Role of the p53-homologue p73 in E2F1-induced apoptosis

Thorsten Stiewe & Brigitte M. Pützer

Most human cancers harbour aberrations of cell-cycle control[1], which result in deregulated activity of the E2F transcription factors with concomitant enhanced cell-cycle progression[2]. Oncogenic signalling by E2F1 has recently been linked to stabilization and activation of the tumour suppressor p53 (refs 1,3,4). The p73 protein shares substantial sequence homology and functional similarity with p53 (refs 5–7). Hence, several previously considered p53-independent cellular activities may be attributable to p73. Here we provide evidence that E2F1 directly activates transcription of *TP73*, leading to activation of p53-responsive target genes and apoptosis. Disruption of p73 function by a tumour-derived p53 mutant reduced E2F1-mediated apoptosis. Thus, p73 activation by deregulated E2F1 activity might constitute a p53-independent, anti-tumorigenic safeguard mechanism.

To investigate the apoptotic activity of E2F1 in p53-null cells, we generated a cell line based on Saos-2 cells (which harbour a homozygous deletion of *TP53*) that constitutively expresses wild-type E2F1 fused to a modified, 4-hydroxytamoxifen (4-OHT)-responsive version of the ligand-binding domain of the mouse oestrogen receptor (ER). The ER–E2F1 protein is activated conditionally with 4-OHT (ref. 8) and at levels comparable to endogenous E2F1 (Fig. 1*c*). Transactivation of a luciferase reporter plasmid containing six E2F DNA binding sites occurred in a manner dependent on 4-OHT (data not shown). In addition, activation of E2F1 in serum-starved cells led to rapid S-phase entry and, subsequently, apoptosis (Fig. 1*a*).

In a cDNA microarray–based screen for potential E2F1-regulated tumour-suppressor proteins, we identified *TP73*. Semi-quantitative RT–PCR analysis revealed an increase in *TP73* mRNA levels following activation of E2F1 (Fig. 1*b*). We detected *TP73* mRNA as early as 4 hours after induction, and it increased up to 20-fold by 8–12 hours after induction. The kinetics of induction were equivalent to those of *CCNE1* (encoding cyclin E) mRNA, which is known to be regulated by E2F1 (Fig. 1*b*). Western-blot

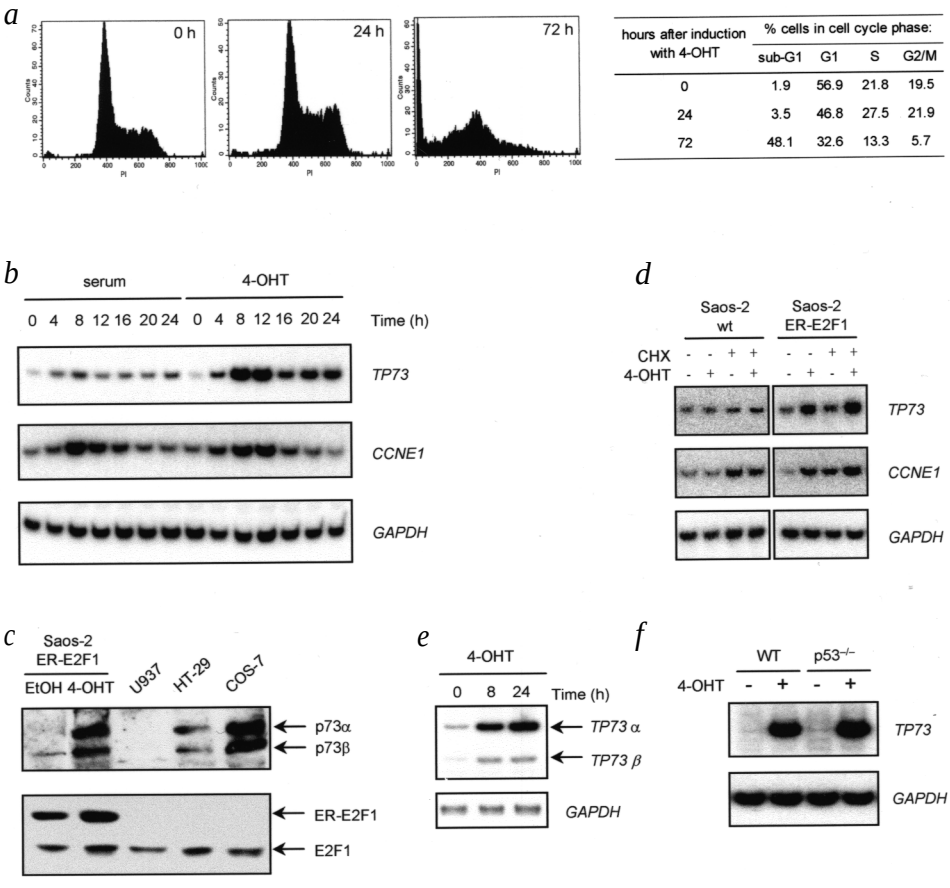


Fig. 1 Induction of p73 by E2F1. ***a***, Flow cytometry profiles showing DNA content (*x* axis), against cell number (*y* axis). The inducible ER–E2F1 Saos-2 cell line was cultured under serum-starvation for 24 h and induced by the addition of 4-OHT. Samples were taken for FACS analysis to determine the cell-cycle profile at indicated hours. ***b***, Serum-starved Saos-2 cells stably transfected with ER–E2F1 were grown for the time indicated. Semiquantitative RT–PCR analysis on total RNA for *TP73*, *CCNE1* and *GAPDH* expression was carried out under linear amplification conditions. ***c***, Western-blot analysis of p73 and E2F1 expression in ER–E2F1 transfected Saos-2 cells grown for 24 h in the absence and presence of 4-OHT. p73-negative U937 and p73-expressing HT-29 and COS-7 cells are shown as negative and positive controls, respectively. ***d***, Semi-quantitative RT–PCR analysis of *TP73*, *CCNE1* and *GAPDH* expression in serum-starved Saos-2 cells expressing ER–E2F1 grown in 4-OHT and/or cycloheximide (CHX) as indicated. ***e***, Semi-quantitative RT–PCR analysis of p73 α- and β-isoform expression in serum-starved Saos-2 cells expressing ER–E2F1 grown for 8 or 24 h in 4-OHT as indicated. *GAPDH* expression is shown as a control. RT–PCR analysis was performed using primers specific for *TP73* exon 12 and exon 14, respectively. ***f***, Semi-quantitative RT–PCR analysis of mouse *Trp73* and *GAPDH* expression in AdER–E2F1 infected wild-type and p53-negative MEFs. Cells were serum-starved for 48 h, infected with 100 multiplicity of infection (MOI) of AdER-E2F1 and induced 24 h postinfection by addition of 4-OHT to a final concentration of 1 μM for 8 h. RT–PCR analysis was carried out using mouse *Trp73* primers.

Institute of Molecular Biology (Cancer Research), University of Essen, Medical School, Essen, Germany (FRG). Correspondence should be addressed to B.M.P. (e-mail: brigitte.puetzer@uni-essen.de).

analysis revealed a concomitant increase in p73 (Fig. 1*c*). In addition, treatment of cells with serum led to induction of *CCNE1* and *TP73* mRNA, suggesting that activation of endogenous E2F1 by growth factor stimulation is sufficient to induce *TP73* transcription. Induction of *TP73* mRNA by serum was less pronounced than that of *CCNE1*. The two major p73 isoforms, produced by alternative splicing, can both activate p53-responsive promoters and induce apoptosis[6]. Hence, both p73α and p73β were similarly induced by activation of E2F1 (Fig. 1*e*).

To discriminate between *TP73* being a direct or indirect E2F1 target, we activated ER–E2F1 in the presence of cycloheximide (CHX), which inhibits *de novo* protein synthesis. Activation of ER–E2F1 in the presence of CHX did not lead to S-phase entry (data not shown), but still upregulated *TP73* mRNA (Fig. 1*d*). We demonstrated 4-OHT–dependent induction of *TP73* mRNA in human p53-null H1299 cells (data not shown) and wild-type or p53-negative primary mouse embryo fibroblasts (Fig. 1*f*) infected with an inducible adenoviral vector expressing ER–E2F1.

These results indicate that E2F1 has a direct effect on *TP73* expression. We then ascertained whether the *TP73* promoter contained E2F-binding sites. We cloned a genomic fragment encompassing exon 1 of *TP73*. Sequence analysis revealed several putative E2F-binding sites, which cluster in the region directly upstream of exon 1 and in the 5′ portion of intron 1 (Fig. 2*a*). Transfection of asynchronously growing Saos-2 cells with a luciferase plasmid containing the complete *Eco*RI fragment of the *TP73* promoter region demonstrated substantial (approximately 400-fold) induction of luciferase activity by co-transfection of E2F1, whereas the E2F1-mutant E132 (defective for DNA binding) did not enhance the basal promoter activity (Fig. 2*b*). We next constructed and tested a panel of reporter plasmids containing the *Eco*RI promoter fragment with increasing 5′ deletions by transient co-transfection of E2F1 in Saos-2 cells (Fig. 2*c*). The highest (approximately 500-fold) activation was observed with the p73P-*Kpn*I construct. Further 5′ deletions gradually reduced the E2F1-responsiveness, which was completely abrogated in the p73P-*Stu*I construct. The main loss in inducibility occurred in the region between the *Bam*HI and *Stu*I sites, indicating that this region spanning *TP73* exon 1 contains the major E2F1-responsive elements. We showed binding of recombinant E2F1 to this promoter region by *in vitro* DNA-binding assay (Fig. 2*d*) and confirmed the specificity of E2F binding by electrophoretic mobility shift assays using oligonucleotides corresponding to the putative E2F-binding sites shown (Fig. 2*a*). These data demonstrate that E2F1 directly activates *TP73* transcription by binding to several E2F-responsive elements in the *TP73* core promoter.

Because ectopic expression of p73 has been shown to activate p53-regulated genes responsible for growth arrest and apoptosis[6,7], we also analysed activation of p53 target genes by E2F1. To exclude activation of p53 targets by

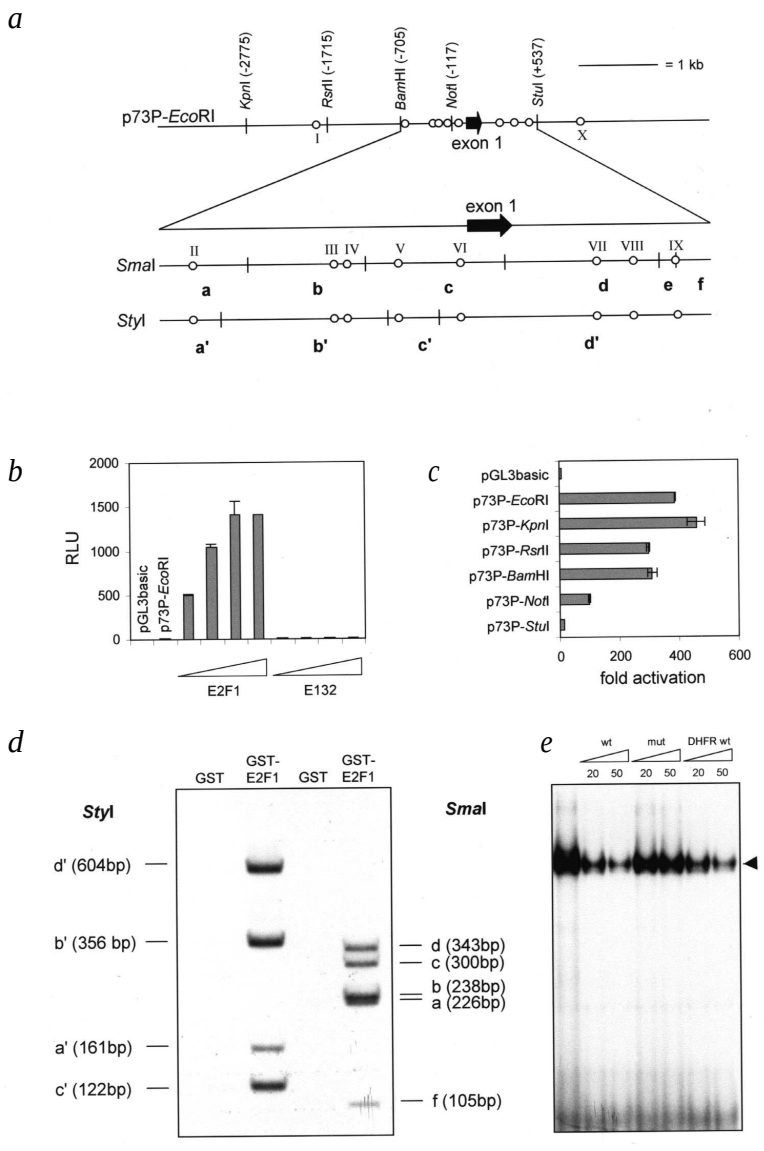
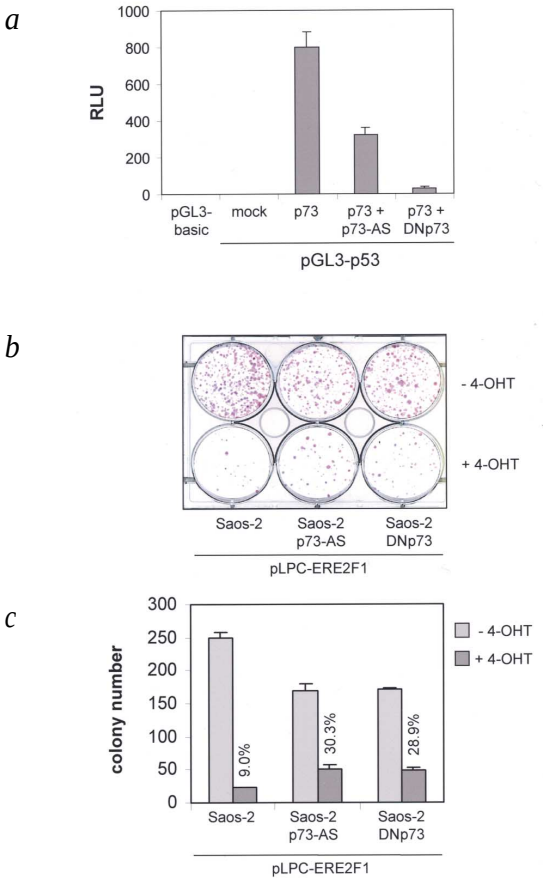


Fig. 2 Transcriptional activation of the human *TP73* promoter by E2F1. ***a***, Schematic representation of the genomic organization of the human *TP73* promoter. Exon 1 is indicated by a filled arrow, putative E2F binding site with the consensus sequence TT(C/G)(C/G)CG(C/G) by open circles. The individual E2F-binding sequences were as follows: I, 5′–TTTGCCGC–3′; II, 5′–CTTGGCGG–3′; III, 5′–GCGCCAAA–3′; IV, 5′–GCGGGAAG–3′; V, 5′–CTTCCCGC–3′; VI, 5′–CCGCGAAG–3′; VII, 5′–CTTGGCGC–3′; VIII, 5′–TTTGGCGCCAAA–3′; IX, 5′–CCGGGAAG–3′; X, 5′–TTTCCCGG–3′. Vertical bars indicate restriction sites used to create 5′ deletion constructs. Positions are labelled relative to the first nucleotide of the published *TP73* cDNA which is considered as nt +1. The core E2F-responsive promoter region is enlarged. Letters (a–f) and (a′–d′) identify *Sma*I or *Sty*I restriction fragments used in (*d*). ***b***, Luciferase assay of Saos-2 cells co-transfected with 1 μg p73P-*Eco*RI or pGL3basic and increasing amounts (100 ng, 500 ng, 1,000 ng, 2,000 ng) of expression plasmid encoding E2F1 or the DNA-binding defective E2F1 mutant E132. p73P-*Eco*RI contains the genomic *Eco*RI fragment between nt –4040 to +3033. Promoter activity of pGL3basic was normalized to 1.0, and the activities of the remaining transfection reactions were expressed relative to this. ***c***, Luciferase assay of Saos-2 cells co-transfected with p73 promoter construct or pGL3basic and 2 μg of E2F1 expression plasmid or empty vector. The different promoter constructs were generated using the restriction sites indicated in (*a*). The resulting luciferase reporter plasmids were termed p73P-*Eco*RI (–4040/+3033), p73P-*Kpn*I (–2775/+3033), p73P-*Rsr*II (–1715/–3033), p73P-*Bam*HI (–705/+3033), p73P-*Not*I (–117/+3033) and p73P-*Stu*I (+537/+3033), respectively. Luciferase activities are expressed as the fold activation relative to co-transfection of the promoter constructs with empty vector. Error bars indicate one standard deviation of the mean. ***d***, Labelled *Sma*I or *Sty*I fragments (a–d,f and a′–d′ in (*a*), respectively) of the *TP73* promoter region (nt –705 to +537) were recovered on glutathione-Sepharose beads after absorption to GST or GST–E2F1 *in vitro* and analysed by polyacrylamide gel electrophoresis. ***e***, EMSA. A labelled oligonucleotide corresponding to the E2F-binding site VIII in the p73 promoter was incubated with recombinant E2F1. A 20- or 50-fold excess of unlabelled wild-type (wt), mutated (mut) or *DHFR* wild-type (DHFR wt) oligonucleotide was added as indicated. The position of the E2F1-complex is indicated (arrowhead).

E2F1-mediated stabilization of p53 by means of p14ARF, we used Saos-2 cells. Co-transfection with wild-type E2F1 expression plasmid and a luciferase reporter plasmid containing a minimal promoter (three p53 binding sites upstream of a TATA box), shown to be inducible by p73 (data not shown), gave increased levels of luciferase activity. In contrast, E2F1 mutant E132 had no effect on luciferase activity (Fig. 3*a*). We next assayed our ER–E2F1-expressing Saos-2 cell line for induction of several p53-responsive genes after treatment with 4-OHT, by RT–PCR analysis (Fig. 4*b*). *TP73* and the *CDKN2A* transcript p14ARF, which are directly activated by E2F1, showed a strong increase in mRNA levels. In addition, p53-responsive genes *CDKN1A*, *MDM2*, *14-3-3σ* and *GADD45A* also showed elevated mRNA levels. These findings indicate that E2F1-induced p73 might in fact be responsible for this effect, in turn indicating that activation of p53 target genes might be responsible for E2F1-induced apoptosis.

Reasoning that inhibition of p73 activity would block E2F1-induced apoptosis, we transfected Saos-2 cells with a p73-antisense expression construct or a p73 mutant with a truncated amino terminus, which is transcriptionally inactive and unable to induce apoptosis[9]. Effective inhibition of high-level p73 expression by both constructs has been demonstrated by luciferase assay using the p53-reporter plasmid described above (Fig. 4*a*). The p73 mutant shows a dominant-negative effect (DNp73). To analyse the effect of p73 inhibition on E2F1-induced cytotoxicity, we transduced Saos-2 cells with retroviral vectors encoding ER–E2F1 and antisense p73 or dominant-negative p73 (Fig. 4*b*). In colony-formation assays, cells with inhibited p73 formed a higher number of colonies in the presence of 4-OHT than did cells with unrestrained p73 function. This result indicates that inhibition of p73 function partially rescues cells from E2F1-induced apoptosis.



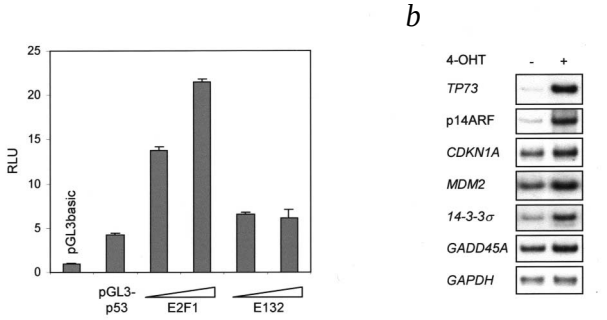


Fig. 3 Induction of p53-responsive targets by E2F1. ***a***, Luciferase assay of Saos-2 cells co-transfected with 1 μg of the p53-responsive luciferase reporter construct pGL3-p53 or pGL3basic and increasing amounts (50 ng and 200 ng) of expression plasmid encoding for E2F1 or the DNA-binding defective E2F1 mutant E132. Promoter activity of pGL3basic was normalized to 1.0, and the activities of the remaining transfection reactions were expressed relative to this. Error bars indicate one standard deviation of the mean. ***b***, Semi-quantitative RT–PCR analysis of *TP73*, *CDKN2A* p14ARF, *CDKN1A*, *MDM2*, *GADD45A* and *GAPDH* expression in ER–E2F1-transfected serum-starved Saos-2 cells grown for 24 h in the absence or presence of 1 μM 4-OHT.

Several groups have recently described inhibition of p73 function by tumour-derived p53 mutants[10,11]. We therefore established Saos-2 cell lines stably expressing the p53 R175H mutant, as E2F1-induced p73 would be neutralized in these cells. Effective inhibition of p73 function in two independently derived cell clones was demonstrated by reduced activation of the p53 reporter plasmid by co-transfected p73α (Fig. 5*a*) and p73β (data not shown). To investigate inhibition of E2F1-induced p73 in these cell clones, we infected parental Saos-2 cells and the two Saos-2 cell clones expressing p53 R175H with a retroviral vector encoding the inducible ER–E2F1. As a control, we infected parental Saos-2 cells with a retroviral vector encoding green fluorescent protein (GFP) and determined that there were high levels of mutant p53 in both cell clones (Fig. 5*b*). Following E2F1 induction for 8 or 24 hours, respectively, *TP73* mRNA levels were increased. A parallel increase in the mRNA levels of *14-3-3σ* and *MDM2* was observed in parental Saos-2 cells expressing ER–E2F1, but not in the p53-mutant cell clones. We tested the effect of p73 inhibition on E2F1-mediated cell killing in colony-formation assays in parental Saos-2 cells and cell clones expressing mutant p53. Consistent with the neutralization of E2F1-induced p73, mutant p53-expressing cell clones were partially resistant to E2F1-induced cytotoxicity, resulting in approximately twice as many colonies as parental Saos-2 cells (Fig. 5*c*).

To determine whether reduction in E2F1-induced cytotoxicity by mutant p53 correlates with diminution of p73-mediated apoptosis, we transfected parental Saos-2 cells and Saos-2 cells expressing mutant p53 with a construct expressing ER–E2F1 and GFP from the same plasmid in the presence or absence of 4-OHT. The fate of transfected GFP-positive cells was followed by fluorescence microscopy. Unlike mutant p53-expressing cells, parental cells were extremely sensitive to ER–E2F1 in the presence of 4-OHT,

Fig. 4 Inhibition of E2F1-induced p73 function. ***a***, Luciferase assay of Saos-2 cells co-transfected with p53-responsive luciferase reporter construct pGL3-p53 or pGL3basic and p73 expression plasmids. For inhibition, p73-antisense (p73-AS) or dominant-negative p73 (DNp73) expression plasmid was co-transfected. The total amount of DNA was equalized with empty vector. Standard deviation is indicated by error bars. ***b***, Colony formation assay. Saos-2 cells, retrovirally transduced with ER-E2F1, p73-antisense (p73-AS) and dominant-negative p73 (DNp73), were seeded at equal cell numbers in 6-well plates and incubated in the absence or presence of 4-OHT. After two weeks, cells were stained with Giemsa and the number of colonies counted. The absolute colony number was obtained from duplicate experiments (bottom). The percentage of viability is as indicated. The error bars represent one standard deviation.

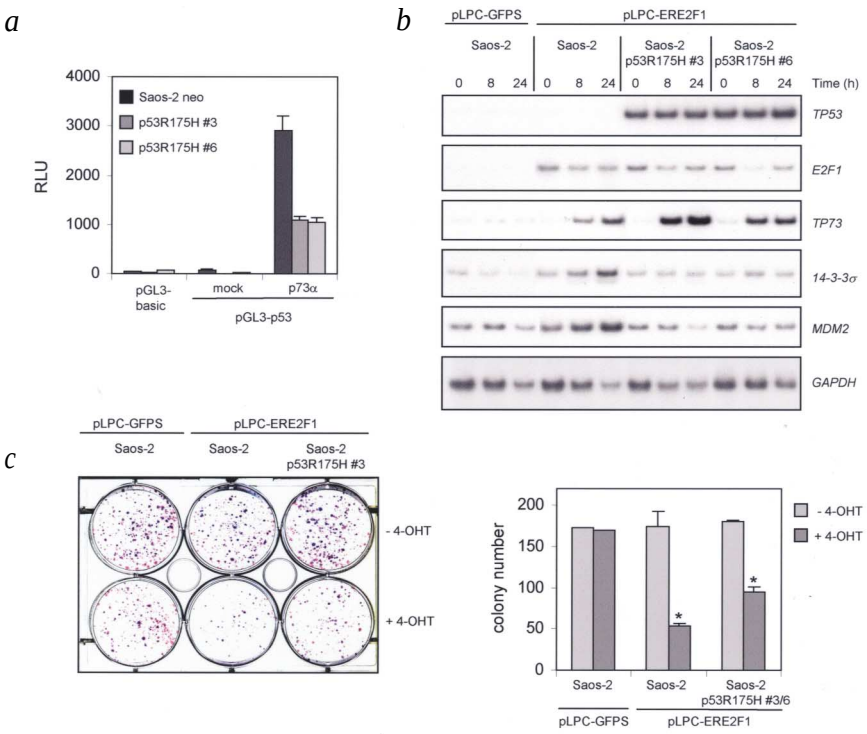


Fig. 5 Inhibition of E2F1-induced p73 function by the tumour-derived p53 mutant R175H. ***a***, Luciferase assay of parental or stably p53 R175H expressing Saos-2 cells co-transfected with the p53-responsive luciferase reporter construct pGL3-p53 or pGL3basic and p73α expression plasmid. ***b***, Semi-quantitative RT–PCR analysis of *TP53*, *E2F1*, *TP73*, *14-3-3σ*, *MDM2* and *GAPDH* expression in parental Saos-2 cells or two independent p53 R175H expressing clones infected with GFPS or ER-E2F1 encoding retroviral vectors as indicated. Infected cells were selected for 5 d in puromycin, serum-starved for 24 h and induced with 4-OHT for the indicated time period before RNA preparation. ***c***, Colony formation assay. Saos-2 cells established as in (*b*) were seeded at equal cell numbers in 6-well plates and incubated in the absence or presence of 4-OHT. After two weeks, cells were stained with Giemsa and the number of colonies counted. In the right panel, the absolute colony number obtained from duplicate experiments is given. The error bars represent one standard deviation. The higher colony number in p53 R175H expressing cells compared with parental cells is highly significant ($P<0.01$).

with cells rounding up and showing cytological features (Fig. 6*a*,*b*) characteristic of apoptotic cells. Expression of mutant p53 resulted in a reduction of apoptotic cells to a level approximately 30% of that observed in parental Saos-2 cells (Fig. 6*c*).

Our data support p73 expression being directly induced by E2F1, leading to activation of p53-responsive target genes and programmed cell death. Two mechanisms of E2F1-induced apoptosis have been described so far: (i) inhibition of survival signals, making tumour cells more sensitive to apoptotic signals such as TNFα (ref. 12); and (ii) activation of the *CDKN2A* transcript p14ARF, resulting in p53-dependent apoptosis[3]. We have shown that part of the apoptotic activity of E2F1 reflects the ability to induce expression of p73. Linking deregulated E2F1 activity to the activation of p73 might constitute a p53-independent, anti-tumorigenic safeguard mechanism. A recent study[2] indicates that the p53-independent apoptotic activity of E2F1 requires DNA binding but not transactivation. Here we show that the DNA-binding domain of E2F1 is required for p73 induction, but we cannot exclude that the mechanism of induction might be independent of transactivation. Expression of mutant p53 overcomes the tumour-surveillance function of this pathway, enabling cells to sustain elevated p73 protein levels, consistent with recent reports demonstrating elevated p73 expression in several human cancers[13–15]. Only approximately 50% of human cancers harbour *TP53* mutations, however, and it will be interesting to investigate how oncogenically stimulated cells evade this failsafe mechanism to promote tumour growth.

Methods

Plasmids and adenoviral contructs. Expression plasmids for wild-type E2F1, the DNA-binding defective E2F1-mutant E132 and ER–E2F1 (pBabeHAERE2F1) have been described[8,16,17]. We constructed the ER–E2F1/GFP expression plasmid by removing the ER–E2F1 cDNA from pBabeHAERE2F1 with an *Eco*RI restriction digest and ligating the blunt-ended cDNA fragment to the blunt-ended, *Bam*HI-restricted E2F1 E138/GFP plasmid[18]. The expression plasmid pC53-175 for human p53 R175H was a gift from B. Vogelstein[19]. Mammalian expression plasmids encoding haemagglutinin (HA)-tagged versions of human p73α and p73β were provided by G. Melino[20]. The N-terminally truncated p73 expression construct was provided by H. Lu[9]. The construction of the ER–E2F1-expressing first-generation adenoviral vector has been described[21].

Cloning the *TP73* promoter region. We cloned the human *TP73* promoter region from the CITB human BAC library (Research Genetics). A 7.0-kb genomic *Eco*RI fragment containing *TP73* exon 1 was isolated and sequenced. To obtain p73P-EcoRI, the *Eco*RI fragment was cloned blunt-ended into the *Sma*I site of pGL3-basic (Promega). We generated 5´-deletion fragments using endogenous restriction sites for *Kpn*I, *Rsr*II, *Bam*HI, *Not*I and *Stu*I of the *Eco*RI *TP73* promoter and subsequently cloned them into pGL3basic.

Retroviral vectors. The ER–E2F1 cDNA from pBabeHAERE2F1 (ref. 8) and the GFPS cDNA encoding the membrane-localized enhanced GFP (ref. 22) were cloned into the *Bam*HI site of pLPC (ref. 23) for expression of the transgene from the CMV IE promoter. The cDNA for the N-terminally truncated p73 mutant was cloned into pLXRN (Clontech). For p73-antisense expression, the 0.75-kb *Nhe*I-*Eco*RI fragment from p73-pcDNA3-HA (ref. 20) was cloned blunt-ended in antisense direction into the *Bam*HI site of pLXRN. High-titre VSV-G pseudotyped retroviruses generated by transient co-transfection of Anjou65 cells (ATCC CRL 11269) with these constructs and VSV-G protein expression plasmid were harvested every 6 h, 48–72 h after transfection. We used pooled, filtered supernatants to infect Saos-2 cells or p53 R175H expressing Saos-2 cell clones (2×10^5 cells plated/100-mm-diameter dishes) in the presence of Polybrene (4 μg/ml). At 12 h postinfection, 10 ml of fresh medium was added, and medium was changed 24 h later. Cells were selected in puromycin (1 μg/ml) for at least 5 d before treatment of surviving cells with 4-OHT (1 μM; Sigma).

Cell culture and transfections. We obtained Saos-2, H1299, U937, COS-7 and HT-29 cell lines from ATCC and prepared primary mouse embryo fibroblasts (MEFs) as described[4]. All cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal calf serum (FCS, Biochrom) and 1% penicillin G/streptomycin sulphate (Life Technologies). Transfections were performed by electroporation[24]. Inducible cell lines were established in Saos-2 cells by transfection with pBabeHAERE2F1 using the calcium phosphate method. Cells were selected in DMEM containing 10% FCS, supplemented with puromycin (1.0 μg/ml) and then cloned by limiting dilution. For starvation conditions, cells were grown in DMEM with 0.5% FCS for 24 h and induced with fresh 15% serum or by the addition of 4-OHT to a final concentration of 1 μM. Cycloheximide (CHX; Sigma) was used at a final con-

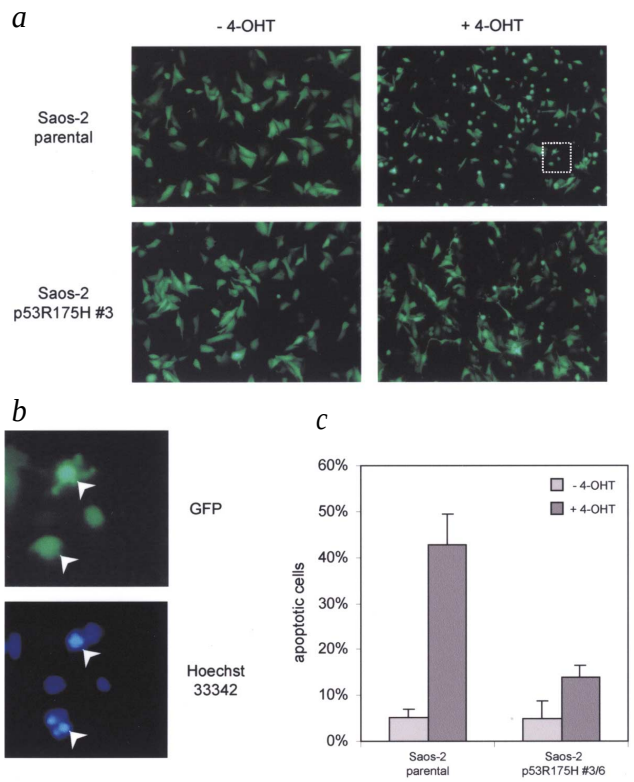


Fig. 6 Reduction of E2F1-induced apoptosis by inhibition of p73. Parental Saos-2 cells or Saos-2 cells expressing p53 R175H were transfected with the ERE2F1/GFP plasmid and incubated in the absence or presence of 4-OHT for 48 h. ***a***, The morphology of GFP-expressing cells is shown on the fluorescence micrographs. ***b***, Higher magnification of GFP-and Hoechst 33342-fluorescence of 4-OHT–induced Saos-2 cells expressing ERE2F1. Transfected cells showing nuclear fragmentation and membrane blebbing, typical features of apoptotic cells, are indicated (arrowheads). ***c***, GFP-stained cells from at least three independent transfections were counted in randomly selected fields from each plate and evaluated for signs of apoptosis. The average percentage of apoptotic cells with standard deviation is shown.

centration of 10 μg/ml. We established mutant p53 R175H expressing Saos-2 cells by transfection of Saos-2 cells with pC53-175. Cell clones stably expressing p53 R175H were selected in medium containing G418 (500 μg/ml; Life Technologies) and analysed by western blot for p53 expression. Adenoviral infections were carried out as described[21].

Semi-quantitative RT–PCR. We carried out RT–PCR on total RNA prepared with the RNeasy Mini Kit (Qiagen). Following DNAse I treatment, RNA (1 μg) was reverse transcribed using oligo-dT and Omniscript RT (Qiagen). PCR was performed on 2 μl of the 50 μl cDNA sample. To obtain a semi-quantitative result, PCR products were labelled with α-[^{32}P]-dCTP and we used the minimum number of cycles to obtain a clear signal in the linear range. Sequences of the primers used are available on request.

Western-blot analysis. Cells were lysed in RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and total protein concentration was quantitated by Bradford assay. Samples (300 μg of protein per lane) were separated by SDS–PAGE, transferred to nitrocellulose membranes (Amersham) and probed with goat anti-human p73 antiserum or anti-human E2F1 monoclonal antibody (both Santa Cruz). Antibody binding sites were visualized using appropriate horseradish-peroxidase conjugated secondary antibodies according to the enhanced chemiluminescence protocol.

Luciferase assay. Saos-2 cells or mutant p53 expressing Saos-2 cell clones were transfected by electroporation. We determined luciferase activities 48 h post transfection using a premanufactured Luciferase Reporter Assay System (Promega) and normalized them to the total protein concentration in the cell extract. Error bars represent the standard deviation within a representative experiment. Each experiment was repeated at least three times.

In vitro DNA binding assay. Recombinant GST–E2F1 fusion protein was generated as described[25]. We tested the presence of potential E2F1-binding sites in the 1.2-kb genomic *Bam*HI-*Stu*I promoter fragment *in vitro* by a GST-pull down assay essentially as described[26]. Briefly, the 1.2-kb *Bam*HI-*Stu*I fragment contained in p73P-*Eco*RI was digested with *Sma*I or *Sty*I, treated with calf intestinal phosphatase and end-labelled with T4 kinase and high specific activity ^{32}P-γATP. We incubated labelled DNA (30 ng) with GST or GST–E2F1 (100 ng) in HMO buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 5% glycerol, 10 mM DTT, 0.1% NP40, 0.5 mg/ml BSA) supplemented with salmon sperm DNA (600 ng). Reactions were incubated at RT for 30 min, bound to glutathione-sepharose for 15 min, then washed 5 times with HMO buffer (500 μl) using MicroSpin GST Purification Modules (Pharmacia). Bound DNA was eluted and analysed on a 5% polyacrylamid gel.

EMSA was performed as described[27]. We used the following oligonucleotides: E2F(VIII), 5′–GGCCGCCTTTGGCGCCAAAGACAGCC–3′; E2F(VIII)mut, 5′–GGCCGCCTTTGGATCCA AAGACAGCC–3′; DHFR wt, 5′–ATTTAAGTTTCGCGCCCTTTCTCAA–3′.

Colony formation assay. We infected Saos-2 cells or mutant p53 expressing Saos-2 cell clones with retroviruses encoding GFPS or ER–E2F1, respectively. Following puromycin selection, equal cell numbers were plated on 6-well plates and cultured in the absence or presence of 4-OHT (1 μM). Two weeks later, the cells were fixed, stained with Giemsa (Sigma) and colonies were counted.

Apoptosis assay. Saos-2 cells or mutant p53 expressing clones were transfected by electroporation with ER–E2F1/GFP expression plasmid (10 μg) and incubated in the absence or presence of 4-OHT (1 μM). To visualize apoptosis in unfixed monolayer cultures, 48 h post-transfection the cells were incubated at 37°C in the presence of 1 μg/ml Hoechst 33342. After 15 min, propidium iodide solution was included to 5 μg/ml and monolayers were observed by epifluorescence microscopy[28]. Cells dissociating from the monolayer exhibiting blue fluorescing masses of chromatin that abutted the nuclear membrane or, at later stages, showed blue fluorescing spherical bodies were identified as apoptotic. Cells from at least three independent transfections were counted in randomly selected fields from each plate.

Flow cytometry. Cells were seeded on 60-mm dishes, serum-starved for 24 h and further incubated in the absence or presence of 4-OHT (1 μM). Cells were collected 24 and 72 h after induction, fixed in 70% ethanol and stained for DNA content with propidium iodide (Sigma). Flow cytometric analysis was carried out (FACSVantage, Becton Dickinson) and analysed as described using CellQuest software[21] (Becton Dickinson).

GenBank accession number. Nucleotide sequence of the *TP73* promoter, AF235000.

Acknowledgements

We thank S. Zimmermann for technical assistance; H. Karsunky for preparing mouse embryo fibroblasts; K. Lennartz for assistance with flow cytometry; and K. Helin, J.R. Nevins, P. Farnham, B. Vogelstein, G. Melino, H. Lu and S. Lowe for providing expression plasmids for E2F1, ER-E2F1, E132 mutant, E2F1-E138/GFP, p53 R175H, p73 and the pLPC retroviral vector, respectively. This work was supported in part by a grant of the Deutsche Krebshilfe, Dr. Mildred Scheel Stiftung (B.M.P.) and the University of Essen, IFORES program of the Medical Faculty (T.S.).

Received 28 August; accepted 4 October 2000.

1. Sherr, C.J. Tumor surveillance via the ARF-p53 pathway. *Genes Dev.* **12**, 2984–2991 (1998).
2. Phillips, A.C., Bates, S., Ryan, K.M., Helin, K. & Vousden, K.H. Induction of DNA synthesis and apoptosis are separable functions of E2F-1. *Genes Dev.* **11**, 1853–1863 (1998).
3. Bates, S. *et al.* p14ARF links the tumor suppressors RB and p53. *Nature* **395**, 124–125 (1998).
4. Zindy, F. *et al.* Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev.* **12**, 2424–2433 (1998).
5. Kaghad, M. *et al.* Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* **90**, 809–819 (1997).
6. Jost, C.A., Marin, M.C. & Kaelin, W.G. p73 is a human p53-related protein that can induce apoptosis. *Nature* **389**, 191–194 (1997).
7. Zhu, J., Jiang, J., Zhou, W. & Chen, X. The potential tumor suppressor p73 differentially regulates cellular p53 target genes. *Cancer Res.* **58**, 5061–5068 (1998).
8. Vigo, E. *et al.* CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. *Mol. Cell. Biol.* **19**, 6379–6395 (1999).
9. Zeng, X. *et al.* The N-terminal domain of p73 interacts with the CH1 domain of p300/CREB binding protein and mediates transcriptional activation and apoptosis. *Mol. Cell. Biol.* **20**, 1299–1310 (2000).
10. Di Como, C.J., Gaiddon, C. & Prives, C. p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. *Mol. Cell. Biol.* **19**, 1438–1449 (1999).
11. Marin, M.C. *et al.* A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. *Nature Genet.* **25**, 47–54 (2000).
12. Phillips, A.C. *et al.* E2F-1 potentiates cell death by blocking anti-apoptotic signaling pathways. *Mol. Cell* **4**, 771–781 (1999).
13. Kovalev, S. *et al.* Expression level, allelic origin, and mutation analysis of the p73 gene in neuroblastoma tumors and cell lines. *Cell Growth Differ.* **9**, 897–903 (1998).
14. Yokomizo, A. *et al.* Overexpression of the wild type p73 gene in human bladder cancer. *Oncogene* **18**, 1625–1629 (1999).
15. Ng, S.-W. *et al.* Analysis of p73 in human borderline and invasive ovarian tumor. *Oncogene* **19**, 1885–1890 (2000).
16. Helin, K., Harlow, E. & Fattaey, A. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol. Cell. Biol.* **13**, 6501–6508 (1993).
17. Cress, W.D., Johnson, D.G. & Nevins, J.R. A genetic analysis of the E2F1 gene distinguishes regulation by Rb, p107, and adenovirus E4. *Mol. Cell. Biol.* **13**, 6314–6325 (1993).
18. Lee, T.A. & Farnham, P.J. Exogenous E2F expression is growth inhibitory before, during, and after cellular transformation. *Oncogene* **19**, 2257–2268 (2000).
19. Baker, S.J., Markowitz, S., Fearon, E., Willson, B. & Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* **249**, 912–915 (1990).
20. DeLaurenzi, V. *et al.* Two new p73 splice variants, γ and δ, with different transcriptional activity. *J. Exp. Med.* **188**, 1763–1768 (1998).
21. Pützer, B.M., Stiewe, T., Crespo, F. & Esche, H. Improved safety through tamoxifen-regulated induction of cytotoxic genes delivered by Ad vectors for cancer gene therapy. *Gene Ther.* **7**, 1317–1325 (2000).
22. Kalejta, R.F., Shenk, T. & Beavis, A.J. Use of a membrane-localized green fluorescent protein allows simultaneous identification of transfected cells and cell cycle analysis by flow cytometry. *Cytometry* **29**, 286–291 (1997).
23. de Stanchina, E. *et al.* E1A signaling to p53 involves the p19ARF tumor suppressor. *Genes Dev.* **12**, 2434–2442 (1998).
24. Van den Hoff, M.J.B., Moorman, A.F.M. & Lamers, W.H. Electroporation intracellular buffer increases cell survival. *Nucleic Acids Res.* **20**, 2902 (1992).
25. Chiang, S.-Y., Azizkhan, J.C. & Beerman, T.A. A comparison of DNA-binding drugs as inhibitors of E2F1- and Sp1-DNA complexes and associated gene expression. *Biochemistry* **37**, 3109–3115 (1998).
26. Wu, K.-J. *et al.* Direct activation of TERT transcription by c-MYC. *Nature Genet.* **21**, 220–224 (1999).
27. Marin, M.C. *et al.* Viral oncoproteins discriminate between p53 and the p53 homolog p73. *Mol. Cell. Biol.* **18**, 6316–6324 (1998).
28. Ormerod, M.G., Sun, X.-M., Brown, D., Snowden, R.T. & Cohen, G.M. Quantification of apoptosis and necrosis by flow cytometry. *Acta Oncol.* **32**, 417–424 (1993).