Rb Regulates DNA Damage Response and Cellular Senescence through E2F-Dependent Suppression of N-Ras Isoprenylation

Rb Regulates DNA Damage Response and Cellular Senescence through E2F-Dependent Suppression of N-Ras Isoprenylation

Awad Shamma,[1,2] Yujiro Takegami,[1] Takao Miki,[1,2] Shunsuke Kitajima[1], Makoto Noda[1], Takao Obara,[3] Takahiro Okamoto,[3] and Chiaki Takahashi [1,2,*]

[1] Department of Molecular Oncology, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan

[2] The 21st Century Center of Excellence Formation, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan

[3] Department of Endocrine Surgery, Tokyo Women's Medical University, Tokyo 166-8666, Japan

[*]Correspondence: shamawad@staff.kanazawa-u.ac.jp and chtakaha@virus.kyoto-u.ac.jp

TEL: +81-75-751-4151

FAX: +81-75-751-4159

Running title: Rb controls Ras isoprenylation

Summary

Oncogene-induced cellular senescence is well documented, but little is known

about how infinite cell prolif- eration induced by loss of tumor suppressor genes is antagonized by cellular functions. Rb heterozygous mice generate Rb-deficient C cell adenomas that progress to adenocarcinomas following biallelic loss of N-ras. Here, we demonstrate that pRb inactivation induces aberrant expression of farnesyl diphosphate synthase, many prenyltransferases, and their upstream regulators sterol regulatory element-binding proteins (SREBPs) in an E2F-dependent manner, leading to enhanced isoprenylation and activation of N-Ras. Conse- quently, elevated N-Ras activity induces DNA damage response and p130-dependent cellular senescence in Rb-deficient cells. Furthermore, Rb heterozygous mice additionally lacking any of Ink4a, Arf, or Suv39h1 generated C cell adenocarcinomas, suggesting that cellular senescence antagonizes Rb-deficient carcino- genesis.

SIGNIFICANCE

Aberration of the RB pathway critically pertains to human carcinogenesis. However, germline RB mutations are detected in a limited variety of malignant tumors, implicating the presence of defense mechanisms antagonizing RB-deficient tumori- genesis. In this study, we attempted to clarify the mechanism by which N-ras loci protect mouse C cells and primary fibro- blasts from Rb loss-induced carcinogenesis and revealed a function of pRb in regulating posttranslational modification of CAAX proteins. Our findings explain the tumor suppressor function of N-Ras in certain types of cells lacking pRb. In addition, since Ras proteins promote carcinogenesis in many cell types, our

findings may provide a rational basis for the application of prenyltransferase inhibitors to human cancers with aberrations in the RB pathway.

Introduction

Carcinogenesis is induced by the breakdown of cellular func- tions that counteract various oncogenic stimuli (Hanahan and Weinberg, 2000). Oncogene-induced cellular senescence (OIS) was first noted as paradoxical growth arrest in human diploid fibroblasts induced by oncogenic Ras (Collado et al., 2007). Currently, OIS is accepted as the cellular function that antago- nizes carcinogenesis despite the presence of oncogenic muta- tion. The study of melanocytic nevi carrying the BRAFV600E muta- tion provided the first evidence of cellular senescence in human premalignant lesions (Mooi and Peeper, 2006). Since then, the number of identified OIS inducers has been increasing rapidly (Di Micco et al., 2007).

OIS is achieved by activating tumor suppressors including p16INK4a, ARF, pRB, and p53 or by inducing DNA damage response (DDR) activation engaged by gH2AX, Chk2, p53, and ATM before critical telomeric attrition occurs (Collado et al., 2007; Di Micco et al., 2007). These cellular functions counteract infinite cell proliferation induced by oncogenic signals. Acceler- ated cell proliferation induced by the loss of tumor suppressors can be counteracted by similar functions. For example, PTEN loss-induced prostate carcinogenesis is antagonized by p53- mediated senescence (Chen et al., 2005), and VHL

loss-induced cellular senescence is mediated by pRB and p400 (Young et al., 2008). In human retinoblastoma, additional aberration in the p53 pathway is required for carcinogenesis (Laurie et al., 2006). Nevertheless, it is suspected that RB deficiency in the human body can be antagonized in many ways because of the diversi- fied functions of pRB and the limited variety of mouse and human cell types that develop tumors with germline RB mutations (Wikenheiser-Brokamp, 2006).

Rb$^{+/-}$ mice generate C cell adenoma in the thyroid following somatic loss of the normal *Rb* allele. Additional loss of *N-ras* alleles induces malignant conversion in *Rb*-deficient C cell adenoma. Moreover, somatic N-ras loss frequently occurs in *Rb*$^{+/-}$;*N-ras*$^{+/-}$ C cells following *Rb* loss, causing spontaneous malignant conversion (Takahashi et al., 2006). The C cell tumor phenotype observed in *Rb*$^{+/-}$;*E2F3*$^{-/-}$ mice (Ziebold et al., 2003) is similar to that in *Rb*$^{+/-}$;*N-ras*$^{-/-}$ mice. It has been proposed that the Rb-E2F complex functions as a transcrip- tional repressor to antagonize Ras signaling during vulval formation in Caenorhabditis elegans (Ceol and Hor- vitz, 2001). The Rb-ras genetic interaction during cell differentiation, embryogen- esis, and tumorigenesis has been exten- sively investigated in mice (Lee et al., 1999; Takahashi et al., 2003, 2004, 2006); however, the core mechanism of this interaction has not yet been clarified. In this study, we sought in vitro and in vivo evidence that *Rb* loss-induced cell proliferation in mouse C cells and primary fibroblasts is counteracted by an N-Ras-dependent senescence pathway and attempted to

elucidate the mechanism by which pRb regulates N-Ras activation.

Results

N-ras-Dependent DDR and Cellular Senescence in Rb-Deficient C Cell Adenoma

To investigate how N-Ras prevents *Rb*-deficient C cell adenoma from progressing to adenocarcinoma (Figure 1A), we analyzed primary C cell tumors developed in $Rb^{+/-}$;$N\text{-}ras^{+/+}$ (average age at examination [AE] ± standard error of the mean [SEM] = 11.0 ± 1.2 months) and $Rb^{+/-}$;$N\text{-}ras^{-/-}$ (AE = 10.6 ± 0.9 months) mice. Proliferating cell nuclear antigen (PCNA) and Ki-67 were frequently expressed in *Rb*-deficient $N\text{-}ras^{-/-}$ adenocarcinomas, but their expression was invariably rare in Rb-deficient $N\text{-}ras^{+/+}$ adenomas (Figure 1B). On the other hand, *Rb*-deficient $N\text{-}ras^{+/+}$ adenomas frequently expressed DDR markers (Shiloh, 2003) including H2AX phosphorylated at serine 139 (gH2AX), ATM phosphorylated at serine 1981 (ATMpS1981), and p53 phosphorylated at serine 15 (p53pS15). These markers were rarely detected in $N\text{-}ras^{-/-}$ adenocarcinomas (Figure 1B). In addition, $N\text{-}ras^{+/+}$ tumors frequently expressed senescence markers (Dimri, 2005) including histone H3 trimethy- lated at lysine 9 (H3K9me3), heterochromatin protein 1g (HP1g), p16Ink4a, and senescence-associated b-galactosidase (SA-b-gal) activity. These markers were rarely detected in $N\text{-}ras^{-/-}$ adenocarcinomas (Figure 1B) or in adenocarcinomas spontaneously developed in $Rb^{+/-}$;$N\text{-}ras^{+/-}$ mice as a consequence of somatic *N-ras* allele loss following *Rb* loss (see Figure S1 available online). These

findings suggest that *N-ras* loci induce DDR and cellular senescence during adenoma formation initiated by *Rb* loss. We there- fore hypothesized that N-Ras prevents *Rb*-deficient C cell adenomas from progressing to adenocarci- nomas by inducing DDR and cellular senescence.

To determine the sequence of events during adenoma development, we compared *Rb*-deficient adenomas developed in younger $Rb^{+/-}$ mice (early adenoma; AE = 6.2 ± 0.3 months) to those in older mice (late adenoma; AE = 11.0 ± 1.2 months). DDR markers were expressed in early and late adenomas with similar frequencies; however, senescence markers were less frequently ex- pressed in early adenomas (Figure S2). In contrast, PCNA and Ki-67 were more frequently expressed in early adenomas than in late adenomas. These findings suggest that DDR may precede cellular senescence during the development of C cell adenoma initiated by *Rb* loss, and that early adenomas grow even in the presence of DDR but cease to grow when cellular senescence is induced.

Role of Senescence-Mediating Genes in *Rb*-Deficient C Cell Adenoma

To examine whether cellular senescence protects *Rb*-deficient C cell adenomas from progressing to adenocarcinoma, we generated $Rb^{+/-}$ mice simultaneously lacking *Ink4a*, *Ink4a* and *Arf*, *Arf*, or *Suv39h1*. All $Rb^{+/-}$;$Ink4a^{-/-}$ mice survived longer than 8 months (n = 12). The average age at onset of sickness or death (ASD) was 9.3 ± 0.4 months in $Rb^{+/-}$;$Ink4a^{+/+}$ (n = 7), 9.5 ± 0.6 months in $Rb^{+/-}$;$Ink4a^{+/-}$ (n = 3), and 9.7 ± 0.4 months in $Rb^{+/-}$;$Ink4a^{-/-}$ (n = 6) mice. No

acceleration of *Rb*-deficient pituitary tumorigenesis was observed in the *Ink4a*$^{-/-}$ background (data not shown). All *Rb*$^{+/-}$;*Ink4a*$^{-/-}$ mice exhibited large macroscopic tumors stemming from the thyroid (Figure 2A; Table 1). *Rb*$^{+/-}$;*Ink4a*$^{+/-}$ mice generated macroscopic tumors, but these were smaller than those in *Rb*$^{+/-}$;*Ink4a*$^{-/-}$ mice. No macroscopic thyroid tumors were observed in *Rb*$^{+/-}$;*Ink4a*$^{+/+}$ mice. Histologically, all thyroid tumors devel- oped in *Rb*$^{+/-}$;*Ink4a*$^{-/-}$ and *Rb*$^{+/-}$;*Ink4a*$^{+/-}$ mice exhibited features of C cell adenocarcinoma (Figure 2B; Table 1) and were similar to tumors developed in *Rb*$^{+/-}$;*N-ras*$^{-/-}$ mice. *Rb*$^{+/+}$;*Ink4a*$^{-/-}$ mice showed no evidence of microscopic thyroid tumors. *Rb*-deficient *Ink4a*$^{-/-}$ cells separated from primary tumors grew in tissue culture and expressed calcitonin (5 successful cultures from 6 attempts with independent tumors) (Figure 2C), as did *Rb*-deficient *N-ras*$^{-/-}$ C cell adenocarcinoma cells. However, no clonally expanding calcitonin-positive cells were derived from thyroids harboring *Rb*-deficient *Ink4a*$^{+/+}$ tumors (0 of 6), suggesting that, like *Rb*-deficient *N-ras*$^{-/-}$ C cells, *Rb*-deficient *Ink4a*$^{-/-}$ C cell tumor cells had acquired the ability to infinitely proliferate. Therefore, we diagnosed tumors developed in *Rb*$^{+/-}$;*Ink4a*$^{-/-}$ mice as C cell adenocarcinomas. These findings suggest that C cell carcinogenesis initiated by *Rb* loss is blocked by *Ink4a*. *Rb*$^{+/-}$;*Ink4a*$^{-/-}$;*Arf*$^{-/-}$ (ASD = 5.3 ± 0.1 months; n = 9) and *Rb*$^{+/-}$;*Arf*$^{-/-}$ (ASD = 4.7 ± 0.2 months; n = 3) mice died of lymphoma, sarcoma, or more often accelerated pituitary tumor growth as described previously (Tsai et al., 2002) before they developed macroscopic thyroid tumors. However, at 4.8 months,

Rb$^{+/-}$;*Ink4a*$^{-/-}$;Arf$^{-/-}$ mice developed large microscopic adenocarcinomas with a morphology different from that of *Rb*-deficient *N-ras*$^{-/-}$ or *Rb*-deficient *Ink4a*$^{-/-}$ tumors (Figure 2B). *Rb*$^{+/+}$;*Ink4a*$^{-/-}$;*Arf*$^{-/-}$ mice of the same age or older showed no evidence of microscopic thyroid tumors (Table 1). In *Rb*$^{+/-}$;*Arf*$^{-/-}$ mice (AE = 5.2 ± 0.3 months), but not in *Rb*$^{+/+}$; *Arf*$^{-/-}$ mice, we often observed large microscopic adenocarcinomas with morphologies different from those in *Rb*-deficient *N-ras*$^{-/-}$, *Rb*-deficient *Ink4a*$^{-/-}$, or *Rb*-deficient *Ink4a*$^{-/-}$;*Arf*$^{-/-}$ tumors. Tumor cells separated from *Rb*$^{+/-}$;*Ink4a*$^{-/-}$;*Arf*$^{-/-}$ (3 of 3) and *Rb*$^{+/-}$;*Arf*$^{-/-}$ (2 of 2) mice grew in tissue culture and expressed calcitonin. However, in the *Arf*$^{-/-}$ background, regardless of Ink4a genotype and despite the frequent PCNA and Ki-67 expression and infinite growth in culture, the frequency of carcinoembryonic antigen (CEA)-positive C cell tumor cells was low (Figure 2B; Table 1). This suggests a specific genetic interaction between Arf and CEA. *Rb*$^{+/-}$;*Suv39h1*$^{-/-}$ mice (AE = 4.6 ± 0.2 months) exhibited macroscopic tumors (Figure 2A; Table 1) that grew in tissue culture (2 of 2) (Figure 2C) and fre- quently expressed CEA, PCNA, and Ki-67 (Figure 2B; Table 1). These findings suggest that *p16*^{Ink4a}, *Arf*, and *Suv39h1* antagonize *Rb*-deficient C cell carcinogenesis.

Role of N-Ras in *Rb*-deficient C-cell carcinogenesis

We generated 11 cell lines from independent primary *Rb*-deficient *N-ras*$^{-/-}$ tumors; all of them were calcitonin positive (data not shown). Six cell lines were less adhesive. Five adhesive cell lines were competent for transfection and

infection. After transduction of N-Ras or N-Ras^{V12}, all the adhesive cell lines grew without immediate growth arrest or cell death for a minimum of 5 days. Moreover, upon subcutaneous inoculation into nude mice, one of the adhesive cell lines, AC61, produced tumors histologically similar to primary *Rb*-deficient *N-ras*$^{-/-}$ tumors. Importantly, this cell line generated smaller subcutaneous tumors with features of *Rb*-deficient adenomas when reconstituted with N-Ras at the level comparable to that of the endogenous N-Ras expressed in the *Rb*-positive and *ret* mutation-positive human C cell adenocarcinoma cell line TT (Tomoda et al., 2008) (Figure 3A).

Nine days after *N-ras* transduction, we observed a marked induction of DDR (Figure 3B). Comet assay detected high frequency of DNA double strands breaks in N-Ras-transduced cells within 10 days (Figure 3C). At day 14, we observed marked induction of senescence markers, including recruitment of Suv39h1, H3K9me3 and HP1γ to the chromatin and induction of p16^{Ink4a}, p19^{Arf} p107 and p130 (Figure 3D and E). We also detected senescence-associated heterochromatin foci (SAHF) (Narita et al., 2003) (Figure 3D) and high SA-β-gal activity (Figure 3F). The other 4 cell lines (AC55, 62, 65 and 69) responded to N-Ras in a manner similar to AC61 (Figure S3). We did not detect telomerase (mTERT) expression in AC61 cells. AC55 cell line was telomerase positive; however, N-Ras transduction did not significantly change mTERT expression nor telomerase activity in AC55 cells, excluding the possibility of replicative

senescence (Figure S4A-C). These findings indicate that in the absence of pRb, N-Ras induces DNA damage and cellular senescence that function as barrier against C cell carcinogenesis.

p130-dependent differential frunctions of wild-type and oncogenic N-Ras in the absence of pRb

When introduced into AC61 cells, N-Ras^{V12} was far less efficient to induce DDR and senescence markers (Figure 3E), growth arrest, and SA-β-gal activity (Figure 3F) compared to N-Ras. Further, we observed only a slight decrease in the proliferation of pRb-reconstituted AC61 cells over 10 days suggesting that AC61 cells were reprogrammed to be no more addicted to *Rb*-loss at least within this period (Figure 3F). The effects of N-Ras and N-Ras^{V12} in inducing cellular senescence were reversed after pRb reconstitution (Figure 3F). These findings suggest that pRb attenuates N-Ras function to induce cellular senescence in AC61 cells and that N-Ras^{V12} fails to activate alternative cellular senescence programs when pRb is unavailable.

We observed that p130 was markedly induced and accumulated in the nuclei of AC61 cells transduced with N-Ras, which was not observed when transduced with N-Ras^{V12} (Figure 3E and G). Nuclear p130 was complexed with Suv39h1, H3K9me3 and HP1γ in the presence of N-Ras but not N-Ras^{V12} (Figure 3G). Consistent with this, we detected the colocalization of p130 and HP1γ signals in

the nucleus of N-Ras-reconstituted cells. N-Ras failed to induce cellular senescence in AC61 cells when p130 was depleted beforehand by specific shRNA (Figure 3H; Figure S5A). Moreover, p130 depletion at day 14 after N-Ras transduction significantly reversed cellular senescence (Figure 3I and Figure S5B). These findings suggest that p130 is required for induction and maintenance of N-Ras-induced cellular senescence in *Rb*-deficient cells. We observed significant p130 induction in primary C cell adenomas developed in $Rb^{+/-}$; $N\text{-}ras^{+/+}$ mice but not in adenocarcinomas developed in $Rb^{+/-}$; $N\text{-}ras^{-/-}$ mice (Figure 3J). Taken together, these findings suggest that induction of p130 and and its recruitment to the chromatin is ciritical for cellular senescence induced by N-Ras in *Rb*-deficient C cell adenocarcinoma cells, and that $N\text{-}Ras^{V12}$ fails to induce cellular senescence in these cells due to inability to induce p130.

$Rb^{-/-}$; $N\text{-}ras^{-/-}$ MEFs escape cellular senescence

To generalize our observation on C cells, we characterized mouse embryonic fibroblasts (MEFs) prepared from $Rb^{-/-}$ embryos with various *N-ras* genotypes. During the serial passages according to 3T3 protocol (Todaro and Green, 1963), $Rb^{-/-}$;$N\text{-}ras^{-/-}$ MEFs achieved infinite proliferation significantly earlier than $Rb^{-/-}$ MEFs (Figure 4A). $Rb^{-/-}$; $Ink4a^{-/-}$ MEFs exhibited proliferative characteristics similar to those of $Rb^{-/-}$; $N\text{-}ras^{-/-}$ MEFs.

$Rb^{-/-}$; $N\text{-}ras^{-/-}$ MEFs but not $Rb^{+/+}$; $N\text{-}ras^{+/+}$, $Rb^{-/-}$; $N\text{-}ras^{+/+}$ or $Rb^{+/+}$; $N\text{-}ras^{-/-}$ MEFs

could form colonies when plated at low cell density (Figure 4B). Moreover, shRNA-directed N-Ras depletion in *Rb*$^{-/-}$ MEFs allowed them to form colonies under the same condition (Figure 4C). Furthermore, *Rb*$^{-/-}$;*Ink4a*$^{-/-}$ MEFs formed colonies when plated at low cell density, which was suppressed by p16^{INK4a}. Colony formation by *Rb*$^{-/-}$;*N-ras*$^{-/-}$ MEFs was suppressed either by N-Ras, pRb or p16^{INK4a} but not by N-Ras^{V12} (Figure 4D). N-Ras^{N17} did not reduce colony numbers suggesting that the function of N-Ras in colony suppression depends on GTP. The colony suppression in *Rb*$^{-/-}$;*N-ras*$^{-/-}$ MEFs induced by N-Ras was due to cellular senescence (Figure 4E and Figure S6). N-Ras^{V12} and other activated Ras isoforms did not induce cellular senescence but induced transofmation in *Rb*$^{-/-}$; *N-ras*$^{-/-}$ MEFs (Figure 4E and F). However, N-Ras^{V12} induced senescence in *Rb*$^{+/+}$;*N-ras*$^{-/-}$ MEFs (Figure 4E) and wild-type MEFs (data not shown). A previous study demonstrated that *Rb*$^{-/-}$;*p107*$^{-/-}$;*p130*$^{-/-}$ MEFs escaped cellular senescence and are efficiently transformed by H-Ras^{V12} (Sage et al., 2000). Similarly, *Rb*$^{-/-}$;*N-ras*$^{-/-}$ MEFs were transformed by all isoforms of Ras^{V12} (Figure 4F). These findings suggest that MEFs can be transformed by N-Ras^{V12} once exempted from senescence program, and thereby support that *Rb*$^{-/-}$;*N-ras*$^{-/-}$ MEFs escape cellular senescence.

Finally, to further elucidate the differential functions of N-Ras and N-Ras^{V12} in the absence of pRb, both proteins were simultaneously expressed in *Rb*$^{-/-}$;*N-ras*$^{-/-}$ MEFs. N-Ras was dominant over N-Ras^{V12} in inducing DDR and most of the

senescence markers, but N-Ras^{V12} antagonized N-Ras activity to induce p16^{Ink4a} in the absence of *Rb* (Figure S6). These findings suggest that N-Ras and N-RasV12 exert many different biological func- tions in the absence of *Rb.* To explain this difference, we analyzed their activation mechanisms in the studies discussed below.

pRb attenuates N-Ras activity in C cell adenocarcinoma

To address why N-Ras and N-Ras^{V12} exert different functions in the absence of pRb, we again analyzed AC61 cells. N-Ras expressed in AC61 cells was significantly activated; however, the activity was decreased to 10.4% (9.6 to 1.0) when pRb was simultaneously expressed under the influence of a strong promoter (Figure 5A). The relative activation level of N-Ras^{V12} (65.1) was 6.8 times higher than that of N-Ras (9.6) in the absence of pRb. The activity of N-Ras^{V12} was also attenuated by pRb, but this attenuation was less efficient (65.1 to 37.5) than that of N-Ras (9.6 to 1.0). Freshly introduced exogenous pRb was hyperphosphorylated when coexpressed with N-Ras^{V12} compared to N-Ras (Figure 5A), as expected from a previous study (Peeper et al., 1997). We therefore considered that hypophosphorylated pRb is more potent than hyperphosphory- lated pRb in suppressing N-Ras activity. These findings suggest that in the absence of pRb, the activation level of N-Ras is moderate yet significantly lower than that of N-Ras^{V12}. This may at least partially explain the differential functions of N-Ras and N-Ras^{V12} in the absence of pRb.

pRb increases unprenylated N-Ras

To explore the mechanism of N-Ras activation control by pRb, we transduced AC61 cells with moderately expressed pRb using a retrovirus of known titer (1.0 multiplicity of infection). We then transiently transduced these cells with the monomeric VenusA207R-tagged N-Ras (Venus-N-Ras). The higher molecular weight endowed by tagging provided us with a better separation of proteins during SDS-PAGE. In addition, rapid analysis immediately after completing transfection and under optimal conditions enabled us to detect cytosolic (unprenylated) Venus- N-Ras proteins by immunoblotting (IB) and image analyses before the majority of these proteins became isoprenylated. In cells growing in 10% FBS, the moderate expression of pRb attenuated Venus-N-Ras activity to almost half (Figure 5B). However, under these conditions, the difference in the migration degree of Venus-N-Ras was barely noticeable. When we decreased FBS to 0.5% in an attempt to enhance pRb activity, we observed an increased proportion of the unprenylated form only in the presence of pRb. Under these conditions, the majority of pRb was hypophosphorylated. This effect of pRb was compa- rable to that of prenyltransferase inhibitors (PTIs) and was reversed by 1 hr serum restimulation. However, Venus-N-Ras activation upon serum restimulation was significantly delayed in the presence of pRb or PTIs (Figure 5B). We observed the same effect of pRb on N-Ras isoprenylation in another Rb-deficient N-ras$^{-/-}$ cell line (AC62) (Figure 5C). These findings suggest that pRb delays N-Ras isoprenylation and activation.

pRb downregulates membrane trafficking of N-Ras

We observed an increased proportion of unprenylated N-Ras by IB in pRb-positive cells only when cultured under low serum conditions. N-Ras proteins in the isoprenylated fractions included not only those anchored to the plasma membrane but also those anchored to endomembranes such as Golgi, endo- plasmic reticulum, and other vesicles. Thus, a considerable proportion of N-Ras is membrane anchored but not always activated. Therefore, we next investigated the effects of pRb on subcellular localization of N-Ras. We cotransfected Venus- N-Ras and red fluorescent protein (RFP)-tagged Rab6A (RFP-Rab6A) as a Golgi marker without the CAAX motif (Goud et al., 1990). Twelve hours after transfection in 10% FBS, Venus-N-Ras overlapping with RFP-Rab6A became detectable in both pRb-negative and -positive cells (Figure 5D). We then decreased the FBS concentration to 0.1% for 12 hr and observed that the signal overlap was barely detectable in pRb-positive cells, whereas it was readily detectable in pRb-negative cells. The abundance and subcellular localization of RFP-Rab6A were not affected by pRb or by serum depletion. PTIs exhibited the same effect as pRb during serum depletion. Under these conditions (0.1% FBS for 12 hr), we observed that in the absence of pRb, about half of the Venus-N-Ras was isoprenylated and enriched in the membrane fraction, whereas in the presence of pRb, most of the Venus-N-Ras was still unprenylated and re- mained in the cytosolic fraction (Figure 5D). After 18 hr

serum depletion, we observed a slight recovery in the signal overlap in pRb-positive cells. Serum restimulation enabled the recovery of signal overlap to the initial level after 2 hr (Figure 5D). These findings suggest that serum concentration influences the pRb function in reducing N-Ras in Golgi at a particular time point. Disappearance of Venus-N-Ras from Golgi can be explained by downregulated transport to Golgi due to the decreased isoprenylation.

Next, to examine whether delayed membrane trafficking attenuates N-Ras activation, we observed AC61 cells cotransfected with Venus-N-Ras and RFP-Rab6A using a confocal laser microscope. After 24 hr culture in 10% FBS and subsequent 48 hr culture in 0.1% FBS, the overlap of Venus-N-Ras and RFP-Rab6A signals was significantly higher in pRb-negative cells than in pRb-positive cells; however, the Venus-N-Ras signal was not detectable at the plasma membrane in either (Figure 5E; Figure S7). Notably, 10 min after serum restimulation, a strong Venus-N-Ras signal was detected in the periphery of pRb-negative, but not pRb-positive, cells (Figure 5E; Figure S7). These observations suggest that pRb delays Venus-N-Ras transport to Golgi by attenuating endomembrane anchoring and thereby delays further trafficking to the plasma membrane. We further speculated that N-Ras-induced cellular senescence in Rb-deficient C cells depends on isoprenylation, as the N-RasSAAX (N-RasS186) mutant that cannot be isopreny- lated completely failed to induce DDR and cellular senescence in AC61 cells (Figure 5F) or to

suppress colony formation by *Rb*$^{-/-}$;*N-ras*$^{-/-}$ MEFs (data not shown). Similarly, treatment with PTIs significantly suppressed N-Ras-induced DDR and cellular senescence in AC61 cells (Figure 5G). Finally, we examined whether *Rb* loss increases N-Ras isoprenylation. Venus-N-Ras was introduced into *Rb*$^{+/+}$ and Rb$^{-/-}$ MEFs. Upon serum starvation initiated 8 hr after transfection, Rb$^{-/-}$ MEFs exhibited faster isoprenylation of Venus-N-Ras protein compared to littermate Rb$^{+/+}$ MEFs (Figure 5H; Figure S8), suggesting that *Rb* loss in MEFs accelerates N-Ras isoprenylation.

pRb downregulates genes involved in isoprenylation

A previous study demonstrated that the influence of pRb on Ras activity depends on de novo protein synthesis (Lee et al., 1999). We therefore performed microarray analysis on AC61 cells freshly transduced with moderately expressed pRb. These cells were maintained unsynchronized in 10% FBS, which allowed the cells to attenuate N-Ras activity to half (Figure 5B). Genes significantly upregulated by pRb included many cell surface anti- gens, lymphokines, interferons, and inflammation-related genes (Figure S9), similar to previous results obtained by analyzing MEFs conditionally lacking pRb (Markey et al., 2007). Moreover, in genes significantly downregulated by pRb, we detected known E2F targets such as *Cdc6*, *N-myc*, and *Mmp-3* with the highest statistical significance (change p value > 0.99998 as determined by Wilcoxon's signed rank test), suggesting that pRb reconstitution was successful (Table S1).

In 396 significantly upregulated genes (change p value < 0.001), we did not detect genes that could be directly involved in suppressing Ras activity such as GTPase-activating protein (Gap). In 270 significantly downregulated genes (change p value > 0.9998), we found that farnesyl diphosphate farnesyltransferase 1 (Fdft1) had the highest rank (Table S1). In addition, we detected a number of genes (Table S2) predicted to be transactivated by sterol regulatory element-binding proteins (SREBPs) via sterol regulatory element (SRE) (Sakakura et al., 2001). Other genes that could be directly involved in stimulating Ras activation, such as GTP exchange factor (Gef) or receptor tyrosine kinase (Rtk), were not detected. We then analyzed pRb-reconstituted AC61 cells by RT-PCR. Surprisingly, in addition to Fdft1, farnesyl diphosphate synthase (Fdps) (Szkopinska and Plochocka, 2005), most of the prenyltransferases (farnesyltransferase a [Fnta], farnesyltransferase b [Fntb], protein geranylgeranyltransferase 1b [Pggt1b], and Rab geranylgeranyltransferase b [Rabggtb]) (Maurer-Stroh et al., 2003), and SREBP-1 and SREBP-2 were significantly downregulated by pRb. All of these genes were induced in MEFs following Rb loss or pRb inactivation using SV40 large T antigen or adenovirus E1A (Figure 6A). A farnesyl- transferase b immunoblot validated the RT-PCR results (Figure 6B). We did not detect a significant change in expression of NF1 (Courtois-Cox et al., 2006) (data not shown).

To investigate the in vivo relevance of the increased expression of genes

involved in protein isoprenylation in Rb-deficient cells, primary Rb-deficient C cell adenomas were immunohistochemically analyzed. These tumors expressed significantly higher levels of Fntb, SREBP-1, and SREBP-2 compared to normal thyroid tissue (Figure 6C). We then compared the expression levels of these proteins between pRb-positive ret mutation-positive human medullary thyroid carcinoma cell line TT (Tomoda et al., 2008) and AC61. AC61 cells expressed significantly higher levels of Fnta, Fntb, SREBP-1, and SREBP-2 compared to TT cells (Figure S10).

E2F-dependent transcription control of genes involved in isoprenylation

To further investigate the mechanism by which pRb controls genes involved in protein isoprenylation, we analyzed the promoter regions of these genes. We detected several E2F-binding consensus sequences in the promoter regions of SREBPs (Figure S11). Moreover, we detected several E2F-binding consensus sequences in each of the promoters of Fdps and prenyltransferases. These findings suggest that E2Fs control Fdps and prenyltransferases via a dual mechanism involving SREBPs and direct binding. Chromatin immunoprecipitation (ChIP) assay detected the direct binding of E2F-1 and E2F-3 to the promoters of these genes specifically in the absence of pRb (Figure 6D). E2F-3 exhibited stronger DNA-binding activity to the Fntb promoter than E2F-1 did. We also detected direct binding of pRb to the Fdps promoter. pRb prevented SREBPs from binding to the Fdps promoter, probably via

downregulation of SREBPs. Furthermore, a pRb mutant derived from a retinoblastoma and lacking the ability to attenuate Ras activation (Lee et al., 1999) could not bind to the Fdps promoter and did not suppress SREBP binding. However, a partially penetrant mutant pRb (Sellers et al., 1998) with the ability to attenuate Ras activation (Lee et al., 1999) bound to the Fdps promoter and suppressed SREBP binding (Figure S12), suggesting that the function of pRb in attenuating Ras activation is not separable from its ability to regulate the Fdps promoter and SREBPs.

Fdps, Fntb, and SREBP-1 promoters were downregulated by pRb. This effect was augmented by serum depletion. These promoters were upregulated by either E2F-1 or E2F-3 (Figure 6E). In particular, E2F-3 was more potent than E2F-1 in transactivating the Fntb promoter, in agreement with the ChIP results (Figure 6D). The truncated mutant E2F containing the DNA-binding domain but lacking the transactivation domain (E2F-DB) slightly but significantly downregulated these promoters, suggesting the involvement of liberated E2Fs in the basal transactivation. Fdps and SREBP-1 promoters were upre- gulated by SREBP-1c, but the Fntb promoter, which lacks SRE, was not. The dominant-negative SREBP-1c downregulated the basal transactivation of these two promoters, indicating increased endogenous SREBP activity in AC61 cells. Further- more, these three promoters were downregulated by pRb in two other pRb-negative cell lines (*Rb*$^{-/-}$;*N-ras*$^{-/-}$ MEFs and Saos2). Transduction of E2F-DB, SV40 large T antigen, or adenovirus E1A in MEFs induced Fdps promoter

transactivation (Figure 6E). E2F-DB is known to affect the functions of E2Fs in multiple ways; one is derepression of active transcriptional silencing by the pRb-E2F complex (Rowland et al., 2002). Thus, we considered that the pRb-E2F complex directly partici- pates in the transcriptional repression of Fdps.

pRb-dependent protein farnesylation

The CAAX motif constitutes a C-terminal tetrapeptide common to all Ras proteins, directing multistep posttranslational modifi- cations initiated by farnesyltransferases (Hancock, 2003). There are approximately 300 proteins with the CAAX motif that are potentially farnesylated. To investigate pRb's effects on these proteins, we analyzed AC61 cells or MEFs metabolically labeled with [3H]farnesol. In 10% FBS, pRb-negative cells showed an increased density of farnesylated protein bands. When serum was depleted, this difference became greater (Figure 6F; Figure S13). These findings suggest that Rb loss induces enhanced global protein farnesylation.

Clinical relevance of pRb-N-Ras pathway in MTCs

Human medullary thyroid carcinomas (MTCs) are pathologically similar to the C cell adenocarcinomas in the mice analyzed in this study. Human C cell adenoma is extremely rare. Familial MTCs are frequently associated with mutation in the ret oncogene; however, almost half of sporadic MTCs are free of the ret mutation (Marsh et al., 2003), indicating a ret-independent mechanism involved in human

C cell carcinogenesis. To address the relevance of the pRb-N-Ras pathway in human tumors, we analyzed 13 sporadic MTC cases, of which 6 (46%) were immu- nohistochemically negative for pRb and 7 (54%) were negative for N-Ras (Figure 7A). Notably, 6 of 7 N-Ras-negative tumors were negative for pRb (Figure 7B). This finding in human sporadic MTC is consistent with the requirement of N-Ras suppression in Rb-deficient C cell carcinogenesis in mice.

Discussion

The current work provides evidence that in the absence of pRb, E2F-1 and E2F-3 participate in the transactivation of most of the genes involved in protein isoprenylation either directly or via SREBPs. This at least partially explains the Ras activity elevation induced by pRb inactivation (Figure S14A). It would be of interest to ascertain whether under physiological conditions, Ras and pRb activities oscillate due to mutual suppression via cyclin D/Cdk4/6 and E2F/isoprenylation (Figure S14B). Furthermore, this study partially clarifies the mechanism of the tumor suppressor function of wild-type Ras. It has previously been shown that loss of the normal ras allele, in addition to activating mutation, is required for tumorigenesis (see references in Zhang et al., 2001). This study indicates that N-Ras and N-RasV12 exert different functions in Rb-deficient cells where only N-Ras can induce p130-dependent cellular senescence, which explains why N-RasV12 requires pRb to induce cellular senescence.

Cell-cycle-dependent Ras activation was noted by Taylor and Shalloway (1996). Later, it was determined that pRb inactivation (Raptis et al., 1997) or genetic loss (Lee et al., 1999) elevates Ras activity. The latter study observed that high N-Ras activation was sharply induced in serum-starved *Rb*$^{-/-}$ MEFs only 10 min after serum restimulation. We suspect that during serum starvation, pRb-negative cells pool greater numbers of N-Ras-GDP mole- cules in the space where GDP to GTP exchange readily occurs upon serum restimulation. Indeed, our results suggest that iso- prenylated Ras-GDP proteins are aberrantly enriched in the Golgi complex in pRb-negative cells during serum starvation. At Golgi, Ras is activated through Src-mediated activation of phospholipase Cg. The nature of Ras signaling generated from Golgi is supposed to be distinct from that generated from the plasma membrane in terms of activation of JNK, ERK, AKT, and Ral pathways (Hancock, 2003; Quatela and Philips, 2006). Therefore, in addition to moderate-level activation, enrichment of N-Ras in Golgi in Rb-deficient cells may assign a specific role to itself.

Simultaneous N-ras or K-ras deletion rescues differentiation defects in Rb null embryos and prolongs life span with no detect- able impact on E2F-dependent ectopic proliferation and apoptosis (Takahashi et al., 2003, 2004). This is in line with our observation that the isoprenylation-dependent regulation of Ras activation is genetically downstream of E2Fs in the absence of pRb (Figure S14A). The limited effect of E2F deletion on differentiation defects in *Rb*$^{-/-}$

embryos can be explained by the redundant functions shared by E2F family members. Indeed, deletion of individual E2Fs in *Rb*$^{-/-}$ embryos results in varying degrees of rescue of erythropoiesis (Wikenheiser-Brokamp, 2006; Dirlam et al., 2007). The extended life span of Rb-deficient embryos achieved by additionally deleting N-ras was apparently due to the rescue of hepatic anemia (Takahashi et al., 2003). Although the etiology of Rb-deficient anemia is still controversial, a significant genetic interaction seems to exist between E2Fs and N-ras in Rb-mediated erythropoiesis. Furthermore, common C cell tumor phenotypes in *Rb*$^{+/-}$;*E2F3*$^{-/-}$ and *Rb*$^{+-}$;*N-ras*$^{-/-}$ mice, the senescence-inducing activity of E2F-3 in vivo (Lazzer- ini Denchi et al., 2005), and the role of E2F-3 in regulating the transcription of genes involved in Ras isoprenylation (this study) suggest that E2F3 and N-ras are genetically linked in *Rb*-deficient C cell carcinogenesis.

Our observation of less frequent senescence markers, compared to DDR, in early *Rb*-deficient adenomas indicates that DDR chronologically precedes the appearance of senescence markers during adenoma formation. However, this does not neces- sarily mean that *Rb* loss-induced DNA damage is a prerequisite for inducing cellular senescence. To further address this issue, we are analyzing *Rb*$^{+/-}$;*ATM*$^{-/-}$ mice (A.S. and C.T., unpublished data). The *RB* pathway has been linked to DDR through E2F-dependent pathways (Pickering and Kowalik, 2006) involving cyclin E (Tort et al., 2006) or Mad2 (Hernando et al., 2004). Our study

proposes that N-Ras or other isoprenylated proteins may participate in accumulating DNA double-strand breaks in *Rb*-deficient cells.

This study also elucidates the tumor suppressor roles of p130, p16Ink4a, Arf, and Suv39h1 in the absence of pRb. Despite the role of p53 in human retinoblastoma, $Rb^{+/-}$;$p53^{+/-}$ mice generate C cell adenocarcinoma at a lower frequency (8%) following spontaneous mutation in the ret oncogene (Coxon et al., 1998) compared to $Rb^{+/-}$;N-$ras^{-/-}$ mice (91.6%). This suggests that, particularly in C cells, the lower p53 activity acts independently to increase genomic instability rather than intimately cooperating with the *RB* pathway.

Our study also explains the tumor-promoting function of wild- type Ras observed in *Rb*-deficient pituitary glands (Takahashi et al., 2004, 2006) and immortalized fibroblasts (Raptis et al., 1997; Fotiadou et al., 2007). Oncogene-induced cellular senes- cence is known to occur in limited cell types, but activated Ras promotes carcinogenesis in many cell types. Furthermore, not only Ras but also other isoprenylated proteins including many small GTPase proteins, CENP-E, and CENP-F (Maurer-Stroh et al., 2003) may be orchestrated by pRB via E2Fs. We have previously detected upregulated RhoA, Rac, and Cdc42 activity in *Rb*-deficient cells (Takahashi et al., 2006). Finally, we empha- size that our study provides a rational basis for applying PTIs to pRB-inactivated tumors without somatic ras mutations such as retinoblastomas, osteosarcomas, and small cell lung carcinomas.

Experimental procedures

Animals

Rb$^{+/-}$ mice with various *N-ras* genotypes were described previously (Takahashi et al., 2006). *Rb*$^{+/-}$ mice were crossed with *Ink4a*$^{-/-}$, *ARF*$^{-/-}$, *Ink4a*$^{-/-}$;*ARF*$^{-/-}$ or *Suv39H1*$^{-/-}$ mice, and the resultant progeny was intercrossed to generate mice used in this study. Average age at examination (AE) was determined irrespective of mouse status. Mouse genotyping is described in Supplemental Experimental Procedures. Animals were handled in accordance with the guidelines of Kyoto University.

Antibodies

Antibodies used are described in Supplemental Experimental Procedures.

Cell Culture

Primary C cell adenocarcinoma cell lines were established as described previously (Takahashi et al., 2006), with minor modifications. MEFs were prepared from E12.5 embryos derived by intercrossing *Rb*$^{+/-}$;*N-ras*$^{+/-}$ or *Rb*$^{+/-}$;*Ink4a*$^{+/-}$ mice.

Comet Assay

CometAssay reagent kit was purchased from Trevigen (4250-050-K).

RNA Interference

MISSION TRC shRNA target sets (TRCN 71271 and 71274 for p130; 34391, 34392, and 34393 for N-Ras) and TurboGFP shRNA control vector (SHC004) were purchased from Sigma-Aldrich.

BrdU incorporation

BrdU incorporation was measured as described previously (Takahashi et al., 2004).

Colony Formation Assays

Colony formation was assessed by plating 1 3 103 cells per 60 mm dish. After 14 days cultivation, Giemsa staining was used to visualize colonies. Suppres- sion of colony formation was observed by transfecting 1 3 105 MEFs with 0.5 mg pLXSB and 5 mg pBabe-puro expressing the indicated proteins, followed by 14 days cultivation with 8 mg/ml blasticidin S.

Ras Activation Assay

Pull-down assay to measure N-Ras activity was performed as described previously (Lee et al., 1999).

Prenylation Assay

Cells transiently transfected with pCAGGS-VenusA207R-N-Ras were lysed as described previously (Miki et al., 2007), sonicated for three cycles of 15 s each, and separated on 8%–10% acrylamide SDS-PAGE gel at a low constant voltage (less than 40V for longer than 36 hr). FTI-I (#344150), FTI-277 (#344555), and GGTI-298 (#345883) were purchased from Calbiochem.

RT-PCR

Total RNA was extracted using a QIAGEN RNeasy Mini Kit (74104), and RT-PCR was performed using a TaKaRa RNA PCR Kit (RR019A) and the sequence-specific primers indicated in Table S3.

Chromatin Immunoprecipitation

ChIP reagents (SC-45000, 45001, 45002, and 45003) were purchased from Santa Cruz Biotechnology. Lysates were reacted with specific antibodies for immunoprecipitation. Released DNA was amplified by PCR using the primers indicated in Table S4.

Global Farnesylation Assay

5 x 10^6 cells were cultured in 10% FBS with 10 mM lovastatin for 4 hr. After lovastatin was removed, the cells were incubated with 1.54 MBq [3H]farnesol in the presence of 10% FBS for 4 hr and then in the presence of 10% or 0.5% FBS for an additional 20 hr. Labeled proteins were analyzed as described previously (Andreas et al., 1999).

Human Tumors

Tumors from patients with sporadic MTC were surgically removed at the Department of Endocrine Surgery and diagnosed at the Department of Pathology, Tokyo Women's Medical University. The protocol of this study was approved by the ethical committee of Tokyo Women's Medical University, and informed consent was obtained from all patients.

ACCESSION NUMBERS

Microarray data described herein have been deposited at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE12637.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, fourteen figures, and four tables and can be found with this article online at http://www.cancercell.org/supplemental/ S1535-6108(09)00076-2.

Acknowledgments

We thank M. Ewen for encouragement and reagents; D. Peeper and T. Taguchi for discussions and reagents; T. Noda and Y. Saiki for critical reading of the manuscript; A. Iwama, T. Jacks, T. Jenuwein, T. Kamijo, R. Kucherlapati, M. Serrano, N. Sharpless, and M. Taketo for animals; S. Gaubatz, W. Hahn, M. Madiredjo, M. Matsuda, W. Sellers, and B. Spiegelman for reagents; H. Futami, Y. Murakami, T. Nishikawa, S. Ogawa, and R. Takahashi for help in analyzing tumors; A. Nishimoto and H. Gu for technical assistance; A. Miyazaki for secretarial assistance; and K. Lee for inspiration. This work was sup- ported by a

Research Grant from the Princess Takamatsu Cancer Research Fund; the Takeda Science Foundation; and the Ministry of Education, Culture, Sports, Science and Technology (Japan).

References

Ceol, C. J., and Horvitz, H. R. (2001). dpl-1 DP and efl-1 E2F act with lin-35 Rb to antagonize Ras signaling in C. elegans vulval development. Mol Cell 7, 461-473.

Chen, Z., Trotman, L. C., Shaffer, D., Lin, H. K., Dotan, Z. A., Niki, M., Koutcher, J. A., Scher, H. I., Ludwig, T., Gerald, W., et al. (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature 436, 725-730.

Collado, M., Blasco, M. A., and Serrano, M. (2007). Cellular senescence in cancer and aging. Cell 130, 223-233.

Cortois-Cox, S., Genther Williamns, S. M., Reczeke, E. E., Johnson, B. W., McGillicuddy, L. T., Johannessen, C. M., Holsten, P. E., MacCollin, M., and Cichowski, K. (2006). A negative feedback signaling network underlies oncogene-induced senescence. Cancer Cell 10, 459-472.

Coxon, A. B., Ward, J. M., Geradts, J., Otterson, G. A., Zajac-Kaye, M., and Kaye, F. J. (1998). RET cooperates with RB/p53 inactivation in a somatic multi-step model for murine thyroid cancer. Oncogene 17, 1625-1628.

Dannenberg, J. H., Schuijff, L., Dekker, M., van der Valk, M., and te Riele, H. (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev 18, 2952-2962.

Di Micco, R., Fumagalli, M., and di Fagagna, F. (2007). Breaking news: high-speed race ends in arrest--how oncogenes induce senescence. Trends Cell Biol 17, 529-536.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A 92, 9363-9367.

Dimri, G. P. (2005). What has senescence got to do with cancer? Cancer Cell 7, 505-512.

Dirlam, A., Spike, B. T., and Macleod, K. F. (2007). Deregulated E2f-2 underlies

cell cycle and maturation defects in retinoblastoma null erythroblasts. Mol Cell Biol 27, 8713-8728.

Fotiadou, P. P., Takahashi, C., Rajabi, H. N., and Ewen, M. E. (2007). Wild-type NRas and KRas perform distinct functions during transformation. Mol Cell Biol 27, 6742-6755.

Goud, B., Zahraoui, A., Tavitian, A., and Saraste, J. (1990). Small GTP-binding protein associated with Golgi cisternae. Nature 345, 553–556.

Hancock, J. F. (2003). Ras proteins: different signals from different locations. Nat Rev Mol Cell Biol 4, 373-384.

Hernando, E., Nahle, Z., Juan, G., Diaz-Rodriguez, E., Alaminos, M., Hemann, M., Michel, L., Mittal, V., Gerald, W., Benezra, R., et al. (2004). Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature 430, 797-802.

Laurie, N. A., Donovan, S. L., Shih, C. S., Zhang, J., Mills, N., Fuller, C., Teunisse, A., Lam, S., Ramos, Y., Mohan, A., et al. (2006). Inactivation of the p53 pathway in retinoblastoma. Nature 444, 61-66.

Lazzerini Denchi, E., Attwooll, C., Pasini, D., and Helin, K. (2005). Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. Mol Cell Biol 25, 2660-2672.

Lee, K. Y., Ladha, M. H., McMahon, C., and Ewen, M. E. (1999). The retinoblastoma protein is linked to the activation of Ras. Mol Cell Biol 19, 7724-7732.

MacPherson, D., Sage, J., Kim, T., Ho, D., McLaughlin, M. E., and Jacks, T. (2004). Cell type-specific effects of Rb deletion in the murine retina. Genes Dev 18, 1681-1694.

Markey, M. P., Bergseid, J., Bosco, E. E., Stengel, K., Xu, H., Mayhew, C. N., Schwemberger, S. J., Braden, W. A., Jiang, Y., Babcock, G. F., et al. (2007). Loss of the retinoblastoma tumor suppressor: differential action on transcriptional programs related to cell cycle control and immune function. Oncogene 26, 6307-6318.

Marsh, D. J., Theodosopoulos, G., Martin-Schulte, K., Richardson, A. L., Philips, J., Roher, H. D., Delbridge, L., and Robinson, B. G. (2003). Genome-wide copy number imbalances identified in familial and sporadic medullary thyroid carcinoma. J Clin Endocrinol Metab 88, 1866-1872.

Maurer-Stroh, S., Washietl, S., and Eisenhaber, F. (2003). Protein prenyltransferases. Genome Biol 4, 212.

Mittnacht, S., Paterson, H., Olson, M. F., and Marshall, C. J. (1997). Ras signalling is required for inactivation of the tumour suppressor pRb cell-cycle control protein. Curr Biol 7, 219-221.

Mooi, W. J., and Peeper, D. S. (2006). Oncogene-induced cell senescence--halting on the road to cancer. N Engl J Med 355, 1037-1046.

Narita, M., Nunez, S., Heard, E., Narita, M., Lin, A. W., Hearn, S. A., Spector, D. L., Hannon, G. J., and Lowe, S. W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113, 703-716.

Peeper, D. S., Upton, T. M., Ladha, M. H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J. A., and Ewen, M. E. (1997). Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature 386, 177-181.

Pickering, M. T., and Kowalik, T. F. (2006). Rb inactivation leads to E2F1-mediated DNA double-strand break accumulation. Oncogene 25,

746-755.

Quatela, S. E., and Philips, M. R. (2006). Ras signaling on the Golgi. Curr Opin Cell Biol 18, 162-167.

Raptis, L., Brownell, H. L., Corbley, M. J., Wood, K. W., Wang, D., and Haliotis, T. (1997). Cellular ras gene activity is required for full neoplastic transformation by the large tumor antigen of SV40. Cell Growth Differ 8, 891-901.

Rowland, B. D., Denissov, S. G., Douma, S., Stunnenberg, H. G., Bernards, R., and Peeper, D. S. (2002). E2F transcriptional repressor complexes are critical downstream targets of p19(ARF)/p53-induced proliferative arrest. Cancer Cell 2, 55-65.

Sage, J., Mulligan, G. J., Attardi, L. D., Miller, A., Chen, S., Williams, B., Theodorou, E., and Jacks, T. (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev 14, 3037-3050.

Sakakura, Y., Shimano, H., Sone, H., Takahashi, A., Inoue, N., Toyoshima, H., Suzuki, S., and Yamada, N. (2001). Sterol regulatory element-binding proteins induce an entire pathway of cholesterol synthesis. Biochem Biophys Res

Commun 286, 176-183.

Schmitt, C. A. (2001). Cellular senescence and cancer treatment. Biochim Biophys Acta 1775, 5-20.

Sellers, W. R., Novitch, B. G., Miyake, S., Heith, A., Otterson, G. A., Kaye, F. J., Lassar, A. B., and Kaelin, W. G., Jr. (1998). Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. Genes Dev 12, 95-106.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 3, 155-168.

Skapek, S. X., Pan, Y. R., and Lee, E. Y. (2006). Regulation of cell lineage specification by the retinoblastoma tumor suppressor. Oncogene 25, 5268-5276.

Szkopinska, A., and Plochocka, D. (2005), Farnesyl diphophate synthase; regulation of product specificity. Acta Biochim Polonica 52, 45-55.

Takahashi, C., Bronson, R. T., Socolovsky, M., Contreras, B., Lee, K. Y., Jacks, T., Noda, M., Kucherlapati, R., and Ewen, M. E. (2003). Rb and N-ras function together to control differentiation in the mouse. Mol Cell Biol 23, 5256-5268.

Takahashi, C., Contreras, B., Bronson, R. T., Loda, M., and Ewen, M. E. (2004). Genetic interaction between Rb and K-ras in the control of differentiation and tumor suppression. Mol Cell Biol 24, 10406-10415.

Takahashi, C., Contreras, B., Iwanaga, T., Takegami, Y., Bakker, A., Bronson, R. T., Noda, M., Loda, M., Hunt, J. L., and Ewen, M. E. (2006). Nras loss induces metastatic conversion of Rb1-deficient neuroendocrine thyroid tumor. Nat Genet 38, 118-123.

Taylor, S. J., and Shalloway, D. (1996). Cell cycle-dependent activation of Ras. Curr Biol 6, 1621-1627.

Todaro, G. J., and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 17, 299-313.

Tomoda, C., Moatamed, F., Naeim, F., Hershman, J. M., and Sugawara, M. (2008). Indomethacin inhibits cell growth of medullary thyroid carcinoma by reducing cell cycle progression into s phase. Exp Biol Med (Maywood) 233, 1433-1440.

Tort, F., Bartkova, J., Sehested, M., Orntoft, T., Lukas, J., and Bartek, J. (2006). Retinoblastoma pathway defects show differential ability to activate the constitutive DNA damage response in human tumorigenesis. Cancer Res 66, 10258-10263.

Tsai, K. Y., Hu, Y., Macleod, K. F., Crowley, D., Yamasaki, L., and Jacks, T. (1998). Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. Mol Cell 2, 293-304.

Wikenheiser-Brokamp, K. A. (2006). Retinoblastoma family proteins: insights gained through genetic manipulation of mice. Cell Mol Life Sci 63, 767-780.

Young, A. P., Nagarajan, R., and Longmore, G. D. (2003). Mechanism of transcriptional regulation by Rb-E2F segregate by biological pathway. Oncogene 22, 7209-7217.

Young, A. P., Schlisio, S., Minamishima, Y. A., Zhang, Q., Li, L., Grisanzio, C., Signoretti, S., and Kaelin, W. G., Jr. (2008). VHL loss actuates a HIF-independent senescence programme mediated by Rb and p400. Nat Cell Biol 10, 361-369.

Zhang, Z., Wang, Y., Vikis, H. G., Johnson, L., Liu, G., Li, J., Anderson, M. W.,

Sills, R. C., Hong, H. L., Devereux, T. R., et al. (2001). Wild-type Kras2 can inhibit lung carcinogenesis in mice. Nat Genet 29, 25-33.

Ziebold, U., Lee, E. Y., Bronson, R. T., and Lees, J. A. (2003). E2F3 loss has opposing effects on different pRB-deficient tumors, resulting in suppression of pituitary tumors but metastasis of medullary thyroid carcinomas. Mol Cell Biol 23, 6542-6552.

Figure legends

Figure 1. Analysis of C-cell tumors in *Rb*$^{+/-}$; *N-ras*$^{+/+}$ and *Rb*$^{+/-}$; *N-ras*$^{-/-}$ mice.

(A) Scheme of C-cell carcinogenesis in *Rb*$^{+/-}$ mice drawn based on previous findings.

(B) Immunohistochemical (IHC) analysis and SA-β-gal assay of thyroid and C-cell tumors in mice with the indicated genotypes. 22 *Rb*$^{+/-}$; *N-ras*$^{+/+}$ (AE= 11.0 ± 1.2) and 28 *Rb*$^{+/-}$; *N-ras*$^{-/-}$ (AE = 10.6 ± 0.9) mice were analyzed. The frequency of immuno-stain positive cells per 200 normal thyroid or tumor cells is quantified. Columns are mean + SE. Scale bars: 100 μm.

Figure 2. C-cell tumor phenotypes in *Rb*$^{+/-}$ mice additionally lacking senescence-related genes.

(A) C-cell tumors (indicated by arrows) developed in mice with the indicated

genotype and age (months). Scale bars: 3 mm.

(B) Histological and IHC analysis of C-cell tumors developed in mice with the indicated genotype and age. Scale bars: 100 μm.

(C) Calcitonin staining of cultured tumor cells prepared from thyroids of the indicated genotype of mice. Scale bars: 100 μm.

Figure 3. Effects of N-Ras reconstitution in *Rb*-deficient *N-ras*$^{-/-}$ C-cell adenocarcinoma.

(A) Immunoblot (IB) analysis of TT and AC61 cells infected with pBabe-puro (vector) or pBabe-puro-N-ras for the indicated proteins (top). Histological and IHC analysis of AC61 cells infected with pBabe-puro or pBabe-puro-N-ras, selected, and cultured subcutaneously in BALB/c-*nu*/*nu* mice for 14 days (middle). Primary C-cell tumors from mice of the indicated genotype are illustrated (bottom). Magnifications are shown. Tumor weights and frequency of immuno-stain positive cells per 200 normal thyroid or tumor cells are quantified (right). Columns are mean + SE from 4 tumors each.

(B) Immunofluorescence (IF) analysis of AC61 cells infected with pBabe-puro vectors expressing the indicated proteins, selected, and cultured for additional 8 days. Blue signals: DAPI.

(C) Comet assay of cells from (B). Cells with DNA double strand breaks per 200 cells are quantified. Numbers are mean ± SE from 3 experiments.

(D) IF analysis of cells made as in (B) and cultured for additional 12 days.

(E) IB of acid-extracted (γH2A.X, Suv39h1, H3K9me3, HP1γ) or whole (others) cell lysates from cells made as in (B) and cultured for additional 9 days. NT: no treatment, H_2O_2: 200 μM for 30 min at 4 ^{o}C, SS: 0.1% FBS for 24 hr.

(F) AC61 cells were infected with pBabe-puro vectors expressing the indicated proteins, and selected (upper). AC61 cells were infected with pLXSB or pLXSB-Rb, and selected. Resultant cells were re-infected with pBabe-puro vectors expressing the indicated proteins, and selected (lower). Cell proliferation was monitored from day 6 onwards and SA-β-gal activity was measured at day 11 after the last infection.

(G) Immunoprecipitation (IP) from whole cell lysates (WCL) or chromatin fraction (CH) of cells made as in (B) and cultured for additional 9 days. HP1γ bound to p130 or Suv39h1 in the chromatin fraction are upper bands. Colocalization of p130 and HP1γ in analyzed cells is demonstrated.

(H) AC61 cells were infected with lentivirus expressing the indicated shRNAs, and selected with puromycin. After 5 days from the initial infection, resultant cells were re-infected with pLXSB or pLXSB-N-ras, selected with blasticidin S, and analyzed. BrdU incorporation and IB at day 6 after the last infection are shown.

(I) AC61 cells were transduced with the indicated shRNAs 14 days after N-Ras transduction and analyzed as in (H).

(J) IHC analysis of thyroid or primary C-cell tumors developed in mice of the indicated genotypes. Scale bars: 100 μm. Quantification was performed as depicted in (A). Columns are mean + SE (n=5 each).

Figure 4. *Rb-N-ras* genetic interaction in MEFs.

(A) 3T3 protocol assay of MEFs with the indicated genotypes. Cumulative multiplicity in the representative cultures at the indicated passage number is illustrated in the graph.

(B) Representative results of colony formation assays of MEFs with the indicated genotypes plated at low density. Results are quantified. Bars are mean + SE (n=6).

(C) $Rb^{-/-}$ MEFs infected with lentivirus expressing the indicated shRNAs, selected, plated at low density, re-infected with the same lentivirus every 5 days for 3 times, and cultured for 21 days from the initial infection (top). Immunoblot analysis of cells infected with the indicated lentiviruses only once is shown (bottom).

(D) Colony suppression assay of MEFs with the indicated genotypes cotransfected with pLXSB, and pBabe-puro vectors expressing the indicated proteins, and selected with blasticidin S. Results are quantified and shown as graph. Bars are mean + SE (n=4).

(E) SA-β-gal assays of MEFs with the indicated genotypes infected with pBabe-puro vectors expressing the indicated proteins, selected, and cultured for 9 days.

(F) Proliferation of MEFs with the indicated genotypes infected with pBabe-puro vectors expressing the indicated proteins, or pMIKcys (K-Ras^{V12}). After selection,

1.0×10^5 viable cells were plated on each 100 mm dish (day 6) and cells were numerated at the indicated time points.

Figure 5. Role of pRb in controlling N-Ras isoprenylation and membrane trafficking.

(A) AC61 cells transfected with 0.25 μg pBabe-puro-N-ras or pBabe-puro-N-ras^{V12} together with 2.5 μg pSG5 or pSG5L-HA-Rb were selected, and immediately analyzed for GTP-bound N-Ras or status of the indicated proteins in the presence of 10% FBS. Pull down of GTP-loaded N-Ras was done using Glutahtione *S*-transferase (GST)-fused Ras-binding domain (RBD) of Raf. Relative N-Ras activity in each cell line was estimated using the N-Ras-GTP/total ratio in the presence of N-Ras and pRb as 1.0 (underlined).

(B) AC61 cells were infected with pLXSB or pLXSB-Rb, and selected. Resultant cells were transiently transfected with pCAGGS-Venus^{A207R}-N-Ras, treated under the indicated conditions, and analyzed for N-Ras prenylation status (upper 3 cases) and activity (lower 3 cases). PTIs represent the mixture of prenyltranfrase inhibitors (FTI-II, FTI-277 at 10 μM and GGTI-298 at 2 μM each). Unprenylated (UP) and prenylated (P) Venus-N-Ras proteins are indicated by arrows (left). The status of transduced pRb in each condition is indicated (right upper). AC62 cells were analyzed under 0.5% FBS as AC61 were (right lower).

(C) The same amount of pCAGGS-Venus^{A207R}-N-Ras and pCXN2-RFP-Rab6A were cointroduced into AC61 cells made as in (B). Subcellular localization of

Venus-N-Ras (green) and RFP-Rab6A (red) was observed under fluorescent microscopy in the indicated cells cultured under the indicated conditions. Merged (yellow) signals are shown (arrows)(top). Relative intensity of signals generated by the indicated molecules in Golgi (RFP-Rab6A-positive area) was measured using NIH image J 1.4 program and quantified at the indicated time points (lower left). S: 10% FBS, SS: 0.1% FBS, RS: restimulation with 10% FBS for the indicated time. Graphs represent mean ± SE from 90 cells observed in 3 experiments. Cells cultured in 0.1% FBS for 12 hr were lysed and fractionated. C: cytosolic, M: membranous, W: whole cell lysates (lower right).

(D) AC61 cells were cotransfected with 0.25 μg pCAGGS-Venus^{A207R}-N-Ras (green) and 0.25 μg pCXN2-RFP-Rab6A (red) together with 2.5 μg pCMV or pCMV-Rb. Cells were grown in 10% FBS for 24 hr, serum starved (SS) in 0.1 % FBS for additional 48 hr, then restimulated (RS) with 10% FBS, and analyzed at the indicated time points using a laser confocal fluorescence microscope. Signal intensity of Venus-N-Ras in the cell periphery (PM) was measured using NIH image J 1.4 program and quantified from observation of 50 cells each. Columns represent mean + SE from 3 experiments

(E) AC61 cells transfected with pBabe-puro, pBabe-puro-N-ras or pBabe-puro-N-ras^{S186} and selected were analyzed after 12 days cultivation by IB. γH2A.X and HP1γ were acid-extracted.

(F) AC61 cells were transfected with pBabe-puro or pBabe-puro-N-ras, seleted, treated with 2.5 μM FTP-II, 2.5 μM FTP-277 and 0.5 μM GGTI-298 or with

vehicle (DMSO), and analyzed as (E). Amido black stain is shown as loading control for acid-extracted proteins.

(G) MEFs with the indicated genotypes were transfected with Venus-N-Ras, cultured under 0.5% FBS for 24 hr or treated by PTIs, and analyzed by IB.

Figure 6. E2F-dependent transcriptional control of enzymes involved in protein isoprenylation.

(A) RT-PCR of the indicated genes in AC61 cells infected with pLXSB or pLXSB-Rb and pBabe-puro or pBabe-puro-N-ras in the indicated combinations, wild-type MEFs infected with pBabe-neo-TAg or control vector, $Rb^{+/+}$ and $Rb^{-/-}$ MEFs cultured under 0.1% FBS for 24 hr, or MEFs freshly infected with pBabe-hygro-E1A or vector control and selected. SV40LT: Simian virus 40 large T antigen (left), E1A: adenovirus E1A.

(B) IB of Fanesyltransferase β in AC61 cells and $Rb^{-/-}$ and $Rb^{+/+}$ MEFs analyzed in (A).

(C) IHC analysis of wild-type thyroid and *Rb*-deficinet $N\text{-}ras^{+/+}$ adenomas. Magnifications are indicated in parenthesis. Quantification was done as Figure 1B.

(D) ChIP assay of binding of the indicated proteins to the indicated gene promoters in AC61 cells infected with pLXSB or pLXSB-Rb. Positions of the primers in mouse genome and sequences are shown in Figure S10 and Table S4.

(E) Luciferase activity in the indicated cells transfected with the indicated pGL3 luciferase reporters together with pCMV-β-gal and following expression vectors: pSG5L-HA-Rb, pcDNA3-HA-E2F-1, pcDNA3-HA-E2F-3, pBabe-puro-E2F-DB, pSV-Sport-SREBP-1c, pSV-Sport-dominant negative (DN)-SREBP-1c, pBabe-neo-TAg or pBabe-hygro-E1A. Results are normalized by β-gal activity. As the control, empty vectors were used under the same conditions. Relative luciferase activities were shown as the controls set to 1.0. Columns are mean + SE ($n \geq 3$).

(F) Global visualization of ^{3}H-farnesylated proteins in AC61 cells made as in (D) treated under the indicated conditions. Coomassie stain is shown as loading control.

Figure 7. Analysis of sporadic human MTCs.

(A) IHC analysis of sporadic human MTCs. Of 13 cases analyzed, 2 representative cases are shown with the magnification indicated in the parenthesis.

(B) Chi-square analysis (Fisher's exact test) showing relationship between the status of pRb and N-Ras expression in 13 sporadic human MTCs. *P* value is indicated. $Chi^2 = 9.55$.