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To cite this version:

Nick Thangue, Laura Ingram, Shonagh Munro, Amanda Coutts. E2F-1 regulation by an unusual DNA damage-responsive DP subunit.. Cell Death and Differentiation, 2010, 10.1038/cdd.2010.70 . hal-00547988

HAL Id: hal-00547988

https://hal.science/hal-00547988v1

Submitted on 18 Dec 2010

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E2F-1 regulation by an unusual DNA damage-responsive DP partner subunit.

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Key words: Cell cycle, E2F-1, DP-4, repression, DNA damage

Running title: DNA damage-responsive DP subunit

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Abstract

E2F activity is negatively regulated by pRb through binding to the E2F-1 subunit. Within the E2F heterodimer, DP proteins are E2F partner subunits that allow proper cell cycle progression. In contrast to the other DP proteins, the newest member of the family, DP-4, down-regulates E2F activity. We report here an unexpected role for DP-4 in regulating E2F-1 activity during the DNA damage response. Specifically, DP-4 is induced in DNA damaged cells, upon which it binds to E2F-1 as a non-DNA binding E2F-1/DP-4 complex. Consequently, depleting DP-4 in cells re-instates E2F-1 activity, which co-incides with increased levels of chromatin-bound E2F-1, E2F-1 target gene expression and associated apoptosis. Mutational analysis of DP-4 highlighted a C-terminal region, outside the DNA binding domain, required for the negative control of E2F-1 activity. Our results define a new pathway, that acts independently of pRb and through a biochemically distinct mechanism, involved in negative regulation of E2F-1 activity.

Introduction

The E2F family of transcription factors regulate a variety of cellular outcomes including cell cycle progression, differentiation and apoptosis [1, 2]. The first member of the family to be identified, E2F-1, is a crucial target of the retinoblastoma pRb tumour suppressor protein in the control of the G1 to S phase transition [3, 4]. pRb physically interacts with DNA bound E2F-1 and thereby prevents transcriptional activity [5]. In turn, cell cycle progression cannot occur, which is important for pRb to exert its tumour suppressor activity [5]. To date, eight E2F subunits have been identified, together with three DP subunits [6]. E2F-7 and E2F-8, the two most recently characterised members of the family, possess unusual properties, with two DNA binding domains that allow DNA binding to occur independently of a DP subunit [7-11]. In mammalian cells, however, most E2F exists as a heterodimer in complex with a DP subunit [6].

Under normal physiological conditions DP-1 is the predominant E2F partner protein [12, 13]. DP-1 is encoded by an essential gene, since *dp-1*$^{-/-}$ mice exhibit embryonic lethality, in part due to defective extra-embryonic tissue development [14]. Further, DP-1 phosphorylation is regulated during cell cycle progression, and its phosphorylation by cyclinA/cdk2 kinase is believed to be involved in controlling the DNA binding activity of the E2F heterodimer and exit from S phase [15]. Another member of the DP family, murine DP-3 (human DP-2), undergoes complex levels of control and a variety of protein isoforms exist in cells which differ in both biochemical and functional properties [16, 17]. The most recent family member to be

described, DP-4, acts negatively on both E2F DNA binding activity and cell cycle progression [18-20].

Here, we describe some unexpected properties of the newest member of the DP family, DP-4, and highlight a novel pathway which allows pRb-independent negative control of E2F-1 activity. We have found that DP-4 is DNA damage-responsive, which leads to the down-regulation of E2F-1 activity by creating an E2F-1/DP-4 complex, which cannot bind to DNA. Consequently, depleting DP-4 re-instates E2F-1 DNA binding activity, which co-incides with increased expression of a variety of E2F-1 target genes and enhanced levels of apoptosis. Significantly, a domain that resides in the C-terminal region of DP-4 is responsible for down-regulating E2F-1 activity. Our results define a new pathway, that acts independently of pRb and through a biochemically distinct mechanism, to regulate E2F-1 activity.

Results

DP-4 is DNA damage-responsive.

An analysis of DP-4 using a DP-4 specific antibody indicated that it is a nuclear protein expressed in a variety of different cancer cell lines (Figure 1a, b and c; SI Figure 1a and b). In characterising the properties of DP-4, we considered that its expression might be influenced by DNA damage. DP-4 was DNA damage-inducible in different cell types (U2OS, HeLa, MCF7, HEK293 and H1299) treated with doxorubicin, etoposide and ultraviolet light (Figure 1c, d, e and f; SI Figure 1c and d), contrasting with DP-1 which was not induced by DNA damage (for example Figure 1d),. Endogenous DP-4 was located in nuclei, although under DNA damage conditions there was a significant increase in protein level and nuclear staining intensity (Figure 1c and SI Figure 1e). Whilst a significant increase was observed at the protein level, DP-4 mRNA levels only underwent a modest increase following DNA damage (SI Figure 1f). Further, the DNA damage-dependent increase in DP-4 protein levels reflected increased levels of the DP-4/E2F-1 heterodimer when either the ectopic or endogenous proteins were measured (Figure 1f and g). These results therefore establish that DP-4 is DNA damage-responsive, and further that DNA damage favours formation of the E2F-1/DP4 heterodimer.

To examine the effect of DP-4 on transcription, we tested the functional consequences of DP-4 expression on a collection of E2F-responsive promoters, namely Apaf1, Cdc6, p73 and cyclin E [5, 21]. Whilst each promoter was activated by E2F-1, co-expressing DP-4 resulted in reduced levels of transcription (Figure 2a). The effect of DP-4 contrasted with DP-1, which augmented transcription (Figure 2a); ectopic protein levels were expressed as expected in transfected cells (Figure 2a).

Thus, DP-4 inactivates transcription, and has the opposite effect compared to DP-1 on E2F site transcription.

To establish that the effect of ectopic DP-4 recapitulated effects on endogenous genes, we used siRNA to deplete endogenous DP-4, and thereafter monitored the effect on E2F target genes. Under conditions of DP-4 depletion (Figure 2b), the level of both RNA and protein encoded by different E2F target genes, including Cdc2, Apaf1, p73 and E2F-1 increased (Figure 2c and d). Thus, DP-4 is a negative regulator of E2F target genes.

DP-4 hinders E2F-1 DNA binding.

To address the mechanisms responsible for down-regulating E2F activity, we considered that DP-4 might compete with DP-1 for binding to the E2F subunit. Under conditions in which E2F-1 and DP-1 were able to activate transcription, there was a titratable decline in transcription as the level of DP-4 increased (Figure 3a). Conversely, the down-regulation of E2F transcription by DP-4 could be overcome by increasing the level of DP-1 (Figure 3b). These results suggest that DP-4 acts competitively with DP-1.

Next, we investigated whether the integrity of the DP-4 DNA binding domain was necessary for DP-4 to down-regulate E2F site transcription. We tested this possibility by manipulating DP-4 in two ways. Firstly, we removed the conserved and essential RRXYD DNA binding consensus motif (RRTYD in DP-4; Figure 4a and b) to create a DP-4 derivative that was defective in DNA binding (Δ162-166). Secondly, an alignment of DP-1 with DP-4 highlighted a number of subunit-specific amino acid residues within the DNA binding and dimerization domains (Figure 4a and b) that, from inspection of the structure of the E2F heterodimer [22], might be

expected to influence DNA binding activity (Figure 4a). We therefore prepared a series of derivatives in which each one of the different residues was serially substituted into DP-4 (the DP-1 residue replacing each of the different DP-4 residues), eventually giving rise to the DP-4*4 where all the relevant DP-1-specific residues were transferred into DP-4 (Figure 4b). Each mutant derivative was subsequently tested for its ability to down-regulate E2F site-dependent transcription. Neither DP-4Δ162-166 or any of the DP-4 derivatives were compromised in ability to down-regulate transcription, and each mutant was expressed at a similar level (Figure 4c and d). The fact that DP-4Δ162-166, which lacks residues that are essential for DNA binding [22], retained the ability to down-regulate transcription strongly suggests that DNA binding is not necessary for the effects of DP-4 on E2F site transcription. This idea is consistent with the properties of the DP-4 substitution mutants (Figure 4d), which similarly were able to down-regulate transcription. Overall, DP-4 down-regulates E2F site transcription in a fashion that does not require the integrity of the DNA binding domain.

We prepared a series of DP-4 deletion mutants to identify the domain in DP-4 that is responsible for the control of E2F activity. Derivatives lacking the N-terminal region of DP-4, up to residue 174, which removed the DNA binding and dimerization domain (Figure 5a), could down-regulate transcription (Figure 5b and c). Further, whilst a C-terminal deletion up to residue 306 could down-regulate E2F-1 activity, further deletion up to residue 206 diminished this activity, and a deletion up to residue 106 completely abolished activity (Figure 5a and b). Significantly, DP-4N174 which lacks DNA binding activity could compete with DP-1 for E2F-1 activity (Figure 5c). These results suggest that the region required for DP-4 to down-regulate E2F site transcription lies between residue 175 and 306. Since the DNA binding and

dimerization domain resides between residue 108 and 174, these results further confirm that DP-4 DNA binding and dimerization activity is unlikely to be involved in the effects on E2F activity. This idea is consistent with the properties of DP-4 N174, which lacks DNA binding and dimerization activity, but retains the ability to reduce transcriptional activation when co-expressed with E2F-1/DP-1 (Figure 5c).

DP-4 hinders E2F DNA binding activity.

It remained possible however that DP-4 affects the DNA binding activity of E2F-1, which we tested by assessing the effect of DP-4 on chromatin bound E2F-1. Firstly, we monitored the effect of ectopic DP-4 on E2F-1 activity by chromatin immunoprecipitation (ChIP) and, secondly, the effect of DP-4 siRNA on chromatin bound endogenous E2F-1. Whilst ectopic DP-1 was detected on the promoter of the E2F-1 and Cdc2 genes, ectopic DP-4 was not (Figure 6a), despite expression of equivalent levels of the ectopic protein (Figure 6a, iii). The effect of DP-4 on E2F-1 contrasted with DP-1, which enhanced E2F-1 binding (Figure 6a). Frequently, ectopic DP-4 even reduced E2F-1 binding to E2F target genes (for example the E2F-1 promoter; Figure 6a). These results suggest that DP-4 fails to locate to the chromatin of E2F target genes, which is compatible with the earlier results indicating that DNA binding is not required for the transcription effects of DP-4.

In order to test whether endogenous DP-4 behaved in a similar fashion to the ectopic protein, we performed a ChIP analysis on DP-4 siRNA treated cells (Figure 6b). Under conditions of DP-4 depletion, E2F-1 underwent increased binding to the promoters of E2F target genes, in both unperturbed and DNA damaged cells (Figure 6b and c). These results support the data obtained with ectopic DP-4 (Figure 6a), and

suggest that endogenous DP-4 down-regulates the expression of E2F target genes through modulating access of E2F-1 to chromatin.

Physiological role of DP-4.

Given the induction of DP-4 under DNA damage conditions, together with its ability to down-regulate E2F-1 activity, we reasoned that DP-4 may take on an important role in mediating the cell cycle effects of DNA damage. This possibility was tested by depleting endogenous DP-4 and thereafter analysing the effect on cell cycle progression. In different cell types (U2OS and SAOS2), depletion of DP-4 caused a decrease in the population of G1 cells, with increased levels of S and G2/M cells (Figure 7a and c; SI Figure 1g). In contrast, under DNA damage conditions reduced levels of DP-4 co-incided with a dramatic increase in sub-G1 (0.79% to 18.4%), together with a decreased population of G1 cells (Figure 7a and c). These results suggest that DP-4 influences cell cycle progression, under both normal and DNA damage conditions.

Further, since E2F-1 activity increases in DP-4 siRNA treated cells, and because E2F-1 can induce apoptosis [23, 24], we tested the effect of DP-4 on E2F-1-induced apoptosis. There was a reduction in the level of apoptosis (measured as PARP cleavage) when DP-4 was co-expressed with E2F-1 (Figure 7d). Overall, therefore, DP-4 is functionally involved in regulating cell cycle progression as well as the outcome of the DNA damage response.

Discussion

DP-4 and the DNA damage response.

An important property that distinguishes DP-4 from other members of the DP family is the ability to down-regulate transcription, and its induction during the DNA damage response. Most interestingly, DP-4 carries a domain in the C-terminal region that is responsible for down-regulating transcription in a fashion that is independent of DNA binding domain. The idea that DP-4 DNA binding activity is not required to down-regulate transcription is compatible with the absence of DP-4 on the promoters of E2F target genes, even in conditions of ectopic protein expression. It is most likely therefore that DP-4 interacts with E2F-1 in a fashion that prevents E2F-1 binding to the promoters of target genes (Figure 7e).

By studying cells in which DP-4 had been depleted, it was apparent that DP-4 regulates the G1 population and contributes to the cell cycle arrest that occurs upon DNA damage. The increased population of apoptotic sub-G1 cells upon depleting DP-4 is consistent with a role for DP-4 in assisting cell survival upon DNA damage. Further, the level of E2F-1-dependent apoptosis was reduced in the presence of DP-4, suggesting that DP-4 activity antagonises apoptosis driven by E2F-1. The ability of DP-4 to augment the G1 population in both unperturbed and DNA damaged cells might therefore reflect decreased E2F-1 activity, and consequently reduced apoptosis (Figure 7e). Together, our results suggest that DP-4 is a functionally distinct member of the DP family, with the ability to negatively regulate E2F-1.

Negative regulation of E2F-1 independently of pRb.

The properties of DP-4 define a new mechanism through which E2F-1 activity can be negatively regulated but, importantly, provide a pathway that operates in a pRb-independent fashion. pRb and the related family members p107 and p130 bind to specific E2F subunits through a domain required for transcriptional activation, and the interaction prevents transcriptional activation by the E2F subunit [5, 24]. Under normal cell cycle conditions, the sequential phosphorylation of pRb directed by cyclin/Cdk complexes releases pRb from E2F-1, thereby facilitating the activation of E2F target genes [25]. It is possible that the ability of DP-4 to hinder E2F-1 DNA binding activity, and thereby limit the level of E2F-1-dependent transcription, provides an alternative biochemically distinct pathway for down-regulating E2F-1 activity.

In conclusion, our results have unearthed an unexpected yet central role for DP-4 in regulating E2F-1 activity. DP-4 down-regulates E2F-1, and the widespread expression of DP-4 suggests that this is a general mechanism utilised in many different types of cells. DP-4 therefore contributes to a new pRb-independent mechanism for regulating cell cycle progression.

Acknowledgements

We thank the MRC, CRUK, EU, LRF and AICR for supporting this work, and Rosemary Williams for assistance in preparing the manuscript.

Materials and methods

Plasmids and expression vectors.

HA-DP-1, HA-DP-4 and HA-E2F-1 have been described previously [18]. pCdc6-luciferase [26], p73-luciferase [27], Apaf1-luciferase [21] and pCyclinE-luciferase [28] have been described previously.

Anti-DP-4 peptide antibody.

The rabbit polyclonal anti-DP-4 antibody was generated by Sigma. Briefly, a synthetic DP-4 peptide was generated containing residues 13-28 (C-ELKVLMDENQTSRPVC-C). This peptide was used to immunise rabbits. The resulting polyclonal antibody was purified by passing the crude antiserum through a DP-4 peptide column.

Tissue culture and transfection.

U2OS, HeLa, HEK293, MCF7, H1299 and SAOS2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% foetal bovine serum (FCS) and penicillin-streptomycin (Gibco) at 37°C in 5% CO_2. Cell lines were transfected with GeneJuice (Novagen). For PARP cleavage, U2OS cells were transfected with expression vectors encoding, HA-DP-4 or empty vector (1µg). After 40h, cells were treated with UV(50J/m^2) for 8h.

Immunoprecipitation and immunoblotting.

Cells were harvested, washed in PBS, and resuspended in TNN buffer [50 mM Tris pH 7.4, 5 mM EDTA, 0.5% Igepal CA-630 (Sigma), 50 mM NaF, 1 mM DTT,

0.2 mM Na_3VO_4, 120 mM NaCl, protease inhibitor cocktail (Roche)]. Total protein concentration was determined by Bradford Assay (Bio-Rad). Typically 1-1.5 mg of cell extract was added to pre-washed Protein-G agarose beads (Sigma) with 1μg of antibody. After overnight incubation the beads were washed 4 times in TNN prior to protein elution with 2xSDS loading buffer and analysis by SDS-PAGE and immuno-blotting with the appropriate antibodies. The following antibodies were used for immunoblotting; anti-HA11 (Covance), anti-PCNA, anti-lamin B, anti-E2F-1, anti-DP-1, anti-Cdc6, anti-Cdc2, anti-GAPDH (Santa Cruz), anti-PARP (BD pharmingen) anti-nucleophosmin and anti-actin (Sigma).

Immunostaining.

U2OS cells were stained according to previously published procedures [29].

Cell fractionation.

Cell fractionation was performed as previously described [30].

Luciferase assays.

For reporter assays, U2OS cells were transfected with 0.2μg of luciferase reporter plasmid, 0.2μg of pCMV-β-galactosidase (β-gal) plasmid as an internal control and the indicated expression plasmids as previously described [31].

Flow cytometry.

U2OS cells were transfected with DP-4 siRNA or non-targeting control siRNA, 72h later cells were treated with UV 50J/m^2. 8h later cells were washed in PBS and fixed overnight in 50% ethanol/PBS at 4ºC. Fixed cells were washed in PBS

and incubated for 30min with 25U/ml RNase A (Sigma) and 50μg/ml propidium iodide (Sigma) in PBS. The analysis of cell cycle profiles was performed as previously described [31].

DP-4 siRNA treatment.

Cells were transfected with 25nM DP-4 siRNA (5'-CAGAAGTGCTGATGTGGAT-3') or non-targeting control #2 siRNA (Dharmacon). Cells were transfected using Oligofectamine (Invitrogen).

Chromatin immunoprecipitation (ChIP).

U2OS cells were maintained in DMEM containing 10% foetal calf serum. Cells were transfected with the indicated plasmids or siRNA. Cells were cross-linked with formaldehyde to a final concentration of 1%. ChIP samples were prepared as described previously [32]. Immunoprecipitations were performed using 2μg anti-E2F-1 (KH95 Santa Cruz), anti-HA11 (Covance) or the relevant non-specific IgG (Santa Cruz). The recovered DNA was analyzed by semi-quantitative or real-time quantitative PCR [33]. The primers used were as follows:

E2F-1 forward 5'-AGGAACCGCCGCCGTTGTTCCCGT-3'; E2F-1 reverse 5'-GCTGCCTGCAAAGTCCCGGCCACT-3'; Cdc2forward 5' –GCTTGCGCTCGCAC TCAGTTGGCC-3'; Cdc2 reverse 5' –CAGATCCCTGACCTCCAGTCC-3' albumin forward 5' –TGGGGTTGACAGAAGAGAAAAGC-3' and albumin reverse 5'-TACATTGACAAGGTCTTGTGGAG-3'

Quantitative PCR.

Real-time PCR for the ChIP was carried out according to the manufacturer's instructions using Brilliant II SYBR Green QPCR Master Mix.

RT-PCR.

Total RNA was isolated using RNeasy Mini kit (QIAGEN). cDNA was synthesized from 1 μg total RNA with SuperScript ™ First-Strand Synthesis System (Invitrogen). Semi-quantitative PCR was performed as described [32]. The primers used were as follows: Cdc2 forward 5'-AATTGGAGAAGGTACCTATGGA-3'; Cdc2 reverse 5'-TGTACTGACCAGGAGGGATA-3'; Apaf1 forward 5'-CACGTT CAAAGGTGGCTGAT-3' Apaf1 reverse 5'-TGGTCAACTGCAAGGACCAT-3'; p73 forward 5'-ACTTCAACGAAGGACAGTCTGCT-3' and p73 reverse 5'-AATTCCGTCCCCACCTGTG-3'

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Figure 1.

DP-4 is DNA damage-inducible.

a) Endogenous DP-4 in HEK293, HeLa, MCF7 and U2OS cells: cell extract (100μg) prepared from each cell type was immunoblotted with peptide purified anti-DP-4 antibody; lamin B served as the loading control.

b) U2OS cells were transfected with expression vector encoding HA-DP-4 (1μg). Immunostaining was performed with anti-HA11 or peptide purified anti-DP-4 antibody as described; DAPI was used to visualize nuclei.

c) MCF7 cells were treated with ultraviolet (UV) light ($50J/m^2$), after 8h cells were fractionated into cytoplasm and nuclei, and thereafter immunoblotted with anti-DP-4 or anti-nucleophosmin as indicated, the latter serving as a control for nuclear fraction.

d) **i)** U2OS cells were treated with doxorubicin (2μM) for 2, 5, 8 and 24h and extracts immunoblotted with anti-DP-4 antibody; PCNA served as the loading control.

ii) U2OS cells treated with etoposide (10μM) for 2, 4, 6 or 8h and extracts immunoblotted with the indicated antibodies; PCNA served as the loading control.

iii) U2OS cells were treated with ultraviolet (UV) light (50J/m^2), extracts prepared at 2.5, 5.0 and 7.5h and immunoblotted with anti-DP-4; PCNA served as the loading control.

e) **i)** HeLa cells were treated with etoposide (10μM) for 2, 5, 8 and 24h and extracts immunoblotted with anti-DP-4 antibody; PCNA served as the loading control.

ii) HeLa cells treated with doxorubicin (2μM) for 2, 4, 6 or 8h and extracts immunoblotted with the indicated antibodies; PCNA served as the loading control.

iii) HeLa cells were treated with ultraviolet (UV) light (50J/m^2), extracts prepared at 2.5, 5.0 and 7.5h and immunoblotted with anti-DP-4; PCNA served as the loading control.

f) U2OS cells were transfected with HA-DP-4 (1μg), and 44h later cells were treated with etoposide (10μM) and left for 4h, harvested, immunoprecipitated with anti-HA11 or a non-specific (NS) antibody and the immunoprecipitates immunoblotted with anti-E2F-1 and HA11 (DP-4) antibody. DP-4 is indicated by the arrow, together with the non-specific IgG heavy chain.

g) U2OS cells were treated with etoposide (10μM), left overnight, harvested and immunoprecipitated with anti-E2F-1 (KH95) or a non-specific (NS) antibody. The immunoprecipitates were immunoblotted with anti-E2F1 and anti-DP-4.

Figure 2.

DP-4 down-regulates E2F site-dependent transcription.

a) U2OS cells were transfected with the indicated expression vectors (1μg) as described and the effect on Apaf1-luciferase, Cdc6-luciferase, p73-luciferase and cyclinE-luciferase measured. The luciferase activity is shown (relative to co-transfected pCMV-βgal expression), and lower panels show the level of ectopic proteins for each experiment.

b) U2OS cells were treated with non-targeting (NT) or DP-4 siRNA as described followed by immunoblotting; actin served as the loading control.

c) U2OS cells were transfected with DP-4 or a non-targeting (NT) control siRNA (25nM) as described. At 72h later cells were harvested, RNA extracted, reverse transcribed and the level of Cdc2, Apaf1, p73 and GAPDH RNA assessed.

d) U2OS cells were transfected with DP-4 or a non-targeting (NT) control siRNA (25nM) as described. At 72h later cells were harvested and extracts immunoblotted with antibodies against Cdc2, Apaf1, DHFR, E2F-1 and actin.

Figure 3.

DP-4 competes with DP-1 to down-regulate E2F site transcription.

a) U2OS cells were transfected with Cdc6-luciferase (1μg), together with expression vectors for E2F-1 (100ng), with DP-4 transfected in increasing amounts (0.1, 0.2, 0.5, 1.0 and 2.0μg) in the presence of a constant level of DP-1 (2μg). The relative luciferase activity is shown together with the level of ectopic protein in the lower panel.

b) U2OS cells were transfected with Cdc6-luciferase (1μg), together with expression vectors for E2F-1 (100ng), with DP-1 transfected in increasing amounts (0.1, 0.2, 0.5, 1.0 and 2.0μg) in the presence of a constant level of DP-4 (2μg). The relative luciferase activity is shown together with the level of ectopic protein in the lower panel.

Figure 4.

Properties of DP-4 mutant derivatives.

a) Outline organisation of DP-4, and an alignment of DP-1 and DP-4 across the DNA binding domain (amino acid residues 108 to 174) indicating the residues important for contacting DNA (red and black circles) and dimerization (green circles; taken from [22]). Residues that differ between DP-1 and DP-4 are indicated by blue (*).

b) Details of the substitution mutants generated in DP-4; the highlighted (red) amino acid residues were introduced into the DP-4 sequence. DP-4Δ162-166 shows the details of the DNA binding deletion mutant, where the conserved DNA binding motif RRTYD has been deleted.

c) U2OS cells were transfected with expression vectors encoding wild-type DP-4 or Δ162-166 together with E2F-1 or DP-1 as indicated, and the effect on Apaf1-luciferase and cyclinE-luciferase measured. Lower panels show levels of ectopic proteins.

d) U2OS cells were transfected with expression vectors, encoding wild-type DP-4 or the indicated DP-4 substitution mutants, together with E2F-1 or DP-1 as indicated, and the effect on Apaf1-luciferase and Cdc6-luciferase reporters measured. Lower panels show levels of ectopic proteins.

Figure 5.

Properties of DP-4 deletion mutants.

a) Schematic representation of the DP-4 derivatives used in b) and c), and a summary of their ability to down-regulate E2F-1 activity.

b) U2OS cells were transfected with expression vectors encoding the indicated DP-4 derivatives together with E2F-1, and the effect on the cyclinE-luciferase and p73-luciferase activity measured. Lower panels show the levels of the ectopic proteins.

c) U2OS cells were transfected with expression vectors encoding E2F-1 or DP-1, together with increasing amounts of DP-4N174 (0.1, 0.2, 0.5, 1.0 or 2.0μg), and the effect on Cdc6-luciferase measured. The level of ectopic protein is shown in the lower panel.

Figure 6.

DP-4 regulates chromatin-bound E2F-1.

a) **i)** U2OS cells were transfected with expression vectors (1μg) encoding HA-DP-1, HA-DP-4 or empty vector (E) as indicated. After 48h chromatin was prepared and ChIP analysis performed using anti-HA, E2F-1 or a non-specific (NS) antibody control, and binding to the E2F-1, Cdc2 and albumin promoters assessed as described.

ii) Quantification of the ChIP analysis in (i) by real-time PCR.

iii) Levels of the ectopic proteins, immunoblotted with anti-HA antibody.

b) **i)** U2OS cells were treated with DP-4 or non-targeting (NT) siRNA (25nM). After 48h chromatin was prepared and ChIP analysis was performed using anti-E2F-1 or a non specific (NS) antibody control as described above. Binding to the E2F-1 (i), Cdc2 (ii) and albumin control (iii) promoter was assessed as described.

iv) Protein levels in cells treated as described in (i) was assessed by immunoblotting with anti-DP-4 and anti-PCNA.

v) and **vi)** Quantification of E2F-1 binding on the E2F-1 and Cdc2 promoters respectively.

c) U2OS cells were treated with DP-4 or non-targeting (NT) siRNA (25nM). 48h later cells were treated with ultraviolet (UV) light ($50J/m^2$). After 8h chromatin was prepared and ChIP analysis performed using anti-E2F-1 or a

non-specific (NS) antibody control. Binding to the p73, Apaf1 and albumin promoters was assessed.

Figure 7.

Cell cycle control by DP-4.

a) U2OS cells treated with DP-4 or non-targeting (NT) siRNA. 48h later cells were treated with ultraviolet (UV) light (50J/m^2). After 8h cells were analysed by flow cytometry (approximately 20,000 cells). The flow cytometry profiles are shown, with the size of the sub-G1 fraction indicated.

b) Level of endogenous DP-4 in U2OS cells treated in (a), with GAPDH as loading control.

c) Graphical representation of the flow cytometry profiles shown in (a) according to cell cycle phase (sub-G1, G1, S or G2/M).

d) U2OS cells were transfected with expression vectors encoding E2F-1 (1µg), DP-4 (1µg) or both, as indicated, 72h later cells were treated with ultraviolet (UV) light (50J/m^2). 8h later cells were harvested, and the level of cleaved PARP (reflecting the level of apoptosis) measured. Actin levels served as a loading control. Quantitation of cleaved PARP relative to actin control is shown in graph below.

e) Model incorporating the properties of DP-4 involved in E2F-1 control. It is envisaged that DP-4 competes with DP-1, which in turn down-regulates E2F-1 DNA binding activity. This occurs both in normal and DNA damaged cells where DP-4 levels increase in response to DNA damage.

SI Figure 1

Properties of DP-4.

a) U2OS cells were transfected with HA-DP-4 (1μg), and 48h later cells were harvested and immunoblotted with anti-HA (lane 1), anti-DP-4 serum (lane 2) or peptide purified anti-DP-4 antibody (lane 3).

b) U2OS cells were transfected with HA-DP-4 (1μg), and 48h later cells were harvested and immunoblotted with anti-HA (lane 1), peptide purified anti-DP-4 antibody (lane 2) or peptide purified anti-DP-4 antibody in the presence of competing DP-4 peptide (2 μg; lane 3).

c) MCF7 cells were treated with doxorubicin (2μM) for 2, 5, 8 and 24h and extracts prepared and immunoblotted with anti-DP-4 or anti-PCNA antibodies.

d) (i) HEK293 cells were treated with doxorubicin (2μM) for 2, 5, 8 and 24h.

(ii) HEK293 cells were treated with etoposide (10μM) for 2, 5, 8 and 24h.

(iii) H1299 cells were treated with ultraviolet (UV light) (50J/m^2), extracts were prepared at 2.5, 5.0 and 7.5h with anti-DP-4 or anti-PCNA antibodies.

e) U2OS cells were untreated or treated with ultraviolet (UV) light (50J/m^2) and examined at 8h as indicated after immunostaining with anti-DP-4 antibody; DAPI staining was used to visualize nuclei.

f) U2OS cells were treated with and without ultraviolet (UV) light (50J/m^2). At 8h later cells were harvested, RNA extracted, reverse transcribed and the level of DP4 and 18S RNA assessed.

g) SAOS2 cells were treated with DP-4 or non-targeting (NT) siRNA with or without ultraviolet (UV light) and analysed by flow cytometry (as described). The graph represents the flow cytometry profiles of each cell cycle phase.

Figure 1

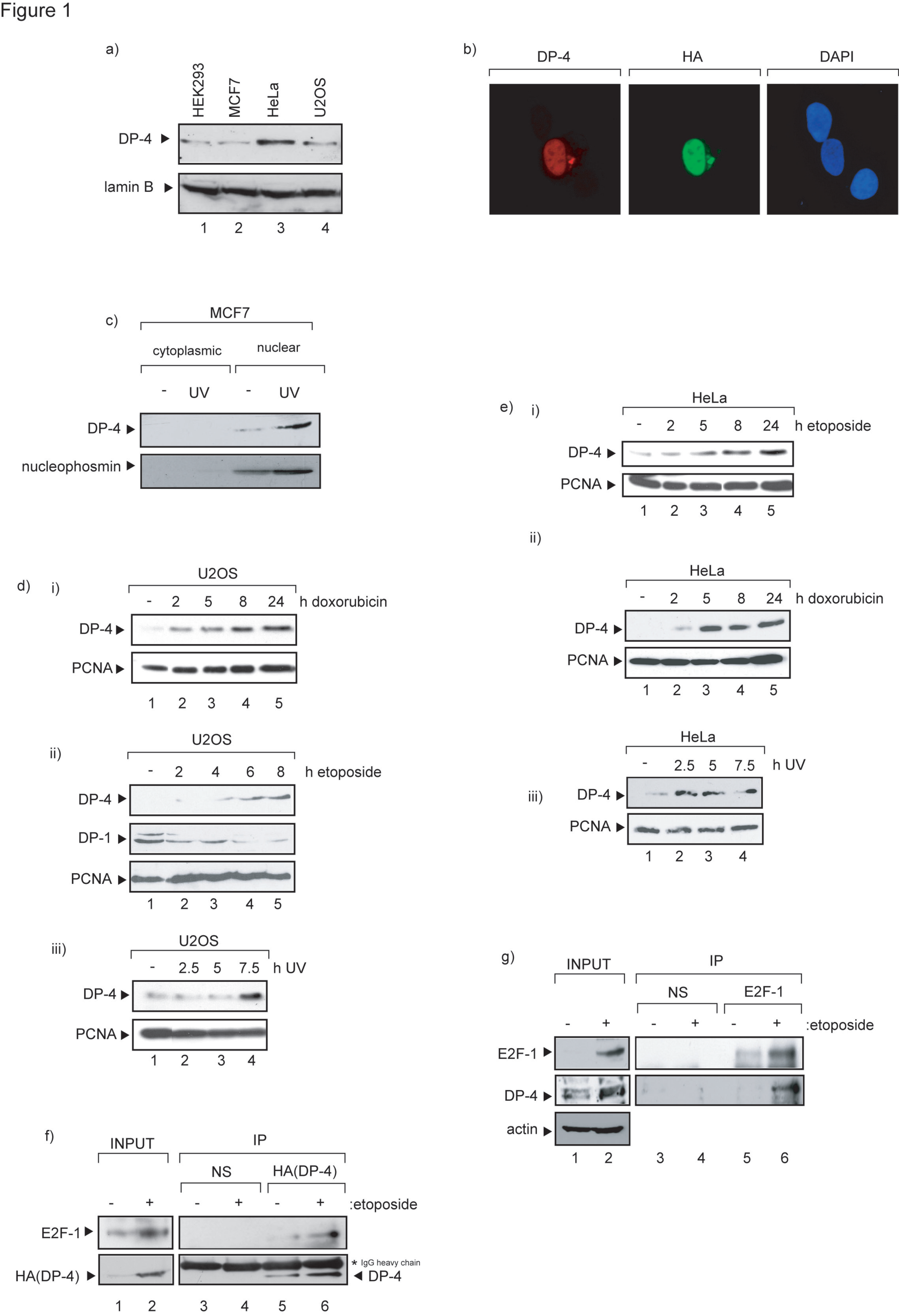
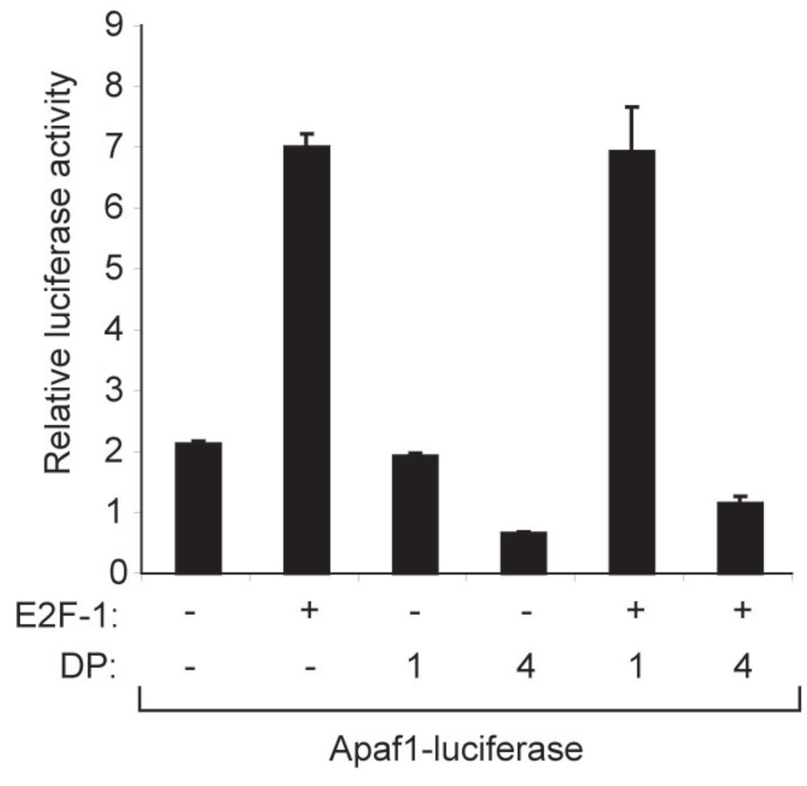
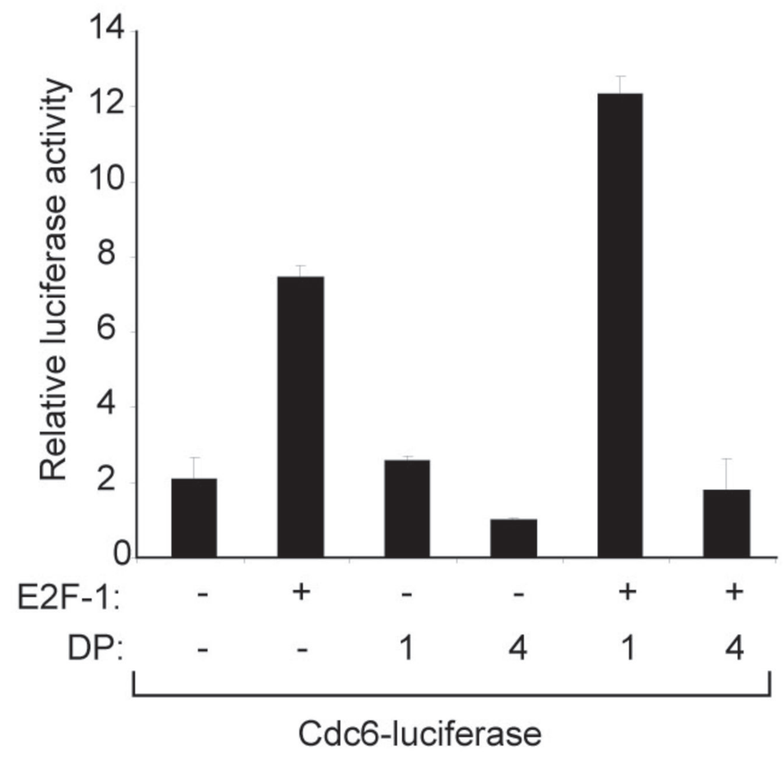
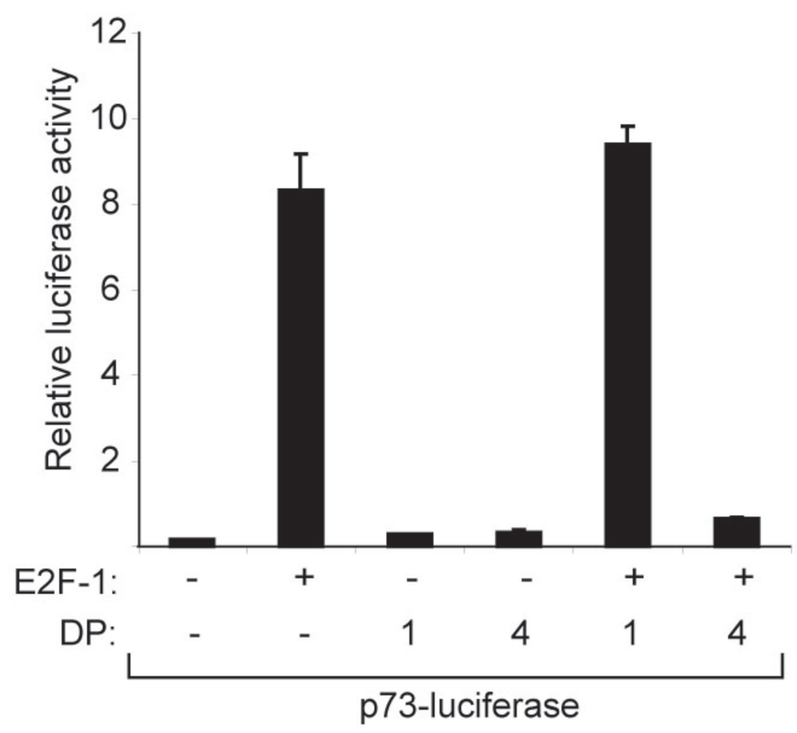

a)
HEK293
MCF7
HeLa
U2OS
DP-4
lamin B
1 2 3 4
b)
DP-4
HA
DAPI
c)
MCF7
cytoplasmic
nuclear
- UV - UV
DP-4
nucleophosmin
d) i)
U2OS
- 2 5 8 24 h doxorubicin
DP-4
PCNA
1 2 3 4 5
ii)
U2OS
- 2 4 6 8 h etoposide
DP-4
DP-1
PCNA
1 2 3 4 5
iii)
U2OS
- 2.5 5 7.5 h UV
DP-4
PCNA
1 2 3 4
e) i)
HeLa
- 2 5 8 24 h etoposide
DP-4
PCNA
1 2 3 4 5
ii)
HeLa
- 2 5 8 24 h doxorubicin
DP-4
PCNA
1 2 3 4 5
iii)
HeLa
- 2.5 5 7.5 h UV
DP-4
PCNA
1 2 3 4
f)
INPUT
IP
NS
HA(DP-4)
- + - + - + :etoposide
E2F-1
HA(DP-4)
* IgG heavy chain
DP-4
1 2 3 4 5 6
g)
INPUT
IP
NS
E2F-1
- + - + - + :etoposide
E2F-1
DP-4
actin
1 2 3 4 5 6

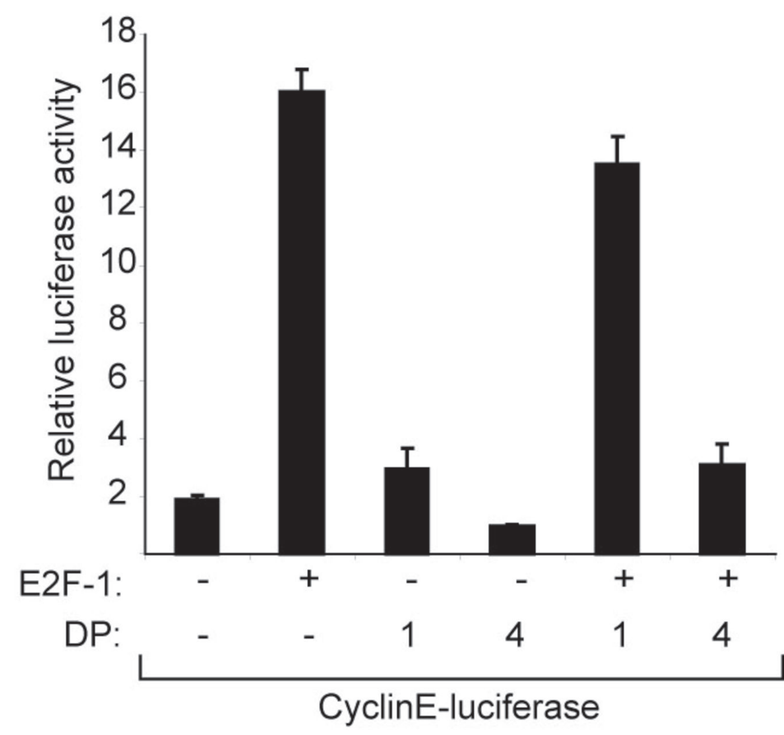
Figure 2

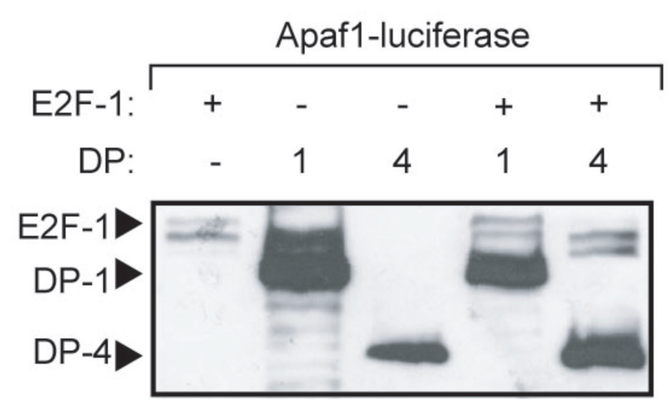
a)

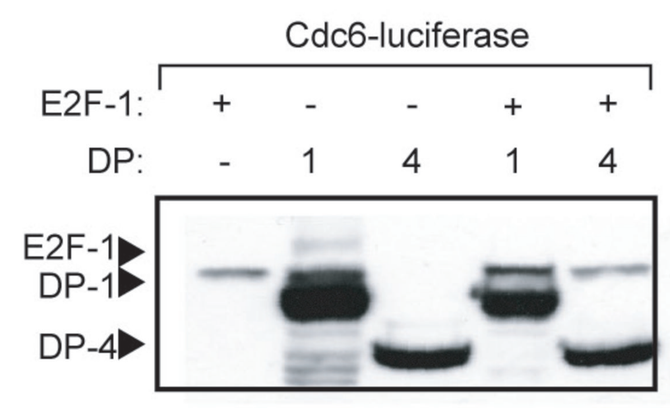


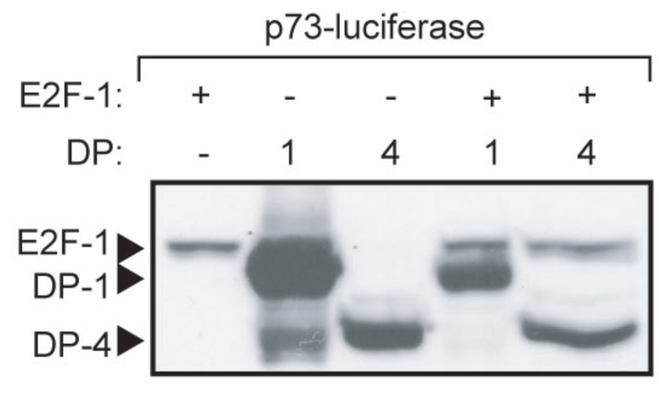


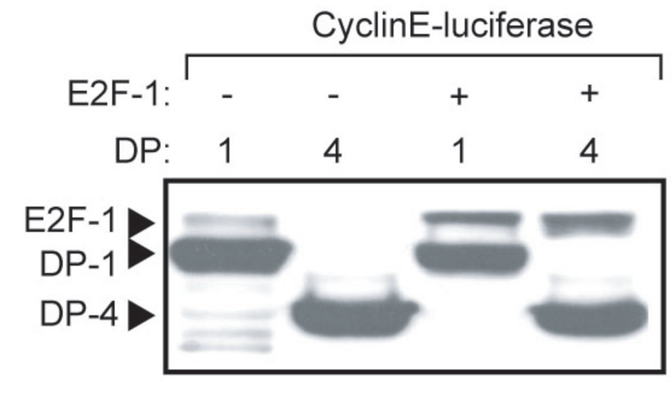












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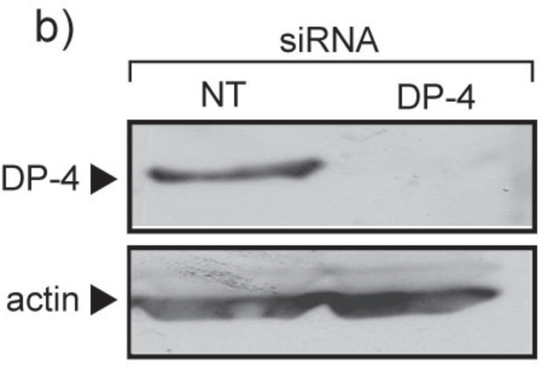

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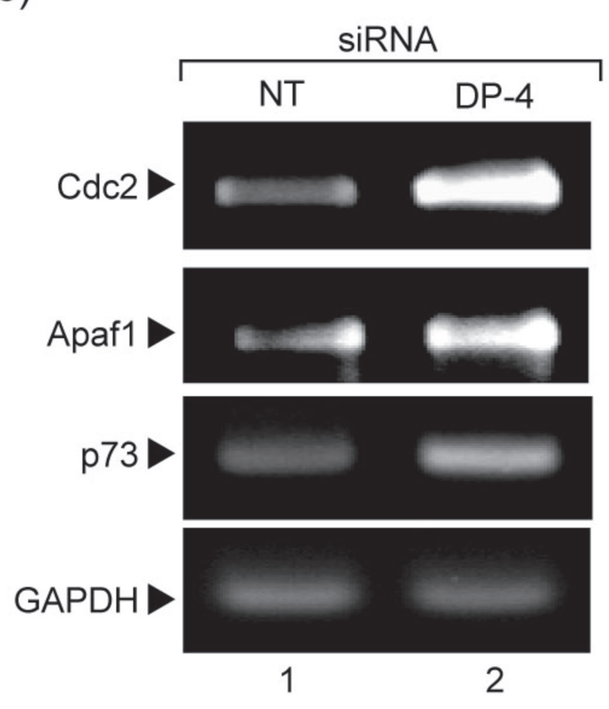

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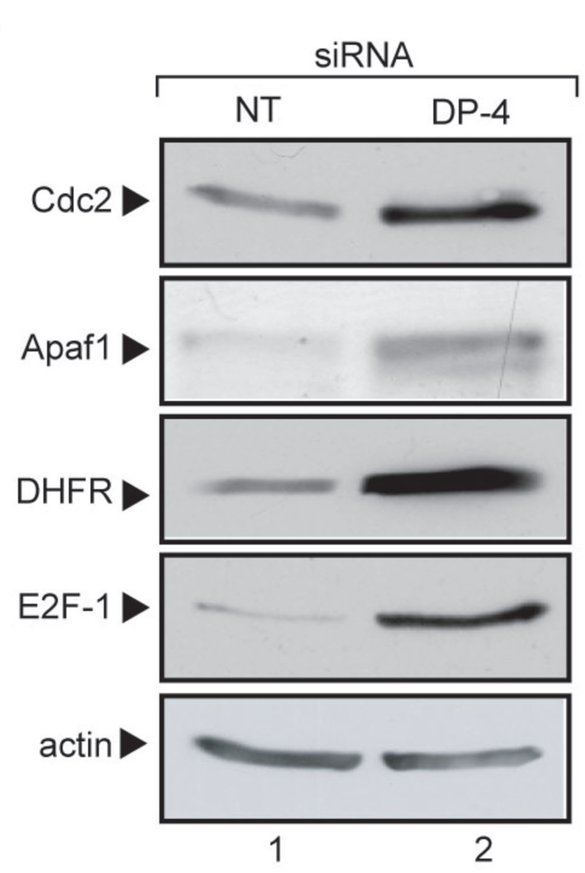
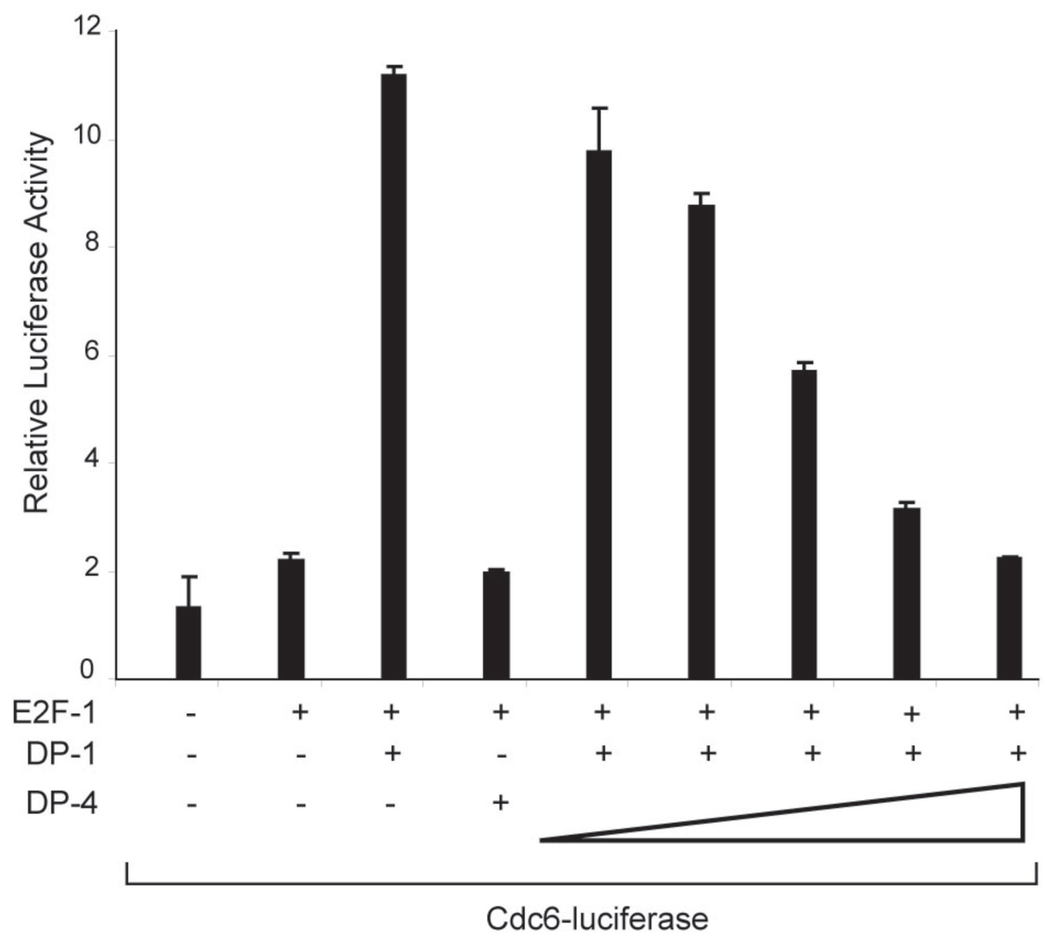
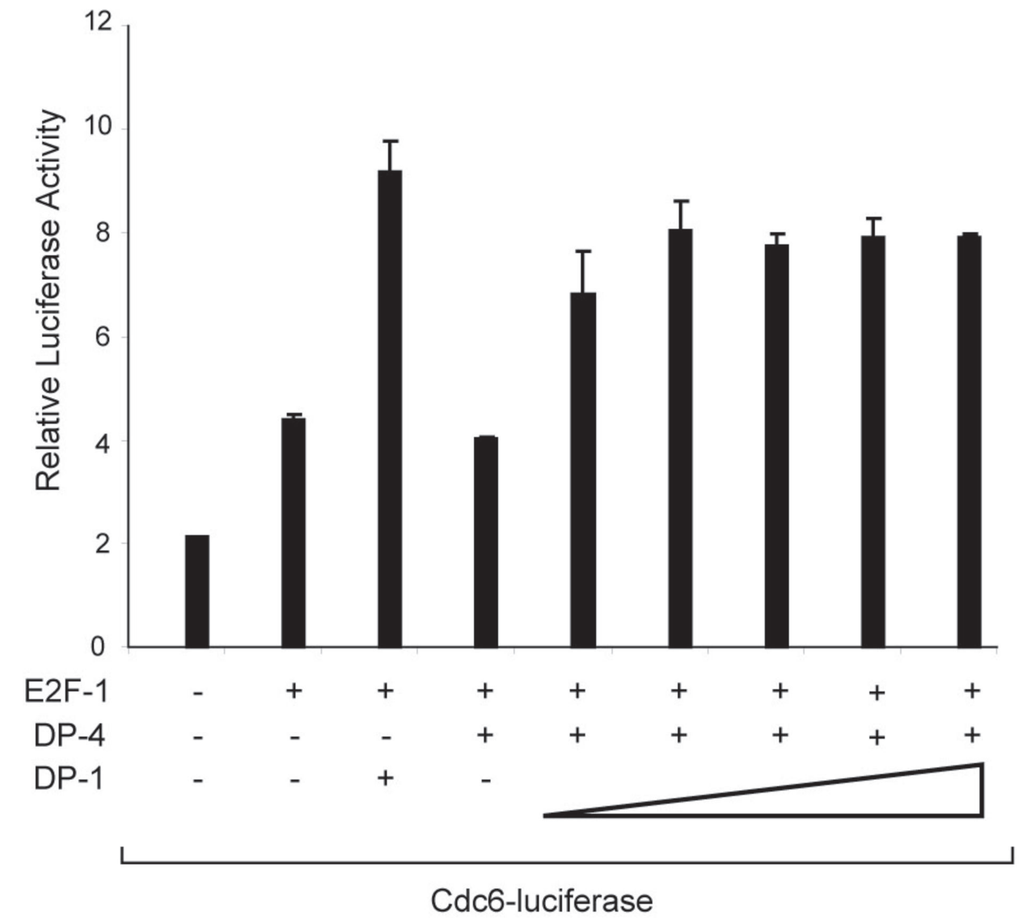


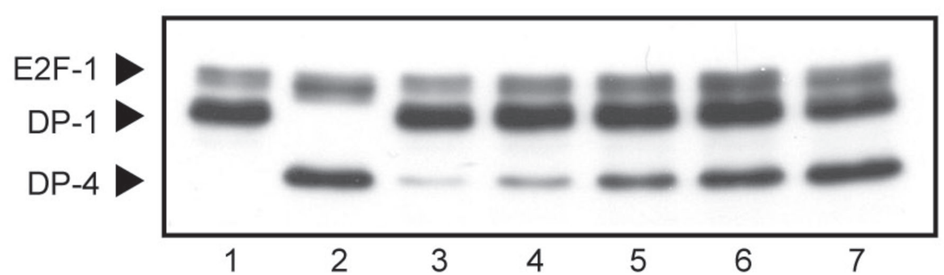
Figure 3

a)


Relative Luciferase Activity
0
2
4
6
8
10
12
E2F-1 - + + + + + + + +
DP-1 - - + - + + + + +
DP-4 - - - +
Cdc6-luciferase

b)


Relative Luciferase Activity
0
2
4
6
8
10
12
E2F-1 - + + + + + + + +
DP-4 - - - + + + + + +
DP-1 - - + -
Cdc6-luciferase


E2F-1
DP-1
DP-4
1 2 3 4 5 6 7

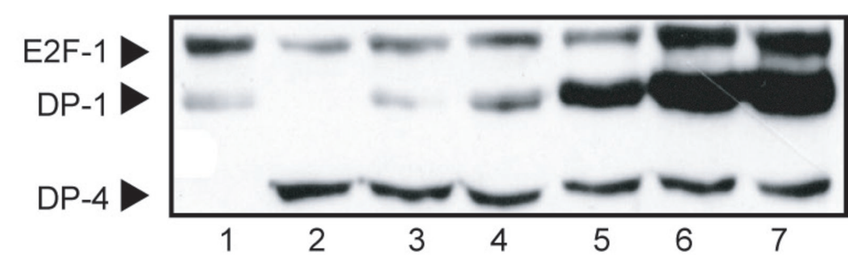

E2F-1
DP-1
DP-4
1 2 3 4 5 6 7

Figure 4

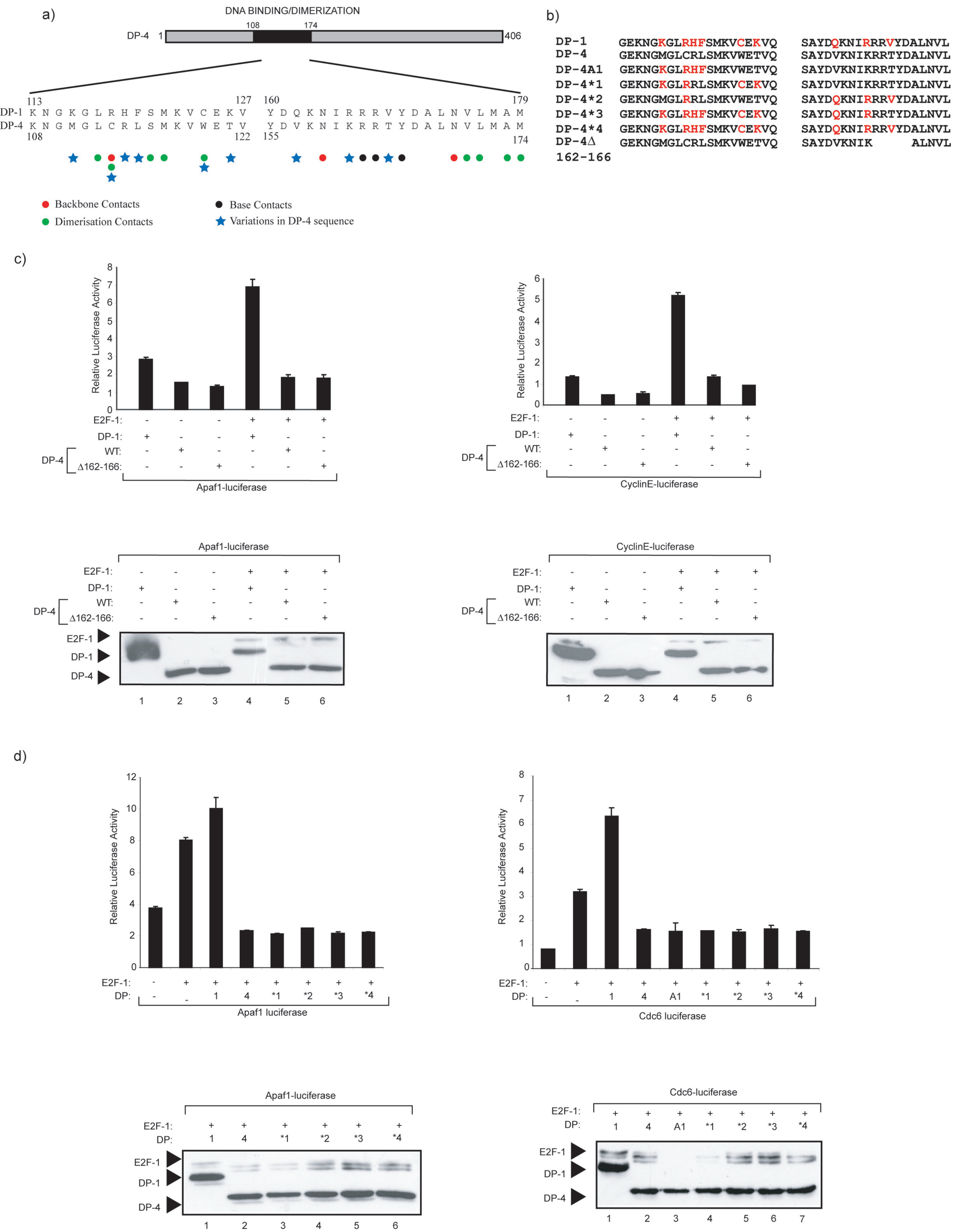

a)
DNA BINDING/DIMERIZATION
DP-4
Backbone Contacts
Dimerisation Contacts
Base Contacts
Variations in DP-4 sequence
b)
DP-1 GEKNGKGLRHFSMKVCEKVQ SAYDQKNIRRRVYDALNVL
DP-4 GEKNGMGLCRLSMKVWETVQ SAYDVKNIKRRTYDALNVL
DP-4A1 GEKNGKGLRHFSMKVWETVQ SAYDVKNIKRRTYDALNVL
DP-4*1 GEKNGKGLRRLSMKVCEKVQ SAYDVKNIKRRTYDALNVL
DP-4*2 GEKNGMGLRRLSMKVWETVQ SAYDQKNIRRRVYDALNVL
DP-4*3 GEKNGKGLRHFSMKVCEKVQ SAYDQKNIRRRTYDALNVL
DP-4*4 GEKNGKGLRHFSMKVCEKVQ SAYDQKNIRRRVYDALNVL
DP-4Δ 162-166 GEKNGMGLCRLSMKVWETVQ SAYDVKNIK ALNVL
c)
Relative Luciferase Activity
E2F-1:
DP-1:
DP-4
WT:
Δ162-166:
Apaf1-luciferase
CyclinE-luciferase
E2F-1
DP-1
DP-4
d)
Relative Luciferase Activity
E2F-1:
DP:
Apaf1 luciferase
Cdc6 luciferase
Apaf1-luciferase
Cdc6-luciferase

Figure 5

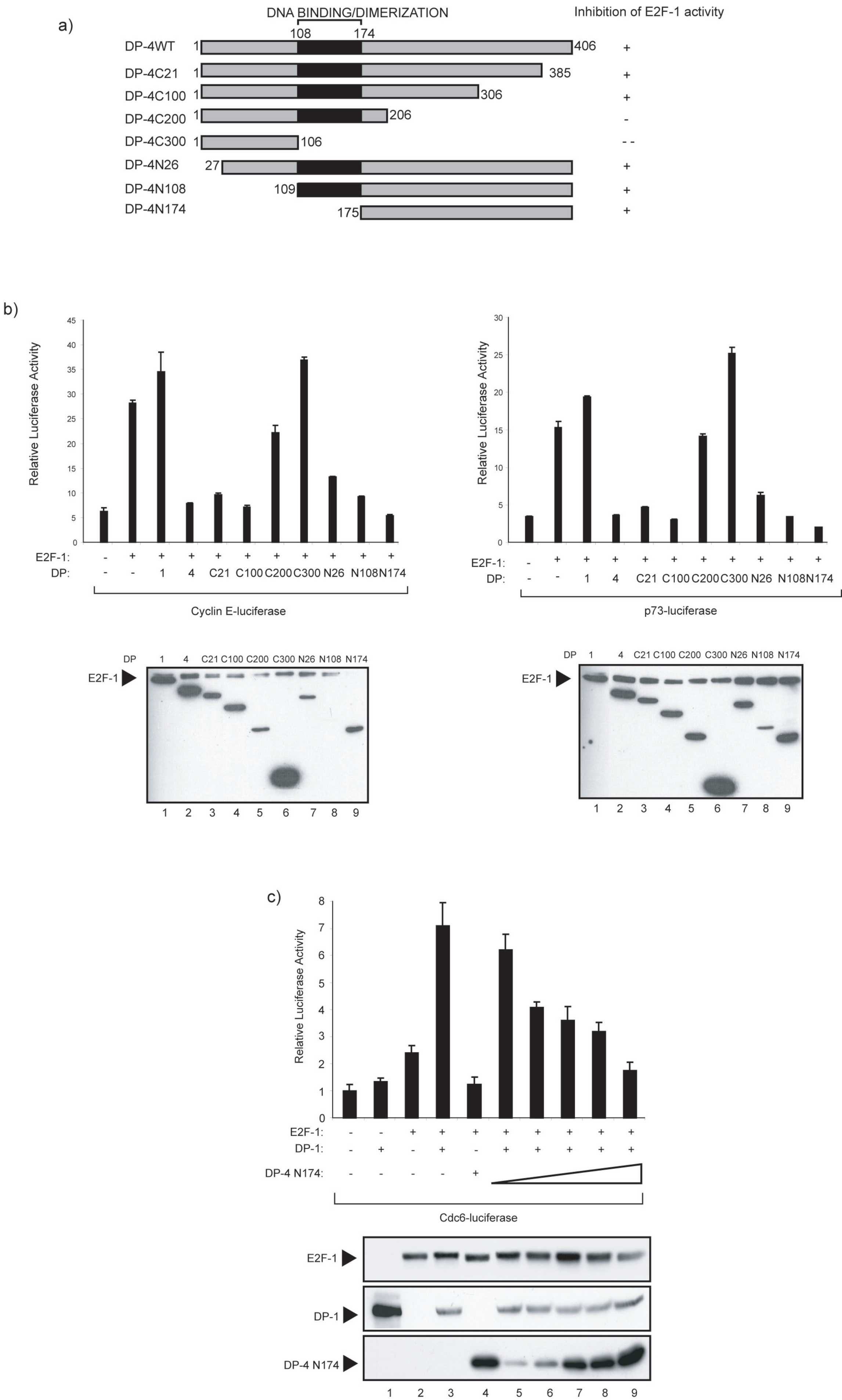
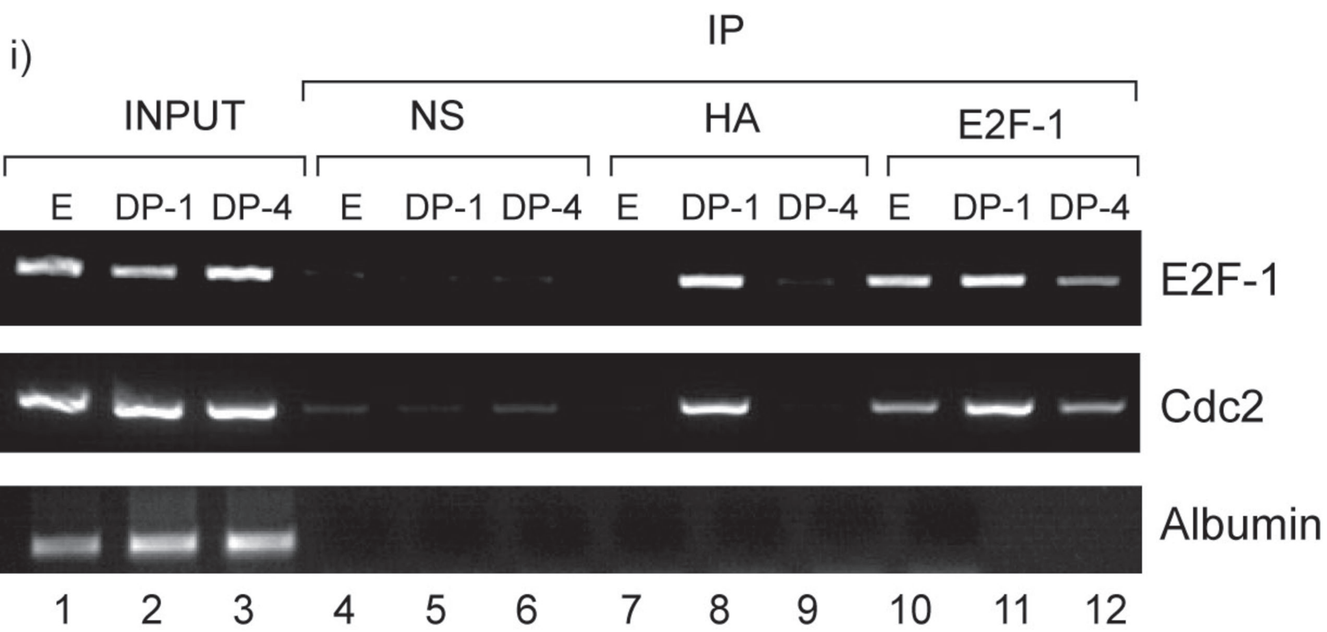

a)
DNA BINDING/DIMERIZATION
Inhibition of E2F-1 activity
108
174
DP-4WT 1 406 +
DP-4C21 1 385 +
DP-4C100 1 306 +
DP-4C200 1 206 -
DP-4C300 1 106 - -
DP-4N26 27 +
DP-4N108 109 +
DP-4N174 175 +
b)
Relative Luciferase Activity
E2F-1:
DP:
Cyclin E-luciferase
p73-luciferase
DP 1 4 C21 C100 C200 C300 N26 N108 N174
E2F-1
1 2 3 4 5 6 7 8 9
c)
Relative Luciferase Activity
E2F-1:
DP-1:
DP-4 N174:
Cdc6-luciferase
E2F-1
DP-1
DP-4 N174

Figure 6

a)

i)



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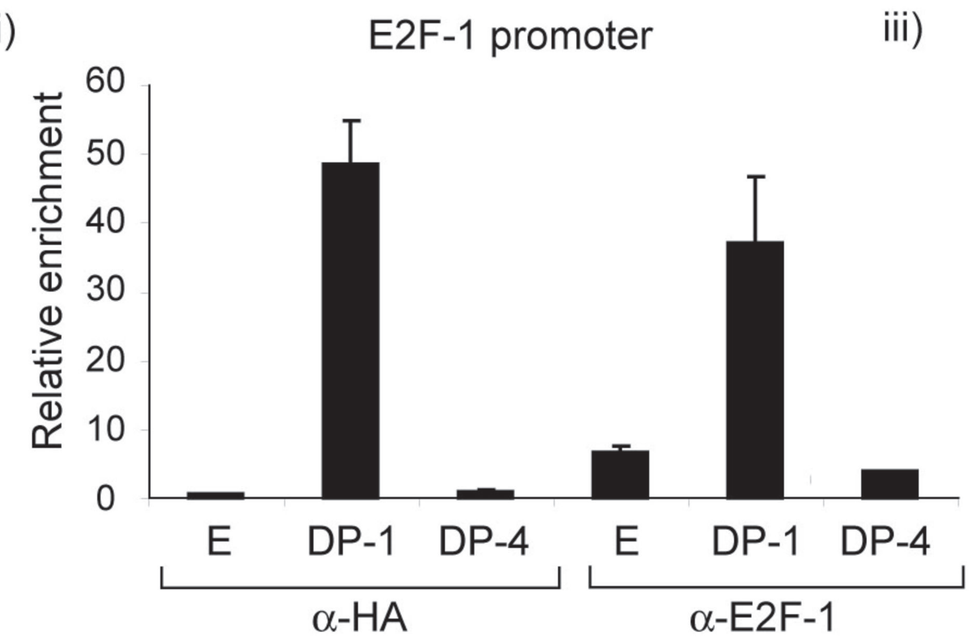

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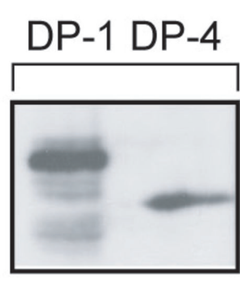
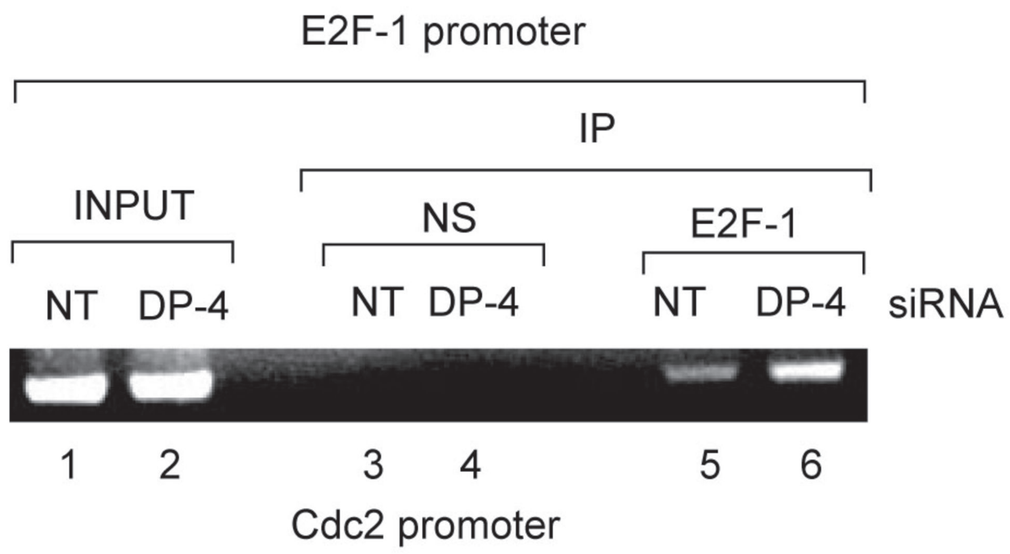


b)

i)



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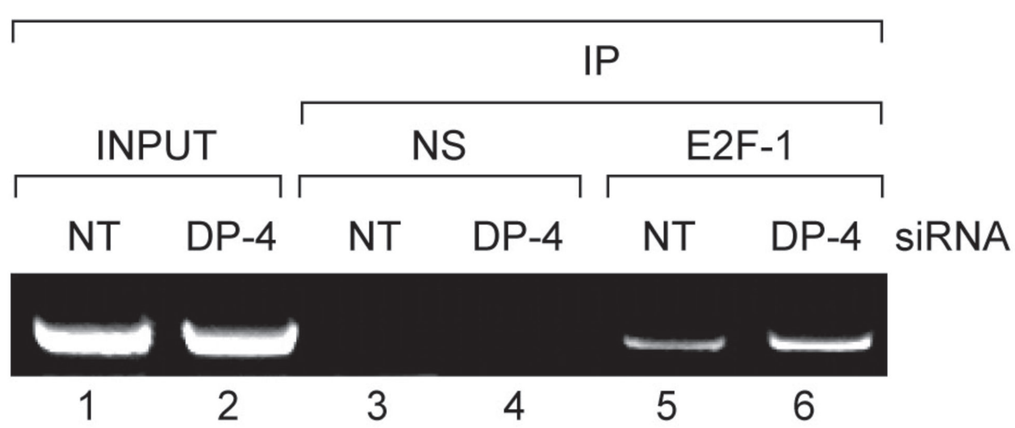

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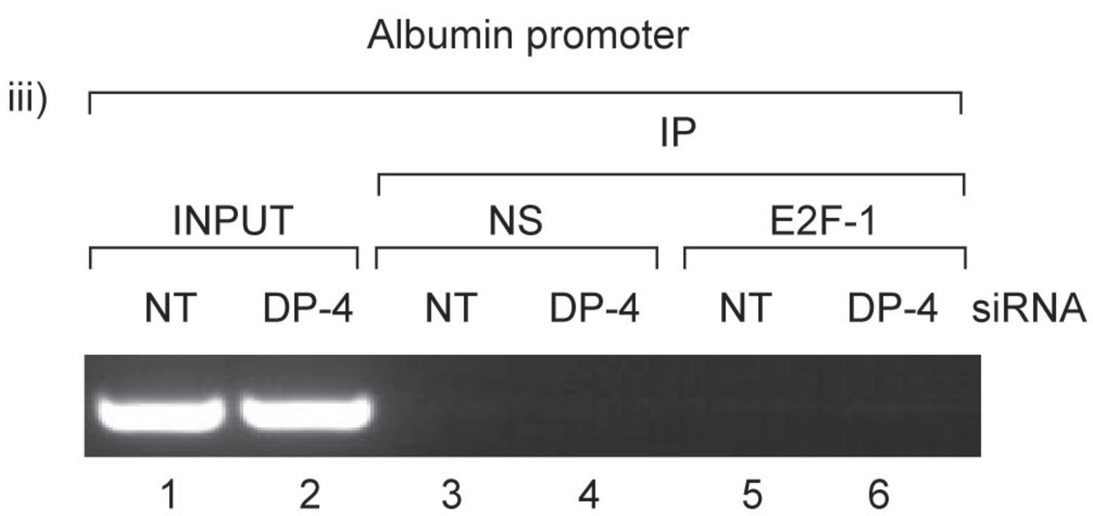

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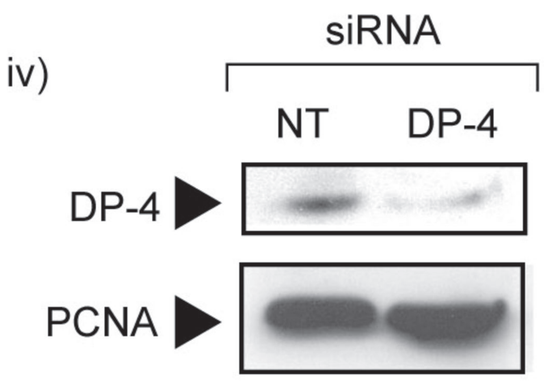

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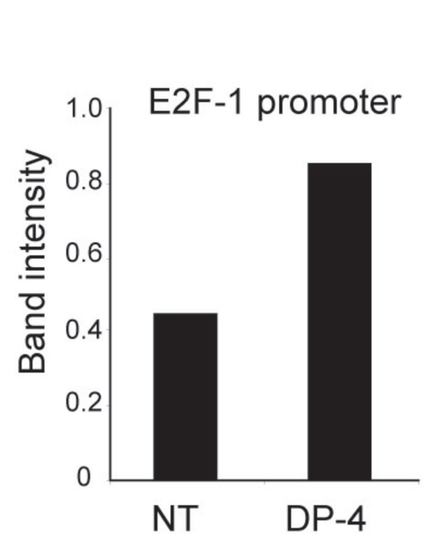

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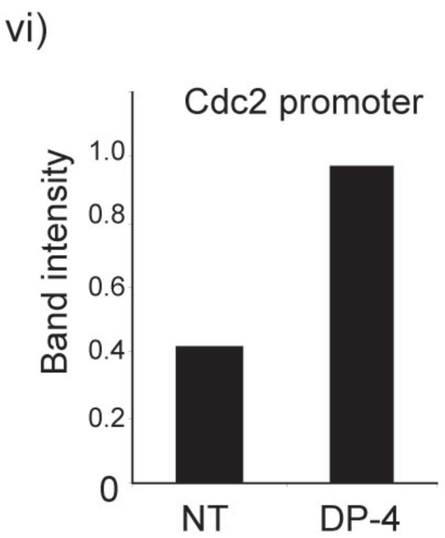

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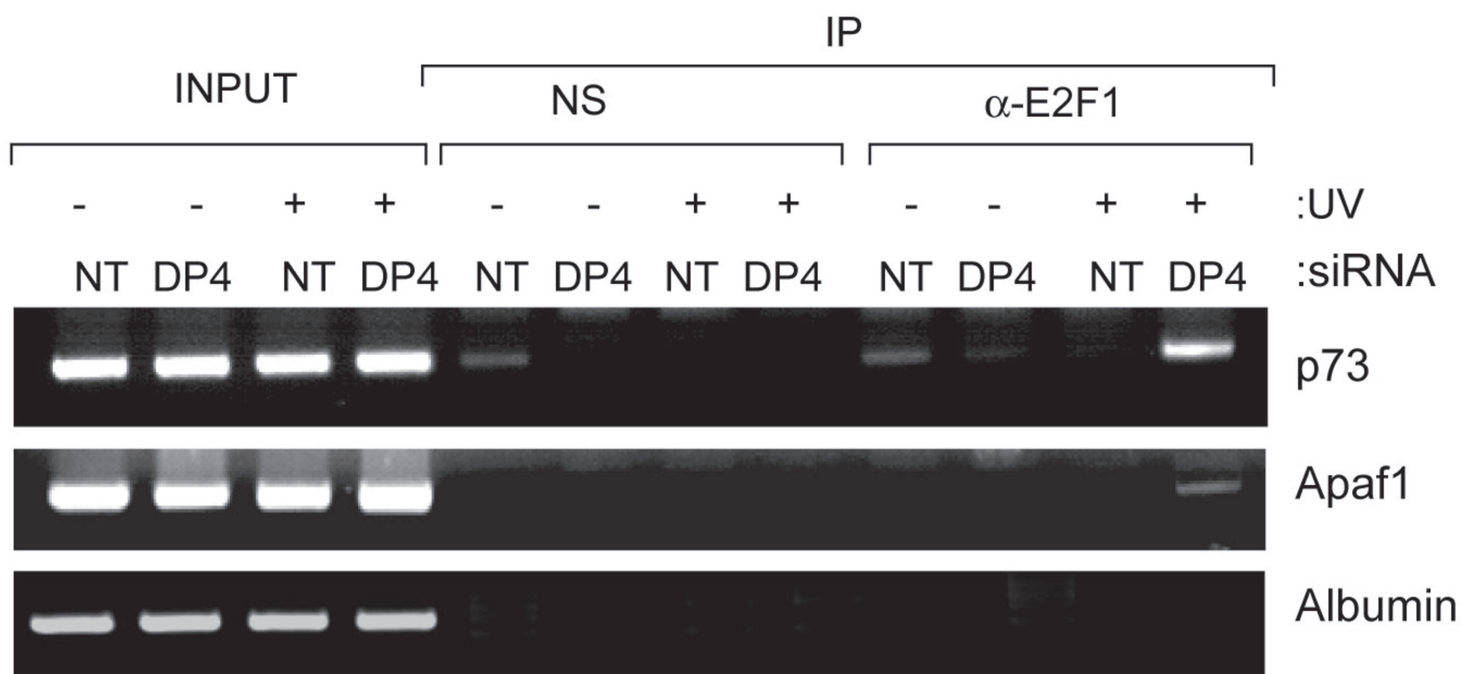
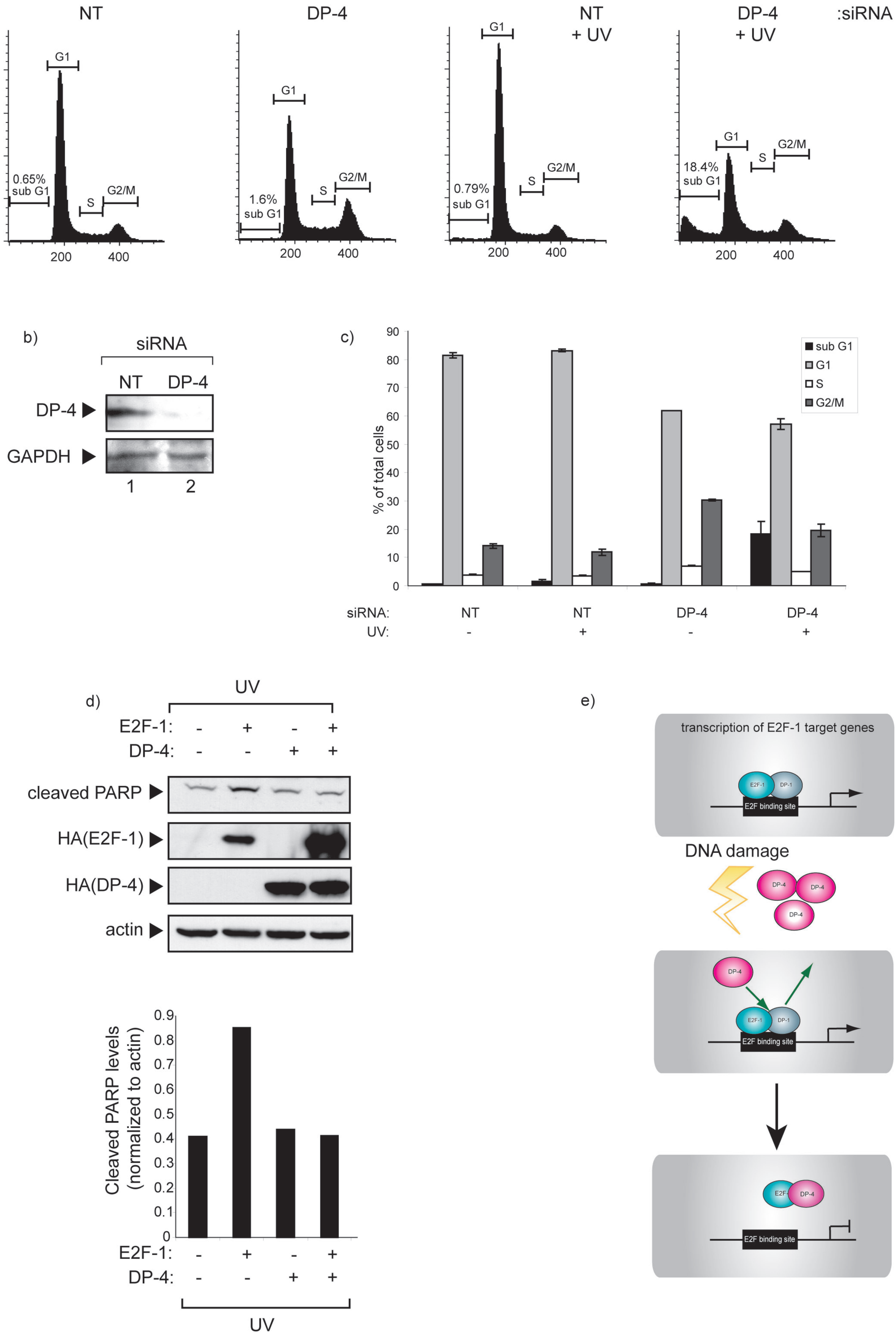


Figure 7


a)
NT
DP-4
NT + UV
DP-4 + UV
:siRNA
G1
S
G2/M
0.65% sub G1
1.6% sub G1
0.79% sub G1
18.4% sub G1
200
400
b)
siRNA
NT
DP-4
DP-4
GAPDH
1
2
c)
% of total cells
sub G1
G1
S
G2/M
siRNA:
UV:
d)
UV
E2F-1:
DP-4:
cleaved PARP
HA(E2F-1)
HA(DP-4)
actin
Cleaved PARP levels (normalized to actin)
e)
transcription of E2F-1 target genes
E2F-1
DP-1
E2F binding site
DNA damage
DP-4