl of protein G-Sepharose beads, the reaction mixtures were incubated for additional 2 h at 4 °C. The beads were then collected by brief centrifugation, washed extensively with the lysis buffer and eluted with 2× SDS-sample buffer by boiling for 5 min. The precipitated proteins were subjected to immunoblotting with anti-E2F1 antibody.

Luciferase reporter assay. Cells were transiently transfected with the indicated expression plasmids. The total amount of the transfected plasmid DNA in each well was kept constant (510 ng) by the addition of the empty plasmid wherever necessary. Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured by Dual-Luciferase reporter assay system (Promega). Results were represented as -fold induction of luciferase activity (normalized to *Renilla*) when compared with the control cells transfected with the empty plasmid.

Half-life determination. HeLa cells were transfected with the expression plasmid for HA-p73α (0.5 μg) or with the expression plasmid encoding HA-p73α (0.5 μg) plus the expression plasmid for E2F1 (0.25 μg). Twenty-four hours after transfection, cells were treated with 100 μg/ml of cycloheximide (Sigma). At the indicated time points after the treatment, cells were harvested and whole cell lysates were analyzed by immunoblotting with anti-p73 or with anti-Actin antibody.

Results

E2F1 acts as a transcriptional activator for p73

To confirm whether E2F1 could act as a transcriptional activator for p73 under our experimental conditions, human cervical carcinoma-derived HeLa and human lung carcinoma-derived H1299 cells were transfected with the empty plasmid (pcDNA3) or with E2F1 expression plasmid. Forty-eight hours after transfection, total RNA was prepared and subjected to RT-PCR. As shown in Fig. 1A, E2F1 significantly induced the transcription of the endogenous *p73α* in both cells. Luciferase reporter assays revealed that E2F1 activates *p73* promoter bearing E2F1-responsive elements (Fig. 1B). Consistent with those results, siRNA-mediated knockdown of the endogenous *E2F1* resulted in a significant down-regulation of the endogenous *p73α* (Fig. 1C). Next, HeLa and H1299 cells were transfected with the empty plasmid, the expression plasmid for HA-p73α or with the expression plasmid encoding E2F1. Forty-eight hours after transfection, cells were transferred into the fresh medium containing G-418 (at a final concentration of 800 μg/ml). Two weeks after the selection, drug-resistant colonies were stained with Giemsa's solution. As shown in Fig. 1D, HA-p73α as well as E2F1 has an ability to reduce number of drug-resistant colonies, indicating that E2F1-dependent apoptotic cell death is mediated at least in part by up-regulation of *p73*.

Interaction between E2F1 and p73

Since it is unclear whether there could exist physical and functional interactions between E2F1 and p73, we performed immunoprecipitation experiments. To this end, HeLa cells were transfected with the expression plasmid for HA-p73α. Forty-eight hours after transfection, cell lysates were immunoprecipitated with normal rabbit serum (NRS) or with polyclonal anti-E2F1 antibody followed by immunoblotting with anti-p73 antibody. As shown in Fig. 2A, exogenously expressed HA-p73α was contained in the anti-E2F1 immunoprecipitates, and also the reciprocal experiments revealed that the anti-p73 immunoprecipitates include endogenous E2F1. Furthermore, the endogenous interaction between them was clearly shown by immunoprecipitation experiments (Fig. 2B). These observations strongly suggest that there could exist functional and physical interaction between them.

E2F1 has an ability to promote proteolytic degradation of p73

Next, we examined a possible effect of E2F1 on p73 expression at protein level and its transcriptional activity. Unexpectedly, the enforced expression of E2F1 in HeLa cells led to a remarkable down-regulation of the endogenous p73α (Fig. 3A), whereas the expression level of the endogenous *p73α* mRNA increased in the presence of the exogenously expressed E2F1. Similarly, the exogenously expressed HA-p73α also degraded in the presence of E2F1 (Supplementary Fig. S1). In accordance with those observations, a half-life of p73α shortened in HeLa cells transfected with the expression plasmid for E2F1 (Supplementary Fig. S2). MG-132 treatment resulted in a significant stabilization of E2F1 and p53 in HeLa cells, whereas E2F1-mediated reduction of p73α was not recovered by MG-132 (Fig. 3B). Under our experimental conditions, MG-132 treatment led to an induction of the endogenous *p73α* mRNA, which might be due to the stabilized E2F1. Similar results

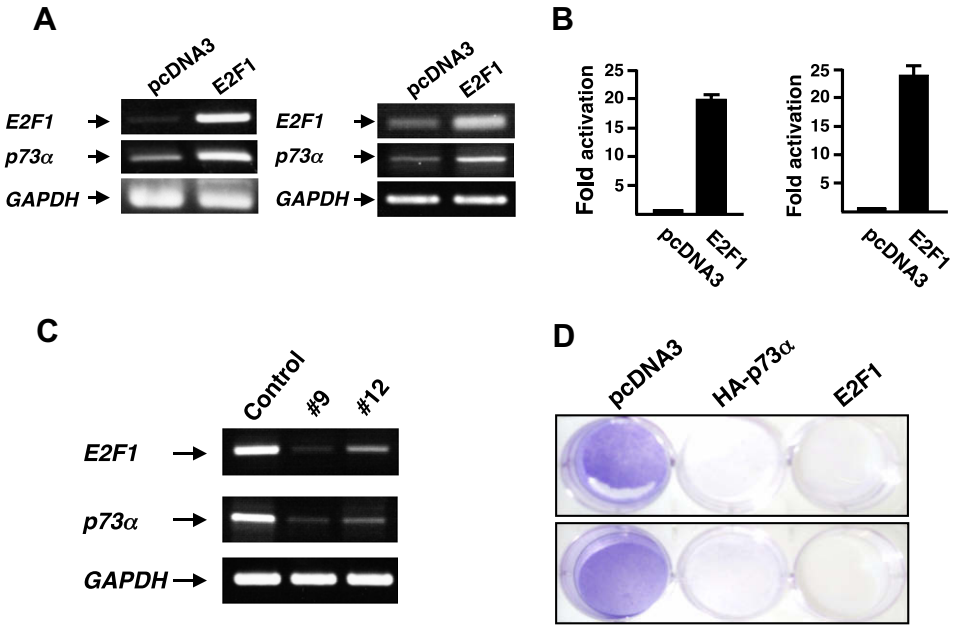

Fig. 1. E2F1 acts as a transcriptional activator for *p73*. (A) Up-regulation of *p73* in the presence of E2F1. Human cervical carcinoma-derived HeLa (right panel) and human lung carcinoma-derived H1299 (left panel) cells were transfected with the empty plasmid or with the expression plasmid for E2F1. Forty-eight hours after transfection, total RNA was prepared and subjected to RT-PCR. *GAPDH* was used as an internal control. (B) E2F1 activates the promoter activity of *p73*. HeLa (left panel) and H1299 (right panel) cells were transfected with the constant amount of the luciferase reporter construct bearing *p73* promoter region (100 ng) and *Renilla* luciferase reporter (10 ng) together with or without the expression plasmid encoding E2F1 (100 ng). Total DNA was kept constant (510 ng) with pcDNA3. Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured by Dual-Luciferase reporter system. The firefly luminescence signal was normalized based on the *Renilla* luminescence signal. The results were obtained at least three independent experiments. (C) siRNA-mediated knockdown of E2F1. HeLa cells were transfected with 20 nM of control siRNA or with siRNA against E2F1 (#9 and #12). Forty-eight hours after transfection, total RNA was prepared and analyzed by RT-PCR. *GAPDH* was used as an internal control. (D) Colony formation assay. HeLa (upper panel) and H1299 (lower panel) cells were transfected with the indicated expression plasmids. Forty-eight hours after transfection, cells were transferred into fresh medium containing G-418 (800 μg/ml). Two weeks after the selection, drug-resistant colonies were stained with Giemsa's solution.

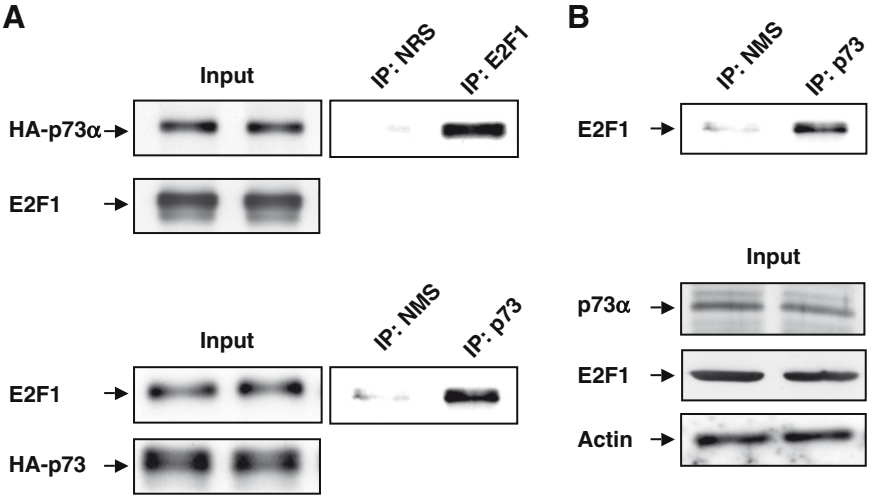

Fig. 2. Complex formation between E2F1 and p73 in cells. (A) Immunoprecipitation. HeLa cells were transfected with the expression plasmid for HA-p73α. Forty-eight hours after transfection, cell lysates were immunoprecipitated with normal rabbit serum (NRS) or with polyclonal anti-E2F1 antibody and the immunoprecipitates were analyzed by immunoblotting with monoclonal anti-p73 antibody. Reciprocal experiments were shown in lower panels. (B) Endogenous interaction. Cell lysates (1 mg of protein) prepared from HeLa cells were immunoprecipitated with normal mouse serum (NMS) or with anti-p73 antibody followed by immunoblotting with anti-E2F1 antibody.

were also obtained in HeLa cells exposed to another potent proteasome inhibitor ALLN (Supplementary Fig. S3), indicating that E2F1-mediated proteolytic degradation of p73 is regulated in a proteasome-independent manner. Recently, Koida et al. found that Plk1 stimulates proteolytic degradation of p73 in a proteasome-independent fashion [14].

To examine whether E2F1 could inhibit p73-mediated transcriptional activation, we performed luciferase reporter assay. HeLa and H1299 cells were transfected with the constant amount of the expression plasmid for HA-p73α and luciferase reporter plasmid bearing p53/p73-responsive element derived from human *MDM2* or *Bax* promoter together with or without the increasing amounts of the expression plasmid encoding E2F1. As shown in Supplementary Fig. S4, E2F1 strongly inhibited p73α-mediated transcriptional activation toward *MDM2* and *Bax* promoters in a dose-dependent manner. As described [1], *MDM2* and *Bax* are the

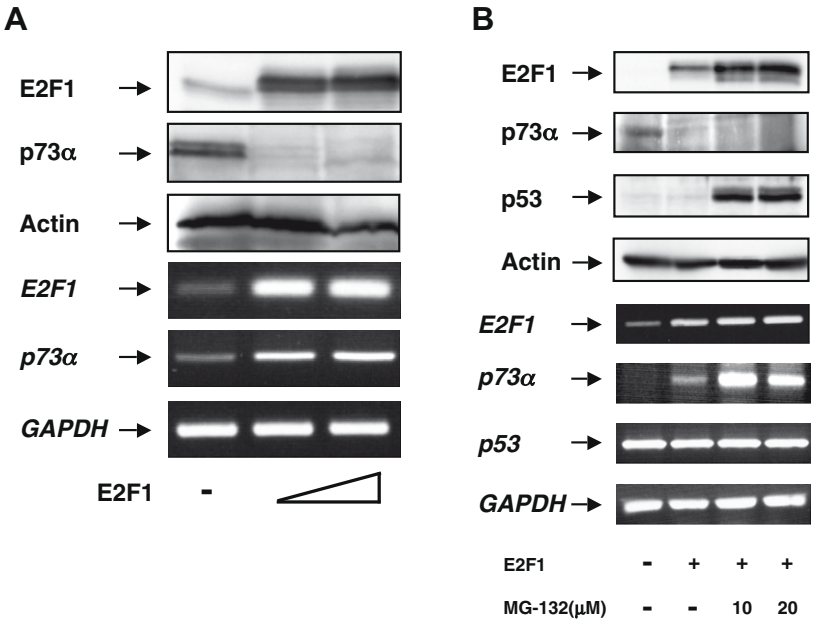

Fig. 3. E2F1 promotes degradation of p73 in a proteasome-independent manner. (A) E2F1-mediated degradation of p73. HeLa cells were transfected with or without the increasing amounts of the expression plasmid for E2F1 (0.5 or 1.0 μg). Forty-eight hours after transfection, cell lysates and total RNA were prepared and subjected to immunoblotting with anti-E2F1, anti-p73 or anti-Actin antibody (upper panels) and processed for RT-PCR (lower panels), respectively. (B) E2F1 induces proteasome-independent degradation of p73. HeLa cells were transfected with or without the constant amount of the expression plasmid for E2F1 (0.5 μg). Forty-eight hours after transfection, cells were treated with the indicated concentrations of MG-132 or left untreated. Six hours after the treatment, cell lysates and total RNA were prepared and subjected to immunoblotting with anti-E2F1, anti-p73, anti-p53 or with anti-Actin antibody (upper panels) and processed for RT-PCR (lower panels), respectively.

direct target genes of p73. Thus, it is likely that the negative effect of E2F1 on the p73α-mediated transcriptional activation is due to E2F1-dependent proteolytic degradation of p73α.

Identification of the region of E2F1 required for the proteolytic degradation and inhibition of p73

Finally, we sought to identify the region of E2F1 required for E2F1-mediated proteolytic degradation and inhibition of p73α. As described [15], the transactivation domain of E2F1 is located at its COOH-terminal region. As shown in Fig. 4A, luciferase reporter assay using luciferase reporter plasmid carrying *p73* promoter revealed that COOH-terminal deletion mutants of E2F1 lack the transactivation function. Immunoblotting experiments demonstrated that E2F1(1–285) and E2F1(1–414) retain an ability to degrade the endogenous p73α, whereas E2F1(1–117) has undetectable effect on the amount of endogenous p73α (Fig. 4B), suggesting that amino acid residues between 118 and 285 of E2F1 play an essential role in the regulation of p73α expression level. Similarly, luciferase reporter assay revealed that, like wild-type E2F1, E2F1(1–285) and E2F1(1–414) reduce p73α-mediated luciferase activities driven by *MDM2* and *Bax* promoters, whereas E2F1(1–117) has a negligible effect on p73α-dependent transcriptional activation (Fig. 4C), suggesting that E2F1-mediated proteolytic degradation of p73 is tightly linked to the transcriptional activity of p73.

Discussion

It has been well established that E2F1 transcriptionally activates ARF which triggers a p53-dependent apoptotic response [16]. ARF binds to MDM2 and prevents MDM2-mediated proteolytic degradation of p53. However, several lines of evidence suggest that ARF binds to E2F1 and inhibits its transcriptional activity [17]. Additionally, E2F1 forms a stable complex with p53 and blocks p53-dependent transactivation [18]. Thus, it is likely that there exists a checkpoint mechanism which inactivates the overactive E2F1 and/or p53. Based on our present observations, the enforced expression of E2F1 resulted in an increase in *p73α* mRNA, however, p73α protein degraded in the presence of excess amount of E2F1 in HeLa and H1299 cells. Similar results were also obtained in human osteosarcoma-derived U2OS and human lung carcinoma-derived A549 cells (data not shown). Like E2F1/ARF/p53 pro-apoptotic pathway, E2F1/p73 complex might provide a checkpoint function to inhibit overactive p73 to allow cell growth. Thus, the proliferative role of E2F1 might be attributed at least in part to its ability to down-regulate p73α at protein level. Since overexpression of E2F1 significantly reduced number of drug-resistant colonies in HeLa in which p53 is inactivated due to the presence of E6-AP [19] and *p53*-deficient H1299 cells (Fig. 1D), E2F1 induced apoptotic cell death might occur in a p53/p73-independent manner under our experimental conditions.

Our deletion analysis demonstrated that amino acid residues between 118 and 285 are required for E2F1-dependent degradation of p73α, and therefore E2F1-targeted degradation of p73α does not require its COOH-terminal transactivation domain, suggesting that E2F1 target gene products might not be involved in this process. In addition to MG-132, Lactacystin which is a potent irreversible inhibitor highly specific for proteasome, did not block E2F1-dependent proteolytic degradation of p73α (data not shown). As described previously [20], Calpain cleaved p73 at two distinct sites. Although ALLN acts as a potent inhibitor for proteasome, Calpain and Cathepsin-like proteases [21], ALLN had an undetectable effect on E2F1-targeted degradation of p73α. Therefore, the precise molecular mechanisms behind E2F1-mediated proteolytic degradation of p73 are still unclear. Further studies should be required to address this issue.

From the clinical point of view, extensive studies revealed that *p73* mRNA was highly expressed in a variety of human tumor tissues as compared with their corresponding normal tissues [2,22]. The deregulation of oncogenic E2F1 is one of the most common ge-

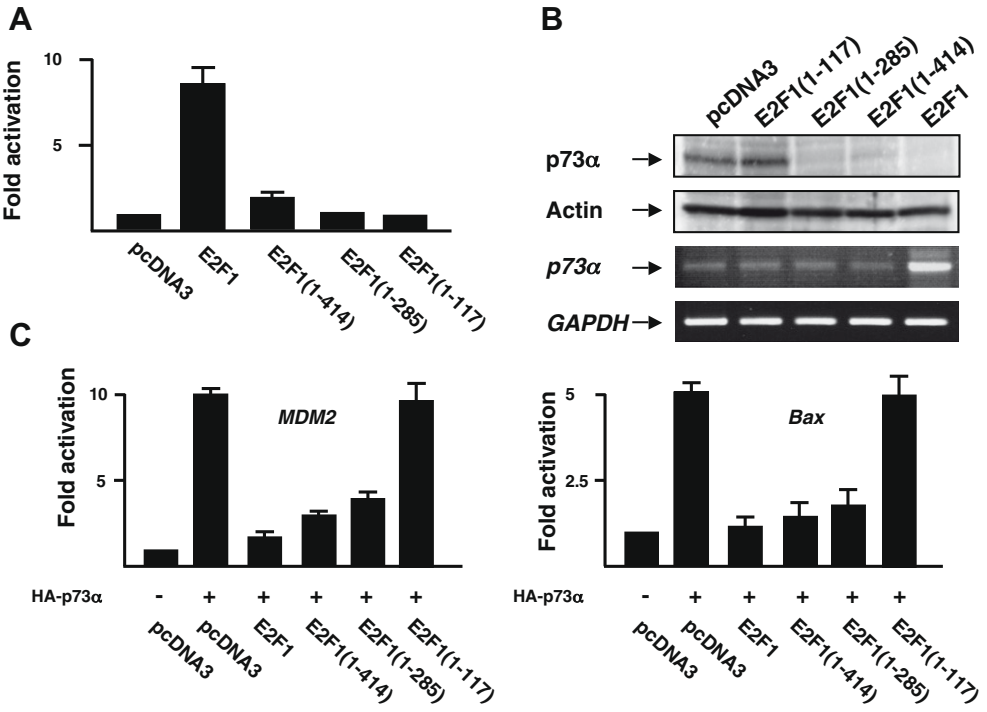

Fig. 4. Identification of the region of E2F1 required for degradation of p73 and inhibition of its transcriptional activity. (A) Transcriptional activity of the indicated E2F1 deletion mutants. HeLa cells were transfected with the constant amount of the luciferase reporter plasmid carrying the promoter region of p73 (100 ng) and *Renilla* luciferase reporter plasmid (10 ng) along with the empty plasmid or with indicated expression plasmids encoding E2F1 deletion mutants (100 ng). Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured as described in Fig. 1B. (B) Expression of the endogenous p73 in the presence of E2F1 deletion mutants. HeLa cells were transfected with 0.5 μg of the indicated expression plasmids. Forty-eight hours after transfection, cell lysates and total RNA were prepared and analyzed by immunoblotting with the indicated antibodies (upper panels) and subjected to RT-PCR (lower panels), respectively. (C) Luciferase reporter assay. HeLa cells were transfected with the constant amount of luciferase reporter plasmid bearing p53/p73-responsive element derived from human *MDM2* or *Bax* promoter, *Renilla* luciferase reporter plasmid and the indicated combinations of the expression plasmids. Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured as described in Fig. 1B.

netic alterations in human tumors [22]. Considering that *p73* is one of direct transcriptional target genes of E2F1, it is conceivable that *p73* is up-regulated in human tumors by deregulated E2F1. The question is why tumor formation is closely associated with the higher expression of tumor suppressor *p73*. In this regard, our present findings provide a clue to understand the complex inactivation mechanisms of p73 in tumor tissues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.141.

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