Reduction of total E2F/DP activity induces senescence-like cell cycle arrest in cancer cells lacking functional pRB and p53

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E2F/DP complexes were originally identified as potent transcriptional activators required for cell proliferation. However, recent studies revised this notion by showing that inactivation of total E2F/DP activity by dominant-negative forms of E2F or DP does not prevent cellular proliferation, but rather abolishes tumor suppression pathways, such as cellular senescence. These observations suggest that blockage of total E2F/DP activity may increase the risk of cancer. Here, we provide evidence that depletion of DP by RNA interference, but not overexpression of dominant-negative form of E2F, efficiently reduces endogenous E2F/DP activity in human primary cells. Reduction of total E2F/DP activity results in a dramatic decrease in expression of many E2F target genes and causes a senescence-like cell cycle arrest. Importantly, similar results were observed in human cancer cells lacking functional p53 and pRB family proteins. These findings reveal that E2F/DP activity is indeed essential for cell proliferation and its reduction immediately provokes a senescence-like cell cycle arrest.

Introduction

As the major downstream mediator of the retinoblastoma protein (pRB) tumor suppressor pathway, the E2F/DP transcription factor complexes play a crucial role in cell cycle regulation (Dyson, 1998). Recent studies suggest that E2F/DP complexes can be broadly classified into two subgroups: a group of "activating" E2Fs (E2F1, E2F2, and E2F3) that are potent transcriptional activators, and a second group of "repressive" E2Fs (E2F4, E2F5, and E2F6) that appear to function primarily as transcriptional repressors (Mann and Jones, 1996; Takahashi et al., 2000; Trimarchi and Lees, 2002). This interpretation is supported by chromatin immunoprecipitation (ChIP) analysis, which reveals that activating E2Fs replace repressive E2Fs at E2F-regulated promoters as cells progress from G0/G1 toward S phase, a change that correlates with the induction of E2F-dependent gene expression (Takahashi et al., 2000).

The combined ablation of E2F1, E2F2, and E2F3 genes in primary mouse embryonic fibroblasts results in the reduction of E2F target gene expression and block of cell proliferation (Wu et al., 2001). However, inactivation of both E2F4 and E2F5 does not inhibit cell proliferation, but instead renders cells resistant to a p16^{INK4a}-induced growth arrest (Gaubatz et al., 2000; Ohtani et al., 2003). These results suggest that activating E2Fs and repressive E2Fs play opposing roles and that the balance between activating E2Fs and repressive E2Fs is likely to regulate cell cycle progression. Consistent with this idea, inactivation of total E2F/DP activity by overexpression of dominant-negative (dn-) forms of E2F or DP did not inhibit cellular proliferation, but rather abolished a variety of growth arrest pathways, such as TGF-β–induced growth arrest, p16^{INK4a}-induced cell cycle arrest, contact inhibition, and cellular senescence (Bargou et al., 1996; Zhang et al., 1999; Rowland et al., 2002). These results suggest that activating E2Fs are only required to counterbalance the effects of repressive E2Fs and that total E2F/DP activity is not essential for cellular proliferation, but rather to promote tumor suppression mechanisms. Thus, inactivation of total E2F/DP activity may actually increase the risk of cancer. Because current therapeutic approaches that target E2F/DP do not inactivate specific E2F family members, but instead block all E2F/DP activity (Bandara et al., 1997), it is vital to clarify the role of E2F/DP activity in normal and cancerous human cells.
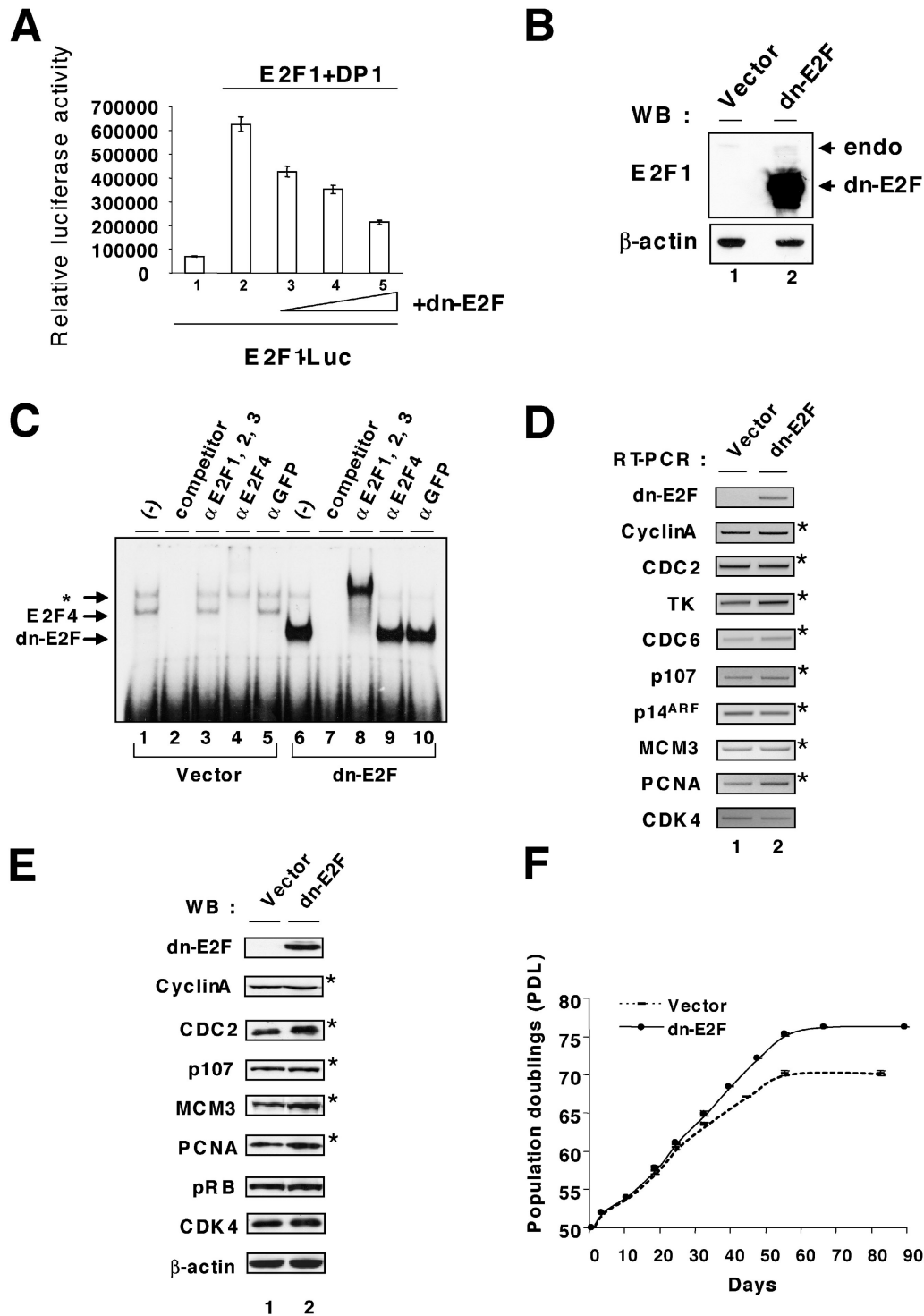
Figure 1. **Effects of dn-E2F on cell growth.** (A) The E2F1 and DP1 expression vectors (0.6 μg) were cotransfected into 60-mm plates of SVts8 cells along with a reporter plasmid and lacZ plasmid. Where indicated, cells were also cotransfected with an increasing amount of expression plasmid encoding dn-E2F (lane 2: 0 μg; lane 3: 0.1 μg; lane 4: 0.3 μg; lane 5; 0.6 μg). Error bars indicate SD. (B) Early passage (45PDLs) TIG-3 cells infected with retrovirus encoding dn-E2F or empty vector. Cells were analyzed for expression of E2F1 and dn-E2F protein by Western blot after selection with hygromycin. The antibody against β-actin was used as a loading control. (C) EMSAs were performed using a radiolabeled probe containing the E2F consensus sequence of the adenovirus E2 gene promoter and extracts from cells infected with retrovirus encoding empty vector (lanes 1–5) or dn-E2F (lanes 6–10) in the absence or presence of antibodies as indicated. The DNA-binding activity, which is not shifted by any available E2F antibodies, was marked by an asterisk. (D and E) Cell extracts were prepared from cells at 60PDLs and subjected to RT-PCR (D) or Western blotting (E) with primers or antibodies shown at left. E2F target genes reported previously were highlighted with an asterisk. CDK4 was used here as a loading control. (F) Early passage (45PDLs) TIG-3 cells infected with retrovirus encoding dn-E2F or empty vector were selected for expression of the hygromycin selectable marker for 5 d and used in proliferation curves performed in triplicate. Error bars indicate SD.
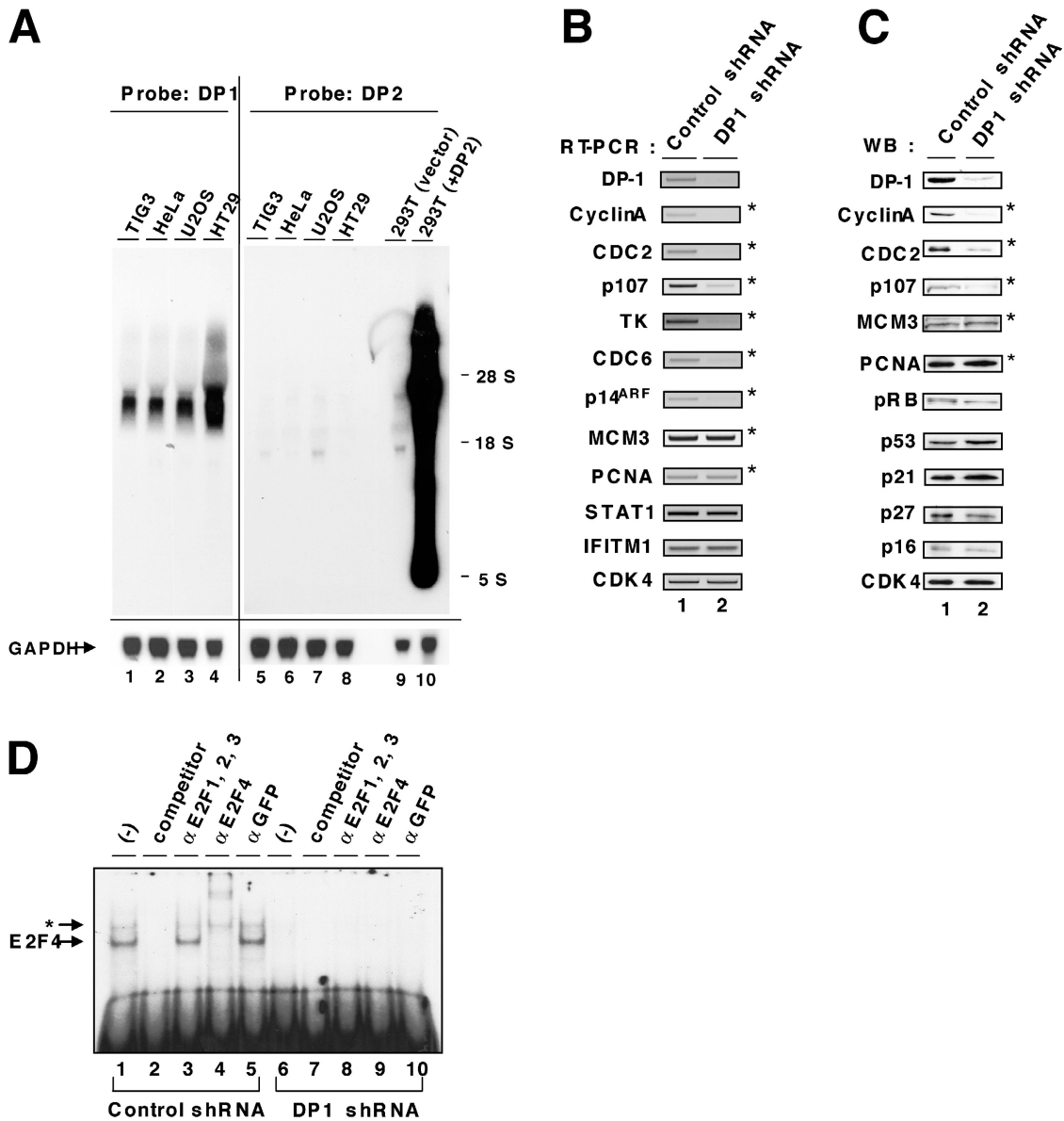
A

Probe: DP1 | Probe: DP2

TIG3 HeLa U2OS HT29 | TIG3 HeLa U2OS HT29 | 293T (vector) 293T (+DP2)

- 28 S
- 18 S
- 5 S

GAPDH▶

1 2 3 4 | 5 6 7 8 | 9 10

B

RT-PCR :   Control shRNA   DP1 shRNA

DP-1
CyclinA *
CDC2 *
p107 *
TK *
CDC6 *
p14^ARF *
MCM3 *
PCNA *
STAT1
IFITM1
CDK4

1   2

C

WB :   Control shRNA   DP1 shRNA

DP-1
CyclinA *
CDC2 *
p107 *
MCM3 *
PCNA *
pRB
p53
p21
p27
p16
CDK4

1   2

D

(-) competitor α E2F1, 2, 3 α E2F4 α GFP (-) competitor α E2F1, 2, 3 α E2F4 α GFP

*
E2F4▶

1 2 3 4 5 6 7 8 9 10

Control shRNA   DP1 shRNA

Figure 2. **Depletion of DP1 causes reduction of E2F target gene expression in HDFs.** (A) 20 µg of total RNA prepared from TIG-3 (50PDLs), HeLa, U2OS, HT29, and 293T cells transfected with or without DP2 expression plasmid were subjected to Northern blot analysis using the same activity of indicated radiolabeled probes. (B and C) Early passage TIG-3 cells (45PDLs) were infected with retrovirus encoding DP1-shRNA (lane 2) or control-shRNA (lane 1). Cell extracts were prepared from cells at 7 d after selection with puromycin, and were subjected to RT-PCR (B) or Western blotting (C) with primers or antibodies shown at left. E2F target genes reported previously were highlighted with an asterisk. CDK4 was used here as a loading control. (D) EMSA was performed using extracts from cells infected with retrovirus encoding control-shRNA (lanes 1 to 5) or DP1-shRNA (lanes 6 to 10) in the absence or presence of antibodies shown at top. The DNA-binding activity, which is resistant to any available E2F antibodies, was marked by an asterisk.

In this paper, we address the role of E2F/DP activity in human primary and cancer cells by comparing two approaches to inactivating total E2F/DP activity: the use of a dn mutant and RNA interference (RNAi) (Brummelkamp et al., 2002).

Results and discussion

To delineate the role of E2F/DP on proliferation of human primary cells, we first tested the effect of overexpressing dn-E2F (E2F-DB), which lacks both the transcriptional activation domain and the pRB-family protein binding domain (Zhang et al., 1999). As reported previously (Zhang et al., 1999; Rowland et al., 2002), increasing amounts of dn-E2F blocked the transactivating activity of cotransfected E2Fs in human fibroblasts (Fig. 1 A). The dn-E2F was then retrovirally transduced into early passage primary human diploid fibroblasts (HDFs), TIG-3 cells. Ectopic dn-E2F expression was more than 100 times greater than that of the endogenous E2F1 (Fig. 1 B, lane 2). As expected, the DNA-binding activity of endogenous E2F4 was abolished and replaced by dn-E2F (Fig. 1 C, lanes 1 and 6). In agreement with previous reports (Zhang et al., 1999; Rowland et al., 2002), overexpression of dn-E2F did not reduce the expression of endogenous E2F target genes in TIG-3 cells (Fig. 1, D and E). In addition, TIG-3 cells expressing dn-E2F grew

Figure 3. **Depletion of DP1 causes severe growth arrest in HDFs.** (A and B) Early passage (45PDLs) TIG-3 cells infected with retrovirus encoding DP1-shRNA or control-shRNA were selected for expression of the puromycin-selectable marker for 7d and used in a proliferation assay performed in triplicate (A) or in cell cycle profile analysis by FACS (B). Error bars indicate SD. (C and D) Early passage TIG-3 cells (45PDLs) were infected with retrovirus encoding flag-tagged wild-type DP1 protein containing a mutated shRNA cleavage site (lanes 3 and 4) or empty vector (lanes 1 and 2). After selection with hygromycin, cells were then super-infected with retrovirus encoding DP1-shRNA (lanes 2 and 4) or control-shRNA (lanes 1 and 3). Expression of DP1, cyclin A, and CDC2 (C) and BrdU incorporation (D) was examined 2 d after super-infection. CDK4 was used here as a loading control. E2F target genes reported previously were highlighted with an asterisk. (E) TIG-3 cells infected with retrovirus encoding DP1-shRNA or control-shRNA were examined for SA-β-gal activity and SAHF.

A

B

	Control shRNA	DP1 shRNA
G1	70.9 %	82.7 %
S	17.2 %	0.0 %
G2/M	11.9 %	17.3 %

C

D

E
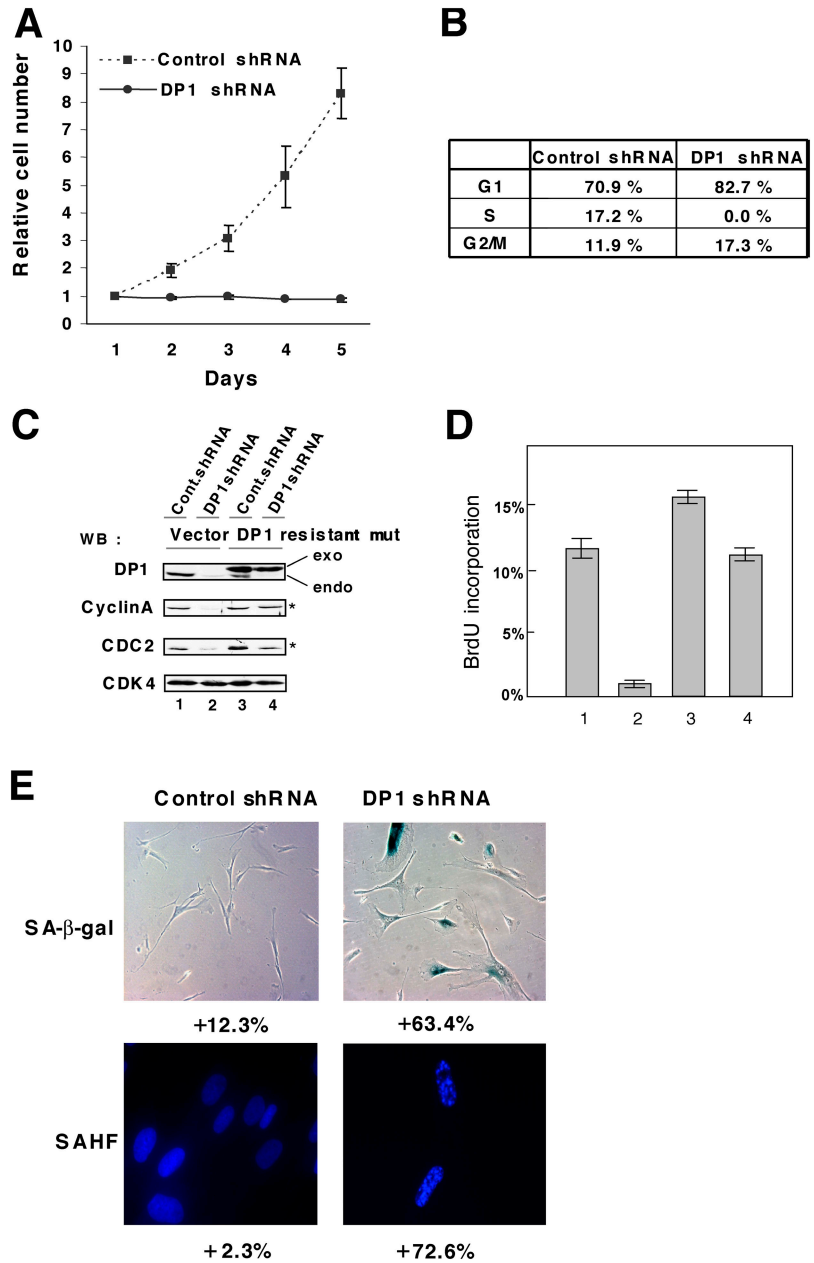
faster than control TIG-3 cells, especially at late passage (Fig. 1 F; unpublished data). These and earlier results (Rowland et al., 2002) suggest that E2F/DP activity may not be essential for cell proliferation in mammalian cells. However, these findings could also be explained by an incomplete inhibition of endogenous E2F/DP activity or by an unforeseen side effect of overexpression of the dn-E2F. Therefore, we sought a different approach to inactivate total E2F/DP activity in HDFs.

Although the E2F family consists of six members, the DP family contains only two (DP1 and DP2) (Trimarchi and Lees, 2002). Because the level of DP2 mRNA is very low in TIG-3 cells (Fig. 2 A) and heterodimerization with a DP protein is essential for E2F activity, we generated a retrovirus vector encoding a small hairpin RNA (shRNA) directed against DP1 to deplete E2F/DP complexes in primary human cells. Early passage TIG-3 cells were infected with retrovirus encoding either a control sequence or shRNA specific for DP1. The levels of DP1 mRNA and protein were significantly reduced within 7 d of infection with the virus expressing the DP1-shRNA, but not the control virus (Fig. 2, B and C). In addition, E2F-DNA–binding activity was almost completely abolished in extracts of TIG-3 cells expressing DP1-shRNA (Fig. 2 D, lanes 1 and 6). In contrast to our results with dn-E2F, the expression level of many E2F target genes required for S phase, such as cyclin A2, thymidine kinase, and cdc6, was dramatically reduced in DP1 knock-down cells (Fig. 2, B and C). Such reduction has also been reported in mouse embryonic fibroblasts lacking the activating E2F genes owing to genetic ablation (Wu et al., 2001). The levels of cdc2 mRNA and protein, an essential component of M phase progression, were also reduced in DP1 knock-down cells. Similar results were also observed within 2 d of viral infection, suggesting that the transcriptional changes directly re-
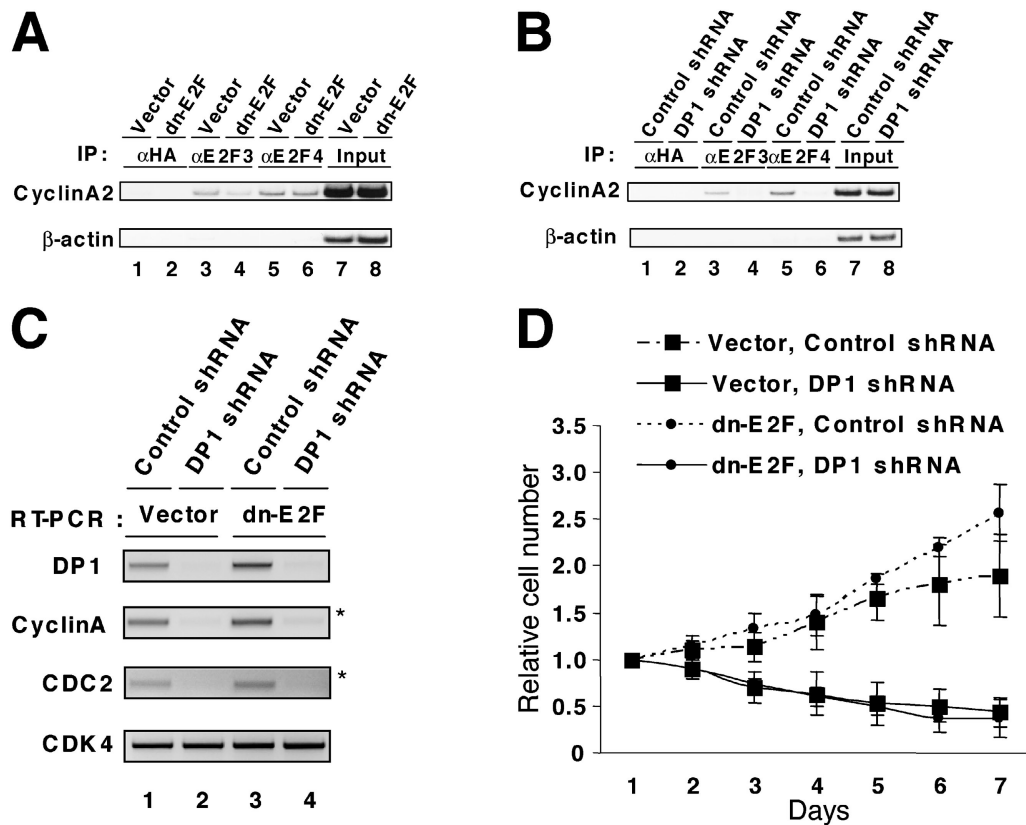
Figure 4. **Incomplete blockage of E2F/DP activity in HDFs expressing dn-E2F.** (A and B) ChIP assays were performed using TIG-3 cells expressing dn-E2F (A), or DP1-shRNA (B) and the antibodies shown. The cyclin A2 promoter was recovered by PCR using primers flanking the E2F-binding sites in the human cyclin A2 promoter. The human β-actin promoter, which does not contain E2F-binding sites, was used as a negative control for PCR. (C) TIG-3 cells (50PDLs) expressing dn-E2F (lanes 3 and 4) or empty vector (lanes 1 and 2) were super-infected with retrovirus encoding DP1-shRNA (lanes 2 and 4) or control-shRNA (lanes 1 and 3). Total RNAs were prepared from cells at 7 d after selection with puromycin, and were subjected to RT-PCR with primers shown on the left of the figure. E2F target genes reported previously were highlighted with an asterisk. (D) Cells described in C were used in a proliferation assay performed in triplicate. Error bars indicate SD.

sult from the decrease of E2F/DP activity (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200411093/DC1). The levels of PCNA and MCM3 were unchanged in DP1 knockdown cells (Fig. 2, B and C). Thus, the expression of MCM3 and PCNA might be regulated by a balance between activating E2Fs and repressive E2Fs, as seen in *Drosophila* cells (Frolov et al., 2003). We failed to see any increase of interferon-inducible genes (STAT1 and IFITM1) or p53 levels (Fig. 2, B and C), arguing against the possibility that the DP1 shRNA induced an interferon response or had nonspecific untargeted effects.

Next, we measured cell proliferation in DP1 knock-down cells. To our surprise, DP1 depletion immediately caused cell cycle arrest (Fig. 3 A) accompanied by a significant decrease in the S phase population and an increase in the G1 phase and G2/M phase populations (Fig. 3 B). To confirm that these effects were actually due to the depletion of DP1 protein, a DP1 cDNA containing a mutated shRNA cleavage site was retrovirally transduced into TIG-3 cells. This mutated cDNA, which is resistant to DP1-RNAi, maintained the level of flag-tagged wild-type DP1 protein, despite expression of DP1-shRNA. Strikingly, it also sustained expression of E2F target genes, such as cyclin A2 and cdc2 (Fig. 3 C, lanes 2 and 4), and entry into S phase (Fig. 3 D, lanes 2 and 4). These results confirm that the

reduction of E2F target gene expression and cell proliferation by DP1-RNAi were specifically dependent on the depletion of DP1 protein. Furthermore, several features of cellular senescence, such as a substantial increase in senescence-associated β-galactosidase (SA-β-gal) activity (Dimri et al., 1995) and senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003) were observed in DP1 knock-down cells (Fig. 3 E), but not in cells expressing flag-tagged DP1 that is resistant to RNAi (unpublished data). These results suggest that the maintenance E2F/DP activity is required for protecting cells from onset of premature or stress-induced senescence.

DP1 knock-down and overexpression of dn-E2F were not equivalent with respect to either E2F target gene expression or cell proliferation. To understand the basis for the apparent discrepancy, we performed ChIP assays using an antibody against E2F3, the most abundant activating E2F in fibroblasts, and examined the precipitated DNA for the presence of cyclin A2 promoter sequences by PCR. Importantly, we found that a significant amount of endogenous E2F3 remained bound to the cyclin A2 promoter in TIG-3 cells expressing dn-E2F (Fig. 4 A, lanes 3 and 4). In contrast, E2F3 binding was significantly reduced in DP1 knock-down TIG-3 cells (Fig. 4 B, lanes 3 and 4). Similar results were observed when we used an anti-E2F4

Figure 5. **Depletion of DP1 causes cellular senescence in human cancer cell lines.** (A–C) U2OS (A), HT-29 (B), and HeLa cells (C) were infected with retrovirus encoding DP1-shRNA or control-shRNA and selected with puromycin for 7 d. Cell numbers were then counted taking a time course in tetraplicate. The levels of DP1 and CDK4 (loading control) were examined by Western blotting at d 3. (D) The HeLa cells infected with retrovirus encoding either control-shRNA or DP1-shRNA were used for SA-β-gal assay 8 d after selection with puromycin. The percentages of average SA-β-gal–positive cells were indicated. (E) ChIP assays were performed in HeLa cells with or without DP1 knocking down as described in Fig. 4, A and B. β-Actin promoter, which does not contain E2F-binding sites, was used as a negative control for PCR. (F) Formation of subcutaneous tumors in nude mice by HeLa cells expressing shRNA against control sequence or DP1. For each injection, 10^6 cells of the indicated populations were injected subcutaneously in a volume of 100 μl. Mice were killed when the tumors reached a diameter of 1 cm or after 10 wk of monitoring.

Cells	Number tumors/ Number injected
HeLa+ control shRNA	19/20
HeLa+ DP1 shRNA	0/20
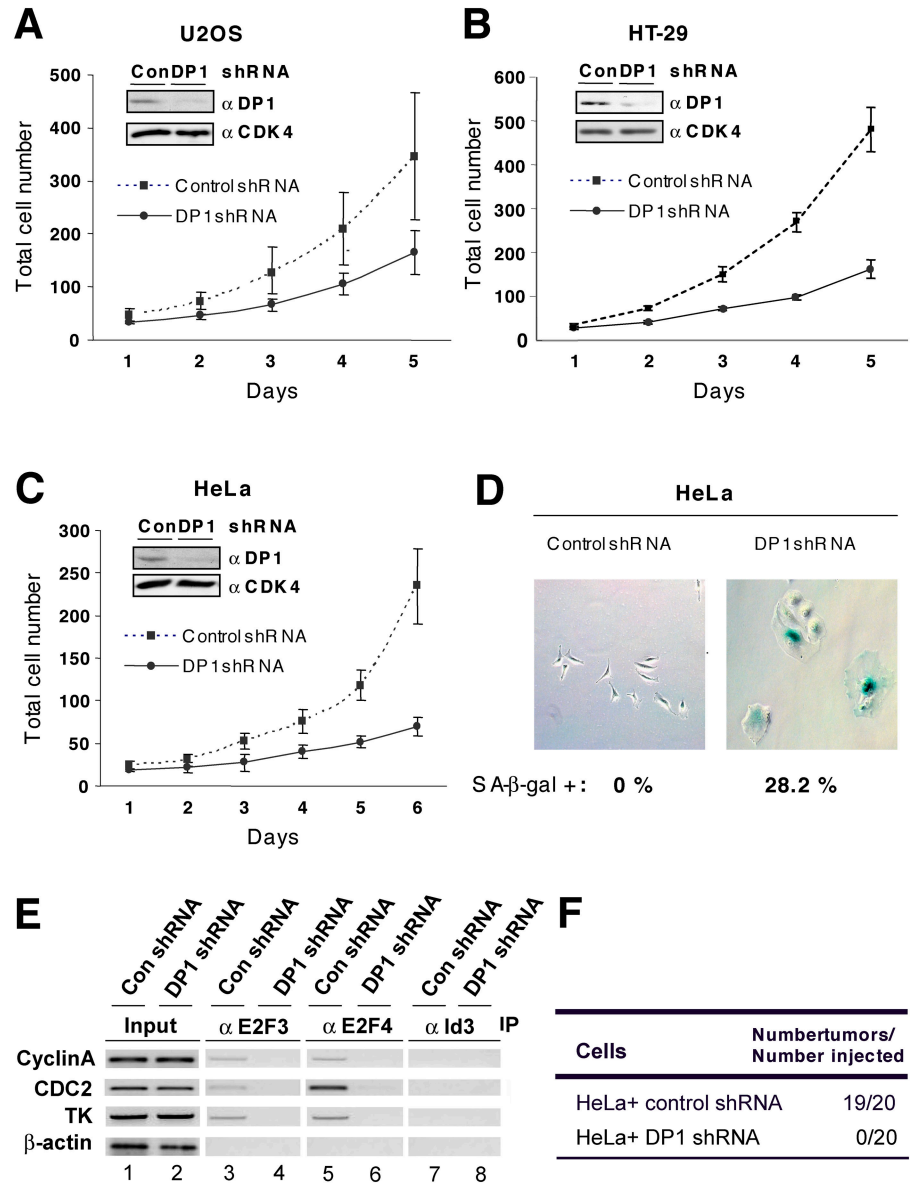
antibody for the ChIP assay (Fig. 4, A and B; lanes 5 and 6). These findings suggest that endogenous E2F/DP activity is effectively blocked by knockdown of DP1, but not by dn-E2F. Indeed, depletion of DP1 from TIG-3 cells expressing dn-E2F caused a significant reduction of E2F target gene expression (Fig. 4 C, lanes 3 and 4) and consequent growth arrest (Fig. 4 D). These results confirm that a substantial level of endogenous E2F/DP activity is indeed present in TIG-3 cells expressing dn-E2F. Thus, the disparate results obtained by the two approaches can be explained by the insufficient blockage of endogenous E2F/DP activity by dn-E2F in HDFs.

To verify our conclusions, we compared the effects of dn-E2F with that of DP1-RNAi in human osteosarcoma U2OS cells, which were used in the previous dn-E2F study (Zhang et al., 1999). Consistent with our results in TIG-3 cells, DP1 knock-down caused a significant inhibition of cell growth (Fig. 5 A), whereas overexpression of dn-E2F had little effect (unpublished data). Additionally, the interpretation of results obtained by dn-E2F has recently been questioned by a study showing that dn-E2F (E2F-DB) contains a previously uncovered pRB-binding domain that could potentially inactivate endogenous pRB-family proteins (Dick and Dyson, 2003). Indeed, we observed significant interaction between dn-E2F and pRB in U2OS cells (Fig. S1 B). Moreover, although overexpression of dn-DP1 in human breast epithelial cells produced effects similar to those produced by dn-E2F, namely an increased incidence of tumors (Bargou et al., 1996), different results were observed depending on the particular dn-DP1 construct that was used (Wu et al., 1996). Therefore, it is important to stress that interpretation of experiments using dn-E2F or DP requires careful evaluation.

A recently reported knock-out study of Kohn et al. (2004) concludes that DP1 is dispensable for growth in various mouse embryonic tissues. Because DP2 levels are very low in human cells tested when compared with DP1 levels (Fig. 2 A; Fig. S2 A, available at http://www.jcb.org/cgi/

content/full/jcb.200411093/DC1), it is possible that the different responses of mouse and human cells to DP1 loss may be due to the DP2 status in the target cells. However, it is equally possible that other explanations exist, such as cell type specificity, acute versus stable target loss, etc.

Next, we asked if DP1 knock-down inhibits the proliferation of other human cancer cell lines. DP1 knock-down significantly inhibited cell proliferation in HT-29 cells, which lack functional p53 (Fig. 5 B), and HeLa cells, in which pRB-family proteins and p53 are inactivated by viral oncoproteins (Fig. 5 C). Interestingly, reduction of DP1 protein levels induced several features of cellular senescence in these cancer cells, including a large, flat morphology and expression of SA-β-gal activity (Fig. 5 D). HeLa cells expressing either DP1-shRNA or control shRNA were injected subcutaneously into immunocompromised mice. Although the control cells efficiently formed tumors in 8 wk (19/20 cases), none of the DP1 knock-down cells developed detectable tumors during the same time period (Fig. 5 F; Fig. S2 B). These results strongly suggest that E2F/DP activity is required for tumor development.

Several lines of evidence suggest that pRB and p53 are critical for induction of cellular senescence (Psyrri et al., 2004). However, we have shown here that a senescence-like cell cycle arrest can be induced by the reduction of total E2F/DP activity without recovering the function of pRB and p53 in HeLa cells (Fig. 5, C and D). Because the p16^{INK4a}/RB tumor suppressor pathway is frequently deregulated in a wide range of human cancers, it is important to identify critical downstream targets of this pathway for cancer therapy (Drayton and Peters, 2002; Lowe and Sherr, 2003). Although additional studies are needed to clarify the precise roles of E2F/DP complexes, our results show that the proliferation of cancer cells could be controlled through targeting the E2F/DP activity.

Materials and methods

Cell culture, transfection, and retrovirus production
TIG-3, SVts8, and HEK 293T cells were grown in DME supplemented with 10% FBS and penicillin/streptomycin. Retrovirus-shRNAs were generated as described previously (Brummelkamp et al., 2002). To generate DP1-resistant mutant against DP1 shRNA, three-point mutations, which do not change encoding amino acids, were introduced into the shRNA cleavage site of the DP1 cDNA. For growth rate analysis, cells were plated on the gridded dish at concentration of 300 cells/1 cm^2. Cell numbers were counted in triplicate. BrdU incorporation was measured as described previously (Ohtani et al., 2003).

Antibodies and protein analyses
Immunoblotting was performed as described previously (Ohtani et al., 2003) with primary antibodies against CDK4 (sc-601; Santa Cruz Biotechnology, Inc.), cyclin A (sc-751; Santa Cruz Biotechnology, Inc.), E2F1 (sc-251; Santa Cruz Biotechnology, Inc.), MCM3 (sc-9849; Santa Cruz Biotechnology, Inc.), PCNA (sc-56; Santa Cruz Biotechnology, Inc.), and p107 (sc-318; Santa Cruz Biotechnology, Inc.), pRB (#554136; BD Biosciences), FLAG (F3165; Sigma-Aldrich), β-actin (A5316; Sigma-Aldrich), DP1 (#W32.3, Cancer Research UK; ab-11834, Abcam), CDC2 (#17; Cancer Research UK), p16 (NA29; Oncogene Research Products), p53 (OP43; Calbiochem), p21 (sc-397; Santa Cruz Biotechnology, Inc.), and p27 (sc-528; Santa Cruz Biotechnology, Inc.)

Electrophoretic mobility shift assays
Electrophoretic mobility shift assays (EMSAs) were performed as described previously (Wu et al., 1995). The specificity of the protein–DNA interactions was conformed by competition with wild-type oligonucleotides

or addition of the antibodies specific for E2F1 (sc-251), E2F2 (sc-633x), E2F3 (sc-879x), E2F4 (sc-866), or GFP (sc-9996).

ChIP assay
ChIP assays were performed based on a modification of previously published methods (Kanemaki et al., 2003; Ohtani et al., 2003). In brief, 5 × 10^6 cells were cross-linked by addition of formaldehyde to 1% final concentration, and then chromatin was sonicated and immunoprecipitation was performed with Dynabeads protein A/G (Dynal), which were incubated with antibody against E2F3 (sc-878x), E2F4 (sc-1082x), or Id3 (sc-490) beforehand. Precipitates were washed and processed for DNA purification. DNA released from precipitated complexes was amplified using sequence-specific primers by PCR (primer sequences: see online supplemental information).

Semiquantitative RT-PCR analyses
Total RNA was isolated using TRIzol (Invitrogen), and 5 μg was reverse transcribed with Super-scriptase (Invitrogen). Hot-start PCR was performed, and the linear range of amplification was determined from PCRs run with serially diluted cDNA. The results were verified by varying the number of PCR cycles for each cDNA and set of primers (see online supplemental information). PCR products were separated on agarose gels and visualized by ethidium bromide staining.

SA-β-gal and SAHF analysis
TIG-3 cells infected with retrovirus encoding DP1-shRNA or control-shRNA were examined for SA-β-gal activity and SAHF as described previously (Dimri et al., 1995; Narita et al., 2003). Slides for SA-β-gal were imaged using a microscope (Axiovert 35M; Carl Zeiss MicroImaging, Inc.) with an Achrostigmat objective (10×, 0.25 NA) and a digital camera (Axiocam MR; Carl Zeiss MicroImaging, Inc.) and software (Zeiss Axiovision). Slides for SAHF were imaged using a microscope (BX51; Olympus) with a Plan Apochromat objective (60×, 1.4 NA) and a digital camera (Colorview 12; Soft-Imaging System) and a software (analysis; Soft-Imaging System). Subsequent processing of TIFF files were undertaken in Adobe Photoshop (version 7.0.1). Images were cropped and assembled into composites for figures after minor adjustments for contrast and color balance were applied to all parts of each image.

Online supplemental material
Fig. S1 A shows the levels of E2F target gene expression after 2 d of DP1 knock-down. Fig. S1 B shows interaction between dn-E2F and pRB. Fig. S2 A shows the levels of DP1 or DP2 mRNA expression in various human cancer cells. Fig. S2 B shows an example of tumor formation assay. Supplemental information shows primer sequences used for RNAi and PCR analysis. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200411093/DC1.

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