The EMBO Journal vol.15 no.22 pp.6178–6188, 1996

Loss of *Rb* activates both *p53*-dependent and independent cell death pathways in the developing mouse nervous system

Kay F.Macleod[1], Yanwen Hu[1] and Tyler Jacks[1,2,3]

[2]Howard Hughes Medical Institute, and [1]Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[3]Corresponding author

Extensive apoptosis occurs in the nervous system of mouse embryos homozygous mutant for a targeted disruption of the retinoblastoma (*Rb*) gene. This cell death is present in both the central (CNS) and peripheral nervous systems (PNS) and is associated with abnormal S phase entry of normally post-mitotic neurons. Aberrant proliferation in the CNS correlates with increased free E2F DNA binding activity and increased expression of *cyclin E*, an E2F target gene and critical cell cycle regulator. Cell death in the CNS is accompanied by increased levels of the p53 tumor suppressor gene product and increased expression of the p53 target gene, *p21*$^{Waf\text{-}1/Cip\text{-}1}$. However, induction of p53 is not observed in the PNS of Rb-mutant embryos, nor does loss of p53 function inhibit cell death in the PNS. Surprisingly, *p21*$^{Waf\text{-}1/Cip\text{-}1}$ is induced in the sensory ganglia of *Rb*-mutant embryos in a p53-independent manner. Although loss of *p53* gene function prevents cell death in the CNS of Rb-mutant embryos, it does not restore normal proliferative control.

Keywords: apoptosis/differentiation/E2F/nervous system/p53/Rb

Introduction

The *Rb* and *p53* tumor suppressor genes are inactivated in a wide variety of human tumors (Friend *et al.*, 1986; Malkin *et al.*, 1990; Weinberg, 1992; Harris and Hollstein, 1993; Malkin, 1993), and several DNA tumor viruses have evolved viral oncoproteins that functionally inactivate both pRB and p53 (Vousden, 1993). Loss of *Rb* or *p53* function in mouse embryonic fibroblasts results in a failure to arrest normally in the G_1 phase of the cell cycle in response to growth inhibitory signals (Kastan *et al.*, 1991, 1992; Slebos *et al.*, 1994; Almasan *et al.*, 1995). Furthermore, mutations in *Rb* and *p53* cooperate in the formation of certain tumor types in the mouse (Williams *et al.*, 1994a).

Induction of p53 can induce growth arrest or programmed cell death depending on cell type (Midgley *et al.*, 1995), the state of differentiation of the cell, the presence of functional pRB (Almasan *et al.*, 1995) and the growth factor environment (Canman *et al.*, 1995). The ability of p53 to induce growth arrest is explained in part by its role as a transcriptional activator of the p21$^{WAF\text{-}1/CIP\text{-}1}$ cyclin/cdk inhibitor (El-Deiry *et al.*, 1993, 1994; Di Leonardo *et al.*, 1994; Brugarolas *et al.*, 1995; Deng *et al.*, 1995), while transactivation by p53 of genes whose products act to induce apoptosis appears to be important to the role of p53 in programmed cell death (Sabbatini *et al.*, 1995; Attardi *et al.*, 1996). The ability of p53 to induce apoptosis can limit tumor formation (Symonds *et al.*, 1994) and strongly influence the response of tumor cells to anti-cancer agents (Lowe *et al.*, 1994). Thus, an elucidation of the mechanism underlying the induction of cell death by p53 is critical to understanding tumor development and treatment.

Recent work has suggested that activated E2F-1 and wild-type p53 can act to induce apoptosis (Wu and Levine, 1994), and this may explain how mutations in *Rb* and *p53* cooperate in the development of certain tumor types in the mouse (Williams *et al.*, 1994a). pRB is thought to regulate passage through the G_1 phase of the cell cycle by binding to and inhibiting the activity of members of the E2F family of transcription factors in a cell cycle-dependent manner (Hiebert *et al.*, 1992; Nevins, 1992; Helin *et al.*, 1993; Lees *et al.*, 1993). Release of free E2F following phosphorylation of pRB is believed to result in the activation of genes required for S phase entry (Means *et al.*, 1992; Lam and Watson, 1993; Slansky *et al.*, 1993; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Muller, 1995). Overexpression of *E2F-1* in established cell lines leads to transformation, and *E2F-1* can transform primary cells in cooperation with activated H-*ras* (Singh *et al.*, 1994; Xu *et al.*, 1995). The overexpression of *E2F-1* by itself in primary cells results in premature entry of cells into S phase (Johnson *et al.*, 1993) and is sufficient to overcome the G_1 arrest induced by wild-type *p53* expression (Wu and Levine, 1994) or γ-irradiation (DeGregori *et al.*, 1995b). *E2F-1*-expressing primary cells eventually undergo programmed cell death (Qin *et al.*, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995).

Mice carrying a targeted disruption of the *Rb* gene die between days 13.5 and 14.5 of gestation, exhibiting defects in fetal liver hematopoiesis as well as in lens and nervous system development (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Morgenbesser *et al.*, 1994). There is extensive cell death in all three tissues. Cell death in the nervous system of *Rb*-mutant embryos appears to occur in neurons that continue to proliferate at a time when they should be post-mitotic (Lee *et al.*, 1994). Cells normally undergo growth arrest as they differentiate (Kiyokawa *et al.*, 1993; Lassar *et al.*, 1994), and *Rb* may play a role in ensuring growth arrest as part of the differentiation program of certain cell types (Richon *et al.*, 1992; Gu *et al.*, 1993; Schneider *et al.*, 1994). For example, retroviral-mediated expression of the HPV *E7* gene in differentiated keratinocytes can promote DNA replication

© Oxford University Press

without altering the state of differentiation of these cells or inducing mitosis (Cheng *et al.*, 1995). Since E7 is thought to function principally by inactivating pRB and related proteins, this is further evidence for the role of pRB-related proteins in preventing differentiated cells from re-entering the cell cycle. Abrogation of *Rb* function by expression of the adenovirus E1A protein leads to the programmed cell death of P19 cells upon differentiation into neuroectoderm (Slack *et al.*, 1995). Similarly, overexpression of cyclin D1 in differentiating neuronal cell lines induces pRB phosphorylation and programmed cell death in a manner which can be blocked by overexpression of $p16^{INK4A}$ (Kranenburg *et al.*, 1996). These observations suggest that *Rb* may have a particularly important role in regulating neuronal differentiation. Failure to establish or maintain growth arrest at a time when the cell is differentiating may trigger programmed cell death.

The development of the ocular lens is also greatly affected by loss of *Rb* function, either by targeted disruption of the *Rb* gene or by expression of a HPV *E7* transgene. Absence of *Rb* function leads to impaired differentiation of lens fiber cells, inappropriate proliferation in regions of the lens which should be post-mitotic and abnormally high levels of apoptosis (Morgenbesser *et al.*, 1994; Pan and Griep, 1994). Loss of *p53* function, either by targeted disruption or by expression of a HPV *E6* transgene, fails to restore normal regulation of proliferation and differentiation in the lens of *Rb*-deficient mice but does rescue cells from apoptotic death (Morgenbesser *et al.*, 1994; Pan and Griep, 1994). More recent evidence suggests that the role of *p53* in programmed cell death in the lens may be a function of developmental age (Pan and Griep, 1995).

We have examined the role of *p53* in the apoptosis induced in the nervous system by loss of *Rb* and shown that cell death is *p53*-dependent in the CNS but *p53*-independent in the PNS. Although cell death can occur by distinct mechanisms in these tissues following *Rb* loss, induction of *cyclin E* expression and increased S phase entry are common features of deregulated growth in the CNS and PNS.

Results

Nervous system cell death in Rb-mutant embryos is p53-dependent in the CNS but p53-independent in the PNS

We and others have previously reported extensive cell death in both the central nervous system (CNS) and peripheral nervous system (PNS) of *Rb*-mutant embryos (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992, 1994). Pyknotic nuclei, indicative of apoptosis, were evident in hemotoxylin and eosin-stained sections through the hindbrain, forebrain, spinal cord (all CNS) and sensory ganglia (PNS) of mutant embryos at 13.5 days of gestation but not in similar sections of wild-type embryos (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992, 1994). Cell death in the ocular lens of *Rb*-mutant embryos has previously been shown to require *p53* function (Morgenbesser *et al.*, 1994). Furthermore, *p53* has also been shown to cooperate with *Rb* in the development of certain tumors in the mouse (Williams *et al.* 1994a). Since cell death induced by overexpression of *E2F-1 in vitro* is *p53*-dependent, we were interested in determining whether *p53* played a role in the cell death that has been observed in the nervous system of *Rb*-mutant embryos and whether *p53* dependence is a consistent feature of apoptosis in *Rb*-deficient tissues.

As shown in Figure 1, there were greatly increased numbers of TUNEL-positive cells in the CNS of *Rb*-mutant embryos at E13.5 compared with wild-type embryos at the same developmental age (Figure 1A and C). Similarly, more apoptotic cells were present in the PNS of *Rb*-mutants than in wild-type embryos (Figure 1B and D). However, the CNS of embryos deficient for both *Rb* and *p53* function (Figure 1E) contained no TUNEL-positive cells, indicating that *p53* was required for death of cells in the CNS of *Rb*-mutant embryos. These observations are consistent with those previously described for the ocular lens of *Rb*-mutant embryos (Morgenbesser *et al.*, 1994; Pan and Griep, 1994) where cell death was also shown to be *p53*-dependent. Although loss of *p53* inhibits cell death in the lens and central nervous system of *Rb*-mutant embryos, it does not affect the survival of the embryo. *Rb–/–;p53–/–* embryos die at approximately the same developmental age as *Rb–/–* embryos, probably due to failed fetal liver hematopoiesis.

In contrast, examination of cell death in the PNS of *Rb–/–;p53–/–* embryos (Figure 1F), revealed an even larger number of TUNEL-positive cells than in *Rb*-mutant embryos (Figure 1D). These results demonstrate that *p53* mutation not only failed to inhibit the abnormal levels of cell death seen in the PNS of *Rb*-mutants but actually resulted in a more severe phenotype. The differing dependence of the cells of the CNS and PNS of *Rb*-mutant embryos on *p53* for the induction of apoptosis strongly indicates that distinct death pathways can be activated in response to loss of *Rb*, in a cell-type-specific manner.

p53 levels and DNA binding activity are induced in the CNS of Rb-mutant embryos

Given the differential requirement for *p53* in the induction of apoptosis in the CNS and PNS of *Rb*-mutant embryos, we examined the expression and activity of *p53* in both tissues. By immunohistochemistry, p53 protein levels were found to be increased in the CNS of *Rb*-mutants (Figure 2C) relative to wild-type littermates (Figure 2A). Western blot analysis showed a 3- to 5-fold induction of p53 levels in the CNS but no detectable change in the electrophoretic mobility of p53 (Figure 3A). Northern blot analysis demonstrated that *p53* is not induced transcriptionally in response to *Rb* loss (data not shown). Although the level of p53 induction in *Rb–/–* brain extracts seen by Western blot was only 3- to 5-fold, it is clear from immunohistochemical studies (Figure 2C) that some cells expressed p53 to a much higher level than other cells in which p53 staining was undetectable.

The increased level of p53 protein in the CNS of *Rb*-mutant embryos was reflected in increased DNA binding activity to a consensus p53 DNA binding site (Figure 3B). *Rb*-mutant brain extracts showed increased levels of a complex bound to the p53 consensus site (complex A), and we also saw the formation of three slower-migrating complexes (complexes B, C and D). Complex A corresponds to monomeric p53 while complexes B, C and D may be the result of p53 oligomerization promoted by the

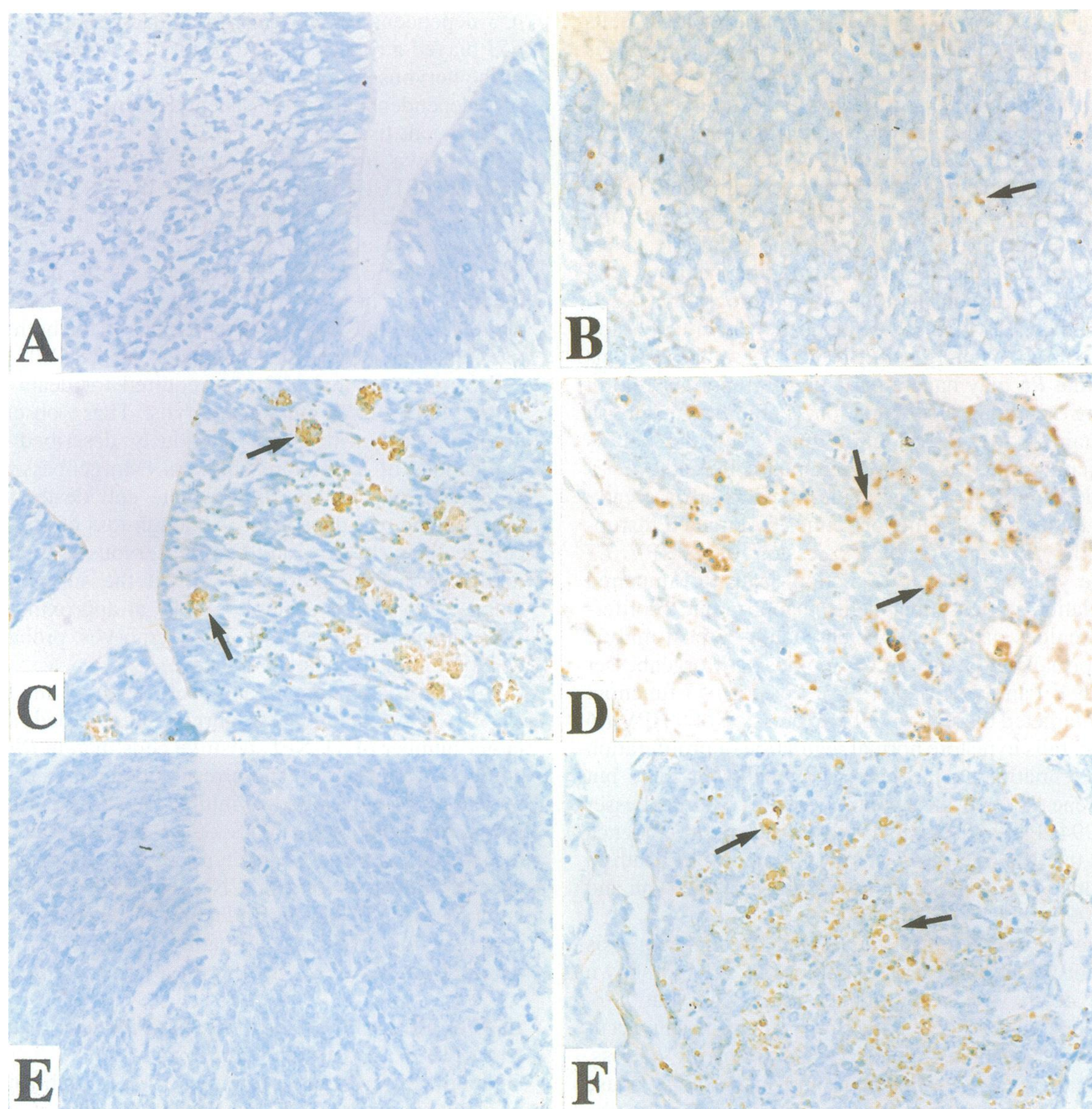

Fig. 1. Cell death in the *Rb*-mutant CNS but not the PNS is suppressed by loss of *p53*. Cell death is visualized by TUNEL assay on sections through the 4th ventricle of the brain (CNS) or the dorsal root ganglia (PNS) of E13.5 mouse embryos. TUNEL-positive cells stain brown (black arrows) against a methyl green-counterstained background of live cells. Cell death in the CNS of the *Rb*-mutant embryo (**C**) is more extensive than in wild-type (**A**) but is absent in the *Rb–/–*;p53–/– brain (**E**). There is increased cell death in the sensory ganglia of the *Rb–/–;p53–/–* embryo (**F**) compared with the *Rb–/–* embryo (**D**), which exhibits more cell death than that seen in the wild-type (**B**).

higher concentrations of p53 found in mutant over wild-type extracts.

Induction of p53 was not apparent in the PNS of *Rb*-mutant embryos. No p53 staining was seen in the dorsal root ganglia of *Rb*-deficient embryos, despite extensive cell death (Figure 2D). The lack of p53 induction in the PNS of *Rb*-deficient embryos is consistent with the failure of *p53* mutation to inhibit cell death in the PNS of these embryos.

Along with increased p53 levels and DNA binding activity, we detected elevated message levels of the p53 target gene, $p21^{Waf\text{-}1/Cip\text{-}1}$ cyclin/cdk inhibitor, in *Rb*-mutant brain (Figure 4C) compared with wild-type (Figure 4A). Induction of $p21^{Waf\text{-}1/Cip\text{-}1}$ in the *Rb*-mutant brain is clearly p53-dependent, as it was not induced in the brain of *Rb–/–;p53–/–* embryos (Figure 4E). Interestingly, despite a dramatic induction of $p21^{Waf\text{-}1/Cip\text{-}1}$ in the hindbrain of *Rb–/–* embryos, increased $\mathrm{p21}^{\mathrm{Waf\text{-}1/Cip\text{-}1}}$ was insufficient to prevent extensive ectopic DNA replication.

$p21^{Waf\text{-}1/Cip\text{-}1}$ was also induced in the sensory ganglia of *Rb*-mutant embryos (Figure 4D) compared with those of wild-type embryos (Figure 4B). However, this induction appeared to occur by a *p53*-independent mechanism, because it was also seen in the dorsal root ganglia of *Rb–/–;p53–/–* embryos (Figure 4F).

Abnormal proliferation in the CNS and PNS of Rb-mutant embryos is not affected by loss of p53

As neuroblasts differentiate into neurons, they migrate away from the neural tube and permanently exit the cell cycle, a process that is defective in *Rb–/–* embryos (Lee *et al.*, 1994). As shown in Figure 5, we observed an increase in the fraction of BrdU-positive neurons in the *Rb*-mutant CNS compared with wild-type (Figure 5A

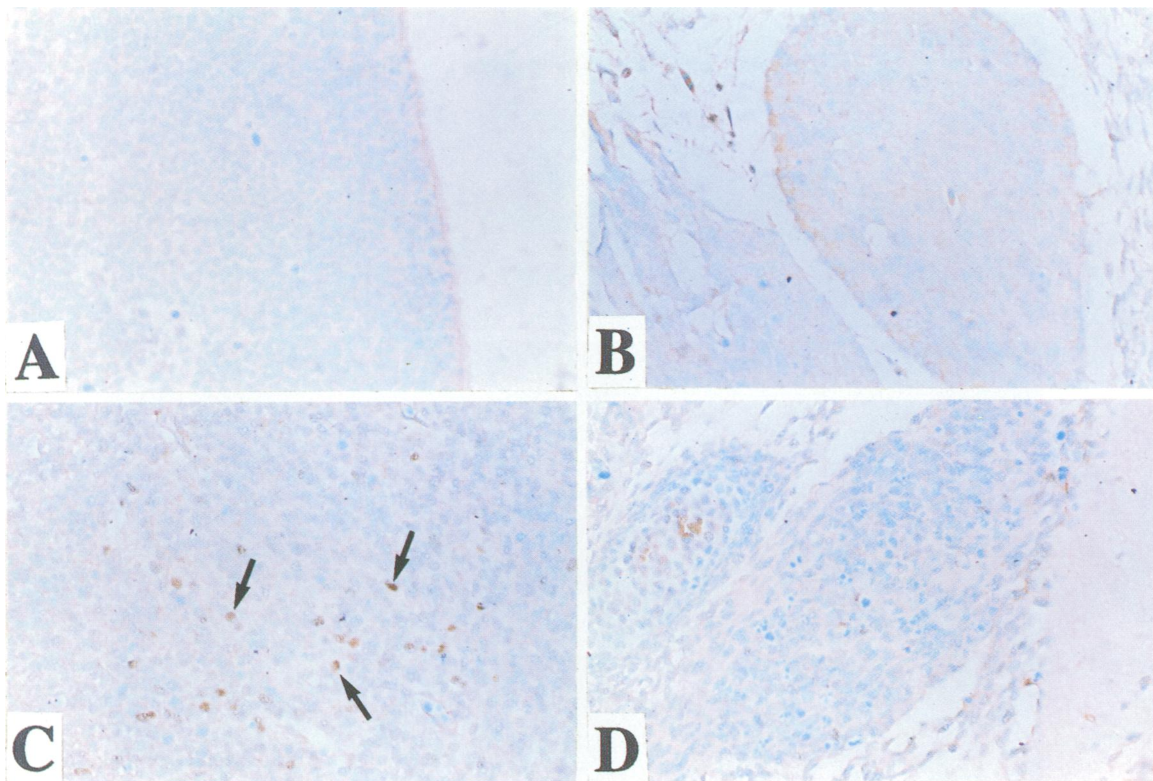

Fig. 2. p53 is induced in the CNS but not in the PNS of *Rb*-mutant embryos. Immunohistochemistry for p53 expression in the CNS (**A** and **C**) and PNS (**B** and **D**) of wild-type (A and B) and *Rb*-mutant (C and D) embryos. The arrows indicate p53-positive cells in the hindbrain of the *Rb*-deficient embryo (C) while no detectable expression is seen in the wild-type embryo (A). p53 is not detectable in the dorsal root ganglia of either wild-type (2B) or *Rb*-mutant embryos (D). Note, there is p53 staining in the vertebral cartilage adjacent to the dorsal root ganglia of *Rb–/–* embryos (D).

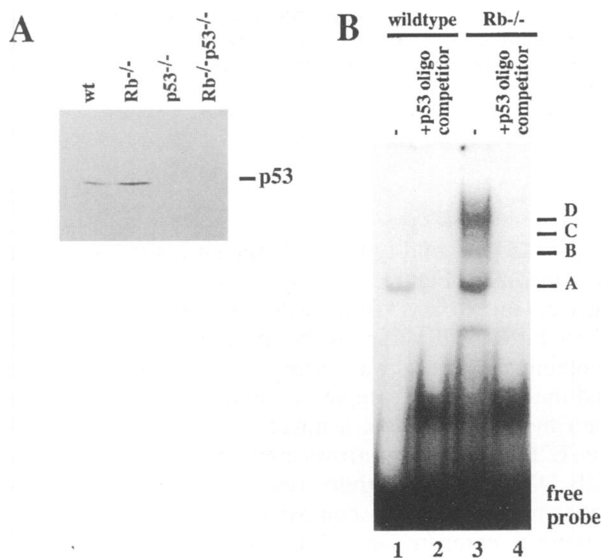

Fig. 3. Up-regulation of p53 levels and DNA binding activity in *Rb–/–* CNS. (**A**) Western blot analysis of brain whole-cell extract from wild-type, *Rb–/–*, *p53–/–* or *Rb–/–;p53–/–* embryos. The figure shows a 3- to 5-fold induction of p53 in *Rb*-mutant brain over wild-type brain and a complete loss of p53 expression, as expected, in both *p53–/–* and *Rb–/–;p53–/–* brains. (**B**) Electrophoretic mobility shift assay for p53 DNA binding activity measured in whole-cell extracts from wild-type or *Rb*-mutant brains using a p53 consensus oligonucleotide probe (Pavletich *et al.*, 1993). Lanes 1 and 2, wild-type brain extracts; lanes 3 and 4, *Rb*-mutant brain extracts; lanes 2 and 4, 100 ng of unlabeled p53 oligonucleotide competitor. Complex A, p53 monomer; complex B, C and D, p53 oligomers.

and C). Importantly, increased BrdU incorporation was observed in areas of the brain normally reserved for post-mitotic neurons (Figure 5C), and these abnormally cycling cells localized to regions of the CNS where increased cell death was observed (Figure 1C). There were also increased numbers of BrdU-positive cells in the sensory ganglia of *Rb*-mutant embryos (Figure 5D) compared with wild-type (Figure 5B). Thus, cell death in both the CNS and PNS of *Rb*-mutant embryos is correlated with aberrant cell cycle regulation (Figure 5C and D).

BrdU labeling of *Rb–/–;p53–/–* embryos demonstrated that loss of *p53* does not restore normal proliferative control to the CNS of *Rb*-mutant embryos (Figure 5E), despite the suppression of cell death in this tissue (Figure 1E). In fact, there were greater numbers of BrdU-labeled cells in the CNS of *Rb–/–;p53–/–* embryos than in the CNS of *Rb–/–* embryos (Figure 5C and E). This may reflect the failure of aberrantly replicating *Rb–/–;p53–/–* neurons to undergo apoptosis. Loss of *p53* also failed to restore normal cell cycle regulation to the *Rb*-mutant PNS (Figure 5F). Furthermore, as observed in the CNS (Figure 5C and E), *p53* loss actually led to a greater number of peripheral neurons aberrantly entering S phase (Figure 5D and F) This result is surprising given that p53 protein levels were not induced in the PNS (Figure 2D) and that loss of *p53* did not suppress cell death in the PNS of *Rb–/–* embryos (Figure 1F). These observations suggest that *p53* may act to limit proliferation of peripheral neurons following *Rb* loss by inducing growth arrest. In this tissue, *p53* mutation exacerbates the proliferative

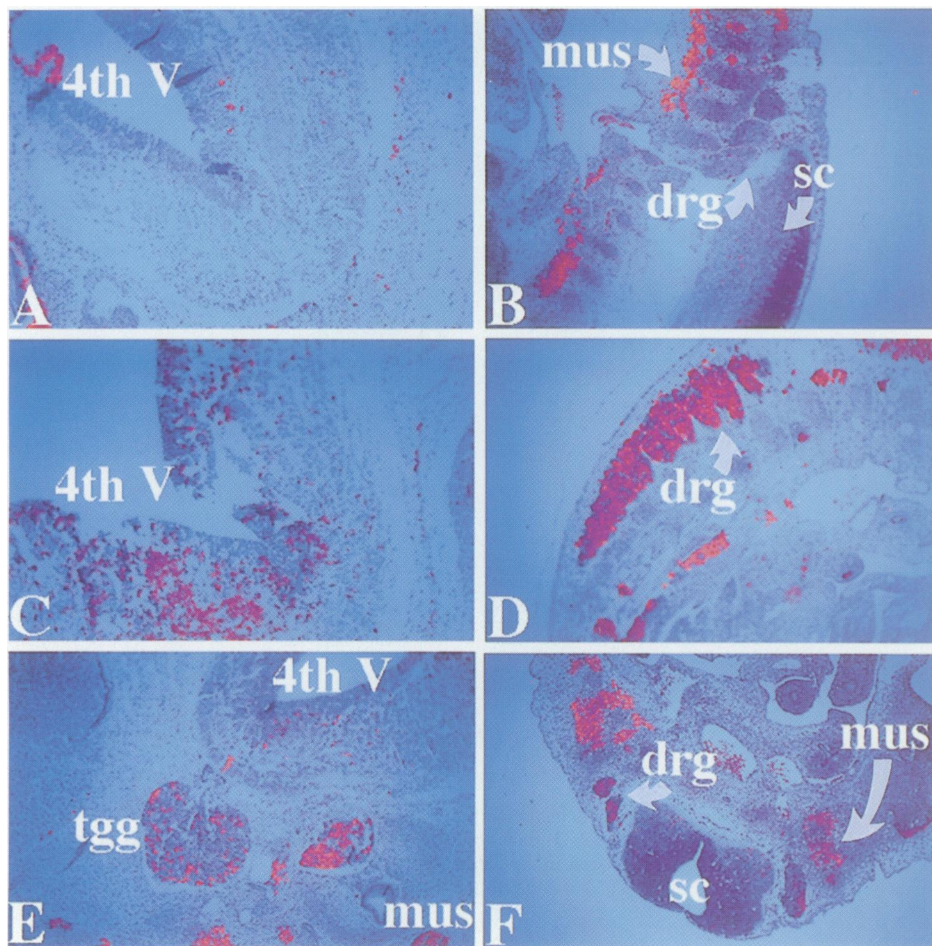

Fig. 4. *p21*$^{Waf\text{-}1/Cip\text{-}1}$ is induced in a *p53*-dependent manner in the CNS and in a *p53*-independent manner in the PNS of *Rb*-mutant embryos. *In situ* hybridization was used to examine p21$^{Waf\text{-}1/Cip\text{-}1}$ expression in the CNS and PNS of wild-type (**A** and **B**), *Rb–/–* (**C** and **D**) and *Rb–/–;p53–/–* (**E** and **F**) embryos. Barely detectable levels of p21 are found in the hindbrain (4th V; panel A) or dorsal root ganglia (drg; panel B) of wild-type embryos. A massive induction of p21$^{Waf\text{-}1/Cip\text{-}1}$ is seen in the hindbrain (4th V; panel C) and dorsal root ganglia (drg; panel D) of the *Rb–/–* embryos. Expression of p21$^{Waf\text{-}1/Cip\text{-}1}$ is lost in the hindbrain (4th V; panel E) and spinal cord (sc; panel F) of *Rb–/–;p53–/–* embryos but we continue to see *p21*$^{Waf\text{-}1/Cip\text{-}1}$ expression in the trigeminal ganglia (tgg; panel 4E) and in the dorsal root ganglia (drg; panel F). *p21*$^{Waf\text{-}1/Cip\text{-}1}$ expression in developing muscle is seen in all four genotypes (mus; panels B, D and F)

defect and consequent apoptosis, which occurs through *p53*-independent mechanisms.

These results illustrate how loss of *Rb* leads to a common cellular defect in the CNS and PNS: deregulated cell cycle control and programmed cell death. However, the mechanism of inducing apoptosis in the two tissues clearly differs. CNS neurons induce p53 and undergo *p53*-dependent death whereas neurons in the PNS do not show increased p53 protein levels and die in a *p53*-independent manner.

Deregulated proliferation of Rb-deficient neurons is associated with increased levels of free E2F and increased expression of cyclin E

Overexpression of the E2F-1 transcription factor in tissue culture cell lines has been shown to induce premature S phase entry and apoptotic cell death (Qin *et al.*, 1994; Wu and Levine, 1994). We sought to determine whether the phenotype observed *in vivo* in *Rb*-mutant embryos was associated with increased levels of free E2F. We used electrophoretic mobility shift assays to characterize E2F DNA binding activity in whole tissue extracts prepared from 13.5-day wild-type or *Rb*-mutant embryonic brains. As shown in Figure 6, a slow mobility complex was present in extracts from wild-type brain, which was supershifted with an antibody specific to pRB. This RB-containing complex was absent from *Rb*-mutant extracts and the loss of this complex in mutant extracts correlated with the appearance of a novel band that migrated with free E2F (indicated by arrows in Figure 6). p107-associated E2F DNA-binding activity remained unchanged in *Rb*-deficient brain extracts compared with wild-type extracts (Figure 6, compare lanes 3 and 9). Extracts from *Rb–/–; p53–/–* brains showed increased levels of free E2F comparable with the *Rb–/–* extracts, while there was no change in *p53–/–* samples compared with wild-type (Figure 6, compare lanes 7 with 19, and 1 with 13).

Increased levels of free E2F-1 are thought to induce premature entry into S phase by inducing the expression of genes encoding functions needed for DNA replication and other S phase events. The *cyclin E* gene is considered to be an important target of E2F-1 and is induced at the G_1/S phase transition in cycling fibroblasts (DeGregori *et al.*, 1995a; Ohtani *et al.*, 1995; Herrera *et al.*, 1996).

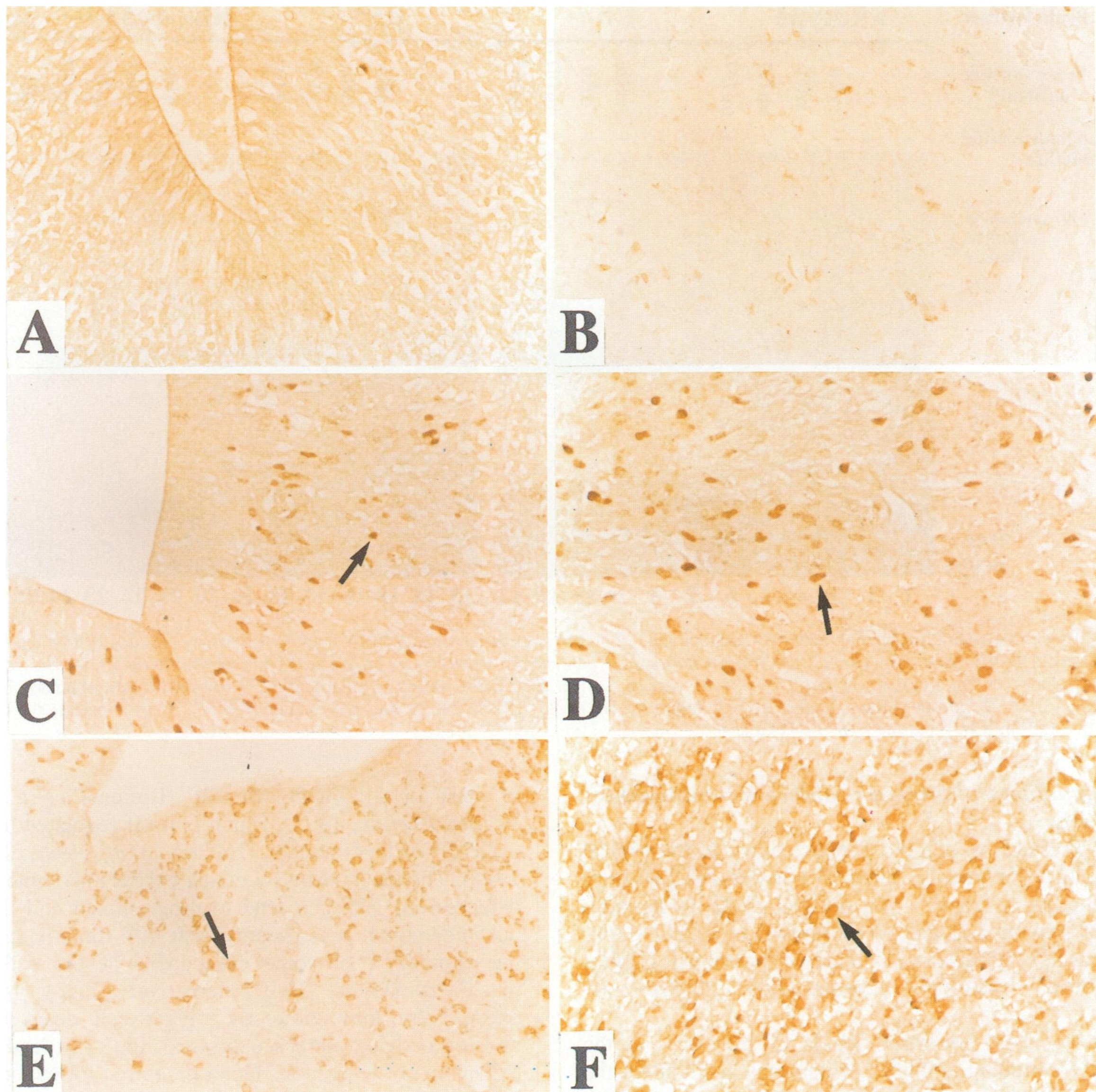

Fig. 5. Increased proliferation in the CNS and PNS of *Rb*-mutant embryos. BrdU incorporation was used as a measure of S phase entry by neurons in the CNS and PNS of wild-type (**A** and **B**), *Rb*-mutant (**C** and **D**) and *Rb–/–;p53–/–* (**E** and **F**) embryos. Arrows indicate some of the BrdU-positive cells. As shown in (A), there is no S phase activity in the hindbrain of wild-type embryos by embryonic age 13.5 days. Nor is there any activity in the trigeminal ganglia (tgg) of these embryos (B). In contrast, both the hindbrain (C) and sensory ganglia (tgg; panel D) of *Rb*-mutant embryos stain incorporate BrdU in a 1-h pulse. Loss of *p53* function results in a more severe perturbation of proliferation in both the CNS (E) and the PNS (F); that is, there are more BrdU-positive cells than in the *Rb*-mutant nervous system (C and D).

Furthermore, cyclin E has been shown to be derepressed in *Rb*-deficient fibroblasts (Herrera *et al.*, 1996). To establish a link between the increased levels of free E2F and the increased proliferation of *Rb*-deficient neurons, we examined the expression of *cyclin E* in the *Rb*-mutant brain by *in situ* hybridization. As shown in Figure 7, we observed increased expression of *cyclin E* in both the CNS and PNS of *Rb*-mutant embryos (Figure 7C and D), compared with wild-type embryos (Figure 7A and B). In particular, there was a striking induction of *cyclin E* in areas away from the ventricular zone where post-mitotic neurons are normally found. These observations are fully consistent with the ectopic DNA replication and associated cell death in this region of the brain.

The induction of *cyclin E* was also apparent in the CNS and PNS of *Rb–/–;p53–/–* embryos (Figure 7E and F), as expected from the increased levels of free E2F in *Rb–/–; p53–/–* embryonic brain (Figure 6) and the abnormal proliferation observed in the CNS and PNS of doubly mutant embryos (Figure 5E and F).

Discussion

We have demonstrated that loss of *Rb* function during mouse development leads to cell death in the CNS and in the PNS by two distinct mechanisms: one *p53*-dependent and the other *p53*-independent. Our results illustrate clearly that the functional interaction of *Rb* with *p53* varies depending on cell type and this may help to explain why loss of *Rb* and *p53* has a cooperative effect on the transformation of some tissues but not others.

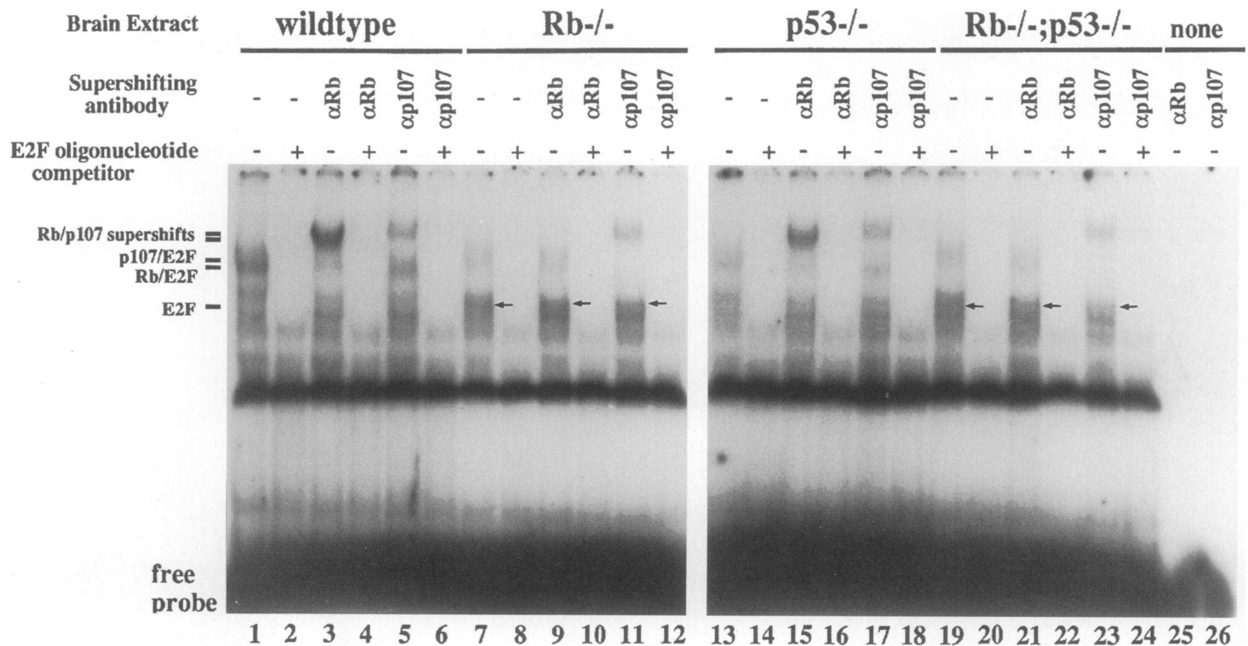

Fig. 6. Increased free E2F DNA binding activity. Electrophoretic mobility shift assay for E2F DNA binding activity in 13.5 day embryonic brain extracts. Lanes 1–6, wild-type brain extract; lanes 7–12, *Rb*-mutant brain extract; lanes 13–18, *p53*-mutant brain extract; lanes 19–24, *Rb–/–;p53–/–* brain extract; lanes 25–26, no extract. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24; 100 ng of unlabeled E2F oligonucleotide competitor. Lanes 3, 4, 9, 10, 15, 16, 21, 22, 25; anti-pRB antibody, 21C9. Lanes 5, 6, 11, 12, 17, 18, 23, 24, 26; anti-p107 antibody, SC-9. Arrows indicate the increased levels of free E2F in *Rb–/–* and *Rb–/–;p53–/–* extracts.

The mechanism of altered proliferation induced by loss of Rb

Loss of *Rb* leads to ectopic S phase entry in the central and peripheral nervous systems. We observed a failure of differentiating neurons to exit the cell cycle, as has also been reported by Lee and co-workers (Lee *et al.*, 1994). The failure to establish or maintain cell cycle exit implies that normal neuronal differentiation requires *Rb* function.

We have noted increased levels of free *E2F* in the brains of Rb-mutant embryos, which may account for the increased numbers of S phase neurons in the CNS. Different E2F family members have been shown to be necessary for the expression of genes involved in S phase entry and progression through S phase (Means *et al.*, 1992; Lam and Watson, 1993; Slansky *et al.*, 1993; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; DeGregori *et al.*, 1995a; Muller, 1995). We have shown that one of these genes, *cyclin E*, is indeed up-regulated and ectopically expressed in the *Rb*-deficient nervous system. In studies of cells in culture, the premature entry of cells into S phase caused by overexpression of E2F-1 results in programmed cell death (Johnson *et al.*, 1993; Qin *et al.*, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995), which is reminiscent of the effect observed here *in vivo*. Although there are clear similarities between the effects of overexpression of E2F-1 in tissue culture and the loss of *Rb* function *in vivo*, it is likely that *Rb* deficiency has additional effects on cell fate that are independent of E2F regulation.

As opposed to initial models that suggested uncomplexed E2F activates certain genes in a cell cycle-dependent manner, recent evidence indicates that promoter E2F sites are occupied by the factor at all times during the cell cycle (Zwicker *et al.*, 1996). These promoters are specifically repressed or derepressed by the state of binding of pRB, or p107/p130, to E2F (Bremner *et al.*, 1995; Weintraub *et al.*, 1995), which is itself regulated in a cell cycle-dependent manner (Hiebert *et al.*, 1992; Nevins, 1992). The mechanism by which excess uncomplexed (or derepressed) E2F can lead to programmed cell death is not understood. It is possible that E2F directly regulates the expression of some component of the cell death machinery (Figure 8A). Alternatively, inappropriate S phase entry may indirectly trigger cell death (Figure 8B), or perhaps increased proliferation may lead to the exhaustion of exogenous factors required for cell survival (Figure 8C).

There is no evidence that E2F regulates the expression of genes involved in apoptosis. However, it has recently been shown that E2F overexpression in *Drosophila* imaginal discs leads to ectopic proliferation, apoptosis and the induction of *reaper*, a known cell death regulator in the fly (Asano *et al.*, 1996). It is not yet clear if induction of *reaper* is direct or indirect. Evidence for the direct regulation of other cell death genes by E2F is lacking, but experiments designed to identify death genes activated following loss of *Rb*, or a determination of which domains of E2F are required for its ability to induce apoptosis may identify such genes. Interestingly, like p53, E2F has been reported to bind mdm2 (Martin *et al.*, 1995). Thus, E2F could induce cell death by titrating mdm2 away from p53 and stimulating transactivation of p53 target genes involved in apoptosis. E2F could also activate p53 indirectly through up-regulation of an activator, such as an S phase kinase (Wang and Prives, 1995).

Cell death in the *Rb*-deficient embryo could also result from activation of a mechanism that detects inappropriate cell cycle regulation (Figure 8B). For example, lack of *Rb* might allow premature S phase entry, followed by inefficient or faulty S phase progression due to the

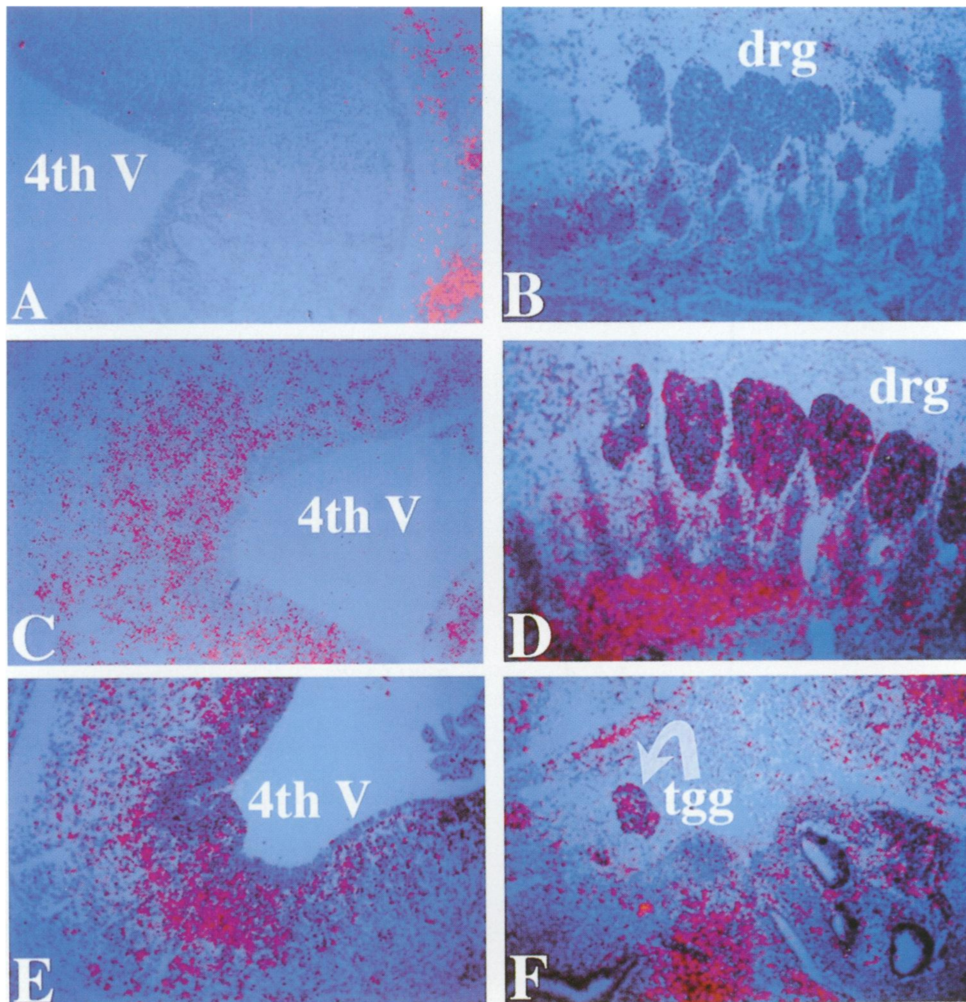

Fig. 7. *Cyclin E* is induced in the CNS and PNS of *Rb–/–* and *Rb–/–;p53–/–* embryos. *In situ* hybridization was used to detect *cyclin E* mRNA expression in the nervous system of wild-type (**A** and **B**), *Rb–/–* (**C** and **D**) and *Rb–/–;p53–/–* (**E** and **F**) embryos. In wild-type embryos, there are undetectable levels of *cyclin E* expression in the CNS (4th V; panel A) and the PNS (drg; panel B). However, the level of *cyclin E* expression in the CNS of *Rb*-mutant embryos (4th V; panel C) is dramatically increased over that of wildtype. Similarly, the level of *cyclin E* expression in the sensory ganglia of *Rb*-mutant embryos (drg; panel D) is highly elevated over wildtype (drg; panel B). Comparing the level of *cyclin E* expression in the CNS of *Rb–/–;p53–/–*embryos (4th V; panel E) with that of *Rb–/–* embryos (panel C), there appears to be equally high levels in both. Similarly, in the PNS of these embryos, the induction of *cyclin E* in the trigeminal ganglia (tgg; panel F) is no greater than that seen in the ganglia of *Rb*-mutant embryos (drg; panel D).

absence or inactivity of other critical factors. Certain DNA replication intermediates might then accumulate in *Rb*-deficient cells, which could trigger a p53-dependent checkpoint mechanism culminating in death (Hartwell and Kastan, 1994). It is also possible that p53 is induced in G_1 independently of E2F or S phase entry, by other cell cycle defects associated with *Rb* deficiency.

The third model (Figure 8C), takes into account the importance of the organismic microenvironment. The model proposes that the rapid proliferation associated with loss of *Rb* might lead to exhaustion of the growth/survival factor supply and indirectly to cell death. The role of survival factors in nervous system development has been extensively analyzed (Barres *et al.*, 1992, 1993; Raff *et al.*, 1993). It is estimated that neurons are produced in excess and compete for survival factors, such as nerve growth factor (NGF) and insulin-like growth factor I (IGF-1). Neurons that fail to receive an adequate supply of growth factors undergo programmed cell death. Different populations of neurons require different growth/survival signals and this requirement may change during development (Buchman and Davies, 1993). Thus, the pattern and timing of growth factor/growth factor receptor expression, is critical for the survival and proliferation of nerve cells (Pittman *et al.*, 1994; Birling and Price, 1995; Silos-Santiago *et al.*, 1995). Abnormal proliferation of neurons in the *Rb*-deficient embryo might upset the balance in this survival system and culminate in apoptosis. Evidence in favor of survival factors regulating apoptosis of *Rb*-mutant cells has come from studies on chimeric embryos created with *Rb*-deficient embryonic stem cells (Maandag *et al.*, 1994; Williams *et al.*, 1994b). Despite extensive contribution of *Rb*-deficient cells in such chimeras, there is relatively little apoptosis (Maandag *et al.*, 1994; Williams *et al.*, 1994b; K.Macleod, B.Williams and T.Jacks, unpublished results). Therefore, the presence of wild-type cells appears to permit survival of the mutant cells, perhaps through the action of secreted survival factors.

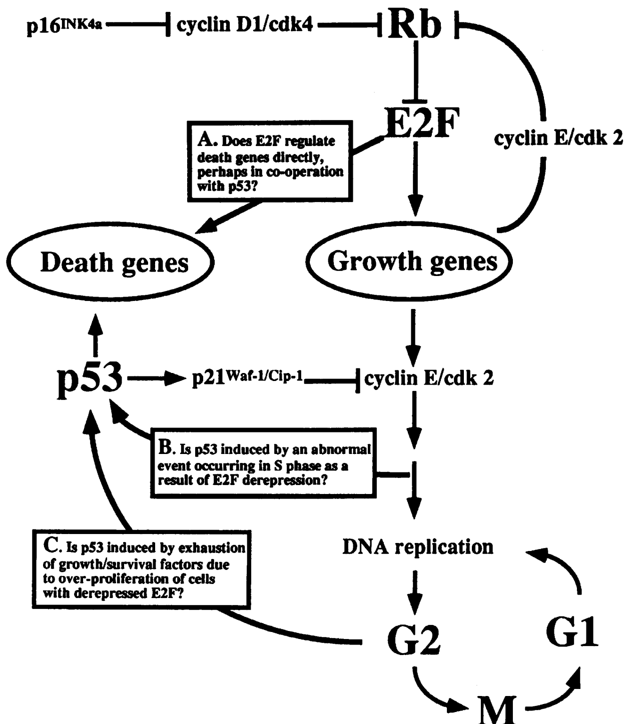

Fig. 8. Mechanisms by which E2F and p53 may cooperate to induce apoptosis. Three mechanisms may play a role in establishing cooperativity between E2F and p53 in the induction of apoptosis following loss of *Rb*. (A) E2F may be directly inducing death genes in a manner dependent on loss of regulation by *Rb* and the presence of a coactivator, such as p53. (B) p53 may be induced indirectly by E2F as a consequence of premature/aberrant S phase entry. (C) p53 may be induced as a consequence of the stress imposed on the cell by overproliferation and exhaustion of growth factors/survival factors.

p53-dependent and independent death

Our work highlights the complexity of the mechanisms that induce apoptosis. In response to the same genetic defect and ostensibly the same cellular abnormalities, neurons in the CNS and PNS initiate apoptosis through distinct mechanisms. This observation is reminiscent of the differential requirement for *p53* in the death of either thymocytes or cycling peripheral T cells following gamma irradiation (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Strasser *et al.*, 1994). At present, we do not have candidates for the mediators of this *p53*-independent form of apoptosis. However, it is noteworthy that the patterns of apoptotic cells in the PNS and CNS are distinct. In the CNS, dying cells are typically present in clusters while in the PNS, the pattern of cell death is more diffuse. The clustering of dying cells in the CNS could result from the local exhaustion of growth/survival factors, which may specifically induce a *p53*-dependent death pathway. Focal stimulation of p53 in the CNS could also contribute to this clustered pattern of cell death. Transcriptional induction of IGF–BP3 by p53 would be expected to reduce the effective local concentration of the IGF-I and -II survival factors, and might lead to the death of neighboring cells (Buckbinder *et al.*, 1995).

The existence of *p53*-independent apoptosis stimulated by the loss of *Rb* function has implications for cancer therapy. There is increasing evidence that *p53* status can affect the efficacy of anti-cancer agents, which is explained in part by the fact that these agents can induce apoptosis more efficiently in *p53*-containing tumor cells (Lowe *et al.*, 1994). Therefore, it is important to discover therapies that induce apoptosis in a *p53*-independent manner and act selectively in abnormal cells. An elucidation of the mechanism of apoptosis in the cells of the PNS of *Rb*-mutant embryos could ultimately lead to such therapies.

Materials and methods

Embryos

Embryos were generated from Rb heterozygous breeding pairs on a 129/Sv genetic background; the presence of a vaginal plug which was taken as day 0. Embryos were harvested on days 11.5, 12.5 or 13.5 of gestation. Most *Rb–/–* embryos are dead by day 14.5. To generate *Rb–/–;p53–/–* embryos, *Rb+/–;p53–/–* females were mated to *Rb+/–;p53+/–* males. *Rb–/–;p53–/–* embryos were expected at a frequency of 1 in 8. Embryos were fixed in 10% neutral buffered saline for 24 h and then transferred to 70% ethanol before processing and sectioning.

TUNEL assays

Tdt dUTP-biotin Nick End Labelling (TUNEL) was carried out on embryo sections as described previously (Morgenbesser *et al.*, 1994). Following the TUNEL assay, sections were counterstained in 0.025% methyl green, 0.1 M sodium acetate, pH 4.0.

BrdU labeling and immunohistochemistry

Embryos were labeled with BrdU *in utero* by injecting the pregnant mother with 100 μg BrdU (5 mg/ml in PBS) per gram body weight, 1 h before sacrifice. Embryos were recovered and fixed for sectioning as described above. Immunohistochemistry was performed on embryo sections as described previously (Morgenbesser *et al.*, 1994), except that the primary anti-BrdU antibody, obtained from Becton Dickinson, was used at a dilution of 1:50, rather than at 1:20 as previously described.

Western blotting

Whole-cell extracts were prepared from dissected mouse brains by passage through a 23-gauge syringe to dissociate cells and then lysed as described previously. Approximately 90 μg of extract was loaded onto a 10% SDS–PAGE gel and electrophoresed at 30 mA for 5 h or overnight at 80 V. The gel was transferred to PVDF membrane by semi-dry blotting. A cocktail of Ab-1 and Ab-3 (Oncogene Science) at a dilution of 1:500 each, was incubated with the pre-blocked filter overnight at 4°C in 5% dried milk, 0.2% Tween, PBS. The blot was washed twice at room temperature for 20 min in PBS, 0.2% Tween, incubated for 4 h at 4°C with secondary antibody (peroxidase linked goat anti-mouse from Amersham) at a dilution of 1:7000 in 5% dried milk, 0.2% Tween, PBS and then washed twice at room temperature in PBS, 0.2% Tween. The blot was developed using ECL reagents (Amersham) and exposed to Kodak X-OMAT 5 film for 3–5 min.

Immunohistochemistry

Sections were deparaffinized and rehydrated as described previously (Morgenbesser *et al.*, 1994). For p53 immunohistochemistry, the epitope was revealed by microwaving sections for 10 min in 0.01 M citrate buffer, pH 6.0, as described by the manufacturers of NCL–p53–CM5p (Novocastra Laboratories). Sections were blocked in 2% horse serum, 5% goat serum, 1% dried spleen extract, PBS for 30 min at room temperature and then incubated for 1 h at room temperature with the primary antibody, diluted 1/1000 in blocking solution. The sections were washed twice in PBS and then incubated for 1 h at room temperature with secondary antibody from the Vectastain *Elite* ABC kit. Sections were washed again and incubated for 30 min with the ABC reagent. The sections were developed using DAB as a substrate. Sections were counterstained with methyl green as described for TUNEL assays.

In situ hybridization

In situ hybridization was carried out as described previously (Wilkinson, 1993). The *p21* probe was generated by cloning a 720 bp *Bam*HI–*Xho*I cDNA fragment into pBS–KS while the mouse cyclin E probe was generated by cloning a 1 kb *Bam*HI–*Hin*dIII cDNA fragment into pBS–KS. Both fragments represented most of the coding sequence of their respective genes. The probes were sequenced and transcribed using T7 polymerase to generate a [^{35}S]UTP-labeled probe which was incubated on sections in hybridization buffer as described (Wilkinson, 1993) in a

Hybaid Omnislide machine at 55°C overnight. High-stringency washes and RNase A digestion were also carried out as described by Wilkinson (1993). Sections were exposed in the dark to NTB3 photographic emulsion (Kodak) for 1 week (cyclin E) or 3 weeks (p21). Sections were developed/fixed in D19 developer/fix (Kodak). Sections were counterstained with toluidine blue, mounted and photographed by double exposure using a red filter for dark-field and blue filters for bright-field photography.

Electrophoretic mobility shift assays

Brain extracts were prepared similarly for both E2F and p53 gel shift assays but the conditions of binding and electrophoresis differed. Whole-cell extracts were prepared from each embryonic brain by lysis in 5× extraction buffer (100 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 2.5 mM EDTA, 20% glycerol, 0.5 M KCl, 0.5 mM PMSF, 20 μM sodium orthovanadate, pH 8.0). The concentrations of lysates were determined by Bradford reagents and 10–15 μg were typically used in a binding reaction. E2F binding reactions were carried out as described by Cao *et al.* (1992) using 1 μl of 0.1 mg/ml of cold E2F oligonucleotide competitor or 1 μl of supershifting antibody (either 21C9 for Rb, kindly provided by the Weinberg laboratory, or SD-15, kindly provided by the Lees laboratory, for p107), as required. p53 binding reactions were carried out similarly except that the binding buffer used was 50% glycerol, 250 mM KCl, 100 mM HEPES, pH 7.4, 5 mM DTT, 5 mg/ml BSA, 0.5% Triton X-100. Also, a lower concentration of non-specific competitor salmon sperm DNA was used (0.3 mg/ml instead of 1 mg/ml) and polyclonal Pab 421 antibody (Oncogene Science) was added to all reactions. Finally, the reactions were separated on a 4% polyacrylamide, 0.33× TBE, 0.1% Triton X-100 gel for 3 h.

The oligonucleotides used were: E2F consensus 5′-ATTTAAGTTT-CGCGCCCTTTCTCAA-3′ (Cao *et al.*, 1992) and p53 consensus 5′-CCTGCCTTGCCTGGACTTGCCTGG-3′ (Pavletich *et al.*, 1993).

Acknowledgements

The authors would like to thank Laura Attardi, Mariana Nacht, Jimmy Brugarolas and Bart Williams for critical reading of the manuscript, and Sharon Morgenbesser and Ron DePinho for helpful discussions. We would also like to thank members of the McMahon, Lees, Weinberg and Hynes laboratories for the gift of reagents or technical advice. K.M. was funded by a long-term fellowship from the Human Frontiers Science Program Organisation during this work. T.J. is an Assistant Investigator of the HHMI; this work was supported by a grant from the NIH. This manuscript is dedicated to the memory of the children and their teacher of Dunblane, Scotland, who died 13 March, 1996.

References

Almasan,A., Yin,Y., Kelly,R., Lee,Y.-H.P., Bradley,A., Li,W., Bertino,J.R. and Wahl,G.M. (1995) Deficiency of retinoblastoma protein leads to inappropriate S-phase entry, activation of E2F-responsive genes and apoptosis. *Proc. Natl Acad. Sci. USA*, **92**, 5436–5440.

Asano,M., Nevins,J.R. and Wharton,R. (1996) Ectopic E2F expression induces S phase and apoptosis in *Drosophila* imaginal discs. *Genes Dev.*, **10**, 1422–1432.

Attardi,L.D., Lowe,S.W., Brugarolas,J. and Jacks,T. (1996) Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene-mediated apoptosis. *EMBO J.*, **15**, 3693–3701.

Barres,B.A., Hart,I.K., Coles,H.S.R., Burne,J.F., Voyvodic,J.T., Richardson,W.D. and Raff,M.C. (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell*, **70**, 31–46.

Barres,B.A., Schmid,R., Sendnter,M. and Raff,M.C. (1993) Multiple extracellular signals are required for long-term oligodendrocyte survival. *Development*, **118**, 283–295.

Birling,M.-C. and Price,J. (1995) Influence of growth factors on neuronal differentiation. *Curr. Opin. Cell Biol.*, **7**, 878–884.

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 Rb and its reversal by specific cyclins. *Mol. Cell. Biol.*, **15**, 3256–3265.

Brugarolas,J., Chandrasekaran,C., Gordon,J.I., Beach,D., Jacks,T. and Hannon,G.J. (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature*, **377**, 552–557.

Buchman,V.L. and Davies,A.M. (1993) Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. *Development*, **118**, 989–1001.

Buckbinder,L., Talbott,R., Velasco-Miguel,S., Takenaka,I., Faha,B., Seizinger,B.R. and Kley,N. (1995) Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature*, **377**, 646–649.

Canman,C.E., Gilmer,T.M., Coutts,S.B. and Kastan,M.B. (1995) Growth factor modulation of p53-mediated growth arrest versus apoptosis. *Genes Dev.*, **9**, 600–611.

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**, 176–179.

Cheng,S., Schmidt-Grimminger,D.C., Murant,T., Broker,T.R. and Chow,L.T. (1995) Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. *Genes Dev.*, **9**, 2335–2349.

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

Clarke,A.R., Purdie,C.A., Harrison,D.J., Morris,R.G., Bird,C.C., Hooper,M.L. and Wyllie,A.H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849–852.

DeGregori,J., Kowalik,T. and Nevins,J.R. (1995a) Cellular targets for activation by the E2F1 transcription factor include DNA synthesis and G1/S regulatory genes. *Mol. Cell. Biol.*, **15**, 4215–4224.

DeGregori,J., Leone,G., Ohtani,K., Miron,A. and Nevins,J.R. (1995b) E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. *Genes Dev.*, **9**, 2873–2887.

Deng,C., Zhang,P., Harper,J.W., Elledge,S.J. and Leder,P. (1995) Mice lacking p21Cip1/Waf1 undergo normal development, but are defective in G1 checkpoint control. *Cell*, **82**, 675–684.

Di Leonardo,A., Linke,S.P., Clarkin,K. and Wahl,G.M. (1994) DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of CIP-1 in normal human fibroblasts. *Genes Dev.*, **8**, 2540–2551.

El-Deiry,W.S. *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

El-Deiry,W.S. *et al.* (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.*, **54**, 1169–1174.

Friend,S.J., 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**, 643–646.

Gu,W., Schneider,J.W., Condorelli,G., Kaushal,S., Mahdavl,V. and Nadal-Ginard,B. (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell*, **72**, 309–324.

Harris,C.C. and Hollstein,M. (1993) Clinical implications of the p53 tumor-suppressor gene. *N. Engl. J. Med.*, **329**, 1318–1327.

Hartwell,L.H. and Kastan,M.B. (1994) Cell cycle control and cancer. *Science*, **266**, 1821–1828.

Helin,K., Harlow,E. and Fattaey,A.R. (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol. Cell. Biol.*, **13**, 6501–6508.

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**, 2402–2407.

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**, 177–185.

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

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

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**, 349–352.

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

Kastan,M.B., Onyekwere,O., Sidransky,D., Vogelstein,B. and Craig,R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304–6311.

Kastan,M., Zhan,Q., El-Deiry,W.S., Carrier,F., Jacks,T., Walsh,W.V., Plunkett,B.S., Vogelstein,B. and Fornace,A.J.,Jr (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia–telangiectasia. *Cell*, **71**, 587–597.

Kiyokawa,H., Richon,V.M., Venta-Perez,G., Rifkind,R.A. and Marks, P.A. (1993) Hexamethylenebisacetamide-induced erythroleukemia cell differentiation involves modulation of events required for cell cycle progression through G1. *Proc. Natl Acad. Sci. USA*, **90**, 6746–6750.

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**, 2491–2500.

Kranenburg,O., van der Eb,A.J. and Zantema,A. (1996) Cyclin D1 is an essential mediator of apoptotic neuronal cell death. *EMBO J.*, **15**, 46–54.

Lam,E.-F. and Watson,R.J. (1993) An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. *EMBO J.*, **12**, 2705–2713.

Lassar,A.B., Skapek,S.X. and Novitch,B. (1994) Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Curr. Opin. Cell Biol.*, **6**, 788–794.

Lee,E.Y.-H.P., Chang,C.-Y., Hu,N., Wang,Y.-C. J., Lai,C.-C., Herrup,K. and Lee,W.-H. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature*, **359**, 288–295.

Lee,E.Y.-H.P., Hu,N., Yuan,S.-S.F., Cox,L.A., Bradley,A., Lee,W.-H. and Herrup,K. (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes Dev.*, **8**, 2008–2021.

Lees,J.A., Saito,M., Vidal,M., Valentino,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**, 7813–7825.

Lowe,S.W., Schmitt,E.S., Smith,S.W., Osborne,B.A. and Jacks,T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847–849.

Lowe,S.W., Bodis,S., McClatchey,A., Remington,L., Ruley,H.E., Fisher,D.E., Housman,D.E. and Jacks,T. (1994) p53 status and the efficacy of cancer therapy *in vivo*. *Science*, **266**, 807–810.

Maandag,E.C.R., 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**, 4260–4268.

Malkin,D. (1993) p53 and the Li–Fraumeni syndrome. *Cancer Genet. Cytogenet.*, **66**, 83–92.

Malkin,D., Li,F.P., Strong,L.C., Fraumeni,J.F.J., Nelson,C.E. and Kim,D.H. (1990) Germline p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*, **250**, 1233–1238.

Martin,K., Trouche,D., Hagemeier,C., Sorensen,T.S., La Thangue,N.B. and Kouzarides,T. (1995) Stimulation of E2F1/DP1 transcriptional activity by mdm2 oncoprotein. *Nature*, **375**, 691–694.

Means,A.L., Slansky,J.E., McMahon,S.L., Knuth,M.W. and Farnham,P.J. (1992) The HIP1 binding site is required for growth regulation of the dehydrofolate reductase promoter. *Mol. Cell. Biol.*, **12**, 1054–1063.

Midgley,C.A., Owens,B., Briscoe,C.V., Thomas,D.B., Lane,D.P. and Hall,P.A. (1995) Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type *in vivo*. *J. Cell Sci.*, **108**, 1843–1848.

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**, 72–75.

Muller,R. (1995) Transcriptional regulation during the mammalian cell cycle. *Trends Genet.*, **11**, 173–178.

Nevins,J. (1992) E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science*, **258**, 424–429.

Ohtani,K., DeGregori,J. and Nevins,J.R. (1995) Regulation of the cyclin E gene by transcription factor E2F1. *Proc. Natl Acad. Sci. USA*, **92**, 12146–12150.

Pan,H. and Griep,A. (1994) Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. *Genes Dev.*, **8**, 1285–1299.

Pan,H. and Griep,A.E. (1995) Temporally distinct patterns of p53-dependent and p53-independent apoptosis during mouse lens development. *Genes Dev*, **9**, 2157–2169.

Pavletich,N.P., Chambers,K.A. and Pabo,C.O. (1993) The DNA-binding domain of p53 contains four conserved regions and the major mutation hot spots. *Genes Dev.*, **7**, 2556–2564.

Pittman,R.N., Mills,J.C., DiBenedetto,A.J., Hynicka,W.N. and Wang,S. (1994) Neuronal cell death: searching for the smoking gun. *Curr. Opin. Neurobiol.*, **4**, 87–94.

Qin,X.Q., Livingston,D.M., Kaelin,W.G. and Adams,P.D. (1994) Deregulated transcription factor E2F1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl Acad. Sci. USA*, **91**, 10918–10922.

Raff,M.C., Barres,B.A., Burne,J.F., Coles,H.S., Ishizaki,Y. and Jacobson,M.D. (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science*, **262**, 695–700.

Richon,V., Rifkind,R.A. and Marks,P.A. (1992) Expression and phosphorylation of the retinoblastoma protein during induced differentiation of murine erythroleukemia cells. *Cell Growth Differ.*, **3**, 413–420.

Sabbatini,P., Lin,J., Levine,A.J. and White,E. (1995) Essential role for p53-mediated transcription in E1A-induced apoptosis. *Genes Dev.*, **9**, 2184–2192.

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**, 1467–1471.

Silos-Santiago,I., Greenlund,L.J.S., Johnson,E.M.,Jr and Snider,W.D. (1995) Molecular genetics of neuronal survival. *Curr. Opin. Neurobiol.*, **5**, 42–49.

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

Slack,R.S., Skerjanc,I.S., Lach,B., Craig,J., Jardine,K. and McBurney,M.W. (1995) Cells differentiating into neuroectoderm undergo apoptosis in the absence of functional retinoblastoma family proteins. *J. Cell Biol.*, **129**, 779–788.

Slansky,J.E., Li,Y., Kaelin,W.G. and Farnham,P.J. (1993) A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. *Mol. Cell. Biol.*, **13**, 1610–1618.

Slebos,R.J.C., Lee,M.H., Plunkett,B.S., Kessis,T.D., Williams,B.O., Jacks,T., Hedrick,L., Kastan,M.B. and Cox,K.R. (1994) p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. *Proc. Natl Acad. Sci. USA*, **91**, 5320–5324.

Strasser,A., Harris,A.W., Jacks,T.J. and Cory,S. (1994) DNA Damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by bcl-2. *Cell*, **79**, 329–339.

Symonds,H., Krall,L., Remington,L., Saenz-Robles,M., Lowe,S., Jacks,T. and Van Dyke,T. (1994) p53-dependent apoptosis suppresses tumor growth and progression *in vivo*. *Cell*, **78**, 703–711.

Vousden,K. (1993) Interactions of human papillomavirus transforming proteins with the products of tumor suppressor genes. *FASEB J.*, **7**, 872–879.

Wang,Y. and Prives,C. (1995) Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. *Nature*, **376**, 88–91.

Weinberg,R.A. (1992) The retinoblastoma gene and gene product. In Levine,A.J. (ed.), *Tumor Suppressor Genes, the Cell Cycle, and Cancer*. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 43–57.

Weintraub,S.J., Chow,K.N.B., Luo,R.X., Zhang,S.H., He,S. and Dean,D.C. (1995) Mechanisms of active transcriptional repression by Rb. *Nature*, **375**, 812–815.

Wilkinson,D.G. (1993) *In situ* hybridisation. In Stern,C.D. and Holland,P.W.H. (eds), *Essential Developmental Biology*. IRL Press, Oxford, pp. 257–274.

Williams,B.O., Remington,L., Bronson,R.T., Mukai,S., Albert,D.M., Dryja,T. and Jacks,T. (1994a) Cooperative tumorigenic effects of germline mutations in Rb and p53. *Nature Genet.*, **7**, 480–484.

Williams,B.O., Schmitt,E.M., Remington,L., Bronson,R.T., Albert,D.M., Weinberg,R.A. and Jacks,T. (1994b) Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. *EMBO J.*, **13**, 4251–4259.

Wu,X. and Levine,A.J. (1994) p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl Acad. Sci. USA*, **91**, 3802–3806.

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. USA*, **92**, 1357–1361.

Zwicker,J., Liu,N., Engeland,K., Lucibello,F.C. and Muller,R. (1996) Cell cycle regulation of E2F site occupation *in vivo*. *Science*, **271**, 1595–1597.

Received on April 16, 1996; revised on July 30, 1996