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Yin Yang 1 induces transcriptional activity of p73 through cooperation with E2F1

Shourong Wu [a,c], Saomi Murai [a], Kazunori Kataoka [b,c], Makoto Miyagishi [d,*]

[a] Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[b] Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[c] Division of Clinical Biotechnology, Center for Disease Biology and Integrative Medicine,The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[d] 21st Century COE Program, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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Abstract

The transcription factor p73 is a structural homologue of p53 and plays an important role in tumorigenesis, differentiation and development. However, the regulation of p73 pathway has not been wholly understood. Here we reported that YY1-silencing resulted in significant reductions in the activities of the p73 promoters and the endogenous p73 expression level, conversely, overexpression of YY1 could induce the activities of them. Furthermore, we showed that YY1 and E2F1 have synergistic effect on p73 promoter activity. The results of YY1-silencing and E2F1-silencing alone revealed that both factors are involved in the doxorubicin-induced activation of p73 promoter. Immunofluorescence staining and co-immunoprecipitation assays demonstrated that cooperation of YY1 and E2F1 is concomitant with physical interaction in nuclei. The results presented here suggested the cooperative transcriptional regulation of p73 by YY1 and E2F1, and might provide a new regulation mechanism by the YY1 network on tumorigenesis, differentiation and development.

Keywords: Yin Yang 1; E2F1; p73; Transcriptional activation; Synergistic effect

Yin Yang 1 (YY1) is a multifunctional transcription factor that exerts its effects on genes involved in various biological processes via its ability to initiate, activate, or repress transcription depending upon the context to which it binds, directly or indirectly via cofactor recruitments [1–3]. Today, YY1 is known to have a fundamental role in normal biological processes such as embryogenesis, differentiation, DNA replication, and cellular proliferation [3]. YY1-deficient embryos died at the time of implantation, and furthermore, its heterozygote knockout mice displayed significant growth retardation and neurological defects, indicating that YY1 plays an indispensable role in embryonic development and neuronal differentiation [4,5]. Recently, the physiologic significance of YY1 activity has also been reported to be associated with tumor biology [3,5,6]. It was known that YY1 is overexpressed in prostate cancer and sarcoma. Therefore, it is helpful to explain the cancer biological function of YY1 via studying its putative interactions with cell cycle regulators, death genes, as well as transcription factors and cofactors in the suppression or progression of various malignancies.

P73 has initially been known as a homologue of p53. They share relatively high sequence homology that includes an N-terminal transactivating (TA) domain and a central DNA binding domain. P73 has been reported to induce cell growth arrest and apoptosis in some cell lines irrespective of p53 status [7,8], and activate some p53 responsive genes, such as bax, cyclin G, IGF-BP3, and 14-3-3σ [9]. However, despite these similarities, p53 and p73 also show some fundamental differences in mechanisms and responses to DNA damage, and furthermore, in the phenotypes of their knockout mice. P73-null mice showed some defects in nervous system, which is not exhibited in p53-null mice

* Corresponding author. Fax: +81 3 5628 3770.
E-mail address: makoto-m@umin.ac.jp (M. Miyagishi).

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[10,11]. On the other hand, p73-null mice do not develop spontaneous tumor formation while p53 knockout mice exhibit high susceptibility to tumorigenesis [3]. Furthermore, a very recent report showed that p73 could promote cell growth in a synergistic manner with the proto-oncogene c-Jun, and conversely, silencing p73 resulted in the reduction of growth rate and decrease of cyclin D1, indicating that p73 could act positively in tumorigenesis [12]. Therefore, p73 has a complex functions in biological processes including apoptosis, differentiation, tumorigenesis, and cell growth.

E2F1 has been identified as a regulation factor for p73 transcription. Pediconi et al. reported that responding to certain DNA damages, E2F1, but not E2F2, E2F3, or E2F4, can recruit the p73 promoter and efficiently and specifically activate its transactivation [13]. However, transcriptional regulation factors of the p73 promoter, excepting E2F1, remain unknown. Identification of other regulators that might control p73 is needed to further understand its functions and roles in tumorigenesis, differentiation and development.

Here, we identified a transcription factor, YY1, as a novel regulator of p73 transcription. Moreover, we showed that YY1 cooperates with E2F1 to induce the transcriptional activity of p73. Taken together, these findings not only shed new light on the nature of YY1's biological activities but also indicate the complex regulation mechanism of p73 in diverse biological processes.

Materials and methods

Cell cultures and chemicals. The U2OS, and HCT116 cells were obtained from the American Type Culture Collection; the SaOS2 cells were from Riken Cell Bank (Tsukuba, Japan). The U2OS and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich) containing 10% fetal bovine serum (FBS, Invitrogen); The HCT116 and SaOS2 cells were maintained in McCoy's 5A medium (Invitrogen) containing 10% FBS or 15% FBS, respectively.

Plasmids and constructs. The long human p73-Luciferase reporter, which contains the p73 gene fragment −4052 to +438, was generously provided by Prof. Levrero (University of Rome 'La Sapienza', Rome, Italy) [13]. The short human p73-Luciferase reporter (−857 to +71) and the human p21-Luciferase reporter were constructed by inserting each PCR products into the BglII site and HindIII site of the pGL4 basic vector (Promega).

To generate the Flag-E2F1 expression vector, pcEF9-Flag-E2F1, the coding regions of human E2F1 were amplified by PCR and inserted into the HindIII and BamHI restriction sites of pcEF9 vector [14]. For the YY1 expression vector, the coding region of human YY1 was inserted into the pcDNA3 vector (Invitrogen). For construction of the HA-YY1 vector for use in immunofluorescence staining, the YY1 coding region in pcDNA3-YY1 was excised and inserted into the BamHI and EcoRI sites of pcDNA3-HA. To construct the Flag-YY1 expression vector for use in co-immunoprecipitation experiments, the Flag-YY1 coding region fragment was generated by PCR using pcDNA3-YY1 as a template, and the PCR product was again inserted into pcDNA3 vector predigested with BamHI and EcoRI.

RNA interference. To construct siRNA expression vectors, oligonucleotides with a hairpin, overhanging sequences and terminator were synthesized, annealed and then inserted into the BspMI sites of the pcENTRhU6 vector [15]. Based on the results of applying our algorithm [16], we identified the target sequences for YY1 and E2F1 genes: siYY1-1 (GCAAGAAGAGTTACCTCAG), siYY1-2 (GGCAGAATTTGCTAGAATG), siE2F1 (GGCTGGACCTGGAAACTGA). We used a T7 siRNA expression vector, which contains a stretch of 7 thymine (Ts) terminator sequences exactly downstream of the U6 promoter, as a control.

Transient transfection and luciferase assays. The U2OS and HCT116 cells were transfected with the different p73 firefly luciferase reporters along with indicated amounts of expression vectors and *Renilla* luciferase expression vector (pRL-SV40, Promega) for transfection normalization. For knockdown experiments, HCT116 and U2OS were transfected with siRNA expression vectors, and 24 h later selection was performed by puromycin. The selected cells were transfected with p73-luciferase reporters and pRL-SV40. FuGENE6 (Roche) were used for all transfections in U2OS cells, and Lipofectamine™ 2000 (Invitrogen) for HCT116 cells. Forty-eight hours after transfection, luciferase assays were performed in triplicate using the Dual Luciferase Assay System (Promega). All relative luciferase activities were determined by calculating the ratio between firefly and *Renilla* luciferase activities, and the results were shown as means ± SDs.

Real-time RT-PCR analysis. The total RNA was isolated using TRIZOL® Reagent (Invitrogen) and treated with DNase I (Qiagen) according to the manufacturer's instructions. The total RNA (1 μg) were then reverse-transcribed using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was carried out using an Applied Biosystems 7500 system (Applied Biosystems) and QuantiTect® SYBR Green PCR Master Mix (Qiagen). All reactions were run in triplicate and expressional results were normalized to actin. Data were expressed as means ± SDs of triplicate wells. The primer sequences are available upon request.

Western blotting analysis. The cells were lysed in whole-cell extract buffer (50 mM Tris–HCl, pH 7.3, 10% glycerol, 250 mM sodium chloride, 2 mM EDTA, 0.1% Nonidet P-40, and 1 mM NaF) with protease inhibitors (complete cocktail; Roche), and the lysate samples proteins were electrophorated on 10% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking, the membrane was incubated with indicated primary antibodies, and then with horseradish peroxidase (HRP)-conjugated secondary antibody IgG (Amersham Biosciences). Detections were performed with ECL Plus™ reagent (Amersham Biosciences). Antibodies used for western blotting were a rabbit polyclonal anti-E2F1 antibody (C-20, Santa Cruz), a mouse monoclonal anti-YY1 antibody (H-10, Santa Cruz), and a rabbit monoclonal anti-actin antibody (Sigma–Aldrich).

Immunofluorescence staining. The U2OS cells were seeded on cover-slips in 6-well plates and transfected with plasmids encoding HA-YY1 and E2F1. Forty-eight hours after transfection, cells were fixed for 20 min at room temperature with 10x PBS containing 4% paraformaldehyde, and permeabilized for 30 min in PBS containing 0.1% Triton X-100. After blocking, cover-slips were incubated at room temperature for 1 h in a 1:250 dilution of rat anti-HA and a 1:50 dilution of rabbit anti-E2F1 (Santa Cruz). Slides were then incubated for 1 h with a 1/1000 dilution of Alexa Fluor® 488 goat anti-rat IgG (H + L) and a 1/1000 dilution of Alexa Fluor® 568 goat anti-rabbit IgG (H + L) (Molecular Probes).

Co-immunoprecipitation. HEK293T cells were transfected with 10 μg of pcDNA3-Flag-YY1 or pcDNA3 using Lipofectamine™ 2000. Transfected cells were harvested 48 h post-transfection for Immunoprecipitation–Western blotting. Cell lysates were solved in lysis buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 0.1% NP-40) with protease inhibitors on ice for 30 min and then cleared by centrifugation at 15,000 rpm. The supernatants were incubated at 4°C for 1 h with protein G-beads in the presence of 3 μg of anti-flag monoclonal antibody. Then, the immunoprecipitated proteins were subjected to western blot analysis using anti-E2F1 antibody as described above.

Results and discussion

YY1-silencing reduces the transcriptional activity of p73

Although YY1 is well known as a p53 inhibitor, recent reports have implied that YY1 has p53-independent

pathway(s) for cell cycle regulation and apoptosis [5,6]. To elucidate novel pathways of YY1, we generated siRNA vectors, siYY1-1 and siYY1-2, targeted against two different sites of YY1 for knock-down experiments. The two siRNA vectors were used to ensure elimination of off-target effect of RNAi. Western blot analysis of YY1 siRNA vectors-transfected cells revealed that both siYY1-1 and siYY1-2 could significantly suppress the endogenous level of YY1 without affecting levels of E2F1 (Fig. 1A). Furthermore, using a p21 promoter/reporter construct, which is essentially dependent on p53, we confirmed the ability of the siYY1 vectors to functionally repress endogenous YY1, which resulted in the up-regulation of p53, and subsequently, the increase of p21 promoter/reporter activities (Supplemental Fig. 1). Thus, the siRNA vectors for YY1 used here were able to knockdown and block the endogenous function of YY1 effectively and specifically.

Next, using a p73 promoter/reporter construct, we found that in contrast to the case of the p21 promoter, YY1-silencing led to a significant reduction of p73 promoter activity in both HCT116 cells and U2OS (Fig. 1B and 1C). To further confirm this finding, we performed real-time RT-PCR using total RNA extracted from YY1-silenced U2OS cells. The results showed that the reduction of p73 mRNA level was observed in the YY1-silenced U2OS cells (Fig. 1D), indicating that in contrast with p53, knockdown of YY1 was able to reduce the transcriptional activity of p73. The results implied the novel regulation of p73 by YY1.

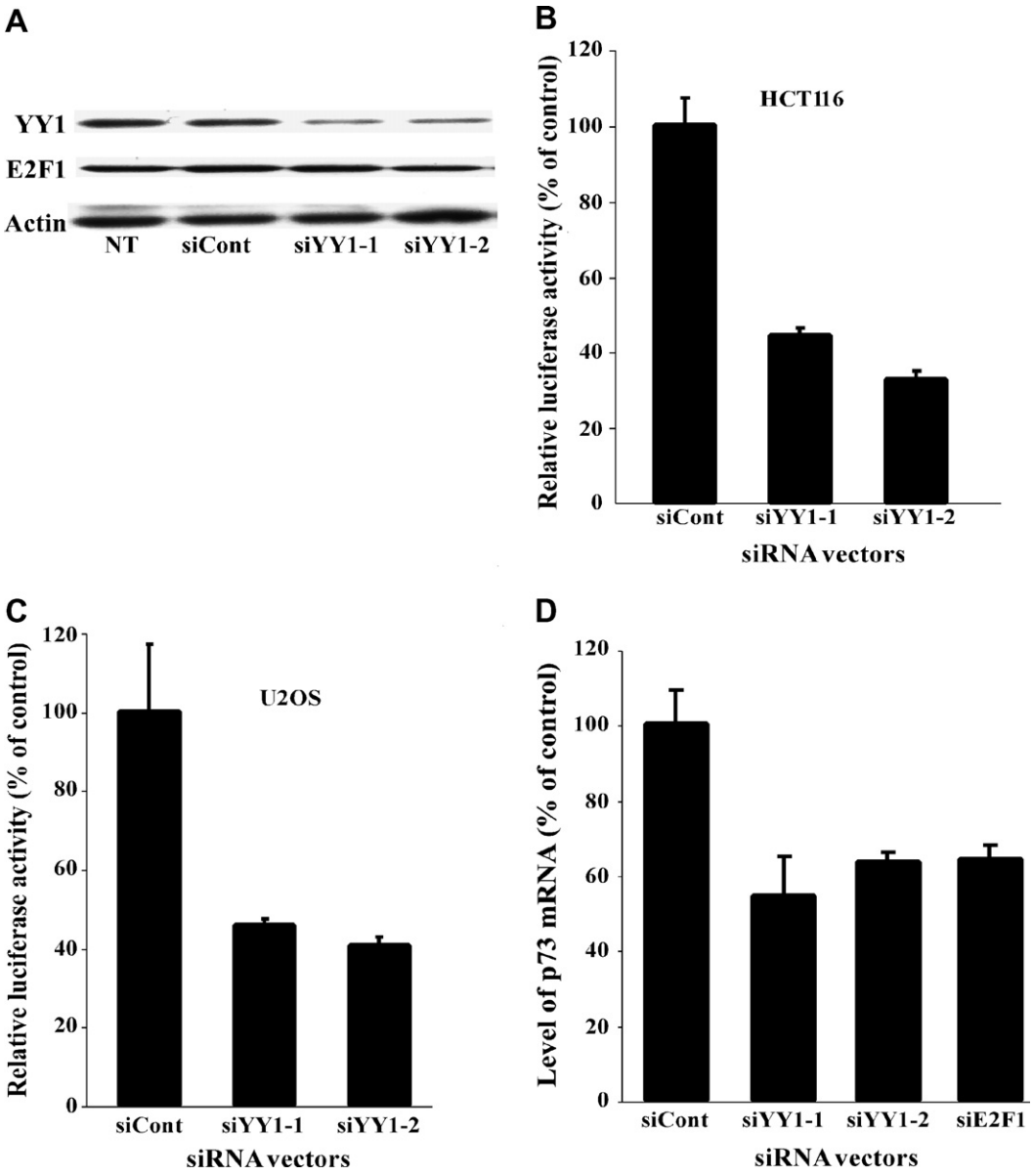

Fig. 1. The knockdown of YY1 reduces the transcriptional activity of p73. (A) HCT116 cells were transfected with siYY1-1 or siYY1-2 vectors or the siCont vector, the Western blotting was performed for detection of YY1 and E2F1. NT: non-transfected cells. (B–C) The effect of YY1-silencing on p73 promoter-driven transcription in HCT116 cells and U2OS cells. The indicated siRNA vectors-transfected HCT116 cells (B) and U2OS cells (C) were co-transfected with p73 luciferase (−4052/+438)/reporter and *Renilla* luciferase expression vector (pRL-SV40). Dual luciferase activity assay was performed 48 h after transfection. (D) Expression of p73 mRNA in YY1, E2F1-knocked down U2OS cells, determined by real-time RT-PCR analysis.

Effect of YY1 and E2F1 overexpression on the transcription activity of p73

Next, to assess the effect of YY1 overexpression and to confirm that of E2F1, a well-known p73 regulator, on p73 promoter activity, we generated a YY1-expression vector (pcDNA3-YY1) and an E2F1 expression vector (pcEF9-Flag-E2F1), and confirmed their expressions by Western blot analysis (Fig. 2A). Furthermore, the p53-inhibition activity of exogenous YY1 was also confirmed by p21 luciferase/reporter (Supplemental Fig. 2). Then, a different dose of the pcDNA3-YY1 was co-transfected into U2OS cells together with the two p73 reporters (Fig. 2B). The results were in agreement with those of knockdown experiments, as overexpression of YY1 led to the activation of the p73 promoter in a plasmid-dose dependent manner. Similar results were also obtained from SaOS2 cells, a p53 deficient human osteocarcinoma cell line (Fig. 2C), indicating that the YY1-induced p73 transcriptional activity is independent on p53 status. On the other hand, overexpressing E2F1 by co-transfecting E2F1 expression vector with the p73 promoter (−4052/+438)/reporter (Fig. 2D) or the p73 promoter (−857/+71)/reporter (Supplemental Fig. 3) resulted in the significant induction of both p73 reporters' activities, as shown previously [13]. Moreover, YY1 overexpression in U2OS cells resulted in the increase of endogenous p73 mRNA (Fig. 2E), similar with the effect of doxorubicin (Fig. 2F), which has been known to induce p73 activity via E2F1.

Collectively, the results from the knockdown and overexpression experiments for YY1 clearly identified the previously unsuspected role of YY1, i.e., the possibility that tumor activator gene YY1 up-regulates the transcriptional activity of p73. Recently, other evidences showed that spontaneous tumor formation did not develop in p73-null mice, and infrequent p73 mutations or overexpression of p73 protein was seen in a variety of tumors [17]; raising

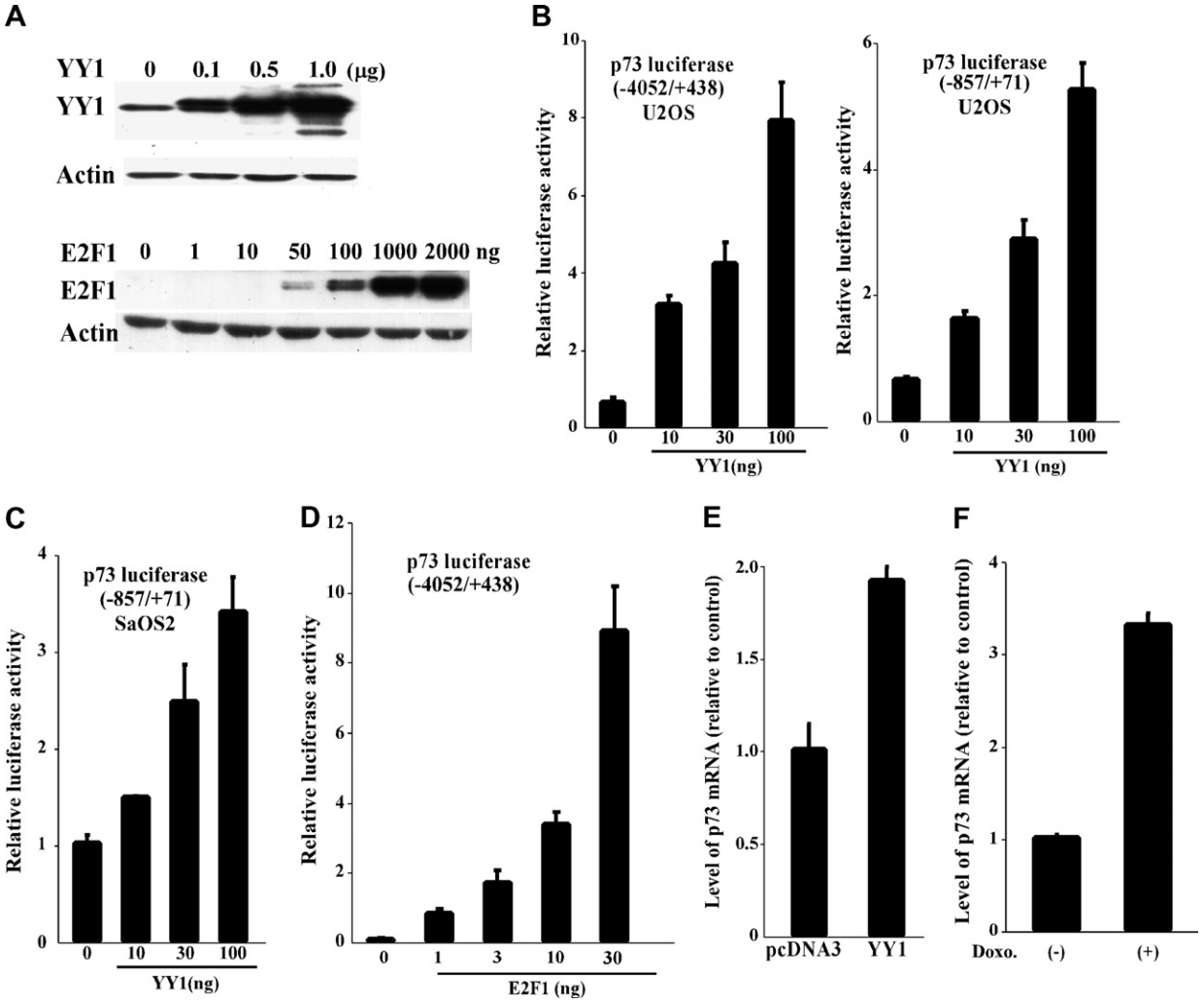

Fig. 2. The induction of p73 promoter activity by YY1 and E2F1. (A) Western blotting analysis of YY1 (upper panel) and E2F1 (lower panel) in HCT116 cells transfected with pcDNA3-YY1 or pcEF9-Flag-E2F1. (B) The effect of YY1 on the activities of p73 luciferase (−4052/+438)/reporter (left) or p73 luciferase (−857/+71)/reporter (right) in U2OS cells, determined by dual luciferase assay 48 h after transfection. (C) The effect of YY1 on the activities of p73 luciferase (−857/+71)/reporter in SaOS2 cells. (D) The effect of E2F1 on the activities of the p73 luciferase (−4052/+438)/reporter in HCT116 cells. (E) Expression of p73 mRNA in U2OS cells transfected with pcDNA3-YY1, determined by real-time RT-PCR analysis 12 h after transfection. (F) Expression of p73 mRNA in U2OS cells treated with doxorubicin (2 μM), determined by real-time RT-PCR analysis 24 h after treatment.

the question of the function of p73 as a tumor suppressor gene. A very recent work by Vikhanskaya et al. provided a potential answer to this question [12]. They found that p73 could promote cellular growth in a synergistic manner with the proto-oncogene c-Jun through AP-1 up-regulation, thus, p73 could positively act for tumorigenesis. Our results were consistent with their results. Moreover, it has also been reported that Yin Yang 1 is essential for oligodendrocyte progenitor differentiation and B-cell development [18,19]. Thus, our findings might also provide an important clue for unveiling molecular functions and mechanisms of YY1 and p73 pathway in neural differentiation and development. Therefore, we next addressed how YY1 regulates p73 gene transcription, specifically, whether there is a relationship between YY1 and E2F1 in regulating the transcriptional activity of p73.

The synergistic effect of YY1 and E2F1 on p73 transactivation

To examine whether there is cooperative regulation of p73 promoter activity by E2F1 and YY1, we performed co-transfection experiments using the p73 promoter (−4052/+438)/reporter, pcDNA3-YY1 and pEF9-E2F1 vectors. The U2OS cells co-transfected with both E2F1-expression and YY1-expression vectors showed a signification enhancement in the activity of p73 promoter compared to cells transfected with either of the vectors alone (Fig. 3A). Furthermore, in experiments with serial doses of YY1-expression vector under the constant presence of pEF9-E2F1, we found that in the presence of E2F1, the activity of p73 promoter increased in a dose-dependent manner with increasing amount of pcDNA-YY1 vector (Fig. 3B). These results demonstrated that YY1 could induce the transcriptional activity of p73 in a synergistic fashion with E2F1.

Previously, Schlisio et al. reported that E2F2 and E2F3, the members of E2F family, could interact with YY1 through mediation of the RYBP protein on the cdc6 promoter; they suggested that the interaction of E2Fs family with YY1 might determine the specificity of E2F2/E2F3 or E2F1 for different promoters [20]. In this study, we observed considerable cooperative transcriptional activation between E2F1 and YY1 on the p73 promoter. However, this functional interaction between YY1 and E2F1 might be not general, as the overexpression of YY1 did not show any significant activation of other well-defined E2F1-dependent promoters tested (DNMT1 and DHFR, data not shown), suggesting that the promoter specificity of E2F1 and E2F2/E2F3 is not simply determined by the

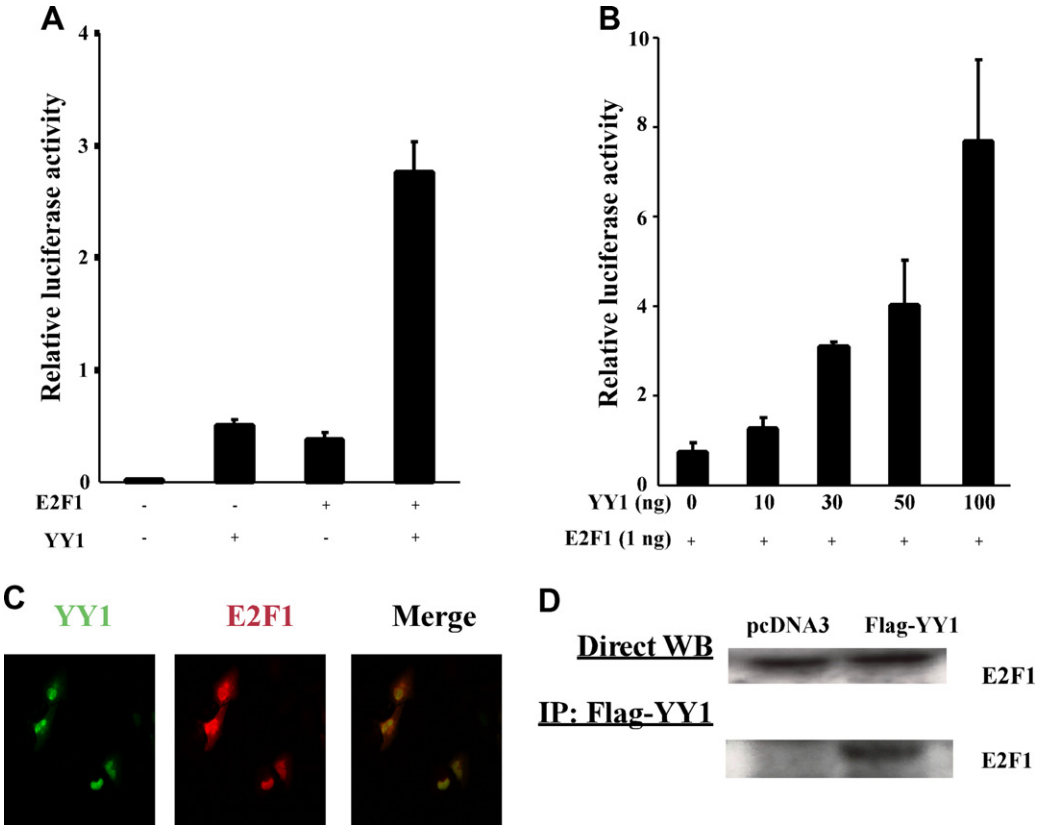

Fig. 3. The synergistic effect of YY1 and E2F1 on the transcriptional activation p73. (A) The activity of p73 luciferase (−4052/+438)/reporter in U2OS cells transfected with mock vector, pcDNA3-Flag-YY1 (50 ng), pcEF9-Flag-E2F1 (1 ng), or both pcDNA3-YY1 (50 ng) and pcEF9-Flag-E2F1 (1 ng). Dual luciferase assay was performed 48 h after transfection. (B) The activity of p73 luciferase (−4052/+438)/reporter in U2OS cells transfected with the indicated amounts of pcDNA3-YY1 and the constant amount of pcEF9-Flag-E2F1 (1 ng). (C) Immunofluorescence staining of YY1 and E2F1 in U2OS cells. (D) Co-immunoprecipitation of E2F1 and YY1 in HEK293T cells. Cells were lysed and pulled-down with anti-Flag antibody.

interaction with YY1, but might be determined by other undefined factors interacting with E2Fs and/or the epigenetic status of the promoters.

Co-localization and interaction between YY1 and E2F1

To understand the cellular and molecular mechanisms of the synergistic effects between YY1 and E2F1 on p73 promoter activation, we first examined the subcellular localization of YY1 and E2F1. As shown in Fig. 3C, YY1 was largely colocalized with E2F1 in the nucleus. Next, we examined the physical interactions between YY1 and E2F1 by performing co-immunoprecipitation experiments. The results showed that E2F1 immunoprecipitated in cells transfected with the Flag-YY1-expression vector, whereas no band was detected in cells transfected with the control vector (Fig. 3D). These observations demonstrated the direct physical association between YY1 and E2F1.

Role of YY1 in DNA damage-induced transcriptional activity of p73 promoter

As reported previously, the p73 promoter was activated E2F1-dependently by doxorubicin, a DNA damaging agent [13]. Therefore, we examined whether YY1 contributed to doxorubicin-induced p73 transcriptional activation. YY1-silenced cells showed significant reduction in the activation of the p73 promoter induced by doxorubicin (Fig. 4A, B), which was similar to that of E2F1-silencing cells.

Furthermore, p73 promoter activation by doxorubicin treatment has been reported to be critically dependent on

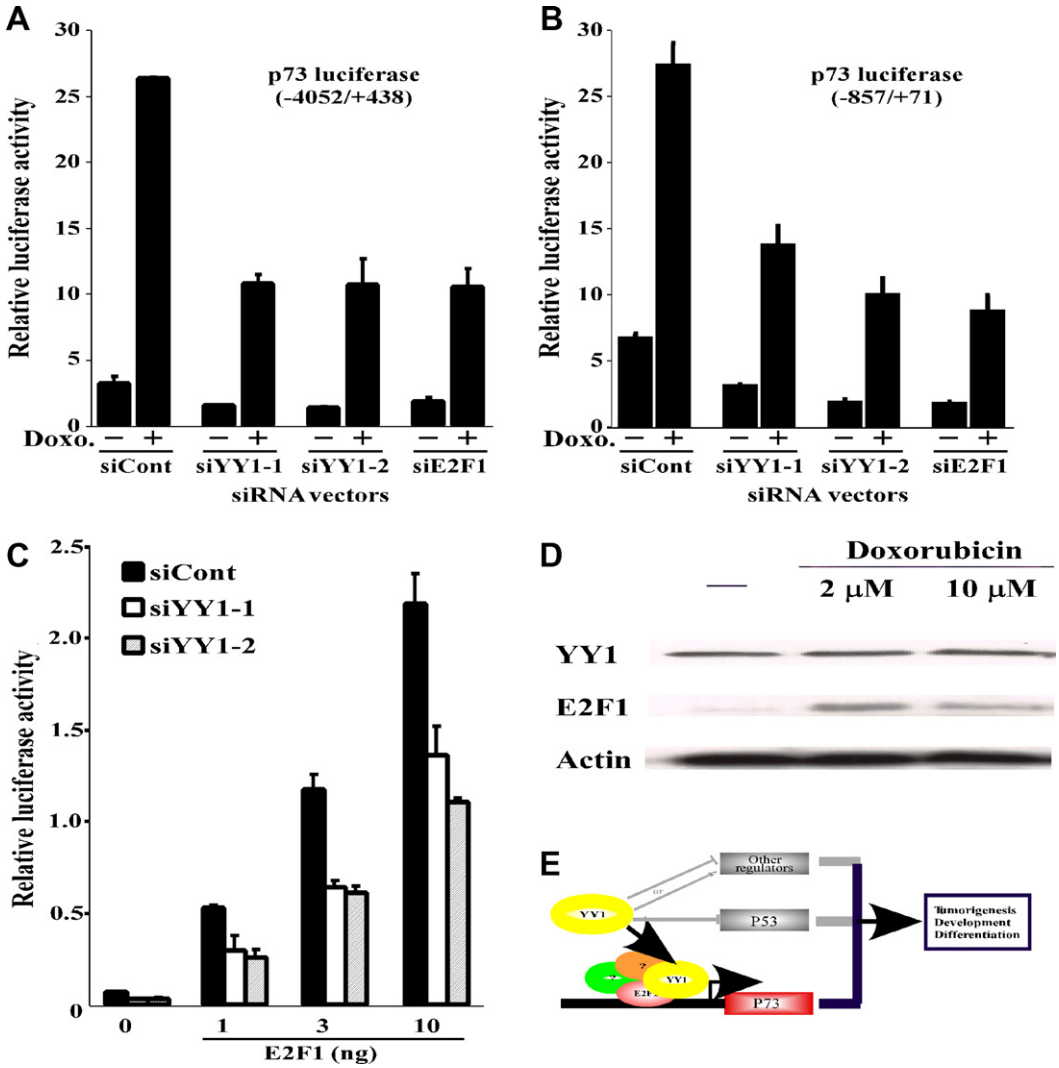

Fig. 4. Involvement of YY1 in transcriptional activation of p73 induced by doxorubicin. (A, B) The activity of p73 promoter in the YY1-silenced U2OS cells under doxorubicin treatment. siYY1-1, siYY1-2 or siE2F1 vectors-transfected U2OS cells were co-transfected with p73 luciferase (−4052/+438)/reporter (A) or (−857/+71)/reporter (B) and pRL-SV40. Twenty-four hours after transfection, U2OS cells were untreated or treated with doxorubicin (2 μM), and 24 h later, dual luciferase assay was performed. (C) The effect of YY1-silencing on p73 promoter activity induced by E2F1 in U2OS cells. The indicated siRNA vectors-transfected U2OS cells were co-transfected with p73 luciferase (−4052/+438)/reporter and increasing amount of pcEF9-Flag-E2F1. (D) Western blot analysis of E2F1 and YY1 in HCT116 cells treated by doxorubicin. (E) A model of the co-regulation of YY1 and E2F1 on p73.

E2F1 [13]. Based on the interaction of E2F1 and YY1, we next tested whether p73 promoter activation by E2F1 overexpression also showed the YY1 dependency. The YY1-knocked down cells were co-transfected with serial doses of E2F1-expression vectors and the p73 reporter vector. As shown in Fig. 4C, in YY1-silenced cells, the p73 promoter activation induced by E2F1 was significantly reduced, which was a comparable suppressive effect as that of doxorubicin-treated cells, suggesting that the YY1 signal might be constitutive during doxorubicin treatment. Together with the results of Western blot analysis (Fig. 4D), which revealed that only endogenous E2F1, not YY1 expression was induced by doxorubicin treatment, these data indicated that cooperative action between the constitutive YY1 and the inducible E2F1 contributes to the activation of the p73 promoter under the treatment with doxorubicin. Furthermore, YY1-silencing did not affect the expression of endogenous E2F1 (Fig. 1A), which also strongly suggested that YY1 induces the transcriptional activity of p73 by cooperation with E2F1, not via increasing E2F1.

Taken together, our results uncovered a novel function of YY1 on E2F1-mediated p73 regulation. We summarized the model in Fig. 4E. The fact that YY1 affects p53 family members opened up an attractive possibility that needs further investigation, that is, YY1 might function as a key integrator or modulator of various pathways in the network that includes p53 family members and regulators concerned with cancer progression, development and differentiation.

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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.2007.10.145.

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