Current Biology 22, 1765–1773, October 9, 2012 ©2012 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2012.07.040

Article

Loss of Rb Cooperates with Ras to Drive Oncogenic Growth in Mammalian Cells

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Summary

Background: The p53, Rb, and Ras/PI3K pathways are implicated in the development of the majority of human cancers. A number of studies have established that these pathways cooperate at the level of the cell cycle leading to loss of normal proliferative controls. Here we have investigated how these signals influence a second critical component of tumor formation—cell growth.
Results: We find that oncogenic Ras is sufficient to drive growth via the canonical growth pathway, PI3K-AKT-TOR; however, it does so relatively weakly and p53 loss does not drive cell growth at all. Importantly, we identify a novel role for the Rb family of tumor suppressors in directing cell growth via a signaling pathway distinct from PI3K-AKT-TOR and via an E2F-independent mechanism. However, we find that strong, sustained growth requires Rb loss together with Ras signaling, identifying an additional mechanism by which these oncogenic pathways cooperate and a critical role for Ras in preserving the uptake of extracellular nutrients required for biogenesis.
Conclusions: We have identified a new role for the Rb family in cell biogenesis and show that, as for other processes associated with tumor development, oncogenic cell growth is dependent on cooperating oncogenes.

Introduction

The maintenance of tissue and body size is achieved by the homeostatic regulation of cell growth (addition of volume/mass), proliferation, and survival [1]. In contrast to unicellular yeast—in which the rates of growth and proliferation are mainly determined by nutrient levels—in animals, additional levels of control are needed to differentially regulate the distinct cell types found within multicellular organisms. So, whereas the bloodstream (or equivalent) aims to supply a relatively constant level of nutrients—cell growth, proliferation, and survival are regulated by additional extracellular factors—growth factors, mitogens, and survival factors, respectively. The formation of a tumor requires the loss of each of these homeostatic controls, but as previous studies have focused primarily on the role of proliferation and apoptosis, the mechanisms underlying loss of growth controls remain poorly understood.

The stringency of growth control has been clearly demonstrated in vitro, in that mammalian cells will only add mass in the presence of an extracellular growth factor—despite being surrounded by nutrients—and that increasing levels of growth factors result in increasing growth rates. In contrast, the removal of growth factors triggers autophagy, resulting in shrinkage and loss of cell mass [2–4]. These findings are mirrored in studies in both mouse and *Drosophila*, which have shown that the genetic manipulation of growth factor pathways leads to changes in tissue and organism size [5, 6]. Thus, homeostasis reflects a level of growth factor signaling sufficient to maintain the mass of a tissue, whereas to produce more tissue—or a tumor— requires either increased growth factor signaling or the deregulation of growth.

The evolutionarily conserved multiprotein kinase complex mammalian target of rapamycin complex 1 (mTORC1) is a central component of the growth regulatory network, controlling diverse processes such as protein and lipid synthesis, mitochondrial metabolism, ribosome biogenesis, and autophagy, which ultimately lead to cell growth. mTORC1 integrates a number of signals that control cell growth, including those from intracellular energy and amino-acid levels and extracellular growth factors. In animals, the most well-characterized growth factor is insulin-like growth factor-1 (IGF1), which acts via phosphatidylinositol-3-kinase (PI3K) and AKT/PKB to activate mTORC1, leading to phosphorylation of downstream effectors such as S6K and 4EBP—which play key roles in translational control and to activation of SREBP1—an important regulator of lipid biogenesis [7, 8].

There is accumulating evidence that specific oncogenic signals deregulate cell growth, many of which—including components of the PI3K-AKT pathway such as PTEN—converge on mTOR signaling [9, 10], and inhibitors of mTOR signaling such as rapamycin are showing clinical potential [11]. Oncogenic Ras constitutively signals through the PI3K pathway and has been shown to control cell growth in animal models, although the downstream pathways are not clearly defined [12]. p53 has been shown to influence metabolic pathways via a number of mechanisms, including the regulation of autophagy and by the transcriptional control of nutrient transporters and glycolytic enzymes [13]. Myc has also been shown to regulate cell growth by controlling ribosome biogenesis and protein synthesis, with increased myc levels resulting in increased cell growth and size [14, 15]. Moreover, the deregulation of both metabolic pathways and the biosynthetic apparatus in cancer highlights the critical importance of biosynthetic pathways in tumor formation [16, 17]. The coincident deregulation of RTK/Ras/PI3K, p53, and Rb signaling is found in the majority of tumors [18]. Consistent with this, activated Ras together with loss of p53 and the Rb family cooperate to drive transformation in both in vitro and in vivo models of tumor formation [19]. The cooperative effects of these oncogenes on the cell cycle are well established but the way in which these pathways interact to result in the loss of growth control required for tumorigenesis remains unclear.

Primary Schwann cells have proven to be a powerful model system for studying the regulation of mammalian cell growth, which has previously been exploited to demonstrate the independent regulation of growth and proliferative pathways and how cell size is determined by the balance of extracellular factors controlling these processes [2, 4]. The advantage of

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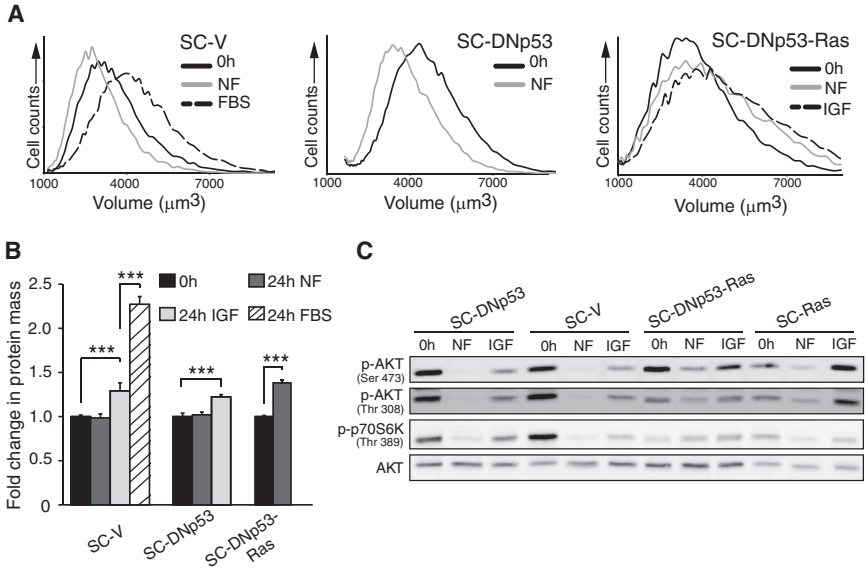


Figure 1. Ras Drives Weak Growth via PI3K-AKT-TOR

(A) Coulter curves showing volume per cell of populations of aphidicolin-cell-cycle-arrested Schwann cells transduced with control vector (SC-V), DNp53 (SC-DNp53), or DNp53 and Ras (SC-DNp53Ras) following factor withdrawal (0 hr) and after 24 hr in SATO medium with no added factors (NF), IGF1 (IGF), or 3% FBS.

(B) Protein mass measurements of cells after 24 hr without factors or with IGF1 or 3% FBS normalized to mass at time 0. Error bars represent means ± SD of three independent experiments.

(C) Western blot analysis of cell lysates collected after 24 hr without factors or in the presence of IGF1. See also Figure S1.

using Schwann cells is that they are primary cells with intact checkpoints that can be cultured indefinitely in defined conditions, permitting the measurement of cell growth following either the addition of an exogenous factor or a genetic change, in the absence of any potentially collaborating exogenous growth factors. In the present study, we have used this system to determine how Ras, p53, and the Rb family affect cell growth networks and found that Ras signaling and disruption of the Rb family cooperate to drive robust cell growth in the absence of signals normally provided by extracellular growth factors. These findings identify a novel role for the Rb family in tumorigenesis and provide further insight into how these ubiquitous tumorigenic pathways interact to drive tumor development.

Results

Loss of p53 Fails to Induce Cell Growth whereas Oncogenic Ras Induces Weak Growth

Previous studies have shown that oncogenic Ras and loss of the tumor suppressor p53 cooperate to stimulate proliferation, and more recent work has described how these two signals synergize to induce a "cancer-related gene signature" [20–22]. To address whether inhibiting p53 is sufficient to induce cell growth, we used a retroviral vector to express a dominant-negative form of p53 (DNp53), which is able to alleviate a cell-cycle arrest induced by γ-irradiation or oncogenic Ras in primary Schwann cells [23] (see Figures S1A and S1B available online) and determined cell growth using two criteria as follows: (1) changes in cell volume, measured in cell-cycle arrested cells and (2) by the addition of protein mass. As in previous studies [3, 4], control Schwann cells infected with the vector construct (SC-V) lost volume and mass once exogenous growth factors were removed, consistent with the onset of autophagy (Figures 1A and 1B; Figure S1C). Somewhat surprisingly, we found that DNp53-expressing cells behaved as control cells, losing volume and mass, at a similar rate, following the removal of extracellular growth factors (Figures 1A and 1B) indicating that loss of p53 is not sufficient to drive cell growth. We next coexpressed constitutively active Ras in both Schwann cells (SC-Ras) and the DNp53-expressing Schwann cells (to avoid the cell-cycle inhibition induced in primary cells by oncogenic Ras [21]) and found that in both cases, oncogenic Ras was able to drive cell growth at similar rates (Figures 1A and 1B; Figures S1C and S1D). However, the growth was relatively weak—when compared to growth induced by fetal bovine serum (FBS), an ill-defined yet potent stimulator of cell growth (Figures 1A and 1B) [2, 4]. The rate of Ras-induced growth was similar to the rate achieved by saturating concentrations of the growth factor IGF1 [2, 4] and IGF1-stimulation failed to potentiate Ras-induced growth indicating that both signals may be acting through the same pathway (Figure 1A). Consistent with this, activation of the PI3K-AKT-TOR pathway was comparable in IGF1-treated control or Ras-expressing cells (Figure 1C) and—as has previously been shown for IGF1 [24]—Ras-driven growth was blocked by the addition of the PI3K inhibitor, LY294002 (Figure S1E).

Loss of the Rb Family Drives Cell Growth

To determine the role of the Rb family in growth control, we initially used SV40 LT antigen (LT) as a tool to remove the entire Rb family and which by sequestering p53 also inactivates the p53 pathway [25]. Interestingly, we found that LT expression was sufficient to drive robust increases in both cell volume and cell mass and at rates consistently higher than stimulated by saturating levels of IGF1 in normal Schwann cells (Figures 2A and 2B; Figures S2A and S2B). We determined mass accumulation in both non-cell-cycle arrested cells and in arrested cells (using two separate methodologies) and obtained similar rates of protein accumulation (Figure 2B; Figures S2A and S2B). Importantly, we were unable to detect induction of the PI3K-AKT-TOR pathway, suggesting that LT does not act by promoting the autocrine production of a factor such as IGF1 and instead is driving cell growth via a distinct pathway (Figure 2C). Moreover, in contrast to Ras-expressing cells, the growth observed in LT cells was potentiated by IGF1. This synergy was not associated with a further increase in signaling through the PI3K-AKT-TOR pathway, indicating that the two signals act through parallel pathways (Figure 2C). Treatment with the MEK inhibitors U0126 or PD184352, previously demonstrated to have no effect on IGF1-induced growth [24], also had no effect on LT-induced growth indicating that signaling through the ERK pathway was not required despite

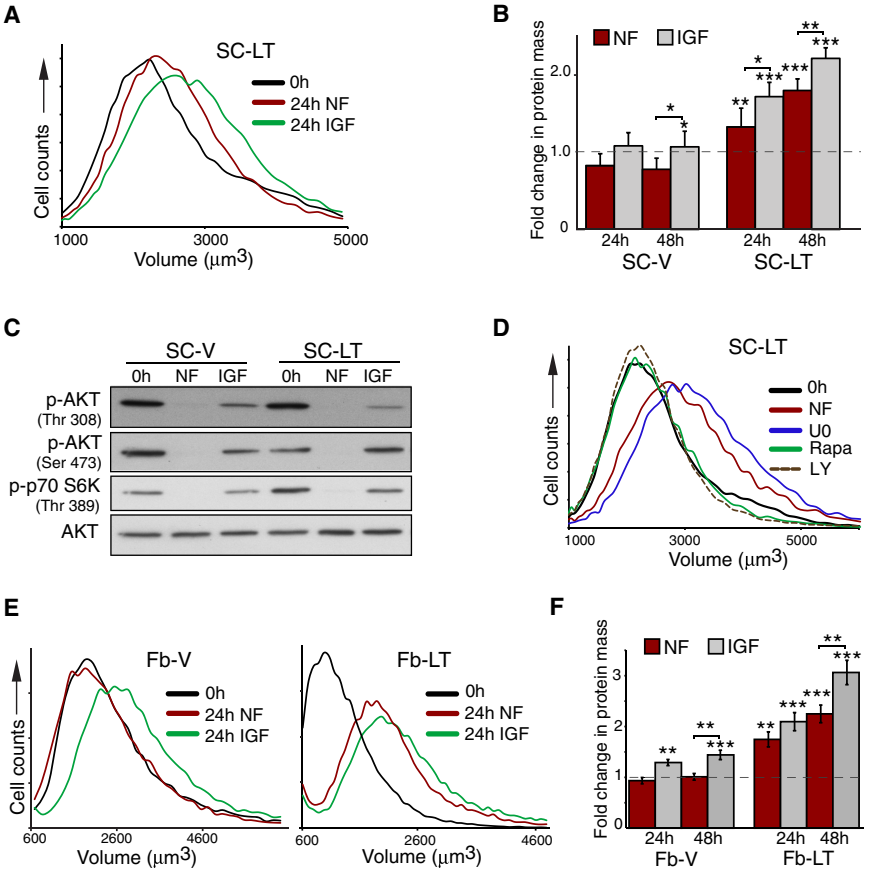


Figure 2. SV40 LT Expression Drives Cell Growth in the Absence of PI3K-AKT-TOR Activation

(A) Coulter curves showing volume per cell of a population of aphidicolin-arrested Schwann cells infected with SV40 LT antigen (SC-LT). Volume measurements were made upon factor withdrawal (0 hr) and after 24 hr without factors (NF) or with IGF1 (IGF).

(B) Protein mass measurements of control (SC-V) and SC-LT cells after 24 and 48 hr without factors or treated with IGF1 were normalized to mass at the time of factor withdrawal. Error bars represent means ± SD of three independent experiments.

(C) Western blot analysis of cell lysates collected following factor removal (0 hr) and after 24 hr without factors (NF) or with IGF1 (IGF).

(D) Aphidicolin-arrested SC-LT cells were treated with 15 μM U0126 (U0), 0.1 μM rapamycin (Rapa), 15 μM LY294002 (LY), or vehicle (NF) and volume per cell was measured after 24 hr without factors.

(E) Coulter curves showing volume per cell of a population of aphidicolin-arrested fibroblasts infected with LT (Fb-LT) or control vector (Fb-V) upon factor withdrawal (0 hr) and after 24 hr without factors (NF) or with IGF1.

(F) Protein mass measurements of control (Fb-V) and LT (Fb-LT) infected fibroblasts after 24 and 48 hr without factors or treated with IGF1. Values were normalized to mass at the time of factor withdrawal. Error bars represent means ± SD of three independent experiments. See also Figure S2.

being required for Schwann cell proliferation (Figure 2D; Figures S2C–S2E). In LT cells treated with rapamycin or LY294002, however, growth was abolished suggesting that although PI3K signaling is not activated, basal flux through mTORC1 is still required for cell growth (Figure 2D). A basal level of signaling PI3K affecting cell growth was further suggested by the observation that control cells treated with LY294002 lose additional volume even in the absence of factors (Figure S2F).

To confirm that the ability of LT to drive cell growth was not specific to Schwann cells, we repeated the experiments in another cell-type—primary rat fibroblasts. We infected the fibroblasts with LT (Fb-LT) or control vector (Fb-V) and measured the ability of the cells to add volume and protein mass in the absence of extracellular growth factors. As for the Schwann cells, we found that LT was able to drive robust growth in the absence of extracellular factors whereas the control cells failed to grow (Figures 2E and 2F; Figure S2G). Moreover, as for the Schwann cells this was not associated with elevated signaling through the PI3K-AKT pathway (Figure S2H).

To determine the role of the Rb family in LT-driven growth, we made use of two well-characterized mutants that differentially affect Rb family interactions (reviewed in [25]). Western blot analysis confirmed that the LT point mutants—one unable to bind the entire Rb family (LT-K1), the second a mutant that differentially inactivates Rb compared to p107/p130 family members (LT-5110)—were expressed at similar levels to wild-type (WT) (LT) (Figure 3A). Cells expressing LT-K1 behaved similarly to control cells, losing volume over time—demonstrating that Rb family inactivation was critical for the growth-promoting function of LT. These results also confirmed the inability of p53 loss to induce cell growth as p53 binding and inhibition is retained by the LT-K1 mutant. In contrast, cells expressing the LT-5110 mutant did grow (Figures 3A and 3B), suggesting that it is loss of Rb function that is mainly responsible for driving cell growth.

We then used the E7 protein from HPV16 (which targets the Rb family of proteins for destruction but leaves p53 intact) to confirm that loss of the Rb family was sufficient to drive cell growth. In both control cells and cells expressing an Rb family-binding defective form of E7, the removal of growth factors led to a decrease in protein mass. In contrast, WT E7 was able to drive the addition of cell mass (Figure S3A), indicating that loss of the Rb family was sufficient to stimulate cell growth. Moreover, growth occurred in the absence of detectable signaling via the PI3-K pathway (Figure S3B).

To directly assess the specific role of Rb, we transfected primary Schwann cells with small interfering RNAs (siRNAs) targeted to Rb, which led to a greater than 70% reduction in Rb protein when compared to cells transfected with the nonspecific control (Figure 3C) and found that Rb knockdown was sufficient to induce an accumulation of protein mass (Figure 3C; Figure S3C). The growth was less than that stimulated by LT, which may reflect incomplete knockdown; however, it may also indicate a role for other members of the Rb family. Depleting Rb in DNp53-expressing cells had no additional effect on growth (Figures S3D and S3E). Taken together, these data demonstrate that loss of Rb is sufficient to drive cell growth, that loss of p53 is neither sufficient nor required for

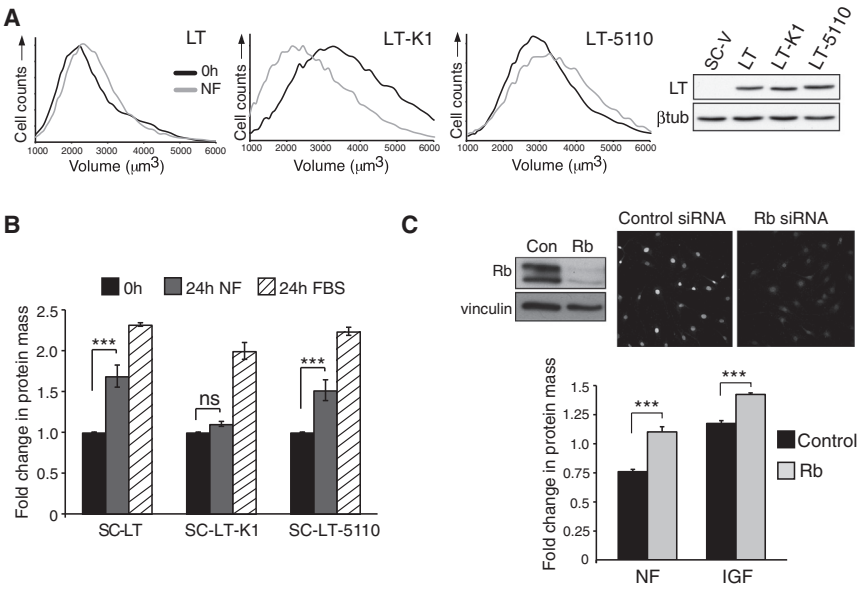


Figure 3. Loss of Rb Is Sufficient to Drive Cell Growth

(A) Representative Coulter curves showing the volume per cell of cell-cycle arrested populations expressing either LT or LT point mutants (LT-K1 and LT-5110) at the time of factor withdrawal (0 hr) and after 24 hr without factors (NF). Western blot analysis of LT is shown.

(B) Protein mass measurements of cells after 24 hr without factors (NF) or supplemented with 3% FBS, normalized to mass at time of factor withdrawal. Error bars represent two independent experiments ± SD.

(C) Rb knockdown. Western blot analysis of total Rb levels in cells transfected with a nonspecific siRNA duplex (Con) or with an oligo specific for Rb. Immunostaining of Rb protein in siRNA transfected cells. Protein mass measurements of control cells and cells with siRNA against Rb after 48 hr without factors or treated with IGF1, normalized to mass at time of factor withdrawal. Error bars show mean of triplicates ± SD. One way ANOVA with Tukey's post hoc test was performed. See also Figure S3.

growth, and that p53 loss does not act synergistically with Rb loss to drive cell growth.

Growth Is Uncoupled from Effects on Cell-Cycle and E2F-Dependent Transcription

The size of proliferating cells is determined by the relative rates of growth and proliferation. Comparison of the effects of LT on growth and proliferation showed that whereas SC-LT cells proliferated at a similar rate in the presence or absence of FBS, the growth rate was significantly lower in cells cultured in the absence of factors resulting in the production of smaller cells (Figure S4A). This is because serum is a more potent growth factor than LT (Figures 1A and 2A), which also means that the more slowly dividing SC-DNp53 cells are larger than the SC-LT cells at time 0 hr of the size experiments, as they have been cultured in serum (Figure 1A). These differential effects also suggested that LT might regulate the cell-cycle and cell growth by distinct mechanisms. Consistent with this, whereas SC-LT cells were able to proliferate and grow in the absence of factors, the LT-5110 mutant, while able to drive cell growth, was unable to induce the expression of cyclin A or trigger entry into the cell-cycle, showing that Rb regulation of cell growth can be uncoupled from effects on the cell cycle (Figures 4A and 4B; Figures 4B and S4C). As cyclin A is a key E2F cell-cycle target, these results suggested that inhibition of Rb causes cell growth via an E2F-independent mechanism. To confirm this, we tested the ability of constitutive E2F activity to drive cell growth and the ability of siRNAs to E2F1 to block LT-induced growth. Because E2F overexpression is reported to induce apoptosis in part via p53-dependent mechanisms, we used cells expressing DNp53 to express E2F1. We introduced hemagglutinin (HA)-tagged E2F1 into these cells and found that whereas E2F1 overexpression was sufficient to efficiently induce cyclin A expression (Figure S4D), it was unable to drive cell growth (Figure 4C). Moreover, we found that despite an efficient knockdown of E2F1, LT-induced growth was unaffected (Figure S4E). These results are consistent with Rb-loss driving growth by an E2F-independent mechanism and independent from effects on the cell cycle.

To determine whether the growth induced by Rb was the result of an increase in biosynthetic pathways, we analyzed the rates of de novo protein and lipid biosynthesis following short-term labeling with radiolabeled methionine and pyruvate and measured their incorporation into protein and lipid fractions, respectively. We found that the SC-LT cells had significantly higher rates of both protein and lipid biosynthesis compared to control cells (Figures 4D and 4E). We then performed pulse-chase experiments to determine the protein degradation rates in the two cell types and found that the half-lives of the proteins were similar in the two cell types, arguing that an increase in biosynthesis rather than an inhibition of degradation pathways was responsible for the growth of the LT-expressing cells (Figures 4F and 4G). We also compared autophagic rates in the two cell types in the absence of factors by both immunofluorescence and western blot analysis of LC3. We found that both SC-V and SC-LT cells showed a dramatic upregulation of LC3-positive autophagic structures following growth factor removal (Figure S4F) indicating the onset of autophagy. To quantify the flux, we compared the levels of LC3-I and LC3-II following growth factor removal and found, in the absence of chloroquine, a similar decrease in LC3-I levels and an increase in LC3-II levels in the SC-V and SC-LT cells. However, upon chloroquine treatment we detected higher levels of LC3-II in the SC-LT cells, arguing that autophagic flux is higher in the LT-expressing cells (Figure S4G). These results clearly demonstrate that a decrease in autophagic rates is not responsible for the increase in cell growth induced by LT expression and that biogenic processes drive the cell growth seen in the LT-expressing cells.

LT Cooperates with Oncogenic Ras to Drive Strong, Sustained Growth

Our findings demonstrate that oncogenic Ras drives weak growth through canonical activation of the PI3K pathway (Figure 1), whereas loss of Rb acts via a distinct pathway (Figure 2). To test the cooperation between these signals, we expressed oncogenic Ras in SC-LT cells. Similarly to the effects of IGF1, we found that the Ras signal synergized with loss of the Rb family to drive stronger growth (Figures 5A and 5B) and without a further increase in signaling through the PI3K pathway compared to SC-DNp53Ras cells, which grow poorly,

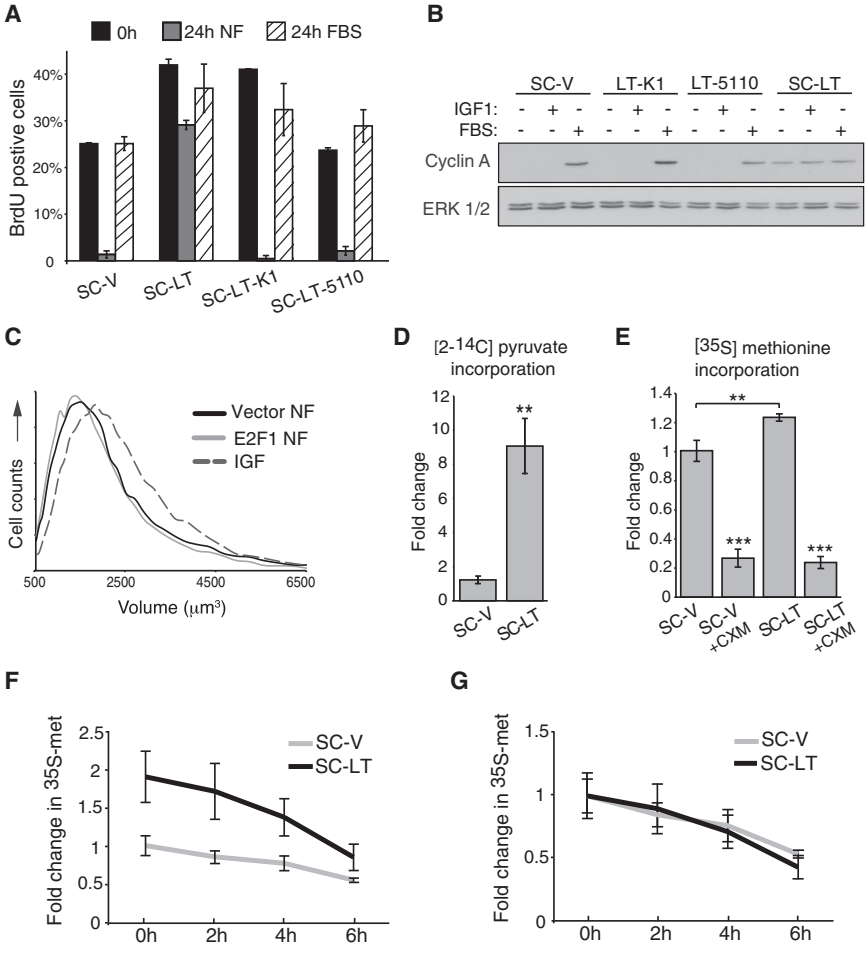


Figure 4. Rb-Induced Growth Is Uncoupled from Effects on Cell Cycle and E2F-Dependent Transcription and Involves an Increase in Biosynthetic Pathways

(A) Counts of bromodeoxyuridine-positive cells after 24 hr without factors (NF) or with 3% FBS. Error bars show mean of triplicates ± SD.

(B) Western blot analysis of cyclin A, 24 hr after withdrawal of factors or 24 hr following the addition of IGF1 or FBS as indicated.

(C) Coulter curves showing volume per cell of arrested SC-DNp53 cells transfected with hE2F1-HA (E2F1) or control vector (Vector) after 24 hr without factors (NF). Cells supplemented with IGF1 were included as positive control.

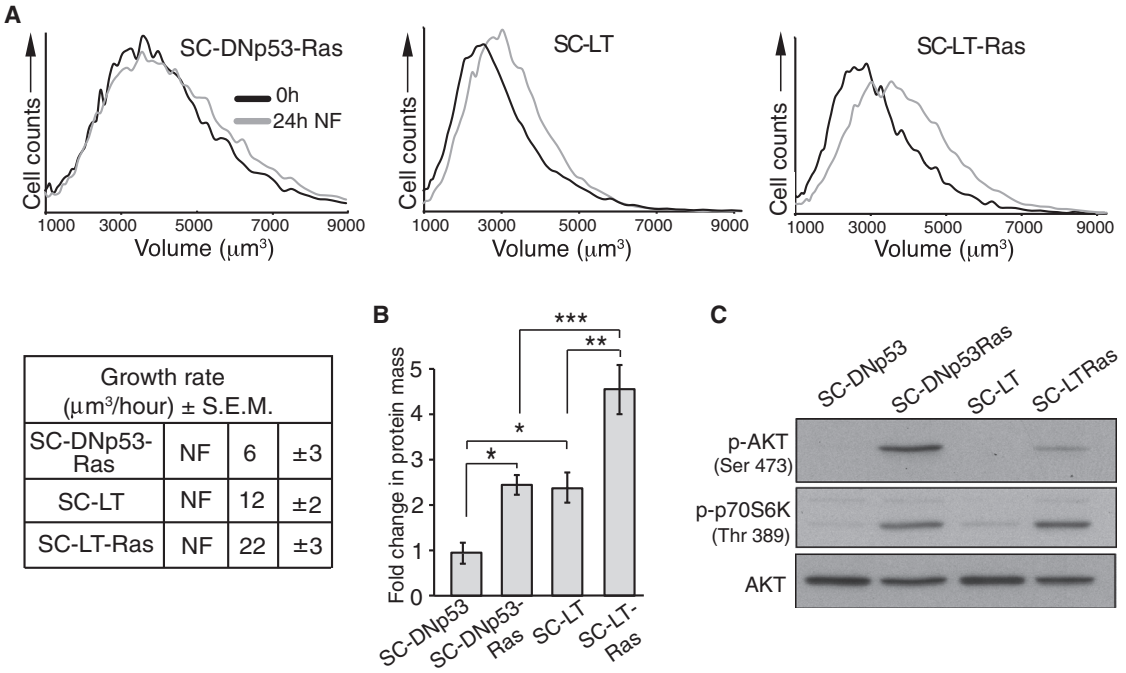
(D) Incorporation of [2-^{14}C] pyruvate into the lipid fraction of SC-V and SC-LT cells following a 4 hr labeling period 24 hr following the removal of factors. Values shown are the mean of three independent experiments ± SEM.

(E) Incorporation of [^{35}S]-methionine into the protein fraction of SC-V and SC-LT cells following a 45 min labeling period, 24 hr following the removal of factors in the absence or presence of cycloheximide (CXM). Values shown are the mean of three independent experiments ± SEM. Student's t test was performed.

(F) Levels of [^{35}S]-methionine in the protein fraction of SC-V and SC-LT cells over time, following a 2 hr pulse, 24 hr following the removal of factors. Numbers are expressed as fold change relative to SC-V cells at 0 hr and show the increased amount of incorporation by the SC-LT cells.

(G) Shown are the data from (F), when the amount at time 0 hr for each cell-type is corrected to 1 and thus shows the relative half-lives of the labeled proteins. Values shown are the mean of three independent experiments ± SEM. One-way ANOVA with Tukey's post hoc test was performed. See also Figure S4.

indicating that the synergy does not act at the level of PI3K signaling (Figure 5C; Figure S5A). Interestingly, we found that Ras and LT also cooperated in a second manner—to drive sustained growth. We found that although SC-LT cells cultured without factors added protein mass progressively for 48 hr, after this point the growth stalled. However, cells coexpressing Ras continued to add mass and stalled SC-LT cells could be reinduced to grow by treatment with IGF1 (Figures 6A and 6B). We noted that stalled SC-LT cells developed a "starved" appearance, similar to that seen in normal cells following removal of glutamine from the medium. As PI3K-AKT-TOR signaling has been shown to regulate nutrient transporter expression and localization at the cell-surface [26, 27], we speculated that the PI3K signal induced by Ras and IGF1 and absent in the LT-expressing cells was required for the maintenance of nutrient uptake that would be predicted to be necessary for sustained cell growth. To determine whether this was the case, we measured the uptake of two radioactively labeled nutrients known to be important for growth—glutamine and glucose (using 2DG; a nonmetabolizable glucose analog) in cells over time, following the removal of factors. In SC-LT cells, we found that whereas nutrient uptake rates were maintained at 24 hr following the removal of factors, by 48 hr the levels had decreased substantially (Figures 6C and 6D), coincident with when the cells stopped growing. In contrast, nutrient uptake was maintained in the Ras-expressing cells, which continued to grow (Figures 6C and 6D). To determine the functional significance of a decrease in nutrient uptake on growth, we initially tested the effects of glutamine removal on LT and LT-Ras cells and found that both required extracellular glutamine to grow (Figure S6A). mTORC1 is regulated by amino acid levels and the complex has recently been reported to relocalize and, as a result, lose activity upon amino acid starvation providing an indicator of intracellular nutrient levels, although the pattern varied between the cell types tested [28, 29]. We therefore used alterations in the subcellular localization of mTOR as a read-out of a significant decrease in nutrient levels within the cell. In Schwann cells, we found that amino acid starvation caused mTOR to redistribute from the periphery to discrete puncta in the perinuclear region (Figure 6E). We then examined the localization of mTOR in SC-LT cells and control vector cells following the removal of factors and found that whereas at early time-points mTOR staining was diffuse throughout the cell, at a later time-point, when nutrient uptake levels had declined, we found a dramatic change in the localization of mTOR to form large clusters near the perinuclear region, although mTOR levels remained constant (Figure 6E; Figures S6B–S6D). Much of the mTOR colocalized with lysosomes, which also moved to the perinuclear region following long-term growth factor removal (Figure S6D). This result is consistent with loss of mTOR activity in response to lowered amino-acid levels within the cell [28], providing a mechanistic explanation for the inhibition of cell growth in these cells.



Growth rate (μm³/hour) ± S.E.M.			
SC-DNp53-Ras	NF	6	±3
SC-LT	NF	12	±2
SC-LT-Ras	NF	22	±3

Figure 5. LT and Oncogenic Ras Cooperate to Drive Strong Growth

(A) Representative volume curves of arrested cell populations at point of factor withdrawal (0 hr) and after 24 hr without factors (NF). Cells coexpress either DNp53 and Ras^{Val12} (SC-DNp53Ras), LT and vector (LT), or LT and Ras^{Val12} (LT-Ras). Mean growth rates (change in mean volume per hr) are given for four independent experiments ± SEM.

(B) Change in total protein mass in these cells after 48 hr without factors compared to time 0 hr for each cell type. Data are presented as mean ± SEM of five independent experiments.

(C) AKT and mTORC1 activation levels after 24 hr without factors as shown by western blot analysis of AKT (Ser 473) and p70S6 kinase phosphorylation. Total AKT is used to assess protein loading. See also Figure S5.

Discussion

The formation of a tumor requires the loss of normal growth controls; therefore, the genetic changes responsible for tumor formation must be capable of driving cell growth. Yet, despite the identification of the key genetic drivers for many tumor types, it remains poorly understood how and which of these genetic changes act to induce the increases in mass required for tumor formation. In the present study, we have characterized the ability of three major oncogenic pathways implicated in the majority of human cancers to induce cell growth and found that two of these, oncogenic Ras and Rb family loss, are each sufficient to drive growth. We found that Ras is a relatively weak driver of growth, whereas loss of Rb induces stronger growth but is unable to produce sustained growth. Instead, as for other aspects of tumor formation (such as proliferation), we find that these two pathways cooperate to produce the strong, sustained growth likely to be required to form a rapidly growing tumor. This novel mechanism of oncogenic cooperation provides further insight into how oncogenic changes may act together to promote cancer and potentially offers further novel strategies for therapeutic targeting.

Loss of the Rb family has been linked to various aspects of tumorigenesis with important roles in cell-cycle control, cell senescence, and changes in differentiation state [30]. Here, we have identified a further consequence of Rb-family loss, in that it is sufficient to directly drive cell growth and acts together with oncogenic Ras signaling to drive robust growth. Interestingly, we find that the effects of Rb loss on the cell cycle and cell growth pathways can be separated, providing another example of the separation of these important and often coordinated cellular processes. Thus, whereas effects on the cell cycle are mediated by E2F-dependent activation of cyclin/cdk expression, the effects on cell growth are independent of E2F activity. This separation is demonstrated most clearly with the mutant of LT (LT-5110) that is able to drive cell growth but not proliferation. Somewhat counterintuitively, we and others find that cells in which the Rb family is inactivated are usually smaller than normal cells [31, 32], however this is readily explained by our findings that LT has a more dramatic effect on the cell cycle than on cell growth, which will lead to cells proliferating at a smaller cell size and/or the potency of serum as a growth factor—conditions in which most cells are cultured in vitro. These differential effects on the cell cycle and cell growth can explain why tumor cells are sometimes smaller than their normal counterparts despite having aberrant growth pathways and emphasize the separate regulation of the growth and proliferative pathways.

We find that LT drives growth without promoting AKT/TOR activity. Very few other growth-promoting pathways have been described, though a direct role for the transcription factor and proto-oncogene c-*myc* in regulating growth is well established [33]. We were unable to detect increased levels of c-*myc* in LT-expressing cells, suggesting that Rb loss does not drive growth by this mechanism—although, consistent with other reports [34], myc was induced by constitutively active Ras (Figure S5B). In *Drosophila*, overexpression of the cyclin D/cdk4 complex, an upstream regulator of Rb, was

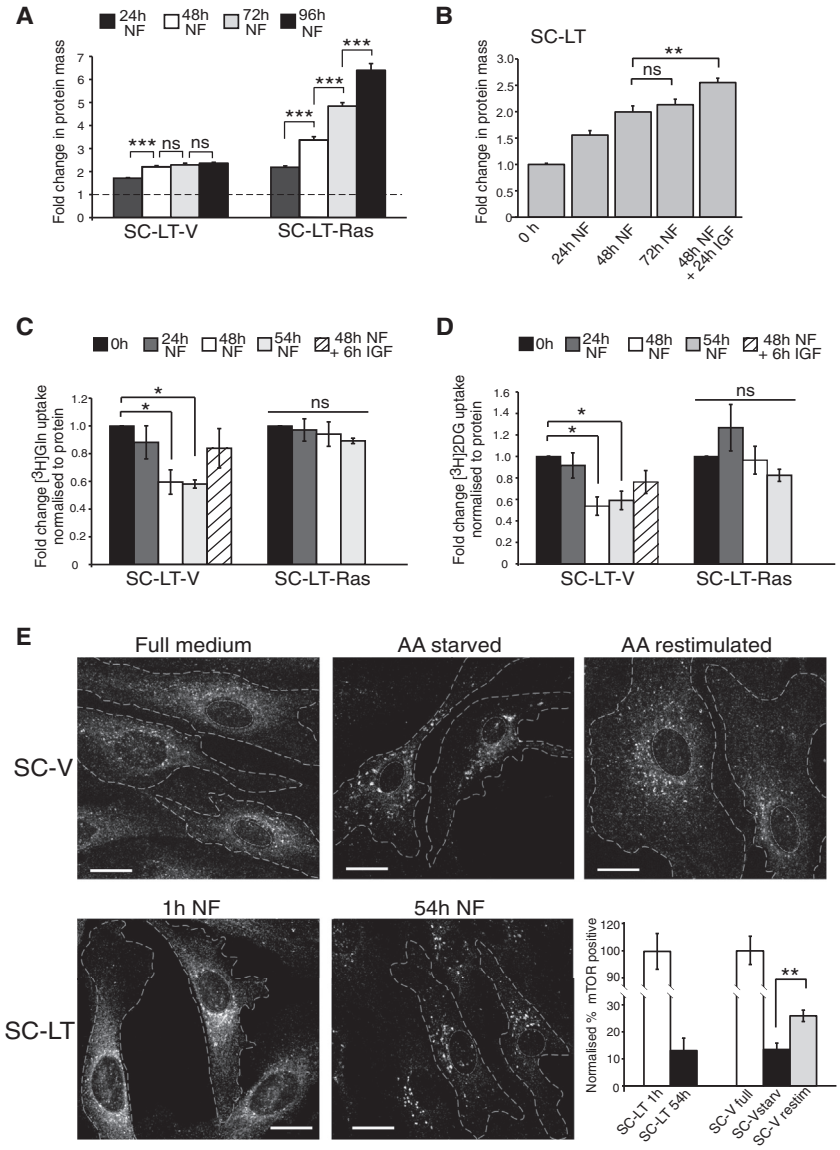


Figure 6. Ras Signaling Maintains Nutrient Uptake and Allows Sustained Growth

(A) Cells coexpressing LT and control vector or LT and Ras^{Val12} cultured in the absence of factors for 96 hr. Protein mass measurements were made at 24 hr intervals and normalized to mass at time of factor withdrawal. Error bars show mean of triplicates ± SD.

(B) Total protein mass of SC-LT cells over time. Cells were cultured without factors for 48 hr and subsequently maintained in the absence of factors or stimulated with IGF1 and measured 24 hr later. Error bars show mean of triplicates ± SD.

(C) Uptake of tritiated glutamine ([^{3}H]Gln) in cells without factors over time, normalized to uptake at time of factor withdrawal. Error bars show means of three independent experiments ± SEM.

(D) Uptake of tritiated 2-deoxyglucose ([^{3}H]2DG) in cells without factors over time, normalized to uptake at time of factor withdrawal. Data are presented as mean ± SEM of four independent experiments.

(E) mTOR localization: Schwann cells in complete culture medium were transferred to medium without amino acids for 40 min, followed by stimulation with amino acids for a further 20 min. SC-LT cells were cultured without factors. Cells were fixed and immunostained for mTOR. The percentage of each cell containing mTOR-positive pixels was quantified and normalized either to SC cells in full medium or, in the case of LT, to cells after 1 hr without factors. Scale bar represents 15 μm. See also Figure S6.

reported to cause cell enlargement [35]; however, this was via an Rb-independent mechanism, which involved the regulation of mitochondrial activity [36]. It has been established that Rb can repress transcription of components of the translational machinery through interactions with subunits of the RNA pol I and III transcription factor complexes [37, 38]. Because protein synthesis is an essential element of cell growth, it is to be expected that disrupted regulation of the synthetic machinery will contribute to the ability of Rb loss to drive cell growth. However, there is no evidence to suggest that dysregulation of RNA pol I and III transcription is sufficient to drive growth, and it is likely that Rb acts at many levels to coordinate cell growth. This is supported by our microarray gene expression analysis (unpublished data), which indicates that loss of Rb also induces E2F-independent global changes in RNA pol II transcription.

Consistent with other studies, we found that oncogenic Ras signaling and activation of the PI3K growth cascade was sufficient to induce cell growth but somewhat to our initial surprise, despite robust activation of the canonical growth pathway, cell growth in the absence of synergizing factors was weak. However, these findings are consistent with other studies in which constitutive activation of the AKT-TOR pathway was also found to drive relatively weak growth [24, 39]. Interestingly, despite previous reports that p53 can act as a metabolic regulator and is able to induce autophagy [13], we found that loss of p53 had no direct effect on cell growth. Although we showed that the Ras-AKT-TOR pathway is relatively weak in driving cell growth, we also showed that the pathway cooperated with the loss of Rb, to drive strong sustained growth. Interestingly, a recent genetic study in *Drosophila* has also found synergy between the mTOR pathway and Rb [40]. Using an in vivo synthetic lethality screen to identify genes that synergistically induced cell death in Rb-negative cells, they identified TSC2—an upstream inhibitor of mTORC1 that is targeted by Ras/PI3K signaling. Significantly, the authors attributed the lethality to a combination of oxidative and endoplasmic reticulum stress signals, which they speculated to be mediated in part by elevated levels of protein synthesis—an in vivo corroboration of our finding that Rb loss and Ras together induce strong growth and perhaps an indication of how these findings may be exploited for therapeutic purposes.

Nutrients are supplied systemically in animals; therefore regulating their uptake can provide a means of cell growth control and has been proposed as a tumor suppressor mechanism [26]. In agreement with other studies, we found that PI3K-AKT-TOR signaling is required to maintain nutrient uptake [27, 41]. We established that Rb loss is capable of driving cell growth but found that this growth pathway is not sustainable—loss of Rb does not activate the PI3K cascade,

and consequently cells lose access to nutrients and eventually stall in their growth. Thus, we have identified regulation of nutrient transport as a novel mechanism by which Rb loss and oncogenic Ras synergize. Our findings underscore the importance of the PI3K pathway in cell growth but demonstrate the necessity for the coactivation of other oncogenic pathways in order to achieve the robust growth associated with aggressive tumor formation.

Experimental Procedures

Cells

Schwann cells and fibroblasts were isolated from P7 rat sciatic nerve and cultured as described previously [23]. Normal culture medium for Schwann cells is Dulbecco's modified Eagle's medium (DMEM) (low glucose) containing 3% FBS, 1 μM forskolin, and 20 ng/ml neuregulin. Fibroblasts were routinely cultured in DMEM containing 10% FBS. For factor withdrawal experiments, Schwann cells were seeded onto dishes coated with poly-L-lysine (PLL), fibronectin and laminin in normal culture medium whereas fibroblasts were seeded onto dishes coated with PLL only. Factors were removed by performing three washes in modified SATO-defined medium (DMEM supplemented with 100 μg/ml BSA, 60 ng/ml progesterone, 16 ug/ml putrescine, 40 ng/ml selenium, 50 ng/ml triiodothyrine, 50 ng/ml thyroxine) and then replacing with SATO containing transferrin (100 μg/ml) with or without IGF1 (100 ng/ml).

Size Measurements

We seeded 10^5 cells onto triplicate wells of six-well PLL-laminin-fibronectin coated dishes in normal culture medium (PLL only for the fibroblasts) and were arrested using 1 μg/ml aphidicolin for 24 hr. Factors were removed by washing as described, and the cells were placed into SATO-defined medium containing transferrin (100 μg/ml) with or without IGF1 (100 ng/ml) and 0.8 μg/ml aphidicolin. Any inhibitors were added at this time point and 15 min prior to the addition of IGF1. The time 0 hr point was then collected by trypsinisation, resuspended in isotonic solution, and measured using a Beckman Multisizer 4 Coulter Counter (10,000 cells per reading). Changes in cell size were then detected 24 hr later.

Statistical Analyses

Unless otherwise indicated in the figure legend, one-way ANOVA with Newman-Keuls post hoc analysis was performed for all tests of significance. In all cases: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not significant.

Supplemental Information

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.07.040.

Acknowledgments

This study was supported by a programme grant from Cancer Research UK. I.N was supported by the Association for International Cancer Research and an EMBO fellowship. S.R. is a GABBA student supported by a fellowship from the Portuguese Foundation for Science and Technology. We thank James DeCaprio for the LT constructs.

Received: December 15, 2011
Revised: June 2, 2012
Accepted: July 18, 2012
Published online: August 9, 2012

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Update

Current Biology
Volume 22, Issue 19, 9 October 2012, Page 1859

DOI: https://doi.org/10.1016/j.cub.2012.08.037

Loss of Rb Cooperates with Ras to Drive Oncogenic Growth in Mammalian Cells

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(Current Biology *22*, 1765–1773; October 9, 2012)

As a result of an author oversight in the version of this article originally published online, the significance bars in Figure 6B were incorrectly represented: the small bar should have been labeled ns, whereas the large bar should have been labeled **. This error has now been corrected in both the print and online versions of the article. The authors apologize for the error and any confusion that may have resulted.

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http://dx.doi.org/10.1016/j.cub.2012.08.037