Regulation of Cell Cycle by E2F1 in Primary Cells

Thesis

REGULATION OF CELL CYCLE BY E2F1

IN PRIMARY CELLS

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I wish to dedicate my thesis to my husband Giorgio, to my parents Rolando and Mariella and to my sister Chiara.

STATEMENT

The experimental work described in this thesis is composed of two related parts. The outcomes of the first session were published in M. Lomazzi et al. "Suppression of the p53- or pRB-mediated G1 checkpoint is required for E2F-induced S-phase entry", *Nature Genetics* 31 (2002) 190-194. All the experiments were performed by me with few exceptions: PMEFs were established by M.R. Jensen, Northern blots were performed by M.C. Moroni, and E. Frittoli provided technical support in micro-injections. I personally performed all the experiments of the second session with technical assistance in FACS analysis by I. Muradore and S. Ronzoni.

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ABSTRACT

Deregulation of the retinoblastoma protein (pRB) pathway is a hallmark of cancer, and in the absence of other genetic alterations, results in lack of differentiation, hyperproliferation and apoptosis. pRB acts as a transcriptional repressor by targeting the E2F transcription factors whose functions are required for S phase entry.

Increased E2F activity can induce S phase in quiescent cells and this fact is a central element of most models for the development of cancer. I provide evidence that E2F1 alone is not sufficient to induce S phase in diploid mouse and human fibroblasts. However, increased E2F1 activity can result in S phase entry in diploid fibroblasts in which the p53-mediated G1 checkpoint is suppressed. Furthermore, I show that E2F1 can induce S phase in primary mouse fibroblasts lacking pRB. These results demonstrate that in addition to working as an E2F-dependent transcriptional repressor, pRB is also required for retaining the G1 checkpoint in response to unprogrammed proliferative signals.

The role of E2F in cell proliferation is not completely understood because it is not known if the E2Fs mainly function as transcriptional repressors or

activators. E2Fs need dimerisation with a DP protein to give rise to functional E2F activity and to regulate promoters containing E2F binding sites. I inactivated endogenous DP in tissue culture by RNA interference providing evidence that loss of DP1 abrogates E2F DNA binding activity. DP is required for tumour and normal cell growth. In addition, the expression of E2F target genes is severely impaired. These results define a crucial role for DP1 in cell proliferation.

INTRODUCTION

THE E2F FAMILY OF TRANSCRIPTION FACTORS

Eight human genes have been identified as components of the E2F transcriptional activity in mammals (Dyson, 1998). On the basis of sequence homology and functional properties, these genes have been divided into two distinct groups: the *E2Fs* (*E2F1– E2F6*) and the *DPs* (*DP1* and *DP2*). Their protein products have highly conserved DNA-binding domains and dimerization domains. The carboxy-terminal portion of E2F1-5 contains a potent transactivation domain but no equivalent activity has been found in E2F6 or in DP proteins (Figure A).

E2F and DP proteins heterodimerize to give rise to functional E2F activity and to regulate promoters containing E2F binding sites. All possible combinations of E2F–DP complexes exist *in vivo*: Chromatin-Immunoprecipitation (ChIP) assays have failed to detect any specificity for the association of individual E2F–DP complexes to various known E2F-responsive promoters (Takahashi et al., 2000). However, the individual E2F–DP species invoke very different transcriptional responses depending on the identity of the E2F moiety and the proteins that are associated with the complex.

On the basis of transcriptional properties, the E2F family can be divided into

three distinct subgroups. E2F1, E2F2 and E2F3 are potent transcriptional activators. By contrast, E2F4 and E2F5 seem to be primarily involved in the active repression of E2F responsive genes by recruiting the pocket proteins and their associated histone-modifying enzymes. Finally, E2F6 acts as a transcriptional repressor, but in a pocket-protein-independent manner.

Much less is known about the roles of the DP1 and DP2 *in vivo*. DP1 is ubiquitously expressed at high levels in tissues and in cell lines. DP2 is 68% identical to DP1 and is expressed at low levels with alternative splicing in a restricted set of tissues and cell lines (Wu et al., 1995). When overexpressed with various E2F partners and pRB family members, DP1 and DP2 function in the same way in *in vitro* assays, such as those for heterodimerization, DNA binding and transactivation.

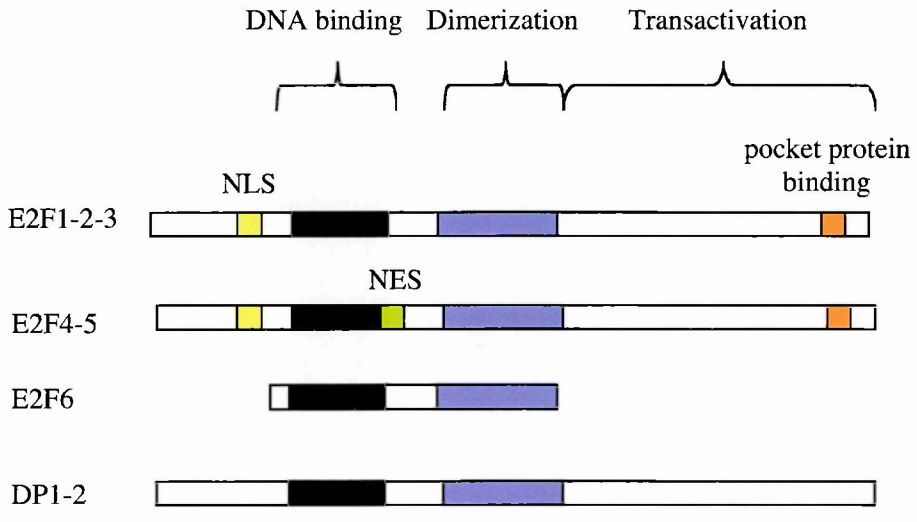


Figure A - The E2F family of transcription factors.

The 'activating' E2Fs

The founding member of this subclass, *E2F1*, was cloned by virtue of its ability to interact with pRB (Helin et al., 1992; Kaelin et al., 1992). E2F1 binds to DNA in a DP-dependent manner, and the resulting complex is a potent transcriptional activator of E2F-responsive promoters (Bandara et al., 1993; Helin et al., 1993b; Krek et al., 1993). E2F2 and E2F3 are highly homologous to E2F1 in the domains that are responsible for DNA binding, DP dimerization and pRB binding (Figure A) and they show similar transactivation properties. The E2F1, E2F2 and E2F3 expression is regulated by cell growth, with maximal accumulation at the G1/S boundary. They associate exclusively with pRB and play a positive role in cell cycle progression.

The *E2f3* locus expresses two distinct transcripts (Leone et al., 2000). The longer transcript encodes the original *E2f3* species, designated *E2f3a*. The second transcript, named *E2f3b*, is transcribed from a previously unrecognized promoter in the first intron of *E2f3a*, and its protein product is identical to E2f3a except that it lacks the amino-terminal domain (Leone et al., 2000). E2F3b is not regulated by cell growth and can be found in both quiescent and proliferating cells, but its properties have yet to be described.

E2F1, E2F2 and E2F3 are potent transcriptional activators of E2F responsive genes (Helin et al., 1992; Lees et al., 1993). Overexpression of any of these

proteins alone or in combination with DPs is sufficient to induce immortalized quiescent cells to re-enter the cell cycle (Johnson et al., 1993; Lukas et al., 1996; Qin et al., 1994). This requires functional DNA-binding and transcriptional activity. Some evidences indicate that endogenous E2F1, E2F2 and E2F3 control cellular proliferation. Microinjection of anti-E2F3 antibodies causes decreased S-phase entry in REF52 cells (Leone et al., 1998). *E2f3*-deficient mouse embryonic fibroblasts (MEFs) are defective in the mitogen-induced activation of many E2F-responsive genes and this reduces the rate of proliferation of both primary and transformed cells (Humbert et al., 2000b). Finally, the combined inactivation of *E2f1*, *E2f2* and *E2f3* blocks cellular proliferation (Wu et al., 2001) suggesting that the activating E2Fs could have overlapping roles in the induction of cell-cycle entry.

E2F1, E2F2 and E2F3 could also contribute to the repression of E2F-responsive genes by recruiting pRB. However, overexpression assays and mutant mouse models indicate that the key role of these three E2Fs is the activation of genes that are essential for cellular proliferation and the induction of apoptosis.

To delineate the functional roles within the E2F family, mice deficient in individual *E2f* genes have been generated. *E2f1*-/- mice are viable and fertile, but they have various tissue-specific abnormalities due to defects in apoptosis (Field et al., 1996; Yamasaki et al., 1998; Yamasaki et al., 1996): for instance,

they have an excess of T cells and develop testicular atrophy between 9 and 12 months of age. Most surprisingly, the *E2f1* deficient mice are tumour-prone and develop a broad spectrum of tumours (lymphoma, lung adenocarcinoma, uterine sarcoma) at an age between 8 and 18 months. However, loss of *E2f1* can reduce the pituary and thyroid tumorigenesis in *Rb*+/- mice (Yamasaki et al., 1998) and can also reduce the nervous system and erythropoietic defects in the *Rb*-/- embryos (Tsai et al., 1998). Inactivation of *E2f2* results in viable adults that, when crossed to *E2f1* deficient mice, are highly tumour prone with deep effects on hematopoietic cell proliferation and differentiation (Zhu et al., 2001). By contrast, a significant proportion of the *E2f3*–/– mice die *in utero*, and most of the adult survivors die prematurely of congestive heart failure without obvious tumour predisposition (Humbert et al., 2000b). Whereas mice null for E2f1 and E2f2 are viable, mice null for E2f1 and E2f3 or E2f2 and E2f3 die early during embryonic development pointing at a central role for E2f3 in mouse development (Cloud et al., 2002; Wu et al., 2001).

The 'repressive' E2Fs

The second subclass of the E2F family includes E2F4 and E2F5. These E2Fs were originally identified and cloned by virtue of their association with p107 and p130 (Hijmans et al., 1995; Vairo et al., 1995). Their sequences diverge considerably from those of the activating E2Fs (Figure A). E2F4 and E2F5 lack most of the sequence that is amino-terminal to the DNA-binding domain and are regulated differently from the activating E2Fs *in vivo*. First, they are

not transcriptionally regulated in cell growth: whereas E2F1, E2F2 and E2F3 are primarily restricted to actively dividing cells, significant levels of E2F4 and E2F5 are detected both in quiescent (G0) and proliferating cells (Ikeda et al., 1996; Moberg et al., 1996). Second, the E2F subgroups bind to different pocket proteins *in vivo*. The activating E2Fs are specifically regulated by pRB, E2F5 is mainly regulated by p130, and E2F4 associates with each of the pocket proteins at different points in the cell cycle. As E2F4 is expressed at higher levels than the other E2F family members, it accounts for at least half of the pRB-, p107- and p130-associated E2F activity *in vivo*. Because of the accumulation of E2F4 or E2F5 complexes in quiescent cells, together with the fact that many E2F target genes are subject to E2F-dependent repression in quiescent cells, these complexes have been suggested to function mainly as repressor.

In contrast to the activating E2Fs, E2F4 and E2F5 are poor transcriptional activators in overexpression assays, and they cannot drive quiescent cells to re-enter the cell cycle (Müller et al., 1997; Verona et al., 1997). The differential activity of the two E2F subgroups results from differences in their sub-cellular localization: E2F1, E2F2 and E2F3 are constitutively nuclear, whereas E2F4 and E2F5 can be found both in the nucleus and in the cytoplasm of quiescent cells, but relocate almost entirely to the cytoplasm once cells reach S phase. The nuclear localization of the activating E2Fs depends on a canonical basic nuclear localization signal (NLS) in their amino-

terminal domain. On the other hand, some experiments suggest that E2F4 and E2F5 localize to the nucleus because of their interaction with pRB or p130 (Verona et al., 1997). E2F4 has two leucine/isoleucine-rich hydrophobic nuclear export signals (NES) and its cytoplasmic localization is dependent on the nuclear export factor CRM1 (Gaubatz et al., 2001).

In G0/G1 cells, E2F4 and E2F5 account for most of the nuclear E2F complexes. As these complexes associate with HDACs *in vivo* (Iavarone and Massague, 1999), they are thought to be crucial for mediating the transcriptional repression of E2F responsive genes. MEFs mutant for *E2f4*, *E2f4* and *E2f5*, or *p107* and *p130* have defects in their ability to exit the cell cycle in response to various growth-arrest signals, including p16 overexpression and contact inhibition (Gaubatz et al., 2000). This correlates with the inappropriate expression of a subset of E2F-responsive genes (Hurford et al., 1997). However, these mutant cells can all respond appropriately to growth-stimulatory signals and there is no detectable change in their proliferative capacity.

E2F4 and E2F5 also play a role in the regulation of differentiation. Overexpression of E2F4 is sufficient to trigger the differentiation of neuronal precursors. Moreover, the developmental defects in the *E2f4* and *E2f5* mutant mouse strains result from lack of differentiation of various cell lineages. Loss of *E2f4* leads to neonatal death with abnormal hematopoiesis and intestinal defects (Humbert et al., 2000a), while the newborn *E2f5*-/- mice die for

abnormal development and function of choroid plexus, where E2F5 is highly expressed (Lindeman et al., 1998). Finally, the simultaneous inactivation of *E2f4* and *E2f5* in mice results in neonatal lethality (Gaubatz et al., 2000).

A third group of the E2F family is defined by E2F6, the most recently identified member. Residues that are crucial for the DNA-binding and dimerization activities of the other E2Fs are conserved in E2F6, but it lacks the carboxy-terminal sequences, required for both pocket-protein binding and transactivation (Figure A).

Overexpression studies have demonstrated that E2F6 represses E2F-responsive genes (Cartwright et al., 1998; Gaubatz et al., 1998). It can behave as a dominant negative inhibitor through competition with other E2F family members (Trimarchi et al., 1998). A complex that contains E2F6, polycomb proteins (PcG) and chromatin modifiers has been shown to occupy target promoters in G0 (Ogawa et al., 2002). Thus, it was suggested that one function of E2F6 is to inactivate E2F-dependent genes in quiescent cells. E2F6 associates with many PcG proteins *in vivo*, including RYBP, Bmi1, MEL-18, Mph1 and Ring1 (Trimarchi et al., 2001). PcG proteins form large multimeric complexes needed to maintain stable transcriptional repression of *Hox* genes that are expressed along the antero-posterior axis in the vertebrate embryo. In addition to this function, PcG proteins also display other activities, for example Bmi1 is a critical regulator of proliferation, senescence and apoptosis (Jacobs et al., 1999).

E2f6 KO mice are viable and healthy, however they appear to be defective in spermatogenesis and, similarly to PcG mutant mice, they display homeotic transformations of their axial skeleton.

E2F AND CELL PROLIFERATION

Progression through cell-cycle phases is controlled by the sequential activation of the cyclin-dependent kinases CDK4/6, CDK2 and CDC2. Their activity is regulated by various mechanisms, including the synthesis and binding of a specific regulatory subunit (cyclin), both inhibitory and activating phosphorylation events, and the association/dissociation of inhibitory molecules called CDK inhibitors (CDIs). There are two families of CDIs: p16INK4a, p15INK4b, p18INK4c, p19INK4d belong to the INK4a family; the CIP/KIP family includes p21, p27, p57 (Sherr and Weber, 2000) (Figure B).

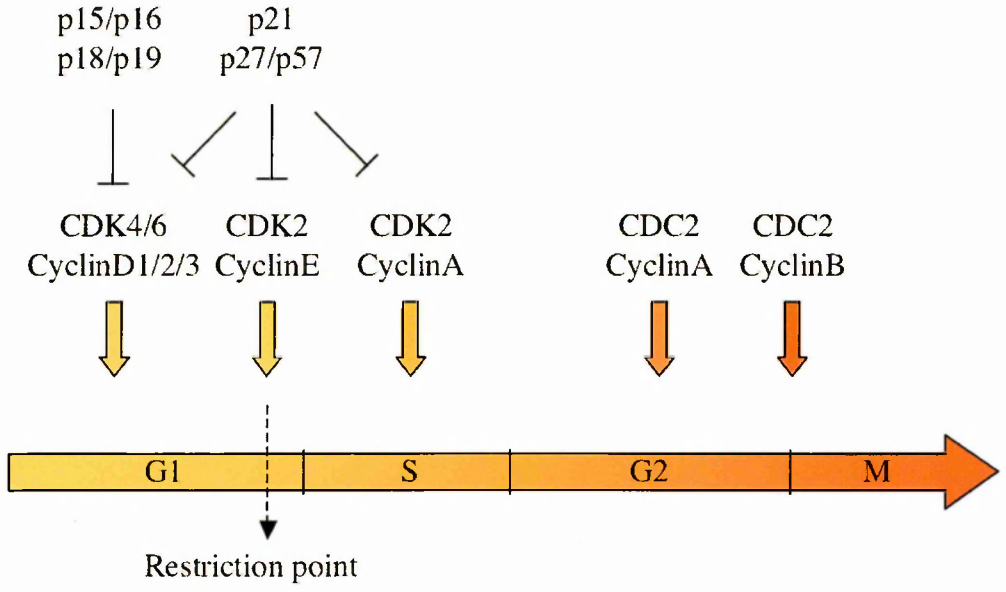


Figure B - The mammalian cell cycle.

In mammalian cells, proliferation control is mainly achieved in the G1 phase of the cell cycle. After G1, cells become independent of extracellular signals and progress through the cell cycle to the next G1. The D-type G1 cyclins, together with their associated kinases, CDK4 and CDK6, initiate the phosphorylation of pRB family members, inactivating their capacity to interact with the E2F transcription factors. This phosphorylation allows the accumulation of E2F1, E2F2, and E2F3 that activate the transcription of a large number of genes (Dyson, 1998; Harbour and Dean, 2000; Nevins, 1998). These include cell-cycle regulators, such as cyclin E, cyclin A, CDC2, CDC25, Myc, B-Myb, and products that are required for DNA replication, such as large subunit of DNA polymerase α, ribonucleotide reductase, proliferating nuclear antigen (PCNA) and minichromosome maintenance proteins (MCMs) (Helin, 1998). In addition, phosphorylation of pRB and p130 disrupts complexes with E2F4 and E2F5 found in quiescent cells that function as transcriptional repressors of S phase genes as well as the genes encoding the E2F1, E2F2, and E2F3 proteins. E2F activation of cyclin E/CDK2 kinase activity leads to the further phosphorylation and inactivation of pRB, thus enhancing E2F activity and increasing the accumulation of cyclin E/CDK2.

One of the most striking properties of E2F proteins is their ability to drive cells into S phase. This is central to most models of E2F function and was first shown for E2F1 (Johnson et al., 1993). E2F1 overexpression overrides many

different types of cell cycle arrest (including the effects of p16, p21, p27, γ-irradiation, TGFβ and dominant negative CDK2) and is able to drive immortalized quiescent cells into S phase (DeGregori et al., 1995a; Johnson et al., 1993; Lukas et al., 1996; Mann and Jones, 1996; Schwarz et al., 1995). The proportion of cells in G_1 is increased by the overexpression of dominant negative mutants of E2F1, DP1 and DP2 (Wu et al., 1996) or by the expression of competitor RNA molecules (Ishizaki et al., 1996). Microinjection of antibodies to E2F3 reduces the percentage of REF52 cells entering S phase (Leone et al., 1998) and E2*f*3 deficient MEFs have low levels of proliferation and deregulation in the expression of E2F responsive genes (Humbert et al., 2000b). Finally, the combined mutation of E2*f*1, E2*f*2 and E2*f*3 blocks cellular proliferation (Wu et al., 2001). Instead, E2F4 and E2F5 are fully dispensable for cellular proliferation (Humbert et al., 2000a; Lindeman et al., 1998; Rempel et al., 2000).

A further indication comes from the *Drosophila* genome that encodes just two E2F genes, *de2f1* and *de2f2*. dE2F1 is a potent activator of transcription: loss of *de2f1* results in the reduced expression of E2F-regulated genes (Frolov et al., 2001) and in low levels of DNA synthesis (Duronio et al., 1995). In contrast, dE2F2 represses the transcription of E2F reporters and the loss of *de2f2* function results in increased gene expression. In the absence of both proteins, larval cell proliferation is relatively normal.

E2F AND THE pRB PATHWAY

A conserved domain near the carboxyl terminus of the E2F proteins mediates binding to pRB-family members (Helin et al., 1993a). This binding domain is embedded in the transactivation domain of the E2F subunit.

The retinoblastoma gene encodes a 928–amino acid phosphoprotein. pRB contains several functional domains. Domains A and B interact with each other along an extended interdomain interface to form the central "pocket" which is critical to the tumour suppressor function of pRB (Qin et al., 1992). Viral oncoproteins and a number of endogenous pRB-binding proteins contain an LXCXE motif that allows them to bind pRB (Lee et al., 1998). The binding site for LXCXE is in domain B. Domain A allows domain B to assume an active conformation. E2Fs do not contain an LXCXE and thus bind pRB at a distinct site with points of contact in both the pocket and in the carboxy-terminal region. This allows E2F to recruit to a promoter the complexes containing pRB and other proteins, such as those with the LXCXE motif. Another functional domain of pRB is located within the carboxy-terminal region. This region contains binding sites for the c-Abl tyrosine kinase and MDM2, which appear to be distinct from the E2F site in the carboxy-terminal region (Xiao et al., 1995).

pRB is phosphorylated and dephosphorylated during the cell cycle: the hyperphosphorylated (inactive) form predominates in proliferating cells,

whereas the hypophosphorylated (active) form is generally more abundant in quiescent or differentiating cells (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1992). Cell cycle progression normally occurs when pRB is inactivated by phosphorylation catalyzed by CDKs in complex with their cyclin partners. Three different cyclin/CDK complexes have been suggested to phosphorylate pRB during the cell cycle. Cyclin D–CDK4/6 phosphorylates pRB early in G1, cyclin E–CDK2 phosphorylates the protein near the end of G1, and cyclin A–CDK2 may maintain phosphorylation of pRB during S phase (Sherr, 1996).

pRB regulates E2F-responsive genes through two distinct mechanisms. First, pRB binds to an 18-amino-acid motif within the transactivation domain of E2F, thereby blocking the ability of E2F to recruit the transcriptional machinery (Helin et al., 1993a). Second, the pRB–E2F complex retains its ability to bind to the promoters of E2F responsive genes and can enlist chromatin remodeling enzymes and lead to transcriptional repression (Zhang and Dean, 2001). These factors include histone deacetylase enzymes (HDACs) which remove acetyl groups from the tails of core histones in the nucleosome and the ATP-dependent remodeling complex SWI/SNF (the human SWI/SNF ATPases are BRG1 and hBRM) (Harbour and Dean, 2000). pRB–E2F complexes can also recruit the histone methyltransferase SUV39H1 creating a high-affinity binding site for the heterochromatin protein 1 (HP1) on E2F-responsive promoters (Nielsen et al., 2001) (Figure C).

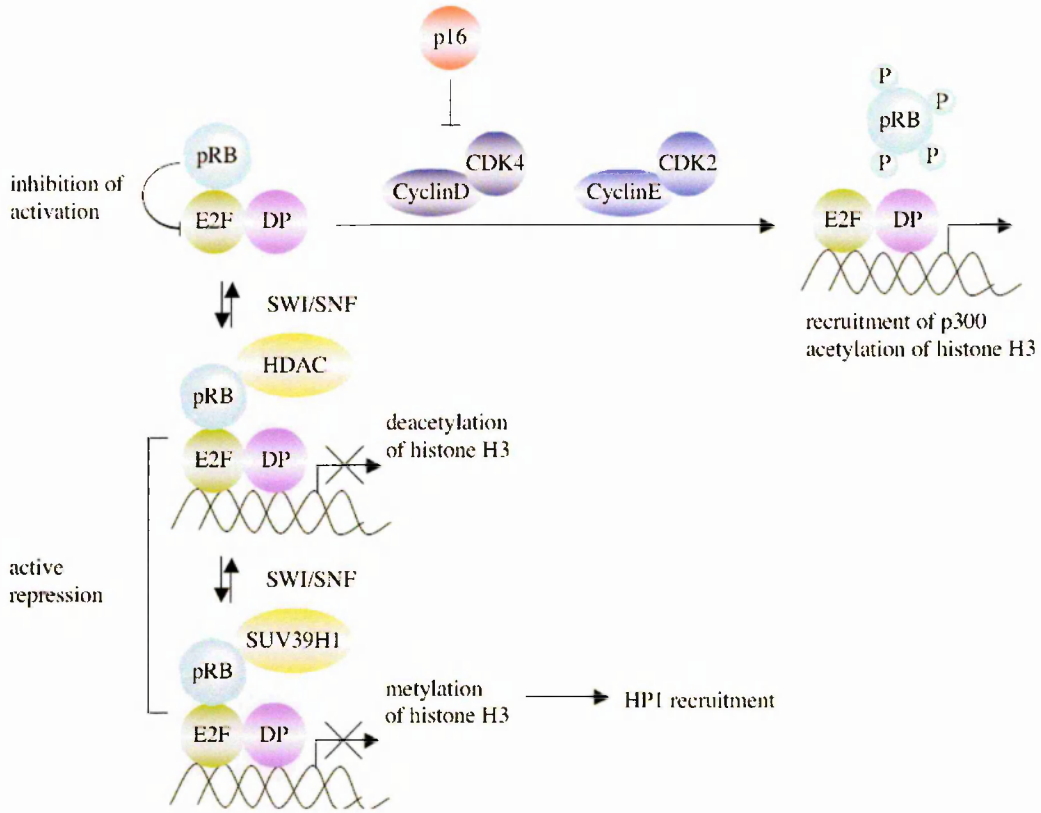


Figure C – pRB controls E2F activity.

Deregulation of the p16INK4a/CDK4/cyclin D/pRB pathway is a prerequisite for oncogenesis. Although mutations in pRB and its upstream regulators (Figure C) are frequently found in human tumours, intragenic mutations in the genes encoding the E2F and DP transcription factors have not been isolated (Bartek et al., 1996; Weinberg, 1995). One reason for this may be that mutations in the pRB pathway are epistatic to E2F1 mutations. Indeed, most tumour-derived pRB mutants show a defect in their ability to regulate E2F function. However, low penetrance alleles of *pRB* have been described which seldom lead to tumour development, despite loss of E2F binding function. Conversely, N-terminal mutants of pRB with preserved E2F binding capability are unable to fully rescue pRB deficiency in mice and give rise to

human tumours, again with low penetrance (Riley et al., 1997). Therefore, loss of pRB function and gain of E2F function do not have equivalent consequences.

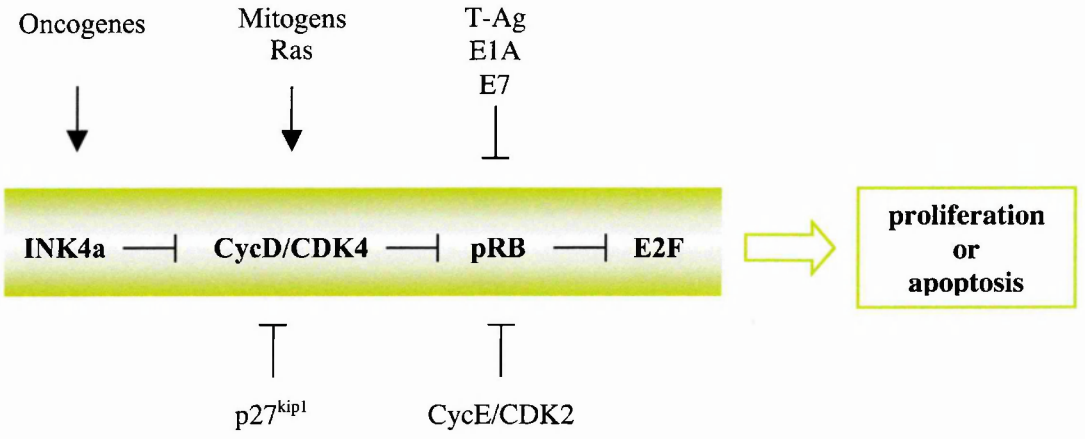


Figure D - The pRB pathway.

Two other pocket proteins, p107 and p130, are homologous with pRB within the pocket, and they also bind viral oncoproteins and E2F. All three pocket proteins interact with histone deacetylases (HDACs) *in vivo*, and can therefore both inhibit transcriptional activation and mediate active repression of E2F responsive genes. They all arrest cells in G1 when overexpressed. The pocket proteins have also unique properties. First, they bind to different members of the E2F family *in vivo*. pRB can bind E2F1–4, whereas p107 and p130 bind to E2F4 and E2F5 (Nevins, 1998). Second, these associations occur at distinct stages of the cell cycle: whereas p130/E2F complexes are found mainly in quiescent or differentiated cells (p130/E2F4 is the most abundant complex in G0), pRB binds to E2F in both quiescent and actively dividing cells, and p107

is mostly associated with E2F during S phase, but can also be found in G1.

The rare incidence of *p107* and/or *p130* mutations in human tumours indicates that p107 and p130, unlike pRB, do not function as tumour suppressors. Mutant mice models have revealed dramatic differences in the biological roles of the pocket proteins (Mulligan and Jacks, 1998).

Rb-deficient embryos die at midgestation with inefficient erythropoiesis as well as abnormal cell cycle entry and cell death in the liver, lens and nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). These defects can be partially rescued through combined mutation of *E2f1*, suggesting that increased E2F activity is responsible for many of the effects of *Rb* deficiency in embryogenesis (Tsai et al., 1998). However recent studies have observed that *Rb*-/- mice show dramatic defects in the labyrinth layer of the placenta, characterized by marked hyperplasia of trophoblast cells and severe dysplasia of the labyrinth architecture, associated with a decrease in placental transport function (Wu et al., 2003). When supplied with a normal placenta, either via tetraploid aggregation or by genetic approaches, *Rb*-/- embryos are able to survive to full term, suggesting that an abnormal placenta is the primary cause for the embryonic lethality of *Rb*-/- animals. Like in Rb knockout embryos, rescued animals show a marked increase in DNA replication and cell division in the CNS. In sharp contrast, the typical widespread neuronal apoptosis is absent in Rb-deficient embryos reconstituted with a normal placenta. In lens fiber cells, however, the inappropriate proliferation and apoptosis that is

normally observed in *Rb*-/- embryos persists (de Bruin et al., 2003b). Rescued animals died at birth with severe skeletal muscle defects.

Rb+/- mice and chimeric animals made with *Rb*-/- ES cells develop pituitary and thyroid tumours but not retinoblastoma or any of the tumours commonly associated with *RB* mutation in humans. In contrast, mice that lack *p107* or *p130* are viable and tumour free at an age of two years. Analysis of double-mutant mice has provided evidence for overlapping roles of the three family members in mouse development and cell cycle control. *Rb*-/-, *p107*-/- and *Rb*-/-, *p130*-/- embryos die earlier during mouse development than *Rb*-/- embryos, with more pronounced cell cycle defects and increased cell death (Lee et al., 1996). However differences in the genetic background of mice have been shown to be important determinants of the developmental consequences of the genetic loss of *p107* and *p130*. On a mixed 129/Sv x C57BL/6 genetic background, *p130*-/-;*p107*-/- mice die just after birth with defects in bone formation and abnormalities in chondrocyte proliferation (Cobrinik et al., 1996). Mice with disruptions in *p107* and *p130* in a BALB/c background have more severe phenotypes (LeCouter et al., 1998a; LeCouter et al., 1998b).

The effect of pRB family mutations has also been examined in mouse embryonic fibroblasts (MEFs) in culture. *Rb*-/- and *p107*-/-; *p130*-/- fibroblasts (Hurford et al., 1997) each have mild defects in cell cycle regulation and show differences in the inappropriate expression of cell cycle regulated genes. *pRb*-deficient MEFs prematurely express both *cyclin E* and *p107*, whereas the combined mutation of *p107* and *p130* causes the inappropriate activation of

the *DHFR*, *B-myb*, *cdc2*, *E2f1*, *TS*, *RRM2* and *cyclin A2* genes during G0/G1 (Mulligan, 1998). In growth-limiting conditions, *Rb*-/- MEFs enter S phase, suggesting that expression of p107 and p130 are not sufficient substitutes for pRb in the arrest of G1 and in the repression of E2F target genes (Almasan et al., 1995). Yet, combined loss of pocket proteins immortalizes MEFs and abolishes G1 arrest after γ-irradiation, contact inhibition or serum starvation, demonstrating that they have some overlapping function *in vivo* (Dannenberg et al., 2000; Peeper et al., 2001; Sage et al., 2000).

E2F AND THE p53 PATHWAY

p53 is mutated in more than 50% of human cancers and accumulates in response to cellular stress from DNA damage, hypoxia and oncogene activation. When stabilized and activated, p53 starts a transcriptional programme that can either arrest the cell cycle allowing the repair of damaged DNA or commit cell to death (Vogelstein et al., 2000).

For example, p53 levels and activity increase after DNA damage (Figure E), in part as a result of *de novo* phosphorylation and conformational changes. Phosphorylation at serine 15 prevents the interaction of p53 with MDM2, which mediates p53 export from the nucleus and targets it for ubiquitin-mediated proteasome degradation. MDM2 is, in turn, negatively regulated by ARF (Sherr and Weber, 2000).

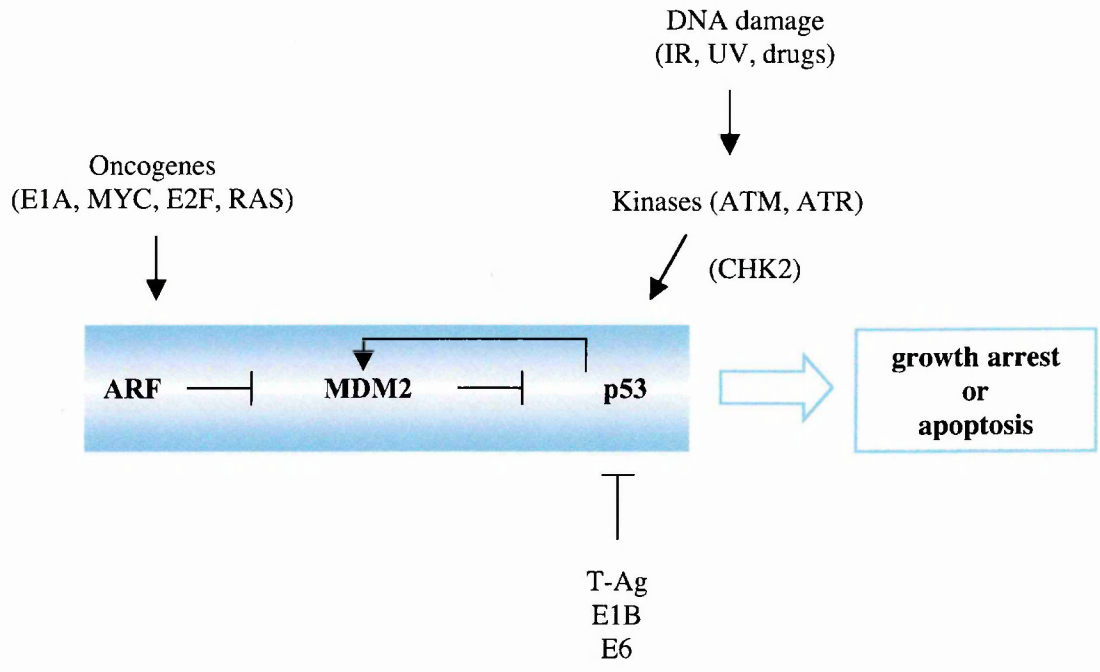


Figure E – The p53 pathway.

Oncogenes can also induce p53 (Figure E), leading to increased apoptosis or premature senescence (Serrano et al., 1997). The adenovirus E1A oncoprotein induces p53 and promotes apoptosis in primary cells, which is reflected by the remarkable ability of E1A to enhance radio- and chemo-sensitivity (Lowe et al., 1993). Although E1A is a mitogenic oncogene, p53 acts to limit its oncogenic potential: p53-deficient primary fibroblasts expressing E1A are resistant to apoptosis and become oncogenically transformed (Lowe et al., 1994). The ability of E1A to activate p53 is not unique, as c-Myc activates p53 to promote apoptosis and oncogenic RAS induces p53, leading to premature senescence (Serrano et al., 1997).

Like p53, ARF is a potent tumour suppressor. As mentioned above, ARF is induced by oncogenes (de Stanchina et al., 1998; DeGregori et al., 1997;

Palmero et al., 1998). This results in p53 activation and commits cells that have sustained oncogenic damage to either growth arrest or apoptosis. ARF provides an important connection between E2F1 and p53. Its expression is slightly elevated in *Rb-/-* cells (de Stanchina et al., 1998), consistent with the possibility that *ARF* is an E2F responsive gene (DeGregori et al., 1997). Indeed, enforced expression of E2F1 induces ARF and conversely, *Arf*-null cells are resistant to E2F1 induced apoptosis (Bates et al., 1998). The *ARF* transcript derives from the same genomic locus as the *p16INK4a* transcript. Even though they share sequences in exons 2 and 3, exon 1 is different and causes translation in different reading frames. Consequently, p16INK4a and ARF are unrelated at the protein level (Sherr, 1998). Nevertheless, they both can mediate cell cycle arrest. While p16-induced cell cycle arrest is dependent on functional pRB, ARF-mediated cell cycle arrest depends on functional p53 (Kamijo et al., 1997). Several observations suggest that ARF may function in a genetic and biochemical pathway that involves p53. The consequences of deleting *p53* and *Arf* are remarkably similar. In either case, the mutant mouse develops normally, but is highly predisposed to malignant tumours of a similar overall pattern and latency. MEFs null for *Arf* or *p53* do not undergo replicative senescence and can be transformed by RAS alone in the absence of an immortalizing oncogene (Harvey and Levine, 1991; Kamijo et al., 1997). Established MEF cell lines that lacked *Arf* preserved p53 function, whereas those that retained *Arf* had sustained p53 mutations. Cells lacking a functional *p53* gene are resistant to ARF induced cell cycle arrest, impling that p53 acts

downstream of ARF (Kamijo et al., 1997). Nevertheless, ARF is not the only activator upstream of p53: cells lacking ARF have an intact p53 checkpoint in response to UV and ionizing radiation. Indeed, p53 is induced upon DNA damage via the ATM and ATR protein kinases, directly or indirectly through the CHK2 kinase (Hirao et al., 2000). The phosphorylation of p53 by ATM/ATR then blocks the ability of MDM2 to target p53 destruction. E2F1 might also be involved in the DNA-damage-response pathway. Treatment of cells with chemotherapeutic agents increases E2F1 protein levels. The induction of E2F1 in response to DNA damage similarly involves the ATM/ATR kinases (Lin et al., 2001), which phosphorylate and stabilize E2F1, inhibiting its degradation. The specificity of ATM and ATR for E2F1, rather than other E2F proteins, reflects a unique phosphorylation site within the N-terminal domain of E2F1. The upregulation of E2F1 in response to DNA damage likely provides a synergistic activation of p53 through the induction of *ARF* or contributes to p53-independent apoptosis, possibly via p73.

DEREGULATED ACTIVITY OF E2F

Oncogenic activity of E2F

The first indications that E2F1 has oncogenic potential come from classical oncogene cooperation studies in vitro. E2F1 cooperates with activated RAS in soft agar assays and the transformed cells produce tumours in nude mice. This effect is more pronounced in cells expressing a pRB-binding deficient E2F1/VP16 chimera that retains transactivation activity (Johnson et al., 1994)

or a point mutant of E2F1 specifically defective in pRB binding (Shan et al., 1996). This suggests that pRB counteracts the oncogenic effect of E2F1. The expression of E2F1-2-3 alone is sufficient to transform NIH3T3 (Xu et al., 1995). Targeted expression of E2F1 in transgenic mice has demonstrated that E2F1 overexpression promotes tumorigenesis in vivo (Pierce et al., 1998). Finally, tumours phenotypes resulting from the inactivation of pRB are impaired when the mice are backcrossed into an *E2f1*-/- background (Tsai et al., 1998; Yamasaki et al., 1996), suggesting that tumour growth depend on the E2F1 that is released when pRB function is blocked. Free E2F1 may become essential for tumour cells by providing them with a proliferative advantage when growth factors are limiting.

Surprisingly, *E2f1* knockout mice develop a broad spectrum of tumours such as lymphomas, lung adenocarcinomas and tumours of the reproductive tract (Yamasaki et al., 1996), suggesting that E2F1 behaves also as a tumour suppressor.

E2F-induced apoptosis

In addition to inducing proliferation, de-regulated E2F activity can trigger apoptosis (Bates et al., 1998; Hsieh et al., 1997; Phillips et al., 1997; Qin et al., 1994; Shan and Lee, 1994). The E2F1-induced apoptosis is potentiated by the presence of wild-type p53. However, both overexpression experiments and mutant mouse models indicate that death can be induced through either p53-dependent or p53-independent mechanisms (Phillips et al., 1997; Phillips et

al., 1999). E2F triggers p53-dependent apoptosis through the transcriptional activation of *ARF*, a known E2F-target gene (Bates et al., 1998; DeGregori et al., 1997). However, studies of mutant mouse models suggest that E2F can induce p53-dependent apoptosis in both embryonic tissues and epithelial brain tumours in the absence of ARF (Tolbert et al., 2002). In addition, ectopic expression of ARF results in cell cycle arrest rather then apoptosis (Sherr, 1998). So alternative mechanisms must exist besides ARF for the p53-dependent apoptosis.

It is widely believed that loss of pRB results in apoptosis as a consequence of higher E2F activity. Rb-deficient mice die in midgestation with widespread apoptosis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), whereas embryos that are mutants for both *Rb* and *E2f1* show a significant reduction in apoptosis and down-regulation of the p53 pathway (Tsai et al., 1998). This suggests that the E2f1 resulting from loss of pRb function mediates most of the p53-dependent apoptosis (Bates et al., 1998), and it could explain why E2F1 overexpression alone is not sufficient for tumorigenesis. A direct link between E2F-induced apoptosis and the apoptosome has been shown. The expression of *APAF1* is regulated by E2F and *APAF1* is required for E2F-induced apoptosis (Moroni et al., 2001). In combination with cytosolic cytochrome c and the caspase 9 protease, APAF1 forms the apoptosome and activates the downstream caspase proteases.

E2Fs can trigger p53-independent apoptosis, but unlike p53-dependent apoptosis, it does not require E2F transactivation and can be triggered by expression of the E2F DNA-binding domain alone (Hsieh et al., 1997; Phillips et al., 1997). This occurs in functionally *RB* negative cells, so the role of the E2F DNA-binding domain may be to displace free E2F rather than pRB-E2F repressor complexes from promoters. At least two distinct pathways have been proposed for the induction of p53-independent apoptosis. These include transcriptional activation of the p53 family member, *p73* (Irwin et al., 2000; Lissy et al., 2000) and a non-transcriptional mechanism that involves inhibition of the tumour-necrosis factor receptor (TNFR)-associated survival response (Phillips et al., 1999). Both p53 dependent and independent apoptosis has been observed *in vivo*. In the central nervous system (CNS) of *Rb-/-* mice apoptosis is p53-dependent, since cells in the CNS of *Rb-/-*, *p53-/-* embryos continue to ectopically enter S-phase, but do not die. The apoptosis that occurs in the peripheral nervous system (PNS) is p53-independent (Macleod et al., 1996).

Previous studies have shown that E2F1 is somewhat unique among the E2F family members in its ability to trigger apoptosis (DeGregori et al., 1997). More recent studies suggest that apoptosis can be triggered by ectopic expression of E2F1, E2F2, or E2F3 (Vigo et al., 1999) and that nuclear localization and DNA binding are required for the apoptotic activity of the E2Fs (Loughran and LaThangue, 2000). Expression of E2F4 at elevated levels induces growth arrest and caspase-dependent apoptosis through a mechanism

distinct from E2F1 (Chang et al., 2000). Mutant mouse embryos that lack *pRb*, and either *E2f1* or *E2f3*, show a significant reduction in the levels of apoptosis, as well as the number of ectopic S-phase cells relative to those seen in mice lacking only *pRb* (Tsai et al., 1998; Ziebold et al., 2001). Restoration of pRb function in extra-embryonic lineages (Wu et al., 2003) is sufficient to rescue many of the embryonic defects of *pRb* knockout fetuses, suggesting that inactivation of E2F1 or E2F3 from *Rb-/-* placental may be sufficient to rescue early lethality.

TRANSCRIPTIONAL ACTIVITY OF E2F

E2F target genes

A role for E2F in the activation of several G1/S transition, S phase and DNA replication genes has been well established. Typical targets include those encoding cell cycle regulators that trigger S-phase entry (such as cyclin E, c-Myb, and CDK2), products involved in the assembly of the pre-replication complex at origins of replication (such as ORC proteins, MCMs and CDC6), and enzymes needed for the direct synthesis of DNA (ribonucleotide reductase, thymidine synthase and DNA polymerase α). (Helin, 1998; Nevins, 1998).

E2F regulates the expression of several genes with mitotic functions. For example, *cyclin B1* and *B2*, *Bub1* and *cdc2*, genes involved in the progression through M-phase, and *RanBMP*, a gene required for centrosome duplication (Ishida et al., 2001; Müller et al., 2001). In the survey of promoters that co-

immunoprecipitate with E2F1 or E2F4 (Ren et al., 2002) were found not only *cdc2* and *cdc25a*, but also promoter fragments for genes with a variety of M-phase functions, including *smc2* and *smc4* (chromosome condensation), *bub3* and *mad2* (spindle checkpoints), *centromere protein* and *securin* (chromosome segregation). Some of these genes are known to induce aberrant spindle behavior when overexpressed, and, potentially, misexpression of these targets may contribute to the chromosomal instability observed in transformed cells.

Continual E2F activity during S phase allows the maintenance of high levels of cyclinA-CDK2 that are responsible for the inactivation of the anaphase promoting complex (APC). APC is a ubiquitin ligase that targets cyclin B1 for degradation. By keeping the APC inactive, E2F allows the accumulation of cyclin B1 at the end of S phase that is required for the progression of mitosis (Lukas et al., 1999) .

Recent studies suggest that E2F1 has a physiological role in DNA-damage responses. Upon DNA damage, E2F1 is directly phosphorylated and stabilized by the ataxia-telangiectasia protein (ATM), a key player of the cellular response to DNA damage (Lin et al., 2001).

E2F1 is known to downregulate the expression of anti-apoptotic factors (Phillips et al., 1999), and several pro-apoptotic genes have been proposed to be induced by E2F1, including *Apaf-1*, *Caspase 3* and *Caspase 7* (Müller et al., 2001). E2F1 expression leads to stabilization of p53, an effect that was thought to be mediated by transcriptional upregulation of $p14/p19^{ARF}$. Mutation in the $p19^{ARF}$, however, failed to suppress the neuronal apoptosis

phenotype of mouse *pRb* mutant embryos (Tsai et al., 2002), indicating that other connections to p53 must also exist. E2F proteins are present at the promoters of *chk1* (Ren et al., 2002), which encodes a kinase that is activate by ATM and required for the cellular response to DNA damage, and *p53*. Other groups found that the transcription of p73, a p53 homologue, is E2F-inducible, and showed that the levels of p73 can influence rates of E2F1-induced apoptosis (Irwin et al., 2000; Lissy et al., 2000; Stiewe and Putzer, 2000).

Xenopus and *Drosophila* E2F activity are required for axis determination in early development (Duronio et al., 1995; Suzuki and Hemmati-Brivanlou, 2000). It has been suggested that the E2Fs regulate axis determination through homeobox-containing proteins in *Xenopus* (Suzuki and Hemmati-Brivanlou, 2000) and an EGF-receptor ligand, Gurken in *Drosophila* (Myster et al., 2000). In mammals, the E2Fs regulate the expression of several proteins that are involved in early development, including homeobox proteins, transcription factors involved in cell fate decision, a number of proteins that determine homeotic gene transcription, and signaling pathways such as the TGFβ and Wnt pathways that are essential for early development. Several PcG genes were identified, like Enhancer of Zeste 2 (*EZH2*), Embryonic Ectoderm Development protein (*EED*) and Homolog of Polyhomeotic (*EDR2/HPH2*) (Müller et al., 2001).

The pRB/E2F pathway is known to be central in the regulation of various types of cellular differentiation (Lipinski and Jacks, 1999). For example, pRB

is required for erythroid, neuronal, eye, muscle, and adipocyte differentiaton (Lipinski and Jacks, 1999). Both p107 and p130 are required for normal endochondrial bone development (Cobrinik et al., 1996). In addition, E2F4 is known to contribute to hematopoetic lineage and to craniofacial development (Humbert et al., 2000a; Rempel et al., 2000), whereas loss of E2f5 leads to overproduction of cerebrospinal fluid and to hydrocephalus (Lindeman et al., 1998). In my laboratory (Müller et al., 2001) a number of transcription factors that are involved in cell fate decisions, such as Hairy/enhancer of split related (*HEY1*), Paired-like homeodomain (*PTX1*), ID4, MAF family members, and Sox9 were found. In addition, E2F activation led to a dramatic change in the expression of genes in the TGFβ pathway.

In summary, the E2F-regulated genes code for proteins whose activity control cell cycle progression, proliferation, apoptosis, differentiation, and development.

E2F-mediated repression

The analysis of E2F-responsive genes shows that E2F–DP–pocket-protein complexes are involved in the repression of E2F target genes (Hateboer et al., 1998; Iavarone and Massague, 1999; Johnson et al., 1994). Mutation of the E2F-binding sites within known E2F-responsive promoters (*B-Myb*, *CDC2*, *E2F1*, *cyclin E*, *CDC25A*, *CDC6* and *ORC1*) leads to increased transcription after serum starvation or treatment with TGFβ. In some cases, deacetylase activity is required for repression of transcription. *In vivo* footprinting studies

with the *B-Myb*, *cyclin A* and *CDC2* promoters detected E2F site occupancy in quiescent cells only in the repressed state (Zwicker and Muller, 1997), while the promoters are unoccupied during the G1/S transition when the genes are actively transcribed. These data have led to the model that E2F can participate in repression of transcription by tethering pocket proteins to E2F target promoters, which in turn recruit chromatin remodeling factors including histone deacetylases (HDACs), members of the ATP-dependent chromatin remodeling complex SWI/SNF, DNA methyltransferase 1 (DMNT1) and the histone-methyltransferase SUV39H1 (Figure C). For a number of genes, the replacement of endogenous E2F by dominant negative E2F1 leads to activation of transcription, meaning that the endogenous E2F complexes normally repress the expression of the gene. Overexpression of this dominant negative form of E2F1 (which can bind to DNA, but cannot transactivate or bind to pocket proteins) compromises the ability of cells to arrest in G1 in response to p16INK4a, TGFβ and contact inhibition (Zhang et al., 1999). In another study the same E2F mutant results in immortalization, bypasses RASV12 induced senescence and rescues ARF- and p53- induced cell cycle arrest (Rowland et al., 2002). This has been interpreted as a result of transcriptional derepression of E2F target genes, whose downregulation is critical for the establishment of G1 arrest.

E2F-mediated transactivation

A number of experiments support the view that E2F is a transcriptional activator. E2F proteins activate transcription of simple reporter constructs with multiple E2F-binding sites (Helin et al., 1992; Shan et al., 1992), they contain conserved domains that activate transcription when transferred to other DNA binding domains (Kaelin et al., 1992) and there is a strong correlation between the ability of E2F to activate transcription and to drive cell cycle progression (Johnson et al., 1993; Qin et al., 1995; Shan and Lee, 1994). Viral oncoproteins target the pocket proteins to release free, transcriptionally active E2F rather than to displace E2F repressor complexes. *In vivo* footprinting studies have detected E2F site occupancy in phases of the cell cycle when pocket proteins are largely inactivated (Hateboer et al., 1998). Finally, E2F-DNA binding activity is downregulated in S phase when DP is phosphorilated by cyclin A-CDK2 and E2Fs are degraded (Krek et al., 1995). The loss of E2F-DNA binding activity correlates with decreased transcriptional activity of a number of E2F target genes at this point of the cell cycle, such as *cyclin E.*

How E2F activates transcription is not known. At least three different mechanisms have been suggested. *In vitro*, E2F1 can bind to TBP (TATA binding protein) (Hagemeier et al., 1993). Biochemical studies show that the transcriptional activation domain of E2F1 can interact with CBP (CREB binding protein), potentially recruiting histone acetylase activity (HAT) to the promoter (Trouche et al., 1996) and the transcriptional activity of E2F1 is potentiated by the overexpression of CBP. Alternatively, the ability of E2F

complexes to bend DNA may be important for transcriptional activation (Cress and Nevins, 1996).

MATERIALS AND METHODS

CLONING TECNIQUES

Agarose gel electrophoresis. DNA samples were loaded on 1% agarose gels along with DNA markers. Gels were made in TAE (Tris-acetate-EDTA) or TBE (Tris-borate-EDTA) buffer containing 0.3 μg/ml ethidium bromide and run at 80V until desired separation was achieved. DNA bands were visualized under a UV lamp.

Minipreps. Cells picked from individual transformed colonies were used to inoculate 2 ml 2xLB (containing ampicillin at 25 μg/ml) and grown overnight at 37°C. 1ml of cells was taken from each tube and pelleted for 4 min at 14000 rpm, resuspended in 100 μl cold solution 1 (50 mM Glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8), vortexed and incubated at room temperature for 5 min. 200 μl of solution 2 (0.2 N NaOH, 1% SDS) was added and the tubes incubated on ice for 5 min. Following the addition of 150 μl 3M potassium acetate, pH 4.8 (solution 3) the tubes were incubated on ice for a further 5 min and then centrifuged at 14000 rpm for 5 min. The supernatants were retained and 400 μl of a 1:1 mix of phenol: chloroform was added to each. After vortexing, the mixtures were separated by centrifugation at 14000 rpm for 2 min. The aqueous layers were removed to fresh tubes and the DNA was ethanol precipitated. Pellets were washed in 70% ethanol and then dried under

vacuum. The DNA was resuspended in 5 μl TE which contained 0.1 μg/ml RNase A (Boehringer Mannheim).

Diagnostic DNA restriction. Between 1-3 μg DNA was digested for 2 hours at 37°C with 10 units of restriction enzyme (New England Biolabs). For digestion, the volume was made up to 20 μl with the appropriate buffer and ddH_2O.

Large scale plasmid preps. Cells containing transfected DNA were expanded into 500 ml cultures overnight. Plasmid DNA was isolated from these cells using the Qiagen Maxi-prep kit according to the manufacturer's instructions.

Transformation of competent cells. Fresh competent cells (Invitrogen) were thawed on ice prior to the addition of 1-2 μl of plasmid DNA in 50 μl of cells. Either water or cut plasmid was included in one transformation as a negative control to determine transformation efficiency. Cells were incubated with DNA on ice for 30 min and then subjected to a heat shock for 1 min at 42°C. The cells were then returned to ice for 2 min and then at 37°C for further 30 min before plating onto ampicillin plates. Two plates for each reaction were used, one treated with 5 μl of the transformed bacterial cells and the remainder plated on the other. Plates were incubated overnight at 37°C.

Site directed mutagenesis. Site directed mutagenesis was performed using the Quick Change mutagenesis kit (Stratagene), following manufacturer's instructions. Briefly, a sense and an antisense oligo of about 30 nucleotides each, carrying the desired mutation, were generated and used in a PCR reaction using the wild type construct (20 ng). PCR was performed using the Turbo Pfu polymerase, to reduce the chance of introducing unwanted mutations. After amplification, 1 µl of DpnI restriction enzyme, which selectively cuts methylated DNA at the GATC sequence, was added to digest the wild type construct. After one-hour incubation at 37°C, the PCR product was used to transform competent *Escherichia Coli* cells and single colonies were sequenced for the presence of the desired mutation and the absence of other, unwanted, base changes. For the generation of the pCMVDP1 and pBabeMYDP1 point mutants the following oligos were synthesized: 5'-GAA TGG CAA GGG CTT ACG GCA TTT CTC-3' as forward primer and 5'-GAG AAA TGC CGT AAG CCC TTG CCA TTC-3' as reverse primer. This results in a silent mutation of DP1 in the target sequence for the siRNA. For the amplification step, 16 PCR cycles were performed with a denaturation step of 30" at 95°C followed by an annealing step of 1' at 55°C and an extension step of 20' at 68°C.

PLASMIDS

pCMVE2F1, pCMVE132, pCMVE2F1(1-374), pCMVE1A12S and pBabePuroHAER-E2F1 were described previously (Fattaey et al., 1993; Helin

et al., 1993a; Helin et al., 1993b; Vigo et al., 1999). I generated pBabeHygro2HA-BMI1 by subcloning the NotI/XhoI fragment of the pMT2HA-BMI1 (a gift of M. van Lohuizen) into pBabehygro2. L. Laimins provided pCB6-E6, and S. Polo provided pCMVMDM2. pCMVDP1 was described in (Helin et al., 1993b). pRetroSUPER-DP1 was generated by annealing of forward primer (5'GATCCCC**TGGCAAGGGCCTACGGCA** TTTCAAGAGA**ATGCCGTAGGCCCTTGCCA**TTTTTGGAAA3') and reverse primer (5'AGCTTTTCCAAAAA**TGGCAAGGGCCTACGGCATT** CTCTTGAA**ATGCCGTAGGCCCTTGCCA**GGG3'). In bold is the sequence of siRNA for DP1 respectively in the sense and anti-sense orientation. The annealed oligos were ligated into pRetroSUPER vector (Brummelkamp et al., 2002a; Brummelkamp et al., 2002b).

PRIMARY MOUSE EMBRYONIC FIBROBLASTS

C. Sherr kindly provided $Arf^{+/-}$ mice (Kamijo et al., 1997). T. Jacks kindly provided $Rb1^{+/-}$ (Jacks et al., 1992), $Trp53^{+/-}$ (Jacks et al., 1994) and $Cdkn1a^{+/-}$ (Brugarolas et al., 1995) mice. All mice were of a mixed C57BL/6-129/Sv genetic background. For preparation of primary mouse embryo fibroblasts (PMEF) we set up matings between heterozygous parents. We considered the morning a vaginal plug was observed as d E0.5. PMEFs were established from 12.5 d embryos. Embryos were harvested, the brain and internal organs were removed and the carcasses were minced and incubated with trypsin for 30-45 min at 37°C. Tissue culture media was added to the cell suspension and the

cells were further disaggregated. Genotyping was done by PCR. We considered plating after disaggregation of embryos as passage 1. PMEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and incubated in a humidified chamber at 37°C, 9% CO_2.

CELL CULTURE

We maintained WI38 human fibroblasts in DMEM supplemented with 10% North American FBS, penicillin/streptomycin and glutamine, in a humidified chamber at 37°C, 5% CO_2. We generated ER-E2F1 pools by infecting early passage (6-7) WI38 cells with retroviruses produced in Phoenix cells transfected with pBabePuroHAER-E2F1 (Vigo et al., 1999). To induce activation of the ER-E2F1 fusion protein, we treated cells for 24 h with 4-hydroxytamoxifen (OHT, 600 nM) after 72 h of starvation in DMEM without serum.

We maintained NIH3T3 in DMEM supplemented with 10% Calf Serum Colorado, penicillin/streptomycin and glutamine, at 37°C, 5% CO_2. We generated ER-E2F1 and ER-E2F1(132) expressing NIH3T3 cells in the same way as WI38 cells. NIH3T3 were starved in 0,1% serum for 24 hours.

HeLa, U2OS and SAOS2 cells were cultured in DMEM supplemented with 10% South American FBS, VA13 and IMR90 fibroblasts in 10% North American FBS.

TRANSFECTIONS

For transfection using the calcium phosphate procedure, 10-15 μg DNA was diluted in 439 μl of ddH_2O, 61 μl of 2 M $CaCl_2$ were added and the solution was added, drop-wise, to 500 μl of 2XHBS. After 15 min incubation, the precipitate was added to cells plated on 10-cm-dishes and removed after 7 h. Transfections using the Lipofectamine (Gibco BRL) method or Fugene method were performed following manufacturer's instructions.

RETROVIRAL INFECTIONS

Retroviruses were produced by transfecting the Phoenix helper cell line (plated at a density of 2 million cells per 10-cm-diameter dish two days before) with 10 μg of DNA. Supernatants were collected 48 h after transfection, filtered (0.45 μm), and used to infect WI38 cells. The viral supernatant was left on the cells for 3 h, and the procedure was repeated twice to increase the efficiency of infection. Two days after infection, the target cell cultures were split and puromycine-resistant cells were selected in medium supplemented with 1 μg/ml of puromycine for 4 d. For the experiments presented in Fig. 6*c*, we infected ER-E2F1 expressing WI38 cells with pBabeHygro2HA-BMI1 and we selected with 100 μg/ml hygromycin B for 10 days.

SMALL INTERFERING RNA (siRNA)

From a given cDNA sequence we selected a targeted region 5'-AA(N21) (Elbashir et al., 2001) with approximately 50% G/C-content beginning 100 nt downstream of the start codon to avoid that regulatory proteins and translation initiation complexes could interfere with binding of the siRNP. The selected siRNA sequences were blasted (NCBI database) against human EST libraries to ensure that only a single gene was targeted. siRNA duplexes were prepared by annealing two pairs of 21-ribonucleotides synthesized by Dharmacon Research in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C, followed by 1 h at 37°C. Tumour cells were transfected with siRNA duplexes using OLIGOFECTAMINE (Invitrogen). For a well of a 12-well plate, we mixed 3 µl 20 µM siRNA duplex (0.84 µg, 60 pmol) with 50 µl OPTI-MEM 1. In a separate tube, we added 3 µl OLIGOFECTAMINE to 12 µl OPTI-MEM 1 and we incubated for 7-10 min at room temperature. The two solutions were combined, mixed gently by inversion and incubated for 20-25 min at room temperature to allow for formation of liposome complexes. Then we added 32 µl fresh OPTI-MEM 1 to obtain a final volume of 100 µl. The liposome complexes were added to cultured cells (50% confluent) seeded the previous day in 500 µl of DMEM supplemented with 10% serum without antibiotics. The plate was incubated for 1-2-3 days at 37°C. If necessary, multiple rounds of transfection were performed.

Diploid fibroblasts were transfected using LIPOFECTAMINE 2000 (Invitrogen). For a well of a 12-well plate, we mixed 3 µl 20 µM siRNA duplex with 50 µl OPTI-MEM 1. In a separate tube, we added 1,5 µl OLIGOFECTAMINE to 48,5 µl OPTI-MEM 1 and we incubated for 5 min at room temperature. The two solutions were combined, mixed gently by inversion and incubated for 20 min at room temperature to allow for formation of liposome complexes. The liposome complexes (100 µl) were added to cultured cells (80% confluent) seeded the previous day in 500 µl of DMEM supplemented with 10% serum without antibiotics.

Immunofluorescence or Western blotting was performed to analyse the depletion of the target protein. When no antibodies were available, the level of the targeted mRNA was monitored by RT/PCR to control for the specificity of the knockdown. As control we transfected cultures with a siRNA duplex targeting firefly luciferase (GL2) or buffer, both of which had no detectable effect on cell growth or morphology. The human targeted sequences (cDNA) were: for DP1 (oligo1: 5'-AATGGCAAGGGCCTACGGCATTT-3', oligo2: 5'-AAGCAGCTCTTGCCAAAAACC-3'), for DP2 (5'-AAA TCC CTG GTG CCA AAG GCT TT-3').

MICROINJECTION EXPERIMENTS

We plated early passage (3-5) PMEFs of the indicated genotypes on 0.5% gelatine coated glass coverslips and made them quiescent by cultivation in

medium containing 0.05% serum for 48-72 h. At the time of microinjection, the cells had reached 60 to 80% confluence. We observed similar levels of S phase induction in wild type PMEFs prepared from littermates of p53, p19ARF, p21 and pRb deficient embryos. We prepared PMEFs and tested them from at least two independent litters. We observed no significant differences between the various litters. We cultured sub-confluent WI38 cells and when specified they were starved in serum free medium for 72 h. We injected cells with 50 ng/µl of expression plasmids (unless otherwise specified) together with 2 µg/µl rabbit IgG (Jackson Laboratories) directly into cell nuclei using a Zeiss automatic injection system. We added BrdU (100 µM) 4 h after injection and fixed cells 20 h after the addition of BrdU. For WI38ER-E2F1, we added 600 nM OHT 6 h after injection, and BrdU 2 h later. We fixed cells 16 h after the addition of BrdU. For each experiment, between 100 and 150 injected cells were counted. The experiments were repeated at least three times.

IMMUNOFLUORESCENCE

Cells grown on coverslips (pre-incubated with 0.5% gelatine at 37°C for 30 min) were fixed in PIPES buffer (PIPES 400 mM pH 6.8, EGTA 500 mM pH 8, $MgCl_2$ 1M) containing 4% paraformaldehyde for 10 min, washed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO_4, pH adjusted to 7.4 with KCl) and permeated with 0.1% Triton X-100, 10% goat serum in PBS. To detect the injection marker (rabbit IgG), cells were incubated in

blocking buffer (10% goat serum in PBS) containing FITC-conjugated donkey anti-rabbit antibody (Jackson Laboratories). After washing in PBS, cells were fixed again in 4% paraformaldehyde. BrdU incorporation was detected by incubation in blocking buffer containing anti-BrdU antibody (Beckton Dickinson BD347580), 3 mM $MgCl_2$ and 100 U/ml DNase I (Roche). Cells were washed extensively before incubation with Cy3-conjugated donkey anti-mouse IgG (Jackson Laboratories). Nuclei were counterstained with DAPI. Some coverslips were stained with antibodies specific for human p14ARF (14PO2, NeoMarkers), anti-p53 (DO-1, Santa Cruz), anti-p21 (CP74, kind gift of E. Harlow) or anti-E2F1 (KH20 or KH95 (Helin et al., 1993b)). Cy3-conjugated donkey anti-mouse IgG (Amersham) was used as secondary antibody.

FLOW CYTOMETRY

At the indicated times, 10^6 cells per sample were trypsinized, combined with any floating cells, pelleted, washed with PBS, repelleted and resuspended in PBS. The cells were fixed in cold ethanol (70%, final concentration) and stored for at least 30 min at 4°C. The fixed cells were centrifuged, washed twice with PBS-BSA 1%, and resuspended in 0.5 ml of PBS containing propidium iodide (50 µg/ml) and RNase A (6.25 µg/ml). Samples were incubated for 3h at room temperature or overnight at 4°C prior to analysis by flow cytometry with a Becton Dickinson FACScan.

For BrdU FACS, $3X10^6$ cells were pulsed for 20 min in medium containing 33 μM BrdU, trypsinized and fixed as above. Cell pellet was incubated in 1 ml of denaturating solution (2 M HCl) for 20 min at room temperature. 2ml of 0,1 M Sodium Borate pH 8,5 was added and cells were incubated for 2 min at room temperature. After two washes in PBS 1% BSA, the pellet was resuspended in 50 μl anti BrdU (Beckton Dickinson BD347580) diluited 1:5 (1 hour incubation at RT), and then in anti-mouse FITC (Sigma) diluited 1:50. Finally PI (2,5 μg/ml overnight at 4 C) was added.

WESTERN BLOTTING

Cells were collected in RIPA lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Sodium Deoxicolate, 0.1% SDS, proteases and phosphatases inhibitors). After clearing of the lysates by centrifugation, the protein content was determined (Biorad Protein Assay). Equal amounts of proteins were separated on a sodium dodecyl sulfate polyacrylamide gel using an appropriate acrylamide concentration (stock 40%, 30:1 mix of acrylamide:bisacrylamide) to resolve the molecular weight of the targeted proteins.

Running gel mix:	**6%**	**8%**	**10%**	**15%**
acrylamide mix (ml)	5	6	7.5	11.25
1.5M Tris pH8.8 (ml)	7.5	7.5	7.5	7.5
Distilled water (ml)	16.9	15.9	14.4	10.65
10% SDS (ml)	0.3	0.3	0.3	0.3
10% APS (ml)	0.3	0.3	0.3	0.3
TEMED (ml)	0.03	0.03	0.03	0.03
TOTAL (ml)	30	30	30	30

Stacking gel mix:	acrylamide mix	1.7
	1M Tris pH6.8 (ml)	1.25
	Distilled water (ml)	6.8
	10% SDS (ml)	0.1
	10% APS (ml)	0.1
	TEMED (ml)	0.01
	TOTAL (ml)	10

Gel running buffer:	Tris-base (pH 8.3)	25 mM
	Glycine	192 mM
	SDS	0.1%

Proteins were transferred onto nitrocellulose membrane and processed for Western Blotting in transfer buffer (20% methanol, 192 mM glicine, 25 mM Tris-base) at 100V for 1 h. We incubated the membrane in 5% milk powder in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2% Tween-20) for 1 h at RT. The blots were probed with the following antibodies: mouse monoclonal anti-DP1 (TFD10), anti-vinculin (Sigma), anti-actin α (Sigma), anti-pRB (PharMingen); rabbit polyclonal anti-CDK2 (Santa Cruz, sc-163). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, the signal was revealed using the ECL (Enhanced Chemiluminescence) method (Amersham).

CDK2 KINASE ASSAY

Infected cultures were lysed by resuspension in lysis buffer (50 mM HEPES pH 7.5, 20 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 0.1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 10 μg/ml of leupeptin, 5 μg/ml of aprotinin, 10 mM β-glycerophosphate) for 30 min at 4°C and cleared by centrifugation at 14.000 rpm for 5 min at 4°C.

Supernatants were assayed for protein concentration (Biorad Protein Assay). Protein samples of 0.2-0.5 mg were then precleared and immunoprecipitated for 2 h at 4°C with protein A-Sepharose beads (Amersham Pharmacia Biotech), precoated with saturating amounts of anti-CDK2 antibody (5 μg of sc-163 from Santa Cruz, 1 h of preincubation at 4°C). Immunoprecipitated proteins on beads were washed twice with 1 ml of lysis buffer and twice with 1 ml of wash buffer (50 mM HEPES pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, plus the protease inhibitors as described above). The beads were resuspended in 25 μl of kinase buffer (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin) containing 1.5 μg of histone H1 (Roche) as substrate, 20 μM ATP, and 10 μCi of [γ-^{32}P]ATP. After incubation for 30 min at 30°C, the samples were boiled in 5X Laemmli buffer, separated by SDS-PAGE 12%, and transferred to a nitrocellulose filter.

GEL RETARDATION ASSAY

Double-stranded oligonucleotides containing a wild type E2F DNA binding site were end labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase, purified on a 12% polyacrylammide gel and used as probe. The oligonucleotide E2F-sense (the E2F binding site is underlined) was 5'-ATTTAAGTTTCGCGCCCTTTCTCAA-3'. We performed gel retardation assays on whole cell extracts (Hepes 20 mM pH 7.5, NaCl 0.42 M, $MgCl_2$ 1.5 mM, EDTA 0.2 mM, PMSF 0.5 mM, DTT 0.5 mM, 25% glycerol) from

interfered HeLa cells by incubating 5-20 μg of cell extract with 1 μg of salmon sperm DNA (sonicated to 500 bp single and double stranded) and 5X gel shift buffer (Hepes 100 mM pH 7.6, $MgCl_2$ 5 mM, EGTA 0.5 mM, NaN_3 0.1%, KCl 200 mM, glycerol 50%) in a 12.5 μl total volume for 10 min at room temperature; 1 μl of ^{32}P-labelled oligonucleotide probe (0.1 ng/μl in TE, 20000 cpm) was then added and the mixture was incubated for a further 20 min. To control for binding specificity, a 100-fold excess of unlabelled oligonucleotide was added to the binding reaction. The DNA-protein complexes were separated on a 4% polyacrilammide gel containing 0.25X Tris-borate-EDTA buffer at 4°C. The gel was dried and autoradiography was performed.

NORTHERN BLOTTING

WI38-ERE2F1 infected cells were grown with or without 600 nM OHT and/or 10 μg/ml cycloheximide (CHX) to inhibit protein synthesis. Cells were harvested in guanidium thiocianate 4 M, sodium acetate 20 mM pH 5.2, Sarkosyl 0.5%, DTT 0.1 mM and lysed by passage through a 20-gauge needle eight times. RNA was isolated by CsCl ultracentrifugation method as described (Ausubel et al., 1988). Poly A+ RNA was isolated with the Oligotex reagents from Quiagen using a batch protocol as described by the manufacturer. 1-4 μg of poly A+ RNA were resolved by elettrophoresis on a 1% agarose gel containing 1.9% formaldehyde and 1X MOPS and they were transferred to a nylon membrane. We sequentially hybridised the blot with

^{32}P-labeled probes (obtained by random primer method) specific for *ARF, CCNE1, CDKN1A, or GAPDH.*

RT-PCR

Total RNA was isolated from cells using the Rneasy extraction kit (Quiagen) according to the manufacturer's instructions. After DNase treatment, 1 μg of RNA was used for cDNA synthesis using the Superscript II Reverse Transcriptase (GIBCO) following manufacture's instruction. PCR was performed in an ABI PRISM 7700 Sequence detection system on 10ng of cDNA, 0.5 μl of a 10 μM primers mix and 2X SYBR Green PCR Master Mix (Applied Biosystem) in a 25 μl volume. The reaction was performed at 50°C for 2 min, 95°C for 10 min, then 40 cycles at 95°C for 15 sec, 60°C for 1 min. We evaluated on agarose gel that the products were of the expected size. GAPDH was used as endogenous control. Quantification was expressed relative to the untreated control. The following sets of primers were designed using Primer Express Software:

gene	amplicon	forward primer	reverse primer
CDC6	111 bp	AGCACTGGATGTTTGCAGGAG	GGGAATCAGAGGCTCAGAAGG
CCNE1	75 bp	TGCAGAGCTGTTGGATCTCTGTG	GGCCGAAGCAGCAAGTATACC
RRM2	113 bp	TTCAGCACTGGGAATCCCTG	GGCTAAATCGCTCCACCAAG
DHFR	66 bp	GAGAACTCAAGGAACCTCCACAAG	AGTTTTAAGGCATCATCTAGACTTCTGG
MCM3	100 bp	TGGAGGGCATTGTCACTAAATG	AGAATAACGTCGCTCTATGGTCTTC
DP1	71 bp	ATTTCCCGGATCTGGTAACATG	TGAAGACCTTGAGTTCTCCGTTG
DP2	79 bp	AAAGAAATCAAGTGGATTGGCC	TCCGCCTCTGCTTCTCTATCTC
E2F1	47 bp	CATCCCTCACCACAGATCCC	AACAGCGGTTCTTGCTCCAG
TK	51 bp	GCCAAAGACACTCGCTACAGC	TGGTGTTCCGGTCATGTGTG
CDC25A	105 bp	TGGCATCTGTTTTGAATGGC	ACTGCACCCTTGATGTGGC
GAPD	87 bp	GCCTCAAGATCATCAGCAATGC	CCACGATACCAAAGTTGTCATGG

Table 1 - Primers used in quantitative PCR.

RESULTS - PART 1

AIM

One of the most striking properties of E2F proteins is their ability to drive cells into S phase. Short-term expression of E2F1, E2F2, or E2F3 is sufficient for the induction of DNA replication in immortalized quiescent rodent fibroblasts in the absence of growth factors (Dimri et al., 1994; Johnson et al., 1993; Lukas et al., 1996; Qin et al., 1994; Shan and Lee, 1994) and requires functional DNA binding and transcriptional activity. This is central to most models of E2F function and was first shown for E2F1 (Johnson et al., 1993). In immortalized cells, E2F1 overexpression overrides many different types of cell cycle arrest, including the effects of p16, p21, p27, γ-irradiation, TGFβ and dominant negative CDK2 (DeGregori et al., 1995b; Lukas et al., 1996; Mann and Jones, 1996; Schwarz et al., 1995). The proportion of cells in G_1 is increased by the overexpression of dominant negative mutants of E2F1, DP1 and DP2 (Wu et al., 1996) or by the expression of competitor RNA molecules (Ishizaki et al., 1996). Despite this, I observed that overexpression of E2F1 in diploid fibroblasts results in cell cycle arrest in G1 and apoptosis. Therefore, I decided to study the effects of inducible E2F1 activation in primary mouse embryo fibroblasts (PMEFs) and non-immortal human diploid fibroblasts (WI38), two well-defined cell types that have been widely used to study normal cell cycle control since they have not accumulated mutations.

RESULTS

E2F1 is not sufficient to induce S phase in diploid fibroblasts

I microinjected serum-starved PMEFs with expression vectors containing E2F1 or adenovirus E1A12S cDNAs driven from the strong cytomegalovirus promoter (Fig. 1*a*). The expression of E2F1 from this promoter has been reported to induce S phase in quiescent Rat1 fibroblasts (Lukas et al., 1996). In agreement with published results, I observed that E1A12S was sufficient to induce S phase in primary rodent cells (Quinlan et al., 1987; Zerler et al., 1987). This indicates that the cells can enter S phase and are not irreversibly blocked by serum starvation. However, the expression of E2F1 in PMEFs did not result in an increase in the number of cells entering S phase (Fig. 1*a*).

To investigate if the lack of S phase induction by E2F1 was specific for primary mouse fibroblasts, I tested whether E2F1 could induce S phase in human diploid fibroblasts. I microinjected serum-starved WI38 cells with E2F1 or E1A12S expression plasmids and I measured S phase entry. As shown in Fig. 1*b*, human diploid fibroblasts expressing E1A12S efficiently entered S phase, whereas cells expressing E2F1 were unable to do the same. These results indicate that E2F1 is not sufficient to induce S phase in diploid fibroblasts, and are in agreement with previous results showing that E2F1 cannot induce S phase in WI38 cells (Dimri et al., 1994).

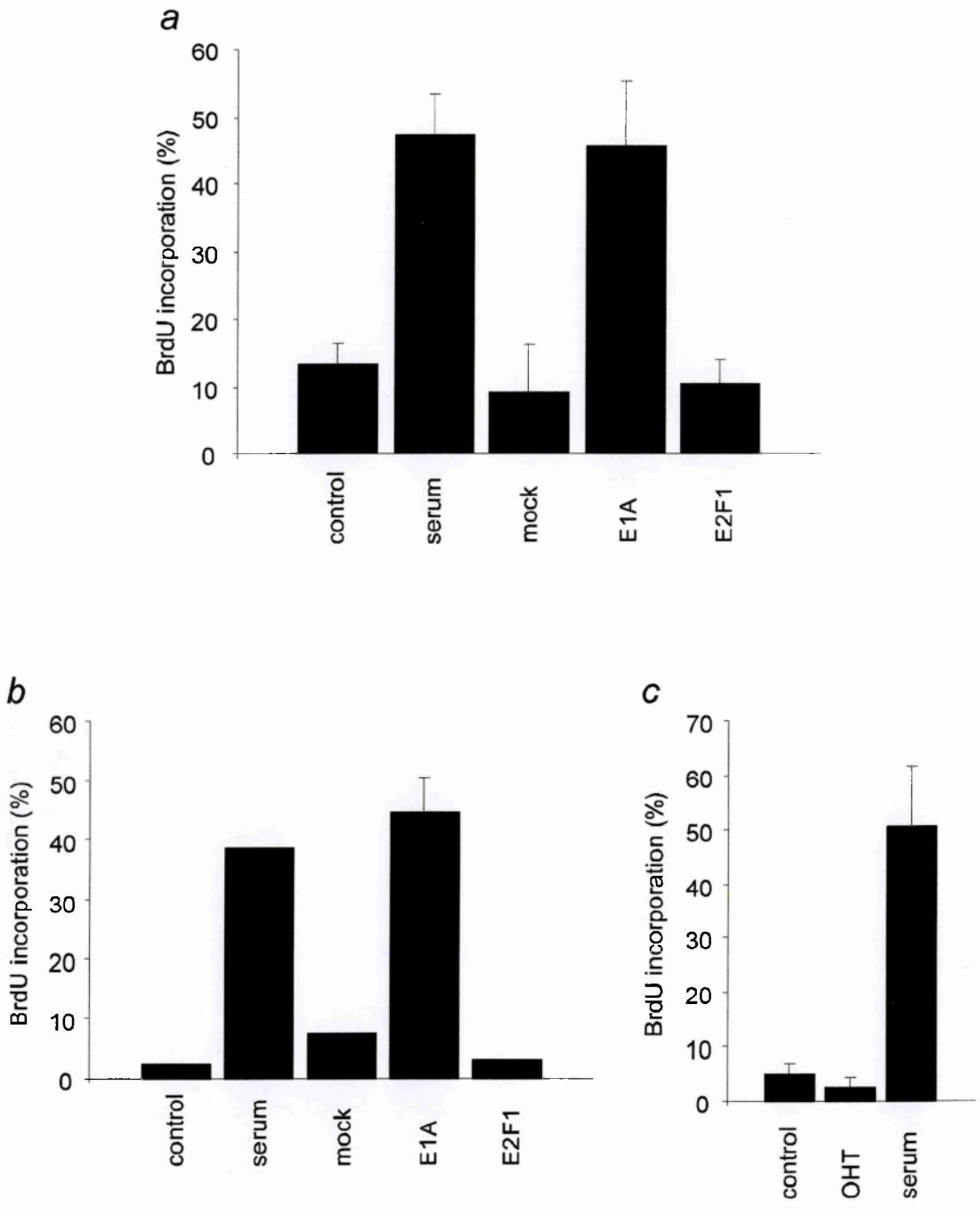


Figure 1 *E2F1 is not sufficient to induce S phase in diploid fibroblasts.* ***a,*** BrdU incorporation in wildtype PMEFs. E2F1 or E1A12S expression plasmids were micro-injected into serum-starved cells along with IgG as an injection marker. Mock-injected or non-injected (Control) cells were negative controls. Serum was added for 24 h as a positive control. DNA synthesis was assessed by BrdU labeling. ***b,*** BrdU incorporation in WI38 fibroblasts. Quiescent cells were injected as in (***a***). ***c,*** BrdU incorporation in WI38 ER-E2F1 fibroblasts. Quiescent cells were either untreated (Control) or treated with OHT or serum for 24 h. Error bars indicate standard deviation of the mean of at least three independent experiments.

My laboratory recently generated cell lines expressing E2F1 fused to the estrogen receptor ligand-binding domain (ER) (Moroni et al., 2001; Müller et al., 2001; Vigo et al., 1999). The ER-E2F1 fusion protein is expressed at relatively low levels as an inactive protein in the cytoplasm. Upon addition of the ligand (4-hydroxytamoxifen, OHT), ER-E2F1 translocates to the nucleus and transactivates E2F-dependent promoters in a DNA-binding and transactivation domain dependent manner. The activation of ER-E2F1 faithfully reproduces all phenotypes associated with expression of native E2F1, including induction of S phase and apoptosis in Rat1 cells (Vigo et al., 1999) and induction of apoptosis in PMEFs and WI38 cells (Moroni et al., 2001). To understand the biochemical mechanisms that prevent S phase induction by E2F1, I tested the ability of ER-E2F1 to induce S phase in quiescent WI38 cells in the absence of serum (Fig. 1*c*). Consistent with the results obtained by microinjection of E2F1 expression plasmid, ER-E2F1 activation was not sufficient to induce S phase in quiescent normal diploid fibroblasts. The expression of ER-E2F1 in the WI38 cell line has been shown previously (Moroni et al., 2001) and I checked it by immunofluorescence (Fig. 2*b*). Furthermore, to verify that ER-E2F1 was activated after OHT addition, I examined the expression of two known E2F target genes. Activation of E2F1 led to a strong increase in *CCNE1* (Cyclin E1) and *ARF* ($p14^{ARF}$) expression independent of de novo protein synthesis, suggesting that these genes are direct targets of E2F1 (Fig. 2*a*). Activation of E2F1 also induced *CDKN1A* (p21) mRNA levels. However, in contrast to the increased expression of *ARF*

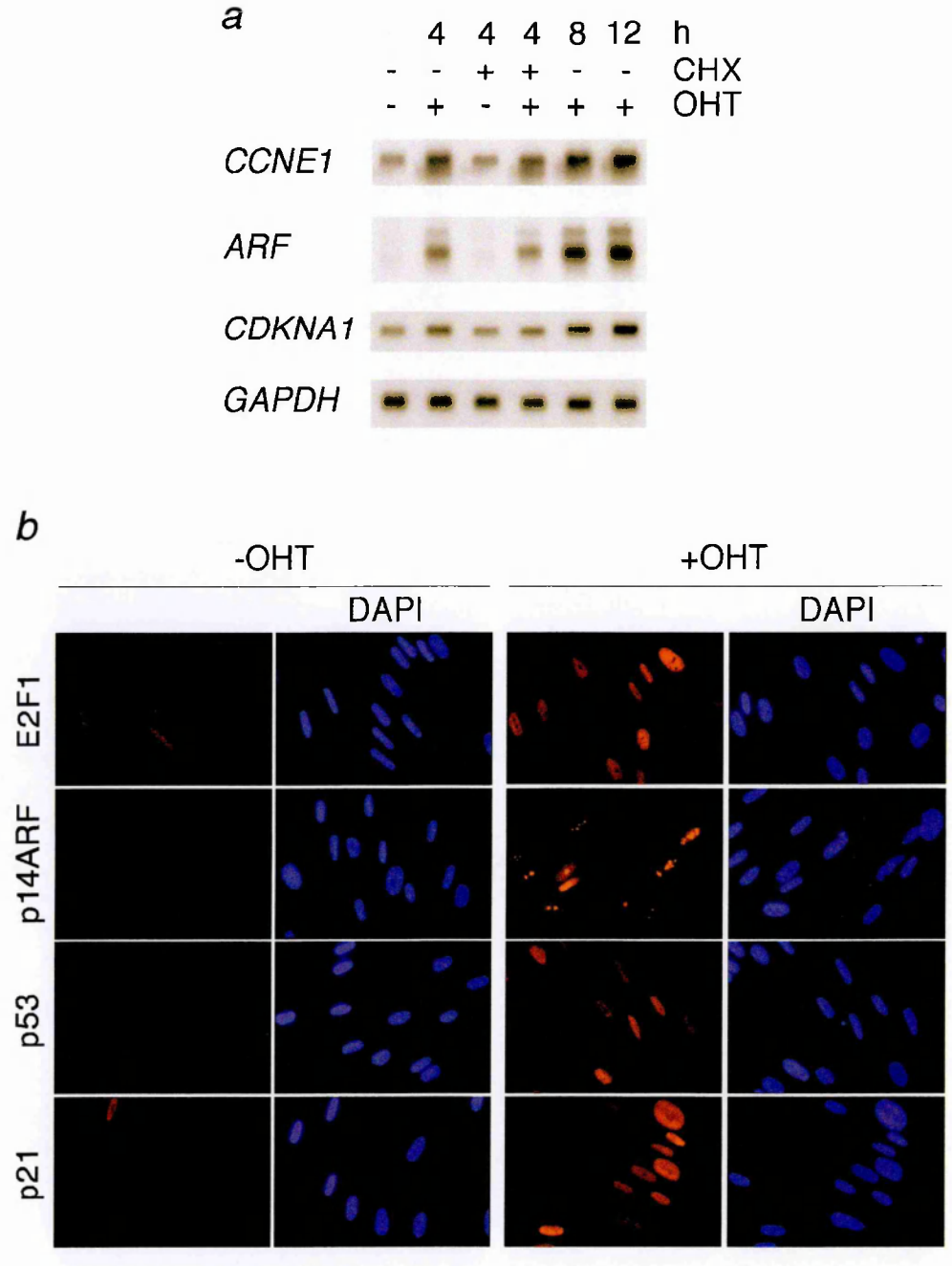


Figure 2 *Activation of E2F1 leads to increased levels of p14^{ARF}, p53 and p21.* ***a,*** Northern blot analysis of mRNA isolated from WI38 cells expressing ER-E2F1. Cells were incubated with OHT, cycloheximide (CHX) or both for the indicated times. The blot (2 µg of poly A+ RNA) was probed for *CCNE1*, *ARF*, *CDKN1A* or *GAPDH* expression. ***b***, Immunofluorescence of WI38 ER-E2F1 cells. Quiescent cells were incubated for 24 h in the absence or presence of OHT. Cells were stained with antibodies specific for E2F1, p14^{ARF}, p53 or p21 on independent coverslips. Nuclei were stained with DAPI.

and *CCNE1*, the increase in *CDKN1A* level was dependent on de novo protein synthesis (Fig. 2*a*; 1.8-fold induced in lane 2 versus 1.1-fold in lane 4). The activation of E2F1 led to increased levels of ARF, p53 and p21 proteins (Fig. 2*b*).

Since the lack of S phase induction by E2F1 could be due to limiting amounts of DP1, the dimerization partner of E2F1, I coexpressed DP1 with E2F1. However, E2F1 did not induce S phase in PMEFs even when co-expressed with DP1 (Fig. 3).

Loss of function in the p53 pathway is required for E2F1-induced S phase

I next sought to understand the genetic changes that allowed E2F1 to induce S phase in immortalized, but not diploid, fibroblasts. p53 is a critical component of the arrest pathway activated by a multitude of DNA damaging agents. Among other genetic changes, either *Arf* or *Trp53* inactivating mutations are the most common single events in the spontaneous conversion of PMEFs into continuously growing cell lines (Sherr, 1998). Since *ARF* is a known E2F1 target gene (Sherr, 1998), and increased ARF levels induce a p53-mediated checkpoint response, I investigated whether inactivation of either *Arf* or *Trp53* would allow E2F1 to induce S phase. I prepared PMEFs from *Arf*–/– or *Trp53*–/– mouse embryos, I serum-starved and microinjected them with E2F1 or E1A12S expression plasmids. As shown in Fig. 4*a*, *b*, expression of E2F1

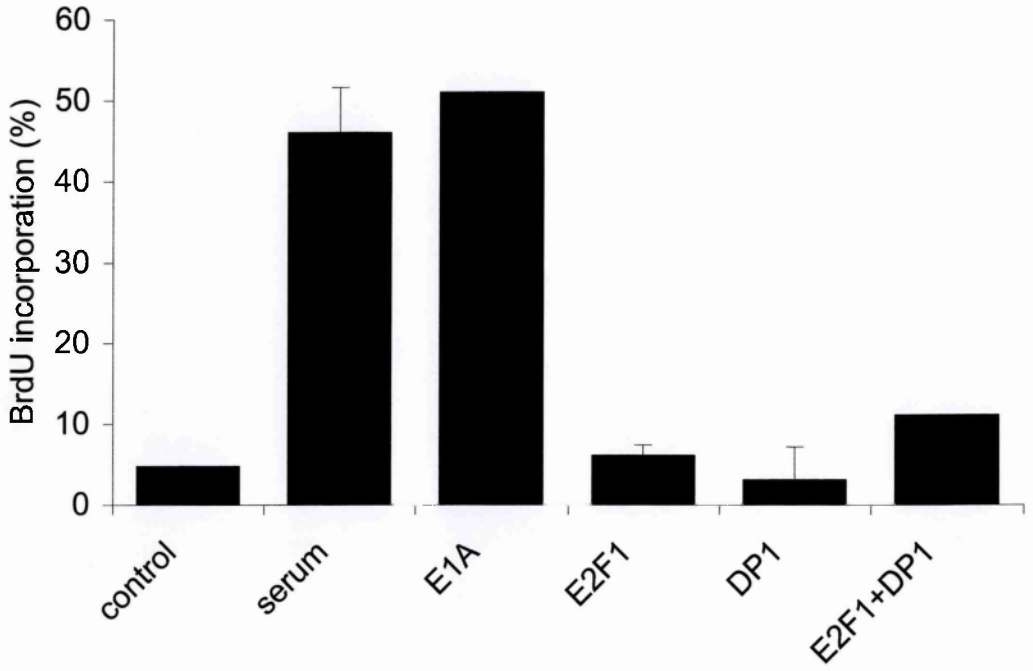


Figure 3 *The E2F1-DP1 heterodimer does not induce S phase in wildtype PMEFs.* Quiescent cells were injected with plasmids expressing E1A12S, E2F1, DP1 or coinjected with E2F1 and DP1. IgG was used as an injection marker. DNA synthesis was assessed by BrdU labeling. Error bars indicate standard deviation of the mean of two independent experiments.

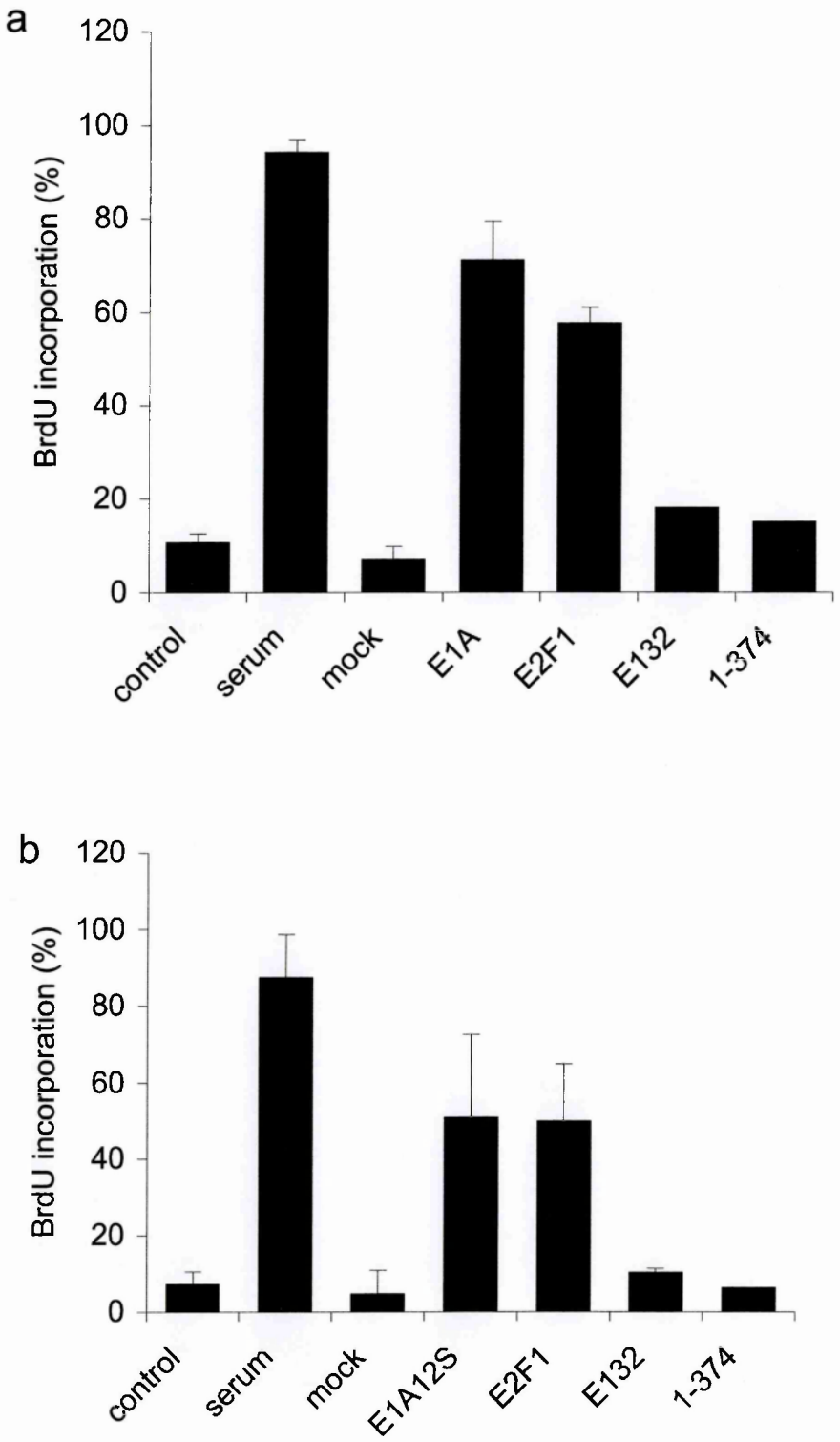


Figure 4 *Loss of function in the ARF-MDM2-p53 pathway allows E2F1-induced S-phase in PMEFs.* E2F1 induces S phase in *Trp53*$^{-/-}$ (***a***) and *ARF*$^{-/-}$ (***b***) PMEFs. Quiescent cells were injected with plasmids expressing E1A12S, E2F1 or E2F1 mutants (E132 and 1-374).

in these cells was as potent as E1A12S at inducing S phase. S phase induction was dependent on the DNA binding and transactivation functions of E2F1, since DNA binding mutant (E132) or transactivation mutant (1-374) alleles did not induce DNA synthesis. Similarly, in quiescent NIH3T3, which lack p19ARF, the microinjection of E2F1 or E1A12S expression plasmid efficiently induced S phase (Fig. 5*a*, *b*). Consistent with this, ER-E2F1 activation was sufficient to induce S phase in quiescent NIH3T3, while ER-E132 was not (Fig. 5*b*), although they both localized into the nucleus upon OHT addition (Fig. 5*c*).

I performed several experiments to confirm these results and to understand the likely mechanism. First, I coexpressed E2F1 and Bmi1. Bmi1 is involved in the regulation of senescence and tumourigenicity (Jacobs et al., 1999; van Lohuizen et al., 1998). It was originally identified as a common insertion site in Moloney murine leukemia virus (MoMLV)-induced B-cell lymphomas in Eμ-Myc transgenic mice (Adams et al., 1985; van Lohuizen et al., 1991) and was only subsequently shown to be a mammalian PcG protein. Overexpression of Bmi1 in PMEFs results in downregulation of *p16INK4a* and *p19ARF*, causing extension of cellular lifespan, increased proliferation and neoplastic transformation in cooperation with oncogenic Ras or Myc (Jacobs et al., 1999). Conversely, the absence of Bmi1 causes de-repression of *p16INK4a* and *p19ARF*, leading to premature senescence of PMEFs and severe proliferation defects in lymphoid organs and cerebellum. When I co-

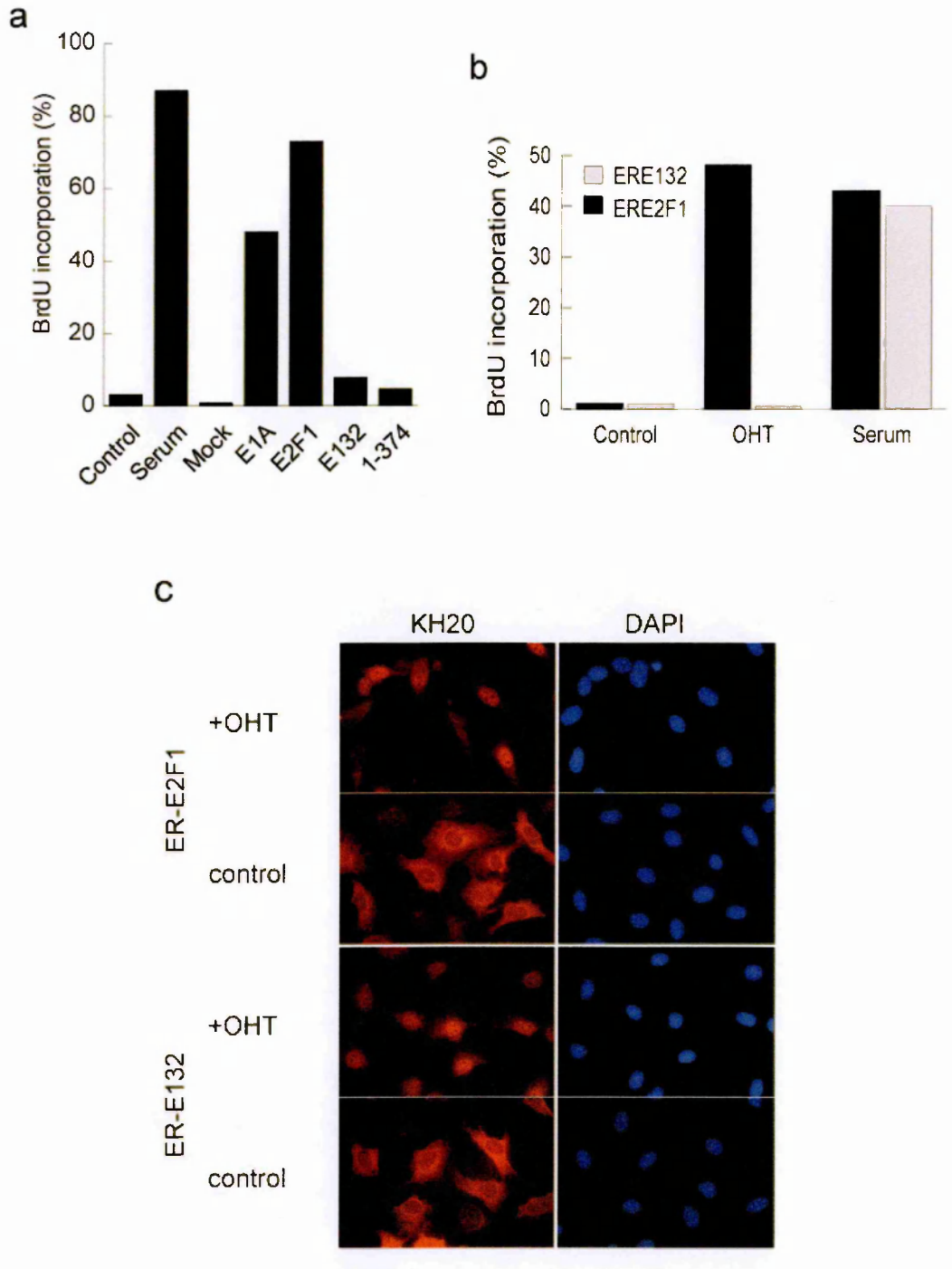


Figure 5 *E2F1 induces S phase in NIH3T3 fibroblasts.* **a,** BrdU incorporation in NIH3T3. E1A12S, E2F1 or E2F1 mutants (E132 and 1-374) expression plasmids were micro-injected into serum-starved cells along with IgG as an injection marker. Mock-injected or non-injected (control) cells were negative controls. Serum was added for 24 h as a positive control. DNA synthesis was assessed by BrdU labeling. **b,** BrdU incorporation in NIH3T3 ER-E2F1 and NIH3T3 ER-E132 fibroblasts. Quiescent cells were either untreated (control) or treated with OHT or serum for 24 h. ***c,*** Immunofluorescence of NIH3T3 ER-E2F1 and NIH3T3 ER-E132. Cells were stained with antibodies specific for E2F1. Nuclei were stained with DAPI.

expressed E2F1 and Bmi1 in PMEFs, I observed S phase induction (Fig. 6*a*). Since Bmi1 is a repressor of ARF expression (Jacobs et al., 1999), my result is consistent with the notion that ARF is required to block E2F1-induced S phase. Second, coexpression of E2F1 and the human papilloma virus E6 protein or the MDM2 oncoprotein, two proteins that target p53 for degradation, induced S phase in serum-starved wildtype PMEFs (Fig. 6*b*). This indicates that p53 is needed to block E2F1 induced S phase. Diploid human fibroblasts also required the presence of functional ARF and p53, since the expression of Bmi1 (Fig. 6*c*), E6 (Fig. 6*d*), or MDM2 (Fig. 6*e*) in WI38 cells cooperated with E2F1 to induce S phase entry.

Loss of p21 allows E2F1 to induce S phase

Activation of p53 in response to unprogrammed growth stimuli results in G1 and G2 cell cycle arrest, and in some circumstances to apoptosis (Vogelstein et al., 2000). The induction of cell cycle arrest is the most common response in diploid fibroblasts, and $p21^{WAF1/CIP1}$, an inhibitor of cyclin dependent kinases, acts as an important effector in the p53-mediated G1 arrest induced by DNA damaging agents (Vogelstein et al., 2000). For instance, cells lacking functional *Cdkn1a* (*p21*) alleles fail to arrest in response to DNA damage (Brugarolas et al., 1995) and exhibit reduced growth factor requirements. p21 and pRb double deficient cells have the ability to grow in soft agar (Brugarolas et al., 1998). The analysis of a single *p21-/-* clone of human

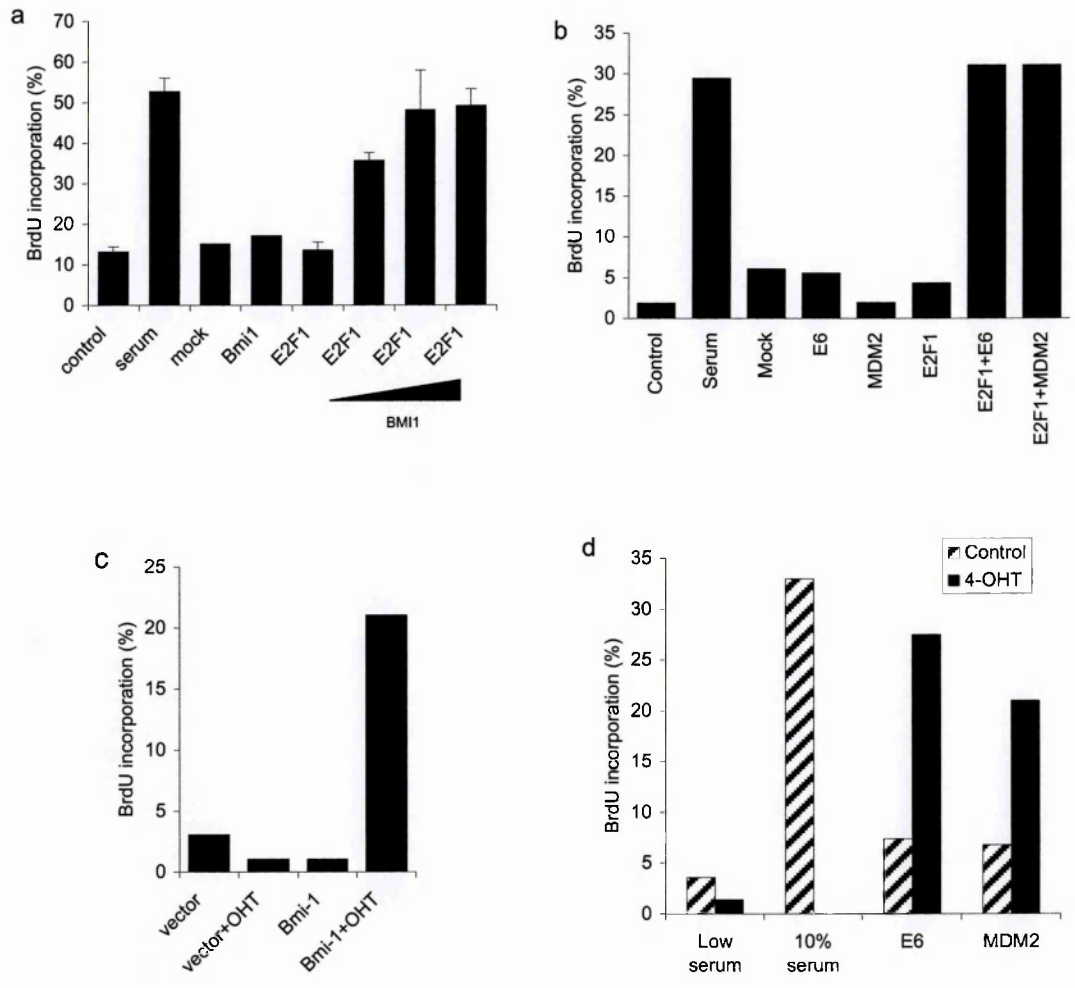


Figure 6 *Loss of function in the ARF-MDM2-p53 pathway allows E2F1-induced S-phase in Wt PMEFs and WI38 cells.* ***a, b*** E2F1 cooperates with BMI1, E6 or MDM2 to induce S phase in wildtype PMEFs. Quiescent cells were injected with E2F1, E6, MDM2 or increasing amounts of BMI1 (10-25-50 ng ml^{-1}). ***c***, ***d*** E2F1 cooperates with BMI1, E6 or MDM2 to induce S phase in WI38 cells. WI38-ER-E2F1 cells were infected with empty vector or pBabeHygro2HA-BMI1, made quiescent and incubated with/without OHT. In (***d***) cells were injected with E6 or MDM2 and incubated with/without OHT. Error bars indicate standard deviation of the mean of at least three independent experiments

fibroblasts obtained after selection for two independent homologous recombination events had led to the conclusion that the loss of *p21* gene is sufficient to bypass senescence (Brown et al., 1997). Finally, p21 is upregulated in association with cell cycle arrest induced by constitutive activation of the Ras/Raf/MEK pathway (Serrano et al., 1997).

To understand if p21 was required to prevent E2F1-induced S-phase, PMEFs were prepared from *Cdkn1a*–/– embryos, serum-starved and microinjected as before. As shown in Fig. 7, E2F1 induced S phase in *Cdkn1a*–/– PMEFs. These results show that p21 is necessary for sustaining a G1 arrest and are consistent with the observation that the G1 arrest mediated by p21 cannot be bypassed either by inactivation of pRB or by overexpression of E2F family members (Mann and Jones, 1996).

Loss of pRB allows E2F1 to induce S phase

Ectopic cell cycle entry and elevated apoptosis levels are apparent in both CNS and PNS of *Rb1*-/- embryos. The inappropriate cell cycle entry is accompanied by elevated activity of free E2F proteins and overexpression of E2F transcription targets, such as cyclin E (Macleod et al., 1996). Additionally, p53 protein levels and p53 DNA binding activity are enhanced in the brains of *Rb1*-/- embryos, leading to increased expression of the p53 transcriptional target p21. Despite higher levels of p53, inactivation of pRB is sufficient for ectopic S phase in *Rb1*-/- embryos (Macleod et al., 1996).

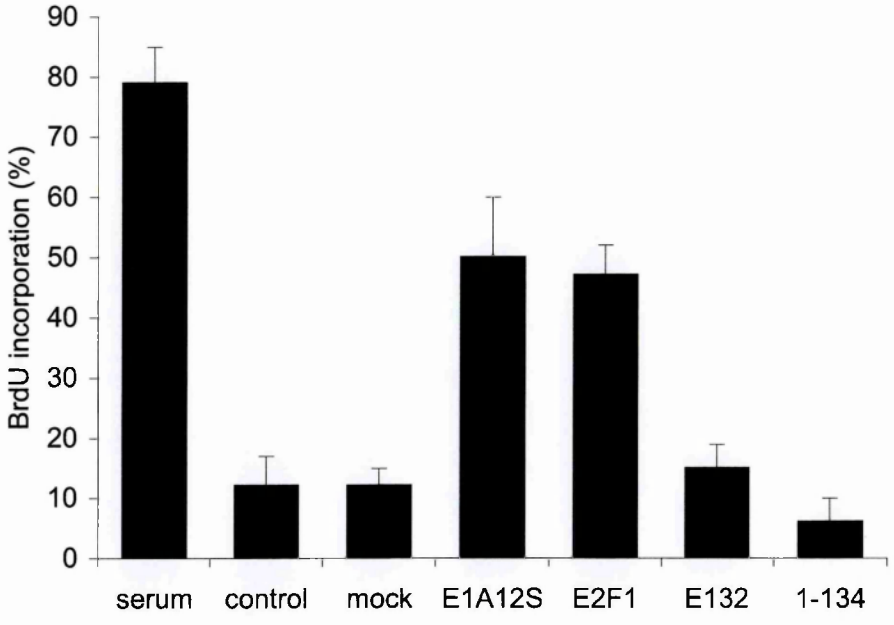


Figure 7 *Loss of p21 is required for E2F1-induced S-phase.* E2F1 induces S phase in *Cdkn1a*$^{-/-}$ PMEFs. Quiescent cells were injected with plasmids expressing E1A12S, E2F1 or E2F1 mutants (E132 and 1-374). Cells injected with IgG (Mock) or non-injected (Control) were negative controls. Serum was added for 24 h as a positive control. DNA synthesis was assessed by BrdU labeling.

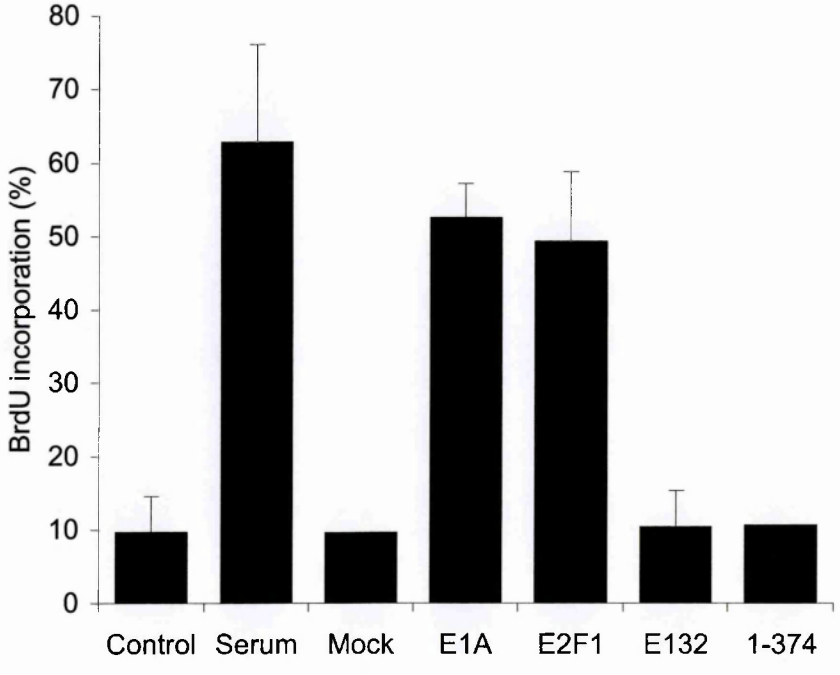


Figure 8 *Loss of pRb is required for E2F1-induced S-phase.* E2F1 induces S phase in *Rb1*$^{-/-}$ PMEFs. Cells were injected as in Fig. 7. Error bars indicate standard deviation of the mean of at least three independent experiments.

To test whether E2F1 was sufficient to induce S phase entry in the absence of pRB, PMEFs were prepared from *Rb1-/-* embryos, serum-starved and microinjected with E1A12S and E2F1 expression plasmids. As demonstrated in Fig. 8, both E1A12S and E2F1 induced S phase in pRb-deficient PMEFs, showing that in addition to causing deregulation of E2F activity, loss of pRB also abrogates the E2F1-induced G1 checkpoint. These data suggest that the G1/S arrest imposed by E2F expression in Wt PMEFs requires p53-triggered, p21-mediated, inhibition of pRB phosphorilation. In addition, the E2F1/VP16 mutant was unable to release quiescent WI38 cells in S phase, suggesting that the interaction between pRB and E2F1 is not required to arrest primary cells in G1 (Fig. 9). The E2F1/VP16 chimera cannot interact with pRB since the transactivation domain of E2F1 is replaced by the transactivation domain of the herpesvirus VP16, but it is fully transcriptionally active in an E2F dependent manner (Johnson et al., 1994). These findings strongly suggest that pRB may regulate the G1/S transition through direct binding to other activities (proteins) in addition to E2Fs such as ID2, HBP1, c/EBPα or MyoD (Lasorella et al., 2000; Lipinski and Jacks, 1999). I focused my attention on ID proteins. They function as dominant negative inhibitors of basic helix-loop-helix (bHLH) transcription factors since they lack a DNA binding domain. In addition to E2F1, ID2 is the only protein described so far able to disrupt the anti-proliferative effect of pocket proteins, thus allowing cell cycle progression (Lasorella et al., 1996). This function correlates with the ability of ID2 to associate with hypophosphorylated pocket proteins. To test whether

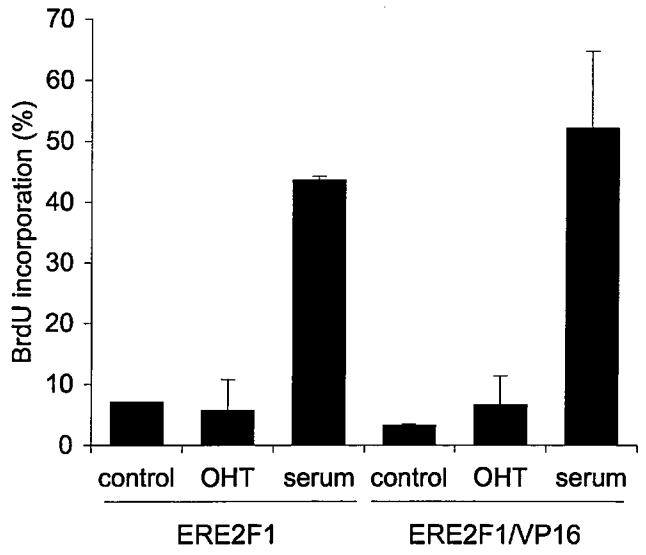


Figure 9 *pRB and E2F interaction is not required to arrest cells in G1.* WI38-ER-E2F1 and WI38-ER-E2F1/VP16 cells were made quiescent and incubated with/without OHT or with serum as positive control for 24h. DNA synthesis was assessed by BrdU labeling. Error bars indicate standard deviation of the mean of two independent experiments.

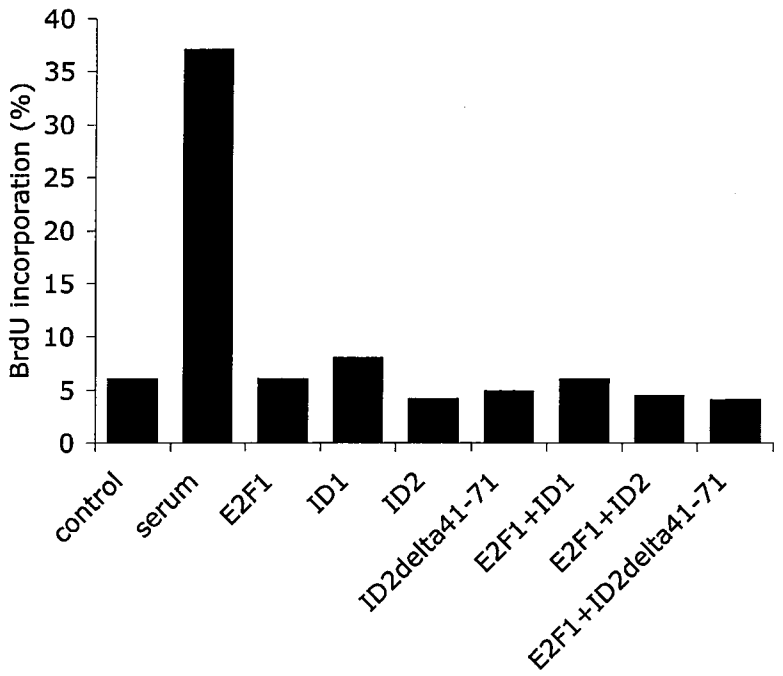


Figure 10 *ID2 does not cooperate with E2F1 to induce S phase in Wt PMEFs.* Quiescent cells were injected with plasmids expressing E2F1, ID1, ID2 or ID2 mutant (delta 41-71). Non-injected cells (Control) were negative control. Serum was added for 24 h as a positive control. DNA synthesis was assessed by BrdU labeling.

pRB could induce G1 arrest through direct binding and regulation of some bHLH transcription factor in addition to E2F1 binding, I expressed E2F1 in quiescent Wt MEFs along with ID2, and, as negative control, ID2Δ41-71 mutant, which lacks HLH domain, or ID1, which is not able to disrupt the anti-proliferative effect of pRB. However, none of the constructs was able to cooperate with E2F1 to induce S phase (Fig. 10).

Biochemical Mechanism

My data suggest that the G1 block imposed by E2F1 overexpression is ultimately mediated by pRB, downstream of p21. The role of p21 in this pathway raised questions regarding the mechanism. Ample evidence suggests that p21 can inhibit both CDK2- and CDK4- associated activity (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). However, after γ irradiation, p21 allows a pRB-mediated G1 arrest (Brugaloras et al., 1999) by inhibiting CDK2- and not CDK4- activity. In *Cdkn1a*-/- PMEFs, CDK2 activity but not CDK4 activity is elevated two to fourfold compared with wildtype cells (Brugarolas et al., 1998).

To elucidate the mechanism more fully, I tested the relative kinase activity of CDK2 when E2F1 is overexpressed in quiescent Wt or *Trp53*-/- PMEFs and the status of pRB phosphorylation in the same cells. Expression of E2F1 did not result in increased level of CDK2 activity in quiescent wildtype PMEFs, whereas E2F1 expression increased CDK2 activity in *Trp53*-deficient PMEFs

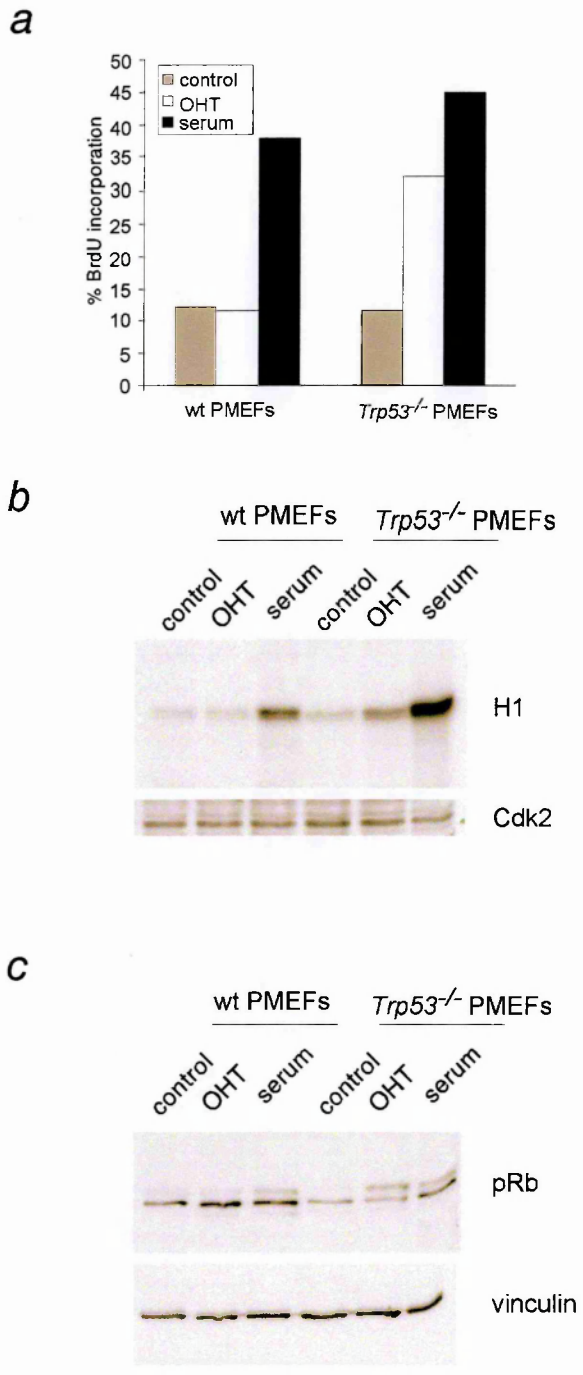


Figure 11 *Abrogation of the p53-dependent G1-checkpoint increases Cdk2 activity and pRb phosphorylation.* Wildtype and *Trp53*$^{-/-}$ PMEFs were infected with pBabePuro HAER-E2F1, made quiescent and incubated with/without OHT or serum for 24 h. ***a,*** DNA synthesis was assessed by BrdU labeling. ***b,*** Relative Cdk2 kinase activity was measured using histone H1 as a substrate. ***c,*** Aliquots of cell lysates were run on a 6% SDS-PAGE and the level of pRb phosphorylation was assessed by probing the Western blot with a specific antibody to pRb (PharMingen). Equal loading was confirmed using an antibody to Vinculin (Sigma).

(Fig. 11*b*). E2F1 expression in p53-deficient, but not in wildtype, PMEFs gave hyperphosphorylation of pRB (Fig. 11*c*).

Cyclin E1 and cyclin A2 have been shown (although in tumour cells) to override a pRB-mediated G1 block (Hinds et al., 1992; Horton et al., 1995). As expected their overexpression along with E2F1, and the consequent increase in E2F activity, is sufficient for S phase induction in wildtype PMEFs (Fig. 12).

E2F2 and E2F3 induce S phase in ARF- and p53- deficient fibroblasts

E2F2 and E2F3 are highly homologous to E2F1 and, if overexpressed, they can induce immortalized quiescent cells to re-enter the cell cycle. When I expressed in normal diploid fibroblasts E2F2 and E2F3 they didn't induce S phase. However, they induced S phase in ARF- and p53-deficient fibroblasts (Fig. 13*a*). Consistent with this, I observed that activation of the three E2Fs directly induces *ARF* expression (Fig. 13*b*).

Quiescent diploid fibroblasts are not apoptotic in response to E2F1

I was also interested in studying why E2F1 blocks quiescent primary fibroblasts in G1. One possibility is that the G1 arrest observed upon E2F1 expression in wildtype cells leads to an increase in apoptosis and that cells go into S phase because they do not die in a p53-/-, ARF-/- or p21-/- background.

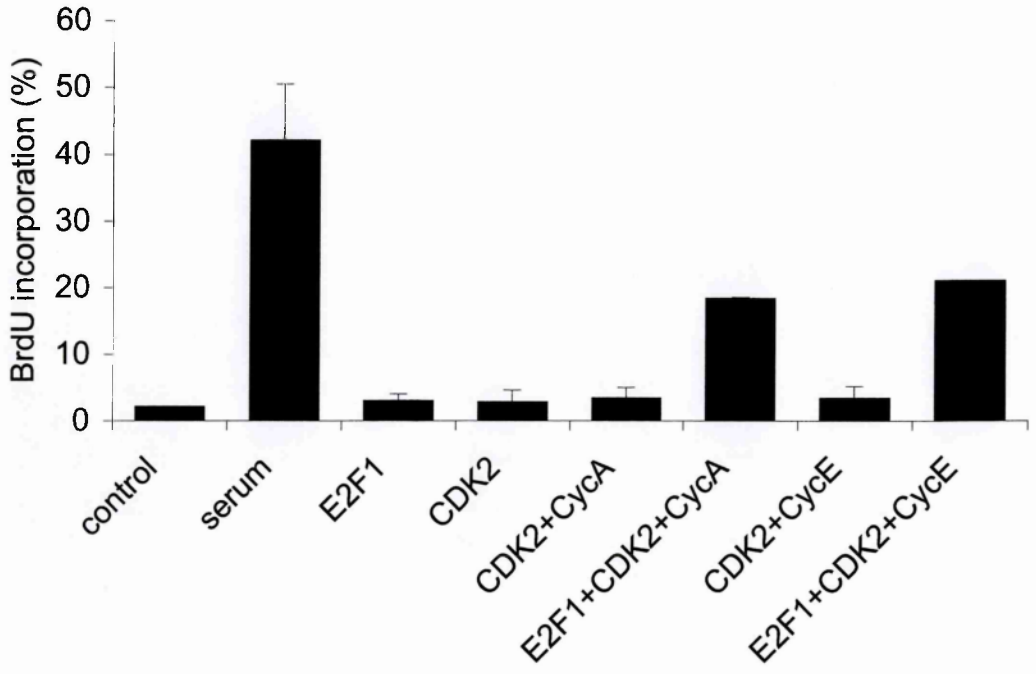


Figure 12 *Coexpression of Cyclin E1 or Cyclin A2 with E2F1 overrides the pRb-mediated G1 block.* Quiescent wildtype PMEFs were injected with plasmids expressing E2F1, CDK2, Cyclin A2 (CycA), or Cyclin E1 (CycE) either alone or in combination. BrdU incorporation was measured 24 h after injection. Error bars indicate standard deviation of the mean of two independent experiments.

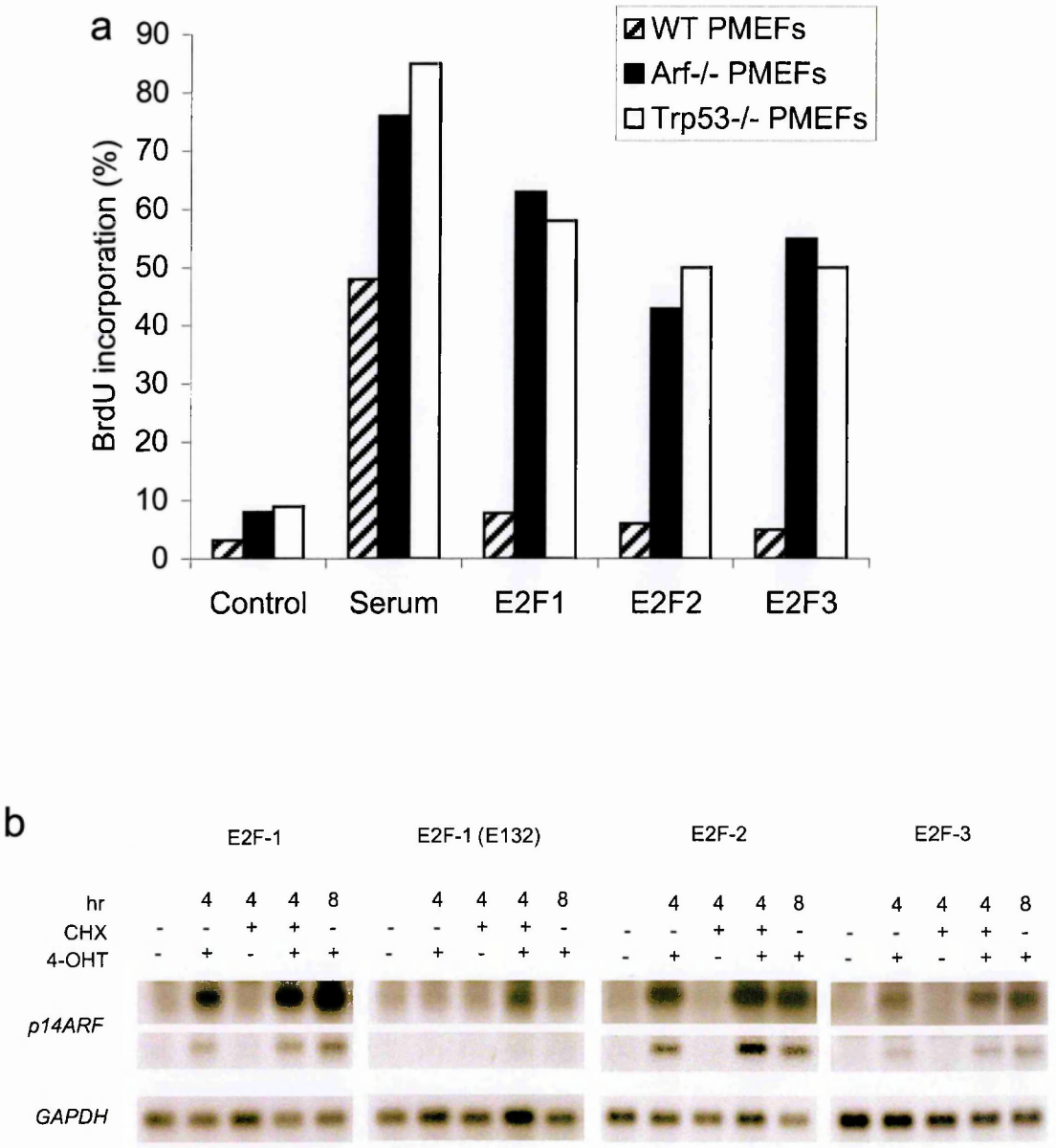


Figure 13 *E2F2 and E2F3 induce S phase when the p53-dependent G1 checkpoint is disabled.* ***a,*** Quiescent wildtype, *Arf*$^{-/-}$ or *Trp53*$^{-/-}$ PMEFs were injected with plasmids expressing E2F1, E2F2 or E2F3 and BrdU incorporation was measured after 24 h. ***b,*** Northern blot analysis of mRNA isolated from WI38 cells expressing ER-E2F1, ER-E2F2, ER-E2F3 or ER-E132. Cells were incubated with OHT, cycloheximide (CHX) or both for the indicated times. The blot (2 μg of poly A+ RNA) was probed for p14ARF (short and long exposure in the pannel) or GAPDH expression.

Another possibility is that the G1 block induced by E2F1 protects cells against cell death, suggesting an anti-apoptotic role of some pathway-regulated gene. In the experiments described above, I didn't see apoptosis within the 24 hours of E2F1 activation. Interestingly, I also observed that quiescent cells (independent of the genetic background), didn't undergo apoptosis within a 72 hours time period, whereas E2F1 induced very efficient apoptosis in asynchronously growing cells even at 24 hours after E2F1 activation (data not shown). Therefore, it is unlikely that the inability of E2F1 to induce S phase in normal diploid fibroblasts is due to induction of apoptosis. This also suggests that E2F1 can induce apoptosis independently of an intact p53 pathway, confirming the results of our and other laboratories. Moreover, it shows that quiescent cells are less prone to apoptotic signals, maybe because they need to be in a phase different from G1 to become apoptotic in response to E2F1, rather than serum supplies some protein(s) that cooperates with E2F1 to induce apoptosis.

DISCUSSION

By analyzing primary cell lines lacking the p53- or pRB-regulated G1 checkpoint, I have investigated the mechanism required for E2F1 to induce S phase (Fig. 14*a*).

E2F1 is fully competent as a transcriptional activator in diploid cells. Its induction in diploid fibroblasts results in the robust activation of several

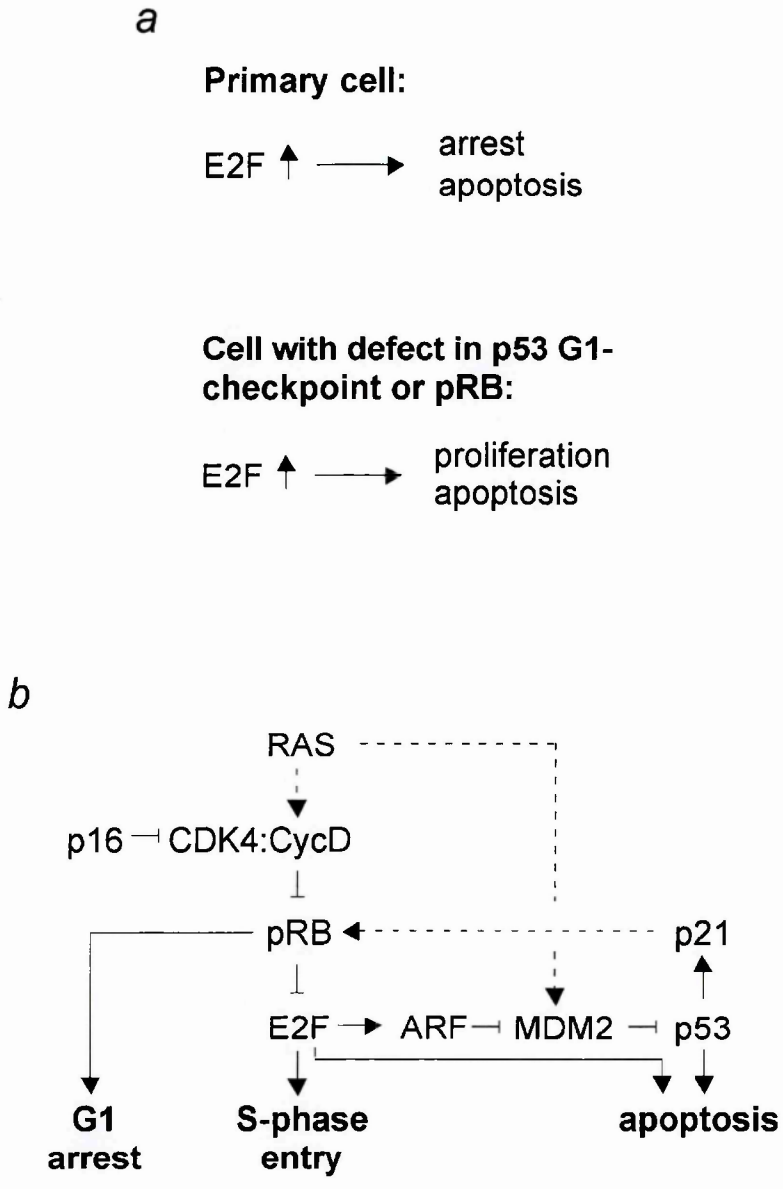


Figure 14 *Model for the regulation of cell proliferation by the pRB pathway.* See text for details. Broken arrows signify genetic interactions, whereas non-broken arrows indicate biochemical and genetic interactions or functions (G1 arrest, S phase entry or apoptosis).

E2F target genes (as shown here for *ARF* and *CCNE1*, and by microarray analysis (M. Ciro', H. Müller and K. Helin, unpublished results) in the absence of S phase entry. It is unlikely that the inability of E2F1 to induce S phase in normal diploid fibroblasts is due to induction of apoptosis. Indeed, I did not observe any apoptotic effects of E2F1 in the experiments presented here (i.e. 24 h of E2F1 expression). However, apoptosis is induced 36-48 h after E2F1 activation in growing diploid mouse and human fibroblasts (Moroni et al., 2001). Hence, the consequence of increased E2F1 activity in diploid cells is G1 arrest or apoptosis, and not DNA replication, unless other genetic alterations occur. In contrast, E2F1 efficiently induced DNA replication in cells that are impaired in the p53- or pRB-mediated G1 checkpoint.

My results are consistent with a model (Fig. 14*b*) whereby increased E2F activity results in direct activation of *ARF* transcription, and subsequent upregulation of p53 and p21 levels. The increased levels of p21 in diploid fibroblasts appears necessary to block cells in G1 since *Cdkn1a–/–* PMEFs efficiently entered S phase after E2F1 activation.

I have performed several experiments to test the validity of the model. First, I have shown that expression of E2F1 did not result in increased level of CDK2 activity in quiescent wildtype PMEFs, whereas E2F1 activity resulted in increased CDK2 activity in p53-deficient PMEFs (Fig. 11*b*). Second, E2F1

expression in p53-deficient, but not in wildtype, PMEFs resulted in hyperphosphorylation of pRB (Fig. 11*c*). Third, I have shown that increased CDK2 activity is sufficient to cooperate with E2F1 in inducing S phase in wildtype PMEFs (Fig. 11*a*). These observations suggest that p21 imposes G1 arrest by inhibiting CDK2 activity and by preventing inactivation of the growth suppressive properties of pRB tumour suppressor.

It has been shown that expression of E2F1 is sufficient to induce DNA synthesis in immortalized REF52 cells (Johnson et al., 1993), even though these cells appear to contain functional ARF and p53. REF52 cells may contain hitherto unidentified genetic alterations that contribute to immortalization and allow the cells to escape the E2F1-induced G1 checkpoint.

My results are in accordance with previous findings that E2F1 does not induce S phase in WI38 cells (Dimri et al., 1994), and that short-term activation of E2F1 in proliferating WI38 cells induces G1 arrest (M. Lomazzi, M.C. Moroni and K. Helin, unpublished results). In contrast to the work of Dimri and colleagues (Dimri et al., 1994), who found that E2F1 was unable to induce S phase in NIH3T3 cells that lack p19ARF, I have shown that expression of E2F1 or activation of ER-E2F1 efficiently induced S phase in NIH3T3 cells (Fig. 5). The reason for this discrepancy is not known.

My results are not in contrast with the observation that inactivation of pRB can result in increased levels of p21 independently of p53, as has been described in the peripheral nervous system in *Rb1–/–* embryos (Macleod et al., 1996). Rather, I show that the presence of wildtype pRB is required for maintaining the G1 arrest imposed in response to unprogrammed E2F1 induction. This result appears mechanistically similar to previous work showing that the DNA damage induced G1-checkpoint, which is dependent on functional p53 and p21, is in part mediated by pRB (Brugaloras et al., 1999; Harrington et al., 1998). However, my findings are significantly different, as they suggest that pRB regulates normal cell proliferation by two independent mechanisms: one that actively represses E2F-dependent promoters, and another one that ensures cells arrest if E2F activity should increase as a result of genetic alterations. Indeed, E2F1 overexpression is not sufficient to overcome the pRB-dependent G1 checkpoint in non-transformed cells, suggesting that pRB may regulate the activity of proteins in addition to E2F that regulate the G1-S transition. Like wildtype E2F1, a pRB-binding deficient but transactivation-competent mutant of E2F1 (E2F1-VP16) is unable to stimulate S phase in diploid fibroblasts (Fig. 9), again suggesting that pRB regulates S phase entry through proteins in addition to E2F. In agreement with previously published data obtained in immortalized fibroblasts (Johnson et al., 1993; Qin et al., 1994; Shan and Lee, 1994), E2F1 induction of S phase in cells deficient in the G1-checkpoint requires both the transactivation and DNA binding function of E2F1, suggesting that entry into S phase is not caused by

sequestration of pRB, but rather is caused by transactivation of E2F-dependent promoters. Since E2F1 can induce S phase in *Cdkn1a–/–* PMEFs, it is likely that the G1 checkpoint function of pRB is regulated by a CDK-dependent phosphorylation mechanism and may involve direct binding of pRB to other potential pRB targets such as ID2, HBP1, C/EBPα, and MyoD (Lasorella et al., 2000; Lipinski and Jacks, 1999). Of these proteins, only ID2 has been connected to the induction of S phase. However, my results show that coexpression of ID2 and E2F1 is not sufficient to induce S phase in serum starved PMEFs (Fig. 10), suggesting that other as yet unidentified pRB-regulated proteins are involved in regulating the G1/S transition.

E2F2 and E2F3, like E2F1, cannot induce S phase in normal diploid fibroblasts. I found that they are capable of inducing S phase in ARF- and p53-deficient fibroblasts to the same extent of E2F1 (Fig. 13*a*). Consistent with this, I have observed that activation of these three E2Fs directly induces *ARF* expression (Fig. 13*b*).

In conclusion, I demonstrate the molecular mechanisms by which p53 and pRB govern E2F activity to control the G1/S transition in mammalian cells. Since the deregulation of the pRB pathway is a common event in cancer (Hanahan and Weinberg, 2000), my results are important for understanding the etiology of uncontrolled cell division in this disease.

RESULTS - PART 2

AIM

"E2F" is a composite activity that is generated by a large number of interrelated complexes. In mammals, six E2Fs (E2F1-6) contain two highly conserved domains that are involved in sequence specific DNA binding and dimerisation with DP proteins. Association of these E2Fs with one of the two known DP proteins is required for high affinity, sequence specific DNA binding, and, in the case of E2F1-5, association with members of the pRB family (Trimarchi and Lees, 2002). The recently identified E2F7 do not bind to the DP transcription factors, and it interacts efficiently with the E2F DNA consensus site without DP. This binding requires both of the two DNA binding domains of E2F7. It lacks also a transcriptional activation and a retinoblastoma-binding domain. E2F7 is able to repress transcription of E2F promoters *in vitro* and it binds to E2F regulated promoters *in vivo* (de Bruin et al., 2003a; Di Stefano et al., 2003).

DP1 is a phosphoprotein ubiquitously expressed at high levels in tissues and cell lines (Girling et al., 1993; Wu et al., 1995), structurally related to E2F, yet devoid of an E2F-like pRB-binding domain (Girling et al., 1993; Helin et al., 1993b). By contrast, DP2 is expressed at low levels with alternative splicing in a restricted set of tissues and cell lines (Rogers et al., 1996; Wu et al., 1995; Zhang and Chellappan, 1995). Despite their distinct pattern of expression,

DP1 and DP2 function indistinguishably in *in vitro* assays, such as those for heterodimerisation, DNA binding and transactivation, when overexpressed with various E2F patterns and pRB family members.

Many evidences indicate that E2F activity is not required for cell proliferation. First, promoters mapping and *in vivo* footprinting studies detected E2F/pRB repressor complexes on promoters at G0/G1 while the promoters were not occupied in S phase. This would suggest that the E2F in complex with pocket proteins represses target genes and keeps cells in G1. Disruption of E2F-mediated transcriptional repression by an E2F-DNA-binding deficient mutant (Rowland et al., 2002; Zhang et al., 1999) has been reported to lead to immortalization of primary MEFs, while control-infected MEFs loose their replicative potential. Derepression of E2F target genes was observed, whose downregulation was critical for the establishment of G1 arrest by either p16 or TGFβ. Importantly, the authors of the paper claim they have knocked out all E2F DNA binding activity, which they show by band shift. It is also not clear whether immortalized clones are rare. In contrast to the milder phenotypes resulting from inactivation of *E2fs*, loss of *Dp1* in mice leads to early embryonic lethality owing to a failure of extra-embryonic tissues development (Kohn et al., 2003). Surprisingly, no differences in DNA synthesis can be seen in the embryonic compartment, suggesting that many cells cycles and DNA replications can occur without DP1. However, the biochemical effect of the absence of DP was not analysed with respect to E2F transactivation. In

Drosophila, where only two *de2f* and one *dDP* exist, the loss of *de2f1* function compromises cell proliferation (Frolov et al., 2001). The defects are due to the unchecked activity of *de2f2*, since they can be suppressed by mutation of *de2f2*. Examination of eye discs from *de2f1;de2f2* double mutant animals reveals that relatively normal patterns of DNA synthesis can occur in the absence of both E2F proteins. Thus, the net effect of E2F on cell proliferation is null. Similarly, the pattern of DNA synthesis and cell proliferation are not severely affected in *dDP* mutant embryos or *dDP* mutant larvae, but they do not survive (Duronio et al., 1998; Royzman et al., 1997).

Other studies suggests that E2F activity is required for cell proliferation. Overexpression of E2F1-2-3 strongly correlates with its ability to drive the cells into S phase (Johnson et al., 1993; Lukas et al., 1996; Qin et al., 1994). TKO cells for E2f1, E2f2, E2f3 (derived from a conditional triple knockout mouse) are defective for S-phase entry and progression through the cell cycle and show a dramatic decrease in the expression of many E2f-regulated genes (Wu et al., 2001). This supports the belief that E2F transactivation activity is required for cell proliferation. However, there is the possibility that the defects observed when E2f1, E2f2, E2f3 are missing are due to a gain in activity of the remaining E2f complexes which are believed to repress transcription and whose inactivation could suppress the proliferation defects. A dominant-negative mutant of DP1 has been reported to inhibit the progression of SAOS2, C33A and U2OS cells into S phase (Wu et al., 1996), supporting the

idea that interaction of E2F/DP with promoters is important for cell cycle progression.

To understand whether E2F transactivating activity is required for cell proliferation and to examine the changes in gene expression that occur when E2F-DNA binding activity is lost, I decided to knock out endogenous DP in normal and tumour cells by RNA interference. To inactivate all E2F complexes I depleted DP, the common heterodimeric partner for E2Fs, required for high affinity DNA binding and functional E2F activity. In addition, DP depletion was more efficient than the co-depletion of the individual E2Fs.

RESULTS

siRNA for DP as a tool to study E2F-DNA binding activity

I designed siRNA oligonucleolides specific for the human sequence of DP1 or DP2 according to Elbashir et al. (see Material and Methods). I transfected the two siRNAs alone or in combination in HeLa cells. A non-specific siRNA targeting the firefly luciferase gene (GL2 siRNA) was used as control. At the mRNA level, DP1 siRNA efficiently inhibited DP1expression (Fig. 15*a*). Similarly DP2 siRNA interfered with the abundance of DP2 mRNA (Fig. 15*b*).

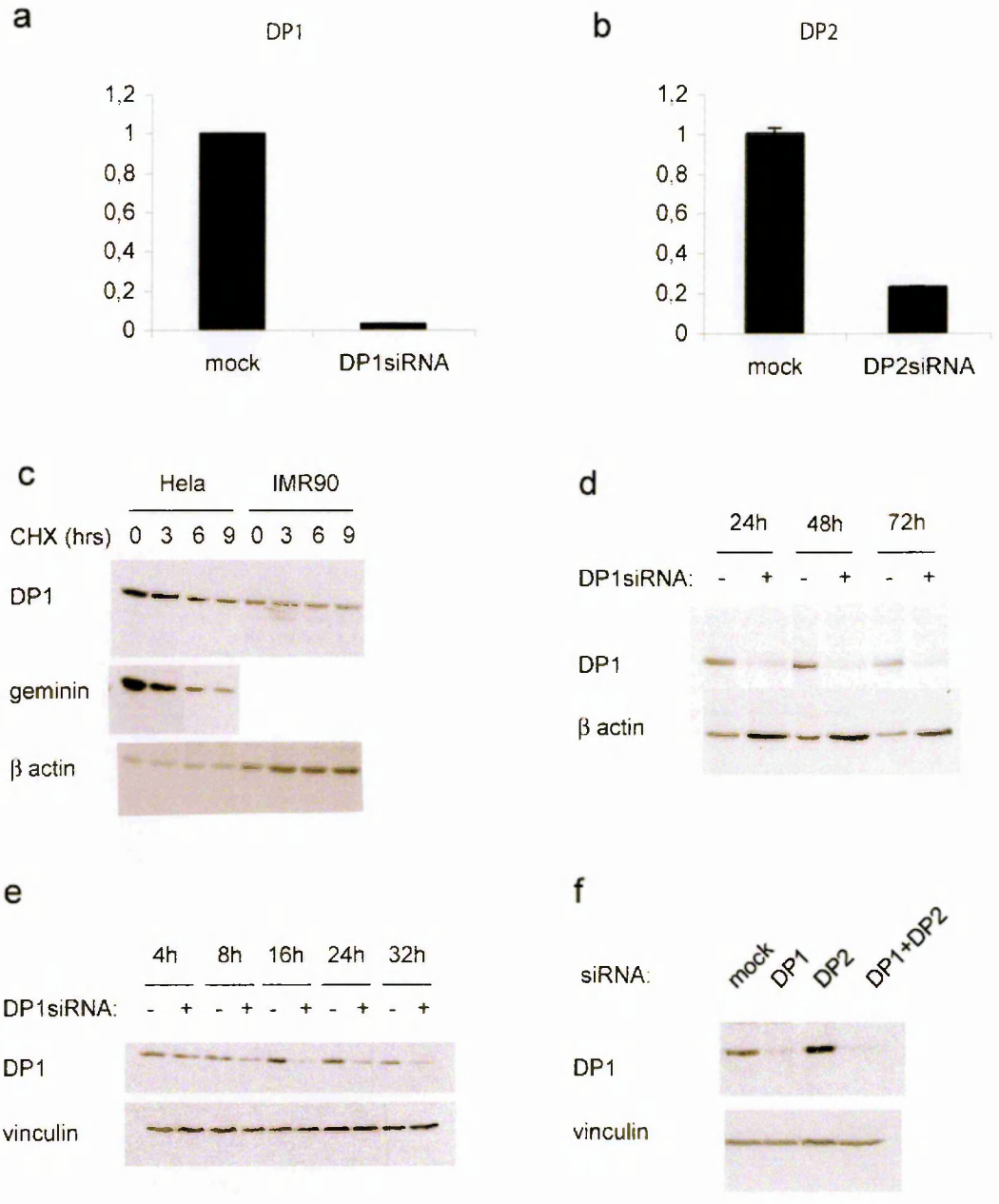


Figure 15 *DP siRNA abolishes DP mRNA and protein expression.*
a, b, DP1 or DP2 siRNA oligos were transfected in asynchronous HeLa cells for 48 h. GL2 siRNA was negative control (mock). qPCR was performed using specific primers for DP1 (***a***) or DP2 (***b***) and GADP to normalize. ***c,*** HeLa cells were incubated with cycloheximide (CHX) for the indicated times. The blot was probed for DP1, geminin and β actin. ***d, e,*** HeLa cells were transfected with DP1 siRNA for the indicated times. Western blot was probed with antibodies for DP1 and β actin. ***f,*** U2OS cells transfected with DP1 siRNA and DP2 siRNA, either alone or in combination.

The efficiency of depletion at protein level was assessed by Western blotting. Fig. 15*c* shows that DP1 is a stable protein. Upon cycloheximide (CHX) treatment (which inhibits proteins synthesis) DP1 half-life is around 10 hours in HeLa cells and 13 hours in IMR90. Geminin, an unrelated protein, was degraded faster (5 hours half-life). The level of DP1 was greatly reduced upon DP1 siRNA treatment compared to cells treated with a non-specific control (luciferase) (Fig. 15*d*, *e*). I was not able to detect endogenous DP2 in Western blot using different commercial antibodies. However, transfection of DP2 siRNA caused a slight increase in DP1 protein level and taken together with the observation that DP2siRNA reduced the mRNA level of DP2, it could suggest that the DP2 siRNA oligo was functional and that loss of DP2 was compensated by DP1 (Fig. 15*f* and Fig. 20*a*).

To investigate whether lack of DP could abrogate E2F DNA binding activity, I performed a gel retardation assay (EMSA) with HeLa cells extracts. HeLa cells express the oncoprotein E7 and thus almost all of E2F is in the free, transactivating form. HeLa cells were transfected with DP1 siRNA and DP2 siRNA either alone or in combination. As negative control the reaction was performed in the absence of lysate (no lysate) or with not transfected cells (mock). As positive control cells were transfected with E2F1 and DP1. An excess of cold probe was added to compete for the hot probe (competitor) (Fig. 16*a*). Little or no DNA-binding activity was generated following transfection with DP1 siRNA either alone or in combination with DP2 siRNA,

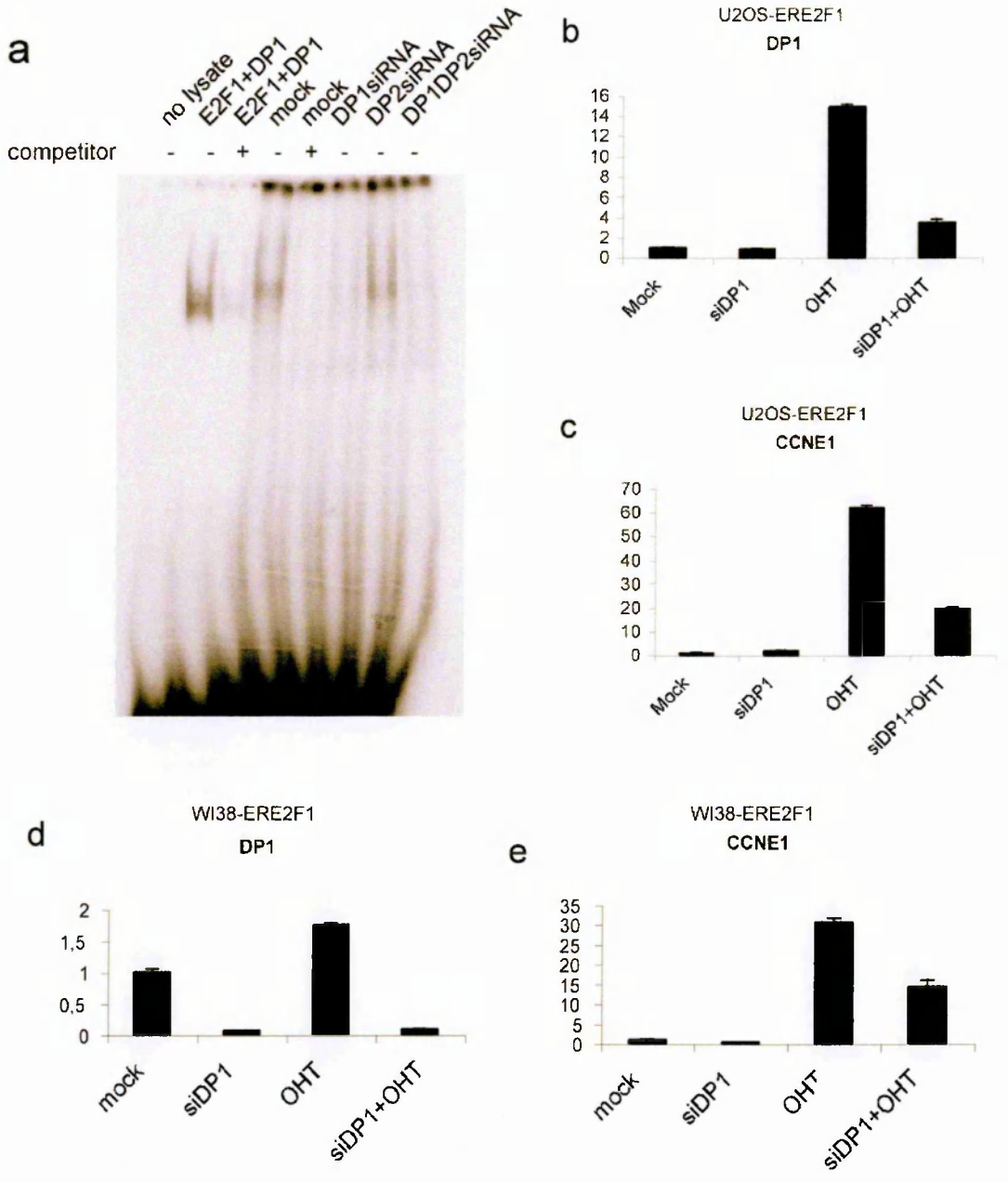


Figure 16 *Loss of DP1 abrogates E2F DNA binding activity.*
a, Gel retardation assay using whole extract from HeLa cells transfected with DP1 siRNA and DP2 siRNA either alone or in combination. As negative control the reaction was performed in the absence of lysate (no lysate) or with not transfected cells (mock). As positive control cells were transfected with E2F1 and DP1. An excess of cold probe was added to compete for the hot probe (competitor). ***b, c*** DP1 oligo was transfected in asynchronous U2OS ER-E2F1 cells for 48 h. GL2 siRNA was negative control (mock). qPCR was performed using specific primers for DP1, CCNE1 and GADP to normalize. Cells were incubated with/without OHT for 24 h. ***d, e*** As in (***b, c***) except that WI38 ER-E2F1 cells were employed.

while upon DP2 siRNA transfection most of E2F DNA binding activity was retained. These results show that loss of DP1, but not of DP2, abolishes E2F DNA binding activity and are in agreement with the fact that DP1 is ubiquitous and is the major protein family expressed.

Recently, our laboratory has generated an efficient system by which E2F1(-2-3) activity can be manipulated. In this systhem E2F1(-2-3) is fused to the estrogen receptor ligand-binding domain (Moroni et al., 2001; Müller et al., 2001; Vigo et al., 1999). To test *in vivo* the ability of DP1 siRNA to abrogate E2F activity in tumour and normal cells, I transfected U2OSER-E2F1 (Fig. 16*b*, *c*) and WI38ER-E2F1 (Fig. 16*d*, *e*) with DP1 siRNA and, after 48 h, I treated them for a period of 24 h with OHT to activate E2F1. In accordance with the result obtained by EMSA, ER-E2F1 activation induced *CCNE1* expression in mock-transfected cells. This activity was significantly reduced in DP1 siRNA interfered cells.

DP is required for tumour cell proliferation

I was interested in studying whether DP had a role in cell proliferation. So, I transfected HeLa cells with DP1 siRNA and DP2 siRNA alone or in combination: cells interfered for DP1, but not for DP2 did not grow compared to mock transfected cells (Fig. 17*a*). The intensity of BrdU signal was

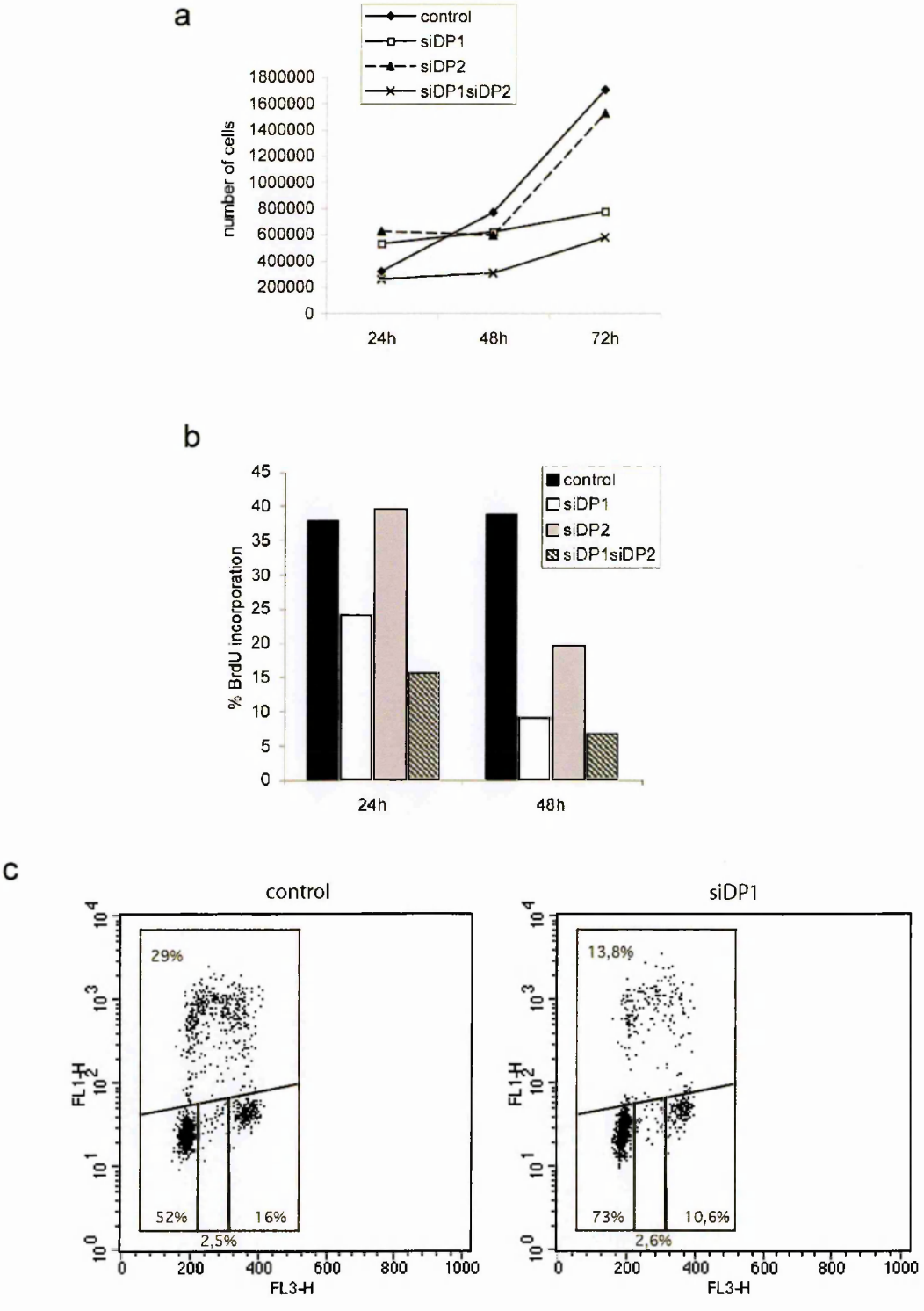


Figure 17 *DP1 is required for HeLa cell proliferation.*
DP1 or DP2 siRNA oligos were transfected in asynchronous HeLa cells alone or in combination. GL2 siRNA was negative control. ***a,*** The number of cells was assessed at the indicated time points by Trypan blue exclusion. ***b,*** DNA synthesis was assessed by BrdU incorporation (1 h pulse) after 24 h / 48 h of siRNA transfection. ***c,*** BrdU FACS analysis at 48 h. The 72 h timepoint has the same profile.

measured in situ and by FACS analysis (Fig. 17*b,c*), suggesting that cells lacking DP1 accumulated in G1 at 48 h and 72 h.

To control the specificity of the siRNA oligo for DP1, I designed another DP1 siRNA oligo and I compared the phenotypes generated by the two oligos in HeLa cells. Both of them downregulated DP1 protein level at 48 h (Fig. 18*a*) led to growth inhibition (Fig. 18*c*) and reduced the rate of BrdU incorporation (Fig. 18*b*). These results show that loss of DP1 impairs cell proliferation due to a defect in cell cycle progression.

It has been reported (Elbashir et al., 2001) that even a single mismatch between a siRNA and the target mRNA sequence abrogates silencing. Thus, I mutagenized DP1 in the target sequence of the siRNA oligo (oligo 1), introducing a silent point mutation (see Material and Methods). I transfected HeLa cells with expression vectors containing mutant DP1 or Wt DP1 cDNAs driven from the strong cytomegalovirus promoter and then I interfered the cells with DP1 siRNA. In transient transfection, DP1siRNA decreased the level of DP1 in cells transfected with Wt DP1, but not with DP1 mutant protein (Fig. 19*a*). By G418 selection, I established stable pools expressing mutant DP1 and then I transfected the cells with DP1 siRNA or with a control oligo. DP1 mutant expression did not vary (Fig. 19*b*), the number of viable

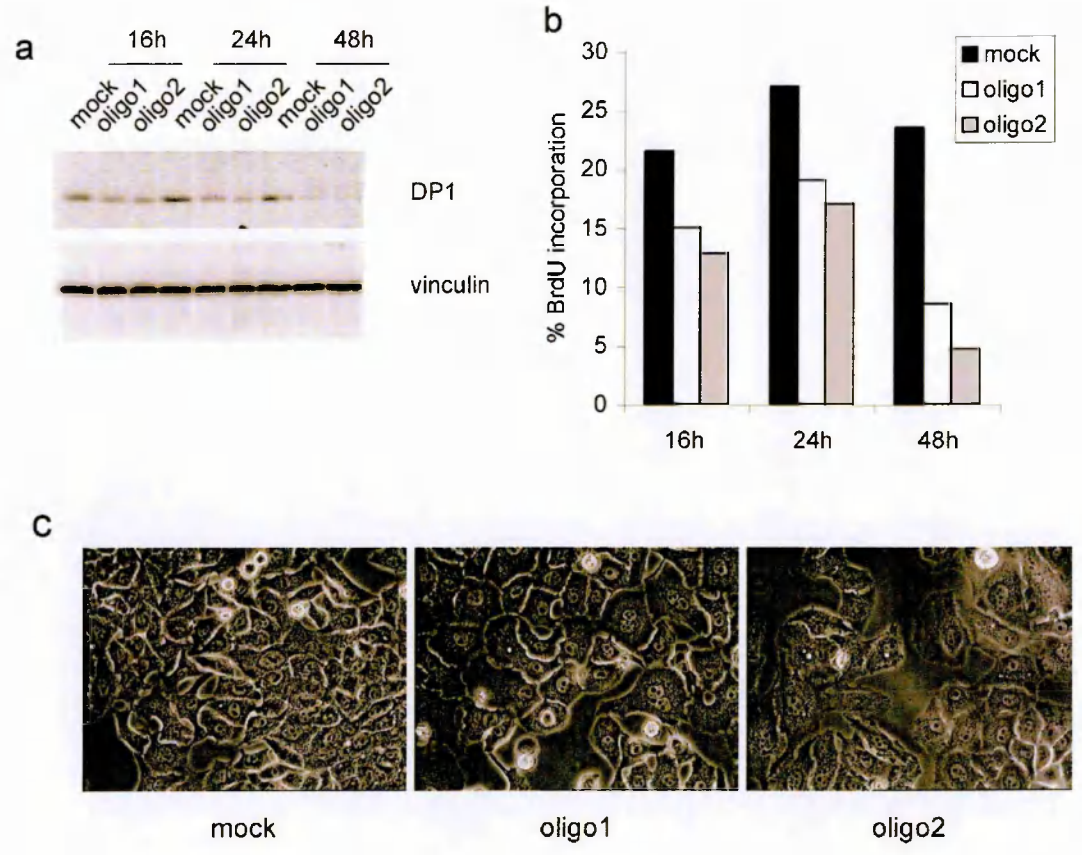


Figure 18 *Different DP1 siRNA show the same phenotype.*
Two DP1 siRNA were transfected in asynchronous HeLa cells for the indicated time points. GL2 siRNA was negative control (mock). ***a***, Western blot on total proteins. The blot was probed for DP1 and vinculin. ***b***, DNA synthesis was assessed by BrdU incorporation (1 h pulse) after 24 h / 48 h of siRNA transfection. ***c***, Cellular phenotype observed at 48 h.

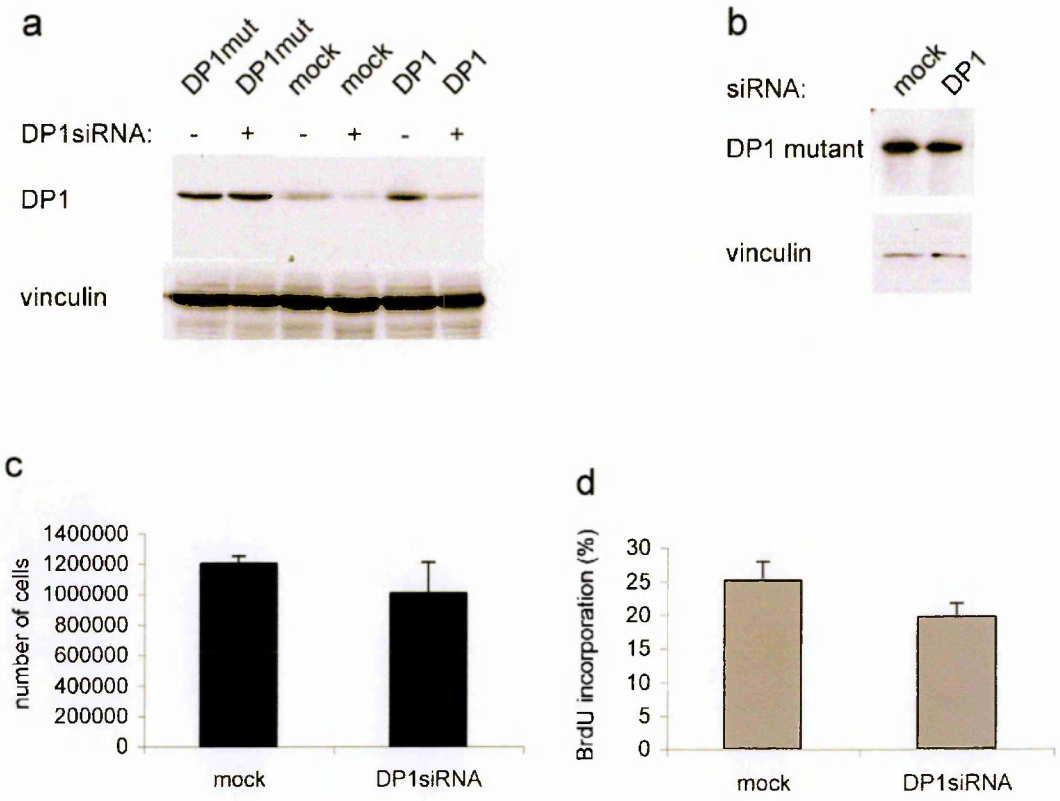


Figure 19 *A DP1 silent mutant rescues the proliferation defect induced by DP1 siRNA.*

a, HeLa cells were transfected with Wt DP1 or with a silent mutant of DP1 and after 24 hours they were interfered with DP1 siRNA (+) or GL2 siRNA (-). Western blot was performed on total proteins. ***b,*** As in (***a***), except that the cells were selected with 750 μg/ml of G418 to obtain a stable pool of cells expressing the DP1 mutant. ***c,*** The number of cells was assessed by Trypan blue exclusion on the pool after transfection with DP1 siRNA or GL2 siRNA (mock). ***d,*** BrdU incorporation (1 h pulse) after transfection with DP1 siRNA or GL2 siRNA.

cells did not vary significantly (Fig. 19*c*), and BrdU incorporation did neither (Fig. 19*d*). This result confirms that the growth arrest observed upon DP1 siRNA transfection is a specific response.

Then, I asked whether the growth arrest due to loss of DP expression was specific to HeLa cells or was a common response in tumour cell lines. SAOS2 cells are transformed cells that express neither pRB nor p53. Loss of pRB increases the level of E2F activity. I transfected SAOS2 cells with DP1 siRNA and DP2 siRNA, either alone or in combination. The protein level of DP1 was strongly reduced at 48 h (Fig. 20*a*). Transfection of DP2 siRNA caused an increase in DP1 protein level: the compensation effect suggests that DP2 siRNA oligo is functional. The number of BrdU positive cells was significantly reduced after DP1 siRNA transfection (48 h and 72 h), alone or in combination with DP2 (Fig. 20*b*), in accordance with the phenotype observed in HeLa cells.

DP is required for normal cell proliferation

I was also interested in studying whether depletion of DP had an effect on normal cell proliferation. I did not succeeded in transfecting human diploid fibroblasts with high efficiency by oligofectamine reagent (Invitrogen). This did not allow me to collect clear evidences that DP was required for cell proliferation in normal cells.

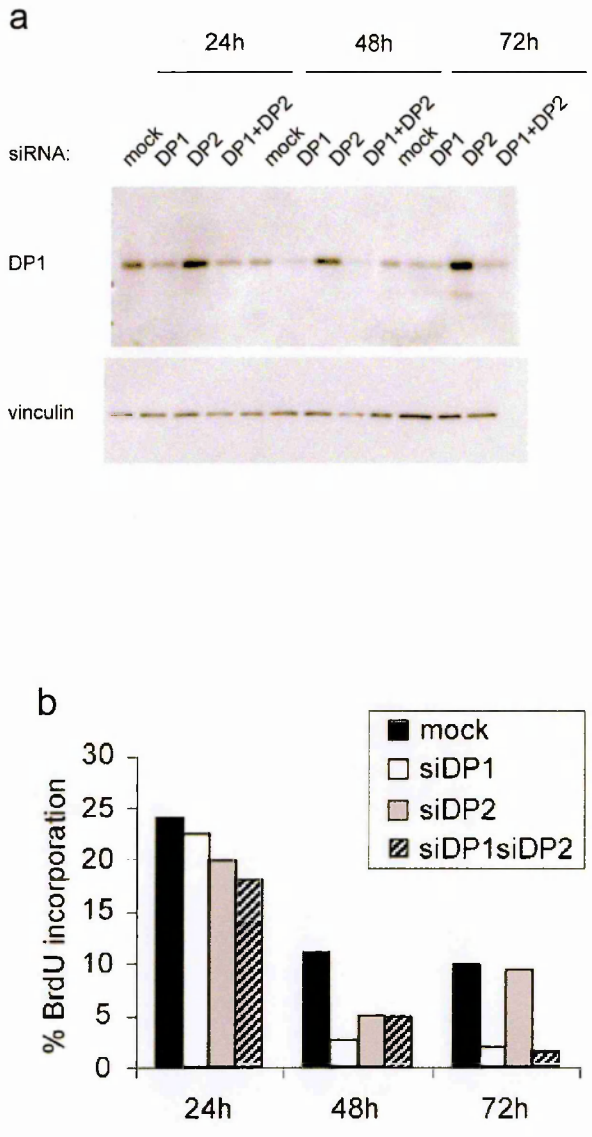


Figure 20 *DP1 is required for cell proliferation in SAOS2 cells.* Asynchronous SAOS2 cells were transfected with DP1 or DP2 siRNA oligos either alone or in combination. GL2 siRNA was negative control (mock). ***a,*** DP1 and vinculin protein levels were assessed by Western blotting. ***b,*** DNA synthesis was assessed by BrdU incorporation (1 h pulse) after 24 h, 48 h, and 72 h of siRNA transfection.

A system for stable expression of short interfering RNAs in mammalian cells has been reported (Brummelkamp et al., 2002a; Brummelkamp et al., 2002b). I infected WI38 and U2OS cells with pRetroSuper DP1 or empty vector but I could not see any variation in DP protein level (Fig. 21*a*). U2OS cells expressing the murine ecotropic receptor were generated (Brummelkamp et al., 2002a) to allow infection by ecotropic virus. This resulted in a 50% reduction of DP1 protein level (Fig. 21*b*), but it was not enough to observe a significant growth arrest (Fig. 21*c*). In my hands stable expression of short interfering RNAs was no as efficient as transient transfection that completely downregulated protein level (Fig. 21*d*) and led to growth arrest (Fig. 21*e,f*). A possible reason for this discrepancy is that during infection there is a selection against the cells that express low level of DP1, since they do not grow.

Transient transfection with lipofectamine 2000 (see Material and Methods) allowed me to achieve a 70% of transfection efficiency in diploid fibroblasts. Recent papers suggest that the specificity of siRNA is concentration dependent (Chi et al., 2003; Semizarov et al., 2003) and a concentration of 100 nM siRNA non specifically induces a significant number of genes, many of which are involved in apoptosis and stress response. I did titration experiments (with 100 nM siRNA, 50 nM and 20 nM) to optimize transfection in TIG3 cells. A concentration of 20 nM siRNA was inefficient in inhibiting protein expression. Using a concentration of 50 nM siRNA allowed me to abolish DP1 protein expression to the same extent of 100 nM (Fig. 22*a*) and to

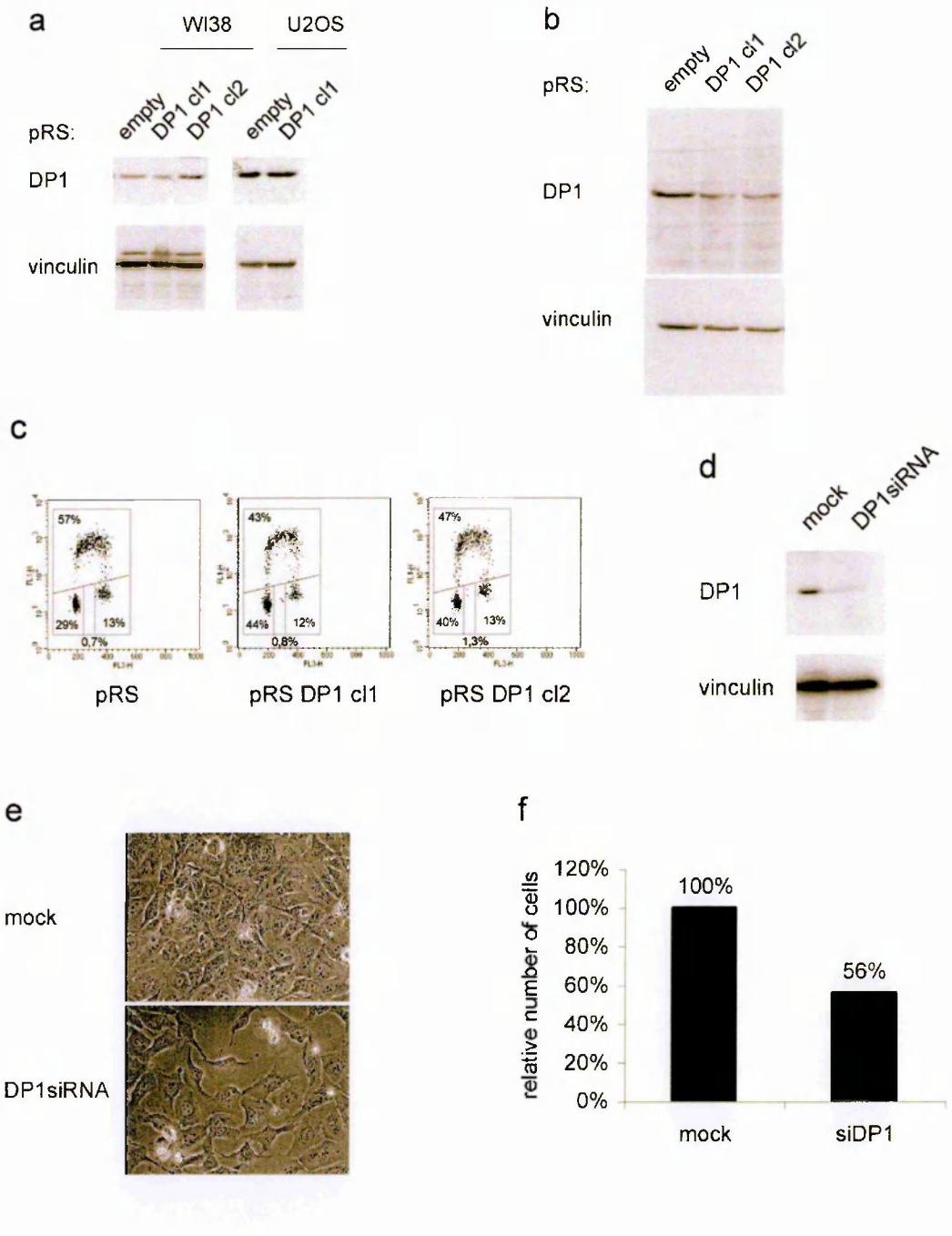


Figure 21 *Expression of pRetroSuper DP1.*
a, WI38 and U2OS cells were infected with pRetroSuper vector expressing DP1 siRNA (two independent clones: cl1 and cl2) or with empty vector (empty) and selected with 1 µg/ml of puromycine for 4 days. DP1 and vinculin protein levels were assessed by Western blotting. ***b,*** U2OS cells expressing ecotropic receptor were infected as in (***a***). ***c,*** BrdU FACS (20 min pulse) in U2OS cells expressing ecotropic receptor infected with two clones of pRetroSuper DP1 (cl.1 and cl.2) or with empty vector. ***d,*** Asynchronous U2OS cells were transfected with DP1 siRNA or GL2 siRNA (mock). DP1 and vinculin protein levels were assessed by Western blotting. ***e,*** Cellular phenotype observed at 72 h and relative number of cells (***f***).

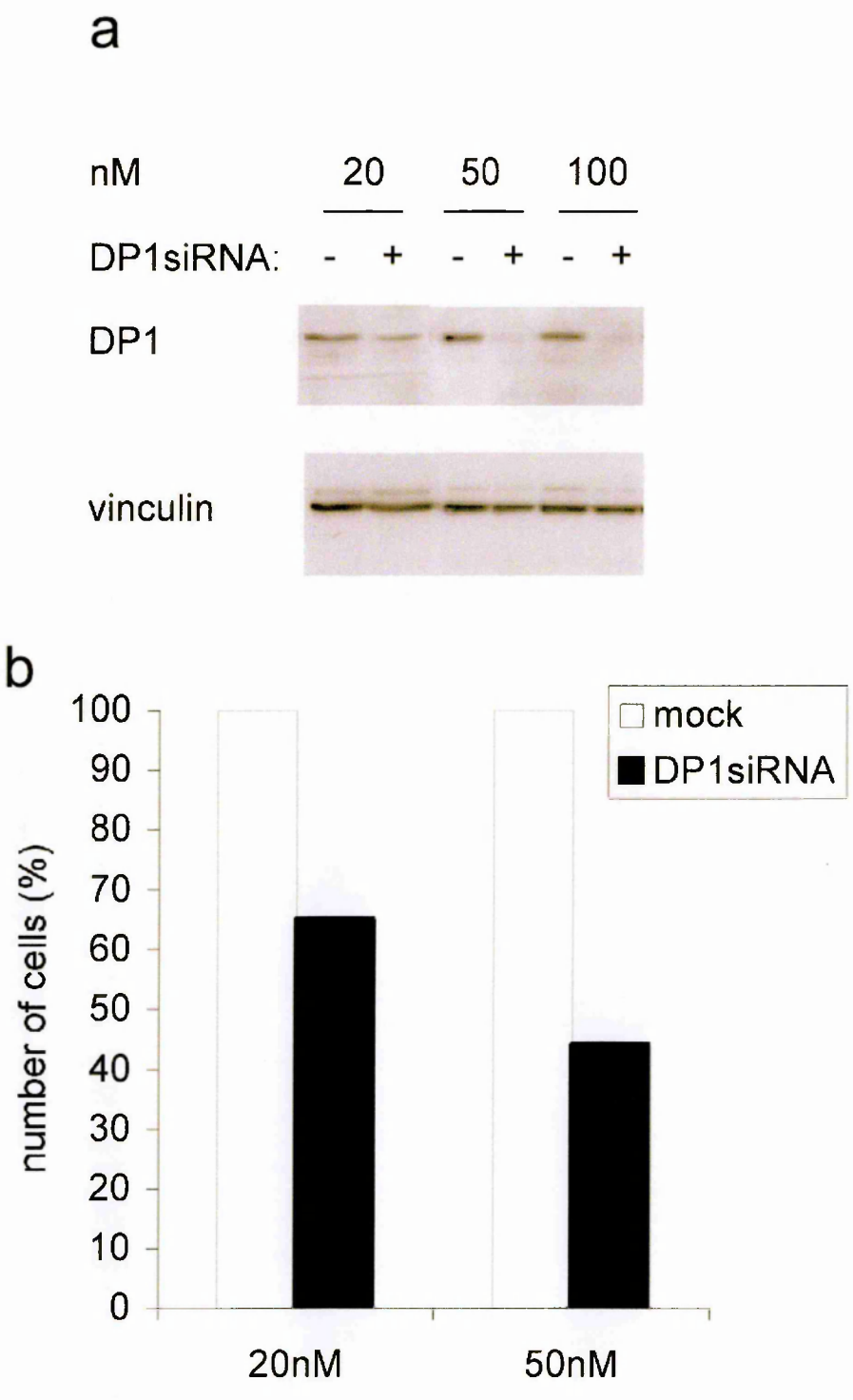


Figure 22 *Transfection of DP1 siRNA in TIG3 cells.*
a, TIG3 cells were transfected by lipofectamine 2000 (Invitrogen) with the indicated amount of DP1 siRNA (+) or GL2 siRNA (-) as control for 48 h. DP1 and vinculin protein levels were detected by Western blotting on total protein extracts. ***b,*** The number of cells was assessed by Tripan blue exclusion.

appreciate differences in cell viability between mock and DP1 siRNA interfered cells (Fig. 22*b*).

IMR90 cells transfected with 50 nM DP1siRNA did not grow compared to control cells (Fig. 23*a*). Loss of DP1 in WI38 (Fig. 23*d*) or TIG3 (Fig. 23*e*) reduced BrdU incorporation (Fig. 23*b,c,f*) and the number of cells (Fig. 23*g*).

As additional control, I compared the effect of suppression of DP1 between WI38 fibroblasts and VA13 cells (which are derived from WI38 after SV40 transformation and thus do not express p53 and pRB) (Fig. 24*a*). In both cases the growth rate of cells lacking DP expression was around 50% compared to control cells (Fig. 24*b,c*).

Loss of DP results in targets repression

Since loss of DP1 compromises E2F-DNA binding activity (Fig. 16*a*), I wished to determine whether it altered the expression of E2F responsive genes. These changes in gene expression identify transcriptional events that depend on the endogenous DP protein. I used parallel cell extracts where I measured BrdU incorporation or cell viability (above experiments). RNA was isolated from depleted and control cells, and changes in gene expression of known E2F target genes were monitored by qPCR analysis. Gene expression was normalized according to the level of *GADP*. I verified that DP1 mRNA was decreased upon DP1 siRNA transfection. Known E2F responsive gene

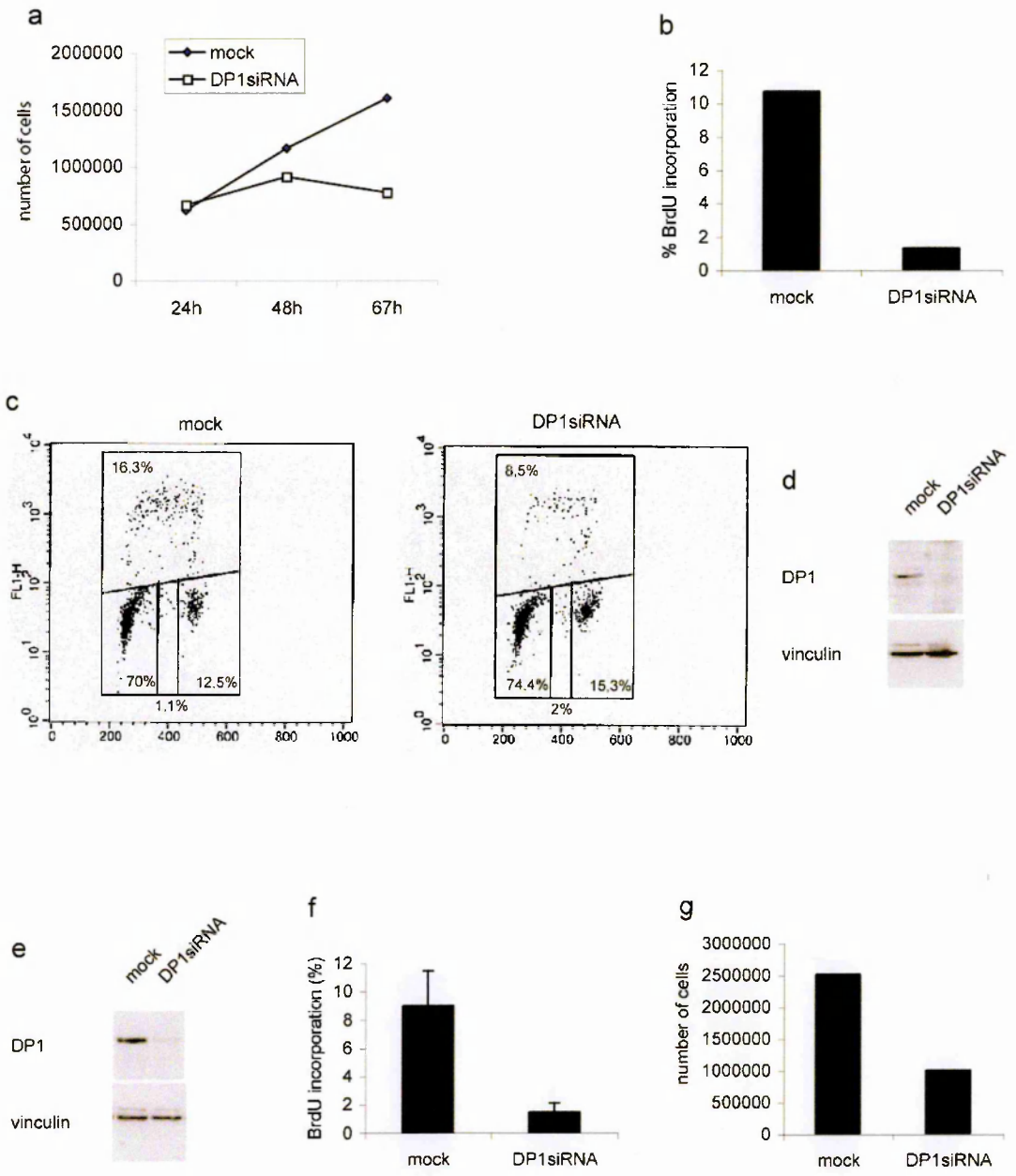


Figure 23 *DP1 is required for cell proliferation in diploid fibroblasts.*
a, IMR90 fibroblasts were transfected with 50 nM of DP1 siRNA or GL2 siRNA (mock) and the number of cells was assessed by Trypan blue exclusion. ***b, c, d*** WI38 fibroblasts were transfected as in (***a***), BrdU incorporation (1 h pulse) was assessed in situ (***b***) and by BrdU FACS (***c***). DP1 and vinculin protein levels were detected (***d***). The results are representative of three independent experiments. ***e, f, g*** TIG3 cells were transfected as in (***a***). DP1 protein level, BrdU incorporation and the number of cells were assessed. The results are representative of two independent experiments.

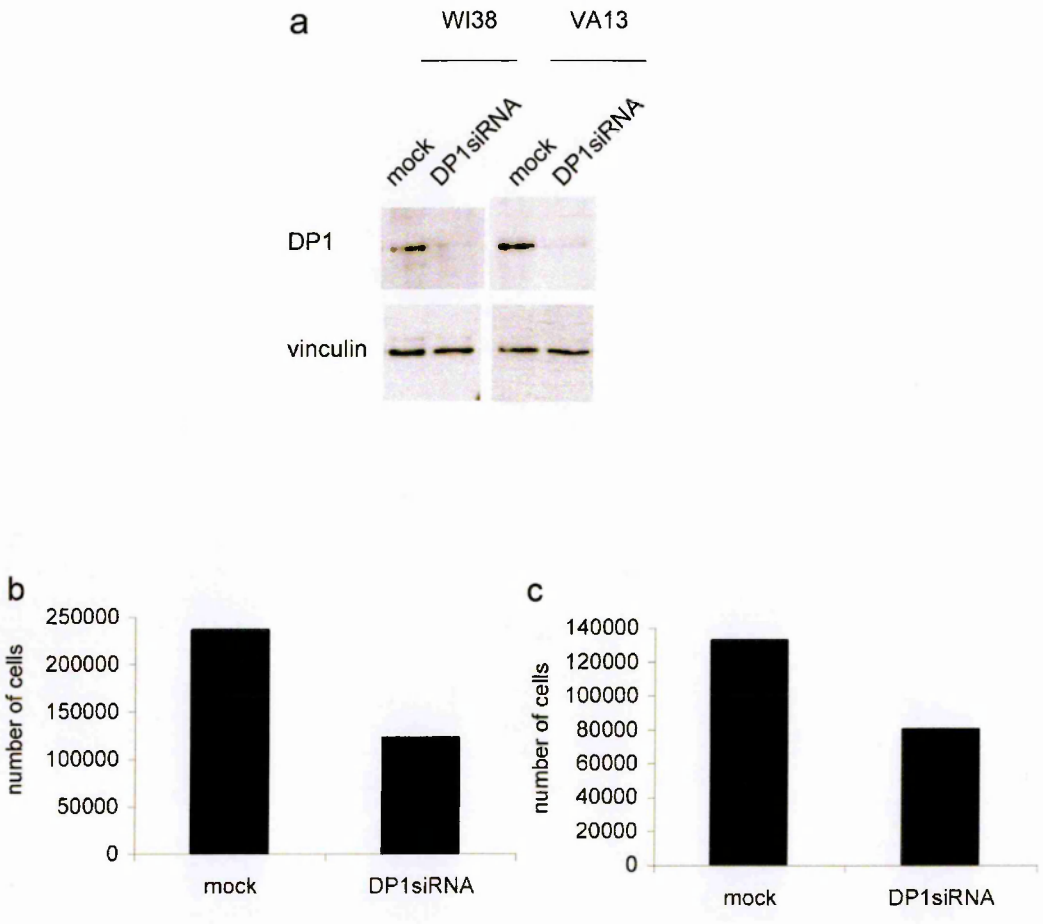


Figure 24 *Lack of p53 and pRB does not rescue the proliferation defect in diploid fibroblasts.*
a, WI38 fibroblasts and the paired cell line VA13 were transfected with DP1 siRNA or GL2 siRNA (mock) for 48 h and DP1 protein level was detected; the number of cells was measured in WI38 (***b***) and in VA13 (***c***) by Trypan blue exclusion.

transcripts were tested, such as *CCNE1*, *CDC25A*, *CDC6*, *RRM2*, *TK*, *DHFR*, and *E2F1*. Loss of DP1 produced a significant reduction in all transcripts compared to control in HeLa cells (Fig. 25*a*), in WI38 (Fig. 25*b*) and TIG3 fibroblasts (Fig. 25*c*). These data indicate that loss of DP1 significantly impairs the expression of most E2F responsive genes both in tumour and in normal cells.

DISCUSSION

I present evidence that loss of DP1 compromises E2F DNA binding activity, impairs the rate of cell proliferation of both primary and transformed cell lines and represses the expression of E2F responsive genes. In stark contrast to the milder phenotypes that result from inactivation of the *E2Fs*, loss of *DP1* in mice leads to death in utero because of dramatic DNA replication defects in extra embryonic tissues (Kohn et al., 2003). Unlike extra-embryonic tissues, no proliferation defects are observed in the DP1 deficient embryos prior to lethality suggesting that many cell cycles and DNA replication can occur without DP1. However, the biochemical effect of DP depletion was not analyzed with respect to E2F transactivating activity, so we do not know the amount of E2F left. In addition, we cannot exclude that DP1/DP2 levels in the embryo can be influenced through a maternal effect. To study the requirement of DP1 in embryonic development it would be useful to get DP1 floxed mice.

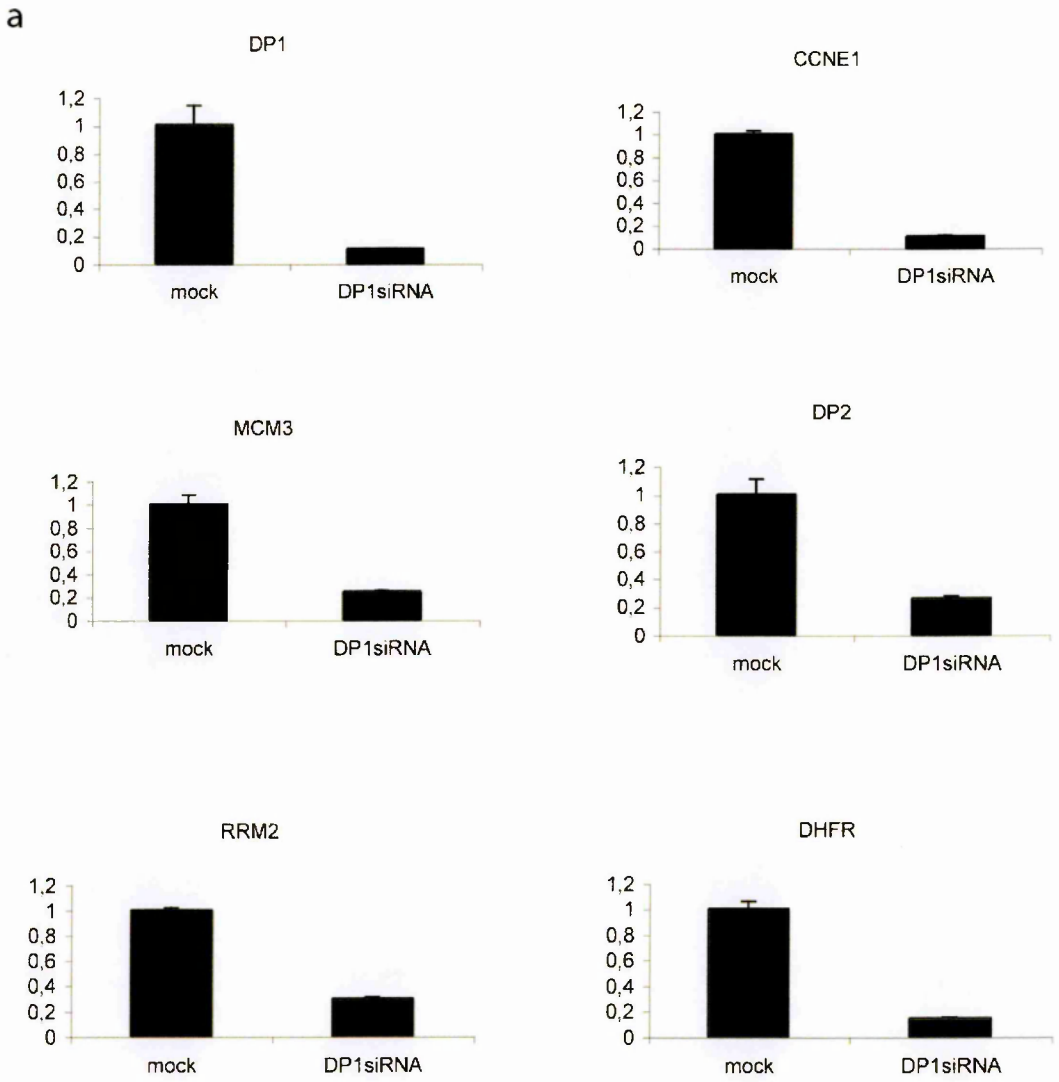
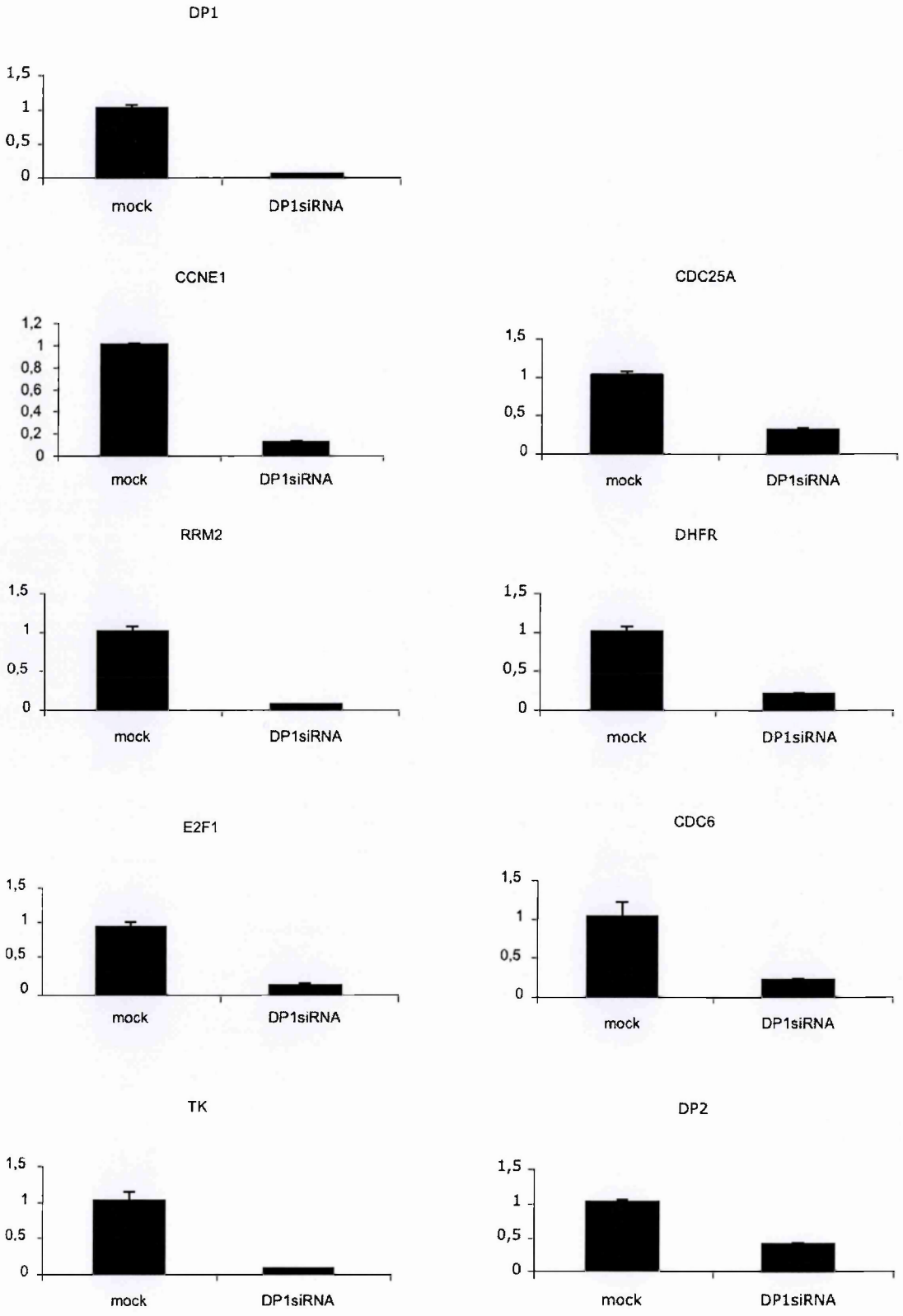


Figure 25 *Loss of DP1 results in E2F targets repression.*
a, DP1 siRNA or GL2 siRNA (mock) were transfected in asynchronous HeLa cells for 48 h. qPCR was performed using specific primers for *DP1*, *DP2* *CCNE1*, *MCM3* and *RRM2*. *GADP* levels were used for normalization. ***b,*** WI38 fibroblasts were transfected as in (***a***) and qPCR was performed with specific primers for *DP1*, *DP2*, *CCNE1*, *CDC25A*, *CDC6*, *RRM2*, *DHFR*, *TK* and *E2F1*. ***c,*** TIG3 cells were transfected and processed as in (***b***). The results are representative of at least two independent experiments.

b


DP1
1,5
1
0,5
0
mock
DP1siRNA
CCNE1
1,2
1
0,8
0,6
0,4
0,2
0
mock
DP1siRNA
CDC25A
1,5
1
0,5
0
mock
DP1siRNA
RRM2
1,5
1
0,5
0
mock
DP1siRNA
DHFR
1,5
1
0,5
0
mock
DP1siRNA
E2F1
1,5
1
0,5
0
mock
DP1siRNA
CDC6
1,5
1
0,5
0
mock
DP1siRNA
TK
1,5
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DP2
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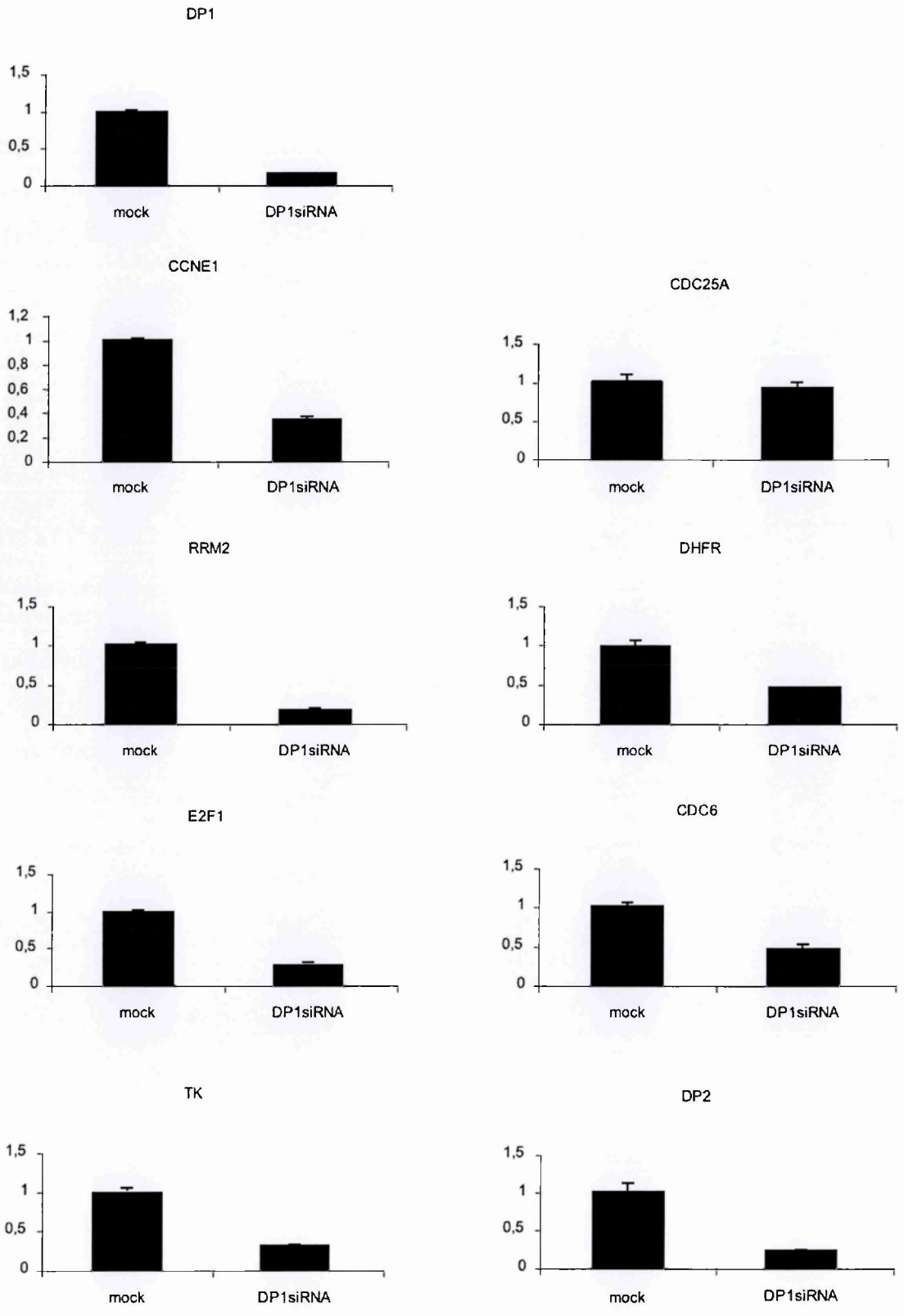
mock
DP1siRNA

C


DP1
1,5
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CDC6
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TK
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DP2
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In the literature several evidences arise stressing that E2F mainly functions as a repressor. Classic promoter mapping and in vivo footprinting studies concluded that repressive E2F complexes regulate many E2F-responsive genes during G0/G1 and that the promoters are unoccupied during G1/S transition when the genes are actively transcribed (Dalton, 1992; Huet et al., 1996; Le Cam et al., 1999; Neuman et al., 1994; Tommasi and Pfeifer, 1995; Zwicker et al., 1996) (Fig 26*a*). Plasmids containing multiple E2F-binding sites were used to titrate RB-E2F repressor complexes (He et al., 2000) and the cells failed to arrest in G1 following accumulation of endogenous hypophosphorylated RB. A dominant-negative mutant of E2F1, which contains the DNA-binding domain but lacks the RB-binding site and transactivation domain, was used to displace RB-E2F complexes from E2F-responsive genes. The expression of this mutant prevented RB-dependent arrest in G1 by either p16 or TGF-β (Zhang et al., 1999). This has been interpreted as a result of transcriptional derepression of E2F target genes, whose downregulation is critical for the establishment of G1 arrest. These studies, however, do not show that E2F does not have a role in transcriptional activation in the cell cycle and it is unclear whether or not the binding of free E2F to endogenous promoters was completely eliminated.

My results agree with previous findings showing, through overexpression systems, that transcriptional activation by E2F is important for the progression of cells through the cell cycle (Johnson et al., 1993; Qin et al., 1995). In these

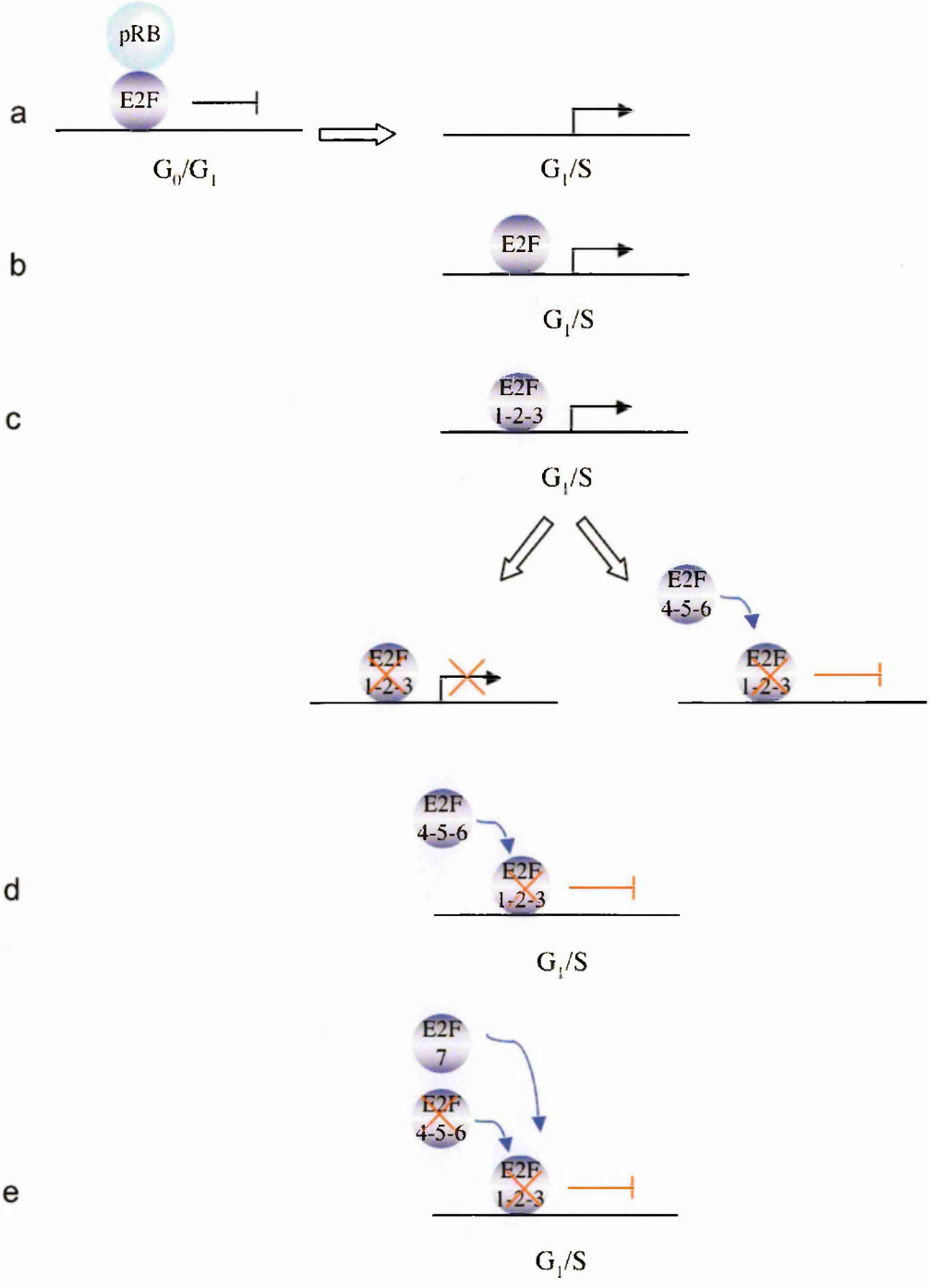

Figure 26 *Models for the regulation of transcription by the E2Fs.*
a, Displacement of repressive pRB-E2F complexes at G1/S transition results in targets derepression. ***b,*** E2F allows a burst of gene expression as cells enter S phase. ***c,*** In E2F1-2-3 TKO MEFs there is no S phase and gene expression is decreased. However, E2F4-5-6 could replace the missing E2Fs on promoters and repress transcription. ***d,*** DP1siRNA knocks down all E2F activity mediated by DP1, arrests the cells and decreases gene expression. ***e***, E2F7 binds DNA in a DP independent manner, may replace the missing E2Fs on promoters and repress transcription.

studies expression of Wt E2F1 induced S phase (Fig. 26*b*). This induction was dependent on the ability of E2F1 to bind DNA and to transactivate E2F dependent promoters, since the DNA binding deficient mutant (E2F1 E132) and the transactivating deficient mutant (E2F1 1-374) did not induce S phase. However, the system leads to loss of target specificity resulting from secondary changes in gene expression due to progression through the cell cycle. My experiments performed in HeLa and in VA13 cells suggest that the transactivation by free E2F is required for proliferation (Fig. 26*b*). Indeed, HeLa cells express the oncoprotein E7, while VA13 are SV40-transformed, thus in both cases almost all of E2F is in the free transactivating form.

Studies in which *E2f* genes have been deleted in mice have failed to demonstrate that transactivation is the primary function of *E2f* in cell cycle regulation, because of redundancy and functional compensation among the *E2f* family members. An evidence that E2f1, E2f2, and E2f3 are required to induce S-phase and activate E2F target gene expression has been provided recently by the generation of a conditional E2f1, E2f2, E2f3 triple knockout (TKO) mouse (Wu et al., 2001). TKO cells are defective for S-phase entry and progression through the cell cycle and show a dramatic decrease in the expression of many E2f-regulated genes. However, the phenotypes seen when E2f1, E2f2, E2f3 are removed could be viewed as a gain in activity of the repressor E2f complexes (Fig. 26*c*). By DP1 siRNA I knocked-out all E2F DNA binding activity mediated by DP proteins including the 'repressive'

E2F4, E2F5 and E2F6 (Fig. 16*a*). My results are in agreement with data obtained by overexpression of a dominant negative form of DP1 (that retained E2F binding, but not DNA binding), which arrested SAOS2, C33A and U2OS cells in the G1 phase of the cell cycle (Wu et al., 1996) (Fig. 26*d*).

I did not observe any apoptotic effect of the DP1 siRNA in the experiments presented here up to 72 hours of DP1 siRNA expression. In tumour cell lines that lack p53 expression (SAOS2, VA13) and thus cannot undergo p53 dependent apoptosis, DP1 produced the same phenotype as observed in diploid fibroblasts (Fig. 20 and 24). These results are in agreement with the fact that inactivation of *p53* in mice is unable to rescue the *DP1*-dependent embryonic lethality (Kohn et al., 2003). Thus, the consequence of loss of DP in diploid and in tumour cells is G1 arrest and not apoptosis or DNA replication. Moreover, intact p53 and pRB are not required to prevent the growth arrest upon loss of DP1 and DP1 is rate limiting both for the proliferation of tumour and normal cells. The G1 arrest induced by DP1 siRNA is rescued by coexpression of a silent DP1 mutant (Fig. 19).

CONCLUDING REMARKS

Although mutation in genes encoding pRB or upstream regulators of pRB is frequently found in human tumours, intragenic mutations in the genes encoding the E2F and DP transcription factors have not been isolated. This may be due to functional compensation by related E2F / DP activity (Dyson, 1998).

Current models of deregulation of DNA replication in cancer cells are based on the observation that increased E2F activity is sufficient to induce DNA replication in immortalized quiescent cells in the absence of growth factors (Dimri et al., 1994; Johnson et al., 1993; Lukas et al., 1996; Qin et al., 1994; Shan and Lee, 1994). I determined the effect of E2F activation in diploid fibroblasts and I found that suppression of the p53- or pRB- mediated G1 checkpoint is required for E2F- induced S phase entry. In addition to act as an E2F-dependent transcriptional repressor, my data suggest that pRB is required to retain the G1 checkpoint in response to unprogrammed proliferative signals. This raises the possibility to investigate whether the mechanism involves direct binding of pRB to other potential pRB targets in addition to E2F, such as HBP1, C/EBPα or MyoD (Lipinski and Jacks, 1999).

To understand how cell proliferation is regulated, it is important to know if the

E2F/DP heterodimers function either as activators or repressors of transcription. My studies provide a further understanding in this direction, defining a crucial role for DP1 in cell proliferation. This is essential for a number of pharmaceutical companies that are developing drugs to the E2F transcription factors. siRNA against DP will be a useful tool to test whether E2F/DP activity is required in biological responses other than proliferation, such as apoptosis and differentiation. We have yet to understand how E2F like proteins that bind DNA in a DP independent manner fits into the model. It is unclear whether, for instance, E2F7 can replace the missing E2F activities and can repress E2F target genes in the absence of DP (Fig. 26*e*).

The results discussed in this thesis enlight two aspects of E2F activity, first demonstrating the molecular mechanism by which p53 and pRB govern E2F activity to control the transition from G1 to S phase, second analysing how cell proliferation is regulated by E2F activity.

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