Cell, Vol. 85, 537–548, May 17, 1996, Copyright ©1996 by Cell Press

Tumor Induction and Tissue Atrophy in Mice Lacking E2F-1

Lili Yamasaki,* Tyler Jacks,† Roderick Bronson,‡ Evelyne Goillot,* Ed Harlow,* and Nicholas J. Dyson*
*Massachusetts General Hospital Cancer Center
Building 149
13th Street
Charlestown, Massachusetts 02129
†Howard Hughes Medical Institute
Department of Biology
Massachusetts Institute of Technology
Center for Cancer Research
Cambridge, Massachusetts 02139
‡USDA Human Nutrition Research Center on Aging
Department of Pathology
School of Veterinary Medicine
Tufts University
Boston, Massachusetts 02111

Summary

The retinoblastoma tumor suppressor protein (pRB) is a transcriptional repressor that regulates gene expression by physically associating with transcription factors such as E2F family members. Although pRB and its upstream regulators are commonly mutated in human cancer, the physiological role of the pRB–E2F pathway is unknown. To address the function of E2F-1 and pRB/E2F-1 complexes in vivo, we have produced mice homozygous for a nonfunctional E2F-1 allele. Mice lacking E2F-1 are viable and fertile, yet experience testicular atrophy and exocrine gland dysplasia. Surprisingly, mice lacking E2F-1 develop a broad and unusual spectrum of tumors. Although overexpression of E2F-1 in tissue culture cells can stimulate cell proliferation and be oncogenic, loss of E2F-1 in mice results in tumorigenesis, demonstrating that E2F-1 also functions as a tumor suppressor.

Introduction

E2F proteins and DP proteins comprise two families of transcription factors that form heterodimeric complexes and confer cell cycle–specific expression to promoters containing E2F-binding sites. The timing of E2F-mediated transcription is regulated through several mechanisms. E2F-mediated transcription is repressed through a physical association of E2F/DP heterodimers with proteins of the retinoblastoma family (pRB, p107, or p130 collectively referred to as the "pocket" proteins). Cyclin-dependent kinase-mediated phosphorylation (Sherr, 1994) or DNA tumor virus antigen binding (Nevins, 1992) inactivates the pocket proteins, leading to dissociation of the pocket protein/E2F/DP complexes, and subsequent activation of E2F-dependent transcription. Additionally, E2F-mediated transcription is stimulated by an E2F-regulatory loop that amplifies the expression of the E2F genes themselves (Hsiao et al., 1994; Johnson et al., 1994b; Neuman et al., 1994). Finally, E2F-mediated transcription is attenuated late in S phase by cyclin-dependent kinase phosphorylation of the E2F/DP heterodimer, which results in the loss of DNA-binding activity (Dynlacht et al., 1994b; Krek et al., 1994; Xu et al., 1994).

Five highly related E2F genes (E2F-1, E2F-2, E2F-3, E2F-4, and E2F-5) and three DP genes have been identified in vertebrates (La Thangue, 1994; Slansky and Farnham, 1995; Weinberg, 1995). Three E2F members (E2F-1, E2F-2, and E2F-3) associate with a DP member and in turn bind pRB through a small 18 amino acid interaction domain located in the carboxyl termini of the E2F polypeptides (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992; Ivey-Hoyle et al., 1993; Lees et al., 1993). E2F-4 and E2F-5 associate with a DP member and then bind the pRB-related proteins, p107 and p130 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995; Vairo et al., 1995).

E2F sites are found in the promoters of many genes intimately involved in cell cycle progression. These include genes required for DNA replication (e.g. *DHFR*, *POL*α, *TK*, *TS* and *RNR*), for cyclin-dependent kinase activity (e.g. cyclins D, E, and A and cdc2), and also the nuclear oncogenes (c-*myc*, N-*myc* and b-*myb*), the pocket protein genes and the E2F-1 gene (Slansky and Farnham, 1995). By deletion or mutation of E2F sites in many of these promoters, it has been demonstrated that E2F sites are important for the correct temporal expression pattern of the target gene. However, given the different times at which these target genes are expressed, it is clear that distinct mechanisms must be responsible for the timing of their activation. For none of these E2F-regulated promoters is it known which E2F/DP complex regulates expression. Also, the pocket protein responsible for repression of specific E2F-target genes is unclear. Consequently, the complexity of the E2F gene family, in conjunction with the breadth of target genes identified, highlights the difficulty in determining the actual physiological role of any individual E2F member.

Clues to the function of E2Fs arise from overexpression or deregulated-expression studies. Microinjection, transient transfection, and viral infection experiments have shown that E2F-1 overexpression is sufficient to induce DNA replication (Johnson et al., 1993; Qin et al., 1994; Shan and Lee, 1994; Kowalik et al., 1995) and that overexpression of dominant negative E2F mutants inhibits cell cycle progression from G1 to S phase (Dobrowolski et al., 1994). Furthermore, studies in Drosophila where only a single E2F has been identified (Dynlacht et al., 1994a; Ohtani and Nevins, 1994) have demonstrated that dE2F is required for entry into S phase (Duronio et al., 1995). These studies suggest that E2F/DP transcriptional activity is rate-limiting for transition from G1 into S phase. Overexpression of E2F-1 or E2F-1/DP-1 cooperates with activated ras in fibroblast transformation assays, and these transformed cells form tumors in nude mice (Johnson et al., 1994a; Singh et al., 1994; Jooss et al., 1995; Xu et al., 1995), demonstrating that E2F-1 can be oncogenic. Apart from the role of E2F-1 in stimulating proliferation, overexpression of E2F-1 in some tissue culture cells can induce apoptosis that is p53-dependent (Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994; Kowalik et al., 1995). Overexpression of E2F-1 may induce apoptosis by regulating genes important for cell survival or perhaps by driving

cells into S phase at an inappropriate time, which may induce apoptosis.

Similar suggestions for the function of E2Fs in regulating proliferation can be inferred from mutations in regulatory proteins upstream of E2F/DP function. $Rb^{+/-}$ mice develop pituitary and thyroid tumors (Jacks et al., 1992; Hu et al., 1994; Williams et al., 1994). Cyclin D overexpression in the mammary epithelium of transgenic mice produces mammary adenocarcinomas (Wang et al., 1994). The frequent isolation of mutations in the genes encoding pRB, cyclin D, cdk4, or the cyclin-dependent kinase inhibitor p16 from human tumors reinforces the importance of the pRB pathway in negative growth control (Hunter, 1994; Kamb, 1995; Weinberg, 1995; Wolfel et al., 1995). Taken together, these data strongly suggest that E2F/DP function may be a major target for pRB-mediated growth suppression. These data have led to a widely accepted model that E2F/DP activity promotes cell-cycle progression, and that pocket proteins suppress growth by repressing E2F/DP-mediated transactivation (Nevins, 1992; La Thangue, 1994; Muller, 1995; Weinberg, 1995).

In addition to suppressing growth, pRB also appears to inhibit apoptosis and facilitate differentiation, suggesting that E2F/DP complexes may participate in these processes as well. $Rb^{-/-}$ mouse embryos die between E14 and E16 of gestation, with increased levels of apoptosis in the central nervous system and fetal liver, and display incomplete erythropoiesis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994). Neuronal differentiation correlates with elevated levels of pRB in embryonic carcinoma cells (Slack et al., 1993) and is impaired in $Rb^{-/-}$ embryos (Lee et al., 1994), and myotubes from pRB-negative cells are able to reenter the cell cycle (Gu et al., 1993; Schneider et al., 1994). These data underscore the delicate balance that pRB may help maintain between proliferation, apoptosis, and differentiation.

To assess the role of a single E2F in normal growth and development and to test whether E2F-1 has a critical function in the regulation of proliferation, apoptosis, and/or differentiation in vivo, we have inactivated the E2F-1 locus in mice by homologous recombination. Mice lacking E2F-1 are viable, yet several tissues are sensitive to the absence of E2F-1. Surprisingly however, the loss of E2F-1 also predisposes many of the homozygous E2F-$1^{-/-}$ mutants to develop tumors. These data strongly suggest that the working model in which E2F-1 functions only to drive cell proliferation is incomplete. In addition to promoting proliferation in some tissues, E2F-1 must act in some manner to suppress proliferation in other tissues. Thus E2F-1 is a previously unrecognized tumor-suppressor protein. This illustrates a novel situation in which E2F-1 can act as an oncogene in some settings and as a tumor suppressor in others.

Results

Inactivation of the E2F-1 Locus

To inactivate the E2F-1 locus by homologous recombination (Mansour et al., 1988; Capecchi, 1989), we constructed a targeting vector in which most of exon 2 and all of exon 3 in the E2F-1 locus were replaced by the neo^R gene in an antisense orientation (Figure 1A). This deletion removes amino acid residues 89–186 of mouse E2F-1 (Li et al., 1994; A. Talis and K. Helin, unpublished data), which correspond to amino acid residues 95–191 of human E2F-1. Exons 2 and 3 encode residues that are known to be critical for cyclin A/cdk2 association and DNA binding, respectively (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992; Dynlacht et al., 1994b; Krek et al., 1994; Xu et al., 1994). Following electroporation and double drug selection of embryonic stem cell clones, Southern analysis revealed that homologous recombination had occurred at the E2F-1 locus at high frequency (1/3 doubly resistant $G418^R$/$gancyclovir^R$ clones or 1/17 singly resistant $G418^R$ clones). Two E2F-1 heterozygous (+/−) clones were used to produce male chimaeras that transmitted the mutated E2F-1 allele through the germline to 50% of their agouti 129 × C57BL/6 offspring. F1 E2F-$1^{+/-}$ males and females were then interbred to produce F2 litters. Genotyping by Southern analysis (Figure 1B) or by genomic polymerase chain reaction (PCR; Figure 1C) demonstrated that E2F-1 homozygous (−/−) mutants, heterozygotes (+/−), and wild-type animals were produced at the expected ratio of 1:2:1.

Since the E2F-$1^{-/-}$ animals were viable, we next confirmed that the E2F-1 protein was indeed absent. Owing to the extremely low levels of endogenous E2F-1 expression in wild type and E2F-$1^{+/-}$ asynchronously growing embryonic fibroblasts, the induction of higher levels of E2F-1 upon serum restimulation was compared between E2F-$1^{-/-}$ and E2F-$1^{+/-}$ fibroblasts. Fibroblasts from E2F-$1^{-/-}$ and E2F-$1^{+/-}$ embryos (E12.5) were synchronized by serum starvation and restimulated with serum for 18–20 hr to induce E2F-1 expression. E2F-1 levels in the resultant lysates were monitored by immunoprecipitation, then Western analysis with anti-E2F-1 antibodies (Figure 1D; all E2F-1 antibody reagents were kindly provided by K. Helin). Either a monoclonal anti-E2F-1 antibody (KH129) or a polyclonal anti-E2F peptide serum (anti-RBP3; Helin et al., 1992) was used for immunoprecipitation, followed by Western blotting with a pool of two anti-E2F-1 specific monoclonal antibodies (KH22 and KH102). E2F-1 was detected with both antibodies in lysates from E2F-$1^{+/-}$ cells, but not in E2F-$1^{-/-}$ cell lysates. Both the polyclonal and monoclonal anti-E2F-1 antibodies recognize C-terminal epitopes of E2F-1 (Helin et al., 1992; K. Helin, unpublished data). Thus, the absence of a signal in E2F-$1^{-/-}$ lysates strongly suggests that a C-terminal fragment of E2F-1 is not expressed. Conceivably, an N-terminal fragment of E2F-1 (amino acid residues 1–88) could be expressed; however, since stop codons were introduced into the altered exon 2, this fragment would lack the DNA-binding, heterodimerization, and pRB-binding domains of E2F-1. Thus, the viability of E2F-$1^{-/-}$ animals occurred in the absence of a functional E2F-1 protein.

E2F-$1^{-/-}$ females and males examined at 2.5 months of age showed no gross anatomical or overt histological abnormalities. However, three abnormalities became obvious with increasing age in the E2F-$1^{-/-}$ animals. These include testicular atrophy, exocrine gland dysplasia, and the development of tumors and are discussed

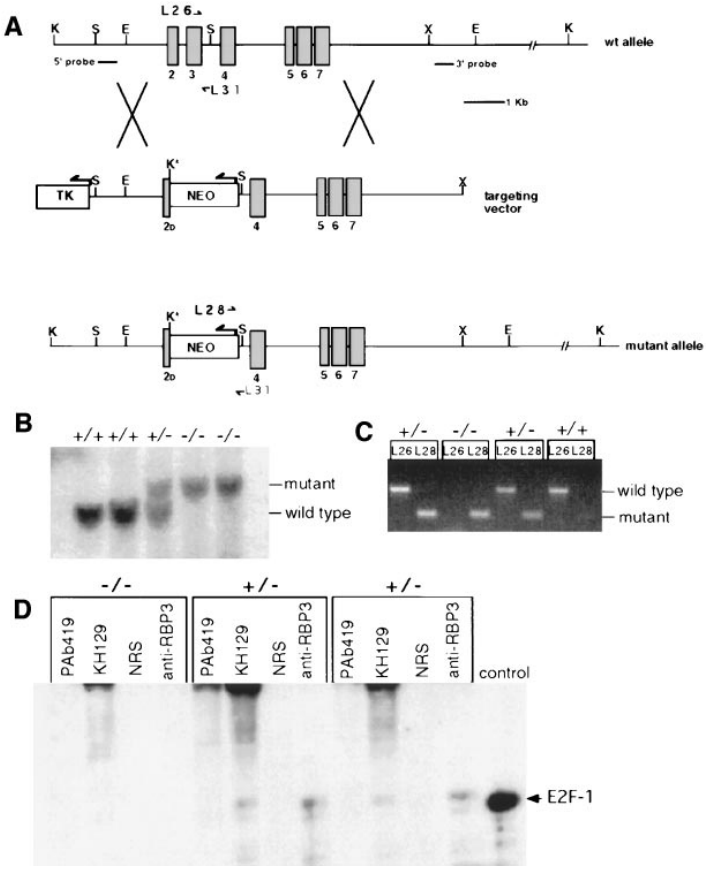

Figure 1. Targeted Disruption of the *E2F-1* Locus

(A) Genomic organization of the wild-type *E2F-1* allele is shown (top) with the positions of coding exons 2–7 indicated. The targeting vector (middle) was constructed to replace a portion of exon 2 and all of exon 3 with the neo^R gene in an antisense orientation. The deleted codons correspond to amino acids 89–186 in mouse E2F-1. These amino acids are equivalent to 94–191 in human E2F-1, which are required for cyclin A binding and DNA binding. Homologous recombination between the wild-type allele and the targeting vector produced the mutant allele (bottom) in which the thymidine kinase gene was excised.

(B) Southern analysis revealed that E2F-1$^{-/-}$ animals were viable and appeared at the expected frequency of 25%. After EcoRI digestion, hybridization with a 3′probe detects a 9.0 Kbp wild-type allele and a 9.8 Kbp mutant allele.

(C) A genomic PCR assay was performed to genotype all progeny. Separate PCR reactions with either the E2F-1 exon 3 primer (L26) or the neo^R gene primer (L28) and an intron primer (L31) were used to detect the wild-type allele (392 bp) or the mutant allele (167 bp), respectively. PCR primer positions are indicated in (A).

(D) Immunoprecipitation–Western analysis for E2F-1. Lysates from serum-starved and restimulated 12.5-day-old mouse embryo fibroblasts were immunoprecipitated with either monoclonal (KH129) or polyclonal (anti-RBP3 peptide) antibody reagents for E2F-1 or control reagents (PAb419 or normal rabbit serum). Western blotting with two anti-E2F-1 monoclonals that recognize unique C-terminal epitopes of E2F-1 (KH22 and KH102) detected E2F-1 only in (+/−) lysates. Recombinant mouse E2F-1 was used as a control for Western blotting (last lane).

in detail below. Other E2F-1$^{-/-}$ tissues were examined and showed no change or only small sporadic differences.

Testicular Atrophy

Both E2F-1$^{-/-}$ males and females were fertile. However, all males autopsied between 9 and 15 months of age displayed testicular atrophy (Figure 2A). The average testis weight for E2F-1$^{-/-}$ males (52 ± 20 mg) was 44% of the average testis weight for wild-type males (118 ± 28 mg). In the most extreme cases, testis weight for E2F-1$^{-/-}$ males was 10% of testis weight for wild-type males. Testicular atrophy had occurred as assessed by bilateral testicular weight reduction (Figure 2B) and by histological appearance (Figure 2C). Histologically, the testes in 2.5-month-old E2F-1$^{-/-}$ males appeared healthy with numerous seminiferous tubules producing abundant sperm (data not shown), which is consistent with the fertility of young E2F-1$^{-/-}$ males. The only apparent histological difference in the testes of young E2F-1$^{-/-}$ males relative to that of young wild-type males was the presence of more interstitial cells (Leydig cells) between the seminiferous tubules. By 9 months and becoming more evident in 12-month-old E2F-1$^{-/-}$ males, the testes contained empty and degenerated seminiferous tubules. The number of empty tubules ranged from several (Figure 2C, middle image) to almost all tubules per cross-section (Figure 2C, right image). Interestingly, the number of Leydig cells did not appear to decrease with age and in some animals appeared to increase in number. Although the testes from E2F-1$^{-/-}$ animals experiencing the most extreme atrophy appeared to contain many more Leydig cells, the apparent increase may have been due to the overall reduction in testicular size.

Testicular atrophy as judged by average testicular weight reduction occurred in 27/27 autopsied E2F-1$^{-/-}$ males and occurred in 1/11 of autopsied wild-type males by 15 months of age. Testicular atrophy has been observed in 11% of C57BL/6 mice (Bronson, 1990), while testicular teratocarcinomas, rather than atrophy, occur frequently in 129 mice (Stevens, 1973). The degeneration of the seminiferous tubules in the presence of Leydig cells was unexpected, since the testosterone production from Leydig cells is considered to be a critical stromal signal necessary for productive spermatogenesis (Russell, 1990). Testicular atrophy in the E2F-1$^{-/-}$ animals may have occurred because of a pituitary or testicular hormonal defect or perhaps a refractory state of the seminiferous tubules to testosterone. Since spermatogenesis in rodents must decrease by 90% to affect the number of progeny produced per litter (Russell, 1990), many of the E2F-1$^{-/-}$ males by 9–12 months of age were most likely still fertile.

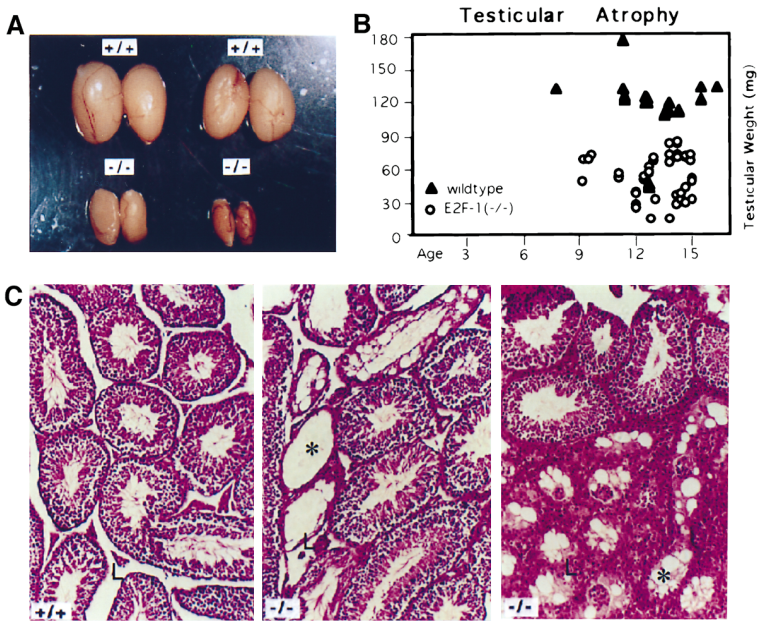

Figure 2. Testicular Atrophy in E2F-1$^{-/-}$ Males

(A) Comparison of wild-type and E2F-1$^{-/-}$ testes. E2F-1$^{-/-}$ testes showed average (bottom left) or severe (bottom right) reduction in size. (B) Testicular atrophy in the E2F-1$^{-/-}$ males. Average testicular weight of E2F-1$^{-/-}$ males (open circles) was reduced bilaterally to 44% the average testicular weight of wild-type animals (closed triangles) by 15 months of age. (C) Histological sectioning (hematoxylin and eosin [H and E]) of the testes shown in (A) revealed degeneration of seminiferous tubules (asterisks) and an increase in Leydig cells (L) between the tubules of E2F-1$^{-/-}$ testes. Wild-type testes (left) display prominent levels of spermatogenesis in all seminiferous tubules. Moderately atrophied E2F-1$^{-/-}$ testes (bottom left in [A]) contained several empty tubules per cross-section (middle). Severely atrophied E2F-1$^{-/-}$ testes (bottom right in [A]) contained mostly collapsed and empty seminiferous tubules (right), surrounded by numerous Leydig cells. This small cluster of active tubules was the only normal region within this entire testis.

Exocrine Tissue Abnormalities

All of the E2F-1$^{-/-}$ mice had submaxillary and parotid salivary glands that were histologically abnormal. In both of these tissues, cell nuclei were abnormally large and swollen, and many cells were binucleate. The submaxillary salivary gland is a mixed seromucous gland, which concentrates salts and produces lubricating glycoproteins and lysozyme. In the submaxillary glands of E2F-1$^{-/-}$ animals, the mucous cells contained irregular, large nuclei (Figure 3A), while the serous cells were normal. The abundance of mucous acini compared to serous acini within the submaxillary glands of E2F-1$^{-/-}$ animals was low relative to that of wild-type animals. This decrease occurred despite an increase in the size of individual mucous cells (Figure 3A). Interestingly, although the mucous cells of the submaxillary gland were abnormal in E2F-1$^{-/-}$ animals, mucous cells of the sublingual salivary gland were normal. In the parotid salivary gland, which produces amylase for the digestion of starch, cells also contained abnormally large nuclei and often were binucleate (Figure 3B). Likewise, the exorbital lacrimal gland of E2F-1$^{-/-}$ animals contained abnormally large nuclei (data not shown). Histological abnormalities in the salivary glands became even more pronounced in older E2F-1$^{-/-}$ animals, and the overall size of the salivary gland complex decreased with age (data not shown).

Other exocrine tissues also appeared sensitive to the loss of E2F-1. Abnormally large nuclei or nuclei doubled in number were observed in the exocrine portion of the E2F-1$^{-/-}$ pancreas (Figure 3C). The zymogen granule content in the pancreas was low and the gross anatomical appearance of the pancreas deteriorated in older E2F-1$^{-/-}$ animals relative to that observed in wild-type animals. The presence of greatly enlarged nuclei in the exocrine tissues of E2F-1$^{-/-}$ animals suggests that E2F-1 helps maintain the normal nuclear structure and possibly the correct DNA content within these tissues. Since nuclear size variation to a lesser extent also occurred in the livers of E2F-1$^{-/-}$ animals, E2F-1 may have a role in maintaining normal nuclear structure in this tissue as well.

Despite the changes in the salivary glands and pancreas of E2F-1$^{-/-}$ animals, body weights measured at 6.7 and 8.7 months of age were not perceptibly different from those of wild-type littermates (data not shown). However, between 12 and 15 months of age, a number of autopsied E2F-1$^{-/-}$ animals lost large amounts of weight, corresponding in some males to as much as 30%–40% of their weight at 8.7 months. Many of these autopsied animals had no obvious abnormalities other than exocrine gland deterioration and fatty changes in the liver that often accompany drastic weight loss.

Tumor Formation in E2F-1$^{-/-}$ Animals

Between 8 and 18 months of age, many E2F-1$^{-/-}$ animals developed tumors (Table 1). Tumors observed include reproductive tract sarcomas, lung tumors, lymphomas, and numerous other tumor types that were found at a lower frequency. E2F-1$^{-/-}$ females and males appeared to be comparably susceptible to tumor formation. A total of 35 tumors were observed out of 102 autopsied E2F-1$^{-/-}$ animals. There are an additional 80 surviving E2F-1$^{-/-}$ animals of this age cohort (originally 182 animals). The expected total tumor incidence for wild-type animals of various genetic backgrounds is only 0%–2% by 12 months and rises to 10% by 18 months (Bronson and Lipman, 1991). Since 34% (35/102) of autopsied E2F-1$^{-/-}$ animals developed tumors mostly within 15 months (Table 1), tumor development was accelerated in the absence of E2F-1. This estimated tumor incidence of 34% is higher than the true tumor incidence, because in most cases (75%) sick E2F-1$^{-/-}$ animals were chosen for autopsy. If no occult tumors are found in the remaining 80 E2F-1$^{-/-}$ animals, then the tumor incidence would be 19% (35/182). The actual tumor incidence lies between these two values (19% and 34%) for animals at 18 months of age.

Since one-third of the tumors isolated from E2F-1$^{-/-}$

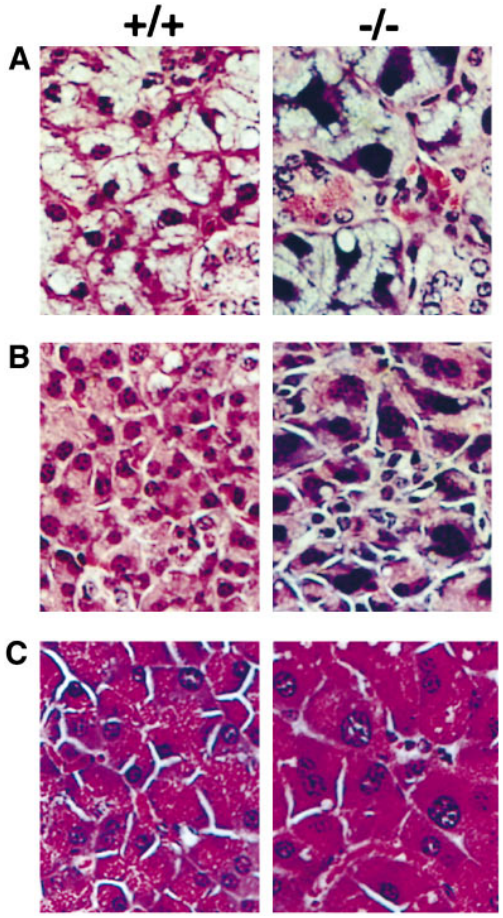

Figure 3. Exocrine Gland Dysplasia in E2F-1$^{-/-}$ Animals

(A) Submaxillary salivary glands of E2F-1$^{-/-}$ mice are abnormal. Histological staining (H and E) shows that the mucous acini of this tissue (blue cytoplasm) contain greatly enlarged nuclei of irregular shape. The serous acini (pink cytoplasm, shown in the bottom right corner) contain normal nuclei of uniform size. (Same magnification.)

(B) Parotid salivary glands of E2F-1$^{-/-}$ mice also are abnormal. Histological staining (H and E) reveals the irregularly enlarged and doubled nuclei. (Same magnification.)

(C) The exocrine portion of the E2F-1$^{-/-}$ pancreas also contains irregularly large nuclei. Histological staining (H and E) shows that nuclei are enlarged or doubled in number per cell. (Same magnification.)

animals were reproductive tract sarcomas (see below)—an extremely rare tumor in the 129 × C57BL/6 hybrid genetic background used in this study—the elevation of the incidence of this tumor is striking. The tumor profile of E2F-1$^{-/-}$ mutants is unique, since the most common tumor is lymphoma for the C57BL/6 mouse strain (Bronson, 1990) and testicular teratocarcinoma for the 129 mouse strain (Stevens, 1973).

Several E2F-1$^{+/-}$ animals also developed tumors (Table 1). Four E2F-1$^{+/-}$ females developed reproductive tract sarcomas and 3 E2F-1$^{+/-}$ animals developed lung adenocarcinomas. The development of rare tumors already observed in the E2F-1$^{-/-}$ animals and subsequently in 7 E2F-1$^{+/-}$ animals suggested that loss of heterozygosity at the E2F-1 locus may have occurred in the tumors of the E2F-1$^{+/-}$ animals. However, loss of heterozygosity at the E2F-1 locus in one of the lung tumors was not demonstrable by either Southern analysis or scrape PCR from histological sections. This may be due to the presence of subtle mutations at the E2F-1 locus that escaped detection or the presence of normal tissue within the examined tumor specimen.

Reproductive Tract Sarcoma

Eleven E2F-1$^{-/-}$ virgin animals (8 females and 3 males) between 12.7 and 16.5 (mean of 13.6; Table 1) months of age developed aggressive sarcomas that appeared grossly as large yellow/white masses in the reproductive tracts. These tumors often attained a remarkable size in the uterine horns (from 0.25 g to 5 g; Figure 4A, right image) and sometimes in the ovaries of females, as well as in the epididymis of males (Figure 4C, right image). Reproductive tract sarcomas represent one-third of the tumors found in the E2F-1$^{-/-}$ animals. In over 1000 mice (*Rb*$^{+/-}$ or *P53*$^{-/-}$ or *Nf1*$^{+/-}$ or control mice) produced with the same hybrid 129 × C57BL/6 genetic background used in this study, no reproductive tract sarcoma has been observed (Jacks et al., 1992, 1994; Williams et al., 1994). In addition, this sarcoma was not seen in studies following 1134 or 1577 mice of various genetic background for 24–30 months (Bronson and Lipman, 1991).

Histologically, the cellular composition of the sarcoma appeared quite uniform and nonglandular in the uterine horns of females (Figure 4C) and epididymis of males (Figure 4D). Tumor cells were densely packed at these sites and occasionally small capillaries could be seen. The most likely histological classification of the reproductive tract sarcoma in females is either endometrial stromal sarcoma (Dr. C. Fletcher, personal communication) or histiocytic sarcoma, between which a histological identification in mice is difficult (Turusov and Mohr, 1994). Both endometrial stromal sarcomas and histiocytic sarcomas in the mouse female reproductive tract can be induced chemically, but spontaneous development is rare (Turusov and Mohr, 1994). Endometrial stromal sarcomas in mice and humans arise from the nonglandular mesenchymal component of the uterine endometrium, which normally proliferates then sloughs in a cyclic manner (every 4–5 days in mice) in response to hormones if embryo implantation has not occurred. Histiocytic sarcomas in mice arise from a macrophage population and commonly present in the liver and spleen; however in humans, histiocytic sarcoma is extremely rare. Furthermore, epididymal tumors in mice and humans are very infrequent (Turusov and Mohr, 1994). Also, the epididymal tumor in E2F-1$^{-/-}$ mice grows between the ducts carrying maturing sperm, in a space normally occupied by smooth muscle and connective tissue without a stromal cell equivalent.

In both females and males, the metastatic potential of these tumors was quite high and could be followed easily by the tumor's canary yellow color (Figures 4E and 4G). Six of the 11 E2F-1$^{-/-}$ animals with this tumor showed numerous metastases in more than 4 abdominal organs, and all of these animals had metastases on the diaphragm and omentum (a membrane to which the liver, spleen, and stomach are attached; Figure 4E). Histologically, the tumor cells were highly invasive, penetrating deep into the pancreas (Figure 4F), kidney, liver,

Table 1. Tumors Arising in E2F-1 Mutant Mice

Tumor Type	No. in E2F-1$^{-/-}$	(F, M)	Age (Months)	No. in E2F-1$^{+/-}$	(F, M)	Age (Months)
Reproductive tract sarcoma	11		13.6[a]	4		17.1[a]
Uterine		(8, −)	12.7, 12.8, 12.9, 12.9, 12.9, 13.6, 15.4, 16.5		(4, −)	15.7, 16.7, 17.0, 19.0
Epididymal		(−, 3)	12.7, 12.9, 14.6			
Lung adenocarcinoma	7	(3, 4)	8.8, 12.0, 12.8, 13.6, 15.9, 17.2, 17.9	3	(1,2)	13.4, 16.7, 17.0
Lymphomas	8	(4, 4)	12, 13.4, 15, 15, 15.4, 15.9, 16.2, 16.5	2	(2, −)	18.8, 19.5
Hemangiosarcoma	2	(1, 1)	8.0, 14.0	1	(1, −)	16.0
Histiocytic sarcoma	2	(−, 2)	11.0, 13.5			
Basal cell tumor	1	(1, −)	11.3			
Leiomyosarcoma	1	(1, −)	12.8			
Teratoma	1	(1, −)	14.1			
Myxosarcoma	1	(1, −)	17.0			
Unidentified tumor	1	(−, 1)	14.1			
Pituitary adenoma	0			1	(1, −)	18.1
Hepatoma	0			1	(−, 1)	20.0
Harderian gland adenoma	0			1	(−, 1)	15.3
Total	35	(20, 15)		13	(9, 4)	

[a] Mean.

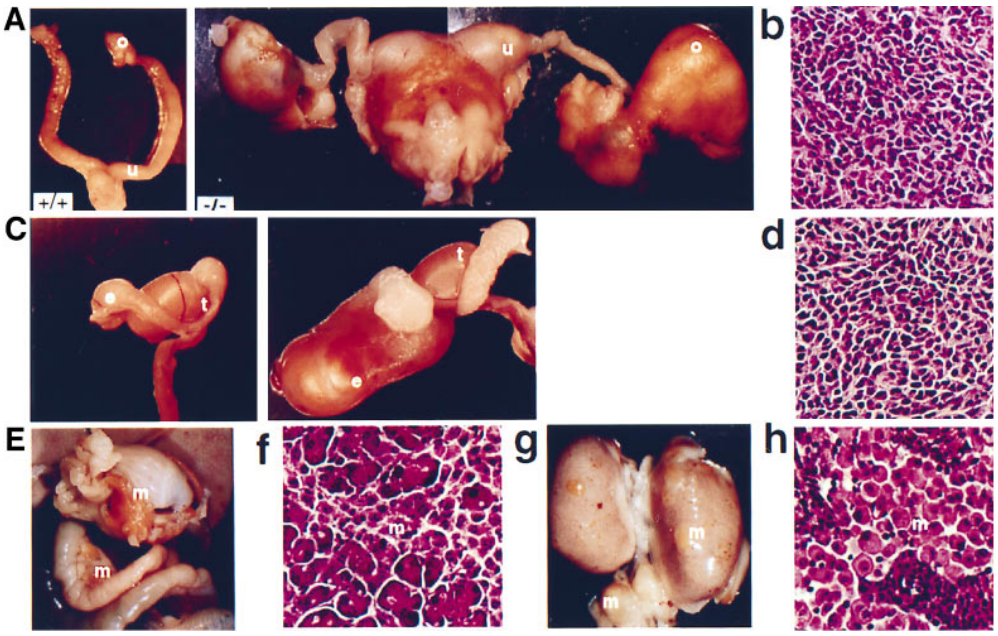

Figure 4. Reproductive Tract Sarcomas in E2F-1$^{-/-}$ Females and Males

(A) Highly aggressive sarcomas (right) developed in the uterine horns (u) and often ovaries (o) of E2F-1$^{-/-}$ females, which massively increased the size of the reproductive tract compared with that from a wild-type female (left). Photos were taken at the same scale. Canary yellow patches were evident on the surface and through this tumor.

(B) Histological sectioning (H and E) of the reproductive tract sarcoma in E2F-1$^{-/-}$ females.

(C) This reproductive tract sarcoma was also found in the lower epididymis of E2F-1$^{-/-}$ males. Left and right images show the epididymis (e) and atrophied testes (t) from the same E2F-1$^{-/-}$ male. The left image shows a normal epididymis, while the right shows the epididymal tumor, with the same canary yellow patches as in the uterine tumor.

(D) Histological sectioning (H and E) of the reproductive tract sarcoma in E2F-1$^{-/-}$ males. The same histological pattern is observed in both the female and male reproductive tract sarcomas.

(E) Numerous canary yellow metastases (m) were observed in the abdominal cavities of E2F-1$^{-/-}$ animals with the reproductive tract sarcoma. The mesenteric nodes near the small intestine and the pancreas and omentum were frequently overtaken by the growth of the sarcoma (shown). Liver and diaphragm also had yellow metastases (not shown).

(F) Histological sectioning (H and E) of metastases in the pancreas of the E2F-1$^{-/-}$ female shown in (A). The sarcoma (pink cells) has grown between two lobes of the exocrine portion of the pancreas (red cells).

(G) Many additional canary yellow metastases (m) can be seen on the surface of the kidneys and in the retroperitoneal nodes.

(H) Histological sectioning (H and E) of the retroperitoneal nodes from the E2F-1$^{-/-}$ female shown in (A) reveals the sarcoma invading the parenchyma of the node. Note the increased size and abundant cytoplasm of the tumor cells (pink).

fat, and regional lymph nodes (Figure 4H). In 2 cases metastases were found in the lungs. While the majority of the metastases appeared histologically similar to the primary tumors in the uterine horns or epididymis, the metastases in fat or in lymph nodes (Figure 4H) contained cells with abundant cytoplasm and distinct borders, which simply may reflect the packing density possible in these tissues. Spontaneous tumor metastases of solid tumors in mice are rare (R. Bronson, unpublished observation), and thus, the ability of the E2F-1$^{-/-}$ reproductive tract sarcomas to metastasize is noteworthy.

Lung Tumors

Seven of the autopsied E2F-1$^{-/-}$ animals between 8.8 and 18 months of age developed lung tumors (up to 1.5 cm in diameter) that often occupied half of the thoracic cavity (Figure 5A; Table 1). These tumors were classified as highly invasive lung adenocarcinomas or generally as non-small-cell lung carcinomas. In most cases, the gross anatomical architecture of the several major lung lobes as well as the architecture of the smaller mediastinal lung lobes was deformed by the tumor growth. In other studies, only 1 lung adenocarcinoma was seen in over 1000 mice (*Rb*$^{+/-}$, *p53*$^{-/-}$, *Nf1*$^{+/-}$, or control mice) with the hybrid 129 x C57BL/6 genetic backgound used in this study (Jacks et al., 1992, 1994; Williams et al., 1994). Similarly, lung adenocarcinomas were not observed in 1134 mice of various genetic backgrounds followed over a 24 month period (Bronson and Lipman, 1991).

Histologically, the normal lace-like alveolar space (Figure 5B, upper image) was overtaken by the expanding tumor (Figure 5B, lower image). From the same mutant animals, lung lobes which at first appeared grossly normal were later identified as containing smaller islands of tumor cells (Figure 5B, middle image) that had most likely metastasized from the neighboring affected lung lobes. Smaller islands of tumor often were surrounded by numerous alveolar histiocytes. Less severe lung adenomas (1–2 mm) were commonly seen in the E2F-1$^{-/-}$ autopsied animals (data not included in Table 1) and also in wild type animals.

Lymphoproliferation and Lymphoma

Between 8 and 15 months, a small fraction of the E2F-1 homozygous (5/32 = 15.6%) and heterozygous (3/23 = 13%) autopsied females developed a lymphoproliferative syndrome that often resulted in death. Affected animals displayed enlarged lymph nodes (Figure 6A), splenomegaly (from 0.1 g up to 1.0–4.3 g; Figure 6B), anemia (hematocrits were reduced from 40%–45% to 8%–15%), and glomerulonephritis of the kidneys (data not shown). This occurred in one wild-type female as well. Although environmental factors may have influenced the development of lymphoproliferation, we examined lymphocyte populations from wild-type and E2F-1$^{-/-}$ animals for possible differences. Fluorescence-activated cell sorter analysis of lymphocytes from thymus, spleen, and lymph nodes of young and old animals demonstrated that most B and T lymphocyte surface markers were normal (data not shown). Moderate differences in the T-cell receptor–associated signaling molecule, CD3ε, which were apparent on thymocytes from

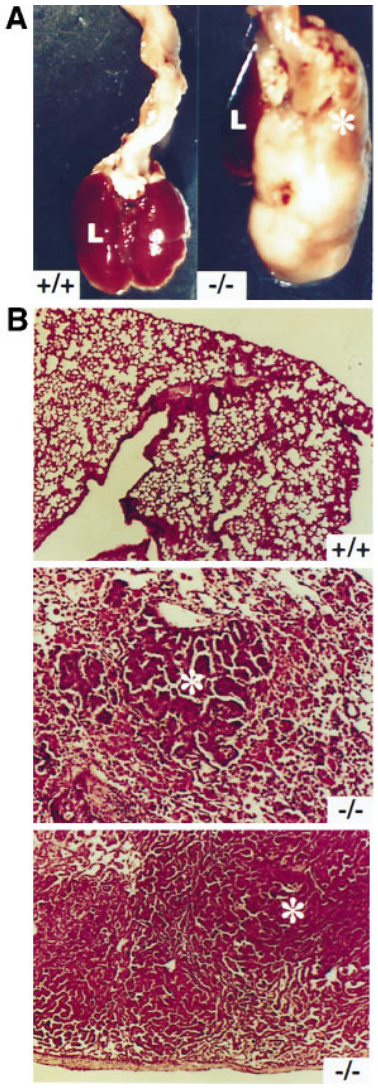

Figure 5. Lung Adenocarcinomas in E2F-1$^{-/-}$ Animals

(A) Massive lung tumors were often isolated from E2F-1$^{-/-}$ animals that occupied more than half the thoracic cavity (right). Lungs from a wild-type animal (left) are shown for comparison.

(B) Histological sectioning (H and E) detected a glandular architecture in these lung tumors consistent with that of a broncho-alveolar adenocarcinoma. The normal lace-like alveolar space in the lung (top) became infiltrated with many small islands of tumor (asterisks) with numerous histiocytes (middle) or became overtaken with tumor mass (lower).

young E2F-1$^{-/-}$ animals, were not detected on peripheral T cells from young animals or on thymocytes from older animals. Although *lpr* mice and *gld* mice, which have naturally occurring mutations in Fas and the Fas ligand respectively, develop a lymphoproliferative state with generalized lymph node enlargement, splenomegaly, and glomerular nephritis, Fas levels in E2F-1$^{-/-}$ animals were not consistently affected (data not shown).

Lymphoproliferation may have been a preneoplastic condition in the E2F-1$^{-/-}$ animals, since 8 E2F-1$^{-/-}$ animals between 12 and 16.5 months of age developed lymphomas (Table 1). Five of these E2F-1$^{-/-}$ animals developed follicular center cell lymphomas in enlarged

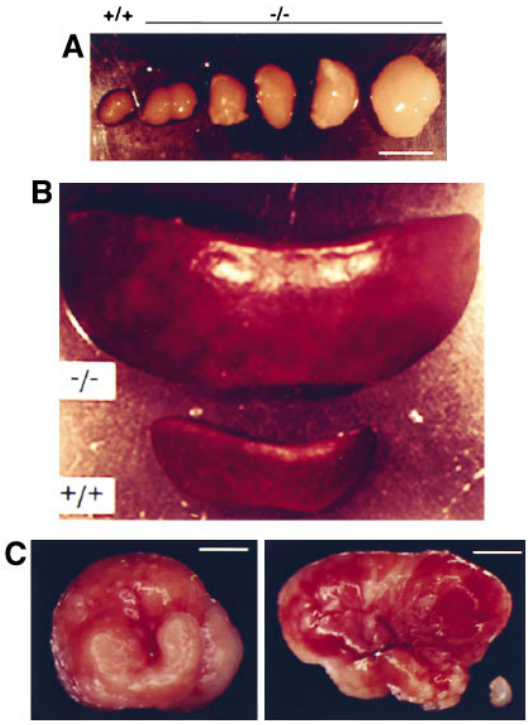

Figure 6. Lymphoproliferation and Lymphoma

(A) Comparison of a wild-type axial lymph node (leftmost) and numerous axial lymph nodes from an E2F-1$^{-/-}$ female. Scale bar is 5 mm. This E2F-1$^{-/-}$ female also had many more enlarged cervical and retroperitoneal lymph nodes.

(B) Splenomegaly (10-fold) in an E2F-1$^{-/-}$ female (top) relative to a spleen from a wild-type female (bottom) is shown. Splenomegaly ranged from 4- to 43-fold in E2F-1$^{-/-}$ and E2F-1$^{+/-}$ females.

(C) Two greatly enlarged mesenteric nodes from two E2F-1$^{-/-}$ males with follicular center cell lymphoma are shown with a normal mesenteric node (bottom right) for comparison.

Scale bars are 5 mm.

mesenteric nodes (up to 1.5 cm in diameter; Figure 6C). The incidence for lymphoma is 7.8% (8/102) of autopsied E2F-1$^{-/-}$ animals. The expected frequency is 1.5% by 18 months in mice of various genetic backgrounds (Bronson and Lipman, 1991).

Discussion

E2F-1 Is Not Required for Viability or Normal Development

The normal development and the fertility of the E2F-1$^{-/-}$ mice demonstrate that the function of E2F-1 is not required for either cell or animal survival. If E2F-1 is important for viability, then this E2F-1 function lost by inactivation of the E2F-1 locus must have been masked, perhaps by the continued action of other E2F family members. Eventually, the production of mice deficient for other E2F family members will allow us to test whether the E2F family is essential. Despite the viability of E2F-1$^{-/-}$ mice, specific tissues in all E2F-1$^{-/-}$ mice were sensitive to the loss of E2F-1. Loss of E2F-1 does not induce grossly abnormal development, but rather in a subset of tissues leads to subtle aberrations that become increasingly more obvious with time, as evident from the testicular atrophy and salivary gland dysplasia observed. Overt malformation is not observed even in normal E2F-1$^{-/-}$ tissues from which tumors and hyperplasia develop. At the simplest level this must indicate that tissues such as the testis and salivary gland rely heavily on the correct function of E2F-1 and that this E2F-1 function cannot be replaced in all tissues by other cellular activities.

Tissue and Temporal Specificity

Given the known capacity of E2F-1 to stimulate cellular proliferation and to act as an oncogene, we anticipated that one possible outcome from inactivating the E2F-1 locus might have been decreased proliferation. Another possible outcome might have been less apoptosis, in light of the capability of E2F-1 to induce apoptosis. Still another possibility is that inactivation of the E2F-1 locus might have compromised the ability of pRB to suppress proliferation, therefore leading to hyperproliferation. While examining the E2F-1$^{-/-}$ mice, we were struck by the radically different cellular and tissue responses to the loss of E2F-1. In exocrine cells, we have detected abnormally large and doubled nuclei, which may have undergone DNA replication without cell division. In the testes of E2F-1$^{-/-}$ mice, tissue atrophy correlates with loss of seminiferous tubules. In a subset of affected mice, dramatic lymphocyte proliferation is seen. Most surprisingly, several different tissues develop aggressive tumors. Discovering the molecular reasons for each of these pathologies will enable us to compare the causes for the different tissue responses. However, even at a phenotypic level it is clear that correct E2F-1 function is important in several types of cells and tissues. Field and coworkers (1996 [this issue of *Cell*]) also have inactivated E2F-1 mice and report a defect in thymocyte apoptosis that results in hyperproliferation. They also observed that their older E2F-1$^{-/-}$ mice undergo testicular atrophy. A single effector function of E2F-1 cannot explain the diverse outcomes seen in all of the affected tissues, and thus eventually we must consider the potential functions of E2F-1 in each of these separate tissues.

There are several possibilities as to why the defects observed in the E2F-1$^{-/-}$ mutants show tissue and temporal specificity. At a biochemical level, the different tissue specific responses to the loss of E2F-1 suggest three possible mechanisms: E2F-1 regulates different genes in different tissues; E2F-1 controls expression of the same genes differently in the various tissues, perhaps in combination with tissue specific factors; or tissues respond differently to the same target genes regulated by E2F-1. Temporal changes in E2F-1-dependent gene regulation add another variable to these mechanisms. To begin to analyze why the affected tissues are sensitive to the loss of E2F-1, it will be important to delineate the expression pattern for all E2F family members relative to one another and to identify the actual E2F-1 target genes in the affected tissues.

An additional level of complexity comes from the realization that a transcriptional response mediated through E2F sites may be either activation or repression, depending on the promoter contexts in which the E2F sites lie (Weintraub et al., 1992). Deletion or mutation of E2F

sites within promoters can lead to decreases (i.e., *DHFR* promoter) or increases (e.g. b-*myb* and E2F-1) in transcription (Blake and Azizkhan, 1989; Means et al., 1992; Lam and Watson, 1993; Hsiao et al., 1994; Johnson et al., 1994b; Neuman et al., 1994). Decreases in transcription following E2F site mutation correspond to the loss of E2F-dependent transactivation, while increases in transcription following E2F site mutation likely result from the loss of repression by pocket protein/E2F complexes.

The dual nature of E2F sites to act positively or negatively may allow E2F-1 to perform vastly different tasks in vivo depending on the function of the target gene. First, consider E2F-1 as an activator. If E2F-1 transactivates target genes that stimulate cell cycle progression (e.g. cyclin E, other E2Fs) or prolong cell survival, then loss of E2F-1 may decrease the cycling cell population. In contrast, if E2F-1 transactivates target genes that increase apoptosis (e.g. c-*myc*) or facilitate differentiation (possibly pRB itself), then loss of E2F-1 may increase the cycling cell population. Alternatively, if we consider pRB/E2F-1 as a repressor, then loss of E2F-1 and the subsequent loss of pRB/E2F-1-mediated repression of target genes may lead to outcomes, which are now opposite to those predicted for E2F-1 as an activator. In tumors, loss of E2F-1 may increase the cycling cell population, which in combination with the accumulation of additional mutations, results in malignancy. At this time however, there is no evidence for either a cell-autonomous or non-cell-autonomous pathway to atrophy or tumor development.

E2F-1 Is a Tumor Suppressor Protein

The development of tumors in E2F-1$^{-/-}$ animals strongly argues that E2F-1 is a previously unrecognized tumor suppressor protein. The increased incidence of tumors in E2F-1$^{-/-}$ animals is impressive, especially considering the age at which tumors were detected and the size, the invasiveness, and the high metastatic potential of the tumors. The frequency of reproductive tract sarcoma suggests that E2F-1 has a key growth suppressive role in the uterine horns of females and the epididymus of males. However, the incidence and long latency for the development of these tumors indicates that additional mutations are required. The extent of the metastasis from the reproductive tract to numerous abdominal organs suggests that E2F-1 normally may repress genes that facilitate, or activate genes that inhibit, metastasis. Alternatively, the reproductive tract sarcoma may be a highly metastatic tumor with or without the loss of E2F-1.

Although no human disease has been mapped to the chromosomal position of human E2F-1 at 20q11, E2F-1 gene amplification with overexpression in an erythroleukemic cell line and translocation of the E2F-1 locus with very low E2F-1 expression in monoblastic cell lines have been identified (Saito et al., 1995). These data suggest that abnormal changes in E2F-1 expression may be involved in human tumor development. Furthermore, the E2F-1 gene at 20q11 lies near the pRB-related p107 gene at 20q11.2 (Saito et al., 1995), so that chromosomal rearrangements involving this region potentially may inactivate two gene products with growth suppressive properties. Interestingly, the E2F-1 gene on mouse chromosome 2 maps near the agouti *A* locus that determines coat color (Li et al., 1994; K. Helin and N. Jenkins, unpublished data). Since the agouti lethal yellow allele, A^y, is associated with spontaneous tumors and has a 120–170 kb deletion (Duhl et al., 1994; Michaud et al., 1994), E2F-1 expression may be altered in mice carrying this rearranged A^y allele.

The tissues affected by tumorigenesis also argue for specific differences in the response to the loss of E2F-1. As observed for testicular atrophy and exocrine gland dysplasia, tumor development occurs only in specific sites. The tissues that are common sites of E2F-1-mediated tumorigenesis must rely more heavily on E2F-1 than other tissues. This same argument fits well with the observed tissue-specific development of tumors seen with inheritance of other tumor suppressor gene mutations.

A pRB/E2F-1 Tumor Suppressor Complex

Tumor development in the E2F-1$^{-/-}$ mice most likely results from the loss of pRB/E2F-1-mediated growth suppression, which is consistent with the biochemical evidence for pRB/E2F-1 complex formation in vitro and in vivo. Loss of growth suppression by the loss of a pRB/E2F-1 complex strongly suggests that pRB/E2F-1 actively represses key promoters by occupying E2F sites. Recent work suggests that repression by pRB/E2F DNA-bound complexes appears to block both E2F-mediated transactivation and also transactivation mediated by other transcription factors (Weintraub et al., 1992, 1995; Flemington et al., 1993; Helin et al., 1993; Adnane et al., 1995; Bremner et al., 1995; Qin et al., 1995; Sellers et al., 1995). We propose that active repression by a pRB/E2F-1/DP tumor suppressor complex bound to key promoters is lost in the E2F-1$^{-/-}$ animals and predisposes to tumor formation.

Clearly, the loss of E2F-1 is not equivalent to the loss of pRB, which in mice is lethal (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Even the tumors seen in the E2F-1$^{-/-}$ mice (Table 1) are distinct from the pituitary adenomas (Jacks et al., 1992) and thyroid tumors (Williams et al., 1994) arising in *Rb*$^{+/-}$ mice. Only one autopsied E2F-1$^{+/-}$ animal developed a pituitary adenoma. While many of the E2F-1$^{-/-}$ mice develop lung adenocarcinomas, *Rb*$^{+/-}$ mice develop only a low level of bronchial epithelium hyperplasia (Williams et al., 1994). The human tumors in which *RB1* mutations are found commonly, such as retinoblastomas, osteosarcomas, small cell lung carcinomas, and carcinomas of breast, bladder, and prostate (Riley et al., 1994; Weinberg, 1995), also were not observed in the E2F-1$^{-/-}$ mice.

We expected the E2F-1$^{-/-}$ phenotype to differ from the *Rb*$^{+/-}$ or *Rb*$^{-/-}$ phenotype in mice for several reasons. First, loss of pRB leads to the derepression of E2F-1, E2F-2, and E2F-3 simultaneously, while loss of E2F-1 may lead to derepression and loss of activation of E2F-1-specific target genes. Secondly, pRB binds many different transcription factors besides E2F (Riley et al., 1994; Horowitz and Udvadia, 1996) and dysregulation of these factors may result in the *Rb*$^{-/-}$ or *Rb*$^{+/-}$ phenotype. Thirdly, *Rb*$^{+/-}$ mice die from pituitary tumors at a mean

age of 11 months (Williams et al., 1994), which may preclude the later development of reproductive tract tumors and lung adenocarcinomas. Thus, a lack of concordance between pRB and E2F mutant phenotypes does not imply necessarily that E2F-1 is suppressing growth through a pRB-independent mechanism.

Malignancy associated with the loss of E2F-1 suggests that loss of the other pRB-regulated E2F proteins, E2F-2 and E2F-3, may predispose to tumor formation as well, perhaps with unique tissue specificities. Since neither p107 or p130 mutations are known to be associated with human tumors, the loss of E2F-4 or E2F-5 may or may not contribute to tumorigenesis. However, given the complex networks connecting the various E2Fs, cyclins, and pocket proteins, many perturbations in this E2F-dependent regulatory matrix may result in tumor formation.

E2F-1 Is an Oncogene and a Tumor Suppressor

While overexpression of E2F-1 was known to transform fibroblasts to be oncogenic, this work demonstrates that loss of E2F-1 also predisposes to tumorigenesis and strongly suggests that we reclassify E2F-1 as a tumor suppressor as well as an oncogene. To our knowledge, this is the first example of a gene product that is able to induce tumors either by overexpression of the wild-type protein or by a loss-of-function mutation.

How can E2F-1 be both an oncogene and a tumor suppressor? If the absolute level of E2F-1 is critical for its correct action, then overexpression of E2F-1 or loss of E2F-1 could be tumorigenic by leading to inappropriate overexpression of the same target gene. Alternatively, E2F-1 may regulate multiple growth-promoting genes, some by E2F-1 activation and some by E2F-1 repression. If either E2F-1-activated genes or E2F-1-repressed genes can drive proliferation, then gain or loss of E2F-1 function is tumorigenic. Formally however, E2F-1 has not been shown to be an oncogene and tumor suppressor gene in the same experimental setting, and tumor formation with transformed fibroblasts in nude mice is significantly different from spontaneous tumor development.

The capacity of E2F-1 to act as an oncogene and a tumor suppressor creates a novel situation that could occur any time a key regulator meets three criteria. It must have both a positive function and a negative function for a rate-limiting step(s) in tumorigenesis, and a null mutation must not block proliferation. E2F-1 fulfills these three criteria, because E2F-1 both transactivates and represses genes required for proliferation, and E2F-1 is not required for viability or proliferation. Therefore, gain-of-function and loss-of-function mutations promote proliferation and enhance tumorigenesis. Any gene whose product can serve this dual role will likely be both an oncogene and tumor suppressor.

Experimental Procedures

Construction of a Targeting Vector

A 129 mouse genomic phage clone containing the E2F-1 gene was mapped to identify exon positions using the mouse E2F-1 cDNA sequence (Li et al., 1994; A. Talis and K. Helin, unpublished data). A 5.9 Kbp SphI–XbaI genomic fragment (Figure 1A), containing 4 exons of the E2F-1 gene downstream of exon 3 (the DNA binding domain exon), was modified with SalI linkers and was subcloned into the XhoI site of the pPNT targeting vector (Tybulewicz et al., 1991). A 2.1 Kbp intron fragment extending from the SphI site upstream of exon 2 to amino acid 89 (equivalent to amino acid 94 in human E2F-1) within exon 2 was generated by PCR, modified with KpnI linkers and subcloned into the KpnI site of pPNT between the neo^R and thymidine kinase genes. The resultant targeting vector has deleted a portion of exon 2 and all of exon 3 that encode the basic domain and DNA binding domains, respectively. Deleted codons correspond to amino acids 89–186 for mouse E2F-1, that are equivalent to amino acids 95–191 for human E2F-1. Since the 3′ end of the neo^R gene contains translational stops in all reading frames, the disrupted exon 2 should cause the premature translational termination of E2F-1. If the disrupted exon 2 is removed by alternative splicing, then a mutant polypeptide may be translated that still lacks cyclin A binding and DNA binding activities. No such product was detected.

Homologous Recombination in ES Cells and Chimaera Production

The E2F-1 targeting vector was linearized and electroporated into D3 embryonic stem cells. Clones doubly resistant to G418 (300 μg/ml) and gancyclovir (2 μM) were tested for homologous recombination by Southern analysis. Two genomic probes were used to confirm that homologous recombination had occurred using KpnI or EcoRI digests. A neo^R gene probe was used to ensure that random integration of the targeting vector had not occurred elsewhere in the genome. Two E2F-$1^{+/-}$ ES cell clones were used to produce chimaeras with >90% agouti coats.

Matings for Mutant Progeny

Male chimaeras from both clones produced F1 agouti animals, 50% of which were F1 heterozygotes. Male and female F1 heterozygotes identified by Southern or genomic PCR analysis were interbred to produce F2 progeny. A genomic PCR assay (Figure 1C) to detect the wild-type allele (392 bp) or the mutant E2F-1 allele (167 bp) was designed using a common L31 intron primer (5′GCTGGAATGGTGT CAGCACAGCG 3′) and the E2F-1 wild-type exon L26 primer (5′TCCAAGAATCATATCCAGTGGCT3′) or the neo^R gene L28 primer (5′CTACCCGGTAGAATTGACCTGCA 3′). PCR reactions (25 μl) were amplified with pyrostase (Molecular Genetic Resources) for 39 cycles (94°C, 1 min; 58°C, 1 min; 72°C, 1 min) and 1 cycle (72°C, 7 min), then analyzed on a 1.6% agarose gel.

Immunoprecipitation–Western Analysis for E2F-1

Mouse embryo fibroblasts from 12.5-day-old embryos were starved with 1%–2% fetal bovine serum in Dulbecco's modified Eagle's medium for 45 hr, then released into 10% fetal bovine serum for 20 hr. Extracts were prepared and mixed with protein A–sepharose to which monoclonal antibodies (PAB419 [anti-SV40 T antigen] or KH129 [anti-E2F-1, K. Helin]) or polyclonal sera (normal rabbit serum or anti-RBP3 peptide anti-serum [Helin et al., 1992]) had been coupled covalently (Harlow and Lane, 1988). After immunoprecipitation for 4 hr at 4°C, beads were washed and bound proteins were analyzed by SDS–polyacrylamide gel electrophoresis and Western blotting with the monoclonal antibodies KH22 and KH102 (K. Helin), which recognize E2F-1. A recombinant mouse E2F-1 bacterial lysate was used as a positive control for Western blotting antibodies.

Autopsy and Histopathology

Between 8 and 18 months, 102 E2F-$1^{-/-}$ animals were autopsied. Animals were autopsied in most cases (75%) because they had lost weight and appeared in poor health or had a palpable abnormality. All tissues were examined from 83 E2F-$1^{-/-}$ animals regardless of their pathologic status. Tissues were formalin fixed, dehydrated, and embedded in paraffin according to standard protocols. Sections (4–5 μm) were stained with hematoxylin and eosin and examined microscopically.

Acknowledgments

We thank Kristian Helin and Andrea Talis for providing the mouse E2F-1 cDNA; Kristian Helin for anti-E2F-1 monoclonal antibodies;

John Mkwandawire for technical assistance in chimaera production; and Jim Koh, Marc Vidal, Nick Heintz, and Joshua La Baer for critical reading of this manuscript. We also thank Dr. Christopher Fletcher (Brigham and Women's Hospital) for his histopathological consultation. L. Y. further thanks Marie Yamasaki, Li-Huei Tsai, Andrea Talis, and Susanne Sternthal for support throughout this project. We are grateful to the past and present members of the Laboratory of Molecular Oncology (Massachusetts General Hospital Cancer Center) and members of the Jacks laboratory for helpful discussions and advice. T. J. is an Assistant Investigator of the Howard Hughes Medical Institute, and E. H. is an American Cancer Society Scholar. This work was supported by National Institutes of Health (NIH) grants CA60636 to E. H., CA64402 to N. D., CA42063 to T. J., and an NIH postdoctoral fellowship CA60451 to L. Y.

References

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

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

Blake, M.C., and Azizkhan, J.C. (1989). Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. *9*, 4994–5002.

Bremner, R., Cohen, B.L., Sopta, M., Hamel, P.A., Ingles, C.J., Gallie, B.L., and Phillips, R.A. (1995). Direct transcriptional repression by pRB and its reversal by specific cyclins. Mol. Cell. Biol. *15*, 3256–3265.

Bronson, R.T. (1990). Chapter 17: Rate of occurrence of lesions in 20 inbred and hybrid genotypes of rats and mice sacrificed at 6 month intervals during the first years of life. In Genetic Effects on Aging II, D.E. Harrison, ed. (Caldwell, New Jersey: Telford Press, Inc.), pp. 279–358.

Bronson, R.T., and Lipman, R.D. (1991). Reduction in rate of occurrence of age related lesions in dietary restricted laboratory mice. Growth Dev. Aging *55*, 169–184.

Capecchi, M.R. (1989). Altering the genome by homologous recombination. Science *244*, 1288–1293.

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

Dobrowolski, S.F., Stacey, D.W., Harter, M.L., Stine, J.T., and Hiebert, S.W. (1994). An E2F dominant negative mutant blocks E1A induced cell cycle progression. Oncogene *9*, 2605–2612.

Duhl, D.M., Stevens, M.E., Vrieling, H., Saxon, P.J., Miller, M.W., Epstein, C.J., and Barsh, G.S. (1994). Pleiotropic effects of the mouse lethal yellow (Ay) mutation explained by deletion of a maternally expressed gene and the simultaneous production of agouti fusion RNAs. Development *120*, 1695–1708.

Duronio, R.J., O'Farrell, P.H., Xie, J.-E., Brook, A., and Dyson, N. (1995). The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. Genes Dev. *9*, 1445–1455.

Dynlacht, B.D., Brook, A., Dembski, M.S., Yenush, L., and Dyson, N. (1994a). DNA-binding and trans-activation properties by *Drosophila* E2F and DP proteins. Proc. Natl. Acad. Sci. USA *91*, 6359–6363.

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

Field, S.J., Tsai, F.-Y., Kuo, R., Zubiaga, A.M., Kaelin, W.G., Jr., Livingston, D.M., Orkin, S.H., and Greenberg, M.E. (1996). E2F-1 Functions in mice to promote apoptosis and suppress proliferation. Cell, this issue.

Flemington, E.K., Speck, S.H., and Kaelin, W.G. (1993). E2F-1 mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc. Natl. Acad. Sci. USA *90*, 6914–6918.

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

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

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Press).

Helin, K., Lees, J.A., Vidal, M., Dyson, N., Harlow, E., and Fattaey, A. (1992). A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell *70*, 337–350.

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

Hijmans, E.M., Voorhoeve, P.M., Beijersbergen, R.L., van 'T Veer, L., and Bernards, R. (1995). E2F-5, a new E2F family member that interacts with p130 in vivo. Mol. Cell. Biol. *15*, 3082–3089.

Horowitz, J.M., and Udvadia, A.J. (1996). Regulation of transcription by the Retinoblastoma (Rb) protein. Mol. Cell. Diff., in press.

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.

Hu, N., Gutsmann, A., Herbert, D.C., Bradley, A., Lee, W.-H., and Lee, E.H.-H.P. (1994). Heterozygous rb-1/+ mice are predisposed to tumors of the pituitary gland with a nearly complete penetrance. Oncogene *9*, 1021–1027.

Hunter, T., and Pines, J. (1994). Cyclins and cancer II: cyclin D and cdk inhibitors come of age. Cell *79*, 573–582.

Ivey-Hoyle, M., Conroy, R., Huber, H., Goodhart, P., Oliff, A., and Heinbrook, D.C. (1993). Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol. Cell Biol. *13*, 7802–7812.

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

Jacks, T., Shih, T.S., Schmitt, E.M., Bronson, R.T., Bernards, A., and Weinberg, R.A. (1994). Tumor predisposition in mice heterozygous for a targeted mutation in NF1. Nature Genetics *7*, 353–361.

Johnson, D.G., Schwartz, 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., Cress, W.D., Jakoi, L., and Nevins, J.R. (1994a). Oncogenic capacity of the E2F-1 gene. Proc. Natl. Acad. Sci. USA *91*, 12823–12827.

Johnson, D.G., Ohtani, K., and Nevins, J.R. (1994b). Autoregulatory control of E2F-1 expression in response to positive and negative regulators of cell cycle expression. Genes Dev. *8*, 1514–1525.

Jooss, K., Lam, E.W.-F., Bybee, A., Girlin, R., Muller, R., and La Thangue, N.B. (1995). Proto-oncogenic properties of the DP family of proteins. Oncogene *10*, 1529–1536.

Kaelin, W.G., Jr., Krek, W., Sellers, W.R., DeCaprio, J.A., Ajchenbaum, F., Fuchs, C.S., Chittenden, T., Li, Y., Farnham, P.J., Blanar, M.A., Livingston, D.M., and Flemington, E.K. (1992). Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell *70*, 351–364.

Kamb, A. (1995). Cell cycle regulators and cancer. Trends Genetics *11*, 136–140.

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.

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

Lam, E.W.-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.

La Thangue, N.B. (1994). DTRF1/E2f: an extending family of heteridimeric factors implicated in cell cycle control. Trends Biochem. Sci. *19*, 108–114.

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

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., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K. (1993). The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. *13*, 7813–7825.

Li, Y., Slansky, J.E., Myers, D.J., Drinkwater, N.R., Kaelin, W.G., and Farnham, P.J. (1994). Cloning, chromosomal location, and characterization of mouse E2F1. Mol. Cell. Biol. *14*, 1861–1869.

Mansour, S.L., Thomas, K.R., and Capecchi, M.R. (1988).Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature *336*, 348–352.

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

Michaud, E.J., Bultman, S.J., Klebig, M.L., van Vugt, M.J., Stubbs, L.J., Russell, L.B., and Woychik, R.P. (1994). A molecular model for the genetic and phenotypic characteristics of the mouse lethal yellow (Ay) mutation. Proc. Natl. Acad. Sci. USA *91*, 2562–2566.

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

Neuman, E., Flemington, E.K., Sellers, W.R., and Kaelin, W.G. (1994). Transcription of the E2F1 gene is rendered cell cycle–dependent by E2F DNA binding sites within its promoter. Mol. Cell. Biol. *14*, 6607–6615.

Nevins, J.R. (1992). E2F; a link betwen the Rb Tumor suppressor protein and viral oncoproteins. Science *258*, 424–429.

Ohtani, K., and Nevins, J.R. (1994). Functional properties of a Drosophila homolog of the E2F1 gene. Mol. Cell. Biol. *14*, 1603–1612.

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

Qin, X.-Q., Livingston, D.M., Ewen, M., Sellers, W.R., Arany, Z., and Kaelin, W.G. (1995). The transcription factor E2F-1 is a downstream target of RB action. Mol. Cell. Biol. *15*, 742–755.

Riley, D.J., Lee, E.Y.-H.P., and Lee, W.-H. (1994). The retinoblastoma protein: more than a tumor suppressor. Ann. Rev. Cell Biol. *10*, 1–29.

Russell, L.D., Ettlin, R.A., SinhaHikim, A.P., and Clegg, E.D. (1990). Histological and Histopathological Evaluation of the Testis, 1st Edition (Clearwater, Florida: Cache River Press).

Saito, M., Helin, K., Valentine, M.B., Griffith, B.B., Willman, C.L., Harlow, E., and Look, A.T. (1995). Amplification of the E2F1 transcription factor gene in the HEL erythroleukemia cell line. Genomics *25*, 130–138.

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

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.

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

Shan, B., and Lee, W.-H. (1994). Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Biol. *14*, 8166–8173.

Shan, B., Zhu, X., Chen, P.-L., Durfee, T., Yang, Y., Sharp, D., and Lee, W.-H. (1992). Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol. Cell. Biol. *12*, 5620–5631.

Sherr, C.J. (1994). G1 phase progression: cycling on cue. Cell *79*, 551–556.

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

Slack, R.S., Hamel, P.A., Bladon, T.S., Gill, R.M., and McBurney, M.W. (1993). Regulated expression of the retinoblastoma gene in differentiating embryonal carcinoma cells. Oncogene *8*, 1585–1591.

Slansky, J.E., and Farnham, P.J. (1995). Introduction to the E2F family: protein structure and gene regulation. In Transcriptional Control of Cell Growth: the E2F Gene Family, P. Farnham, ed. (New York, New York: Springer-Verlag).

Stevens, L.C. (1973). A new inbred strain in mice (129/ter Sv) with a high incidence of spontaneous congenital testicular teratomas. J. Natl. Cancer Inst. *50*, 235–242.

Turusov, V., and Mohr, U. (1994). Pathology of Tumors in Laboratory Animals (Lyon, France: IARC Scientific Publications).

Tybulewicz, V.L.J., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligan, R.C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell *65*, 1153–1163.

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

Wang, T.C., Cardiff, R.D., Zukerberg, L., Lees, E., Arnold, A., and Schmidt, E.V. (1994). Mammary hyperplasia and carcinoma in MMTV–cyclin D1 transgenic mice. Nature *369*, 669–671.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323–330.

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

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

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

Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer, K.-H. B., and Beach, D. (1995). A $p16^{ink4a}$-insensitive cdk4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science *269*, 1281–1284.

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

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

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.