MOLECULAR ANALYSIS OF THE ROLE OF E2F PROTEINS IN THE pRB PATHWAY

by

Kenneth H. Moberg, Jr.

B.S., Biology
Haverford College, 1989

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of

Ph. D. in Biology

at the
Massachusetts Institute of Technology
June, 1998
[September 1998]

Signature of Author: _____
Kenneth H. Moberg, Jr.

Thesis Supervisor: _____
Jacqueline A. Lees
Assistant Professor of Biology

Certified by: _____
Terry Orr-Weaver
Professor of Biology
Chairman, Committee on Graduate Students

For my parents, Zandra and Ken, who knew what really mattered,

and set me on my way

ABSTRACT

The E2F family of transcription factors appear to represent the primary cellular target of the tumor suppressive properties of the retinoblastoma protein. E2F therefore functions in a pathway which is a frequent target in human cancer, and the tumorigenicity of these mutations may be mediated at the transcriptional level by E2F. E2F is also regulated by cell cycle-dependent interactions with the pRB-related proteins p107 and p130. Unlike pRB, mutations in p107 or p130 are not associated with cancer. The different properties of the pRB family may result from the manner in which each protein regulates E2F. To determine how individual E2Fs contribute to the cell cycle regulatory properties of pRB, p107 and p130, we have examined the regulation of individual members of the E2F family. Our data suggest that the induction of E2F responsive genes is primarily due to the loss of nuclear repressor complexes at G1/S. This loss correlates with the disappearance of nuclear forms of E2F-4 protein, which represents the majority of pRB-bound nuclear E2F during G1. These data suggests that E2F-4, the most abundant E2F *in vivo*, acts primarily as the DNA-binding component of a G1 transcriptional repressor complex. In contrast, we find that E2F-1, -2 and -3 are present at low levels *in vivo* and localize to the nucleus by virtue of a nuclear localization signal sequence in the N-terminal domain of these proteins. Their constitutive nuclear localization suggests that these E2F family members will contribute to the activation of responsive gene transcription during S-phase. Together, these data suggest that induction of E2F-responsive genes at G1/S is triggered both by the loss of an abundant transcriptional repressor, E2F-4•pRB, and by the presence of nuclear forms of E2F capable of transcriptional activation. These functional differences among E2Fs may underlie the oncogenic consequences specifically associated with pRB loss. Inactivation of pRB is predicted to both abrogate repression of E2F-responsive genes, and relieve inhibition of nuclear, activatory E2Fs. The combined effect of these forms of transcriptional deregulation of the E2F pathway may be sufficient to promote transformation *in vivo*.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

A. The cell division cycle: an overview

Replication of genetic material in one cell and its proper segregation into each of two daughter cells is the central function of the cell division cycle. Proper control of this cycle is of fundamental importance to the viability of all organisms. It ensures the fidelity of DNA duplication and is required for proper regulation of cell multiplication in both single-cell populations and in complex multicellular organisms. Deregulation of cell division can lead to lethality or, in the case of multicellular eukaryotes, loss of developmental control of growth and differentiation. Consequently, cell division is a tightly controlled process subject to many forms of regulation which ensure that daughter cells receive exact copies of the genome, and that the cell division process only initiates under appropriate conditions.

The eukaryotic cell division cycle is divided into four discrete stages, termed Gap1 (G1), DNA Synthesis (S), Gap2 (G2) and Mitosis (M), which follow in that order (reviewed in Nasmyth, 1996). Each stage is marked by a distinct set of cellular processes and structures. Early G1 cells are comparatively small and have a low ratio of cytoplasmic-to-nuclear volume as a result of partitioning during the previous mitosis. As cells progress through G1, active synthesis of cellular components necessary for completion of the next cycle results in expansion in cell size. Eventually, the process of DNA replication is triggered and the cell enters S-phase, during which the enzymatic process of DNA replication produces a copy of the entire genome. During the following G2 phase, the cell prepares for mitosis by synthesizing components of the mitotic apparatus. Chromosome condensation, formation of a mitotic spindle and pairing of homologs along the metaphase plate mark entry into Mitosis (M), during which separation of homologs and

cytokinesis produces two daughter cells. This G1-S-G2-M cycle represents the various stages a proliferating population of cells moves through. However, most cells in a developed organism are actually in a state which corresponds to a cessation of division and entry into a quiescent state called "G0". Cells in G1 which have either failed to receive the required growth factor stimulation or have been signaled to cease division enter this G0 state from G1, and so G0 is considered to be an adjunct stage of G1. In some cell types, G0 represents a state of transient arrest, exit from which is dependent upon proper stimulation - in the case of resting B and T cells of the peripheral immune system, G0 exit is dependent upon antigen binding and cytokine signaling that normally occur in the context of an immune response (Nourse et al., 1994). In other cell types, entry into G0 is thought to represent a irreversible withdrawal from the cell cycle necessary for terminal differentiation.

Progression through the various stages of the cell cycle is subject to control by signals from the extracellular environment as well as those emanating from within the cell. Eukaryotic cells arrest the cell cycle in response to a variety of stimuli, including secreted inhibitors of cell growth, nutrient starvation, loss of attachment to substratum, high cell density, and absence of necessary growth factors in the extracellular environment (reviewed in Murray, 1993). Signals which originate from within the cell are also capable of inducing pathways which result in cell cycle arrest. DNA damage incurred during the process of replication, or caused by exposure to genotoxic agents, results in the activation of cell-autonomous "checkpoint" pathways which transiently arrest progression and allow the cell to mount a proper response (reviewed in Elledge, 1996). Checkpoint pathways monitor multiple components of the cell cycle and can likewise arrest the cycle when the integrity of these components is compromised. The specific mechanisms that underlie some checkpoint arrests remain unclear. However, studies of checkpoint mechanisms in single-celled eukaryotes have begun to reveal a conserved mode of action which is dependent upon the ability of checkpoint-specific molecules to target and modulate the activities of more basic components of cell cycle control (eg. Furnari et al., 1997; Hwang et al., 1998; Sanchez et al., 1997; Sidorova and Breeden, 1997).

A1. Molecular mechanisms of cell cycle control in yeast and frog

Current models of the molecular mechanisms of cell cycle control have their origins in studies of the genetic requirements for cell cycle progression in the yeasts *Sacchromyces cerevisiae* and *Schizosacchromyces pombe*, and in the biochemical analysis of the regulation of oocyte maturation in the amphibian *Xenopus laevis* (reviewed in Nasmyth, 1996). Genes required for cell cycle progression in budding and fission yeast were identified as temperature-sensitive mutations that gave a uniform arrest at a particular cell cycle stage(s) when shifted to the restrictive temperature (Hartwell et al., 1974; Nurse and Thuriaux, 1980). These mutations were designated as cell-division cycle (CDC) genes due to their apparent roles in regulating the cell division process.

One such CDC gene was identified as necessary for cell cycle progression in both budding and fission yeast. This gene, termed *cdc28* in *S. cerevisiae* and CDC2 in *S. pombe*, encodes a serine/threonine protein-kinase that appears to play a fundamental role in cell cycle control as an S and M phase "promoting factor" (Hindley and Phear, 1984; Lorincz and Reed, 1984; Reed et al., 1985). This kinase represents the catalytic moiety of a multimer, and is only active when complexed to a class of regulatory proteins called *cyclins*, so named because their levels oscillate throughout the cell cycle (Evans et al., 1983). The abundance of these cyclins subunits is subject to cell cycle-dependent control at both the transcriptional level and at the post-transcriptional level of proteolytic degradation (Aerne et al., 1998; Cross et al., 1994; Walker et al., 1997; reviewed King et al., 1996). Once assembled with catalytic cyclin-dependent kinase (CDK) subunits, the activity of cyclin•cdk complexes is subject to a further level of regulation by both inhibitory and activating phosphorylations on specific cdk residues mediated by yet other kinases and phosphatases (reviewed in Elledge, 1996). Concurrent studies of the regulation of entry into mitosis in *Xenopus* identified a biochemical activity capable of promoting entry in M-phase in immature G_2-arrested oocytes which was called Maturation Promoting Factor (MPF). Identification of the components of MPF activity in *Xenopus* oocytes revealed that it contained a CDK activity homologous to that identified in yeast CDC screens (Draetta et al., 1989; Dunphy et

al., 1988; Gautier et al., 1988). This concordance between yeast genetics and *Xenopus* biochemistry revealed a conserved role for CDKs as regulators of the eukaryotic cell division cycle and has significantly advanced understandings of how eukaryotic cells regulate progression through specific stages of the cell cycle.

The cell cycle events catalyzed by cyclin•cdk kinases represent irreversible changes in the state of the cell, when it ceases performing tasks associated with one stage of the cell cycle and begins to execute functions associated with the next stage. *In vivo*, different cyclin•cdk complexes promote specific cell cycle stages. The cell cycle function of the single *S. cerevisiae* CDK, cdc28, was first uncovered by temperature-sensitive arrest of a *cdc28-ts* allele at two points in the yeast cell cycle: in G1 just prior to S-phase, and at G2/M (Piggott et al., 1982). The role of a single yeast CDK in the regulation of multiple cell cycle transitions reflects the ability of Cdc28 to associate successively with different cyclin subunits to promote different cell cycle events. Activation of Cdc28 by the G1 cyclins (Cln1, Cln2 and Cln3) drives cells through G1 and into S, while the Clb5 and Clb6 cyclins promote S-phase progression and the G2/M cyclins (Clbs 1, 2, 3, & 4) drive cells into and through mitosis (reviewed in Nasmyth, 1996). The ability of different cyclin subunits, in association with the same catalytic Cdc28 molecule, to promote different cell cycle phases suggests that the functional specificity of the various different cyclin•Cdc28 complexes may largely lie with the cyclin molecule. This appears to be at least partially the case, as the cyclin subunits have been shown to contribute to the target specificities of intact cyclin•cdk complexes *in vitro* and *in vivo* (Huang et al., 1998; Levine et al., 1996; Peeper et al., 1993).

A2. Mammalian CDKs

Identification of CDK activities in a wide range of organisms has verified the fundamental role these serine/threonine kinases play as basic elements of cell cycle control. Human homologs of the yeast cyclins were cloned in part by virtue of their ability to functionally compensate for loss of G1 cyclin (Cln) function in *S. cerevisiae* (Lew et al., 1991). This finding pointed towards a degree of cyclin conservation extending to the level of functional cooperation with Cdc28 in the

promotion of G1 in yeast, and suggested that human cyclins would likewise function to promote specific cell cycle transitions. Subsequent work has demonstrated this to be the case. In mammals, cyclins D and E promote G1 progression and entry into S, cyclin A is required primarily for passage through S-phase and G2/M, and cyclins B1 and B2 function during mitosis (reviewed in Nasmyth, 1996). As in yeast, the periodic accumulation of these cyclins triggers the activation of their catalytic cdk partners, which are a multigene family in higher eukaryotes (Meyerson et al., 1992). The specificity of the cyclin-cdk interaction results in the formation of active cyclin D•cdk4 (or cdk6) during G1, active cyclin E•cdk2 during late G1 and G1/S, active cyclin A•cdk2 during S, and active cyclin A/B•cdk1(cdc2) during G2/M.

In higher eukaryotes, the activity of G1 cyclin•cdks is subject to a further level of control by members of the *CIP/KIP* (cdc2/kinase inhibitory proteins) and *INK4* (inhibitor of cdk4) gene families . The protein products of these families, called cyclin-dependent kinase inhibitors (CKIs), bind directly to cyclin•cdks and inhibit their enzymatic activity. The *CIP/KIP* family is comprised of three separate CKI proteins called p21, p27 and p57 (reviewed in Hengst and Reed, 1998). Each of these CKIs is capable of stable binding to and inhibition of G1-phase cyclin•cdk complexes, and overexpression of members of the CIP/KIP family produces a G1 arrest which correlates with a decrease in the activity of G1 cyclin•cdks (Harper et al., 1995; Lee et al., 1995; Matsuoka et al., 1995; Toyoshima and Hunter, 1994; Xiong et al., 1993). The upregulation of CIP/KIP proteins in many differentiating tissues suggests that the primary role of these proteins will be in cell cycle withdrawal at the onset of differentiation (reviewed in Zavitz and Zipursky, 1997). Genetic analysis of the function of the single *Drosophila* CIP/KIP, *dacapo* , reveals that it is required for cell cycle exit during embryogenesis (de Nooij et al., 1996; Lane et al., 1996). Similarly, targeted inactivation of p27 and p57 in the mouse results in proliferation defects in a variety of developing tissues which appear to correlate with impaired ability to arrest at the onset of differentiation (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; Yan et al., 1997; Zhang et al., 1997). However, the role of each CIP/KIP in developmental control of proliferation is likely to be tissue specific. The expression patterns of *p21*, *p27*, and *p57* are different and in

some cases non-overlapping, (Lee et al., 1995; Nakayama et al., 1996; Parker et al., 1995; Yan et al., 1997) and the consequences of homozygous deficiency for each of the CIP/KIPs in the mouse are distinct. Thus, the role of the p21, p27 and p57 CKIs in cell cycle control is likely to vary between different tissues and cell types.

Like the CIP/KIPs, the *INK4* gene family consists of multiple CKIs and includes p15, p16, p18 and p19 (reviewed in Carnero and Hannon, 1998). Each of these proteins is capable of producing cell cycle arrest in G1 when overproduced, by virtue of their ability to bind cdk4 and prevent the formation of active kinase (Chan et al., 1995; Guan et al., 1994; Guan et al., 1996; Hirai et al., 1995; Koh et al., 1995). Evidence to date suggests that the INK4 proteins may play a role more in proliferation control in the adult animal, rather than during development. Mice homozygous for a targeted mutation of the murine $p16^{INK4A}$ develop normally, but show an increased susceptibility to tumors (Chin et al., 1997; Serrano et al., 1996). In cultured cells, accumulation of p16 is associated with cessation of cell division and entry into cellular senescence (Alcorta et al., 1996; Hara et al., 1996; Serrano et al., 1997), suggesting that this INK4 member may function in "homeostatic" cell cycle control in the adult, as opposed to a developmental role in proliferation control. The tissue restricted patterns of expression of the different INK4 proteins (Zindy et al., 1997) suggests that whereas they seem equally capable of cdk4 inhibition, each member of the family fulfills a distinct role in the control of G1/S progression in the whole animal.

The combined effects of regulation by transcriptional and proteolytic control of cyclin abundance, cdk phosphorylation state, and levels of enzymatic inhibitors, ensure that full activation of individual cyclin-dependent kinases is restricted to discrete temporal intervals during the cell cycle. These multiple mechanisms of regulation also provide numerous points at which progression through the cell cycle is rendered responsive to signaling pathways which modulate cdk activity. Whereas the activation of such pathways can be rate-limiting for cell cycle progression, persistent cdk activation by deregulation of these pathways might then constitute the loss of an important mechanism which normally limits cell division.

A3. Regulating transitions: the R point in eukaryotes

Many signaling pathways which are capable of regulating the rate of cell division do so by altering the activity of cyclin•cdks (reviewed in Elledge, 1996). By modulating the activity of these kinases, these pathways are able to control the rate at which cells progress through the cell cycle transitions which cyclin•cdks control. In higher eukaryotes, the most highly regulated transition is that between a quiescent, resting cell to one which is actively engaged in the process of DNA replication and is committed to completion of mitosis. This transition from a G0/G1 state to S-phase (G1/S) represents the point at which most extracellular signals converge to regulate progression through the cell cycle and to control the overall rate of cell division.

Regulation of G1/S progression occurs at a point termed the restriction point (R), which was originally defined empirically as a discrete time point in mid-to-late G1 at which cells commit to entry into S-phase and completion of the remaining phases of the cell cycle (S, G2 and M) (reviewed in Planas-Silva and Weinberg, 1997). Cells in the pre-R stages of G1 require the continued action of mitogenic stimuli to prevent arrest in a G0-like state, while those that have passed R are committed to completion of the cell division cycle and show a dramatically reduced requirement for extracellular growth factors. Passage through R is accompanied by the induction of a G1/S transcriptional program that readies the cell for completion of the division cycle. The targets of this program include enzymatic activities required for DNA replication and regulatory components that drive cell cycle progression. Thus, the R point represents a crucial regulatory interval during which the cell integrates signals from the extracellular environment with the basic cell cycle machinery to effect alterations in the transcriptional state of the cell.

Proper regulation of G1/S is required for execution of cell fate decisions such as continued division, transient arrest, senescence and differentiation. Regulated progression through G1/S ensures that cell division does not occur in the absence of proper signals and that it remains developmentally regulated in the context of the whole organism. Widespread deregulation of G1/S entry during embryonic development often has lethal consequences, while clonal loss of G1/S

control later in development is thought to represent a fundamental step in the origin of overproliferative disorders such as cancer.

B. Cancer & control of G1/S

The hallmark of cancer is uncontrolled cell proliferation leading to the formation of tumors. Cellular phenotypes associated with cancer include mitogen-independent cell cycle progression and a compromised ability to exit the cell cycle (reviewed in Sherr, 1996). *In vivo,* this inability to withdrawal from cycle can preempt the execution of differentiative pathways; similarly, impaired differentiation pathways can leave a cell in a state of incomplete cell cycle withdrawal in which it remains sensitive to the mitogenic effects of growth factors which then drive inappropriate cell division. As a result of these sorts of disruptions of normal cellular growth and differentiative programs, tumors are often composed of rapidly dividing, poorly-differentiated cells.

The proliferative potential of primary cells in culture is normally limited by entry in a period of growth arrest called "crisis". Entry into crisis occurs after a defined number of cell divisions called the "Hayflick limit" (Hayflick, 1961; reviewed in Hayflick, 1997), and is marked by a cessation of cell division in the G1 phase and the acquisition of cellular phenotypes indicative of replicative senescence (reviewed in Goldstein, 1990). Cell populations in crisis display elevated levels of a number of CKI proteins known to act as inhibitors of G1 cyclin•cdks (Alcorta et al., 1996; Hara et al., 1996; Noda et al., 1994; Reznikoff et al., 1996; Tahara et al., 1995; Tsao et al., 1995). This accumulation is causally linked to entry into senescence, as human fibroblasts engineered to lack both copies of the $p21^{CIP1}$ gene, or murine embryo fibroblasts (MEFs) deficient for the $p16^{INK4A}$ gene, no longer encounter features of replicative senescence in culture (Serrano et al., 1996; Brown et al., 1997). These findings suggest that entry into replicative senescence represents a cell cycle regulatory mechanism which is normally enforced *in vivo* to limit the growth potential of cells. At very low frequency, cells in culture appear capable of escaping crisis and growing out into "immortal" clones. The appearance of this heritable, immortal phenotype correlates with the acquisition of genetic alterations that permit the cell to

escape the controls that would normally enforce a crisis arrest (reviewed in Duncan and Reddel, 1997; Yeager et al., 1998). Further passage of immortal cells generally results in the outgrowth, at very low frequency, of fully "transformed" cells that have become largely mitogen-independent and capable of tumor formation when injected into immuno-compromised mice. The transition from the immortal to the transformed phenotype is associated with the acquisition of further mutations that activate mitogenic pathways and inactivate growth inhibitory mechanisms, rendering the cell progressively more refractory to the action of anti-mitogenic factors and increasingly more growth factor independent (reviewed in Sherr, 1996).

This model of progression in culture from a primary to a fully transformed state is thought to in many ways recapitulate the genetic and phenotypic changes associated with the progression from a normal cell to a cancerous one in the context of the whole organism. As cancer is in many respects a disease of the cell cycle, the genetic alterations which underlie the acquisition of tumorigenic phenotypes often occur in genes whose products regulate the cell division process. The central role of cyclin•cdk activity in promoting cell division suggests that mutations that produce persistent cdk activation could lead to a cell-autonomous fate of continued cell division and contribute to overproliferative phenotypes. The likely importance of R-point control in both proliferation and differentiation programs in higher eukaryotic systems points to pathways involving the G1 cyclin•cdks as especially good candidates for harboring the sorts of mutations that contribute to the process of tumorigenesis.

C. The *RB-1* gene

The heritable genetic changes associated with cancer have provided an excellent opportunity to identify genes whose products represent crucial proliferation control elements. The study of the molecular genetics of these mutations has revealed that they are broadly divisible into two classes: those that promote cell division in the heterozygous state, and therefore act dominantly to disrupt normal proliferation control pathways; and those for which homozygous mutation of both alleles is required to reveal oncogenic phenotypes. The requirement for the homozygous inactivation of

the latter class of genes suggests that they normally act to inhibit tumorigenic processes, and that only by the complete loss of cellular function are the relevant proliferation control pathways deregulated sufficiently to promote transformation. It is now established that dominant, gain-of-function mutations of the former class can also occur in genes which normally function to inhibit tumorigenesis *in vivo*. Many of these tumor suppressors, and their counterpart oncogenes, are known or predicted to contribute to the regulation of the G1/S cell cycle transition (reviewed in Bartkova et al., 1997). There is considerable evidence to suggest that these cell cycle roles underlie the link between mutations of these genes and promotion of tumorigenic processes. One of the best studied tumor suppressors is the product of the human retinoblastoma susceptibility locus (*RB-1*). The *RB-1* tumor suppressor gene was originally cloned by virtue of its absence in retinoblastoma, a childhood cancer of the retina (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). *RB-1* encodes a 105kD nuclear phosphoprotein, pRB, which normally functions to restrain S-phase entry by inhibiting the transcription of genes required for completion of G1 and entry into S-phase (reviewed in Beijersbergen and Bernards, 1996). This function likely underlies its role in tumor suppression, as expression of wildtype *RB-1*, but not tumor derived alleles, in cells that lack functional pRB protein results in an arrest in the G1 phase of the cell cycle (Bookstein et al., 1990; Goodrich et al., 1991; Huang et al., 1988).

Following its identification as the tumor suppressor involved in both familial and sporadic retinoblastoma, it has become evident that *RB-1* is mutated in a wide variety of other human cancers (reviewed in Weinberg, 1992). Inactivation of the growth-suppressive function of pRB is therefore likely a frequent event during the process of transformation of a broad spectrum of cell types *in vivo*. The creation of mouse strains carrying only one wildtype copy of the murine retinoblastoma gene, *Rb*, has confirmed this model of pRB tumor suppressor function (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). *Rb*+/- mice are predisposed to tumors of the thyroid and pituitary glands (60% and 100% penetrance respectively) and tumor formation correlates with loss of the remaining wildtype *Rb* allele.

The phenotype of *Rb*+/- mice significantly does not include retinoblastoma, the human cancer predisposition associated with germline transmission of a mutant *RB-1* allele. However, examination of the retina of *Rb*-/- embryos reveals inappropriate S-phase entry and increased apoptosis in cells which are normally in a post-mitotic state (Morgenbesser et al., 1994). These observations suggest that loss of pRB may compromise the ability of differentiating cells to exit the cell cycle, and that this aberrant proliferation can lead to the induction of cell death. A role for pRB in differentiation control is further suggested by additional phenotypes associated with *Rb* deficiency. These animals die *in utero* around day 14 of gestation, showing extensive apoptosis in the developing nervous system and impaired hematopoesis in the fetal liver. pRB also seems to be required for terminal cell cycle exit in certain myogenic lineages, as *Rb*-/- myoblasts are able to form syncitial myotubes when cultured in low serum, but unlike wildtype cells, the nuclei in these myotubes can be induced to undergo DNA replication by readdition of serum (Schneider et al., 1994). The compromised ability of myogenic cells to fully commit to a differentiated state in the absence of pRB is further indicated by the finding that homozygosity for a hypomorphic allele of *Rb* results in a reduction in skeletal muscle mass in the limbs, which demonstrates a requirement for complete pRB function in some aspect of myogenic development or maintenance (Zacksenhaus et al., 1996).

The role of pRB as a key regulator of the mammalian cell cycle whose inactivation contributes to the acquisition of a fully transformed phenotype is further highlighted by the finding that pRB is a critical target of the small DNA tumor viruses (reviewed in Dyson and Harlow, 1992). The high-risk forms of adenovirus type12, SV40, and human papilloma virus (HPV) each encode oncogenic proteins (E1A, large T_{ag}, E7 respectively) which are capable of collaborating with other oncogenes in the transformation of cultured cells. In each case, cellular transformation induced by these viral oncogenes overlaps with their ability to bind cellular pRB protein. These viral proteins interact with pRB via a conserved "LxCxE" motif (amino acid designations in single-letter code where "x" is any residue) common to most, but not all, pRB-interacting proteins (Figge et al., 1988). Binding of these viral gene products to pRB is therefore thought to inactivate

the growth suppressive function of pRB and permit entry into S-phase. This ability to deregulate G1/S via pRB-binding is crucial to the ability of these oncoproteins to contribute to transformation in culture and tumorigenesis *in vivo*.

C1. Regulation of pRB by the cell cycle apparatus

The ability of overexpressed pRB to enforce a G1 arrest in susceptible cell types suggests that one function of this protein is to inhibit entry into the cell division cycle. As wildtype cells are readily capable of G1/S progression following appropriate stimuli, it is clear that cell cycle entry can however occur in the context of wildtype pRB. This indicates the existence of cellular pathways that function to inactivate the growth suppressive properties of pRB, and suggests that these pathways act in the course of the normal cell cycle to permit transition through G1/S. Such pathways could operate at transcriptional, and/or post-transcriptional levels to modulate pRB function. The observation that pRB protein persists at all stages of the cell cycle suggests that such a mechanism(s) is post-translational in nature, and that it likely involves modification of preexisting pRB protein, rather than, for instance, its specific degradation in late G1.

Examination of the state of pRB in synchronized cultures reveals that the protein is expressed throughout the cell cycle, but that it undergoes a dramatic change in phosphorylation state at or about the R point. During early and mid-G1 pRB is hypophosphorylated, but it becomes highly phosphorylated in late G1 and G1/S (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Furukawa et al., 1990; Mihara et al., 1989). The temporal character of these modifications suggests that they may serve to inactivate of the G1/S-inhibitory function of pRB, and that this event represents an important element of R-point control. The finding that the E7 and T_{ag} viral oncogenes bind specifically to the underphosphorylated forms of pRB also supports the hypothesis that these species represent the active forms of pRB necessary for growth inhibition (Imai et al., 1991; Ludlow et al., 1989).

The primary amino acid sequence of pRB contains a number of copies of the predicted cdc2-family consensus phosphorylation site, minimally defined as S/T-P (Langan et al., 1989).

Phospho-peptide and phospho-amino analysis of ^{32}P-labeled pRB confirms that the protein is phosphorylated on multiple sites, and that it is highly enriched in phospho-serine and phospho-threonine residues (DeCaprio et al., 1992; Lees et al., 1991). A direct assessment of the ability of cdc2 to phosphorylate pRB reveals that cdc2 is able to efficiently and specifically recapitulate *in vitro* a number of pRB phosphopeptides which occur *in vivo* (Lees et al., 1991). This suggests that pRB represents a physiological substrate of cdc2-associated kinase activity. However, the G1-cell cycle timing of pRB modification *in vivo* (reviewed in Mittnacht, 1998) is not consistent with a role for cdc2 in the initiation of this process, but supports a model in which cdc2 contributes to the maintenance of pRB phosphorylation during G2/M (Hu et al., 1992). The cell cycle-regulated phosphorylation of pRB is more consistent with a model in which the G1-phase kinases, cdk2 and cdk4, are responsible for the *de novo* phosphorylation of pRB during late G1.

A considerable amount of data now support a role for both G1 cyclin•cdks, cyclin D•cdk4/6 and cyclin E•cdk2, in the cell cycle-dependent phosphorylation of pRB (reviewed in Mittnacht, 1998). In both cases, this activity underlies the ability of these kinases to overcome pRB growth suppression (Hatakeyama et al., 1994; Hinds et al., 1992). The precise role of each of these cell cycle kinases in the functional inactivation of the retinoblastoma protein remains unclear. However, accumulating data suggests that these kinases are not completely functionally equivalent (Hatakeyama et al., 1994; Resnitzky and Reed, 1995; Zarkowska and Mittnacht, 1997), and that they collaborate to functionally inactivate pRB through a mechanism which involves sequential phosphorylation, first by cyclin D•cdk4/6 in mid-G1, and then by cyclin E•cdk2 in late G1 and G1/S (Connell-Crowley et al., 1997; Ezhevsky et al., 1997; Kitagawa et al., 1996; Lundberg and Weinberg, 1998; see Chapter 5). This inactivation of pRB appears to be partially rate-limiting for progression through G1, as mouse embryo fibroblasts (MEFs) lacking both copies of the *Rb* gene display a reduced dependence on extracellular growth factors for proliferation in culture, and have a shortened G1 phase compared to wildtype cells (Brugarolas et al., 1998; Herrera et al., 1996).

The elucidation of this functional relationship between the activity of G1 cdks and the functional state of pRB has broad reaching implications. First, the many known growth

stimulatory and inhibitory pathways which converge to modulate the activity of G1 cdks are predicted to alter the phosphorylation state, and thereby the growth suppression function, of pRB. Second, the loss of pRB function during the course of tumorigenesis is predicted to deprive any such growth inhibitory pathways of a potentially necessary downstream effector. Third, it is now clear that pRB, as a target of cdk pathways, is itself a basic component of the cell cycle control machinery, and that the tumor suppressive properties of this protein arise in large part as a consequence of this role.

C2. Cellular regulators of pRB & tumorigenesis

The importance of the retinoblastoma protein as a tumor suppressor suggests that deregulation of G1/S may also occur via mutation of other genes whose products are required for proper functioning of the pRB pathway. In fact, work on cellular components of cell division pathways has uncovered a number of genes that are frequently mutated in human cancers, and whose primary function appears to be modulation of the tumor suppressive properties of pRB.

C2.1 *Cyclin D*

Cyclin D-associated kinase activity is predicted to play an important role in the inactivation of the growth suppressive function of pRB during the G1-phase of the cell cycle (reviewed in Sherr, 1995). In mammals, cyclin D activity is the product of three highly related genes *Cyclin D1*, *D2*, and *D3*. These proteins appear equally capable of directing the cdk4-catalyzed phosphorylation of pRB (Dowdy et al., 1993; Ewen et al., 1993; Matsushime et al., 1994), and may be to some degree functionally redundant. The members of the *cyclin D* family are expressed in largely overlapping patterns during murine development (Bartkova et al., 1994; Inaba et al., 1992). This suggests that in most tissues all three D-type cyclins participate in the inactivation of pRB, and that loss of one would likely be compensated by the others. However, a few tissues display expression of one particular D-type cyclin to the exclusion of the others. In these cases, it is predicted that this D-type cyclin plays a required role in the inactivation of pRB and the promotion of proliferation. This hypothesis is supported by studies of the biological consequences

associated with deficiency for the murine *cyclin D1* and *D2* genes. Cyclin D1-deficient animals in many respects develop normally, but show reduced body size, reduced viability and symptoms of neurological impairment (Fantl et al., 1995; Sicinski et al., 1995). Dramatic hypoproliferative defects are apparent in tissues which are normally the sites of highest *cyclin D1* expression: the developing retina, and the mammary epithelium during pregnancy. Similarly, animals deficient for cyclin D2 display phenotypes consistent with underproliferation of tissues which normally express high levels of *cyclin D2* transcript (Sicinski et al., 1996).

The role of D-type cyclin-kinases in the inactivation of a negative regulator of G1/S with tumor suppressive properties suggests that components of these biochemical activities could have oncogenic potential. Indeed, *cyclin D1* was originally cloned as *PRAD1*, the product of a translocation in parathyroid tumors between *cyclin D1* coding sequences and the parathyroid hormone locus (Motokura et al., 1991). It was subsequently found that *cyclin D1* is also amplified in human B-cell neoplasia as a result of a t(11:14) chromosomal translocation which juxtaposes *cyclin D1* with the immunoglobulin heavy chain enhancer Eμ (Medeiros et al., 1990; Withers et al., 1991). Further work has demonstrated that both *cyclin D1* and *cyclin D2* are overexpressed in a number of human tumor types (reviewed in Hamel and Hanley-Hyde, 1997). The transforming potential of *cyclin D* genes are demonstrated by their ability to cooperate with other oncogenes in the transformation in cultured cells, and in the production of tumors in animal models (Hinds et al., 1994; Jiang et al., 1993; Kerkhoff and Ziff, 1995; Lovec et al., 1994).

Among the G1 cyclins, cyclin D protein is unique in its ability to form a stable interaction with pRB *in vivo* via an "LxCxE" motif present near the amino terminus of the protein (Dowdy et al., 1993; Kato et al., 1993). This physical interaction may underlie target specificity of cyclin D-kinases observed *in vitro*. Unlike other cyclin•cdks, whose enzymatic activity is readily detected *in vitro* using a variety of both physiological and non-physiological protein substrates, the activity of cyclin D•cdk4/6 kinases are most apparent when the retinoblastoma protein is used as substrate (Matsushime et al., 1992; Matsushime et al., 1994; Meyerson et al., 1994). Together, these data suggest that the retinoblastoma protein is a specific substrate of cyclin D-kinases, and that such

specificity may arise in part due to a direct interaction between cyclin D and the retinoblastoma protein. Additional data implies that pRB represents the primary target of the growth promoting activity of D-type kinases *in vivo*. Injection of α-cyclin D1 neutralizing antibodies produces a G1 arrest in cells with intact pRB, but has no affect upon cells which lack functional pRB protein (Lukas et al., 1995), strongly suggesting that cyclin D1-associated kinase activity is dispensable in cells in which pRB is already functionally inactivated. Thus, evidence supports a model in which phosphorylation of the retinoblastoma protein is the only function of the *cyclin D1* proto-oncogene which is absolutely required for cell cycle progression, and that pRB is the critical cell cycle target through which oncogenic forms of *cyclin D1* mediate their tumorigenic effect.

Accumulating data suggest that cyclin D-kinase activity represents the component of the pRB pathway which is directly inducible by growth factor signaling in pre-R cells. D-type cyclin transcript levels are induced rapidly upon restimulation of G0/G1 cells with serum or with specific growth factors (Surmacz et al., 1992; Winston and Pledger, 1993; Won et al., 1992). This transcriptional induction seems to represent a required element of cell cycle reentry, as cyclin D-associated kinase activity is required by multiple receptor-mediated mitogenic signal transduction pathways for induction of S-phase (Lukas et al., 1996). The molecular basis of this dependence is likely to involve the Ras/Raf signaling pathway, which activates G1 kinases via induction of *cyclin D1* transcription and degradation of $p27^{KIP1}$ (Agrawal et al., 1996; Aktas et al., 1997; Filmus et al., 1994; Liu et al., 1995; Takuwa et al., 1997; Weber et al., 1997). In fact, recent work has demonstrated a direct link between $p21^{ras}$-dependent mitogenic signaling and the pRB cell cycle control pathway (Mittnacht et al., 1997; Peeper et al., 1997; Serrano et al., 1995; Stacey et al., 1994). Anti-pan-Ras antibodies block Ras signaling and produce a G1 arrest in wildtype MEFs. However, blocking Ras function in *Rb-/-* MEFs does not result in a discernible cell cycle phenotype. This result implies that the primary function of the Ras pathway in cell cycle control is to promote G1/S through a pRB-dependent mechanism, and that inactivation of pRB renders this aspect of Ras function dispensable. Together, these data have established a causal link between

growth factor-receptor activation, *cyclin D1* transcription and formation of cyclin D-cdk species capable of contributing to the phosphorylation-mediated inactivation of pRB.

C2.2 The *p16^{INK4A}/MTS1* locus

Analysis of the genetic basis of human cancer predisposition has provided another tumor suppressor that regulates cell cycle progression through the pRB pathway. The *p16^{INK4A}/MTS1* (inhibitor of cdk4/multiple tumor suppressor-1) gene was originally cloned as a cDNA which encoded a protein capable of interacting with the cdk4 catalytic subunit of the G1 kinase cyclin D•cdk4 (Serrano et al., 1993). Soon thereafter, *p16* was found to lie within the 9p21 genomic region commonly deleted in gliomas, lung cancers, leukemias and both sporadic and familial forms of melanoma (Kamb et al., 1994). The p16 gene has been found mutated in a wide range of human tumors and transformed cell lines (Nobori et al., 1994; reviewed in Carnero and Hannon, 1998). Modeling the function of p16 in mice confirms the tumor suppressive properties of this locus: mice homozygous for a targeted deletion of the second exon of the *p16* gene are highly predisposed to fibrosarcomas and lymphomas by ~30 weeks of age, and administration of carcinogenic agents accelerates this process (Serrano et al., 1996). MEFs derived from *p16-/-* embryos show growth properties consistent with progression towards the transformed state. Wild-type diploid MEFs normally enter crisis after about ~20 population doublings. However, *p16-/-* cells do not enter a growth crisis period during culture and appear to have become immortalized by loss of p16. The increased susceptibility of immortal *p16-/-* cells to neoplastic transformation is demonstrated by the finding that introduction of Ha-*ras*^{val12} alone into *p16-/-* MEFs is sufficient to induce anchorage-independence and formation of tumors in immunocompromised mice (Serrano et al., 1996). These data are consistent with a model in which loss of both alleles of *p16^{INK4A}/MTS1* represents a growth deregulatory event that accelerates the process of oncogenesis *in vivo* .

The *p16* gene encodes a small molecular weight cyclin-dependent kinase inhibitor (CKI). Expression of *p16* produces a G1 arrest in some cell lines, which correlates with the ability of p16

protein to block the formation of active cyclin D•cdk4 kinase by binding and sequestering cdk4 (Serrano et al., 1993). Importantly, tumor derived alleles of *p16* are deficient in their ability to bind cdk4 and to produce cell cycle arrest (Koh et al., 1995; Ranade et al., 1995), and enforced expression of wildtype *p16* in pRB-deficient cell lines also fails to arrest proliferation (Lukas et al., 1995; Medema et al., 1995). These data demonstrate that p16 anti-proliferative activity is entirely dependent upon both its ability to bind cdk4 and the presence of intact retinoblastoma protein. Significantly, there are instances of germline mutations within *cdk4* exons that cosegregate with melanoma susceptibility within a kindred (Wolfel et al., 1995; Zuo et al., 1996). These mutations render cdk4 refractory to inhibition by INK4 proteins. These data demonstrate that like cyclin D, the catalytic subunit of the cyclin D•cdk4 G1 kinase also has oncogenic potential, and that this potential is revealed by escape from regulation by the p16 tumor suppressor protein.

Participation of pRB and p16 in a common G1/S growth regulatory pathway is likely to underlie the observation that concurrent loss of *both* tumor suppressors in the same clonal line is a rare event (reviewed in Elledge and Harper, 1994; and in Sherr and Roberts, 1995), as it would predict no growth advantage for a compound mutant cell. Loss of the *p16* CKI during the course of tumorigenesis consequently deprives the cell of a critical negative regulator of G1/S that normally acts in a post-translational manner to regulate the activity of the proto-oncogenic cyclin D•cdk4(or cdk6) kinase and modulate the phosphorylation-dependent growth suppressive properties of pRB. The frequency of mutations in components of this pathway suggests that it may be among the most commonly disrupted pathways in human cancer (reviewed in Sherr, 1996). In fact, accumulating data regarding genetic lesions in various cell lines and cancers suggest that this p16•cyclin D•pRB G1/S regulatory pathway may even be a *required* mutational target during human tumorigenesis.

Since the generation of genetic models of the phenotypic consequences of *p16* loss, it has become evident that a second important tumor suppressor gene lies within the *p16* locus (reviewed in Haber, 1997). This gene, called $p19^{ARF}$ (alternative reading frame), has its own unique 5' exon (exon-1α) distinct from the first exon of *p16* (exon -1β), but shares its second exon (exon-2) with

p16 . Translation of p19ARF in a different frame produces a polypeptide which is capable of producing cell cycle arrest in both the G1 and G2 phases, but with no homology to the *INK4* family or to any other cellular protein (Quelle et al., 1995).

While some of the mutations described in the *p16* region in human cancers specifically target coding sequences unique to p16, most of the lesions of this region are predicted to disrupt the coding sequences of both $p16^{INK4A}$ and $p19^{ARF}$. In a number of these cases, it has been demonstrated that these mutations specifically disrupt the ability of p16, but not p19, to arrest the cell division cycle (Quelle et al., 1997). However, the p19 product of the *INK4a* locus does appear to fulfill an important growth regulatory function. Recent work has demonstrated that $p19^{ARF}$ functions as a tumor suppressor in mice (Kamijo et al., 1997). This property is likely due to the ability of $p19^{ARF}$ to promote the degradation of the mdm2 oncoprotein (murine double minute-2) (Pomerantz et al., 1998; Zhang et al., 1998). Mdm2 is in turn a negative regulator of the p53 tumor suppressor, which is involved in both checkpoint control and induction of apoptosis (reviewed in Levine, 1997). As the targeted disruption of murine $p16^{INK4A}$ removed exon-2 sequences shared with $p19^{ARF}$ (Serrano et al., 1996), it is now clear that the organismal and cell culture phenotypes associated with "p16-deficiency" represent the consequence of deficiency for *both* these tumor suppressors. These data reveal an unexpected level of complexity in the biology of the *INK4a* locus, and support the emerging concept that it encodes growth inhibitory proteins that individually act upstream of either pRB or p53. Given that *p53* mutation is thought to be the most frequent genetic alteration in human cancers (Hollstien et al., 1991; Levine et al., 1991), this model raises important questions regarding which of these gene products, p16 or p19, plays a more important role in tumor suppression. However, a clear role for the p16 protein in G1/S regulation suggests that the tumor suppressive properties of the *INK4a* locus are in part attributable to the cell cycle-regulatory effects of the p16 via a pRB-dependent pathway of growth control.

C2.3 *CyclinE*

Evidence to date suggests that cyclin E•cdk2 kinase activity fulfills a required role in the promotion of S-phase entry. Constitutive expression of *cyclin E* shortens the duration of G1, decreases cell size, and diminishes the serum requirement for the transition from G1 to S phase (Ohtsubo and Roberts, 1993). Inhibition of endogenous cyclin•cdk2 kinase by overexpression of either the *p21* CKI or a dominant-negative form of cdk2, results in G1-phase cell cycle arrest (Harper et al., 1995; Lundberg and Weinberg, 1998; van den Heuvel and Harlow, 1993; Xiong et al., 1993). Specific inhibition of cyclin E-associated kinase by microinjection of α-cyclin E antibodies inhibits S-phase entry (Ohtsubo et al., 1995). Genetic analysis of function in *Drosophila melanogaster* demonstrates a strict requirement for cyclin E in S-phase entry during development (Knoblich et al., 1994). Fly embryos homozygous for a mutation in *cyclin E* encounter cell cycle arrest at cycle 17 of embryogenesis. Inhibition of cyclin E-kinase in the developing eye by ectopic *dacapo* expression delays S-phase entry; *DmcycE* is likely to represent a critical target of the cell cycle inhibitory effects of *dacapo*, as the phenotype of ectopic *dacapo* expression is enhanced by heterozygosity for *DmcycE* but not for *DmcycA* or *B* (deNooij et al., 1996). This proliferative potential of cyclin E is apparently incompatible with developmentally programmed arrest, as ectopic expression of *DmcycE* in cells of the dorsal epidermis prevents cell cycle exit and stimulates additional cell division (Lane et al., 1996). These observations show that cyclin E activity can be rate-limiting for S-phase entry in eukaryotic cells, and suggest that cyclin E targets a molecule(s) with an important and conserved G1/S regulatory role.

It is likely that cell cycle phenotypes associated with alterations in cyclin E activity arise in part due to the role of cyclin E-associated kinase in the phosphorylation-mediated inactivation of pRB. *In vivo*, the inactivation of pRB by G1 cyclin•cdks is accomplished by both cyclin D- and cyclin E-associated kinase activity (reviewed in Mittnacht, 1998). The staggered cell cycle-dependent accumulation of active cyclin D- and cyclin E-associated kinase activities suggests that cyclin D•cdk4 initiates this process in mid-G1 and cyclin E•cdk2 completes it in late G1 and G1/S. However, overexpression and *in vitro*-kinase systems have failed to effectively demonstrate

significant differences in the manner in which these two kinases regulate pRB. Given this apparent functional overlap between cyclin E•cdk2 and cyclin D•cdk4 in the inactivation of pRB, it is of note that alterations in the *cyclin E* locus have not been found associated with any human cancer syndromes. The prevalence of amplifications and translocations involving the *cyclin D* loci suggests that amplification of *cyclin E* might facilitate constitutive inactivation of pRB and deregulation of G1/S via a similar pathway. However, the absence of such mutations strongly suggests that cyclin E- and cyclin D-associated kinase activities are not completely functionally equivalent with respect to the mechanisms through which they regulate cell cycle progression.

It is clear from multiple eukaryotic systems that one target of cyclin E•cdk2 activity is the retinoblastoma protein (Du et al., 1996a; Hatakeyama et al., 1994; Hinds et al., 1992). However, unlike cyclin D, cyclin E plays a role in both pRB-dependent and pRB-independent cell cycle regulatory pathways (Alevizopoulos et al., 1997; Leng et al., 1997; Lukas et al., 1997). This is best illustrated by the finding that injection of neutralizing α-cyclin E antibodies arrests both pRB-wildtype and pRB-deficient cells in G1 (Ohtsubo et al., 1995). Therefore, in addition to pRB, cyclin E•cdk2 kinase is required for phosphorylation of other unknown target(s) that regulate G1/S. This additional role would seem to suggest that *cyclin E* has the potential to be even more oncogenic than *cyclin D*, as its activity is predicted to regulate both pRB-dependent and -independent G1/S control pathways.

This prediction is in direct contrast to the lack of *cyclin E* mutations in human cancer. There are a number of possible explanations of this apparent incongruity. It is possible that deregulation of this second, undetermined cyclin E-dependent pathway is not compatible with continued cellular viability; alternatively, the combined deregulation of pRB-dependent and -independent G1/S control pathways may produce a shortening of G1 so pronounced that affected cells eventually succumb to minimum size limitations. The lack of *cyclin E*-mediated oncogenicity may also be a direct reflection of the manner in which it regulates pRB function. Recent work from a number of sources has begun to suggest that cyclin D and cyclin E phosphorylate different sites within the pRB sequence (Kitagawa et al., 1996). Although not a confirmed property of the

endogenous kinases, such site-specificity may underlie the apparent cooperativity between cyclins D and E in the functional inactivation of pRB *in vivo*. Confirmation of such cooperativity by the sequential activation of these kinases would strongly suggest that, in the absence of prior cyclin D-mediated phosphorylation of pRB, cyclin E•cdk2 is insufficient to independently inactivate the growth suppressive properties of pRB. This hypothesis is supported by the finding that specific inhibition of cdk4-, but not cdk2-kinase activity, results in the accumulation of hypophosphorylated pRB which does not appear to be phosphorylated by cyclin E•cdk2 (Lundberg and Weinberg, 1998; Resnitzky and Reed, 1995). In this regard, each kinase may be individually required, but not sufficient, for phosphorylation-mediated inactivation of pRB. In the absence of growth factor-stimulation of *cyclin D* transcription *in vivo*, increased levels of cyclin E-kinase may be unable to promote cell cycle progression because a necessary upstream event - the cyclin D-mediated phosphorylation of pRB - has not occurred. Thus, amplification of *cyclin E* would not relieve the growth factor-dependence of pRB inactivation and R-point control. In contrast, proliferative signals generated by amplification of *cyclin D* would be propagated by the mitogen-independent accumulation of cyclin E-kinase.

C3. Targets of pRB: transcriptional control of cell cycle & differentiation

Analysis of the post-translational regulators of pRB function has uncovered proteins with oncogenic and tumor suppressive activities that correlate with their roles in cell cycle control. The pRB-dependence of the cell cycle phenotypes associated with each of these proteins suggests that pathways downstream of pRB include proteins with central roles in tumorigenesis and cell cycle control. The identification of these pRB targets is necessary to understand fully the molecular mechanisms of carcinogenesis, and to permit the development of models of the true physiological function of the pRB gene product.

C3.1 The A•B pocket

Analysis of pRB mutations found in human tumors suggests that the function of a region within the *RB-1* gene called the "A•B pocket" is required for pRB growth suppression (Hiebert,

1993; Qin et al., 1992). The "pocket" is composed of the A and B domains separated by a region known as the "spacer". This region of pRB is an important mutational target in the process of tumorigenesis (reviewed in Dyson and Harlow, 1992). In retinoblastoma, inactivation of the remaining of *RB-1* wildtype allele normally occurs by small-scale deletion or point mutation which results in disruption of either the A or B domain, or in some cases the loss of the entire A•B pocket (reviewed in Sellers and Kaelin, 1996). The importance of the A•B pocket for pRB function is further emphasized by the finding that this region falls within the domain recognized by the E1A, Tag, and E7 oncoproteins, suggesting that the oncogenic and cell cycle phenotypes induced by these viral proteins may depend upon their ability perturb the normal function of this domain.

Extensive work has resulted in a lengthy list of cellular and viral proteins capable of interacting with pRB *in vitro* via sequences in the A•B pocket (reviewed in Mulligan and Jacks, 1998). For only a subset of these has an *in vivo* interaction been demonstrated which represents a biologically significant property of the endogenous proteins. From these, it has become clear that the biological properties of pRB are likely a result of its ability to modulate the transcriptional state of certain cellular promoters. This function of pRB is directly linked to its ability to interact physically with transcription factors in nuclear complexes bound to DNA. This connection between transcription control and regulation of G1 progression provides a link between the activity of G1 cyclin•cdks and modulation of transcriptional programs required for cell cycle progression. As the commitment to G1/S progression is at one level a transcriptional decision whether or not to synthesize the enzymatic, structural and regulatory components necessary for entry into and completion of S phase, the G1/S regulatory role of pRB may reflect its ability to interfere with induction of this program. By extension, the transformation and differentiation phenotypes associated with pRB-deficiency are likely to correlate with deregulation of transcriptional programs normally regulated by the retinoblastoma protein.

C3.2 Differentiation control

Deletion analysis of the pRB cDNA has demonstrated that the A•B pocket is required for the G1 arrest which results from overexpression of pRB in the SAOS-2 human osteosarcoma cell line (Qian, 1992 ; Qin, 1992). Interestingly, in addition to this cell-cycle phenotype, the integrity of the A•B pocket is also required for a "differentiation" function of pRB seen in these cells. Reintroduction of pRB in SAOS-2 cells is associated with the induction of a distinctive phenotype in which cells flatten out and grow quite large (Hinds et al., 1992; Qin et al., 1992; Templeton et al., 1991). These large cells are no longer cycling, yet they appear to remain metabolically active and display signs of osteoblast differentiation as measured by induction of bone-alkaline phosphatase activity and mineral deposition (Sellers et al., 1998). Certain tumor-derived alleles of *RB-1* associated with high-penetrance retinoblastoma are deficient in this differentiation-promotion activity (Sellers et al., 1998). The ability of pRB to drive under-differentiated SAOS-2 osteosarcoma cells towards a bone-differentiation fate may reflect the ability of pRB to interact with one or more endogenous transcription factors involved in bone differentiation. That tumor-derived alleles may be deficient in this function(s) imply that tumorigenicity as a result of pRB loss may involve both deregulation of G1/S cell cycle control and impairment of differentiation pathways.

One cellular factor that interacts with pRB via the B-domain of the pocket region and has an established role in cellular differentiation is the fate determining transcription factor MyoD (Gu et al., 1993). Expression of MyoD during development is linked to the activation of muscle-specific genes and the execution myogenic differentiation programs (reviewed in Molkentin and Olson, 1996). Stable expression of MyoD in human fibroblasts is sufficient to trigger the expression of muscle markers, such as myogenin, and the formation of myotube-like syncitia in culture. The inability of cultured *Rb-/-* murine myotubes to commit to terminal cell cycle exit correlates with reduced expression of MyoD-responsive genes (Gu et al., 1993; Novitch et al., 1996), suggesting that pRB is required for MyoD function. Recent experiments show that pRB is required for MyoD-mediated transactivation of the muscle creatine kinase (MCK) promoter

(Sellers et al., 1998). While a reporter driven by the MCK promoter is normally induced dramatically by coexpression of MyoD, expression of MyoD in *Rb-/-* MEFs has little or no effect on the activity of the this promoter. Consistent with a physical and functional interaction between pRB and MyoD, the lack of MyoD-mediated activation of the MCK reporter in *RB-1-/-* cells is rescued by co-expression of a wildtype, but not a tumor-derived, allele of *RB-1* carrying an internal deletion in the A•B pocket. In addition to activation of genes associated with differentiation programs, expression of MyoD is also associated with the induction of a G1 arrest (Crescenzi et al., 1990). This arrest correlates with the ability of MyoD to induce the *CIP/KIP* family member p21 (Halevy et al., 1995) and suggests that MyoD may induce cell cycle exit during skeletal muscle differentiation by upregulation of the p21 CKI.

It is likely that the involvement of pRB in the execution of G0 differentiation programs by virtue of its ability to interact with fate determining transcription factors like MyoD contributes to tumorigenesis in the absence of pRB. However, it is clear that these interactions are insufficient to fully explain the tumorigenic consequences of pRB loss and are unlikely to play a role in a more generalized function of pRB as a G1 regulator. Firstly, whereas all tumor-derived alleles of *RB-1* tested to date are deficient in the induction of a G1 arrest in primary cells, certain tumor-derived alleles are unaffected in their ability to functionally interact with MyoD (Sellers et al., 1998), suggesting that tumor suppression is to some degree dissociable from differentiation control. Secondly, the ability of pRB and components of the pRB pathway to either inhibit or promote the progression of human and mouse primary cells through the G1 phase of the cell cycle occurs in a cellular context lacking detectable levels of differentiation-specific factors like MyoD, and production of these cell cycle phenotypes does not correlate with the induction of such activities. Lastly, the generation of chimeric mice reveals that *Rb-/-* cells are capable of contributing to most fully differentiated tissues and cell types in these animals (Maandag et al., 1994; Williams et al., 1994). As the juxtaposition of wildtype and pRB-deficient cells in this system more closely recapitulates the clonal loss of pRB function during tumorigenesis, the absence of a differentiation deficit in these *Rb-/-* cells suggests this function may not underlie tumorigenic phenotypes

associated with pRB loss in human tumors. Together, these observations indicate that interactions with transcriptional activities associated with differentiation do not account for the central role of pRB as a regulator of G1/S. Clearly, the critical target(s) of pRB-mediated G1/S control is likely to have a more generalized role in cell cycle control which is directly linked to the basic machinery regulating cell division.

C3.3 Cell cycle control: E2F

Considerable evidence now suggests that a class of pRB-binding proteins called E2F/DRTF (Early-2 Factor/ Differentiation Regulated Transcription Factor; henceforth referred to as E2F) represent the target of pRB-mediated growth suppression *in vivo* (reviewed in Bernards, 1997). These DNA-binding transcription factors interact with pRB via the A•B pocket and normally function to regulate the expression of genes required for G0/G1 exit and S-phase progression. The role of E2F as an important transcriptional effector of the p16•cyclin D•pRB suggests that the growth regulatory properties of this pathway will in large part be determined by the transcriptional properties of E2F.

E2F displays many of the expected characteristics of a major target of pRB growth suppression. E2F is reported to bind specifically to the unphosphorylated, growth-suppressive form of pRB during G1 (Bagchi et al., 1991; Bandara and La Thangue, 1991; Cao et al., 1992; Chellappan et al., 1991; Mudryj et al., 1991; Bandara, 1991; Bagchi, 1991; Chellappan, 1991 ; Cao, 1992 ; Kaelin, 1992). This association does not prevent E2F from binding DNA but results in repression of E2F transactivation activity (Helin et al., 1993a; Hiebert et al., 1992). Moreover, the resulting pRB•E2F complex can act as an active repressor of E2F responsive promoters (Adnane et al., 1995; Bremner et al., 1995; Chow and Dean, 1996; Weintraub et al., 1995; Weintraub et al., 1992). Consistent with an important role in proliferation control, the pRB•E2F interaction is disrupted during the course of tumorigenesis, as all tumor-derived alleles of pRB tested to date are deficient in E2F binding (Sellers et al., 1998). *In vivo* the pRB•E2F interaction is regulated by the cell cycle-dependent kinases. Phosphorylation of pRB by G1 cyclin•cdks

dissociates pRB from DNA-bound E2F complexes, simultaneously relieving pRB-mediated repression, and permitting "free" E2F that remains at the promoter to transactivate target genes at G1/S. Expression of the adenovirus E1A transforming protein activates E2F-dependent transcription by binding pRB and disrupting the pRB•E2F complex (Bandara and La Thangue, 1991; Chellappan et al., 1992; Chellappan et al., 1991; Raychaudhuri et al., 1991).

As would be expected of a critical transcriptional regulator of G1/S, a list of E2F-responsive genes includes many whose products are implicated in or directly linked to the process of cell proliferation. These genes can be grouped into two classes: those whose products are involved in cell cycle regulation (eg. *RB-1*, *p107*, *cdc2*, *cyclin A2*, *cyclin E*, *E2F-1*, *b-myb*) or those that encode activities directly linked to the enzymatic process of DNA replication (eg. dihydrofolate reductase [*DHFR*], *mcm3*, *cdc6*, ribonucleotide reductase subunits 1 and 2 [*RNR1/2*], DNA ligase, thymidine kinase [*TK*], and the large subunit of DNA polymerase α [*Polα*]) (reviewed in Bernards, 1997). Together, these data suggest that pRB functions to inhibit E2F activity and that inactivation of pRB, either by mutation, viral proteins or phosphorylation, activates E2F and the transcriptional program(s) it controls.

D. The E2F and DP family proteins: heterodimerization and structure

In vivo, E2F activity is composed of a heterodimer of two distantly related polypeptides called E2F and DP (DRTF protein) (Wu et al., 1995). Heterodimerization is absolutely required for high affinity binding to E2F-site DNA sequences and for transactivation (Bandara et al., 1993; Helin et al., 1993b; Krek et al., 1993). Once bound to DNA, the transactivation and regulatory properties of E2F•DP complexes are primarily determined by the E2F subunit. There are currently six members of the E2F family in human and mouse (E2F-1 through E2F-6) (Trimarchi et al., 1998; reviewed in Bernards, 1997), which are grouped together based upon high homology throughout their DNA, heterodimerization, transactivation and pRB-binding domains. Mammalian DP activity is also represented by a family of genes, of which there are currently two known members (DP-1 and DP-2) (Girling et al., 1993; Wu et al., 1995; Bandara, 1994). It

appears that any member of the E2F family of polypeptides can complex with any member of the DP family to give rise to functional E2F activity.

The E2F family of polypeptides can be divided into three subclasses based upon domain structure. E2F-1, -2 and -3 all contain an extended amino-terminal domain which is absent in the other E2Fs. This N-terminus is rather divergent at the amino acid level, although there appears to be some conserved function within this domain, as it is responsible for the cyclin A-binding and nuclear localization properties (see Chapter 4) of E2F-1, -2 and -3 (Adams et al., 1996; Krek et al., 1994). E2F-4 and -5 represent the second subclass of E2F proteins; these E2Fs lack the N-terminus present in E2F-1, -2 and -3. Further, at a few amino positions within the DNA binding domain that are identical between the first E2F subclass (E2F-1,- 2 & -3), *E2F-4* and *-5* encode residues that are divergent with respect to the first group, but conserved between each other. To date, the biological consequences of some of these sequence differences remain unclear.

E2F-6 is currently the only representative of the third structural group. Human and mouse *E2F-6* encode a protein containing consensus E2F DNA binding and dimerization domains, but lacking the entire C-terminal domain present in the other E2Fs that encodes the pRB-binding and transactivation functions (Morkel et al., 1997; Trimarchi et al., 1998). Further, E2F-6 protein contains an N-terminal domain of intermediate length between the other two E2F subgroups. These structural differences suggest that while E2F-6 protein may be competent to heterodimerize with DP proteins and bind DNA, it does not interact with the pRB tumor suppressor protein or participate in transcriptional regulation of responsive genes in a manner strictly analogous to the other members of the E2F family.

Like the E2F family, the DP-1 and DP-2 proteins are fairly well conserved throughout their entire length. The regions of highest DP homology are the DNA binding and heterodimerization domains, which are required for E2F-binding and contribute to the DNA binding affinity of the intact E2F•DP complex. Both DP proteins contain a highly acidic C-terminal stretch of approximately 20 residues which makes a small contribution to the transactivation potential of the heterodimer (Bandara et al., 1994). Alternative splicing of the *DP-2*

transcript produces a number of different DP-2 polypeptides *in vivo* (Ormondroyd et al., 1995; Rogers et al., 1996). The functional roles of the coding regions whose inclusion is regulated by alternative splicing remain unclear, although the relative expression levels of the DP-2 isoforms appears to vary between different cell lines and tissues (Rogers et al., 1996). There are no apparent differences between these isoforms in their ability to interact with E2F proteins and produce a heterodimer competent to bind DNA.

E. The pRB-related proteins p107 and p130

The physical and functional interactions between E2F•DP heterodimers and the tumor suppressor pRB suggest that E2F activity plays a critical role in the regulation of cell cycle progression. As a result, this transcription factor family is predicted to represent an important target of proliferation control pathways that regulate the G1/S transition. Elucidation of the molecular mechanisms of pRB regulation suggests that cell cycle regulatory pathways stimulate E2F activity through the inactivation of proteins which normally function to repress E2F. It is now clear that pRB does not represent the only cellular protein that potentially falls into this E2F-repressive class. Examination of the properties of endogenous proteins has revealed that E2F activity is subject to cell cycle-dependent control by two other proteins called p107 and p130. (Cobrinik et al., 1993; Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). Both p107 and p130 contain an "A•B pocket" domain and are capable of interacting with E2F proteins in DNA-bound complexes; like pRB, this interaction results in a repression of E2F transactivation activity (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995; Vairo et al., 1995). Consistent with the high degree of homology between the pRB family members, experiments suggest that E2F•p107 and E2F•p130 complexes can also function as active repressors of E2F-responsive promoters in a manner similar to pRB (Chow et al., 1996; Starostik et al., 1996).

Foremost, these observations suggest that the transcriptional role of mammalian E2F proteins is unlikely to be restricted exclusively to the pRB growth regulatory pathway. They imply

that E2F is also the target of p107/p130-dependent pathways of transcriptional regulation, and that E2F will play an important role in mediating the biological properties of these pathways. Understanding the roles of p107/p130 in proliferation control, and characterizing the cellular pathways that regulate p107/p130 function therefore become necessary for the development of models of E2F function in cell cycle control and tumorigenesis.

The p107 and p130 proteins are highly homologous to pRB throughout portions of their coding regions. This conservation indicates that these three genes are evolved from a common ancestral locus, and has led to the collective designation of pRB, p107 and p130 as the "pocket proteins". This evolution has apparently also produced some degree of divergence, as the p107 and p130 proteins are more highly homologous to each other than they are to pRB, particularly with the spacer region. A region of p107/p130 homology within the spacer region is predicted to harbor an important functional domain. This region contains a motif, which is homologous to regions within the CIP/KIP family proteins, through which p107/p130 interact stably with cyclin A•cdk2 and cyclin E•cdk2 (Adams et al., 1996; Cobrinik et al., 1993; Li et al., 1993). This interaction, which does not prevent p107 and p130 from interacting with E2F, or E2F from binding DNA, results in an inhibition of the activity of these kinases (De Luca et al., 1997; Woo et al., 1997; Zhu et al., 1995).

The regions of conservation between members of the pRB family also include a number of the consensus cdc2 family-phosphorylation sites that are known to be modified in the retinoblastoma polypeptide *in vivo*. This conservation suggests that p107/p130 activity may be regulated through cell cycle-dependent phosphorylation in a manner similar to pRB. Consistent with this hypothesis, both p107 and p130 are phosphoproteins *in vivo* and can serve as efficient substrates for cdc2-type kinases *in vitro* and *in vivo* (Beijersbergen et al., 1995; Li et al., 1993; Xiao et al., 1996). Studies of the relevance of these phosphorylation events to p107/p130 function have lagged behind those of pRB. However, recent work has begun to suggest that cyclin D-dependent phosphorylation of p130 during G0 exit represents an important transcriptional regulatory event in cell cycle reentry (Dong et al., 1998; Mayol et al., 1996).

The shared structural and E2F-binding properties of the pocket protein family indicate that p107 and p130 are capable of growth suppression through a mechanism similar to pRB. Consistent with such a role as negative regulators of cell proliferation, overexpression of p107 and p130 in certain cell lines produces a G1-phase arrest (Beijersbergen et al., 1995; Vairo et al., 1995; Zhu et al., 1993). Like pRB, this growth suppression activity appears to be subject to negative regulation by components of the cell cycle machinery, as co-expression of D-type cyclins is capable of blocking p107-mediated cell cycle arrest (Beijersbergen et al., 1995). Unlike pRB, mapping of the regions in p107 which are required for this activity has revealed that two distinct domains of this protein can function independently in growth suppression (Zhu et al., 1995). The first is the conserved A•B pocket, which presumably enforces arrest by binding and inhibiting E2Fs. The other p107 growth suppression-domain corresponds to the cyclin•cdk interaction motif within the "spacer" region, and likely functions via sequestration and/or inactivation of cell cycle kinases. These molecular genetic data demonstrate that p107 and p130 can regulate cell cycle progression via inhibition of both E2F and cyclin•cdk activities.

The finding that all three members of the pRB family are sequestered by viral oncoproteins such as large T antigen and E1A (reviewed in Dyson and Harlow, 1992), implies that inactivation of the growth suppression functions of p107/p130 contributes to virally-induced cellular transformation. Indeed, the ability of SV40 large T antigen to transform primary murine cells is dependent upon the pocket-protein binding domain even in *Rb-/-* cells (Christensen and Imperiale, 1995; Zalvide and DeCaprio, 1995). This strongly suggests that inactivation of p107 and p130 is necessary for full disruption of growth control pathways. In the case of SV40 large T, this inactivation appears to be dependent both on direct binding, and on induction of post-translational changes in phosphorylation state and protein stability (Sheng et al., 1997; Stubdal et al., 1997; Zalvide et al., 1998). As with pRB, these forms of regulation result in the disruption of E2F•p107 and E2F•p130 complexes, and are therefore predicted to have deregulatory effects upon E2F-responsive gene transcription. Because the regions of viral oncoproteins required to bind p107/p130 overlap with those that bind pRB, it has been difficult to establish by standard

molecular techniques whether, or to what extent, these pathways differ from those deregulated by disruption of E2F•pRB complexes.

F. The unique tumor suppressive properties of pRB

The apparent functional conservation of pRB, p107 and p130 as regulators of E2F, and as targets of viral oncogenes whose inactivation is necessary for the production of transformed phenotypes, suggests that these proteins play similar roles in negatively regulating the cell division cycle. These similarities suggest that p107 and p130 may also function to inhibit tumorigenesis in the context of the whole organism. However, genetic evidence from both human tumors and mouse strains carrying targeted disruptions of the genomic loci of pRB-family members argues against functional equivalence between pRB, p107 and p130.

The different biological properties of pRB and p107/p130 are apparent at both the tumorigenic and developmental levels. While *RB-1* clearly functions as a tumor suppressor in the human, neither *p107* or *p130* has been found mutated in any type of cancer (reviewed in Weinberg, 1995). This finding suggests that in humans, pRB fulfills a proliferation control function which is not redundant with, or compensated by, the p107 and p130 gene products. This result is reiterated in the mouse, which carries a complement of E2F and pRB family genes highly homologous to their human counterparts: while *Rb-/+* animals are highly predisposed to thyroid and pituitary tumors, heterozygosity for disruption of *p107* or *p130* in the mouse is without tumorigenic consequence in the 129/Sv mouse strain (Cobrinik et al., 1996; Lee et al., 1996).

Significantly, nullizygous *p107* or *p130* 129/Sv mice are completely viable and do not display any developmental or tumorigenic phenotypes (Cobrinik et al., 1996; Lee et al., 1996). As is the case with tumor suppression, this result demonstrates that pRB plays a required role during murine development that is not shared with p107/p130. The apparent lack of required individual developmental roles for p107 and p130 may actually occur as a result of functional overlap at the molecular level between these two E2F regulatory proteins. The p107 and p130 proteins bind largely overlapping subsets of E2F proteins *in vivo* (reviewed in Bernards, 1997), and cells

deficient for p130 display elevated levels of p107 with increased contribution to E2F DNA-binding complexes (Hurford et al., 1997; Mulligan et al., 1998). This molecular phenotype apparently reflects functional compensation at the transcriptional level, as compound deficiency for both *p107* and *p130* results in the deregulation of known E2F-responsive genes (Hurford et al., 1997). This p107/p130 pathway of transcriptional control appears to be required for proper regulation of proliferation in some developing tissues: compound *107-/-;p130-/-* mice die neonataly and show long-bone developmental defects that appear to correlate at the cellular level with chondrocyte overproliferation (Cobrinik et al., 1996). This phenotype is similar to that seen in mice deficient for the $p57^{KIP2}$ cdk inhibitor, suggesting that this protein may function together with p107/p130 to regulate E2F activity in these cells (Yan et al., 1997). However, p107 and p130 do not appear to be completely functionally equivalent within this pathway. The presence of a single copy of the *p107* gene in *p107+/-; p130-/-* animals restores development to near wildtype; in contrast, *p107-/-;p130+/-* animals are dramatically runted and show increased perinatal lethality (Cobrinik et al., 1996). This runted phenotype is similar to that observed in *p107-/-;Rb+/-* mice (Lee et al., 1996). While this correlation is clearly not a demonstration of equivalence, it may reflect the involvement of pRB, p107 and p130 in a shared developmental pathway(s). However, the lack of an increased rate of oncogenesis in (*p107-/-;p130+/-*), (*p107+/-; p130-/-*) and (*p107-/-;Rb+/-*) animals suggests that the functional redundancy of these pocket protein family members may not extend to a collaborative suppression of tumorigenesis.

A recent report suggests that phenotypes associated with deficiency for murine pRB family members are highly strain dependent. In contrast to the 129/Sv phenotypes described above, homozygous inactivation of the *p107* and *p130* loci in a BALB/c genotypic background produces severe developmental defects which appear to correlate at the transcriptional level with deregulation of E2F-responsive transcription (LeCouter et al., 1997). The origins of this phenotypic variabilty remain unclear. It may reflect strain differences in the developmental expression patterns of p107 and p130, or it may arise as a direct consequence of the presence of modifier genes in specific murine strains. Clearly, the identification of the molecular origins of this phenotypic variability is

now a paramount concern, and will likely broaden our understanding of the molecular mechanisms through which p107 and p130 function to restrain cellular proliferation.

G. E2F as cell cycle regulator

The different biological properties of pRB and p107/p130 contrast significantly with the apparently shared role of these proteins as negative regulators of E2F activity. The apparent lack of critical G1/S regulatory or tumor suppressive roles for p107/p130 indicates that E2F may not represent the critical target of pRB, and that the growth regulatory properties of pRB may be mediated through E2F-independent pathways. However, the number of important cell cycle regulatory proteins and DNA replication enzymes whose promoters are E2F-inducible suggests that E2F-mediated activation of target promoters may be sufficient for entry into S-phase. Such sufficiency would imply that E2F is endowed with significant oncogenic potential, and that the tumor suppressive properties of pRB derive in large part from its ability to inhibit this potential. While this model is attractive in its simplicity, it fails to explain why p107 and p130, two other proteins that also inhibit E2F activity, are not tumor suppressors themselves. Resolution of this issue is significantly dependent upon the characterization of the cell cycle regulatory properties of the E2F family proteins. If experiments demonstrate that E2Fs are insufficient to override R-point control, it would then be likely that this class of transcription factors is not the critical target of pRB's growth regulatory properties. However, if E2F proteins prove sufficient to drive the G1/S transition, the lack of p107/p130-mediated tumor suppression suggests an important level of functional specificity in the pocket protein•E2F pathway.

G1. Mammalian E2F overexpression

The structural differences between the multiple members of the mammalian E2F family suggests that functional distinctions may exist between E2Fs in their ability to promote proliferation. However, the precise roles of individual E2Fs in the p16•cyclin D•pRB growth regulatory pathway remains unclear. At one extreme, it is possible that a property exclusive to a single E2F (for instance a unique target gene) renders it more important to G1/S control in certain

tissues than its fellow E2Fs, and that it is therefore *the* critical E2F through which pRB mediates growth suppression. At the other extreme, it is not the deregulation of a single E2F, but the combined effect of deregulating the transcriptional potential of multiple E2Fs, which represents the crucial event in deregulation of growth.

The absence of cancer-associated oncogenic mutations in *E2F* loci suggest that deregulation of individual E2Fs is not sufficient for the oncogenic processes promoted by pRB loss in humans. This suggests that it may be the combined effects of deregulating multiple E2Fs which causes loss of G1/S control. In order to determine how individual E2Fs might contribute to phenotypes associated with pRB deficiency, it is therefore necessary to establish the ability of individual mammalian E2F proteins to regulate progression through G1/S .

To study the ability of human E2Fs to contribute to oncogenic processes, many groups have characterized phenotypes associated with overexpression of E2F family members in cultured cells. In these systems, E2F displays the expected characteristics of a positive regulator of G1/S with oncogenic potential. Enforced expression of E2F-1, -2 or -3 will drive quiescent cells back into the cell cycle (Johnson et al., 1993; Qin et al., 1994; Shan and Lee, 1994). E2F-4 and -5 are also capable of inducing S-phase when expressed in cells, although to a lesser extent than E2F-1, -2 and -3 (Beijersbergen et al., 1994; DeGregori et al., 1997; Lukas et al., 1996). Consistent with the proposed role for E2Fs as the primary transcriptional effectors of the p16•cyclin D•pRB pathway, the expression of E2F-1 ,-2 or -3 is sufficient to overcome G1-phase cell cycle blocks associated with overexpression of pRB, or of inhibition of G1 phase kinases (DeGregori et al., 1995; Lukas et al., 1996; Mann and Jones, 1996; Qin et al., 1995). As in the experiments above, E2F-4 and E2F-5 are less efficient than E2F-1, -2 and -3 in overriding cell cycle blocks imposed through the pRB pathway: E2F-4 or -5 efficiently bypass a p16-mediated G1 block only when coexpressed with DP-1 (Lukas et al., 1996). In some circumstances, overexpressed E2F-1 is capable of inducing transformed phenotypes in primary cells (Johnson et al., 1994; Singh et al., 1994; Xu et al., 1995), and can cooperate with E6 and a pRB-binding-deficient form of E7 in the transformation of normal human foreskin keratinocytes (Melillo et al., 1994). These mitogenic

effects of E2F can apparently be reproduced in the context of the whole organism: transgenic overexpression of one E2F family member, *E2F-1*, under the control of the skin-specific *keratin-5* promoter, promotes epidermal hyperplasia and can cooperate with an v-Ha-*ras* transgene to induce skin tumors in mice (Pierce et al., 1998).

In certain settings, overexpression of oncogenes, such as *myc* and adenoviral *E1A* , is associated with an increased sensitivity to apoptotic stimuli (reviewed in Evan, 1997). While overexpression of E2F-1 in serum-starved cells drives S-phase entry, the ultimate fate of these cells is also programmed cell death (Hiebert et al., 1995; Kowalik et al., 1995; Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994). This apoptotic response can be explained by a "conflicting signals" model in which the simultaneous activation of mitogenic pathways by E2F, and growth arrest pathways by a second stimulus (eg. serum/growth factor deprivation), produce antagonistic and incompatible signals that are resolved by the induction of cellular suicide. However, in some cases E2F-1 overexpression induces actively dividing cells to undergo apoptotic death even in the presence of abundant growth factors (Hsieh et al., 1997; Krek et al, 1995; Phillips et al., 1997). This response to overexpression of E2F-1 is reminiscent of the apoptotic cell death seen in *Rb* -/- mice and raises the possibility that apoptosis is a physiologically relevant response to deregulation of cellular E2F-1. This hypothesis is supported by the finding that cell death induced by ectopic E2F-1 expression is largely dependent upon p53 status (Hiebert et al., 1995; Qin et al., 1994; Wu and Levine, 1994), and that some of the cell death observed in *Rb*-/- embryos is rescued by crossing into a *p53*-/- background (Macleod et al., 1996; Morgenbesser et al., 1994).

Genetic analysis of the regions of E2F necessary for the induction of cellular transformation and apoptosis suggests that the DNA binding and transactivation domains are required for the production of these cell biological phenotypes (Hsieh et al., 1997; Johnson et al., 1993; Krek et al., 1995; Melillo et al., 1994; Phillips et al., 1997; Shan and Lee, 1994; Singh et al., 1994; Xu et al., 1995). This requirement strongly implies that the biological effects of E2F overexpression occur mainly as a consequence of the induction of E2F target genes. These same

targets are predicted to become deregulated by the loss of pRB in a manner sufficient to trigger premature entry into S-phase in the absence of upstream signals.

While the experiments described in this section provide compelling evidence in favor of the "sufficiency" of overexpressed E2Fs for S-phase entry, the important issue of the extent to which individual E2Fs are "necessary" for cell cycle progression has not been as well addressed. The abilities of dominant-negative versions of E2F-1 and DP-1 to block S-phase entry when overexpressed in certain cultured cell lines (Dobrowolski et al., 1994; Wu et al., 1996) suggest that transcriptional activation by E2Fs is necessary for S-phase entry. However, these data contrast with results obtained by microinjection experiments in a different cell type that suggest that S-phase entry can be triggered by cyclin E•cdk2 in the absence of E2F activation (Lukas et al., 1997). This difference may reflect the observation that activation of cyclin E-kinase is a downstream consequence of E2F transcription (see below, section **G3**) and may therefore bypass a requirement for E2F in cell cycle progression; alternatively, it may simply be indicative of cell type-specific requirements for S-phase entry. Regardless, these experiments have not significantly clarified the extent to which E2F-driven transcription is required for G1/S progression in wildtype cells. Proper resolution of this question is clearly dependent upon the ability to assess the requirement(s) for endogenous E2F function in cell cycle regulation from a genetic perspective.

G2. Drosophila E2F function

The conservation of components of the pRB•E2F pathway in other eukaryotic organisms, such as *Drosophila melanogaster*, has provided the opportunity to conduct genetic analysis of the requirement for E2Fs in cell cycle control. While these model systems do not permit a direct assessment of E2F function in mammalian tumorigenesis, they have confirmed a requirement for E2F in developmental control of S-phase entry and progression in flies.

Drosophila E2F and *DP* (dE2F and dDP) were originally identified by low-stringency screening of a *Drosophila* imaginal disc cDNA library with a fragment of the human *E2F-1* cDNA corresponding to the highly conserved C-terminal portion of the DNA binding domain

(Dynlacht et al., 1994; Ohtani and Nevins, 1994). *Drosophila* E2F and DP are structurally and functionally highly analogous to their mammalian homologs. dE2F bears most resemblance to the E2F-1,-2 and -3 subgroup at the amino acid level and is capable of DNA binding and transactivation from a heterologous reporter. dE2F also retains the pRB-binding domain and physically and functionally interacts with the *Drosophila melanogaster* pRB homolog *RBF* (Du et al., 1996a).

Genetic analysis of the function of E2F in *Drosophila* has provided evidence that dE2F and dDP promote cell cycle progression during development. Flies homozygous for mutations in *dE2F* or *dDP* encounter lethality in the late larval or early pupal stages of development (Royzman et al., 1997). In *dE2F* animals, this lethality is apparently the result of dramatic developmental delay that arises due to a prolongation of S-phase and a slowed overall rate of cell cycling. This effect is apparently directly linked to transcriptional deficiency at E2F-responsive promoters, as the G1/S pulse of expression normally associated with two E2F responsive genes, *RNR2* and *PCNA*, is absent in these cells. These phenotypes demonstrate a requirement for dE2F and dDP in timely progression through S-phase and confirm a cell cycle regulatory role for E2F activity which is linked to the transcriptional induction of target genes. Significantly, cells in *dE2F* null larvae are capable of S-phase entry, as measured by BRDU-incorporation (Royzman et al., 1997). This result suggests that the induction of E2F-responsive genes at G1/S is not absolutely required for cell cycle progression. However, it is possible that other E2F-like activities persist in these animals, or that loss of *dE2F* is sufficient to relieve the repression of certain promoters during G1 whose products are minimally sufficient for S-phase entry.

G3. *CyclinE* as a critical E2F target

Similar to human E2F, ectopic expression of dE2F•dDP during *Drosophila* development drives S-phase entry and apoptosis (Du et al., 1996b). Analysis of the genetic requirements of S-phase induction by ectopic dE2F expression reveals that, in some tissues, *cyclin E* is a necessary downstream target of dE2F activity (Duronio et al., 1996; Duronio and O'Farrell, 1995). The role

of mammalian cyclin E•cdk2 in the functional inactivation of pRB suggests that cyclin E•cdk2 is functionally upstream of E2F, however, the inability of overexpressed dE2F to drive S-phase in the absence of *cyclin E* suggests that this target gene directly couples E2F activity to G1/S control in some *Drosophila* tissues. The discovery of multiple E2F binding sites in the promoters of both human and mouse *cyclin E* suggest that E2F is responsible for regulating the periodic expression of cyclin E in mammals as well (Botz et al., 1996; Geng et al., 1996; Ohtani et al., 1995). The link between E2F activity and the periodic transcription of a G1 cyclin•cdk subunit which is necessary for cell cycle progression places E2F within a basic cell division control pathway and suggests that the role of E2F in this pathway includes participation in a positive feedback loop through *cyclin E* to enhance the phosphorylation-driven inactivation of pRB during late G1.

The connection between E2F and *cyclin E* transcription may represent an important mechanism which contributes to the loss of proper G1/S control in *RB-1-/-* cells. While it is clear that one target of cyclin E•cdk2 activity is the retinoblastoma protein, experiments in cultured cells demonstrate cyclin E•cdk2 functions in both pRB-dependent and pRB-independent G1/S control. This demonstrates that in addition to pRB, cyclin E•cdk2 kinase targets an unknown protein(s) whose phosphorylation is likely required for entry into S-phase. The finding that exogenous E2F-1 is capable of activating the transcription of the endogenous human *cyclin E* gene (Ohtani et al., 1995) suggests that loss of pRB renders one G1 cyclin, cyclin D, dispensable and transcriptionally activates cyclin E, the other. This model is further supported by the finding that *cyclin E* expression is specifically deregulated in *Rb-/-* MEFs, but not in either *107-/-* or *p130-/-* cells (Herrera et al., 1996; Hurford et al., 1997; Mulligan et al., 1998). Where such a mechanism operates, E2F-driven activation of cyclin E transcription as a result of pRB inactivation may facilitate the functional inactivation of as yet unknown proteins whose normal function, as targets of cyclin E•cdk2, is to restrain G1/S progression. In such a manner, deregulation of G1/S as a result of pRB loss may also involve deregulation of "pRB-independent" pathways of G1/S control by virtue of the ability of E2F to induce cyclin E.

H. E2F regulation *in vivo*

Studies such as those discussed above confirm the ability of E2F proteins to promote cell cycle progression, and tend to indicate that E2F activity is likely to be the critical target of pRB growth suppression. However, while overexpression studies have made important contributions to models of the mitogenic effects of E2F activity, they have not been able to fully address the important questions of: (1) how E2F in general, and the transcriptional activity of individual E2F proteins in particular, contribute to cell cycle progression; and (2) what are the precise regulatory mechanisms that function to restrict the activity of E2F proteins to specific stages of the cell division cycle.

It is well established that direct, inhibitory interactions with members of the pRB family represent a critical E2F regulatory mechanism. However, the timing and extent to which individual E2F proteins are subject to this inhibition remains unclear. In contrast, the cell cycle-dependent E2F-binding properties of each pRB family protein are well characterized, and have provided important clues regarding the regulation of E2F activity *in vivo*. These studies of the cell cycle-dependent patterns of E2F DNA binding complexes and have found that pRB, p107 and p130 interact with E2F at distinct stages of the cell cycle (Chittenden et al., 1993; Schwarz et al., 1993; Shirodkar et al., 1992). This demonstrates that one level at which the pRB proteins differ in their E2F regulatory properties is the timing of these inhibitory interactions, and suggests that this difference may in part underlie the differences in the biological properties of pRB, p107 and p130.

The most abundant form of E2F activity in G0 cells is a kinase-deficient E2F•p130 complex (Chittenden et al., 1993; Cobrinik et al., 1993; Vairo et al., 1995). In contrast, pRB and p107 do associate with E2F until the cells have reached the G1/S transition, and during S-phase the E2F•p107 complex also contains stably bound cyclin A•cdk2 kinase (Schwarz et al., 1993; Shirodkar et al., 1992). Patterns of E2F•pocket protein binding in actively dividing cells differ somewhat from those observed in cells reentering the cell cycle from G0. In these cells, the E2F•pRB complex is most abundant prior to S-phase, supporting the model of pRB-mediated repression of E2F activity during G1 (Chellappan et al., 1991; Shirodkar et al., 1992). E2F

complexes containing p130 and p107 are present in late G1 and S-phase respectively, and include stably bound forms of cyclin E•cdk2 or cyclin A•cdk2 (Bandara et al., 1991; Devoto et al., 1992; Lees et al., 1992; Mudryj et al., 1991; Pagano et al., 1992). Considered together, the data regarding cell cycle-dependent interactions between E2F and pocket proteins, in both actively dividing cells and those reentering the cell cycle, suggest that pRB, p107 and p130 act in concert to restrict the activity of E2F, and therefore E2F-responsive genes, to precise stages of the cell cycle. The role of pRB, as the only tumor suppressor in this group of highly related E2F-regulatory proteins, would suggest that the retinoblastoma plays a critical role in this pathway by virtue of an important level of functional specificity.

A number of recent studies suggest that E2F-1, -2 and -3 interact specifically with pRB, and not with p107/p130, *in vivo*, and that E2F-4 and E2F-5 proteins participate in complexes with p107/p130 (Beijersbergen et al., 1994; Dyson et al., 1993; Ginsberg et al., 1994; Hijmans et al., 1995; Lees et al., 1993; Sardet et al., 1995). This suggests that the unique biological properties of pRB versus p107/p130 may arise as a consequence of their ability to regulate distinct subsets of cellular E2F. This hypothesis has not been tested by a comprehensive characterization of E2F complexes found *in vivo*. In fact, comparatively little is known regarding extent to which specific members of the E2F family participate in the various "free" E2F and E2F•pocket-protein complexes which display cell cycle-dependent patterns of regulation. As a result, it has been difficult to assign specific cell cycle functions to individual E2F gene products. In this regard, overexpression studies have made important contributions to models of mammalian E2F function. However, it is clear that cellular phenotypes associated with these experiments are to some degree the result of non-physiological levels of E2F expression which may escape normal regulatory mechanisms and overcome functional specificity. This concern makes uncertain the link between ectopic expression and the cell cycle regulatory roles of individual E2Fs *in vivo*. Similarly, it is unclear the extent to which genetic analysis of a single *Drosophila* E2F reveals specific cell cycle functions of the multiple mammalian E2Fs, some of which are more structurally related to dE2F than others, particularly since the expansion of the E2F family in higher eukaryotes may represent

a division, or even diversification, of the function(s) of dE2F. These concerns do not detract from the contributions such data make towards the construction of basic models of E2F function. However, together they emphasize the need for analysis of endogenous mammalian E2F species in order understand the role of individual E2Fs play in mediating the growth regulatory properties of the pocket protein family in general, and of pRB in particular.

References

Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nalin, C. M., and Kaelin, W. G., Jr. (1996). Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. Mol. Cell. Biol. *16*, 6623-33.

Adnane, J., Shao, Z., Robbins, P. D. (1995). The retinoblastoma susceptibility gene product represses transcription when directly bound to the promoter. J. Biol. Chem. *270*, 8837-43.

Aerne, B. L., Johnson, A. L., Toyn, J. H., and Johnston, L. H. (1998). Swi5 controls a novel wave of cyclin synthesis in late mitosis. Mol. Biol. Cell. *9*, 945-56.

Agrawal, D., Hauser, P., McPherson, F., Dong, F., Garcia, A., and Pledger, W. J. (1996). Repression of p27kip1 synthesis by platelet-derived growth factor in BALB/c 3T3 cells. Mol. Cell. Biol. *16*, 4327-36

Aktas, H., Cai, H., and Cooper, G. M. (1997). Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. Mol. Cell. Biol. *17*, 3850-7.

Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J. C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc. Natl. Acad. Sci. U S A. *93*, 13742-7.

Alevizopoulos, K., Vlach, J., Hennecke, S., and Amati, B. (1997). Cyclin E and c-Myc promote cell proliferation in the presence of p16INK4a and of hypophosphorylated retinoblastoma family proteins. EMBO J. *16*, 5322-33.

Bagchi, S., Weinmann, R., and Raychaudhuri, P. (1991). The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. Cell. *65*, 1063-72.

Bandara, L. R., Adamczewski, J. P., Hunt, T., and La Thangue, N. B. (1991). Cyclin A and the retinoblastoma gene product complex with a common transcription factor. Nature. *352*, 249-51.

Bandara, L. R., Buck, V. M., Zamanian, M., Johnston, L. H., and La Thangue, N. B. (1993). Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. EMBO J. *12*, 4317-24.

Bandara, L. R., and La Thangue, N. B. (1991). Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature. *351*, 494-7.

Bandara, L. R., Lam, E. W., Sorensen, T. S., Zamanian, M., Girling, R., and La Thangue, N. B. (1994). DP-1: a cell cycle-regulated and phosphorylated component of transcription factor DRTF1/E2F which is functionally important for recognition by pRb and the adenovirus E4 orf 6/7 protein. EMBO J. *13*, 3104-14.

Bartek, J., Bartkova, J., and Lukas, J. (1996). The retinoblastoma protein pathway and the restriction point. Curr. Opin. Cell. Biol. *8*, 805-14.

Bartkova, J., Lukas, J., and Bartek, J. (1997). Aberrations of the G1- and G1/S-regulating genes in human cancer. Prog. Cell Cycle Res. *3*, 211-20.

Bartkova, J., Lukas, J., Strauss, M., and Bartek, J. (1994). Cell cycle-related variation and tissue-restricted expression of human cyclin D1 protein. J. Pathol. *172*, 237-45.

Beijersbergen, R. L., and Bernards, R. (1996). Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. Biochim. Biophys. Acta. *1287*, 103-20.

Beijersbergen, R. L., Carlee, L., Kerkhoven, R. M., and Bernards, R. (1995). Regulation of the retinoblastoma protein-related p107 by G1 cyclin complexes. Genes & Dev. *9*, 1340-53.

Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M., and Bernards, R. (1994). E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes & Dev. *8*, 2680-90.

Bernards, R. (1997). E2F: a nodal point in cell cycle regulation. Biochim. Biophys. Acta. *1333*, 33-40.

Bookstein, R., Shew, J. Y., Chen, P. L., Scully, P., and Lee, W. H. (1990). Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. Science. *247*, 712-5.

Botz, J., Zerfass-Thome, K., Spitkovsky, D., Delius, H., Vogt, B., Eilers, M., Hatzigeorgiou, A., and Jansen-Durr, P. (1996). Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. Mol. Cell. Biol. *16*, 3401-9.

Bremner, R., Cohen, B. L., Sopta, M., Hamel, P. A., Ingles, C. J., Gallie, B. L., and Phillips, R. A. (1995). Direct transcriptional repression by pRB and its reversal by specific cyclins. Mol. Cell. Biol. *15*, 3256-65.

Brown, J. P., Wei, W., and Sedivy, J. M. (1997). Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. Science. *277*, 831-4.

Brugarolas, J., Bronson, R. T., and Jacks, T. (1998). p21 is a critical CDK2 regulator essential for proliferation control in Rb-deficient cells. J. Cell Biol. *141*, 503-14.

Buchkovich, K., Duffy, L. A., and Harlow, E. (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell. *58*, 1097-105.

Cao, L., Faha, B., Dembski, M., Tsai, L. H., Harlow, E., and Dyson, N. (1992). Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature. *355*, 176-9.

Carnero, A., and Hannon, G. J. (1998). The INK4 family of CDK inhibitors. Curr. Top. Microbiol. Immunol. *227*, 43-55.

Chan, F. K., Zhang, J., Cheng, L., Shapiro, D. N., and Winoto, A. (1995). Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. Mol. Cell. Biol. *15*, 2682-8.

Chellappan, S., Kraus, V. B., Kroger, B., Munger, K., Howley, P. M., Phelps, W. C., and Nevins, J. R. (1992). Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. U S A. *89*, 4549-53.

Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991). The E2F transcription factor is a cellular target for the RB protein. Cell. *65*, 1053-61.

Chen, P. L., Scully, P., Shew, J. Y., Wang, J. Y., and Lee, W. H. (1989). Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell. *58*, 1193-8.

Chin, L., Pomerantz, J., Polsky, D., Jacobson, M., Cohen, C., Cordon-Cardo, C., Horner, J. W. n., and DePinho, R. A. (1997). Cooperative effects of INK4a and ras in melanoma susceptibility in vivo. Genes & Dev. *11*, 2822-34.

Chittenden, T., Livingston, D. M., and DeCaprio, J. A. (1993). Cell cycle analysis of E2F in primary human T cells reveals novel E2F complexes and biochemically distinct forms of free E2F. Mol. Cell. Biol. *13*, 3975-83.

Chow, K. N., and Dean, D. C. (1996). Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. Mol. Cell. Biol. *16*, 4862-8.

Chow, K. N., Starostik, P., and Dean, D. C. (1996). The Rb family contains a conserved cyclin-dependent-kinase-regulated transcriptional repressor motif. Mol. Cell. Biol. *16*, 7173-81.

Christensen, J., and Imperiale, M. (1995). Inactivation of the retinoblastoma susceptibility protein is not sufficient for the transforming function of the conserved region 2-like domain of simian virus 40 large T antigen. J. Virol. *69*, 3945-3948.

Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M., van der Valk, M., Hooper, M. L., Berns, A., and te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development [see comments]. Nature. *359*, 328-30.

Cobrinik, D., Lee, M. H., Hannon, G., Mulligan, G., Bronson, R. T., Dyson, N., Harlow, E., Beach, D., Weinberg, R. A., and Jacks, T. (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. Genes & Dev. *10*, 1633-44.

Cobrinik, D., Whyte, P., Peeper, D. S., Jacks, T., and Weinberg, R. A. (1993). Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes & Dev. *7*, 2392-404.

Connell-Crowley, L., Harper, J. W., and Goodrich, D. W. (1997). Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol. Biol. Cell. *8*, 287-301.

Crescenzi, M., Fleming, T. P., Lassar, A. B., Weintraub, H., and Aaronson, S. A. (1990). MyoD induces growth arrest independent of differentiation in normal and transformed cells. Proc. Natl. Acad. Sci. U S A. *87*, 8442-6.

Cross, F. R., Hoek, M., McKinney, J. D., and Tinkelenberg, A. H. (1994). Role of Swi4 in cell cycle regulation of CLN2 expression. Mol. Cell. Biol. *14*, 4779-87.

De Luca, A., MacLachlan, T. K., Bagella, L., Dean, C., Howard, C. M., Claudio, P. P., Baldi, A., Khalili, K., and Giordano, A. (1997). A unique domain of pRb2/p130 acts as an inhibitor of Cdk2 kinase activity. J. Biol. Chem. *272*, 20971-4.

de Nooij, J. C., Letendre, M. A., and Hariharan, I. K. (1996). A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during Drosophila embryogenesis. Cell. *87*, 1237-47.

DeCaprio, J. A., Furukawa, Y., Ajchenbaum, F., Griffin, J. D., and Livingston, D. M. (1992). The retinoblastoma-susceptibility gene product becomes phosphorylated in multiple stages during cell cycle entry and progression. Proc. Natl. Acad. Sci. U S A. *89*, 1795-8.

DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C. M., and Livingston, D. M. (1989). The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell. *58*, 1085-95.

DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. Proc. Natl. Acad. Sci. U S A. *94*, 7245-50.

DeGregori, J., Leone, G., Ohtani, K., Miron, A., and Nevins, J. R. (1995). E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. Genes & Dev. *9*, 2873-87.

Devoto, S. H., Mudryj, M., Pines, J., Hunter, T., and Nevins, J. R. (1992). A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. Cell. *68*, 167-76.

Dobrowolski, S. F., Stacey, D. W., Harter, M. L., Stine, J. T., and Hiebert, S. W. (1994). An E2F dominant negative mutant blocks E1A induced cell cycle progression. Oncogene. *9*, 2605-12.

Dong, F., Cress, W. D., Jr., Agrawal, D., and Pledger, W. J. (1998). The role of cyclin D3-dependent kinase in the phosphorylation of p130 in mouse BALB/c 3T3 fibroblasts. J. Biol. Chem. *273*, 6190-6195.

Dowdy, S. F., Hinds, P. W., Louie, K., Reed, S. I., Arnold, A., and Weinberg, R. A. (1993). Physical interaction of the retinoblastoma protein with human D cyclins. Cell. *73*, 499-511.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell. *56*, 829-38.

Du, W., Vidal, M., Xie, J. E., and Dyson, N. (1996a). RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in Drosophila. Genes & Dev. *10*, 1206-18.

Du, W., Xie, J. E., and Dyson, N. (1996b). Ectopic expression of dE2F and dDP induces cell proliferation and death in the Drosophila eye. EMBO J. *15*, 3684-92.

Duncan, E. L., and Reddel, R. R. (1997). Genetic changes associated with immortalization. A review. Biochemistry (Mosc). *62*, 1263-74.

Dunphy, W. G., Brizuela, L., Beach, D., and Newport, J. (1988). The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell. *54*, 423-31.

Duronio, R. J., Brook, A., Dyson, N., and O'Farrell, P. H. (1996). E2F-induced S phase requires cyclin E. Genes & Dev. *10*, 2505-13.

Duronio, R. J., and O'Farrell, P. H. (1995). Developmental control of the G1 to S transition in Drosophila: cyclin Eis a limiting downstream target of E2F. Genes & Dev. *9*, 1456-68.

Dynlacht, B. D., Brook, A., Dembski, M., Yenush, L., and Dyson, N. (1994). DNA-binding and trans-activation properties of Drosophila E2F and DP proteins. Proc. Natl. Acad. Sci. U S A. *91*, 6359-63.

Dyson, N., Dembski, M., Fattaey, A., Ngwu, C., Ewen, M., and Helin, K. (1993). Analysis of p107-associated proteins: p107 associates with a form of E2F that differs from pRB-associated E2F-1. J. Virol.. *67*, 7641-7.

Dyson, N., and Harlow, E. (1992). Adenovirus E1A targets key regulators of cell proliferation. Cancer Surv. *12*, 161-95.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science. *274*, 1664-72.

Elledge, S. J., and Harper, J. W. (1994). Cdk inhibitors: on the threshold of checkpoints and development. Curr. Opin. Cell. Biol. *6*, 847-52.

Evan, G. (1997). Cancer--a matter of life and cell death. Int. J. Cancer. *71*, 709-11.

Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D., and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell. *33*, 389-96.

Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J., and Livingston, D. M. (1993). Functional interactions of the retinoblastoma protein with mammalian D- type cyclins. Cell. *73*, 487-97.

Ewen, M. E., Xing, Y. G., Lawrence, J. B., and Livingston, D. M. (1991). Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell. *66*, 1155-64.

Ezhevsky, S. A., Nagahara, H., Vocero-Akbani, A. M., Gius, D. R., Wei, M. C., and Dowdy, S. F. (1997). Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. Proc. Natl. Acad. Sci. U S A. *94*, 10699-704.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes & Dev. *9*, 2364-72.

Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Firpo, E., Polyak, K., Tsai, L. H., Broudy, V., Perlmutter, R. M., Kaushansky, K., and Roberts, J. M. (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell. *85*, 733-44.

Figge, J., Webster, T., Smith, T. F., and Paucha, E. (1988). Prediction of similar transforming regions in simian virus 40 large T, adenovirus E1A, and myc oncoproteins. J. Virol.. *62*, 1814-8.

Filmus, J., Robles, A. I., Shi, W., Wong, M. J., Colombo, L. L., and Conti, C. J. (1994). Induction of cyclin D1 overexpression by activated ras. Oncogene. *9*, 3627-33.

Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature. *323*, 643-6.

Fung, Y. K., Murphree, A. L., T'Ang, A., Qian, J., Hinrichs, S. H., and Benedict, W. F. (1987). Structural evidence for the authenticity of the human retinoblastoma gene. Science. *236*, 1657-61.

Furnari, B., Rhind, N., and Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase [see comments]. Science. *277*, 1495-7.

Furukawa, Y., DeCaprio, J. A., Freedman, A., Kanakura, Y., Nakamura, M., Ernst, T. J., Livingston, D. M., and Griffin, J. D. (1990). Expression and state of phosphorylation of the retinoblastoma susceptibility gene product in cycling and noncycling human hematopoietic cells. Proc. Natl. Acad. Sci. U S A. *87*, 2770-4.

Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988). Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene cdc2+. Cell. *54*, 433-9.

Geng, Y., Eaton, E. N., Picon, M., Roberts, J. M., Lundberg, A. S., Gifford, A., Sardet, C., and Weinberg, R. A. (1996). Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. Oncogene. *12*, 1173-80.

Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z. X., Xu, G., Wydner, K. L., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994). E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes & Dev. *8*, 2665-79.

Girling, R., Partridge, J. F., Bandara, L. R., Burden, N., Totty, N. F., Hsuan, J. J., and La Thangue, N. B. (1993). A new component of the transcription factor DRTF1/E2F [published erratum appears in Nature 1993 Sep 30;365(6445):468]. Nature. *362*, 83-7.

Goldstein, S. (1990). Replicative senescence: the human fibroblast comes of age. Science. *249*, 1129-33.

Goodrich, D. W., Wang, N. P., Qian, Y. W., Lee, E. Y., and Lee, W. H. (1991). The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell. *67*, 293-302.

Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell. *72*, 309-24.

Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes & Dev. *8*, 2939-52.

Guan, K. L., Jenkins, C. W., Li, Y., O'Keefe, C. L., Noh, S., Wu, X., Zariwala, M., Matera, A. G., and Xiong, Y. (1996). Isolation and characterization of p19INK4d, a p16-related inhibitor specific to CDK6 and CDK4. Mol. Biol. Cell. *7*, 57-70.

Haber, D. A. (1997). Splicing into senescence: the curious case of p16 and p19ARF. Cell. *91*, 555-8.

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD [see comments]. Science. *267*, 1018-21.

Hamel, P. A., and Hanley-Hyde, J. (1997). G1 cyclins and control of the cell division cycle in normal and transformed cells. Cancer Invest. *15*, 143-52.

Hannon, G. J., Demetrick, D., and Beach, D. (1993). Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes & Dev. *7*, 2378-91.

Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. (1996). Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Mol. Cell. Biol. *16*, 859-67.

Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., and et al. (1995). Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell. *6*, 387-400.

Hartwell, L. H., Culotti, J., Pringle, J. R., and Reid, B. J. (1974). Genetic control of the cell division cycle in yeast. Science. *183*, 46-51.

Hatakeyama, M., Brill, J. A., Fink, G. R., and Weinberg, R. A. (1994). Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. Genes & Dev. *8*, 1759-71.

Hayflick, L., and P.S. Moorhead. (1961). Experimental Cell Research. *25*,

Hayflick, L. (1997). Mortality and immortality at the cellular level: A review. Biochemistry. *62*, 1180-90.

Helin, K., Harlow, E., and Fattaey, A. (1993a). Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. *13*, 6501-8.

Helin, K., Wu, C. L., Fattaey, A. R., Lees, J. A., Dynlacht, B. D., Ngwu, C., and Harlow, E. (1993b). Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. Genes & Dev. *7*, 1850-61.

Hengst, L., and Reed, S. I. (1998). Inhibitors of the Cip/Kip family. Curr. Top. Microbiol. Immunol. *227*, 25-41.

Herrera, R. E., Sah, V. P., Williams, B. O., Makela, T. P., Weinberg, R. A., and Jacks, T. (1996). Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. Mol. Cell. Biol. *16*, 2402-7.

Hiebert, S. W. (1993). Regions of the retinoblastoma gene product required for its interaction with the E2F transcription factor are necessary for E2 promoter repression and pRb-mediated growth suppression. Mol. Cell. Biol. *13*, 3384-91.

Hiebert, S. W., Chellappan, S. P., Horowitz, J. M., and Nevins, J. R. (1992). The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes & Dev. *6*, 177-85.

Hiebert, S. W., Packham, G., Strom, D. K., Haffner, R., Oren, M., Zambetti, G., and Cleveland, J. L. (1995). E2F-1:DP-1 induces p53 and overrides survival factors to trigger apoptosis. Mol. Cell. Biol. *15*, 6864-74.

Hijmans, E. M., Voorhoeve, P. M., Beijersbergen, R. L., van 't Veer, L. J., and Bernards, R. (1995). E2F-5, a new E2F family member that interacts with p130 in vivo. Mol. Cell. Biol. *15*, 3082-9.

Hindley, J., and Phear, G. A. (1984). Sequence of the cell division gene CDC2 from Schizosaccharomyces pombe; patterns of splicing and homology to protein kinases. Gene. *31*, 129-34.

Hinds, P. W., Dowdy, S. F., Eaton, E. N., Arnold, A., and Weinberg, R. A. (1994). Function of a human cyclin gene as an oncogene. Proc. Natl. Acad. Sci. U S A. *91*, 709-13.

Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell. *70*, 993-1006.

Hirai, H., Roussel, M. F., Kato, J. Y., Ashmun, R. A., and Sherr, C. J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol. Cell. Biol. *15*, 2672-81.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991). p53 mutations in human cancers. Science. *253*, 49-53.

Hsieh, J. K., Fredersdorf, S., Kouzarides, T., Martin, K., and Lu, X. (1997). E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. Genes & Dev. *11*, 1840-52.

Hu, Q.J., Lees, J.A., Buchkovich, K.J., and Harlow, E. (1992). The retinoblastoma protein physically associates with the human cdc2 kinase. Mol. Cell. Biol. *12*, 971-980.

Huang, D., Moffat, J., Wilson, W. A., Moore, L., Cheng, C., Roach, P. J., and Andrews, B. (1998). Cyclin partners determine pho85 protein kinase substrate specificity In vitro and In vivo: control of glycogen biosynthesis by pcl8 and pcl10. Mol. Cell. Biol. *18*, 3289-99.

Huang, H. J., Yee, J. K., Shew, J. Y., Chen, P. L., Bookstein, R., Friedmann, T., Lee, E. Y., and Lee, W. H. (1988). Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science. *242*, 1563-6.

Hurford, R. K., Jr., Cobrinik, D., Lee, M. H., and Dyson, N. (1997). pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes & Dev. *11*, 1447-63.

Hwang, L. H., Lau, L. F., Smith, D. L., Mistrot, C. A., Hardwick, K. G., Hwang, E. S., Amon, A., and Murray, A. W. (1998). Budding yeast Cdc20: a target of the spindle checkpoint. Science. *279*, 1041-4.

Imai, Y., Matsushima, Y., Sugimura, T., and Terada, M. (1991). Purification and characterization of human papillomavirus type 16 E7 protein with preferential binding capacity to the underphosphorylated form of retinoblastoma gene product. J. Virol.. *65*, 4966-72.

Inaba, T., Matsushime, H., Valentine, M., Roussel, M. F., Sherr, C. J., and Look, A. T. (1992). Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. Genomics. *13*, 565-74.

Ivey-Hoyle, M., Conroy, R., Huber, H.E., Goodhart, P.J., Oliff, A., and Heimbrook, D.C. (1993). Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol. Cell. Biol. *13*, 7802-12.

Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. (1992). Effects of an Rb mutation in the mouse . Nature. *359*, 295-300.

Jiang, W., Kahn, S. M., Zhou, P., Zhang, Y. J., Cacace, A. M., Infante, A. S., Doi, S., Santella, R. M., and Weinstein, I. B. (1993). Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. Oncogene. *8*, 3447-57.

Johnson, D. G., Cress, W. D., Jakoi, L., and Nevins, J. R. (1994). Oncogenic capacity of the E2F1 gene. Proc. Natl. Acad. Sci. U S A. *91*, 12823-7.

Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature. *365*, 349-52.

Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S. r., Johnson, B. E., and Skolnick, M. H. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science. *264*, 436-40.

Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell. *91*, 649-59.

Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes & Dev. *7*, 331-42.

Kerkhoff, E., and Ziff, E. B. (1995). Cyclin D2 and Ha-Ras transformed rat embryo fibroblasts exhibit a novel deregulation of cell size control and early S phase arrest in low serum. EMBO J. *14*, 1892-903.

King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. (1996). How proteolysis drives the cell cycle. Science. *274*, 1652-9.

Kitagawa, M., Higashi, H., Jung, H. K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yoshida, E., Nishimura, S., and Taya, Y. (1996). The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J. *15*, 7060-9.

Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohman, L. A., and Koff, A. (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell. *85*, 721-32.

Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C. F. (1994). Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell. *77*, 107-20.

Koh, J., Enders, G. H., Dynlacht, B. D., and Harlow, E. (1995). Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature. *375*, 506-10.

Kowalik, T. F., DeGregori, J., Schwarz, J. K., and Nevins, J. R. (1995). E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J. Virol.. *69*, 2491-500.

Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., Jr., and Livingston, D. M. (1994). Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell. *78*, 161-72.

Krek, W., Livingston, D. M., and Shirodkar, S. (1993). Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. Science. *262*, 1557-60.

Krek, W., Xu, G., and Livingston, D. M. (1995). Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. Cell. *83*, 1149-58.

Lane, M. E., Sauer, K., Wallace, K., Jan, Y. N., Lehner, C. F., and Vaessin, H. (1996). Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during Drosophila development. Cell. *87*, 1225-35.

Langan, T. A., Gautier, J., Lohka, M., Hollingsworth, R., Moreno, S., Nurse, P., Maller, J., and Sclafani, R. A. (1989). Mammalian growth-associated H1 histone kinase: a homolog of cdc2+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells. Mol. Cell. Biol. *9*, 3860-8.

LeCouter, J.E., Megeney, L.A., Kablar, B., Parker, M., Hardy, R., Singh, G., and Rudnicki, M.A. (1997). Severe growth and differentiation deficits in mice lacking p107 and p130. *Proceedings of the Conference on Tumor Suppressor Genes*, Vancouver, B.C.

Lee, E. Y., Chang, C. Y., Hu, N., Wang, Y. C., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis [see comments]. Nature. *359*, 288-94.

Lee, M. H., Reynisdottir, I., and Massague, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes & Dev. *9*, 639-49.

Lee, M. H., Williams, B. O., Mulligan, G., Mukai, S., Bronson, R. T., Dyson, N., Harlow, E., and Jacks, T. (1996). Targeted disruption of p107: functional overlap between p107 and Rb. Genes & Dev. *10*, 1621-32.

Lee, W. H., Shew, J. Y., Hong, F. D., Sery, T. W., Donoso, L. A., Young, L. J., Bookstein, R., and Lee, E. Y. (1987). The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature. *329*, 642-5.

Lees, E., Faha, B., Dulic, V., Reed, S. I., and Harlow, E. (1992). Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. Genes & Dev. *6*, 1874-85.

Lees, J. A., Buchkovich, K. J., Marshak, D. R., Anderson, C. W., and Harlow, E. (1991). The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J. *10*, 4279-90.

Lees, J. A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K. (1993). The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. *13*, 7813-25.

Leng, X., Connell-Crowley, L., Goodrich, D., and Harper, J. W. (1997). S-Phase entry upon ectopic expression of G1 cyclin-dependent kinases in the absence of retinoblastoma protein phosphorylation. Curr. Biol. *7*, 709-12.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell. *88*, 323-31.

Levine, A. J., Momand, J., and Finlay, C. A. (1991). The p53 tumour suppressor gene. Nature. *351*, 453-6.

Levine, K., Huang, K., and Cross, F. R. (1996). Saccharomyces cerevisiae G1 cyclins differ in their intrinsic functional specificities. Mol. Cell. Biol. *16*, 6794-803.

Lew, D. J., Dulic, V., and Reed, S. I. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell. *66*, 1197-206.

Li, Y., Graham, C., Lacy, S., Duncan, A. M., and Whyte, P. (1993). The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes & Dev. *7*, 2366-77.

Liu, J. J., Chao, J. R., Jiang, M. C., Ng, S. Y., Yen, J. J., and Yang-Yen, H. F. (1995). Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. Mol. Cell. Biol. *15*, 3654-63.

Lorincz, A. T., and Reed, S. I. (1984). Primary structure homology between the product of yeast cell division control gene CDC28 and vertebrate oncogenes. Nature. *307*, 183-5.

Lovec, H., Grzeschiczek, A., Kowalski, M. B., and Moroy, T. (1994). Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice. EMBO J. *13*, 3487-95.

Ludlow, J. W., DeCaprio, J. A., Huang, C. M., Lee, W. H., Paucha, E., and Livingston, D. M. (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell. *56*, 57-65.

Lukas, J., Bartkova, J., and Bartek, J. (1996). Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb-controlled G1 checkpoint. Mol. Cell. Biol. *16*, 6917-25.

Lukas, J., Bartkova, J., Rohde, M., Strauss, M., and Bartek, J. (1995). Cyclin D1 is dispensable for G1 control in retinoblastoma gene- deficient cells independently of cdk4 activity. Mol. Cell. Biol. *15*, 2600-11.

Lukas, J., Herzinger, T., Hansen, K., Moroni, M. C., Resnitzky, D., Helin, K., Reed, S. I., and Bartek, J. (1997). Cyclin E-induced S phase without activation of the pRb/E2F pathway. Genes & Dev. *11*, 1479-92.

Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature. *375*, 503-6.

Lukas, J., Petersen, B. O., Holm, K., Bartek, J., and Helin, K. (1996). Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. Mol. Cell. Biol. *16*, 1047-57.

Lundberg, A. S., and Weinberg, R. A. (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol. Cell. Biol. *18*, 753-61.

Maandag, E. C., van der Valk, M., Vlaar, M., Feltkamp, C., O'Brien, J., van Roon, M., van der Lugt, N., Berns, A., and te Riele, H. (1994). Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. EMBO J. *13*, 4260-8.

Macleod, K. F., Hu, Y., and Jacks, T. (1996). Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. EMBO J. *15*, 6178-88.

Mann, D. J., and Jones, N. C. (1996). E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest. Curr. Biol. *6*, 474-83.

Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W., and Elledge, S. J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes & Dev. *9*, 650-62.

Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.-Y., Hanks, S.K., Roussel, M.F., and Sherr, C.J. (1992). Identification and properties of an atypical catalytic subunit ($p34^{PSK-J3}$/cdk4) for mammalian D type cyclins. Cell. *71*, 323-334.

Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J., and Kato, J. Y. (1994). D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. *14*, 2066-76.

Mayol, X., Garriga, J., and Grana, X. (1996). G1 cyclin/CDK-independent phosphorylation and accumulation of p130 during the transition from G1 to G0 lead to its association with E2F-4. Oncogene. *13*, 237-46.

Mayol, X., Grana, X., Baldi, A., Sang, N., Hu, Q., and Giordano, A. (1993). Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene. *8*, 2561-6.

Medeiros, L. J., Van Krieken, J. H., Jaffe, E. S., and Raffeld, M. (1990). Association of bcl-1 rearrangements with lymphocytic lymphoma of intermediate differentiation. Blood. *76*, 2086-90.

Medema, R. H., Herrera, R. E., Lam, F., and Weinberg, R. A. (1995). Growth suppression by p16ink4 requires functional retinoblastoma protein. Proc. Natl. Acad. Sci. U S A. *92*, 6289-93.

Melillo, R. M., Helin, K., Lowy, D. R., and Schiller, J. T. (1994). Positive and negative regulation of cell proliferation by E2F-1: influence of protein level and human papillomavirus oncoproteins. Mol. Cell. Biol. *14*, 8241-9.

Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E., and Tsai, L. H. (1992). A family of human cdc2-related protein kinases. EMBO J. *11*, 2909-17.

Meyerson, M., and Harlow, E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. *14*, 2077-2086.

Mihara, K., Cao, X. R., Yen, A., Chandler, S., Driscoll, B., Murphree, A. L., T'Ang, A., and Fung, Y. K. (1989). Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science. *246*, 1300-3.

Mittnacht, S. (1998). Control of pRB phosphorylation. Curr. Opin. Genet. Dev. *8*, 21-7.

Mittnacht, S., Paterson, H., Olson, M. F., and Marshall, C. J. (1997). Ras signalling is required for inactivation of the tumour suppressor pRb cell-cycle control protein. Curr. Biol. *7*, 219-21.

Molkentin, J. D., and Olson, E. N. (1996). Defining the regulatory networks for muscle development. Curr. Opin. Genet. Dev. *6*, 445-53.

Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. (1994). p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature. *371*, 72-4.

Morkel M., Wenkel, J., Bannister, A.J., Kouzarides, T., and Hagemeier, C. (1997). An E2F-like repressor of transcription. Nature(scientific correspondence). *390*, 567-568.

Motokura, T., Bloom, T., Kim, H. G., Juppner, H., Ruderman, J. V., Kronenberg, H. M., and Arnold, A. (1991). A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature. *350*, 512-5.

Mudryj, M., Devoto, S. H., Hiebert, S. W., Hunter, T., Pines, J., and Nevins, J. R. (1991). Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. Cell. *65*, 1243-53.

Mulligan, G.J., and Jacks T. (1998). The retinoblastoma gene family: cousins with overlapping interests. Trends in Genetics. *14*, 223-229.

Mulligan, G. J., Wong, J., and Jacks, T. (1998). p130 is dispensable in peripheral T lymphocytes: evidence for functional compensation by p107 and pRB. Mol. Cell. Biol. *18*, 206-20.

Murray, A., and T. Hunt. (1993). The Cell Cycle: an introduction. Oxford University Press. *New York, NY.*

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D. Y., and Nakayama, K. (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell. *85*, 707-20.

Nasmyth, K. (1996). Viewpoint: putting the cell cycle in order. Science. *274*, 1643-5.

Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature. *368*, 753-6.

Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp. Cell. Res. *211*, 90-8.

Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M. H., Massague, J., Crabtree, G. R., and Roberts, J. M. (1994). Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. Nature. *372*, 570-3.

Novitch, B. G., Mulligan, G. J., Jacks, T., and Lassar, A. B. (1996). Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. J. Cell. Biol. *135*, 441-56.

Nurse, P., and Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe. Genetics. *96*, 627-37.

Ohtani, K., DeGregori, J., and Nevins, J. R. (1995). Regulation of the cyclin E gene by transcription factor E2F1. Proc. Natl. Acad. Sci. U S A. *92*, 12146-50.

Ohtani, K., and Nevins, J. R. (1994). Functional properties of a Drosophila homolog of the E2F1 gene. Mol. Cell. Biol. *14*, 1603-12.

Ohtsubo, M., and Roberts, J. M. (1993). Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science. *259*, 1908-12.

Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol. Cell. Biol. *15*, 2612-24.

Ormondroyd, E., de la Luna, S., and La Thangue, N. B. (1995). A new member of the DP family, DP-3, with distinct protein products suggests a regulatory role for alternative splicing in the cell cycle transcription factor DRTF1/E2F. Oncogene. *11*, 1437-46.

Pagano, M., Draetta, G., and Jansen-Durr, P. (1992). Association of cdk2 kinase with the transcription factor E2F during S phase. Science. *255*, 1144-7.

Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W., and Elledge, S. J. (1995). p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells [see comments]. Science. *267*, 1024-7.

Peeper, D. S., Parker, L. L., Ewen, M. E., Toebes, M., Hall, F. L., Xu, M., Zantema, A., van der Eb, A. J., and Piwnica-Worms, H. (1993). A- and B-type cyclins differentially modulate substrate specificity of cyclin-cdk complexes. EMBO J. *12*, 1947-54.

Peeper, D. S., Upton, T. M., Ladha, M. H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J. A., and Ewen, M. E. (1997). Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature. *386*, 177-81.

Phillips, A. C., Bates, S., Ryan, K. M., Helin, K., and Vousden, K. H. (1997). Induction of DNA synthesis and apoptosis are separable functions of E2F- 1. Genes & Dev. *11*, 1853-63.

Pierce, A. M., Fisher, S. M., Conti, C. J., and Johnson, D. G. (1998). Deregulated expression of E2F1 induces hyperplasia and cooperates with ras in skin tumor development. Oncogene. *16*, 1267-76.

Piggott, J. R., Rai, R., and Carter, B. L. (1982). A bifunctional gene product involved in two phases of the yeast cell cycle. Nature. *298*, 391-3.

Planas-Silva, M. D., and Weinberg, R. A. (1997). The restriction point and control of cell proliferation. Curr. Opin. Cell. Biol. *9*, 768-72.

Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., Cordon-Cardo, C., and DePinho, R. A. (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell. *92*, 713-23.

Qin, X. Q., Chittenden, T., Livingston, D. M., and Kaelin, W. G., Jr. (1992). Identification of a growth suppression domain within the retinoblastoma gene product. Genes & Dev. *6*, 953-64.

Qin, X. Q., Livingston, D. M., Ewen, M., Sellers, W. R., Arany, Z., and Kaelin, W. G., Jr. (1995). The transcription factor E2F-1 is a downstream target of RB action. Mol. Cell. Biol. *15*, 742-55.

Qin, X. Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. U S A. *91*, 10918-22.

Quelle, D. E., Cheng, M., Ashmun, R. A., and Sherr, C. J. (1997). Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. Proc. Natl. Acad. Sci. U S A. *94*, 669-73.

Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell. *83*, 993-1000.

Ranade, K., Hussussian, C. J., Sikorski, R. S., Varmus, H. E., Goldstein, A. M., Tucker, M. A., Serrano, M., Hannon, G. J., Beach, D., and Dracopoli, N. C. (1995). Mutations associated with familial melanoma impair p16INK4 function [letter]. Nat. Genet. *10*, 114-6.

Raychaudhuri, P., Bagchi, S., Devoto, S. H., Kraus, V. B., Moran, E., and Nevins, J. R. (1991). Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes. Genes & Dev. *5*, 1200-11.

Reed, S. I., Hadwiger, J. A., and Lorincz, A. T. (1985). Protein kinase activity associated with the product of the yeast cell division cycle gene CDC28. Proc. Natl. Acad. Sci. U S A. *82*, 4055-9.

Resnitzky, D., and Reed, S. I. (1995). Different roles for cyclins D1 and E in regulation of the G1-to-S transition. Mol. Cell. Biol. *15*, 3463-9.

Reznikoff, C. A., Yeager, T. R., Belair, C. D., Savelieva, E., Puthenveettil, J. A., and Stadler, W. M. (1996). Elevated p16 at senescence and loss of p16 at immortalization in human papillomavirus 16 E6, but not E7, transformed human uroepithelial cells. Cancer Res. *56*, 2886-90.

Rogers, K. T., Higgins, P. D., Milla, M. M., Phillips, R. S., and Horowitz, J. M. (1996). DP-2, a heterodimeric partner of E2F: identification and characterization of DP-2 proteins expressed in vivo. Proc. Natl. Acad. Sci. U S A. *93*, 7594-9.

Royzman, I., Whittaker, A. J., and Orr-Weaver, T. L. (1997). Mutations in Drosophila DP and E2F distinguish G1-S progression from an associated transcriptional program. Genes & Dev. *11*, 1999-2011.

Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25 [see comments]. Science. *277*, 1497-501.

Sardet, C., Vidal, M., Cobrinik, D., Geng, Y., Onufryk, C., Chen, A., and Weinberg, R. A. (1995). E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. U S A. *92*, 2403-7.

Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994). Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. Science. *264*, 1467-71.

Schwarz, J. K., Devoto, S. H., Smith, E. J., Chellappan, S. P., Jakoi, L., and Nevins, J. R. (1993). Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. EMBO J. *12*, 1013-20.

Sellers, W. R., and Kaelin, W. G. (1996). RB as a modulator of transcription. Biochim. Biophys. Acta. *1288*, M1-5.

Sellers, W. R., Novitch, B. G., Miyake, S., Heith, A., Otterson, G. A., Kaye, F. J., Lassar, A. B., and Kaelin, W. G., Jr. (1998). Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. Genes & Dev. *12*, 95-106.

Serrano, M., Gomez-Lahoz, E., DePinho, R. A., Beach, D., and Bar-Sagi, D. (1995). Inhibition of ras-induced proliferation and cellular transformation by p16INK4. Science. *267*, 249-52.

Serrano, M., Hannon, G. J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature. *366*, 704-7.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R. A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell. *85*, 27-37.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. *88*, 593-602.

Shan, B., and Lee, W. H. (1994). Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Biol. *14*, 8166-73.

Sheng, Q., Denis, D., Ratnofsky, M., Roberts, T. M., DeCaprio, J. A., and Schaffhausen, B. (1997). The DnaJ domain of polyomavirus large T antigen is required to regulate Rb family tumor suppressor function. J. Virol.. *71*, 9410-6.

Sherr, C. J. (1995). D-type cyclins. Trends Biochem. Sci. *20*, 187-90.

Sherr, C. J. (1996). Cancer cell cycles. Science. *274*, 1672-7.

Sherr, C. J., and Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes & Dev. *9*, 1149-63.

Shirodkar, S., Ewen, M., DeCaprio, J. A., Morgan, J., Livingston, D. M., and Chittenden, T. (1992). The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell. *68*, 157-66.

Sicinski, P., Donaher, J. L., Geng, Y., Parker, S. B., Gardner, H., Park, M. Y., Robker, R. L., Richards, J. S., McGinnis, L. K., Biggers, J. D., Eppig, J. J., Bronson, R. T., Elledge, S. J., and Weinberg, R. A. (1996). Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature. *384*, 470-4.

Sicinski, P., Donaher, J. L., Parker, S. B., Li, T., Fazeli, A., Gardner, H., Haslam, S. Z., Bronson, R. T., Elledge, S. J., and Weinberg, R. A. (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell. *82*, 621-30.

Sidorova, J. M., and Breeden, L. L. (1997). Rad53-dependent phosphorylation of Swi6 and down-regulation of CLN1 and CLN2 transcription occur in response to DNA damage in Saccharomyces cerevisiae. Genes & Dev. *11*, 3032-45.

Singh, P., Wong, S. H., and Hong, W. (1994). Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. EMBO J. *13*, 3329-38.

Stacey, D. W., Dobrowolski, S. F., Piotrkowski, A., and Harter, M. L. (1994). The adenovirus E1A protein overrides the requirement for cellular ras in initiating DNA synthesis. EMBO J. *13*, 6107-14.

Starostik, P., Chow, K. N., and Dean, D. C. (1996). Transcriptional repression and growth suppression by the p107 pocket protein. Mol. Cell. Biol. *16*, 3606-14.

Stubdal, H., Zalvide, J., Campbell, K. S., Schweitzer, C., Roberts, T. M., and DeCaprio, J. A. (1997). Inactivation of pRB-related proteins p130 and p107 mediated by the J domain of simian virus 40 large T antigen. Mol. Cell. Biol. *17*, 4979-90.

Surmacz, E., Reiss, K., Sell, C., and Baserga, R. (1992). Cyclin D1 messenger RNA is inducible by platelet-derived growth factor in cultured fibroblasts. Cancer Res. *52*, 4522-5.

Tahara, H., Sato, E., Noda, A., and Ide, T. (1995). Increase in expression level of p21sdi1/cip1/waf1 with increasing division age in both normal and SV40-transformed human fibroblasts. Oncogene. *10*, 835-40.

Takuwa, N., and Takuwa, Y. (1997). Ras activity late in G1 phase required for p27kip1 downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. Mol. Cell. Biol. *17*, 5348-58.

Templeton, D. J., Park, S. H., Lanier, L., and Weinberg, R. A. (1991). Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. Proc. Natl. Acad. Sci. U S A. *88*, 3033-7.

Toyoshima, H., and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell. *78*, 67-74.

Trimarchi, J. M., Fairchild, B., Verona, R., Moberg, K., Andon, N., and Lees, J. A. (1998). E2F-6, a member of the E2F family that can behave as a transcriptional repressor. Proc. Natl. Acad. Sci. U S A. *95*, 2850-5.

Tsao, Y. P., Kuo, S. W., Li, S. F., Liu, J. C., Lin, S. Z., Chen, K. Y., and Chen, S. L. (1995). Differential regulation of cyclin A, cyclin B and p21 concentrations in a growth-restricted human fibroblast cell line. Biochem. J. *312*, 693-8.

Vairo, G., Livingston, D. M., and Ginsberg, D. (1995). Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes & Dev. *9*, 869-81.

van den Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. Science. *262*, 2050-4.

Walker, S. S., Shen, W. C., Reese, J. C., Apone, L. M., and Green, M. R. (1997). Yeast TAF(II)145 required for transcription of G1/S cyclin genes and regulated by the cellular growth state. Cell. *90*, 607-14.

Weber, J. D., Hu, W., Jefcoat, S. C., Jr., Raben, D. M., and Baldassare, J. J. (1997). Ras-stimulated extracellular signal-related kinase 1 and RhoA activities coordinate platelet-derived growth factor-induced G1 progression through the independent regulation of cyclin D1 and p27. J. Biol. Chem. *272*, 32966-71.

Weinberg, R. A. (1992). The retinoblastoma gene and gene product. Cancer Surv. *12*, 43-57.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell. *81*, 323-30.

Weintraub, S. J., Chow, K. N., Luo, R. X., Zhang, S. H., He, S., and Dean, D. C. (1995). Mechanism of active transcriptional repression by the retinoblastoma protein. Nature. *375*, 812-5.

Weintraub, S. J., Prater, C. A., and Dean, D. C. (1992). Retinoblastoma protein switches the E2F site from positive to negative element. Nature. *358*, 259-61.

Williams, B. O., Schmitt, E. M., Remington, L., Bronson, R. T., Albert, D. M., Weinberg, R. A., and Jacks, T. (1994). Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. EMBO J. *13*, 4251-9.

Winston, J. T., and Pledger, W. J. (1993). Growth factor regulation of cyclin D1 mRNA expression through protein synthesis-dependent and -independent mechanisms. Mol. Biol. Cell. *4*, 1133-44.

Withers, D. A., Harvey, R. C., Faust, J. B., Melnyk, O., Carey, K., and Meeker, T. C. (1991). Characterization of a candidate bcl-1 gene. Mol. Cell. Biol. *11*, 4846-53.

Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K. H., and Beach, D. (1995). A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science. *269*, 1281-4.

Won, K. A., Xiong, Y., Beach, D., and Gilman, M. Z. (1992). Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. U S A. *89*, 9910-4.

Woo, M. S., Sanchez, I., and Dynlacht, B. D. (1997). p130 and p107 use a conserved domain to inhibit cellular cyclin- dependent kinase activity. Mol. Cell. Biol. *17*, 3566-79.

Wu, C. L., Classon, M., Dyson, N., and Harlow, E. (1996). Expression of dominant-negative mutant DP-1 blocks cell cycle progression in G1. Mol. Cell. Biol. *16*, 3698-706.

Wu, C. L., Zukerberg, L. R., Ngwu, C., Harlow, E., and Lees, J. A. (1995). In vivo association of E2F and DP family proteins. Mol. Cell. Biol. *15*, 2536-46.

Wu, X., and Levine, A. J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. U S A. *91*, 3602-6.

Xiao, Z. X., Ginsberg, D., Ewen, M., and Livingston, D. M. (1996). Regulation of the retinoblastoma protein-related protein p107 by G1 cyclin-associated kinases. Proc. Natl. Acad. Sci. U S A. *93*, 4633-7.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature. *366*, 701-4.

Xu, G., Livingston, D. M., and Krek, W. (1995). Multiple members of the E2F transcription factor family are the products of oncogenes. Proc. Natl. Acad. Sci. U S A. *92*, 1357-61.

Yan, Y., Frisen, J., Lee, M. H., Massague, J., and Barbacid, M. (1997). Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. Genes & Dev. *11*, 973-83.

Yeager, T. R., DeVries, S., Jarrard, D. F., Kao, C., Nakada, S. Y., Moon, T. D., Bruskewitz, R., Stadler, W. M., Meisner, L. F., Gilchrist, K. W., Newton, M. A., Waldman, F. M., and Reznikoff, C. A. (1998). Overcoming cellular senescence in human cancer pathogenesis. Genes & Dev. *12*, 163-74.

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J. D., Phillips, R. A., and Gallie, B. L. (1996). pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. Genes & Dev. *10*, 3051-64.

Zalvide, J., and DeCaprio, J.A. (1995). Role of pRB-related proteins in simian virus 40 large-T-antigen mediated transformation. Mol. Cell. Biol. *15*, 5800-5810.

Zalvide, J., Stubdal, H., and DeCaprio, J. A. (1998). The J domain of simian virus 40 large T antigen is required to functionally inactivate RB family proteins. Mol. Cell. Biol. *18*, 1408-15.

Zarkowska, T., and Mittnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem. *272*, 12738-46.

Zavitz, K. H., and Zipursky, S. L. (1997). Controlling cell proliferation in differentiating tissues: genetic analysis of negative regulators of G1-->S-phase progression. Curr. Opin. Cell. Biol. *9*, 773-81.

Zhang, P., Liegeois, N. J., Wong, C., Finegold, M., Hou, H., Thompson, J. C., Silverman, A., Harper, J. W., DePinho, R. A., and Elledge, S. J. (1997). Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature. *387*, 151-158.

Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell. *92*, 725-34.

Zhu, L., Enders, G., Lees, J. A., Beijersbergen, R. L., Bernards, R., and Harlow, E. (1995). The pRB-related protein p107 contains two growth suppression domains: independent interactions with E2F and cyclin/cdk complexes. EMBO J. *14*, 1904-13.

Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N., and Harlow, E. (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes & Dev. *7*, 1111-25.

Zindy, F., Quelle, D. E., Roussel, M. F., and Sherr, C. J. (1997). Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. Oncogene. *15*, 203-11.

Zuo, L., Weger, J., Yang, Q., Goldstein, A. M., Tucker, M. A., Walker, G. J., Hayward, N., and Dracopoli, N. C. (1996). Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. Nat. Genet. *12*, 97-9.

CHAPTER 2

E2F-4 switches from p130 to p107 and pRB in response to cell cycle re-entry.

Ken Moberg, Michelle A. Starz and Jacqueline A. Lees

Molecular and Cellular Biology, April 1996, V. 16 (No. 4), 1436-1449.
(Author's contribution: All data, with the exception of Figure 1)

A. Abstract

The E2F transcription factor couples the coordinate expression of cell cycle proteins to their appropriate transition points. Its activity is controlled by the cell cycle regulators pRB, p107 and p130. These bind to E2F at defined but distinct stages of the cell cycle. Using specific antisera, we have identified the DP and E2F components of each of these species. Although present at very different levels, DP-1 and DP-2 are evenly distributed amongst each of these complexes. In contrast, the individual E2Fs have distinctly different binding profiles. Consistent with previous studies, E2F-1, -2 and -3 bind specifically to the retinoblastoma protein. In each case, their expression and DNA binding activity is restricted to post G_1/S fractions. Surprisingly, E2F-1 and E2F-3 make unequal contributions to the pRB-associated and free E2F activity, suggesting that these proteins perform different cell cycle functions. Most significantly, this study showed that E2F-4 accounts for the vast majority of the endogenous E2F activity. In arrested cells, E2F-4 is sequestered by the p130 protein. However, as the cells pass the G_1/S transition, the levels of pRB and p107 increase and E2F-4 now associates with both of these regulators. In spite of this, a considerable amount of E2F-4 exists as free E2F. In G_1 cells, this accounts for almost all of the free activity. Once the cells enter S-phase, free E2F is comprised of an equal mixture of both

E2F-4 and E2F-1.

B. Introduction

E2F is a cellular transcription factor that plays a pivotal role in the regulation of cell division. Its responsive genes are either strongly implicated in or directly linked to the induction of cellular proliferation. E2F appears to act to tether the expression of these genes to the point in the cell cycle at which their products are known to act. E2F activity is tightly regulated by the physical association of key components of the cell cycle machinery. The best characterized of these is a known tumor suppressor, the retinoblastoma protein (pRB). The retinoblastoma gene (*RB-1*) was originally identified and subsequently cloned by virtue of its absence in retinoblastomas (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). However, further studies have identified *RB-1* gene mutations in 30% of all human tumors and, in each case, these result in either loss or functional inactivation of the retinoblastoma protein (reviewed in Weinberg, 1992). Moreover, the transforming potential of the small DNA tumor viruses correlates closely with their ability to bind, and presumably sequester, pRB (reviewed in Dyson and Harlow, 1992).

In normal cells, the growth-inhibitory properties of pRB are inactivated by phosphorylation (Dynlacht et al., 1994; Hinds et al., 1992). This modification appears to be mediated by one or more of the cell cycle dependent kinases, cyclin D/cdk4 or 6 (mid-G1 specific), cyclin E/cdk2 (G1/S specific) or cyclin A/cdk2 (S-phase specific) (Ewen et al., 1993; Hinds et al., 1992; Hu et al., 1992; Lees et al., 1991; Matsushime et al., 1994; Meyerson et al., 1994). This provides a simple mechanism to ensure that the growth inhibitory properties of pRB become inactivated once cells are triggered to re-enter the cell cycle.

In 1991, a number of laboratories demonstrated that the retinoblastoma protein binds to E2F in vivo (Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991). Although many other pRB-binding proteins have been reported, E2F has all of the predicted characteristics of a major pRB-target. It binds specifically to the un-phosphorylated form of pRB (Chellappan et al.,1991; Mudryj et al., 1991). This association does not effect its DNA binding activity but is sufficient to inhibit its transcriptional properties in a manner that can be specifically relieved by pRB phosphorylation (Dynlacht et al., 1994; Helin et al., 1993a; Hiebert et al., 1992;

Lees et al., 1993). In addition, E2F is capable of acting as an oncoprotein. E2F overexpression will drive quiescent cells to re-enter the cell cycle (Johnson et al., 1993) and, in some situations, is sufficient to bring about transformation (Singh et al., 1994; Xu et al., 1995). More often, E2F expression induces cells to undergo apoptosis suggesting that E2F is sufficient to induce cell cycle re-entry even in the presence of conflicting growth signals (Melillo et al., 1994; Qin et al., 1994; Shan et al., 1994; Wu and Levine, 1994). In each case, genetic analyses suggest that these effects are directly dependent upon the inappropriate activation of one or more target genes.

E2F is also regulated by two other proteins called p107 and p130 (Cao et al., 1992; Cobrinik et al., 1993; Devoto et al., 1992; Shirodkar et al., 1992). These were originally identified and cloned by virtue of their association with the adenovirus E1A protein and both share considerable sequence homology with the retinoblastoma protein (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). Unlike pRB, these proteins also associate stably with the cell cycle dependent kinases, cyclin A/cdk2 and cyclin E/cdk2, and this association does not prevent them from binding to E2F (Cao et al., 1992; Cobrinik et al., 1993; Devoto et al., 1992; Ewen et al., 1992; Faha et al., 1992; Lees et al., 1992; Mudryj et al., 1991). Several studies have shown that pRB, p107 and p130 each bind to E2F at defined but different stages of the cell cycle (Chittenden et al., 1993; Schwarz et al., 1993; Shirodkar et al., 1992). In arrested cells, the predominant E2F species appears to be a kinase deficient form of the p130/E2F complex (Chittenden et al., 1993; Cobrinik et al., 1993). In contrast, pRB and p107 do not associate with E2F until the cells reach the G_1/S-transition (Lees et al., 1992; Schwarz et al., 1993). In each case, formation of the p130/E2F/kinase or p107/E2F/kinase complexes coincides exactly with the timing of appearance of either cyclin E/cdk2 at the G_1/S transition or cyclin A/cdk2 during S-phase (Lees et al., 1992; Shirodkar et al., 1992). Although p107 and p130 are not tumor suppressor proteins, they appear to repress E2F in a similar manner to pRB (Hijmans et al., 1995; Zhu et al., 1993), and are also targeted by the small DNA tumor viruses (reviewed in Dyson and Harlow, 1992). These data therefore suggest that pRB, p130 and p107 act in concert to confine the activation of E2F, and therefore E2F-responsive genes, to precise stages of the cell cycle.

In the last three years, we and others have cloned at least seven genes that encode components of E2F (Ginsberg et al., 1994; Girling et al., 1993; Helin et al., 1992; Ivey-Hoyle et al., 1993; Kaelin et al., 1992; Lees et al., 1993; Sardet et al., 1995; Wu et al., 1995). These genes fall into two distinct classes, termed E2F and DP, that share little or no sequence homology. E2F and DP proteins heterodimerize in vivo and this association appears to be essential for both high affinity DNA binding and transcriptional activation (Bandara et al., 1993; Helin et al., 1993b; Krek et al., 1993; Wu et al., 1995). Several groups have shown that the individual E2F/DP heterodimers differ in their regulatory protein binding properties. Although both subunits are required for high affinity binding, this specificity appears to be determined by the E2F moiety. We have shown that complexes containing E2F-1, -2 or -3 bind specifically to pRB and not p107 in vivo (Dyson et al., 1993). In contrast, E2F-4 and -5 complexes have been reported to bind specifically to p107 and/or p130 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Vairo et al., 1995). Since the regulatory proteins bind to E2F at defined but different stages of the cell cycle, this specificity is likely to play an essential role in determining both the timing and length of activation of the individual E2F/DP heterodimers. It has also been suggested that differences in either the intrinsic properties of the E2F/DP heterodimers and/or the modulating effects of the associated regulatory protein might cause the pRB- and p107-/p103-specific E2Fs to activate different classes of responsive genes. Despite this speculation, we actually know very little about the cell cycle regulation of the individual E2F/DP complexes. To help address this issue, we have used specific antisera to analyze the temporal expression patterns and binding properties of the individual family members. These studies have revealed striking differences in the cell cycle regulation of E2F-1, -2, -3 and -4.

C. Results

C1. Generating anti-E2F antibodies

In the last three years it has become clear that the endogenous E2F activity arises from the concerted action of multiple E2F/DP heterodimers. Although there is considerable information about the basic properties of the E2F and DP proteins, we have yet to understand whether the individual E2F/DP complexes are required to mediate distinct functions in vivo or whether they are functionally redundant. In order to address this question, we need to be able to identify and follow each of these complexes in vivo. To this end, BALB/c mice were immunized with purified, bacterially-expressed proteins in which a tag of six histidines is fused to either full-length E2F-2, amino acids 1-244 of E2F-3 or amino acids 147-413 of E2F-4. The response of these mice was monitored by assaying successive bleeds for their ability to detect E2F/DP complexes in either gel shift or immunoprecipitation assays. For these experiments, C33-A cells were transiently transfected with pCMV-E2F-1, -2, -3, -4 or -5 expression vectors in combination with pCMV-HA-DP-1. These cells were either labeled with ^{35}S-methionine for immunoprecipitation experiments or used to generate whole cell lysates for gel retardation assays. The polyclonal antisera were tested for their ability to either supershift E2F/DP/DNA bound complexes or co-precipitate both the labeled E2F and its associated DP protein. In each case, these antisera specifically recognized the relevant E2F-2, E2F-3 (data not shown) or E2F-4 (Figure 1a) containing complexes in both gel retardation and immunoprecipitation assays.

In the case of E2F-2 and -3, the polyclonal antisera were of sufficiently high titer (a strong positive reaction with a 1μl of a 1/100 dilution) to conduct hybridoma fusions. The resultant lines were initially screened by enzyme-linked immunosorbent assay (ELISA) for their ability to secrete antibodies that recognized the relevant E2F immunogen. These primary screens identified multiple wells that produced either anti-E2F-2 or -3 antibodies. Subsequently, these ELISA-positive tissue culture supernatants were tested for their ability to specifically recognize the relevant E2F/DP complexes in either gel shift or immunoprecipitation assays. Exactly as described above,

these experiments were conducted using extracts from C33-A cells transiently transfected with E2F and DP expression vectors. Most of the ELISA positive E2F-2 and -3 antisera specifically recognized either E2F-2 or E2F-3 in immunoprecipitation and/or gel retardation assays (data not shown). Figure 1 confirms that the monoclonal antibodies utilized in this manuscript [KH20 (anti-E2F-1), LLF2-1 (anti-E2F-2) and LLF3-1 (anti-E2F-3)] specifically recognize their respective E2Fs in both gel retardation assays (Figure 1a) and western blots (Figure 1b) . In the former case, neither the binding capacity or specificity of these antibodies was affected when DP-1 was replaced by DP-2 or when the regulatory proteins, pRB or p107, were individually included in the DNA-bound complexes (data not shown).

C2. E2F-4 associates with pRB *in vivo*

To date, five distinct members of the E2F gene family have been identified (Beijersbergen et al., 1994; Ginsberg et al., 1994; Helin et al., 1992; Ivey-Hoyle et al., 1993; Kaelin et al., 1992; Lees et al., 1993; Sardet et al., 1995). Although these proteins share similar DNA binding and transcriptional properties, numerous studies have reported distinct differences in their specificity of regulatory protein binding. E2F-1, -2 and -3 each bind specifically to the retinoblastoma protein *in vivo* (Dyson et al., 1993; Lees et al., 1993). In contrast, E2F-4 interacts *in vivo* with both p107 and p130 while E2F-5 associates specifically with the G_0-regulatory protein, p130 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Vairo et al., 1995). Since there is extensive evidence that pRB, but not p107 and p130, is a tumor suppressor, it is essential that we understand the functional significance of the different binding specificities of the individual E2F/DP complexes.

Initially, we tested our antibodies for their ability to detect the individual E2F/DP complexes *in vivo*. For these experiments pRB or p107 were immunoprecipiated from asynchronous ML-1 cells, associated E2F activity was released by treating the precipitates with deoxycholate (DOC) and then detected in E2F gel shift assays in the absence or presence of our anti-E2F antibodies (Figure 2a). While the control (anti-large T antigen) monoclonal antibody

failed to bring down any proteins capable of binding the E2F probe (lane 1), a considerable amount of E2F activity was recovered from both the pRB- and p107-immunoprecipitates (lanes 2 and 10 respectively). In both cases, we were able to resolve this E2F DNA binding activity into a series of bands, all of which can be specifically competed by a 50-fold molar excess of wild-type but not mutant unlabeled E2F probe (data not shown). Consistent with our previous findings, the monoclonal antibodies against E2F-1 (KH20 ; lanes 4 & 12), E2F-2 (LLF2-1; lanes 5 & 13) and E2F-3 (LLF3-1; lanes 6 & 14) supershifted E2F-DNA binding activity from DOC-treated pRB- but not p107-immunoprecipitates. Both E2F-1 and E2F-3 were easily detectable in these assays and seemed to be present at roughly similar levels. In contrast, the anti-E2F-2 supershift was extremely weak (Figure 2a, lane 5), although clearly detectable when the input level of pRB-associated E2F activity is increased (Figure 2b). Since several other E2F-2 monoclonal antibodies detected similarly low levels (data not shown), these data suggest that E2F-2 comprises a small proportion of the pRB-associated E2F activity.

To date, E2F-1, -2 and -3 are the only known pRB-associated E2Fs. To determine whether these three proteins comprise all of the pRB-associated E2F activity, we tested how a mixture of these three antibodies affected the pRB-associated E2F activity. Surprisingly, the antibody cocktail supershifted less than half of the pRB-associated E2F activity (Figure 2a, lane 7). Moreover, this supershift had no detectable effect upon the most abundant, upper species of E2F activity. Since control experiments (using transfected E2F/DP complexes) were able to confirm that all three antibodies were present in at least 10 fold excess (data not shown), these data indicate that pRB must associate with one or more additional E2F species in vivo.

In an effort to identify this additional activity, we tested the effect of the E2F-4 polyclonal antisera in this assay. Consistent with previous studies, the anti-E2F-4 sera supershifted a significant proportion of the p107-associated E2F activity (lane 16), confirming that we can detect the association between E2F-4 and p107 *in vivo*. In addition, this antisera also specifically supershifted a large proportion of the E2F activity released from the pRB-immunoprecipitates (Figure 2a, lane 8). In this case, the presence of this antibody selectively depleted the prominent

upper complex that had been unaffected by the E2F-1, -2 and -3 specific monoclonal antibodies (Figure 2a, lane 7). To determine whether E2F-1, -2 , -3 and -4 were sufficient to account for all of the pRB-associated E2F, we included a mixture of all four antibodies in the DNA binding reaction (Figure 2a, lanes 9 & 17). Together, these reagents recognized all of the upper complex as well as a portion of the lower complexes. However, a significant fraction of the lower species was not supershifted by the antibodies, suggesting that they represented novel E2F species. Since forms of equal mobility also persisted when the p107-associated E2F preparation was treated with the E2F-1, -2 , -3 and -4 antibody cocktail, these novel E2Fs appeared to associate with both pRB and p107 in a manner similar to E2F-4. Obviously, E2F-5 is a good candidate to be one or both of these species and we are currently raising E2F-5 specific antisera to determine whether this accounts for the remaining E2F activity or whether the cell contains other, as yet unidentified E2F species.

Although E2F-4 is generally considered to be a p107/p130-associated protein, these data suggest that it also comprises a significant proportion of the pRB-associated E2F activity. Consistent with this finding, Vairo et al. have previously reported that their anti-E2F-4 polyclonal antisera also disrupts a portion of the pRB-associated E2F activity (Vairo et al., 1995). However, both of these studies have been conducted using polyclonal antisera. We therefore wished to confirm that our E2F-4 antisera was unable to supershift the endogenous E2F-1, -2 or -3 species. Exactly as above, we tested the different combinations of E2F antisera for their ability to supershift E2F activity that had been released from pRB-immunoprecipitates. However, in this experiment, we increased the levels of pRB-released E2F activity by over 5 fold (Figure 2b). Although this reduced our ability to detect discrete E2F complexes, we were now able to supershift significant levels of E2F-1 (lane 3), E2F-2 (lane 5) or E2F-3 (lane 7) from the pRB precipitates. Co-addition of the E2F-4 antisera had no effect upon these E2F-1 , E2F-2 or E2F-3 supershifts (Figure 2b, lanes 4, 6 and 8), confirming that our E2F-4 polyclonal antibody did not crossreact with any of these pRB-specific E2Fs. On the basis of this and other studies, we therefore conclude that E2F-4 comprises a significant proportion of the pRB-associated E2F activity.

C3. Differential cell-cycle expression of E2F proteins

Previous studies have shown that pRB, p107 and p130 bind to E2F at defined but distinct stages of the cell cycle. Having confirmed that our antisera effectively recognize endogenous E2F activity, we were able to compare the cell cycle regulation of the individual E2F/DP complexes with that of the regulatory proteins. Human T cells were selected for these experiments for three reasons. Firstly, these cells re-enter the cell cycle in a highly synchronous manner and the staging is maintained throughout the first round of division. Secondly, the timing of pRB-, p107- and p130-complex formation has already been well documented in this system (Chittenden et al., 1993). Finally, because these are primary cells, their cell cycle regulation should most closely parallel that found in vivo.

Human T cells were isolated from peripheral blood and stimulated to proliferate by the addition of phytohemagglutinin (PHA). At each time point the level of DNA replication was monitored by assessing the uptake of tritiated thymidine (Figure 3a). In this experiment [^{3}H]thymidine incorporation peaked 36 hours-post stimulation and declined to near basal levels by the 66 hr time point. The sharp increase between the 30 hr and 36 hr time points indicates the majority of cells traversed the G_1/S transition in this time interval; the narrowness of the peak and the rapid decline to near basal levels suggested that the cells responded to the proliferative signal in a synchronous manner.

Expression of the E2F proteins was assessed by Western blot analysis of whole cell extracts from each of the time points (Figure 3b). In a similar manner to the ML-1 cells, E2F-2 was expressed at extremely low levels in the T cells (see the gel shift assays below) and was undetectable in the Western blot experiments. In contrast, E2F-1, -3 and -4 were all present at reasonable levels in the T-cell fractions. E2F-1 was first detected at 30 hours, coincident with the beginning of S-phase. At this time point, it existed as a clear doublet. The level of the more prominent, upper species reached a maximum at 42 hours and then declined to a moderate level that was maintained throughout the remainder of the cell cycle. In contrast, the smaller species was only detected in fractions undergoing DNA replication. Both the timing and the rapid rate

induction of E2F-1 protein synthesis were consistent with the known E2F-dependent, G_1/S-specific transcriptional activation of this gene (Hsiao et al., 1994; Johnson et al., 1994; Neuman et al., 1994).

In contrast to E2F-1, our studies show that the E2F-3 mRNA is present in both the arrested and PHA-stimulated T cell fractions at similar, low levels (data not shown). We were therefore surprised to find that expression of the E2F-3 protein required cell cycle re-entry. In fact, E2F-3 was not detected until 24 hours after PHA stimulation, just 6 hours prior to the initiation of DNA synthesis and the induction of E2F-1 expression. Following its appearance, the level of E2F-3 rose quickly to reach a maximum at 36 hours that was maintained throughout the remainder of the cell cycle. Although the profile of E2F-3 expression closely mirrored that of E2F-1, this protein was detected as a single species throughout the time course of the experiment.

Consistent with previous studies (Vairo et al., 1995), the E2F-4 protein was detected at significant levels in all of the time points. In G_0 cells, E2F-4 was present as a single species whose levels increased steadily as the cells progressed through G_1 and S phase and then returned to the G_0 levels towards the end of the cell cycle. At 30 hours, we first detected two additional E2F-4 species, the presence of which appeared to be specific to fractions that were undergoing DNA synthesis. In fact, the appearance of these species was entirely coincident with the appearance of the minor form of E2F-1.

For comparison, we also screened the T-cell fractions for the presence of the regulatory proteins, pRB and p107. As previously described, pRB was detected as a single species in G_0 cells that corresponds to the underphosphorylated form (Figure 3b). Once the cells re-entered the cell cycle the overall level of pRB increased and slower migrating species were also detected. These first appeared 24 hours after PHA-addition and persisted through the remainder of the cell cycle and are entirely consistent with the cell cycle dependent phosphorylation of pRB that is initiated at the G_1/S transition.

The p107 protein was first detected 24 hours after PHA addition. From this time on, its levels continued to rise to reach a peak at 42 hours and then dropped to undetectable levels at 66

hours. Since the level of this species was only just within the detection limits of the experiments, we cannot conclude that p107 was absent from the early and late time points. However, these experiments clearly show that expression of the p107 protein is induced upon cell cycle re-entry. The concomitant increase in the levels of the E2Fs and their regulatory proteins, was consistent with recent reports that the *RB-1* and *p107* genes both contain E2F-responsive elements within their promoters (Shan et al., 1994; Zhu et al, 1995).

C4. Cell cycle-dependent E2F DNA-binding activities

The western blotting data indicated that E2F-1, -3 and -4 were each expressed at defined but different points of the cell cycle. However, since monomeric E2Fs are unable to bind to DNA in the absence of an associated DP protein, it was important to determine when each of the E2F proteins became competent to bind to DNA. In order to abolish any possible influence of the regulatory proteins, the T cell extracts were treated with deoxycholate (DOC) to dissociate the higher order complexes and the total "free" E2F activity was assayed by gel shift (Figure 4). E2F activity was detected in all of the time points, but the levels increased significantly as the cells re-entered the cell cycle to reach a maximum in the S-phase fractions. Wild-type but not mutant E2F oligonucleotides competed all of the upper species (labeled as free E2F in Figure 4) but failed to alter the strong lower band indicating that this corresponds to a non-specific binding activity (data not shown).

As expected, addition of a control monoclonal antibody (PAb419), had no effect on the DNA binding properties of any of these bands. In contrast, the E2F-1, -2, -3 or -4 specific antibodies were all capable of supershifting a proportion of the free E2F activity from one or more of the T-cell fractions. The three pRB-specific E2Fs, E2F-1, -2 and -3, were each detected in a cell cycle dependent manner. E2F-1 activity was first supershifted in the 30 hr extract, its levels peaked in the 36 and 42 hr samples and then declined to a low level in the remainder of the time points. E2F-2 DNA binding activity was also detected in this assay but at extremely low levels, beginning at the 36 hour time point and declining to almost undetectable levels in the later time

points. Finally, an-LLF3-1 specific supershift was first identified 24 hours after PHA addition. Although this activity did increase during S-phase, E2F-3 remained clearly detectable in all subsequent fractions.

In each case, the timing and level of these DNA-bound complexes were nearly identical to the timing and level of the individual E2Fs detected in our western blotting experiments. [The apparent discrepancy in our ability to detect E2F-2 in the DNA binding assay and not the western blot simply reflects the greater sensitivity of the gel shift assay and the extremely low levels of this protein and is entirely consistent with our analysis of other cell lines, including ML-1 and C33-A (data not shown).] In light of these findings, these experiments strongly suggest that there is little delay between the synthesis of the individual E2Fs and their ability to associate with a DP to form competent DNA binding complexes.

Consistent with its expression pattern, E2F-4 dependent DNA binding activity was detected in all of the time points (Figure 4). In the early stages of the cell cycle, E2F-4 comprised almost all of the total E2F activity. However, as the cells progressed through the cell cycle, significant levels of non-supershifted bands appeared in those time points in which E2F-1, -2 and/or -3 activity had previously been detected. As with our analysis of ML-1 cells (see Figure 2), E2F-4 seemed to correspond to the major, higher mobility species while E2F-1, -2 and -3 corresponded to the collection of bands migrating immediately below.

Our preliminary experiments in ML-1 cells indicated that E2F-1, -2, -3 and -4 were not sufficient to account for all of the endogenous E2F activity (Figure 2). We were therefore interested to determine whether the unidentified E2Fs are present at all stages of the cell cycle, like E2F-4 or whether their presence is also cell cycle regulated in a similar manner to the pRB-specific E2Fs. To this end, the gel shift reactions were performed in the presence of a cocktail of all three monoclonal reagents and the polyclonal sera (Figure 4). Consistent with the results in ML-1 cells, supershifting E2F-1, -2, -3 and -4 revealed the presence of two distinct bands that were unaffected by the presence of these antisera. One of these novel T cell E2F activities is present in all of the time points, suggesting that its expression is regulated in a similar manner to E2F-4, and may

therefore correspond to the recently described p130-specific E2F, E2F-5. The second, slower migrating activity is completely absent in G_0/G_1 extracts, appears strongest in the S-phase extracts (30 hr through 54 hr) and declines to low levels in the remainder of the time points in a similar to the three pRB-specific E2Fs.

C5. E2F-1, -3 and -4 associate with regulatory proteins in a cell cycle-dependent manner

Having determined the timing of synthesis and DNA binding activity of the individual E2Fs, it was important to establish whether these DNA binding complexes were free to activate transcription or whether their activity was blocked by the association of regulatory protein(s). We therefore used gel shift assays to examine the pattern of complexes arising from each of the T-cell time points (Figure 5a). For clarity, we have labeled each of these complexes in order of their appearance. In G_0 cells, E2F activity was detected as two discrete complexes, labeled A and B respectively (Figure 5a, lane 1). During the first twelve hours the level of the A and B complexes declined steadily. At the same time, we began to detect a third novel species, labeled C, whose levels increased steadily (Figure 5a, lane 3). At the G_1/S transition (between 30-36 hours after PHA addition) this pattern altered considerably (compare lanes 6 and 7). Firstly, we detected the formation of two novel complexes that migrated with increased mobility. The larger complex (labeled D) was present at reasonably high levels while the smallest complex (labeled E) was barely detectable. At the same time we detected significant changes in both the A and the B complex. Despite the previous steady decline, the levels of the upper A complex appeared to increase dramatically between 30 and 36 hours. In fact, the antibody experiments described below indicate that the increase in the level of this band was actually caused by the appearance of a novel, similarly sized species (labeled F) that replaced the G_0-A complex. In a similar manner, the levels of the B complex declined and disappeared between 30 and 36 hours to be replaced by another novel species (labeled G) that has increased mobility. These five complexes (C through G) persisted in all of the fractions (36, 42, 48 and 54 hr) containing S-phase cells (Figure 5a, lanes 7-10). However, as the cells exited S-phase (between 54 and 60 hours) the pattern of these

complexes altered once more. The S-phase specific G complex disappeared rapidly and was replaced by an additional species, labeled H, that migrates with the same mobility as the original G_0-B complex. At the same time the level of other complexes declined. Although this reduction affected all four of these species, the E and F complexes appeared to be lost preferentially. The strong peak of E2F activity in S-phase coincided exactly with the increased levels of E2F detected in both the western blotting (Figure 3b) and DOC-release gel shift (Figure 4) assays.

To determine the identity of each of these complexes, the T-cell extracts were analyzed in gel shift assays in the presence of antibodies against the regulatory proteins. The most interesting time points are shown in Figure 5b. Consistent with previous studies, a p130 polyclonal antisera specifically supershifted both the A and the B complexes (data not shown), confirming that p130 is contained within both of the G_0 complexes and free E2F is absent at this time (Chittenden et al., 1993; Cobrinik et al., 1993). As the cells re-entered the cell cycle, the level of these p130 complexes declined steadily and disappeared between 30 and 36 hours after PHA addition. Low levels of p107 and pRB-specific supershifts were first detected at 30 hours. These supershifts caused a partial reduction in the upper two bands, indicating that they both contain a mixture of two species, A (p130) + F (p107) and B (p130) + G (pRB). By the time DNA replication had reached maximum levels (42 hours), the p107- and pRB-specific antibodies were able to supershift all of the upper two bands (lanes 10 -12) indicating that the p130 complexes (A and B) have been completely replaced by p107 (complex F) and pRB (complex G). By 54 hours the majority of the cells had completed DNA replication (Figure 3a). Although the pRB specific antibodies continued to supershift a significant amount of E2F activity from this later time point, they only shifted the lower half of the second band (Figure 5b, compare lanes 13 and 14) indicating that once again it derived from two distinct species, G (pRB) and H. Consistent with this observation, antibodies against p107 suddenly generated two distinct supershifted species; one abolishing the F complex and the other affecting the upper half of this G (pRB) + H (p107) band. Throughout the experiment, antibodies against either pRB, p107 or p130 failed to alter the C and D complexes, indicating that they comprise the free, transcriptionally active E2F.

Having determined the regulatory protein component of each of the T cell complexes, we wished to establish the identity of their associated E2Fs. The fractions were therefore treated with antisera specific to either the DP (Figure 5c) or E2F proteins (Figure 5d). We have previously investigated the relative levels of DP-1 and DP-2 in asynchronous ML-1 cell extracts (Wu et al., 1995). This study showed that DP-1 is the major species within these cells and together with DP-2 is sufficient to account for all of the endogenous E2F DNA binding activity. Our analysis of the T cell fractions was highly consistent with these data (Figure 5c). In fact, DP-1 seemed to comprise at least 80% of the B, C, D, E, G and H complexes. Although the DP-1 supershift co-migrates with the higher mobility complexes (A and F), two distinct supershifted species were detected in G_0 time point (compare lanes 1 and 2) suggesting that the A complex also contained a large amount of this protein. These data therefore suggest that the DP-1 was equally distributed among all of the complexes and not limited to particular species. Because it is a polyclonal antisera, the DP-2 antibody do not give rise to a discrete supershifted band(s). However, we did detect a reduction in the level of some early complexes (for example B and C in lanes 4 and 6) as well as a minor supershift in the later time points (compare lanes 16 and 18; 19 and 21), indicating that DP-2 makes some contribution to many of these species. Together these data suggest that DP-1 and DP-2 do not participate preferentially in any specific complex(es).

In contrast to the DP data, we detected clear differences in the identity of the E2F component of these complexes. In the early time points (prior to 30 hours), we failed to detect any evidence of the pRB-specific E2Fs. In contrast, E2F-4 was present at high levels. In G_0, the E2F-4 polyclonal antisera specifically supershifted all of the B complex (Figure 5s, lane 5), indicating that this corresponds to p130/E2F-4. In addition, this antisera also recognized the complex that first appeared in mid G_1. This C-complex persisted in all subsequent time points, and in each case was fully supershifted by the E2F-4 antisera. Since this complex did not contain an associated regulatory protein, these experiments suggest that E2F-4 is transcriptionally active from mid-G_1 to late S-phase. In addition to E2F-4, our analysis of DOC-treated cell extracts detected a second G_0/G_1-specific E2F of unknown identity. Consistent with this observation, the

G_0/G_1-specific p130-A complex was unaffected by the E2F-1, -2 -3 or -4 antisera. In light of recent observations that E2F-5 binds specifically to p130 (Hijmans et al., 1995) it seems likely that the A complex corresponds to p130/E2F-5.

At the G_1/S-transition (30 hours after PHA addition), we detected major changes in the binding properties of both the regulatory proteins (as described above) and the E2Fs. Consistent with both the western blotting and DOC-release data, E2F-1 and E2F-3 specific supershifts were first detected at this time (see Figure 5d, lanes 12 and 14). Although the initial supershifts were weak, the levels of E2F-1 and E2F-3 continued to rise as the proportion of S-phase cells increased. By 42 hours it was clear that the E2F-1 antisera specifically supershifted almost all of the D complex (Figure 5d, compare lanes 16 and 17). This finding is consistent with previous data (Chittenden et al., 1993) and suggests that E2F-1 is a significant component of the free E2F and is likely to play an important role in activating G_1/S and S-phase specific transcription. Although E2F-1 and E2F-3 seem to be present at similar levels, it was almost impossible to identify the origin of the E2F-3 supershift. Even in the peak S-phase fractions, the presence of the E2F-3 antisera did not significantly alter the intensity of any of the original complexes (Figure 5d, compare lanes 16 and 19) suggesting that E2F-3 is a minor component of one or more of these species. Consistent with its low level of expression (see Figure 3b and 4), we were also unable to detect any E2F-2 supershift in the presence of the undissociated complexes.

Although our previous experiments had shown that E2F-4 also bound pRB in vivo, we were extremely surprised by the extent to which E2F-4 continued to dominate the latter E2F activity. In both the G_1/S and S-phase fractions (30 and 42 hours), E2F-4 was detected as the predominant component of both the p107 (complex F) and pRB (complex G) species (Figure 5d, lanes 15 and 20). At the same time, the level of the free E2F-4 complex (C) also continued to rise as the proportion S-phase cells increased. Together, these data indicate that the E2F-4 protein exists in excess of the pRB-specific E2Fs at all stages of the cell cycle. These high levels may help to explain why E2F-4 binds pRB in the presence of E2F-1, -2 and -3.

C6. pRB does not bind E2F activity during G0/G1

Our cell cycle experiments clearly demonstrate that the G_1 T cell fractions contain both pRB (as judged by western blotting) and free E2F-4 (as judged by gel retardation assay). In light of these findings, we might expect pRB and E2F-4 to associate during the early cell cycle stages. However, our gel retardation assays failed to detect any pRB-containing complexes until after the cells have traversed the G_1/S transition. We therefore used the immunoprecipitation-DOC release assay to screen the early T cell fractions for any evidence of pRB-E2F complexes. To ensure that we would detect rare complexes, a second batch of PHA-activated T cells was prepared specifically for this experiment. In this instance, the profile of E2F complexes was almost identical to the previous batch of T cells (data not shown) with maximum DNA synthesis being detected approximately 48 hours after PHA addition (Figure 6a). A significant proportion of each time point was then immunoprecipitated with either a control (PAb419) or an anti-pRB (XZ55) monoclonal antibody. Any associated E2F activity was released by treating the precipitates with deoxycholate (DOC) and detected in E2F gel shift assays (Figure 6b). While the control (anti-large T antigen) monoclonal antibody failed to bring down any proteins capable of binding the E2F probe (lanes 1-11), we were able to detect E2F activity within some of the pRB-immunoprecipitates. However, consistent with our gel shift assays, this activity was specifically detected in post G_1/S fractions (lanes 19-22) and was most abundant in late S-phase cells. The appearance of these S-phase specific pRB/E2F complexes is highly consistent with apparent increase in the rate of pRB synthesis that occurs at this point in the cell cycle. Although we cannot rule out that pRB/E2F complexes are present at low levels, these data suggest that the pRB species that are present in G_0/G_1 cells are unable to associate with any E2F DNA binding activity.

C7. E2F-1 and -3 display different pRB-binding properties

Our cell cycle experiments suggest that the pRB-specific E2Fs also act after the cells have traversed the G_1/S boundary (Figures 3b, 4 and 5d). However, there is some indication that these proteins participate in different complexes. The E2F-1 specific monoclonal antibody produces a

clear supershift that arises at the expense of the S-phase specific, free E2F complex (Figure 5d, lane 17). Since this antibody had little detectable effect upon the pRB/E2F complexes, these data suggest that E2F-1 is present in the predominantly free form. In the same experiment, the E2F-3 specific monoclonal antibody also produced a clear supershifted species. However, in this case, we were unable to detect a significant reduction in any of the E2F complexes. We have therefore used two distinct assays to try and identify the nature of the E2F-3 complex. Initially, we tested whether the pRB-specific monoclonal antibody, XZ55, was able to alter the mobility of the individual E2F supershifts. In this experiment, the T cell fractions were tested in a gel shift assay with a mix of E2F and pRB specific antibodies. Since similar results were obtained at each time point, only data from the peak S-phase fraction is shown (Figure 7a). As before, the E2F-1 and E2F-3 specific antibodies both gave rise to a discrete supershifted band (lanes 2 and 8). However, these bands were differentially affected by the addition of the anti-pRB monoclonal. Inclusion of both KH20 and XZ55 (lanes 4) gave rise to two discrete supershifted species that comigrated with the single E2F-1 (lane 2) and pRB (lanes 2) shifts respectively. This therefore supports our previous conclusion that pRB is not a component of the E2F-1 complex. In contrast, a mixture of LLF3-1 and XZ55 gave rise to a novel complex (lane 10) that migrated with reduced mobility relative to the individual E2F-3 (lane 8) or pRB (lane 9) supershifted species. The formation of this band suggests that, unlike E2F-1, the majority of the E2F-3 protein is associated with pRB. Moreover, this partitioning was apparent at every stage of the cell cycle at which the E2F-1 and E2F-3 DNA bound complexes were detected (data not shown).

To confirm this difference, we have used the immunoprecipitation-DOC release assay to examine the E2F content of the pRB associated T-cell E2F activity. For this experiment, we pooled the two T-cell fractions (54 and 60 hrs) that were previously shown to contain the majority of the pRB-associated E2F activity (Figure 6b) and immunoprecipitated either pRB or p107 complexes. The associated E2F activity was then released by DOC-treatment and detected in gel shift assays in the presence of the E2F antisera (Figure 7b). Consistent with our previous experiments, antibodies against E2F-1 (lanes 2 and 7) or E2F-2 (lanes 3 and 8) failed to supershift

any of the pRB- or p107-associated E2F activity. In contrast, LLF3-1 specifically detected a significant amount of E2F-3 in the pRB- (lane 4) but not the p107- (lane 9) immunoprecipitate. Not surprisingly, the E2F-4 polyclonal antisera recognized the majority of both the pRB- (lane 5) and p107- (lane 10) associated E2F activity, giving rise to a large smear of supershifted bands. Although the apparent lack of association between pRB and either E2F-1 or E2F-2 may be due to the detection limits of this assay, these experiments reveal a major difference in the binding profile of E2F-1 and E2F-3. Despite the fact that these two proteins are present at similar levels, E2F-1 remains predominantly free in all fractions in which it was detected, while the majority of E2F-3 seems to remain bound to the retinoblastoma protein.

D. Discussion

E2F is known to play a pivotal role in coupling the coordinate expression of cell cycle proteins to their appropriate transition points. Although the field has made considerable progress in establishing both the properties and regulation of the individual E2F/DP heterodimers, we do not understand how these complexes bring about the differential activation of different target genes. Several groups have reported that the individual E2F/DP heterodimers bind specifically to either pRB (E2F-1, -2 and -3) or p107/p130 (E2F-4 and -5) in vivo (Biejersbergen et al., 1994; Dyson et al., 1993; Ginsberg et al., 1994; Hijmans et al., 1995; Lees et al., 1993; Sardet et al., 1995Vairo et al., 1995). It is generally accepted that this specificity will play a major role in determining both the timing and length of activation of these E2F complexes. It has also been suggested that differences in either the intrinsic properties of the E2F/DP heterodimers and/or the modulating effect of the associated regulatory protein(s), would be sufficient to somehow ensure that these different E2F subclasses target different E2F responsive genes. Differences in E2F target gene specificity could also explain why pRB and not p107 or p130 is a tumor suppressor.

In this study, we document the cell cycle regulation of the individual E2F/DP heterodimers and their associated regulatory proteins. Both the components of the complexes and the timing of their appearance is summarized in Figure 8. These findings considerably alter our interpretation of the relative roles of the individual E2Fs. Most surprising, our data indicate that a single E2F/DP complex, E2F-4/DP-1, is responsible for the majority of the endogenous E2F DNA binding activity. Moreover, this complex interacts in turn with each of the known E2F regulatory proteins, including the retinoblastoma protein. In G_0 cells, E2F-4 and an unidentified E2F that has properties similar to those described for E2F-5, were sufficient to account for all of the endogenous E2F activity. In both cases, these complexes were associated with p130 and presumably transcriptionally inactive. Once the cells re-entered the cell cycle the unidentified E2F complex disappeared rapidly while the levels of E2F-4 increased continually. By mid-G_1, we were able to detect significant quantities of free, presumably transcriptionally active E2F-4/DP-1.

Although the levels of the p130/E2F-4 complex were in decline, this was insufficient to account for the rise in levels of free E2F-4/DP-1. The mid-G_1 "free" E2F must therefore be composed of a combination of p130-released and newly synthesized protein. Most known E2F responsive genes are first transcribed at the G_1/S-transition (reviewed in Nevins, 1992), considerably later than the appearance of the free E2F-4. In fact, northern analysis confirms that the transcription of at least one known E2F-responsive gene, *E2F-1,* is not detected until 12 hours after the appearance of the free E2F-4 (our unpublished data). This suggests that this initial free E2F-4 does not activate the transcription of the known G_1/S-responsive genes. Obviously, it will be important to determine whether this E2F-4 complex activates a different set of responsive genes or whether it requires an additional modification (for example phosphorylation) to give rise to a transcriptionally active complex.

Once cells reach the G_1/S boundary, we detected a significant increase in the total level of E2F activity that continues throughout S-phase. Consistent with the induction of p107 and pRB expression, this increase arises largely from the appearance of p107- and pRB-containing complexes. Surprisingly, both of these complexes are almost entirely composed of E2F-4/DP-1 and this occurs without any apparent reduction in the level of free E2F-4 species. This suggests that the increased synthesis of this protein is sufficient to match the increasing demand for E2F-4. Our data have suggested that the mid-G_1, free E2F-4 (consisting of p130-released and newly synthesized protein) does not activate the "G_1/S" class of E2F responsive genes. This raises questions about the target specificity of the S-phase free E2F-4 complex. At this time, we assume that the free E2F-4 may include forms that have been released from association with either p107 and/or pRB. If the regulatory proteins influence the target specificity of their associated E2Fs, this S-phase E2F-4/DP-1 activity could activate the transcription of responsive genes that the mid-G_1 complex was unable to target.

The S-phase activity is further complicated by the appearance of E2F-1 -2 and -3 as well as an additional E2F that is clearly detectable in DOC-release experiments. Unlike the G_0 complex, there are no good candidates for this novel species suggesting that it may correspond to an as yet

unidentified, sixth E2F. The E2F-1, -2 and -3 proteins are absent in arrested cells and are not expressed until the G_1/S-transition. Once synthesized, these proteins are quickly detected in DNA-dependent assays suggesting that DP association is not a rate limiting step in the formation of active E2F/DP complexes. In all cell types examined (ML-1, C33-A and human thymocytes), E2F-2 seems to be a very minor component of the endogenous E2F activity and we were unable to determine the relative levels of the bound and free forms of this protein. In contrast, E2F-1 and E2F-3 were both clearly detectable. Strikingly, although these proteins were present at roughly similar levels (as judged by both westerns and DOC-release assays) they appear to be present in very different forms. Through S-phase, G_2 and M, the majority of E2F-3 remains associated with the retinoblastoma protein in a presumably inactive form. In contrast, E2F-1 is predominately detected within a free E2F complex in agreement with previous studies (Chittenden et al., 1993). In fact, although the total S-phase levels of E2F-4 vastly exceed those of E2F-1, these proteins seem to contribute almost equally to the amount of free E2F that is present at this stage of the cell cycle.

These observations raise important questions as to the underlying mechanism(s) that determines whether or not a particular E2F is free. At this time, it is not clear how the p130/E2F and p107/E2F complexes are regulated. In contrast, it is generally accepted that dissociation of the pRB/E2F is induced by the cell cycle dependent phosphorylation of the retinoblastoma protein. Since our data show that pRB is being continually synthesized throughout S-phase, the presence of these late pRB/E2F complexes does not challenge the model. However, it does not help to address why we detect major differences in the bound:free ratio of E2F-1, -3 and -4 or why pRB does not bind to E2F during G_1. It is possible that the level of these E2Fs exceeds that of the unbound retinoblastoma protein and these differences reflect differences in its affinity/avidity for the three E2Fs. Although we cannot rule this out, both the levels of pRB and the in vitro binding properties of the individual E2F/DP species are inconsistent with this model. Alternatively, one has to argue that these complexes are modified in different ways. It has recently been reported that phosphorylation of E2F can increase its affinity for pRB (Peeper et al., 1995). If this is true in

vivo, differential phosphorylation of the individual E2F/DPs would significantly influence their relative pRB-binding properties. Finally, one could imagine that the E2F/pRB complexes are formed with equal efficiency but dissociated at different rates because the cdks somehow recognize the individual E2F/pRB complexes with varying degrees of efficiency. Understanding what determines the bound:free ratio of the individual E2F/DPs will become increasingly important if these complexes are found to activate different target genes.

In light of the models discussed above, it is of note that additional forms of E2F-1 and E2F-4 appeared in the western blots in the S-phase fractions. At the same time, E2F-3 was consistently detected as a single species. It is interesting to speculate that these differences reflect changes in the phosphorylation of these proteins that specifically effect the free E2F species. This would be consistent with the recent finding that the free E2F-1/DP complex is specifically phosphorylated by the S-phase kinase, cyclin A/cdk2, and that this is sufficient to inhibit its DNA binding and transcriptional activity (Dynlacht et al., 1994; Krek et al., 1994). Obviously, further studies are required to determine both the nature and the functional consequences of these in vivo modifications.

A long term goal of the field has been to understand why pRB, but not p107 or p130, is a tumor suppressor. This is particularly confusing because these proteins display many of the same characteristics: all three contribute to the regulation of E2F and all are targeted by the transforming proteins of the small DNA tumor viruses. The finding that the individual E2Fs bound with high specificity to either pRB or p107/p130 offered a possible explanation for pRB's unique properties. This model proposes that the pRB- and p107/p130-specific E2Fs activate different E2F responsive genes and the pRB-E2F targets are the ones that confer a growth advantage. The observation that E2F-4 is also regulated by the retinoblastoma protein suggests that pRB may be the tumor suppressor because only its loss is sufficient to mobilize the vast majority of the endogenous E2F/DP species.

Figure 1A.

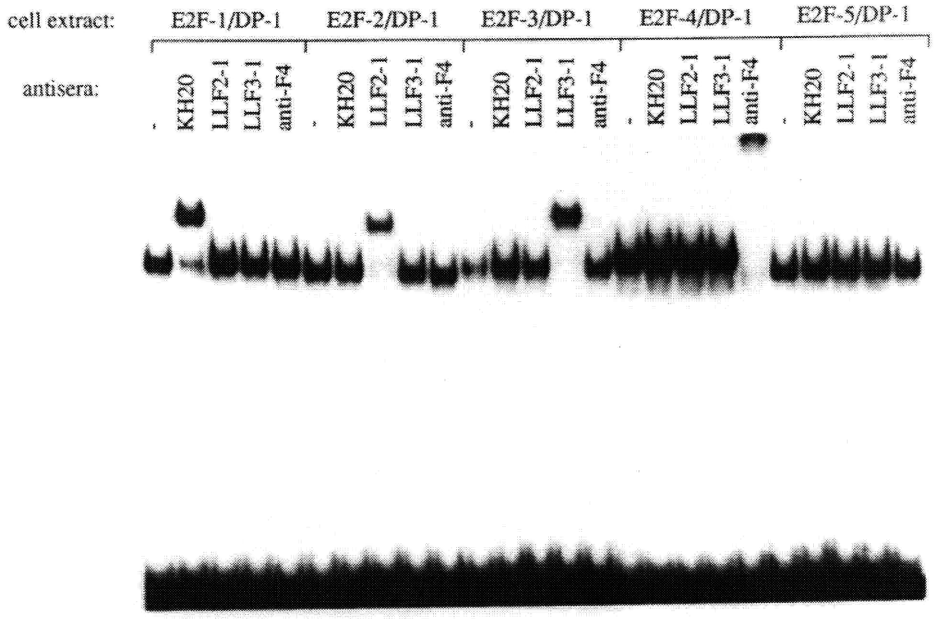


Figure 1B.

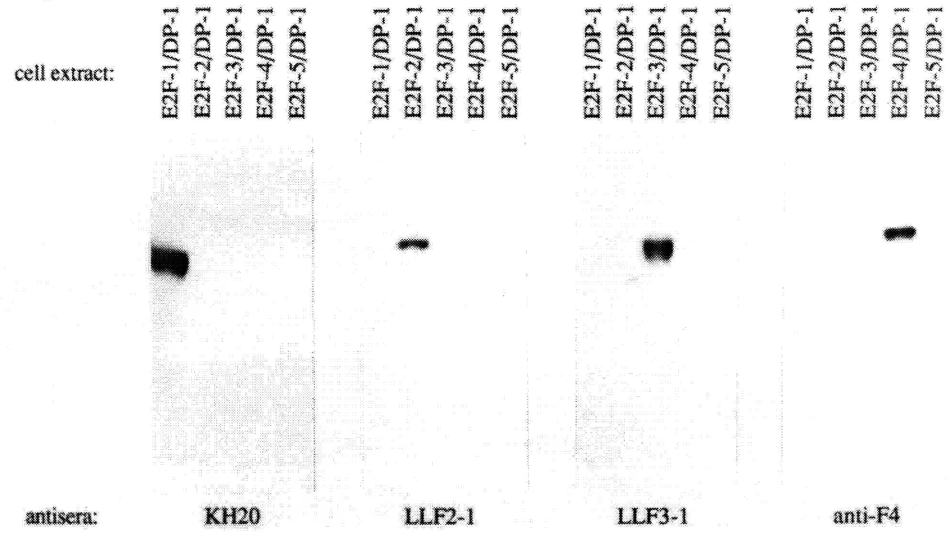


Figure 2A.

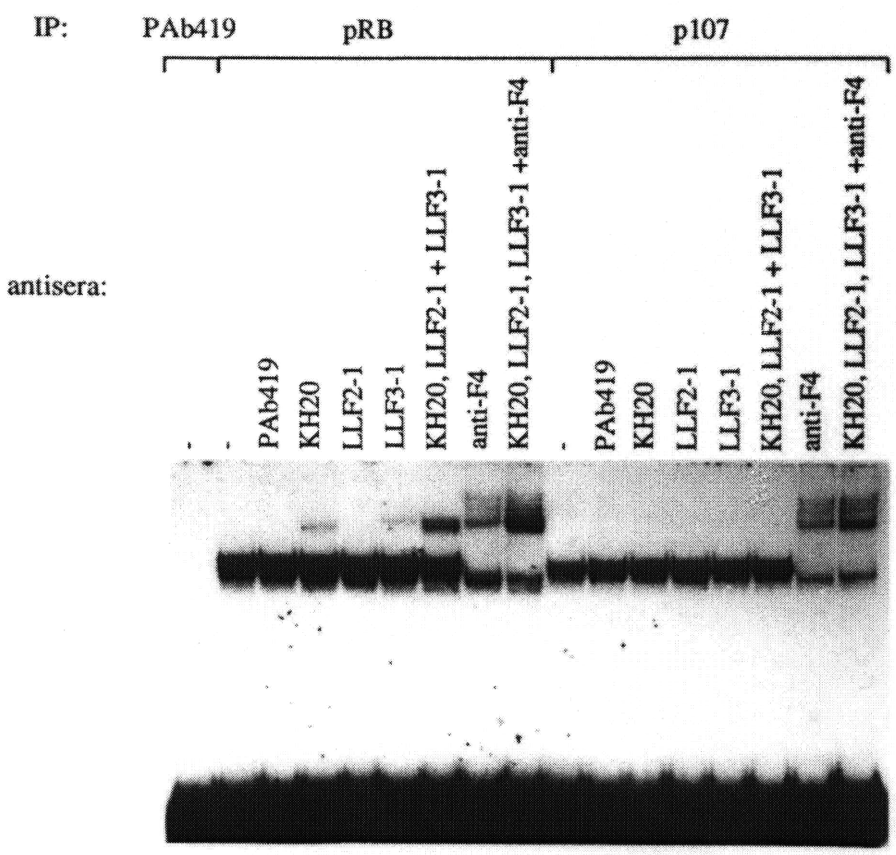


Figure 2B.

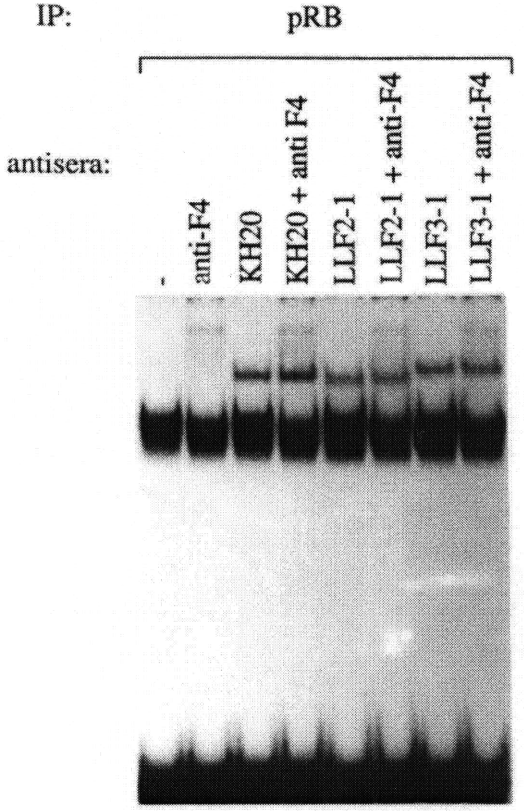


Figure 3A.

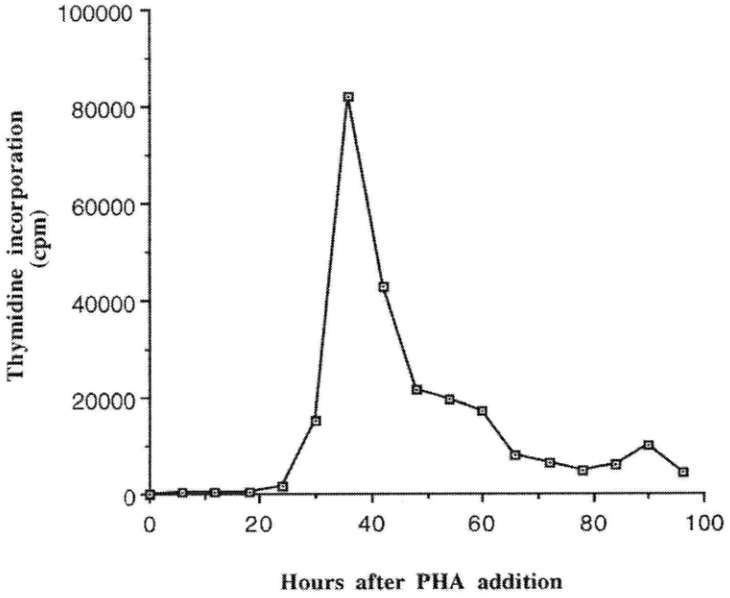


Figure 3B.

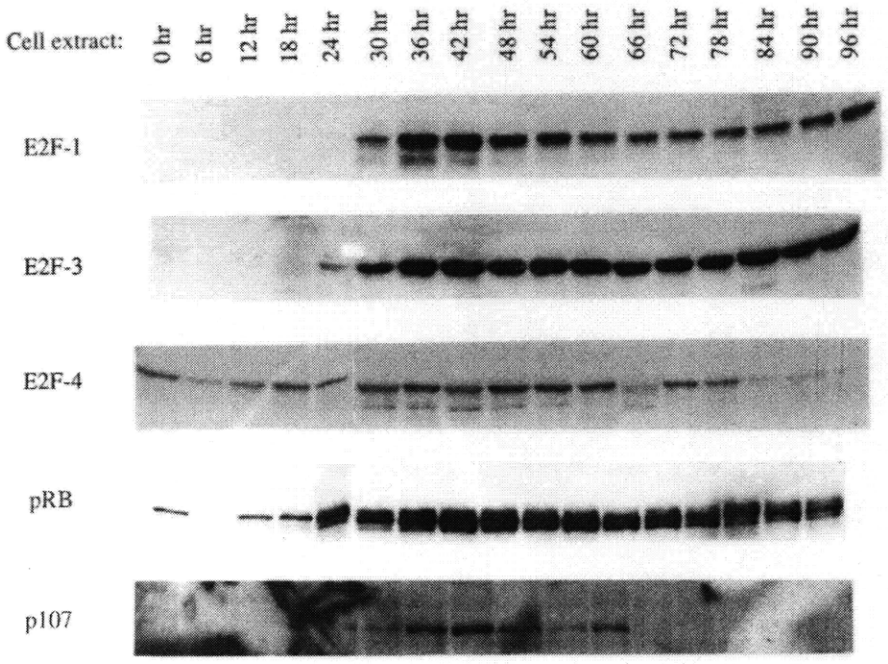


Figure 4.

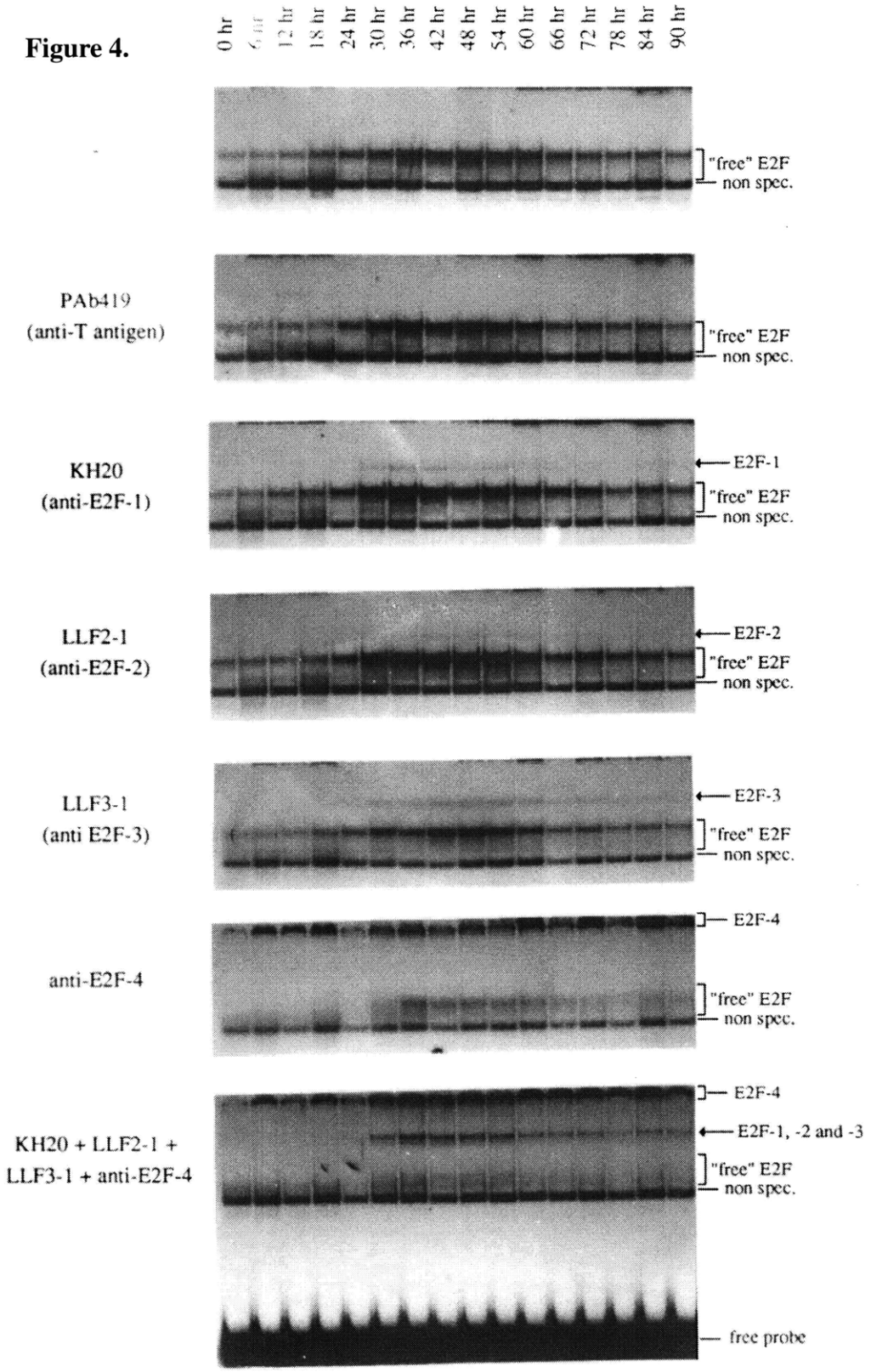

0 hr
12 hr
18 hr
24 hr
30 hr
36 hr
42 hr
48 hr
54 hr
60 hr
66 hr
72 hr
78 hr
84 hr
90 hr
"free" E2F
non spec.
PAb419
(anti-T antigen)
"free" E2F
non spec.
KH20
(anti-E2F-1)
E2F-1
"free" E2F
non spec.
LLF2-1
(anti-E2F-2)
E2F-2
"free" E2F
non spec.
LLF3-1
(anti E2F-3)
E2F-3
"free" E2F
non spec.
anti-E2F-4
E2F-4
"free" E2F
non spec.
KH20 + LLF2-1 +
LLF3-1 + anti-E2F-4
E2F-4
E2F-1, -2 and -3
"free" E2F
non spec.
free probe

Figure 5A.

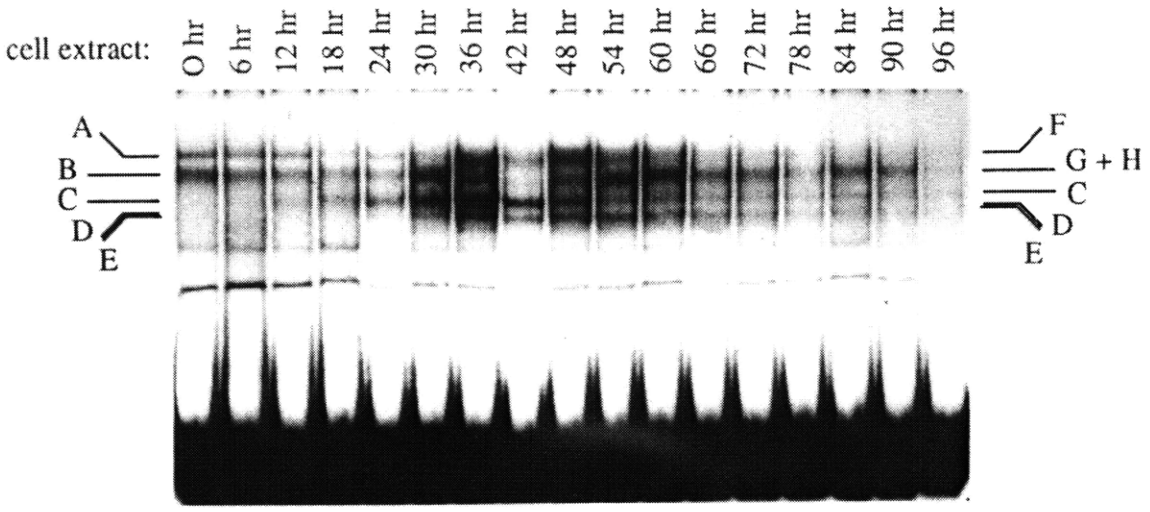


Figure 5B.

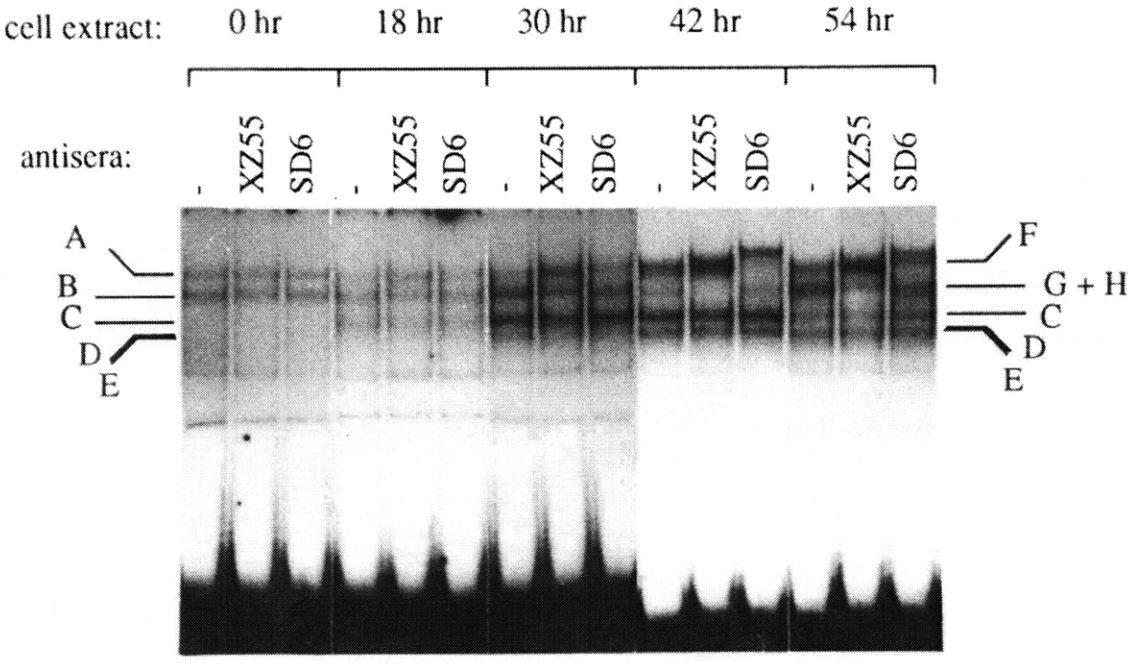


Figure 5C.

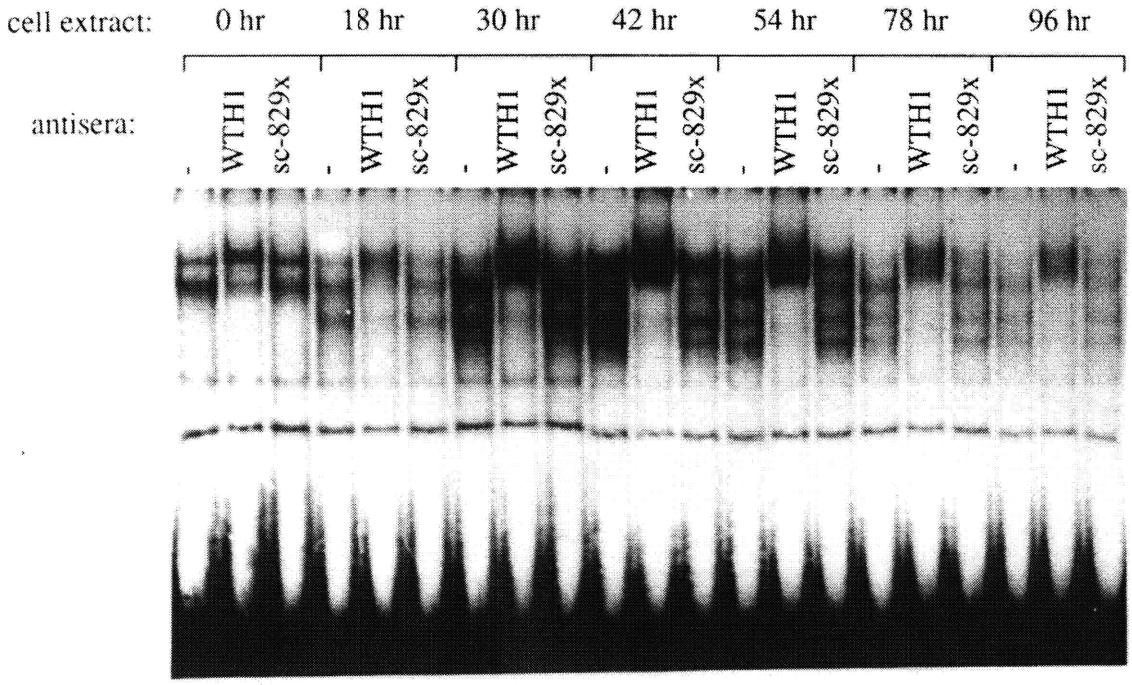


Figure 5D.

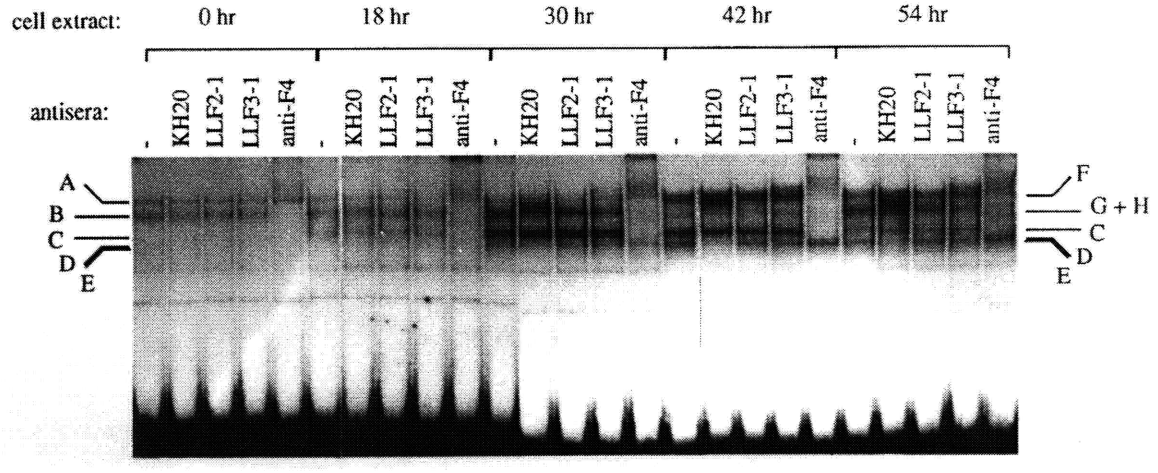


Figure 6A.

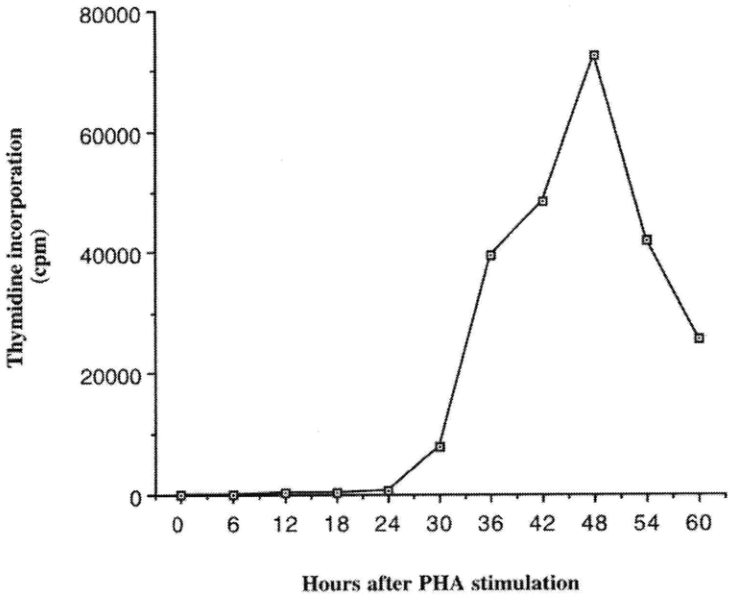
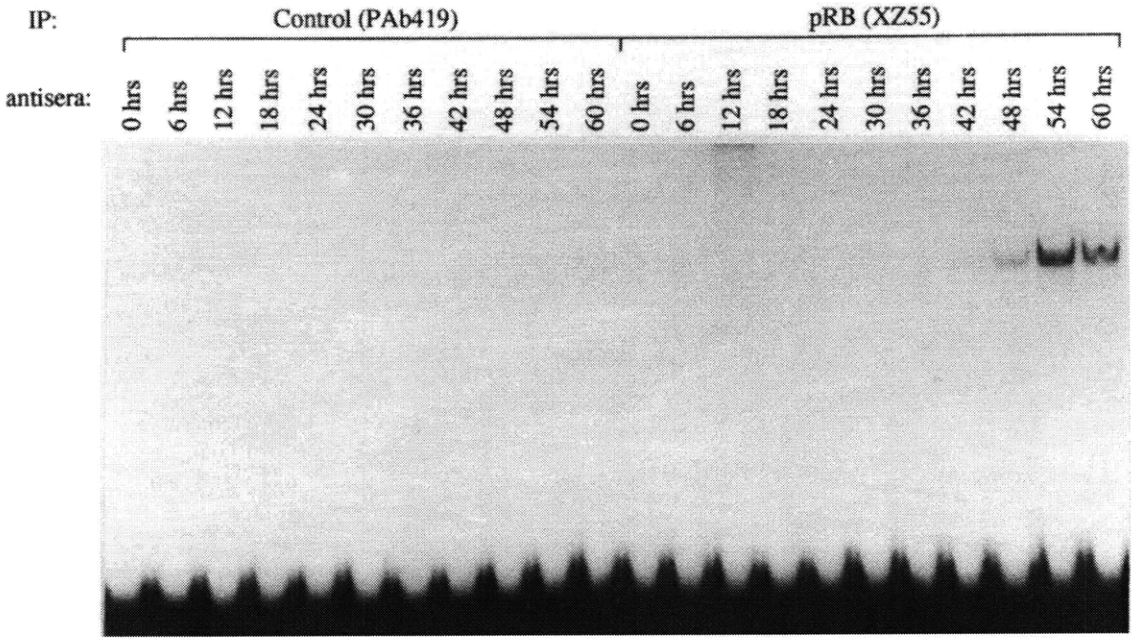


Figure 6B.



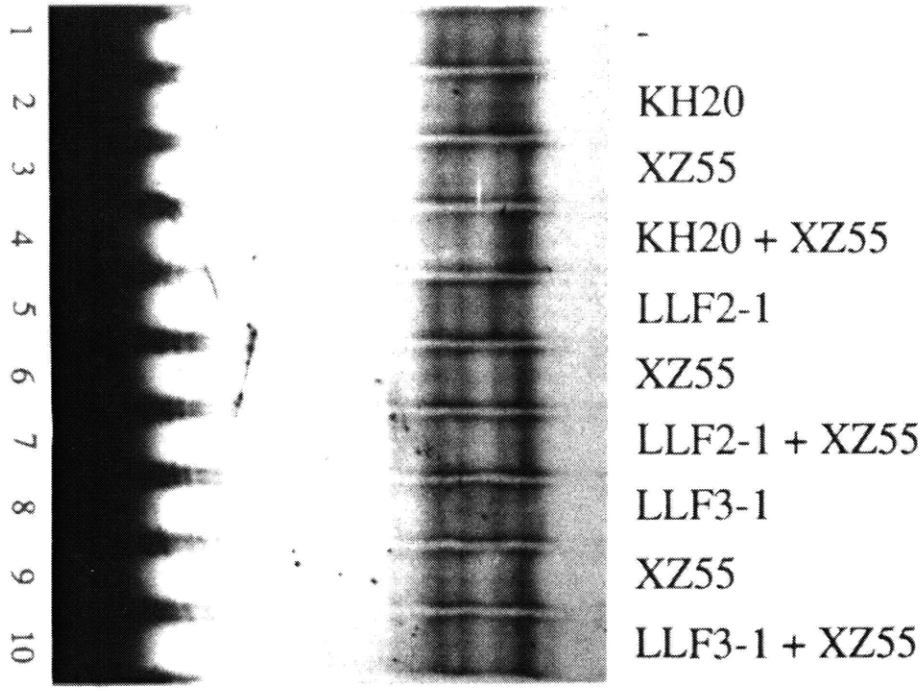


Figure 7A.

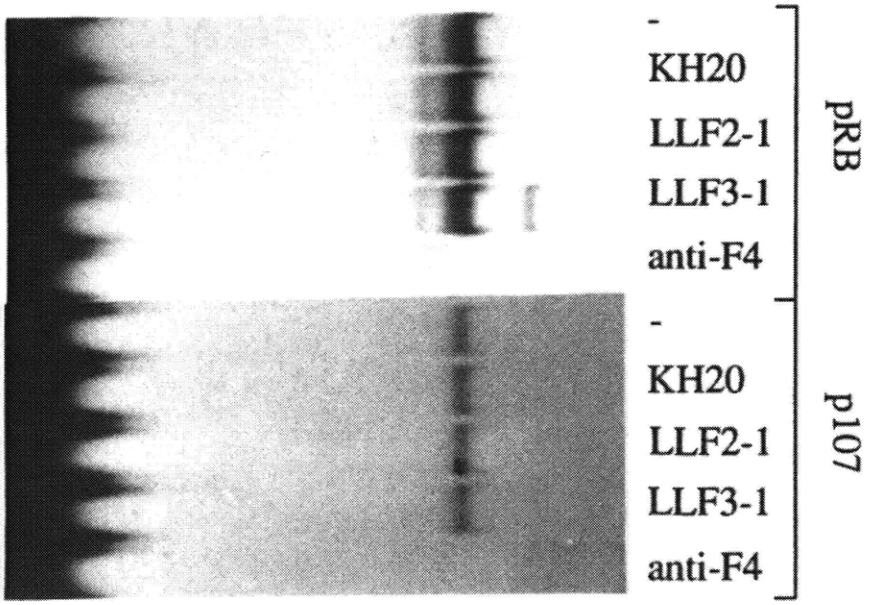
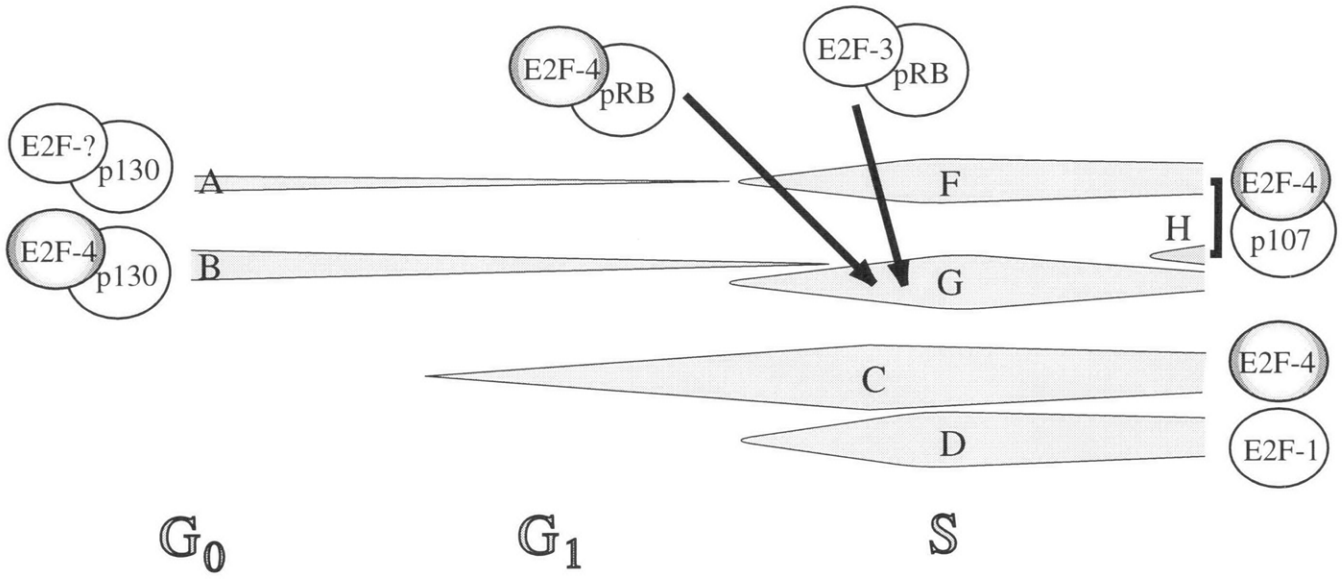


Figure 7B.

Figure 8.


E2F-4
pRB
E2F-3
pRB
E2F-?
p130
E2F-4
p130
A
B
F
G
H
E2F-4
p107
C
D
E2F-4
E2F-1
G0
G1
S

E. Figure Legends

Figure 1. Specificity of the E2F antisera. **(A)** Anti-E2F antibodies were tested in gel shift assays using whole cell extracts (5μg per lane) of C33-A cells that were transiently transfected with the indicated CMV-E2F and CMV-DP expression vectors. Where noted, gel shift reactions contained 1μl of monoclonal antibody supernatant specific for E2F-1 (KH20), E2F-2 (LLF2-1) or E2F-3 (LLF3-1). The anti-E2F-4 lanes contain 1μl of a 1:10 dilution of mouse polyclonal sera. **(B)** Anti-E2F antibodies were tested in western assay using whole cell extract (20μg per lane) derived from C33-A cells transiently transfected with the indicated CMV-E2F and CMV-DP expression vectors. Blots were probed with antisera specific to E2F-1 (KH20), E2F-2 (LLF2-1), E2F-3 (LLF3-1) or E2F-4 (Santa Cruz #sc-512x) as indicated.

Figure 2. E2F-1, -2, -3 and -4 associate with pRB in vivo. **(A)** Asynchronous ML-1 whole cell extract (800 μg per lane) was immunoprecipitated by either PAb419 (anti-T antigen), XZ55 (anti-pRB) or SD6 (anti-p107) and the immune complexes treated with 0.72% sodium deoxycholate (DOC) to release associated E2F activity. The supernatants were then analyzed in E2F gel shift assays in the presence of 1μl of the anti-E2F-1 (KH20), anti-E2F-2 (LLF2-1) or anti-E2F-3 (LLF3-1) tissue culture supernatant or 1μl of the diluted anti-E2F-4 polyclonal sera. Binding reactions containing multiple antibodies included 1μl of each of the indicated antibodies. **(B)** Asynchronous ML-1 whole cell extract (5000 μg per lane) was immunoprecipitated with XZ55 (anti-pRB) and the immune complexes released as described above. Supernatants were analyzed in E2F gel shift assays in the presence of 1 μl of anti-E2F-1 (KH20), anti-E2F-2 (LLF2-1), anti-E2F-3 (LLF3-1) or 1μl of the diluted anti-E2F-4 polyclonal sera. Reactions containing multiple antibodies included 1μl of each of the indicated antibodies.

Figure 3. Cell cycle expression of the E2F proteins. **(A)** [^{3}H] thymidine incorporation into T cells at the indicated times following addition of PHA. **(B)** The levels of E2F-1, -3, -4, pRB and p107 in each of the T cell fractions was assessed by western blotting. Each lane contained 15μg of whole cell extract. The filters were probed with a 1/5 dilution of the E2F-1 (KH20), E2F-3

(LLF3-1), pRB (cocktail of XZ77 and XZ91) or p107 (cocktail of SD2, SD6, SD9 and SD15) tissue culture supernatant or a 1:1000 dilution of the anti-E2F-4 mouse polyclonal antisera, #sc-512x.

Figure 4. Cell cycle dependent E2F DNA binding activity. The T cell whole cell extracts (6μg per lane) were preincubated with 0.6% sodium deoxycholate in order to dissociate higher order complexes. These samples were then analyzed in E2F gel shift assays in the presence of 2μl of tissue culture supernatant [PAb419 (anti-Tag), KH20 (anti-E2F-1), LLF2-1 (anti-E2F-2), LLF3-1 (anti-E2F-3)] and/or 1μl of the diluted polyclonal anti-E2F-4 as indicated. The bracket denotes the position of the "free" E2F, as determined by competition with unlabeled wildtype E2F binding site. The non-competable activity is also indicated. In each case, the position of the supershifted complex(es) is marked with an arrow. All gels were exposed for 4 days, except the anti-E2F-2 gel which was exposed for 8 days.

Figure 5. Cell cycle regulation of the individual E2F complexes. The T-cell whole cell extracts (8μg per lane) were analyzed in the E2F gel shift assay in either the absence **(A)** or the presence of the following antisera: **(B)** 2μl of the anti-pRB (XZ55) or anti-p107 (SD6) tissue culture supernatant; **(C)** 2μl of the anti-DP-1 (WTH1) tissue culture supernatant or 1μl of the anti-DP-2 (Santa Cruz #sc-829x) polyclonal antisera; **(D)** 2μl of the anti- E2F-1 (KH20), anti- E2F-2 (LLF2-1) or anti- E2F-3 (LLF3-1) tissue culture supernatant or 1μl of the diluted polyclonal anti-E2F-4. In each case, complexes are labeled as described in the text.

Figure 6. Cell cycle regulation of the pRB-associated E2F activity. **(A)** Time course of [^{3}H] thymidine incorporation into human T cells at the indicated times following addition of PHA. **(B)** T cell whole cell extract (500 μg) from each of the indicated time points was subject to immunoprecipitation with anti-pRB (XZ55) tissue culture supernatant. Associated E2F activity was released by incubation of the precipitates in 0.72% sodium deoxycholate and the supernatant was analyzed by E2F gel shift assay.

Figure 7. Identification of the pRB-associated E2F activity. **(A)** Whole cell extracts (8μg per lane) from the 54 hr time point of the first batch of T cells were analyzed in E2F gel shift assays in the presence of 2μl of the indicated anti-E2F monoclonal antibody and/or the pRB-specific XZ55. **(B)** Whole cell extracts containing the peak pRB-associated E2F activity of the second batch of T cells (54 hr and 60 hr) were pooled and 500 μg total protein was subject to immunoprecipitation with either the anti-pRB (XZ55) or the anti-p107 (SD6) tissue culture supernatant. Associated E2F activity was released by incubation in the presence of 0.72% sodium deoxycholate and the supernatant was analyzed in E2F gel shift assays in the absence or presence of either 1μl of the anti- E2F-1 (KH20), anti- E2F-2 (LLF2-1) or anti- E2F-3 (LLF3-1) tissue culture supernatant or 1μl of the diluted polyclonal anti-E2F-4.

Figure 8. Diagrammatic representation of the cell cycle dependence and identity of the various E2F complexes. Complexes are labeled A-H according to the nomenclature used in the text; their constituent E2F and pocket-protein moieties are indicated.

F. Materials and Methods

Plasmid construction.

The cDNA clones encoding E2F-1, -2, -3, and -5 and DP-1 and -2 have been described previously (Helin et al., 1992; Hijmans et al., 1995; Lees et al., 1993; Sardet et al., 1995; Wu et al., 1995). The E2F-4 cDNA clone was isolated by screening a Nalm-6 human pre-B cell library with a degenerate probe derived from sequences encoding the C-terminal 15 amino acids of the minimal DNA binding domains of E2F-1, -2 and -3 (our unpublished data). The sequence of this clone is identical to those described previously (Beijersbergen et al., 1994; Ginsberg et al., 1994). Plasmids encoding the 6x His-tagged E2F proteins were each constructed using the vector pQE8 (Qiagen). pQE8-E2F-2 (1-437) was generated by subcloning a *Bgl*II fragment encompassing the complete E2F-2 open reading frame from pCMV-E2F-2 into the *Bam*HI site of pQE8. Sequences encoding amino acids 1-244 of E2F-3 and 147-413 of E2F-4 were amplified by polymerase chain reaction with Vent polymerase (NEB) using the following primers: 31.6 [GATCGGATCCATGGTGAGAAAGGGAATCCAGCCC] & 31.3 [GATCGGATCCT CAGCCCATCCATTGGACGTTG] (E2F-3) and TPF4 [GATCGGATCCAGATGCTTTGCT GGAGATAC] & 4.15 [GATCGGATCCTCAGAGGTTGAGAACAGGCAGATC] (E2F-4). The resulting products were digested with *Bam*HI and subcloned into pQE8 to generate pQE8-E2F-3 (1-244) and pQE8-E2F-4 (147-413) respectively. The eukaryotic expression vectors were constructed in pCMV-Neo-Bam (Baker et al., 1990). pCMV-E2F-1 (1-437), pCMV-E2F-2 (1-437) and pCMV-HA-hDP-1 (1-410) have been described previously (Helin et al., 1992; Lees et al., 1993; Wu et al., 1995). The E2F-5 eukaryotic expression vector pcDNA3-E2F5 has been described previously (Sardet et al., 1995) and was a kind gift of C. Sardet. Sequences encompassing the complete E2F-3 open reading frame (425 amino acids) were prepared by polymerase chain reaction with Vent polymerase (NEB) using the primers 31.6 (as above) & 31.15 [CTAGGATCCGGATCGAAGGAGAGTTCACACGAAGC]. The amplified fragment was digested with *Bam*HI and subcloned into pCMV-Neo-Bam to generate pCMV-E2F-3 (1-

425). The complete E2F-4 open reading frame was excised from pBKS-E2F-4 as a *Eco*RV-*Bam*HI fragment and transferred to pCMV-Neo-Bam using *Bam*HI linkers (NEB).

Polyclonal and monoclonal antibody production

6x His-tagged E2F-2 (amino acids 1-437), E2F-3 (amino acids 1-244) or E2F-4 (amino acids 147-413) polypeptides were expressed in bacteria, purified over Ni^{2+}-NTA-Agarose resin (Qiagen) and used to immunize female BALB/c mice. The resultant polyclonal antisera was monitored by testing its ability to specifically supershift transfected E2F-2, -3 or -4/DP-1 complexes in gel shift assays or recover transfected E2F-2, -3 or -4/DP-1 complexes in immunoprecipitation assays.

In the case of both E2F-2 and E2F-3, as little as 1μl of a 1:100 dilution of the polyclonal antisera was sufficient to detect the correct E2F/DP complex. These mice were sacrificed and the spleens removed. Hybribomas were generated by PEG-mediated fusion of the recovered splenocytes to the SP2/O cell line. Eight days post fusion the tissue culture supernatants were screened for their ability to detect the relevant 6xHis-tagged purified proteins by ELISA. These positive supernatants were then screened for their ability to specifically supershift transfected E2F-2 or -3/DP-1 complexes in gel shift assays, recover transfected E2F-2 or -3/DP-1 complexes in immunoprecipitation assays or western blot assays. The positive hybridoma cell lines were separated from other hybridomas using limiting dilution and then single cell cloning.

The monoclonal antibodies KH20 (anti-E2F-1), WTH1 (anti-DP-1), XZ55, XZ77 and XZ91 (anti-pRB), and SD2, SD6, SD9 and SD15 (anti-p107) (Dyson et al., 1993) were a gift of Nick Dyson and Ed Harlow.

Tissue culture

The human lines ML-1 (premyeloid leukemia) and C33-A (cervical carcinoma) were grown under standard conditions in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Transient transfections into C33-A cells were carried out by standard $CaPO_4$ precipitation

methods (Ausubel et al., 1988). Ten cm dishes of C33-A cells at 50% confluence were transfected with 10μg of each of the E2F and DP expression plasmids plus 10μg of carrier DNA. Cells were washed 16 hours post-transfection and re-fed with fresh media. Cells were harvested 24 hours later and whole cell extracts prepared as described below.

For T cell preparations, buffy coats from human blood were obtained from the Massachusetts General Hospital Blood Bank. The mononuclear cell layer was isolated by centrifugation on a Ficoll-Paque cushion (Pharmacia) and then washed twice with phosphate buffered saline. Mononuclear cells were then resuspended at 2.0 X 10^6 cells per ml in RPMI supplemented with 10% heat-inactivated fetal calf serum and 1 μg of phytohemagglutinin (PHA, Murex UK) per ml to stimulate T-cell proliferation. Cells were harvested at the indicted times and the majority were used to prepare whole cell extracts as described below. At each time point, thymidine incorporation was also assayed by incubating 1ml of cultured T cells for 30 min in the presence of 10 μCi of [^{3}H]thymidine. After washing, these cells were lysed in 0.3N NaOH, spotted onto GF/C glass filters (Whatman), TCA precipitated and counted.

Gel shift assays

Whole cell extracts were prepared from ML-1, C33-A and T cells using standard procedures. Briefly, cells were lysed at 5 x 10^6/0.1 ml in 0.5 M KCl, 35% glycerol, 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 5 mM $MgCl_2$, 0.5 mM ethylenedinitrilo-tetraacetic acid [EDTA; pH 8.0], 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol [DTT], 5 μg/ml aprotinin, 5 μg/ml leupeptin and then spun at 4^o C for 45 min at 40,000rpm in a TLA45 rotor (Beckman) to remove cell debris. Supernatants were removed and protein concentrations determined (Protein Assay Reagent, BioRad). Gel shift reactions were performed as follows. The initial DNA binding mixtures contained 1 μg of sonicated salmon sperm DNA and 5-8 μg of whole cell extract (as indicated in the figure legends) in 20 μl of 50 mM KCl, 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 8.5% glycerol, 1 mM EDTA, pH8.0. For experiments involving T cell extracts, 75 ng of double-stranded mutant E2F

oligonucleotide (ATTTAAGTTTCGatCCCTTTCTCAA) was also included in the binding reaction to inhibit the formation of non-specific DNA complexes. Samples were incubated 10 min on ice prior to the addition of 1 ng of ^{32}P-end-labeled, double-stranded, wildtype E2F oligonucleotide (ATTTAAGTTTCGCGCCCTTTCCAA). Binding reactions were incubated a further 10 min on ice followed by 15 min at room temperature. The samples were then analyzed by electrophoresis at 180V at 4° C in 4% polyacrylamide gels buffered with 0.25X TBE (22 mM Tris, 22 mM borate, 0.5 mM EDTA).

Deoxycholate (DOC)-treated gel shift reactions were performed in an identical manner except for the presence of 0.6% sodium deoxycholate (SIGMA) in the initial binding mixture and the addition of Nonidet P-40 to 1% at the beginning of the room temperature incubation step. In both standard and DOC-treated gel shift reactions, competitions were carried out by the addition of 100 ng of unlabeled double-stranded wildtype or mutant E2F oligonucleotide prior to the addition of cell extract. Where indicated, hybridoma supernatant or diluted polyclonal antisera was also added to the DNA binding reactions prior to the addition of cell extract.

Immunoprecipitation-DOC release assays

Immunoprecipitation-deoxycholate-released proteins were generated from the standard ML-1 or T-cell whole cell extracts. 500-5000 µg extracts were incubated on a rocking platform for 60 min at 4° C with 200 µl of the indicated hybridoma supernatants in 1X IP-DOC buffer (20 mM HEPES, pH 7.4, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0) containing 3 mg bovine serum albumin (BSA) per ml. Immune complexes were recovered on Protein A-Sepharose beads (Pharmacia) and then washed three times in 1X IP-DOC buffer. The associated proteins were released by the addition of 10 µl of 0.72% sodium deoxycholate in 1X IP-DOC buffer. Nonidet P-40 was added to a final concentration of 1.5% and the supernatants assayed in the gel shift protocol described above.

Western blots

Whole cell extract was prepared from T cells as described above. The indicated amounts of cellular protein were separated by 8% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore) by electrophoresis for 16 h at 20V in 390mM glycine, 50mM Tris, 20% methanol. The membranes were blocked in 1X TBST (10mM Tris pH 8.0, 150mM NaCl, 0.2% Tween-20) containing 5% dry milk for 2 h at room temperature and then immunoblotted with the indicated antibodies. Horseradish peroxidase-linked sheep anti-mouse Ig or HRP-linked donkey anti-rabbit Ig (Amersham) were used as secondary antibodies and the blots developed using the ECL system (Amersham).

G. Acknowledgments

We are grateful to S. Keightley, B. Fairchild, J.P. Vernon, A. Singh, E. Chen, T.P. Singh, S. Morel and P. Kumar for technical assistance in the preparation of antisera. We thank S. Estes, B. Fairchild and R. Verona for helpful discussion and critical reading of the manuscript. We wish to particularly thank E. Farrell for her contribution to the preparation of this manuscript. This work is supported by a grant from the National Cancer Institute (NIH/NCI S.P30-CA14051-23).

References

Ausubel, F.M., R. Brent, R.E. Kington, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (ed.). 1988. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.

Bagchi, S., R. Weinmann, and P. Raychaudhuri. 1991. The Retinoblastoma Protein Copurifies with E2F-I, an E1A-Regulated Inhibitor of the Transcription Factor E2F. Cell *65*, 1063-1072.

Baker, S.J., S. Markowitz, E.R. Fearon, J.K.V. Willson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science *249*,912-915.

Bandara, L.R., V.M. Buck, M. Zamanian, L.H. Johnston, and N.B. La Thangue. 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. EMBO J. *12*, 4317-4324.

Bandara, L.R. and N.B. La Thangue. 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature *351*, 494-497.

Beijersbergen, R.L., R.M. Kerkhoven, L. Zhu, L. Carlee, P.M. Voorhoeve, and R. Bernards. 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes & Dev. *8*, 2680-2690.

Cao, L., B. Faha, M. Dembski, L. Tsai, E. Harlow, and N. Dyson. 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature *355*, 176-179.

Chellappan, S.P., S. Hiebert, M. Mudryj, J.M. Horowitz, and J.R. Nevins. 1991. The E2F Transcription Factor Is a Cellular Target for the RB Protein. Cell *65*, 1053-1061.

Chittenden, T., D.M. Livingston, and J.A. DeCaprio. 1993. Cell Cycle Analysis of E2F in Primary Human T Cells Reveals Novel E2F Complexes and Biochemically Distinct Forms of Free E2F. Mol. and Cell Bio. *13*, 3975-3983.

Cobrinik, D., P. Whyte, D.S. Peeper, T. Jacks, and R.A. Weinberg. 1993. Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes and Dev. *7*, 2392-2404.

Devoto, S.H., M. Mudryj, J. Pines, T. Hunter, and J.R. Nevins. 1992. A Cyclin A-Protein Kinase Complex Possesses Sequence-Specific DNA Binding Activity: $p33^{cdk2}$ Is a Component of the E2F-Cyclin A Complex. Cell *68*, 167-176.

Dynlacht, B.D., O. Flores, J.A. Lees, and E. Harlow. 1994. Differential regulation of E2F *trans*- activation by cyclin / cdk2 complexes. Genes & Dev. *8*, 1772-1786.

Dyson, N., and E.Harlow. 1992. Adenovirus E1A Targets Key Regulators of Cell Proliferation. Cancer Surveys *12*, 161-195.

Dyson, N., M. Dembski, A. Fattaey, C. Ngwu, M. Ewen, and K. Helin. 1993. Analysis of p107- Associated Proteins: p107 Associates with a Form of E2F That Differs from pRB-Associated E2F-1. Journal of Virology *67*, 7641-7647.

Ewen, M.E., Y. Xing, J.B. Lawrence, and D.M. Livingston. 1991. Molecular Cloning, Chromosomal Mapping, and Expression of the cDNA for p107, a Retinoblastoma Gene-Product-Related Protein. Cell *66*, 1155-1164

Ewen, M.E., B. Faha, E. Harlow, and D.M. Livingston. 1992. Interaction of p107 with Cyclin A Independent of Complex Formation with Viral Oncoproteins. Science *255*, 85-87.

Ewen, M.E., H.K. Sluss, C.J. Sherr, H. Matsushime, J.Y. Kato, and D.M. Livingston. 1993. Functional Interactions of the Retinoblastoma Protein with Mammalian D-type Cyclins. Cell *73*, 487-497.

Faha, B., M.E. Ewen, L.H. Tsai, D.M. Livingston, and E. Harlow. 1992. Interaction Between Human Cyclin A and Adenovirus E1A-Associated p107 Protein. Science *255*, 87-90

Friend, S.H., R. Bernards, S. Rogelj, R.A. Weinberg, J.M. Rapaport, D.M. Albert, and T.P. Dryja. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature *323*, 643-646.

Fung, Y.K.T., A.L. Murphree, A. T'Ang, J. Qian, S.H. Hinrichs, and W.F. Benedict. 1987. Structural Evidence for the Authenticity of the Human Retinoblastoma Gene. Science *236*, 1657-1661.

Ginsberg, D., G. Vairo, T. Chittenden, Z.X. Xiao, G. Xu, K.L. Wydner, J.A. DeCaprio, J.B. Lawrence, and D.M. Livingston. 1994. E2F-4, a new member of the E2F trancription factor family, interacts with p107. Genes & Dev. *8*, 2665-2679.

Girling, R., J.F. Partridge, L.R. Bandara, N. Burden, N.F. Totty, J.J. Hsuan, and N.B. La Thangue. 1993. A new component of the transcription factor DRTF1/E2F. Nature *362*, 83-87.

Hannon, G., D. Demetrick, and D. Beach. 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes & Dev. *7*, 2378-2391.

Helin, K., J.A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey. 1992. A cDNA Encoding a pRB-Binding Protein with Properties of the Transcription Factor E2F. Cell *70*, 337-350.

Helin, K., E. Harlow, and A. Fattaey. 1993a. Inhibition of E2F-1 Transactivation by Direct Binding of the Retinoblastoma Protein. Mol. and Cell. Biol. *13*, 6501-6508.

Helin, K., C. Wu, A.R. Fattaey, J.A. Lees, B.D. Dynlacht, C. Ngwu, and E. Harlow. 1993b. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative *trans*- activation. Genes & Dev. *7*, 1850-1861.

Hiebert, S.W., S.P. Chellappan, J.M. Horowitz, and J.R. Nevins. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes & Dev. *6*, 177-185.

Hijmans, E.M., P.J. Voorhoeve, R.L. Beijersbergen, L.J. VAN'T Veer, and R. Bernards. 1995. E2F-5, a New E2F Family Member that interacts with p130 In Vivo. Mol. and Cell. Biol. *15*, 3082-3089.

Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.A. Weinberg. 1992. Regulation of Retinoblastoma Protein Functions by Ectopic Expression of Human Cyclins. Cell *70*, 993-1006.

Hsiao, K.M., S.L. McMahon, and P. Farnham. 1994. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. Genes & Dev. *8*, 1526-1537.

Hu, Q., J.A. Lees, K.J. Buchkovich, and E. Harlow. 1992. The retinoblastoma protein physically associates with the human cdc2 kinase. Mol. Cell. Biol. *12*, 971-980.

Ivey-Hoyle, M., R. Conroy, H.E. Huber, P.J. Goodhart, A. Oliff, and D.C. Heimbrook. 1993. Cloning and Characterization of E2F-2, a Novel Protein with the Biochemical Properties of Transcription Factor E2F. Mol. and Cell. Biol. *13*, 7802-7812.

Johnson, D.G., J.K. Schwarz, W.D. Cress, and J.R. Nevins. 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature *365*, 349-352.

Johnson, D.G., K. Ohtani, and J.R. Nevins. 1994. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes & Dev. *8*, 1514-1525.

Kaelin, W.G.,Jr., W. Krek, W.R. Sellers, J.A. DeCaprio, F. Ajchenbaum, C.S. Fuchs, T. Chittenden, Y. Li, P.J. Farnham, M.A. Blanar, D.M. Livingston, and E.K. Flemington. 1992. Expression Cloning of a cDNA Encoding a Retinoblastoma-Binding Protein with E2F-like Properties. Cell *70*, 351-364.

Krek, W., M.E. Ewen, S. Shirodkar, Z. Arany, W.G. Kaelin, Jr., and D.M. Livingston. 1994. Negative Regulation of the Growth-Promoting Transcription Factor E2F-1 by a Stably Bound Cyclin A-Dependent Protein Kinase. Cell *78*, 161-172.

Krek, W., D.M. Livingston, S. Shirodkar. 1993 . Binding to DNA and the Retinoblastoma Gene Product Promoted by Complex Formation of Different E2F Family Members. Science *262*, 1557-1560.

Lee, H.W., R. Bookstein, F. Hong, L.J. Young, J.Y. Shew and E.Y.H.P. Lee. 1987. Human Retinoblastoma Susceptibility Gene: Cloning, Identification and Sequence. Science *235*, 1394-1399.

Lees, E., B. Faha, V. Dulic, S.I. Reed, and E. Harlow. 1992. Cyclin E / cdk2 and cyclin A / cdk2 kinases associate with p107 and E2F in a temporally distinct manner. Genes and Dev. *6*, 1874-1885.

Lees, J.A., K.J. Buchkovich, D.R. Marshak, C.W. Anderson, and E. Harlow. 1991. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J. *10*, 4279-4290.

Lees, J.A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin. 1993. The Retinoblastoma Protein Binds to a Family of E2F Transcription Factors. Mol. Cell Bio. *13*, 7813-7825.

Li, Y., C. Graham, S. Lacy, A.M.V. Duncan, and P. Whyte. 1993. The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes & Dev. *7*, 2366-2377.

Matsushime, H., D.E. Quelle, S.A. Shurtleff, M. Shibuya, C.J. Sherr, and J.Y. Kato. 1994. D-Type Cyclin-Dependent Kinase Activity in Mammalian Cells. Mol. and Cell. Biol. *14*, 2066-2076.

Mayol, X., X. Grana, A. Baldi, N. Sang, Q. Hu, and A. Giordano. 1993. Cloning a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene *8*, 2561-2566.

Melillo, R.M., K. Helin, D.R. Lowy, and J.T. Schiller. 1994. Positive and Negative Regulation of Cell Proliferation by E2F-1: Influence of Protein Level and Human Papillomavirus Oncoproteins. Mol. and Cell. Biol. *14*, 8241-8249.

Meyerson, M., and E. Harlow. 1994. Identification of G_1 Kinase Activity for cdk6, a Novel Cyclin D Partner. Mol. and Cell. Biol. *14*, 2077-2086.

Mudryj, M., S.H. Devoto, S.W. Hiebert, T. Hunter, J. Pines, and J.R. Nevins. 1991. Cell Cycle Regulation of the E2F Transcription Factor Involves an Interaction with Cyclin A. Cell *65*, 1243-1253.

Neuman. E., E.K. Flemington, W.R. Sellers, and W.G. Kaelin, Jr. 1994. Transcription of the E2F-1 Gene is Rendered Cell Cycle Dependent by E2F DNA-Binding Sites within Its Promoter. Mol. and Cell. Biol. *14*, 6607-6615.

Nevins, J.R. 1992. A closer look at E2F. Nature *358*, 375-376.

Peeper, D.S., P. Keblusek, K. Helin, M. Toebes, A.J. van der Eb, and A. Zantema. 1995. Phosphorylation of a specific cdk site in E2F-1 affects its electrophoretic mobility and promotes pRB-binding *in vitro*. Oncogene *10*, 39-48.

Qin, X.Q., D.M. Livingston, W.G. Kaelin, Jr., and P.D. Adams. 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. USA *91*, 10918-10922.

Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R.A. Weinberg. 1995. E2F-4 and E2F-5, two members of the E2F family , are expressed in early phases of the cell cycle. Proc. Natl. Acad. Sci. USA *92*, 2403-2407.

Schwarz, J.K., S.H. Devoto, E.J. Smith, S.P. Chellappan, L. Jakoi, and J.R. Nevins. 1993. Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. EMBO J. *12*, 1013-1020.
Shan, B., and W.H. Lee. 1994. Deregulated Expression of E2F-1 Induces S-phase Entry and Leads to Apoptosis. Mol. and Cell. Biol. *14*, 8166-8173.

Shan, B., C.Y. Chang, D. Jones, and W.H. Lee. 1994. The Transcription Factor E2F-1 Mediates the Autoregulation of RB Gene Expression. Mol. and Cell. Biol. *14*, 299-309.

Shirodkar, S., M. Ewen, J.A. DeCaprio, J. Morgan, D.M. Livingston, and T. Chittenden. 1992. The Transcription Factor E2F Interacts with the Retinoblastoma Product and a p107-Cyclin A Complex in a Cell Cycle-Regulated Manner. Cell *68*, 157-166.

Singh, P., S.H. Wong, and W. Hong. 1994. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. EMBO J. *13*, 3329-3338.

Vairo, G., D.M. Livingston , and D. Ginsberg. 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma family members. Genes & Dev. *9*, 869-881.

Weinberg, R.A. 1992. Tumor Suppressor Genes. Science *254*, 1138-1146.

Wu, C.L., L.R. Zukerberg, C. Ngwu, E. Harlow, and J.A. Lees. 1995. In Vivo Association of E2F and DP Family Proteins. Mol. and Cell. Biol. *15*, 2536-2546.

Wu, X., and A.J. Levine. 1994. p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA *91*, 3602-3606.

Xu, G., D.M. Livingston, and W. Krek. 1995. Multiple members of the E2F transcrpition factor family are the products of oncogenes. Proc. Natl. Acad. Sci. USA *92*, 1357-1361.

Zhu, L., S. van der Heuvel, K. Helin, A. Fattaey, M. Ewen, D.M. Livingston, N. Dyson, and E. Harlow. 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes & Dev. *7*, 1111-1125.

Zhu, L., L. Zhu, E. Xie, and L.S Chang. 1995. Differential Roles of Two Tandem E2F Sites in Repression of the Human p107 Promoter by Retinoblastoma and p107 Proteins. Mol. and Cell. Biol. *15*, 3552-3562.

CHAPTER 3

E2F activity is regulated by cell-cycle-dependent changes in subcellular localization.

Raluca Verona[1], Ken Moberg[1], Scott Estes, Michelle Starz,
Juan P. Vernon and Jacqueline A. Lees

[1]These authors made an equal contribution to this work.

Molecular and Cellular Biology, December 1997, Vol.17, No.12, p.7268-7282
(Author's contribution: Figures 3A, 4, and 5)

A. Abstract

E2F directs the cell-cycle dependent expression of genes that induce or regulate the cell division process. In mammalian cells, this transcriptional activity arises from the combined properties of multiple E2F•DP heterodimers. In this study, we show that the transcriptional potential of individual E2F species is dependent upon their nuclear localization. This is a constitutive property of E2F-1, -2 and -3, whereas the nuclear localization of E2F-4 is dependent upon its association with other nuclear factors. We have previously shown that E2F-4 accounts for the majority of endogenous E2F species. We now show that the subcellular localization of E2F-4 is regulated in a cell-cycle-dependent manner that results in the differential compartmentalization of the various E2F complexes. Consequently, in cycling cells, the majority of the p107•E2F, p130•E2F and free E2F complexes remain in the cytoplasm. In contrast, almost all of the nuclear E2F activity is generated by pRB•E2F. This complex is present at high levels during G_1 but disappears once the cells have passed the restriction point. Surprisingly, dissociation of this complex causes little increase in the levels of nuclear, free E2F activity. This observation suggests that the repressive properties of the pRB•E2F complex will play a critical role in establishing the temporal regulation of E2F responsive genes. How the differential subcellular

localization of pRB, p107 and p130 contributes to their different biological properties is also discussed.

B. Introduction

E2F is a transcriptional regulator that plays a pivotal role in the regulation of cellular proliferation (reviewed in Nevins, 1992 and Beijersbergen and Bernards, 1996). Many E2F-responsive genes have been identified and their products are components of either the cell cycle control (e.g. cyclin E, cyclin A and cdc2) or DNA synthesis (e.g. dihydrofolate reductase, thymidine kinase or DNA polymerase α) machinery. In each case, E2F is thought to restrict the expression of these genes to the point of the cell cycle at which their products act (Hsiao et al., 1994).

E2F is regulated by the retinoblastoma protein (Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991), a tumor suppressor that is functionally inactivated in a large proportion of all human tumors (reviewed in Weinberg, 1992). Consistent with its anti-proliferative role, the retinoblastoma protein (pRB) blocks the ability of E2F to activate transcription (Hiebert et al., 1992; Helin et al., 1993b). In addition, over-expression studies indicate that the resultant pRB•E2F complex can act as a transcriptional repressor, in which E2F provides the sequence specific DNA binding activity and pRB inhibits transcription by sequestrating adjacent transcription factors (Adnane et al., 1995; Bremner et al., 1995; Sellers et al., Weintraub et al., 1992; Weintraub et al., 1995). This suggests that E2F participates in both the activation and inhibition of cellular proliferation. Consistent with this hypothesis, homozygous deletion of the murine E2F-1 gene causes atrophy in some tissues and tumors in others (Field et al., 1996; Yamasaki et al., 1996).

The growth inhibitory properties of pRB are regulated by its cell cycle dependent phosphorylation (reviewed in Bartek et al., 1996). Phosphorylation is catalyzed by one or more of the cell cycle dependent kinases (Ewen et al., 1993; Hinds et al., 1992; Hu et al., 1992; Lees et al., 1991; Matsushime et al., 1994; Meyerson et al., 1994) and over-expression studies indicate that this modification is essential for S-phase entry (Hinds et al., 1992). *In vivo* studies confirm that the phosphorylation of pRB is sufficient to induce the release of free, presumably transcriptionally

active E2F (Chellappan et al., 1991). Because of the dual role of the E2F complex, phosphorylation of the retinoblastoma protein provides a simple mechanism to switch E2F-responsive genes from the fully repressed to the fully induced state. Consistent with this model, the timing of transcriptional activation of E2F responsive genes correlates closely with the induction of pRB phosphorylation at the G_1/S transition.

Our understanding of E2F is complicated by the finding that this activity is regulated by two other proteins, p107 and p130 (Cao et al., 1992; Cobrinik et al., 1993; Devoto et al., 1992; Shirodkar et al., 1992). These two proteins share significant sequence similarity with the retinoblastoma protein (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993) and over-expression studies confirm that they can regulate E2F in a similar manner (Starostik et al., 1996; Zhu et al., 1993). Despite these similarities, pRB, p107 and p130 interact with E2F at different stages of the cell cycle (Cao et al., 1992; Chittenden et al., 1993; Cobrinik et al., 1993; Lees et al., 1992; Mudryj et al., 1991; Shirodkar et al., 1992). Moreover, unlike the pRB•E2F complex, the timing of appearance or disappearance of the p130•E2F and p107•E2F species does not correlate with the timing of repression or activation of known E2F responsive genes. These findings suggest that pRB, p107 and p130 do not regulate E2F in the same way *in vivo* and genetic analyses confirm that these proteins have different biological consequences. While pRB is mutated in 30% of all human tumors, neither p107 or p130 is a tumor suppressor (Weinberg, 1995). Similarly, the mutation of pRB, p107 or p130 within otherwise isogenic mouse strains gives rise to very different phenotypes (Clarke et al., 1992; Cobrinik et al., 1996; Jacks et al., 1992; Lee et al., 1992; Lee et al., 1996). Clearly, the different biological consequences of pRB, p107 and p130 action could reflect differences in their regulation of E2F or of non-E2F targets.

To date, at least seven human genes have been identified that encode components of the E2F transcriptional activity (reviewed in Beijersbergen and Bernards, 1996). These can be divided into two distinct groups, termed E2F (1 through 5) and DP (1 and 2), that share little sequence similarity. E2F and DP proteins heterodimerize and this association is essential for high affinity DNA binding, transcriptional activity and interaction with pRB, p107 or p130 (Bandara et al.,

1993; Helin et al., 1993a; Krek et al., 1993; Sardet et al., 1995; Wu et al., 1995). *In vivo* studies have confirmed that the endogenous E2F activity arises from the concerted action of multiple E2F•DP complexes (Moberg et al., 1996; Wu et al., 1995). These individual E2F•DP complexes have different pRB, p107 and p130 binding properties. Complexes containing E2F-1, -2 or -3 associate with pRB, but not p107 or p130 *in vivo* (Dyson et al., 1993; Lees et al., 1993). In contrast, E2F-4 and -5 complexes have been reported to bind preferentially to p107 and p130 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Vairo et al., 1995). Consistent with these findings, sequence comparisons indicate that E2F-1, -2 and -3 are more closely related to each other than they are to E2F-4 and -5. Taken together, these observations suggest that the two E2F subsets (E2F-1, -2 and -3 versus E2F-4, and -5) will play distinct roles *in vivo* that will at least partially account for the different biological consequences of pRB, p107 and p130 action. Overexpression assays have revealed some differences in the properties of the individual E2F•DP complexes (DeGregori et al., 1995; Hofmann et al., 1996; Lukas et al., 1996) but the mechanistic distinction(s) between these species remains unclear.

We have previously characterized the cell cycle regulation of the individual E2F•DP complexes (Moberg et al., 1996). This study revealed clear differences in the relative contribution and potential activity of these species. E2F-1, -2, -3 and -5 exist at low levels *in vivo* and together comprise less than a third of the endogenous E2F species. In contrast, E2F-4 accounts for the majority of E2F complexes at every stage of the cell cycle. Moreover, in addition to binding p107 and p130, E2F-4 was found to be the major component of the pRB-associated E2F activity. These findings suggest that E2F-4 plays a pivotal role in establishing the biological properties of the cellular E2F activity. Nevertheless, we also found that the appearance of free E2F-4•DP, which occurs early in G_1, is insufficient to induce the activation of known E2F-responsive genes. This observation can be explained in two distinct ways; (i) the transcriptional activity of free E2F-4 is regulated by an unknown mechanism and/or (ii) it is directed at an unknown set of target genes. Since E2F-4 accounts for most of the endogenous E2F species, either mechanism will have a profound effect upon the biological consequences of E2F action.

In this study, we have used a combination of *in vitro* and *in vivo* assays to investigate these two hypotheses. These experiments did not allow us to examine whether E2F-4 has a different target specificity from the other E2Fs. However, our data indicate that, unlike E2F-1, -2 and -3, the transcriptional activity of E2F-4 is regulated at the level of subcellular localization. *In vivo*, the nuclear localization of E2F-4 is restricted to certain stages of the cell cycle and is limited to a specific subset of the E2F-4 complexes. This novel mode of E2F-4 regulation provides new insight into the molecular mechanism(s) that establish the different biological properties of the individual E2F family members and may offer important insight into the *in vivo* roles of the E2F regulators, pRB, p107 and p130.

C. Results

C1. *In vivo* expression of E2F-4 inhibits its transcriptional activity

Our previous studies indicate that the appearance of free E2F-4 complexes is not sufficient to trigger the activation of known E2F-responsive genes (Moberg et al., 1996). This observation suggests two potential models of E2F-4 action; either its transcriptional activity is regulated by an unknown mechanism or it is directed at an unknown set of target genes. To distinguish between these two models, we generated stable cell lines that express the individual E2F proteins in an inducible manner. In this system, the expression of a given transgene is controlled by a transcriptional regulator, the hybrid VP16 -tetracycline repressor, whose activity is inhibited in the presence of tetracycline (Gossen and Bujard, 1992). Using this approach, we selected two cell lines (called U2F2 and U2F4) that expressed either E2F-2 or E2F-4 in a strictly regulated manner (Figure 1a). When cultured in the presence of tetracycline, the levels of E2F-2 or E2F-4 in the U2F2 or U2F4 cell lines were similar to those detected in the parental cell line. In contrast, tetracycline withdrawal produced a modest increase in E2F-2 (between 3 and 8 fold) in the U2F2 cells and a dramatic increase in E2F-4 (greater than 40 fold) in the U2F4 cells.

To establish the biological properties of the induced E2F proteins, we compared the level of E2F DNA binding activity and transcriptional activity present in the U2tTA10, U2F2 or U2F4 cells after culture in either the absence or presence of tetracycline. DNA binding was assessed by screening whole cell lysates for their ability to bind to the consensus E2F site in a gel retardation assay (Figure 1b). Consistent with our expression data, the uninduced cells contained similar levels of E2F DNA binding activity. In contrast, induction of the U2F2 and U2F4 cell lines increased the levels of a single E2F complexes, the fastest migrating, free E2F species. Supershift experiments confirmed that this was caused by a direct increase in the level of either free E2F-2•DP (U2F2) or E2F-4•DP (U2F4, Figure 1b). These data indicate that the induction of E2F-2 or E2F-4 resulted in an increase in the level of free E2F-2 or E2F-4 complexes without altering the other, endogenous E2F species. The transcriptional activity of these induced E2F complexes was

tested by transiently transfecting these cells with an artificial E2F-responsive reporter, called $E2F_4$-CAT, in which the expression of the chloramphenicol acetyl transferase (CAT) gene is controlled by a minimal promoter containing the E1B TATA box and four consensus E2F sites (Helin et al., 1993a). This reporter was selected because it has been shown to respond with similar efficiency to free E2F-2•DP and E2F-4•DP complexes in *in vitro* transcription assays (Dynlacht et al., 1997). Although induction of the U2F2 cell line produced a relatively small increase in the levels of E2F-2•DP complexes (Figure 1b), the E2F transcriptional activity increased by more than 11 fold (Figure 1c). In contrast, tetracycline withdrawal caused little change in the transcriptional activity of the U2F4 cells, despite the much greater increase in the level of free E2F-4•DP complex (Figures 1b and c). Since this complex can efficiently activate this reporter *in vitro*, we conclude that there are additional factors *in vivo* that prevent activation of transcription by the free E2F-4. Although this finding does not rule out the possibility that the individual E2F proteins activate different target genes *in vivo*, it suggests the existence of an unidentified regulatory mechanism that controls the activity of E2F-4 differently from that of E2F-2.

C2. The transcriptional activity of E2F-4 is inhibited by its cytoplasmic localization.

To determine the mechanism responsible for the inhibition of E2F-4 transcriptional activity, we investigated whether there were any obvious differences in the regulation of the individual E2F proteins. As part of this study, we examined the localization properties of E2F proteins that have been expressed in micro-injection assays (Figure 2a). Consistent with their role as transcriptional regulators, the three pRB-specific E2Fs, E2F-1, -2 and -3, were all detected in the nucleus. In contrast, the vast majority of the E2F-4 protein localized to the cytoplasm. Although we could not rule out the possibility that the localization of this protein was an artifact of its over-expression and presumably monomeric state, this finding strongly suggested that the differential localization of E2F-2 and E2F-4 might account for the differences in their transcriptional activity revealed in the inducible cell lines. To test this hypothesis, we used indirect immunofluorescence to examine the localization of E2F-2 or E2F-4 that had been induced in the

U2F2 or U2F4 cells (Figure 2b). These proteins are expressed at considerably lower levels than those produced in the micro-injection assays and therefore a much greater proportion form productive E2F•DP heterodimers. However, their localization was identical to that observed by micro-injection; E2F-2 was detected in the nucleus while E2F-4 was predominantly cytoplasmic (Figure 2b). These findings suggest that the transcriptional activity of exogenously expressed E2F-4 is inhibited *in vivo* by its cytoplasmic localization.

C3. Localization of the endogenous E2F-4 protein is regulated in a cell cycle dependent manner.

We have previously shown that the appearance of endogenous free E2F-4 complexes does not induce the transcription of known E2F responsive genes (Moberg et al., 1996). Our data now suggest that the transcriptional activity of these complexes could be blocked by their sequestration in the cytoplasmic compartment. To test this hypothesis, we compared the subcellular localization of the endogenous E2F proteins. Initially, we used standard methods to prepare nuclear and cytoplasmic extracts for a wide variety of human cell lines. These fractions were then screened by western blotting for the presence of either E2F-1 or E2F-4 (Figure 3a). Regardless of the cell line, E2F-1 was consistently detected in the nuclear fraction. Similar results were also observed with E2F-2 and E2F-3 (data not shown). In contrast, the majority of the endogenous E2F-4 protein was contained within the cytoplasm. These findings confirm that the endogenous E2F proteins localize to different subcellular compartments in a similar manner to the overexpressed E2Fs. To reinforce these data, we also examined the localization of the endogenous E2F-4 by indirect immunofluorescence. Initially, we screened for E2F-4 in an asynchronous population of U2OS cells (Figure 3b). Within this population, the individual cells had one of two distinct staining patterns; the E2F-4 was either predominantly cytoplasmic or it was present in both the cytoplasm and the nucleus. This dual staining pattern was observed with multiple E2F-4 monoclonal antibodies in several different cell types (data not shown).

The presence of two distinct E2F-4 staining patterns within asynchronous cells suggested that the localization of E2F-4 might change through the cell cycle. To test this idea, we compared the pattern of E2F-4 staining at different cell cycle stages. Initially, U2OS cells were released from a drug induced G_2/M arrest and harvested at 6 hour intervals for both FACS analysis and E2F immunofluorescence (Figure 3b). Consistent with our hypothesis, E2F-4 was detected in both the nucleus and the cytoplasm in the enriched G_1 population but was predominantly cytoplasmic in cells that had entered S-phase. Since the pRB pathway is known to be disrupted in most if not all tissue culture cell lines, we also examined the localization of E2F-4 in a primary human diploid fibroblast cell line, WI-38 cells. In this case, the cells were arrested in G_0/G_1 by contact inhibition and serum starvation and then stimulated to re-enter the cell cycle. The localization of E2F-4 was then examined in the peak G_0/G_1 and S-phase fractions (as judged by FACS analysis) using immunofluorescence (Figure 3c). In this primary cell line, the cell cycle dependent change in subcellular localization E2F-4 was even more pronounced. Almost all of the E2F-4 protein was retained in the nucleus in the G_0/G_1 population. However, by the time that the cells had entered S-phase, the vast majority of E2F-4 was detected in the cytoplasm.

These experiments yield several important findings. First, our data suggest that E2F-4, but not E2F-1, -2 or -3, is regulated at the level of subcellular localization. Second, these changes appear to be linked to the state of cell cycle progression. In either G_0 or G_1 cells, a significant proportion of the endogenous E2F-4 is retained in the nucleus but this protein is almost entirely cytoplasmic by S-phase. The dramatic alteration in the relative levels of nuclear to cytoplasmic E2F-4 could be caused by either the translocation of preexisting E2F-4 or the combined effect of the degradation of nuclear E2F-4 and appearance of newly synthesized cytoplasmic protein. Finally, our analysis of the E2F inducible cell lines suggests that the cytoplasmic form(s) of E2F-4 are unable to activate transcription. By extension of this logic, our data suggest that the transcriptional effects of the endogenous E2F-4 are primarily exerted during G_0 and G_1.

C4. The subcellular localization of the endogenous E2F complexes changes through the cell cycle.

The identity and regulation of the endogenous E2F complexes has been analyzed in a wide variety of cell types and growth conditions (Cao et al., 1992; Chittenden et al., 1993; Devoto et al., 1992; Moberg et al., 1996; Shirodkar et al., 1992). However, all of these studies have been conducted using whole cell rather than nuclear extracts. We have previously shown that E2F-4 comprises more than 80% of the endogenous E2F species and makes a major contribution to each of the pRB•E2F, p107•E2F and p130•E2F complexes (Moberg et al., 1996). The localization properties of this protein suggested that a significant proportion of the endogenous E2F complexes exist in the cytoplasm at certain stages of the cell cycle. To address this issue, we used counterflow centrifugal elutriation to generate populations of a human lymphoma cell line, HL60, that were highly enriched in either G_1 (94%), S (78%) or G_2/M (81%) cells. These cells were then fractionated to yield nuclear and cytoplasmic extracts. Initially, we used western blotting to compare the subcellular localization of the E2F-1 and E2F-4 proteins (see Figure 4a). Regardless of the cell cycle staging, the vast majority of the endogenous E2F-1 protein was detected in the nuclear fraction. In contrast, this experiment confirmed that the localization of E2F-4 changed through the cell cycle; this protein was present in both the nucleus and cytoplasm during G_1 but became increasingly cytoplasmic as the proportion of G_1 cells declined. These changes strictly mirrored those detected in our immunofluorescence studies.

To determine the localization of the E2F•DP complexes, we used gel retardation assays to compare the level of E2F DNA binding activity in the cytoplasmic and nuclear fraction of each cell cycle population (Figure 4b). These experiments demonstrated that localization has a profound effect upon the profile of E2F complexes. In the first cell cycle fraction, comprised of 94% G_1 cells, the nucleus contained considerably more E2F activity than the cytoplasm. However, the ratio of cytoplasmic to nuclear E2F activity increased dramatically as the HL60s progressed through the cell cycle. This change arose from two distinct effects: a steady increase in the levels of cytoplasmic E2F activity and a dramatic reduction in the levels of the nuclear E2F complexes.

By the last three elutriation fractions (which were almost free of contaminating G_1 cells) less than 10% of the E2F DNA binding activity was retained in the nucleus. Consistent with our finding that E2F-4 accounts for the majority of the endogenous E2F activity (Moberg et al., 1996), the change in the relative levels of the nuclear and cytoplasmic E2F complexes closely mirrored the changes in the localization of the E2F-4 protein detected by either western blotting or immunofluorescence. This experiment yielded one other critical finding: the cytoplasmic and nuclear E2F complexes migrated with different mobilities. Of the three major E2F complexes, two (labeled A and C) were predominantly cytoplasmic while the third (labeled B) accounted for almost all of the nuclear E2F activity. This strongly suggested that the cytoplasmic and nuclear E2F activities were generated by different E2F species.

C5. The pRB•E2F, p107•E2F and p130•E2F complexes localize preferentially to either the cytoplasm or the nucleus.

The retinoblastoma protein, a known tumor suppressor, and its related proteins, p107 and p130, are thought to play a pivotal role in determining the biological properties of the endogenous E2F complexes (reviewed in Beijersbergen and Bernards, 1996). Although it is widely accepted that these three proteins must regulate E2F in different ways *in vivo*, over-expression assays have failed to reveal any obvious differences in the properties of the pRB•E2F, p107•E2F and p130•E2F complexes. The experiments described above raised the possibility that these complexes are preferentially sequestered in different subcellular compartments *in vivo*. To address this hypothesis, we used specific monoclonal antibodies to identify the components of the nuclear and cytoplasmic complexes in each cell cycle fraction. Our analysis of the peak G_1 and S-phase fractions is shown in Figure 5.

Initially, we focused our attention on identifying the E2F species that localized to the nucleus and therefore likely participate in the transcriptional regulation of E2F-responsive genes. In G_1 cells most, if not all, of the nuclear activity arose from a single complex, Complex B (Figure 4b and 5). The supershift experiments revealed that this band comprised a mixture of E2F

species, of which E2F-4 was the most prevalent (Figure 5, nuclear G_1 phase). These nuclear, G_1 complexes also contained an associated regulatory protein. Although p107 accounted for a small subset of these nuclear E2F species, the vast majority (>90%) contained the retinoblastoma protein. As described above, the level of nuclear E2F DNA binding activity dropped dramatically as cells entered S-phase. Even though present at low level, it was important that we identify these nuclear species. To achieve this goal, we used five fold higher levels of the S-phase, nuclear extract in the gel retardation assays (Figure 5, nuclear S-phase). Under these conditions, we were able to demonstrate that the constituent E2F activity was generated by three distinct E2F complexes. First, these S-phase cells contained a low level of the pRB•E2F complex (complex B). The reduction in the level of this species (relative to G_1 cells) is entirely consistent with the known dissociation of the pRB•E2F complex at the G_1/S transition (reviewed in Beijersbergen and Bernards, 1996). Whether the remaining pRB•E2F complexes are a true component of S-phase cells or whether they are derived from the low level of contaminating G_1 cells is unclear. The other two species, labeled A and C, were both identified as E2F-4 complexes. This is consistent with our previous finding that the nucleus contains low levels of E2F-4 protein at this stage of the cell cycle (Figure 4a). Further analysis identified these species as free E2F-4 (complex A) and p107•cyclin A•E2F-4 (complex C; Figure 5).

Having characterized the nuclear E2F activity, we turned our attention to the cytoplasmic complexes. Regardless of the cell cycle stage, we found that E2F-4 accounted for all of the cytoplasmic activity (Figure 5). The G_1 cells contained high levels of three different E2F-4 complexes, A, C and C'. Consistent with our analysis of the nuclear E2F activity, the A and C species were identified as free E2F-4 and p107•E2F-4 respectively (Figure 5, cytoplasmic G_1 phase). The remaining complex, complex C', was specifically recognized by antibodies against p130. Although pRB•E2F was present at high levels in the nucleus of G_1 cells, this complex was absent from the cytoplasmic fraction. Upon S-phase entry, we detected changes in the cytoplasmic complexes that were consistent with the known cell cycle regulation of p130•E2F and p107•E2F. Specifically, p130•E2F-4 disappeared while the level of p107•E2F-4 increased steadily. Notably,

this p107 complex was enriched in cytoplasmic fractions and now included the S-phase kinase, cyclin A•cdk2, whose subcellular localization has been shown to be cell cycle-regulated (Pines and Hunter, 1991). Finally, the level of cytoplasmic, free E2F-4 also increased considerably in cells that had entered S-phase.

In summary, our analysis of the cell cycle fractions confirms that the endogenous E2F complexes are regulated at the level of subcellular localization. Most importantly, the individual E2F species were found to be preferentially located in either the cytoplasm or the nucleus. The vast majority of the nuclear E2F activity is generated by a single species, the pRB•E2F complex, that is present at high levels in the G_1 population In contrast, the vast majority of the remaining E2F complexes, p130•E2F, p107•E2F and free E2F are predominantly located in the cytoplasm. Consistent with previous studies, the p130•E2F complex is only detected during the early stage of the cell cycle. In contrast, the level of both the p107•E2F and free E2F species increases as cells progress through the cell cycle and this results in a steady increase in the level of cytoplasmic E2F activity. In each case, the cytoplasmic localization of these three E2F species correlates with the presence of E2F-4 within the complex. This directly supports our conclusion that cytoplasmic localization is a particular property of E2F-4 and not E2F-1, -2 or -3. However, our data also indicate that E2F-4 is not sufficient to ensure cytoplasmic localization, since this E2F family member is also the major E2F component of the nuclear, pRB•E2F complex.

C6. E2F-1, -2 and -3 contain a nuclear localization signal that is absent in E2F-4.

Our data indicate that a significant proportion of the endogenous E2F species are localized in the cytoplasm and not the nucleus. This observation suggested that subcellular localization could have a profound influence upon the biological properties of the individual E2F complexes. To address this issue, we need to identify the molecular mechanism(s) that control this process. Our previous experiments suggest that this may be determined by two distinct factors. First, localization of the E2F complexes appears to be partially dependent upon the localization properties of the constituent E2F proteins. Complexes containing E2F-1, -2 or -3 are exclusively nuclear,

while the cytoplasmic localization of the endogenous E2F complexes seems to be dependent upon the presence of E2F-4. Second, although monomeric E2F-4 is predominantly cytoplasmic, this E2F family member participates in both cytoplasmic and nuclear E2F complexes *in vivo*. This suggests that the localization of the E2F-4 may be altered by the presence of one or more of its associated proteins.

Our first goal was to identify the signal(s) that establish the localization of the monomeric (i.e. non-DP associated) E2F proteins. To address this issue, we generated a series of chimeras in which one or more domains (the N-terminal, DNA binding, dimerization/transactivation or pRB/p107/p130-binding) were exchanged between these proteins (see Figure 6). The resultant mutants were named to indicate the origin of each domain; for example, 2224 contains the N-terminal, DNA binding and dimerization/transactivation domains of E2F-2 and the pRB/p107/p130-binding domain of E2F-4. The localization properties of each chimera was determined by indirect immunofluorescence after transient transfection into U2OS cells (Figure 6). Consistent with our micro-injection studies, the transiently transfected E2F-2 was predominantly nuclear while the exogenous expressed E2F-4 preferentially localized to the cytoplasm. We next examined the localization of mutants in which various functional domains of E2F-2 had been replaced with the corresponding region of E2F-4 (2224, 2242, 2244 and 2444). In each case, these mutants localized to the nucleus with similar or greater efficiency than the parental E2F-2 protein (Figure 6). In fact, it was possible to exchange all of the E2F-2 sequences from the start of the DNA binding domain to the end of the protein (amino acids 118 - 437) with the corresponding region of E2F-4 (amino acids 2 - 416) without impairing nuclear import. We therefore conclude that E2F-2 contains a nuclear localization signal (NLS) within its N-terminal domain that, when fused to E2F-4, can induce this normally cytoplasmic protein to enter the nucleus.

To precisely map the E2F-2 nuclear localization signal, we examined the localization properties of N-terminal deletion mutants (Figure 6). Deletion of the first 83 amino acids of E2F-2 did not affect its nuclear localization. However, deletion of an additional 5 amino acids (to generate F2Δ88) caused the protein to shift from being predominantly nuclear (75% of cells) to

being predominantly cytoplasmic (69% of cells) in a similar manner to E2F-4. Deletion of additional N-terminal sequences (F2Δ117) did not further increase cytoplasmic localization. We therefore concluded that E2F-2 contains a single NLS that encompasses residues 83 and 88. This region encompasses a short motif, PAKRKLDL (residues 84-91), that is closely related to the nuclear localization signal of the c-myc protein (Dang et al., 1988). Moreover, this sequence is highly conserved in the other nuclear E2F proteins, E2F-1 (PVKRRLDL) and E2F-3 (PAKRRLEL), and represents the only region of homology in the N-terminal domain of these three E2F family members. To directly demonstrate the importance of this domain, we used site directed mutagenesis to alter the basic residues within this motif. When tested in the transient transfection assay, the resulting mutant (named F2ΔNLS) localized to the cytoplasm in a similar manner to either F2Δ88 or E2F-4 (Figure 6). We therefore conclude that the nuclear localization of E2F-1, E2F-2 and E2F-3 is mediated by the P(A/V)KR(K/R)L(D/E)L motif.

In addition to the mutants shown in Figure 6, we have tested the localization properties of several other chimeric and deletion mutants (data not shown). The localization properties of these mutants did not yield any evidence for the existence of a nuclear export signal (NES) within E2F-4. Although we cannot rule out the possibility that E2F-4 contains a weak NES, our data argues that the predominant cytoplasmic localization of this protein results from the lack of a nuclear localization signal. This conclusion is supported by the finding that E2F-2 mutants that lack the NLS (F2Δ88, F2Δ117, F2ΔNLS) localize to the cytoplasm with a similar efficiency to E2F-4.

C7. Associated proteins can mediate the nuclear localization of E2F-4.

Our data suggest that monomeric E2F-4 is unable to enter the nucleus because it lacks a nuclear localization signal. However, our analysis of the cellular E2F complexes indicates that E2F-4 participates in both cytoplasmic and nuclear E2F complexes *in vivo* (Figure 5). What mediates the localization of these nuclear E2F-4 complexes? One likely possibility is associated proteins. Indeed, our analysis of the endogenous E2F complexes revealed a clear difference in the localization of the E2F-4 complexes that were associated with p107 or p130 (predominantly

cytoplasmic) rather than pRB (exclusively nuclear; Figure 5). To determine whether any of the known associated proteins could influence E2F-4 localization, we used micro-injection assays to compare the localization of E2F-4 that had been expressed in either the absence or presence of these proteins (Figure 7). Since functional E2F activity requires the formation of an E2F•DP heterodimer, we initiated this study by examining the effect of the known E2F heterodimeric partners, DP-1 and DP-2. The monomeric DP-1 protein was found to be predominantly cytoplasmic and this protein caused little or no change in the nuclear uptake of the co-expressed E2F-4 protein. In contrast, E2F-4 became almost exclusively nuclear when co-expressed with DP-2 and this localization clearly reflects the nuclear localization properties of the monomeric DP-2 protein. We therefore conclude that the association of DP-2, but not DP-1, is sufficient to trigger the nuclear localization of E2F-4.

Since our *in vivo* studies indicate that the association of pRB, but not p107 or p130, also correlates with the nuclear localization of E2F-4, we also investigated whether the retinoblastoma protein was able to induce nuclear uptake of the cytoplasmic E2F-4•DP-1 complex (Figure 7). When co-expressed in the micro-injection assays, the retinoblastoma protein had no effect upon the localization of either the E2F-4•DP-1 (predominantly cytoplasmic) or the E2F-4•DP-2 (nuclear) species. Although we cannot rule out the possibility that the retinoblastoma protein will play some role in determining the localization of E2F-4 *in vivo*, these experiments indicate that it is not sufficient to induce the nuclear uptake of this E2F family member. In contrast, our data suggest that the differential localization properties of the two DP proteins could provide the underlying basis for the differential localization of the individual E2F-4 complexes.

C8. Nuclear localization of the individual E2F proteins correlates with their ability to activate transcription.

The transcriptional role of the individual E2F species has been the focus of extensive study. Over-expression experiments suggest that the free E2F•DP complexes each induce the activation of E2F-responsive genes while complexes containing either pRB, p107 or p130 repress their

transcription. In this study, we have shown that a significant proportion of these complexes are localized in the cytoplasm and not the nucleus *in vivo*. This observation raised clear questions about how these species' subcellular localization effects their ability to regulate transcription. To directly address this issue, we have examined the functional properties of various nuclear or cytoplasmic versions of the E2F-2 and E2F-4 proteins. Initially, we wanted to verify that the construction of these mutants did not disrupt their ability to dimerize with DP and/or bind to DNA. To this end, the relevant chimeric and deletion mutants were transiently transfected into C-33A cells, along with CMV-DP1, and whole cell extracts were then screened in gel retardation assays (Figure 8a). In each case, we recovered a significant proportion of E2F DNA binding activity confirming the structural integrity of these proteins. We then examined the transcriptional activity of these mutants by transiently transfecting their eukaryotic expression vectors into C-33A cells along with the E2F reporter plasmid, $E2F_4$-CAT, and an internal control for transfection efficiency, pRSV-LUC (Figure 8b). That each of these proteins were expressed at similar levels was confirmed by western blotting (data not shown). Consistent with our analysis of the E2F-inducible cell lines (Figure 1), E2F-2 activated the transcription of the reporter with a much greater efficiency than E2F-4. However, the deletion or mutation of the E2F-2 nuclear localization signal (mutants F2Δ88 and F2ΔNLS) significantly reduced its transcriptional activity. In contrast, the transcriptional activity of E2F-4 was significantly increased when this protein was fused in frame to either the N-terminal domain of E2F-2 (mutant 2444) or the nuclear localization signal of the SV40 large T antigen (mutant F4+NLS). In fact, the transcriptional activity of the latter mutant significantly exceeded that of the wild-type E2F-2 protein.

In every case, the transcriptional activity of the deletion and chimeric proteins correlated with their localization properties rather than the origin (either E2F-2 or E2F-4) of their transactivation domain. This suggests that the mutants each have a similar capacity to activate transcription but this is restricted by their ability to localize to the nucleus. If this hypothesis is true, we would predict that the association of DP-2, which was sufficient to induce the nuclear uptake of E2F-4 (Figure 7), should active the transcriptional potential of the cytoplasmic mutants.

To test this idea, we compared the transcriptional activity of each mutant after co-transfection with DP-2. The presence of DP-2 was sufficient to mediate the nuclear localization of all of the mutants (data not shown). Under these conditions, the chimeric and deletion mutants activated transcription with similar efficiency (Figure 8b). We therefore conclude that E2F-2 and E2F-4 have a similar potential to activate transcription but these activities are dependent upon their subcellular localization.

D. Discussion

The cellular transcription factor E2F plays a critical role in directing the cell cycle dependent transcription of the genes that control cellular proliferation. It is well established that E2F activity arises from the combined properties of multiple E2F•DP heterodimers. However, despite extensive study, the precise role of the individual E2F•DP species is not well understood. We have focused our attention on one member of the E2F family, E2F-4, for the following reasons. First, this E2F protein accounts for the vast majority of the endogenous E2F complexes, including most of pRB-, p107- or p130-associated E2F activity (Ikeda et al., 1996; Moberg et al., 1996). This observation suggests that E2F-4 plays a pivotal role in establishing the properties of the endogenous E2F activity. Second, free E2F-4•DP complexes accumulate early in the cell cycle but they are unable to induce the transcription of known E2F responsive genes (Moberg et al., 1996). This finding led us to propose two possible, although not mutually exclusive, models of E2F-4 action; either the transcriptional activity of E2F-4 is directed at an unknown set of target genes or it is regulated by a previously unknown mechanism.

To distinguish between these two models, we generated a stable cell line that showed inducible E2F-4 DNA binding activity but, surprisingly, did not show inducible E2F transcriptional activity. Although this prevented us from addressing whether or not E2F-4 activates a novel set of target genes *in vivo*, this observation led us to the finding that monomeric E2F-4 is sequestered in the cytoplasm. The transcriptional activity of this protein was rescued by the induction of its nuclear uptake. Moreover, under these conditions E2F-4 can activate transcription with similar efficiency to the other E2F family members. Taken together, these data lead us to conclude that E2F-4 may activate transcription *in vivo* but, in contrast to E2F-1, -2 and -3, this is dependent upon an additional step that mediates the nuclear localization of this protein.

These findings suggest the need to re-evaluate previous studies concerning the biological properties of the individual E2F proteins. Over-expression studies have revealed several differences in both the transcriptional and cell cycle effects of E2F-4 versus those of E2F-1, -2 and -3 (Dimri et al., 1996; Hofmann et al., 1996; Lukas et al., 1996). For example, Lukas et al. (1996)

have shown that micro-injection of either E2F-1, -2 or -3 is sufficient to induce quiescent cells to initiate DNA synthesis whereas E2F-4 is unable to trigger these events. That study and another (Dimri et al., 1996) further showed that over-expression of E2F-1 but not E2F-4 can override the G_1-arrest induced by either p16 or p21. Our data suggest that these biological differences arise as a direct consequence of the differential localization properties of these over-expressed proteins. In addition, our data also affect the interpretation of E2F-1 mutants. Previous studies have identified a short motif within the N-terminus of E2F-1, -2 and -3 that mediates their cyclin A•cdk2 binding properties (Adams et al., 1996; Krek et al., 1994). This kinase is thought to ensure the S-phase specific inactivation of the E2F-1•DP, E2F-2•DP and E2F-3•DP species through its interaction with the E2F subunit and phosphorylation of the associated DP protein (Dynlacht et al., 1994; Dynlacht et al., 1997; Krek et al., 1995; Xu et al., 1994). Interestingly, our mapping studies indicate that this domain is also responsible for mediating the nuclear localization of these three E2Fs. At this point, the biological consequences of the co-localization of the cyclin A binding and nuclear localization functions are unclear. However, it will be important to re-evaluate studies that have used deletion mutagenesis to assess the role of cyclin A binding in E2F regulation (for example see Krek et al., 1995), given our finding that these mutations also disrupt the nuclear localization of the E2F proteins.

D1. Cell cycle regulation of the endogenous E2F-4 localization.

The localization properties of E2F-4 are only relevant if they extend to the endogenous protein. Both subcellular fractionation and immunofluorescence confirmed that a significant proportion of the endogenous E2F-4 protein is localized in the cytoplasm. Moreover, the relative levels of nuclear to cytoplasmic E2F-4 protein alter dramatically as cells progress through the cell cycle. While cytoplasmic E2F-4 exists throughout the cell cycle, nuclear E2F-4 protein is primarily detected during G_0 and G_1. These observations strongly suggest that the regulation of E2F-4 subcellular localization plays a critical role in controlling the activity of the endogenous E2F-4 complexes.

Our previous studies have shown that E2F-4 accounts for a large proportion of the endogenous E2F complexes, including the majority of pRB-, p107- or p130-associated E2F activity (Moberg et al., 1996). This result raised the possibility that a significant proportion of these complexes might localize to the cytoplasm at one or more stages of the cell cycle. We addressed this issue by examining the localization of the individual E2F complexes in actively dividing cells (Figure 9). As predicted by our overexpression experiments, the endogenous free E2F-4 complexes were found to be predominantly cytoplasmic. Surprisingly, we did not detect any obvious redistribution of free E2F-4 from the cytoplasm to the nucleus in any of the cell cycle fractions. This observation suggests that we can rule out a model in which the activation of E2F-responsive genes is triggered by the wholesale nuclear import of free E2F-4.

The p107•E2F and p130•E2F complexes, which consist primarily of E2F-4 are also predominantly cytoplasmic in actively dividing cells. As with free E2F-4, we did not detect any obvious change in the ratio of nuclear to cytoplasmic forms of these species at any particular stage of the cell cycle. This finding seems at odds with the recent report that coexpression with p107 or p130 can increase the nuclear uptake of E2F-4 in transient transfection assays (Lindeman et al., 1997). We have conducted similar experiments and, under these overexpression conditions, have also found that p107 and p130 can trigger the nuclear localization of coexpressed E2F-4 (unpublished data). Given the difference between transient transfection and *in vivo* assays, we assume that the overexpression of these proteins must somehow perturb the mechanism(s) that establishes their subcellular localization. The analysis of this difference may provide the key to understanding how the localization of endogenous E2F complexes is regulated. As with the E2F proteins, these observations suggest that we need to be cautious in interpreting the results of experiments performed with overexpressed p107 or p130 protein.

Consistent with the role of E2F as a cellular transcription factor, some of the endogenous E2F complexes were detected in the nucleus. However, supershift experiments indicated most of the nuclear E2F activity was generated by a single E2F species, the pRB•E2F complex. In contrast to the other E2F complexes, pRB•E2F was found to be exclusively nuclear despite the

fact that E2F-4 was its major component. The nuclear pRB•E2F complex was present at high levels during G_1 but not in S-phase cells. The disappearance of this species correlates closely with the known timing of phosphorylation of pRB and is sufficient to account for the reduction in nuclear E2F-4 protein at later stages of the cell cycle.

Our finding that the transcriptional potential of a given E2F complex is dependent upon its nuclear localization strongly suggests that the endogenous E2F-responsive genes are regulated by the subset of E2F species that can localize to the nucleus. We therefore conclude that the transcriptional regulation of E2F-responsive genes in actively dividing cells is largely dependent upon the properties of the pRB•E2F complex. Overexpression studies indicate that the pRB•E2F complex mediates the basal repression of E2F responsive genes through sequestration of other transcription factors that are bound at the promoter (Adnane et al., 1995; Bremner et al., 1995; Sellers et al., 1995; Starostik et al., 1996; Weintraub et al., 1992; Weintraub et al., 1995). Therefore, the current model of E2F action suggests that dissociation of the pRB•E2F complex leads to the induction of target genes by both relieving basal repression and releasing free, transcriptionally active E2F. Our observation that the pRB•E2F complex is present in the nucleus at high levels during G_1 strongly supports the notion that the dissociation of the pRB•E2F complex contributes to the induction of E2F-responsive genes by relieving their repression. However, although we did detect low levels of free E2F activity in the nuclei of S-phase cells, the dissociation of the pRB•E2F complex did not lead to a commensurate increase in the levels of free, nuclear E2F activity. Moreover, supershift experiments indicate that free E2F-4 is selectively lost from the nuclear compartment after release from pRB. It is unclear whether the this free E2F-4 is translocated to the cytoplasm or is subject to ubiquitin-mediated proteolysis (Hofmann et al., 1996; Hateboer et al., 1996). Whatever the mechanism, dissociation of the high levels of nuclear pRB•E2F complex results in surprisingly little nuclear free E2F activity. The reduction in the level of nuclear E2F activity is consistent with *in vivo* footprinting studies which demonstrate that the E2F-responsive elements of known target genes are only occupied during G_0/G_1 (Zwicker et al., 1996). We assume that the low levels of nuclear, free E2F species contribute to the activation of

E2F responsive genes. However, our data suggest that we need to reconsider the relative contributions that pRB•E2F repression and free E2F activation make to the transcriptional regulation of E2F-responsive genes.

D2. The pRB•E2F, p107•E2F and p130•E2F complexes are preferentially sequestered in different subcellular compartments.

Analysis of both human tumors and mutant mouse strains suggests that pRB plays a critical role in the regulation of cellular proliferation that cannot be fulfilled by either p107 and/or p130. Our investigation of the endogenous E2F activity has led to the unexpected finding that the p107•E2F and p130•E2F species are preferentially localized in a different subcellular compartment from the pRB•E2F complex. As discussed above, the nuclear localization of the pRB•E2F complex suggests that it will play a major role in repressing the transcription of E2F responsive genes prior to the G_1/S transition. In contrast, it seems likely that the cytoplasmic retention of the p130•E2F and p107•E2F species significantly reduces the ability of these complexes to repress transcription. These data raise the possibility that the different localization properties of these species contribute to the distinct biological consequences of pRB, p107 and p130 action.

At this point, we have restricted our analysis of E2F complexes to actively dividing cells. The resultant findings raise questions about the role of E2F in quiescent cells. It has been shown that p130•E2F is the sole E2F species in G_0 cells, and it was therefore assumed that this complex would mediate the repression of E2F-responsive genes in this setting (Chittenden et al., 1993; Moberg et al., 1996; Smith et al., 1996). Given the findings of this study, it will be important to determine the localization of p130•E2F in quiescent cells. Similarly, when these cells are stimulated to reenter the cell cycle, the pRB•E2F complex accumulates at high levels during S, G_2, and M (Moberg et al., 1996; Smith et al., 1996). If nuclear, this complex may contribute to the down-regulation of E2F-responsive genes after the G_1-S transition. Alternatively, it is possible that localization is regulated by cell cycle staging rather than being an intrinsic property of the individual E2F complexes and that the pRB•E2F complex will be found to be cytoplasmic when

cells reenter the cell cycle from G_0. Further analysis of quiescent cells should lead to a better understanding of both the mechanism and biological consequences of the subcellular localization of the individual E2F complexes.

In addition to E2F, pRB, p107 and p130 have been reported to regulate many other transcription factors (reviewed in Beijersbergen and Bernards, 1996). Clearly our data only address the localization of the pRB•E2F, p107•E2F, and p130•E2F species. Consistent with our findings, immunofluorescence studies have confirmed that the endogenous pRB protein localizes to the nucleus (Mittnacht et al., 1994). In contrast, we are unaware of any analysis of the localization of the endogenous p107 and p130 proteins. It is therefore unclear whether significant proportions of these proteins are localized in the cytoplasm *in vivo* or whether this property is specific to the pool of p107 and p130 that is bound to E2F. A comparison of the relative levels and localization of E2F-associated versus total p107 and p130 proteins may provide critical information about the relative importance of E2F in either p107 or p130 function.

D3. Potential mechanisms of E2F-4 localization.

There are two mechanisms that could account for the cytoplasmic localization of the E2F-4 protein, the lack of a nuclear localization signal (NLS) or the presence of a nuclear export signal (NES). Although we cannot rule out the possibility that E2F-4 contains a weak NES, our analysis of both E2F-4 deletion mutants and chimeric proteins suggest that the predominant cytoplasmic localization of this protein results from its lack of an NLS. In contrast, this deletion strategy identified a short motif, P(A/V)KR(K/R)L(D/E)L(D/E), that is both necessary and sufficient to mediate the nuclear localization of E2F-1, -2 and -3. The presence or absence of these motifs explains the localization properties of the monomeric E2F proteins. However, in some situations, most noticeably when pRB is associated with the complex, E2F-4 complexes are able to enter the nucleus. How this occurs is unclear, but associated proteins almost certainly play a role in this process. Our preliminary studies suggest that the association of the retinoblastoma protein is unable to induce the nuclear uptake of E2F-4. Instead, our experiments implicate the DP proteins

in the control of E2F-4 localization. While interaction with DP-1 does not significantly alter the localization of E2F-4, DP-2 binding is sufficient to drive E2F-4 into the nucleus. The monomeric DP proteins also localize to different compartments of the cell and this correlates with the presence of a stretch of basic residues within DP-2 that is absent in DP-1. These observations suggest that the DP proteins will act to establish the localization of the associated E2F-4 complex. Future experiments will focus on comparing the DP components of the nuclear and cytoplasmic E2F-4 complexes.

Figure 1A.

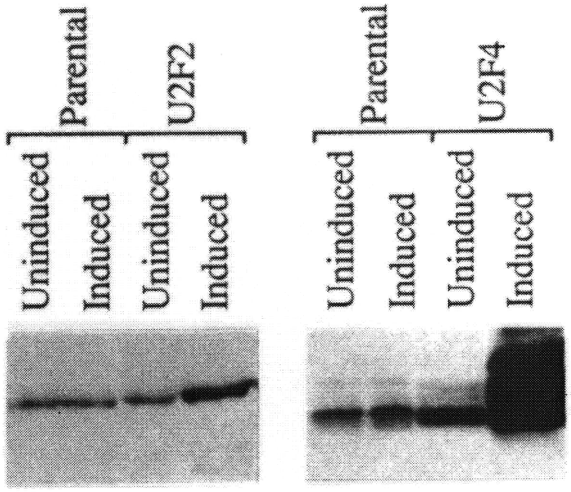


Figure 1B.

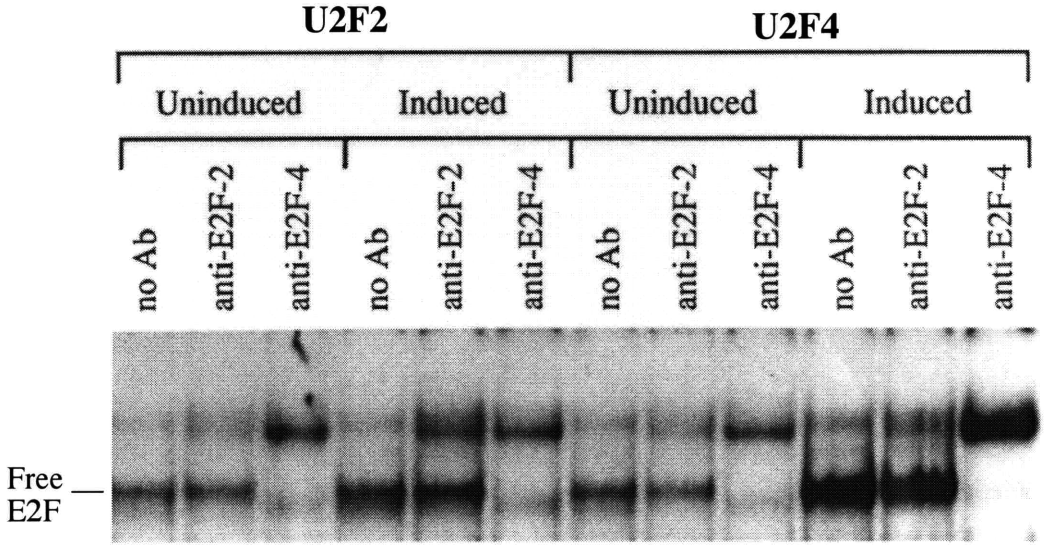


Figure 1C.

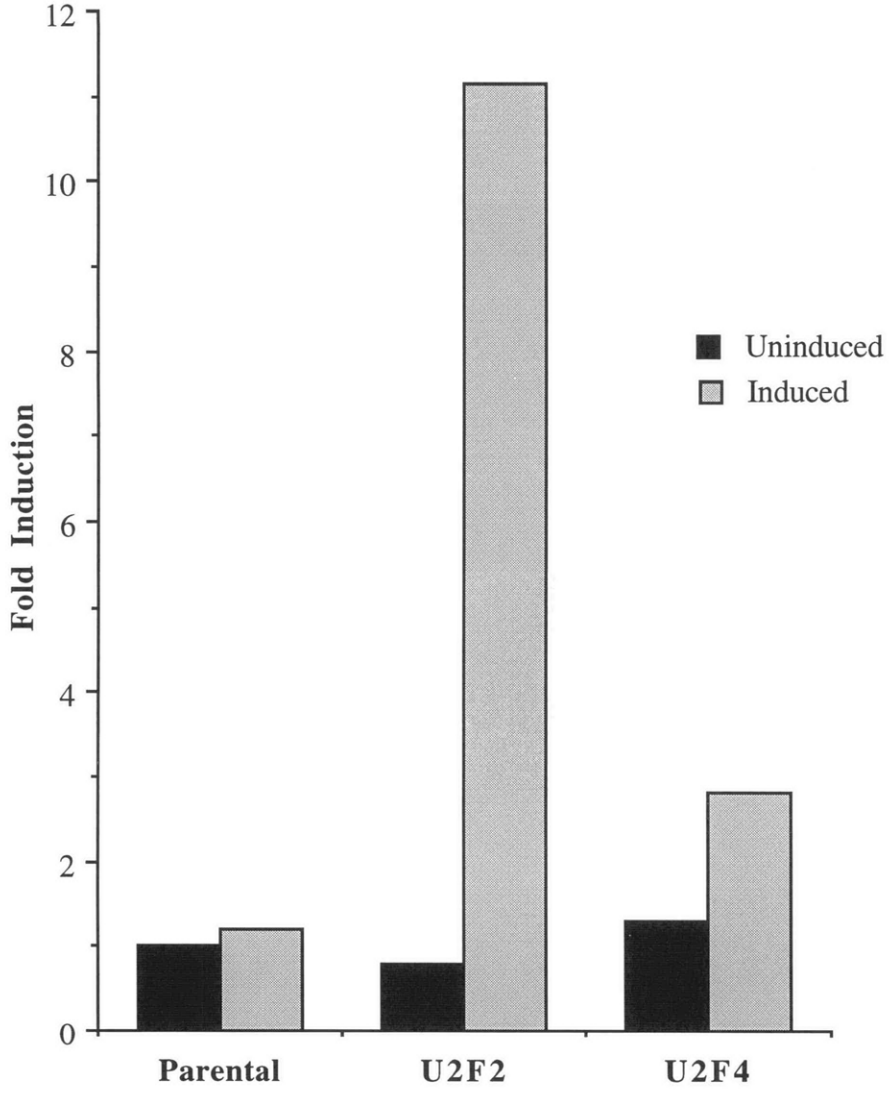

12
10
8
6
4
2
0
Fold Induction
Uninduced
Induced
Parental
U2F2
U2F4

Figure 2A.

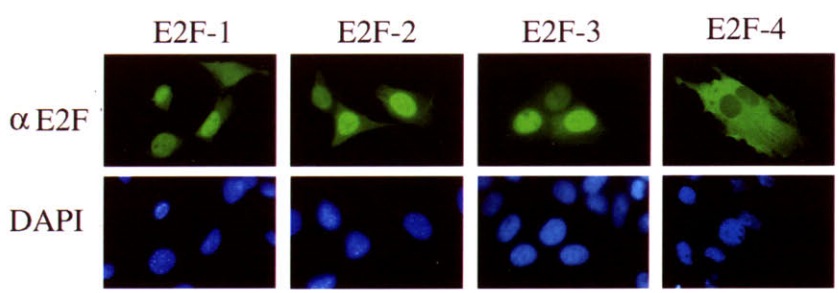


Figure 2B.

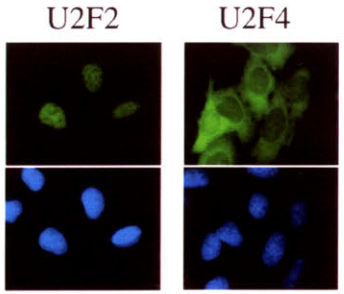


Figure 3A.

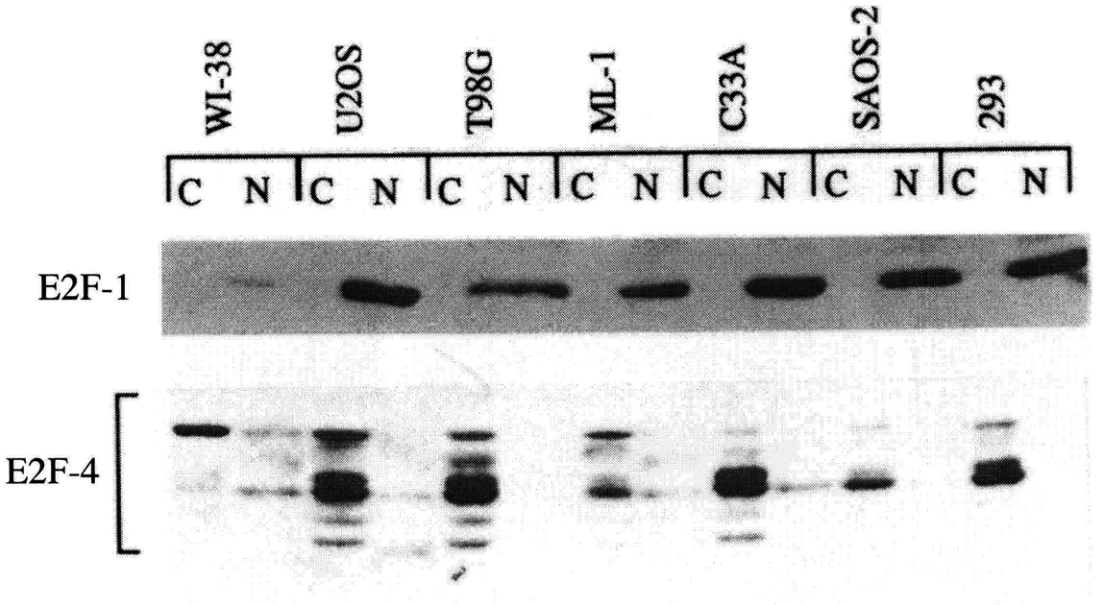


Figure 3.

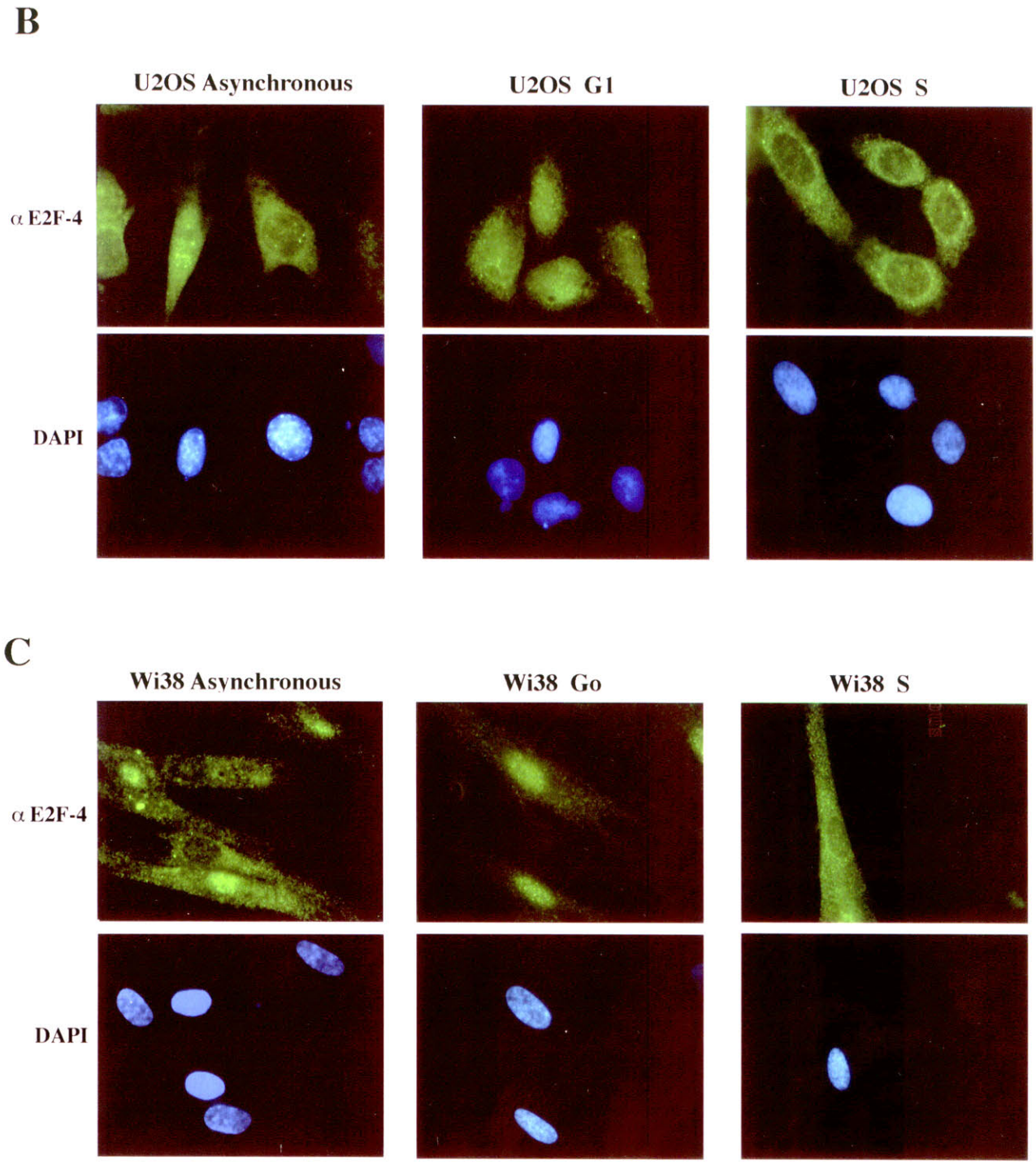

B
U2OS Asynchronous
U2OS G1
U2OS S
α E2F-4
DAPI
C
Wi38 Asynchronous
Wi38 Go
Wi38 S
α E2F-4
DAPI

Figure 4A.

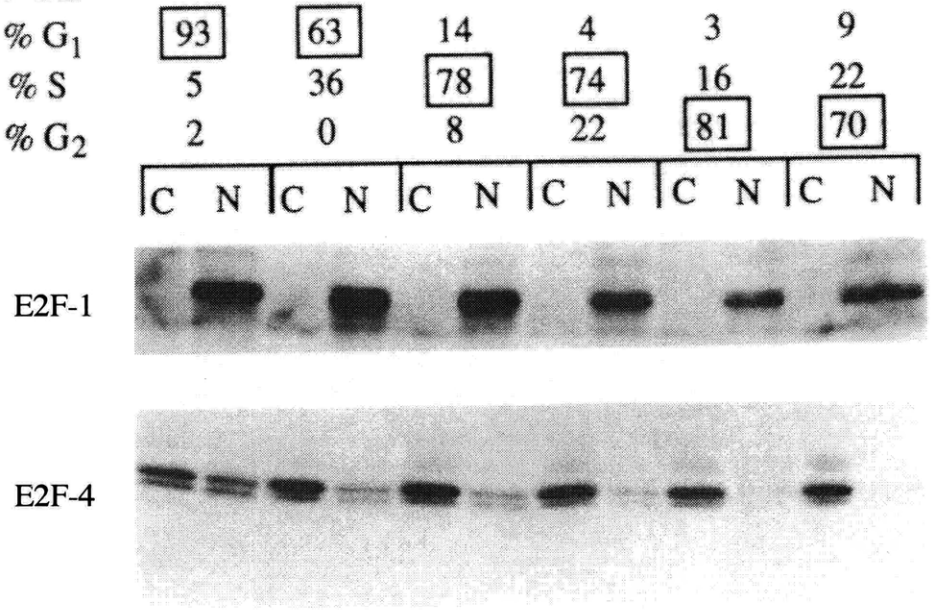


Figure 4B.

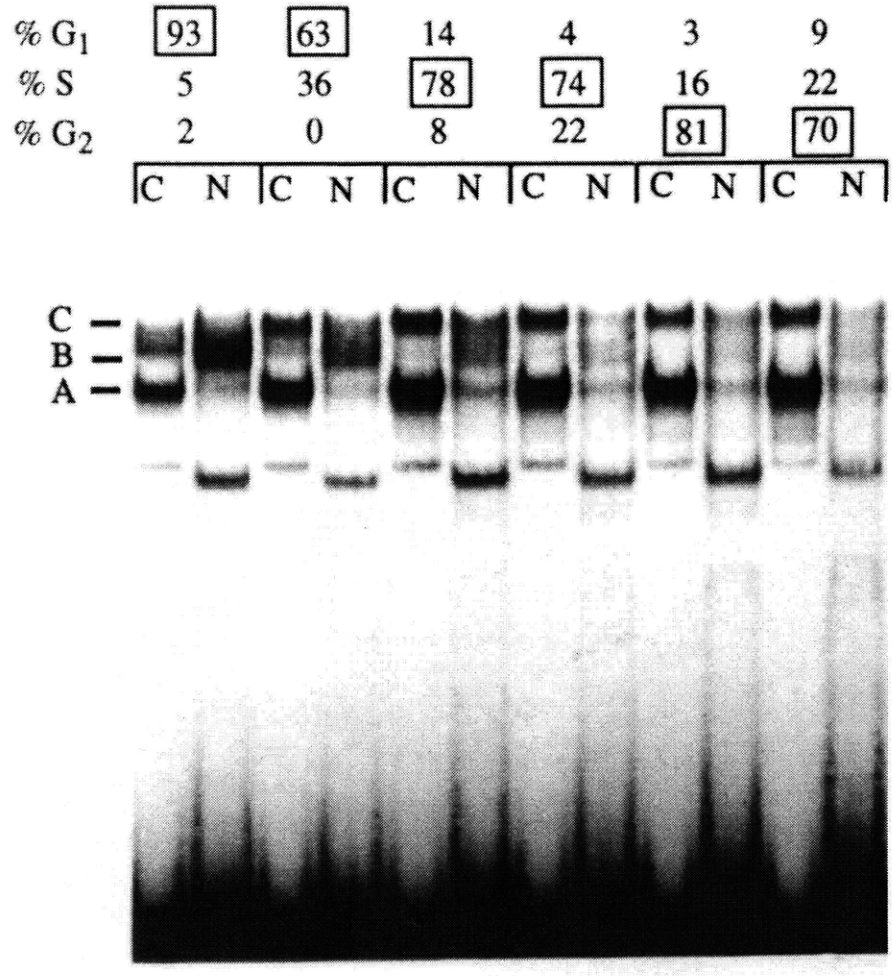


Figure 5.

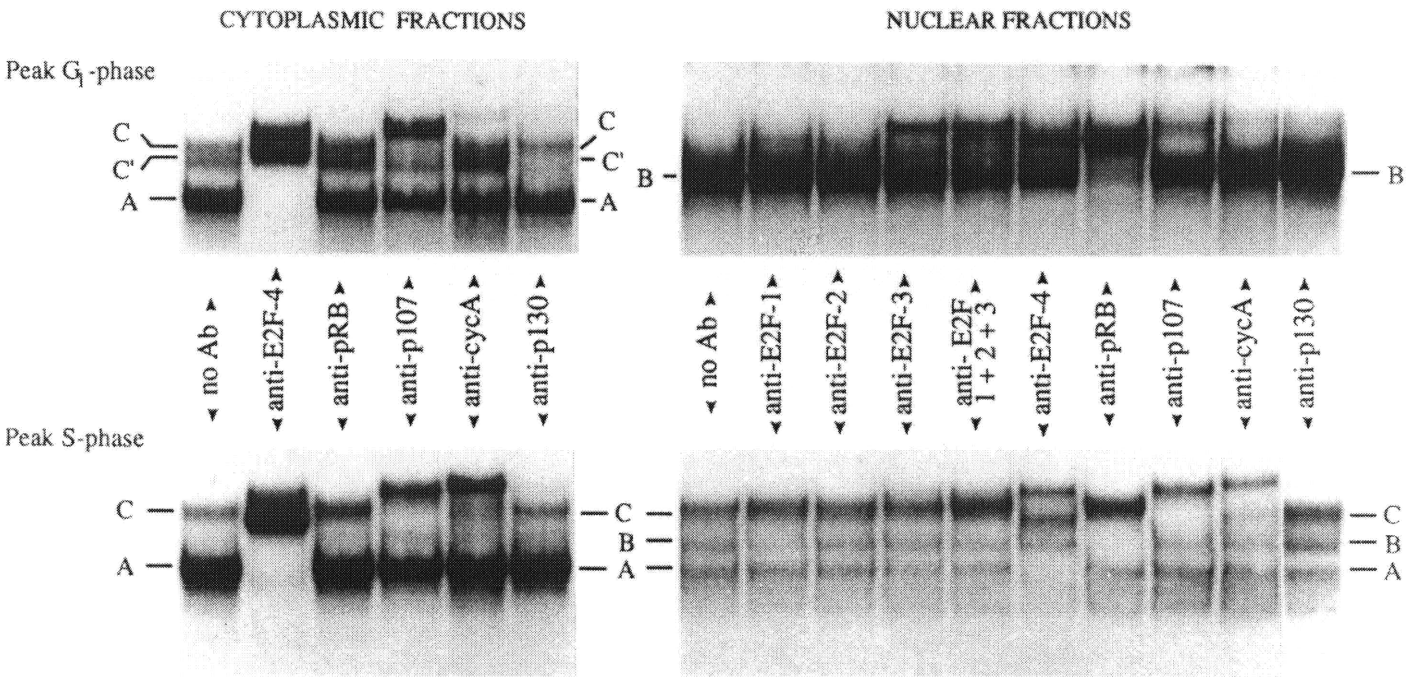

CYTOPLASMIC FRACTIONS
NUCLEAR FRACTIONS
Peak G1-phase
C
C'
A
B
no Ab
anti-E2F-4
anti-pRB
anti-p107
anti-cycA
anti-p130
anti-E2F-1
anti-E2F-2
anti-E2F-3
anti- E2F 1 + 2 + 3
anti-E2F-4
Peak S-phase

Figure 6.

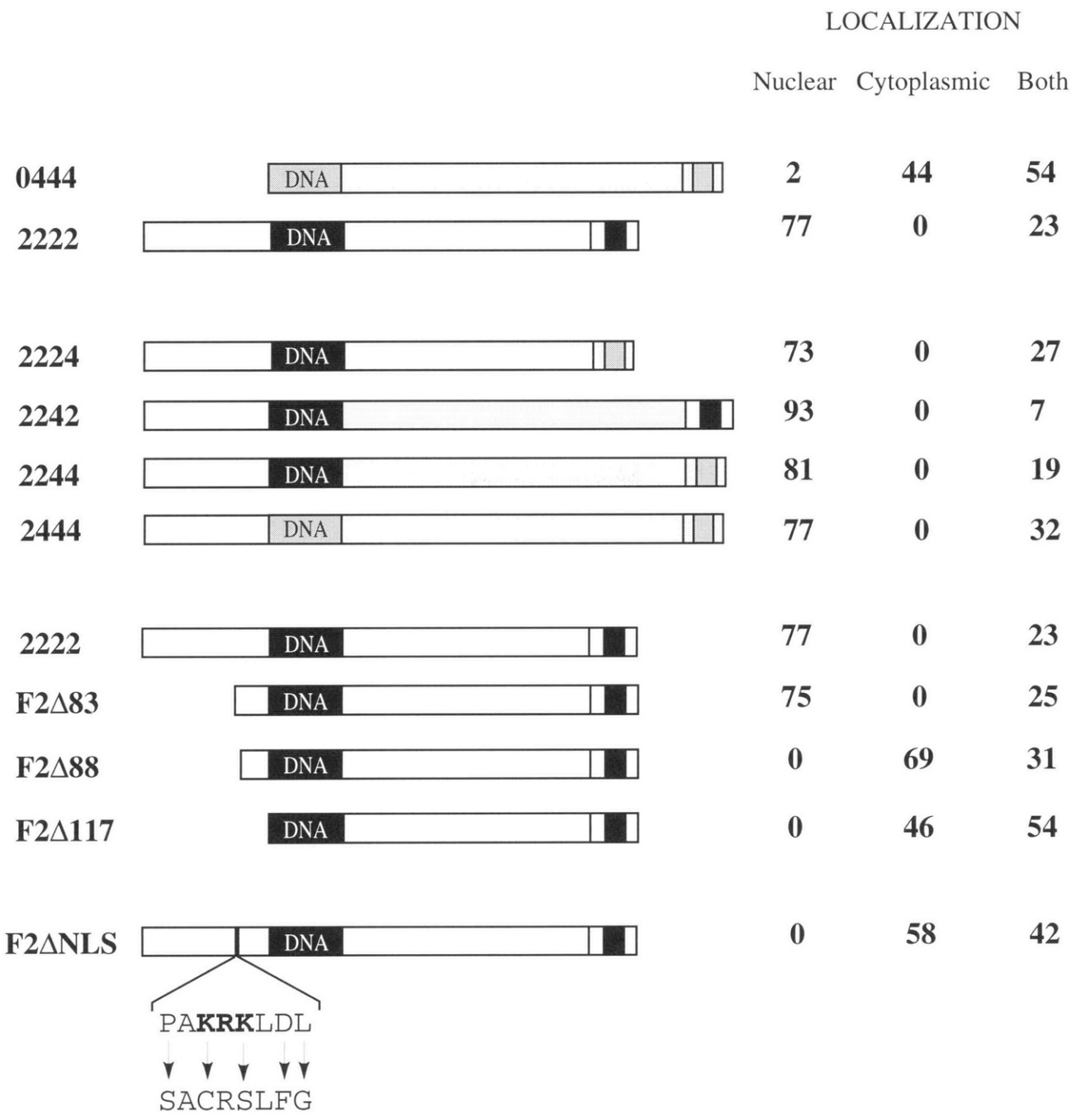

LOCALIZATION
Nuclear Cytoplasmic Both
0444
DNA
2 44 54
2222
DNA
77 0 23
2224
DNA
73 0 27
2242
DNA
93 0 7
2244
DNA
81 0 19
2444
DNA
77 0 32
2222
DNA
77 0 23
F2Δ83
DNA
75 0 25
F2Δ88
DNA
0 69 31
F2Δ117
DNA
0 46 54
F2ΔNLS
DNA
0 58 42
PAKRKLDL
SACRSLFG

Figure 7.

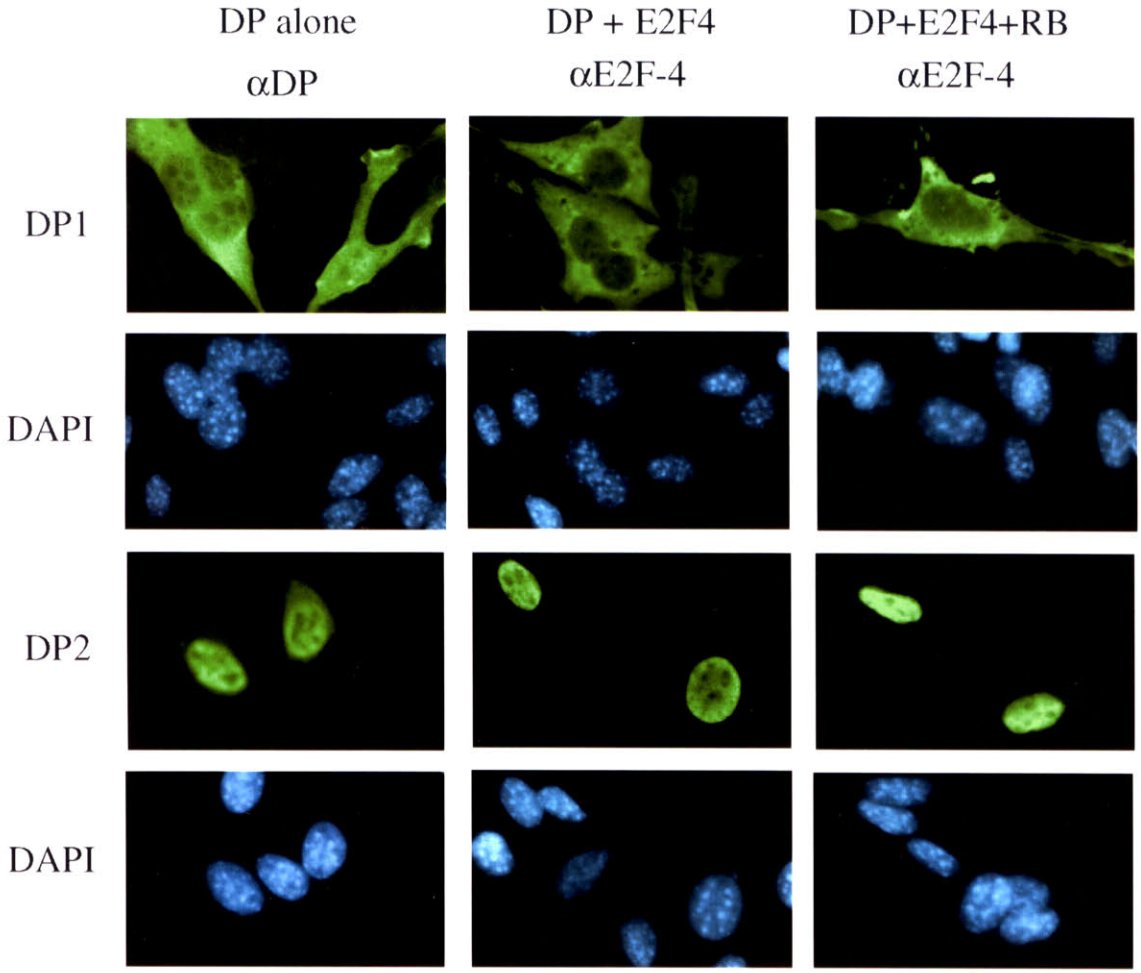
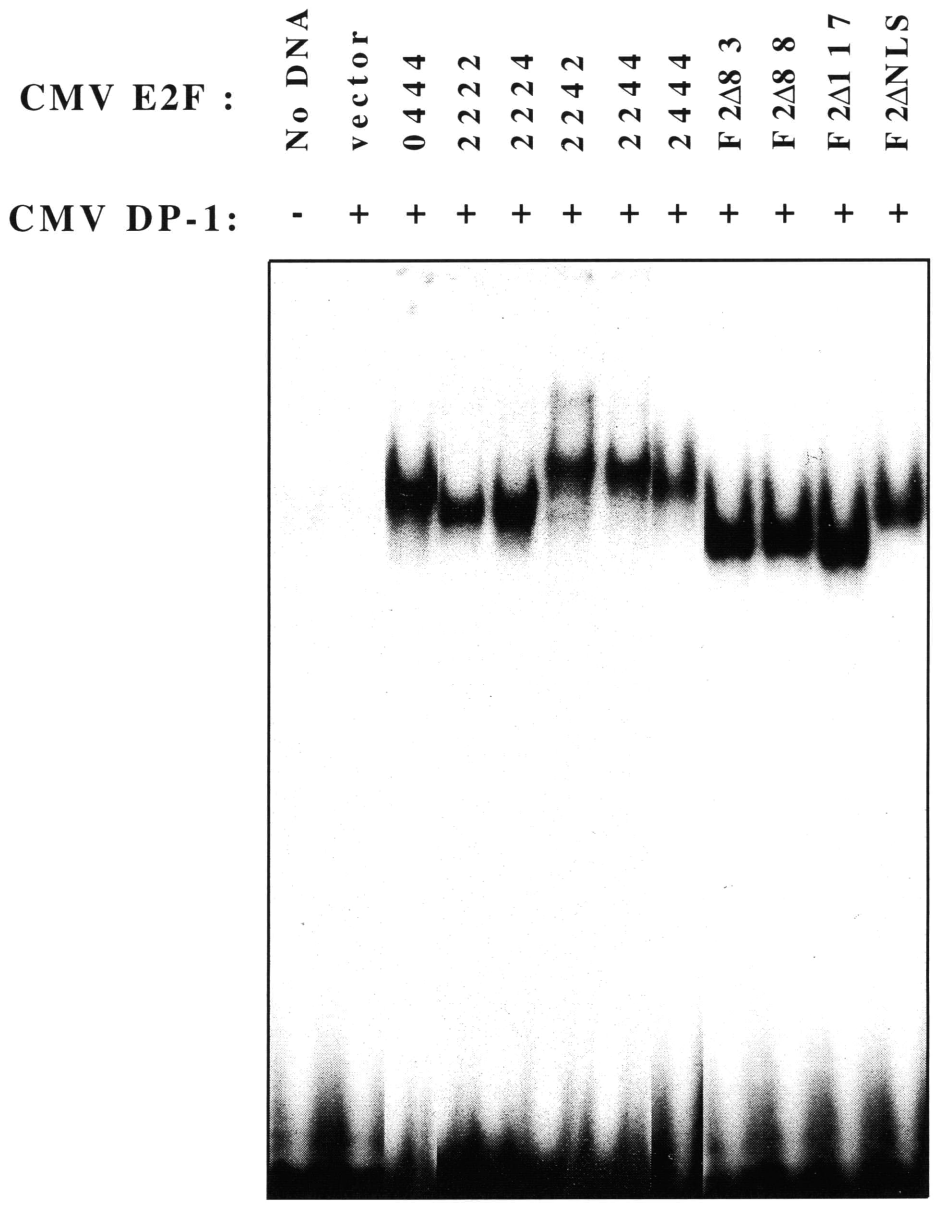


Figure 8A.


CMV E2F :
No DNA
vector
0444
2222
2224
2242
2244
2444
F2Δ83
F2Δ88
F2Δ117
F2ΔNLS
CMV DP-1:
- + + + + + + + + + + +

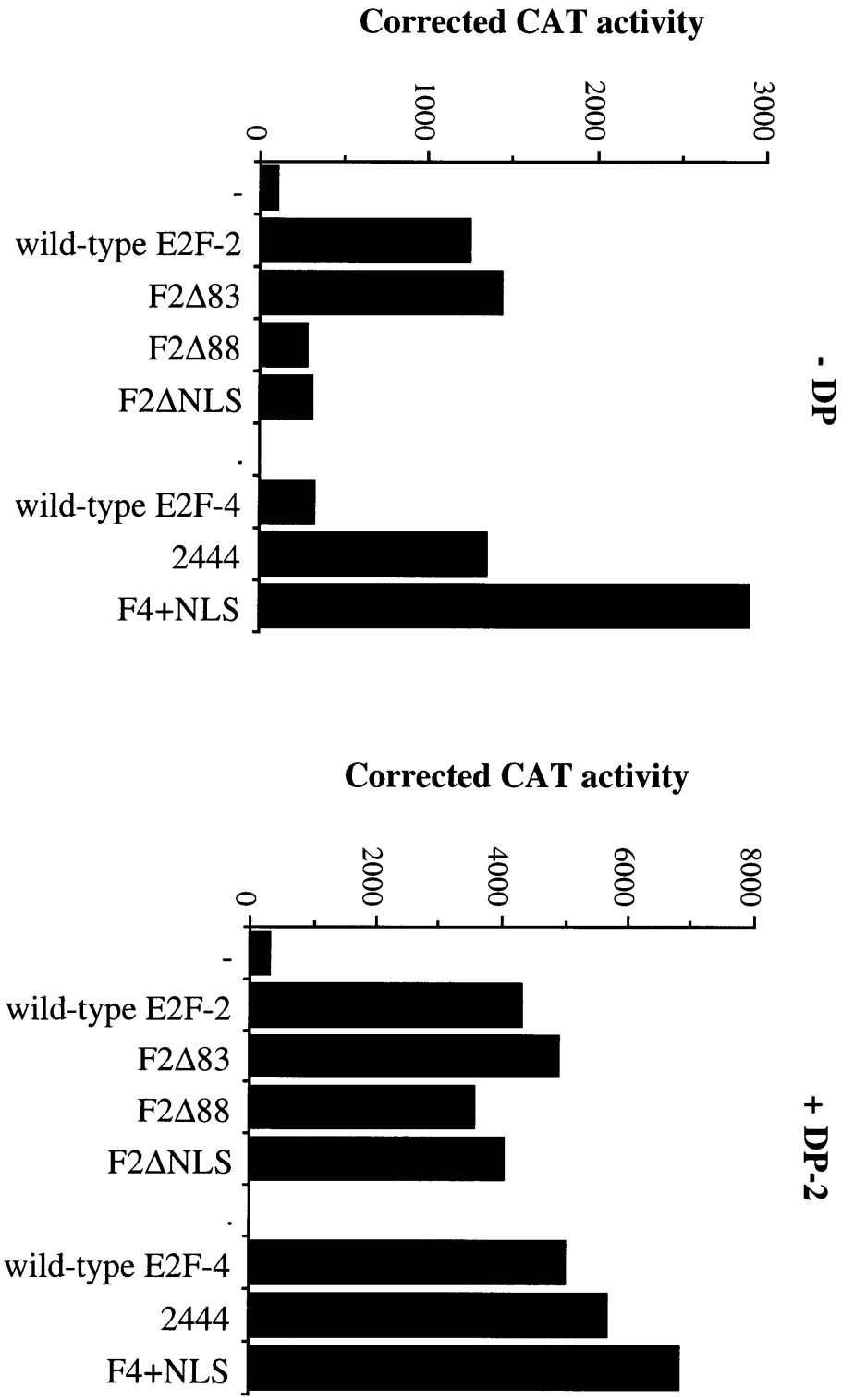
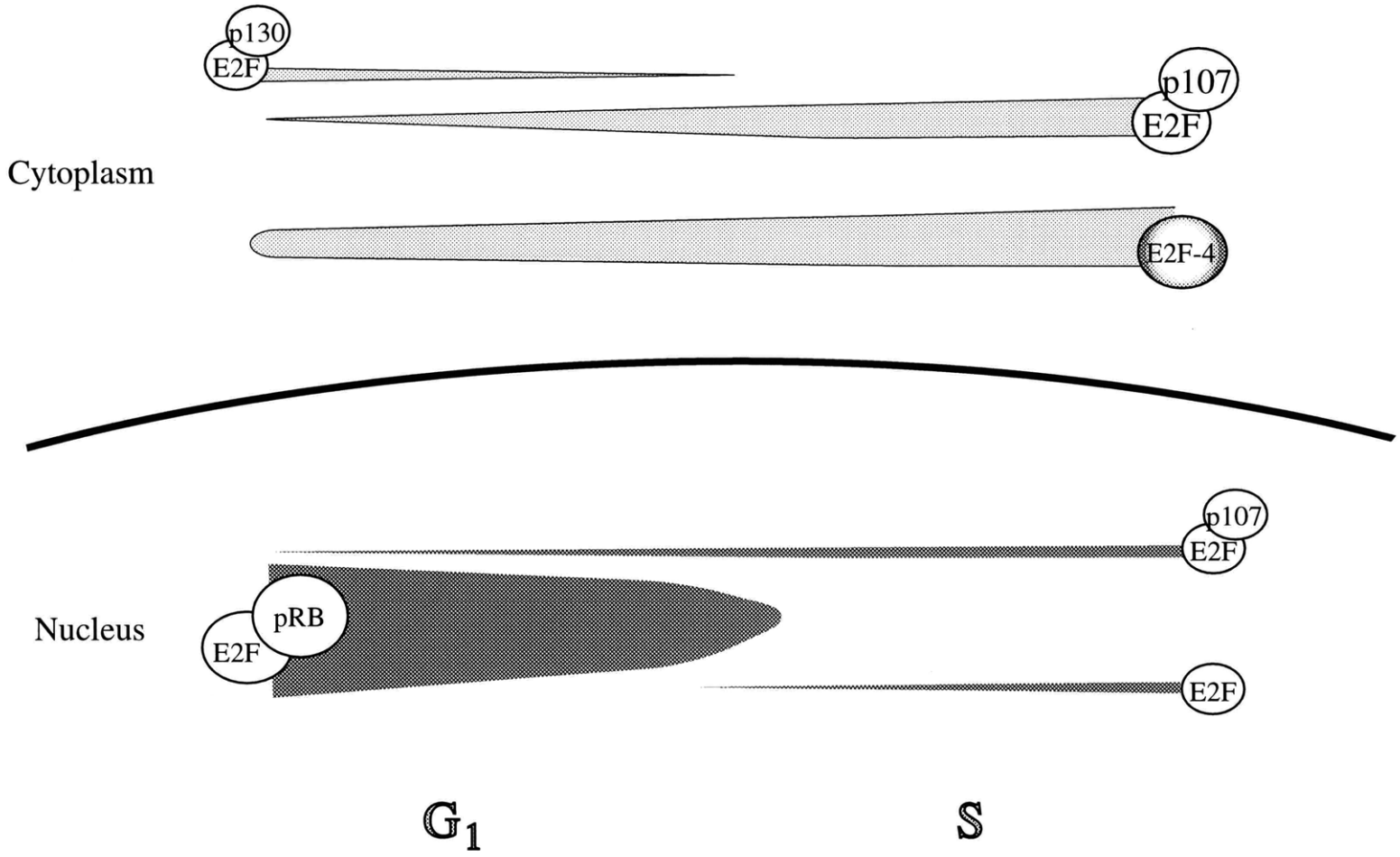

Corrected CAT activity
0
1000
2000
3000
-
wild-type E2F-2
F2Δ83
F2Δ88
F2ΔNLS
.
wild-type E2F-4
2444
F4+NLS
- DP
Corrected CAT activity
0
2000
4000
6000
8000
-
wild-type E2F-2
F2Δ83
F2Δ88
F2ΔNLS
.
wild-type E2F-4
2444
F4+NLS
+ DP-2

Figure 8B.

Figure 9.


p130
E2F
p107
E2F
Cytoplasm
E2F-4
p107
E2F
Nucleus
pRB
E2F
E2F
G1
S

E. Figure Legends

Figure 1. The transcriptional activity of E2F-4 is impaired by its *in vivo* expression . Parental U2tTA10, U2F2 or U2F4 cell lines were cultured in either the presence (uninduced) or absence (induced) of tetracycline for 36 hours and cell extracts were generated as previously described (Moberg et al, 1996). The levels of E2F-2 or E2F-4 protein and DNA binding activity were then analyzed in either western blots **(A)** or gel retardation assays **(B)** using monoclonal antibodies against either E2F-2 (LLF2-1) or E2F-4 (LLF4-1) as indicated. **(C)** The parental U2tTA10, U2F2 or U2F4 cell lines were transiently transfected with 5 μg of the E2F-responsive reporter, $E2F_4$-CAT, and 2 μg of pRSV-Luciferase, as an internal control of transfection efficiency. The cells were then cultured in duplicate in either the presence (uninduced) or absence (induced) of tetracycline and the levels of CAT and luciferase activity were measured after 24 hours. The fold induction represents the average of three transfections.

Figure 2. Exogenously expressed E2F-4 localizes to the cytoplasm. **(A)** R12 cells were micro-injected with CMV expression constructs, encoding the indicated E2Fs, along with CMV-β-galactosidase, to mark injected cells (data not shown). Immunofluorescence was carried out using either control (data not shown), anti-E2F-1 (KH20), anti-E2F-2 (LLF2-1), anti-E2F-3 (LLF3-1) or anti-E2F-4 (LLF4-1) antibodies along with DAPI as indicated. **(B)** The U2F2 or U2F4 cell lines were plated on coverslips and then cultured in the absence (induced) of tetracycline for 36 hours. Immunofluorescence was carried out using control (data not shown), anti-E2F-2 (LLF2-1) or anti-E2F-4 (LLF2-1) antibodies along with DAPI as indicated.

Figure 3. Localization of the endogenous E2F-4 protein is regulated in a cell cycle dependent manner. **(A)** Asynchronous WI-38, U2OS, T98G, ML-1, C33-A, SAOS-2 and 293 cells were fractionated using standard methods. Equivalent volumes of cytoplasmic or nuclear extracts were resolved by 10% SDS-PAGE and transferred to nitrocellulose. These blots were probed as indicated with antibodies that specifically recognize either E2F-1(KH20) or E2F-4 (LLF4-1). **(B)** U2OS cells were cultured in the presence of thymidine (for 12 hours) and then nocodazole (for 24

hours) to generate a synchronized cell population. These were plated on coverslips and cultured in normal media. Fractions were removed every 6 hours and the cell cycle staging was assessed by FACS analysis. Immunofluorescence was carried out on asynchronous, peak G_1 or S-phase populations using either control (data not shown), or anti-E2F-4 (LLF4-1) antibodies along with DAPI as indicated. **(C)** WI38 cells synchronized in G_0/G_1 by starvation or released in to S-phase by readdition of serum were subject to indirect immunofluorescence localization of E2F-4 protein as described above.

Figure 4. Localization of the endogenous E2F-4 complexes is regulated in a cell cycle dependent manner. Human HL60 cells were separated by centrifugal elutriation and the cell-cycle distribution was determined by FACS analysis of a proportion of the resultant populations (as indicated). The remainder of the cells were then fractionated to yield nuclear and cytoplasmic extracts. **(A)** Equivalent volumes of cytoplasmic and nuclear extracts were subject to Western blot analysis of E2F-1 (KH20 monoclonal) and E2F-4 (LLF4-1 monoclonal). **(B)** Cytoplasmic and nuclear extracts from the elutriated fractions were screened for the presence of E2F DNA binding activity in gel retardation assays.

Figure 5. The pRB•E2F, p107•E2F and p130•E2F complexes preferentially localize to either the cytoplasm or the nucleus in actively dividing cells. To identify the constituent components, the cytoplasmic and nuclear fractions of the peak G_1 (93% G_1, 5% S and 2% G_2/M) and S-phase (14% G_1, 78% S and 8% G_2/M) populations were analyzed in the gel retardation assays in the absence or presence of monoclonal antibodies specific for the individual E2F or regulatory proteins as indicated. The anti-E2F antibodies all supershift pocket protein-containing E2F complexes as efficiently as their free E2F•DP counterparts (Moberg et al. 1996). Similar results were obtained with multiple other anti-E2F-1, -2 -3 or -4 monoclonal or polyclonal antisera.

Figure 6. Localization properties of chimeric E2F proteins. U2OS cells were transiently transfected as described previously with 1 μg of the indicated CMV-E2F constructs along with

CMV-β–galactosidase to mark transfected cells. Immunofluorescence was performed as described (Materials and Methods) using anti-E2F-2, anti-E2F-4, and anti-β-gal antibodies (data not shown). Two hundred β-galactosidase positive cells were scored for E2F protein localization and the percent of cells displaying exclusively nuclear, exclusively cytoplasmic or both nuclear and cytoplasmic staining was determined.

Figure 7. Associated proteins modulate the localization of E2F-4. Murine 3T3 fibroblasts were microinjected with CMV expression constructs encoding HA-tagged human DP-1 or DP-2 either alone or in combination with the indicated plasmids. CMV-β–galactosidase was included to mark injected cells. Immunofluorescence was performed as described (Materials and Methods) using anti-E2F-4, anti-HA and anti-β–galactosidase antibodies.

Figure 8. Localization of E2F proteins determines their ability to activate transcription *in vivo*. The properties of the chimeric E2F molecules were assayed by transfection into human C33-A cells. **(A)** DNA binding activity was determined by transfection of 10 μg of the relevant CMV-E2F expression constructs together with 10 μg CMV-HA-DP-1 as indicated. Whole cell extracts were generated as described (Moberg et al. 1996) and assayed for E2F DNA binding activity in gel shift assays (Materials and Methods). **(B)** The transcriptional activity of these chimeric E2F molecules *in vivo* was tested by cotransfection of 200ng of the relevant CMV-E2F, 4μg of $E2F_4$-CAT, 2μg RSV-Luciferase in the presence or absence of 1μg CMV-HA-DP-2. Normalized CAT values were used as a measure of transcriptional induction.

Figure 9. Summary of the nuclear and cytoplasmic E2F complexes through the cell cycle.

F. Materials and Methods

Cell Culture.

Human cell lines ML-1 (premyeloid leukemia), C33-A (cervical carcinoma), WI-38 (normal diploid lung fibroblast), 293 (renal adenocarcinoma), T98G (glioblastoma), R12 cells (Rat1A derivative cell line), U2OS and SAOS-2 (osteosarcomas) were grown under standard conditions of 5% CO_2 in DMEM supplemented with 10% heat-inactivated fetal bovine serum. Murine 3T3 fibroblasts were grown in DMEM supplemented with 10% calf serum. The HL60 (human lymphoma) cell line was cultured in RPMI medium supplemented with 20% fetal bovine serum. Transient transfections were carried out exactly as described previously (Moberg et al., 1996).

Cell Synchronization.

U20S cells were arrested at metaphase by culturing sequentially in media containing 2mM thymidine (12 hours) and then 1.7 μM nocodazole (24 hours), washed several times and re-plated in fresh media on coverslips. Cells were collected every 6 hours and processed for either immunofluorescence or FACS analysis. HL60 were separated by centrifugal elutriation using standard procedures and a small proportion of each fraction was subjected to FACS analysis to determine cell cycle staging. Synchronized G_0/G_1 WI38 human diploid fibroblasts were obtained by starvation of early passage cells in 0.1% serum for 48hrs; enriched S-phase populations were generated by replating arrested cells in 20% fetal bovine serum for 14hrs (Mittnacht et al., 1994).

Plasmids.

The following plasmids have been described previously: pCMV-E2F-1, -2, -3, and -4 (Moberg et al., 1996); pCMV-DP-1, -2 and pCMV-pRB (Wu et al., 1995). The E2F-responsive reporter $E2F_4$-CAT have pRSV-Luciferase have been described in Helin et al. (1993). Chimeric E2F cDNAs were constructed in a derivative of the pBKSII$^+$ vector (Strategene) in which the XbaI site had been deleted. Briefly, domains of either E2F-2 or E2F-4 were amplified by PCR using primers containing silent restriction sites which did not alter codon usage within the relevant ORF.

Hybrid cDNAs were created by the ligation of these individual domains together in the pBKSII^{+}ΔXba vector using the engineered restriction sites. Full-length chimeric cDNAs were then subcloned as BamHI fragments into the BamHI site of the pCMV-Neo-Bam vector (Baker et al., 1990). The E2F-2 and E2F-4 specific primer pairs are as follows.

(i) E2F-2: 10.18: 5'-GGATCCATGCTGCAAGGGCCCCGGGCCTTG-3' and 10.24Xba: 5'-CCT-AAGCTTCTAGAACGTTGGTGATGTCATAG-3'; 10.23Xba: 5'-CGTTCTAGAAGGCACT-CAGCTCATC-3' and 10.22HIII: 5'-GAGAAGCTTATCAGAGGGGAG-3'; 10.21HIII: 5'-GATAAGCTTCTCCCCATCCTTG-3' and 10.20: 5'-GGTGGTACCGGATCCTCAATTAATC-AACAGGTCC-3'.

(ii) E2F-4. 4.13 :5'-GGATCCATGGCGGAGGCCGGGCCACAG-3' and 4.19Xba: 5'-CCTAAG-CTTCTAGAACATTGGTAATGTCGTA-3'; 4.18Xba: 5'-TGTTCTAGAAGGTATC-GGGCTAATC-3' and 4.17HIII: 5'-GAAAAGCTTAGCAGAGGGGCAAACACT-3'; 4.16HIII: 5'-GCTAAGCTTATCTCCACCCCCGGGAGAC-3' and 4.15: 5'-GGTGGTACCGGATCC-TCAGAGGTTGAGAACAGG-3'

For pCMV-2444, the N-terminus of E2F-2 was amplified by PCR using the primers 10.18 (above) and 10.2N: 5'-GATGGATCCGAGGCCATCCACTCTGAT-3'. The N-terminal deletions of E2F-2, Δ83, Δ88, and Δ117 were constructed by PCR amplification of wildtype E2F-2 cDNA template using the following primers together with primer 10.20 (above): 10.83: 5'-GGTGGATCCAT-GGCCAAAAGGAAGCTGG-3', 10.88: 5'-GGTGGATCCATGCTG-GATCTGGAGGG-GATTG-3' and 10.117: 5'-GGTGGATCCATGGGCCCCAA-AACCCCCAAATC-3'. The F2ΔNLS construct was generated by PCR amplification of E2F-2 cDNA using the primer pairs 10.18 (above) & 10.ΔNLS2: 5'- CTTCAAGCTTCTACAGGC-ACTCAGCCGTCCTG-CCGGCAG-3' and 10.20 (above) & 10ΔNLS1: 5'-GTTGAAGCTTG-

TTTGTGGCGGGGATTGGGAGGCC-3'. The two fragments were then ligated together to yield an E2F-2 cDNA containing a novel HindIII site within the altered NLS sequences.

E2F Transactivation Assays.

CAT and luciferase assays were performed as described in Helin et al. (1993). Briefly, cells were harvested 36h post-transfection and lysed in 0.025M Tris-HCl (pH 8.0) by three rapid freeze-thaw cycles. Extracts were clarified by a 15,000*g* spin for 10 min and the supernatants were assayed for CAT and luciferase activities.

E2F Inducible Cell Lines.

E2F-2 or E2F-4 inducible cell lines were generated using the technique of Gossen and Bujard (1992). U2OS cells were cotransfected with pUHD15-1 and pCMVneo by the calcium phosphate method. After selection in G418 (250μg/ml), stable cell lines were assayed by transient transfection for tetracycline regulated activity of a luciferase reporter (pUHC13-3). One line, U2tTA10, which showed a $2x10^3$ increase in luciferase activity upon tetracycline withdrawal, was transfected with pTK-HYG and the pUHD10-3 expression vector containing either the E2F-2 or E2F-4 cDNA. After selection in the presence of hygromycin (100μg/ml) and tetracycline (0.1μg/ml), extracts from stable cell lines were screened by western blotting for the induction of the relevant E2F.

Microinjection and Immunofluorescence.

3T3 and R12 cells were plated on glass coverslips and grown to 70% confluency. E2F expression plasmids (25 μg/ml) were coinjected with a plasmid encoding ß-galactosidase (5 μg/ml) to mark injected cells. After injection, the cells were grown in DME-HEPES supplemented with 10% serum for 3-4 hours. Following fixation and permeabilization, cells were incubated for 30 min with rabbit anti-β-galactosidase antibodies (1:50 dil.; 5 Prime-3 Prime, Inc.) and a cocktail of mouse anti-E2F monoclonals (1:25 dil). E2F antibodies used were KH20 (anti E2F-1), LLF2-1 (anti E2F-2), LLF3-1 (anti E2F-3), and LLF4-1 (anti E2F-4). KH20, LLF2-1 and LLF3-1 have

been described previously. The LLF4-1 monoclonal hybridoma cell line was isolated from BALB/c mice immunized with 6X His-tagged E2F-4 (amino acids 147-413) exactly as described by Moberg et. al. (1996). Following incubation in primary antibody, cells were washed with PBS and then incubated for 30 min in secondary antibody (FITC-conjugated goat anti-mouse at a 1:1000 dil, Cappel) and rhodamine-conjugated goat anti-rabbit at a 1:1000 dil, Cappel). The cells were then washed four times with PBS, incubated with DAPI (0.1 mg/ml) for 5 min, washed again and mounted on glass slides with Mowiol.

For detection of transfected proteins, 50% confluent U2OS cells, plated on coverslips 48h prior, were transfected with the indicated expression constructs together with CMV-β-galactosidase to mark transfected cells and processed 24h later for immunofluorescence exactly as described above. The murine anti-influenza hemagglutinin antibody 12CA5 was used to detect HA-tagged proteins.

For detection of endogenous E2F-4, synchronous or asynchronous U2OS cells or WI38 cells were plated on glass coverslips and immunofluorescence was performed as described above with the following modifications. After fixing and permeabilizing, block solution (5% goat serum, 0.2 % fish skin gelatin (Sigma), and 0.2% Tween-20) was added for 60 min. Cells were then incubated with mouse anti-E2F-4 monoclonals (dil 1:25 in blocking solution) for 60 min, washed twice with PBS+0.2% Tween-20, and incubated with FITC-conjugated goat anti-mouse for 30 min. After several washes with PBS+Tween-20 and DAPI staining for 5 min, the cells were mounted on glass slides with VectaShield (Vector).

Subcellular Fractionation, Western Blots and Gel Shift Assays.

Fractionation of cultured cells was performed as follows. Cell pellets were resuspended in two packed-cell volumes (PCV) of hypotonic buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM EDTA, pH 8.0, 10mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 1mg/ml aprotinin, 1mg/ml leupeptin) and incubated 5 min on ice. Nuclei were separated by a 500*g* spin for 5 min and washed twice more in hypotonic buffer. Nuclei were

then lysed in 3 PCV of Lysis Buffer and clarified by a 45 min spin at 100,000 *g* as described previously (Moberg et al., 1996). The cytoplasmic supernatant of the original 500*g* spin was supplemented with glycerol to 35% (3 PCV final volume) and clarified by a 20,000*g* spin for 10 min. Protein concentrations were determined with Protein Dye Reagent (BioRad). As the final volumes of cytoplasmic and nuclear extract from a given cell population were equivalent, the ratio of protein concentrations was taken as a measure of the per-cell ratio of cytoplasmic and nuclear proteins.

Western blots and gel shift assays were performed exactly as described previously (Moberg et al., 1996). Briefly, equal volumes of cytoplasmic and nuclear extract were assayed for E2F protein (western blotting) or E2F DNA binding activity (gel shift) in the presence of the indicated antibodies. E2F-4 was detected in both assays with the LLF4-1 monoclonal supernatant described above. Additional antibodies used were KH20 (anti-E2F-1), XZ55 (anti-pRB), SD15 (anti-p107), and sc-317X (anti-p130; Santa Cruz).

G. Acknowledgments

We thank Laura Attardi and Barbara Panning for technical assistance with the microinjection and immunofluorescence assays and E. Nunnerley for her contribution to the preparation of the figures. We are also grateful to members of the Lees laboratory for helpful discussion and to Steve Bell, Nicholas Dyson and David Housman for critical reading of the manuscript. S.E. was supported by an Anna Fuller fellowship. This work was supported by the Harcourt General Charitable Foundation.

References

Adams, P.D., W.R. Sellers, S.K. Sharma, A.D. Wu, C.M. Nalin, and W.G. Kaelin Jr.. 1996. Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. Mol. Cell Biol. *16*, 6623-6633.

Adnane, J., Z. Shao, and P.D. Robbins. 1995. The retinoblastoma susceptibility gene product represses transcription when bound directly to the promoter. J. Biol.Chem. *270*, 8837-8843.

Bagchi, S., R. Weinmann, and P. Raychaudhuri. 1991. The Retinoblastoma Protein Copurifies with E2F-I, an E1A-Regulated Inhibitor of the Transcription Factor E2F. Cell *65*, 1063-1072.

Baker, S.J., S. Markowitz, E.R. Fearon, J.K.V. Willson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science *249*, 912-915.

Bandara, L.R. and N.B. La Thangue. 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature *351*, 494-497.

Bandara, L.R., V.M. Buck, M. Zamanian, L.H. Johnston, and N.B. La Thangue. 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. EMBO J. *12*, 4317-4324.

Bartek, J., J. Bartkova and J. Lukas. 1996. The retinoblastoma protein pathway and the restriction point. Current Opin. in Cell Biol. *8*, 805-814.

Beijersbergen, R.L., and R. Bernards. 1996. Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. Biochem. Biophys. Acta. *1287*, 103-120

Beijersbergen, R.L., R.M. Kerkhoven, L. Zhu, L. Carlee, P.M. Voorhoeve, and R. Bernards. 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes & Dev. *8*, 2680-2690.

Bremner, R., B.L. Cohen, M. Sopta, P.A. Hamel, C.J. Ingles, B.L. Gallie, and R.A. Phillips. 1995. Direct Transcriptional Repression by pRB and Its Reversal by Specific Cyclins. Mol. Cell Biol. *15*, 3256-65.

Cao, L., B. Faha, M. Dembski, L. Tsai, E. Harlow, and N. Dyson. 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature *355*, 176-179.

Chellappan, S.P., S. Hiebert, M. Mudryj, J.M. Horowitz, and J.R. Nevins. 1991. The E2F Transcription Factor Is a Cellular Target for the RB Protein. Cell *65*, 1053-1061.

Chittenden, T., D.M. Livingston, and J.A. DeCaprio. 1993. Cell Cycle Analysis of E2F in Primary Human T Cells Reveals Novel E2F Complexes and Biochemically Distinct Forms of Free E2F. Mol. and Cell Biol. *13*, 3975-3983.

Clarke, A.R., E.R. Maandag, M. van Roon,N.M.T. van der Lugt, M. van der Valk, M.L. Hooper, A. Berns, and H. te Riele. 1992. Requirement for a functional Rb-1 gene in murine development. Nature *359*, 328-330.

Cobrinik, D., M.-H. Lee, G. Hannon, G. Mulligan, R.T. Bronson, N. Dyson, E. Harlow, R.A. Weinberg and T. Jacks. 1996. Shared role of the pRB-related p130 and p107 proteins in limb development. Genes and Dev. *10*, 1633-1644.

Cobrinik, D., P. Whyte, D.S. Peeper, T. Jacks, and R.A. Weinberg. 1993. Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes and Dev. *7*, 2392-2404.

Dang, C.V., and W.M.F. Lee. 1988. Identification of the human c-myc protein nuclear translocation signal. Mol. and Cell. Biol. *8*, 4048-4054.

DeGregori, J., T. Kowalik, and J.R. Nevins. 1995. Cellular targets for activation by the E2F-1 transcription factor include DNA-synthesis and G1/S-regulatory genes. Mol. Cell Biol. *15*, 4215-4224.

Devoto, S.H., M. Mudryj, J. Pines, T. Hunter, and J.R. Nevins. 1992. A Cyclin A-Protein Kinase Complex Possesses Sequence-Specific DNA Binding Activity: $p33^{cdk2}$ Is a Component of the E2F-Cyclin A Complex. Cell *68*, 167-176.

Dimri, G.P., M. Nakanishi, P-.Y. Desprez, J.R. Smith, and J. Campisi. 1996. Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor p21 in cells expressing or lacking a functional retinoblastoma protein. Mol. Cell Biol. *16*, 2987-2997.

Dynlacht, B.D., O. Flores, J.A. Lees, and E. Harlow. 1994. Differential regulation of E2F *trans*-activation by cyclin/cdk2 complexes. Genes and Dev. *8*, 1772-1786.

Dynlacht, B.D., K. Moberg, J.A. Lees, E. Harlow and L. Zhu. 1997. Specific regulation of E2F Family members by cyclin-dependent kinases. Mol. Cell Biol. *17*, 3867-75.

Dyson, N., M. Dembski, A. Fattaey, C. Ngwu, M. Ewen, and K. Helin. 1993. Analysis of p107- Associated Proteins: p107 Associates with a Form of E2F That Differs from pRB-Associated E2F-1. Journal of Virology *67*, 7641-7647.

Ewen, M.E., H.K. Sluss, C.J. Sherr, H. Matsushime, J.Y. Kato, and D.M. Livingston. 1993. Functional Interactions of the Retinoblastoma Protein with Mammalian D-type Cyclins. Cell *73*, 487-497.

Ewen, M.E., Y. Xing, J.B. Lawrence, and D.M. Livingston. 1991. Molecular Cloning, Chromosomal Mapping, and Expression of the cDNA for p107, a Retinoblastoma Gene-Product-Related Protein. Cell *66*, 1155-1164

Field, S.J., F-.Y. Tsai, F. Kuo, A.M. Zubiaga, W.G. Kaelin, Jr., D.M. Livingston, S.H. Orkin, and M.E. Greenberg 1996. E2F-1 Functions in Mice to Promote Apoptosis and Surpress Proliferation. Cell *85*, 537-548.

Ginsberg, D., G. Vairo, T. Chittenden, Z.X. Xiao, G. Xu, K.L. Wydner, J.A. DeCaprio, J.B. Lawrence, and D.M. Livingston. 1994. E2F-4, a new member of the E2F trancription factor family, interacts with p107. Genes & Dev. *8*, 2665-2679.

Gossen, M. and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA *89*, 5547-5551.

Hannon, G., D. Demetrick, and D. Beach. 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes & Dev. *7*, 2378-2391.

Hateboer, G. R.M. Kerkhoven, A. Shvarts, R. Bernards and R.L. Beijersbergen. 1996. Degradation of E2F by the ubiquitin-proteasome pathway: regulation by the retinoblastoma family proteins and adenovirus transforming proteins. Genes & Dev. *10*, 2960-2970.

Helin, K., C. Wu, A.R. Fattaey, J.A. Lees, B.D. Dynlacht, C. Ngwu, and E. Harlow. 1993a. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative *trans*- activation. Genes & Dev. *7*, 1850-1861.

Helin, K., E. Harlow, and A. Fattaey. 1993b. Inhibition of E2F-1 Transactivation by Direct Binding of the Retinoblastoma Protein. Mol. and Cell. Biol. *13*, 6501-6508.

Hiebert, S.W., S.P. Chellappan, J.M. Horowitz, and J.R. Nevins. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes & Dev. *6*, 177-185.

Hijmans, E.M., P.J. Voorhoeve, R.L. Beijersbergen, L.J. van't Veer, and R. Bernards. 1995. E2F-5, a New E2F Family Member that interacts with p130 In Vivo. Mol. and Cell. Biol. *15*, 3082-3089.

Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.A. Weinberg. 1992. Regulation of Retinoblastoma Protein Functions by Ectopic Expression of Human Cyclins. Cell *70*, 993-1006.

Hofmann, F. and D. Livingston. 1996. Differential effects of cdk2 and cdk3 on the control of pRB and E2F function during G_1 exit. Genes & Dev. *10*, 851-861.

Hofmann, F., F. Martelli, D. Livingston and Z. Wang. 1996. The retinoblastoma protein product protects E2F-1 from degradation by the ubiquitin-proteosome pathway. Genes & Dev. *10*, 2949-59.

Hsiao, K.M., S.L. McMahon, and P. Farnham. 1994. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. Genes & Dev. *8*, 1526-1537.

Hu, Q., J.A. Lees, K.J. Buchkovich, and E. Harlow. 1992. The retinoblastoma protein physically associates with the human cdc2 kinase. Mol. and Cell. Biol. *12*, 971-980.

Ikeda M., L. Jakoi, and J.R. Nevins. 1996. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. Proc. Natl. Acad. Sci. USA *93*, 3215-3220.

Jacks, T., A. Fazeli, E.M. Schmitt, R.T. Bronson, M.A. Goodell, and R.A. Weinberg. 1992. Effects of an *Rb* mutation in the mouse. Nature *359*, 295-300.

Krek, W., D.M. Livingston, S. Shirodkar. 1993. Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. Science *262*, 1557-1560.

Krek, W., M.E. Ewen, S. Shirodkar, Z. Arany, W.G. Kaelin Jr., and D.M. Livingston. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell *78*, 161-172..

Krek, W., G. Xu, and D.M. Livingston. 1995. Cyclin A-Kinase Regulation of E2F-1 DNA Binding Function Underlies Suppression of an S Phase Checkpoint. Cell *83*, 1149-1158.

Lee, E.Y., C.Y. Chang, N. Hu, Y.C. Wang, C.C. Lai, K. Horrup, W.H. Lee, and A. Bradley. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature *359*, 288-294.

Lee, M.-H., B. Williams, G. Mulligan, S. Mukai, R.T. Bronson, N. Dyson, E. Harlow, and T. Jacks. 1996. Targeted disruption of p107: functional overlap between p107 and pRb. Genes and Dev. *10*, 1621-1632.

Lees, E., B. Faha, V. Dulic, S.I. Reed, and E. Harlow. 1992. Cyclin E / cdk2 and cyclin A / cdk2 kinases associate with p107 and E2F in a temporally distinct manner. Genes and Dev. *6*, 1874-1885.

Lees, J.A., K.J. Buchkovich, D.R. Marshak, C.W. Anderson, and E. Harlow. 1991. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J. *10*, 4279-4290.

Lees, J.A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin. 1993. The Retinoblastoma Protein Binds to a Family of E2F Transcription Factors. Mol. Cell Bio. *13*, 7813-7825.

Li, Y., C. Graham, S. Lacy, A.M.V. Duncan, and P. Whyte. 1993. The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes & Dev. *7*, 2366-2377.

Lindeman, G.J., S. Gaubatz, D.M. Livingston, and D. Ginsberg. 1997. The subcellular localization of E2F-4 is cell-cycle dependent. Proc. Natl. Acad. Sci. USA *94*, 5095-5100.

Lukas, J., B. O. Peterson, K. Holm, J. Bartek, and K. Helin. 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. Mol. Cell Biol. *16*, 1047-1057.

Matsushime, H., D.E. Quelle, S.A. Shurtleff, M. Shibuya, C.J. Sherr, and J.Y. Kato. 1994. D-Type Cyclin-Dependent Kinase Activity in Mammalian Cells. Mol. and Cell. Biol. *14*, 2066-2076.

Mayol, X., X. Grana, A. Baldi, N. Sang, Q. Hu, and A. Giordano. 1993. Cloning a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene *8*, 2561-2566.

Meyerson, M., and E. Harlow. 1994. Identification of G_1 Kinase Activity for cdk6, a Novel Cyclin D Partner. Mol. and Cell. Biol. *14*, 2077-2086.

Mittnacht, S., J.A. Lees, D. Desai, E. Harlow, D.O. Morgan, and R.A. Weinberg. 1994. Distinct sub-populations of the retinoblastoma protein show a distinct pattern of phosphorylation. EMBO J. *13*, 118-127.

Moberg, K., Starz, M.A., and J. A. Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle re-entry. Mol. and Cell. Biol. *16*; 1436-1449.

Mudryj, M., S.H. Devoto, S.W. Hiebert, T. Hunter, J. Pines, and J.R. Nevins. 1991. Cell Cycle Regulation of the E2F Transcription Factor Involves an Interaction with Cyclin A. Cell *65*, 1243-1253.

Nevins, J.R. 1992. A closer look at E2F. Nature *358*, 375-376.

Pines, J., and T. Hunter. 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J. Cell Biol. *115*, 1-17.

Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R.A. Weinberg. 1995. E2F-4 and E2F-5, two members of the E2F family , are expressed in early phases of the cell cycle. Proc. Natl. Acad. Sci. USA *92*, 2403-2407.

Sellers, W.R., J.W. Rodgers, and W.G. Kaelin, Jr. 1995. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. USA *92*, 11544-11548.

Shirodkar, S., M. Ewen, J.A. DeCaprio, J. Morgan, D.M. Livingston, and T. Chittenden. 1992. The Transcription Factor E2F Interacts with the Retinoblastoma Product and a p107-Cyclin A Complex in a Cell Cycle-Regulated Manner. Cell *68*, 157-166.

Smith, E.J., G. Leone, J. Degregori, L. Jakoi and J.R. Nevins. 1996. The accumulation of an E2F-p130 transcription repressor distinguishes a Go cell state from a G1 cell state. Mol. and Cell. Biol. *16*, 6965-6976.

Starostik, P., K.N.B. Chow and D.C. Dean. 1996. Transcriptional repression and growth suppression by the p107 pocket protein. Mol. Cell Biol. *16*, 3606-3614.

Vairo, G., D.M. Livingston , and D. Ginsberg. 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma family members. Genes & Dev. *9*, 869-881.

Weinberg, R.A. 1992. Tumor Suppressor Genes. Science *254*, 1138-1146.

Weinberg, RA. 1995. The Retinoblastoma Protein and Cell Cycle Control. Cell *81*, 323-330.

Weintraub, S.J., C.A. Prater, and D.C. Dean. 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature *358*, 259-261.

Weintraub, S.J., K.N.B. Chow, R.X. Luo, S.H. Zhang, S. He, and D.C. Dean. 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature *375*, 812-815.

Wu, C.L., L.R. Zukerberg, C. Ngwu, E. Harlow, and J.A. Lees. 1995. In Vivo Association of E2F and DP Family Proteins. Mol. and Cell. Biol. *15*, 2536-2546.

Xu, M., K.-A. Sheppard, C.-Y. Peng, A.S. Yee, and H. Piwnica-Worms. 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. and Cell. Biol. *14*, 8420-8431.

Yamasaki, L., Jacks, T, Bronson, R., Goillot, E., Harlow, E., and N.J. Dyson. 1996. Tumor Induction and Tissue Atrophy in Mice Lacking E2F-1. Cell *85*, 549-560.

Zhu, L., S. van der Heuvel, K. Helin, A. Fattaey, M. Ewen, D.M. Livingston, N. Dyson, and E. Harlow. 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes & Dev. *7*, 1111-1125.
Zwicker, J., N. Liu, K. Engeland, F.C. Lucibello, and R. Müller. 1996. Cell cycle regulation of E2F site occupancy in vivo. Science *271*, 1595-1597.

CHAPTER 4

"Specific Regulation of E2F family members by cyclin-dependent kinases"

Brian D. Dynlacht, Ken Moberg, J.A. Lees, E. Harlow, and L. Zhu

Molecular and Cellular Biology, July 1997, Vol.17, No.7, p.3867-3875

(Author's contribution: Figure 4, and additional data in Figure 2, Appendix)

A. Abstract

The transcription factor E2F-1 interacts stably with cyclin A via a small domian near its amino terminus and is negatively regulated by the cyclin A-depnedent kinases. Thus, the activities of E2F, a family of transcription factors involved cell proliferation, are regulated by at least two types of cell growth regulators: the retinoblastoma protein family and the cyclin-dependent kinase family. To investigate further the regulation of E2F by cyclin-dependent kinases, we have extended our studies to include additional cyclins and E2F family members. Using purified components in an in vitro system, we show that the E2F-1•DP-1 heterodimer, the functionally active form of the E2F activity, is not a substrate for the active cyclin D-dependent kinases but is efficiently phosphorylated by the cyclin B-dependent kinases, which do not form stable complexes with the E2F-1•DP-1 heterodimer. Phosphorylation of the E2F-1•DP-1 heterodimer by cyclin B-dependent kinases, however, did not result in down-regulation of its DNA-binding activity, as is readily seen after phosphorylation by cyclin A-dependent kinases, suggesting that phosphorylation per se is not sufficient to regulate E2F DNA-binding activity. Furthermore, heterodimers

containing E2F-4, a member of the family lacking the cyclin A-binding domain found in E2F-1, are not efficiently phosphorylated or functionally down-regulated by cyclin A-dependent kinases. However, addition of the E2F-1 cyclin A binding-domain to E2F-4 conferred cyclin A-dependent kinase-mediated down-regulation of the E2F-4•DP-1 heterodimer. Thus, both enzymatic phosphorylation and stably physical interaction are necessary for the specific regualtion of E2F family members by cyclin-dependent kinases.

B. Introduction

Regulation of the mammalian cell cycle involves the concerted interplay of both positive and negative regulators. Often , the central targets of these regulators are transcription factors. One such example is the transcription factor E2F, which is believed to play an important role in the controlled expression of genes that are essential for the G1/S phase transition and DNA replication.

The E2F family encompasses two distantly related subfamilies, E2F and DP. One subunit of E2F and one subunit of DP combine to form the heterodimers that are the active E2F activities in the cell. The E2F family thus far includes six E2F members and two DP members. For clarity in this report, we will refer to a particular E2F member using a numerical designation, and we will use the term E2F for the active E2F•DP heterodimer. Evidence for an important role for E2F in cell cycle progression stems from several findings. First, the promoters of a number of genes whose products are required for cell proliferation contain E2F sites (including the genes encoding B-myb, DNA polymerase α, dihydrofolate reductase, thymidine kinase, and E2F-1). Secondly, E2F can act as a potent growth-promoting factor. Overexpression of E2F-1 prevents cell cycle arrest of fibroblasts following serum withdrawl and stimulates quiescent cells to enter S phase (Johnson et al., 1993). Under other cirumstances, overexpression of E2F promotes cellular transformation (Bieijersbergen et al., 1994; Ginsberg et al., 1994; Singh et al., 1994; Xu et al., 1995). In still other cases, E2F drives cells into S phase, then induces apoptosis (Kowalik et al., 1995; Qin et al., 1994; Shan and Lee, 1994; Wu et al., 1994). Third, the importance of E2F in cell growth regulation is strongly implicated by its regulators. The retinoblastoma protein, pRB, inhibits the activity of E2F, and mutations in pRBthat render the protein incapable of inhibiting E2F are frequently found in human tumors. Surprisingly, a growth-suppressive role for E2F was uncovered by loss of E2F-1 function in mice, which results in proliferative defects and tumor formation (Field et al., 1996; Yamasaki et al., 1996)

The best-studied upstream regulators of E2F are the retinoblastoma protein family of proteins, including pRB, p107, and p130. The first E2F, E2F-1, was cloned based on its

interaction with pRB (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992). Members of the pRB family of proteins stably associate with E2F and repress its *trans* activation activity, as determined by transient transfection of E2F binding site-containing promoter constructs into cells and by in vitro transcription assays (Dynlacht et al., 1994; Helin et al., 1993; Hiebert et al., 1992; Weintraub et al., 1992; reviewed in Dyson, 1994). The ability of pRB and related proteins to repress E2F activity correlates with their growth suppression function, suggesting that E2F repression may be an essential component of pRB-mediated growth suppression (reviewed in Helin and Harlow, 1993; and in Nevins, 1992). Different members of the pRB family apparently regulate specific E2F family members as pRB primarily binds to E2F-1, E2F-2, and E2F-3, and E2F-4 (Ikeda et al., 1996; Moberg etal., 1996). In contrast, p107 and p130 interact specifically with E2F-4 and E2F-5 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Vairo et al., 1995). Based on these findings it has been suggested that different E2F members, together with their primary pRB protein regulators, control different sets of genes at different stages of the cell cycle.

A second regulatory mechanism for E2F connects E2F directly with the cell cycle machinery. E2F-1 stably interacts with cyclin A•cdk2 via a small domain near the amino terminus of the transcription factor, and one consequence of this interaction is the inhibition of E2F transcription factor activity by the cyclin A-dependent kinases (Dynlacht et al., 1994; Krek et al., 1994; Xu et al., 1994). The cyclin A binding domain of E2F-1 is conserved in E2F-2 and E2F-3 but not in E2F-4 or E2F-5. E2F-4 and E2F-5 can, however, interact with cyclins through p107 or p130, and possible regulation of E2F-4 by cyclin A•cdk2 in the cyclin A•cdk2•p107•E2F-4 complex has been demonstrated in vitro (Zhu et al., 1995). This level of E2F regulation has the great potential of revealing important, and remarkably direct, actions of the basic cell cycle machinery on the celluar transcription apparatus and may provide exciting targets for therapeutic intervention. At this stage, however, our understanding of this important level of regulation is limited. Most notably, the specificities of different cyclins towards different E2F members and the molecular mechanisms for this cyclin-dependent kinase-mediated regulation have not been systematically examined.

To address these issues, we have tested substrate phophorylation and functional regulation of additional E2F and cyclin•kinase pairs. Based on the functional relationships between different E2F members and different cyclins, we then performed domain fusion experiments to demonstrate that both enzymatic phosphorylation and stable physical interaction are the necessary, and sufficient, underlying mechanisms for this cyclin-E2F regulatory relationship.

C. Results

C1. Phosphorylation of E2F-1•DP-1 by specific cyclin•kinases

Using the in vitro system we have developed (Dynlacht et al., 1994), we first extended our studies on E2F-1 regulation by cyclin•kinases. Previously, we tested cyclin E•cdk2 and cyclin A•cdk2 complexes, kinases that are active primarily during late G1 and S phases. Here, we continued our studies to include the cyclin•kinases that functioned in other stages of the cell cycle. Cyclin D•cdk4 is an important player in early G1 while cyclin B•cdc2 is activated during the G2/M phase transition (reviewed in Hunter and Pines, 1994). The kinetics of cell cycle-dependent activation of these kinases is therefore considerably different from that of cyclin E- and cyclin A-dependent kinases.

Each of the kinases was produced by co-infection of insect cells with recombinant baculoviruses and purified by affinity chromatography (data not shown). In this manner, we produced both cdk2 and cdc2 complexes with cyclins A and B, as well as cdk4 complexes with cyclin D1. In addition, cyclin D1 complexes containing cdk2 or cdk6 and cyclin D2 complexes with cdk6 were also purified using immunoaffinity methods (data not shown).

Unlike most cyclin•cdks, cyclin D•cdk4 and cyclin D•cdk6 phosphorylate pRB preferentially to histone H1 (Matsushime et al., 1992; Matsushime et al., 1994; Meyerson and Harlow, 1994). Therefore we tested these cyclin D•cdk complexes in parallel kinase reactions containing either pRB or E2F. In each case, reactions were normalized so that similar levels of pRB or histone H1 kinase units (compared to cyclin A•cdk2) were used. In Figure 1A, the cyclin D complexes were purified by immunoprecipitation and kinase assays were performed using a GST fusion protein containing a carboxy-terminal fragment of pRB or the E2F-1•DP-1 heterodimer as substrates. Although the cyclin D2•cdk6 and cyclin D1•cdk4 complexes were potent pRB kinases, neither of these complexes was able to phosphorylate the E2F-1•DP-1 heterodimer. In addition, we used a purified, soluble form of cyclin D1•cdk4 complex to avoid possible problems with immunopurified, solid-phase complexes (Figure 1B). Again, the results

show that cyclin D1•cdk4 complexes were not able to phosphorylate the E2F-1 heterodimer. In each of these experiments, an amount of cyclin A•cdk2 that gives rise to similar levels of pRB phosphorylation relative to purified cyclin D kinases efficiently phosphorylated the E2F-1 heterodimer, and a mixing experiment ruled out the presence of inhibitors in the E2F-1 preparations (Figure 1B lane 13). We conclude that the E2F-1 heterodimer is not a substrate for the cyclin D kinases.

In experiments shown in Figure 2, we tested the cyclin B-dependent kinases in a similar manner as described above. Interestingly, cyclin B•cdc2 and cyclin B•cdk2 behaved almostly identically with cyclin A•cdk2 in their ability to phosphorylate E2F-1 heterodimers. Further, cyclin A•cdc2 phosphorylated the heterodimer with a specific activity indistinguishable from that of cyclin A•cdk2.

From this series of experiments, we have learned that (1): E2F-1 is phosphorylated by specific cyclin dependent kinases, since cyclin D-associated kinases are clearly unable to use E2F-1 as substrates; and (2): phosphorylation of E2F-1 apparently does not require stable protein-protein interaction, since cyclin B-dependent kinases efficiently phosphorylated E2F-1 without stably binding to it (see Figure 5 below).

C2. Functional regulation of E2F-1•DP-1 by specific cyclin •kinases

The next important question was to determine the effects of each of these cyclin kinases on the DNA-binding activity of the E2F-1 heterodimer. To do so, each of the purified kinases was tested in gel mobility shift assays containing purified E2F-1 and DP-1 (Figure 3). To avoid the possibility that the differences in the extent of phosphorylation of the E2F-1 heterodimer may be the cause of different effects, the amounts of cyclin A- and cyclin B-dependent kinases in the E2F gel shift reactions were equalized based on their kinase activities toward the E2F-1 dimer. Although cyclin D1•cdk4 did not phosphorylate this substrate, we still used an equivalent amount of pRB kinase units relative to cyclin A•cdk2.

As expected, cyclin A•cdk2, as well as cyclin A•cdc2, effectively abolished E2F DNA-binding activity in this assay (Figure 3, lanes 3 and 7). Cyclin D1•cdk4, which did not phosphorylate E2F, had no effect on its DNA-binding activity (lane 12). Notably, however, cyclin B•cdc2 and cyclin B•cdk2 failed to down-regulate the DNA-binding activity of E2F-1, despite the fact that the cyclin B-associated kinases could efficiently phosphorylate the heterodimer.

To understand the reason for the different effects of the cyclin A- and cyclin B-associated kinases on E2F-1•DP-1 dimer, we first sought to determine whether these two kinases phophorylated the same sites on E2F-1 and DP-1. We performed tryptic 2-dimensional gel electrophoresis on E2F-1 and DP-1, which were phosphorylated by either cyclin A•cdk2 or cyclin B•cdk2. As shown in Figure 4, the phosphotryptic patterns of E2F-1 and DP-1 phosphorylated by cyclin A•cdk2 are the same as those phosphorylated by cyclin B•cdk2.

We next compared the abilities cyclin A•cdk2 and cyclin B•cdk2 to stably associate with E2F-1. We immunoprecipitated either cyclin B•cdk2 or cyclin A•cdk2 complexes from reactions containing E2F-1•DP-1 and probed the resulting immunoblots with antibodies against E2F-1 and DP-1. Clearly, while cyclin A•cdk2 formed stable complexes with E2F-1•DP-1, no significant interaction between cyclin B•cdk2 and E2F-1•DP-1 was detected (Figure 5A, compare lanes 5 and 7). Furthermore, interactions between the cyclin A-associated kinases and the E2F-1•DP-1 heterodimer were productive, as demonstrated by kinase assays performed after immunoprecipitation of these kinases (Figure 5B). In this assay, which is considerably more sensitive than the immunoblotting experiment shown in Figure 5A, interactions between either cyclin A•cdk2 or cyclin A•cdc2 and E2F-1•DP-1 resulted in the phosphorylation of this heterodimer, while the cyclin B•cdk2 complex was without effect, since it did not stably interact with the heterodimer (Figure 5B, compare lanes 2, 4, and 5).

We went further to ask whether the stable association between cyclin A and E2F-1 was sufficient for down-regulation of E2F-1. A complex containing cyclin A and a catalytically inactive, dominant-negative version of cdk2 (cdk2-DN; van den Heuvel and Harlow, 1993) was purified and tested in an assay similar to the one described in Figure 3. Purified cyclin A•cdk2-

DN formed a stable association with E2F-1, resulting in a slower mobility complex (Figure 6, compare lanes 1 and 2). However, the DNA binding activity of this complex was not affected by the inactive cyclin A•cdk2-DN complex, as it was with the corresponding wild-type kinase (compare lanes 2 and 7). Furthermore, we wanted to learn whether down-regulation of E2F-1 could be achieved by providing the enzymatic phosphorylation through cyclin B•cdk2 and the stable physical interaction through cyclin A•cdk2-DN in trans. As shown in Figure 6 lanes 4 and 5, the DNA-binding activity of E2F-1 was not affected in this way, either when these two kinase complexes were added together or when cyclin B•cdk2 was used to phosphorylate E2F-1 first and cyclin A•cdk2-DN was added subsequently. When the E2F heterodimer was first treated with cyclin A•cdk2 and then subsequently incubated with cyclin A•cdk2-DN, the DNA binding activity of E2F-1 was abolished, as expected. We conclude that the function of E2F-1 is specifically regulated by cyclin A-associated kinases in a manner dependent upon coupled phosphorylation and stable interaction.

C3. Regulation of specific E2F members by cyclin A kinases

In the next series of experiments we investigated the regulation of different members of the E2F family by cyclin-dependent kinases. Each of the E2F polypeptides was produced in bacteria and purified extensively via a carboxy-terminal tubulin tag (Figure 1 and data not shown) (Dynlacht et al., 1994; Huber et al., 1993; Ivey-Hoyle et al., 1993). Because of difficulties related to expression of E2F-2 in bacteria (B.D.D. and J.L., unpublished data), we did not include E2F-2 in this study. Each of these purified proteins was shown to bind E2F site-containing oligonucleotides in a DP-1-dependent manner (Figure 7A and data not shown). For E2F-4, we further demonstrated its ability to form a higher order complex with p107 and cyclin A•cdk2 (Figure 7A, and 53), and to stimulate transcription, in a DP-1-dependent manner, from a template containing four E2F-binding sites using a reconstituted transcription assay (Figure 7B, compare lanes 2-4 with 5-7). Thus, these recombinant proteins are fully functional.

We tested the effects of cyclin A•cdk2 on the DNA-binding activities of various E2F proteins (Figure 7C). Treatment with cyclin A•cdk2 promoted the loss of DNA-binding by E2F-3•DP-1, as well as E2F-1•DP-1, but not E2F-4•DP-1 or E2F-5•DP-1. In fact, the DNA-binding activity of E2F-5 even increased slightly upon treatment with cyclin A•cdk2, although the significance of this finding, if any, remains to be clarified. The non-specific DNA-binding proteins present in reactions containing E2F-3 and E2F-5 served as good internal controls since their activities were not affected (lanes 4-6 and 10-12). In other experiments, E2F-3, but not E2F-4 or E2F-5 (Figure 8B below and data not shown), served as a good substrate for phosphorylation by cyclin A•cdk2, further suggesting a link between phosphorylation and regulation of DNA-binding.

Protein sequence analysis of the five E2F members has revealed extensive homology among them, but this homology does not extend to amino-terminal sequences. E2F-1, E2F-2, and E2F-3 share a short sequence near their amino-termini that was previously shown to be the cyclin A-binding domain of E2F-1 (Krek et al., 1994). This sequence is conspicuously absent from E2F-4 and E2F-5 (Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995). We therefore compared the ability of E2F-4 to bind cyclin A•cdk2 with that of E2F-1. As shown in Figure 5A, E2F-4•DP-1 was not co-immunoprecipitated with cyclin A•cdk2, while in parallel reactions, a large fraction of E2F-1•DP-1 was co-immunoprecipitated with this kinase (compare lanes 1 and 5 with 2 and 6). These results were corroborated by subsequent incubation of identical immunoprecipitates under kinase assay conditions (Figure 5B). Under these conditions, E2F-4•DP-1 complexes are not co-immunoprecipitated with cyclin A•cdk2, unlike the corresponding E2F-1-containing complexes (compare lanes 2 and 3). These experiments and the DNA-binding experiments shown in Figure 4 suggest that cyclin A•cdk2 specifically regulates only those members of the E2F family that can stably associate with it.

C4. The cyclin A binding domain of E2F-1 as both a necessary and sufficent mechanism of regulation

The studies described above point to cyclin A-binding as an important determinant in the specific regulation of E2F by cyclin A-dependent kinases. We next asked whether cyclin A binding was sufficient for E2F regulation. We addressed this question by fusing the previously defined cyclin A binding domain of E2F-1 (residues 67-108; Krek et al., 1994) to the amino terminus of E2F-4 and testing the regulation of this fusion protein, called E2F-4(+A), by cyclin A•cdk2. A mutant form of E2F-1 (termed E2F-1(DA)), in which the cyclin A binding domain was deleted, was also constructed to further demonstrate the role of this domain in E2F-1 regulation by cyclin A•cdk2.

We over-produced and purified these two recombinant E2F proteins with carboxy-terminal tubulin tags as before (Figure 8A). In kinase assays shown in Figure 8B, the E2F-1•DP-1 heterodimer was potently phosphorylated by cyclin A•cdk2, while the E2F-4•DP-1 complex was not. Strikingly, however, when the cyclin A binding domain was deleted from E2F-1 (E2F-1(DA)) and added to E2F-4 (E2F-4(+A)), the substrate specificity was switched. Phosphorylation of the E2F-4(+A)•DP-1 heterodimer by cyclin A•cdk2 was comparable to that of E2F-1•DP-1. In parallel experiments, regulation of the DNA-binding activities of these proteins was also tested (Figure 8C). Both the E2F-4(+A) and E2F-1(DA) proteins heterodimerized with DP-1 and bound DNA in the absence of added kinase with a specific activity similar to the parental proteins (Figure 8B). In the presence of increasing amounts of cyclin A•cdk2, the activity of full-length E2F-1, but not E2F-4, was inhibited (lanes 1-4 and 9-12). In contrast, the E2F-1 mutant, E2F-1(DA), was not affected by the kinase (lanes 5-8). Conversely, addition of the cyclin A-binding domain to E2F-4 (to produce E2F-4(+A)), generated a protein that was down-regulated by kinase treatment (lanes 13-16) in a manner similar to E2F-1. Therefore, we conclude that the cyclin A binding domain of E2F-1 is not only necessary but also sufficient in determining the specific regulation by cyclin A-dependent kinases.

D. Discussion

In this report, we have investigated the regulation of E2F family members by various cyclin-dependent kinases. We demonstrate that this regulation specifically pertains to certain E2F family members and particular cyclin-dependent kinases and that the underlying mechanism for this specificity involves both physical association and enzymatic phosphorylation. These findings have significant implications for a number of important issues.

D1. Substrate specificity of cyclin•kinases determined by a novel enzyme-substrate relationship

When the E2F-1•DP-1 heterodimer was tested for regulation by various cyclin•kinases, three distinct outcomes were obtained: no phosphorylation and no functional regulation by cyclin D-dependent kinases, phosphorylation and functional down-regulation by cyclin A-dependent kinases, and phosphorylation but no functional regulation by cyclin B-dependent kinases. While this set of results convincingly indicates that the cyclin D-dependent kinases do not recognize E2F-1•DP-1 as substrates, the difference between the effects by cyclin A-dependent kinases and cyclin B dependent kinases was unexpected. Cyclin B-associated kinases phosphorylated E2F-1•DP-1 to the same extent as cyclin A-dependent kinases and on similar phosphorylation sites as determined by phosphotryptic mapping, yet they were without effect on the DNA-binding activity of the heterodimer.

Clearly, the inability of cyclin B-associated kinases to affect the DNA-binding activity of E2F-1•DP-1 could be attributed to its lack of stable association with E2F-1. This lack of stable interaction could not be corrected in trans by treatment with cyclin A•cdk2-DN, which binds to E2F-1 but cannot phosphorylate it (Figure 6). In this respect, it is interesting to note that the E2F-1-A mutant was not phosphorylated efficiently by the cyclin A•cdk2 kinase (Figure 8). This would sugget that cyclin A•cdk2 needs to stably interact with E2F-1 in order to phosphorylate it efficiently, while cylcin B•cdk2 can efficiently phosphorylate E2F-1 without stably bound to it. Furthermore, when the cyclin A-binding domain of E2F-1 was fused to the amino-terminus of

E2F-4, a protein that does not contain this domain and is not phosphorylated or functionally regulated by cyclin A•cdk2, both phosphorylation and down-regulation were transferred from E2F-1 to E2F-4 (Figure 8).

The mechanisms underlying this requirement of stable interaction between cyclin A•cdk2 and E2F-1 for efficient phosphorylation and functional regulation, as opposed to the classic enzyme-substrate relationship between cyclin B•cdk2 and E2F-1, needs to be further investigated. Stuctural features of cyclin A and cylcin B that contribute to these different relationships with E2F-1 are currently being determined.

In general, our currently study suggest that certain targets may require two events in order to be functionally regulated by cyclin-dependent kinases. In addition to phosphorylation, stable interaction between a cyclin•cdk complex and its target is also required for a particular functional regulation. This additional level of complexity could confer a second layer of specificity. We suggest that this previously unrecognized mechanism may be involved in other enzyme-substrate relationships as well. Indeed, a related situation may exist for cyclin D•cdk4 and pRB, since pRB interacts with cyclin D•cdk4 and is thought to be the principal substrate for cyclin D•cdk4 (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993).

D2. Cyclin A-dependent kinases directly regulate only a subset of E2F members

By systematically examining the members of the E2F family we demonstrate a remarkable distinction in their regulation by cyclin A•kinases. E2F-1 and E2F-3 (E2F-2 was not tested because of difficulties in expression but most likely would share similarities with E2F-1 and E2F-3 due to the presence of the cyclin A-binding domain) are regulated by cyclin A-dependent kinases, while E2F-4 and E2F-5 are not. As discussed above, this specificity is achieved through a novel mechanism involving a domain of E2F-1 and E2F-3 that is absent in E2F-4 and E2F-5. Thus, different E2F family members have evolved to use a separate domain to ensure the specificity of regulation of cyclin A-dependent kinases.

The direct regulation of E2F-1, E2F-2 and E2F-3, but not E2F-4 and E2F-5, by cyclin A-associated kinases strongly suggests that these two groups of E2Fs have distinct functional properties. The group represented by E2F-1 probably has certain functions that must be down-regulated efficiently by cyclin A-dependent kinases during S phase. It was recently reported that when an E2F-1 mutant that could not bind cyclin A was ectopically expressed in fibroblasts, S phase progression was blocked (Krek et al., 1995). It will be of interest to compare the effects of overexpression of other members of the E2F family. In order to understand the mechanisms of E2F function, it is essential to elucidate the functional differences among the different E2Fs. Investigation of their regulation by cyclin A•kinases may provide an entry to this difficult, and until now unyielding, question.

Although E2F-4 and E2F-5 do not directly interact with cyclin A-dependent kinases, they are nevertherless indirectly connnected to cyclins through the p107 and p130 proteins (Cao et al., 1992; Devoto et al., 1992; Faha et al., 1992; Lees et al., 1992; Shirodkar et al., 1992; Zhu et al., 1995). Thus, these pRB-related proteins are found in complexes containing E2F-4•DP-1 and cyclin A•cdk2 or cyclin E•cdk2, although the functional consequences of this association have yet to be fully determined.

D3. The cyclin A binding domain of E2F-1 is conserved in other cyclin binding proteins

Finally, it is important to point out that the cyclin A binding domain of E2F-1 is related to a number of other cyclin binding domains identified in p21, p27 and p107, and it is an essential component for cyclin binding to these proteins (Chen et al., 1996a; Chen et al., 1996b; Lin et al., 1996; Luo et al., 1995; Zhu et al., 1995). Moreover, the crystal structure of a p27•cyclin A•cdk2 ternary complex has recently been solved, and this conserved domain forms the p27-cyclin A binding interface (Russo et al., 1996). While the interaction between p21 or p27 with cyclin-dependent kinases confers negative regulation upon the kinase, an interaction between cyclin A-dependent kinases and E2F-1 serves to negatively regulate E2F-1 activity. It appears, therefore, that stable physical interaction is an important mechanism for regulation both of, and by, the

cyclin-dependent kinases. We anticipate that our current study will lead to the discovery of other examples of regulation whereby cyclin-binding domains may be used to stably recruit substrates for phosphorylation.

Figure 1.

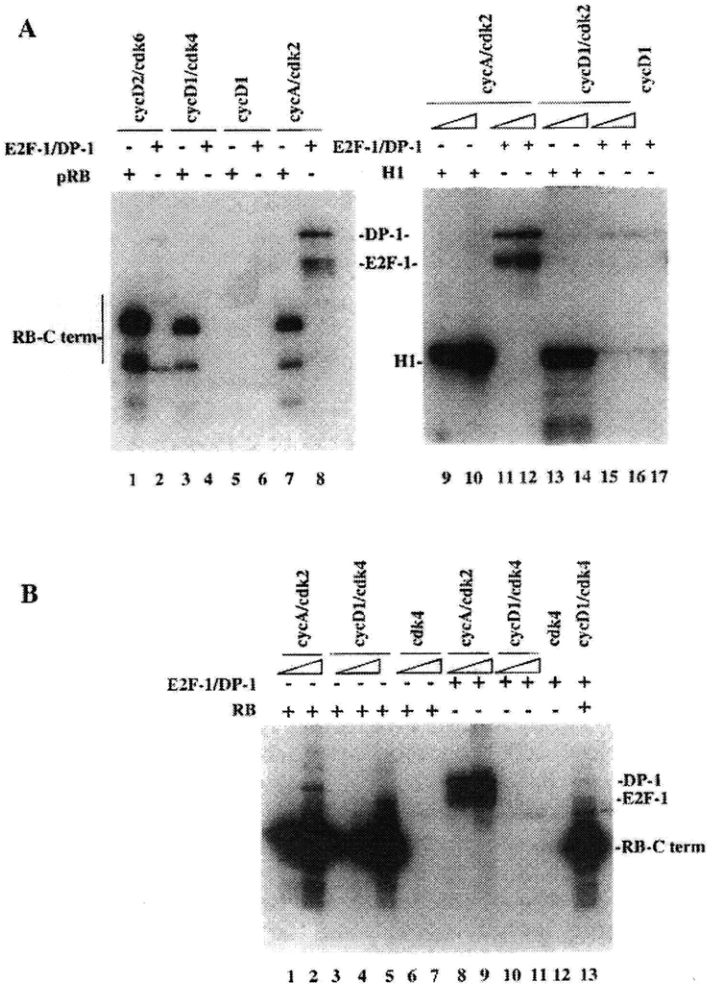


Figure 2.

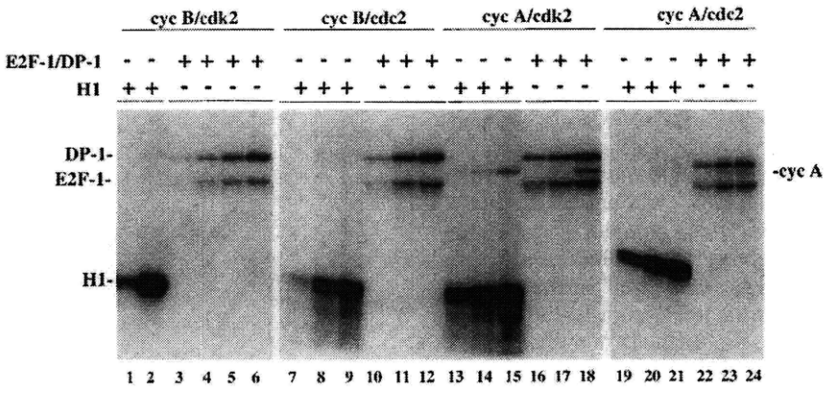


Figure 3.

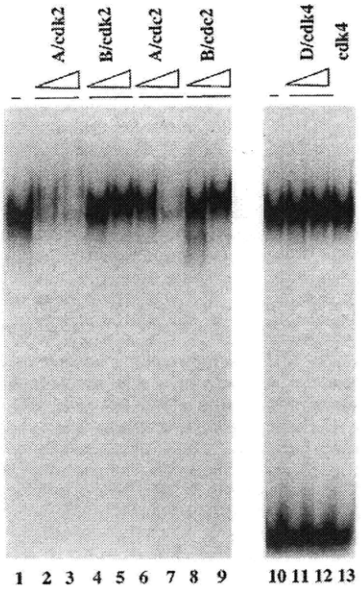


Figure 4.

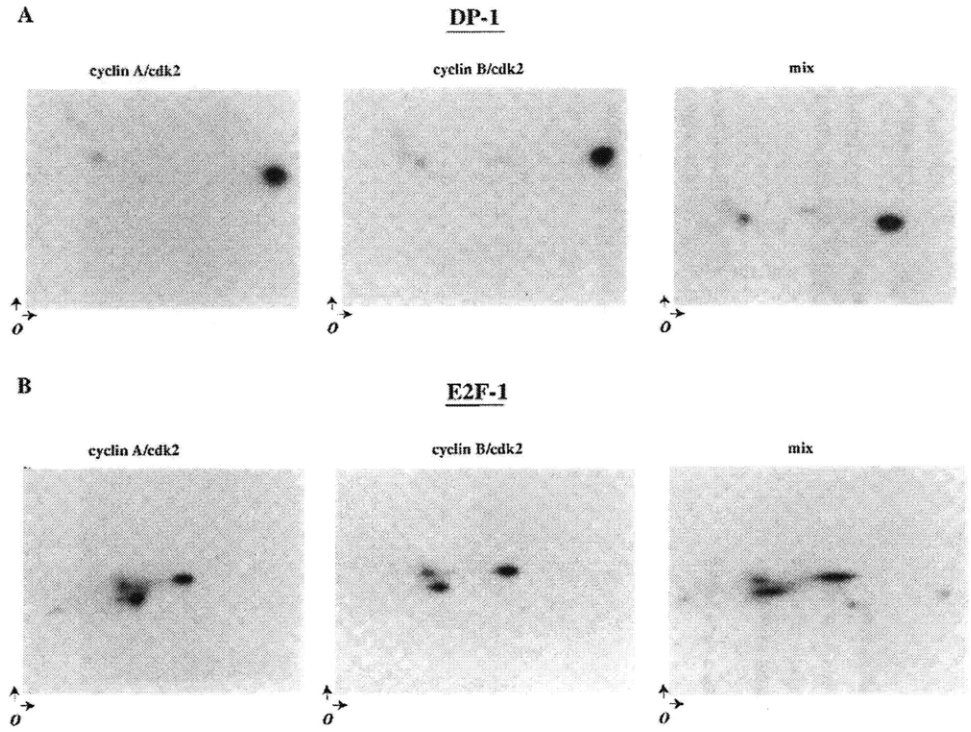


Figure 5.

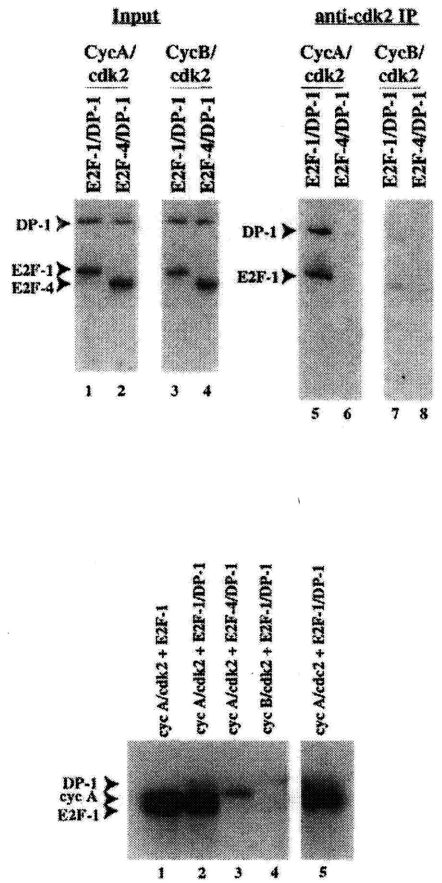


Figure 6.

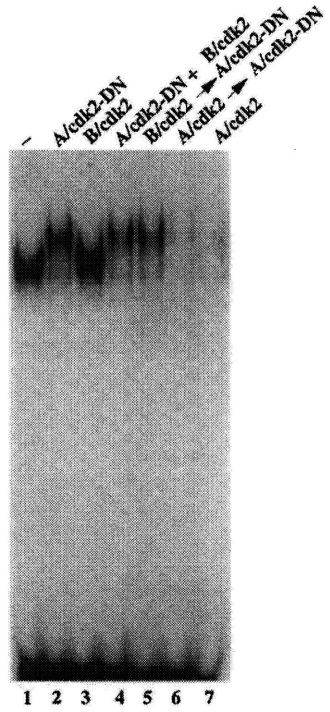


Figure 7.

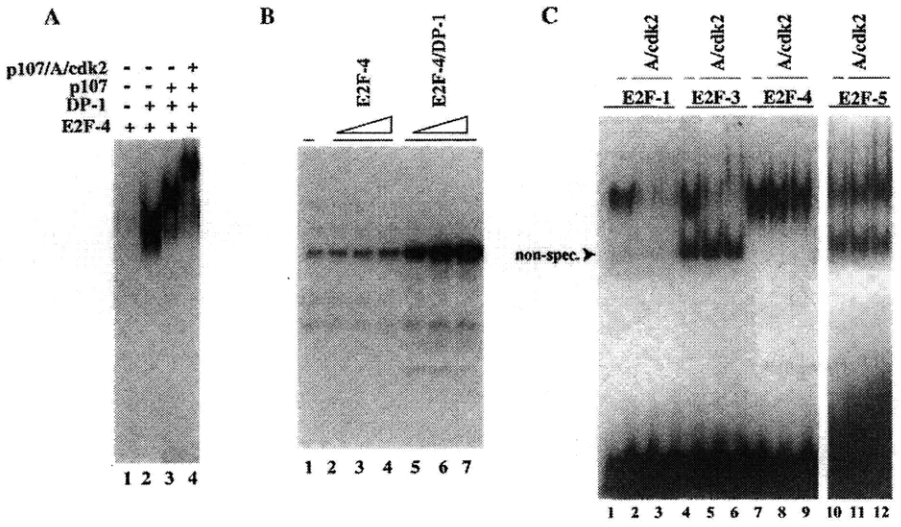
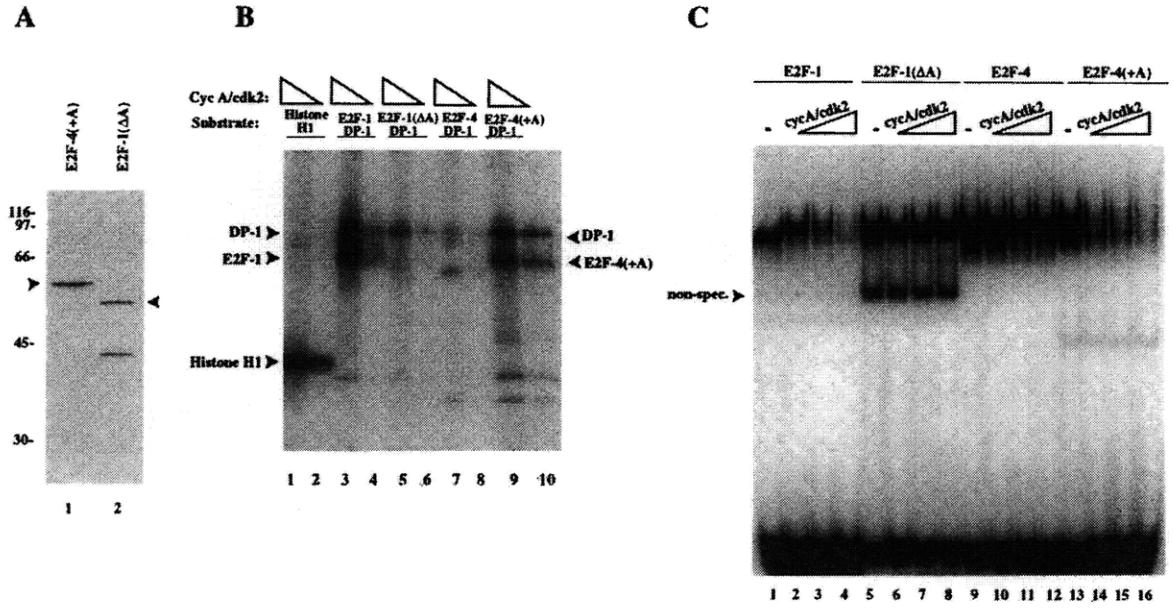


Figure 8.



E. Figure legends

Figure 1: Cyclin D kinases do not phosphorylate E2F-1•DP-1 heterodimers.
Various recombinant cyclin D•cdk complexes were purified from over-expressing insect cells either in the form of immune complexes **(A)** or soluble protein **(B)** and tested in parallel kinase reactions containing either E2F-1•DP-1, pRB, or histone H1 as indicated above each panel. **(A)** Immuno-purified cyclin D complexes were prepared by purification on protein A sepharose beads containing affinity-purified antibodies against either cyclin D1 (lanes 3-6 and 13-17) or cdk6 (lanes 1-2). The purity of each cyclin D immune complex was assessed by Coomassie-staining SDS-PAGE gels; in each case, complexes were greater than 90% pure (data not shown). The mobility of phosphorylated E2F-1, DP-1, pRB, and histone H1 are indicated to the left or right of each panel. As a positive control, parallel reactions were performed with 0.1 (lanes 9 and 11) or 0.25 ng of cyclin A•cdk2 (lanes 10 and 12). In addition, purified cyclin D1 (approximately 50 ng, lanes 5-6, and 17) alone failed to phosphorylate either substrate. **(B)** Phosphorylation assays with kinases purified by an alternate method. The gel in this panel was over-exposed to show the inability of purified cyclin D1•cdk4 (1, 3, and 10 ng, lanes 10-11 and 13, in that order) to phosphorylate E2F, although it is a potent pRB kinase (same amounts, lanes 3, 4, and 13). Nor does purified cdk4 (15 and 60 ng, lanes 6-7, respectively) have pRB or E2F (60 ng cdk4, lane 12) kinase activity. In contrast, 0.5 ng (lanes 1and 8) or 2.5 ng (lanes 2 and 9) of cyclin A•cdk2 potently phosphorylates both E2F-1•DP-1 (lanes 8-9) and pRB (lanes 1-2). A shorter exposure indicates that similar amounts of cyclin A•cdk2 (lanes 1-2) and cyclin D1•cdk4 (lanes 3-5) RB kinase units were added to reactions containing E2F-1•DP-1 (lanes 8-11).

Figure 2: E2F is a substrate for cyclin A- and cyclin B-type kinases.
Kinase reactions were reconstituted as described in Figure 2 using purified cyclin A and cyclin B kinases. Reactions contained the kinases and substrates indicated above each panel: 0.5 (lanes 1 and 3), 1 (lane 4), 2.5 (lanes 2 and 5), or 10 ng (lane 6) of cyclin B•cdk2; 1 (lanes 7 and 10), 5 (lanes 8 and 11), or 15 ng (lanes 9 and 12) of cyclin B•cdc2; 1 (lanes 13 and 16), 2.5 (lanes 14 and

17), or 10 ng (lanes 15 and 18) of cyclin A•cdk2; and 1, 5, or 10 ng of cyclin A•cdc2 (lanes 19-21 and 22-24, in that order) were used in each reaction. As indicated on the right-hand side of the figure, cyclin A becomes phosphorylated in these kinase reactions, which accounts for the phosphorylated protein migrating between DP-1 and E2F-1 (lanes 13-18).

Figure 3: E2F-1•DP-1 heterodimers are down-regulated solely by the cyclin A kinases.
(A) Gel mobility shift assays were performed using 5-10 ng of E2F-1•DP-1 and each of the cyclin•kinases shown at the top of the figure. Reactions contained either no added kinase (lanes 1 and 13), 2.5 or 10 ng of cyclin A•cdk2 (lanes 2 and 3, respectively), 2.5 or 10 ng of cyclin B•cdk2 (lanes 4 and 5 respectively), or 1 or 10 ng of cyclin A•cdc2 (lanes 6 and 7, respectively), 1 or 10 ng of cyclin B•cdc2 (lanes 8 and 9, respectively), 12 or 60 ng of cyclin D1•cdk4 (lanes 10 and 11, respectively), or 300 ng of cdk4 alone (lane 12). Inhibition of DNA-binding activity by cyclin A•cdc2 is linear with respect to amount of kinase added (data not shown) and is not due to a threshold effect as suggested here (lanes 6 and 7).

Figure 4: Two-dimensional phosphopeptide mapping of E2F-1 and DP-1 phosphorylated *in vitro* by cyclin A•cdk2 and cyclin B•cdk2.
DP-1 **(A)** and E2F-1 **(B)** phosphorylated by the indicated kinases were isolated and trypsinized, and the resulting peptides were separated by two-dimansional electrophoresis. Maps were also obtained from a mixture (Mix) of these phosphopeptides. The origin (*O*) and direction of both electrophoresis (horizontal arrow) and chromatography (vertical arrow) are indicated.

Figure 5: Interaction between E2F-1 or E2F-4 and cyclin-dependent kinases.
(A) Purified cyclin A•cdk2 or cyclin B•cdk2 was incubated with either purified E2F-1•DP-1 or purified E2F-4•DP-1. In the two panels on the left, the input E2F and DP proteins (indicated at the top of each panel) were checked by western blot analysis with, sequentially, anti-DP-1, anti-E2F-1 and anti-E2F-4 antibodies (lanes 1, 2, 3, and 4). On the right, after incubation of the cyclin A or cyclin B kinases with E2F heterodimers, complexes were immunoprecipitated with anti-

hemagglutinin (HA) antibodies that recognize the HA-tagged cdk2 protein, and Western blots of the resulting immunoprecipitates were probed sequentially with antibodies against E2F-1, E2F-4, and DP-1 (lanes 5, 6, 7, and 8). **(B)** Each of the immunoprecipitates obtained in **(A)** was incubated in kinase assays. The labeled band in lane 3 is cyclin A, which is potently phosphorylated (see also Figure 3); the faint band in lane 4 is most likely cyclin B since it becomes phosphorylated during kinase reactions, and its mobility is indistinguishable from that of recombinant GST-DP-1.

Figure 6: Inhibition of E2F activity by cyclin A kinases requires both binding and phosphorylation.

Down-regulation of E2F-1•DP-1 DNA-binding activity requires a cyclin A-binding and active kinase domain in *cis*. Reactions were carried out with E2F-1•DP-1 heterodimers as described in Figure 4 and contained either no additional protein (lane 1), cyclin A•cdk2-DN (lane 2), cyclin B•cdk2 (lane 3), a mixture of each of cyclin A•cdk2-DN and cyclin B•cdk2 (lane 4), or cyclin A•cdk2 alone (lane 7). Order-of-addition reactions (order is indicated by an arrow; lanes 5 and 6) were carried out as follows. E2F complexes were first incubated with cyclin B•cdk2 (lane 5) or cyclin A•cdk2 (lane 6) and subsequently treated with cyclin A•cdk2-DN.

Figure 7: Cyclin A•cdk2 selectively inhibits the DNA-binding activity of certain E2F family members.

(A and B) E2F-4•DP-1 is a fully functional transcription factor. **(A)** Gel shift reactions were reconstituted with 5 ng of E2F-4 alone (lane 1), 5 ng each of E2F-4 and DP-1 (lane 2), or the E2F-4•DP-1 heterodimer with approximately 100 ng of recombinant p107 (lane 3) or p107•cyclin A•cdk2 complex (lane 4) purified from over-expressing insect cells. Addition of these proteins generates "supershifted" p107-E2F and p107•cyclin A•cdk2•E2F complexes. **(B)** In vitro transcription reactions were reconstituted either without added E2F-4•DP-1 (lane 1), with 8, 25, and 50 ng of E2F-4 alone (lanes 2-4, respectively) or 8, 25, and 50 ng each of E2F-4 and DP-1 (lanes 5-7, respectively). **(C)** Gel shift reactions were reconstituted with 10 ng of E2F-1•DP-1, 10

ng E2F-3•DP-1, 3 ng of E2F-4•DP-1, or 10 to 20 ng of E2F-5•DP-1 containing either no added kinase (lanes 1, 4, 7 and 10) or 2 (lanes 2, 5, 8, and 11) or 15 (lanes 3, 6, 9, and 12) ng of cyclin A•cdk2. A nonspecific DNA-binding activity apparent in some reactions (indicated at left; see also Fig. 8) serves as a good internal control, since it was unaffected by any treatment (lanes 4 through 6, and 10 through 12).

Figure 8: The cyclin A binding domain confers the ability of cyclin A•cdk2 to phosphorylate and negatively regulate E2F heterodimers.

(A) A tubulin-tagged version of E2F-4 with an amino-terminal fusion containing the minimal cyclin A binding domain of E2F-1 [residues 67 to 108; termed E2F-4(+A)] (lane 1) and a deletion mutant of E2F-1 lacking this domain [in which residues 79 to 103 were removed; termed E2F-1(DA)] (lane 2) were expressed in *E. coli* and purified by using affinity chromatography as described for E2F-1, -3, -4 and -5 in Materials and Methods. An arrowhead indicates the position of each recombinant protein. **(B)** Kinase reaction mixtures containing purified recombinant proteins were reconstituted as in the experiment shown in Fig. 2. Increasing amounts of cyclin A (CycA)•cdk2 were titrated into the reaction mixtures. For each heterodimer tested, 5 (lanes 2, 4, 6, 8, and 10) or 25 (lanes 1, 3, 5, 7, and 9) ng of purified kinase was used. Reaction mixtures also included50ng of each E2F and DP-1 protein indicated at the top of the panel. We confirmed by immunoblotting that equal amounts of each E2F heterodimer were added to the reaction mixtures (data not shown). Histone H1 (1 μg) was also added to parallel reaction mixtures as a control for kinase activity. The mobility of each phosphoryklqted protein is indicated. Reaction products were seperated on SDS-10%PAGE gels and autoradiographed. **(C)** Each of these recombinant E2F proteins [2 ng of E2F-1(DA) in lanes 5 through 8, or 25 ng of E2F-4(+A) in lanes 13 through 16] was tested in gel mobility shift assays in the presence of increasing amounts of cyclin A•cdk2 in parallel with intact E2F-1 (lanes 1 through 4) and E2F-4 (lanes 9 through 12). Recombinant proteins were tested in the absence (lanes 1, 5, 9, and 13) or presence of 3 ng (lanes 2, 6, 10, 14), 10 ng (lanes 3, 7, 11, and 15), or 20 ng (lanes 4, 8, 12, and 16) of purified cyclin

A•cdk2. A non-specific DNA-binding protein present in preparations of E2F-1(DA) shifts the mobility of the labeled probe, as indicated on the left, in a manner independent of DP-1 addition (data not shown).

F. Materials and Methods

Plasmid construction

Bacterial expression plasmids encoding full length E2F-3, E2F-4 , and E2F-5 with a three amino acid carboxy-terminal tubulin epitope were generated essentially as described (Ivey-Hoyle et al., 1993). For E2F-3, PCR reactions included the oligos (5') CCAGAG GATCCA TTGGAG GATGAT TAAATG AGAAAG GGAATC CAGCCC and (3') GAGAGC AAGCTT AGAACT CCTCAC TACACA TGAAGT CTTCCA CCAG; for E2F-4, (5') CCAGAG GATCCA TTGGAG GATGAT TAAATG GCGGAG GCCGGG CCACA and (3') GAGAGC AAGCTT AGAACT CCTCGA GGTTGA GAACAG GCACAT CAAA; for E2F-5, (5') CCAGAG GATCCA TTGGAG GATGAT TAAATG GCGGCG GCAGAG CCCGC and (3') GAGAGC AAGCTT AGAACT CCTCAT AATTTA GTATCT GGACAT CAAACA G. cDNAs of E2F-4 and E2F-5 in pBluescript were kind gifts of R. Beijersbergen and R. Bernards (E2F-4) and C. Sardet and R. Weinberg (E2F-5).

pT5T-E2F-1(DA) was constructed by replacing the BssHII-SmaI fragment (spanning E2F-1 amino acids 23 to 122) of pT5T-E2F-1 (Dynlacht et al., 1994; Huber et al., 1993) with the corresponding fragment from pRcCMV-HA-E2F-1(D24) (Krek et al., 1994) that contains a deletion in the cyclin A binding domain of E2F-1. pT5T-E2F-4(+A) was constructed in several steps as follows. Amino acid sequences in E2F-1 necessary for cyclin A binding (residues 67 to 108; Krek et al., 1994) were amplified by PCR using the following primers: 5' primer (L43) 5' CCG GGA TCC ATT GGA GGA TGA TTA ACC ATG GCC ACA CCG CAG GCG CCC 3' and 3' primer (L42) 5' GA AGA TCT AGC TGG CCC ACT GCT CTC 3'. The PCR product was digested with BamHI and BglII and inserted into the BamHI site of pCMV-E2F-4 (Ginsberg et al., 1994) so that the cyclin A binding domain of E2F-1 was fused in-frame with full-length E2F-4. This fusion product was then amplified with 5' primer (L44) 5' CCG GGA TCC ATT GGA GGA TGA T 3' and 3' primer (E2F-4-2) 5' GAG AGC AAG CTT AGA ACT CCT CGA

GGT TGA GAA CAG GCA CAT CAA A 3', cut with BamHI and HindIII and inserted into BamHI/HindIII digested pT5T vector.

Expression and purification of recombinant proteins

Insect cells (High Five; Invitrogen) were infected with recombinant baculoviruses, harvested 40-48 hours post infection, lysed in NP-40 buffer, and proteins were purified as described (Dynlacht et al., 1994), except for the cyclin D•cdk4 complexes. GST-cyclin A, GST-cyclin B, cdk6, cyclin D1 and D2, cdk2-HA, cdc2-HA, and cdk2-dn-HA viruses have been described (Desai et al., 1992; Dynlacht et al., 1994; Meyerson et al., 1994; Peeper et al., 1993). GST-cdk4 virus was a kind gift of W. Harper. Cyclin D1•GST-cdk4 complexes were isolated after disruption of infected cells by sonication in D buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 1mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% glycerol, 1 mM DTT, 0.2 mM AEBSF, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 10 mM NaF, and 50 mM sodium β-glycerophosphate; Matsushime et al., 1994) instead of NP-40-containing lysis buffer. Eluates from glutathione-agarose beads were then dialyzed against D buffer. Immuno-affinity purification of cyclin D1 and cdk6 complexes was carried out as follows. Cell lysates in D buffer were incubated with affinity-purified polyclonal antibodies against cyclin D1 or cdk6 pre-bound to protein A for 1 hour and washed with D buffer. The purity of these complexes was assessed by Coomassie and silver staining electrophoresed proteins. A recombinant p107 baculovirus that expresses a p107 fragment has been described (Zhu et al., 1995). p107 protein was purified in a single step by affinity chromatography on columns bearing an HPV E7 peptide exactly as described for the purification of recombinant pRB (Dynlacht et al., 1994). Recombinant p107•cyclin A•cdk2 complexes were generated by triply infecting insect cells with each of these viruses and purification on glutathione-agarose beads using methods for other GST proteins listed above. Recombinant E2F-1, E2F-3, E2F-4, and E2F-5 were produced and purified by anti-tubulin antibody affinity chromatography followed by heparin sepharose chromatography as previously described, as was GST-tagged DP-1 (Dynlacht et al., 1994). In order to obtain active E2F-4 and the E2F-4(+A) derivative, the purified protein was

solubilized at room temperature in 4.5 M guanidine-HCl (Gd-HCl) in the presence or absence of purified GST-DP-1. The proteins were renatured either by dialysis against 0.1 HEMG (0.1 M KCl, 25 mM Hepes, pH 7.6, 0.1 mM EDTA, 10 mM MgCl2, 10% glycerol, and 1 mM DTT) containing 0.01% NP-40 or by dilution into 0.1 HEMG buffer with 0.1% NP-40. Purification of a bacterially produced, GST-tagged, carboxy-terminal fragment of RB (Meyerson et al., 1994) was achieved as for all other GST-tagged proteins except that D Buffer was used in place of 0.1 HEMGN lysis buffer. Protein eluted from the glutathione-agarose beads was subsequently dialyzed against D buffer and concentrations determined by staining with Coomassie (GST-RB) or silver (all other proteins).

In vitro binding, immunoprecipitations, western blotting, and kinase reactions

Protein association and coupled association/kinase reactions were performed by incubating 10 to 20 ng of various cyclin•kinase complexes with E2F and DP-1 proteins in 0.1 HEMGN containing 0.1% NP-40 for 20 minutes at room temperature and then immunoprecipitating complexes with 12CA5-protein A beads. Immune complexes were washed three times with 0.1 HEMGN. To perform kinase reaction, the immunoprecipitates were washed once more with kinase buffer. Kinase reactions with [γ-32P] ATP were carried out as described below.

Kinase assays

Kinase assays were performed in buffer containing 50 mM Hepes, pH 7.0, 5 mM MnCl2, 10 mM MgCl2, 0.2 mg/ml BSA, 1mM DTT, 1 μM ATP, 2.5 μCi of [γ-32P]ATP (3000 Ci/mmole), and either 100 ng of E2F-1•DP-1, 2.5 μg of histone H1, or 1-2 μg of GST-pRB substrate by incubation at 37^{o}C for 30 minutes, unless otherwise noted.

Gel mobility shift assays

Gel mobility shift assays with recombinant E2F were carried out essentially as described (Dynlacht et al., 1994). Reactions contained approximately 5-10 ng of DP-1 and 5-10 ng E2F-1, E2F-3, E2F-5, E2F-1(DA), E2F-4(+A), or 5 ng of E2F-4•DP-1, as indicated.

In vitro transcription assays

In vitro transcription reactions were reconstituted and performed using recombinant TFIIB and TFIIE as well as partially purified HeLa cell TFIIA, TFIID, TFIIF/H, and RNA polymerase II fractions exactly as described (Dynlacht et al., 1994). Reactions contained recombinant E2F-4 or E2F-4•DP-1 as indicated.

Two dimensional tryptic phosphopeptide mapping

For phosphopeptide mapping experiments, approximately 5 ng of either purified, recombinant cyclin A•cdk2 or cyclin B•cdk2 (the amounts of each enzyme were normalized using histone H1 as a substrate; data not shown) was incubated with 100 ng of the E2F-1•DP-1 heterodimer using kinase assay conditions described above. Phosphorylated substrates were resolved on a 10% SDS-PAGE gel, transferred to Immobilon, and visualized by autoradiography. The relevant bands were excised, and two dimensional tryptic phosphopeptide mapping was performed essentially as described (Dynlacht et al, 1994).

G. Acknowledgments

We are grateful to Drs. C. Sardet, R. Weinberg, D. Ginsberg, D. Livingston, R. Beijersbergen, R. Bernards for plasmids encoding the E2F-1 D24 deletion, E2F-4, and E2F-5. We are also grateful to Drs. C-L. Wu, A. Fattaey, M. Meyerson, D. Morgan, L. Parker, H. Piwnica-Worms, J.W. Harper, and D. Beach for the gifts of recombinant baculoviruses and plasmids. Antibodies against DP-1, E2F-4, cyclin D1, and cdk6 were kind gifts of Drs. C-L. Wu, L. Zukerberg, and M. Meyerson. We thank Osvaldo Flores (Tularik, Inc.) for the initial gift of HeLa cell and recombinant transcription factor fractions. We also thank S. Woo for excellent technical help. The manuscript was improved by the suggestions of I. Sanchez. B.D.D. was supported by a Damon Runyon-Walter Winchell Cancer Fund postdoctoral fellowship and is currently supported by a Damon Runyon Scholar Award generously donated by E. and K. Langone. L.Z. was a Fellow of the Leukemia Society of America. E.H. is supported by grants from the NIH and American Cancer Society.

References

Beijersbergen, R. L., R. M. Kerkhoven, L. Zhu, L. Carlee, P. M. Voorhoeve, and R. Bernards. 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes and Dev. *8*, 2680-2690.

Cao, L., B. Faha, M. Dembski, L.-H. Tsai, E. Harlow, and N. Dyson. 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature *355*, 176-179.

Chen, I.-T., M. Akamatsu, M. L. Smith, F.-D. T. Lung, D. Duba, P. P. Roller, J. Fornace, A.J., and P. M. O'Conner. 1996a. Characterization of p21cip1/waf1 peptide domains required for cyclin E/cdk2 and PCNA interactions. Oncogene *12*, 595-607.

Chen, J., P. Saha, S. Kornbluth, B. D. Dynlacht, and A. Dutta. 1996b. Cyclin-binding motifs are essential for the function of p21cip1. Mol. Cell. Biol. *16*, 4673-4682.

Desai, D., Y. Gu, and D. Morgan. 1992. Activation of human cyclin-dependent kinases in vitro. Mol. Biol. Cell *3*, 571-582.

Devoto, S. H., M. Mudryj, J. Pines, T. Hunter, and J. R. Nevins. 1992. A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F- cyclin A complex. Cell *68*, 167-176.

Dowdy, S. F., P. W. Hinds, K. Louie, S. I. Reed, A. Arnold, and R. A. Weinberg. 1993. Physical interaction of the retinoblastoma protein with human D cyclins. Cell *73*, 499-511.

Dynlacht, B. D., O. Flores, J. A. Lees, and E. Harlow. 1994. Differential regulation of E2F trans-activation by cyclin-cdk2 complexes. Genes & Dev. *8*, 1772-1786.

Dyson, N. 1994. pRB, p107, and the regulation of the E2F transcription factor. J. Cell Sci. Suppl. *18*, 81-87.

Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J. Kato, and D. M. Livingston. 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell *73*, 487-497.

Faha, B., M. Ewen, L.-H. Tsai, D. Livingston, and E. Harlow. 1992. Interaction between human cyclin A and adenovirus E1A-associated p107 protein. Science *255*, 87-90.

Field, S. J., F.-Y. Tsai, F. Kuo, A. M. Zubiaga, J. Kaelin, W.G., D. M. Livingston, S. H. Orkin, and M. E. Greenberg. 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell *85*, 549-561.

Ginsberg, D., G. Vairo, T. Chittenden, Z. X. Xiao, G. Xu, K. L. Wydner, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston. 1994. E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes and Dev. *8*, 2665-79.

Helin, K., and E. Harlow. 1993. The retinoblastoma protein as a transcriptional repressor. Trends in Cell Biol. *3*, 43-46.

Helin, K., E. Harlow, and A. R. Fattaey. 1993. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. *13*, 6501-6508.

Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey. 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell *70*, 337-350.

Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins. 1992. The interaction of pRB with E2F inhibits the transcriptional activity of E2F. Genes and Dev. *6*, 177-185.

Hijmans, E. M., P. M. Voorhoeve, R. L. Beijersbergen, L. van 'T Veer, and R. Bernards. 1995. E2F-5, a new E2F family member that interacts with p130 in vivo. Mol. Cell. Biol. *15*, 3082-3089.

Huber, H. E., G. Edwards, P. J. Goodhart, D. R. Patrick, P. S. Huang, M. Ivey-Hoyle, S. F. Barnett, A. Oliff, and D. C. Heimbrook. 1993. Transcription factor E2F binds DNA as a heterodimer. Proc. Natl. Acad. Sci. USA *90*, 3525-3529.

Hunter, T., and Pines, J. 1994. Cyclins and Cancer II: cyclin D and cdk inhibitors come of age. Cell *79*, 573-582.

Ikeda, M.-A., L. Jakoi, and J. R. Nevins. 1996. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. Proc. Natl. Acad. Sci. USA *93*, 3215-3220.

Ivey-Hoyle, M., R. Conroy, H. Huber, P. Goodhart, A. Oliff, and D. C. Heinbrook. 1993. Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol. Cell. Biol. *13*, 7802-7812.

Johnson, D. G., J. K. Schwartz, W. D. Cress, and J. R. Nevins. 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature *365*, 349-352.

Kaelin, W. G., Jr., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington. 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell *70*, 351-64.

Kato, J.-Y., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr. 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRB) and pRB phosphorylation by the cyclin D dependent kinase CDK4. Genes and Dev. *7*, 331-342.

Kowalik, T. F., J. DeGregori, J. K. Schwarz, and J. R. Nevins. 1995. E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J. Virol. *69*, 2491-2500.

Krek, W., M. Ewen, E., S. Shirodkar, Z, Z. Arany, W. Kaelin, G., and D. Livingston. 1994. Negative Regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell *78*, 161-172.

Krek, W., G. Xu, and D. M. Livingston. 1995. Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. Cell *83*, 1149-1158.

Lees, E., B. Faha, V. Dulic, S. I. Reed, and E. Harlow. 1992. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. Genes and Dev. *6*, 1874-1885.

Lin, J., C. Reichner, X. Wu, and A. Levine. 1996. Analysis of wild-type and mutant p21WAF activities. Mol. Cell. Biol. *16*, 1786-1793.

Luo, Y., J. Hurwitz, and J. Massague. 1995. Cell cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. Nature *375*, 159-161.

Matsushime, H., M. E. Ewen, D. K. Strom, J.-Y. Kato, S. K. Hanks, M. F. Roussel, and C. J. Sherr. 1992. Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type cyclins. Cell *71*, 323-334.

Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J.-Y. Kato. 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. *14*, 2066-2076.

Meyerson, M., and E. Harlow. 1994. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell Biol. *14*, 2077-2086.

Moberg, K., M. A. Starz, and J. A. Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell. Biol. *16*, 1436-1449.

Muller, R. 1995. Transcriptional regulation during the mammalian cell cycle. Trends in Genet. *11*, 173-178.

Nevins, J. R. 1992. E2F: A link betwen the Rb Tumor suppressor protein and viral oncoproteins. Science *258*, 424-429.

Peeper, D. S., L. L. Parker, M. E. Ewen, M. Toebes, F. L. Hall, M. Xu, A. Zantema, A. J. van der Eb, and H. Piwnica-Worms. 1993. A- and B-type cyclins differentially modulate substrate specificity of cyclin-cdk complexes. EMBO J. *12*, 1947-54.

Qin, X.-Q., D. M. Livingston, W. G. J. Kaelin, and P. Adams. 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. U.S.A. *91*, 10918-10922.

Russo, A. R., P. D. Jeffrey, A. K. Patten, J. Massague, and N. P. Pavletich. 1996. Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. Nature *382*, 325-331.

Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R. A. Weinberg. 1995. E2F-4 and E2F-5, two novel members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. USA *92*, 2403-2407.

Shan, B., and W.-H. Lee. 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Molec. Cell. Biol. *14*, 8166-8173.

Shan, B., X. Zhu, P.-L. Chen, T. Durfee, Y. Yang, D. Sharp, and W.-H. Lee. 1992. Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol. Cell. Biol. *12*, 5620-5631.

Shirodkar, S., M. Ewen, J. A. DeCaprio, D. Morgan, D. Livingston, and T. Chittenden. 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell *68*, 157-166.

Singh, P., S. H. Wong, and W. Hong. 1994. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. EMBO J. *13*, 3329-38.

Vairo, G., D. M. Livingston, and D. Ginsberg. 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes and Dev. *9*, 869-881.

van den Heuvel, S., and E. Harlow. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. Science *262*, 2050-2054.

Weintraub, S. J., C. A. Prater, and D. C. Dean. 1992. Retinoblastoma protein swithches the E2F site from positive to negative element. Nature *358*, 259-261.

Wu, X., and A. J. Levine. 1994. p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA *91*, 3602-3606.

Xu, G., D. M. Livingston, and W. Krek. 1995. Multiple members of the E2F transcription factor family are the products of oncogenes. Proc. Natl. Acad. Sci. USA *92*, 1357-1361.

51. Xu, M., K. A. Sheppard, C. Y. Peng, A. S. Yee, and H. Piwnica-Worms. 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. Cell. Biol. *14*, 8420-31.

Yamasaki, L., T. Jacks, R. Bronson, E. Goillot, E. Harlow, and N. J. Dyon. 1996. Tumor induction and tissue atrophy in mice lacking E2F-1. Cell *85*, 537-548.

Zhu, L., E. Harlow, and B. D. Dynlacht. 1995. p107 uses a p21CIP1-related domain to bind cyclin/cdk2 and regulate interactions with E2F. Genes and Dev. *9*, 1740-1752.

CHAPTER 5

"Inhibition of CDK2 by p21 is necessary for pRB-mediated G_1 Arrest following γ-Irradiation"

James Brugarolas, Ken Moberg, Scott D. Boyd, Yoichi Taya, Tyler Jacks and Jacqueline A. Lees

Genes and Development, in press

(Author's contribution: Figure 3)

A. Abstract

In mammalian cells, DNA damaging agents result in a G1 arrest that is dependent upon the tumor suppressor p53 and its transcriptional target p21. Using primary cell lines lacking specific cell cycle regulators, we demonstrate that this pathway functions through the growth suppressive properties of the pRB tumor suppressor. Specifically, γ-irradiation inhibits the phosphorylation of pRB at cdk2- but not cdk4-specific sites in a p21-dependent manner. Most importantly, we show that pRB is a critical component of this DNA damage checkpoint. These data indicate that the p53 $\Rightarrow$ p21 checkpoint pathway uses the normal cell cycle regulatory machinery to induce the accumulation of the growth suppressive form of pRB.

B. Introduction

The retinoblastoma gene (*Rb*) was originally cloned by virtue of its absence in retinoblastomas (for review, see Weinberg 1992). Subsequent studies showed that *Rb* gene mutations exist in approximately one third of all human tumors (for review, see Weinberg 1992). These mutations result in either complete loss or functional inactivation of the retinoblastoma protein and reintroduction of the wild-type gene is sufficient to reverse the tumorigenicity of several *Rb* negative cell lines.

The growth suppressive properties of the retinoblastoma protein (pRB) are thought to be dependent upon its ability to regulate the cellular transcription factor E2F (for review, see Nevins 1997). pRB binds to E2F *in vivo* and this association is sufficient to inhibit its transcriptional activity. Moreover, the resulting pRB•E2F complex is capable of mediating the transcriptional repression of E2F-responsive genes (Weintraub et al. 1992; Bremner et al. 1995; Weintraub et al. 1995). Many E2F-responsive genes have been identified and they each play a critical role in the control of cellular proliferation (for review, see Nevins et al. 1997). In addition, E2F binding maps to the "growth suppression" domain of pRB and mutant, tumor derived-forms of the retinoblastoma protein all lack the ability to bind to E2F (for review, see Nevins et al. 1997).

The retinoblastoma protein is phosphorylated in a cell cycle dependent manner and these modifications are sufficient to inactivate its ability to bind to E2F and to block cell division (for review, see Dyson and Harlow 1992). Several pRB phosphorylation sites have been identified and they each match the consensus recognition sequence of the cyclin-dependent kinases (cdks; Lees et al. 1991). The G_1 cdks, cyclin D•cdk4/6 and cyclin E•cdk2, can both phosphorylate pRB *in vitro* (Hinds et al. 1992; Ewen et al. 1993; Matsushime et al. 1994; Meyerson and Harlow 1994). In these *in vitro* assays, cyclin D•cdk4 and cyclin E•cdk2 preferentially phosphorylate distinct but overlapping pRB sites (Connell-Crowley et al. 1997; Zarkowska and Mittnacht 1997). Cyclin D•cdk4 is the first cdk to be activated in response to growth factors (Matsushime et al. 1994) and *in vivo* studies confirm that it is essential for pRB inactivation (Lukas et al. 1995a; Lukas et al.

1995b). Indeed, pRB appears to be the only essential target of this kinase (Lukas et al. 1995a; Lukas et al. 1995b). Studies addressing the role of cyclinE•cdk2 in pRB regulation have yielded conflicting conclusions (Connell-Crowley et al. 1997; Ezhevsky et al. 1997; Lundberg and Weinberg 1998). One study showed that cyclin D1/cdk4 was sufficient to inactivate both the E2F binding and growth suppressive properties of pRB (Connell-Crowley et al. 1997). However, two other labs have reported that inhibition of cdk2 (by either treatment with TGFβ or over-expression of dominant negative cdk2) resulted in the accumulation of an under-phosphorylated form of pRB that can still bind to E2F (Ezhevsky et al. 1997; Lundberg and Weinberg 1998). These latter studies suggest that cyclinE•cdk2 contributes to inactivation of the growth suppressive properties of pRB. In contrast to the D-type kinases, cyclinE•cdk2 is known to have at least one other substrate whose phosphorylation is essential for S-phase entry (Ohtsubo et al. 1995).

Superimposed on the normal cell cycle regulation are a number of checkpoint mechanisms. These are not required for normal cell cycle progression but are critical for the cellular response to stress (for review, see Paulovich et al. 1997). One of the best characterized of the mammalian checkpoint pathways is the DNA damage-induced G_1 arrest. This checkpoint is dependent upon the tumor suppressor p53 (Kastan et al. 1992). The loss of p53 abrogates the DNA damage response and this is thought to contribute to tumorigenesis by permitting the propagation of mutations (for review, see Lane 1992). The mechanism by which p53 imposes the DNA-damage induced G_1 arrest has been partially elucidated. In response to irradiation, p53 induces the transcription of the *p21* gene (El-Deiry et al. 1993), which encodes an inhibitor of cyclin-dependent kinases (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993). Analysis of *p21*-deficient cells confirms that the p21 protein is essential for the integrity of the DNA damage-induced G_1 arrest (Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995). In normal cells, this p21-dependent arrest correlates with the accumulation of hypo-phosphorylated pRB (Dulic et al. 1994; Slebos et al. 1994). Although this change in pRB phosphorylation could arise as an indirect consequence of the G_1 arrest, the presence of the pRB-binding, papilloma virus E7 protein has been shown to abrogate the DNA damage checkpoint (Slebos et al. 1994). These data

suggest that pRB is involved in the DNA damage response, however, the multifunctional nature of E7 makes it difficult to eliminate other possible mechanisms (Funk et al. 1997; Jones et al. 1997).

In this study, we investigate how the p53 $\Rightarrow$ p21 DNA damage checkpoint pathway interfaces with the normal cell cycle machinery. Using cell lines derived from mutant mouse strains, we demonstrate that the p21-mediated arrest is dependent upon the presence of active, growth suppressive pRB. Induction of the checkpoint by low doses of γ-irradiation, results in the downregulation of cdk2, but not cdk4, kinase activity and the accumulation of partially phosphorylated pRB. Phosphopeptide specific antibodies confirm that this form of pRB has been phosphorylated on cdk4- but not cdk2-specific sites. Thus, p21 arrests cells by blocking the inactivation of pRB that normally occurs as cells progress through the G_1 phase of the cell cycle. Our data supports a model whereby this checkpoint blocks cell cycle progression by co-opting normal cell cycle regulatory mechanisms.

C. Results

γ-irradiation of fibroblasts results in the activation of the DNA damage checkpoint pathway and thereby induces cell cycle arrest (Kastan et al. 1991). It is well documented that p21 acts as an important downstream target of this p53-dependent radiation response but the mechanism of p21 action is unknown (Dulic et al. 1994; Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995). Because the pRB-binding, papilloma virus E7 protein is sufficient to override the DNA damage checkpoint (Slebos et al. 1994), we tested if pRB is required for the p53 ⇒ p21-mediated arrest. To address this question, we generated primary mouse embryo fibroblasts (MEFs) from wild-type, *p53-/-*, *p21-/-* and *Rb-/-* mouse strains and tested their response to DNA damage. Wild-type and mutant cells were exposed to γ-irradiation and the degree of G_1 arrest was assessed by comparing the proportion of S-phase cells in irradiated versus unirradiated populations (Fig. 1). Consistent with previous studies, loss of p53 dramatically impairs the G_1 cell cycle block. In contrast, loss of p21 significantly reduced, but did not abolish, this γ-irradiation induced arrest. This supports previous conclusions that p21 is a critical downstream target of p53 but it is not the only mechanism by which p53 can mediate the DNA damage-induced G_1 arrest (Brugarolas et al. 1995; Deng et al. 1995).

When tested in this assay, *Rb*-deficient fibroblasts were also impaired in their ability to arrest in G_1 in response to DNA damage. Significantly, the magnitude of this defect was similar to that observed in the *p21-/-* cells, suggesting that p21 and pRB act in the same DNA damage response pathway. To test this hypothesis, we generated mouse embryos that were deficient for both *p21* and *Rb* and then compared the irradiation response of single and double mutant MEFs derived from littermate embryos (Fig. 1). The G1 arrest response of the *p21-/-;Rb-/-* cells was indistinguishable from that of either of the single mutant MEFs. We therefore conclude that p21 and pRB likely act in the same p53-dependent checkpoint pathway.

To investigate the mechanism by which p21 and pRB participate in the p53-dependent G_1 arrest, we first tested whether the absence of these proteins affects the regulation of the cell cycle

dependent kinases that control the G_1/S-transition. p21 is known to inhibit the activity of both cyclin D•cdk4 and cyclin E•cdk2 (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993) and these G_1 kinases are downregulated in response to a variety of DNA damaging agents (Terada et al. 1995; Poon et al. 1996). We therefore compared the level of cdk4 and cdk2 kinase activity in wild-type and *p21*-deficient cells either with or without γ-irradiation treatment (Fig. 2A). We did not detect any effect of γ-irradiation on the level of cdk4 kinase activity in either cell type. In contrast, γ-irradiation significantly reduced the level of cdk2 kinase activity in wild-type but not *p21-/-* cells. These data suggest that the observed p21-dependent, radiation induced G_1 arrest is mediated through the specific inhibition of cdk2 but not cdk4 kinase activity.

As pRB is known to control the expression of the cdk2 regulatory subunit, cyclin E, it seemed possible that the loss of pRB could impair the DNA damage response by altering the regulation of cdk2 kinase activity. To address this possibility, we directly compared the level of cdk2 kinase activity in wild-type and *pRB-/-* cells both before and after γ-irradiation (Fig. 2A). Although the radiation response was significantly impaired in the *Rb-/-* fibroblasts (Fig. 1), both the basal level of cdk2 kinase activity and the degree of its downregulation in response to γ-irradiation were similar to those observed in the wild-type cells (Fig. 2A). Moreover, the radiation induced inhibition of cdk2 kinase activity was completely abolished in the *p21-/-;Rb-/-* MEFs confirming that downregulation of this kinase in the absence of pRB is still dependent upon p21 (Fig. 2A). We therefore conclude that loss of pRB does not interfere with either the induction of p21 or the specific inhibition of the cdk2 kinase activity in response to γ-irradiation. These observations strongly suggest that pRB acts downstream of p21 in the p53-dependent checkpoint pathway.

There is good evidence to suggest that the cdk2 kinase contributes to the phosphorylation of the retinoblastoma protein and that this is required to inactive its growth suppressive properties (Ezhevsky et al. 1997; Lundberg and Weinberg 1998). To test the role of pRB phosphorylation in the DNA damage response, we immunoprecipitated pRB from wild-type (downregulated cdk2 kinase) and *p21-/-* (normal cdk2 kinase) irradiated cells that had been metabolically labeled with

$^{32}P_i$ (Fig. 2B). Consistent with the continued presence of cdk4 kinase activity (see Fig. 2A), we detected phosphorylated pRB in extracts of wild-type irradiated cells (Fig. 2B). This phosphorylated pRB was present in two distinct forms, a slower migrating species (labeled band A) and a faster migrating species (labeled band B), that were detected at roughly equal levels. Both of these pRB species were also present in the irradiated p21-deficient cells. In these cells, however, the slower migrating form of pRB (band A) was present at much higher levels than the faster migrating form (band B). This suggested that γ-irradiation results in a change in the degree of pRB phosphorylation in a p21-dependent manner.

To assess the relative levels of each pRB species (instead of the degree of ^{32}P incorporation), we also performed immunoprecipitation followed by immuno-blotting of whole cell extracts of wild-type and *p21-/-* irradiated cells (Fig. 2C). In this assay, we were able to detect three distinct pRB species. The fastest migrating form (band C) was not detected by ^{32}P labeling, indicating that it represents unphosphorylated pRB. This was present at a similar low level in both the wild-type and *p21-/-* irradiated cells. The remaining bands co-migrated with the ^{32}P-labeled band A and band B. Comparison of the ^{32}P and immunoblotting signals for bands A and B suggested that band A corresponds to the hyperphosphorylated form of pRB whereas band B results from the partial phosphorylation of the pRB protein. Significantly, whereas the protein levels of partially phosphorylated pRB (band B) were similar in the wild-type and mutant irradiated cells, the fully phosphorylated form of pRB was significantly reduced in wild-type cells. We therefore conclude that γ-irradiation blocks the conversion of partially- to hyper-phosphorylated pRB in a p21-dependent manner.

The difference between the partially- and hyper-phosphorylated forms of pRB could be due to differences in either the specific sites of phosphorylation or the extent to which pRB is phosphorylated. To distinguish between these two possibilities, we compared the two dimensional tryptic phosphopeptide maps of pRB species isolated from either wild-type (predominantly band B) or *p21-/-* (band A and B) irradiated cells. In wild-type irradiated cells, we detected six prominent pRB tryptic phosphopeptides (1-6; Fig. 3). Thus, pRB is phosphorylated at multiple

sites in wild-type cells after γ-irradiation. The two-dimensional phosphopeptide map of pRB from *p21-/-* cells was considerably more complex, containing more than 15 major phosphopeptides (1-15; Fig. 3). Six of these phosphopeptides (1-6) were identical to those detected in the map of pRB from wild-type cells. The remainder (phosphopeptides 7-15) were either greatly under-represented or completely absent from the two dimensional map of pRB derived from wild-type cells (Fig. 3). The simplest interpretation of these data is that the novel phosphopeptides are derived from the *p21-/-* specific species of pRB, band A, and that transition from partially- to hyper-phosphorylated pRB must therefore involve the phosphorylation of a novel set of sites. By extension of this logic, we conclude that the *p21-/-* cells must contain at least two pRB-kinases with different site specificities; one that is similarly active in wild-type and *p21-/-* irradiated cells and results in phosphorylation of a subset of pRB sites (phosphopeptides 1-6) and one that is exclusively active in *p21-/-* irradiated cells and results in the hyper-phosphorylation of pRB through the specific modification of a distinct set of sites (phosphopeptides 7-15).

Our kinase assays (Fig. 2A) showed that cdk4 kinase activity was present at similar levels in both wild-type and *p21-/-* irradiated cells but cdk2 activity was only present in *p21-/-* irradiated cells. This suggested that cdk4 accounts for the partial phosphorylation of pRB (phosphopeptides 1-6), whereas cdk2 is responsible for phosphorylating the second set of sites that switch pRB from the partially- to the hyper-phosphorylated form (phosphopeptides 7-15). To test this hypothesis, we employed a panel of antibodies that specifically recognize individual pRB-phosphopeptides that are preferentially phosphorylated by either cyclinD•cdk4 or cyclinE•cdk2 *in vitro* (Kitagawa et al. 1996). These antibodies were used to screen immunoblots of pRB-immunoprecipitates from either wild-type or *p21-/-* irradiated cells (Figure 4). CyclinD•cdk4 is known to specifically phosphorylate Ser780 of human pRB with a 20-60 fold higher efficiency than either Cyclin E•cdk2 or Cyclin A•cdk2 (Kitagawa et al. 1996). Antibodies specific for phospho-Ser780, recognized both the partially and the hyper-phosphorylated form of pRB indicating that the cdk4 kinase contributes to the phosphorylation of both of these species (Fig. 4). In contrast, an antibody directed against a cdk2-specific pRB site, α-phospho-Ser811 of human pRB (Connell-Crowley et

al. 1997; Y. Taya, unpublished data), recognized the hyper-phosphorylated form of pRB in *p21-/-* irradiated cells but failed to detect the partially phosphorylated pRB species in either wild-type or *p21-/-* irradiated cells (Fig. 4). Similar results were obtained with a second cdk2-specific phosphorylation site antibody, α-phospho-Thr350 (Fig 4). Taken together, these data indicate that the cdk4 kinase is responsible for the partial phosphorylation of pRB *in vivo*, but active cdk2 kinase is required to phosphorylate the additional sites specifically modified in hyper-phosphorylated pRB. Most importantly, our data suggest that the p53 ⇒ p21 pathway arrests cells in response to low doses of γ-irradiation by specifically inhibiting the cdk2 kinase and thereby preventing the transition of pRB from the partially phosphorylated to the hyper-phosphorylated form. Given the requirement of pRB for the integrity of the G_1 arrest, we conclude that partially phosphorylated pRB is essential for this growth arrest.

D. Discussion

The ability of cells to arrest in G_1 in response to DNA damage is dependent upon the accumulation of the tumor suppressor, p53 (Kastan et al. 1992). This p53-dependent G_1 arrest is largely mediated through the induction of the cdk inhibitor p21 (Brugarolas et al. 1995; Deng et al. 1995). By analyzing primary cell lines lacking specific cell cycle regulators, we have investigated the mechanism by which p21 brings about a G_1 arrest following treatment with low dose γ-irradiation. Our data indicate that the induction of p21 results in the specific inhibition of cdk2 but not cdk4 kinase activity. Moreover, the consequent G_1 arrest is dependent upon the presence of functional retinoblastoma protein. Absence of pRB does not significantly alter either the basal level of cdk2 kinase activity or the ability of p21 to downregulate it in response to γ-irradiation. Instead, in wild-type cells γ-irradiation results in a p21-dependent alteration in the phosphorylation status of pRB. This arises from the loss of phosphorylation at cdk2- but not cdk4-specific sites. Although we cannot rule out the possibility that pRB participates in the p53-dependent checkpoint pathway in an indirect manner, these observations strongly suggest that pRB acts as a downstream target of p21. We therefore favor a model in which p21 acts to impose the G_1 arrest by specifically inhibiting the cdk2 kinase and thereby preventing inactivation of the growth suppressive properties of the pRB tumor suppressor (Fig. 5). In this manner, pRB plays a critical role in determining whether or not a cell will initiate DNA replication in the presence of DNA damage.

Our observations also provide considerable insight into the role of pRB in normal cell cycle control (see Figure 5). Recent studies suggest that pRB is phosphorylated in a two step process during the normal cell cycle (Kitagawa et al. 1996; Ezhevsky et al. 1997; Lundberg and Weinberg 1998). CyclinD•cdk4/6 specifically phosphorylates pRB at a subset of its phosphorylation sites. However, complete phosphorylation of pRB requires cyclinE•cdk2 to specifically target the remaining phosphorylation sites. In each case, inhibition of cdk2 resulted in a G_1 block that correlates with inhibition of the second step of pRB phosphorylation (Ezhevsky et al. 1997;

Lundberg and Weinberg 1998). Our current data are entirely consistent with the notion that cyclinD•cdk4/6 and cyclinE•cdk2 mediate the sequential phosphorylation of pRB through the phosphorylation of distinct subsets of sites within this protein. These findings further indicate that the p53 $\Rightarrow$ p21 checkpoint pathway is able to impose a G_1 block by specifically inhibiting cdk2 kinase activity and thereby only the second step of pRB phosphorylation. These findings do not rule out the possibility that there are other cdk2 substrates whose phosphorylation is critical for S-phase entry. Indeed, Serrano et al. (1995) have shown that over-expression of p21 can inhibit cell cycle entry in an pRB-negative tumor cell line. However, our data strongly suggest that partially phosphorylated pRB retains the ability to prevent cell cycle progression. By extension of this logic, cyclinD•cdk4-dependent phosphorylation is insufficient to inactivate the growth suppressive properties of pRB. Although these conclusions were derived from the analysis of the DNA damage response, it seems highly likely that the same mechanisms will control the timing of S-phase entry in the normal cell cycle.

This model raises clear questions about the role of cyclinD•cdk4 in the regulation of pRB. It is possible that this kinase affects a pRB function that is unrelated to the control of cell cycle entry. However, there is extensive data to suggest that cyclinD•cdk4 plays a critical role in overriding the growth suppressive properties of pRB (Hinds et al. 1992; Matsushime et al. 1994; Lukas et al. 1995a; Lukas et al. 1995b). Alternatively, the inactivation of pRB by cyclinE•cdk2 may be entirely dependent upon the prior phosphorylation of pRB by cyclinD•cdk4. This mechanism would provide two distinct points at which extracellular signals and/or checkpoint pathways could influence the state of pRB phosphorylation and therefore the cell division process. Significantly, very high doses of γ-irradiation can result in the inhibition of both cdk2 and cdk4 kinase activity (Terada et al. 1995). Together with our data, this observation suggests that the DNA damage checkpoint can block pRB phosphorylation by specifically inhibiting either one (cyclinE•cdk2) or both (cyclinD•cdk4 and cyclinE•cdk2) of the pRB kinases depending on the severity of the DNA damage. The mechanism of inactivation of cyclinD•cdk4 in this response has yet to be established. However, it is now clear that the modulation of the site-specific

phosphorylation state of pRB is a critical control point in both normal cell cycle regulation and the DNA damage checkpoint.

Our observation that pRB is a key component of the p53-dependent G_1 arrest may also help to explain how chemotherapeutic agents target tumor versus normal cells. Many anti-neoplastic treatments cause DNA damage that results in the activation of p53. Significantly, these events have a differential effect on normal and tumor cells; the tumor cells are more likely to undergo p53-dependent apoptosis whereas their normal cellular counterparts preferentially activate the p53-dependent G_1 arrest pathway (Lowe et al. 1993). Given the high frequency of pRB inactivation in human tumors (for review, see Weinberg 1992), our data suggest that the propensity of tumor cells to undergo p53-dependent apoptosis could arise from their inability to enforce a pRB-dependent cell cycle arrest. This hypothesis is directly supported by the observation that certain antineoplastic drugs cause *Rb+/+* and *Rb+/-* fibroblasts to arrest but induce *Rb-/-* fibroblasts to apoptose (Almasan et al. 1995). Taken together, these findings suggest that the clinical efficacy of chemotherapeutic agents will be influenced by both the *p53* and *pRB* status of the target tumor.

Figure 1.

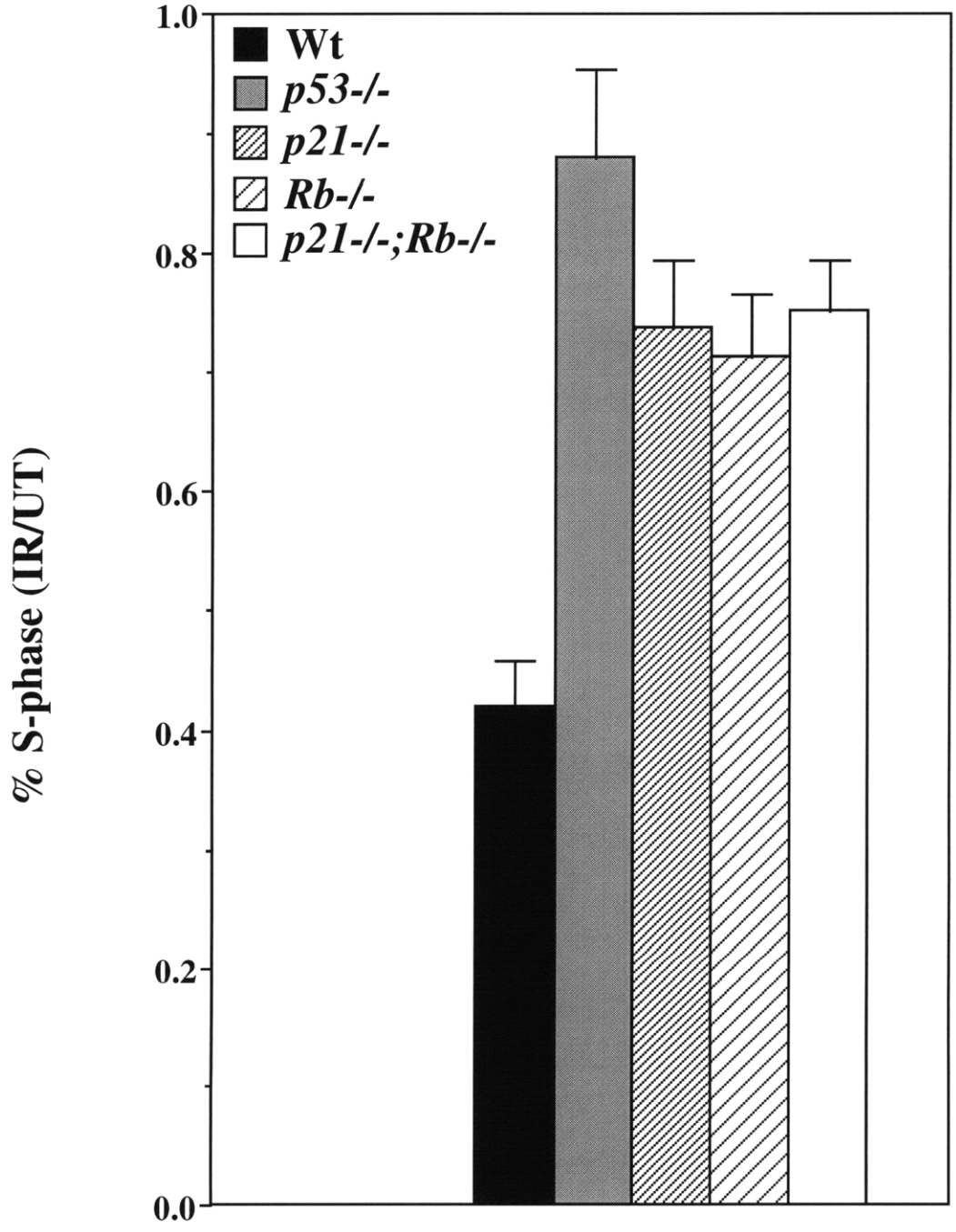


Figure 2.

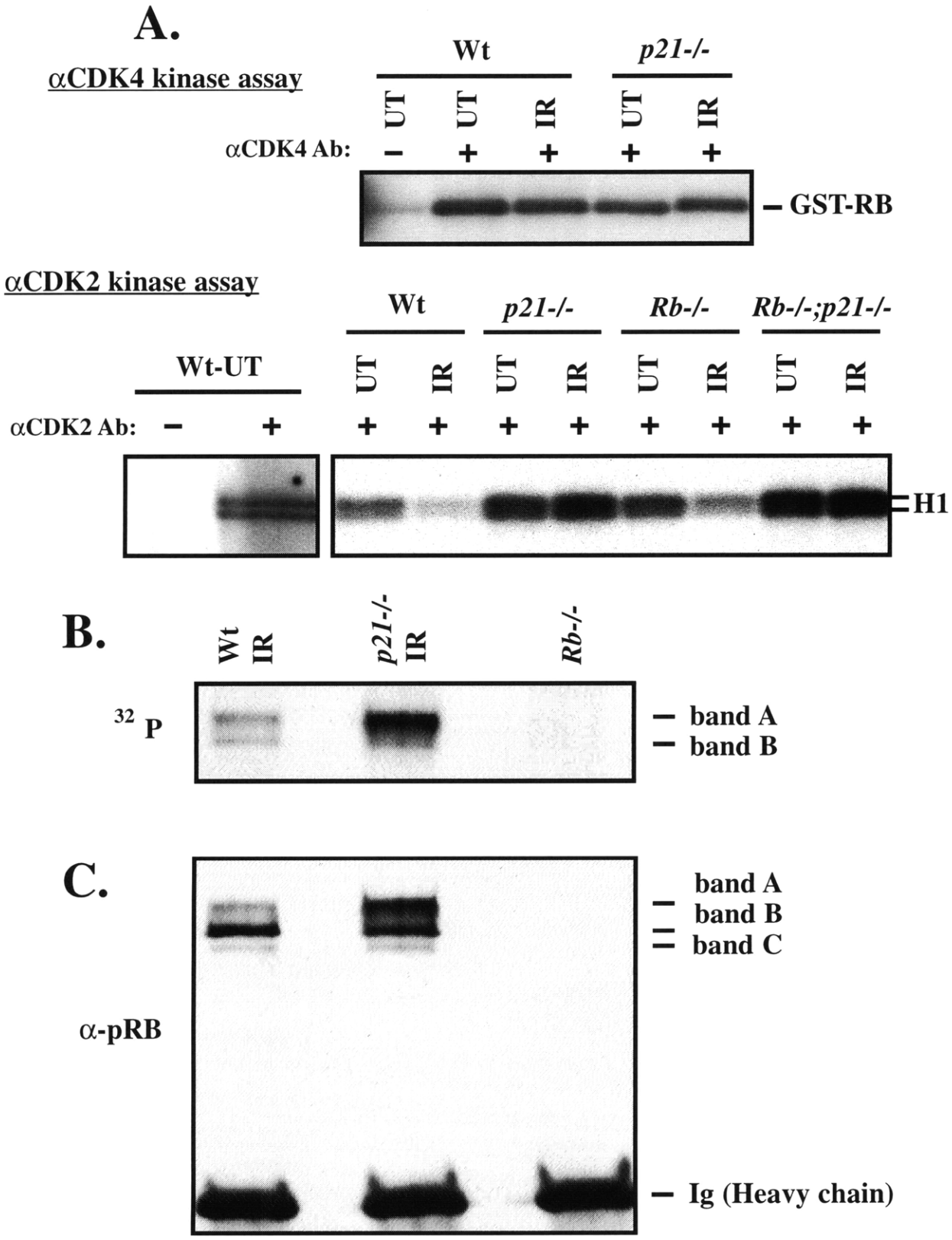

A.
αCDK4 kinase assay
Wt
p21-/-
UT
UT
IR
UT
IR
αCDK4 Ab:
–
+
+
+
+
GST-RB
αCDK2 kinase assay
Wt
p21-/-
Rb-/-
Rb-/-;p21-/-
Wt-UT
UT
IR
UT
IR
UT
IR
UT
IR
αCDK2 Ab:
–
+
+
+
+
+
+
+
+
+
H1
B.
Wt
IR
p21-/-
IR
Rb-/-
32 P
band A
band B
C.
band A
band B
band C
α-pRB
Ig (Heavy chain)

Figure 3.

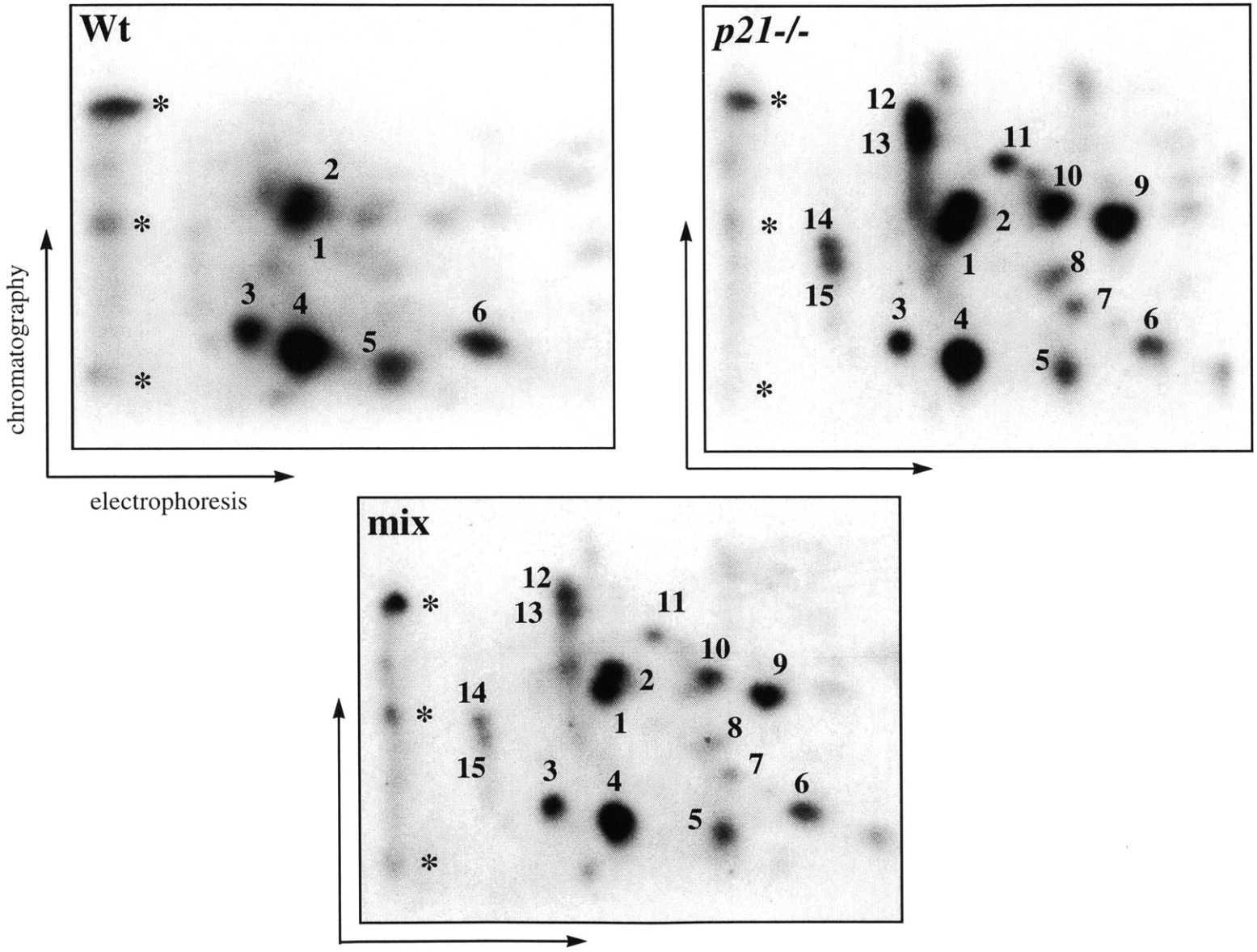

Wt
p21-/-
mix
chromatography
electrophoresis
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
*

Figure 4.

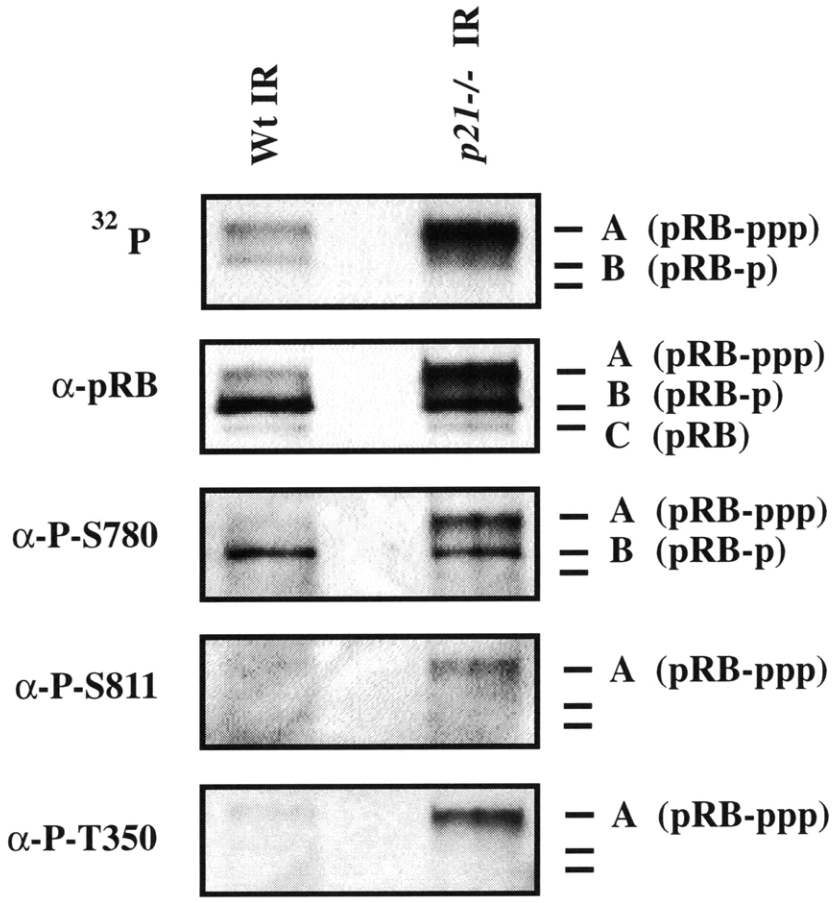
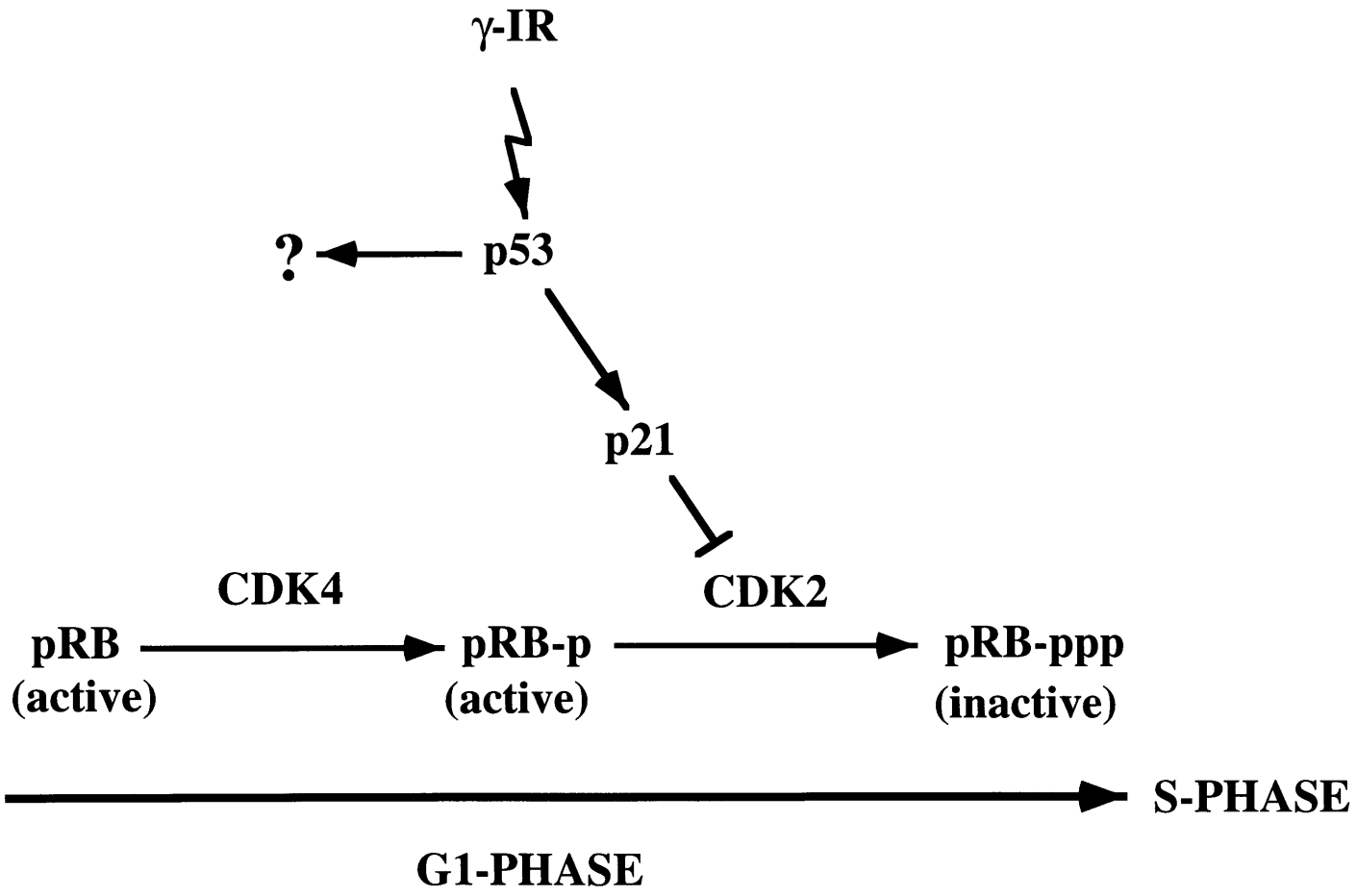

Wt IR
p21-/- IR
32 P
α-pRB
α-P-S780
α-P-S811
α-P-T350
A (pRB-ppp)
B (pRB-p)
C (pRB)

Figure 5.


γ-IR
?
p53
p21
CDK4
CDK2
pRB
(active)
pRB-p
(active)
pRB-ppp
(inactive)
S-PHASE
G1-PHASE

E. Figure Legends

Figure 1. Analysis of the genetic requirements of the DNA damage-induced G_1 arrest response. Asynchronous cultures of wild-type, *p53-/-* , *p21-/-*, *Rb-/-* and *p21-/-;Rb-/-* fibroblasts were irradiated with a dose of 5.5 Gy and labeled with BrdU for 4 hours beginning 14 hours after irradiation. Histogram shows the S-phase fraction of irradiated versus untreated samples with the mean and standard deviations (error bars) from four independent experiments.

Figure 2. Effect of γ-irradiation upon G_1 cdk kinase activity and pRB phosphorylation. (*A*) Normalized protein extracts from untreated or γ-irradiated (18 hours) cells were precleared with protein A beads, immunoprecipitated with the indicated antibodies and then assayed for kinase activity by incubation with an excess of [γ-^{32}P]ATP and substrate (histone H1 or a C-terminal fragment of pRB). (*B*) SDS-PAGE analysis of [^{32}P]pRB from wild-type and *p21-/-* cells 18 hours after irradiation. (*C*) Western blot analysis of [^{32}P]pRB with an α-pRB monoclonal antibody.

Figure 3. Two-dimensional phospho-tryptic mapping of pRB derived from wild-type or *p21-/-* irradiated cells. pRB was immunoprecipitated from either wild-type or *p21-/-* irradiated cells and subjected to trypsin digestion. The resulting phosphopeptides were resolved by electrophoresis and ascending chromatography and visualized by autoradiography. (The asterisk denotes phosphopeptides that were present in maps from *Rb-/-* cells and are therefore not derived from pRB.)

Figure 4. Phosphorylation status of cdk2- and cdk4-specific pRB phosphorylation sites. Immunoblot analysis of immunporecipitated pRB with α-P-S780 (cdk4 site), α-P-S811 (cdk2 site) and α-P-T350 (cdk2 site).

Figure 5. Model of DNA damage induced G_1-arrest.

F. Materials and Methods

Irradiation and cell cycle analysis

Sparse cultures of MEFs were irradiated with 5.5 Gy using a γ-cell irradiator with a *Cs* source. Untreated and irradiated cultures were harvested for cell cycle analysis 18 hours after γ-irradiation. Cell cycle analysis were performed as described (Brugarolas et al. 1995).

In vitro kinase assays

Cdk2 and cdk4 *in vitro* kinase assays were performed as described (Brugarolas et al. 1998). Briefly, cell lysates were precleared with equilibrated protein A beads (Pierce) and incubated with anti-cdk4 (Santa Cruz, C-22) or anti-cdk2 antibody (kindly provided by G. J. Hannon, CSH, NY) for 4 hours. Immune complexes were precipitated with protein A beads (Pierce) and incubated in the kinase buffer containing 4 mM ATP, 20 μCi [γ-^{32}P] ATP (NEN-DuPont) and 6 μg of GST-RB (GST fusion with amino acids 792-928 from the C-terminus of pRB) or 2 μg of histone H1 (Sigma), for 30-60 minutes at 30^{o}C. Quantitation was performed by phosphorimager analysis.

^{32}P-labeling and pRB immunoprecipitation

Subconfluent MEFs cultures were labeled with 5 mCi per ml HCl free ^{32}P-orthophosphate (NEN-DuPont) in DME supplemented with 5% dialyzed fetal bovine serum. Labeling proceeded for 4 hours starting 14 hours after γ-irradiation. Protein extracts were prepared as described (Brugarolas et al. 1998), normalized for ^{32}P incorporation and used for pRB immunoprecipitation using monoclonal antibodies XZ104, XZ133 and 21C9 (kindly provided by E. Harlow). pRB was then resolved in a 6% polyacrylamide SDS gel, blotted onto Immobilon-P (Millipore) and visualized by autoradiography.

Anti-phospho pRB antibodies.

α-phospho-Ser870 and α-phospho-Ser811 have been previously described (Kitagawa et al., 1996). These antisera were raised against phospho-peptides derived from human pRB that are absolutely conserved in the mouse protein (Ser 773 and Ser 804 respectively). α-phospho-Thr350

was raised against the murine pRB sequence. The phosphopeptide, SFETERT(PO_3)PRKNNPC, was chemically synthesized, conjugated with KLH and then injected into rabbits as previously described (Kitagawa et al., 1996). The resulting polyclonal antibodies were purified by column chromatography with the same phosphopeptide linked to Sepharose CL-4B followed by a column of Sepharose CL-4B coupled to the corresponding unphosphorylated peptide, SFETERTPRKNNP. Purified antibodies specifically recognized the phosphopeptide in ELISA assays (data not shown).

Immunoblotting

Membranes were blocked in TBS-T (10 mM Tris (pH 7.5), 150 mM NaCl, 0.03% Tween-20) containing 5% non-fat dry milk. pRB was detected using mouse monoclonal G3-245 (Pharmingen) at a dilution 1:175 and a three step protocol using a rabbit anti-mouse secondary antibody and an anti-rabbit tertiary antibody conjugated to HRP. Phosphoserine 780, phosphoserine 811 and phosphothreonine 350 (Kitagawa et al. 1996) were detected using rabbit polyclonal antibodies at 1:300 and 1:100 dilution respectively and a secondary anti-rabbit antibody conjugated to HRP. Detection was performed by enhanced chemiluminescence.

Two-dimensional phosphopeptide mapping

[^{32}P]pRB was immunoprecipitated, blotted and visualized as described above. Both band A and band B were excised and subjected to two dimensional tryptic phospho-peptide mapping as described (Lees et al. 1991).

G. Acknowledgments

We thank M. Natch for GST-RB; A. Amon and S. Bell for discussions and critical reading of the manuscript. T. Jacks is an Associate Investigator of the Howard Hughes Medical Institute. This work was funded in part by grants from the NIH to T. Jacks and J.A. Lees.

References

Almasan, Y., Y. Yin, R.E. Kelly, E.Y. Lee, A. Bradley, W. Li, J.R. Bertino, and G.M. Wahl. 1995. Deficiency of retinoblastoma protein leads to inappropriate S-phase entry, activation of E2F responsive genes and apoptosis. Proc. Natl. Acad. Sci. *92*, 5436-40.

Bremner, R., B. L. Cohen, M. Sopta, P. A. Hamel, C. J. Ingles, B. L. Gallie, and R. A. Phillips. 1995. Direct transcriptional repression by pRB and its reversal by specific cyclins. Mol. Cell. Biol. *15*, 3256-65.

Brugarolas, J., C. Chandrasekaran, J. I. Gordon, D. Beach, T. Jacks, and G. J. Hannon. 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature *377*, 552-557.

Brugarolas, J., R. T. Bronson, and T. Jacks. 1998. p21 Is a Critical CDK2 Regulator Essential for Proliferation Control in Rb-deficient Cells. J. Cell Biol. *141*, 503-14.

Connell-Crowley, L., J. W. Harper, and D. W. Goodrich. 1997. Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol. Biol. Cell *8*, 287-301.

Deng, C., P. Zhang, J. W. Harper, S. Elledge, and P. Leder. 1995. Mice Lacking p21 $^{CIP1/WAF1}$ Undergo Normal Development, but Are Defective in G1 Checkpoint Control. Cell *82*, 675-684.

Dulic, V., W. K. Kaufmann, S. J. Wilson, T. D. Tlsty, E. Lees, J. W. Harper, S. J. Elledge, and S. I. Reed. 1994. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell *76*, 1013-1023.

Dyson, N., and E. Harlow. 1992. Adenovirus E1A targets key regulators of cell proliferation. Cancer Surv. *12*, 161-95.

El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W.E. Mercer, K. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.

Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J. Kato, and D. M. Livingston. 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell *73*, 487-497.

Ezhevsky, S. A., H. Nagahara, A. M. Vocero-Akbani, D. R. Gius, M. C. Wei, and S. F. Dowdy. 1997. Hypo-phosphorylation of the retinoblastoma protein (pRB) by cyclin D:Cdk4/6 complexes results in active pRB. Proc. Natl. Acad. Sci. U S A *94*, 10699-704.

Funk, J. O., S. Waga, J. B. Harry, E. Espling, B. Stillman, and D. A. Galloway. 1997. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. Genes & Dev. *11*, 2090-100.

Gu, Y., W. Turck, and D. O. Morgan. 1993. Inhibition of cdk2 activity in vivo by an associated 20K regulatory subunit. Nature *366*, 707-710.

Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-816.

Hinds, P. W., S. Mittnacht, V. Dyulic, A. Arnold, S. I. Reed, and R. A. Weinberg. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell *70*, 993-1006.

Jones, D. L., R. M. Alani, and K. Munger. 1997. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. Genes & Dev. *11*, 2101-11.

Kastan, M., Q. Zhan, W. S. El-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein, and A. J. Fornace Jr. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell *71*, 587-597.

Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig. 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. *51*, 6304-6311.

Kitagawa, M., H. Higashi, H. K. Jung, I. Suzuki-Takahashi, M. Ikeda, K. Tamai, J. Kato, K. Segawa, E. Yoshida, S. Nishimura, and Y. Taya. 1996. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J. *15*, 7060-9.

Lane, D. P. 1992. p53, guardian of the genome. Nature *358*, 15-16.

Lees, J. A., K. J. Buchkovich, D. R. Marshak, C. W. Anderson, and E. Harlow. 1991. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J. *10*, 4279-4289.

Lowe, S.W., H.E. Ruley, T. Jacks, and D.E. Housman. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957-967.

Lukas, J., D. Parry, L. Aagaard, D. J. Mann, J. Bartkova, M. Strauss, G. Peters, and J. Bartek. 1995a. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature *375*, 503-506.

Lukas, J. J. B., M. Rohde, M. Strauss, and J. Bartek. 1995b. Cyclin D1 is dispensable for G1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. Mol. Cell. Biol. *15*, 2600-2611.

Lundberg, A. S., and R. A. Weinberg. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol. Cell. Biol. *18*, 753-61.

Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J. Y. Kato. 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. *14*, 2066-76.

Meyerson, M., and E. Harlow. 1994. Identification of a G1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Bio. *14*, 2077-2086.

Nevins, J., G. Leone, J. Degregori, and L. Jakoi. 1997. Role of the Rb/E2F pathway in cell growth control. J. Cell Physiol. *173*, 233-236.

Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol. Cell. Biol. *15*, 2612-24.

Paulovich, A. G., D. P. Toczyski, and L. H. Hartwell. 1997. When checkpoints fail. Cell *88*, 315-21.

Peng, C. Y., P. R. Graves, R. S. Thoma, Z. Wu, A. S. Shaw, and H. Piwnica-Worms. 1997. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501-5.

Poon, R., W. Jiang, H. Toyoshima, and T. Hunter. 1996. Cyclin-dependent kinases are inactivated by a combination of p21 and Thr-14/Tyr-15 phosphorylation after UV-induced DNA damage. J. Biol. Chem. *271*, 13283-91.

Slebos, R. J. C., M. H. Lee, B. S. Plunkett, T. D. Kessis, B. O. Williams, T. Jacks, L. Hedrick,M. B. Kastan, and K. R. Cho. 1994. p53-dependent G1 arrest involves pRB related proteins and is disrupted by the human papilloma virus 16 E7 oncoprotein. Proc. Natl. Acad. Sci. USA *91*, 5320-5324.

Terada, Y., Tatsuka, M., Jinno, S., and Okayama, H. 1995. Requirement for tyrosine phosphorylation of cdk4 in G1 arrest induced by ultraviolet radiation. Nature *376*, 358-362.

Waldman, T., K. W. Kinzler, and B. Vogelstein. 1995. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res. *55*, 5187-90.

Weinberg, R. A. 1992. The retinoblastoma gene and gene product. Cancer Surveys *12*, 43-57.

Weintraub, S. J., K. N. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean. 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature *375*, 812-815.

Weintraub, S. J., C. A. Prater, and D. C. Dean. 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature *358*, 259-261.

Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. Science *366*, 701-704.

Zarkowska, T., and S. Mittnacht. 1997. Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem. *272*, 12738-46.

CHAPTER 6

SUMMARY COMMENTS

We have focused our efforts on analysis of the cell cycle-dependent properties of endogenous E2F proteins. Our work reveals novel modes of E2F regulation that operate at multiple levels to restrict E2F-dependent transcription to specific cell cycle stages, and has expanded our understanding of the role of E2F activity downstream of pRB. Most significantly, the data presented here clarify models of the transcriptional roles of individual E2F proteins, and provide a molecular understanding on which to base genetic analysis of the role of E2F in pocket protein-dependent pathways of growth control and tumor suppression.

A. Previous model of E2F function

Prior to these experiments, the prevailing model of the unique biological properties of pRB versus p107/p130 was based upon the observation that pRB interacts with a subset of E2Fs distinct from those regulated by p107/p130. Both *in vivo* and *in vitro*, the E2F-1, -2 and -3 proteins physically interact with pRB, and not p107/p130 (Dyson et al., 1993; Lees et al., 1993). This specificity is a property of endogenous, and to some extent overexpressed, E2F•pRB complexes, and is apparent in both DNA-dependent and -independent assays. Conversely, overexpressed E2F-4 and -5 have been observed to interact specifically with p107/p130, and not pRB (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995; Vairo et al., 1995). These findings lead to a molecular model of E2F function in which E2F-1, -2 and -3 function downstream of pRB, and E2F-4 and -5 function downstream of p107/p130. The unique tumor suppressive property of pRB might then actually be a reflection of a transcriptional role(s) specific to E2F-1, -2 and -3. This specificity could possibly arise at the level of distinct target gene(s), or in the timing of the regulation of shared target promoters. In any case, this

model predicted that deregulation of E2F-1, -2 and -3-specific transcriptional events promotes tumorigenesis, whereas deregulation of E2F-4 and -5, -dependent transcription does not.

This model places E2F-1, -2 and -3 at the bottom of the p16•cyclin D•pRB pathway, and makes specific predictions regarding the ability of these E2Fs to drive cell cycle transitions. A number of published studies support a specific role for E2F-1, -2 and -3 in driving G1/S, which are not shared by E2F-4 or E2F-5 (reviewed in Bernards, 1997) . These data suggest, consistent with known physical interactions, that E2F-1, -2 and -3 are functionally downstream of pRB and that each of these transcription factors is equally capable of inducing the transcription of genes sufficient to drive entry into S-phase. By extension, it is inferred from these data that the transcriptional activity of overexpressed E2F-4 is insufficient to drive G1/S progression. Together, these observations tend to support the contention that accumulation of active, "free" E2F-4 or -5 activity is insufficient to bypass the molecular mechanisms underlying critical G1 regulatory pathways.

B. Implications for the model: Inactivity of E2F-4

Prior to a discussion of the implications of this work and the prospects for further research, it is necessary to examine the effect of our findings on the model of E2F function outlined above. The demonstration that endogenous, free E2F-4•DP complexes localize predominantly to the cytoplasm strongly suggests that these complexes do not participate directly in transcriptional regulation. This does not mean that these heterodimers are intrinsically incapable of driving transcription, but that this activity may be preempted by their cytoplasmic location. In this regard, the observation that overexpressed E2F-4 protein localizes to the cytoplasm in all cell types tested to date has an immediate impact upon the interpretation of previous studies of the transcriptional properties of E2F-4. It is now apparent that the failure of E2F-4 to drive G1 progression in transfection assays does not represent a measure of the ability of E2F-4 to drive the transcription of genes required for G1/S, but rather is direct consequence of its failure to localize to the nucleus. In fact, when E2F-4 is directed to the nucleus by addition of the SV40 Tag NLS, or by fusion with

the NLS-containing N-terminal domain of either E2F-1 or E2F-2, this transcription factor is now able to overcome both p16-mediated G1 arrest, and cell cycle withdrawal induced by serum starvation (Muller et al., 1997; S. Estes, unpub.). These data tend to undermine the contention that, because the pRB-specific E2Fs drive G1/S and E2F-4 does not, the different E2F classes are predicted to regulate a different subset of genes, and that the failure of overexpressed E2F-4 to drive G1/S is a direct reflection of E2F functional differences which underlie the tumor suppressive function of pRB. Clearly, E2F-4 is capable of driving entry into S-phase when it is overexpressed in a nuclear form. This observation does not by any means prove that the endogenous population of E2Fs will all regulate the same responsive gene-promoters *in vivo*, although it suggests that the transcriptional properties of overexpressed, nuclear E2Fs are sufficiently similar as to promote similar biological outcomes.

C. Molecular evidence of function

C1. E2F-dependent repression and activation

In spite of the extent to which our findings have provided valuable insight into the biological effects of overexpression, the more significant ramifications of our findings are the extent to which they strongly suggest functional roles of endogenous mammalian E2F species. The potential for non-equivalence between E2F proteins is first suggested by our analysis of the cell cycle-dependent regulation, abundance, and pocket-protein binding properties of individual E2Fs (summarized in Fig. 1). One of the most salient features of this data is the extent to which E2F-4 accounts for the majority of cellular E2F activity. The abundance of E2F-4, and its *in vivo* interactions with all three members of the retinoblastoma family, suggest that E2F-4 will play an important role in E2F/pocket protein-regulated proliferation control pathways. Specifically, our demonstration of a direct and significant interaction between E2F-4 and pRB *in vivo* suggests that E2F-4 functions in pRB-mediated growth suppression pathways. The timing and subcellular compartmentalization of this interaction in cycling cells supports a model in which E2F-4 is the DNA-binding component of an abundant, G1 transcriptional repressor complex. Thus, E2F-4,

Figure 1. Summary of the cell cycle-dependent regulation of E2Fs

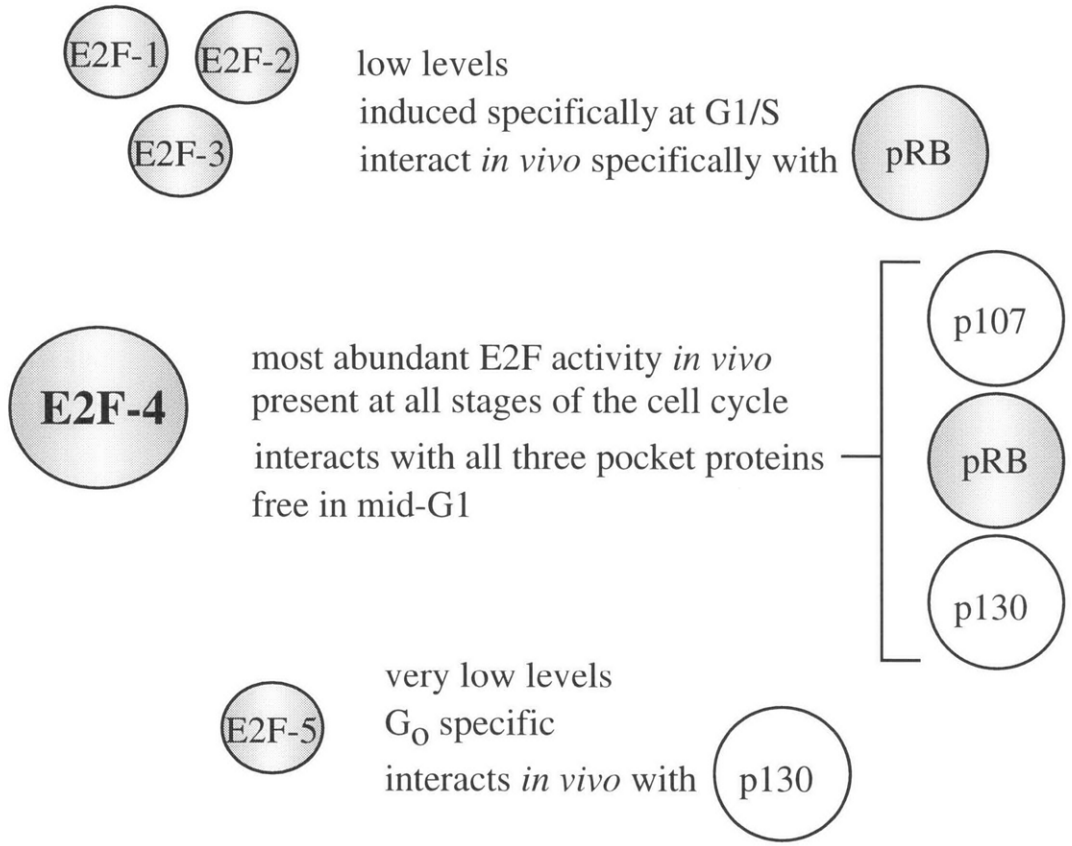
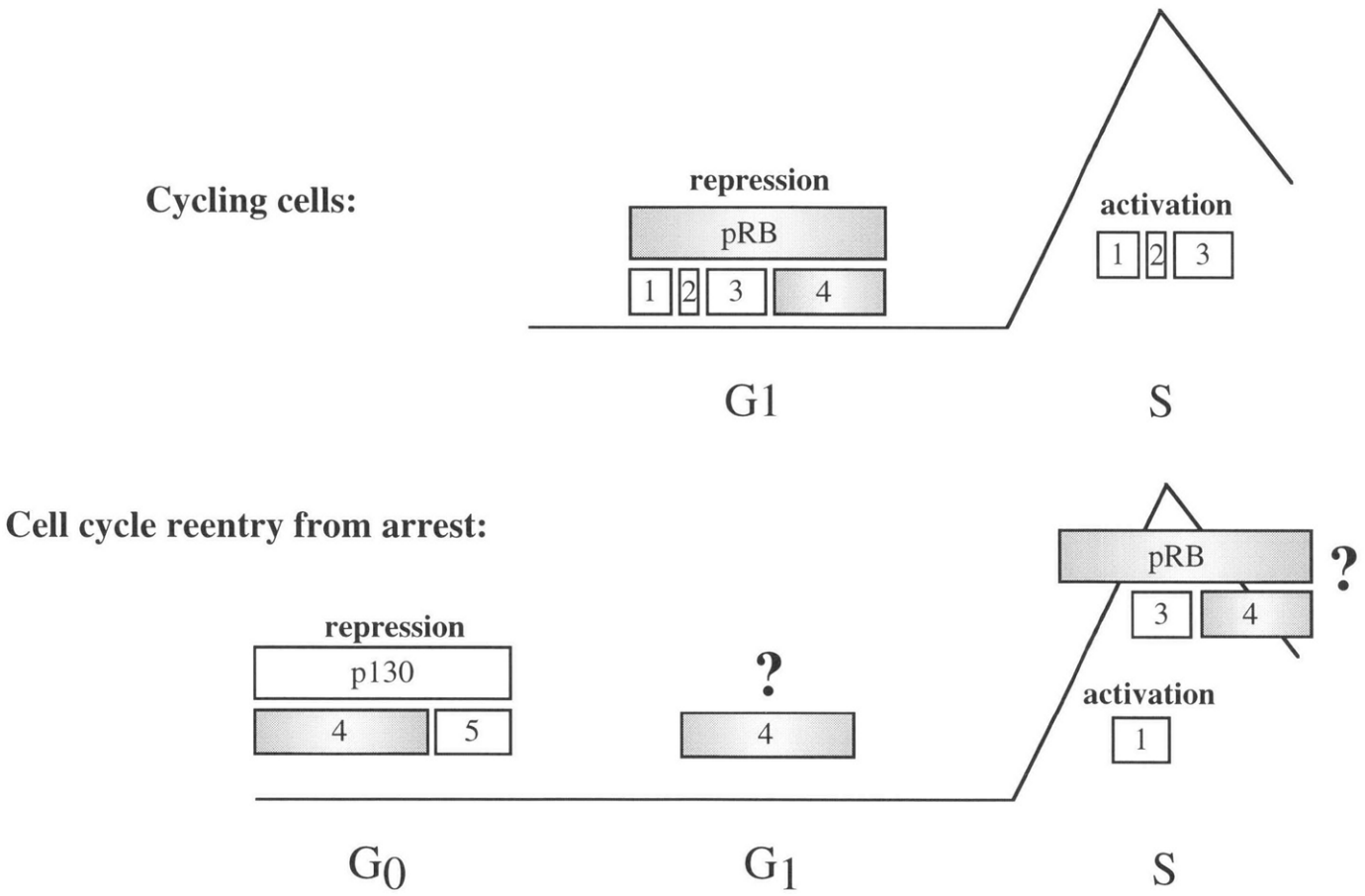


Figure 2. Summary of critical E2F complexes



together with E2F-1, -2 and -3, functions in a pRB-dependent pathway of growth control. The role of each of these E2Fs in this pathway is linked to their ability to act as sequence-specific, DNA binding proteins. However, the different localization properties of E2F-1, -2 and -3 versus E2F-4 are predicted to directly influence the precise transcriptional role each of these proteins plays in pRB-dependent mechanisms of G1/S control (see Fig. 2). The cell cycle-dependent nuclear localization of E2F-4 suggests this protein is necessary for full repression of responsive gene transcription during G1, but that it plays a minimal role in the induction of these promoters during S-phase. In contrast, the localization and relative abundance of E2F-1, -2 and -3 suggests that these E2Fs also act as limiting activators of transcription during S-phase. The unique role of pRB in controlling the activity of both of these classes of E2Fs suggests that loss of pRB results in simultaneous derepression, and inappropriate activation, of E2F-responsive genes in a manner which does not occur following inactivation of p107 or p130. Thus, the specificity of the "activatory" E2Fs for pRB provides one explanation for the non-tumorigenic consequences associated with p107/p130 inactivation, as loss of p107/p130 may only lead to derepression of transcription and not inappropriate activation. This concept in and of itself is not necessarily novel. What is novel is our contention that these transcriptional effects of pRB-loss are a consequence of deregulation of groups of functionally distinct E2F species, and that both elements may be required for tumorigenicity. According to this model, each of these effects is primarily mediated by distinct E2F species. If this proves to be the case, genetic analysis of E2F function in the pRB pathway will allow us to separate these two processes, and to assess the extent to which each contributes to tumorigenesis.

A requirement for "compound" deregulation of the E2F pathway in tumorigenesis could have a number of potential explanations. First and foremost, it is possible that distinct classes of E2F-responsive genes are differentially susceptible to deregulation by the two different mechanisms; increased expression of either "set" alone is required, but not sufficient, to promote tumorigenicity. At the other extreme, it may be that all E2F responsive genes are equally susceptible to deregulation by both mechanisms, but that only by the combined effect of both are

the levels of responsive-gene transcription sufficiently elevated as to constitutively promote G1 progression and S-phase entry. Clearly, both of these models assume that transformation processes downstream of E2F require the transcriptional deregulation of multiple cellular gene products. It is highly likely that all E2F target genes are not equal in their transforming potential, and that only a few particular promoters (eg. *cyclin E*) represent the crucial oncogenic targets of the pRB•E2F tumor suppression pathway. In this case, it will be necessary to determine which of the above models more accurately describes the E2F-dependent transcriptional regulation of these specific promoters. Resolution of these issues will likely have to await improved biochemical analysis of the promoter specificity of endogenous E2Fs, and ultimately will have to be confirmed by genetic dissection of the role of E2F and its targets in the pRB pathway of growth control.

C2. E2F-1, -2 and -3

Analysis of the endogenous populations of E2F has also revealed potentially significant differences among the pRB-specific E2Fs. These differences are predicted to have a significant impact upon the functional consequences of E2F activity. First, it is now apparent that E2F-2 is an extremely rare protein *in vivo*. The protein is undetectable by immunoblotting, and E2F-2 DNA binding activity is only barely detectable by EMSA. The timing of its appearance in primary human T cells reentering the cell cycle mirrors that of the considerably more abundant E2F-1 and -3, suggesting that these E2Fs are all active during the same cell cycle interval. Unless E2F-2 has a function which is non-overlapping with the other E2Fs, these data suggest that this protein plays little role in the cell cycle-dependent expression of responsive genes.

The differences we have observed between the E2F-1 and E2F-3 proteins are likely to have significant impact upon the transcriptional roles of these proteins. In the post-G1/S samples from the human T cell system, E2F-1 is detected primarily in the free form, while E2F-3 is primarily complexed with pRB (Fig. 2). This molecular observation immediately suggests that these proteins do not fulfill the same transcriptional function during this stage of the cell cycle, and that they are differentially targeted for inhibition by pRB. The presence of E2F-1 in the free form

suggests that this E2F is primarily an activator of responsive gene transcription during cell cycle reentry in human T cells. Significantly, it has recently been reported that *E2F-1-/-* MEFs take longer to reach S-phase following G0-exit than do wildtype cells (Yamasaki et al., 1998). While this deficiency has not been demonstrated to correlate with the altered expression of E2F-responsive genes, it is highly likely that this prolonged G1-transit reflects a requirement for E2F-1 in the timely expression of target genes at G1/S. Thus, data from both human T cells and murine fibroblasts support a role for E2F-1 as an activator of transcription during cell cycle reentry. In contrast, the appearance of E2F-3 in complexes with pRB at G1/S suggests that this E2F functions as the DNA binding-component of a transcriptional repressor complex during S-phase. Given the G1/S induction of most known E2F responsive genes, it is likely that the targets of this repressive complex are as yet unidentified. In this regard, it is possible that E2F-3•pRB functions to repress certain E2F-responsive promoters required for G1 transit in human T cells, but which must be down-regulated upon S-phase entry.

The different pRB binding properties of E2F-1 and E2F-3 in human T cells contrasts sharply with numerous reports that document approximately equivalent binding of E2F-1 and -3 to pRB. This result therefore implies that the pRB-binding capacity of E2Fs can be modulated at the post-translational level. Two published reports suggest that cdk-mediated phosphorylation of E2F-1 can alter its pRB-binding properties (Fagan et al., 1994; Peeper et al., 1995). If such a mechanism were to selectively target different E2Fs, it would have a significant impact upon the relative roles of individual E2Fs *in vivo*. E2Fs rendered refractory to the inhibitory effects of pRB would be predicted to contribute exclusively to the activation of target genes, whereas those competent to bind pRB would be predicted to participate in transcriptional repression and, upon cyclin•cdk mediated inactivation of pRB, activation. Determination of the exact nature of such modification may therefore enable one to predict the extent to which a given E2F functions to promote proliferation, or functions in collaboration with pRB to inhibit cell cycle progression.

C3. Differences between the pocket proteins

Our cellular fractionation data in the human HL-60 cell line suggests that the retinoblastoma family members p107 and p130 localize primarily to the cytoplasm in cycling cells. This finding is indirectly supported by immunofluorescence analysis of the localization of E2F-4 protein during S-phase in both U2OS and Wi38 human cells. It is well established that p107 interacts *in vivo* specifically with E2F-4, and that the E2F-4•p107 complex is most abundant during S-phase (Schwarz et al., 1993; Shirodkar et al., 1992). Strong αE2F-4 cytoplasmic staining, and the relative lack of nuclear signal in S-phase cells, implies this E2F-4-containing complex is also cytoplasmic. If true, this might help explain the long standing paradox between high levels of E2F-responsive gene transcription during S-phase correlating temporally with high levels of an apparent transcriptional repressor complex E2F-4•p107.

If p107 and p130 are indeed primarily cytoplasmic proteins in these cells, it is logical to suggest that these proteins are not direct transcriptional regulators, and that only pRB plays a significant role in regulation of E2F-dependent transcription. However, our immunofluorescence data also reveal that E2F-4 protein is localized to the nuclei of G0 cells. In fact, the staining pattern in these experiments shows that the majority of E2F-4 is nuclear during G0 in the human Wi38 cell line. It is well established, by our work and others', that the most abundant G0 complex in a wide variety of both human and murine cell types is E2F-4•p130, and that little if any free E2F exists in quiescent cells(Chittenden et al., 1993; Cobrinik et al., 1993; Vairo et al., 1995). As in cycling cells, this places E2F-4 in the nucleus at a time when E2F-responsive genes are transcriptionally inactive, and implies a role for E2F-4 in this process in cooperation with pocket protein(s). As loss of p130 (or p107) derepresses a subset of E2F-responsive genes in MEFs reentering the cell cycle from G0 (Hurford et al., 1997), E2F-4 and p130 appear to form a DNA-binding repressor complex which is localized to the nucleus of quiescent cells.

This apparent inconsistency between the cytoplasmic localization of p107/p130 in HL-60 cells, and the inferred nuclear localization of p130 in quiescent Wi38s and MEFs, may actually represent an important difference between cycling and non-cycling cells. It is well established that

the patterns of E2F complexes in cycling cells and in those stimulated to reenter the cell cycle from G0 are quite different (Chellappan et al., 1991; Chittenden et al., 1993; Schwarz et al., 1993). Despite this, many E2F-responsive genes are induced at G1/S in both systems. It is therefore possible the molecular mechanisms underlying the E2F-dependent aspects of G1/S induction of responsive genes may actually be different in these two systems, and that p107/p130 make a more significant contribution to the regulation of E2F activity in non-cycling, quiescent cells due to an increased affinity for the nuclear compartment. Regardless of the validity of this somewhat speculative model, the original observation of p107 and p130 in the cytoplasm has yet to be confirmed or refuted by immunofluorescent localization studies of these proteins. Such experiments will clearly represent an important test of this model of the unique biological properties of the different members of the pRB family of proteins.

D. Future directions

D1. E2F-4 mislocalization

An improved molecular understanding of the transcriptional roles of individual E2Fs has led to a number of significant predictions regarding the manner in which each E2F contributes to the G1/S regulatory properties of pRB. The extent to which these transcriptional properties are predicted to depend upon subcellular localization suggests that disruption of the normal localization properties of individual E2Fs may affect the rate of G1/S progression. In particular, loss of E2F-4 from the nuclei of G1 cells may sufficiently relieve pRB-mediated repression so as to accelerate G1 exit and S-phase entry. A test of this prediction in cell culture clearly depends upon understanding the cellular pathways which normally act to enforce the cell cycle dependent localization of E2F-4. Such data would inform attempts to develop reagents which cause the mislocalization of endogenous E2F-4 protein complexes. A considerable amount of effort was directed towards determining the pathway(s) responsible for the G1/S-regulated nuclear exit of E2F-4 (see Appendix, Section D). This work was did not uncover such a mechanism, and to date we have been unable to test our predictions of E2F-4 function by gene transfection into cultured cells. A recent report demonstrates that the HPV type 16 E7 oncoprotein is able to cause the accumulation of free, *nuclear* E2F-4•DP-1 complexes, and that this accumulation correlates with the anchorage-independent induction of the *cyclin A* gene (Schulze et al., 1998). This observation suggests that cellular pathways that regulate the cell cycle-dependent localization of E2F-4•DP-1 are disrupted by E7 expression, and raises the possibility that E7-driven mislocalization of free E2F-4 represents an important cell cycle deregulatory event during the course of HPV-mediated transformation. In order to address these questions, it will first be necessary to define the regions of E7 required for nuclear accumulation of free E2F-4. Given the well documented functional properties of the E7 protein, this data may provide important clues as to the mechanisms which normally regulate E2F-4•DP-1 localization in non-HPV infected cells. Genetic disruption of this region(s) may also permit an assessment of the extent to which E2F-4 mislocalization is required for E7-induced phenotypes (eg. apoptosis and transformation) in cultured cells.

D2. Genetic analysis of E2F function in mice

Our predictions regarding the manner in which each E2F contributes to the growth and tumor suppressive properties of pRB are clearly best addressed within the context of preexisting genetic models of murine pocket protein-mediated growth suppression. As a step towards this, the field has now begun to develop genetic models of E2F function in an effort to understand the role these proteins play in proliferation control in murine development and tumorigenesis.

D2.1 *E2F-1*

The *E2F-1* gene has already been inactivated by targeted deletion in the mouse (Field et al., 1996; Yamasaki et al., 1996). This has allowed an direct assessment of the role of this member of the E2F family in developmental control of cell proliferation, and in oncogenic processes promoted by loss of pRB. Interestingly, *E2F-1* seems to be largely dispensable during murine development: homozygous null mice are viable and fertile. This suggests that the activity of the E2F-1 protein is not necessary during the many highly regulated cell divisions between early embryogenesis and adulthood. This phenotype, however, is not altogether unexpected. It is in some ways reminiscent of phenotypes associated with *dE2F* null mutations in the fly (Royzman et al., 1997). Flies deficient for dE2F survive well into late larval stages, and S-phase occurs, however slowly, in all tissues examined. As was discussed previously, this may occur due to partial compensation by an additional dE2F activity (present in *Drosophila* EST database; B. Fairchild, pers. comm.), or by derepression of transcription by loss of a DNA-bound dE2F•RBF complex on relevant promoters. Either mechanism may also apply to the *E2F-1-/-* animals. However, the smaller fraction of total E2F activity which E2F-1 represents in the mouse suggests that the potential for compensation by other E2F activities is even greater than in the fly; this raises the possibility that the variable effect of E2F-1 deficiency on different murine tissues reflects to some degree the extent of functional overlap between E2F family members. Clearly, such compensation would only be revealed by compound deficiency for additional E2F family members. Given the current model of pRB-specific E2F function as a necessary element of a

G1/S transcriptional program which is intrinsic to almost all types of cell cycles, it is unlikely that the pRB-specific E2Fs are collectively dispensable for proper development. However, the intercrossing necessary to test this hypothesis will have to await the generation of these strains.

The adult growth phenotypes associated with a null E2F-1 mutation can be interpreted to support the proposed molecular role of this protein in both transcriptional activation and repression of genes required for S-phase entry. Adult E2F-1 null mice display atrophy in some tissues and infrequently, tumors in others. These findings demonstrate that E2F-1 can not only act as a promoter of cell proliferation, but that E2F-1 also inhibits proliferation to the extent that it fulfills a tumor suppressor function during adulthood. That *E2F-1* heterozygous mice are as susceptible to these lesions as are their homozygous litter mates strongly suggests that loss of the remaining wildtype E2F-1 allele is not rate limiting for this process, but that following the accumulation of additional genetic alterations, E2F-1 deficiency accelerates oncogenesis. Whereas the role of E2F-1 in promoting cell proliferation is likely a direct result of its ability to transactivate genes necessary for S-phase entry, there are two quite different mechanistic explanations for the tumor suppressive properties of E2F-1. Firstly, it may reflect the fact that E2F-1 can participate in a growth inhibitory complex with pRB, and that loss of this E2F-1•pRB complex represents a growth deregulatory event which is not compensated for, or redundant with, other E2Fs. If this is the case, then one might expect some overlap between the tissues which develop tumors in *E2F-1-/-* animals and those which are tumor prone in pRB heterozygotes. While there is no such overlap, it is possible that late-onset tumors, of the sort seen in E2F-1 null condition, would eventually also occur in *pRB+/-* mice if they were able to survive to that age. Alternatively, the tumor suppressor function of E2F-1 may be attributable to an apoptosis promoting function specific to E2F-1 (Hsieh et al., 1997; Phillips et al., 1997). In this case, it is of note that *E2F-1-/-* animals show an increased incidence of lymphoma (Field et al., 1996). Lymphomas are common in mice deficient for the *p53* gene (Jacks et al., 1994), whose tumor suppressor function has been directly linked to its ability to promote apoptotic cell death in response to specific signaling pathways (reviewed in Levine, 1997). The finding that certain apoptotic pathways seem to be partially compromised in

E2F-1 null lymphoid cells, suggests that loss of E2F-1 compromises an apoptotic program whose normal function is to inhibit cellular transformation *in vivo* (Field et al., 1996).

The ability of pRB to inhibit E2F-1, and the demonstration of the constitutively nuclear localization of this protein, implies that in the absence of pRB, E2F-1 may be a potent oncogene. The oncogenicity of E2F-1 is confirmed by genetic analysis of the requirement of E2F-1 in the pRB tumorigenesis pathway. Generation of compound *pRB+/-;E2F-1-/-* mice completely rescues the thyroid tumors, and partially rescues the pituitary tumors which normally occur in *pRB+/-* mice (Yamasaki et al., 1998). Thus, genetic analysis of E2F-1 function demonstrates a requirement for E2F-1-driven activation of target genes during tumorigenesis. The extent to which specific deregulation of E2F-1 is sufficient to promote tumorigenesis has been assessed rather crudely by tissue-specific transgenic mouse models (Pierce et al., 1998). In these systems, ectopic E2F-1 expression can induce hyperplasia and is capable of cooperating with other activated oncogenes to produce tumors. A more relevant method to assess the tumorigenic potential of endogenous E2F-1 would be to create, by targeted mutation of the *E2F-1* genomic locus, a strain of mice carrying a version of E2F-1 that is refractory to inhibition by pRB. Such an experiment is currently ongoing, and promises to reveal the set of pRB-loss developmental and tumor phenotypes specifically due to deregulation of E2F-1.

Interestingly, the *pRB-/-* x *E2F-1-/-* cross also rescues some forms of the developmental apoptosis observed in pRB nullizygous mice. This provides direct evidence that apoptosis is a physiologically relevant consequence of deregulated E2F-1 activity and raises the possibility that the widespread developmental apoptosis seen in pRB-null embryos is a product of elevated E2F activity. In this regard, it is significant that *pRB-/-;E2F-1-/-* animals display an extended survival time compared to *pRB-/-* alone (d13.5 p.c. to d15.5 p.c.). Whereas the developmental process(es) rescued by this cross remains unclear, this finding clearly suggests that one function of pRB during development is to inhibit E2F-1, and that ectopic E2F-1 activity perturbs a growth regulatory pathway(s) necessary for proper development.

The requirement for E2F-1 in full-penetrance of the developmental and tumorigenic phenotypes in pRB-deficient mice is in direct contrast to its dispensability during murine development. It demonstrates that E2F-1 plays a non-redundant function during tumorigenesis, but that a developmental role, if it has one, overlaps with that of other E2Fs whose activity suffices during development. This differential requirement for E2F-1 in these two types of proliferative processes may simply reflect the difference between overlapping and non-overlapping expression patterns. However, it is clear that E2F-3, for example, is expressed at levels comparable to E2F-1 in *pRB-/-* tumor cell lines (K. Moberg, unpub.). Alternatively, this differential requirement may also arise if E2F-1 performs distinct transcriptional roles during development and tumorigenesis. This may occur due to differences in the extent to which E2F-1 contributes to repression and activation during the two processes; or it may reflect the ability of pRB to alter/modulate the spectrum of target genes regulated by E2F-1. With regards to the former possibility, it is clear that E2F-1 is very likely to fulfill a required role as an activator of transcription in *pRB-/-* tumors. The partial rescue of *pRB-/-* developmental lethality by homozygous inactivation of *E2F-1* suggests that these two proteins do cooperate in a developmentally regulated repressor complex; the lack of developmental phenotype in E2F-1-/- mice suggests that loss of this E2F-1 repressive function is either redundant with, or compensated by, other E2Fs. According to this model, E2F-1-driven transactivation in pRB null cells is required to deregulate G1/S progression and its loss is not rescued by the activity of other E2Fs, while loss of E2F-1•pRB repressive complexes from those same promoters is either insufficient to deregulate G1/S, or is compensated by other E2F•pocket protein complexes. Such a model makes many predictions which are testable by both molecular and genetic means. It also emphasizes the fact that establishing the biological properties of a single E2F is dependent upon the development of molecular and genetic models of the role of other E2Fs in the pRB pathway.

D2.2 *E2F-3*

The failure of the E2F-1 deficiency to completely rescue the developmental and tumor phenotypes of *pRB+/-* mice suggests that the deregulated activity of other proteins is partially responsible for these effects. Due to the very low abundance of E2F-2, it is likely that one source of this activity is E2F-3. Obviously, the proper test of this hypothesis would be to cross the *pRB+/-* genotype into an E2F-3 deficient background. As *E2F-3-/-* mice are viable and fertile (P. Humbert, pers. comm.), this cross is underway. There are a number of possible phenotypic consequences of combining these genotypes, each of which would be quite informative regarding the roles of E2F-1 and -3 in tumorigenesis.

(1): E2F-3 deficiency rescues the same pRB-loss phenotypes as does loss of E2F-1. This would suggest that E2F-1 and E2F-3 activity cooperate to produce this subset of phenotypes, and that the deregulated activity of each is necessary, but not sufficient, for these effects. This result implies that these E2Fs function in the same tissues to promote proliferation in the absence of pRB. Such cooperativity would be evident if E2F-1 and E2F-3 function in distinct proliferative pathways within these tissues, but would also be evident if E2F-1 and E2F-3 collaborate in a shared pathway. This outcome would therefore immediately raise the question of whether E2F-1 and -3 have overlapping function(s) in cell cycle control and tumorigenesis. In this regard, analysis of the expression of E2F target genes in *pRB-/-*, *pRB-/-;E2F-1-/-* and *pRB-/-;E2F-3 -/-* MEF lines might reveal differences in the spectrum of target genes deregulated in each genotypic background. Alternatively, since mice deficient for E2F-1 and E2F-3 individually develop quite normally (P. Humbert pers. comm. and (Field et al., 1996; Yamasaki et al., 1996) resolution of this issue of functional specificity might also be accomplished at the organismal level by intercrossing *E2F-1-/-* and *E2F-3-/-* animals to reveal any potential functional redundancy between these proteins during development. Further, the generation *E2F-1-/-*, *E2F-3-/-* and *E2F-1-/-;E2F-3-/-* MEFs would provide the opportunity to identify E2F target genes whose expression is specifically regulated by E2F-1, by E2F-3, or which are only deregulated by loss of both E2F-1

and E2F-3. *In situ* analysis of the expression of these genes in affected tissues might then enable one to correlate the developmental functions of E2F-1 and E2F-3 to transcriptional effects at specific promoters.

(**2**): E2F-3 deficiency rescues pRB-loss phenotypes distinct from those rescued by E2F-1 deficiency. This result would immediately suggest that E2F-1 and E2F-3 play tissue-restricted roles in tumorigenesis, and that each is sufficient to promote this process in the absence of the other. In the case that each protein is comparably expressed in the relevant tissues, it also suggests that E2F-1 and E2F-3 are not functionally equivalent in their ability to promote proliferation *in vivo*. Such differences might arise as a result of intrinsic properties of each protein, or from the tissue-dependent expression of proteins that modify the activity of these transcription factors. Interestingly, *E2F-1-/-* MEFs in culture are indistinguishable from wildtype MEFs in their growth characteristics, while in contrast, *E2F-3-/-* MEFs in culture display a dramatically reduced proliferative rate relative to wildtype (P.Humbert, pers. comm.). While issues of expression, complex formation, and compensation at the molecular level have yet to be addressed in either genetic background, this observation strongly suggests that in fibroblasts, E2F-3 is required for the expression of genes involved in the timely execution of proliferative pathways and E2F-1 is not. Determining the identity of these genes may significantly advance our ability to predict the biological outcomes associated with E2F-1 and E2F-3 activity.

(**3**): E2F-3 deficiency does not rescue any of the tumor phenotypes promoted by pRB loss. This result would support a model in which E2F-3 transcriptional activity is dispensable for tumorigenic processes in the mouse, and that E2F-1 represents the primary tumor promoting E2F activity in *pRB-/-* cells. If further study of the role of E2F-3 in these tissues were to confirm its expression and DNA-binding properties, this would strongly suggest that E2F-1 and E2F-3 are functionally non-equivalent. Given the similar cell cycle-dependent expression patterns of these E2Fs and their generally accepted roles as activators of E2F-dependent transcription, the different

biological outcomes of these activities would then most likely arise due to differences in the identity of E2F-1 and E2F-3 target genes.

D2.3 *E2F-4*

Molecular analysis of E2F-4 protein has led to very specific predictions regarding the role of this protein in the transcriptional regulation of E2F-responsive genes. These data suggest that E2F-4 functions as a necessary cofactor for pocket protein-mediated repression. Examination of the expression patterns of this transcriptional regulator reveals that E2F-4 is the most abundant E2F activity in all cell types yet tested, regardless of the tissue of origin. E2F-4 is therefore likely to play an important role in regulating cell proliferation in multiple tissues by virtue of its interactions with pRB, p107 and p130.

Genetic analysis of the function of pRB, p107 and p130 has revealed overlapping functions for these E2F regulators during murine development (reviewed in Mulligan and Jacks, 1998). Individually, p107 and p130 are dispensable for normal development in the 129/Sv strain. Similarly, a reduction to heterozygosity of the dosage of the pRB gene is without developmental effect. However, intercrossing these genotypes produces phenotypes consistent with disruption of developmentally controlled proliferative pathways. *p107-/-;p130-/-* mice die neonatally, showing disturbed bone development (Cobrinik et al., 1996). This phenotype at the cellular level is apparently linked to chondrocyte overproliferation and differentiation defects. Similarly, the generation of *pRB+/-;p107-/-* and *p107+/-;p130-/-* animals reveals phenotypes not apparent in either of the individual genotypes (reviewed in Mulligan and Jacks, 1998). Both of these compound mutant strains exhibit similar developmental defects: they are severely runted and are susceptible to both embryonic and neonatal death. It is likely that the overlapping developmental functions of pRB, p107 and p130 revealed in these crosses derives from the shared role of these proteins as E2F regulators. However, inactivation of the pRB-specific E2Fs has yet to uncover evidence of a developmental requirement for these proteins. In fact, as E2F-1, -2 and -3 do not interact with p107/p130 *in vivo*, the overlapping developmental functions of pRB, and especially

p107 and p130, are likely linked to their shared role as transcriptional repressors in a DNA-binding complex containing E2F-4.

The generation of mice carrying a targeted inactivation of the *E2F-4* locus has begun to deomonstrate the primary role of E2F-4 as a component of a transcriptional regulatory complex with a necessary role in development. *E2F-4* null animals are runted and show poor viability; additionally, *E2F-4-/-* and +/- animals are recovered at reduced frequency, apparently as a result of embryonic lethality at an undetermined developmental stage (P. Humbert, pers. comm.). This organismal phenotype is somewhat similar to the *pRB+/-;p107-/-* and *p107+/-;p130-/-* compound mutant phenotypes, which also display reduced recovery of mutant animals and runted adults. The generalized similarity of these two phenotypes does not prove a common molecular defect. However, it is possible that it reflects the common role of E2F-4, and p107/p130 as necessary components of repressors of E2F-responsive transcription. In this regard, further analysis of the cellular and molecular defect(s) associated with E2F-4 deficency may support our molecular model of E2F-4 function as primarily a repressor, but not an activator, of E2F-responsive genes.

The *E2F-4-/-* phenotype reveals that E2F-4 protein is required for proper development, and that it functions during this process in a manner which is not redundant with, or cannot be compensated by, the other E2Fs. Clearly, this role is likely linked to the ability of E2F-4 to interact with all three pocket protein family members. Unlike the case E2F-1 and pRB, our current understanding of E2F-4 function downstream of the pocket-proteins predicts that crossing E2F-4-deficiency into the *pRB-/-*, *pRB+/-;p107-/-* and *p107+/-;p130-/-* genotypes will fail to rescue developmental defects or tumor progression. In contrast, our data suggest that loss of E2F-4 in the context of these genotypes will exacerbate these defects, as it may effectively mimic loss of additional pRB family function. While a full study of the extent to which pocket-protein growth regulatory pathways are dependent upon E2F-4 is ongoing, the apparent requirement for this protein in full transcriptional repression by pRB indicates that E2F-4 may also be necessary for the tumor suppression function of this protein. If this is the case, then E2F-4 deficiency may actually accelerate oncogenesis. However, if our model of E2F-4 function is in some way incorrect, or if

the relative contribution of E2F-4 to repression and activation is tissue-dependent, then loss of E2F-4 may actually rescue pocket protein-mutant phenotypes. For instance, if the chondrocyte overproliferation defects of *p107-/-;p130-/-* mice are due to inappropriate activation of E2F target genes by free E2F-4, the *E2F-4-/-* x *p107-/-;p130-/-* cross may to some extent rescue this defect. However, given the embryonic phenotypes associated with each of these genotypes, this cross may produce more severe embryonic defects which compromise the ability to observe cellular phenotypes at particular developmental stages. In this case, the ability to establish the extent to which E2F-4 deficiency rescues or modifies cellular *p107-/-;p130-/-* defects may be dependent upon the generation of chimeric animals bearing cells triply mutant for *E2F-4*, *p107*, and *p130*. A chimeric analysis may also be necessary to study the manner in which *E2F-4* loss exacerbates or rescues *Rb* mutant phenotypes. Clearly, these crosses promise to shed significant light on the role of E2F-4 downstream of pRB, p107 and p130, and represent the ultimate test of models of E2F-4 function in the whole animal.

D3. E2F and checkpoint control

The use of genetically defined MEF lines has enabled us to demonstrate that pRB is required for the efficient induction of G1 arrest in response to ionizing radiation. This requirement is directly linked to changes in the phosphorylation state of the protein, which immediately suggests that the effect of this checkpoint pathway is to maintain pRB in an underphosphorylated, growth suppressing form. Underphosphorylated pRB is known to interact with E2F, and the ability of pRB to induce G1 arrest strongly correlates with its ability to sequester, and inhibit E2F. In light of this, it is likely that the E2F•pRB interaction represents the critical target of this checkpoint pathway, and that arrest is imposed in part through inhibition of E2F-responsive gene transcription.

A clear prediction of this hypothesis is that irradiation-induced arrest correlates with the accumulation of E2F•pRB transcriptional repressor complexes. Examination of the E2F DNA binding complexes in treated and untreated populations of wildtype MEFs confirms the

accumulation of a novel E2F activity specifically in treated cells (see Appendix). Consistent with our model, this complex has the electrophoretic mobility characteristic of E2F•pRB complexes, and its appearance is entirely dependent upon the status of *p21^{CIP1}*. Surprisingly, this complex is not dependent upon the status of the *RB-1* gene, and antibodies which recognize murine pRB fail to supershift the complex. Additional antibody experiments suggest that the irradiation-inducible complex contains E2F-4 and p130, and provides yet another example of cell cycle arrest which correlates with high levels of E2F-4•pocket protein complexes. The relevance of such a complex to checkpoint arrest is unclear. Our genetic analysis clearly suggests that pRB is entirely epistatic to p21 in this pathway, yet the biochemical data demonstrate that this putative E2F-4•p130 complex is induced in a p21-dependent manner. This apparent inconsistency may reflect the existence of two independent pathways downstream of cyclin E•cdk2, one operating through pRB and one through p130, each of which is required for arrest. The absence of a dramatic change in the E2F binding profile of pRB in untreated and treated cells may suggest that the relevant target of pRB growth suppression in this response is not E2F; or that the ability of pRB to induce arrest in this checkpoint pathway correlates with the sequestration of a small fraction of total cellular E2F. Finally, it may be that this E2F-4•p130 complex is unrelated to the induction of arrest. In this regard, a single attempt to examine the integrity of this checkpoint pathway in *p130-/-* MEFs revealed no deficiency in the G1 arrest-response to DNA damage (G. Mulligan, pers. comm.).

We have used genetically defined MEF cells to extend the G1 arrest checkpoint pathway from p53 and p21, through to cdk2 and pRB. However, our biochemical methods have proven insufficient to resolve the precise role of pRB and E2F proteins in this pathway. Clearly, the next step is to assay the integrity of this response in MEF populations specifically deficient for different E2F family members. Such an analysis might reveal further functional specificity between E2F proteins in the extent to which they are required targets of a p53-dependent checkpoint pathway, which acts through pRB to regulate a E2F-dependent G1/S transcriptional program.

E. Where it's all going

The ultimate goal of this work is to understand the role of individual E2Fs in pathways of pRB-mediated growth suppression. The generation of E2F-deficient mouse strains promises to advance this effort in two distinct, yet complementary, ways. Whereas the animals will reveal the developmental and tumorigenic roles of these proteins, the generation of MEF lines from these animals will permit characterization of the cell biological and molecular phenotypes associated with E2F deficiency. By a combination of these two approaches, the opportunity exists to fully describe the consequences of E2F function, from the level of the whole organism, all the way to the transcriptional effects of DNA binding complexes. As these sorts of models of E2F function are improved and tested, it may become possible to ascribe specific pRB-loss phenotypes, at both the organismal and the transcriptional levels, to the deregulated transcriptional activity of particular E2Fs.

However, this genetic approach will bear principally upon questions of necessity, and not of sufficiency. In this regard, improved models of E2F function may eventually permit a fundamental test of the role of individual E2Fs in the oncogenic process: reproduction of pRB-loss phenotypes in the context of wildtype *pRB*. The emerging concept that different E2Fs may fulfill distinct transcriptional roles in the pRB pathway may eventually provide just such an opportunity. One step towards this is the creation of mice carrying "activated" alleles of *E2F-1* engineered to be refractory to inhibition by pRB. These animals promise to reveal phenotypes specifically induced by inappropriate activation of endogenous E2F-1. However, it is unlikely that transcriptional activation by E2F-1 alone is sufficient to recapitulate the full tumorigenic effect of pRB mutations. The repressive interactions between pRB and the other relevant E2Fs will still be intact in these animals, as will the pathways which act though these interactions to restrict progression through G1/S. Our data suggests that these pathways in cycling cells are largely dependent upon the ability of E2F-4 to cooperate with pRB to repress transcription during G1. Consequently, loss of E2F-4 may mimic loss of pRB so as to sufficiently deregulate these pathways and accelerate oncogenesis. In the context of an otherwise unperturbed E2F•pRB pathway, loss of E2F-4 may have minimal

effect upon cell cycle progression and tumorigenesis. However, a more significant issue is the extent to which this mutation cooperates with "activating" E2F mutations to deregulate E2F-responsive promoters in the whole animal. Such a compound genotype is predicted to have similar effect on shared targets as does loss of pRB: simultaneous derepression, and inappropriate activation, of responsive gene transcription. The combined effect of these E2F mutations may therefore be sufficient to independently reproduce organismal phenotypes associated with mutational inactivation of pRB. While the actual outcome of such a cross will not be known for some time, the ability to replicate phenotypes associated with pRB loss by manipulation of the activities of its downstream targets will represent a fundamental advance in our understanding of the biological properties of the pRB•E2F interaction, and will verify the role of E2F as the primary transcriptional effector of the cell cycle and tumorigenic properties of the p16•cyclin D•pRB G1/S regulatory pathway.

References

Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M., and Bernards, R. (1994). E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes & Dev. *8*, 2680-90.

Bernards, R. (1997). E2F: a nodal point in cell cycle regulation. Biochim. Biophys. Acta. *1333*, 33-40.

Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991). The E2F transcription factor is a cellular target for the RB protein. Cell. *65*, 1053-61.

Chittenden, T., Livingston, D. M., and DeCaprio, J. A. (1993). Cell cycle analysis of E2F in primary human T cells reveals novel E2F complexes and biochemically distinct forms of free E2F. Mol. Cell. Biol. *13*, 3975-83.

Cobrinik, D., Lee, M. H., Hannon, G., Mulligan, G., Bronson, R. T., Dyson, N., Harlow, E., Beach, D., Weinberg, R. A., and Jacks, T. (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. Genes & Dev. *10*, 1633-44.

Cobrinik, D., Whyte, P., Peeper, D. S., Jacks, T., and Weinberg, R. A. (1993). Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes & Dev. *7*, 2392-404.

Dyson, N., Dembski, M., Fattaey, A., Ngwu, C., Ewen, M., and Helin, K. (1993). Analysis of p107-associated proteins: p107 associates with a form of E2F that differs from pRB-associated E2F-1. J. Virol. *67*, 7641-7.

Fagan, R., Flint, K.J., and Jones, N. (1994). Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenoviral E4 19kDa protein. Cell *78*, 799-811.

Field, S. J., Tsai, F. Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell. *85*, 549-61.

Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z. X., Xu, G., Wydner, K. L., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994). E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes & Dev. *8*, 2665-79.

Hijmans, E. M., Voorhoeve, P. M., Beijersbergen, R. L., van 't Veer, L. J., and Bernards, R. (1995). E2F-5, a new E2F family member that interacts with p130 in vivo. Mol. Cell. Biol. *15*, 3082-9.

Hsieh, J.K., Fredersdorf, S., Kouzarides, T., Martin, K., and Lu, X. (1997). E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. Genes & Dev. *11*, 1840-52.

Hurford, R. K., Jr., Cobrinik, D., Lee, M. H., and Dyson, N. (1997). pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes & Dev. *11*, 1447-63.

Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmı, S., Bronson, R. T., and Weinberg, R. A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr. Biol. *4*, 1-7.

Lees, J. A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K. (1993). The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. *13*, 7813-25.

Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-31.

Muller, H., Moroni, M. C., Vigo, E., Petersen, B. O., Bartek, J., and Helin, K. (1997). Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol. Cell. Biol. *17*, 5508-20.

Mulligan, G., amd Jacks T. (1998). The retinoblastoma gene family: cousins with overlapping interests. Trends in Genetics. *14*, 223-229.

Peeper, D.S., Keblusek, P., Helin, K., Toebes, M., van der Eb, A.J., and Zantema, A. (1995). Phosphorylation of a specific cdk site in E2F-1 affects its electrophoretic mobility and promotes pRB-binding in vitro. Oncogene *10*, 39-48.

Phillips, A.C., Bates, S., Ryan, K.M., Helin, K., and Vousden, K.H. (1997). Induction of DNA synthesis and apoptosis are seperable functions of E2F-1. Genes & Dev. *11*, 1853-63.

Pierce, A. M., Fisher, S. M., Conti, C. J., and Johnson, D. G. (1998). Deregulated expression of E2F1 induces hyperplasia and cooperates with ras in skin tumor development. Oncogene. *16*, 1267-76.

Royzman, I., Whittaker, A. J., and Orr-Weaver, T. L. (1997). Mutations in Drosophila DP and E2F distinguish G1-S progression from an associated transcriptional program. Genes & Dev. *11*, 1999-2011.

Sardet, C., Vidal, M., Cobrinik, D., Geng, Y., Onufryk, C., Chen, A., and Weinberg, R. A. (1995). E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. U S A. *92*, 2403-7.

Schulze, A., Mannhardt, B., Zerfass-Thome, K., Zwerschke, W., and Jansen-Durr, P. (1998). Anchorage-independent transcription of the cyclin A gene induced by the E7 oncoprotein of human papillomavirus type 16. J. Virol. *72*, 2323-2334.

Schwarz, J. K., Devoto, S. H., Smith, E. J., Chellappan, S. P., Jakoi, L., and Nevins, J. R. (1993). Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. EMBO J. *12*, 1013-20.

Shirodkar, S., Ewen, M., DeCaprio, J. A., Morgan, J., Livingston, D. M., and Chittenden, T. (1992). The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell. *68*, 157-66.

Tao, Y., Kassatly, R. F., Cress, W. D., and Horowitz, J. M. (1997). Subunit composition determines E2F DNA-binding site specificity. Mol. Cell. Biol. *17*, 6994-7007.

Vairo, G., Livingston, D. M., and Ginsberg, D. (1995). Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes & Dev. *9*, 869-81.

Yamasaki, L., Bronson, R., Williams, B. O., Dyson, N. J., Harlow, E., and Jacks, T. (1998). Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-)mice. Nat Genet. *18*, 360-4.

Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N. J. (1996). Tumor induction and tissue atrophy in mice lacking E2F-1. Cell. *85*, 537-48.

APPENDIX

A. Loss of E2F•pocket protein specificity in overexpression

Our concerns regarding the ability of overexpression-based systems to faithfully function *in vivo* were confirmed by simple co-transfection of E2F-2, HA-DP-1 and p107 CMV expression constructs into C33A cells (10μg each). Cells were labeled and subject to immunoprecipitation using the anti-hemaglutinin (HA) monoclonal antibody, 12CA5. Precipitates were washed and resolved by 9% SDS-PAGE. The result of this experiment clearly demonstrates that this assay detects non-physiological complexes of E2F-2•HA-DP-1•p107 produced by overexpression (Appendix Fig.1). By careful manipulation of the input amounts of overexpressed protein, this result is reproducible in the EMSA interaction assay. I have used this autoradiograph many times to remind myself of these pitfalls, and I think that it is as important as any other piece of published data. Beware those who never look at endogenous protein.

B. Antibody production

B1. Anti-human E2F antibodies

The description of the production of antibodies specific to E2F-2, -3 and -4 is described in the Materials and Methods sections of Chapters 1 and 2. A complete summary of the known characteristics of these antibodies may be found in the "Anti-E2F Antibodies" book in Dr. Lees' laboratory. All of the antibodies in this section are derived from BALB-c mice.

* Symbols indicate whether or not an antibody recognizes the indicated antigen in an overexpression assay in C33A cells. A "-" symbol indicates an antibody does not work in that assay. The strongest antibodies in a given assay are noted with "+++"; medium antibodies are noted with "++", and the weakest are noted with an "+".
* Overexpression used 10μg of CMV-E2F and CMV-DP expression constructs per 10cm dish of cells. For IPs, cells were labelled with 0.25 mCi/ml of NEN ^{35}S-Express Labeling Mix for 3-4 hours. IPs were performed in ELB lysis buffer.

* Western blot analysis of transfected protein was performed by loading ~10μg total cellular protein in a single 12cm well SDS-PAGE gel. Hybridoma supernatants were screened by slot-blot at a 1:10 dilution.
* The comments in the "Notes" category refer to specific characteristics of individual antibodies. Where possible, comments have been made regarding reactivity to murine E2Fs.
* All isotypes unknown; however, all "IP+" lines must be IgG, either of subclass 2a, 2b, or 3.
* All epitopes undetermined, except for indicated anti-E2F-2 hybridomas
* Frozen stocks of all of these lines in $N_2(l)$ freezer (see nitrogen freezer log book)

B2. Anti-human DP-1 antibodies

All comments in section A1 are relevant here, with the exception of:

* full-length DP-1 was used to immunize animals
* Swiss Webster mice used for anti-DP-1 antibody generation
* All clones taken through serial-dilution step (ie. NOT single-cell cloned) and frozen in $N_2(l)$ (see nitrogen freezer book for location)
* Since clones are not pure, only "-/+" designations are used
* All clones mycoplasma-free by the BoehringerMannheim PCR assay "BM Cyclin".

C. Cyclin E- vs. Cyclin A-mediated phosphorylation of E2F-1•DP-1

While we have resolved the issue of the ability of cyclin A and cyclin B-kinase activity to mediate site-specific phosphorylation of E2F-1•DP-1 heterodimers, we have not published our analysis of the differences between cyclin A and cyclin E. Partially because of a bit of a dispute in the field, this data was withheld from publication. Like cyclin B, cyclin E does not interact stably with E2F heterodimers. However, reports from other labs suggest that phosphorylation by cyclin E•cdk2 actually enhances DNA binding of E2F-1•DP-1 at high levels of input kinase; this effect was not reported for cyclin A•cdk2. The ability of cyclin E•cdk2 to produce this effect correlated

with the presence of a novel E2F-1-derived phosphopeptide which was not produced by the activity of cyclin A•cdk2. We also included cyclin E•cdk2 in our comparison of the ability cyclin-dependent kinases to phosphorylate the E2F-1•DP-1 heterodimer *in vitro*. Like cyclin B, cyclin E associated kinase was observed to phosphorylate this substrate 50% less efficiently than cyclin A, based upon a normalization of kinase activity versus histone H1 substrate. Significantly, our assessment of the ability of these kinases to phosphorylate E2F-1•DP-1 was performed in the presence of limiting input kinase (1:10 molar ratio of cyclin•cdk:E2F•DP; see Mat. & Meth., Chapter 4). In this way, the experiment was intended to reveal the *preferred* sites of phosphorylation, and not those which *can* become modified by the individual kinases. Using these input levels, two-dimensional phosphopeptide analysis of E2F-1•DP-1 heterodimers incubated with either cyclin A•cdk2 or cyclin E•cdk2 failed to reveal major differences in the major sites of phosphorylation (see Appendix Fig.2). There are, however, minor "spots" which are differentially apparent in each of the maps. These could represent incomplete trypsin digestion, or they could indicate minor differences in the site-preferences of cyclin A vs. cyclin E. The physiological relevance of such *in vitro* differences was not investigated.

We have also taken advantage of the generation of phosphorylation site mutants in the DP-1 cDNA (generated by R. Verona) to map the site of the "off-switch" phosphorylation in DP-1 . We have mapped this site to serine residue 23, and show that this residue represents the only site of DP-1 phosphorylation *in vivo* (Appendix Fig.5).

D. Mechanism(s) of E2F-4 localization

D1. Phosphorylation

A considerable amount of time and effort was directed towards determining the molecular mechanisms responsible for the cell cycle-dependent localization of E2F-4. It is immediately apparent from the examination of the timing of this process that it may be linked directly to the accumulation of active cyclin-dependent kinases in late G1. The ability of these kinases to phosphorylate members of the retinoblastoma, E2F, and DP families suggests that

phosphorylation of these proteins may trigger the disappearance of nuclear E2F-4. Examination of the phosphorylation state of cytoplasmic and nuclear forms of endogenous E2F-4•DP-1 suggested that these forms were distinguished by a novel phosphopeptide present only in the cytoplasmic population of DP-1. After much work it was determined that this peptide was actually derived from E2F-4 (a minor fraction of ^{32}P-labeled E2F-4 co-migrates with ^{32}P-labeled DP-1 in 8-10% SDS-PAGE), and that the abundance of this phosphopeptide does not correlate with subcellular localization. However, in the course of this work, we actually learned quite a bit about the relative abundance of DP-1 and -2 bound to E2F-4 in each cellular compartment (Appendix Fig.4). All three major isoforms of DP-2 were found exclusively in nuclear E2F-4•DP complexes. Phosphopeptide mapping also suggests that a significant portion of endogenous, E2F-4-associated DP-1 is phosphorylated on serine 23 (Appendix Fig.5). Current models suggest that this phosphorylated E2F-4•DP-1 heterodimer may represent a non-DNA binding form of cellular E2F.

D2. 14-3-3 protein binding

An considerable amount of recently published work suggests that the 14-3-3 family of cytoplasmic signaling molecules are site-specific phosphoserine binding proteins (Muslin et al., 1996). These proteins are known to bind specifically to serine-phosphorylated forms of important signaling molecules, like Raf and CDC25 (Muslin et al., 1996; Yaffe et al., 1997), and to be necessary for the inhibition of the activity of these proteins. In the case of CDC25 family proteins, this inhibition is thought to occur in large part by cytoplasmic sequestration. The 14-3-3 family is now known to recognize a phosphoepitope which is conserved in all of its targets. This sequence is RSxSxP, in which the fourth-position serine represents the site of regulatory phosphorylation. Inspection of the primary amino acid sequence reveals a similar motif present in the E2F-4/5 subclass of E2Fs: KSxSxP This sequence is present within the conserved "marked-box" region of E2F-4 and -5; the critical fourth-position residue is not present in the other E2Fs. This observation suggested that 14-3-3 binding might directly bind to, and regulate the localization of,

E2F-4/5. Attempts to uncover complexes containing 14-3-3 isoforms and E2F-4 were unsuccessful (Appendix Fig.6); however, these IP-Western experiments tend to suggest that E2F-2 binds 14-3-3 proteins *in vivo*. This relevance of this result is unclear, although from the perspective of potential E2F-4 localization mechanisms, it was completely uninformative.

D3. Half-life of cytoplasmic and nuclear E2F-4 species

One possible explanation of the absence of E2F-4 protein from the nuclei of S-phase cells, and its persistence in the cytoplasm, is that these two forms are differentially affected by proteolytic degradation pathways. It has been reported that free E2F is more susceptible to such destruction than are pocket-protein bound forms of E2F proteins (Hateboer et al., 1996; Hofmann et al., 1996). The phosphorylation-triggered disruption of the E2F•pRB complex in late G1 may then expose E2F-4 to a nuclear proteolytic process which effectively eliminates E2F-4 from this cellular compartment. This model predicts that levels of free E2F-4 are elevated in the cytoplasm because such a proteolytic mechanism does not operate in this subcellular compartment. To test for differential susceptibility to proteolysis, wildtype, constitutively nuclear (NLS-E2F-4), and constitutively cytoplasmic (NES-E2F-4) forms of E2F-4 were transfected into U2OS cells and their half-life assessed by pulse-chase ^{35}S-methionine/cysteine labeling (Fig.7). In this experiment, free, nuclear E2F-4 was not significantly less stable than cytoplasmic E2F-4; if anything, the cytoplasmic protein had a shorter half-life than did nuclear protein.

E. Cell cycle-dependent properties of E2F-3 protein

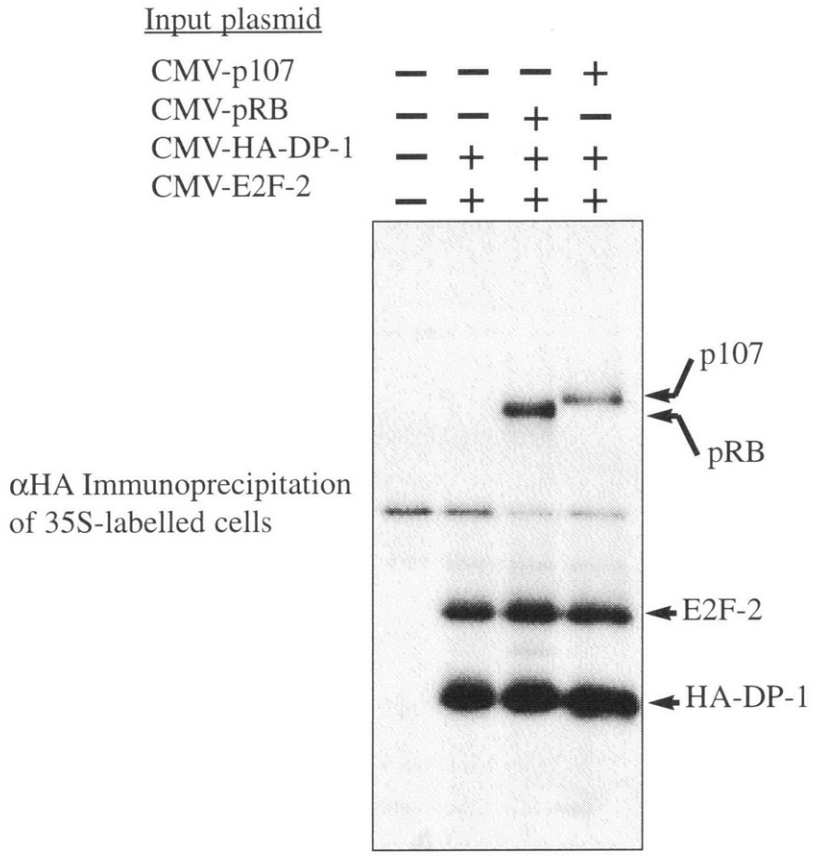
In the course of our analysis of the localization properties of endogenous E2F proteins in elutriated fractions of HL-60 cells (see Chapter 3), it was observed that approximately 50% of the total cellular E2F-3 protein undergoes an S-phase-specific modification in electrophoretic mobility which correlates with a reduced affinity for the nuclear compartment (Appendix, Fig. 8). This modification is specific to E2F-3, and reduces the mobility of the E2F-3 polypeptide in SDS-PAGE. The nature of this modification was not investigated (ie. phosphatase treatment). However, given that E2F proteins are targets of cyclin•cdks *in vitro* and *in vivo*, it is likely that this

mobility shift represents a novel phosphorylation state. Two aspects of E2F function have been demonstrated to be regulated by site-specific phosphorylation of E2F•DP heterodimers: interaction with pRB (Fagan et al., 1994; Peeper et al., 1995) and DNA-binding activity (Dynlacht et al., 1994; Krek et al., 1994; Xu et al., 1994). Either mechanism may underlie the E2F-3 mobility shift observed here.

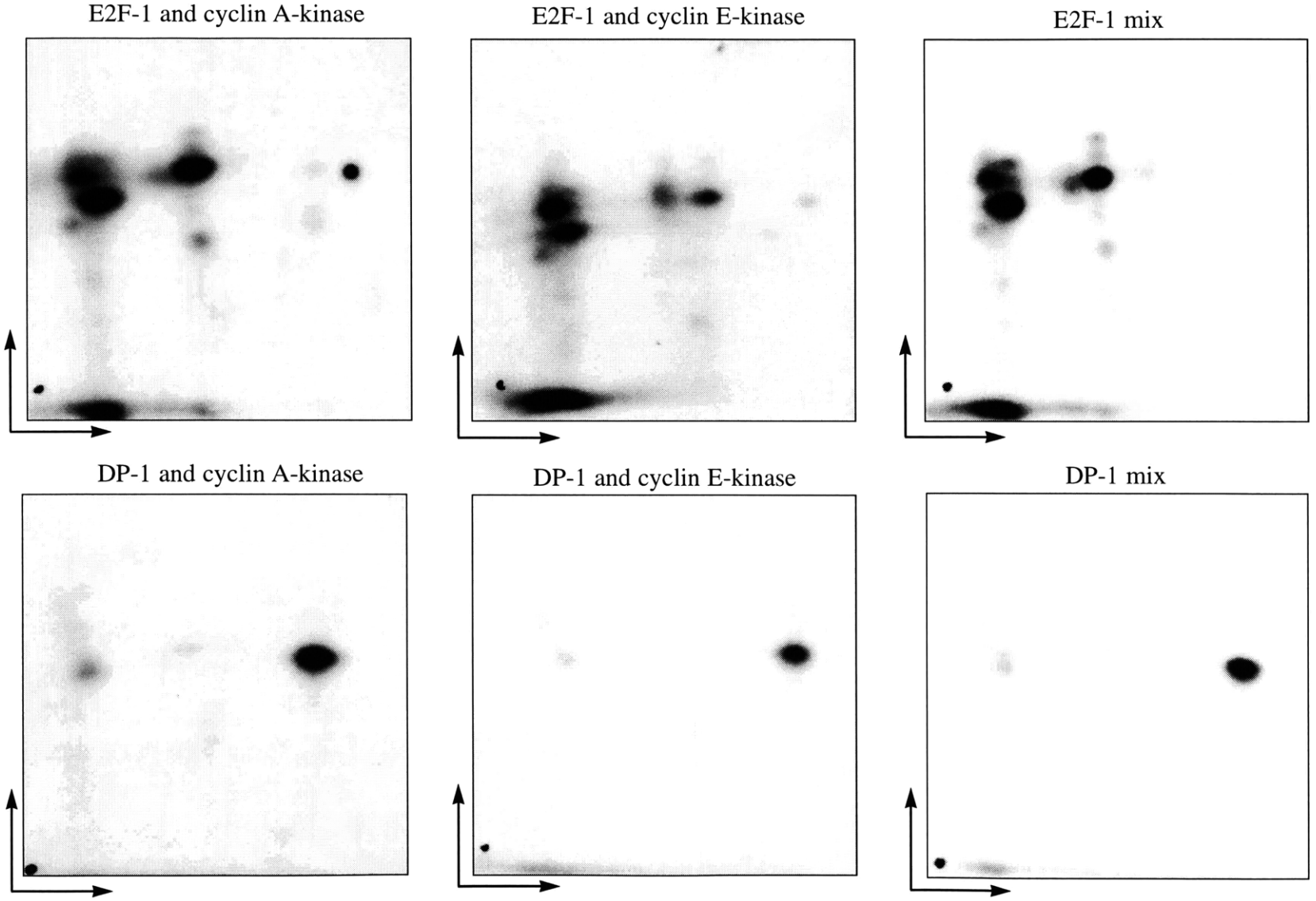
F. An irradiation inducible E2F•pocket protein complex

Given our genetic and biochemical data regarding the role of pRB in the G1 arrest pathway in response to DNA damage, it was logical to assume that this pathway induced the accumulation of transcriptionally repressive E2F•pRB complexes. To test this, whole cell extracts of treated/untreated MEFs of either wildtype, *p21-/-* or *pRB-/-* genotypes were subject to analysis in gel shift assays. An irradiation inducible E2F•pocket protein complex was clearly detectable which migrates above free E2F, but below E2F•p107. Antibodies to E2F-4 (LLF4-2) specifically supershift this complex; so does the Santa Cruz polyclonal rabbit antibody to p130 (sc317x), which can cross react to p107 when added in excess (Appendix Fig.9).

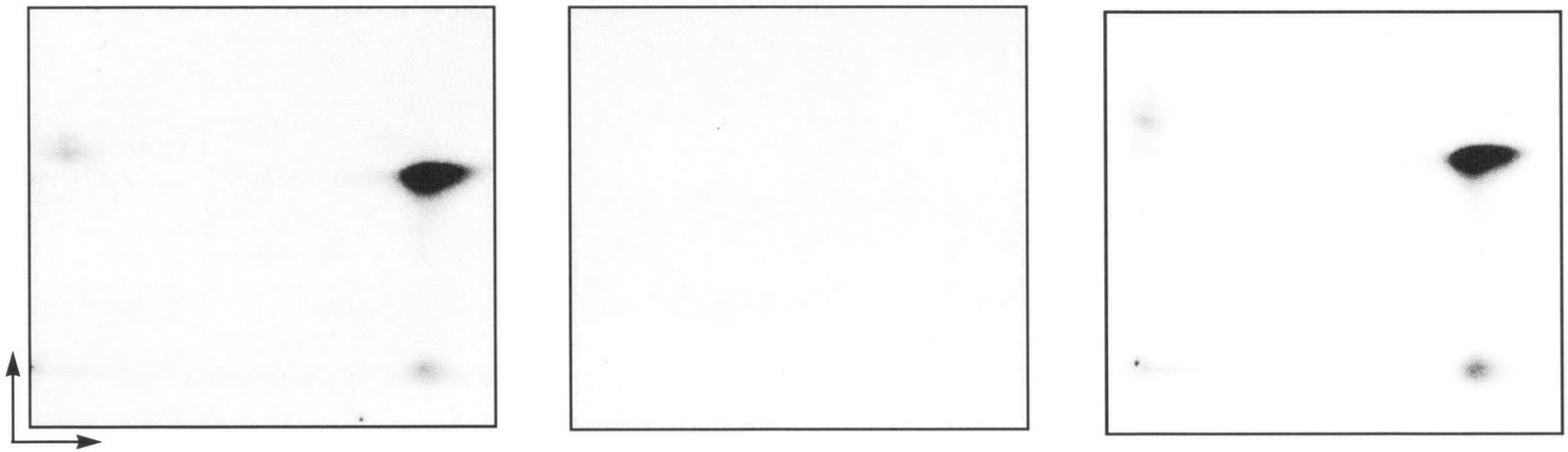
Appendix Figure 1: Interaction of E2F-2 and pRB/p107 in transfected C33A cells



Appendix Fig. 2: E2F-1/DP-1 as substrates of CyclinA and Cyclin E kinases

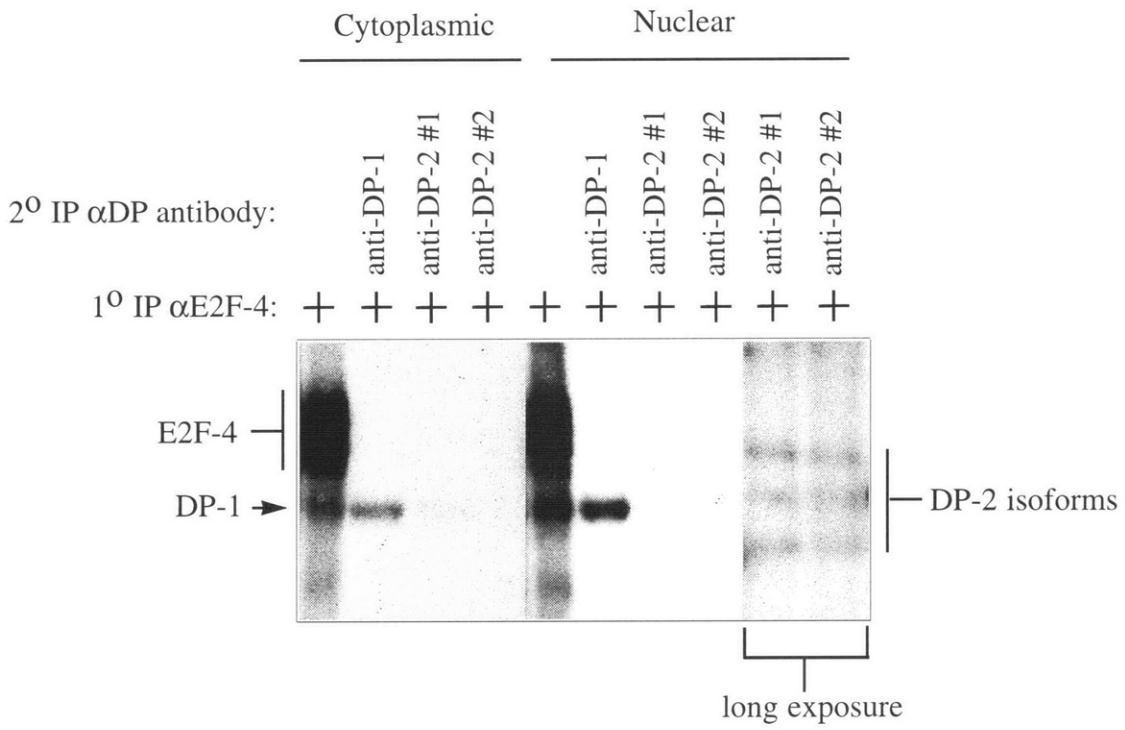


Appendix Figure 3: "Off-switch" phosphorylation maps to Serine 23 in E2F-1-bound DP-1



wildtype DP-1 DP-1 S23A DP-1 T66A

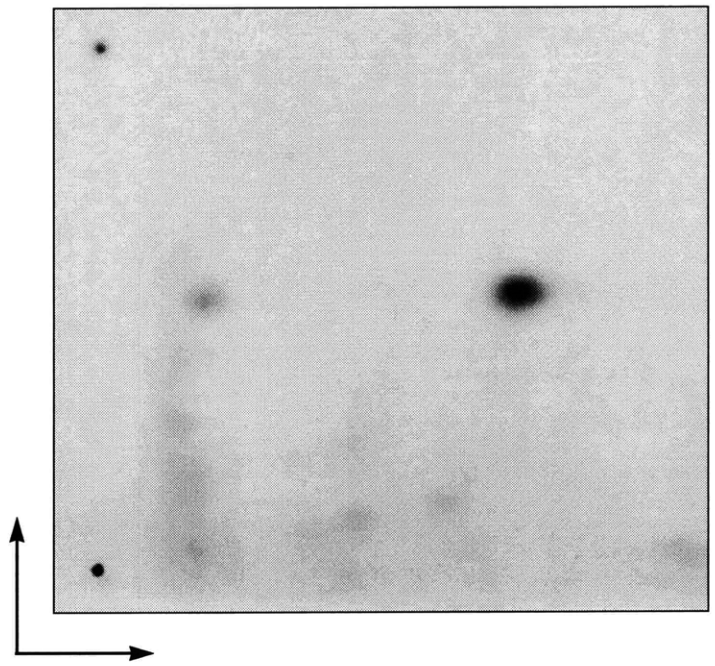
Appendix Fig. 4: E2F-4 asscociated forms of DP proteins



Immunoprecipitation-reimmunoprecipitation analysis from ^{32}P-labelled, fractionated U2OS cells

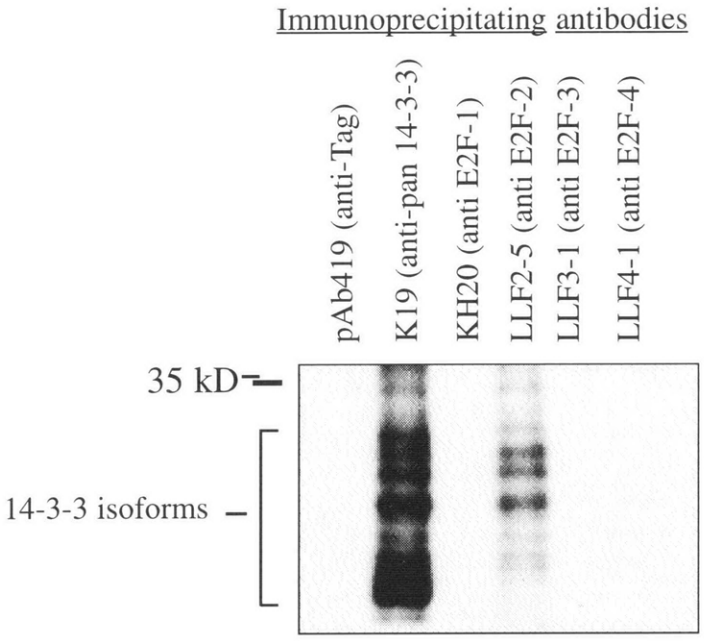
Appendix Fig. 5: Phosphopeptide analysis of DP-1 reimmunoprecipiated from E2F-4 containing complexes *in vivo*

Endogenous DP-1 from ^{32}P-labelled U2OS cells



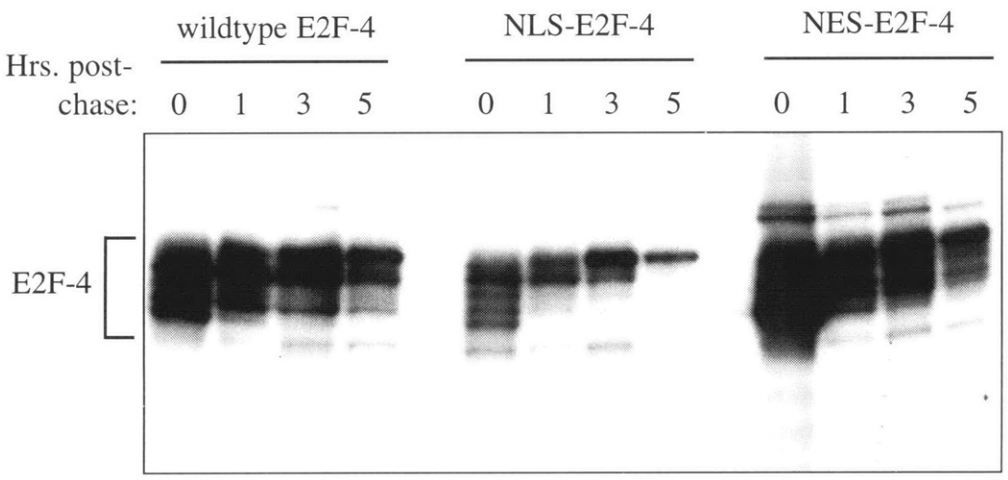
Migration position of single, major DP-1-derived phophopeptide corresponds to position of Serine23-containing"spot"

Appendix Fig. 6: Association of endogenous E2F and 14-3-3 proteins in the U2OS cell line

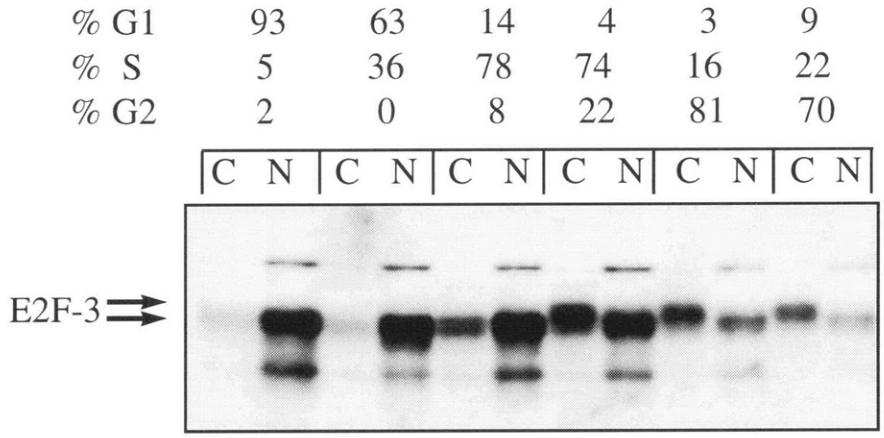


Immunoblot developed with K19 α-pan-14-3-3 rabbit antibody (Santa Cruz)

Appendix Figure 7: Half-life comparison of transfected forms of E2F-4 in U2OS cells

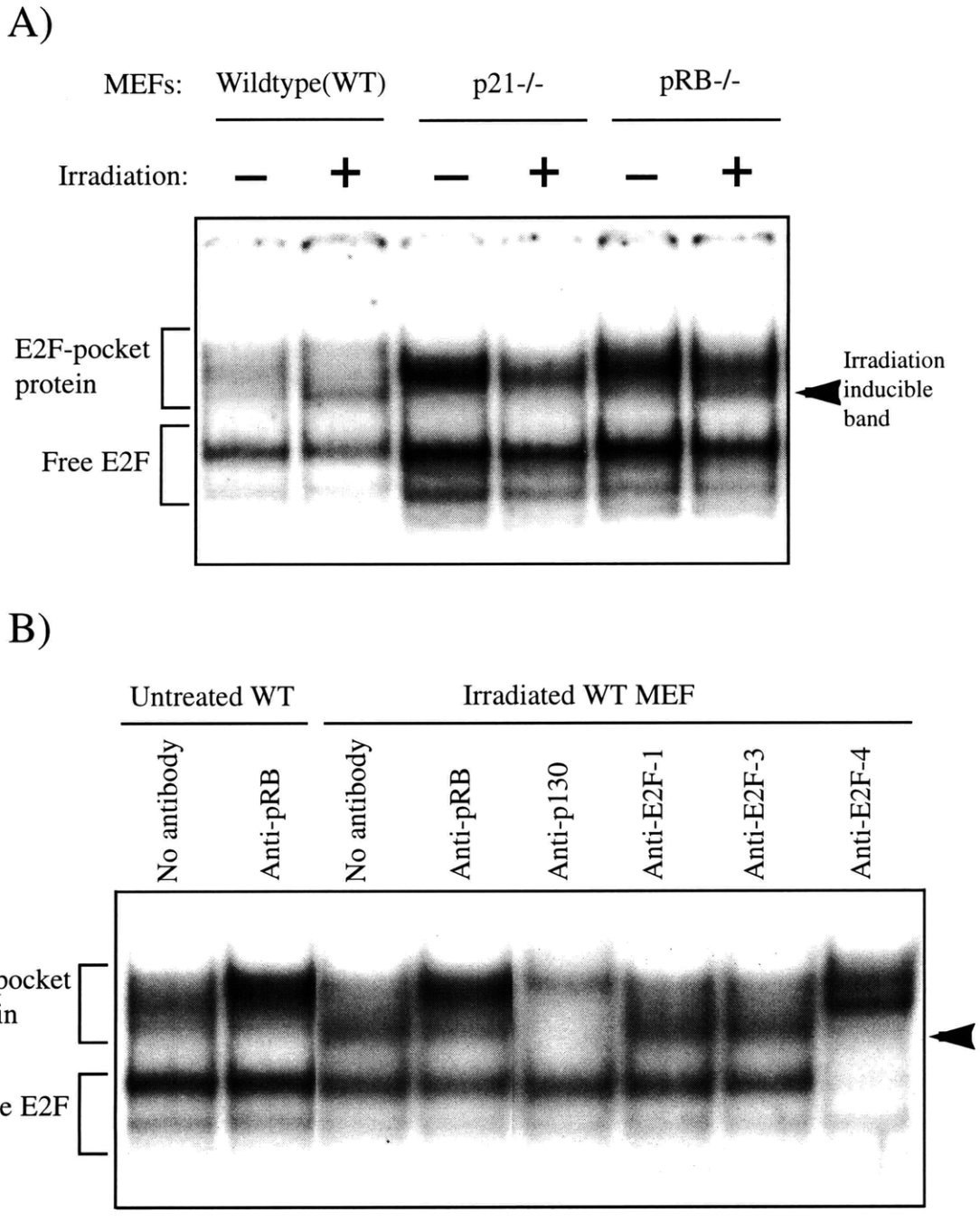


Appendix Fig. 8: Cell cycle-dependent changes in mobility and nuclear affinity of E2F-3 protein in HL-60 cells



Western Immunoblot analysis (LLF3-1)

Appendix Fig.9: An irradiation-inducible E2F complex



References

Dynlacht, B. D., Flores, O., Lees, J. A., and Harlow, E. (1994). Differential regulation of E2F transactivation by cyclin/cdk2 complexes. Genes & Dev. *8*, 1772-86.

Fagan, R., Flint, K. J., and Jones, N. (1994). Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenoviral E4 19 kDa protein. Cell. *78*, 799-811.

Hateboer, G., Kerkhoven, R. M., Shvarts, A., Bernards, R., and Beijersbergen, R. L. (1996). Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. Genes & Dev. *10*, 2960-70.

Hofmann, F., Martelli, F., Livingston, D. M., and Wang, Z. (1996). The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. Genes & Dev. *10*, 2949-59.

Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., Jr., and Livingston, D. M. (1994). Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell. *78*, 161-72.

Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996). Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell. *84*, 889-97.

Peeper, D. S., Keblusek, P., Helin, K., Toebes, M., van der Eb, A. J., and Zantema, A. (1995). Phosphorylation of a specific cdk site in E2F-1 affects its electrophoretic mobility and promotes pRB-binding in vitro. Oncogene. *10*, 39-48.

Xu, M., Sheppard, K. A., Peng, C. Y., Yee, A. S., and Piwnica-Worms, H. (1994). Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. Cell. Biol. *14*, 8420-31.

Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997). The structural basis for 14-3-3:phosphopeptide binding specificity. Cell. *91*, 961-71.

ACKNOWLEDGEMENTS

There are many people to whom I would like to express my gratitude and respect. Most of all, to Jackie, who like Judy, never let me off the hook. To all my colleagues and collaborators, who made me realize I have something to contribute. To Michelle, Raluca, Brenda, Brian, Sandra, Pat, Cass, Elad, Riki and Scott who forvever changed my musical tastes and made lab feel like home. To the entire Lees lab, past and present, who showed me that science should be fun. To J.P. and Ameet, who suffered my attempts to manage them, and still got things done. And, because nothing ever comes easy, to those lost on the way. Thank you.

"One only becomes real at the point of action"

-Chinese proverb