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# **E2F-1 regulation by an unusual DNA damage-responsive DP partner 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-occurs 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<sup>1, 2</sup>. 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<sup>3, 4</sup>. pRb physically interacts with DNA bound E2F-1 and thereby prevents transcriptional activity<sup>5</sup>. In turn, cell cycle progression cannot occur, which is important for pRb to exert its tumour suppressor activity<sup>5</sup>. To date, eight E2F subunits have been identified, together with three DP subunits<sup>6</sup>. 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<sup>7-11</sup>. In mammalian cells, however, most E2F exists as a heterodimer in complex with a DP subunit<sup>6</sup>.

Under normal physiological conditions DP-1 is the predominant E2F partner protein<sup>12, 13</sup>. DP-1 is encoded by an essential gene, since *dp-1*<sup>-/-</sup> mice exhibit embryonic lethality, in part due to defective extra-embryonic tissue development<sup>14</sup>. 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<sup>15</sup>. 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<sup>16, 17</sup>. The most recent family member to be

described, DP-4, acts negatively on both E2F DNA binding activity and cell cycle progression<sup>18-20</sup>.

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 reinstates 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<sup>5, 21</sup>. 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 RXYD DNA binding consensus motif (RRTYD in DP-4; Figure 4a and b) to create a DP-4 derivative that was defective in DNA binding ( $\Delta$ 162-166). 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<sup>22</sup>, 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-4 $\Delta$ 162-166 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-4 $\Delta$ 162-166, which lacks residues that are essential for DNA binding <sup>22</sup>, 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-occurred 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<sup>23, 24</sup>, 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<sup>5, 24</sup>. 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<sup>25</sup>. 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**

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## **Materials and methods**

### **Plasmids and expression vectors.**

HA-DP-1, HA-DP-4 and HA-E2F-1 have been described previously<sup>18</sup>. pCdc6-luciferase<sup>26</sup>, p73-luciferase<sup>27</sup>, Apaf1-luciferase<sup>21</sup> and pCyclinE-luciferase<sup>28</sup> 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<sub>2</sub>. 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<sup>2</sup>) 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<sub>3</sub>VO<sub>4</sub>, 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 immunoblotting 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<sup>29</sup>.

#### **Cell fractionation.**

Cell fractionation was performed as previously described<sup>30</sup>.

#### **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<sup>31</sup>.

#### **Flow cytometry.**

U2OS cells were transfected with DP-4 siRNA or non-targeting control siRNA, 72h later cells were treated with UV 50J/m<sup>2</sup>. 8h later cells were washed in PBS and fixed overnight in 50% ethanol/PBS at 4°C. 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<sup>31</sup>.

#### **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<sup>32</sup>. 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<sup>33</sup>. The primers used were as follows:

E2F-1 forward 5'-AGGAACCGCCGCGTTGTTCCCGT-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<sup>32</sup>. The primers used were as follows: Cdc2 forward 5'-AATTGGAGAAGGTACCTATGGA-3'; Cdc2 reverse 5'-TGTA CTGACCAGGAGGGATA-3'; Apaf1 forward 5'-CACGTTCAAAGGTGGCTGAT-3' Apaf1 reverse 5'-TGGTCAACTGCAAGGACCAT-3'; p73 forward 5'-ACTTCAACGAAGGACAGTCTGCT-3' and p73 reverse 5'-AATTC CGTCCCCACCTGTG-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 (100 $\mu$ g) 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 (1 $\mu$ g). 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<sup>2</sup>), 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 (2 $\mu$ M) 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 (10 $\mu$ M) 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 ( $50\text{J}/\text{m}^2$ ), 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 ( $10\mu\text{M}$ ) 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 ( $2\mu\text{M}$ ) 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 ( $50\text{J}/\text{m}^2$ ), 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 ( $1\mu\text{g}$ ), and 44h later cells were treated with etoposide ( $10\mu\text{M}$ ) 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 ( $10\mu\text{M}$ ), 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 (1 $\mu$ g) 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- $\beta$ gal 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 (1 $\mu$ g), together with expression vectors for E2F-1 (100ng), with DP-4 transfected in increasing amounts (0.1, 0.2, 0.5, 1.0 and 2.0 $\mu$ g) in the presence of a constant level of DP-1 (2 $\mu$ g). 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 (1 $\mu$ g), together with expression vectors for E2F-1 (100ng), with DP-1 transfected in increasing amounts (0.1, 0.2, 0.5, 1.0 and 2.0 $\mu$ g) in the presence of a constant level of DP-4 (2 $\mu$ g). 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 <sup>22</sup>). 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-4 $\Delta$ 162-166 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  $\Delta$ 162-166 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 2.0 $\mu$ g), 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 (1 $\mu$ g) 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<sup>2</sup>). 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 ( $50\text{J}/\text{m}^2$ ). 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 ( $1\mu\text{g}$ ), DP-4 ( $1\mu\text{g}$ ) or both, as indicated, 72h later cells were treated with ultraviolet (UV) light ( $50\text{J}/\text{m}^2$ ). 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 (1 $\mu$ g), 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 (1 $\mu$ g), 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  $\mu$ g; lane 3).
- c)** MCF7 cells were treated with doxorubicin (2 $\mu$ M) 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 (2 $\mu$ M) for 2, 5, 8 and 24h.  
(ii) HEK293 cells were treated with etoposide (10 $\mu$ M) for 2, 5, 8 and 24h.  
(iii) H1299 cells were treated with ultraviolet (UV light) (50J/m<sup>2</sup>), 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<sup>2</sup>) 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 ( $50\text{J}/\text{m}^2$ ). 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

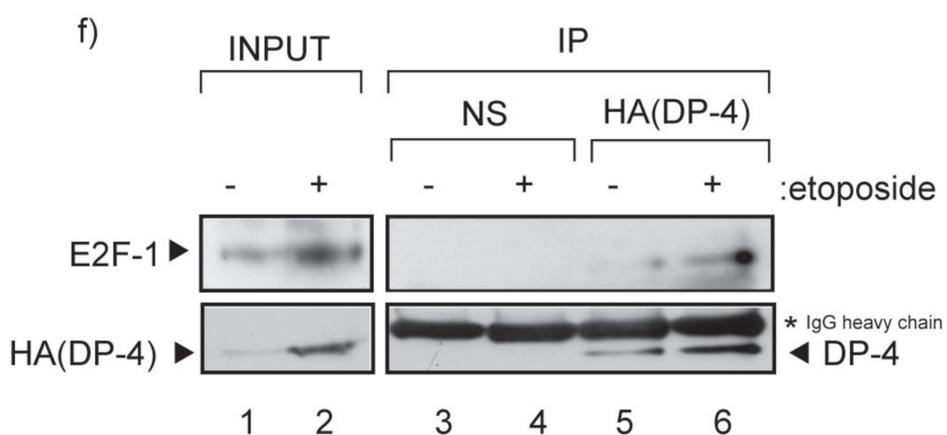
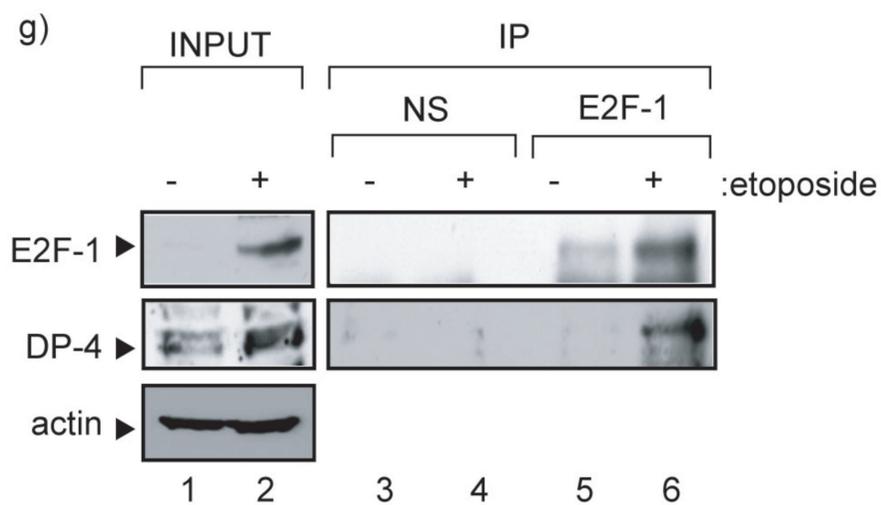
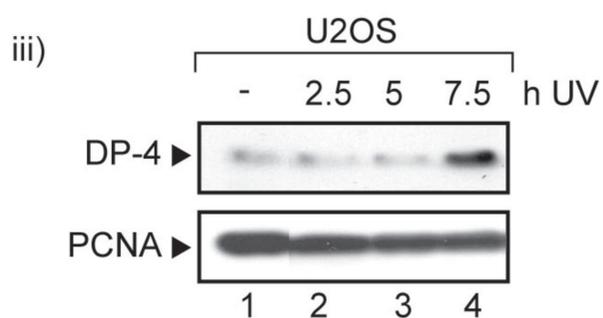
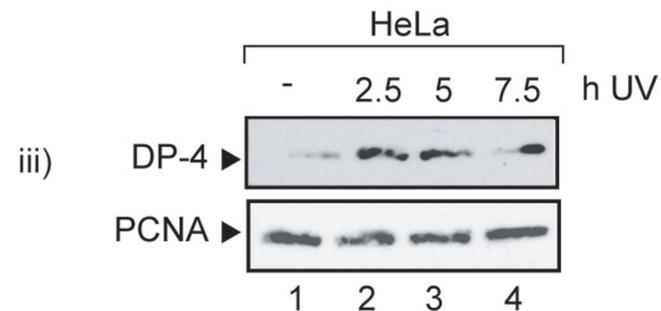
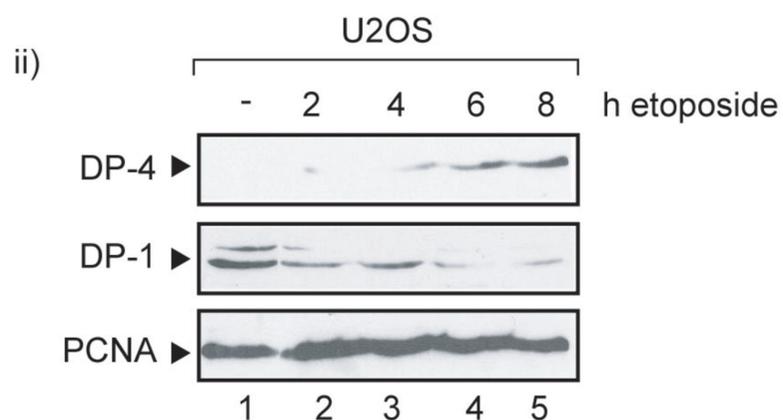
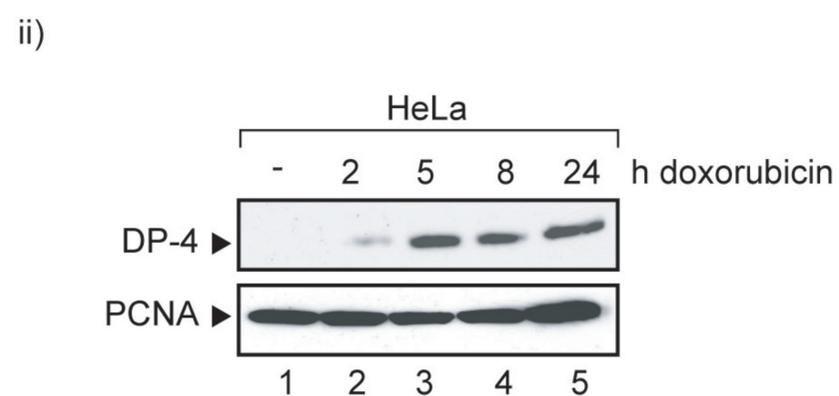
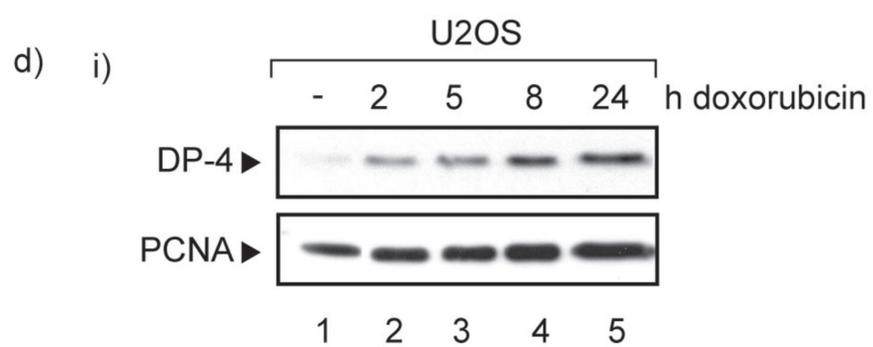
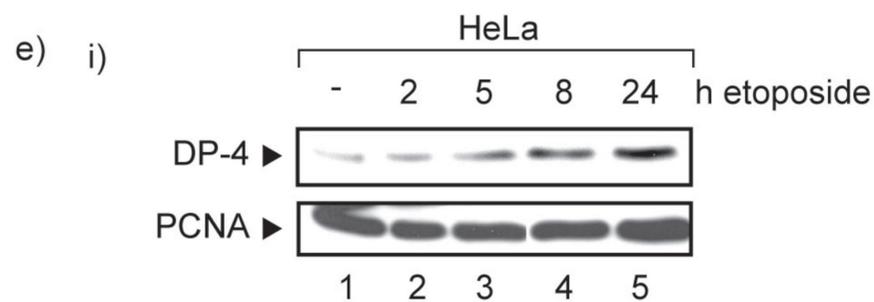
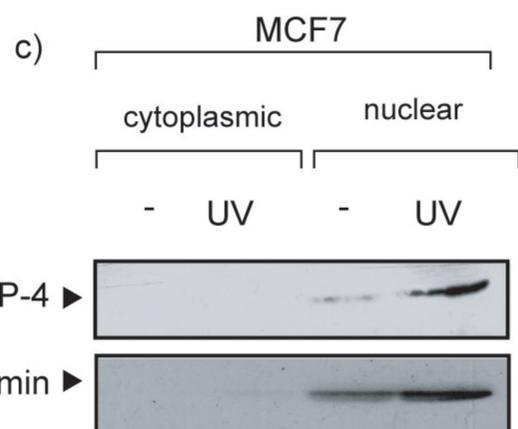
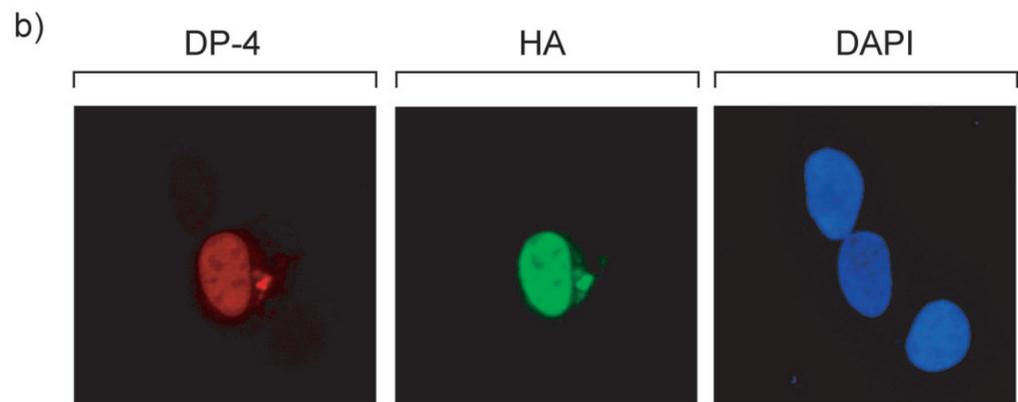
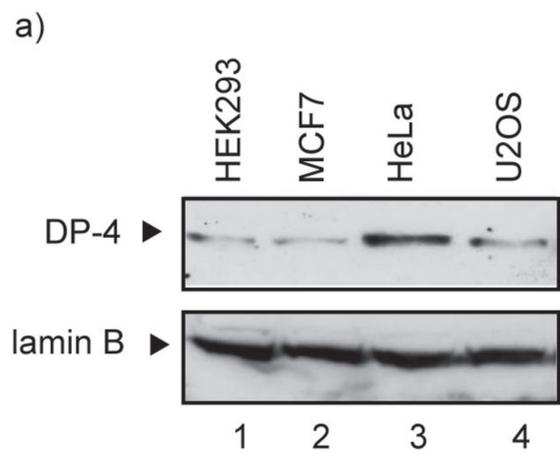


Figure 2

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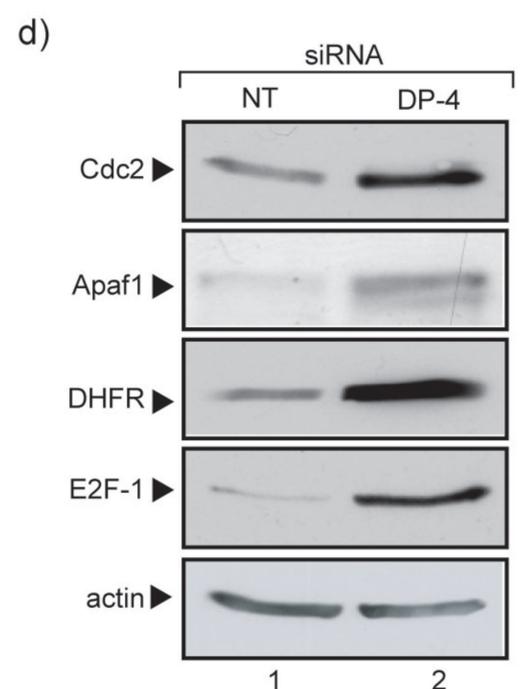
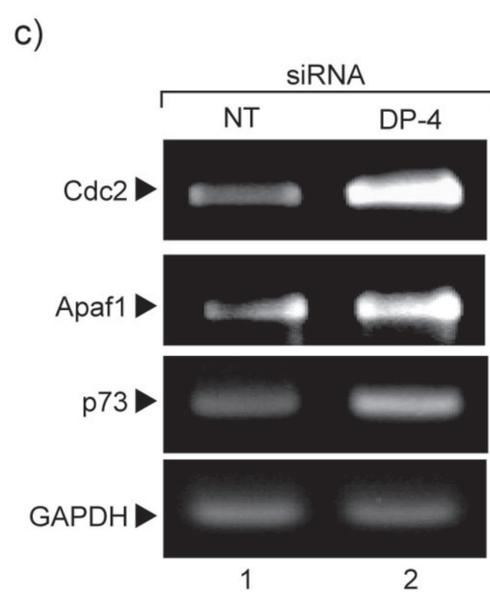
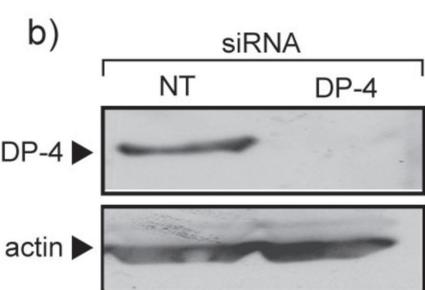
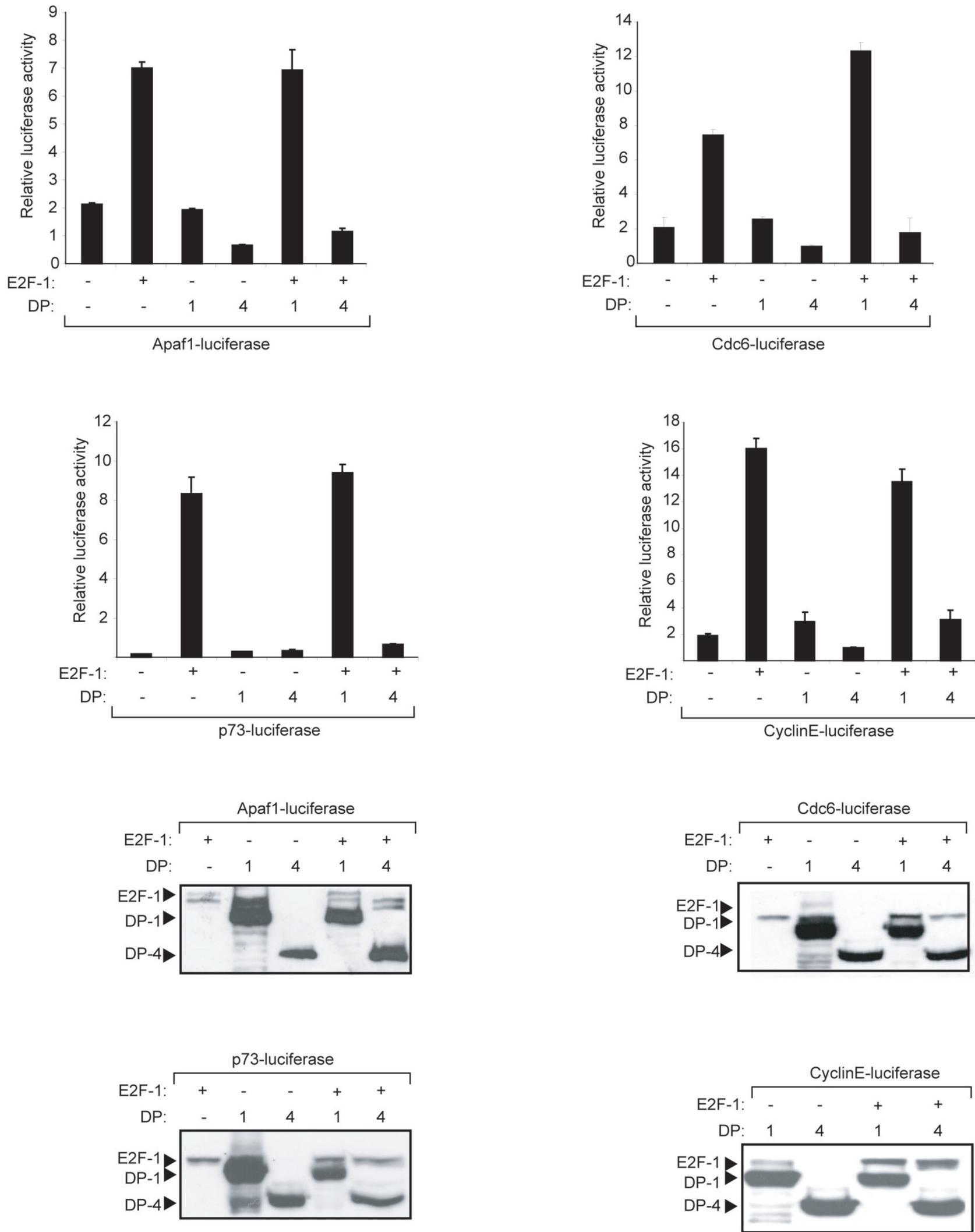
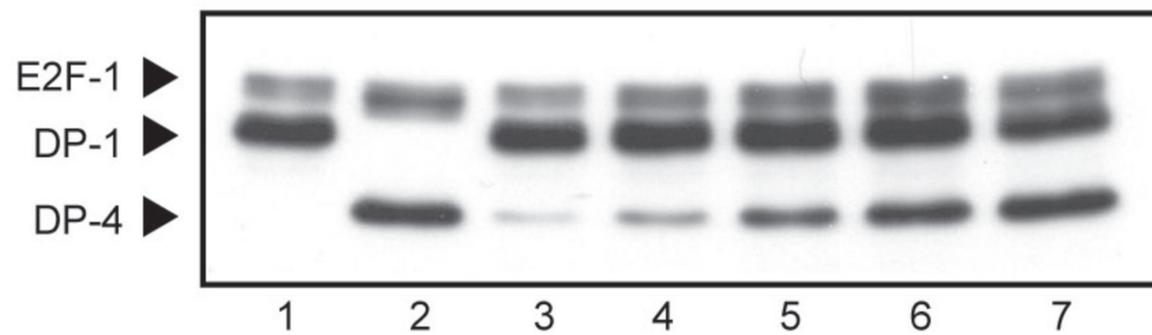
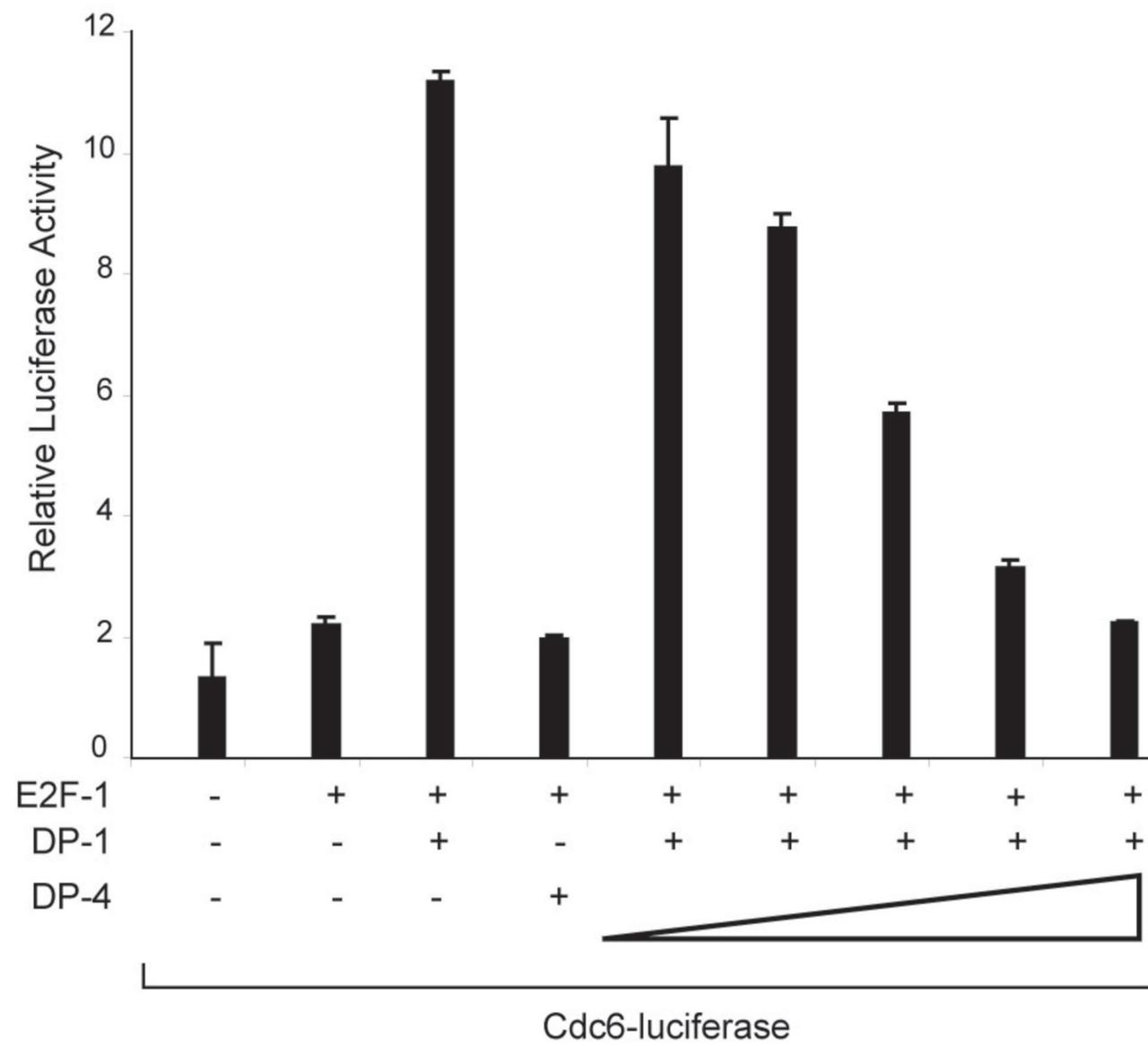


Figure 3

a)



b)

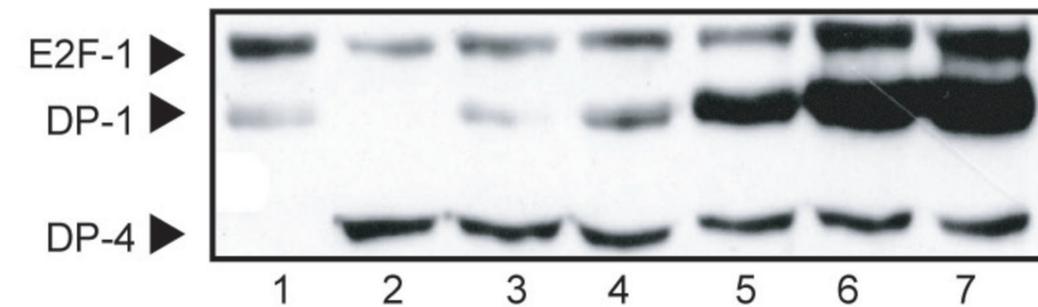
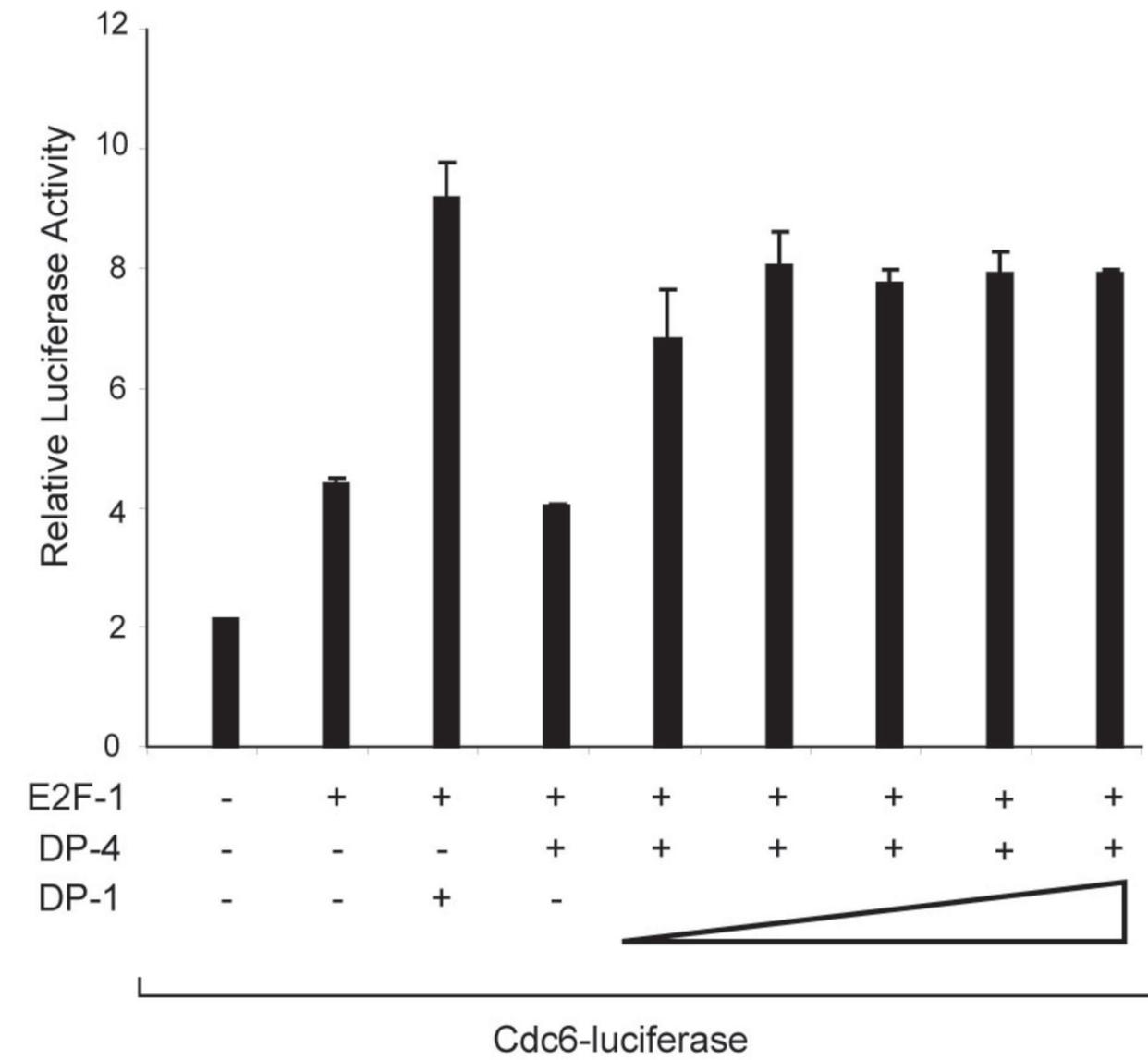


Figure 4

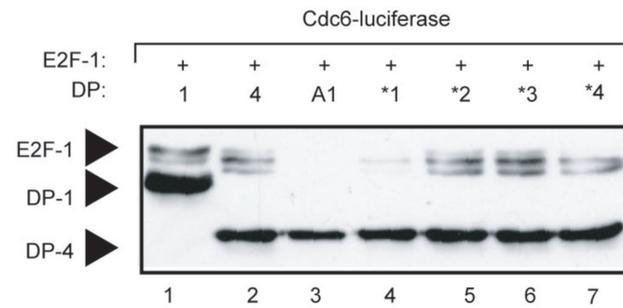
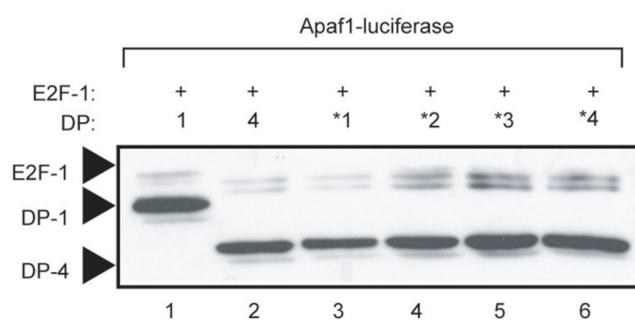
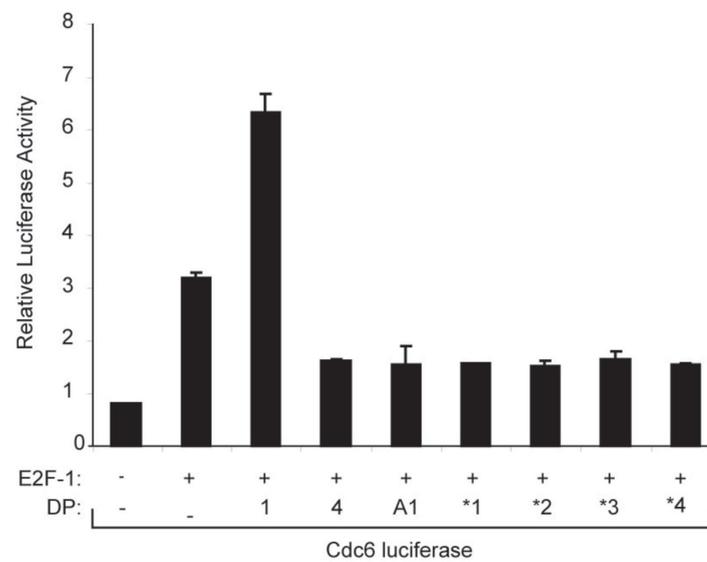
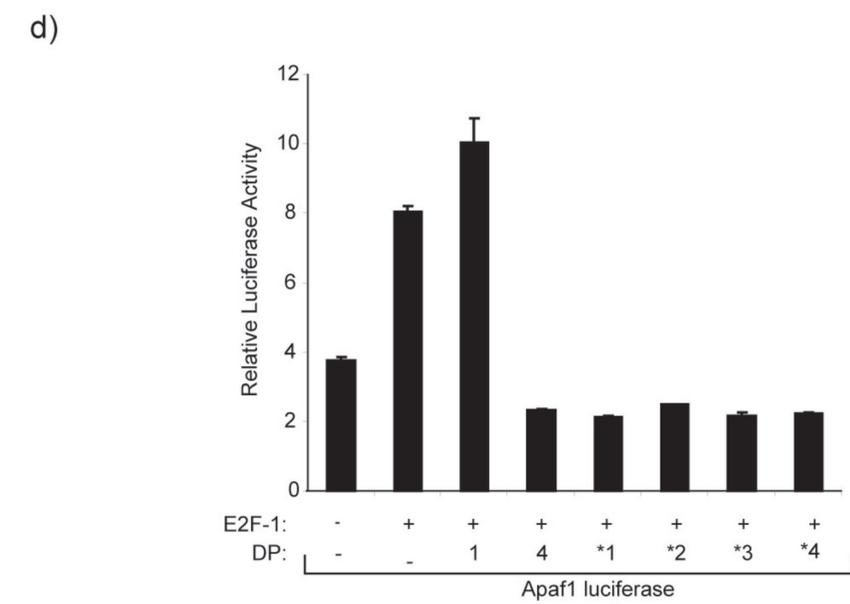
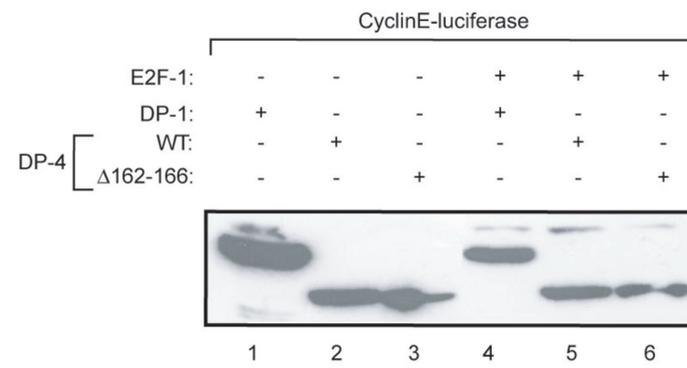
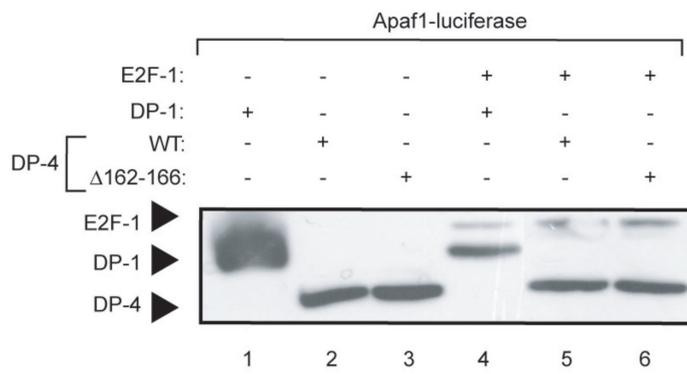
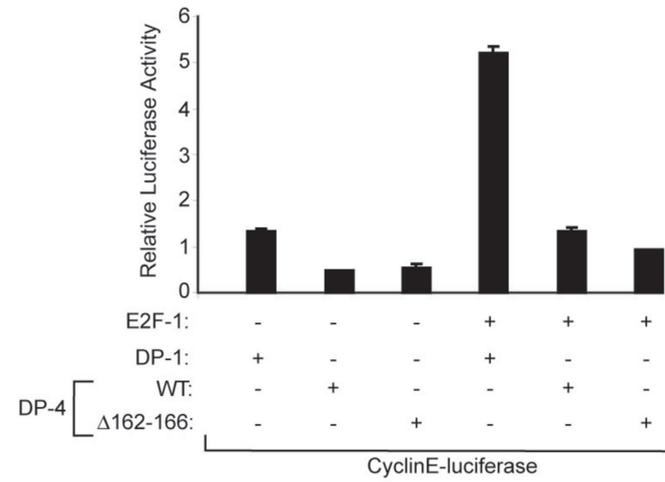
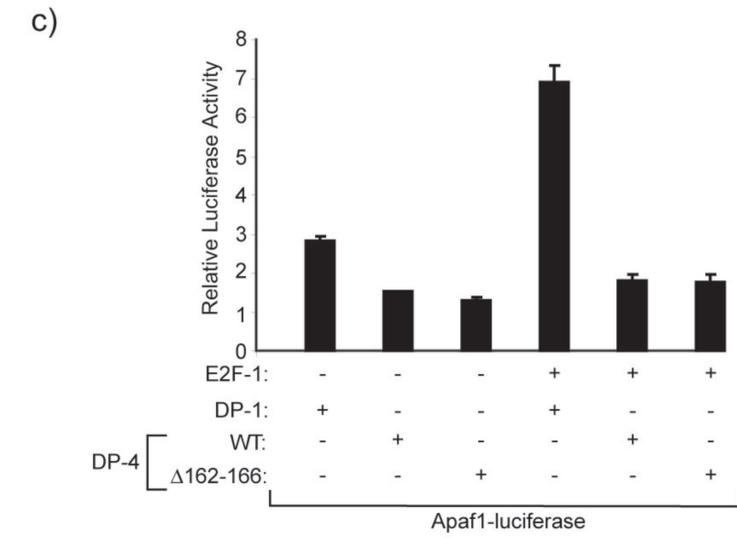
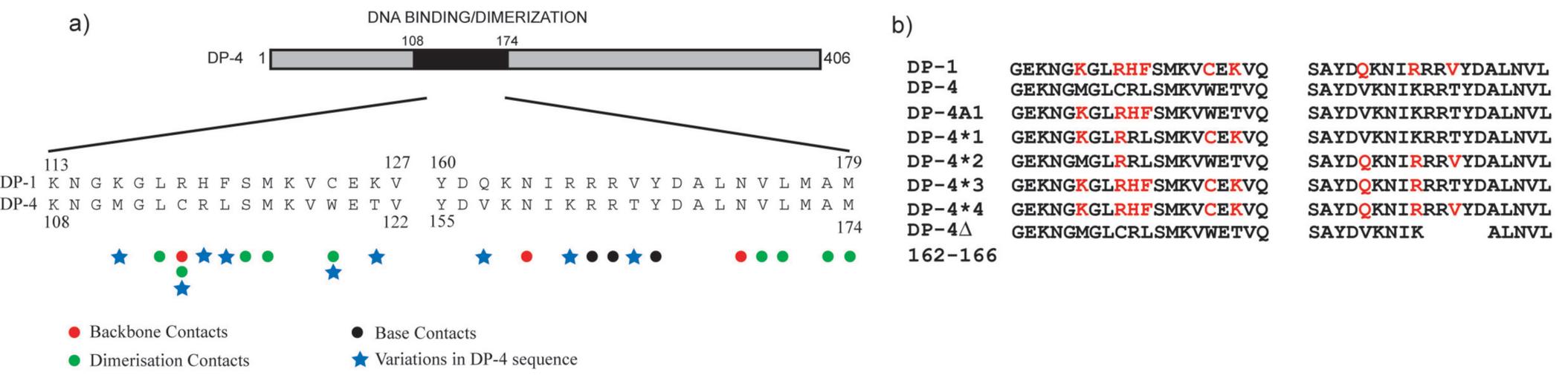
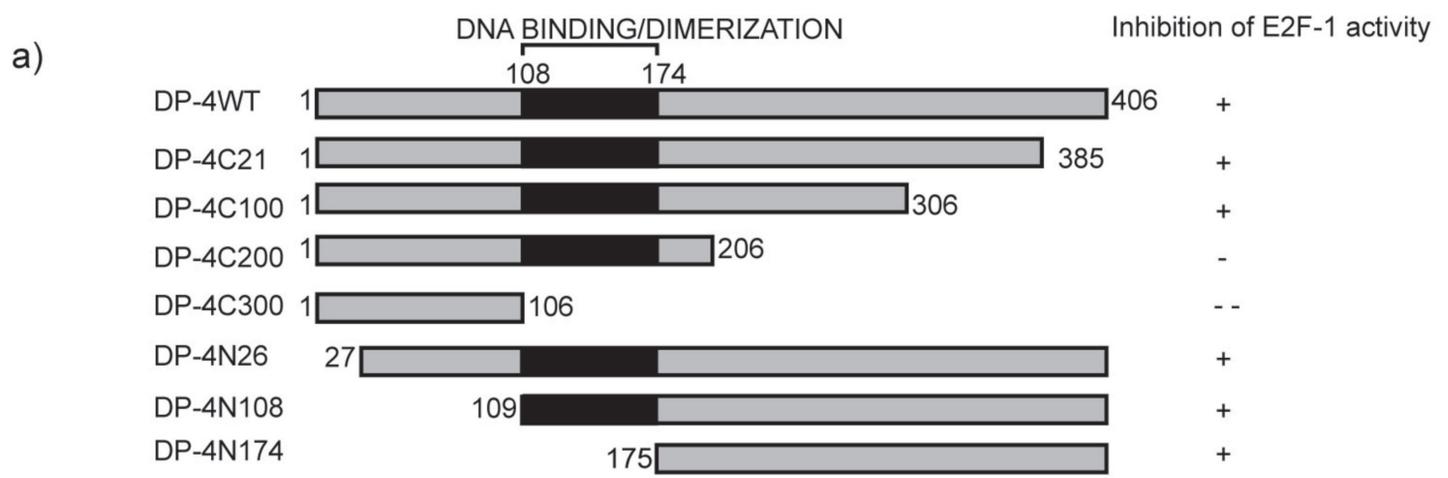
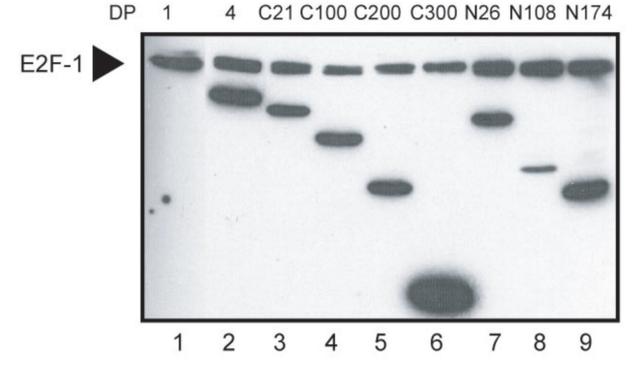
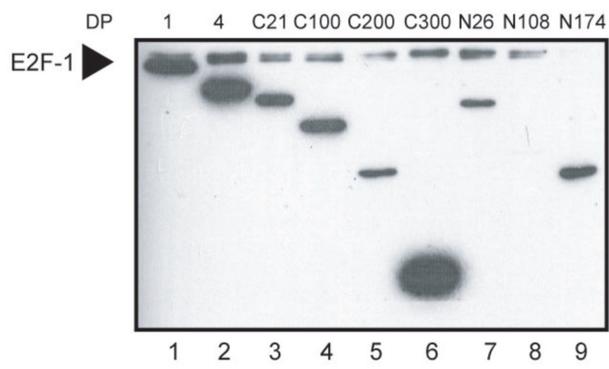
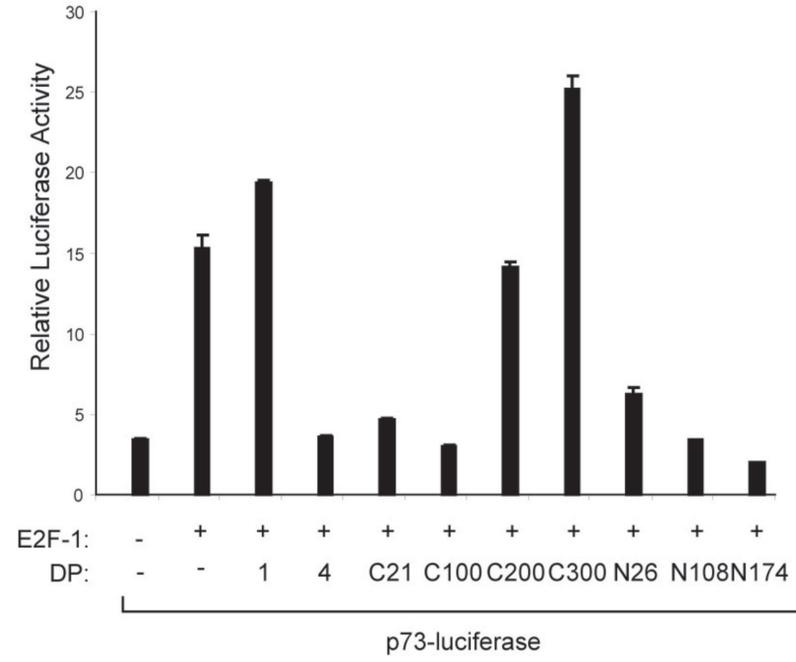
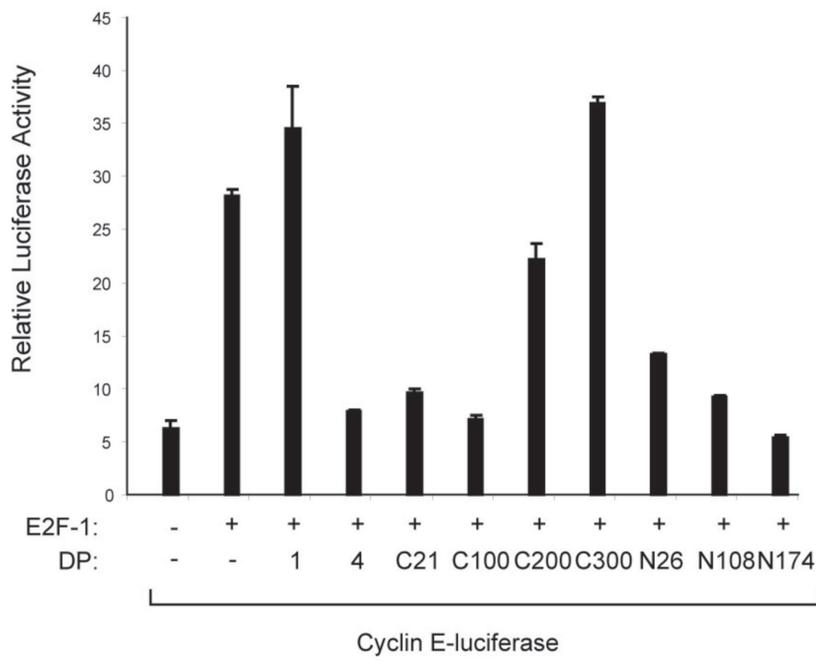


Figure 5



b)



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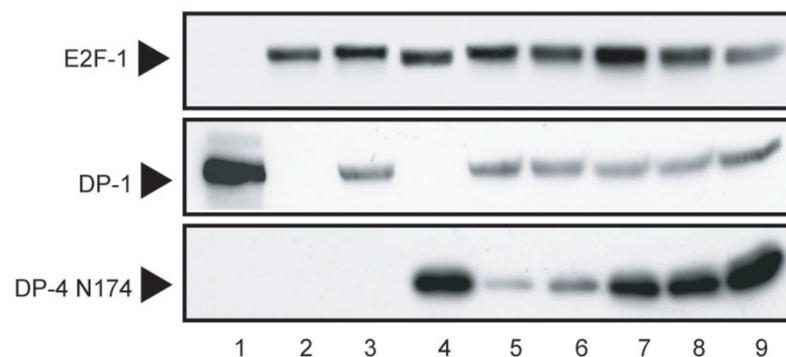
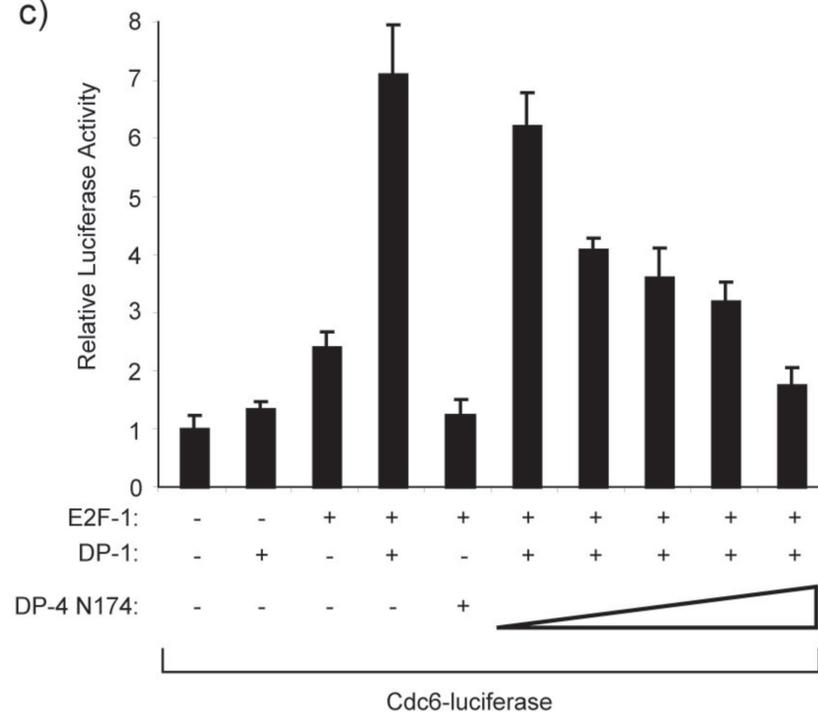
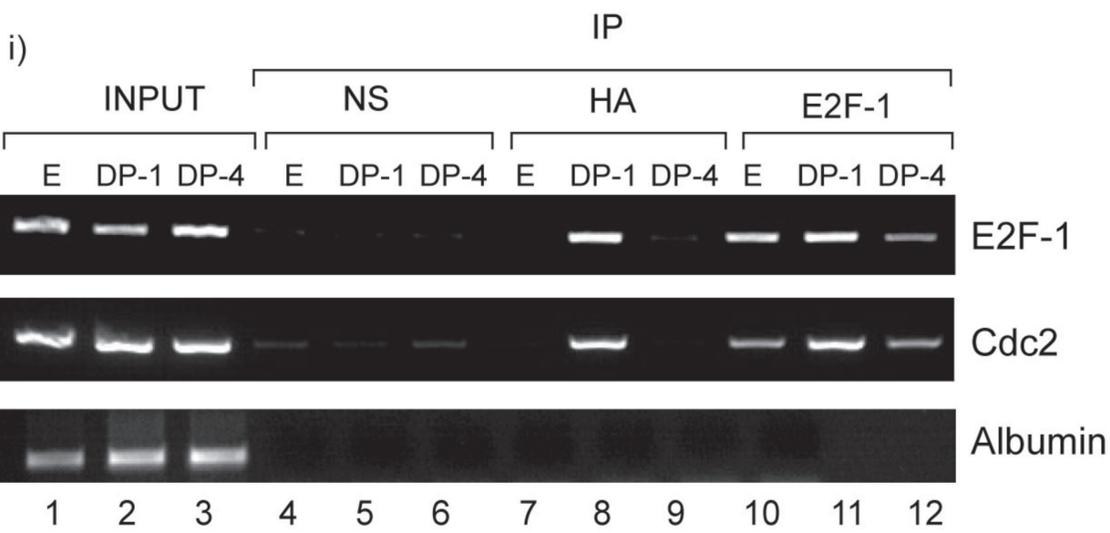
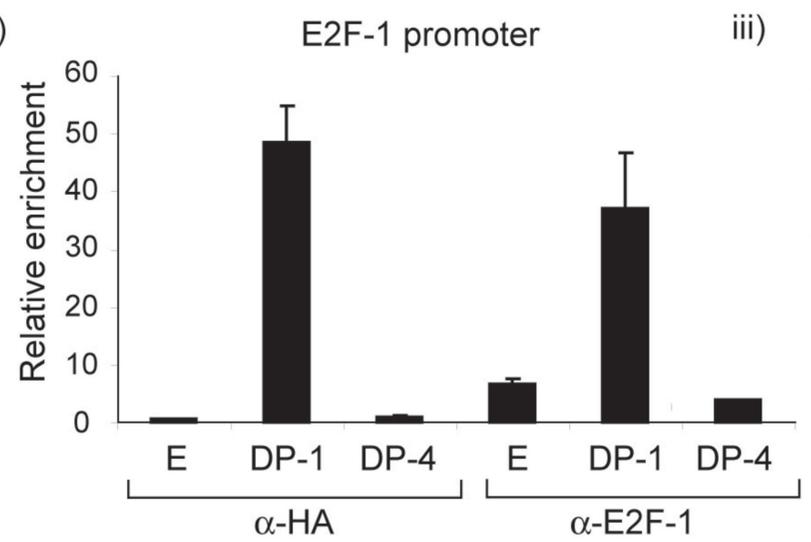


Figure 6

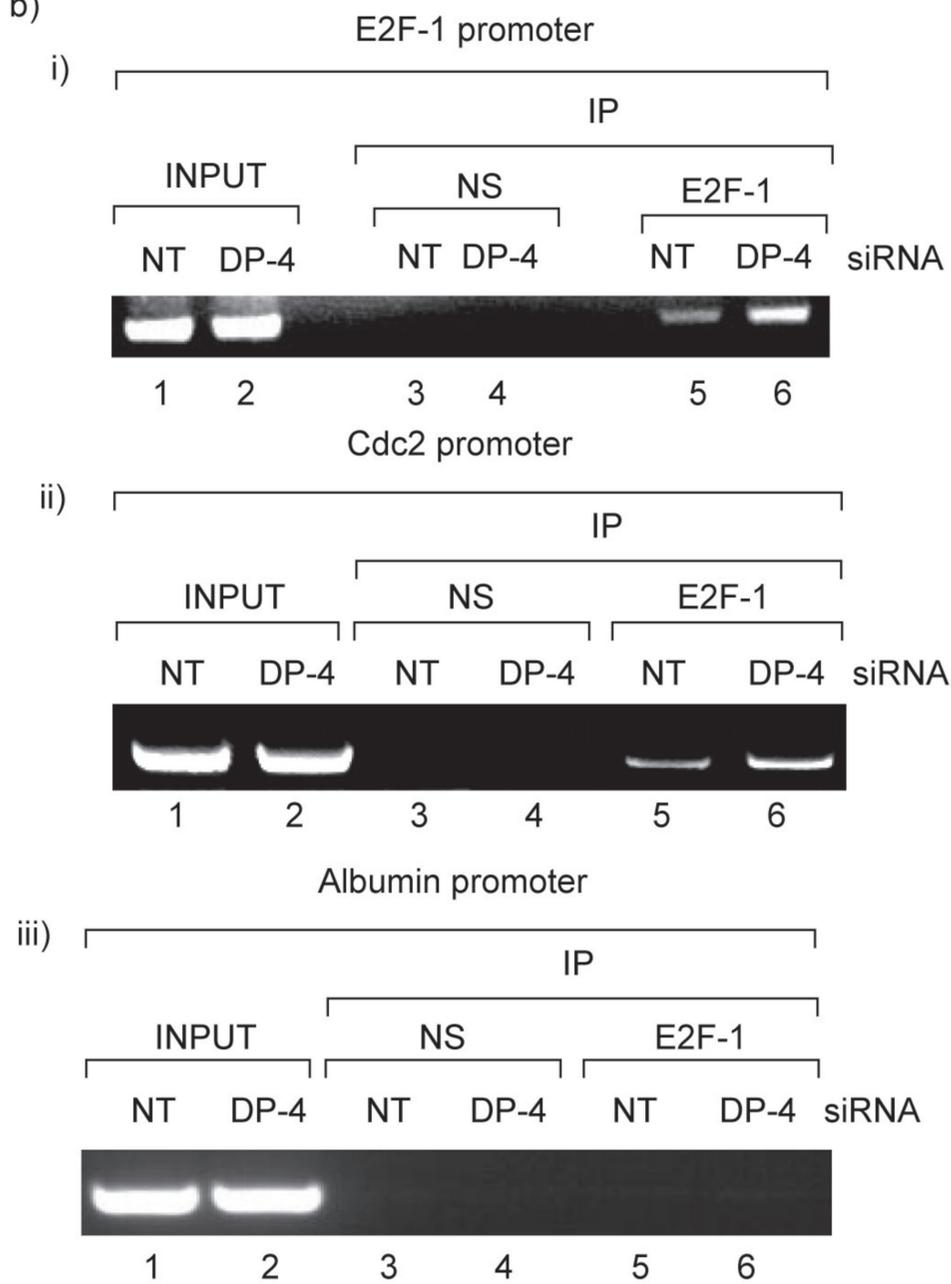
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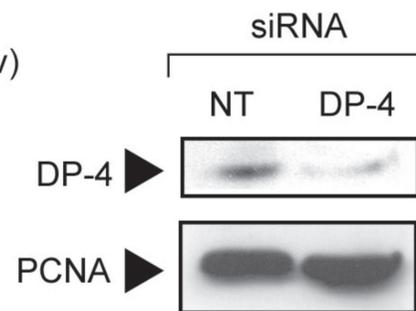
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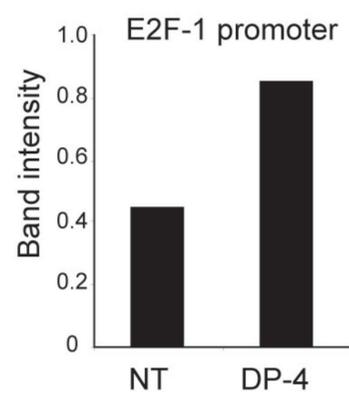
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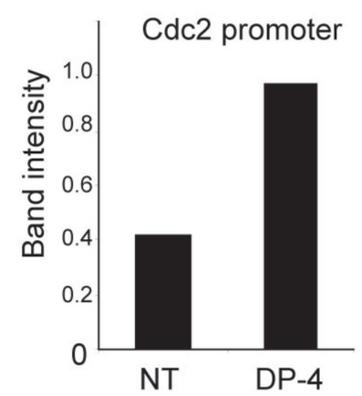
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c)

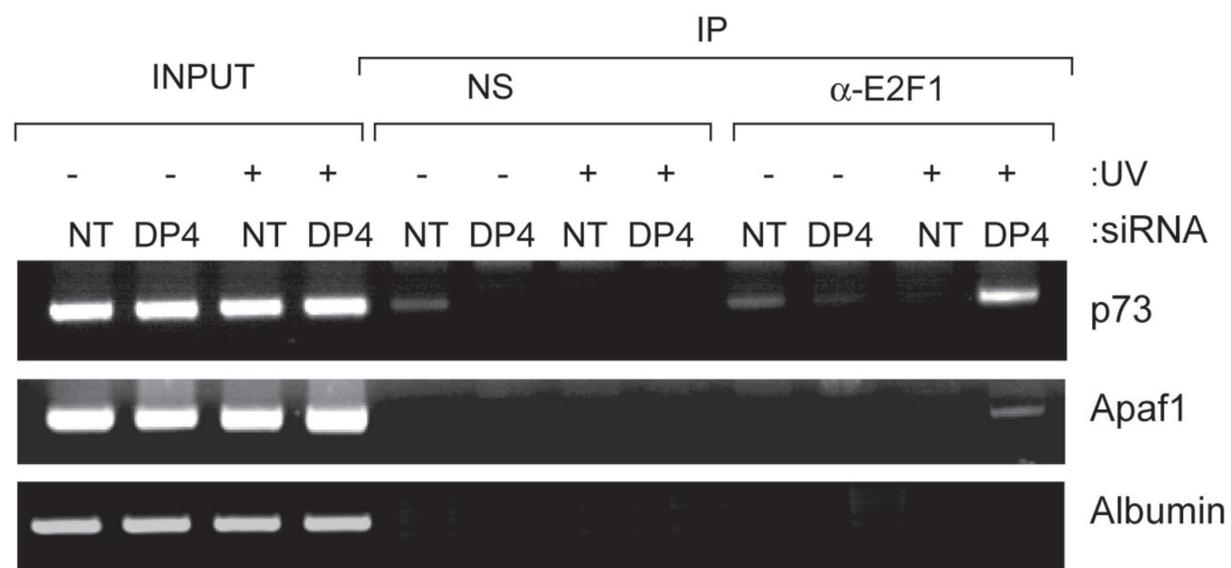


Figure 7

