Escape from premature senescence is not sufficient for oncogenic transformation by Ras

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Resistance of primary cells to transformation by oncogenic Ras has been attributed to the induction of replicative growth arrest[1–3]. This irreversible 'fail-safe mechanism' resembles senescence and requires induction by Ras of p19^{ARF} and p53 (refs 3–5). Mutation of either *p19ARF* or *p53* alleviates Ras-induced senescence and facilitates oncogenic transformation by Ras[3,6,7]. Here we report that, whereas *Rb* and *p107* are each dispensable for Ras-induced replicative arrest, simultaneous ablation of both genes disrupts Ras-induced senescence and results in unrestrained proliferation. This occurs despite activation by Ras of the p19^{ARF}/p53 pathway, identifying pRb and p107 as essential mediators of Ras-induced antiproliferative p19^{ARF}/p53 signalling. Unexpectedly, in contrast to *p19ARF* or *p53* deficiency, loss of *Rb/p107* function does not result in oncogenic transformation by Ras, as Ras-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ fibroblasts fail to grow anchorage-independently *in vitro* and are not tumorigenic *in vivo*. These results demonstrate that in the absence of both *Rb* and *p107* cells are resistant to p19^{ARF}/p53-dependent protection against Ras-induced proliferation, and uncouple escape from Ras-induced premature senescence from oncogenic transformation.

The oncogenic mutant Ras^{V12} protein lacks transforming activity in normal rodent fibroblasts, which have a limited lifespan, but can fully transform fibroblasts that have been immortalized by oncogenes or carcinogens[1,2,8]. Introduction of Ras^{V12} into primary fibroblasts results in the induction of various antiproliferative proteins, including p16^{INK4a} and p53 (ref. 3). The accompanying cell-cycle arrest resembles normal senescence, as it is irreversible and involves upregulation of senescence-associated markers such as β-galactosidase. *INK4a*$^{-/-}$ and *p53*$^{-/-}$ fibroblasts fail to undergo Ras^{V12}-induced (as well as normal) senescence. Instead, these genetic deficiencies facilitate oncogenic transformation by Ras^{V12} in the absence of an immortalizing oncogene (reviewed in ref. 5). *INK4a* loss and Ras^{V12} also cooperate in the acceleration of melanoma development in mice[9]. Similar to loss of the *INK4a* locus, loss of the alternative *INK4a* product p19^{ARF} alone collaborates with Ras^{V12} in oncogenic transformation[6], and p19^{ARF} is required for induction of p53 by Ras^{V12} (ref. 4). In addition to deficiency in tumor-suppressor genes, co-expression of specific oncogenes such as those encoding c-Myc and adenovirus E1A can also bypass Ras^{V12}-induced senescence and facilitate oncogenic transformation[10–12]. Together, these observations help to explain why immortalizing events can disrupt the normal anti-oncogenic response to excessive Ras^{V12} signalling, and indicate that premature senescence may act as a tumor-suppressing mechanism[3,8]. Thus, it seems that disruption of the normal senescence programme (by either co-expression of oncogenes or inactivation of tumor-suppressor genes) in these genetically defined settings not only results in escape from Ras^{V12}-induced senescence, but also greatly facilitates complete oncogenic transformation, indicating that the first event may be sufficient for the second. We have shown previously that the retinoblastoma tumor-suppressor protein pRb is required for cells to undergo cell-cycle arrest in G1 phase in response to downregulation of normal Ras-dependent mitogenic signalling[13]. Here, we investigate the function of the retinoblastoma family of proteins in protection of cells against Ras^{V12}-dependent oncogenic transformation.

To monitor the requirement of pRb-family members in cellular protection against Ras^{V12}-dependent oncogenic transformation, we infected primary mouse embryonic fibroblasts (MEFs) deficient for *Rb, p107* or both with retroviruses encoding the oncogenic mutant Ras^{V12} protein. After a brief selection for successfully infected cells, we monitored their proliferative capacity. As expected[3], Ras^{V12} expression in wild-type MEFs led to premature senescence and cessation of proliferation, even in the presence of 10% serum (Fig. 1a). Disruption of either *Rb* or *p107* did not allow MEFs to escape from Ras^{V12}-induced premature senescence and they adopted a flat-cell morphology typical of senescent cells (Fig. 1b and data not shown). These observations indicate that either *Rb* or *p107*, alone, is dispensable for Ras^{V12}-induced senescence and is not required for protection against Ras^{V12} transformation. In contrast, when we infected several independent populations of *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs derived from independently targeted embryonic stem (ES) cells with Ras^{V12} retrovirus, they continued to proliferate and reached a cell number at least as great as that of the control-infected population (Fig. 1a). We observed no significant difference in proliferation rate between Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ cells and Ras^{V12}-expressing *INK4a*$^{-/-}$ cells, which do not exhibit Ras^{V12}-induced premature senescence and undergo oncogenic transformation much more efficiently than wild-type MEFs[3,14]. To exclude the possibility that the observed escape from Ras^{V12}-induced premature senescence was independent of *Rb/p107* loss, but rather was caused by an unrelated (genetic) event, we re-introduced either gene. Restoration of either *Rb* or *p107* function in Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs led to the expected correction in a dosage-dependent manner and cells underwent growth arrest (Fig. 1c). Together, these results demonstrate that in the absence of both *Rb* and *p107*, but not of either gene alone, cells do not adequately respond to oncogenic Ras^{V12}, that is, they fail to undergo premature senescence.

In a long-term analysis we also observed no difference in proliferation rate between vector- and Ras^{V12}-retrovirus-infected *Rb*$^{-/-}$/*p107*$^{-/-}$ populations (Fig. 2a). Note that at the end of the experiment vector-infected *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs were at passage 12

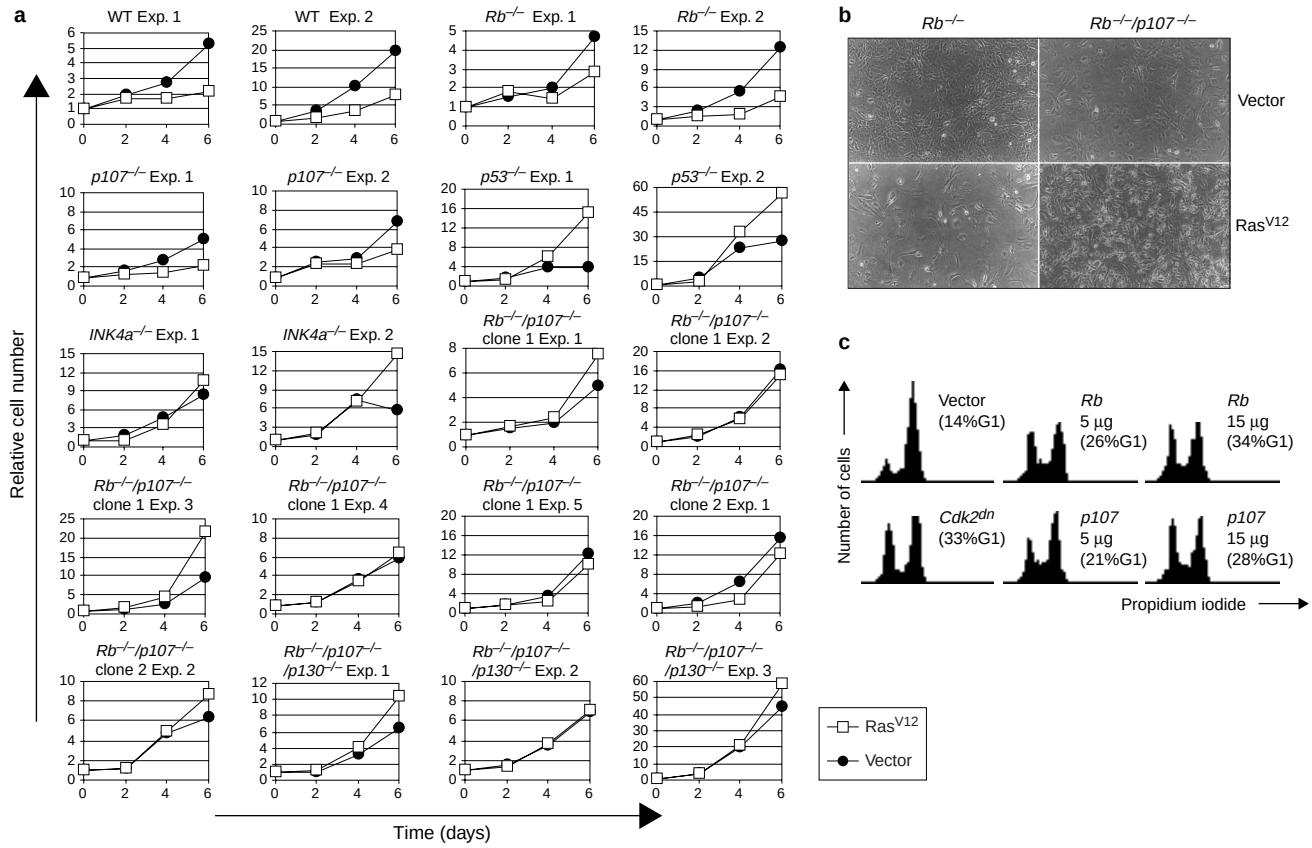

Figure 1 **Primary *Rb*$^{-/-}$/*p107*$^{-/-}$ fibroblasts fail to undergo Ras^{V12}-induced premature senescence. a**, Primary MEFs of the indicated genotype (for *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs two batches were used (clones 1 and 2), derived from independently targeted ES cells) were infected with either empty (filled circles) or Ras^{V12}-containing (open squares) pBABE-puro or LZRS-zeo (triple-null cells only) retroviral vectors. Two days after infection, successfully infected cells were selected with puromycin or zeocin for 4 days and subsequently used to obtain growth curves. Several embryos of each genotype were analysed, and at least two representative examples are shown from at least three independent experiments, all carried out in duplicate. WT, wild type. **b**, Illustration of the flat-cell, senescence-like phenotype of Ras^{V12}-expressing primary *Rb*$^{-/-}$ MEFs, in contrast to that of Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs. **c**, Ras^{V12}-expressing expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs were electroporated with expression vectors for the indicated complementary DNAs, along with a plasmid driving expression of H2B–GFP, in a 10:1 ratio. One day later, nocodazole was added to the cultures to reduce the background fraction of cells in G1 phase. After 48 h, the cell-cycle distribution of the GFP^{+} population was determined by FACS analysis. A representative example of three independent experiments is shown; data are expressed as absolute percentages of cells in G1 phase. Cdk2^{dn} is a dominant negative mutant of Cdk2 that was previously shown to inhibit proliferation in a pRb-independent manner[26].

and that they, as well as their Ras^{V12}-expressing counterparts, were still proliferating exponentially. We were able to propagate Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs well beyond this stage, and we have never observed senescence or even a reduction in proliferative potential at later passages (data not shown). Such an escape from either normal or Ras^{V12}-induced senescence was never observed for wild-type, *Rb*$^{-/-}$ or *p107*$^{-/-}$ MEFs (Fig. 1a and data not shown). These results therefore also indicate that *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs are immortal, or become readily immortalized. To determine directly whether *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs can be established as immortal cultures without acquiring mutations in the genes that are frequently mutated during spontaneous immortalization, we propagated five independent populations for twenty passages and then analysed p19^{ARF}, p53 and p21^{CIP1} levels as well as p53 function. Figure 2b shows that four out of five immortal *Rb*$^{-/-}$/*p107*$^{-/-}$ MEF clones expressed normal, low levels of p53 and showed no loss of p19^{ARF} expression, whereas one clone (no. 5) expressed highly elevated levels of p53, indicative of mutational stabilization. Indeed, when these populations were exposed to γ-irradiation, only the latter population failed to induce p21^{CIP1}, whereas p53/p21^{CIP1} signalling was intact in the four remaining clones (Fig. 2b). Moreover, DNA-sequence analyses of the *p19ARF* open reading frame revealed that the gene was not mutated in any of the five immortal *Rb*$^{-/-}$/*p107*$^{-/-}$ MEF clones. We conclude that mutation of the p19^{ARF}/p53 pathway is not a prerequisite for the immortalization of *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, in contrast to normal MEFs which require mutation in *p53* or loss of *p19ARF* (or of the entire *INK4a* locus) to become immortalized[6]. Together, these data show that either pRb or p107 is required for the induction of both normal and Ras^{V12}-induced senescence.

Both Ras^{V12}-induced premature senescence and normal senescence are accompanied by the induction of p16^{INK4a}, p19^{ARF}, p53 and p21^{CIP1} (refs. 4–6, 15, 16). To determine whether this proliferation-restraining response is intact in *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, we analysed the levels of these proteins after infection with Ras^{V12} retrovirus. Ras^{V12} expression led to the induction of p16^{INK4a}, p19^{ARF}, p53 and p21^{CIP1} in all three genotypes that undergo premature senescence upon Ras^{V12} expression, that is, wild type, *Rb*$^{-/-}$ and *p107*$^{-/-}$ MEFs (Fig. 3). Strikingly, *Rb*$^{-/-}$/*p107*$^{-/-}$ cells accumulated all four proteins to similar levels in response to Ras^{V12}. The Ras^{V12}-induced levels of p53 were at least as high in *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs as in the other genotypes, along with a clear induction of p21^{CIP1}, indicating that p53 was functional and not mutated. This result

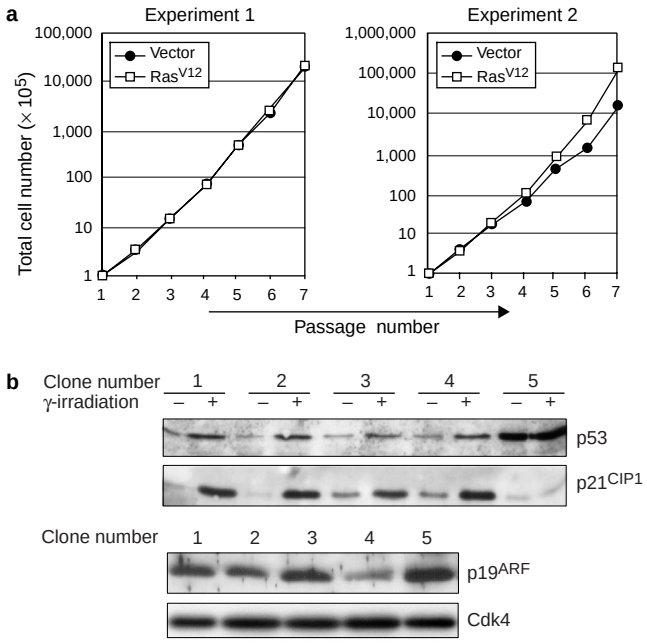

Figure 2 **Continued proliferation of parental and Ras^{V12}-expressing primary *Rb*$^{-/-}$/*p107*$^{-/-}$ fibroblasts. a**, Two independent populations of *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs were infected and selected as described in Fig. 1a and used to obtain a long-term growth curve. The number indicated on the x-axis refers to the number of passages after infection with and selection for expression of the retroviral vector. Altogether, upon explantation, propagation, infection and selection these MEFs had undergone roughly five passages before the start of the growth curve. At the end of the experiment the four populations shown here were also used in a soft-agar assay, the results of which are shown in Table 1b. **b**, Five independent populations of *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs were propagated according to a 3T9 protocol, for 20 passages. Cell extracts (50 μg) were then prepared and analysed by western blotting for expression of the indicated proteins, either directly (lower panel), or 24 h after exposure to γ-irradiation (20 Gy; upper panel).

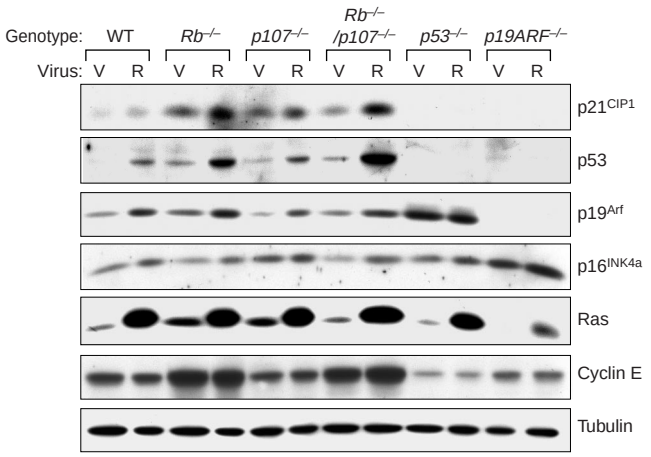

Figure 3 **Signalling from Ras^{V12} to the p16^{INK4a} and p19^{ARF}/p53 pathways is intact in primary *Rb*$^{-/-}$/*p107*$^{-/-}$ fibroblasts.** Low-passage MEFs of the indicated genotype were infected with either empty (V) or Ras^{V12}-containing (R) pBABE-puro retroviral vectors. Two days after infection, cells were selected with puromycin for 4 days, after which cell extracts were prepared for western blotting with the indicated antibodies. In each case, 50 μg of protein extract was used; tubulin served as a loading control.

indicates that the senescence programme was initiated, and that the failure of *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs to undergo Ras^{V12}-induced growth arrest was not due to an intrinsic defect in signalling from Ras^{V12} to various downstream antiproliferative factors. Indeed, it indicates that pRb and p107 may represent essential effector proteins that mediate the growth-inhibitory effect of the Ras^{V12}-activated p19^{ARF}/p53 pathway. Thus, although *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, upon expression of Ras^{V12}, exhibit a normal biochemical response, including induction of the p19^{ARF}/p53 pathway, they fail to show the expected biological response — rather than undergoing premature senescence as part of a failsafe mechanism to protect against Ras^{V12}, they continue to proliferate.

Levels of cyclin E are de-repressed in *Rb*$^{-/-}$ MEFs relative to wild-type MEFs, which may partly explain the selective growth advantage conferred by deletion of the *Rb* gene during tumorigenesis[17]. Figure 3 shows that cyclin E levels in *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs were similar to those in *Rb*$^{-/-}$ MEFs, irrespective of Ras^{V12} expression. As *Rb*$^{-/-}$ MEFs, in contrast to *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, undergo Ras^{V12}-induced replicative arrest, these results show that increased cyclin E levels cannot account for their distinct proliferative response to Ras^{V12}.

We next investigated whether *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs are under selective pressure to lose expression of Ras^{V12} during prolonged passage in culture. We infected *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs with Ras^{V12} retrovirus and subsequently cultured them for six passages, without applying positive (puromycin) selection for continued expression of Ras^{V12}, to increase the sensitivity of the experiment. As a control, we infected *p53*$^{-/-}$ MEFs, which oncogenically transform upon Ras^{V12} expression[7]. Ras^{V12} was expressed at high levels in both genotypes (Fig. 4). Neither Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ nor *p53*$^{-/-}$ MEFs, however, exhibited a reduction in Ras^{V12} levels over time, compared to the tubulin loading control. Expression of oncogenic Ras^{V12} *in vivo* selects for mutation in p53 (ref. 18). However, we did not observe an accumulation of p53 as a result of a stabilizing mutation, nor a reduction in p21^{CIP1} levels at any stage during the propagation of Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, indicating that the p53 pathway had not been mutated during the course of the experiment (Fig. 4; see also below). We conclude that *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs are not under selective pressure to eliminate either Ras^{V12} expression or to acquire mutations in the p53 pathway. This further supports the idea that *Rb*/*p107* deficiency ablates the normal antiproliferative response upon expression of mutant Ras^{V12} in primary cells.

To date, only three defined genetic deficiencies allow for an escape from Ras^{V12}-induced premature senescence, that is, *INK4a*, *p19ARF* and *p53* (refs 3, 6, 14). Importantly, in all these cases the escape is accompanied by the adoption of an oncogenically transformed phenotype, as illustrated by the ability of such cells to proliferate in an anchorage-independent manner and to form tumours *in vivo*. We therefore expected that the escape from Ras^{V12}-induced premature senescence in *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs would likewise be coupled to the acquisition of an oncogenically transformed phenotype. Table 1a shows that Ras^{V12}-expressing *Rb*$^{-/-}$ or *p107*$^{-/-}$ MEFs were unable to form overt colonies in soft agar. Surprisingly, Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, though clearly not growth-arrested under adherent conditions, were also unable to grow anchorage-independently. In contrast, Ras^{V12}-expressing *p53*$^{-/-}$ and *p19ARF*$^{-/-}$ MEFs formed many colonies, which is consistent with previous reports[6,7]. The lack of anchorage-independent growth of Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs is a stable phenotype, as prolonged passage of these cells in culture did not lead to a selective outgrowth of anchorage-independent variants (Table 1b). Co-infection of *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs with Ras^{V12} and c-Myc retroviruses led to the formation of a significant number of colonies in soft agar, an observation that excludes the possibility that *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs are intrinsically unable to become oncogenically transformed (Table

Table 1a Ras^{V12}-expressing, *Rb*–family-deficient MEFs are unable to grow anchorage-independently

Virus	Genotype						
	WT	*Rb*$^{-/-}$	*p107*$^{-/-}$	*Rb*$^{-/-}$/p107$^{-/-}$	*Rb*$^{-/-}$/*p107*$^{-/-}$/*p130*$^{-/-}$	*p19*$^{-/-}$	*p53*$^{-/-}$
Vector	0	0	0	0	1	0	0
Ras^{V12}	0	0	0	0	2	>1,000	>1,000
Ras^{V12} + c-Myc	–	–	–	27	–	–	–
Ras^{V12} + HPV E6	–	–	–	–	25	–	–
Ras^{V12} + MDM-2	–	–	–	–	97	–	–

Table 1b Ras^{V12}-expressing, *Rb*–family-deficient MEFs are unable to grow anchorage-independently

Pools of MEFs	Genotype
Vector	*Rb*$^{-/-}$/p107$^{-/-}$
1	0
2	0
3	0
4	0
5	0
6	0*
7	0*
Ras^{V12}	
1	2
2	2
3	0
4	0
5	0
6	0*
7	0*

a, Primary MEFs at passage 3–4 were infected with retrovirus encoding the indicated cDNA and a puromycin-selectable marker, selected with puromycin for 4 days and seeded (2.5 X 10^4 cells per well on a 6-well plate) into soft agar. After 2 weeks, foci visible to the naked eye (that is, containing at least 10–50 cells) were scored. Upon infection with either empty or Ras^{V12}-containing retroviruses, very small foci of triple null MEFs were sometimes observed consisting of <10 cells. However, these colonies remained small and did not lead to sustained growth. Results are representative of at least three independent experiments, all carried out in duplicate. Western blotting was carried out to confirm equal expression of Ras^{V12} in all genotypes (data not shown). See Supplementary Information for photographs of these cells. **b**, Independent pools of primary MEFs at passage 3–4 were infected with retroviruses and selected as described in **a**. They were then passaged for 4 weeks, during which they underwent ~17 population doublings. At this time, cells were transferred to soft agar (in duplicate). Very small foci of either control or Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, consisting of <10 cells, were sometimes observed. However, these colonies remained small and did not lead to sustained growth.
*The proliferative potential of two vector-infected and two Ras^{V12}-infected populations was also measured during the 4 weeks before seeding into soft agar (see Fig. 2a).

1a; see also below). These results indicate that *Rb/p107* loss is insufficient to confer an anchorage-independent phenotype onto Ras^{V12}-expressing primary fibroblasts.

To extend these observations to an *in vivo* setting, we examined the ability of Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs to form tumours in athymic nude mice. Table 2 shows that 0 out of 12 injections with Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs yielded tumours within 2 months. In contrast, *p53*$^{-/-}$ MEFs expressing similar levels of Ras^{V12} formed large tumours in all cases within 1–2 weeks of injection. These results further support our findings described above and indicate that by neither criterion examined, anchorage-independent proliferation *in vitro* and tumorigenicity *in vivo*, are Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs oncogenically transformed. These data also support the idea that in Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs no selection occurs for a mutation (for example, in the p19^{ARF}/p53 pathway), as this would have led to collaboration with Ras^{V12} in oncogenic transformation.

Finally, we determined whether in the absence of both the *Rb* and *p107* genes the third pocket-protein gene, *p130*, is responsible for protection against Ras^{V12}-dependent transformation. Similar to Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$/*p130*$^{-/-}$ triple-null MEFs proliferated continuously under normal adherent conditions, even though p53 was again induced by Ras^{V12} (Fig. 1a and data not shown). Moreover, in line with observations of *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$/*p130*$^{-/-}$ MEFs failed to grow anchorage-independently (Table 1a). However, upon co-expression of Ras^{V12} and either human papilloma virus (HPV) E6 or MDM-2 (both of which target p53 for degradation), triple-null cells readily formed many foci in soft agar. This result shows that triple-null fibroblasts are not directly transformed upon Ras^{V12} expression, but that at least one cooperating event still is required.

We have shown that in primary fibroblasts, pRb and p107 provide an essential barrier that protects against the proliferative effects of a continuously firing Ras^{V12} protein, even in the presence of elevated levels of p19^{ARF}, p53 and p21^{CIP1}. Our results argue that the mechanism by which the p19^{ARF}/p53 pathway protects cells against Ras^{V12}-induced proliferation depends on the presence of functional retinoblastoma-family members, implicating these proteins as critical mediators of physiologic, antiproliferative p19^{ARF}/p53 signalling. The observed essential function of *p107* in protecting cells in the absence of *Rb* against the proliferative effects of Ras^{V12} is consistent with and extends previous data showing that *p107* is a suppressor of retinoblastoma development in *Rb*-deficient mice[19]. Immortal rodent cell lines that are unable to undergo Ras^{V12}-induced senescence adopt an oncogenically transformed phenotype upon introduction of Ras^{V12} (ref. 1). As described above, immortal *INK4a*-, *p19ARF*- or *p53*-deficient cells, as well as cells immortalized by c-Myc or adenovirus E1A, become oncogenically transformed by Ras^{V12} (although the requirement for further mutations cannot formally be excluded). However, we have shown that Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs, though unable to undergo Ras^{V12}-induced premature senescence, lack tumorigenic potential. This observation demonstrates that escape from Ras^{V12}-induced premature senescence is not sufficient for oncogenic transformation. Thus, a critical difference between deficiency of p19^{ARF}/p53 compared with that of pRb/p107 concerns the phenotype of such cells in the presence of Ras^{V12} — the former are efficiently oncogenically transformed whereas the latter are merely immortalized (or partly transformed). In keeping with this, we have shown that

Table 2 Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs are not tumorigenic

Virus	Genotype	
	Rb$^{-/-}$/*p107*$^{-/-}$	*p53*$^{-/-}$
Experiment 1		
Vector	0/6	0/6
Ras^{V12}	0/6	6/6
Experiment 2		
Vector	0/6	nd
Ras^{V12}	0/6	6/6

Nude, athymic mice were injected subcutaneously in both flanks with 10^6 MEFs selected to express the proviral insert. For each sample, three mice (six flanks) were injected. Data represent numbers of tumours per injection, 2 months after injection. In all cases, Ras^{V12}-expressing *p53*$^{-/-}$ MEFs produced tumours within 1–2 weeks, at which time the mice were sacrificed. Western blotting was carried out to confirm equal expression of Ras^{V12} in both genotypes immediately before injection (data not shown).
nd, not determined.

Ras^{V12}-cooperating oncogenes such as those encoding c-Myc, MDM-2 or HPV E6 are still required for *Rb*-family-deficient cells to adopt a transformed phenotype, just as they are needed for transformation of wild-type cells. Thus, in the context of Ras^{V12}, inactivation of two or more pRb-family proteins disrupts the normal proliferative control, but does not directly contribute to transformation beyond that. Apparently, disruption of p19^{ARF}/p53 signalling simultaneously deregulates both anti-immortalizing and anti-tumorigenic events, whereas loss of *Rb/p107* causes disruption of the former mechanism exclusively, at least in the context of oncogenic Ras. The pRb-family-regulated transcription factor E2F-1 has been implicated as an upstream activator of Arf[20]. Our data indicate that, conversely, retinoblastoma-family proteins, in a pathway leading to Ras^{V12}-dependent premature senescence, may be downstream effectors of p19^{ARF}/p53. This model is compatible with recent data showing that the antiproliferative activity of p19^{ARF} depends not uniquely on p53 but also on the p16^{INK4a} pathway[21].□

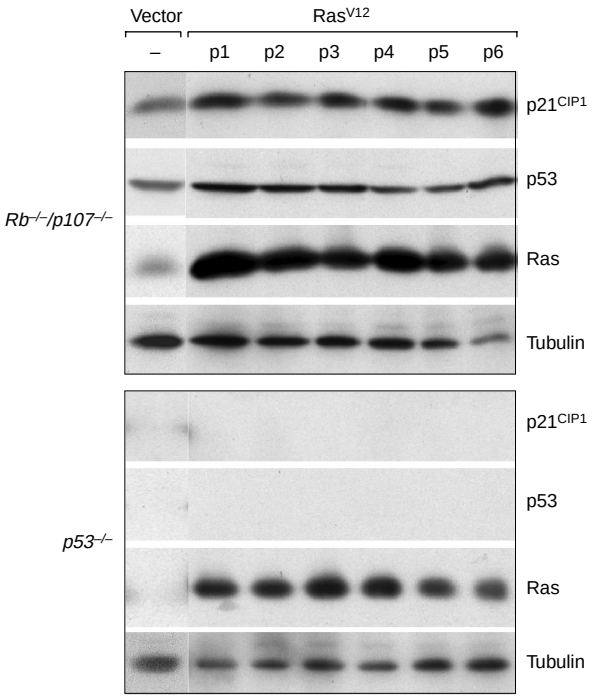

Figure 4 ***Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs are under no selective pressure to lose expression of Ras^{V12} or mutate p53 during prolonged culture.** Primary *Rb*$^{-/-}$/*p107*$^{-/-}$ or *p53*$^{-/-}$ fibroblasts at passage 3–4 were infected with either empty (vector) or Ras^{V12}-containing pBABE-puro retroviral vectors. Cells were subsequently cultured during 6 passages (~12 population doublings). This occurred without puromycin selection for successfully infected cells, to eliminate any possible positive selective pressure to maintain Ras^{V12} expression, thereby increasing the sensitivity of the experiment. Cell extracts were prepared and 50 μg of each lysate was subjected to western blotting with the indicated antibodies.

Methods

Preparation of MEFs, cell culture and retroviral infection.

Chimaeric embryos were generated by injection of blastocysts with ES cells with the following genotypes: wild type, *Rb*$^{-/-}$, *p107*$^{-/-}$ and *Rb*$^{-/-}$/*p107*$^{-/-}$ (refs 19, 22). Chimaeric embryos lacking all three Rb-family members were generated as described[23]. To obtain primary MEFs from 15-day-old chimaeric embryos, organs and head were removed and the remaining tissue was washed in PBS and minced. After a second PBS wash, the tissue was incubated with 100 μl trypsin/EDTA (Gibco) overnight on ice. The next morning, 100 μl trypsin/EDTA was added and the tissue was incubated at 37 °C for 30 min. The tissue was then dissociated to near-homogeneity in complete medium and transferred to a 100-mm dish. MEFs were maintained in DMEM (Gibco) supplemented with 10% FBS (PAA Laboratories) and 0.1 mM β-mercapto ethanol. Taking advantage of a neomycin selection marker introduced in all genotypes, 48 h of selection with 800 μg ml^{-1} G418 yielded MEF cultures that were exclusively derived from ES cells. These cells were designated as passage 1. For all genotypes, several embryos were analysed.

Phoenix packaging cells were used to generate ecotropic retroviruses, as described[3]. Low-passage (1–3) MEFs were infected with filtered (0.45 μm) viral supernatant, supplemented with 4–8 μg ml^{-1} polybrene. In general, a single infection round of 6 h was sufficient to infect at least 90% of the population.

Proliferation curves and cell-cycle analysis.

To obtain proliferation curves, MEFs were infected with either empty or human H-Ras^{V12}-containing pBABE-puro retroviral vectors[24], and after 1–2 days were selected with puromycin (1–3 μg ml^{-1}) for 4 days. As triple-null cells are puromycin-resistant, they were infected with LZRS-zeo retroviruses and selected with zeocin (250 μg ml^{-1}). After confirming that all mock-infected cells were dead, we plated 2.5×10^4 cells per well in 12-well plates and processed them for a colorimetric analysis of cell proliferation in time, as described[3]. For cell-cycle analysis, Ras^{V12}-expressing *Rb*$^{-/-}$/*p107*$^{-/-}$ MEFs were electroporated with cytomegalovirus (CMV) expression plasmids encoding human pRb or p107 or a dominant negative mutant of cyclin-dependent kinase 2 (Cdk2), together with an expression plasmid encoding H2B tagged with green fluorescent protein (GFP)[25], in a 10:1 ratio. After 1 day, nocodazole (50 ng ml^{-1}) was added to the cultures to reduce the background proportion of cells in G1 phase. After a further 2 days, cells were permeabilized and stained with propidium iodide, and the cell-cycle distribution of the GFP$^+$ population was measured by fluorescence-activated cell sorting (FACS).

Western blotting.

Cell extracts were prepared in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC and 50 mM Tris–HCl pH 8.0, supplemented with a protease-inhibitor cocktail (Boehringer)). They were then assayed for protein concentration, and 50 μg of clarified extract was resolved on 11% SDS–polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Millipore). Primary antibodies used for western blotting were R562 (Abcam) for p19^{ARF}, R02120 (Transduction Laboratories) for Ras, and M-20 for cyclin E, M-156 for p16^{INK4a}, FL-393 for p53 and C-19 for p21^{CIP1} (all from Santa Cruz). Proteins were detected by enhanced chemiluminescence (Amersham).

Soft-agar assays and tumour growth in mice.

Cells were infected and briefly selected as described for the proliferation curves. Six days after infection, cells were used for either assay. To monitor the capacity of MEFs to grow in semi-solid medium *in vitro*, cells were transferred to 2 ml complete DMEM containing 0.4% low-gelling agarose (Sigma type VII, cat. A-4018). Then, 2.5×10^4 cells were seeded in duplicate into 6-well plates already containing a 2-ml layer of solidified 1% agar in complete medium. After 2 weeks, the number of foci was determined. For analysis of the *in vivo* tumorigenic capacity of MEFs, athymic nude mice were injected subcutaneously in each flank with 1×10^6 cells, and mice were inspected weekly. Mice were sacrificed at the time tumours appeared.

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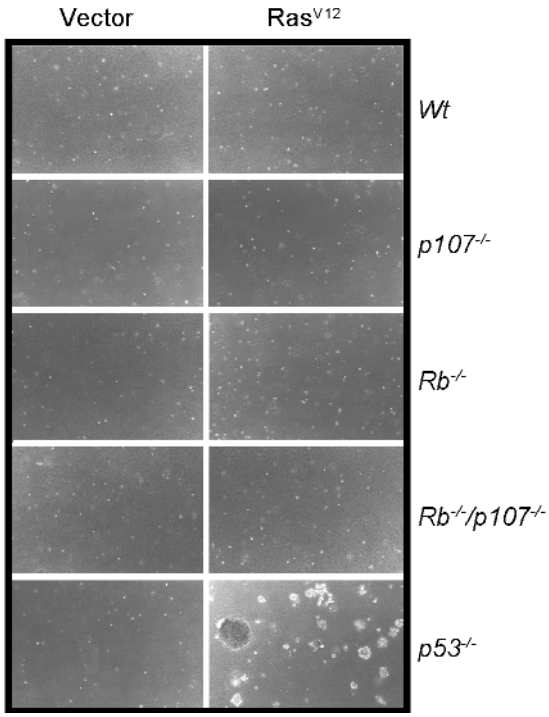

Figure S1 RasV12-expressing, *Rb*-family-deficient MEFs are unable to grow anchorage-independently. Primary MEFs of the indicated genotypes were infected at passage 3–4 with control or Ras^{V12}-expressing retrovirus, which also encoded a puromycin selectable marker, selected with puromycin for 4 days and seeded (2.5 X 104 cells per well on a 6-well plate) into soft agar. After 2 weeks, foci were photographed at X40 magnification.