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Inhibition of pituitary tumors in Rb mutant chimeras through E2f4-loss reveals a key suppressive role for the pRB/E2F pathway in urothelium and ganglionic carcinogenesis

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Abstract

The retinoblastoma protein pRB suppresses tumorigenesis largely through regulation of the E2F transcription factors. E2F4, the most abundant E2F protein, is thought to act in cooperation with pRB to restrain cell proliferation. In this study, we analyze how loss of E2f4 affects the tumorigenicity of pRB-deficient tissues. Since $Rb^{-/-};E2f4^{-/-}$ germline mice die in utero, we generated $Rb^{-/-};E2f4^{-/-}$ chimeric animals to allow examination of adult tumor phenotypes. We found that loss of E2f4 had a differential effect on known Rb-associated neuroendocrine tumors. It did not affect thyroid and adrenal glands tumors but partially suppressed lung neuroendocrine hyperplasia. The most striking effect was in the pituitary where E2F4-loss delayed the development, and reduced the incidence, of Rb mutant tumors. This tumor suppression increased the longevity of the $Rb^{-/-};E2f4^{-/-}$ chimeric animals allowing us to identify novel tumor types. We observed ganglionic neuroendocrine neoplasms, lesions not previously associated with mutation of either Rb or E2f4. Moreover, a subset of the $Rb^{-/-};E2f4^{-/-}$ chimeras developed either low or high-grade carcinomas in the urothelium transitional epithelium supporting a key role for Rb in bladder cancer.

Keywords

E2f4; Rb; neuroendocrine tumors; urothelial cancer

INTRODUCTION

The retinoblastoma tumor suppressor gene RB is mutated in approximately 30% of all human cancers and in more than 90% of retinoblastomas, osteosarcomas and small cell lung carcinomas (Weinberg, 1995). pRB belongs to the family of pocket proteins that includes p107 and p130. These two proteins share structural and functional similarities with pRB, but are rarely mutated in human tumors (Du and Pogoriler, 2006; Wikenheiser-Brokamp, 2006).

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A large part of the tumor suppressor activity of pRB derives from its ability to interact with the E2F transcription factors and, together with the other pocket proteins, control the balance between quiescence and proliferation (Harbour and Dean, 2000). E2Fs control the expression of genes crucial for cell cycle re-entry, DNA replication and mitosis. pRB binds to the E2Fs in its active under-phosphorylated form, and inhibits the transcription of E2F target genes through two distinct mechanisms (Dyson, 1998; Trimarchi and Lees, 2002). The first involves sequestration of E2F1, 2, and 3, and inhibition of their transcriptional activity, thereby preventing progression from the G1 to the S phase of cell cycle. The second involves formation of E2F4- or E2F5-pocket protein complexes that bind to E2F-responsive promoters and actively repress their transcription, thereby promoting quiescence. Consistent with these dual roles, $Rb^{-/-};p107^{-/-};p130^{-/-}$ (Dannenberg et al., 2000; Sage et al., 2000) and $E2f4^{-/-};E2f5^{-/-}$ (Gaubatz et al., 2000) mouse embryonic fibroblasts (MEFs) fail to respond to a variety of growth inhibitory signals, while MEFs lacking E2f1, E2f2, and/or E2f3 have impaired proliferative capacity (Humbert et al., 2000b; Wu et al., 2001). While these results have led to the designation of E2F1, E2F2 and E2F3 as “activators” and E2F4 and E2F5 as “repressors”, accumulating evidence suggest that this division is not so clear-cut especially with regard to E2F4.

E2F4 is ubiquitously expressed throughout cell cycle, and accounts for most of the E2F endogenous activity (Moberg et al., 1996). E2F4 has a transactivation domain but it is primarily localized to the cytoplasm in its free form due to the presence of strong nuclear export signals and thus its transcriptional activity is restrained (Gaubatz et al., 2001; Verona et al., 1997). In the G0/G1 phase of cell cycle E2F4, by virtue of its interaction with the pocket proteins, accumulates in the nucleus where chromatin immunoprecipitation studies suggest that these E2F/pocket protein complexes play a major role actively repressing E2F-target genes by recruiting histone deacetylases (Rayman et al., 2002; Ren et al., 2002). In agreement with a function of E2F4 in the G0/G1 phase of cell cycle, E2f4 null mice often die shortly after birth with defects in terminal differentiation including craniofacial, respiratory epithelium abnormalities and altered hematopoietic lineages that may result from an inability to establish quiescence (Humbert et al., 2000a; Rempel et al., 2000). Concordantly, $E2f4^{-/-};E2f5^{-/-}$ MEFs have a normal proliferation capacity but are unable to arrest in G1 in response to growth inhibitory signals (Gaubatz et al., 2000). These observations all fit with the hypothesis that E2F4 is a repressive E2F. However, analysis of E2F’s role in the context of Rb mutant tumors challenges this conclusion. $Rb^{+/-}$ mice die from intermediate lobe pituitary tumors and develop c-cell thyroid tumors at high frequency (Clarke et al., 1992; Jacks et al., 1992). Loss of E2f4 suppresses development of both tumor types and thus, significantly expands the lifespan (Lee et al., 2002). There are a number of possible explanations for E2F4 apparent oncogenic activity. First, E2F4 could behave as a trascriptional activator in the context of these tumor cells. Second, there is evidence that E2F4 may influence tumorigenesis in a indirect manner: we found that the absence of E2F4 in $Rb^{-/-}$ cells allows p107 and p130 to associate with E2F1, E2F2 and E2F3 and presumably substitute for pRB in preventing these activator E2Fs from promoting proliferation and therefore tumorigenesis (Lee et al., 2002). Third, as the $Rb^{+/-};E2f4^{-/-}$ germline mouse model requires Rb-loss of heterozygosity (LOH) for tumorigenesis, it is possible that E2f4-deficiency reduces the frequency of Rb LOH or the viability of the resulting $Rb^{-/-};E2f4^{-/-}$

cells. Finally, in the $Rb^{+/-};E2f4^{-/-}$ animals, tumor onset is analyzed not in a wildtype context, but in tissues lacking E2f4, a situation that does not reflect the normal tumor environment and may cause non-cell autonomous effects on Rb-deficient tumor growth. These four models are not mutually exclusive.

To analyze tumorigenesis induced by combined loss of E2f4 and Rb, in this study we generate $Rb^{-/-};E2f4^{-/-}$ chimeric mice. This model system overcomes the lethality of the $Rb^{-/-}$;E2f4-/- germline animals and therefore eliminates the requirement for Rb LOH. Moreover, the stochastic nature of the chimeric system allows for studying the effects of gene mutation in a wildtype context and importantly, due to its mosaic nature, to identify phenotypes that would unlikely be uncovered by the use of a tissue-specific conditional system. Consistent with previous observations in $Rb^{+/-};E2f4^{-/-}$ germline animals, we found that loss of E2f4 suppresses formation of pituitary tumors that are the predominant cause of death of the $Rb^{-/-}$ chimeras (Parisi et al., 2007; Williams et al., 1994) and therefore greatly expands the lifespan. This extended longevity allowed the identification of two novel tumor types, ganglionic neuroendocrine neoplasms and papillary urethelial carcinomas. Altogether our observations indicate that E2F4 and pRB functionally interact in specific neuroendocrine tissues and establish a role for these proteins in the urogenital epithelium.

RESULTS

Isolation of mutant $E2f4^{-/-};Rb^{-/-}$ ES cells and analysis of their contribution to embryonic and adult tissues

We have previously shown that E2f4-loss suppresses the formation of pituitary and thyroid tumor in $Rb^{+/-}$ germline animals (Lee et al., 2002). To study the contribution of E2f4 to the tumors that arise in Rb null tissues, we generated $E2f4^{-/-};Rb^{-/-}$ chimeric mice. To obtain these animals, we intercrossed $E2f4^{+/-};Rb^{+/-}$ Rosa26 germline mutant animals and isolated $E2f4^{-/-};Rb^{-/-}$Rosa26 embryonic stem (ES) cells by de novo derivation. Two $E2f4^{-/-};Rb^{-/-}$ Rosa26 ES cell lines were injected into blastocysts giving rise to E2f4;Rb chimeras. We screened E18.5 stage embryos for the presence of β-galactosidase activity (expressed from the Rosa26 allele) and found that both cell lines were able to contribute to the entire embryo with high efficiency indicating that the ES cells were pluripotent (Figure 1a, data not shown). $E2f4^{-/-};Rb^{-/-}$ ES cells also contributed to a variety of adult tissues (Figure 1b, data not shown). However, we observed that on average, our cohort of adult $Rb^{-/-};E2f4^{-/-}$ chimeric animals, had a lower degree of chimerism than the $Rb^{-/-}$ chimeric mice as judged by coat color (Table 1, Parisi et al., 2007). Since our $Rb^{-/-};E2f4^{-/-}$ ES cells are pluripotent, and we know that $E2f4^{-/-}$ animals can be viable (Humbert et al., 2000a; Rempel et al., 2000), these findings suggest that additional loss of E2f4 decreases the viability of $Rb^{-/-}$ cells in chimeric mice.

E2f4-loss suppresses pituitary tumors in $Rb^{-/-}$ chimeric mice

To study the effects of E2f4-loss in Rb tumorigenesis we aged our cohort of $Rb^{-/-};E2f4^{-/-}$ chimeric animals and compared them to $Rb^{-/-}$ chimeric mice that we generated in parallel (Parisi et al., 2007). We found a striking difference in the lifespan of the $Rb^{-/-};E2f4^{-/-}$ versus $Rb^{-/-}$ chimeras. $Rb^{-/-}$ chimeric animals die by the age of 9 months due to pituitary tumors

(Parisi et al., 2007). In stark contrast, after nine months more than 70% of the $Rb^{-/-};E2f4^{-/-}$ chimeras were still alive (Figure 2a, b). Accordingly, we found that less than half of the $Rb^{-/-};E2f4^{-/-}$ chimeric mice developed pituitary tumors and the affected animals were all greater than ten months of age (Figure 2b, Table 1). Histologically, the pituitary tumors that do develop in the $Rb^{-/-};E2f4^{-/-}$ animals are similar to the ones found in the $Rb^{-/-}$ chimeras. They originate from the pars intermedia of the pituitary, have the morphology of adenocarcinomas, and all derive from $Rb^{-/-};E2f4^{-/-}$ mutant cells as shown by β-galactosidase staining (Figure 3a, b). Thus, E2f4-loss does not change the nature of the pituitary tumors that arise in the $Rb^{-/-}$ chimeric mice, but rather delays their development and decreases their incidence. Analysis of pituitary tumor samples recovered from $Rb^{-/-}$ and $Rb^{-/-};E2f4^{-/-}$ chimeras at necropsy showed that there was a general absence of both apoptotic and proliferative cells (as judged by staining for TUNEL and Ki67 respectively) in both genotypes (data not shown). Thus, it remains an open question how E2F-4 loss suppresses pituitary tumor development. Since we detect pituitary tumors in Rb-deficient mice with chimerism as low as 5% (Parisi et al., 2007), we can be sure that the suppression of tumor development in $Rb^{-/-};E2f4^{-/-}$ chimeras is not merely the result of insufficient contribution of $Rb^{-/-};E2f4^{-/-}$ cells to the pituitary. Thus, we can now conclude that the effect of E2f4-loss on the development of tumors in this organ is not due to a change in the propensity of Rb LOH.

Differential effects of E2f4-deficiency in other Rb-dependent neuroendocrine tumors

E2f4-loss has been shown to completely suppress c-cell carcinomas of the thyroid in $Rb^{+/-};E2f4^{-/-}$ germline mice (Lee et al., 2002). These tumors arise at high frequency in $Rb^{+/-}$ germline mice, but are less commonly detected in $Rb^{-/-}$ chimeras where only a minority of the animals shows these neoplastic lesions (Table 1, Parisi et al., 2007). When we screened $Rb^{-/-};E2f4^{-/-}$ chimeric animals, we found that they also bore thyroid tumors at a similar frequency to $Rb^{-/-}$ chimeras (Figure 4a, Table 1) leading us to conclude that E2f4 is dispensable for the development of c-cell tumors in $Rb^{-/-}$ chimeric thyroids. This is in contrast to the suppression of thyroid tumors that occurs in the germline $Rb^{+/-};E2f4^{-/-}$ animals suggesting that this latter phenotype is likely due to a change in the rate of Rb LOH, or to non-cell autonomous effects caused by the lack of E2f4 in other tissues.

$Rb^{-/-}$ chimeras develop additional neuroendocrine lesions in adrenal glands and in the lung (Parisi et al., 2007; Williams et al., 1994). We therefore also examined these organs in the $Rb^{-/-};E2f4^{-/-}$ chimeric mice at the histological level. We observed that these animals presented uni- or multifocal foci of hyperplasia in the adrenal medulla (pheochromocytoma) at a similar frequency to $Rb^{-/-}$ chimeras (Figure 4b, Table 1), indicating that E2f4 is also dispensable for this tumor type. Notably, we observed a very different effect in the lung. We have previously observed neuroendocrine cell hyperplasia in the lung of 10 out of 13 $Rb^{-/-}$ chimeras (Parisi et al., 2007). We found that $Rb^{-/-};E2f4^{-/-}$ chimeric animals also presented hyperplastic areas in the lung, but the severity of these lesions as well as the percentage of animals bearing them, was much lower when compared to than seen in the $Rb^{-/-}$ chimeras (Figure 4c, Table 1). Thus, our findings suggest that E2f4-loss has differential effects in a variety of Rb-deficient neuroendocrine tumors. It is dispensable for adrenal gland and thyroid tumors, while playing an essential role in pituitary tumors and in $Rb^{-/-}$ pulmonary neuroendocrine cells.

Novel tumor phenotypes in $Rb^{-/-};E2f4^{-/-}$ chimeras

The increased lifespan of the $Rb^{-/-};E2f4^{-/-}$ animals due to suppression of pituitary tumors, together with the ability to generate adult tissues simultaneously lacking Rb and E2f4, gave us the unique opportunity to identify new tumor types that might be modulated by these proteins. We performed necropsy and whole histology on all the $Rb^{-/-};E2f4^{-/-}$ chimeric mice and found a distinct tumor spectrum in the older animals (Table 1). Six mice, all but one older than ten months, showed neoplastic lesions within the ganglia in different areas of the body such as the neck, kidney region and testes. These lesions appeared to be composed by groups of very dark cells embedded within neurons whose morphology was reminiscent of neuroendocrine cells (Figure 5a). To confirm that these cells were indeed of neuroendocrine origin, we performed immunohistochemistry for the markers GCRP and Synaptophysin and found that all of them were positive (Figure 5a). As neuroendocrine cells are not normally detected within ganglia we looked for any sign of metastases originating from other neuroendocrine tumors present in these animals, but were unable to find any. This suggests that these cells were indeed arising from within the ganglia. We did not detect any TUNEL-positive cells in the neoplastic lesions of the $Rb^{-/-};E2f4^{-/-}$ chimeras or the normal ganglia of either $Rb^{-/-}$ or $Rb^{-/-};E2f4^{-/-}$ chimeras (data not shown). Thus, we have no evidence that E2f4-deficiency acts to suppress the induction of apoptosis by Rb-loss, but these negative data do not rule this out. We also did not detect any Ki67-positive cells in either the neoplastic or normal $Rb^{-/-};E2f4^{-/-}$ chimeric ganglia (data not shown). Thus, it remains an open question whether the ganglionic tumors result from a direct effect (e.g. via differences in proliferative and/or apoptotic capacity of $Rb^{-/-};E2f4^{-/-}$ versus Rb mutant tissues) or an indirect effect (the extended lifespan of the Rb mutant chimeras) of E2f4 loss.

In addition to these ganglionic neoplasms, we also identified tumors in the urogenital epithelium of the $Rb^{-/-};E2f4^{-/-}$ chimeric mice (Table 1). We noticed that three animals, 16 months of age or older, had polyps within the uroepithelium that lines the ureter. This papillary form of urothelial cancer was hyperplastic non invasive in two animals, but highly dysplastic and invasive in a third animal (Figure 5b). The severity of these lesions prevented normal exchange of urine between the kidney and the bladder causing nephrosis (not shown). Urothelial cancer does not spontaneously arise in mice and there is no evidence of this tumor in $Rb^{-/-}$ chimeras (Parisi et al., 2007; Williams et al., 1994). Histological comparison of tumorigenic versus normal urothelium in the $Rb^{-/-};E2f4^{-/-}$ chimeras, revealed no apoptosis, but high levels of proliferation specifically within circumscribed regions of the tumors but not the normal urothelium (data not shown). Thus, as above, these studies provide no evidence for, but also do not rule out, the possibility that E2f4-loss plays a direct role in promoting formation of these tumors.

Finally, we wished to determine if urothelium and ganglionic neoplasms were cell or non-cell autonomous. As we could not take advantage of β-galactosidase staining due to problems of high background (kidney) or poor penetrability of the dye (ganglion) in the tissues surrounding the tumors, we assessed the contribution of mutant cells to the novel tumors by screening the tumor areas for the presence of the E2f4 mutant allele. The mutant band was clearly present in both neoplasms and its intensity, relative to the wildtype band, correlated well with the heterogeneity of the tumors (Figure 5c). Specifically, the mutant

band was relatively weak in the urothelium neoplasm which had a high non-epithelial component and much stronger in the ganglionic neoplasm which was more homogenous. Although correlative, these results strongly suggest that these novel tumor types are derived from the $Rb^{-/-};E2f4^{-/-}$ cells. Similar to the ganglionic neurendocrine neoplasms, urothelial cancer was found at a time when the vast majority of $Rb^{-/-}$ chimeric animals are dead. Thus, it is an open question whether these tumor types arise as a consequence of loss of both Rb and E2f4 or whether they can arise with long latency through inactivation of Rb in an E2f4-independent manner. Nevertheless, the finding that $Rb^{-/-};E2f4^{-/-}$ chimeric mice develop a papillary form of urothelial cancer represents the first direct evidence of a role for Rb in this cancer type and is concordant with the high incidence of Rb mutation in urothelial carcinoma of the bladder.

DISCUSSION

pRB's prominent role in tumorigenesis is due, at least in part, to its ability to bind the E2Fs and control cell division. Nevertheless, the extent to which the interaction of pRB with the E2Fs influences its role in cancer remains unclear due in part to the embryonic lethality of $Rb^{-/-}$ and $Rb^{-/-};E2f^{-/-}$ mutant animals. In this study we generated $Rb^{-/-};E2f4^{-/-}$ chimeric mice to analyze the interplay between pRB and E2F4. These animals are viable at levels of chimerism up to 60% thus allowing us to study the contribution of E2f4 to Rb-dependent tumorigenesis. $Rb^{-/-}$ chimeric mice die of pituitary tumors between 2 and 9 months of age (Parisi et al., 2007; Williams et al., 1994). We found that loss of E2f4 increased the lifespan of these animals by delaying the development and decreasing the incidence of pituitary tumors. Therefore, our studies show conclusively that E2f4-loss inhibits pituitary tumors independently of the rate of Rb LOH. Furthermore, the ability to generate mice with a wide range of chimerism allows us to extrapolate that the suppressive effect of E2f4 on this tumor type is cell autonomous as we could detect pituitary tumors in Rb null mice with chimerism as low at 5% (Parisi et al., 2007). The function of E2F4 in cell division is thus still unclear; in vitro E2F4 acts as a proliferation inhibitor, while in vivo E2F4 functions to promote pituitary tumorigenesis. We believe that two models may reconcile these results. The first, called the "pocket protein reshuffling" model, stems from our finding that p107 and p130, normally unable to bind to the activator E2Fs, associate with these proteins in Rb;E2f4 deficient tissues (Lee et al., 2002). These novel repressor complexes could prevent E2F1, E2F2 and E2F3 from activating target genes responsible for cell proliferation thus inhibiting tumor formation in $Rb^{+/-};E2f4^{-/-}$ mice. The second model proposes that E2F4 functions as a transcriptional activator in certain contexts, including Rb mutant tumors, and this explains its oncogenic activity. E2F4 does in fact have a strong activation domain and it is certainly capable of activating E2F responsive promoters when localized to the nucleus by over-expression or addition of nuclear localization signals (DeGregori et al., 1997; Verona et al., 1997). Notably, we have recently found that E2F4 associates with the promoter of E2F-responsive genes in tumor cells, concordant with their transcriptional activation, raising support for this hypothesis (Iaquinta and J. A. L., unpublished). Based on our in vivo observations (Lee et al., 2002; Iaquinta and J. A. L., unpublished), we suspect that both the pocket protein reshuffling and transcriptional activation mechanisms contribute to E2F4's

oncogenic properties. Our chimeric system does not address these two possibilities, and additional experiments will be required to tease out the underlying mechanisms.

Our data also show that Rb$^{-/-}$ and Rb$^{-/-}$;E2f4$^{-/-}$ chimeric mice developed thyroid tumors at a similar frequency. This is in contrast to what previously found in Rb$^{+/-}$;E2f4$^{-/-}$ germline animals where the thyroid tumors are fully suppressed (Lee et al., 2002). It is hard to envisage how either the reshuffling or transcriptional activation mechanisms could account for tumor suppression in Rb$^{+/-}$;E2f4$^{-/-}$ germline mice, but not in Rb$^{-/-}$;E2f4$^{-/-}$ chimeras. Thus, two possibilities remain to explain the specific suppression of tumors in the Rb$^{+/-}$;E2f4$^{-/-}$ thyroids. One is that E2f4-deficiency may decrease the rate of Rb LOH in the c-cells of Rb$^{+/-}$ mice. The other is that cell non-autonomous effects may operate in Rb$^{+/-}$;E2f4$^{-/-}$ germline. These current and past findings highlight the utility of employing chimeric mouse models to identify direct requirements for particular E2Fs in tumorigenesis. Interpretation of data generated in traditional germline mice is confounded by possible non-cell autonomous effects as well as a requirement for Rb LOH. In fact, while previous Rb;E2f germline mutant mice assigned opposing roles for E2f3 and E2f4 in the development of thyroid tumors (Lee et al., 2002; Ziebold et al., 2003) our studies in chimeric mice suggest that these E2fs are both fully dispensable for this tumor type (Parisi et al., 2007).

Irrespective of the mechanisms that operate in the pituitary and the thyroid, Rb$^{-/-}$;E2f4$^{-/-}$chimeric mice allowed the examination of the effects that E2f4-loss has on other tumor types present in Rb$^{-/-}$ chimeras. These animals develop neuroendocrine lesions in the adrenal gland and in the lung (Parisi et al., 2007; Williams et al., 1994) and we find that E2f4 differentially affects these tumor types. It is dispensable for the adrenal gland tumors, while is required for the hyperplasia of the neuroendocrine cells. The latter lesions are believed to be the precursors of small cell lung carcinomas, a highly metastatic and therefore lethal tumor type (Meuwissen et al., 2003). Therefore, our findings indicate that E2f4 may play a role in the hyperplastic stage of small cell lung carcinomas. Interestingly this property is not unique to E2F4 as Rb$^{-/-}$;E2f3$^{-/-}$ chimeric mice have no detectable signs of neuroendocrine lung hyperplasia (Parisi et al., 2007). Thus, this suggests that E2F3 and E2F4 play a prominent role in the hyperplastic stage of small cell lung carcinoma. As with the pituitary, The E2F4's oncogenic function could result from the "pocket protein reshuffling" and/or the "activating" function mechanisms described above.

The extended longevity of Rb$^{-/-}$;E2f4$^{-/-}$ chimeric mice due to suppression of pituitary tumors allowed us to identify two novel tumor types in these mice, ganglionic neuroendocrine neoplasms and urethelial cancer. The presence of ectopic neuroendocrine cells within ganglions confirms once more that Rb plays a key role in neuroendocrine lineage, which include all the tumor types listed above, and has very interesting implications for the biology of these cells. During development neuroendocrine and neuronal cells share a common progenitor, called sympathoadrenal cells that originate from the neural crest (Huber, 2006). This progenitor cell migrates from the dorsal aorta to its final destination where it adopts either a neuronal or neuroendocrine fate. The mature neuroendocrine cells maintain the expression of markers specific of the immature common progenitors while the mature neurons downregulate these markers. Thus, we speculate that the neuroendocrine-like neoplastic cells that we found within the ganglia of Rb$^{-/-}$;E2f4$^{-/-}$ chimeras are not derived

from neurendocrine cells, but represent immature, neural crest-like stem cells that either failed to differentiate into neurons or represent a physiological population of neuronal stem cells that proliferate inappropriately. Unfortunately, to our knowledge there are no markers that discriminate between a blastic stage neuronal cell and a differentiated neuroendocrine cell, and would therefore allow us to show that the lesions found in the ganglia of $Rb^{-/-};E2f4^{-/-}$ chimeras are neuronal stem cell-like cells. Nevertheless, as the determinant factors that govern the fate choice are still not well understood, our findings suggest that Rb and E2f4 play a role in the development of the neuronal-neuroendocrine cells and may potentially add another tile to the complex mosaic that specifies the sympathoadrenal lineage.

The other novel tumor in $Rb^{-/-};E2f4^{-/-}$ chimeric animals is urothelial transitional cell carcinoma. Urothelial carcinomas are tumors of the urogenital tract and represent the fifth most common cancer type in humans (Dinney et al., 2004). They manifest as two variants, papillary or non-papillary. The papillary form accounts for about 80% of urethelial cancers, is generally low grade and has been documented to give rise to invasive transitional cell carcinoma in 15% of cases of human bladder cancers. The non papillary transitional cell carcinoma, is high grade, accounts for the remaining 20% cases of human urethelial carcinomas, and originates de novo or from preexisting carcinoma in situ. The observation that this non papillary form is present in patients with no previous history of papillary carcinomas, and the fact that the non papillary and papillary forms have a distinct genetic signature, has lead to the hypothesis that these two types of transitional cell carcinoma are unrelated (Wu, 2005; Schulz, 2006). There is a strong correlation between Rb mutations and urothelial cancer. In humans Rb has been found inactivated in about 60% cases of human bladder cancer, although there is still a debate as to whether Rb mutation is associated with the low grade, non papillary form (Dinney et al., 2004; Wu, 2005). Despite the strong association between Rb mutations and urothelial cancer, only one animal model has investigated the role of pRB in this tumor type. In this mutant mouse Rb and p53 are both inactivated in urothelial cells by transgenic expression of the SV40 Large T antigen (Zhang et al., 1999), and this causes invasive carcinoma in situ. In $Rb^{-/-};E2f4^{-/-}$ chimeras we found both invasive and non invasive papillary carcinomas, suggesting that Rb mutation may facilitate the switch from low grade to dysplastic, high grade tumors. Thus, together the Large T antigen and Rb;E2f4 mouse models recapitulate the non papillary and papillary variants of human transitional cell carcinomas and implicate a key role for Rb in the development of these tumors.

To conclude, we have learnt from the analysis of $Rb^{-/-};E2f4^{-/-}$ chimeric animal that E2f4 plays a role in the pituitary tumors as well as in lung neuroendocrine hyperplasia caused by loss of Rb, but not in thyroid and adrenal gland tumors. In addition our chimeric mouse model gives us the opportunity to identify pRB and E2F unknown functions. We have shown here that E2f4 and Rb have a role in derivatives of neural crest cells and in the urogenital epithelium. We are now pursuing the significance of these findings by generating E2f4 conditional mice. We will use these mice in combination with the Rb conditional-urethelial or neural crest specific Cre-Recombinase expressing mice to dissect the relative roles of Rb and E2f4 in these tissue types and to generate novel cancer mouse models.

MATERIAL AND METHODS

Generation of ES cells and chimeric animals

Mouse colonies were maintained in compliance with Institutional Animal Care and Use Committee guidelines. E2f4 and Rb mutant mice, as well as the primers used for genotyping were previously described (Humbert et al., 2000a; Jacks et al., 1992). $E2f4^{+/-}Rb^{+/-}$ 129/Sv mice were crossed to 129/Sv Rosa β-geo 26 (Rosa26) animals (Friedrich and Soriano, 1991). The $E2f4^{+/-}Rb^{+/-}$ Rosa26 mice so obtained were mated and the females sacrificed at 3.5 days post-coitum. ES cells were derived by E3.5 blastocysts as previously described (Parisi et al, 2007). $E2f4^{-/-}Rb^{-/-}$ Rosa26 chimeric mice were generated by injecting 10-12 mutant ES cells into C57Bl/6 blastocysts.

Histology, immunohistochemistry and X-gal staining

Tissues were processed for X-gal staining or directly fixed in phosphate-buffered formalin as previously described (Parisi et al., 2007). To visualize the pituitary glands, adult heads were fixed for a week in Bouin's fixative. 5μm sections of paraffin embedded tissues were stained with H&E, or processed for immunohistochemistry. For antibody staining sections were processed as previously described (Parisi et al, 2007) with 1:500 mouse anti-synaptophysin (Chemicon, clone SY38), and 1:5000 rabbit anti-neuroendocrine cell marker calcitonin-related peptide (anti-CGRP, Sigma). Antigen-antibody complexes were detected with diaminobenzidine (DAB).

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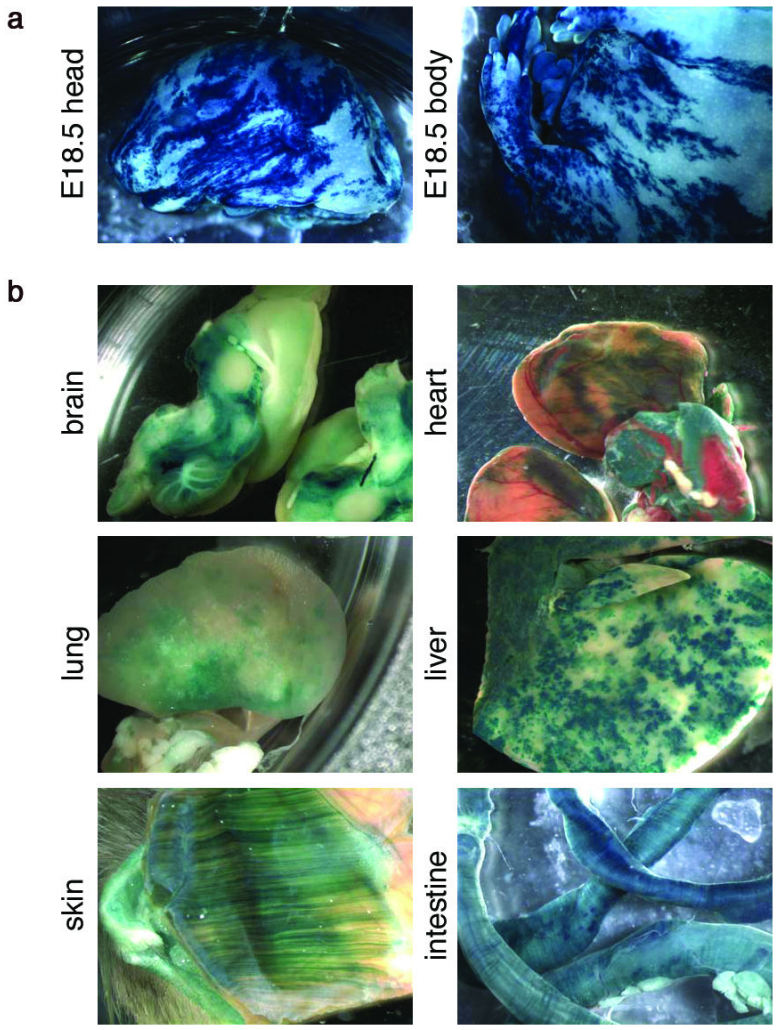

Figure 1.

$\underline{Rb^{-/-};E2f4^{-/-}}$ ES cells contribute to chimeric embryos and to adult tissues. (**a**) E18.5 $\underline{Rb^{-/-};E2f4^{-/-}}$ embryos stained for β-galactosidase activity (blue color) show high levels of chimerism. (**b**) $\underline{Rb^{-/-};E2f4^{-/-}}$ ES cells contribute to a wide variety adult tissues as shown by β-galactosidase activity (blue color) in representative examples.

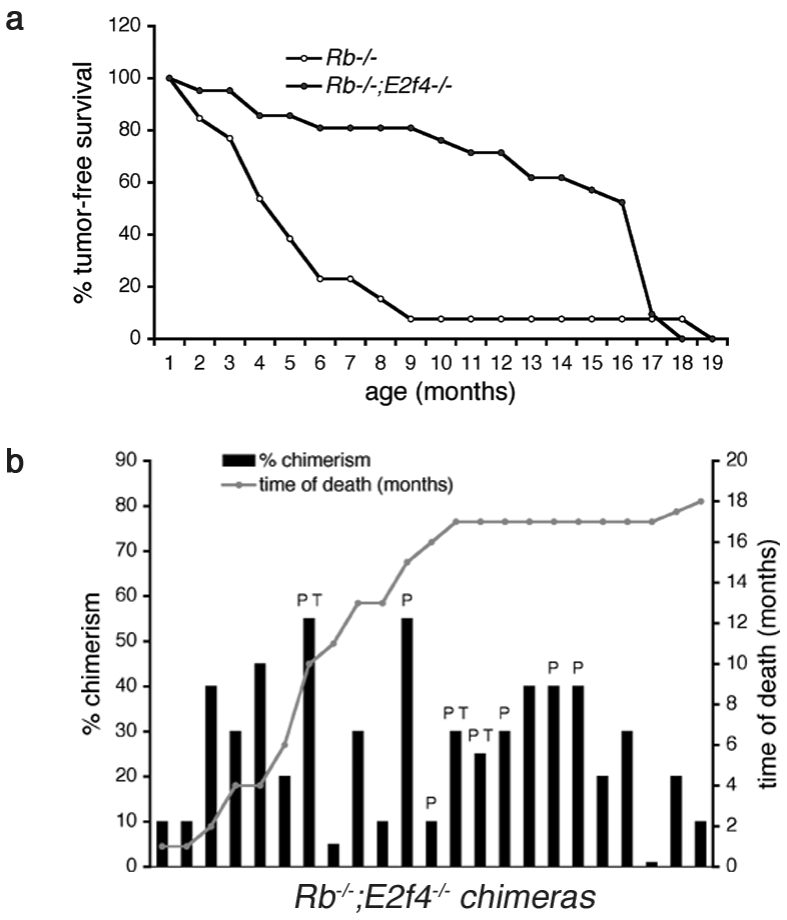

Figure 2.

E2f4-loss increases the lifespan of $Rb^{-/-}$ chimeras by delaying the onset and the incidence of pituitary tumors. (**a**) Kaplan-Meyer curve showing a significant difference in survival of $Rb^{-/-};E2f4^{-/-}$ versus $Rb^{-/-}$ chimeras. (**b**) Schematic representation of the relations between percentage of chimerism, time of death and tumors found in $Rb^{-/-};E2f4^{-/-}$ chimeric animals. Each chimeric animal is represented by a bar reporting the percent chimerism, and is arranged according to the time of death (from youngest to oldest, grey line, second Y axis). Animals presenting pituitary or thyroid tumors are indicated with (P) and (T) respectively. Note that the pituitary tumors manifest only after 10 month of age.

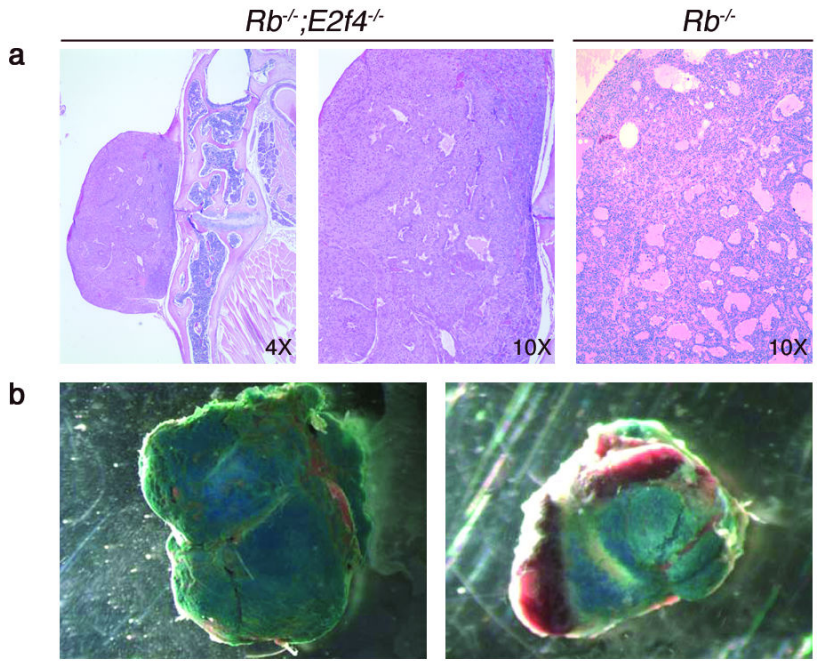

Figure 3.

The pituitary tumors that do develop in $Rb^{-/-};E2f4^{-/-}$ chimeric pituitaries are morphologically similar to the ones that originate in $Rb^{-/-}$ chimeras and exclusively derive from mutant cells. (**a**) Histological analysis of $Rb^{-/-}$ and $Rb^{-/-};E2f4^{-/-}$ pituitary tumors shows that these tumors are adenocarcinomas and arise from the intermediate lobe of the pituitary. (**b**) β-galactosidase staining (blue color) of pituitary tumors derived from $Rb^{-/-};E2f4^{-/-}$ indicates that these tumors specifically derive from mutant cells.

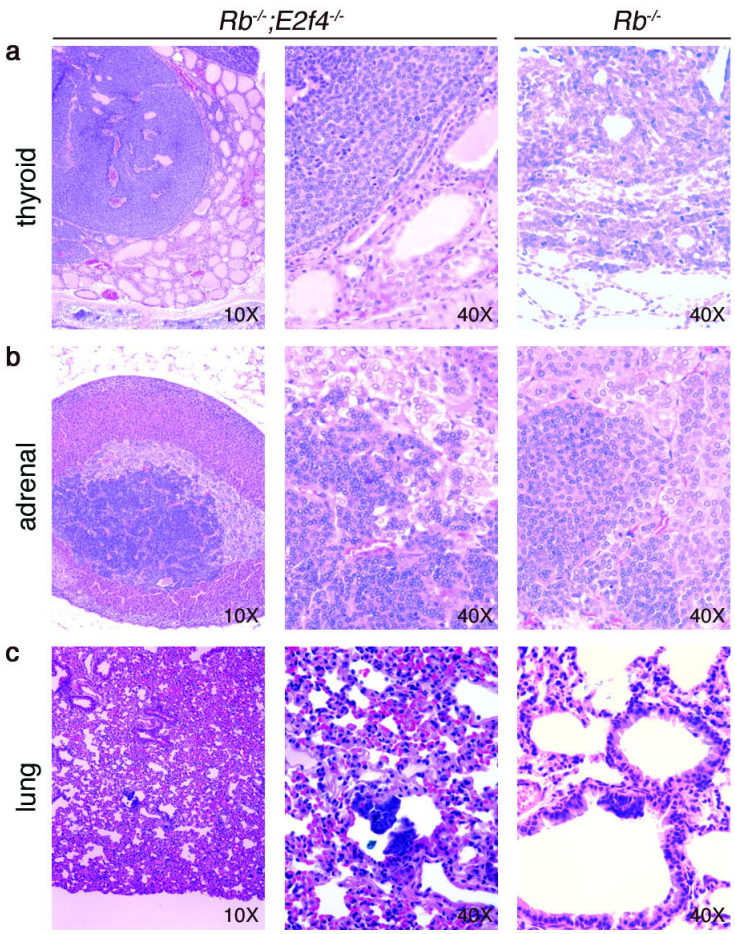

Figure 4.

E2f4-loss partially suppresses pulmonary neuroendocrine hyperplasia while being dispensable for thyroid and adrenal glands tumors that arise in $Rb^{-/-}$ chimeras (a-c). H&E staining of sections containing the various neuroendocrine tumors found in $Rb^{-/-}$ and $Rb^{-/-}$;$E2f4^{-/-}$ chimeric mice (**a**) c-cell carcinoma of the thyroid, (**b**) tumor in the adrenal gland medullaand (**c**) hyperplastic lung neuroendocrine cells also showing positivity for β-galactosidase activity (blue color).

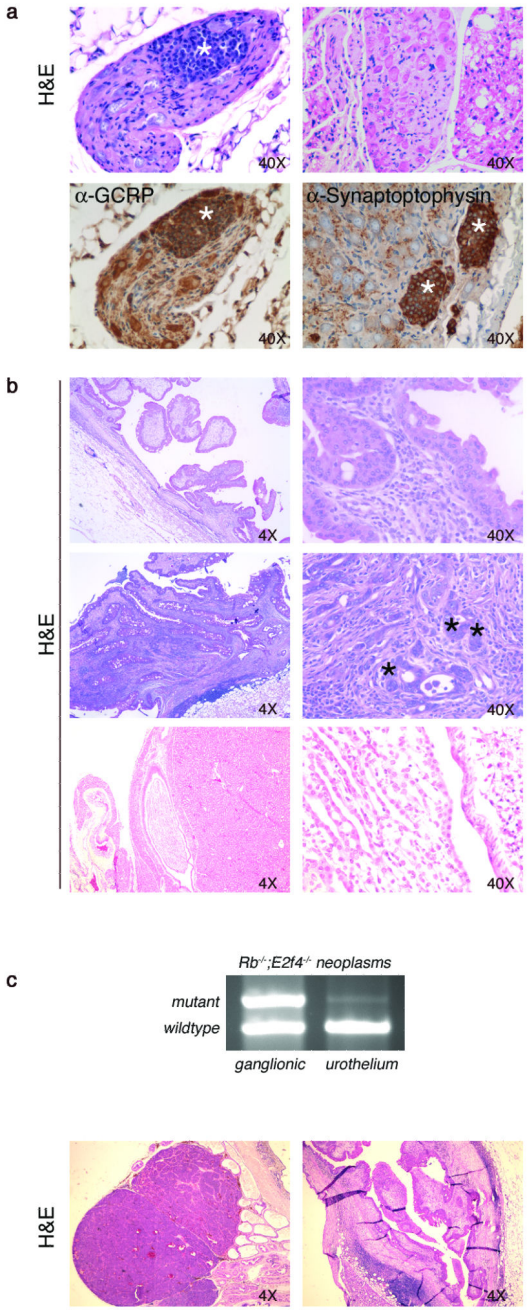

Figure 5.

E2f4-loss causes the appearance of novel tumors in $Rb^{-/-}$ chimeric mice. (**a**) Ganglionic neuroendocrine neoplasms found in $Rb^{-/-};E2f4^{-/-}$ chimeras. H&E staining of ganglia containing a very dark group of neuroendocrine-like cells in $Rb^{-/-};E2f4^{-/-}$ chimeras (top left, asterisk) but not in $Rb^{-/-}$ chimeras (top right). Bottom panel: examples of ganglionic neuroendocrine neoplasms (asterisks) showing strong positivity for the neuroendocrine marker GCRP and Synaptophysin. Note that the positivity to GCRP and Synaptophysin also extends to the neurons. (**b**) Loss of E2f4 leads to urothelium transitional cell carcinomas in

$Rb^{-/-}$ chimeric mice. Polyps originating from the urogenital epithelium protrude into the lumen of the ureter to give rise to non invasive (top panel) and invasive papillary carcinomas (middle panel) where the epithelium has penetrated the muscle wall (asterisks). The urothelium of $Rb^{-/-}$ chimerics is completely normal (bottom panel). (**c**) The novel tumors derive from $Rb^{-/-}$;$E2f4^{-/-}$ cells. Top: PCR analysis of genomic DNA isolated from the ganglionic and urothelium tumors (shown below) and assayed for the presence of the mutant and wildtype allele of E2f4. Note that the ganglionic lesion is very homogeneous compared to the urothelium polyps where the epithelium represents only a small fraction of the tumor.

TABLE 1

Tumorigenic phenotypes in Rb-/- versus Rb-/-;E2f4-/- chimeric mice.

	$Rb^{-/-}$ %, N animals	**$Rb^{-/-}$;$E2f4^{-/-}$ %; N animals**
Average chimerism	62%; 13	28%; 21
Pituitary tumors	69%; 13	38%; 21
Thyroid tumors	31%; 13	16%; 19
Adrenal gland lesions	58%; 12	67%; 18
Pulmonary neuroendocrine cell hyperplasia	77%; 13	20%; 20
Neuroendocrine ganglionic neoplasms	0%; 12	30%; 20
Uroepithelium lesions	0%; 12	15%; 20