Oncogene (2000) 19, 2257–2268

www.nature.com/onc

Exogenous E2F expression is growth inhibitory before, during, and after cellular transformation

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To gain insight into the tumor suppressor properties of E2F1, we investigated growth inhibition by the E2F family of transcription factors using a tissue culture model system. We first show that exogenous E2F expression causes an 80% decrease in NIH3T3 colony formation and activated c-Ha-Ras-mediated focus formation. Inhibition of Ras-mediated transformation was dependent upon E2F DNA binding activity but did not require amino- or carboxy-terminal E2F1 protein interaction domains. Because E2F upregulation has been suggested to be associated with a neoplastic phenotype, it was possible that increased E2F activity would not be inhibitory to previously transformed cells. However, we found that exogenous E2F was also inhibitory to growth of NIH3T3 cells previously transformed by Ras or Neu. Further characterization revealed that exogenous E2F expression is inhibitory at very early times after transfection, causing dramatic losses in transfected cell populations. Interestingly, those few cells which do establish appear to be unaffected by the overexpressed E2F. Therefore, we propose that increased E2F activity may only be tolerated in a subset of cells which have acquired specific alterations that are dominant over E2F-mediated growth inhibition. *Oncogene* (2000) **19,** 2257–2268.

Keywords: E2F; Ras; Neu; NIH3T3; transformation; tumor suppressor

Introduction

E2F is a family of transcription factors which function as heterodimers that regulate transcription by binding to a sequence (TTTSSCGC; S=C or G) known as an E2F site. To date, six different mammalian E2F cDNAs (E2F1, E2F2, E2F3, E2F4, E2F5 and E2F6) have been cloned; each of these can heterodimerize with either DP1 or DP2. The E2F components contain a central DNA binding and dimerization domain and, except for E2F6, a carboxy terminal transactivation domain. Nested within the transactivation domain of the E2F proteins is an interaction domain called the pocket binding domain which mediates contact with the Retinoblastoma (Rb), p107, and p130 proteins. Rb, p107, and p130 repress transcription of promoters which contain E2F sites via recruitment of histone deacetylases (Ferreira *et al*., 1998). Rb must be released from the E2F/promoter complex for the gene to be transcribed. The disruption of the E2F/Rb protein complex is due to phosphorylation of Rb by cell cycle-regulated kinases, in particular Cyclin D1- and Cyclin E-dependent kinases. Hyperphosphorylated Rb cannot bind to E2F. Thus, as cells progress through G1 into S phase, the increased cyclin-dependent kinase (CDK) activity causes the release of Rb and activation of E2F target genes. The action of the cyclin-dependent kinases is kept under control by the p16 and p21 families of CDK inhibitors. In turn, p21 is controlled by the activity of the p53 tumor suppressor protein, whose levels can be regulated by p19ARF. Many studies have shown that negative regulators of E2F activity, such as Rb and p53, are lost in a variety of human tumors while the positive regulators of E2F activity, such as Cyclin D1 and Cyclin E, are upregulated in cancer.

The frequent deregulation of the Rb/cyclin signal transduction pathway in human cancers has led to the hypothesis that increased E2F activity is a critical determinant of the neoplastic phenotype. If so, intentional overexpression of E2F family members should be able to cause neoplastic transformation. Accordingly, several studies have demonstrated the ability of E2F family members, in cooperation with activated Ras, to transform both primary rat embryo fibroblasts grown in culture and keratinocytes of transgenic animals (Johnson *et al*., 1994; Pierce *et al*., 1998a,b). Although these overexpression studies clearly indicate that E2F1 has the potential to be an oncogene under certain conditions, the creation of the E2F1 nullizygous mouse has provided an alternative view. Mice lacking E2F1 develop tumors in specific organs later in life, suggesting that loss of E2F1 enhances tumor development, a hallmark of a tumor suppresor gene, not an oncogene (Field *et al*., 1996; Yamasaki *et al*., 1996). In addition, targeted overexpression of E2F1 in skin cells of transgenic mice led to resistance to skin tumor development following treatment with the promoting agent *O*-tetradecanoyl-phorbol-13-acetate (TPA) (Pierce *et al*., 1999). Thus, E2F1 can act as both an oncogene and a tumor suppressor gene. It is likely that the different functional consequences of high levels of E2F1 are determined by the levels and/or activity of other growth promoting or growth inhibitory proteins expressed in the cells being analysed.

In order to gain a better understanding of the different growth properties of E2F, we tested E2F family members in two assays using NIH3T3 cells: (i) a colony formation assay, which determines if the exogenously expressed protein promotes or inhibits growth, and (ii) a focus formation assay, which determines if the exogenously expressed protein

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Received 12 October 1999; revised 24 February 2000; accepted 1 March 2000

cooperates or interferes with transformation mediated by an oncoprotein. We find that E2F family members are growth inhibitory and interfere with transformation mediated by the Ras oncoprotein in NIH3T3 cells. We also show that prior transformation of cells by Ras or Neu cannot override the growth inhibitory properties of E2F in NIH3T3 cells. Finally, we delineate the domains of E2F1 necessary for inhibition of growth and transformation, and characterize the nature of this inhibition.

Results

E2F1 is growth inhibitory to NIH3T3 cells in a colony formation assay

Depending on the model system examined, E2F1 can either exhibit the growth promoting properties of an oncoprotein or the inhibiting effects of a tumor suppressor protein. As a first step in ascertaining the growth properties of E2F, we analysed colony formation upon transfection by E2F1 in Swiss NIH3T3 cells. E2F1 expression constructs were cotransfected into NIH3T3 cells with a limiting amount of plasmid which confers hygromycin B resistance. After the cells were allowed to grow under hygromycin B drug selection for 2 weeks, the culture dishes were formalin-fixed, stained, and scored for drug resistant colony formation. If E2F1 promotes growth, an increase in colony number and/or size compared to dishes transfected with empty vector should be detected. If E2F1 is growth inhibitory, a decrease in the colony number and/or size should be seen. We found that expression of E2F1 caused an 80% reduction in the number of colonies formed, suggesting that exogenous expression of E2F1 is inhibitory to NIH3T3 cells growth (Figure 1a,b). This colony assay result is characteristic of results obtained using tumor suppressor proteins, such as Rb and p107 (Huang *et al*., 1988; Zhu *et al*., 1993).

To further characterize this growth suppressive property of E2F1, we evaluated the importance of three major functional domains of E2F1 in the inhibition of colony formation: the DNA binding domain, the transactivation domain, and the Rb binding domain. When E2F1 E138, a mutant E2F1 protein which contains an inactivating mutation within the DNA binding domain (Cress *et al*., 1993), was tested in this assay, there was less inhibition of colony formation. This result indicated that DNA binding properties of E2F1 are important for growth inhibition (Figure 1a,b). To determine if E2F growth inhibition was dependent on the ability of E2F to transactivate or to bind to Rb, the E2F1 Δ417–437 protein, which contains a 20 amino acid carboxy-terminal deletion, was tested in the assay (Helin *et al*., 1993a). Despite the removal of the pocket binding domain and inactivation of the transactivation domain, E2F1 Δ417–437 effectively inhibited colony formation indicating that Rb binding and transactivation by E2F1 are not necessary for growth inhibition (Figure 1a,b). The difference in activity of the three E2F1 expression constructs was not due to differences in protein expression, as demonstrated by Western blot analysis (Figure 1c). Thus, we conclude that the growth inhibition required DNA binding ability, but did not require the transactivation or the Rb binding domain of E2F1.

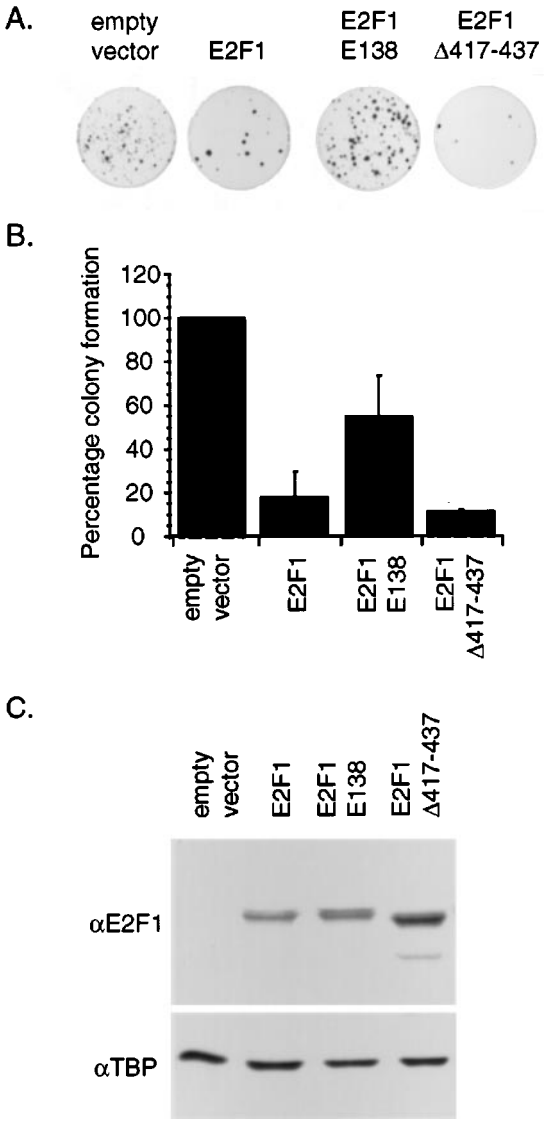

Figure 1 Inhibition of colony formation by exogenous E2F1 expression. Ten μg E2F1 construct or empty vector plus 1 μg hygromycin resistance vector were cotransfected into NIH3T3 cells. Cells were grown for 2 weeks in growth media supplemented with hygromycin B (1 mg/ml) following transfection. Plates were harvested by formalin fixation and methylene blue staining. (**a**) Representative plates. (**b**) Bar graph representing the percent of colonies obtained relative to empty vector transfectants for wildtype and mutant E2F1 constructs. Values represent the mean of three independent experiments conducted in duplicate. Standard error for each value is given. (**c**) Western blot probed with anti-E2F1 and anti-TBP antibodies. NIH3T3 cells were lipofected with 10 μg wildtype or mutant E2F1 expression constructs. One day following lipofection, cells were harvested for nuclear extracts (NE) and 100 μg NE was analysed by Western blot analysis

E2F transcription factors inhibit NIH3T3 focus formation mediated by activated c-Ha-Ras

Although overexpressed E2F was inhibitory to normal NIH3T3 cell growth, it was possible that coexpression of E2F with a transforming oncoprotein could alter the E2F inhibitory response in these cells. Therefore, we conducted a focus formation assay in which an empty

vector control plasmid or plasmids driving expression of E2F1, E2F1 E138, or E2F1 Δ417–437 were cotransfected into NIH3T3 cells with limiting amounts of plasmid expressing the activated c-Ha-Ras oncoprotein (Figure 2a,b). Cells were allowed to grow for 2 weeks, after which time, culture dishes were fixed, stained, and scored for transformed foci. We found that E2F1 and E2F1 Δ417–437 inhibited Ras-mediated focus formation, but E2F1 E138, which lacks DNA binding activity, did not. In the experiment shown in Figure 2a, a 50-fold excess of the E2F1 plasmid over the Ras plasmid was used. We have

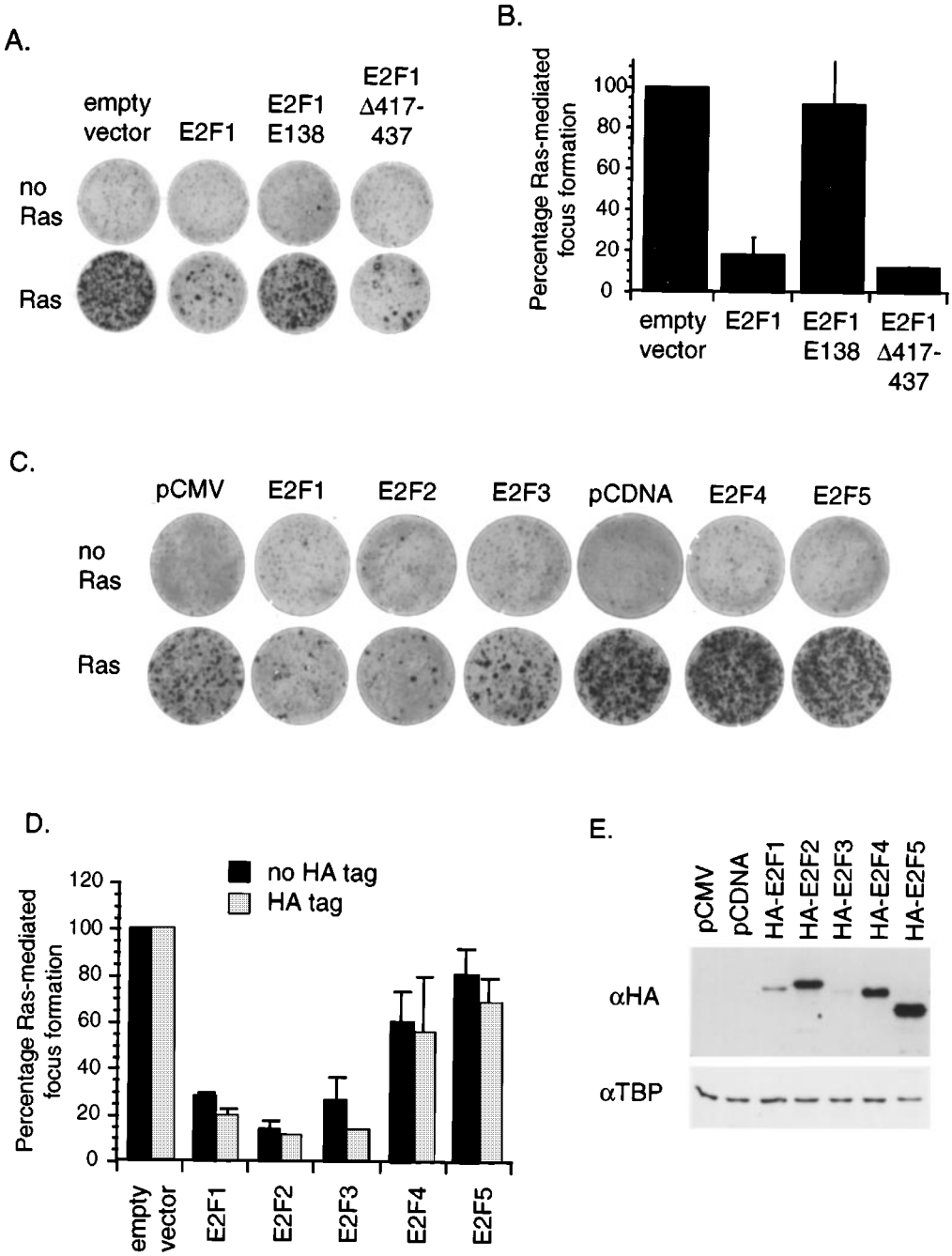

Figure 2 E2F transcription factors inhibit NIH3T3 focus formation mediated by activated c-Ha-Ras. (**a**) NIH3T3 cells were transfected with 10 μg E2F1 expression constructs or empty vector plus or minus 0.0, 0.1, or 0.2 μg Ras construct. Cells were grown for 2 weeks in growth media following transfection and harvested by formalin fixation and methylene blue staining. Shown are representative plates from a single experiment using 0.0 μg ('no Ras') or 0.2 μg Ras ('Ras') construct. (**b**) Bar graph representing the per cent of Ras-transformed foci obtained relative to empty vector transfectants. Values represent the mean of three independent focus formation assays, each conducted in duplicate. Standard error for each value is shown. (**c**) NIH3T3 cells were transfected with 10 μg E2F expression constructs plus or minus 0.0, 0.1 or 0.2 μg Ras construct. Cells were grown for 2 weeks in growth media following transfection and harvested by fixing plates with formalin and staining with methylene blue. Shown are representative plates from a single experiment using 0.0 μg ('no Ras') or 0.2 μg Ras ('Ras') construct. (**d**) Bar graph representing the percentage of Ras-transformed foci relative to empty vector transfectants. Black bars represent values for untagged E2F constructs and the gray bars represent values for hemagglutinin (HA)-tagged E2F constructs. With the exception of HA-E2F2 and HA-E2F3, values for all constructs represent the mean of three independent focus formation assays, each conducted in duplicate. Standard error for each value is given. Values for HA-E2F2 and HA-E2F3 constructs represent one experiment conducted in duplicate. (**e**) Western blot probed with anti-HA and anti-TBP antibodies. NIH3T3 cells were lipofected with 10 μg HA-E2F1, HA-E2F2, HA-E2F3, HA-E2F4, HA-E2F5 or empty constructs. One day following lipofection, cells were harvested for nuclear extract (NE) and 100 μg NE was analysed by Western blot analysis

found that gradually decreasing this ratio results in a gradual loss of E2F1 inhibition (data not shown). As shown in Figure 1c, the loss of inhibition by E2F1 E138 was not due to a lack of protein expression. We next tested whether other E2F family members had a similar inhibitory effect on Ras-mediated transformation. Plasmids driving expression of E2F1, E2F2, E2F3, E2F4 and E2F5 were transfected into NIH3T3 cells with or without a plasmid expressing activated Ras. In the absence of transfected Ras, no foci were observed in plates transfected with E2F expressing plasmids, indicating that E2F family members alone are not oncogenic in this assay (Figure 2c). However, cotransfection of all E2F family members with Ras caused a decrease in Ras-mediated focus formation (Figure 2c,d). E2F1, E2F2, or E2F3 caused an approximately 80% decrease in the number of Ras-induced foci, with less inhibition detected in the presence of E2F4 or E2F5. Although all E2F expression plasmids contain E2F cDNAs driven by the cytomegalovirus (CMV) promoter, the E2F4 and E2F5 expression constructs have a slightly different plasmid backbone than the E2F1, E2F2, and E2F3 expression constructs. If this difference resulted in lower levels of E2F4 and E2F5 expression, it may explain the lack of inhibition by these two E2F proteins. Relative expression levels cannot be determined using antibodies to the individual E2F family members due to differences in antibody affinity. However, relative expression levels could be determined when the E2F proteins were fused to a hemagglutinin (HA) epitope. As shown in Figure 2d, the HA-tagged E2F constructs had similar Ras inhibitory effects as the original E2F plasmids in a focus formation assay. To determine the relative levels of the exogenously expressed E2F proteins, Western blot analysis was conducted on nuclear extracts from NIH3T3 cells lipofected with constructs expressing HA-tagged E2F proteins. Western blot analysis indicates that the inability of E2F4 and E2F5 to inhibit focus formation is not due to low protein expression; the amounts of E2F4 and E2F5 were as high or higher than amounts of E2F1, E2F2 and E2F3 (Figure 2e, top panel). There were relatively low levels of E2F1 and E2F3 protein detected. This is consistent with studies which state that the E2F1 and E2F3 proteins are unstable due to ubiquitin-mediated proteolysis (Flores *et al*., 1998; Marti *et al*., 1999). The same blot was probed with anti-TATA binding protein (TBP) antibodies as a loading control for nuclear protein (Figure 2e, bottom panel).

DNA binding ability appears to be necessary and sufficient for inhibition of Ras-mediated transformation by E2F1

In addition to the domains which mediate DNA binding, Rb binding, and transactivation, E2F1 contains other domains which mediate different protein-protein interactions. To determine whether these other domains of E2F are important for inhibition of transformation, we tested several previously characterized mutant E2F1 expression vectors in the focus formation assay. E2F1, E2F2 and E2F3 share similar amino-terminal sequences not found in E2F4 or E2F5. These domains mediate interactions with proteins such as Cyclin A and the Sp1 transcription factor (Krek *et al*., 1994; Karlseder *et al*., 1996; Lin *et al*., 1996). It was possible that such protein-protein interactions specify stronger inhibition by E2F1, E2F2 and E2F3, compared to E2F4 and E2F5. To address whether amino-terminal sequences are important in the inhibition of Ras-mediated transformation, we utilized an E2F1 amino-terminal deletion mutant, E2F1 $\Delta 1-88$, which contains a truncation of the first 88 amino acids, and E2F1 $\Delta 79-103$, which contains an internal deletion within the Cyclin A binding domain. Our results indicate that Cyclin A binding is not required for inhibition of Ras-mediated transformation (Figure 3a). In fact, loss of Cyclin A binding causes E2F1 to be a more effective inhibitor of transformation. Since Cyclin A is thought to negatively regulate DNA binding by E2F1 through the cell cycle (Krek *et al*., 1994; Xu *et al*., 1994; Kitagawa *et al*., 1995), loss of Cyclin A binding may cause an increase in E2F1 DNA binding activity, especially at inappropriate times during the cell cycle. While deletion of the first 88 amino acids of E2F1 does decrease the effectiveness of E2F1 as an inhibitor of transformation, E2F1 $\Delta 1-88$ still retains the ability to reduce Ras-mediated foci to 37.5% of levels seen with Ras alone (Figure 3a). The first 88 amino acids contain the Sp1 transcription factor interaction domain as well as amino acids which contribute to nuclear localization (Karlseder *et al*., 1996; Lin *et al*., 1996; Müller *et al*., 1997). Either of these functions may contribute to the inhibition of focus formation.

We next analysed the region downstream of the DNA binding domain. While consecutive carboxy-terminal deletions up to the DNA binding domain of E2F1 caused decreases in inhibition, deletion of sequences from amino acids 191–437 still resulted in inhibition of Ras-mediated transformation to 30% of the number of foci seen with Ras alone. Thus, the minimal region common to all inhibitory E2F1 proteins is amino acids 103–191 (Figure 3a). Included within this region are amino acids 110–191 which have been shown to be the minimal DNA binding domain using *in vitro* gel mobility shift assays (Ivey-Hoyle *et al*., 1993). Our data suggests that the DNA binding domain of E2F1 is critical in mediating the growth inhibitory effects. Accordingly, cotransfection of E2F1 with DP1, a heterodimeric DNA binding partner of E2F, results in greater inhibition of foci formation than the addition of E2F1 alone (data not shown).

As noted above, the different E2F family members inhibit Ras-mediated transformation to varying extents. For example, E2F1 is a better inhibitor of Ras-mediated foci formation than is E2F4. However, our deletion analysis indicates that the DNA binding domain of E2F1, which is very similar to the DNA binding domain of E2F4, appears to be the critical component for inhibition of Ras-mediated transformation. Using this criterion, E2F4 should be as effective an inhibitor of Ras-mediated transformation as E2F1. It has been previously noted that during S phase, when E2F target genes are expressed, E2F1 is nuclear whereas E2F4 is mostly cytoplasmic (Magae *et al*., 1996; Lindeman *et al*., 1997; Müller *et al*., 1997; Verona *et al*., 1997). To determine if the difference in cellular localization decreases the effectiveness of E2F4 as a Ras inhibitor, we tested an E2F4 protein fused to

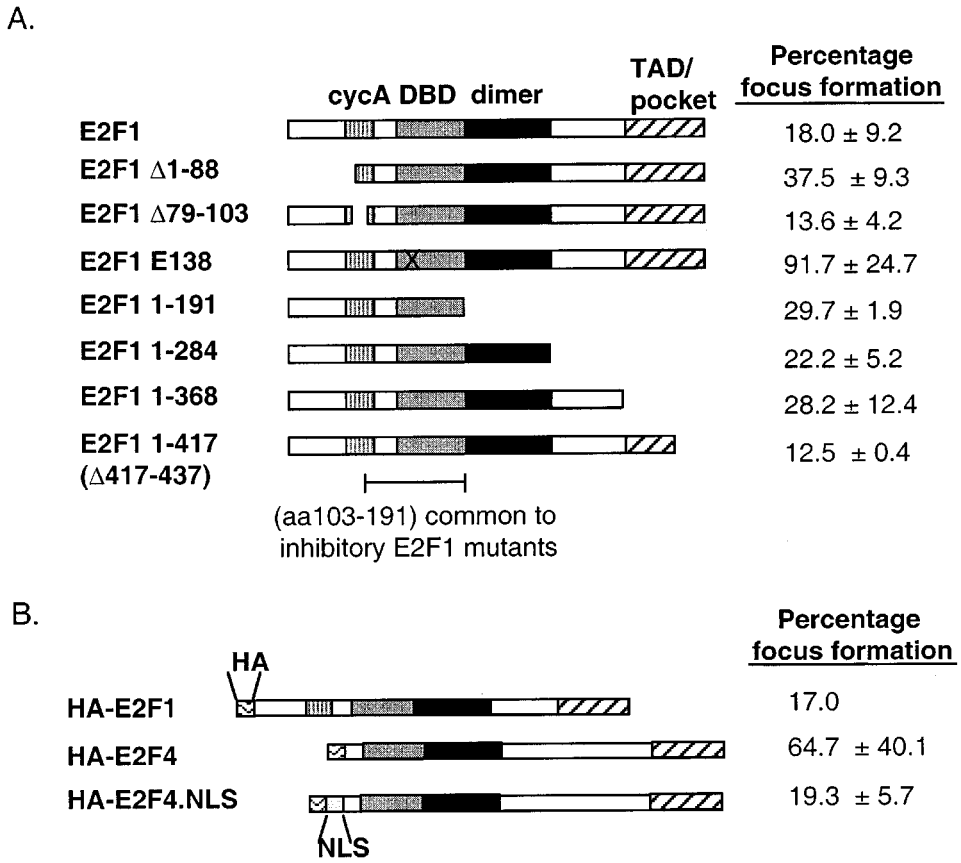

Figure 3 DNA binding ability appears to be necessary and sufficient for inhibition of Ras-mediated transformation by E2F1. (**a**) The percentage of focus formation observed in Ras-mediated focus formation assays with wildtype or mutant E2F1 relative to empty vector is shown. Endpoints at bottom of graph indicate the region common for the inhibitory E2F1 deletion proteins. (**b**) The percentage of focus formation observed in Ras-mediated focus formation assays with HA-E2F1, HA-E2F4, and HA-E2F4.NLS relative to empty vector is shown. HA-E2F4.NLS contains a nuclear localization signal from SV40 large T antigen at the amino-terminus. Values for (**a**) and (**b**) represent the mean of three independent focus formation assays, each conducted in duplicate; standard error for each value is given

a consensus nuclear localization signal from the SV40 large T antigen in the focus formation assay (Lindeman *et al*., 1997). As shown in Figure 3b, the addition of a nuclear localization signal enables E2F4 to inhibit Ras-mediated transformation as effectively as E2F1. Thus it seems that the critical determinant in inhibition of Ras-mediated focus formation is the ability to obtain sufficient quantities of a DNA binding-competent E2F in the nucleus at stages of the cell cycle when E2F4 is normally cytoplasmic.

E2F1 inhibits NIH3T3 cells previously transformed by Ras or Neu

E2F overexpression is clearly inhibitory in NIH3T3 cells before and during the Ras transformation process. Since E2F activity is believed to be increased in cancer, overexpression of E2F may be tolerated only after the transformation process. To determine if overexpressed E2F was inhibitory to NIH3T3 cells previously transformed by Ras, multiple foci derived from Ras-transformed NIH3T3 cells were expanded into cell populations. Two expanded foci were tested for the effects of E2F1 in a colony formation assay using E2F expression constructs. We found that colony formation of Ras-transformed cells continues to be inhibited by wildtype E2F1, E2F2, and E2F3, but not by the E2F DNA binding mutant, E2F1 E138 (Figure 4a). Therefore, the growth inhibitory effects of E2F are dominant in NIH3T3 cells before, during, and after the Ras transformation process.

Finally, we wished to determine if NIH3T3 cells transformed by other oncogenes were sensitive to E2F-mediated growth inhibition. We conducted a focus formation assay using an activated form of HER-2/Neu. HER-2/Neu is a receptor tyrosine kinase that is amplified and/or overexpressed in several types of human cancers, particularly breast and ovarian cancer. The activated form of Neu used in this assay contains a point mutation within the transmembrane domain, allowing more efficient Neu dimerization and kinase activation (Bargmann *et al*., 1986). Titration experiments indicated that, in contrast to Ras which caused foci formation with only 0.1 μg of plasmid, a minimum of 5 μg of Neu plasmid was required to obtain foci. We do not know if the requirement for a larger amount of Neu plasmid was due to differences in protein expression or protein activity. For the Ras focus assays, at least a 50-fold excess of E2F1 plasmid was added, ensuring that all cells receiving Ras would also receive E2F1. When the excess of E2F1 plasmid was lowered, inhibition of focus formation decreased with a complete loss of inhibition witnessed using only a 10-fold excess of E2F1 plasmid (data not shown). Due to the high amount of Neu plasmid required for foci formation, we could not obtain a 50 : 1 ratio in the Neu

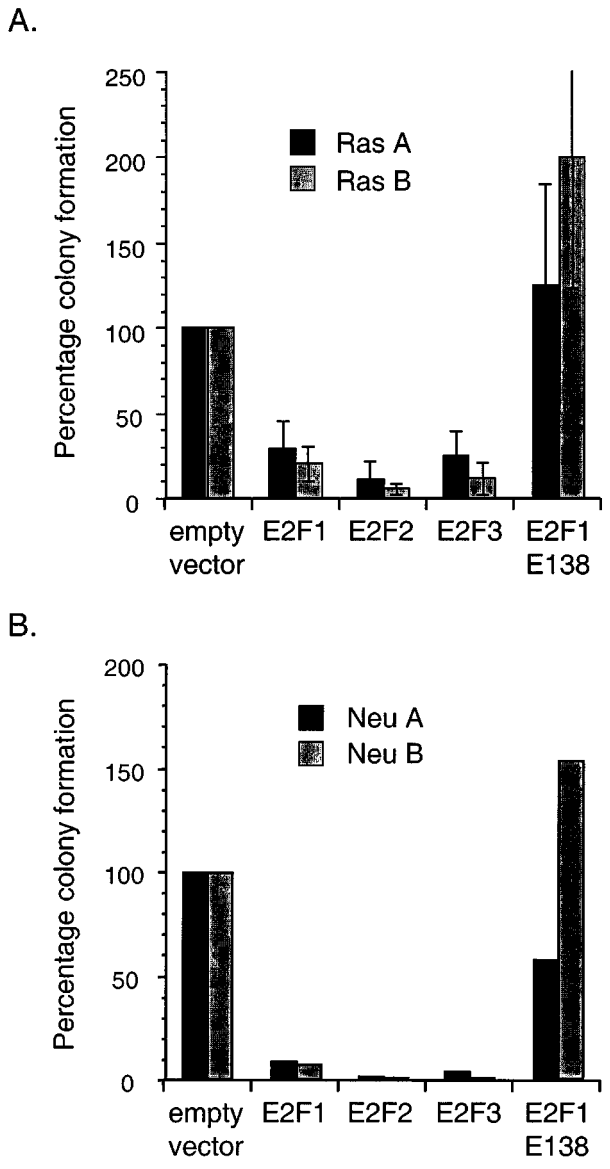

Figure 4 E2F1 inhibits growth of transformed cells. (**a**) Ten μg E2F construct or empty vector plus 1 μg hygromycin resistance vector were cotransfected into two different clonal populations of NIH3T3 cells previously transformed by Ras, designated 'Ras A' (black bars) and 'Ras B' (gray bars). Cells were grown for 2 weeks in growth media supplemented with hygromycin B (1 mg/ml) following transfection. Plates were harvested by formalin fixation and methylene blue staining. The bar graph represents the per cent of colonies obtained relative to empty vector transfectants for wildtype and mutant E2F constructs. Values represent the mean of three independent experiments, each conducted in duplicate. Standard error for each value is shown. (**b**) Ten μg E2F construct or empty vector plus 1 μg hygromycin resistance vector were cotransfected into two different clonal populations of NIH3T3 cells previously transformed by Neu, designated 'Neu A' (black bars) and 'Neu B' (gray bars). Cells were grown for 2 weeks in growth media supplemented with hygromycin B (1 mg/ml) following transfection. Plates were harvested by formalin fixation and methylene blue staining. The bar graph represents the per cent of colonies obtained relative to empty vector transfectants for wildtype and mutant E2F constructs. Data is shown for two different clones of Neu-transformed cells, with each experiment being performed in duplicate

experiments. Therefore, we transformed cells with Neu, picked foci, expanded the foci and used the cells in a colony formation assay. As shown in Figure 4b, E2F1, E2F2, and E2F3 were growth inhibitory to Neu-transformed cells. Consistent with our studies of Ras-transformed cells, an E2F1 lacking the ability to bind to DNA (E2F1 E138) was not inhibitory to Neu-transformed cells.

E2F1 is inhibitory immediately after introduction into cells

Several studies have reported that E2F1 can cause apoptosis in certain cell types (Hsieh *et al*., 1997; Phillips *et al*., 1997). To determine if E2F1 decreases NIH3T3 colony formation through an apoptotic mechanism, we conducted standard apoptosis assays which use expression plasmids for CD20 or a membrane-localized GFP protein as markers to analyse the apoptotic cell population, which contains a sub G1 DNA content. CD20 and the membrane-localized GFP are cell surface markers that are not lost after cell death, allowing analysis of both live and dead cell populations. We found the same percentage of sub G1 cells in the presence of E2F1 or empty vectors using this assay for either marker (Figure 5a). Therefore, this experiment did not support the hypothesis that increased apoptosis resulted from E2F1 exogenous expression. These results are in agreement with previous studies which show NIH3T3 cells to be resistant to E2F1 cell cycle effects when analysed for apoptosis or inappropriate DNA synthesis (Dimri *et al*., 1994; Krek *et al*., 1995).

To further analyse the mechanism by which E2F overexpression decreases colony and focus formation, we analysed the cell cycle profile of NIH3T3 cells transfected with wildtype E2F1, E2F1 E138 and E2F1 Δ417–437 by flow cytometry at 2 and 4 days following transfection. The E2F1 transfected populations were selected by transfecting the cells with a GFP expression plasmid in a 1 : 5 ratio with E2F1 expression plasmids. Percentages from the DNA profiles for the GFP positive population, which should also be E2F1 positive, suggested that introduction of wildtype or mutant E2F1 did not cause a change in cell cycle profiles (Figure 5b) and, thus, did not arrest cells in a particular stage of the cell cycle. However, it was difficult to reconcile the absence of apoptosis and the lack of alteration in cell cycle profiles with the profound effect on colony and focus formation. Therefore, further investigations were performed. Interestingly, we detected a small difference in the percentage of GFP positive cells within the total cell population for the different transfected groups at both 2 and 4 days following transfection. While cotransfection of GFP with empty vector gave rise to a 12.5% GFP positive population 2 days following transfection, we witnessed an 8 and 9% GFP positive population for wildtype E2F1 and E2F1 Δ417–437, respectively, and 18.6% for E2F1 E138 (Figure 5c). Because GFP only allows detection of live cells, decreases in the percentage of GFP positive cells may be related to the ability of E2F1 to inhibit colony and focus formation. However these results are not as dramatic as the results seen in the colony and focus formation assay. To further investigate this decrease in GFP positive cells, we introduced constructs into cells which express both E2F1 and GFP from the same plasmid and analysed the GFP positive population by flow cytometry. When E2F1 and GFP were delivered on the same plasmid, exogenous E2F1 expression effectively eliminated the transfected population at 16 h post-

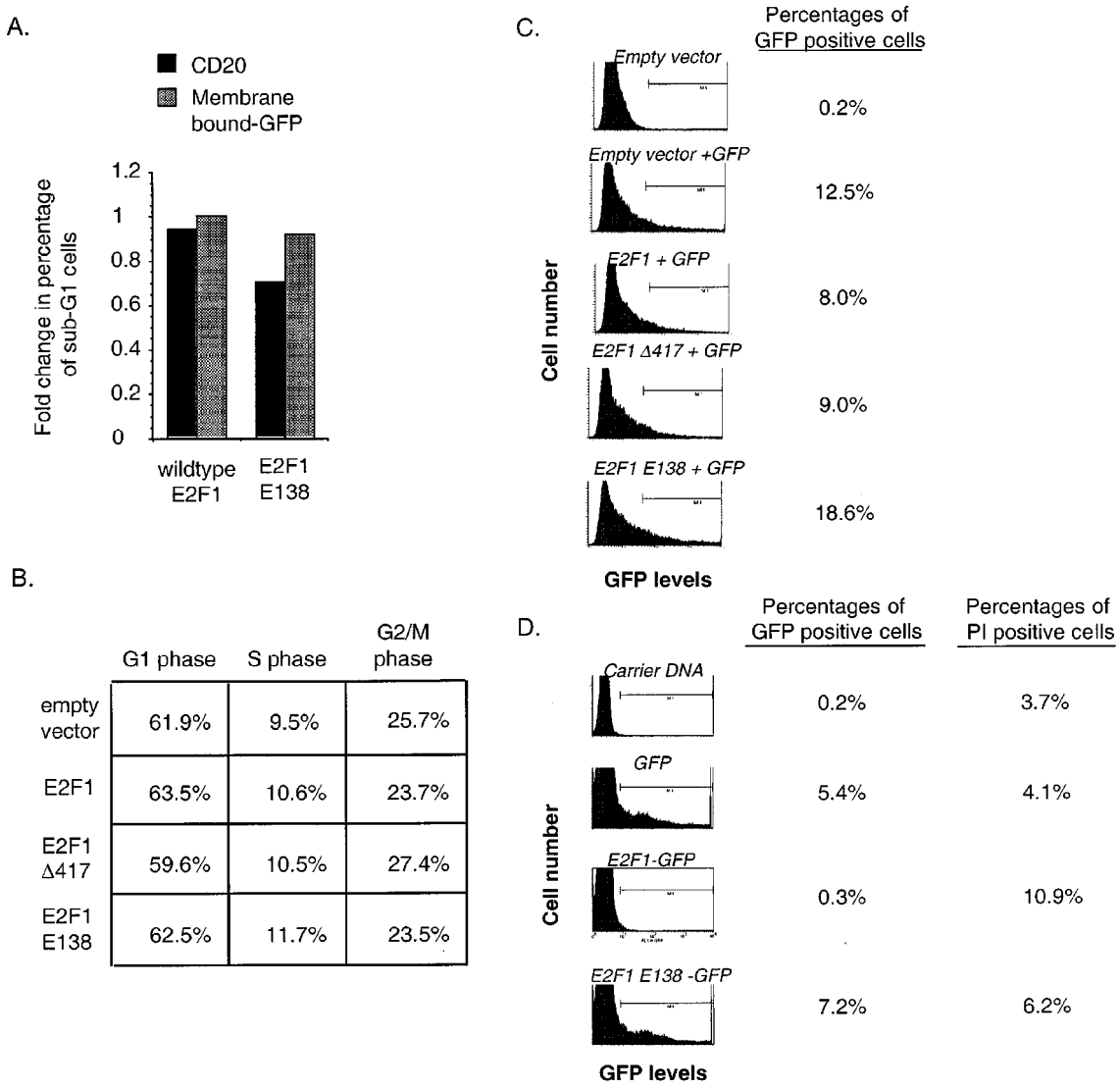

Figure 5 Flow cytometry analysis of E2F1-mediated growth inhibition in NIH3T3 cells. (**a**) NIH3T3 cells were transfected with 10 μg of E2F1 expression construct or empty vector and 2 μg of CD20 or membrane-bound GFP (Us9-GFP) expression vector. Cells were grown for 2 days in growth media following transfection and then harvested for flow cytometry analysis. The transfected population was selected by gating on and analysing CD20 or GFP positive events. Bar graph indicates fold-change in percentage of sub G1 cells transfected with E2F1 compared to cells transfected with empty vector. (**b**) NIH3T3 cells were transfected with 10 μg of E2F1 expression construct or empty vector and 2 μg of green fluorescent protein (GFP) expression vector. Cells were harvested for flow cytometry at 2 days following transfection. The transfected population was analysed by sorting out the GFP positive cells and analysing DNA profiles of the sorted cells. Shown are the percentages of cells corresponding to G1 phase, S phase, or G2/M phase of the cell cycle for the GFP positive population. (**c**) Shown are profiles of green fluorescence for the total cell populations. Y-axis measures cell number; X-axis measures GFP levels. Percentages of GFP positive cells are indicated next to profiles. (**d**) NIH3T3 cells were transfected with 10 μg of E2F1/GFP expression vector and harvested for flow cytometry analysis at 16 h following transfection. Shown are profiles of green fluorescence for the total cell populations. Y-axis measures cell number; X-axis measures GFP levels. Percentages of GFP positive cells and percentages of propidium iodide stained/dead cells are indicated next to profiles

transfection while vectors which express either GFP alone or the combination of E2F1 E138 and GFP gave rise to a 5 or 7% GFP positive population, respectively (Figure 5d). The E2F1/GFP plasmid has been shown to produce both exogenous E2F1 (as assayed by Western blot analysis) and fluorescent GFP (as analysed by microscopy) (data not shown). Therefore, the lack of GFP in cells containing the E2F1/GFP plasmid was not due to a lack of expression. Samples were also exposed to propidium iodide which only stains dead cells. Analysis of the propidium iodide staining indicates that 11% of the total population in plates transfected with E2F1/GFP were dead compared to only 4 and 6% of the total population dead in GFP alone and E2F1 E138/GFP transfected plates. These data suggest that the E2F1 positive cells are eliminated immediately after transfection. This rapid elimination explains why the cell cycle profiles of vector versus E2F1 transfected cells were so similar in Figure 5b. Because the experiment shown in Figure 5b utilized separate E2F and GFP constructs, it was possible for cells to receive only GFP plasmid or to receive both the GFP and the E2F1 plasmid. It is likely that the experiment of Figure 5b reflected the cell cycle profile of cells which contained only the GFP plasmid due to the rapid loss of the E2F1 plus GFP positive cells. Due to the speediness of elimination, it was not possible to obtain enough E2F1 positive cells for determining the mode of cell death.

E2F1 protein is functionally expressed in Ras plus E2F1 expanded foci

Although E2F1 is able to cause a fivefold decrease in NIH3T3 focus formation by activated Ras, a small number of transformed foci are still detected. Three possibilities for the appearance of these foci are as follows: (i) the foci have selected for increased expression of Ras, the transforming activity, (ii) the foci have selected against the expression of E2F1, the inhibiting activity, or (iii) the foci arose from cells which could bypass the E2F1 inhibition, without affecting E2F1 or Ras expression. To determine if there is a disproportionate expression of the Ras protein in the foci that arose in the presence of inhibitory E2F1, Western blot analysis on whole cell extracts from a panel of E2F1 plus Ras or E2F1 E138 plus Ras expanded foci was performed using an anti-pan-Ras monoclonal antibody. Results indicate similar levels of Ras expression in all the expanded foci regardless of the expression of E2F1 (data not shown). This suggests that E2F1-containing foci did not arise due to increased Ras expression. This also fits with our colony formation assay observations in which a few colonies arise in the presence of E2F1 and in the absence of exogenous Ras. To investigate the second possibility, foci were analysed for the expression of E2F1. Multiple foci arising after transfection with Ras plus E2F1, or Ras plus E2F1 E138 were picked and expanded. As a control, foci arising after transfection with Ras alone were also analysed. Nuclear extracts from the expanded foci were then analysed in Western blots. Since the E2F1 expression vectors produce human E2F1 protein and the E2F1 monoclonal antibody used does not effectively detect mouse E2F1, we were able to distinguish which foci maintained exogenous expression of E2F1. Nuclear extract from the HeLa human cell line was used as a positive control for the detection of human E2F1 protein (Figure 6a; lane H). When foci arising from transfection with Ras alone were analysed by Western blot analysis, no exogenous E2F1 was detected (Figure 6a top panel). However, five of the 11 expanded foci arising from plates transfected with wildtype E2F1 plus Ras expressed E2F1 (Figure 6a middle panel; lanes 1, 3, 5, 8 and 9). Correspondingly, six of 11 Ras/E2F1 E138 expanded foci expressed E2F1 E138 (Figure 6a bottom panel; lanes 2, 3, 4, 5, 7 and 10). Because a similar number of expanded foci continue to express either E2F1 or E2F1 E138, there appears to be no selection against the inhibiting form (wildtype E2F1) versus the non-inhibiting form (E2F1 E138) of E2F1 in the Ras-mediated transformation process. Therefore, it appears that the cells that escaped E2F1-mediated growth inhibition did so via a mechanism independent of E2F1 or Ras levels.

To determine if the exogenous E2F1 expressed in the foci is functional, foci containing HA-tagged E2F1 were expanded. E2F DNA binding activity in nuclear extract from an expanded focus overexpressing HA-E2F1 and from an expanded focus containing Ras but lacking exogenously expressed E2F was tested in an electrophoretic mobility shift assay. An additional band of E2F gel shift activity was detected in extracts from the expanded HA-E2F1/Ras focus as compared to extract from the Ras alone focus (Figure 6b, arrow). This band represents an E2F-DNA interaction since it is competed by an E2F site, but not by a binding site for the LEF transcription factor. The mobility of this band suggests that E2F must be in a complex with other proteins. In an attempt to identify the components of this new complex, we included antibodies against E2F1, E2F4 and the hemagglutinin epitope in the gel mobility shift assay. The anti-E2F4 antibody clearly eliminated the two lower bands which are found in all extracts but did not eliminate the high mobility weight complexes. However, neither the anti-HA or anti-E2F1 antibodies could supershift the new band in the HA-E2F1/Ras extracts. Even though this additional gel shift complex was not disrupted or supershifted with anti-E2F1 or anti-hemagglutinin antibodies, it is possible that the exogenous E2F1 is a part of the complex but the epitopes for the antibodies are masked by other proteins in the complex. Consistent with this idea, Singh *et al.* (1994) reported a band having a similar mobility using extracts of rat embryo fibroblasts transformed by E2F1 which could not be supershifted by E2F1 antibodies. Therefore, while it is not definitive that the new DNA binding complex contains the exogenous E2F1 protein, the change in the gel mobility shift profile suggests that the exogenous E2F1 protein expressed in foci from the E2F1/Ras focus assays remained functional.

Discussion

Alterations in the Rb pathway are commonly found in several types of human cancers such that the negative regulators of E2F activity (e.g. Rb and p16) are lost or inactivated and the positive regulators (e.g. Cyclin D1 and Cyclin E) are increased. If E2F regulators are common targets for genetic alterations in tumorigenesis, it follows that E2F itself may also be a target. However, despite extensive searches for amplification of E2F family members in human tumors, the only reported amplification of the *E2F1* locus is in one erythroleukemia cell line (Saito *et al.*, 1995). The absence of reports showing overexpression of E2F1 in human tumors suggested that it was possible that exaggerated expression of E2F is detrimental to cell proliferation. We show that increased levels of E2F are tolerated only in a small subset of normal and transformed cells.

Our studies of the detrimental effects of E2F overexpression are not contradictory to other reports suggesting that E2F activity is necessary for proper cell cycle proliferation. Rather, our studies suggest that it is crucial that the cell precisely controls the amount of E2F activity. In fact, there are several reported mechanisms by which E2F activity is controlled. For example, E2F activates its negative regulators, such as Rb, p107, p16, and p19, at the transcriptional level. Also, several groups have observed that E2F4 is regulated by cellular localization, being shuttled between the cytoplasm and the nucleus in a cell cycle-dependent fashion (Magae *et al.*, 1996; Lindeman *et al.*, 1997; Müller *et al.*, 1997; Verona *et al.*, 1997). In addition, E2F1 and E2F3 proteins have short half lives due to ubiquitin-mediated proteolysis (Flores *et al.*, 1998; Marti *et al.*, 1999). These different mechanisms of regulation all suggest that the levels of E2F activity

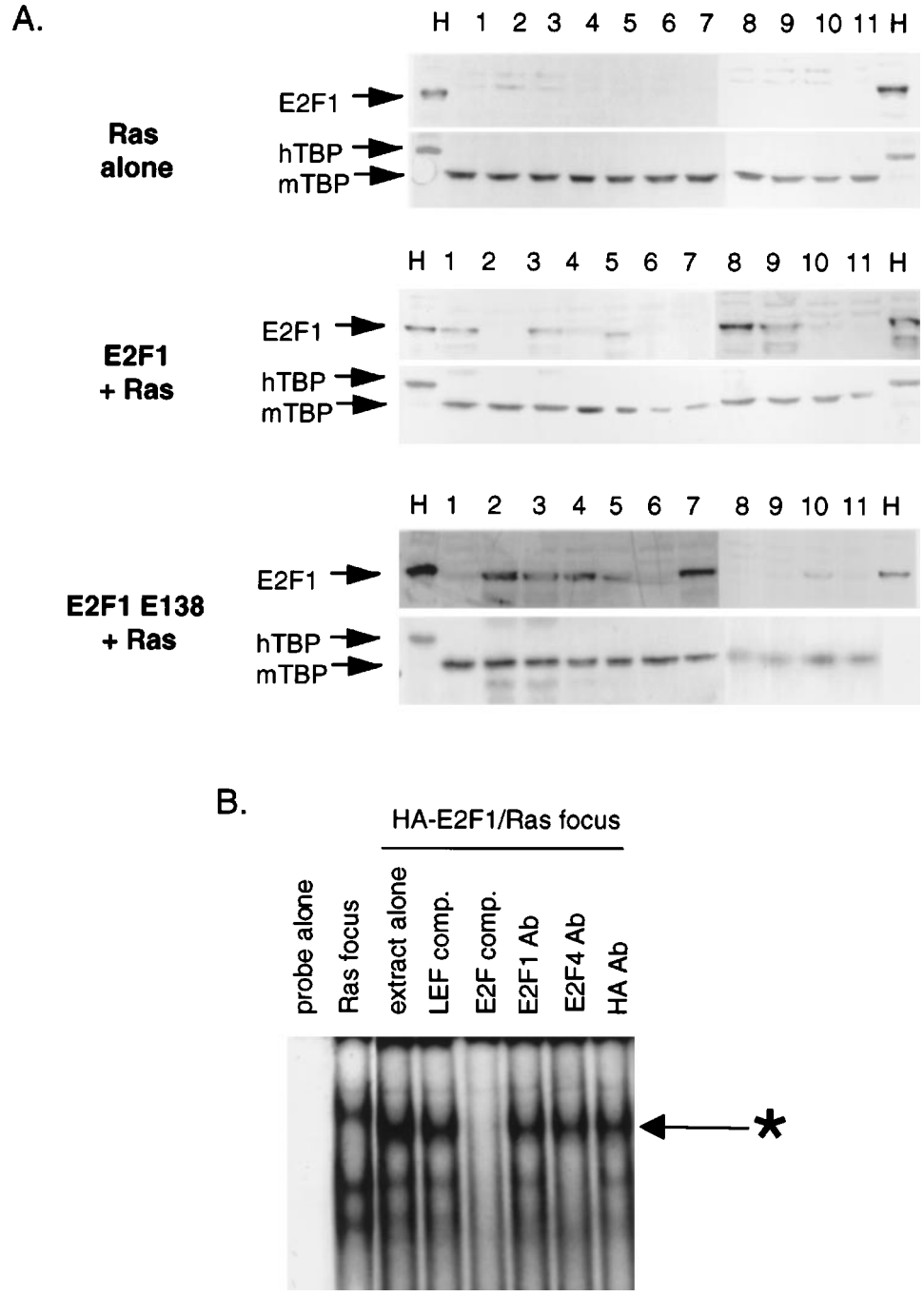

Figure 6 E2F1 protein is functionally expressed in Ras plus E2F1 expanded foci. 10–11 foci from Ras-mediated focus formation assays with E2F1 were selected and expanded. (**a**) Cells were harvested for nuclear extracts (NE) and 100 μg of NE was analysed by Western blot analysis using anti-E2F1 and anti-TBP antibodies. (**b**) Extracts from one Ras plus empty vector expanded focus, one Ras plus HA-E2F1 expanded focus was analysed for gel shift activity using a probe containing the overlapping E2F sites from the dihydrofolate reductase (DHFR) promoter. The DHFR E2F site was used as a E2F specific competitor and a binding site for the LEF transcription factor was used as a nonspecific competitor, designated 'E2F comp.' and 'LEF comp.', respectively. Supershifts were conducted with anti-E2F1, anti-E2F4, and anti-hemagglutinin antibodies, designated 'E2F1 Ab', E2F4 Ab' and 'HA Ab' respectively. The additional E2F binding activity detected in extracts from the Ras plus E2F1 expanded focus is indicated by an arrow with an asterisk

need to be kept under tight control for proper cell growth.

Since the DNA binding activity of E2F1 appears to be a major determinant for inhibition of focus and colony formation, it is possible that overexpressed E2F inhibits cell growth by interfering with the transcription of certain genes required either for cell proliferation or matrix adhesion. Perhaps overexpressed E2F displaces another transcription factor from binding to its normal site within a promoter, thereby preventing normal transcription of that gene. For example, E2F sites overlap with an Ets site in the c-*myc* promoter such that binding of Ets and E2F is mutually exclusive (Roussel *et al*., 1994). Increased E2F may displace Ets from the c-*myc* promoter or affect a different promoter in a similar fashion. In such a model, endogenous E2F would not be produced at a high enough level to cause interference.

We have attempted to identify genes which are deregulated by overexpression of E2F1 by comparing

the levels of various E2F targets and cell cycle regulators in cells derived from foci which express Ras alone or Ras plus wildtype or mutant E2F1. Unfortunately, none of the target genes investigated have expression patterns which correlate with the growth inhibitory properties of the E2F proteins (data not shown). However, it is important to note that the expanded foci are not the proper cellular environment in which to determine the mechanism of inhibition since these cells represent a population which have overcome E2F inhibition. The physiologically relevant population to study would be the cells that have received Ras and E2F1 but did not give rise to foci. Unfortunately, low transfection efficiency and the difficulty in selecting large sample populations make such an analysis technically difficult. Our laboratory has also performed subtractive hybridization, using inducibly expressed E2F1, in an attempt to clone mRNAs which respond to high levels of E2F1; these experiments have not yet identified an mRNA which is reproducibly affected by E2F1. However, it remains possible that E2F-mediated growth inhibition is caused by changes in gene expression and that a deregulated mRNA will eventually be identified.

Materials and methods

Plasmids, cell culture, and transfection

The GFP expression parental plasmid used in Figure 5d was a generous gift from Phillip Watson and Michael Gould (Watson and Gould, unpublished data). This vector is similar in structure to the pJLR plasmid except for the replacement of the neomycin phosphotransferase gene with the green fluorescent protein gene as a selection marker (Wang *et al*., 1991a). The E2F1/GFP and E2F1 E138/GFP vectors were made by removing the human E2F1 cDNAs from pCMV-E2F1 and pCMV E2F1 E138 (Cress *et al*., 1993) with a *Bam*HI restriction digest and ligating the cDNA fragments to the *Bam*HI-digested GFP expression vector. Standard cloning techniques were used to make these vectors (Maniatis *et al*., 1982).

All other expression plasmids used in this study have been previously described. Previously described E2F expression constructs contain E2F cDNA sequences that are driven from the cytomegalovirus [CMV] immediate early promoter. Details of their construction can be found in the following original references: pCMV E2F1 wt, pCMV E2F1 E138, pCMV E2F1 Δ1–88 (Cress *et al*., 1993); pCMV/Rc HA E2F1, pCMV/Rc HA E2F1 Δ24 (referred to as E2F1 Δ79–103 in this study), pCMV/Rc HA E2F2, pCMV/Rc HA E2F3 (Krek *et al*., 1994); pCMV E2F1 Δ417–437 (Helin *et al*., 1993a); pCMV E2F1 1–284, pCMV E2F1 1–191 (Helin *et al*., 1993b); pCMV E2F1 1–368 (Sellers *et al*., 1995); pCMV E2F2 (Wu *et al*., 1995); pCMV E2F3 (Lees *et al*., 1993); pCDNA E2F4 (Beijersbergen *et al*., 1994); pCDNA mE2F5 (gift from Nicholas La Thangue); pCDNA-HA E2F4, pCDNA HA-E2F5, pCDNA HA-E2F4-NLS (Lindeman *et al*., 1997); priboEJras (Finlay *et al*., 1989); pJLR-neu (Wang *et al*., 1991b); pCMV-HygroR, a hygromycin resistance vector equivalent in structure to the pCMV-BNLF-1 empty vector (Hammerschmidt *et al*., 1989); pGFPemd-c (Packard Instrument Co.; Meriden, CT, USA); pCMV-CD20 (Zhu *et al*., 1993); pUs9-GFP (Brideau *et al*., 1998; Kalejta *et al*., 1999).

Swiss NIH3T3 cells (American Type Culture Collection; Rockville, MD, USA) that have retained their ability to be contact inhibited were cultured in Dulbecco's modified Eagle's media (GIBCO–BRL; Grand Island, NY, USA), supplemented with 10% bovine calf serum (Hyclone; Logan, UT, USA) and 1% penicillin/streptomycin, and incubated at 37°C in a humidified 5% CO_2 incubator. For focus formation assays, 5×10^5 exponentially grown cells were plated into 100 mm tissue culture dishes and grown overnight. Indicated amounts of plasmid DNA, as described in each figure legend, were transfected with sonicated salmon sperm carrier DNA amounting to a total of 20 μg DNA per 100 mm dish using the calcium phosphate method previously described in Chen and Okayama (1987). Cells continued to grow for 14 days with media changes every 3–4 days, after which time cells were rinsed with phosphate buffered saline (PBS), fixed with 10% buffered formalin, and stained with 0.14% methylene blue. All plates were scored blindly. The same amount of transfected c-Ha-Ras or Neu expression plasmid could give rise to slightly different numbers of foci from experiment to experiment due to differences in transfection efficiency. This would create a problem if the number of foci on a plate were too numerous to count. To control for this variation, titration experiments were performed to find the optimal amount of Ras or Neu expression plasmid. For example, we found that 0.1 or 0.2 μg of Ras expression plasmid produced approximately 100 foci per plate. Parallel experiments were then performed in which either 0.1 or 0.2 μg of the Ras plasmid was cotransfected with 10 μg of the E2F constructs. We found that 5 or 10 μg of Neu expression plasmid was required to detect foci. Transformed foci were scored as intensely stained areas larger than 3 mm in diameter.

NIH3T3 cells used in colony formation assays were transfected as described for the focus formation assays. One day following transfection, cells were then expanded to 150 mm tissue culture dishes and grown for 14 days in media supplemented with 1 mg/ml hygromycin B. Plates from the colony formation assays were fixed and stained as indicated in the focus formation protocol and colonies were scored as any stained areas on the culture dish.

Flow cytometry

In order to analyse the sub G1 cell population, cells were colipofected with 8 μg E2F expression plasmid and either 2 μg CD20 plasmid, pCMV-CD20, or 2 μg of the integral membrane green fluorescent fusion protein expression plasmid, Us9-GFP, per 100 mm culture dish with 12.5 μl lipofectamine using the recommended product protocol (GIBCO–BRL; Grand Island, NY, USA). Two days following transfection, cells were harvested as described previously (Zhu *et al*., 1993). CD20-transfected cells were exposed to FITC-conjugated anti-CD20 antibodies (Becton Dickinson; San Jose, CA, USA) as described previously (Zhu *et al*., 1993). The PI DNA profiles were determined with a FACSscan benchtop flow cytometer (Becton Dickinson; San Jose, CA, USA) and analysed for the percentage of subG1 DNA. Ten thousand events were acquired with CELLQuest software and cell doublets were excluded from the analysis.

In order to analyse cell cycle kinetics, 2×10^5 NIH3T3 cells were transfected by either calcium phosphate as described above using 10 μg E2F expression plasmid and 2 μg green fluorescent protein (GFP) expression plasmid, pGFPemd-c, or by lipid-mediated transfection using 10 μg E2F1/GFP constructs. At the indicated times following transfection, cells were harvested by trypsinization and resuspended in PBS at 1.5×10^6 cells per ml PBS. Cells were filtered through a 40 μm nylon mesh and in order to gate out dead cells, propidium iodide (PI) was added to a final concentration of 5 μg/ml. The GFP signals were excited by a 488 nm line generated by an argon laser (Coherent; Palo Alto, CA, USA) on a FACStar Plus flow cytometer (Becton Dickinson; San Jose, CA, USA). The GFP signal was collected through a 530/30 BP filter and the PI signal was collected through a 660 long pass filter. At least 10 000 events were acquired using CELLQuest software and analysed for the percentage of

GFP positive events. In addition, cells were sorted for the GFP positive population. The sorted cells were treated with detergent, RNaseA, and PI (DNA QC particle kit, Becton Dickinson; San Jose, CA, USA) and PI DNA profiles of the sorted population were determined with a FACSscan bench-top flow cytometer (Becton Dickinson; San Jose, CA, USA). Pulse width and area were also determined and analysis was restricted to singlet events.

Western analysis

To obtain nuclear protein for Western blot analysis, a modified version of a previously described extraction protocol was used (Dignam *et al*., 1983). Briefly, the rinsed cell pellet was resuspended in 100–200 μl buffer A (10 mM 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid [HEPES]-KOH, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol [DTT]; 0.2 mM phenylmethylsulfonyl fluoride [PMSF]). After a 20 min incubation on ice and a brief centrifugation, the supernatant, containing the cytoplasmic fraction, was removed. The pellet was resuspended in 50–100 μl of Buffer C (20 mM HEPES-KOH, pH 7.9; 1.5 mM $MgCl_2$; 420 mM NaCl; 0.5 mM DTT; 0.2 mM PMSF; 0.2 mM EDTA; 25% glycerol). After a 30 min incubation on ice and a 10 min centrifugation at 14 000 r.p.m., the supernatant, containing nuclear protein, was collected. To obtain whole cell protein for Western blot analysis, a modified version of a previously described extraction protocol was used (Gumerlock *et al*., 1989). Briefly, the rinsed cell pellet was resuspended in 150–300 μl lysis buffer (10 mM Na_2HPO_4; 154 mM NaCl; 12 mM deoxycholic acid, sodium salt; 1 mM NaF; 3.5 mM sodium dodecyl sulfate [SDS]; 31 mM sodium azide; 1% Triton X-100; 1 mM PMSF). After a 10 min incubation on ice, samples were vortexed three times for 10 s each and centrifuged for 10 min at 14 000 r.p.m., and the supernatant containing whole cell protein was collected.

Quantifying protein expression from the cells transfected with E2F expression constructs was done as follows. E2F expression plasmids were introduced into NIH3T3 cells via lipid-mediated transfection using lipofectamine. One to two days after lipofection, nuclear or whole cell protein was extracted, and Westerns were then conducted using 150 μg of whole cell extract or 100 μg of nuclear extract. Western blots were probed with either an antibody for E2F1 (KH95; Santa Cruz Biotechnology Inc.; Santa Cruz, CA, USA) or an anti-hemagglutinin (HA) antibody (HA.11; Berkeley Antibody Co.; Richmond, CA, USA). Western blots were also probed with an anti-TATA binding protein (TBP) antibody (gift from Richard Burgess) as a loading control for nuclear protein.

Gel mobility shift assay

Nuclear protein extraction and gel mobility shift assays were performed as previously described (Wells *et al*., 1996). Briefly, scraped cells were placed in 100–200 μl buffer A (10 mM 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid (HEPES)-KOH, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol (DTT); 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). After a 20 min incubation on ice and a brief centrifugation, the supernatant, containing the cytoplasmic fraction, was removed. The pellet was resuspended in 50–100 μl of 5× buffer (100 mM HEPES, pH 7.4; 500 mM KCl; 5 mM $MgCl_2$; 0.5 mM EDTA; 35% glycerol; 5 mM NaF; 2 μg/ml PMSF; 0.1 μg/ml of aprotinin; 0.1 μg/ml of leupeptin; 1 mM DTT) and freeze-thawed in liquid nitrogen three times, cooled on ice for 30 min and spun in a microfuge at 14 000 r.p.m. for 15 min at 4°C. For binding reaction mixtures, equal amounts of nuclear extract were incubated with 3 μg herring sperm DNA and 50 ng of specific competitor DNA or 1 μl of anti-E2F1 (50% C-20, 50% KH95; Santa Cruz Biotechnology Inc.; Santa Cruz, CA, USA), anti-E2F4 (C-20; Santa Cruz Biotechnology Inc.; Santa Cruz, CA, USA), or anti-hemagglutinin (HA) (HA.11; Berkeley Antibody Co.; Richmond, CA, USA) antibodies where indicated for 10 min at room temperature. Then, 1 ng of end-labeled double-stranded oligonucleotide probe was added and extracts were allowed to incubate at room temperature for an additional 20 min. Protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel in 0.25× Tris-borate-EDTA. Gels were then dried and exposed to film. The sequences of the individual double-stranded probes and competitors are listed below: mouse DHFR probe/E2F specific competitor top strand, 5′-CTCAGGGCTGCGATTTCGCGCCAAACTTGAGGCA-3′; bottom strand, 5′-GGATTGCCTCAAGTTTGGCGCGAAATCGCAGCCC-3′; LEF nonspecific double-stranded competitor, 5′-CCCTTTGATCTTACC-3′.

Acknowledgments
We are grateful to Richard Burgess, Doug Cress, LW Enquist, Doron Ginsberg, Michael Gould, Ed Harlow, Kristen Helin, Wilhelm Krek, Jackie Lees, David Livingston, Joseph Nevins, Bill Sugden and Phillip Watson for providing plasmids and reagents and to Kathy Schell and Kristen Elmer for assistance with flow cytometry. We also thank members of the Farnham laboratory for critical readings of this manuscript. This work was supported in part by Public Health Service Grants CA07175, CA22484 and predoctoral training grants CA09135 and DAMD17-96-1-6109.

References

Bargmann CI, Hung M-C and Weinberg RA. (1986). *Cell,* **45,** 649–657.
Beijersbergen RL, Kerkhoven RM, Zhu L, Carlée L, Voorhoeve PM and Bernards R. (1994). *Genes Dev.,* **8,** 2680–2690.
Brideau AD, Banfield BW and Enquist LW. (1998). *J. Virol.,* **72,** 4560–4570.
Chen C and Okayama H. (1987). *Mol. Cell. Biol.,* **7,** 2745–2752.
Cress WD, Johnson DG and Nevins JR. (1993). *Mol. Cell. Biol.,* **13,** 6314–6325.
Dignam JD, Lebovitz RM and Roeder RG. (1983). *Nucleic Acids Res.,* **11,** 1475–1489.
Dimri GP, Hara E and Campisi J. (1994). *J. Biol. Chem.,* **269,** 16180–16186.
Ferreira R, Magnaghi-Jaulin L, Robin P, Harel-Bellan A and Trouche D. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 10493–10498.
Field SJ, Tsai F-Y, Kuo F, Zubiaga AM, Kaelin Jr WG, Livingston DM, Orkin SH and Greenberg ME. (1996). *Cell,* **85,** 549–561.
Finlay C, Hinds P and Levine A. (1989). *Cell,* **57,** 1083–1093.
Flores AM, Kassatly RF and Cress WD. (1998). *Oncogene,* **16,** 1289–1298.
Gumerlock PH, Meyers FJ, Kokoris SP, Wong G, McCormick FP and de Vere White RW. (1989). *Anal. Biochem.,* **180,** 158–168.
Hammerschmidt W, Sugden B and Baichwal VR. (1989). *J. Virol.,* **63,** 2469–2475.

Helin K, Harlow E and Fattaey A. (1993a). *Mol. Cell. Biol.,* **13,** 6501 – 6508.

Helin K, Wu C-L, Fattaey AR, Lees JA, Dynlacht BD, Ngwu C and Harlow E. (1993b). *Genes Dev.,* **7,** 1850 – 1861.

Hsieh J-K, Fredersdorf S, Kouzarides T, Martin K and Lu X. (1997). *Genes Dev.,* **11,** 1840 – 1852.

Huang H-JS, Yee J-K, Shew J-Y, Chen P-L, Bookstein R, Friedmann T, Lee EY-HP and Lee W-H. (1988). *Science,* **242,** 1563 – 1566.

Ivey-Hoyle M, Conroy R, Huber HE, Goodhart PJ, Oliff A and Heimbrook DC. (1993). *Mol. Cell. Biol.,* **13,** 7802 – 7812.

Johnson DG, Cress WD, Jakoi L and Nevins JR. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 12823 – 12827.

Kalejta RF, Brideau AD, Banfield BW and Beavis AJ. (1999). *J. Exp. Cell Res.,* **248,** 322 – 328.

Karlsreder J, Rotheneder H and Wintersberger E. (1996). *Mol. Cell. Biol.,* **16,** 1659 – 1667.

Kitagawa M, Higashi H, Suzuki-Takahashi I, Segawa K, Hanks SK, Taya Y, Nishimura S and Okuyama A. (1995). *Oncogene,* **10,** 229 – 236.

Krek W, Ewen ME, Shirodkar S, Arany Z, Kaelin WGJ and Livingston DM. (1994). *Cell,* **78,** 161 – 172.

Krek W, Xu G and Livingston DM. (1995). *Cell,* **83,** 1149 – 1158.

Lees JA, Saito M, Vidal M, Valentine M, Look T, Harlow E, Dyson N and Helin K. (1993). *Mol. Cell. Biol.,* **13,** 7813 – 7825.

Lin SY, Black AR, Kostic D, Pajovic S, Hoover CN and Azizkhan JC. (1996). *Mol. Cell. Biol.,* **16,** 1668 – 1675.

Lindeman GJ, Gaubatz S, Livingston DM and Ginsberg D. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 5095 – 5100.

Magae J, Wu C-L, Illenye S, Harlow E and Heintz NH. (1996). *J. Cell Sci.,* **109,** 1717 – 1726.

Maniatis T, Fritsch EF and Sambrook J. (1982). *Molecular cloning; a laboratory manual.* Cold Spring Harbor Laboratory: Cold Spring Harbor, New York.

Marti A, Wirbelauer C, Scheffner M and Krek W. (1999). *Nature Cell Biol.,* **1,** 14 – 19.

Müller H, Moroni M, Vigo E, Peterson BO, Barteck JH and Helin K. (1997). *Mol. Cell. Biol.,* **17,** 5508 – 5520.

Phillips AC, Bates S, Ryan KM, Helin K and Vousden KH. (1997). *Genes Dev.,* **11,** 1853 – 1863.

Pierce AM, Fisher SM, Conti DJ and Johnson DG. (1998a). *Oncogene,* **16,** 1267 – 1276.

Pierce AM, Gimenez-Conti IB, Schneider-Broussard R, Martinez LA, Conti CJ and Johnson DG. (1998b). *Proc. Natl. Acad. Sci. USA,* **95,** 8858 – 8863.

Pierce AM, Schneider-Broussard S, Gimenez-Conti IB, Russell JL, Conti CJ and Johnson DG. (1999). *Mol. Cell. Biol.,* **19,** 6408 – 6414.

Roussel MF, Davis JN, Cleveland JL, Ghysdael J and Hiebert SW. (1994). *Oncogene,* **9,** 405 – 415.

Saito M, Helin K, Valentine MB, Griffith BB, Willman CL, Harlow E and Look AT. (1995). *Genomics,* **25,** 130 – 138.

Sellers WR, Rodgers JW and Kaelin Jr WG. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 11544 – 11548.

Singh P, Wong SH and Hong W. (1994). *EMBO J.,* **13,** 3329 – 3338.

Verona R, Moberg K, Estes S, Starz M, Vernon JP and Lees JA. (1997). *Mol. Cell. Biol.,* **17,** 7268 – 7282.

Wang B, Kennan WS, Yasukawa-Barnes J, Lindstrom MJ and Gould MN. (1991a). *Cancer Res.,* **51,** 2642 – 2648.

Wang B, Kennan WS, Yasukawa-Barnes J, Lindstrom MJ and Gould MN. (1991b). *Cancer Res.,* **51,** 5649 – 5654.

Wells J, Held P, Illenye S and Heintz NH. (1996). *Mol. Cell. Biol.,* **16,** 634 – 647.

Wu CL, Zukerberg LR, Ngwu C, Harlow E and Lees JA. (1995). *Mol. Cell. Biol.,* **15,** 2536 – 2546.

Xu M, Sheppard KA, Peng CY, Yee AS and Piwnica-Worms H. (1994). *Mol. Cell. Biol.,* **14,** 8420 – 8431.

Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E and Dyson NJ. (1996). *Cell,* **85,** 537 – 548.

Zhu L, van den Heuvel S, Helin K, Fattaey A, Ewen M, Livingston D, Dyson N and Harlow E. (1993). *Genes Dev.,* **7,** 1111 – 1125.