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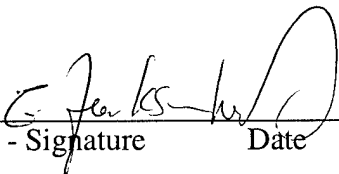
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**IV. TABLE OF CONTENTS**

I. FRONT COVER	1
II. STANDARD FORM 298	2
III. FOREWORD	5
IV. TABLE OF CONTENTS	6
V. INTRODUCTION	6
VI. BODY	7
VII. KEY RESEARCH ACCOMPLISHMENTS	15
VIII. REPORTABLE OUTCOMES	15
IX. CONCLUSIONS	16
X. LEGENDS TO FIGURES	17

**V. INTRODUCTION**

The tumor suppressor Rb exerts its effects on cell growth by modulating the activity of transcription factors such as E2F1. Activity of Rb is regulated by phosphorylation by G1 cyclins, their associated kinases (Cdk) and Cdk inhibitors such as p16<sup>Ink4a</sup> (1). Germ-line mutations in Rb predispose individuals to retinoblastoma, whereas somatic mutations are frequently associated with the progression of most human cancer (2). In many tumors, however, such as breast adenocarcinoma, other components of the Rb pathway (i.e. Cdk4, cyclin D1 or p16<sup>Ink4a</sup>) are preferentially mutated or deregulated, but this tumor suppressor is intact and amenable to therapeutical activation (3). In such tumors, re-activation of endogenous Rb (e.g. by Cdk inhibitors) may provide an approach to suppress cell proliferation. Accordingly, introduction of phosphorylation-resistant, constitutively active Rb alleles, RbΔKs, into many cell types efficiently inhibits cell proliferation *in vitro* (4) and *in vivo* (5).

A potential caveat to the use of the Rb pathway to control cell proliferation is that this pathway restricts not only cell proliferation but also apoptosis (6,7). Long term suppression of apoptosis by the Rb pathway could result in the accumulation of oncogenic alterations and neoplastic transformation. Indeed, in mouse models, both over-expression and inactivation of E2F1, the major partner of Rb, induce cancer by disrupting different aspects (proliferation versus apoptosis, respectively) of cell physiology (8-11). In this report, we directly addressed the consequences of activating the Rb pathway *in vivo* by targeting RbΔK alleles to the mammary gland of transgenic mice. We show that transgenic females initially display ductal growth suppression but later exhibit enlargement of the alveolar compartment, precocious differentiation,

reduced apoptosis and ultimately develop breast tumors. The implications of these results to cancer therapy are discussed.

## VI. BODY

**Targeted expression of phosphorylation-resistant Rb alleles to the mammary gland**  
Rb $\Delta$ p34 (12) and Rb $\Delta$ K11 (see Methods) contain Serine/Threonine to alanine substitutions in 8 and 11 Cdk phosphoacceptor sites, respectively (Fig. 1A, a). These Rb $\Delta$ K alleles outperform wildtype Rb in multiple assays due to their resistance to phosphorylation by Cdks and are viewed as constitutively active, under-phosphorylated, native Rb alleles (12-16). The two Rb $\Delta$ K alleles were targeted to the mammary gland under control of the mouse mammary tumor virus long terminal repeat promoter (MMTV)(17)(Fig. 1A). Four independent MMTV-Rb $\Delta$ p34 transgenic lines in mixed C57BL/6xSJL background and three MMTV-Rb $\Delta$ K11 lines in uniform FVB background were established (Fig. 1B, Table 1).

MMTV-Rb $\Delta$ p34#24 transcripts were detected in the mammary gland as well as the salivary gland and spleen but not in the brain, kidney and liver (Fig. 1B, a, d-e and data not shown). The HA-Rb $\Delta$ K transgene was also detected by western blots with antibodies specific to the HA epitope (Fig. 1B, b-c). We were unable so far to detect the transgenes by immunohistochemistry with anti-HA antibodies. RT-PCR analysis showed that the MMTV-Rb $\Delta$ p34 and MMTV-Rb $\Delta$ K11 transgenes (collectively referred to as MMTV-Rb $\Delta$ K) were expressed throughout mammogenesis (Fig. 1B, d-e). In contrast, both *in situ* hybridization and immunohistochemical analyses of wildtype mammary glands revealed that expression of endogenous Rb was very low and sporadic in virgin females, elevated in early pregnancy and mid-pregnancy, reaching a peak at lactation and involution (Fig. 1C).

### MMTV-Rb $\Delta$ K transgenes suppress ductal growth in pubescent females

During the ductal stage, the mammary epithelium infiltrates the fat pad by proliferation at the terminal end buds (TEBs) and dichotomous side-branching (18-20). Whole mount analysis of pubescent MMTV-Rb $\Delta$ K transgenic females revealed moderate suppression of TEB progression (Fig. 2A). The growth suppression coincided with reduced expression of PCNA, a marker for cell proliferation (Fig. 2B, a-d). On average, MMTV-Rb $\Delta$ K TEBs exhibited 3-4 fold less PCNA-positive nuclei compared to control wildtype littermates (Fig. 2B, e). The suppression of ductal growth was most evident at 5-7 weeks of age and reduced thereafter (Fig. 2A, e). Accordingly, there were no obvious histological differences between the mammary glands of transgenic and normal littermates during pregnancy and lactation (data not shown).

### **MMTV-Rb $\Delta$ K transgenes induce alveolar outgrowth and precocious differentiation in nulliparous females**

Following puberty, the mammary epithelium undergoes cyclic proliferation, limited differentiation and cell death during the estrous cycle (21). Interestingly, fully-developed nulliparous MMTV-Rb $\Delta$ K transgenic females exhibited consistent alveolar outgrowth relative to control littermates (Fig. 3A, a-b). PCNA-staining revealed very few cycling cells in both wildtype and transgenic mice (Fig. 3A, c-d), suggesting that enlargement of the alveolar compartment was not associated with overt cellular proliferation. Intriguingly, multiple nulliparous transgenic but not wildtype females expressed  $\beta$ -Casein, a milk protein, suggesting precocious differentiation of the mammary gland (Fig. 3A, e-f).  $\beta$ -Casein was also detected by RT-PCR in ~30% (10 of 35) of 9-20 week-old transgenic females (Fig. 3C). Notably,  $\beta$ -Casein transcripts were observed, albeit at lower levels, in some wildtype females at the RNA (Fig. 3C), but not the protein level (Fig. 3A,e; B,h).

Alveolar outgrowth and precocious expression of  $\beta$ -Casein became more pronounced in older nulliparous MMTV-Rb $\Delta$ K transgenic females (Fig. 3B). Some MMTV-Rb $\Delta$ K transgenic nulliparous females exhibited pregnant- or lactating-like glands and appeared to be filled with milk (not shown). RT-PCR analysis of a group of three transgenic and control nulliparous females revealed  $\beta$ -Casein expression in all transgenic females; WAP, a later marker of differentiation, was detected in two of them (not shown). Histological examination of additional transgenic mice revealed hyperplastic alveoli, dilated ducts as well as enlarged lobules that resembled normal lobuloalveoli in pregnant females, with characteristic vacuoles and acinar morphology (Fig. 3B, b-d, f-g). Immunohistochemistry revealed high levels of  $\beta$ -Casein expression in the mammary gland of four out of five transgenic females but not in control littermates (Fig. 3B, h-j). Thus, persistent expression of the Rb $\Delta$ K transgene in nulliparous MMTV-Rb $\Delta$ K transgenic females induces precocious differentiation of the mammary epithelium both at the molecular and cellular levels.

### **MMTV-Rb $\Delta$ K transgenic females develop hyperplastic nodules**

Strikingly, 22% of the transgenic females (18 out of 81) from independent MMTV-Rb $\Delta$ K lines developed focal, hyperplastic nodules by 10-15 months of age (Fig. 4A, Table 1). Both nulliparous and multiparous females developed these lesions indicating that pregnancy was not required for neoplastic transformation. In many cases there were multifocal lesions per gland (Fig. 4Ab, e, f), suggesting independent, stochastic transformations of the mammary epithelium. The incidence of hyperplastic nodules may be underestimated because only one mammary gland per animal (of the ten glands in a female mouse) was subject to whole mount analysis. No such lesions were observed in over 57 wildtype littermate females (Fig. 4Aa, c; Table 1).

**WAP-Rb $\Delta$ p34 transgenic females also develop hyperplastic nodules**

To further corroborate these observations, we targeted Rb $\Delta$ p34 to the mammary gland under control of the Whey Acid Protein (WAP) promoter/3'UTR (Fig. 4Ba). The WAP regulatory unit directs linked transgenes exclusively to differentiated mammary epithelial cells during the estrous cycle and pregnancy and WAP-transgenes are expressed and exert a phenotype in both nulliparous and pregnant females (21-23). Three WAP-Rb $\Delta$ p34 transgenic lines were established in C57BL/6xSJL background. Expression of the WAP-Rb $\Delta$ p34 transgenes was low in all three lines and transcripts could only be detected during pregnancy (Fig. 4B, b). Remarkably, 38% (8 of 21) of the transgenic females from the three independent WAP-Rb $\Delta$ p34 lines developed multiple focal hyperplastic nodules within 10-15 months (Fig. 4B, c-f; Table 1). As with the MMTV-Rb $\Delta$ K lines, both nulliparous and parous females developed these micro lesions. No similar lesions were found in over 21 wildtype littermate females (Fig. 4B, c, Table 1).

**MMTV-Rb $\Delta$ K and WAP-Rb $\Delta$ p34 transgenic females develop full-blown mammary adenocarcinomas**

A fraction (7 in 102 = ~7%) of 10-15 month old MMTV-Rb $\Delta$ p34, MMTV-Rb $\Delta$ K11 and WAP-Rb $\Delta$ p34 transgenic females developed visible, palpable masses in their breasts (Table 1). Pathology analysis indicated that these masses represented full-blown mammary adenocarcinomas (Fig. 5A, see legend for pathology). None of over 100 wildtype littermate females in our colony developed breast tumors (Table 1 and not shown). The incidence of breast tumors in the MMTV-Rb $\Delta$ K11 #29 line was highest (Table 1). We have recently analyzed two retired MMTV-Rb $\Delta$ K11 #29 transgenic breeders and two wildtype control littermates at 20 months of age. Both transgenic females but none of the control mice developed palpable breast tumors in several mammary glands (not included in Table 1). Histology analysis revealed that each of these mammary glands contained 1-4 breast tumors. Fig. 5A, g-k shows one such gland with a medium-size tumor and several additional lesions. Thus, with time some of the hyperplastic nodules seen in one-year-old transgenic females (Fig. 4, Table 1) might develop into full-fledged breast tumors.

**Transgene expression is lost in mammary adenocarcinomas from MMTV-Rb $\Delta$ K transgenic mice**

PCNA immunohistochemistry revealed a moderate to high proliferation index in different breast tumors (Fig. 5B, a-b). This increase in cell proliferation prompted us to determine whether the Rb $\Delta$ K transgene was lost during tumor progression. Semi-quantitative RT-PCR analysis was performed on two tumor biopsies, as well as breast tumor cells derived from the mammary adenocarcinoma shown in Figure 5A, f and successfully propagated *in vitro*. Remarkably, Rb $\Delta$ K transgene expression was virtually lost in both the tumor biopsies and the cultured tumor cells (Fig. 5B, panel d).

### **MMTV-Rb $\Delta$ K transgenes extend the survival of differentiated mammary epithelial cells following post-lactational involution**

The aforementioned results suggest that Rb $\Delta$ K may induce breast tumors by acting as a survival factor to extend the life span of mammary epithelial cells, allowing the accumulation of oncogenic alterations, and that the loss of the transgene leads the progression into full-blown breast tumors. To directly test the effect of Rb $\Delta$ K on cell survival, we examined the first post-lactational involution in MMTV-Rb $\Delta$ K transgenic mice. During this process the extra-cellular matrix is degraded and the mammary epithelium undergoes massive apoptosis and remodeling back to a virgin-like state. After six days, most of the differentiated epithelial cells are eliminated and the expression of milk genes is greatly reduced (24). At this stage, there were remarkably more  $\beta$ -Casein expressing lobuloalveoli in transgenic females compared to wildtype littermates (Fig. 6A, d and data not shown). By RT-PCR, expression of both WAP and  $\beta$ -Casein was slightly, but consistently, elevated in the involuting mammary glands from MMTV-Rb $\Delta$ K11 transgenic females (Fig. 6B). *In situ* apoptosis assay (TUNEL) revealed very few apoptotic nuclei in the involuting glands in both wildtype and transgenic females. However, invariably, lobules that did not express  $\beta$ -Casein exhibited apoptosis (Fig. 6A, a-b), whereas lobules that maintained  $\beta$ -Casein expression did not contain apoptotic nuclei (Fig. 6A, c-d), suggesting that the persistent expression of  $\beta$ -Casein was due to inhibition of apoptosis by Rb $\Delta$ K11.

To test whether the defect in the first involution had a compounded effect following multiple pregnancies, we performed serial cross-section analyses on multiparous, retired, transgenic MMTV-Rb $\Delta$ K breeders. Intriguingly, none-regressing areas with lobuloalveolar-like structures were detected in each of five transgenic females (Fig. 6C, b, d) but not in five wildtype control breeders (Fig. 6C, a, c). These lobuloalveolar-like structures stained positively for collagen by Gomori Trichrome indicating the presence of extra-cellular matrix (Fig. 6C, f).

### **Aberrant expression of survival factors in multiparous MMTV-Rb $\Delta$ K transgenic breeders**

To determine whether the extended survival of the mammary epithelium in multiparous retired transgenic breeders was associated with alterations in the expression of survival factors, we performed western blot analyses on protein lysates extracted from whole mammary glands. While expression of cyclin D1, Bcl-2 and Bcl-X1 was not obviously affected, E2F1 was slightly reduced; IGFR-1R $\alpha$  and phospho-PKB/Akt were slightly elevated; and p21 exhibited an additional, faster migrating form (Fig. 6D, a). In contrast, four-month-old nulliparous transgenic females did not show significant alterations in the expression profiles of these factors (Fig. 6D, b). Taken together, the delayed involution, the appearance of lobulo-alveolar-like structures in retired breeders, the

reduced level of E2F1 and increased expression of IGFR-1R $\alpha$  and phospho-PKB/Akt suggest that expression of the Rb $\Delta$ K transgene suppresses cell death and extends the survival of the mammary epithelium, thereby leading to neoplastic transformation.

## DISCUSSION

Targeted expression of phosphorylation-resistant, constitutively active Rb proteins in the mammary gland is shown herein to lead to growth suppression, precocious differentiation, extended survival of the mammary epithelium and ultimately breast cancer. The growth suppression is moderate and represents the only phenotype that is consistent with the role of Rb as a tumor suppressor.

The precocious differentiation and co-induction of Rb and  $\beta$ -casein at mid-pregnancy (Fig. 1 and data not shown) suggest a direct effect of Rb on transcriptional activation of milk genes (Fig. 6E, b). Rb is required for terminal differentiation of certain tissues and for transcriptional activation by C/EBP protein family (25) and the glucocorticoid receptor (26). We were, however, unable so far to detect any effect of Rb on the  $\beta$ -casein promoter in transient reporter assays. Alternatively, active Rb may promote differentiation indirectly by extending the life span of differentiated mammary epithelial cells in each estrous cycle. The "survival model" is consistent with the reduced apoptosis and persistent expression of  $\beta$ -casein and WAP following post-lactational involution; the appearance of lobuloalveolar-like structures, decreased E2F1 and increased IGFR-1R $\alpha$  and phospho-PKB/Akt expression in multiparous, retired transgenic breeders (Fig. 6); and the development of breast tumors in MMTV-Rb $\Delta$ K and WAP-Rb $\Delta$ p34 transgenic mice (Figs. 4-5). The two models are not mutually exclusive and Rb may contribute to both the onset and survival of the differentiation state (Fig. 6E).

We propose that persistent activation of the Rb pathway in the mammary gland induce mammary adenocarcinoma in three steps. First, growth suppression by Rb $\Delta$ K may exert a selection pressure in favor of transforming mutations that accelerate cell proliferation. Second, the suppression of apoptosis by the Rb $\Delta$ K transgenes may promote the survival and accumulation of transformed cells. Third, decreased expression of the transgene (Fig. 5B, d-e) may permit rapid clonal expansion and the development of full-blown breast tumors. The dual effects of the Rb-E2F1 complex on cell cycle progression and apoptosis may underlie the symmetry in the phenotypes of Rb knockout mice and transgenic E2F1 mice on the one hand, and the activated Rb transgenic mice, reported herein, and E2F1 null mice on the other hand (Fig. 6E)(8-11).

Our results challenge two paradigms of cancer biology. First, the reversal of an oncogenic or tumor suppressor pathway may also induce cancer. Second, activation of oncogenic or anti-oncogenic pathways may have different outcomes depending on the extent of the activation. Thus, forced expression of high levels of Rb $\Delta$ K can efficiently inhibit cell proliferation. Moreover, stable activation of Rb by amplification, chromosomal rearrangement, etc., is not compatible with the

progression of cancer, which invariably involves the loss of Rb (3). However, low and/or transient activation of the Rb pathway, as in our experimental models, may allow cells to proliferate, albeit slowly, and the protection from apoptosis be consequential. The latter scenario has direct implications for cancer therapy. Systemic activation of the Rb pathway (e.g. by Cdk inhibitors) may inadvertently extend the survival of cells destined to die by apoptosis, leading to the accumulation of oncogenic alterations, clonal expansion and ultimately cancer. Administration of Flavopiridol, a Cdk inhibitor, to ischemic animals inhibits Rb phosphorylation and blocks apoptosis (27). Yet, Flavopiridol and other drugs that directly or indirectly target the Rb pathway are in pre-clinical and clinical development (28). In view of our results, these drugs ought to be examined to determine the outcome of long term treatment in animal models. Inasmuch as that the progression of cancer often involves the inactivation of both Rb and p53, long-term cancer therapy may require combinatorial drugs that would induce the Rb pathway but also antagonize its survival effect. Alternatively, the activation of other tumor-suppressor pathways (e.g. p53), which inhibit cell proliferation but induce (rather than suppress) apoptosis, might be favored as targets for long-term adjuvant therapy of cancer.

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## VII. KEY RESEARCH ACCOMPLISHMENTS

- We showed that expression of Rb is upregulated during pregnancy and peaks at lactation and involution.
- We generated transgenic mice expressing two alleles of unphosphorylatable Rb in the mammary glands under control of the MMTV and WAP promoters.
- We showed that despite the fact that early expression of unphosphorylatable Rb suppresses ductal growth, the ultimate effect of activating the Rb pathway is the induction of breast cancer.
- We are currently analyzing mammary gland development in E2F1<sup>-/-</sup> mutant mice and generating and analyzing composite transgenic mice including MMTV-Rb $\Delta$ K:E2F1<sup>-/-</sup> and

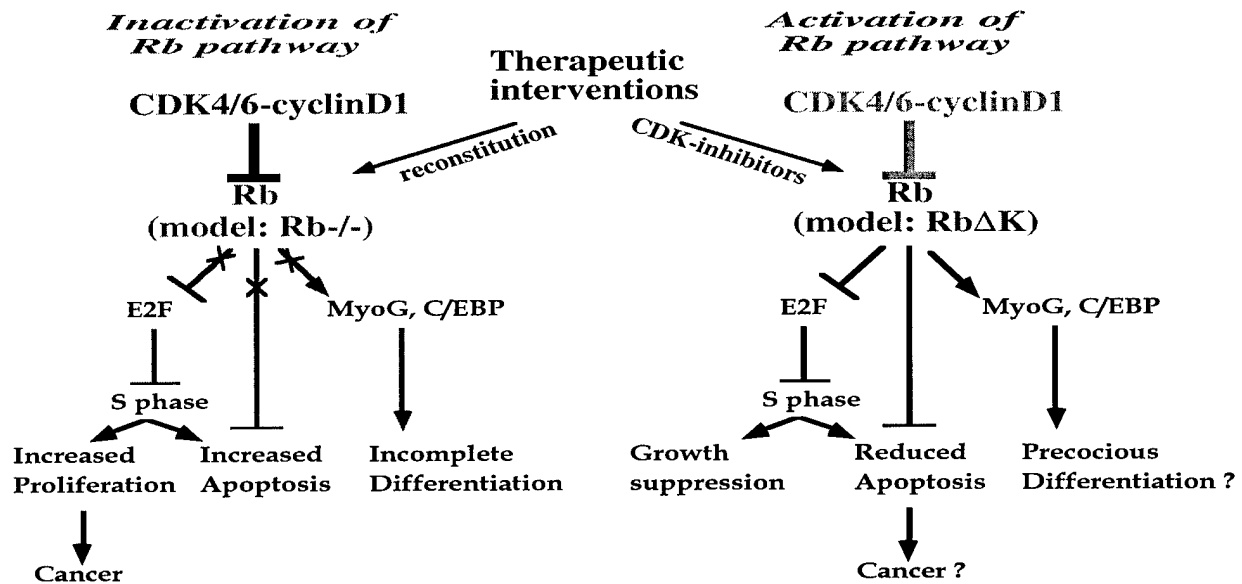
MMTV-Rb $\Delta$ K:MMTV-neu. Due to the complexity of the phenotypes we obtained with the MMTV-Rb $\Delta$ K transgenic mice, this analysis (technical objective 3) will be completed after this grant period.

### VIII. REPORTABLE OUTCOMES

- A manuscript describing these results was reviewed twice by Science but finally rejected (see attached note from the editor regarding second review). In the first round, one reviewer liked it very much but the other reviewer misunderstood several points. In the second round, again one reviewer was enthusiastic but the other was negative. We have made several additional experiments and submitted the manuscript to Nature Medicine.
- We developed the following transgenic lines: MMTV-Rb $\Delta$ p34, MMTV-Rb $\Delta$ K11, WAP-Rb $\Delta$ p34.
- Since E2F1 expression is strongly induced during involution, we are examining E2F1-/- knock-out females for defect in involution. This analysis plus the expression analysis of the Rb and E2F gene family should yield additional publication.
- I gave an oral presentation on part of the work in an CBCRI organized conference on breast cancer on June, 1999 and May, 2001. I was invited to give talks on this work at Sunnybrook Research Institute (Jan. 17, 2001) and at the Medical Science Building, University of Toronto, Feb. 5, 2001).
- We presented posters on this work:
  - Zhe Jiang and Eldad Zacksenhaus. Mammary tumors induced by activated retinoblastoma protein in transgenic mice. Era of Hope. Department of Defence Breast Cancer Research Program meeting. Atlanta, Georgia. June 2000.
  - Zhe Jiang and Eldad Zacksenhaus. Effects of targeted expression of constitutively active retinoblastoma proteins in the mammary gland. Keystone Symposia, Cell cycle. Taos, New Mexico. Jan. 9-14 2001.

### IX. CONCLUSIONS

Studies on Rb-deficient mice revealed that several cell types undergo ectopic DNA synthesis and apoptosis and display incomplete differentiation (Fig. I, below). In addition Rb $\pm$  heterozygote mice develop pituitary tumors and other malignancies. Since the Rb pathway is so often disrupted in human cancer, activation of the pathway by expression of unphosphorylatable Rb or by soluble CDKIs is an attractive approach for reversing malignancy by gene therapy. Although we understand in great detail the consequences of inactivating Rb, little is known about the outcome of activating this tumor suppressor in vivo.



**Fig. I.** A model for activation of the Rb pathway.

Unexpectedly, our analysis of transgenic mice expressing unphosphorylatable Rb alleles in the mammary gland revealed that MMTV-RbΔK transgenic mice display initial growth suppression but later develop breast tumors. Furthermore, WAP-RbΔK transgenic mice also developed breast tumors. These results suggest that since Rb regulates both cell proliferation and apoptosis, targeting this pathway for cancer therapy should be exercised with caution. The effect of upregulating the Rb pathway may vary depending on the tissue and developmental stage. The most obvious effect of upregulating the Rb pathway in most tissues may be inhibition of cell proliferation. In tissues such as mammary epithelium, where cells undergo cyclic proliferation and apoptosis the ultimate effect of activating the Rb pathway may be inhibition of apoptosis and cancer. In as much as cancer often involves the inactivation of both Rb and p53, the protection of cells from tumorigenicity may require the activation of both Rb and p53 (or another pro-apoptotic factor). If we identify the mechanisms by which Rb suppresses apoptosis in the mammary gland and find a way to block the apoptosis by RbΔK the ultimate effect may be inhibition of breast cancer. Alternatively, the lesson from our study is that perhaps the Rb pathway is not a good target for cancer therapy as it inhibits both proliferation and apoptosis. Perhaps other targets, i.e. p53, which inhibit cell proliferation but induce (rather than suppress) apoptosis should be favored for cancer therapy.

## X LEGENDS TO FIGURES

**1. Targeted expression of constitutively active Rb to the mammary gland.** (A, a) Schematic structure of Rb showing the E1A/large T binding domain (A and B), relative location of 16 Cdk phosphoacceptor sites and mutated residues in Rb $\Delta$ p34 and Rb $\Delta$ K11. (b) Schematic presentation of the MMTV-Rb $\Delta$ p34 and MMTV-Rb $\Delta$ K11 transgenes. (B) Detection of MMTV-Rb $\Delta$ K transgenes in the mammary gland. (a) Northern blot analysis. The transgene has a shorter 3'UTR and migrates faster. (b) Western blots of protein lysates (50  $\mu$ g) from mammary glands of 13 week-old virgin transgenic and wildtype females were developed with anti-HA monoclonal antibody. (c) Protein lysates (1.2 mg) from 14 month-old transgenic and control mammary glands were immuno-precipitated with anti-HA antibodies and western blotted with anti-Rb antibody. (d) RT-PCR analysis of the MMTV-Rb $\Delta$ p34#24 and MMTV-Rb $\Delta$ K11#29 transgenes relative to keratin 18 in virgin (V), pregnant (P), lactating (Lac) and involuting (Inv) glands. w, weeks; d, days; + and - signs indicate the presence or absence of reverse transcriptase. (C) Temporal expression analysis of endogenous Rb during mammatogenesis in wildtype females. (a-f) *In situ* hybridization analysis of endogenous Rb. Shown are bright field (BF, a,c,e) and dark field (DF, b,d,f) micrographs of representative sections. (b) Immunohistochemical analysis of endogenous Rb in wildtype mammary glands.

**2. Suppression of ductal growth in pubescent MMTV-Rb $\Delta$ K transgenic mice.** (A) Whole-mount staining of pubescent MMTV-Rb $\Delta$ p34 (a-b) and MMTV-Rb $\Delta$ K11 (c-d) transgenic and control females. LN, lymph node. (e) The distance of the TEBs to lymph-nodes in 7-week-old wildtype females was set at 100%. The average growth rates of endbuds in three transgenic females and three wildtype littermates were plotted against this value for each time point. (B) PCNA-staining of sections through endbuds of 6-week old MMTV-Rb $\Delta$ p34 (a-b) and MMTV-Rb $\Delta$ K11 (c-d) transgenic females and their control wildtype littermates. Arrows indicate PCNA-positive nuclei. (e) Quantitative analysis of the relative percentage of PCNA-positive nuclei in TEBs at the indicated stages. For each time point, two sections from three transgenic and three wildtype mice were analyzed. The average number of PCNA-positive nuclei in the control wildtype females was set at 100%.

**3. Precocious differentiation of the mammary epithelium in nulliparous MMTV-Rb $\Delta$ K transgenic females.** (A, a-b) Whole-mount analyses of 13-week-old nulliparous

transgenic and control females; **(c-d)** PCNA staining; **(e-f)** immunohistochemistry with  $\beta$ -Casein. **(B, a-c)** Whole-mount hematoxylin staining of 14 month-old control **(a)** and two nulliparous transgenic females **(b-c)**(40x). **(d)** Higher magnification (70x) of the mammary gland in panel **c**. Note the lobuloalveolar-like structure (LA), the hyperplastic alveoli (HP) and dilated duct. **(e, g)** H&E staining of the mammary gland of 14-month-old transgenic **(g)** and wildtype **(e)** littermate female. **(f)** H&E staining of a mammary gland from 17.5 day pregnant wildtype female (P17.5). Note the similarity of nulliparous transgenic gland **(g)** with 17.5 day-pregnant control gland **(f)**. **(h-j)** Immunohistochemical analysis of  $\beta$ -Casein in mammary glands of 14 month-old nulliparous wildtype **(h)** and two transgenic **(i-j)** females. **(C)** RT-PCR analysis of  $\beta$ -Casein expression in 9-20 week-old nulliparous transgenic and control females.

**4. Hyperplastic nodules in the mammary gland of MMTV-Rb $\Delta$ K and WAP-Rb $\Delta$ p34 transgenic mice.** **(A, a-b)** Low power views (8x) of whole-mount staining of #4 right inguinal mammary glands from 13.5 month-old nulliparous wildtype littermate and MMTV-Rb $\Delta$ p34#16 transgenic female. Arrows indicate multiple hyperplastic nodules. **(c-d)** 13.5 month-old multiparous wild type and MMTV-Rb $\Delta$ K11#29 transgenic littermate with a hyperplastic nodule. **(e-f)** High-power views of representative hyperplastic nodules (arrows) in independent MMTV-Rb $\Delta$ p34 transgenic lines. **(B, a)** Schematic structure of the WAP-Rb $\Delta$ p34 transgene. **(b)** RT-PCR analysis of WAP-Rb $\Delta$ p34 in 18.5 day-pregnant transgenic female. **(c-d)** Whole mount view of #4 inguinal mammary glands from 12 month-old nulliparous wildtype and WAP-Rb $\Delta$ p34#44 transgenic females (8x). **(e)** Representative, high-power view of hyperplastic nodules in a WAP-Rb $\Delta$ p34 transgenic female. **(f)** H&E staining of cross-section through a hyperplastic nodule.

**5. Mammary adenocarcinomas in MMTV-Rb $\Delta$ K and WAP-Rb $\Delta$ p34 transgenic mice and loss of transgene expression.** **(A)** Histology of mammary adenocarcinomas: **(a)** Whole mount staining of a palpable mammary adenocarcinoma in a 13.5-month-old nulliparous MMTV-Rb $\Delta$ p34#27 transgenic female, showing the periphery of the tumor with invasion into the ducts. **(b)** Mammary adenocarcinoma with multiple cystic areas in a 12 month-old, multiparous, MMTV-Rb $\Delta$ p34#24 transgenic female. **(c)** Well-differentiated tubulo-acinar mammary adenocarcinoma in

a 12 month-old, nulliparous WAP-Rb $\Delta$ p34#44 transgenic female. **(d)** Well-differentiated papillary mammary adenocarcinoma with peripheral growth of basaloid cells and radiating keratinization in a 14 month-old multiparous WAP-Rb $\Delta$ p34#61 transgenic female. **(e-f)** Papillary mammary adenocarcinoma and mammary adenocarcinoma in the first and third glands, respectively, from a multiparous 18 month-old MMTV-Rb $\Delta$ K11#29 transgenic female. **(g)** Multiple small breast tumors and hyperplastic nodules in MMTV-Rb $\Delta$ K11#29 retired breeder. **(h-k)** high power magnifications of the lesions shown in **g**. **h-j** are breast tumors; **k** is an enlarged lobuloalveolar-like structure. Tumors in **a-f** were about 1 cm in length and significantly larger than in **h**. **(B)** Analysis of mammary adenocarcinomas. **(a-b)** PCNA-staining demonstrating high mitotic index (**b**, arrows) in tumors relative to a tumor-free gland in the same transgenic female (**a**). **(c)** Positive staining of the mammary adenocarcinomas with a wide spectrum screening anti-Keratin antibody. **(d-e)** RT-PCR analysis of MMTV-Rb $\Delta$ K11 transgene expression (**d**) and keratin 18 (**e**) in tumor biopsies and a derived cell line. **(1-2)** Adenocarcinoma biopsys from the tumors shown in panel A,e and f; **(3)** mammary gland from a MMTV-Rb $\Delta$ K11 transgenic female; **(4)** cell line derived from the mammary adenocarcinoma in panel A,f; **(5)** mammary gland from a wildtype female. The + and – signs indicate the presence or absence of reverse transcriptase.

**6. Extended survival of the mammary epithelium in MMTV-Rb $\Delta$ K transgenic mice.** **(A-B)** Effects of activated Rb on first involution. **(A)** TUNEL analysis (**a**, **c**) and  $\beta$ -Casein immunohistochemistry (**b**, **d**) were performed on adjacent sections (**a-b**) and (**c-d**) from MMTV-Rb $\Delta$ K11 transgenic females at day six of first involution. Note the inverse correlation between the presence of apoptotic nuclei (black arrows in **a**) and the expression of  $\beta$ -Casein (white arrows in **d**) in the same lobules. **(B)** RT-PCR analysis of WAP and  $\beta$ -Casein in two transgenic and two wildtype littermates at day six of first involution. Expression of these milk genes was slightly higher in the transgenic females. **(C)** Lobulo-alveolar-like structures in multiparous, retired MMTV-Rb $\Delta$ K11 transgenic breeders (**b**, **d**, **f**) but not in control littermates (**a**, **c**, **e**). **(a-d)** H&E staining (a-b, 100x; c-d, 400x). **(e-f)** Gomori Trichrome staining for collagen (e, 1260x; f, 630x). ECM is present in transgenic (black arrow) but not wildtype females (white arrow). **(D)** Western blot analyses of **(a)** multiparous retired and **(b)** 4 month-old nulliparous females for the indicated proteins. **(E)** Models for the effects of inactivation versus activation of Rb. **(a)** Loss of Rb results in ectopic DNA synthesis, increased cell death and

incomplete differentiation. Unscheduled cell proliferation is counter-balanced by apoptosis. Suppression of apoptosis signal accelerates neoplastic transformation. Impaired differentiation in Rb-deficient mice may be a consequence of ectopic cell proliferation and apoptosis that perturbs the differentiation program or may reflect a direct requirement for Rb in the onset of terminal differentiation. **(b)** As shown herein, expression of constitutively active Rb in the mammary gland results in suppression of cell proliferation, extended survival, precocious differentiation and breast tumors. The precocious differentiation may reflect the ability of activated Rb to extend the survival of differentiated cells and/or to directly promote differentiation. We propose that transient activation of the Rb suppress apoptosis and lead to the accumulation of oncogenic alterations and neoplastic transformation of susceptible cells (see text for details).

**Table 1. Incidence of preneoplastic lesions and adenocarcinomas in transgenic mice expressing constitutively active Rb in the mammary gland**

Transgenic line		Number of mammary glands analyzed <sup>a</sup>		Number of mammary glands with tumors <sup>b</sup>		Incidence per line		Incidence combined
MMTV-LTR-Rb $\Delta$ p34(K8)	#24	38	63	8 <sup>c</sup>	14	21%		22%
	#16	7		4		57%		
	#8	16		1		6.25%		
	#27	2		1 <sup>c</sup>		50%		
Control littermates <sup>d</sup>		50		0		0%		0%
MMTV-LTR-Rb $\Delta$ K11	#29	18		4 <sup>3c</sup>		22%		22%
Control littermates <sup>d</sup>		7		0		0%		0%
WAP-Rb $\Delta$ p34(K8)	#9	7	21	2	8	28.6%		38%
	#44	5		4 <sup>c</sup>		80%		
	#61	9		2 <sup>c</sup>		22.2%		
Control littermates <sup>d</sup>		21		0		0%		0%

a. Preneoplastic lesions were detected by whole mount staining of the right inguinal mammary gland in 10 to 14 month old females. Mean 12 months.

Adenocarcinomas were detected visually and confirmed by histopathology.

b. Numbers include both preneoplastic lesions and adenocarcinomas.

c. Indicates one full-blown mammary adenocarcinoma in this group.

d. Control littermates were caged and analyzed together with transgenic females.

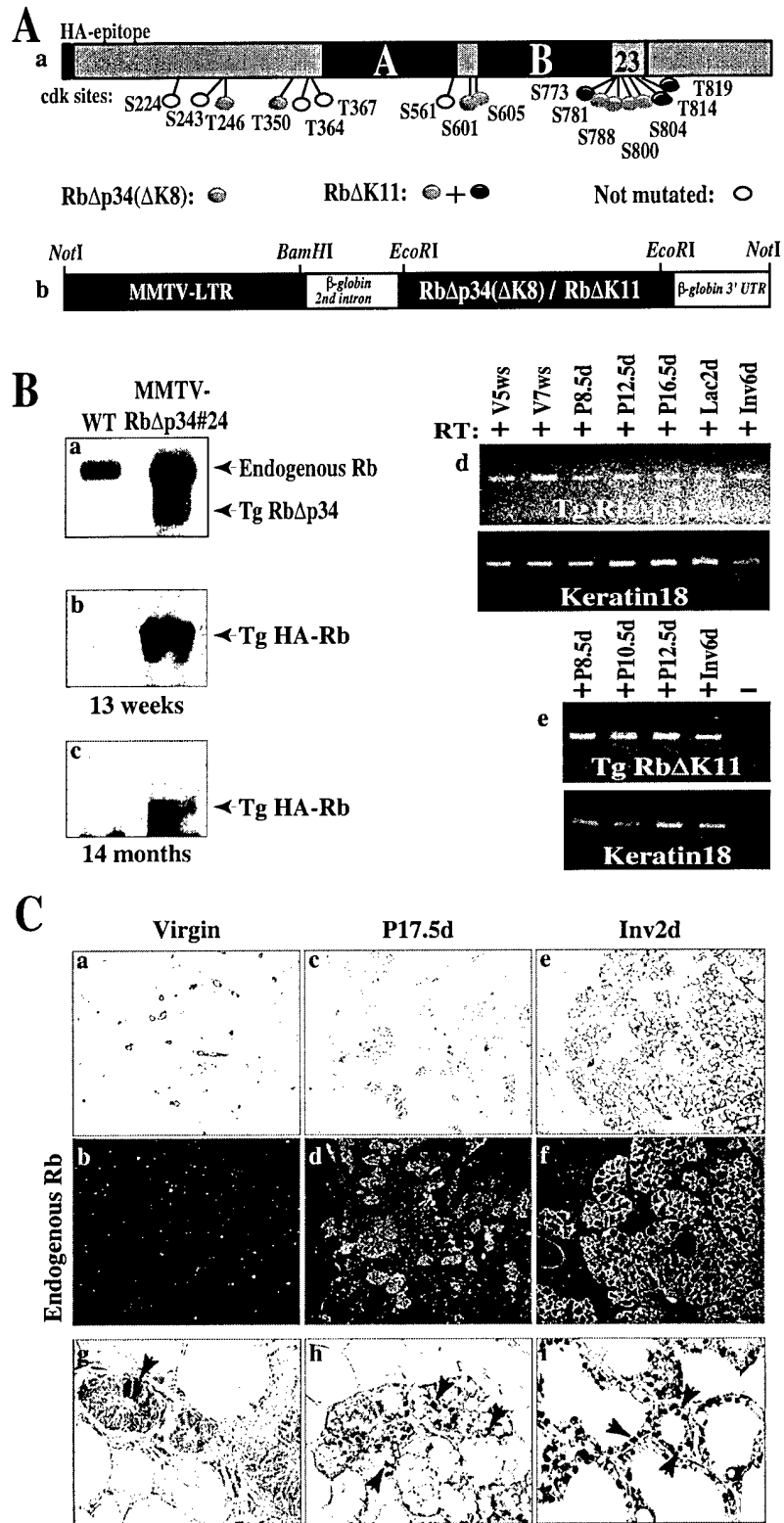


Fig. 1 Jiang & Zacksenhaus

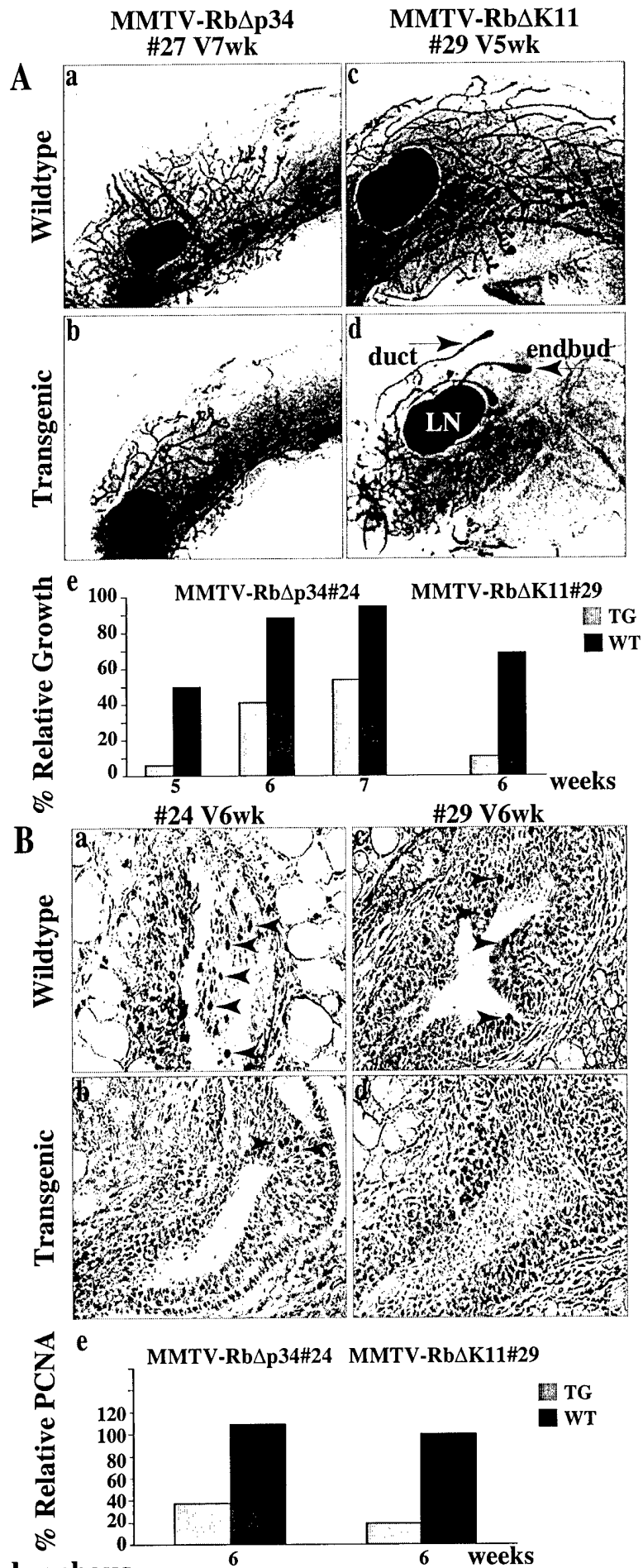


Fig. 2 Jiang & Zacksenhaus

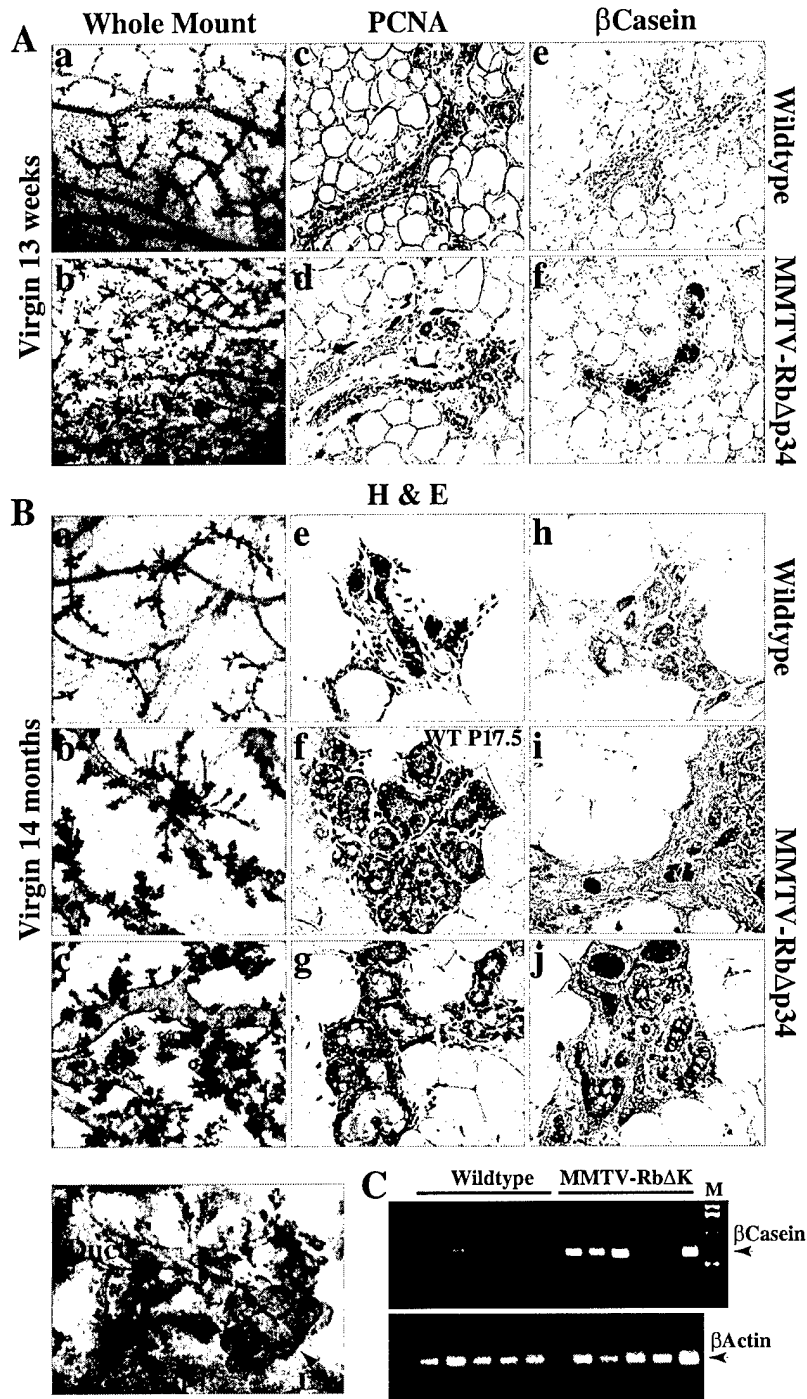
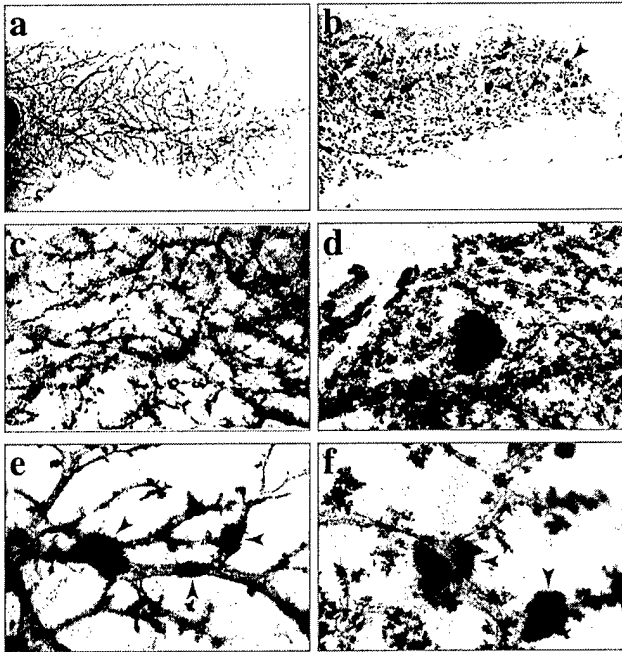
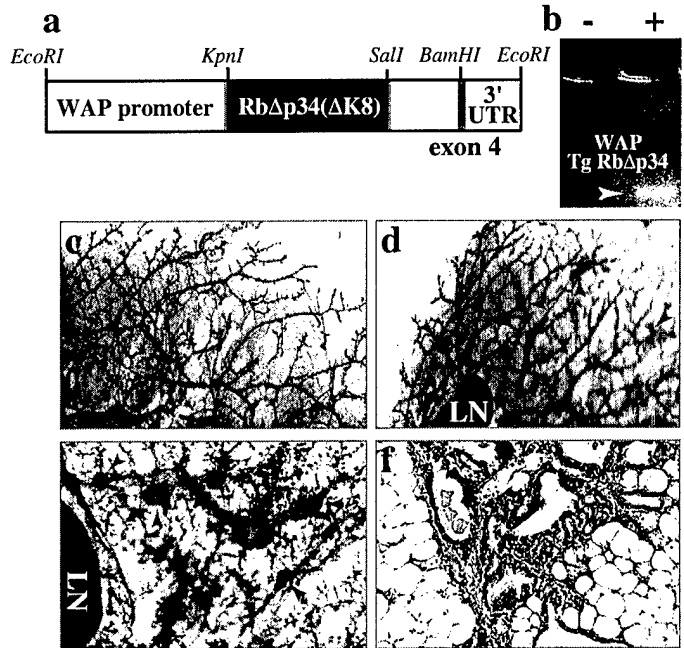


Fig. 3 Jiang & Zacksenhaus

**A**

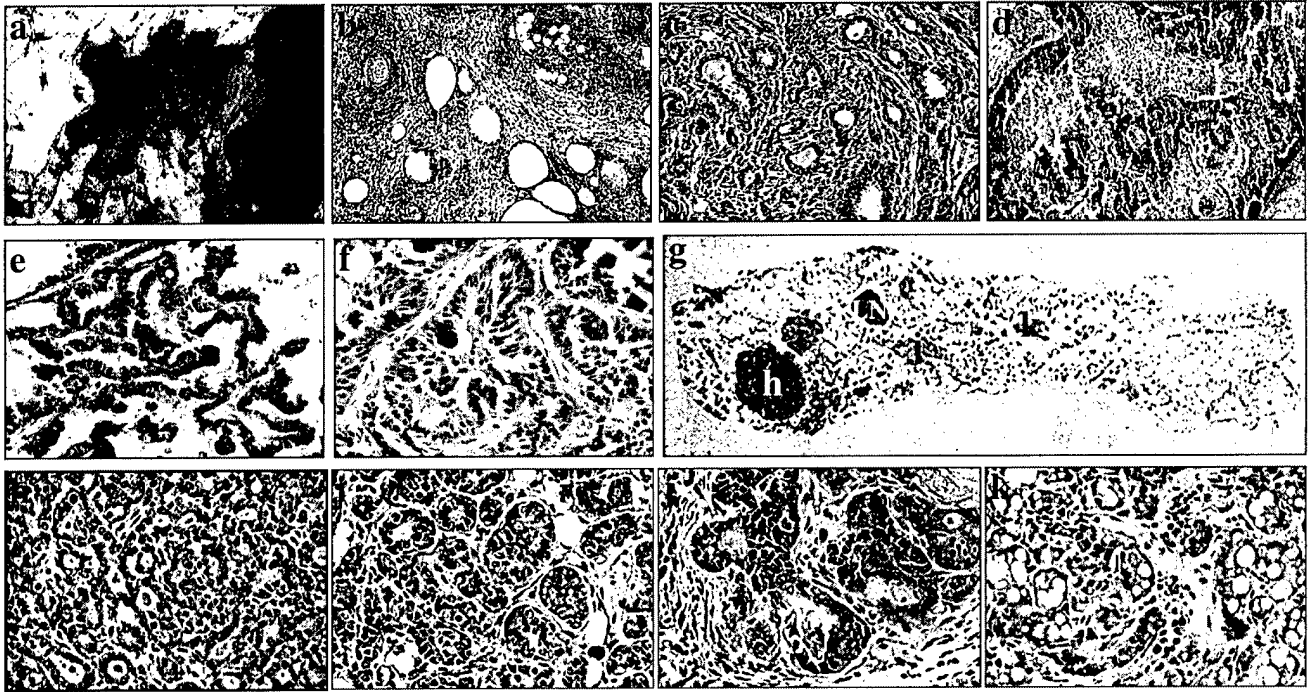


**B**



**Fig. 4 Jiang & Zacksenhaus**

A



B

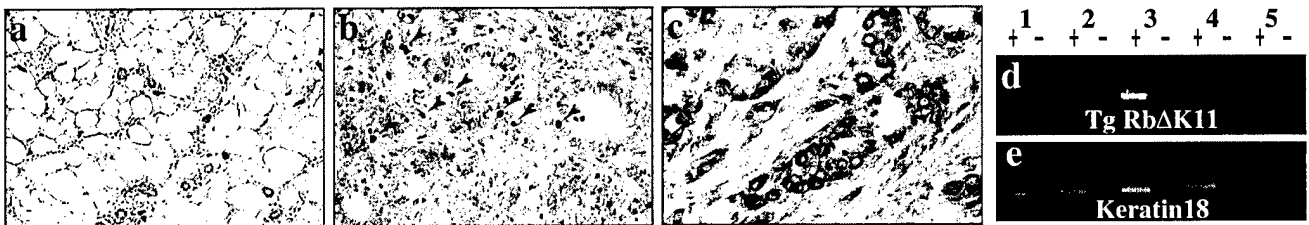


Fig. 5 Jiang & Zacksenhaus

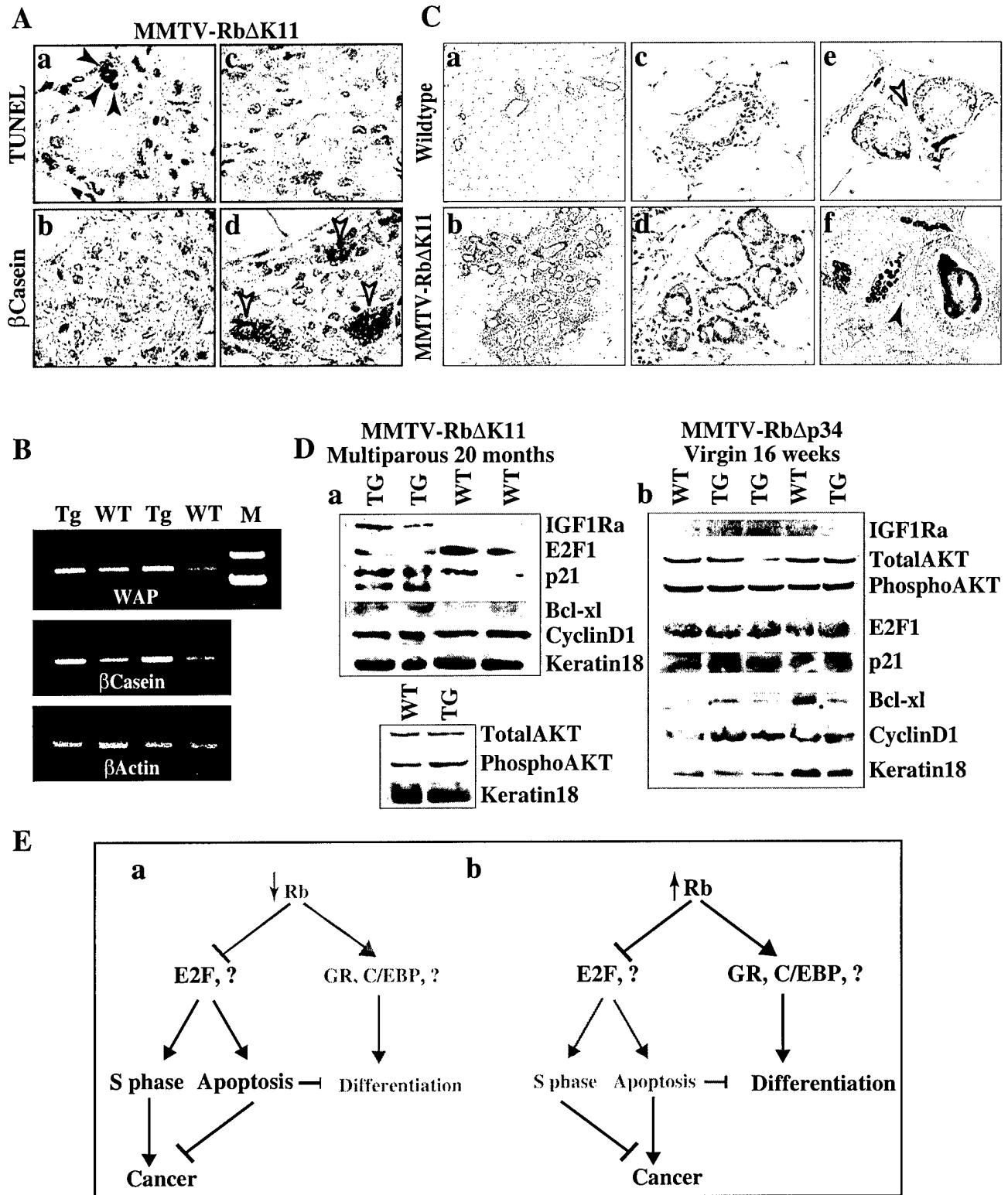


Fig. 6 Jiang & Zacksenhaus