Genes to Cells

Tumor suppressor *TAp73* gene specifically responds to deregulated E2F activity in human normal fibroblasts

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Discrimination of oncogenic growth signals from normal growth signals is crucial for tumor suppression. The transcription factor E2F, the main target of pRB, plays central role in cell proliferation by activating growth-promoting genes. E2F also plays an important role in tumor suppression by activating growth-suppressive genes such as pro-apoptotic genes. The regulatory mechanism of the latter genes is not known in detail, especially in response to normal and oncogenic growth signals. E2F is physiologically activated by growth stimulation through phosphorylation of pRB. In contrast, upon dysfunction of pRB, a major oncogenic change, E2F is activated out of control by pRB, generating deregulated E2F activity. We show here that the tumor suppressor *TAp73* gene, which can induce apoptosis independently of p53, responds to deregulated E2F activity, but not to physiological E2F activity induced by growth stimulation in human normal fibroblasts. We identified E2F-responsive elements (ERE73s) in TAp73 promoter that can specifically sense deregulated E2F activity. Moreover, *RB1*-deficient cancer cell lines harbored deregulated E2F activity that activated ERE73s and the *TAp73* gene, which were suppressed by re-introduction of pRB. These results underscore the important role of deregulated E2F in activation of the *TAp73* gene, a component of major intrinsic tumor suppressor pathways.

Introduction

Cell cycle progression is promoted not only by normal growth stimulation but also by abnormal growth stimulation induced by oncogenic changes. In response to such inappropriate cell cycle promotion, cells can induce apoptosis or senescence to avoid tumorigenesis (Lowe *et al.* 2004). The tumor suppressors pRB and p53 play major roles in the intrinsic tumor suppression mechanism (Sherr & McCormick 2002). Accordingly, defects in the RB pathway and the p53 pathway are observed in almost all cancers (Sherr 1996).

The transcription factor E2F, the main target of pRB, plays essential roles in cell proliferation by regulating a group of growth-promoting genes (Dyson 1998). E2F consists of eight family members that are divided into transcriptional activators (E2F1 to E2F3) and transcriptional repressors (E2F4 to E2F8) (DeGregori & Johnson 2006). Growth stimulation activates cyclin-dependent kinases (CDKs), which phosphorylate and inactivate pRB family proteins and physiologically activates E2F to promote cell cycle progression. In contrast, E2F can also induce expression of pro-apoptotic and growth-suppressive genes, whose expression is inconvenient for cell proliferation

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(Dimri *et al.* 2000; Muller *et al.* 2001). It is not known in detail how E2F regulates pro-apoptotic and growth-suppressive genes in response to normal and abnormal growth stimulation.

We previously reported that the tumor suppressor $p14^{ARF}$ (hereafter *ARF*) gene, an upstream regulator of p53, and the CDK inhibitor $p27^{Kip1}$ gene, an upstream regulator of pRB and E2F, are activated by deregulated E2F activity caused by dysfunction of pRB, but not by physiological E2F activity induced by serum stimulation, that is, normal growth stimulation, in human normal fibroblasts (Komori *et al.* 2005; Ozono *et al.* 2009). These observations suggest that there is a mechanism that discriminates deregulated E2F activity from physiological E2F activity to activate the tumor suppressor genes, which activate the two major tumor suppressor pathways. The distinct regulation of the genes seems to be important to avoid tumorigenesis in response to dysfunction of pRB, although allowing normal cell proliferation in response to normal growth stimulation.

The *TP73* gene encodes two isoforms with opposite functions. The TAp73 isoform, which contains transactivation (TA) domain, is a homologue of p53 and can induce apoptosis independently of p53 (Kaghad *et al.* 1997; Stiewe & Putzer 2000). In contrast, the ΔNp73 isoform, which lacks the TA domain, counteracts TAp73 and p53 and is anti-apoptotic. Different promoters regulate the expression of these two isoforms. The *TAp73* gene is thought to be a tumor suppressor gene and is known to be a direct target of E2F1 (Irwin *et al.* 2000; Lissy *et al.* 2000; Stiewe & Putzer 2000). However, the regulatory mechanism of the *TAp73* gene by E2F1 has been examined using cancer cell lines with over expression of E2F1 in most cases and has not been analyzed in normal cells in detail. In addition, it is not known how the *TAp73* gene is regulated by E2F, especially in response to physiological and deregulated E2F activity. In this report, we examined the regulation of the *TAp73* gene by physiological and deregulated E2F activity in human normal fibroblasts (HFFs) to further elucidate the role of deregulated E2F in tumor suppression, focusing on the pathway independent of the RB and p53 pathways. We show that the *TAp73* gene is activated by deregulated E2F activity, but not by physiological E2F activity induced by growth stimulation in HFFs. Moreover, isolated TAp73 promoter is activated in *RB1*-deficient cancer cell lines but not in HFFs, underscoring the role of deregulated E2F in tumor suppression.

Results

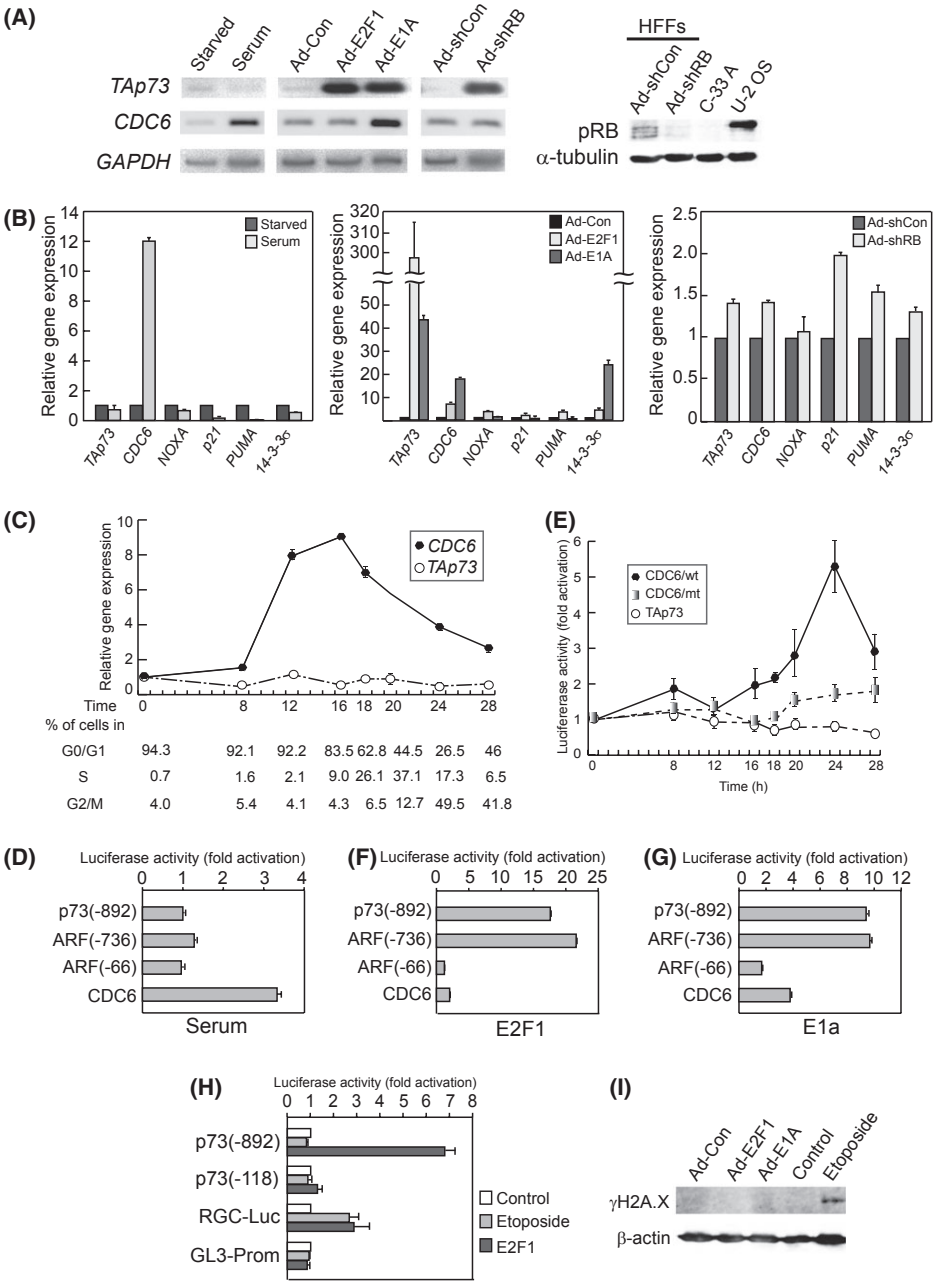
TAp73 gene is activated by deregulated E2F activity, but not by physiological E2F activity

To explore the regulatory mechanism of the *TAp73* gene by E2F, we examined the responsiveness of the *TAp73* gene to physiological and deregulated E2F activity. In order to examine the responsiveness of the *TAp73* gene to dysfunction of pRB, a prototype of oncogenic changes, we used normal cells (human foreskin fibroblasts; HFFs), and not cancer cell lines, which have defects in the RB pathway in most cases. To induce physiological E2F activity, we used serum for normal growth stimulation of fibroblasts. To induce deregulated E2F activity, we used ectopic expression of E2F1 and forced inactivation of pRB by the Δ2-11 form or 2RG form of adenovirus E1a, which binds to and inactivates all pRB family members but does not interfere with CBP/p300. We also used knockdown of pRB expression by shRNA against *RB1* (shRB), which has been successfully used in previous studies (Komori *et al.* 2005; Ozono *et al.* 2009).

We first examined the responsiveness of the endogenous *TAp73* gene to physiological E2F activity. HFFs were starved of serum, restimulated with serum, and the *TAp73* mRNA levels were examined by RT-PCR (Fig. 1A, left panel) and quantitative (q)RT-PCR (Fig. 1B, left panel). Remarkably, *TAp73* gene expression was scarcely induced by serum stimulation at 18 h after serum stimulation. In contrast, expression of the *CDC6* gene, whose growth-regulated expression is mainly mediated by E2F (Ohtani *et al.* 1998), was clearly induced, indicating that E2F was physiologically activated under the serum-stimulated condition. These results suggest that physiological E2F activity induced by serum stimulation scarcely activates the *TAp73* gene in HFFs.

We next examined whether the *TAp73* gene is activated by deregulated E2F activity in HFFs. The cells were infected with recombinant adenovirus expressing E2F1 or the Δ2-11 form of adenovirus E1a, and the *TAp73* mRNA level was similarly examined by RT-PCR and qRT-PCR. The expression of the *TAp73* gene was dramatically induced by ectopically expressed E2F1 and adenovirus E1a (Fig. 1A, left panel and Fig. 1B, middle panel). This suggests that not only ectopically expressed E2F1, but also endogenous E2F deregulated by forced inactivation of pRB family proteins can activate the *TAp73* gene. To examine whether forced inhibition of pRB alone generates deregulated E2F activity that activates the *TAp73* gene, HFFs were infected with recombinant adenovirus

expressing shRB, which successfully down-regulated pRB expression (Fig. 1A, right panel). *TAp73* gene expression was induced by shRB (Fig. 1A, left panel and Fig. 1B, right panel), indicating that forced down-regulation of pRB expression alone is sufficient to activate endogenous E2F to activate the *TAp73* gene. We also examined whether expression of the *NOXA*, *p21*, *PUMA,* and *14-3-3σ* genes, which are targets of TAp73, is specifically induced by deregulated E2F activity in HFFs (Fig. 1B). Expression of all the genes was not induced at all by serum stimulation (Fig. 1B, left panel) and was induced, although slightly at this time point, by ectopically expressed E2F1 (Fig. 1B, middle panel) and shRB (Fig. 1B, right panel) in HFFs. Expression of the *14-3-3σ* gene was induced more than 20-fold and that of the *NOXA* gene was slightly induced by adenovirus E1a (Fig. 1B, middle panel). However, expression of the *p21* and *PUMA*



genes was not induced by adenovirus E1a. This could be due to additional functions of E1a to inactivation of pRB family proteins. E1a is reported to suppress activation of p21 promoter by TAp73 (Das *et al.* 2003). Similar effect of E1a for *PUMA* gene expression is expected, because E1a is reported to suppress p53-mediated induction of *p21* and *PUMA* gene expression (Savelyeva & Dobbelstein 2011), and TAp73 is a p53 family member.

To confirm that expression of the *TAp73* gene is not induced by serum stimulation all through the cell cycle, we monitored *TAp73* gene expression by qRT-PCR until 28 h after serum stimulation (Fig. 1C). *TAp73* gene expression was not induced till 28 h, when percentage of cells in G2/M decreased and that in G0/G1 increased (next G1 phase) (Fig. 1C). These results indicate that *TAp73* gene expression is not induced by serum stimulation in HFFs.

To further analyze E2F regulation of the *TAp73* gene in normal cells, we also examined regulation of TAp73 promoter by E2F in HFFs. For this purpose, we isolated TAp73 promoter (−892 to +52) and examined its responsiveness to serum stimulation, ectopically expressed E2F1 and the 2RG form of adenovirus E1a. Consistent with the endogenous gene expression, the TAp73 promoter was not activated by serum stimulation at all under conditions in which CDC6 promoter was clearly activated at 20 h after serum stimulation (Fig. 1D). We also monitored TAp73 promoter activity until 28 h after serum stimulation (Fig. 1E). Consistent with the endogenous gene expression, TAp73 promoter activity was not induced at any time points till 28 h. In contrast, the TAp73 promoter was dramatically activated by ectopically expressed E2F1 and adenovirus E1a to a far greater extent than that of the CDC6 promoter

Figure 1 *TAp73* gene is activated by deregulated E2F activity but not by physiological E2F activity in human normal fibroblasts**.** (A) Left panel: Endogenous *TAp73* gene expression is induced by deregulated E2F activity but not by physiological E2F activity. For serum stimulation experiments, human foreskin fibroblasts (HFFs) were starved of serum for 2 days, either left serum-starved (Starved) or re-stimulated with serum (Serum) for 18 h, and harvested. For experiments with ectopically expressed E2F1 and the Δ2-11 form of adenovirus E1a, which inactivates all pRB family proteins but not p300/CBP, HFFs were infected with recombinant adenovirus expressing E2F1 (Ad-E2F1) or the Δ2-11 form of E1a (Ad-E1A), or control virus (Ad-Con) at MOI 200, cultured under serum-starved conditions for 1 day and harvested. For pRB knockdown experiments, HFFs were infected with recombinant adenovirus expressing shRNA against *RB1* (Ad-shRB) or control virus (Ad-shCon) at MOI 500, cultured under serum-starved conditions for 2 days, restimulated with serum for 18 h, and harvested. The mRNA levels were examined by RT-PCR. *CDC6* is a positive control for serum stimulation and *GAPDH* is an internal control. Right panel: Down-regulation of pRB expression by shRB. pRB expression was examined by Western blotting. U-2 OS and C-33 A are positive and negative controls, respectively. α-tubulin is an internal control. (B) qRT-PCR analysis of the same set of samples in (A), including the *NOXA*, *p21*, *PUMA* and *14-3-3σ* genes. (C) *TAp73* gene expression is not induced by serum stimulation in HFFs all through the cell cycle. The expression levels of the *TAp73* and *CDC6* genes were examined by qRT-PCR and were normalized by that of *GAPDH* (upper panel). Cell cycle distribution of HFFs was examined by FACS analysis based on DNA content (lower panel). (D) The TAp73 promoter is not activated by serum stimulation. The TAp73 reporter plasmid, p73(-892)-Luc, was transfected into HFFs with pCMV-β-gal as an internal control by lipofection, cultured under serum-starved conditions for 2 days, restimulated with serum for 20 h and harvested. Luciferase activity was normalized to β-galactosidase activity. pCDC6-Luc/wt and pARF (-66)-Luc were used as positive and negative controls, respectively, for serum stimulation. (E) The TAp73 promoter is not activated by serum stimulation all through the cell cycle of HFFs. HFFs were transfected with p73(-892)-Luc, pCDC6-Luc/wt or pCDC6-Luc/mt along with pCMV-β-gal, starved of serum for 2 days and restimulated with serum. The cells were harvested at indicated time points and assayed for luciferase activity normalized by β-galactosidase activity. (F, G) The TAp73 promoter is activated by deregulated E2F activity induced by dysfunction of pRB. HFFs were transfected with reporter plasmids, 20 ng of expression vector for E2F1 (F), or the 2RG form of adenovirus E1a (G), which inactivates all pRB family proteins but not p300/CBP, and pCMV-β-gal, cultured under serum-starved conditions for 1 day, and assayed. pCDC6-Luc/wt and pARF(-736)-Luc were used as positive controls and pARF(-66)-Luc was used as a negative control to monitor deregulated E2F activity. (H) The TAp73 promoter is not activated by etoposide treatment in HFFs. HFFs were transfected with p73(-892)-Luc and pCMV-β-gal along with the expression vector for E2F1, or control plasmid, cultured in the presence of serum for 1 day. The cells transfected with the control plasmid were treated with etoposide (50 μM) or DMSO (vehicle), and harvested after 20 h. A p53-responsive reporter, pRGC-Luc, was used as a positive control and p73(-118)-Luc and pGL3-Prom were used as negative controls. (I) γH2A.X is induced by etoposide treatment but scarcely induced by deregulated E2F activity in HFFs. HFFs were infected with recombinant adenovirus expressing E2F1 or the Δ2-11 form of E1a at MOI 200 that activates the TAp73 promoter to a similar extent to that in transient transfection assay, or treated with etoposide (50 μM) as described in the legend to Fig. 1G. γH2A.X was detected by Western blotting. β-actin is an internal control.

(Fig. 1F,G). Thus, regulation of the TAp73 promoter by E2F is similar to that of the ARF(-736) promoter, which has been shown to be activated by deregulated E2F activity, but not by physiological E2F activity induced by serum stimulation in human normal fibroblasts (Komori *et al.* 2005).

Taken together, these results indicate that the *TAp73* gene is activated by deregulated E2F activity caused by forced inactivation of pRB, but not by physiological E2F activity induced by serum stimulation in HFFs. Thus, regulation of the *TAp73* gene is distinct from that of growth-related E2F targets, which are activated by both physiological and deregulated E2F activity.

TAp73 promoter is not activated by etoposide treatment in HFFs

It has been reported that ectopically expressed E2F1 can induce DNA damage (Rogoff *et al.* 2004) and that TAp73 promoter is activated by treatment with DNA damaging reagent in cancer cell lines (Pediconi *et al.* 2003). These reports suggest a possibility that deregulated E2F activity may induce DNA damage, which in turn activates the TAp73 promoter. To address this possibility, we examined the responsiveness of the TAp73 promoter to treatment of HFFs with etoposide, a DNA damaging reagent, which is known to activate the *TAp73* gene in some cancer cell lines (Pediconi *et al.* 2003; Urist *et al.* 2004). As a positive control, which is known to be activated by DNA damage, we used the p53-responsive reporter RGC-Luc, which contains p53 binding sites from ribosomal gene cluster (RGC) (Kern *et al.* 1991) upstream of murine c-fos core promoter.

Under the condition that RGC-Luc was clearly activated by etoposide treatment in HFFs, the TAp73 promoter was not activated by etoposide treatment at all (Fig. 1H). However, ectopically expressed E2F1 dramatically activated the TAp73 promoter. RGC-Luc was activated by ectopically expressed E2F1 to a similar extent as that by etoposide treatment. This activation of RGC-Luc by ectopically expressed E2F1 is likely to be mediated through induction of ARF, an upstream activator of p53 (Komori *et al.* 2005).

In addition, γH2A.X, a marker of DNA damage, was detected after the treatment of HFFs with etoposide, but not upon generation of deregulated E2F activity by ectopically expressed E2F1 or adenovirus E1a under these experimental conditions (Fig. 1I). These results suggest that activation of the TAp73 promoter by deregulated E2F activity is mainly mediated through deregulated E2F itself, and not through DNA damage in HFFs.

Identification of E2F responsive elements of the TAp73 promoter

To further analyze E2F regulation of the TAp73 promoter, we explored E2F responsive elements of the TAp73 promoter (ERE73s), which may play important roles in discriminating between physiological and deregulated E2F activity in HFFs. We constructed a series of 5′ deletion mutants of the TAp73 promoter and examined their responsiveness to ectopically expressed E2F1 by a reporter assay. A remarkable decrease in E2F1-responsiveness was observed between (−295) to (−218) and (−218) to (−118), suggesting that E2F responsive elements are located in these two regions (Fig. 2A). To narrow down the location of E2F responsive elements, we isolated each region and divided them into two parts (Fig. 2B, middle), connected to a heterologous core promoter of ARF, ARF(-66) (Komori *et al.* 2005), and examined their E2F1-responsiveness (Fig. 2C). Regions (−295 to −263) and (−159 to −112) were highly activated, suggesting that the main E2F responsive elements are located in these regions. We further divided each of these regions into two parts (Fig. 2B, bottom) and found that all of the four subregions were activated by ectopically expressed E2F1 (Fig. 2D) and/or adenovirus E1a (Fig. 2E). These regions contain GC stretches, which have been shown to be important for responsiveness of the ARF promoter to deregulated E2F activity (Fig. 3A) (Komori *et al.* 2005). Two point mutations in the consecutive GC sequence (Fig. 3B) almost abolished the responsiveness of each element to ectopically expressed E2F1 (Fig. 2F) and adenovirus E1a (Fig. 2G), suggesting that these GC stretches are important for responsiveness of ERE73s to deregulated E2F activity.

We refer to sequences (−295 to −278), (−277 to −263), (−159 to −136) and (−135 to −112) as ERE73-1 to 4, respectively. Three identified ERE73s (1, 3, and 4) contain the functional E2F sites in TAp73 promoter that were showed to bind to E2F1 by electrophoretic mobility shift assay (Seelan *et al.* 2002). Because the degree of activation of each ERE73 was relatively small and may hinder further analyses, we used the (−295 to −263) and (−159 to −112) regions, referred to as ERE73-(1+2) and ERE73-(3+4), respectively, for further analyses (Figs 2B and 3C).

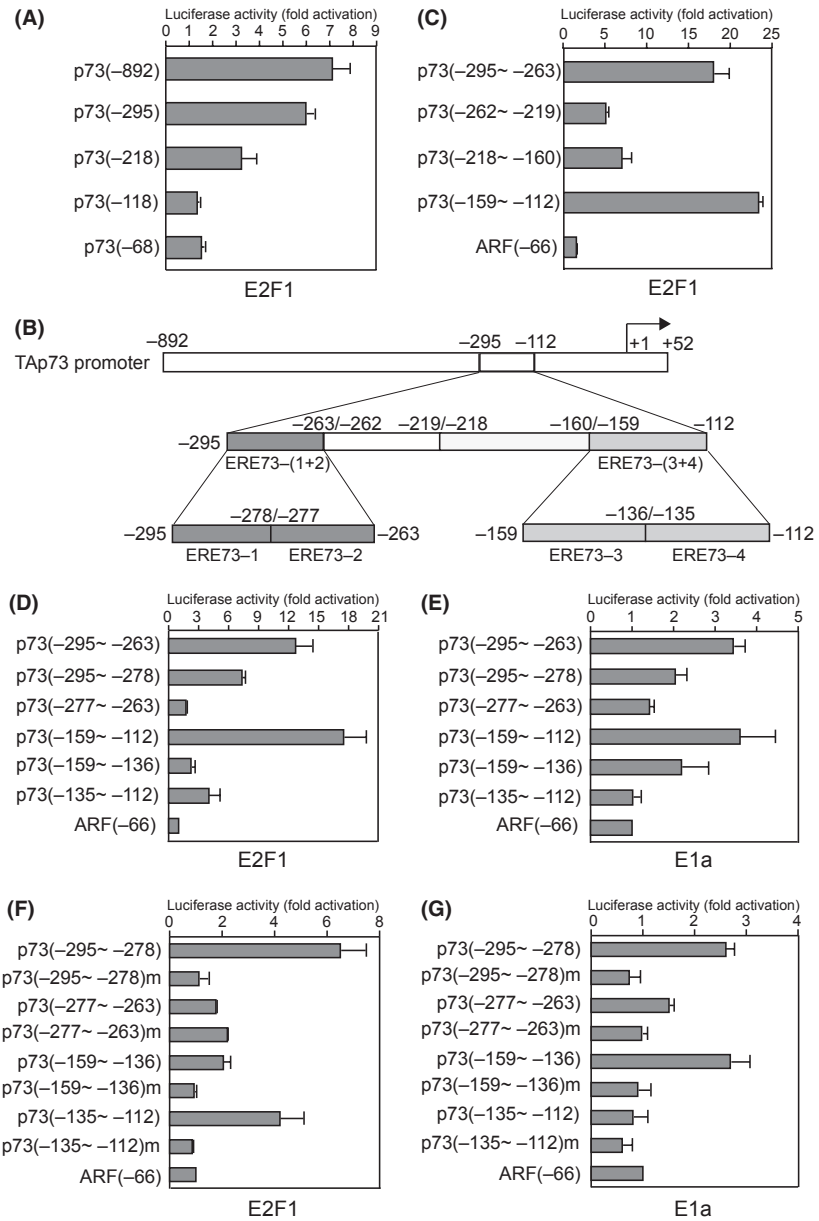


Figure 2 Identification of the E2F-responsive elements of the TAp73 promoter (ERE73s). (A) A set of 5′ deletion mutants of the TAp73 promoter was examined for E2F1-responsiveness as described in the legend to Fig. 1F. (B) Location of ERE73s in the TAp73 promoter. (C–E) Isolated fragments of the TAp73 promoter were combined with pARF(-66)-Luc, and their responsiveness to E2F1 (C, D) and E1a (E) was examined as described in the legend to Fig. 1F,G. (F, G) Mutant forms (m) of the isolated fragments were similarly examined for responsiveness to E2F1 (F) and the 2RG form of adenovirus E1a (G).

ERE73s sense deregulated E2F activity but not physiological E2F activity in HFFs

To explore whether ERE73s are responsible for discriminating between physiological and deregulated E2F activity, we examined the responsiveness of ERE73-(1+2) and ERE73-(3+4) to deregulated and physiological E2F activity in HFFs. ERE73-(1+2) and ERE73-(3+4) were activated by ectopically expressed E2F1 and by adenovirus E1a, and the introduction of the same mutations as that of each ERE73 mutant almost abolished the responsiveness

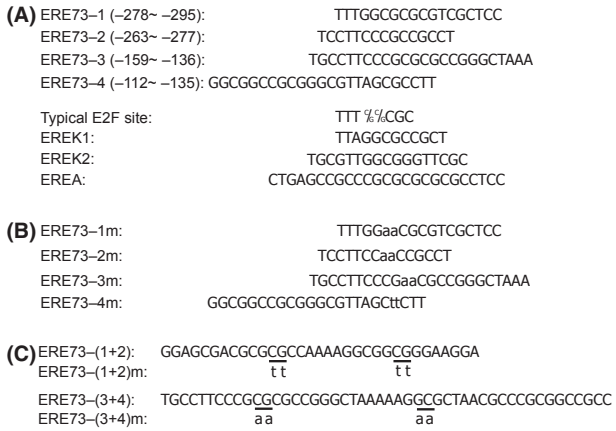


Figure 3 Sequences of ERE73s compared with other E2F-responsive elements. (A) Sequences of ERE73s were compared with those of typical E2F sites, E2F responsive element of $p27^{Kip1}$ (EREKs) and $p14^{ARF}$ (EREA). (B, C) Mutations introduced into ERE73s.

(Fig. 4A,B). Consistent with the TAp73 promoter, ERE73-(1+2) and ERE73-(3+4) did not show any clear responsiveness to serum stimulation (Fig. 4C).

To examine whether ERE73s are necessary for sensing deregulated E2F activity in the TAp73 promoter, we introduced the same mutations as that of each ERE73 mutant into the TAp73 promoter (Fig. 3B). Introduced mutations almost abolished the responsiveness of the TAp73 promoter to ectopically expressed E2F1 (Fig. 4D) and adenovirus E1a (Fig. 4E). Taken together, ERE73s play major roles in discriminating between physiological and deregulated E2F activity in the TAp73 promoter.

Among the eight E2F family members, E2F1 through E2F3 are thought to be activator E2Fs. We thus examined contribution of the each activator E2F to activation of the TAp73 promoter. We examined the responsiveness of the TAp73 promoter to E2F1 through E2F3, which were expressed from the same amount of expression vectors with the same backbone in HFFs (Fig. 4F). The TAp73 promoter was dramatically activated by ectopically expressed E2F1, slightly activated by E2F2 and scarcely activated by E2F3, in a similar manner to ARF promoter, a pro-apoptotic E2F target promoter, which specifically senses deregulated E2F activity. The results seem reasonable because, in general, E2F1 is regarded as a strong activator for pro-apoptotic genes and E2F3 for growth-related gene, E2F2 being at the middle.

To investigate binding of deregulated and physiological E2F1 to TAp73 promoter *in vivo*, we carried out chromatin immunoprecipitation (ChIP) assay, using serum-starved HFFs (Fig. 4G). Upon serum stimulation, binding of E2F1 to TAp73 promoter was not increased compared with serum-starved condition. Under the same condition, binding of E2F1 to CDC6 promoter was clearly increased. In contrast, binding of E2F1 to both TAp73 promoter and CDC6 promoter was clearly increased upon ectopic expression of E2F1 or adenovirus E1a, which forcedly inactivates all pRB family members and deregulates endogenous E2F. These results suggest that deregulated E2F1, but not physiologically activated E2F1 induced by serum stimulation, can bind to TAp73 promoter.

RB1-deficient cancer cell lines harbor deregulated E2F activity that activates the *TAp73* gene

The *TAp73* gene is activated by deregulated E2F activity caused by dysfunction of pRB. However, accumulation of oncogenic changes may tolerate *TAp73* gene activation, leading to tumorigenesis. If this is the case, cancer cells may retain deregulated E2F activity, which activates the *TAp73* gene. To address this issue, we examined whether *RB1*-deficient cancer cell lines (5637, Saos-2, and C-33 A) harbored the deregulated E2F activity.

We first examined whether ERE73s, which specifically sense deregulated E2F activity in HFFs, are activated in the cancer cell lines by reporter assay. ERE73-(1+2) and ERE73-(3+4) showed higher activity than those mutants, which almost lost the E2F responsiveness, in all cancer cell lines tested, but not in HFFs (Fig. 5A). These results suggest that ERE73s are activated in the cancer cell lines but not in HFFs. When we introduced a constitutively active form of pRB (PSM.7-LP) (Knudsen & Wang 1997), the activity of ERE73-(1+2) and ERE73-(3+4) was suppressed in the cancer cell lines but not in HFFs (Fig. 5A). Under the same conditions, CDC6 promoter activity was suppressed in HFFs, indicating that physiological E2F activity was suppressed by PSM.7-LP in HFFs. Similarly, the activity of the TAp73 promoter was suppressed by PSM.7-LP in the cancer cell lines, but not in HFFs (Fig. 5B). These results suggest that the *RB1*-deficient cancer cell lines, but not HFFs, retain deregulated E2F activity that activates ERE73s and the TAp73 promoter. To confirm the biological significance of the deregulated E2F activity retained in the *RB1*-deficient cancer cell lines, we next examined whether the endogenous *TAp73* gene is activated by deregulated E2F activity in the cancer cell lines. For this purpose, we introduced PSM.7-LP into the cancer cell lines using recombinant adeno-

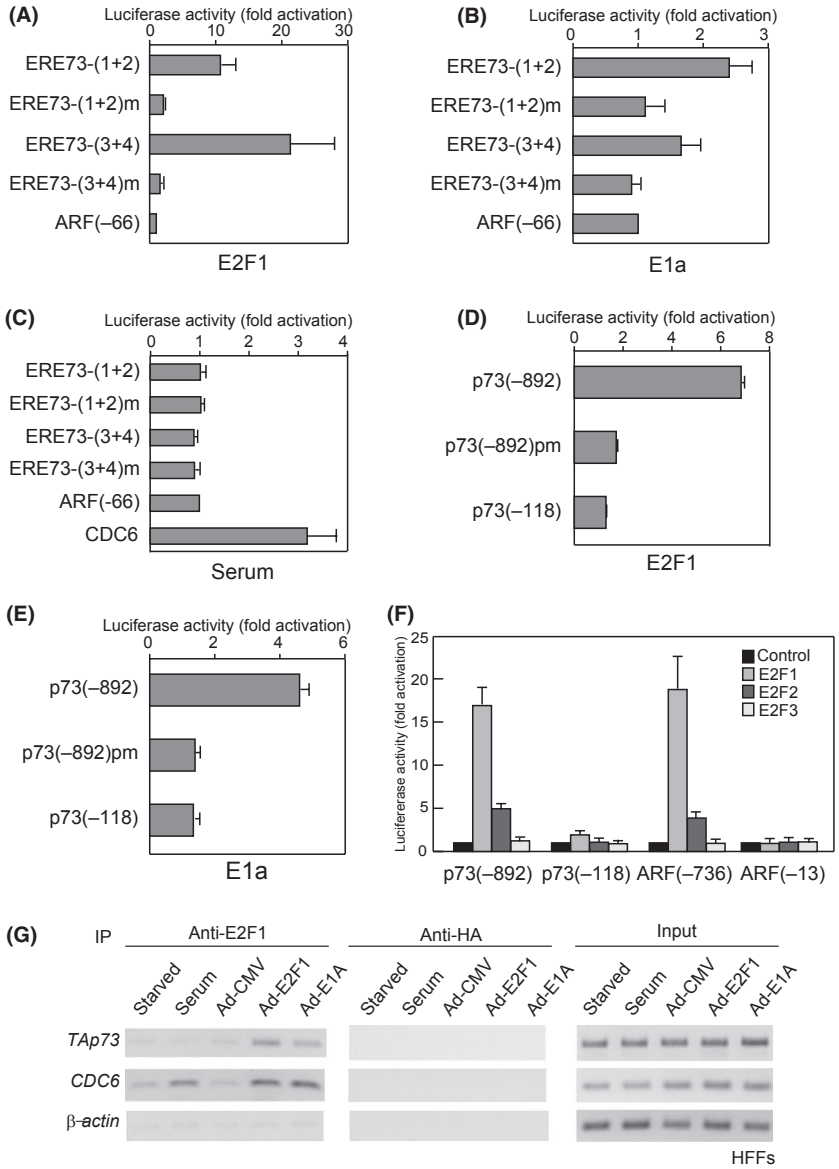


Figure 4 E2F responsive elements of the TAp73 promoter are activated by deregulated E2F activity but not by physiological E2F activity. (A–C) ERE73-(1+2), ERE73-(3+4), and their mutants were examined for responsiveness to E2F1 (A), E1a (B), and serum stimulation (C), as described in the legend to Fig. 1F,G, and D, respectively. (D, E) ERE73s are responsible for sensing deregulated E2F activity. p73(-892)-Luc and its point mutant, p73(-892)pm-Luc, were examined for responsiveness to E2F1 (D) and E1a (E) as described in the legend to Fig. 1F,G. (F) The TAp73 promoter is preferentially activated by E2F1. p73(-892)-Luc was examined for responsiveness to activator E2Fs (E2F1-E2F3), expressed from 5 ng of pENTR-CMV expression vector, along with its core promoter p73(-118)-Luc, in HFFs. ARF(-736)-Luc, which specifically responds to deregulated E2F activity, was used as a positive control. ARF(-13)-Luc was used as a negative control. (G) Binding of E2F1 to TAp73 promoter was increased by either ectopic expression of E2F1 or adenovirus E1a, which inactivates all pRB family members and induces endogenous deregulated E2F activity, but not by serum stimulation that physiologically activates E2F. HFFs were starved of serum, restimulated with serum or infected with recombinant adenovirus as in the legend to Fig. 1A. ChIP assay was carried out with anti-E2F1 antibody. anti-HA antibody is a negative control. Input is one 60th of the lysates.

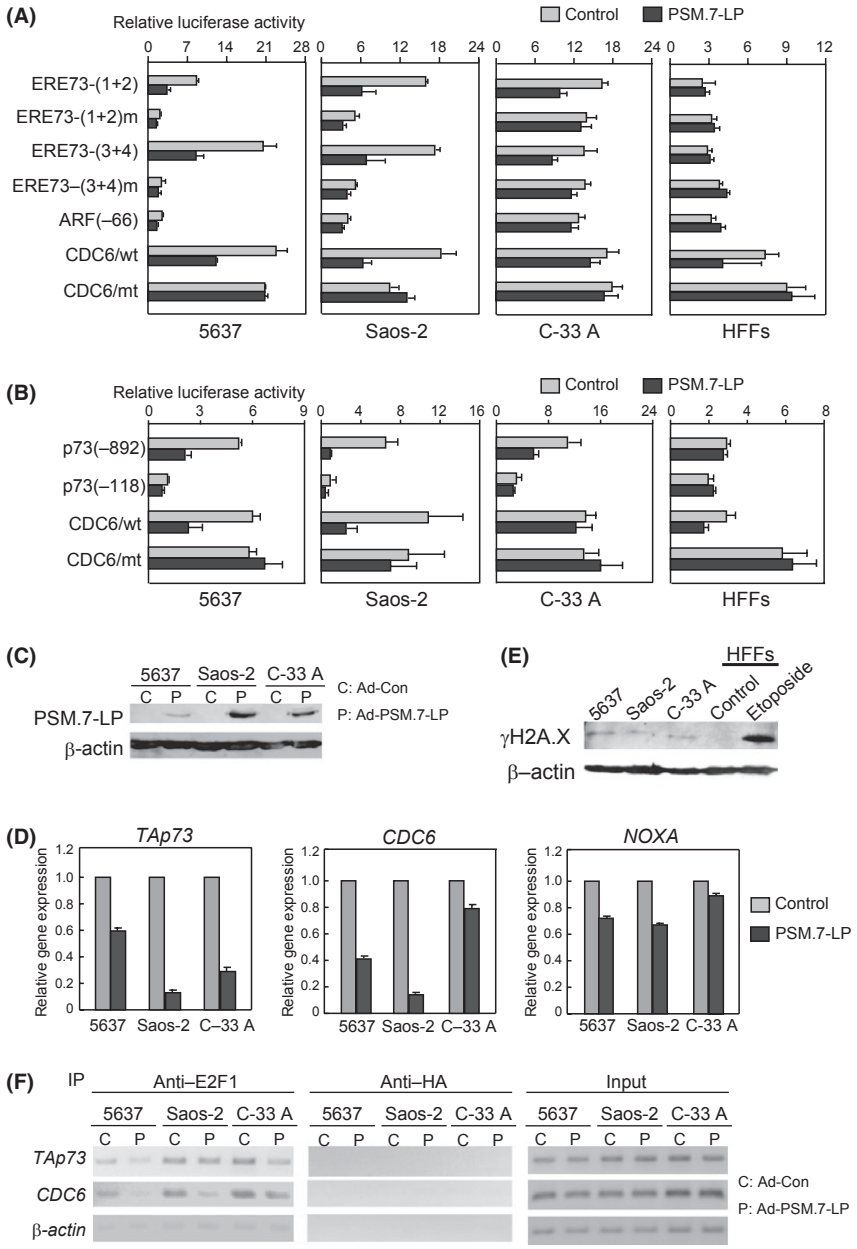


Figure 5 *RB1*-deficient cancer cell lines, but not human normal fibroblasts, harbor deregulated E2F activity that activates the *TAp73* gene. (A, B) ERE73s and the TAp73 promoter are activated in *RB1*-deficient cancer cell lines but not in HFFs. *RB1*-deficient cancer cell lines (5637, Saos-2 and C-33 A) and HFFs were transfected with ERE73s or p73(-892)-Luc reporter plasmids with an expression vector for the constitutively active form of pRB (PSM.7-LP). The cells were cultured for 1 day in the presence of serum and harvested. pCDC6-Luc/wt and pCDC6-Luc/mt were used as a positive and negative control, respectively. (C) Expression of PSM.7-LP was examined by Western blot analysis. (D) The endogenous *TAp73* gene is activated in the *RB1*-deficient cancer cell lines. The *RB1*-deficient cancer cell lines were infected with recombinant adenovirus expressing PSM.7-LP or control virus at MOI 50, cultured in the presence of serum for 1 day, and harvested. The *TAp73, CDC6,* and *NOXA* mRNA levels were examined by qRT-PCR. *CDC6* is a positive control. The expression levels were adjusted by that of *GAPDH* as an internal control. (E) The levels of γH2A.X in the *RB1*-deficient cancer cell lines were lower than that in HFFs treated with etoposide. HFFs were treated with etoposide as described in the legend to Fig. 1H. γH2A.X was detected by Western blotting. β-actin is an internal control. (F) Binding of E2F1 to endogenous TAp73 promoter was decreased by PSM.7-LP in the *RB1*-deficient cancer cell lines. ChIP assay was carried out with anti-E2F1 antibody using the cancer cell lines introduced with PSM.7-LP as in the legend to (C). *CDC6* and *β-actin* are positive and negative control, respectively.

virus (Fig. 5C) and examined TAp73 gene expression by qRT-PCR (Fig. 5D). As expected, the expression of the *TAp73* gene was clearly suppressed by PSM.7-LP in all the cancer cell lines. We also examined whether TAp73 target gene expression was reduced by PSM.7-LP in accordance with suppression of TAp73 gene expression. NOXA gene expression was reduced, at least to some extent, by PSM.7-LP in all the cancer cell lines. These results suggest that the *TAp73* gene was activated by deregulated E2F in the cancer cell lines and that the activation was reduced upon re-introduction of pRB.

Most cancer cell lines are thought to harbor DNA damage, which may activate the *TAp73* gene. Therefore, we compared the amount of γH2A.X in the cancer cell lines with that in HFFs treated with etoposide. The result showed that the levels of γH2A.X in the *RB1*-deficient cancer cell lines were lower than that induced by etoposide treatment of HFFs (Fig. 5E), which did not activate the TAp73 promoter at all (Fig. 1H). These results suggest that the *TAp73* gene is mainly activated by deregulated E2F activity, and not mainly through DNA damage, in the cancer cell lines.

To further examine deregulated E2F activity in *RB1*-deficient cancer cell lines, we examined binding of deregulated E2F1 to endogenous TAp73 promoter using ChIP assay (Fig. 5F). When we introduced PSM.7-LP, binding of E2F1 to TAp73 promoter was clearly decreased in 5637 and C-33 A cells and slightly decreased in Saos-2 cells. These results suggest that, in the *RB1*-deficient cancer cell lines, deregulated E2F1 bound to TAp73 promoter, which was suppressed by re-introduction of pRB.

Taken together, these observations indicate that the *RB1*-deficient cancer cell lines harbor deregulated E2F activity that binds and activates the *TAp73* gene.

Discussion

Our results clearly indicate that the tumor suppressor *TAp73* gene, a previously known pro-apoptotic E2F target, is activated by deregulated E2F activity induced by ectopically expressed E2F1 and forced inactivation of pRB by adenovirus E1a or shRB, but not by physiological E2F activity induced by serum stimulation in human normal fibroblast HFFs. The isolated TAp73 promoter reflected the responsiveness of the *TAp73* gene to deregulated and physiological E2F activity. We showed that four ERE73s could discriminate deregulated E2F activity from physiological E2F activity. ChIP assay showed that deregulated E2F1, but not physiological E2F1, bound to TAp73 promoter *in vivo*. Moreover, the *RB1*-deficient cancer cell lines harbored deregulated E2F activity, which activated ERE73s, the TAp73 promoter and the endogenous *TAp73* gene.

We previously reported that the tumor suppressor *ARF* gene, the upstream regulator of the tumor suppressor p53, and the CDK inhibitor $p27^{Kip1}$ gene, an upstream regulator of the tumor suppressor pRB are specifically activated by deregulated E2F activity in human normal fibroblasts (Komori *et al.* 2005; Ozono *et al.* 2009). These results suggest that, upon loss of pRB function, deregulated E2F activates the two major tumor suppressor pathways (p53 and RB pathways) to protect cells from tumorigenesis. In this study, we discovered that the tumor suppressor *TAp73* gene is activated by deregulated E2F activity, but not by physiological E2F activity in HFFs. Accumulating evidence indicates that the *TAp73* gene also plays major roles in tumor suppression. TAp73 can induce apoptosis independently of p53 and plays major role in E2F1-induced apoptosis in $p53^{-/-}$ cells (Irwin *et al.* 2000). $TP73^{+/-}$ mice show an increased rate of spontaneous tumors (Flores *et al.* 2005). Specifically, $TAp73^{-/-}$ mice are tumor prone and sensitive to chemical carcinogenesis (Tomasini *et al.* 2008). Taken together, these observations indicate that the *TAp73* gene is a *bona fide* tumor suppressor gene. Our results underscore the role of deregulated E2F in tumor suppression by activating the TAp73 pathway in addition to the p53 and RB pathways.

It is reported that *TAp73* gene expression is induced by treatment with etoposide, a DNA damaging agent, in the H116 colon cancer cell line through stabilization of E2F1 (Urist *et al.* 2004). In contrast, the TAp73 promoter was not activated by etoposide treatment in HFFs (Fig. 1H) under the condition where γH2A.X, a DNA damage marker, was detected (Fig. 1I) and where a p53 reporter (RGC-Luc) was activated (Fig. 1H). There seems to be a difference in the regulation of the *TAp73* gene in response to DNA damage between cancer cell lines and normal cells. Our results that the *TAp73* gene is activated by deregulated E2F activity in the *RB1*-deficient cancer cell lines suggests that the *TAp73* gene is also activated by deregulated E2F activity in other cancer cell lines including ones retaining pRB. It may be that stabilization of E2F1 by DNA damage augments deregulated E2F activity and further activates *TAp73* gene expression in cancer cell lines but not in normal cells, which do not harbor deregulated E2F activity. Alternatively, response of E2F1 to

treatment with DNA damaging reagents may differ between cancer cell lines and normal growing cells. Further studies are required to elucidate differential regulatory mechanism of *TAp73* gene expression by DNA damage in cancer cell lines and in normal growing cells.

We identified the main E2F responsive elements in the TAp73 promoter, ERE73s, which play central role in discriminating between physiological and deregulated E2F activity in HFFs. Unexpectedly, the core sequences of ERE73s were similar to that of a typical E2F site (Fig. 3A), which is activated by both physiological and deregulated E2F activity. This is in contrast to the E2F-responsive element of ARF promoter (EREA), which lacks T-stretches and is composed of GC repeats and is similar to that of $p27^{Kip1}$ promoter (EREK) (Fig. 3A). Indeed, the core sequence of ERE73-1 completely fits the sequence of a typical E2F site. Accordingly, we could not find consensus sequences to specifically sense deregulated E2F activity. It might be difficult for an E2F binding site alone to discriminate between deregulated and physiological E2F activity. The flanking sequence around the E2F binding site could be important for discriminating deregulated E2F activity from physiological E2F activity. Alternatively, the location of the E2F-responsive element in the promoter may be important for the distinct regulation. In the case of growth-related genes, E2F-responsive elements are located very close to transcription start sites, mostly within 100 bp. All of ERE73s are located more than 100 bp upstream of transcription start site. The location of ERE73s may hinder activation by physiologically activated E2F. Another possibility is the presence of other sequences in the promoter that bind a factor(s), which cooperates with deregulated E2F to activate transcription. Further studies are required to address these issues.

The TAp73 promoter and ERE73s were activated in the *RB1*-deficient cancer cell lines, but not in HFFs. These results suggest that deregulated E2F activity may exist specifically in cancer cells, but not in normal growing cells. Current treatments of cancer such as radiation and anti-cancer drugs preferentially damage growing cells. Accordingly, they damage not only cancer cells but also normal growing cells, which may cause side effects that hamper radical treatment of cancer. To avoid these side effects, it is crucial to specifically target cancer cells. For this purpose, it is important to discriminate cancer cells from normal growing cells. Our findings that deregulated E2F activity exists in the *RB1*-deficient cancer cells but not in growing HFFs suggest that deregulated E2F activity may serve as a useful indicator to discriminate cancer cells from, at least, normal growing fibroblasts. It might also be possible to apply deregulated E2F activity to specifically target cancer cells. Defects in the RB pathway are observed in almost all cancers. It has yet to be determined whether cancer cells, which retain pRB but have defect(s) in upstream of pRB, also harbor deregulated E2F activity that activates the tumor suppressor genes.

Experimental procedures

Cell culture

Human normal fibroblasts (HFFs) and *RB1*-deficient cancer cell lines (5637, Saos-2, and C-33 A) were maintained as described previously (Ozono *et al.* 2009). To synchronize the cell cycle, HFFs were cultured in Dulbecco's modified Eagle medium (DMEM) containing 0.1% fetal calf serum (FCS) for 2 days and were restimulated with serum by adding FCS to a final concentration of 20%.

Plasmids

p73(-892)-Luc was generated by cloning the −892 to +52 region of the TAp73 promoter (AF235000) into pGL3-Basic (Promega) using *Sma* I and *Hin*d III sites. p73(-892)pm-Luc, which has point mutations in all four E2F-responsive elements in the TAp73 promoter (Fig. 3), was generated by site-directed mutagenesis. Expression vectors for E2F1, the 2RG form of E1a, shRNA against *RB1* (shRB), control shRNA (shCon), a constitutively active form of pRB (PSM.7-LP), β-galactosidase, and control plasmids were described previously (Ohtani *et al.* 1998; Komori *et al.* 2005; Iwanaga *et al.* 2006). A series of expression vectors for E2F1 through E2F3, pENTR-E2F1 through E2F3 were generated by cloning full length cDNAs for E2F1 through E2F3 into pENTR/CMV, which were made by cloning CMV promoter to poly(A) addition signal cassette from pcDNA3 (Invitrogen, Carlsbad, CA, USA) into pENTR/D-TOPO (Invitrogen). pGL3-Prom, pARF(-736)-Luc, pARF(-66)-Luc, pCDC6-Luc/wt, and pCDC6-Luc/mt were described previously (Komori *et al.* 2005). pARF(-13)-Luc was generated by deleting the region between *Bgl* II and *Pst* I of pARF(-66)-Luc. pRGC-Luc contains ribosomal gene cluster (RGC) p53 binding sites (Kern *et al.* 1991), upstream of murine c-fos core promoter-driven luciferase gene.

Transfection and reporter assay

Lipofection and luciferase assay were carried out as described previously (Ohtani *et al.* 1998; Komori *et al.* 2005). All assays were carried out at least three times and results are presented as means ± SE.

Recombinant adenovirus

Ad-Con, Ad-E2F1, Ad-12SE1A (Δ2-11), Ad-shCon and Ad-shRB were described previously (Komori *et al.* 2005; Ozono *et al.* 2009). Ad-PSM.7-LP was generated from the expression vector for PSM.7-LP using ViraPower Adenoviral Expression System (Invitrogen) according to the supplier's protocol.

RNA interference

The target sequence for *RB1* was described previously (Komori *et al.* 2005).

Reverse transcription-PCR and quantitative (q) RT-PCR

mRNA isolation and reverse transcription (RT)-PCR were carried out as described previously (Komori *et al.* 2005). The *TAp73* cDNA was amplified by nested PCR, using primer sets specific for *TAp73*. The first round amplification was 20 cycles with the primer set 5′-GGAGGCCGGCGTGGGGAA GAT-3′ and 5′-GCTGGGTTGTGCGTAGGGCGAGTG-3′ (annealing temperature: 64.2 °C). The second round amplification was 35 cycles with the primer set 5′-AGT CCACCGCCACCTCCCCTGAT-3′ and 5′-CATTATTC CCCCGGCTTGACTGG-3′ (annealing temperature: 58.8 °C). Primer sets for *CDC6* and *GAPDH* were described previously (Komori *et al.* 2005). The PCR products of *TAp73* and *CDC6* were detected using ethidium bromide staining and that of *GAPDH* was detected using ^{32}P incorporation using image analyzer BAS 1500 (Fuji Film, Tokyo, Japan). qPCR analysis was carried out using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) for *CDC6*, *NOXA* and *GAPDH,* and SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) for *TAp73*. Primer sets for *CDC6*, *GAPDH*, *TAp73*, *NOXA*, *p21*, *PUMA* and *14-3-3σ* are described previously (Ferguson *et al.* 2000; Kartasheva *et al.* 2002; Yakovlev *et al.* 2004; Komori *et al.* 2005; Zhao *et al.* 2007; Sun *et al.* 2009).

Fluorescence-activated cell sorting analysis

Cells were fixed with 1x Fluorescence-activated cell sorting (FACS) lysing solution (Becton Dickinson Franklin Lakes, NJ, USA), washed with phosphate-buffered saline containing 0.5% bovine serum albumin and 15 mM NaN_3 and stained with propidium iodide (50 μg/mL) containing RNase (50 μg/mL). Cell samples were analyzed with a FACSCalibur (Becton Dickinson).

Immunoblot analysis

Immunoblot analysis was carried out as described previously (Iwanaga *et al.* 2001). The antibodies used were anti-pRB [sc-50, Santa Cruz, 1:1000 with Canget Signal (TOYOBO, La Jolla, CA, USA)], anti-phospho-Histone H2A.X (Ser139) [05-636, Millipore, 1:1000 with Canget Signal (TOYOBO)], anti-α-tubulin (DM1A, Oncogene Research Products, 1:500 with skim milk) and anti-β-actin (A1978, SIGMA, St Louis, MO, USA; 1:2000 with skim milk).

Chromatin immunoprecipitation assay

ChIP assay was carried out as described (Komori *et al.* 2005) using specific primer sets for TAp73 promoter (annealing temperature: 55 °C) (Lau *et al.* 2009), CDC6 promoter (annealing temperature: 60 °C) (Komori *et al.* 2005) and β-actin promoter (annealing temperature: 59.2 °C) (Komori *et al.* 2005). The amplification was 35 cycles for each PCR. Antibodies for immunoprecipitating protein-DNA complexes were anti-E2F1 (sc-56662) and anti-HA (sc-7392) as a negative control (all from Santa Cruz). Input is one 60th of the lysates.

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