HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2016 May 18.

Published in final edited form as:

Oncogene. 2015 November 26; 34(48): 5890–5899. doi:10.1038/onc.2015.30.

Inactivation of the retinoblastoma gene yields a mouse model of malignant colorectal cancer

Tiziana Parisi[1], **Roderick T. Bronson**[2], and **Jacqueline A. Lees**[1,3]

[1]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[2]Department of Pathology, Tufts Cummings School of Veterinary Medicine, North Grafton, MA 01536, USA

[3]Dept of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Abstract

The retinoblastoma gene (*Rb*) is mutated at significant frequency in various human epithelial tumors, including colorectal cancer, and is strongly associated with metastatic disease. However, sole inactivation of *Rb* in the mouse has so far failed to yield epithelial cancers. Here, we specifically inactivate *Rb* and/or *p53* in the urogenital epithelium and the intestine. We find that loss of both tumor suppressors is unable to yield tumors in the transitional epithelium lining the bladder, kidneys and ureters. Instead, these mice develop highly metastatic tumors of neuroendocrine, not epithelial, origin within the urogenital tract to give prostate cancer in the males and vaginal tumors in the females. Additionally, we discovered that the sole inactivation of *Rb* in the intestine was sufficient to induce formation of metastatic colorectal adenocarcinomas. These tumors closely mirror the human disease in regard to age of onset, histological appearance, invasiveness and metastatic potential. Like most human colorectal carcinomas, our murine *Rb*-deficient tumors demonstrate genomic instability and they show activation of β-catenin. Deregulation of the Wnt/β-catenin pathway is specific to the intestinal tumors, as genomic instability but not activation of β-catenin was observed in the neuroendocrine tumors. To date, attempts to generate genetically engineered mouse models of colorectal cancer tumors have yielded mostly cancer of the small intestine, which rarely occurs in humans. Our system provides the opportunity to accurately model and study colorectal cancer in the mouse via a single gene mutation.

Keywords

Rb; colorectal cancer; intestine; neuroendocrine; bladder; β-catenin

Corresponding Author: Jacqueline A. Lees, ; Email: jalees@mit.edu, **Tel:** 617 252 1972, **Fax:** 617 253 9863, **Mail:** MIT, 77 Massachusetts Avenue, Building 76-461B, Cambridge, MA 02139

CONFLICT OF INTEREST: The authors declare no financial conflict of interest.

Supplementary Material has been submitted.

INTRODUCTION

Inactivation of the retinoblastoma gene occurs in a variety of human cancers including retinoblastoma, small cell lung carcinomas, osteosarcomas, bladder cancer, prostate cancer and a subset of colorectal cancers[1]. However, while mouse models have established a causal correlation between *Rb* mutation and multiple cancers, the role of this tumor suppressor in epithelial cancer development is unclear. In the mouse, germline *Rb* loss causes embryonic lethality, while inactivation of one *Rb* allele leads exclusively to neuroendocrine tumors of the pituitary and thyroid[2]. Similarly, chimeras bearing *Rb* null cells develop only neuroendocrine tumors[3, 4]. This narrow tumor spectrum suggests that transformation of other cell types requires additional mutations, or that the short lifespan of the neuroendocrine tumor-bearing *Rb* mutant mice (one year maximum) precludes the possibility of slower developing tumors.

The tumor suppressive activity of the retinoblastoma protein, pRB, is at least partially dependent upon its ability to inhibit the E2F transcription factors and thereby prevent cell cycle entry[5]. By generating $Rb^{-/-}$;$E2f4^{-/-}$ chimeric animals, we have previously shown that *E2f4* loss inhibits early onset pituitary tumors, and increases the lifespan of *Rb* mutant animals[6]. Moreover, these older animals developed additional tumor types dependent on *Rb* loss. Specifically, 15% of $Rb^{-/-}$;$E2f4^{-/-}$ chimeras sixteen months or older had cancer of the urothelium, which is the transitional epithelium lining the bladder, ureters and kidney calyces. Notably, we never observed urothelial tumors in $Rb^{+/-}$;$E2f4^{-/-}$ or $E2f4^{-/-}$ germline mice[7, 8]. Thus, we hypothesized that *E2f4* loss is not a direct driver of urothelial tumors but acts instead to extend the lifespan of $Rb^{-/-}$ chimeras and enable formation of slower developing *Rb* mutant bladder tumors. Importantly, over 50% of human urothelial cancers display *Rb* mutations[9], and our analysis of $Rb^{-/-}$;$E2f4^{-/-}$ chimeras provided the first evidence that *Rb* loss is a driver of this tumor type.

Urothelial (or bladder) cancer does not spontaneously occur in mice and is still very understudied due to the difficulties in creating valid models that do not involve surgery[10, 11]. Given the complexity of the $Rb^{-/-}$;$E2f4^{-/-}$ chimeras, we wished to generate a more tractable model of *Rb* mutant urothelial cancer. Previously, He et al. used Uroplakin-Cre to delete *Rb* and also *p53* (mutated in more than 50% of human urothelial cancers), and found no bladder cancer[12]. Since Uroplakin-Cre is expressed at low penetrance in the bladder, we reasoned that a stronger *Cre* might boost the frequency of *Rb* and *p53* mutation and thus the possibility of transformation. Thus, in this study, we crossed *Rb* and *p53* conditional mutant mice with the *Fabpl-Cre* transgene. This is expressed very efficiently in urothelial cells, and is also active in the prostate and the intestinal epithelium[13]. Notably, human data indicate that a small but significant fraction of colorectal cancers carry *Rb* mutation (0.5–4%) but the significance of this is unclear[14, 15]. Our analysis of this new mouse model shows that inactivation of *Rb* and *p53* is insufficient to initiate tumorigenesis in the urogenital epithelium even when nearly every cell is mutated. Instead, double mutant animals develop highly metastatic neuroendocrine tumors in the prostate and the female reproductive tract. Finally, we discovered that *Rb* mutation is sufficient to initiate intestinal tumors that closely resemble human colorectal cancer. This reveals a fundamental role for this tumor suppressor in the intestine, and provides a novel model system to study colon cancer.

RESULTS

Combined loss of Rb and p53 leads to urogenital neuroendocrine metastatic cancer

To assess the contribution of *Rb* to the tumorigenesis of multiple epithelia, we used the *Fabpl-Cre* transgenic in which *Cre* recombinase is driven by the liver fatty acid binding protein promoter. Previous reports indicate that this transgene is expressed in the urothelium, intestine and anterior prostate[13, 16]. In agreement, by crossing *Fabpl-Cre* to mice carrying a conditional *LacZ* reporter allele, we observed transgene expression at nearly 100% penetrance in the transitional epithelium cells lining the kidneys and the bladder (supplementary Fig. 1). Additionally, we observed *LacZ* expression in most of the colon (large intestine), in limited areas of the small intestine (mostly the distal part), and in the dorsal prostate (supplementary Fig. 1). We crossed *Fabpl-Cre* with *Rb* and *p53* conditional mice (respectively $Rb^{c/c}$ and $p53^{c/c}$) to generate cohorts of animals lacking *Rb*, *p53* or both genes, which were aged alongside appropriate controls ($Rb^{c/c}$, $p53^{c/c}$, and $Rb^{c/c}p53^{c/c}$ without *Fabpl-Cre*). Between six and eight months, 7/8 $FabplCre^{tg/+}Rb^{c/c}p53^{c/c}$ males had to be euthanized (Fig. 1a, grey lines). Gross and microscopic analysis revealed that these animals all displayed highly aggressive prostate tumors that had colonized lungs, liver and kidneys (Fig. 1b). *Rb* mutations exist in over 30% of human prostate cancers, and they associate strongly with androgen therapy resistance[17]. Human prostate tumors are typically epithelial, but often contain a neuroendocrine component that correlates with androgen therapy resistance, and a subfraction of human tumors are exclusively neuroendocrine[18]. *Probasin-Cre* induced deletion of *Rb* and *p53* in the mouse prostate was found to yield malignant tumors co-expressing neuroendocrine and epithelial markers[19]. In contrast, the histological appearance (Fig. 1b) and staining for neuroendocrine (chromogranin A) and prostate epithelial (keratin 17/19) cell markers (Fig. 1c), showed that all but one of the tumors in our model were neuroendocrine. Likewise, morphological and immunochemical analyses detected exclusively neuroendocrine cells in the metastases (not shown). Since the presence of neuroendocrine cells in human prostate tumors associates with androgen therapy resistance, we examined the levels of androgen receptor (AR) in our *Rb;p53* deficient tumors and metastases. We found that 3/8 expressed physiological levels of this receptor and 5/8 tumors showed low or no AR expression (Fig. 1d). Furthermore, all the metastases, even ones originating from AR positive tumors, had downregulated AR expression (Fig. 1d). Therefore, these tumors model neuroendocrine prostate tumors, the subset of human cancers that is both metastatic and therapy resistant.

Neuroendocrine tumors also appeared to be the primary cause of death of $FabplCre^{tg/+}Rb^{c/c}p53^{c/c}$ females, between eight and fourteen months (Fig. 1a, black lines). Histological and immunochemical analysis showed these tumors originated in the vagina and spread to lung, liver and kidneys (Fig. 1e). There are no previous reports of *Cre* expression from this transgene in neuroendocrine vaginal cells. Thus, there must be rare and scattered neuroendocrine cells in the female genital tract, underscoring the extreme sensitivity of this cell type to transformation upon concomitant *Rb* and *p53* loss.

Rb and p53 deficiency is insufficient for urothelial carcinogenesis

Histological examination of the *FabplCre*$^{tg/+}$*Rb*$^{c/c}$*p53*$^{c/c}$ mutant males showed complete absence of tumors in the transitional epithelium that lines bladder, ureters and kidneys. This finding was unsurprising since the vast majority of these animals succumbed before eight months, and we have previously shown that *Rb*$^{-/-}$;*E2f4*$^{-/-}$ chimeras do not display detectable tumors until sixteen months[6]. However, given the high penetrance of the *FabplCre*-driven recombination, we were disappointed by the complete absence of either malignant or benign bladder tumors in the mutant females, since these lived up to fourteen months. Instead, we found that the males and females both developed atypia of the umbrella cells along the entire urothelium with 94% penetrance (Fig. 2a). These cells had much bigger nuclei and higher DNA content than their corresponding controls. This phenotype had been previously described in the *Uroplakin-Cre* transgene model although, consistent with the lower expression of *Cre* in the urothelium, the authors detected umbrella cell atypia in only 20% of the *Rb*$^{-/-}$*p53*$^{-/-}$ mutant animals[12]. Histological analysis of bladders harvested from *p53* and *Rb* single deficient animals showed that this neoplasm arose from mutation of *p53* alone (11 out of 13 animals), but not *Rb* alone (0 out of 26 animals), although combined mutations of *Rb* and *p53* clearly increased its severity (Fig. 2a). The physiological implication of the umbrella cells atypia remains unclear as *p53* single mutants lived 22 months on average and showed no signs of urogenital disease (Fig. 2b). These results suggest that in our previous *Rb*$^{-/-}$;*E2f4*$^{-/-}$chimeric model, loss of E2F4 was playing a contributing role in the development of *Rb*-deficient urothelial tumors.

Inactivation of Rb causes development of intestinal adenocarcinomas

Despite the lack of urothelial tumors, *FabplCre*$^{tg/+}$*Rb*$^{c/c}$ mice died on average six months earlier than their corresponding controls (Fig. 3a). The primary cause of death was masses that occluded the intestinal tract in 11 out of 26 mice. These malignancies were found typically in twelve to eighteen months old mice, independent of gender, and were located in the small intestine (10/26; 38% of animals) and colon (6/26; 23% of animals). With the exception of a few relatively benign adenomas, these tumors were aggressive adenocarcinomas (Table 1). Indeed, most of the *FabplCre;Rb*$^{c/c}$ mice bore multiple tumors with pathological stages varying from well-circumscribed polyps (stage II) to moderately or poorly differentiated tumors invading through the submucosa and the muscle wall (stage III and IV, Fig. 3b,c, Table 1). Importantly, we detected tumors that had traveled through mesenteric lymphatics and blood vessels and entered the hepatic portal system to colonize the liver, indicating that the mutant cells were metastastic (Fig. 3d). Prior studies, where *Rb* was deleted in the intestine using *VillinCre* and *FabplCre* transgenes, reported that *Rb* mutation alone is insufficient to cause cancer in animals younger than one year (Ref [20, 21] and Haigis, personal communication). We only detect intestinal tumors after this time point, explaining this apparent discrepancy. Interestingly, this time of onset essentially mirrors that at which humans sporadically develop the disease, around or after middle age. Most importantly, cancer of the colon has been very difficult to model genetically in mice. With few exceptions, existing models develop tumors in the small intestine, not the large intestine. Furthermore, those lesions are usually benign and unable to metastasize[22]. Our studies show for the first time that *Rb* ablation alone can cause aggressive, metastatic tumors in the intestine that closely mirror the human disease thus providing a model for colorectal cancer.

p53 inactivation is not a prerequisite for the development of Rb-deficient intestinal tumors

The late onset of intestinal tumors in *Rb*-deficient mice suggests that, similar to human colon cancers, multiple mutations are required for tumor development. As *p53* is mutated in about 50% of human colon cancers, we analyzed $Rb^{-/-}$;$p53^{-/-}$ double and $p53^{-/-}$ single mutants for evidence of intestinal tumors. In agreement with a previous study[23], we observed no intestinal tumors in the $p53^{-/-}$ single mutants, even though they lived 22 months on average, indicating that *p53* loss is insufficient to initiate intestinal tumorigenesis. We did observe a single tumor in three of the sixteen $Rb^{-/-}$;$p53^{-/-}$ animals: a high-grade adenocarcinoma of the small intestine (PT IV), a low-grade adenocarcinoma of the large intestine (PT II), and a benign polyp (data not shown). This low incidence suggested that *p53* inactivation does not obviously accelerate intestinal tumors arising from *Rb* loss. To test this hypothesis, we screened the *Rb* single mutant intestinal tumors for the presence of p53. Remarkably, every one (n=53) retained p53, and those examined showed concomitant expression of its downstream target $p21^{Cip1}$ (Fig. 4). These findings strongly suggest that the *Rb* mutant tumors express functional p53 and therefore lack any selective pressure to inactivate this tumor suppressor. Altogether, our data argue that inactivation of *Rb*, and not *p53*, is promoting intestinal tumors in our model.

Genomic instability in Rb-deficient tumors

Human colorectal cancers are very heterogeneous and they accumulate mutations mostly through two types of genomic instability: microsatellite instability (MIN) or chromosomal instability (CIN). Microsatellite instability leads to amplification of short repetitive DNA sequences caused by loss of function mutations in mismatch repair (MMR) genes. Inactivation of MMR genes can occur sporadically in 15% of colorectal cancers resulting from somatic mutations of the MLH1 gene or germline mutations of the genes MSH2, MSH6 or MLH1 genes[24, 25]. In contrast, chromosomal instability occurs in the majority of human colon cancers, and involves whole chromosome gain or loss that is typically associated with large chromosomal rearrangements[24, 25]. Notably, *Rb*-deficiency has been shown to cause chromosomal instability by altering chromosome condensation and cohesion[26–28]. Thus, we hypothesized that *Rb* loss might contribute to the development of intestinal tumors in our *FabplCre;*$Rb^{c/c}$ mice by promoting CIN and thus genomic instability. Since MIN and CIN pathways both lead to double stranded DNA breaks that can be identified via phosphorylated H2AX (γ-H2AX), an early marker of DNA damage, we performed immunochemical analysis of γ-H2AX on our tumor sections. This revealed DNA breaks in the tumorigenic intestinal epithelium, which supports the presence of genomic instability and explains p53 activation in the tumors (Fig. 4, left panels). To address CIN versus MIN, we examined the expression of MLH1 (that is in sporadic MIN) by immunohistochemistry. We readily detected MLH1 protein in the tumors as well as in *Rb*-deficient normal epithelium indicating that the mutant cells are unlikely to undergo genomic instability through the mechanism of microsatellite instability (Fig. 4, middle panels). On the contrary, the presence of high levels of MLH1 protein, together with upregulation of another component of the MMR system, MSH2, in our *Rb*-deficient intestinal tumors, indicates that the function of mismatch repair genes is activated rather than lost (Fig. 4, right panels). This is consistent with reports of other human tumor types, where the mismatch repair genes are upregulated upon DNA damage caused by genomic rearrangements. Therefore, altogether

our results strongly suggest that *Rb*-deficient intestinal tumors undergo genomic rearrangements through chromosomal instability.

Activation of the Wnt pathway in Rb-deficient intestinal tumors

A nearly universal feature of human colorectal cancers is deregulation of the Wnt signaling pathway with consequent activation of β-catenin. This event typically occurs through direct mutation of the β-catenin gene or through truncating mutations of APC. These mutations allow β-catenin to translocate to the nucleus and cooperate with the TCF/LEF transcriptional factors to activate genes responsible for cell transformation[29]. To assess whether *Rb* loss impinges on the Wnt pathway, we analyzed the localization of β-catenin in the intestinal tumors by immunohistochemistry. We found that indeed *Rb* inactivation influences β-catenin status. As expected, β-catenin was localized to the cell membrane in the non-tumorigenic *Rb*-deficient intestinal epithelium, but it showed a highly heterogeneous pattern in the tumors. Analysis of both small and large intestinal tumors (n=35) identified regions covering 5–80% of the cross-sectional area where areas of membranous β-catenin co-exist with areas of nuclear and/or cytoplasmic accumulation (Fig. 6a). This pattern has been described in human colorectal cancer where the presence of diffuse nuclear+cytoplasmic or nuclear β-catenin is associated with invasive or potentially invasive regions of the tumor and is predictive of poor prognosis[30]. In agreement, immunochemical analysis of cells colonizing mesenteric lymph nodes shows diffuse nuclear+cytoplasmic β-catenin staining (Fig. 6a). Notably, all the tumors showed upregulation of Sox9, a known β-catenin-TCF target[31], confirming that the Wnt pathway is activated in *Rb*-deficient intestinal tumors (Fig. 6b). To determine if activation of the Wnt pathway resulted from loss of the β-catenin upstream regulator APC, we examined the levels of APC in the intestinal tumors. Immunochemical analysis revealed that the presence or absence of APC was a poor predictor of β-catenin status in *Rb*-deficient tumors (Fig. 6c). Specifically, in a subset of tumors (n=19/35) absence of the APC protein coincided with activated β-catenin, but other tumors and metastases (n=16/35) retained, or partially retained, APC expression despite the presence of nuclear/cytoplasmic β-catenin (Fig. 6c). Moreover, irrespective of the levels of APC expression, we found that nuclear β-catenin consistently co-localized with areas of DNA damage and DNA repair (Fig. 6c). This indicates the Wnt pathway is typically activated within the *Rb*-deficient intestinal tumors, but the underlying mutations vary, presumably as a result of the ensuing genomic instability. *KRas* is also frequently activated in early stages human colorectal cancer[25]. To determine whether this occurs in our *Rb* deficient intestinal tumors, we screened for the presence of phosphorylated MEK1 (P-MEK1 T286), and showed that this marker of *kRas* signaling was upregulated in 27/40 tumors analyzed (Fig. 6d). Taken together, these data show that our *Rb* deficient intestinal tumors have upregulated signaling pathways, including Wnt/β-catenin and *kRas*, similar to human colorectal tumors.

Nuclear accumulation of β-catenin is specific to Rb-deficient intestinal, not neuroendocrine tumors

We wanted to establish whether deregulation of β-catenin is strictly associated with the onset of intestinal tumors or represents a more general phenomenon related to *Rb* inactivation. Thus, we analyzed the $Rb^{-/-}p53^{-/-}$ mutant neuroendocrine tumors (n=8) and found that they all lacked detectable nuclear β-catenin, and generally retained both membranous β-catenin

and detectable APC protein (Fig. 7a, b). This indicates that the Wnt/β-catenin pathway remains intact in the neuroendocrine tumors, while being abrogated in the intestinal tumors. By extension of this logic, it appears that distinct oncogenic signaling events are driving the *Rb*-intestinal versus the *Rb;p53*-deficient neuroendocrine tumors. Importantly, our analyses showed that the neuroendocrine tumors display high levels of MLH1 and MSH2 expression in a comparable manner to the intestinal tumors (Fig. 7b), arguing that the MMR pathway is activated in both settings. Accordingly, we found that the neuroendocrine tumors also contained areas positive for γ-H2AX, revealing the presence of DNA damage (Fig. 7b). These results strongly suggest that genomic instability represents a common event in the *Rb*-deficient tumors, but loss of *Rb* in neuroendocrine versus intestinal epithelial cells must activate distinct molecular pathways to induce tumorigenesis.

DISCUSSION

This study provides key insights into the consequences of *Rb* loss into a subset of epithelial tissues. We show that ablation of *Rb*, with or without *p53* mutation, is unable to initiate cancer of the murine urothelium. Conversely, simultaneous inactivation of these proteins causes rapid onset of metastatic prostate and vaginal cancer. Most importantly, our model system yields the first evidence that *Rb* inactivation is sufficient to promote intestinal tumorigenesis. Below, we discuss the significance of each of these findings.

We found that despite nearly complete inactivation of *Rb* and/or *p53* in urothelial cells, no tumors develop in aged animals indicating that these mutations are insufficient for tumor initiation. Given that about 50% of human tumors have mutation in *Rb* and *p53*, our results and the study from He et al[12] imply that inactivation of *Rb* and *p53* contributes to the progression, but not the initiation, of bladder cancer, or that additional mutations are required for tumor onset. In support of the requirement for additional mutations is the presence of tumors in chimeric animals lacking both *Rb* and *E2f4* (Ref. [6]) and a study reporting that large T antigen, which targets *p53* and the entire *Rb* family, causes development of invasive tumors in the mouse bladder[32].

Human neuroendocrine prostate tumors occur at low frequency[18]. Generally these cancers are epithelial but as they progress they show a substantial fraction of neuroendocrine cells. These cells express low levels of androgen receptor and they associate with acquisition of resistance to hormone therapy[33]. The formation of aggressive, AR low neuroendocrine tumors in the $Rb^{-/-}p53^{-/-}$ mutants, clearly establishes that the combined loss of *Rb* and *p53* differs in its ability to transform urothelial versus neurendocrine cell types. Moreover, as *Rb* mutations are generally associated with androgen therapy resistance[34], it also suggests that our model may offer a useful tool to study prostate neuroendocrine tumors. We note that our system differs from the TRAMP model, which typically yields epithelial prostate cancers that give rise to AR therapy-resistant metastases containing neuroendocrine cells[35]. We believe that our model could be used together with TRAMP mice and other models of epithelial prostate cancer to dissect the neuroendocrine component present in many advanced stage human cancers[19].

Our discovery that a significant number of *Rb* mutant mice develop high-grade intestinal tumors residing in the colon, provides novel insights into the role of *Rb* in colon cancer. Importantly, these *Rb*-deficient intestinal tumors have similar features to human colorectal cancer; the masses range from well to poorly differentiated, can be highly invasive and even metastasize to the liver, the first target organ in human colorectal cancer. Furthermore, the *Rb*-deficient colon tumors, similar to human colorectal cancer, have a mosaic distribution of β-catenin with cytoplasmic/nuclear staining interspersed with membranous localization. Finally, these tumors bear marks of genomic instability and upregulate the MMR system suggesting that they result from CIN, not MIN, mechanisms.

Notably, unlike humans, most genetically defined mouse models of intestinal tumorigenesis develop lesions in the small intestine rather than the colon[36]. The few existing systems of colon carcinogenesis develop tumors incapable of metastasizing, and thus cannot reproduce the primary cause of mortality in human colorectal cancer[37]. Only recently, combinations of surgical procedures in *kRas;apc* double mutant mice have yielded model systems in which tumors develop in the colon and can be metastatic[38]. However, these models require technical expertise, specific instrumentation and aseptic settings. Our model involves a single gene mutation and recapitulates the key features of human colon cancer.

Mutations in the *Rb* locus have been found in a significant fraction of colorectal carcinomas[14, 15]. Therefore, the function of *Rb* in the mouse intestine has already been investigated in different systems but due to the nature of the system utilized and/or the age ranges employed, its role in colorectal carcinoma had not been revealed previously. The only prior aging study was performed in *Villin-CreRb*$^{-/-}$ mice, which die by one year of age of pituitary and thyroid tumors and therefore preclude evaluation of *Rb*'s role in intestinal tumorigenesis[21]. Notably, studies in embryos and younger animals hinted at tumor enabling events from *Rb* loss in the intestine. In embryos, *Rb* ablation using a *CollagenIA-Cre* transgene causes an increase in number of enterocytes, paneth, goblet and endocrine cells which indicates a general effect on proliferation[39]. Using the *Fabpl-Cre* transgene, Haigis et al. showed that this ectopic proliferation becomes restricted to enterocytes in young adults[20]. Moreover, they found that simultaneous deletion of *Rb* and either one of the pRB-related proteins, *p107* or *p130*, disrupted the intestinal architecture although no tumors were detected. While we cannot explain the lack of tumors in the Haigis study versus the presence in our own, we speculate that this may result from changes in the representation of the cells and/or their relative niches that are the targets of transformation in the context of normal tissue architecture.

In the present study we definitively show a requirement for *Rb* in suppression of colorectal carcinoma in mice. Moreover, our model suggests that tumorigenesis occurs with, and thus likely requires, synergistic activation of β-catenin, as we find nuclear accumulation in our tumors and upregulation of Sox9. We hypothesize that this Wnt pathway deregulation occurs through genomic rearrangements induced by *Rb* inactivation. In fact, the variation in staining of APC among tumors is suggestive of random mutations in the *apc* gene itself and/or in other members of the pathway. Thus, our data indicate that *Rb* loss is a driver of tumorigenesis in the colon that leads to Wnt/β-catenin activation. We note that Kucherlapati et al. found that *Rb* loss in mice accelerates intestinal tumors initiated by *apc* mutation[40]. In

that study, additional deletion of *Rb* shifts the spectrum of *apc*-deficient tumors from small to large intestine and augments the overall tumor burden. This finding argues that *Rb* has targets beyond the Wnt/β-catenin pathway that could reflect the known effect of *Rb* inactivation in promoting cell cycle entry and/or additional cooperating events resulting from *Rb* loss. In line with the cell cycle hypothesis, while direct mutation of *Rb* is relatively rare in colorectal carcinoma (0.5–4%), inactivation of the *Rb* pathway is a seemingly universal feature of colorectal cancer[14, 15]. Several studies have shown that in addition to showing mutation in *apc* or the Wnt/β-catenin pathway, most intestinal tumors typically carry mutations in growth signaling pathways expected to inactivate *Rb* function and promote proliferation. For example, a comprehensive analysis of mutations in human cancer showed a significant frequency of inactivating mutations (17%) in the ubiquitin ligase FBXW7, which positively regulates *Rb* by targeting its inhibitor cyclin E for degradation[14]. One potential exception to this role is a report, conducted on a very small sample size and prior to the availability of deep sequencing, of super-physiological levels of *Rb* correlating with chromosome 13 amplification[41]. This phenomenon has been explained through the inhibitory effect of E2F1 on β-catenin. E2F1 has been shown to promote β-catenin degradation and inhibit its transcriptional activity in an APC-independent manner in Drosophila and in cell lines[42]. Therefore, the authors speculate that maintenance of *Rb* expression would be required to prevent E2F1 inhibition of β-catenin function. While this mechanism may operate in the small subset of tumors that retain *Rb*, data from this current study and others lead us to conclude that loss or inactivation of *Rb* can certainly enable the development of intestinal tumors.

Another feature that highlights the significance and broad applicability of our model is that chromosomal instability is the most likely mechanism operating in the tumors. Our results show that *Rb*-deficient tumors do not undergo microsatellite instability. On the contrary, they contain double stranded DNA breaks and up-regulate the mismatch repair system suggesting the presence of foci of DNA damage that recruit DNA repair components in line with the presence of genomic instability. These observations dovetail nicely with prior reports showing that *Rb*-deficiency alters chromosome condensation and promotes chromosome missegregation by altering centromeric structure through changes in chromosome cohesion[26–28]. Taken together, these studies and ours, argue for a causal effect between *Rb* deficiency and chromosomal rearrangements through different mechanisms and in different settings, suggesting that chromosomal instability is the driving force of the *Rb*-deficient tumors.

In conclusion, here we show that *Rb* deficiency in the intestine causes the onset of aggressive, invasive intestinal tumors that share numerous histological and biological mechanisms with human colorectal cancer, indicating that this mouse system will prove useful for the modeling of this deadly cancer type.

MATERIAL AND METHODS

Mouse strains and histological analysis

Mouse colonies were maintained in compliance with IACUC guidelines. *Fabp*$l^{-4\times@132}$-*Cre*[13], *Rb2*^{lox}[43], p53$^{lox/lox}$[44], and Rosa26-LSL-lacZ (Jackson Labs) conditional mutant mice

(all defined as $^{c/c}$ in the text) were maintained on a mixed 129/Sv;C57Bl/6 genetic background. Animals were dissected and tissues were fixed in formalin except when they were stained for X-gal as described previously[45]. 5μm paraffin sections were used for all analyses.

Immunohistochemistry

Immunostaining was performed with antigen retrieval (20–30′ in boiling 10mM sodium citrate pH 6.0, 0.05% Tween 20) and by incubating primary antibodies overnight at 4°C. Detection and staining was performed with Vectastain ABC kit and DAB kits (Vector laboratories). Slides were counterstained with Hematoxylin. Antibodies used: anti-APC #sc-896 and anti-AR #sc-816, from Santa Cruz Technology; anti-β-catenin #610181 (BD); anti-chromogranin A #ab1560-1, anti-MLH1 #ab92312, anti-Sox9 # ab5535, and anti-P-MEK1 ab59329 from Abcam; anti-ck17/19 #3984, anti-MSH2 #D24B5, and anti-γ-H2AX #9718, from Cell Signaling; anti-p21 #LS-C88585 (Lifespan Biosciences), and anti-p53 #CM5 (Vector Laboratories).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are indebted to Omer Yilmaz for scientific suggestions and professional evaluation of the pathology slides, Kevin Haigis for unpublished information and reagents, A. Amsterdam and S. Frank for critical reading of the manuscript, and the Koch Institute Swanson Biotechnology Center for technical support, particularly the Histology Core. This work was supported by NIH/NCI grants to the Koch Institute (P30-CA14051), and to JAL (P01-CA42063 and RO1-CA121921), a Ludwig Scholar at MIT.

References

1. Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer. 2008; 8:671–682. [PubMed: 18650841]
2. Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, et al. Requirement for a functional Rb-1 gene in murine development. Nature. 1992; 359:328–330. [PubMed: 1406937]
3. Williams BO, Schmitt EM, Remington L, Bronson RT, Albert DM, Weinberg RA, et al. Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. Embo J. 1994; 13:4251–4259. [PubMed: 7925270]
4. Maandag EC, van der Valk M, Vlaar M, Feltkamp C, O'Brien J, van Roon M, et al. Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. Embo J. 1994; 13:4260–4268. [PubMed: 7925271]
5. Henley SA, Dick FA. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. Cell Div. 2012; 7:10. [PubMed: 22417103]
6. Parisi T, Bronson RT, Lees JA. 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. Oncogene. 2009; 28:500–508. [PubMed: 18997819]
7. Lee EY, Cam H, Ziebold U, Rayman JB, Lees JA, Dynlacht BD. E2F4 loss suppresses tumorigenesis in Rb mutant mice. Cancer Cell. 2002; 2:463–472. [PubMed: 12498715]
8. Humbert PO, Rogers C, Ganiatsas S, Landsberg RL, Trimarchi JM, Dandapani S, et al. E2F4 is essential for normal erythrocyte maturation and neonatal viability. Mol Cell. 2000; 6:281–291. [PubMed: 10983976]

9. Wu XR. Urothelial tumorigenesis: a tale of divergent pathways. Nat Rev Cancer. 2005; 5:713–725. [PubMed: 16110317]
10. Dinney CP, McConkey DJ, Millikan RE, Wu X, Bar-Eli M, Adam L, et al. Focus on bladder cancer. Cancer Cell. 2004; 6:111–116. [PubMed: 15324694]
11. Rosenberg JE, Hahn WC. Bladder cancer: modeling and translation. Genes Dev. 2009; 23:655–659. [PubMed: 19299556]
12. He F, Mo L, Zheng XY, Hu C, Lepor H, Lee EY, et al. Deficiency of pRb family proteins and p53 in invasive orothelial carcinogenesis. Cancer Res. 2009; 69:9413–9421. [PubMed: 19951992]
13. Saam JR, Gordon JI. Inducible gene knockouts in the small intestinal and colonic epithelium. J Biol Chem. 1999; 274:38071–38082. [PubMed: 10608876]
14. Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. Nature. 2014; 505:495–501. [PubMed: 24390350]
15. Zack TI, Schumacher SE, Carter SL, Cherniack AD, Saksena G, Tabak B, et al. Pan-cancer patterns of somatic copy number alteration. Nat Genet. 2013; 45:1134–1140. [PubMed: 24071852]
16. Yoo LI, Liu DW, Le Vu S, Bronson RT, Wu H, Yuan J. Pten deficiency activates distinct downstream signaling pathways in a tissue-specific manner. Cancer Res. 2006; 66:1929–1939. [PubMed: 16488991]
17. Sharma A, Yeow WS, Ertel A, Coleman I, Clegg N, Thangavel C, et al. The retinoblastoma tumor suppressor controls androgen signaling and human prostate cancer progression. J Clin Invest. 2010; 120:4478–4492. [PubMed: 21099110]
18. Humphrey PA. Histological variants of prostatic carcinoma and their significance. Histopathology. 2012; 60:59–74. [PubMed: 22212078]
19. Zhou Z, Flesken-Nikitin A, Corney DC, Wang W, Goodrich DW, Roy-Burman P, et al. Synergy of p53 and Rb deficiency in a conditional mouse model for metastatic prostate cancer. Cancer Res. 2006; 66:7889–7898. [PubMed: 16912162]
20. Haigis K, Sage J, Glickman J, Shafer S, Jacks T. The related retinoblastoma (pRb) and p130 proteins cooperate to regulate homeostasis in the intestinal epithelium. J Biol Chem. 2006; 281:638–647. [PubMed: 16258171]
21. Kucherlapati MH, Nguyen AA, Bronson RT, Kucherlapati RS. Inactivation of conditional Rb by Villin-Cre leads to aggressive tumors outside the gastrointestinal tract. Cancer Res. 2006; 66:3576–3583. [PubMed: 16585182]
22. Nandan MO, Yang VW. Genetic and Chemical Models of Colorectal Cancer in Mice. Curr Colorectal Cancer Rep. 2010; 6:51–59. [PubMed: 20376303]
23. Schwitalla S, Ziegler PK, Horst D, Becker V, Kerle I, Begus-Nahrmann Y, et al. Loss of p53 in enterocytes generates an inflammatory microenvironment enabling invasion and lymph node metastasis of carcinogen-induced colorectal tumors. Cancer Cell. 2013; 23:93–106. [PubMed: 23273920]
24. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med. 2009; 361:2449–2460. [PubMed: 20018966]
25. Moran A, Ortega P, de Juan C, Fernandez-Marcelo T, Frias C, Sanchez-Pernaute A, et al. Differential colorectal carcinogenesis: Molecular basis and clinical relevance. World J Gastrointest Oncol. 2010; 2:151–158. [PubMed: 21160823]
26. Coschi CH, Martens AL, Ritchie K, Francis SM, Chakrabarti S, Berube NG, et al. Mitotic chromosome condensation mediated by the retinoblastoma protein is tumor-suppressive. Genes Dev. 2010; 24:1351–1363. [PubMed: 20551166]
27. Manning AL, Longworth MS, Dyson NJ. Loss of pRB causes centromere dysfunction and chromosomal instability. Genes Dev. 2010; 24:1364–1376. [PubMed: 20551165]
28. Manning AL, Yazinski SA, Nicolay B, Bryll A, Zou L, Dyson NJ. Suppression of genome instability in pRB-deficient cells by enhancement of chromosome cohesion. Mol Cell. 2014; 53:993–1004. [PubMed: 24613344]
29. Burgess AW, Faux MC, Layton MJ, Ramsay RG. Wnt signaling and colon tumorigenesis–a view from the periphery. Exp Cell Res. 2011; 317:2748–2758. [PubMed: 21884696]

30. Horst D, Reu S, Kriegl L, Engel J, Kirchner T, Jung A. The intratumoral distribution of nuclear beta-catenin is a prognostic marker in colon cancer. Cancer. 2009; 115:2063–2070. [PubMed: 19298008]
31. Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, et al. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. J Cell Biol. 2004; 166:37–47. [PubMed: 15240568]
32. Zhang ZT, Pak J, Shapiro E, Sun TT, Wu XR. Urothelium-specific expression of an oncogene in transgenic mice induced the formation of carcinoma in situ and invasive transitional cell carcinoma. Cancer Res. 1999; 59:3512–3517. [PubMed: 10416618]
33. Terry S, Beltran H. The many faces of neuroendocrine differentiation in prostate cancer progression. Front Oncol. 2014; 4:60. [PubMed: 24724054]
34. Aparicio A, Den RB, Knudsen KE. Time to stratify? The retinoblastoma protein in castrate-resistant prostate cancer. Nat Rev Urol. 2011; 8:562–568. [PubMed: 21811228]
35. Kaplan-Lefko PJ, Chen TM, Ittmann MM, Barrios RJ, Ayala GE, Huss WJ, et al. Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. Prostate. 2003; 55:219–237. [PubMed: 12692788]
36. Zeineldin M, Neufeld KL. Understanding phenotypic variation in rodent models with germline Apc mutations. Cancer Res. 2013; 73:2389–2399. [PubMed: 23580574]
37. Johnson RL, Fleet JC. Animal models of colorectal cancer. Cancer Metastasis Rev. 2012; 32:39–61. [PubMed: 23076650]
38. Hung KE, Maricevich MA, Richard LG, Chen WY, Richardson MP, Kunin A, et al. Development of a mouse model for sporadic and metastatic colon tumors and its use in assessing drug treatment. Proc Natl Acad Sci U S A. 2010; 107:1565–1570. [PubMed: 20080688]
39. Yang HS, Hinds PW. pRb-mediated control of epithelial cell proliferation and Indian hedgehog expression in mouse intestinal development. BMC Dev Biol. 2007; 7:6. [PubMed: 17257418]
40. Kucherlapati MH, Yang K, Fan K, Kuraguchi M, Sonkin D, Rosulek A, et al. Loss of Rb1 in the gastrointestinal tract of Apc1638N mice promotes tumors of the cecum and proximal colon. Proc Natl Acad Sci U S A. 2008; 105:15493–15498. [PubMed: 18832169]
41. Gope R, Christensen MA, Thorson A, Lynch HT, Smyrk T, Hodgson C, et al. Increased expression of the retinoblastoma gene in human colorectal carcinomas relative to normal colonic mucosa. J Natl Cancer Inst. 1990; 82:310–314. [PubMed: 2405172]
42. Morris EJ, Ji JY, Yang F, Di Stefano L, Herr A, Moon NS, et al. E2F1 represses beta-catenin transcription and is antagonized by both pRB and CDK8. Nature. 2008; 455:552–556. [PubMed: 18794899]
43. Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature. 2003; 424:223–228. [PubMed: 12853964]
44. Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet. 2001; 29:418–425. [PubMed: 11694875]
45. Parisi T, Yuan TL, Faust AM, Caron AM, Bronson R, Lees JA. Selective requirements for E2f3 in the development and tumorigenicity of Rb-deficient chimeric tissues. Mol Cell Biol. 2007; 27:2283–2293. [PubMed: 17210634]

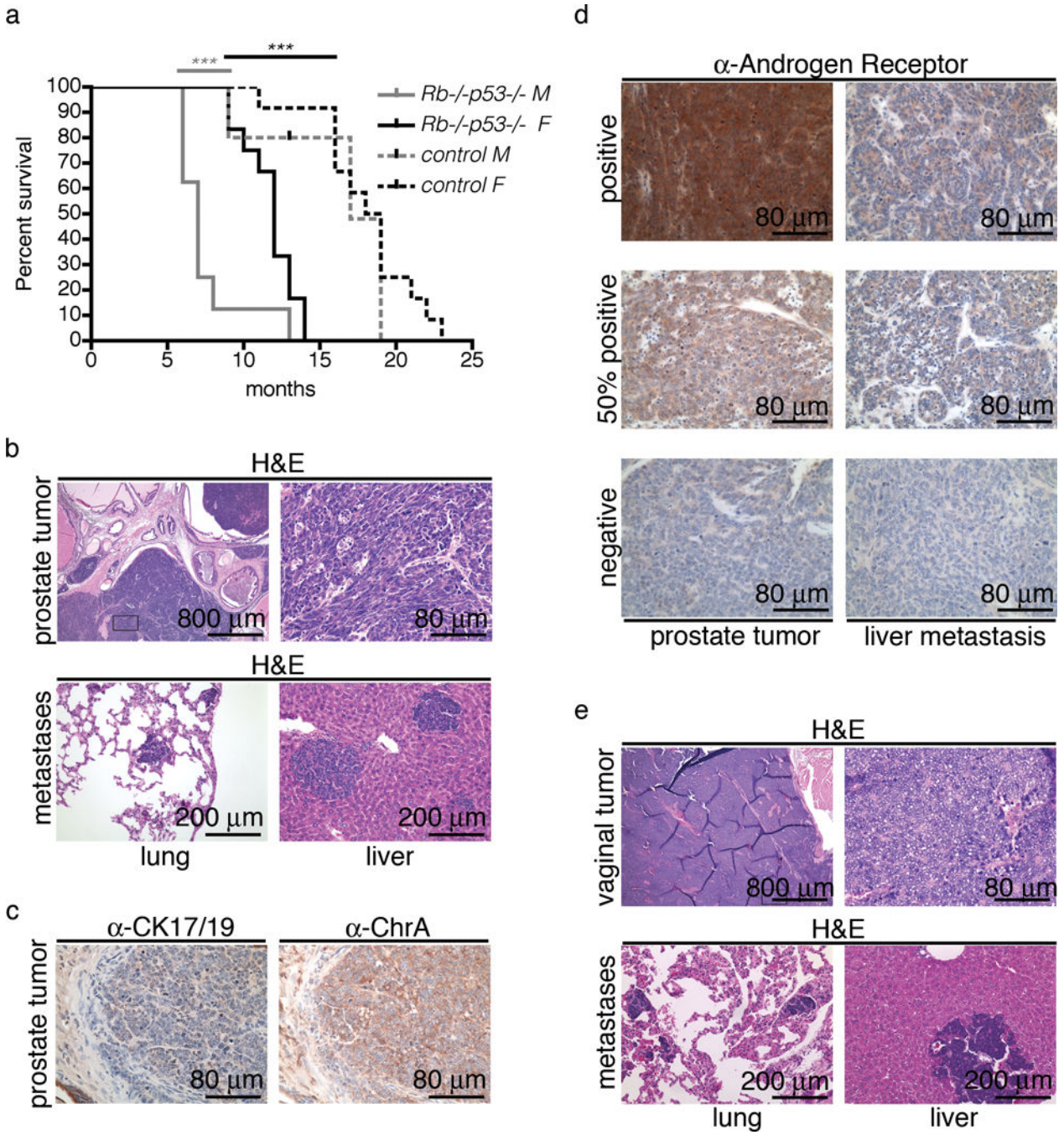

Figure 1. *Rb* and *p53* deficiency causes metastatic neuroendocrine tumors in the urogenital tract

(a) Kaplan-Meyer curves show reduced survival of $Rb^{-/-};p53^{-/-}$ double mutant animals, with males shown in grey (N=8), females in black (N=8), and p<0.0001 for both genders. **(b)** Histological analysis reveals the presence of multiple neuroendocrine-like tumors in the prostate (top) and their metastases in the lungs and livers (bottom). **(c)** Immunohistochemistry with neuroendocrine and epithelial-specific antibodies shows that the prostatic primary tumors are immunoreactive only to neuroendocrine (chromogranin A), not epithelial markers (CK17/19). (**d**) Immunological staining for androgen receptor of prostate

tumors (left) indicates that most of them have under-physiological levels of the protein. The right panels show that the expression of the receptor dramatically decreases in the metastases compared to the primary tumors. (**e**) H&E staining of the vaginal tumors (top panels) and their metastases (bottom panels) shows that there are neuroendocrine.

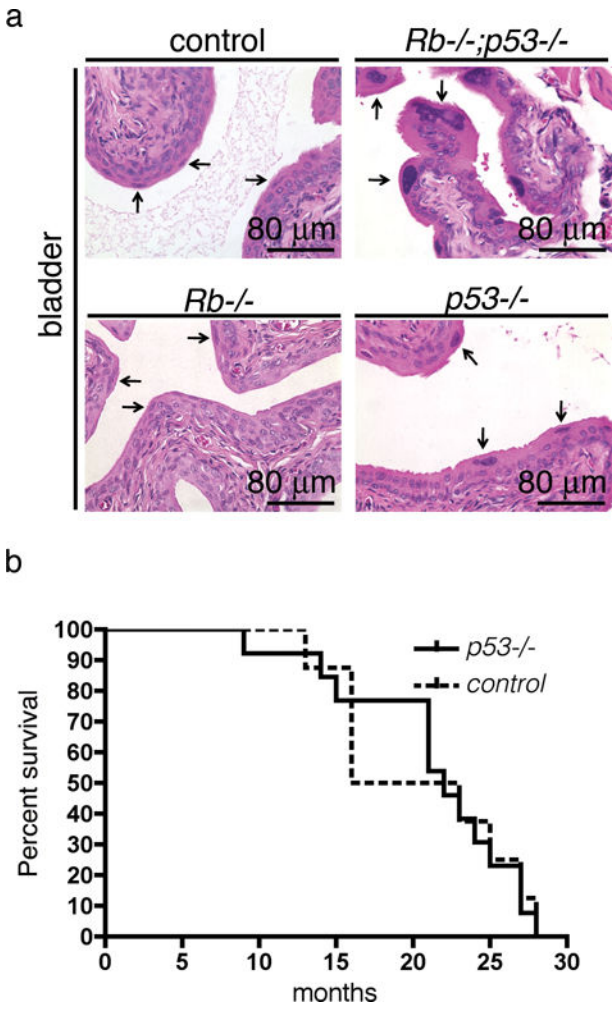

Figure 2. Urothelial cells are not susceptible to tumorigenesis upon inactivation of *Rb* and/or *p53*

(a) H&E staining of bladders isolated from double $Rb^{-/-};p53^{-/-}$ mutant as well as $Rb^{-/-}$ and $p53^{-/-}$ single mutant reveals absence of tumors. Instead, anomalies in the umbrella cells, with macronuclei containing higher levels of chromatin (indicated by the arrows), are seen in $Rb^{-/-};p53^{-/-}$ double and $p53^{-/-}$ single mutant animals. **(b)** The absence of differences in the lifespan among $p53^{-/-}$ mutants and their controls shows that umbrella cell atypia does not overall affect survival (p=0.91).

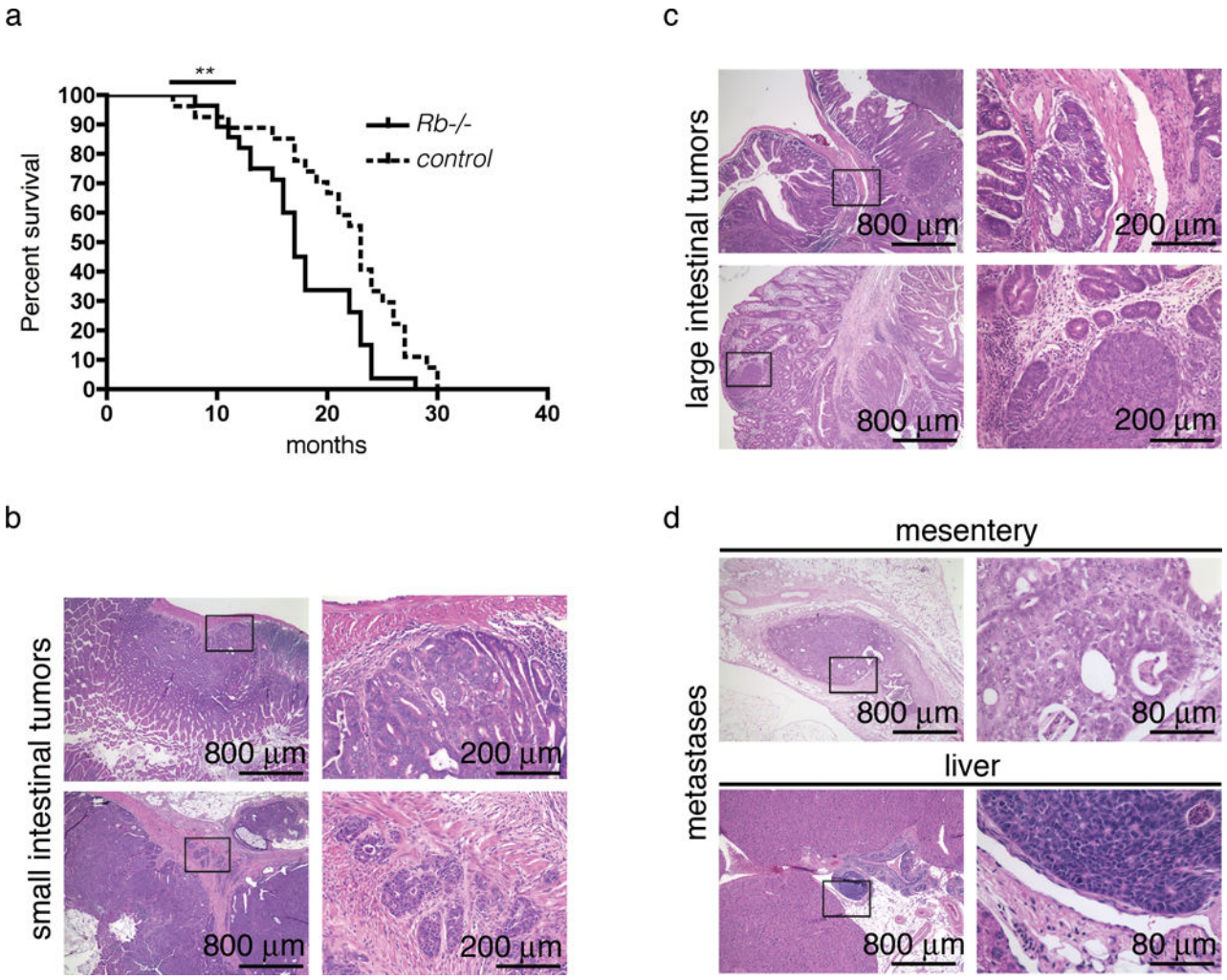

Figure 3. *Rb*-deficiency leads to invasive, poorly differentiated intestinal adenocarcinomas

(a) *Rb* mutant mice have a significantly reduced lifespan relative to control animals (p=0.0047). **(b)** H&E staining of different grades of small intestinal tumors in *Rb* mutant mice. The top panels show a low grade tumor while the bottom panels represent a poorly differentiated high grade (stage IV) and invasive tumor that traveled through the muscle wall to invade the mesentery. **(c)** The tumors found in the colon are highly invasive (top panels), and undifferentiated (bottom panels, note in both cases cells breaking through the muscle). **(d)** *Rb*-deficient intestinal tumors have metastatic potential. The top panels show a mesenteric vessel containing intestinal tumor cells; the bottom panel shows the portal vein in the liver clogged by intestinal cells, indicating that these cells, like their human counterpart can metastasize to the liver.

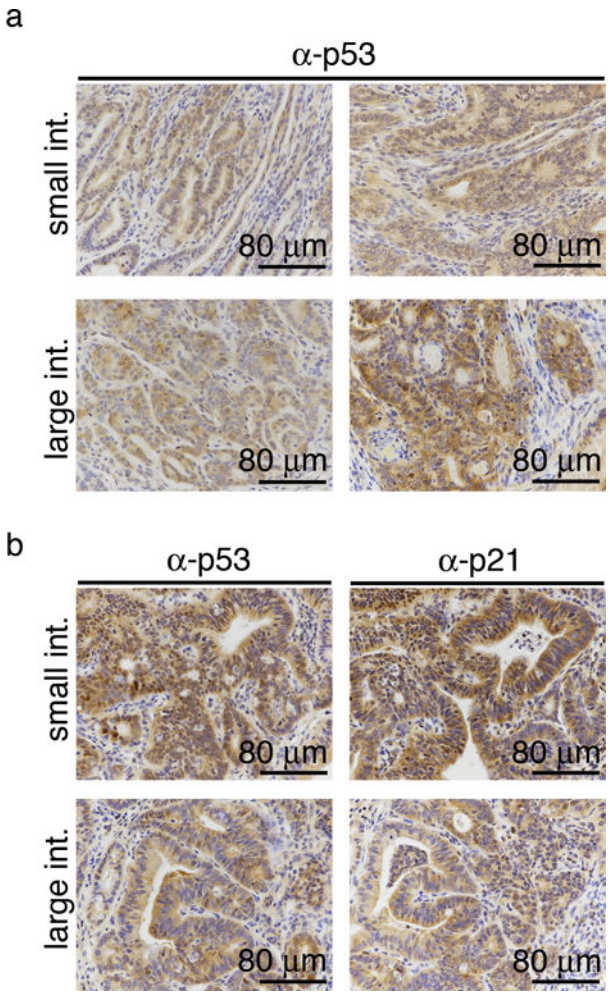

Figure 4. *Rb*-deficient intestinal tumors show activation of p53

(a) Analysis of p53 expression by immunostaining shows high levels of the protein in *Rb*-deficient small and large intestinal tumors. **(b)** Concomitant upregulation in the tumors of $p21^{Cip1}$, a p53 direct target, shows that p53 is present in its active form. This indicates that *p53* inactivating mutations are unlikely to play a role in the development of $Rb^{-/-};p53^{-/-}$ or $Rb^{-/-}$ intestinal tumors.

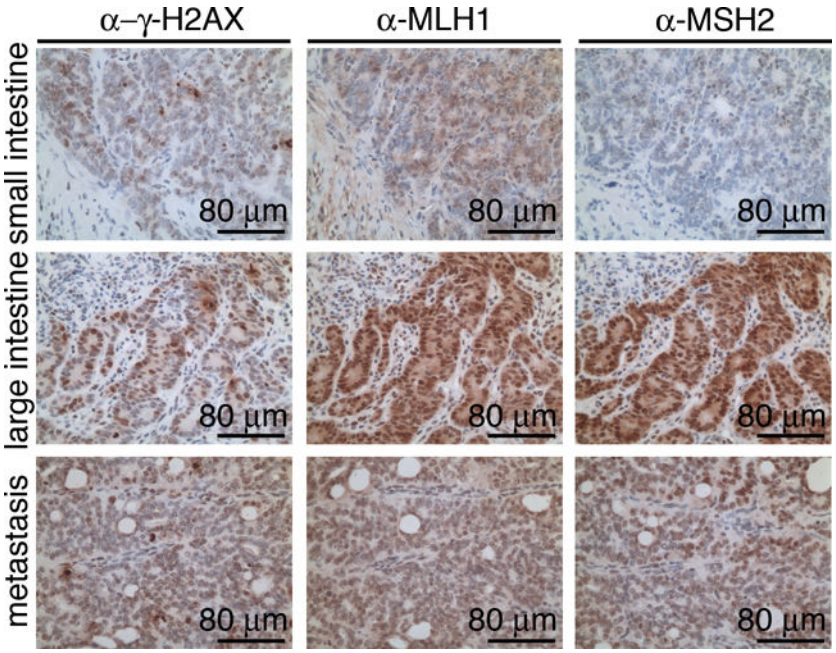

Figure 5. *Rb*-deficient intestinal tumors undergo genomic instability and upregulate the mismatch repair pathway

Left panels, immunostaining with anti γ-H2AX antibodies shows extensive DNA damage in primary small and intestinal tumors as well as in metastases. Upregulation of MLH1 (middle panels) and MSH2 (right panels) proteins coincides with areas of DNA damage and indicates activation of the mismatch repair system.

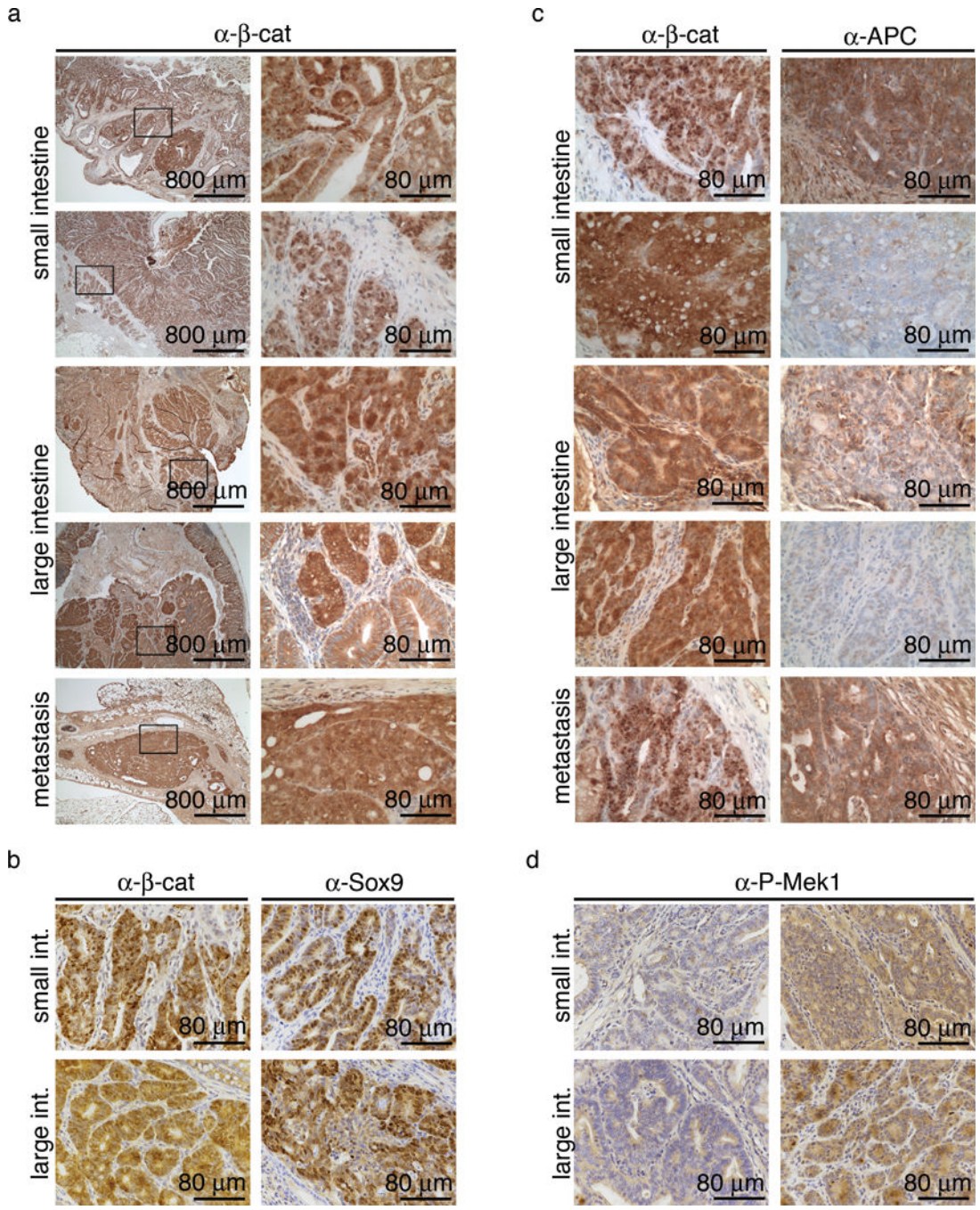

Figure 6. Nuclear accumulation of β-catenin in intestinal tumors indicates deregulation in the Wnt pathway

(a) IHC for β-catenin shows diffuse cytoplasmic staining as well as areas of nuclear accumulation in small and large intestinal tumors that generally associate with the invasive front of the tumors. The bottom panels show diffuse cytoplasmic+nuclear staining in a mesenteric metastasis. (**b**) The areas of β-catenin accumulation are coincident with Sox9 upregulation indicating that the Wnt pathway is activated in the intestinal tumors. **(c)** Analysis of *Rb*-deficient intestinal tumors shows variable APC expression. The top two

panels represent small intestinal tumors, one with uniform APC expression and one with no expression despite the presence of nuclear β-catenin. The mid two panels show colon tumors expressing nuclear β-catenin, one with patchy localization of APC and the other lacking expression of the protein. The first, fourth and last panels are from the same tumors shown in Figure 4, allowing comparison of APC and β-catenin staining respectively. Note that the expression of APC is heterogenous, while the pattern of nuclear β-catenin mirrors the ones of DNA damage and DNA repair. **(d)** Immunostaining for phosphorylated MEK1 (P-MEK1 T286) shows expression of the protein in a proportion of *Rb*-deficient intestinal tumors (right panels) indicating activation of *kRas* signaling pathway.

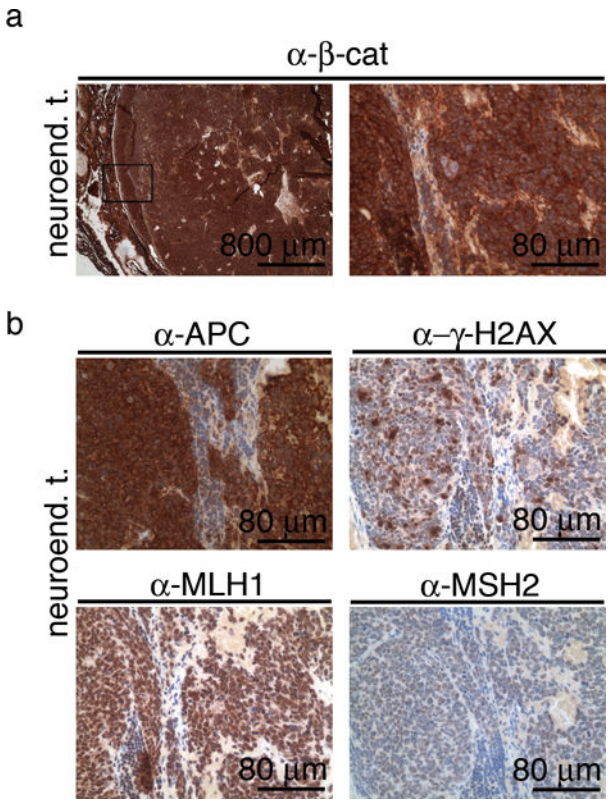

Figure 7. Neuroendocrine tumors undergo genomic instability without deregulating the Wnt pathway

(a) β-catenin staining of neuroendocrine tumors shows membranous localization of the protein. **(b)** IHC for APC indicates that this protein is not lost in the neuroendocrine tumors. However, the presence of DNA damage together with upregulation of MLH1 and MSH2 indicate that these tumors undergo genomic instability.

Table 1

Phenotypes of Fabpl-Cre$^{tg/+}$Rb$^{c/c}$ tumor-bearing mice.

AGE (months)	TUMORS IN THE SMALL INTESTINE	TUMORS IN THE LARGE INTESTINE	TUMORS IN OTHER ORGANS
MALES			
10	none	4 Tumors. PT I	
13	None	2 Tumors. 1 PT I, 1 PT II, invasive carcinoma	
16	4 Tumors. 1 PTI, 1 PT II, 1 PT III, 1 PT IV blood and lymphovasculature invasion	1 PT III moderately differentiated non mucinus, invasion into mesenteric small vessel	intestinal metastases in the portal vein
17	9 Tumors. PT II	6 Tumors. 3 PTI, 2 PT II, 1 PT III, poorly differentiated, potentially invasive	
17	2 Tumors. PT III and PT IV, lymphovasculature invasion	benign	
17	1 PT III, carcinoma, vasculature invasion	none	
22	none	none	liver tumor
23	none	none	lungs
FEMALES			
8	2 Tumors. PT 1	none	
12	5 Tumors. 4 PT II and 1 PT III, carcinoma with lymphovasculature invasion	none	
13	none	none	thyroid
16	2 Tumors. PT II	4 Tumors. 3 PT II, 1 PT III-IV poorly diff. with neuroendocrine features	
17	4 Tumors. PT I	none	
18	6 Tumors. PT IV, poorly diff (neuroend and epithelial), metastatic in lymph nodes and mesentery	benign	
18	none	none	pituitary
22	none	none	thyroid
23	7 Tumors. 5 PTI, 2 PTII	1 PT III	

PT= pathological stage. PT II: Tumor invades muscularis propria. PT III: Tumor invades through the muscularis propria into pericolorectal tissues. PT IV: Tumor penetrates to the surface of the visceral peritoneum and/or directly invades or is adherent to other organs or structures.