Loss of E2F-1 reduces tumorigenesis and extends the lifespan of *Rb1*(+/–) mice

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Mutation of the retinoblastoma tumour-suppressor gene (*RB*) leads to the deregulation of many proteins and transcription factors that interact with the retinoblastoma gene product (pRB), including members of the E2F transcription factor family[1,2]. As pRB is known to repress E2F transcriptional activity and overexpression of E2F is sufficient for cell cycle progression, it is thought that pRB suppresses growth in part by repressing E2F-mediated transcription[3]. Previously, we reported that loss of *E2f1* in mice results in tissue-specific tumour induction and tissue atrophy[4], demonstrating that E2F-1 normally controls growth both positively and negatively in a tissue-specific fashion[4,5]. To determine whether E2F-1 deregulation—as a result of loss of pRB—promotes proliferation *in vivo*, we have tested whether loss of *E2f1* interferes with the pituitary and thyroid tumorigenesis that occurs in *Rb1(+/–)* mice[6–9]. We have found that loss of *E2f1* reduces the frequency of pituitary and thyroid tumours, and greatly lengthens the lifespan of *Rb1(+/–); E2f1(–/–)* animals, demonstrating that E2F-1 is an important downstream target of pRB during tumorigenesis. Furthermore, loss of *E2f1* reduces a previously reported strain-dependent difference in *Rb1(+/–)* lifespan[9,10], suggesting that *E2f1* or an E2F-1–regulated gene acts as a genetic modifier between the 129/Sv and C57BL/6 strains.

Almost all human tumours contain either loss-of-function mutations in *RB* or mutations in genes encoding upstream regulators of pRB (cyclin D1, cdk4 and p16; ref. 11). These mutations are thought to deregulate the numerous transcription factors and cellular proteins that interact with pRB (refs 1,2), including members of the E2F transcription factor family[3]. Deregulation of E2F activity *via* loss of pRB is predicted to result in proliferation, because overexpression of E2F-1 is sufficient to drive entry into S phase[12–15]. In fact, E2F-1 can act as an oncoprotein when tested in cooperation assays with activated Ras, and the resultant transformed cells are able to form tumours in nude mice[16–18]. Similar to the function of other nuclear oncoproteins, prolonged overexpression of E2F-1 in some settings drives cells to undergo p53-dependent[13–15,19] or p53-independent apoptosis[20–22].

As overexpression of E2F-1 can induce cell proliferation in some experimental settings and cell death in others, we have begun to define the role of E2F-1 *in vivo* by inactivating the *E2f1* gene by homologous recombination in mouse embryonic stem cells. Inactivation of *E2f1* in mice leads to tissue-specific atrophy and tumour induction, demonstrating that *E2f1* normally participates in aspects of cell proliferation and survival and that *E2f1* can itself function as a tumour-suppressor gene[4]. The conclusion that E2F-1 suppresses growth in some *in vivo* settings is in agreement with the reduction in thymocyte apoptosis observed in the absence of E2F-1 (ref. 5). The exact mechanism by which specific tumour types develop in *E2f1*-deficient mice is currently unknown, but it may reflect a tissue-specific lack of pRB/E2F-1–mediated growth suppression, or a reduction in E2F-1–induced p53-mediated apoptosis. As no *E2F1* mutations have been described in human tumours to date, and loss of pRB potentially deregulates many different transcription factors and cellular proteins, it is important to determine the contribution of E2F-1 deregulation to tumorigenesis and apoptosis in the absence of pRB.

Rb1 mutant mice provide an excellent opportunity to test the functional significance of the interaction between pRB and E2F-1. *Rb1(+/–)* mice are viable, but all of them develop intermediate lobe pituitary tumours[6–8]. A large subset of the *Rb1(+/–)* mice also develop C-cell adenomas or C-cell hyperplasia in the thyroid[7,9]. For *Rb1(+/–)* mice of mixed 129/Sv×C57BL/6 genetic background, the mean age of survival is 11.2 months (ref. 9, and data presented here). For *Rb1(+/–)* mice of the inbred 129/Sv background, the mean age of survival is 8.4 months (ref. 10, and data presented here). Analysis of the pituitary (melanotrophic) tumours and thyroid (C-cell) tumours revealed that loss of the wild-type *Rb1* allele has occurred during tumour development[7–9]. Consistent with this observed loss of heterozygosity, chimaeric mice produced with *Rb1(–/–)* ES cells develop pituitary tumours more rapidly than *Rb1(+/–)* animals[10,23]. In contrast, *Rb1(–/–)* mice die during mid-gestation from E13.5–E15.5, with increased apoptosis in various tissues and incomplete haematopoiesis in the foetal liver[6,24,25]. In the *Rb1(–/–)* central nervous system, this increased apoptosis correlates with increases in S-phase entry, free E2F DNA binding activity, and cyclin E expression[26].

Table 1 • Reduced tumorigenesis in *Rb1(+/–)* mice following inactivation of *E2f1*

	total population		inbred 129/Sv		mixed 129/Sv×C57BL/6	
genotype	Pit T	Thyr T/H	Pit T	Thyr T/H	Pit T	Thyr T/H
Rb1(+/–)	19/20 (95%)	10/19 (53%)	12/12	5/12	7/8	5/7
Rb1(+/–);E2f1(+/–)	36/36 (100%)	2/34 (6%)	19/19	0/18	17/17	2/16
Rb1(+/–);E2f1(–/–)	16/26 (62%)	0/22 (0%)	8/13	0/12	8/13	0/10

Mice were examined upon dissection and histologically for pituitary and thyroid abnormalities. For the pituitary tumours (Pit T), the ratios below are the number of animals with grossly detectable pituitary enlargement (which later scored histologically as intermediate lobe adenocarcinomas) over the number of animals examined. For thyroid C-cell adenomas and C-cell hyperplasia (Thyr T/H), the ratios below are the number of animals with histologically detectable C-cell hyperplasia or C-cell tumours over the number of animals examined. The 'total' ratios are the combined 129/Sv inbred and mixed 129/Sv×C57BL/6 animal populations with the denoted lesion over the total number of animals of the given genotype. These total ratios are also given as percentages in parentheses.

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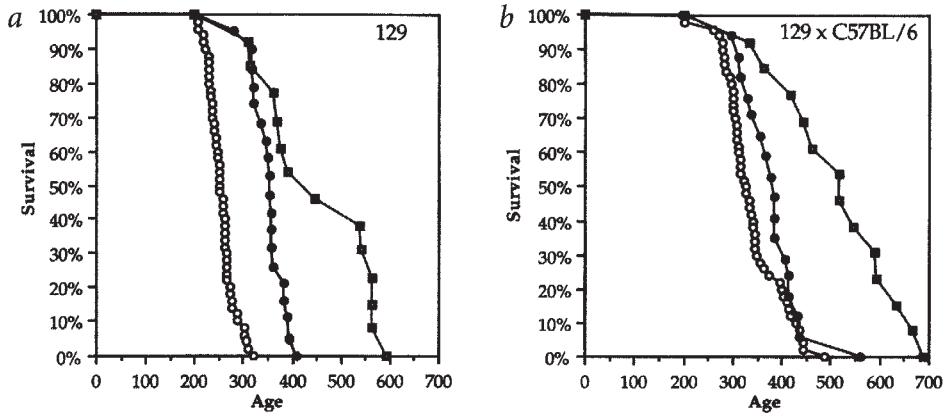

Fig. 1 Increased lifespan of *Rb1(+/–)* mice with the loss of *E2f1*. Animals died or were killed just prior to natural demise. Survival curves were constructed for animals (Table 3) of either the inbred 129/Sv strain (***a***) or the mixed 129/Sv×C57BL/6 strain (***b***). *Rb1(+/–)* control animals are denoted by open circles (○), *Rb1(+/–);E2f1(+/–)* animals by filled circles (●) and *Rb1(+/–); E2f1(–/–)* animals by filled squares (■). % survival represents the percentage of the total population surviving at a given age (in days) for any single genotype. Loss of *E2f1* significantly extends the lifespan of *Rb1* (+/–) animals of either genetic background.

Tumorigenesis in the *Rb1(+/–)* mice or embryonic lethality of the *Rb1(–/–)* mice may result from the deregulation of E2F transcriptional activity, dissociation of pRB from other transcription factors or other cellular proteins, or a combination of these.

To examine the significance of pRB-mediated growth suppression through E2F-1, we crossed *Rb1(+/–)* mice with *E2f1(–/–)* mice to generate double mutants. We obtained *Rb1(+/–); E2f1(+/–)* and *Rb1(+/–);E2f1(–/–)* mice, but consistently were unable to generate *Rb1(–/–);E2f1(–/–)* or *Rb1(–/–);E2f1(+/–)* animals. This was the case whether the background of the animals was mixed (129/Sv×C57BL/6) or inbred (129/Sv). As no *Rb1(–/–)* progeny were recovered, we conclude that the inactivation of *E2f1* is not sufficient to rescue the lethality caused by the *Rb1(–/–)* mutation. The lethal phenotype of the *Rb1(–/–);E2f1(–/–)* double mutants suggests that the lethality in the absence of pRB does not result solely from the deregulation of the E2F-1 transcription factor. In related experiments, we have observed that loss of *E2f1* can delay embryonic lethality caused by the *Rb1(–/–)* mutation, which supports a role for E2F-1 in *Rb1(–/–)* lethality (K.T., M.H., L.Y. & T.J., unpublished observation).

To determine if deregulation of E2F-1 contributes to pituitary tumorigenesis in the *Rb1(+/–)* adults, we monitored *Rb1(+/–); E2f1(+/–)* and *Rb1(+/–);E2f1(–/–)* animals for the development of pituitary tumours, using *Rb1(+/–)* animals as controls (Table 1). Nearly all of the *Rb1(+/–)* animals (19/20) and all of the *Rb1(+/–);E2f1(+/–)* double heterozygotes (36/36) of either mixed or inbred background developed grossly detectable pituitary tumours, which histologically were classified as intermediate lobe pituitary adenocarcinomas. In contrast, only 62% of *Rb1(+/–); E2f1(–/–)* animals (16/26) developed grossly detectable pituitary tumours (Table 1). The reduced penetrance of macroscopic pituitary tumors in *Rb1(+/–);E2f1(–/–)* animals demonstrates that inactivation of both wild-type *E2f1* alleles significantly affects the penetrance of pituitary tumorigenesis in *Rb1(+/–)* mice ($P<0.05$ when compared to *Rb1(+/–)* mice, and $P<0.001$ when compared to *Rb1(+/–);E2f1(+/–)* mice by Fisher's Exact Test). Loss of one wild-type *E2f1* allele did not alter the frequency of pituitary tumour formation in the *Rb1(+/–)* mice. In addition, the pituitary tumours that arose in the *Rb1(+/–);E2f1(–/–)* animals were often much smaller (approximately 8-fold) than pituitary tumours from the *Rb1(+/–)* and *Rb1(+/–);E2f1(+/–)* animals, although they were histologically indistinguishable. Two pituitaries from older *Rb1(+/–);E2f1(–/–)* animals (at 19.4 and 22.7 months) showed no gross enlargement, but contained small histological abnormalities suggesting the development of early neoplastic lesions. The reduced severity of the pituitary lesions in the *Rb1(+/–);E2f1(–/–)* animals suggests that loss of E2F-1 may delay the initation or the progression of pituitary tumours.

To investigate whether deregulation of E2F-1 contributed to thyroid tumorigenesis in the *Rb1(+/–)* adults, we also monitored *Rb1(+/–);E2f1(–/–)* and *Rb1(+/–);E2f1(+/–)* animals for the development of C-cell adenomas, using *Rb1(+/–)* animals as controls (Table 1). Strikingly, we found that none of the *Rb1(+/–);E2f1(–/–)* animals (0/22) and only 6% of *Rb1(+/–); E2f1(+/–)* animals (2/34) developed C-cell adenomas or C-cell hyperplasia based on histological examination, whereas 53% of the *Rb1(+/–)* animals (10/19) presented with these thyroid lesions. The reduction of C-cell adenomas and hyperplasia with the inactivation of one or both wild-type *E2f1* alleles is highly significant when compared to the frequencies found in the

Table 2 • Comparison of lesions in *Rb1(+/–)* mice following inactivation of *E2f1*

	Rb1(+/–)	*Rb1(+/–);E2f1(+/–)*	*Rb1(+/–);E2f1(–/–)*
Pituitary adenocarcinoma	19/20	36/36	16/26
C-cell hyperplasia/adenoma	10/19	2/34	0/22
Lung adenocarcinoma	0/19	0/34	3/24 (19.5*, 19.6, 22.0)
Lymphoma	0/19	0/34	1/24 (19.4)
Uterine sarcoma	0/19	0/17	1/10 (19.5*)
Haemangioma	0/19	0/34	3/24 (12.0*, 15.2*, 22.7*)
Uterine haemorrhage	0/19	0/17	5/10 (10.2*, 12.3*, 14.5*, 14.6*, 20.9*)
Gastric polyp	0/19	1/34 (11.7)	1/24 (14.5*)
Fungioma	0/19	3/34 (11.0, 11.5, 11.7)	2/24 (11.9, 12.9)
Testicular teratoma	0/19	1/17 (13.5)	0/16
Thyroid degeneration	0/19	8/34	14/19
Adrenal medullary hyperplasia	6/13	12/23	19/20

Mice were examined upon dissection and histologically for the presence of tumours and other tissue abnormalities. Incidence of each lesion is expressed as the number of mice displaying each lesion over the total number of animals examined. The age upon sacrifice or death at which these lesions were detected in months is given in parentheses. *denotes *Rb1(+/–);E2f1(–/–)* animals that died without a grossly detectable pituitary tumour.

Table 3 • Prolonged survival in *Rb1(+/–)* mice with inactivation of *E2f1*

	inbred 129/Sv background			mixed 129/Sv×C57BL/6 background		
genotype	n	mean survival	survival range	n	mean survival	survival range
Rb1(+/–)	50	8.4±0.9 (254±26)	6.8 to 10.5	50	11.2±1.9 (340±58)	6.6 to 16.0
Rb1(+/–);E2f1(+/–)	19	11.6±1.1 (352±32)	9.2 to 13.5	17	12.6±2.1 (383±63)	9.7 to 18.4
Rb1(+/–);E2f1(–/–)	13	15.2±3.6 (456±107)	10.2 to 24.4	13	17.1±3.7 (521±113)	11.0 to 22.7

Mice were monitored closely for a precipitous decline in overall body condition and then killed at approximately the same morbidity, in order to preserve their tissues for histological examination. The number of animals which have died or were killed of each genotype is denoted by 'n'. Mean survival is the average longevity±the standard deviation given in months and also in days (in parentheses) for these populations. Survival range in months is the earliest age at which death occurred up to the oldest age of any surviving animals for a given genotype.

Rb1(+/–) control animals ($P<0.001$ for both comparisons by Fisher's Exact Test), and occurred regardless of the genetic background examined. No statistically greater reduction in C-cell abnormalities on the *Rb1(+/–);E2f1(–/–)* animals is observed over the reduction seen with the inactivation of a single *E2f1* allele in the *Rb1(+/–);E2f1(+/–)* animals. This demonstrates that although pituitary tumorigenesis in *Rb1(+/–)* mice is unaffected by the loss of one wild-type *E2f1* allele, the occurrence of thyroid tumours is lowered dramatically by such a mutation. Loss of E2F-1 may interfere with thyroid tumorigenesis by reducing proliferation of the C-cell precursor population. Substantial thyroid degeneration is apparent in both *Rb1(+/–);E2f1(–/–)* and *Rb1(+/–);E2f1(+/–)* animals relative to the *Rb1(+/–)* controls (Table 2). We also observed thyroid degeneration in homozygous (6/12) and heterozygous *E2f1* mutants (15/19) older than 14 months.

Other lesions were observed in the *Rb1(+/–);E2f1(–/–)* animals, suggesting that the *Rb1(+/–)* mutation did not interfere with the previously defined phenotypes associated with the loss of *E2f1* (ref. 4). Testicular atrophy and exocrine gland dysplasia still developed with 100% penetrance in the *Rb1(+/–);E2f1(–/–)* animals. Furthermore, several of the *Rb1(+/–);E2f1(–/–)* animals which developed pituitary tumours also developed lung adenocarcinoma or lymphoma (Table 2), tumours frequently observed in *E2f1*-deficient animals[4]. Ten *Rb1(+/–);E2f1(–/–)* animals (six females and four males) did not develop grossly detectable pituitary tumours. Of these, five females died with severe uterine haemorrhages and three males developed haemangiomas (Table 2). We also observed that *E2f1*-deficient animals (4/15) developed haemangiomas or haemangiosarcomas and *E2f1(+/–)* females (10/15) developed uterine haemorrhages. Finally, adrenal medullary hyperplasia was observed at very high frequency in the *Rb1(+/–);E2f1(–/–)*, *Rb1(+/–);E2f1(+/–)* and *Rb1(+/–)* animals (Table 2). While this is not a common lesion in wild-type mice[27], older homozygous (10/12) and heterozygous (8/13) *E2f1* mutants also displayed adrenal medullary hyperplasia.

Diminished pituitary and thyroid tumorigenesis in the *Rb1(+/–);E2f1(+/–)* and *Rb1(+/–);E2f1(–/–)* animals was accompanied by a significantly prolonged survival (as assessed by Student's two-tailed t-test). Lifespan was extended most dramatically in animals of the inbred 129/Sv background, and influenced by the number of wild-type *E2f1* alleles present. For inbred 129/Sv animals (Table 3 and Fig. 1*a*), the mean age of survival increased markedly from 8.4 months for *Rb1(+/–)* animals to 11.6 months for *Rb1(+/–);E2f1(+/–)* animals ($P<0.001$) and from 8.4 months for *Rb1(+/–)* to 15.2 months for *Rb1(+/–);E2f1(–/–)* animals ($P<0.001$). Survival of *Rb1(+/–);E2f1(–/–)* animals was prolonged by 3.6 months over that of *Rb1(+/–);E2f1(+/–)* animals (from 11.6 to 15.2 months, $P<0.01$), demonstrating that lifespan was sensitive to *E2f1* gene dosage for animals of the inbred 129/Sv background. Viewed another way, fewer than 4% of *Rb1(+/–)* animals of the 129/Sv background survive beyond 10 months (304 days), whereas the majority (90%) of *Rb1(+/–);E2f1(+/–)* animals and all of *Rb1(+/–);E2f1(–/–)* animals were still alive at this age. The oldest 129/Sv *Rb1(+/–);E2f1(–/–)* animal is presently alive at 24.4 months of age. Taken together, these results demonstrate that the inactivation of *E2f1* greatly reduces the severity of the *Rb1(+/–)* phenotype.

The loss of E2f1 also prolonged survival of *Rb1(+/–)* animals of mixed (129/Sv×C57BL/6) genetic background (Table 3 and Fig. 1*b*). With the loss of one *E2f1* allele, the mean lifespan

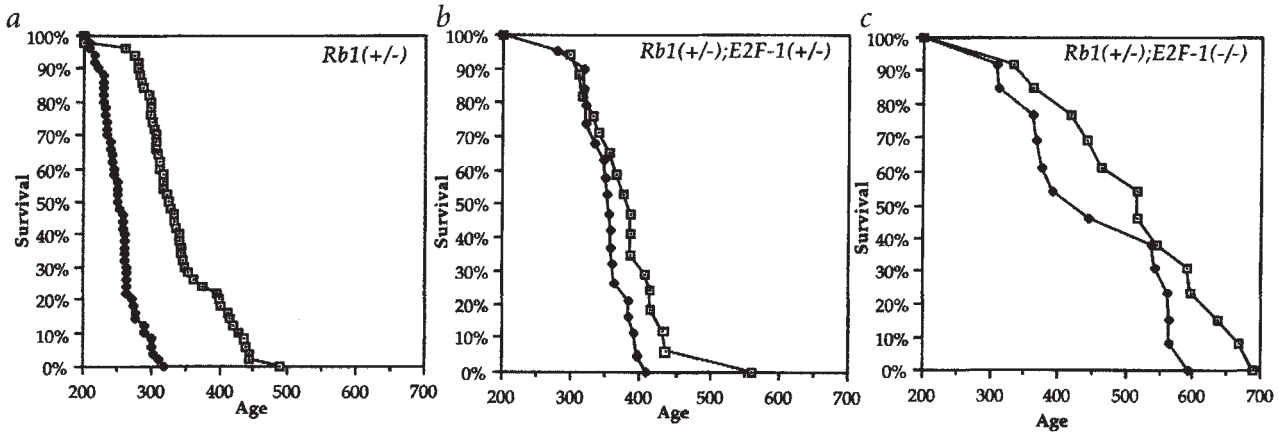

Fig. 2 Strain-specific difference in lifespan decreases with the loss of *E2f1*. Survival of inbred 129/Sv animals (◆) is now compared to survival of mixed 129/Sv×C57BL/6 animals (❑) from Table 3 and from Fig. 1 for each genotype. ***a***, *Rb1(+/–)*. ***b***, *Rb1(+/–);E2f1 (+/–)*. ***c***, *Rb1(+/–); E2f1(–/–)*. % survival represents the percent of the total population surviving at a given age (in days) for any single genotype. (Note that the x-axis begins at 200 days.) The strain-specific difference in survival of *Rb1(+/–)* animals is highly significant ($P<0.001$), while there is no significant ($P>0.05$) strain-specific difference in survival of either *Rb1(+/–);E2f1(+/–)* animals or *Rb1(+/–);E2f1(–/–)* animals.

increased moderately from 11.2 months for *Rb1(+/–)* animals to 12.6 months for *Rb1(+/–);E2f1(+/–)* animals ($P<0.05$ by Student's t-test, but $P>0.05$ by Tukey-Kramer's multiple comparisons test). The loss of both alleles of *E2f1* increased lifespan from 11.2 months for *Rb1(+/–)* to 17.1 months for *Rb1(+/–);E2f1(–/–)* animals ($P<0.001$ by Student's t-test). As observed for animals of the inbred background, the loss of both wild-type *E2f1* alleles further extends lifespan over that obtained with the loss of one *E2f1* allele in the *Rb1(+/-)* animals of mixed background (from 12.6 months to 17.1 months, $P<0.001$ by Student's t-test). On the mixed background at 14.6 months (443 days) when fewer than 4% of *Rb1(+/–)* animals survive, 75% (12/16) of *Rb1(+/–);E2f1(–/–)* animals are still alive. The two oldest mixed-background *Rb1(+/–);E2f1(–/–)* animals lived to 22.3 and 22.7 months. Loss of *E2f1*, therefore, reduces the lethality of the *Rb1(+/–)* phenotype regardless of the genetic background of the animal. Lifespan can be affected by many physiological factors, one of which is tumour development and it is not possible to conclude that the extended lifespan results solely from the reduced occurrence of pituitary and thyroid tumours in the *Rb1(+/–);E2f1(–/–)* animals.

The genetic background of the *Rb1(+/–)* animals influenced the extent to which the loss of one wild-type *E2f1* allele increased longevity. As shown previously[9,10] as well as in this study (Table 3 and Fig. 2*a*), a great increase in lifespan occurs in *Rb1(+/–)* animals of mixed 129/Sv×C57BL/6 genetic background (n=50, mean survival=340±58 days) compared with those of the inbred 129/Sv background (n=50, mean survival=254±26 days). This strain-specific difference in survival is highly significant ($P<0.001$). The lifespan of the 12 *Rb1(+/–)* animals of the inbred 129/Sv background (Table 1) falls within the range of the 50 *Rb1(+/–)* animals of the inbred 129/Sv background (Table 3 and Fig. 2*a*). Surprisingly, however, no substantial increase in lifespan occurs in *Rb1(+/–);E2f1(+/–)* animals of mixed background (n=17, mean survival=383±63 days) over those double heterozygotes which are 129/Sv inbred (n=19, mean survival=352±32 days, Table 3 and Fig. 2*b*). Thus, the inactivation of a single *E2f1* allele prolonged the survival of the 129/Sv strain *Rb1(+/–)* animals to a similar extent as crossing the 129/Sv *Rb1(+/–)* animals to the C57BL/6 strain. As all of the *Rb1(+/–);E2f1(+/–)* animals still develop pituitary tumours regardless of their genetic background, the increased lifespan seen on the 129/Sv inbred background may be due to a delay in pituitary tumorigenesis or reduced thyroid tumorigenesis in the absence of one wild-type *E2f1* allele.

Similar to the double heterozygotes, no significant increase in lifespan occurred in *Rb1(+/–);E2f1(–/–)* animals of mixed background (n=13, mean survival=521±113 days) compared with those *Rb1(+/–);E2f1(–/–)* of the 129/Sv background (n=13, mean survival=456±107 days, Table 3 and Fig. 2*c*). Taken together, these data demonstrate that *E2f1* can act as a genetic modifier of survival for *Rb1(+/–)* animals on the 129/Sv inbred background. Furthermore, our results suggest that *E2f1* or an E2F-1 target gene may be responsible for the genetic differences between the 129/Sv and C57BL/6 strains with regard to the *Rb1(+/–)* phenotype. We have detected no DNA rearrangements at the *E2f1* locus in the 129/Sv or C57BL/6 strains by Southern analysis (data not shown).

It had been demonstrated previously that pituitary and thyroid tumorigenesis in *Rb1(+/–)* animals involves the loss of the wild-type *Rb1* allele[7–9,23]. Potentially, this can lead to the deregulation of a number of transcription factors and cellular proteins known to interact with pRB (refs 1,2). However, because inactivation of *E2f1* can reduce the penetrance of the pituitary and thyroid tumour phenotypes in *Rb1(+/–)* mice and greatly extend their lifespan, loss of *Rb1* appears to deregulate at least E2F-1. Thus, E2F-1 normally contributes to the development of these tumours in the *Rb1(+/–)* animals. This is the first genetic demonstration that E2F-1 functions downstream of pRB in tumour development *in vivo*. The results presented here, together with previous data on *E2f1*-deficient mice, demonstrate that *E2f1* functions as an oncogene or a tumour-suppressor gene in a tissue-specific manner.

Methods

Generation and genotyping of mutant mice. *E2f1*-deficient animals of either mixed 129/Sv×C57BL/6 or inbred 129/Sv background were mated to 129/Sv *Rb1(+/–)* animals to generate *Rb1(+/–);E2f1(+/–)* animals. Subsequent intermating of *Rb1(+/–);E2f1(+/–)* males and females produced *Rb1(+/–);E2f1(–/–)* animals of either mixed or inbred background. Animals were genotyped from tail DNA using previously published PCR assays with primers that specifically amplified the wild-type and mutant alleles for *Rb1* (ref. 6) and *E2f1* (ref. 4). In light of 129/Sv substrain differences[28], a description of the exact 129/Sv substrains used in our experiments follows. D3 ES cells derived from the 129/SvPas substrain were used to generate the original *E2f1(+/–)* chimaeras and *Rb1(+/–)* chimaeras that were subsequently propagated using inbred 129/SvJae animals. Both the 129/SvPas and 129/SvJae substrains belong to the 129/Sv Steel subdivision, which show minimal SSLP marker variation (indistinguishable at 84 out of 86 loci examined, or 97.6% identical; ref. 28).

Survival and tumour scoring. Mice were monitored closely for a precipitous decline in overall body condition and then died or were killed just prior to their natural demise. All animals were examined during dissection, and tissues were subsequently analysed histologically. Pituitary tumours were scored as macroscopically enlarged pituitaries upon dissection and were categorized histologically as intermediate lobe adenocarcinomas. Thyroid C-cell hyperplasia and adenomas were scored histologically after inspection of the thyroid gland (in most cases two glands per animal). All tissues were routinely analysed histologically to characterize all other lesions.

Statistics. Differences in pituitary and thyroid tumour frequency were evaluated using Fisher's Exact test. Differences in mean survival were analysed using the two-tailed Student's t-test. Statistical significance is reported as $P<0.001$ highly significant, $P<0.01$ very significant, $P<0.05$ significant, or $P>0.05$ not significant.

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