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SHORT COMMUNICATION

A functionally distinct member of the DP family of E2F subunits

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E2F transcription factors regulate genes involved in cell-cycle progression. In mammalian cells, physiological E2F exists as an E2F/DP heterodimer. Currently, eight E2F and two DP subunits have been characterized. We report here the characterization of a new member of the DP family, DP-4. While DP-4 exhibits certain similarities with members of the DP family, it also possesses a number of significant differences. Thus, DP-4 forms a heterodimer with E2F subunits, binds to the E2F site and associates with pocket proteins including pRb. In contrast to DP-1, however, DP-4/E2F-1 complexes exhibit reduced DNA binding activity. Furthermore, DP-4 interferes with E2F-1-dependent transcription and delays cell-cycle progression. These results highlight an emerging complexity in the DP family of E2F subunits, and suggest that DP-4 may endow E2F heterodimers with distinct transcription properties.
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The E2F family of transcription factors regulate genes linked to cell-cycle progression (Stevens and La Thangue, 2003). In mammalian cells, physiological E2F is an E2F/DP heterodimer, which binds to a specific DNA sequence in the transcriptional control region of target genes (Frolov and Dyson, 2004). Currently, eight E2F subunits and two DP subunits have been well characterized. Moreover, it is generally believed that E2F-1, E2F-2 and E2F-3 are transcriptional activators, whereas E2F-4 to E2F-8 act in transcriptional repression (Frolov and Dyson, 2004). The family of pocket proteins, exemplified by the retinoblastoma tumour suppressor protein (pRb), bind to and inactivate E2F-1 to E2F-5, whereas E2F-6, E2F-7 and E2F-8 mediate transcriptional repression through pocket protein-independent mechanisms.

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The pathway that controls E2F activity becomes aberrant in most if not all human tumour cells either through mutation and inactivation of the Rb gene, or by altered Cdk activity, which results in deregulated E2F activity. The activation of E2F target genes prompts entry into S-phase and, in some cases, increased apoptosis (Stevens and La Thangue, 2003). For example, *E2F-1*$^{-/-}$ mice display defective apoptosis (Field *et al*., 1996), and the loss of E2F-1 activity suppresses apoptosis and S-phase entry in *Rb*$^{-/-}$ mice (Tsai *et al*., 1998). Furthermore, E2F-1 loss reduces the incidence of tumours and extends the lifespan of *Rb*$^{+/-}$ mice (Yamasaki *et al*., 1998). Thus, E2F activity is intimately connected with proliferation control, and its deregulation contributes significantly to the abnormal growth of tumour cells.

DP subunits are heterodimeric partner proteins for E2F subunits. The first member to be characterized, DP-1, is a widespread component of physiological E2F activity (Girling *et al*., 1993; Bandara *et al*., 1994). It is an essential gene because *DP-1*$^{-/-}$ mice exhibit embryonic lethality, in part owing to defective extra-embryonic tissue development (Kohn *et al*., 2003, 2004). DP-1 is a target for cyclinA/cdk2 kinase, and the phosphorylation of DP-1 blocks the DNA binding activity of the E2F heterodimer that assists exit from S-phase (Krek *et al*., 1994). The other member of the family, murine DP-3, is a rarer component of physiological E2F activity (Ormondroyd *et al*., 1995; Zhang and Chellappan, 1995; Rogers *et al*., 1996). DP-3 is subject to complex regulatory cues that give rise to a variety of alternatively spliced RNA variants and protein isoforms (Ormondroyd *et al*., 1995). For example, one protein isoform, known as β, lacks the nuclear localization signal (NLS) present in the δ isoform and locates primarily to the cytoplasm (de la Luna *et al*., 1996; Allen *et al*., 1997), whereas the α-isoform possesses an *N*-terminal extension, which binds the BTB/POZ domain protein DIP (de la Luna *et al*., 1999). In general, therefore, DP subunits are essential components of E2F activity that integrate important regulatory signals with the E2F heterodimer.

To acquire a thorough understanding of the role of E2F in cell-cycle control it is necessary to clarify the extent and molecular complexity of the two families of subunits. With this objective in mind, we have searched

for new members of the DP family, and describe here a previously uncharacterized member of the DP family, referred to as DP-4. Our results indicate that DP-4 exhibits certain properties in common with other DP proteins but also differences that establish it as a unique and interesting member of the family.

The complete human DP-4 sequence contains 405 amino-acid residues (Figure 1a). The DP-4 gene is

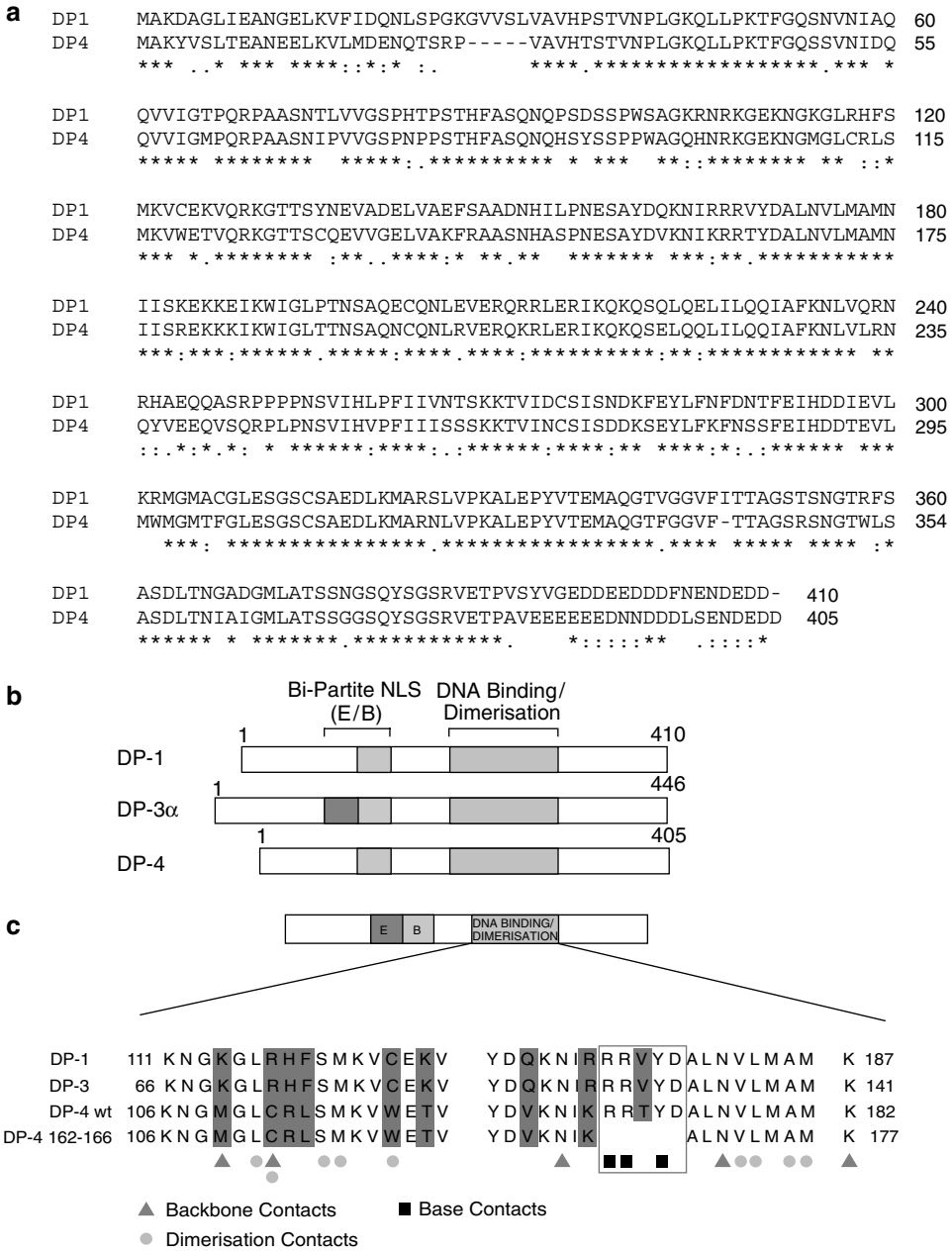

Figure 1 Sequence of DP-4 and comparison to other members of the DP family. (**a**) Complete sequence of human DP-4 and comparison to DP-1 using single-letter amino-acid code. Identical residues are indicated by * and similar residues by •. Human DP-4 was isolated from a human cDNA library using the following primers, SalIDP4, 5′-GATCGTCGACGATGGCAAAATATGT CAGTCTCACTGAAGC-3′ and EcoRIDP4, 5′-GATCGAATTCTCAGTCATCCTCGTCATTCTCACTGAGG-3′. The PCR product was digested with EcoRI and SalI and then cloned into pcDNA3.1+ to create the vector pCMV-HA-DP4wt. The vector pCMV-HA-DP4mt was created by deletion of the DNA-binding domain of pCMV-HA-DP4wt by use of the QuikChange Mutagenesis Kit (Stratagene) using the following primers, DP4For, 5′-GACGTGAAAAACATAAAAGCCTTAAACGTGCTGATGGCC3′ and DP4Rev, 5′-GGCCATCAGCACGTTTAAGGCTTTTATGTTTTTCACGTC3′. (**b**) Diagram of members of the DP family indicating the DNA binding and dimerization domains (green) and bi-partite NLS (red and blue; de la Luna *et al*., 1996). Note that human DP-2 (Zhang and Chellappan, 1995) is equivalent to one of the four isoforms of murine DP-3 (Ormondroyd *et al*., 1995). (**c**) Sequence alignment of the DNA binding and dimerization domain of DP-4 with other members of the family. The sequence of DP-4Δ162–166, which lacks the conserved RRXYD motif (show by the box), is indicated at the bottom. Red colour highlights residues that are conserved across DP-1 and DP-3 but differ in DP-4. The residues involved in DNA binding and dimerization are taken from Zheng *et al*. (1999).

located on the X chromosome and is widely expressed in, for example, skin, testis, ovary, lung and brain tissue. Sequence comparison indicated that DP-4 is closely related to other members of the family, with greater than 90% identity to DP-1. The DNA-binding domain, located in the central region of the protein (Figure 1b), is highly conserved. Most of the residues in human DP-2 (equivalent to murine DP-3; Ormondroyd *et al*. (1995), Zhang and Chellappan (1995)) deduced from the E2F-4/DP-2 structure that contact DNA bases and backbone (Zheng *et al*., 1999) are conserved in DP-4 including the RRXYD motif,

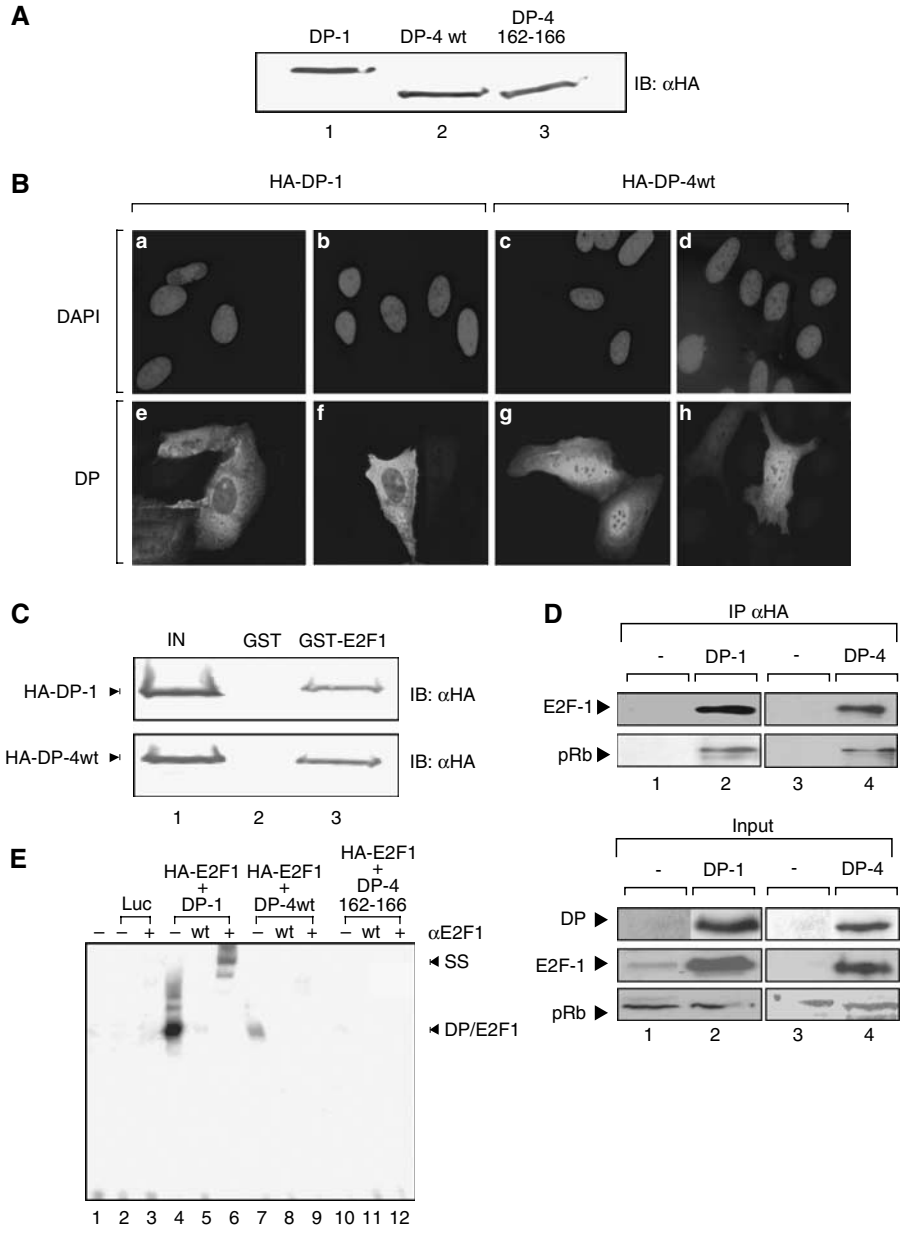

Figure 2 Properties of DP-4. (**A**) U2OS cells were transfected with HA-tagged DP-1 (10 μg), DP-4 (10 μg) or DP-4Δ162–166 (10 μg) as described (Logan *et al*., 2004) and immunoblotted with anti-HA. Ectopic DP-4 resolves as a polypeptide of 45 000 molecular weight. (**B**) U2OS cells were transfected with HA-tagged DP-1 (2 μg) or DP-4 (2 μg) and immunoblotted with anti-HA antibody as previously described (Logan *et al*., 2004). Note that the nuclear accumulation of DP-4 is significantly greater than DP-1. The top images (**a–d**) represent DAPI staining. (**C**) U2OS cells were transfected with expression vectors encoding DP-1 and DP-4 (50 μg) and cell extracts assessed for binding activity to GST-E2F-1 (5 μg) as previously described (Logan *et al*., 2004). The input levels of DP-1 and DP-4 are indicated in track 1. (**D**) U2OS cells were transfected as above with expression vectors encoding HA-tagged DP-1, HA-tagged DP-4, E2F-1 or pRb (μg) as indicated and immunoprecipitated with anti-HA followed by immunoblotting with the relevant antibodies as previously described (Bandara *et al*., 1994). (**E**) *In vitro*-translated HA-tagged DP-1, DP-4, or DP-4Δ162–166 together with E2F-1 were assessed for DNA-binding activity on the E2F site taken from the adenovirus E2A promoter (Logan *et al*., 2004). Track 1 shows the E2F binding site alone and the presence of the E2F-1 in the DNA-binding complex was confirmed with anti-HA (indicated by +). WT indicates the presence of competing E2F binding site (non-radiolabelled 20-fold excess).

present as RRTYD (Figure 1c). Similarly, many of the residues that make dimerization contacts with the E2F component are conserved (Figure 1c).

The expression of ectopic DP-4 indicated that the polypeptide is about 45 kD (Figure 2A). Immunostaining indicated that DP-4 is primarily located in the nuclei of transfected cells (Figure 2B). The nuclear accumulation of DP-4 contrasts with the intracellular distribution of DP-1, which is mostly cytoplasmic (Figure 2B).

However, because there are some differences between DP-4 and human DP-2 in the residues that contact the E2F partner and DNA sequence (Figure 1c), we compared their dimerization and DNA-binding activity, and ability to form pocket protein complexes. Using an *in vitro* assay, we found that DP-4 and DP-1 bind with similar efficiency to E2F-1 (Figure 2C) and, like the DP-1/E2F-1 heterodimer (Stevens and La Thangue, 2003), DP-4/E2F-1 could form ternary complexes with pRb (Figure 2D). Interestingly, however, the DNA-binding activity of the DP-4/E2F-1 heterodimer on the E2F DNA recognition site was considerably lower than the DNA-binding activity of the DP-1/E2F-1 heterodimer (Figure 2E).

We investigated the transcription regulating properties of DP-4 on a panel of E2F responsive-promoter constructs. As it is known that DP-1 assists the

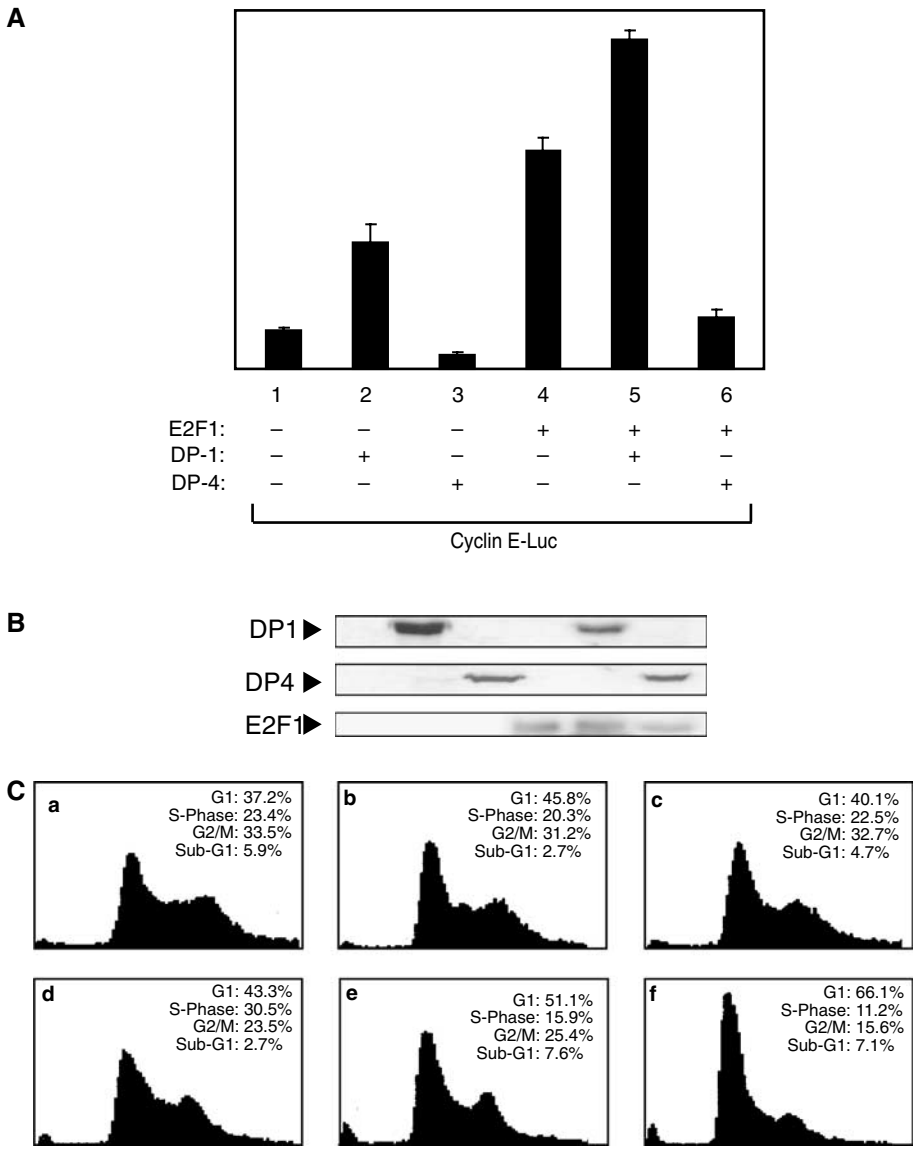

Figure 3 Transcription and cell-cycle regulating properties of DP-4. (**A**) U2OS cells were transfected with expression vectors encoding E2F-1 (100 ng) together with DP-4 or DP-1 (1 μg) as indicated with the cyclinE-luciferase reporter (0.5 μg) and pCMV-β-gal (200 ng) as previously described (Logan *et al*., 2004). The results represent the ratio of luciferase to β-gal activity. (**B**) U2OS cells were transfected with expression vectors (as described in (**A**)) for HA-tagged DP-1 or HA-tagged DP-4 together with E2F-1 as indicated and immunoblotted with anti-HA or anti-E2F-1 (C20). (**C**) Flow cytometry was performed with U2OS cells that had been transfected with pcDNA3 (20 μg; (a)), CD20 (10 μg; (**a–d**)), E2F-1 (10 μg; (**b**), (**d**) and (**f**)), DP-1 (10 μg; (**c**) and (**d**)) or DP-4 (10 μg; (**e**) and (**f**)). Flow cytometry was performed as described (Logan *et al*., 2004). The level of G1, S and G2/M phase cells is indicated in parenthesis.

E2F-1-dependent activation of E2F site-dependent transcription (Bandara *et al*., 1994), we compared the activity of DP-4 to DP-1 when coexpressed with E2F-1. DP-4 was less efficient than DP-1 in activating E2F-dependent transcription of a cyclinE-luciferase reporter; in fact, we frequently observed that DP-4 lowered the constitutive activity of the reporter (Figure 3A). This effect was most clear when DP-4 was coexpressed with E2F-1. Thus, whereas coexpressing E2F-1 with DP-1 enhanced transcriptional activity, the effect of DP-4 was to override E2F-1 activity, causing a reduced level of transcription (Figure 3A). A similar relative difference between DP-4 and DP-1 was observed on a variety of other E2F-responsive promoters (data not shown). These results suggest that DP-4 is a less-potent activator than DP-1 and, when coexpressed with E2F-1, can reduce E2F site-dependent transcription.

To establish that DP-4 can bind to E2F regulated genes in cells, we performed chromatin immunoprecipitation (ChIP). In the presence of E2F-1, ChIP analysis detected DP-4 on E2F sites in the E2F-1 and Cdc2 promoters (Figure 4a) – two genes that are known to be regulated by the E2F pathway (Stevens and La Thangue, 2003). The level of DP-4 associated with the E2F-1 and Cdc2 promoters was less than the level of DP-1, which mirrors the observations made in the band shift analysis, where DP-4/E2F-1 bound less efficiently than the DP-1/E2F1 heterodimer (Figure 2E). We conclude that DP-4 locates to endogenous E2F target genes, although, when assessed on E2F-1 and Cdc2, it is less efficient than DP-1.

Next, we studied the growth-regulating properties of DP-4 by introducing DP-4 either alone or together with E2F-1 into U2OS cells, which represents a cell-type that has been used extensively to study the cell-cycle-regulating properties of E2F subunits. The expression of E2F-1 caused an accumulation of cells in G1 phase, and the coexpression of DP-1 resulted in an increase in the S-phase population (from 20.3–30.5%; Figure 3B compare (a), (b) and (d)). In contrast, the introduction of DP-4 alone caused an accumulation of cells in G1, and when coexpressed with E2F-1, there was a further increase in the G1 population, which differed significantly from the effect of DP-1 and E2F-1 (66.1% G1 cells with DP-4/E2F-1, compared to 43.3% for DP-1/E2F-1; Figure 3B). These results indicate that the effects of DP-4 on cell-cycle progression can be distinguished from the effects of DP-1, and suggest that DP-4 and E2F-1 act to regulate the G1 population, in contrast to DP-1 and E2F-1 that increases the proportion of S-phase cells.

In order to verify that the DNA-binding activity of DP-4, in the context of the DP-4/E2F-1 heterodimer, is required for cell-cycle regulation, we prepared a mutant derivative of DP-4 that lacks the conserved RRXYD DNA-binding motif (Figure 1c). While DP-4Δ162–166 retained the ability to form a heterodimer with E2F-1 (Figure 5A), and was expressed at a similar level to wild-

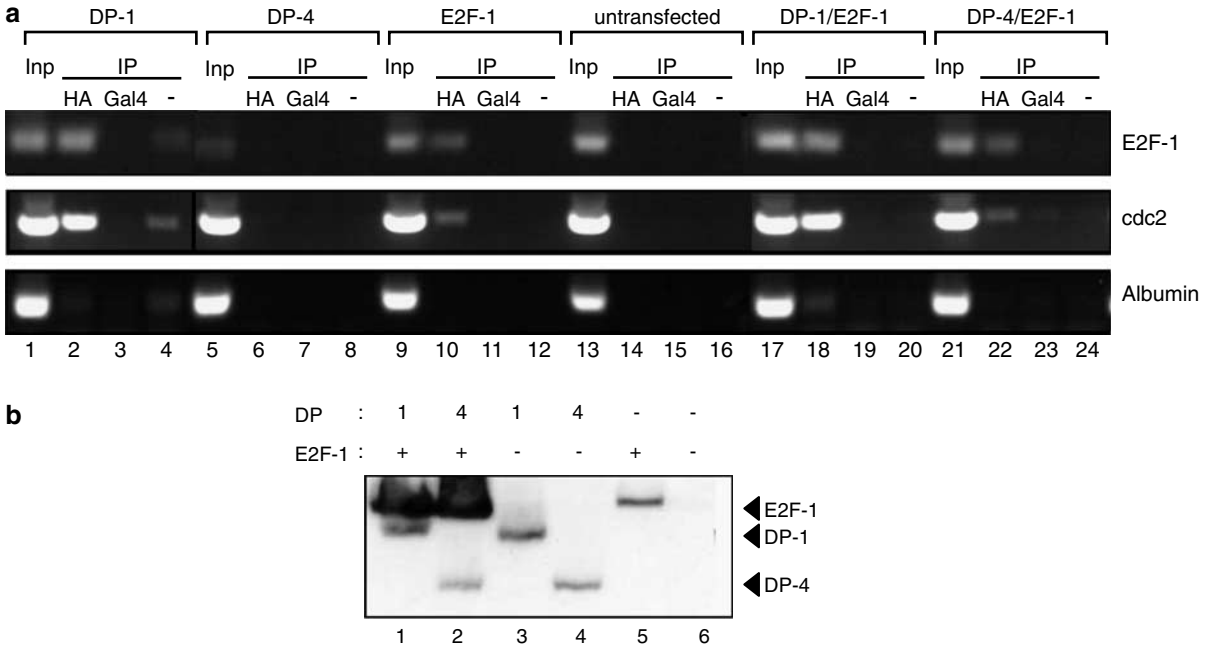

Figure 4 DP-4 locates to the promoters of endogenous E2F target genes. (**a**) Chromatin was prepared from U2OS cells transfected with expression vectors encoding HA-E2F-1 (tracks 9, 10, 11 and 12), non-tagged E2F-1 (tracks 17–24), HA-DP-1 (tracks 1–4, and 17–20) or HA-DP-4 (tracks 5–8 and 21–24) as described (Zhu *et al*., 2004). Chromatin immunoprecipitation was carried out as previously described using anti-HA, anti-GaL4 (the control antibody) or in the absence of an antibody (indicated by –). The level of input chromatin is indicated (Inp). The primers surrounding the E2F sites in the E2F-1 and Cdc2 promoters were as described (Ogawa *et al*., 2002; Zhu *et al*., 2004). ChIP performed on the albumin promoter using primers as previously described (Zhu *et al*., 2004) served as a negative control. (**b**) Immunoblot on extracts prepared from U2OS cells transfected as described in (**a**). The level of DP-1, DP-4 and E2F-1 is indicated.

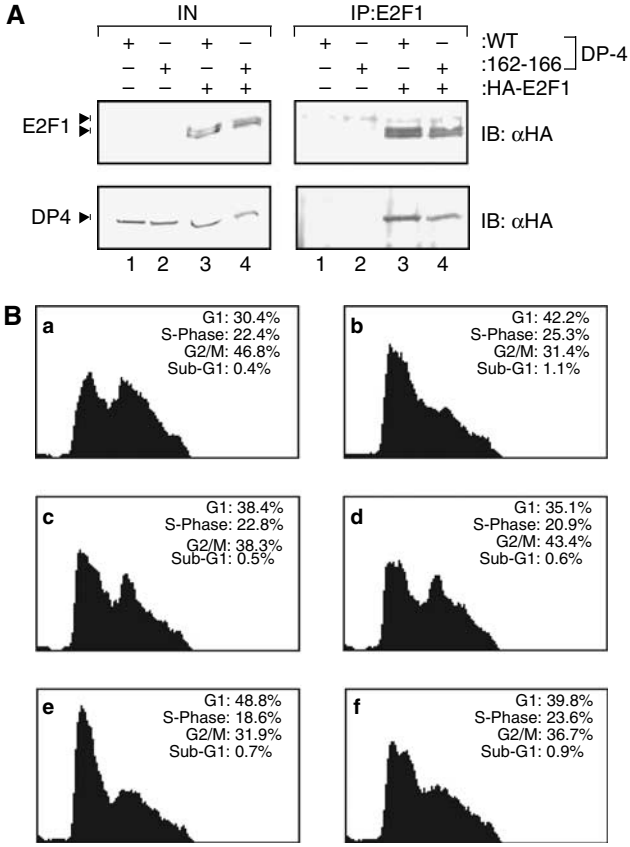

Figure 5 The cell-cycle effects of DP-4 require DNA-binding activity. (**A**) U2OS cells were transfected with HA-tagged DP-4 or DP-4Δ162-166 (20 μg) together with E2F-1 (20 μg), immunoprecipitated with anti-E2F-1 (KH95) followed by immunoblotting with anti-HA. The input levels are represented in tracks 1–4. (**B**) Flow cytometry in U2OS cells transfected with pcDNA3 (20 μg; (**a**)), CD20 (10 μg; (**a**–**f**)), E2F-1 (10 μg; (**b**), (**e**) and (**f**)), DP-4 (10 μg; (**c**) and (**e**)) or DP-4Δ162-166 (10 μg; (**d**) and (**f**)). Cells were harvested and treated as described in Figure 3B.

type DP-4 (Figure 2A), it failed to bind to the E2F DNA recognition site (Figure 2D). Thus, DP-4Δ162–166 is a DNA-binding-deficient mutant derivative of DP-4.

We compared the cell-cycle regulating properties of wild-type DP-4 to DP-4Δ162–166. As expected, DP-4 coexpressed with E2F-1 caused an increase in the G1 population, which contrasted with the effect of coexpressing DP-4Δ162–166, where the cell-cycle profile resembled that of E2F-1 alone (Figure 5B, compare (b), (e) and (f)). These results therefore suggest that DNA-binding activity is required for the DP-4 heterodimer to affect cell-cycle progression.

Our results define a new member of the DP family of subunits, DP-4, and suggest that, whereas DP-4 shares some properties with DP-1, there are some notable differences between the two proteins. Thus, although DP-4 binds to E2F subunits and forms ternary complexes with pRb, the DNA-binding activity of the DP-4/E2F-1 heterodimer is significantly less than that of DP-1/E2F-1. Although the molecular details that are responsible for the reduced DNA-binding activity remain to be determined, we noticed that DP-4 possesses a number of residue differences across the conserved DNA-binding domain. For example, arginine 72, which in the DP-2/E2F-4 crystal structure contacts the DNA backbone (Zheng *et al*., 1999), is replaced by a cysteine in DP-4. Similarly, cysteine 79, which in DP-1 is involved in dimerization with the E2F partner (Zheng *et al*., 1999), is a tryptophan in DP-4. It is possible that differences such as these explain the reduced DNA-binding activity of the DP-4/E2F-1 heterodimer. However, we note that our DNA-binding studies were performed on the E2F recognition site taken from selective E2F target genes and, therefore, we cannot rule out that DP-4 heterodimers preferentially bind to other E2F sites.

Most significantly, our results suggest that the cell-cycle-regulating properties of the DP-4/E2F-1 heterodimer are distinct from those of DP-1/E2F-1. Whereas DP-1/E2F-1 has a well-established effect on cell-cycle profile, augmenting entry into S-phase (Stevens and La Thangue, 2003), the properties of DP-4/E2F-1 appear to be more related to the induction of G1 cells. Furthermore, the DNA-binding activity of DP-4/E2F-1 is necessary for the cell-cycle effects, which in turn suggests that DP-4 may direct the E2F heterodimer to a population of target genes involved in G1 progression. In this respect, it will be interesting to study the status of the DP-4 gene in tumour cells.

In conclusion, our results suggest that DP-4 is involved in regulating cell-cycle progression and further indicate that DNA-binding activity is required for the cell-cycle effects. A key question will be to clarify the physiological role of DP-4, particularly with respect to the different roles played by members of the expanding DP family, and the nature of the target genes regulated by each DP heterodimer.

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