Hypophosphorylated pRb knock-in mice exhibit hallmarks of aging and vitamin C-preventable diabetes

Zhe Jiang1,*, Huiqin Li1, Stephanie A Schroer1, Veronique Voisin2, Youngjun Ju1, Marek Pacal3,4, Natalie Erdmann5, Wei Shi1, Philip E D Chung1,4, Tao Deng1, Nien-Jung Chen5, Giovanni Ciavarra1,4, Alessandro Datti5,7, Tak W Mak6, Lea Harrington6, Frederick A Dick9, Gary D Bader2, Rod Bremner3,4, Minna Woo1,10 & Eldad Zacksenhaus1,4,10,**

Abstract

Despite extensive analysis of pRB phosphorylation in vitro, how this modification influences development and homeostasis in vivo is unclear. Here, we show that homozygous RbΔKK and RbΔK7 knock-in mice, in which either four or all seven phosphorylation sites in the C-terminal region of pRb, respectively, have been abolished by Ser/Thr-to-Ala substitutions, undergo normal embryogenesis and early development, notwithstanding suppressed phosphorylation of additional upstream sites. Whereas RbΔKK mice exhibit telomere attrition but no other abnormalities, RbΔK7 mice are smaller and display additional hallmarks of premature aging including infertility, kyphosis, and diabetes, indicating an accumulative effect of blocking pRb phosphorylation. Diabetes in RbΔK7 mice is insulin-sensitive and associated with failure of quiescent pancreatic β-cells to re-enter the cell cycle in response to mitogens, resulting in induction of DNA damage response (DDR), senescence-associated secretory phenotype (SASP), and reduced pancreatic islet mass and circulating insulin level. Pre-treatment with the epigenetic regulator vitamin C reduces DDR, increases cell cycle re-entry, improves islet morphology, and attenuates diabetes. These results have direct implications for cell cycle regulation, CDK-inhibitor therapeutics, diabetes, and longevity.

Keywords aging; diabetes; knock-in mice; pRB; retinoblastoma; senescence; vitamin C

Introduction

The retinoblastoma tumor suppressor, pRB, regulates diverse biological processes including cell cycle progression, survival, metabolism, and differentiation and is frequently lost in cancer (Dyson, 2016; Zacksenhaus et al., 2017; Dick et al., 2018; Kitajima et al., 2020). pRB exerts its effects by binding to and modulating the activity of multiple cellular proteins including members of the E2F family of transcription factors (Kent & Leone, 2019). When bound to E2F1-3, pRB represses transcription by hindering their trans-activation domains and by recruiting chromatin-modifying enzymes. In cycling cells, E2F-bound pRB transiently silences cell cycle genes by recruiting histone deacetylases (Harbour & Dean, 2000), whereas in differentiating cells, pRB engages histone methyltransferases and other histone modifiers that stably suppress cell cycle genes, establishing an epigenetic differentiation state that is more resistant to mitogenic signals (Narita et al., 2003; Ait-Si-Ali et al., 2004; Uchida, 2016).

A major form of regulation of pRB activity is phosphorylation, mediated by cyclin-dependent kinases (CDKs): pRB is monophosphorylated at single sites by type D cyclins-CDK4/6 during most...
G1 phase of the cell cycle and then abruptly hyper-phosphorylated on the remaining sites by E cyclins-CDK2 at the restriction point, just prior to S phase (Narasimha et al., 2014). Hyper-phosphorylated pRb dissociates from E2Fs, allowing transcriptional activation of genes required for G1-to-S transition and cell cycle progression.

The RB gene is often disrupted in certain cancers by mutations, deletions, or promoter silencing; in other cancers, RB is intact but the pRB protein is inactivated by hyper-phosphorylation through amplification/activation of D type Cyclins-CDK4/6 and E type Cyclins/CDK2 complexes (Koboldt et al., 2012; Nik-Zainal et al., 2016). In the latter tumors, hyper-phosphorylated pRB can be reactivated by CDK4/6 inhibitory drugs such as palbociclib that improve survival outcome of cancer patients (Knudsen & Witkiewicz, 2017; Otto & Sicinski, 2017; Pernas et al., 2018; Turner et al., 2018; Roberts et al., 2020).

pRB phosphorylation is also implicated in tissue regeneration. The CDK4/6 inhibitor p16INK4A is induced in aging tissues including pancreatic islets in mice (Jansen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006) and humans (Nielsen et al., 1999; Kanavaros et al., 2001; Chkhotua et al., 2003; Krishnamurthy et al., 2004; Liu et al., 2011; Aguayo-Mazzucato et al., 2017) as well as in type 2 diabetes (T2D) (Kong et al., 2016). Other CDK inhibitors such as p21cip1 and p27kip1 are induced by various genotoxic stresses to halt cell cycle progression in part by blocking pRB phosphorylation (Chicas et al., 2010; Chien et al., 2011; Kang et al., 2015; Georgakilas et al., 2017). These CDK inhibitors act not only through pRB but also through other targets (e.g., Kollmann et al., 2013; Klein et al., 2018; Zhang et al., 2018), and therefore the specific impact of blocking pRB phosphorylation on development and homeostasis, and whether the consequences of inhibiting pRB phosphorylation can be therapeutically mitigated are not known.

pRB has 14 CDK phospho-acceptor sites that are clustered in the N terminus, spacer/linker region, and exon 23 at the C terminus (Lees et al., 1991; Buckley et al., 1993; Mittnacht, 1998; Fu et al., 2004) (Fig 1A). These phosphorylation sites can be classified into four groups on the basis of their effect on specific pRB-protein interactions (Sanidas et al., 2019): S224-S243-T246, T350-T367-S601, S605-S773-S781-S788, and S800-S804-T814-T819 (mouse pRB numbering; Fig 1A). Phosphorylation of the exon 23 cluster triggers a conformation change that excludes E2F (Rubin, 2013) and destabilizes interaction with chromatin-modifying enzymes, allowing G1-to-S cell cycle progression (Goodrich et al., 1991; Knudsen & Wang, 1996, 1997; Connell-Crowley et al., 1997; Burkhart & Sage, 2008; Rubin, 2013; Narasimha et al., 2014; Dick et al., 2018; Sanidas et al., 2019). Substitutions of all seven sites lead to constitutive repression of E2F1-regulated genes in over-expression experiments in culture (Brown et al., 1999).

To determine the role of pRB phosphorylation in vivo, we herein engineered knock-in mice with Ser/Thr-to-Ala substitutions in the four most distal or all seven sites in the C terminus of endogenous Rb. Unexpectedly, homozygous RbΔK4 mice were fertile, showed apparent normal myogenesis and T-cell development, and displayed no obvious external or histo-pathological defects (Fig 1C and Appendix Fig S1A).

To determine the effect of pRB phosphorylation in vivo, we first generated knock-in mice in which the four most distal Ser/Thr residues (S800, S804, T814, and T819) were substituted to Alanine, a related but unphosphorylatable amino acid (Figs 1A and EV1A-D). Homozygote RbΔK4 mice (henceforth referred to as RbΔK4ΔK7) were obtained with normal Mendelian inheritance and appeared normal. Sequencing analysis confirmed the Ser/Thr-to-Ala substitutions introduced into the Rb locus in these mice. pRB migrates on SDS–PAGE gels as a hypo-phosphorylated protein of ~110 KDa as well as slow-migrating phosphorylated species. The slow migration and level of phosphorylation are inhibited by substitutions on Ser800/Ser804 in vitro (Knudsen & Wang, 1996; Brown et al., 1999).

Consistent with this, lysates from RbΔK4 thymocytes exhibited a single band of hypo-phosphorylated pRB (Fig 1B).

Despite the blockade of phosphorylation of these important sites, homozygous RbΔK4 mice were fertile, showed apparent normal myogenesis and T-cell development, and displayed no obvious external or histo-pathological defects (Fig 1C and Appendix Fig S1A).

The four most distal Ser/Thr sites in exon 23, which are substituted in RbΔK4 mice, were shown to mediate interaction with LxCxE-binding factors through E2F1-dependent (e.g., HDAC1, SUV39H) and independent (SUV4-20H) mechanisms. The latter interaction is critical for trimmingethylation of H4K20 and telomere maintenance (Benetti et al., 2011). Indeed, triple knockout fibroblasts with mutations in Rb and its two relatives, p107 and p130 (RbΔK4ΔK7/p107ΔK7/p130ΔK7), exhibit abnormally long telomeres (Garcia-Cao et al., 2002). To determine telomere length in RbΔK4 mice, we backcrossed them six generations into C57BL/6 mice, commonly used to analyze telomere length (Snow et al., 2007). Metaphase spreads prepared from five pairs of splenocytes from 3- to 4-month-old mice revealed that telomeres in RbΔK4 mice were significantly shorter compared to control littermates (Mann–Whitney–Wilcoxon test \( P < 0.001 \); Fig 1D; Appendix Fig S2A). GST-SUV4-20H1 and GST-SUV4-20H2 preferentially precipitated hypo-phosphorylated, not hyper-phosphorylated pRB species from wild-type thymocytes (Appendix Fig S2B), thus supporting the idea that constitutive inhibition of H4K20 trimethylases by pRbΔK4 through-out the cell cycle underlies short telomeres in these mice (Discussion).

RbΔK7 knock-in mice also exhibit apparent normal embryogenesis and early neonatal growth

Although phosphorylation of single CDK4/6 sites in pRb can affect specific cellular functions in culture (Sanidas et al., 2019), there is
Figure 1.
clear evidence that inhibition of pRb phosphorylation on multiple sites has an accumulative effect on its transcriptional repression in vitro (Bremner et al., 1995; Brown et al., 1999). We therefore asked whether disruption of all seven phosphorylation sites in exon 23 would impact normal development and homeostasis (Figs 2A and EV1A–D). Surprisingly, like homozygote RbΔK7 mice, RbΔK7/K7 knocked-in mice (henceforth RbΔK7) were obtained by interbreeding RbΔK7/+ heterozygotes with the same Mendelian inheritance as RbΔ+/− littermates (not shown).

BrdU incorporation analysis revealed robust cell proliferation in multiple RbΔK7 embryonic tissues at comparable levels to those seen in control RbΔKT littermates (Fig 2B). Moreover, RbΔK7 mice exhibited apparent normal myogenesis in vivo and in vitro, as well as early T-cell development (Figs 2C; Appendix Fig S1B). As germline mutation in Rb predisposes to retinoblastoma, we specifically analyzed the effect of RbΔK7 on proliferation of retinal progenitors. Staining for Ki67 (dividing cells), BrdU incorporation (S-phase cells), or Ser10 phosphorylated histone 3 (PH3, mitotic cells) revealed indistinguishable levels of progenitor cell proliferation in RbΔK7 versus control retina (Fig 2D). Hematology and innate immunity were also apparently normal in RbΔK7 mice (Appendix Figs S3 and S4). Thus, expression of hypo-phosphorylated pRbΔK at endogenous levels does not attenuate cell proliferation in multiple tissues.

Phospho-specific antibody analysis of lysates from proliferating RbΔK7 thymocytes revealed that in addition to the substituted Serine/Threonine amino acids in exon 23 (e.g., Ser773), phosphorylation of upstream Thr350 and Ser601 sites was completely inhibited, while Ser243/Thr246 and Ser605 remained phosphorylated (Fig 2E). Interestingly, in RbΔK7 thymocytes, Thr350 phosphorylation was also completely suppressed, whereas Ser601 phosphorylation was detectable at a low level (Fig 2F, arrowhead). Phospho-antibodies for mouse Ser224- and Thr367-pRb, the remaining two sites, are not currently available. Thus, phosphorylation of at least 9 of 14 DK sites in pRb (seven sites in exon 23 plus Thr350 and Ser601) is not essential for embryogenesis or early neonatal growth.

**Embyronic fibroblasts from RbΔK7 knock-in mice exhibit reduced sensitivity to CDK2 inhibitors and increased spontaneous senescence**

One mechanism by which cells may escape cell cycle inhibition by pRbΔK7 could be through compensating phosphorylation by other kinases. To begin to address this possibility, we performed focused kinase screens (360 drugs) on mouse embryonic fibroblasts (MEFs) isolated from pRbΔK7 or control embryos. 32 drugs that showed some differential effects in the initial screen were subject to secondary screens, but no kinase inhibitor significantly suppressed the growth of RbΔK7 over control littermate MEFs (Fig 3A). As p38-MAPK inhibits the N terminus of pRb (Gubert et al., 2016), we examined the effect of inhibitors of this kinase. However, three different inhibitors, SB-203580 (Fig 3A), VX-702, and SB-202190 (Fig 3B), which target p38α or p38β plus p38γ, respectively, had no significant effects on RbΔK7 relative to control MEFs. In addition, the CDK4/6 inhibitor palbociclib (PD-0332991), and the checkpoint kinase CHK1 inhibitor, TCS-2312, had no differential effect on RbΔK7 versus normal cells.

Instead, RbΔK7 MEFs appeared more resistant to CDK2 inhibition. Indeed, RbΔK7 MEFs were significantly more resistant to GW8510 (P = 0.008) and showed a trend toward significant resistance to a pan-CDK inhibitor, AT-751, which targets CDK2, as well as CDK1, CDK4, CDK6, and CDK5 (P = 0.07; Fig 3B). The specific resistance of RbΔK7 MEFs to CDK2 inhibitors is in stark contrast to the increased sensitivity of CyclinD1/D2/D3 triple-mutant MEFs to CDK2 inhibition (Kozar et al., 2004). This uncovers a clear difference between CyclinD1/2/3 null cells, in which pRb can be phosphorylated (by CDK2), and RbΔK7 cells in which phosphorylation of multiple CDK sites is blocked by Ser/Thr-to-Ala substitutions, rendering the cells resistant to CDK2 inhibition compared to control RbΔ− cells.

Early passage RbΔK7 MEFs exhibited a slight but significant increase in cell survival and a longer G1-phase of the cell cycle (Fig 3C and D). Moreover, whereas control MEFs became readily immortalized after ~10 passages, RbΔK7 MEFs underwent cell senescence and failed to spawn colonies of immortalized cells (Fig 3E). In accordance, RbΔK7 MEFs at passage 9 showed stronger senescence-associated β-galactosidase (SA-βGAL) staining than control MEFs (Fig 3F). Considering all SA-βGAL positive-stained cells (both low and high), 82% of RbΔK7 MEFs were positive compared to 51.5% in control MEFs (n = 4, P = 0.039 by 2-tailed Student t-test). Thus, in accordance with its tumor-suppressive function, RbΔK7 MEFs showed modest but significantly reduced cell proliferation and apoptosis and increased cellular senescence.

**RbΔK7 mice exhibit severe diabetes and accelerated aging**

Strikingly, as RbΔK7 mice matured, they exhibited smaller size and various signs of premature aging. On average, RbΔK7 male mice weighed 21% less than their littermates (Fig 4A). Males were infertile, with small testes and reduced number of matured sperm, indicating hyper-sensitivity of meiosis to constitutive expression of hypo-phosphorylated pRb (Fig 4B–D). At 8–11 months of age,
Figure 2.

A diagram illustrating Ser/Thr-to-Ala substitutions.

B: Comparison of control (ctrl) and RbΔK7 conditions with BrdU labeling.

C: Flow cytometry analysis of CD3 and CD4/CD8 expression in ctrl and RbΔK7 conditions.

D: Immunostaining with Ki67, PH3, and BrdU markers in E16.5 and P0 stages.

E: Western blot analysis of Rb phosphoforms in ctrl and RbΔK7 conditions.

F: Western blot analysis of pThr350, pSer601, and Tubulin in ctrl and RbΔK7 conditions.
RbΔK7 mice became moribund with severe kyphosis (Fig 4E). Hair regrowth two weeks post-plucking was delayed in RbΔK7 mice compared to control littersmates (Fig 4F). While hair follicles in control mice were in both telogenic (resting) and anagenic (growing) phases, hair follicles in RbΔK7 mice were mostly in the telogen phase and expressed low levels of keratin 14 (a stem cell marker) and PCNA (a cell cycle marker) relative to control (Fig 4G and expressed low levels of keratin 14 (a stem cell marker) and PCNA (a cell cycle marker) relative to control (Fig 4G–I).

Most strikingly, adult RbΔK7 mice developed severe diabetes, and all mice succumbed to the disease by one year of age (Fig 4I). Diabetes was observed within a shorter window of time in males than in females, and therefore, male mice were analyzed henceforward. Blood glucose levels in fasting 8- to 12-month-old RbΔK7 males, but not in as old as 18-month RbΔK4 mice, was on average over 25 mM compared to 6–8 mM in wild-type animals (n = 6 each, P = 1.8 × 10⁻⁵). Complete pathological analysis of six RbΔK7 mice with severe diabetes revealed mostly normal histology of other tissues, with no evidence for gross abnormalities or cancer (not shown). Thus, systemic inhibition of pRb phosphorylation does not interfere with normal embryogenesis but attenuates cell proliferation and tissue regeneration and accelerates hallmarks of aging such as diabetes in adult mice as further described below.

Diabetes in RbΔK7 mice is marked by reduced cell cycle re-entry and reduplication of pancreatic β-cells

Diabetes is caused by defects in glucose metabolism, which is controlled by pancreatic α- and β-islet cells that secrete glucagon or insulin, respectively (Ashcroft & Rorsman, 2012; Talchai et al., 2012). Type 1 diabetes (T1D) is characterized by loss of β-cells through autoimmune-mediated destruction and cellular senescence (Thompson et al., 2019) and is insulin-sensitive, whereas type 2 diabetes (T2D) is characterized by failure of tissues such as muscle and fat cells to absorb blood glucose in response to insulin, but also involves senescence and progressive loss of β-cells (Kong et al., 2016; Aguayo-Mazzuccato et al., 2019).

To determine the mechanism underlying high serum glucose in RbΔK7 knock-in mice, hyperglycemic animals were injected with insulin. This led to a rapid decrease in blood glucose, indicating insulin responsiveness and type 1-like diabetes (Fig 5A). RbΔK7 mice were also unable to clear blood glucose as efficiently as control mice following exogenous glucose injection, and this deficiency became more severe with age (Fig 5B), suggesting a progressive degeneration of insulin-producing pancreatic β-cells. The primary defect was not in insulin secretion per se as revealed by Glucose-Stimulated Insulin Secretion (GSIS) assays of young RbΔK7 mice versus control littersmates (Fig 5C). Instead, pancreatic islets progressively deteriorated, becoming ~3 fold smaller in 1-year-old RbΔK7 mice compared to control littersmates (Fig 5D, left; center; images below and Fig 5E). RbΔK7 islets were not only smaller but also disorganized, and insulin-positive β-cells were larger with aberrant morphology. Length of RbΔK7 insulin+ β-cells was on average 1.73-fold greater than control cells (Fig 5D, right). In addition, whereas control wild-type islets stained relatively uniformly for insulin, pancreatic β-cells from diabetic RbΔK7 mice exhibited a mixture of intensely and weakly stained insulin+ β-cells (Fig 5D, left). Finally, fasting circulating insulin levels in 6- to 10-month-old diabetic RbΔK7 mice (17 mM average blood glucose compared with 3.2 mM in control mice, n = 8 each, P = 0.0001) was 1.5 fold lower than in control wild-type littersmates (0.13 vs. 0.2 ng/ml; P = 0.037, one-sided t-test; Fig 5E).

Analysis of cell death ruled out apoptosis as the cause of pancreatic islet degeneration and reduced islet mass; there were very rare TUNEL+ apoptotic β-cells in control islets and even less in RbΔK7 mice (Fig 5F). β-cells expand during embryogenesis and neonatal growth through proliferation of progenitor cells, and in adults through duplication of pre-existing β-cells (Dor et al., 2004; Nir et al., 2007; Teta et al., 2007; Smukler et al., 2011). Staining for phospho-Serine 800/804-pRb, a site that is substituted to alanine in RbΔK7 mice, revealed positive β-cells in control but not, as expected, knock-in islets, indicating active regulation of pRb phosphorylation in dividing pancreatic β-cells (Fig 5F). BrdU incorporation and phospho-histone H3 staining showed significant reduction in cell division in pancreatic RbΔK7 β-cells at 2–3 weeks of age compared to control (Fig 5F and G). Decreased level of histone H3K36 trimethylation (H3K36me3) coincides with short life span (Pu et al., 2015) and reduced homologous recombination (HR) repair (Pai et al., 2017). Consistent with this, H3K36me3 expression was significantly reduced in RbΔK7 islets compared to control littersmates (Fig 5F and G).

To determine whether mature RbΔK7 β-cells could be stimulated to re-enter the cell cycle in vivo, BrdU incorporation analysis or ki67 staining was performed during pregnancy, which triggers β-cell proliferation (Rieck & Kaestner, 2010), or after injecting adult mice with...
Figure 3. RbΔK7 MEFs exhibit enhanced resistance to CDK2 inhibitors and spontaneous senescence in vitro.

A Effects of selected kinase inhibitors with differential effects on RbΔK7 vs. control MEFs from initial kinome screens. Average proliferation rate of untreated MEFs is shown on the right (white column). Arrows point to pan-CDK- and CDK2/7/9-inhibitors. Bars represent mean ± SD, n = 3 independent MEFs.

B Increased resistance of RbΔK7 vs. control MEFs to CDK2 inhibition (P = 0.008 by two-tailed unpaired Student’s t-test). Effect of a pan CDK inhibitor was near significance (P = 0.07). Bars represent mean ± SD, n = 3 independent MEFs.

C Left, representative flow cytometric analysis with 7-AAD and AnnexinV, showing a significant decrease in apoptosis in RbΔK7 MEFs relative to control. Right, significant differences in average percentage of live (7-AAD+AnnexinV-) vs. apoptotic (7-AAD+AnnexinV+) cells. Bars represent mean ± SD; P value calculated by two-tailed unpaired Student’s t-test; n = 3 independent MEFs.

D Increased G1 phase in RbΔK7 MEFs. Mean ± SD of propidium iodide flow cytometry analysis showing a significant increase in G1 and decrease in S phase in synchronized RbΔK7 MEFs relative to control (n = 3 independent MEFs).

E Representative bright-field images of multi-passage MEFs showing immortalized control cells but large senescing RbΔK7 cells (arrows, original magnification: 200×; n = 4–5).

F Representative images showing increased senescence in RbΔK7 MEFs (arrows) determined by senescence-associated β-galactosidase assay (SA-βGAL); original magnification: 200×; P = 0.0395 by two-tailed unpaired Student’s t-test, n = 4 technical replicates (see text).
Exendin-4, a glucagon-like peptide-1, which stimulates glucose-dependent insulin secretion and \(\beta\)-cell proliferation (Xu et al., 1999). In both cases, \(\beta\)-cells from control wild-type mice re-entered the cell cycle, whereas \(\beta\)-cells from \(Rb^{\Delta K7}\) mice failed to reduplicate (Figs 5H and EV2A). Thus, the primary defect in \(Rb^{\Delta K7}\) islets can be attributed to a reduced capacity of pre-existing, differentiated \(\beta\)-cells to...
re-enter the cell cycle and regenerate in response to mitogenic sig-
als, leading to features of both TID (Thompson et al, 2019) and
t2D (Kong et al, 2016). These results demonstrate a critical role for
pRb phosphorylation during the transition of β-cells from a quies-
cent/differentiated state back into the cell cycle (G0-to-G1) in
response to mitogens in vivo.

Pancreatic β-cells in RbK7 mice exhibit increased DDR and
senescence

To examine the fate of pancreatic β-cells in RbK7 mice, we con-
goled global gene expression profiling on isolated pancreatic
islets. RbK7 islets are extremely fragile; using reduced collagenase
concentrations and pooled islets from several RbK7 mice, we were
nonetheless able to extract sufficient amount of RNA for dual-color
agilent microarray analysis from 8 RbK7 and 4 control mice in two
independent experiments: 1 (10 weeks of age) and 2 (4 weeks). 241
genes were differentially (> 2 fold) and significantly (P < 0.05)
between RbK7 and control islets (228 up; 13 down; Table EV1).

Most down-regulated in RbK7 islets were the islet regeneration
factors Reg3b and Reg3g (Xiong et al, 2011). Among upregulated
genes were Rad51 and Brca2, involved in DDR; GINS complex
subunit 2 and MCM-2, MCM-4, and MCM-5, involved in initiation
of DNA replication, DNA replication licensing, and progression of the
DNA replication fork; and aldehyde dehydrogenase family 1 (A1
and A3), TGFβ2, TGFβ3, and SOX9. Induction of some of these
genes is likely in response to DNA replication stress (Byun et al,
2005; Drissi et al, 2015) and “transcriptional noise”, observed in
aging pancreas (Enge et al, 2017), as well as compensatory mecha-
nisms to diminished β-cell proliferation in RbK7 islets. Indeed,
SOX9, which is required for bi- and multi-potent pancreatic progeni-
tors, initiation of endocrine differentiation during embryogenesis
and maintenance of pancreatic ductal identity in adults (Seymour,
2014), was highly expressed as revealed by immuno-staining in duc-
tal epithelial but not islet cells in adult RbK7 pancreas relative to
control (Fig 5I; Appendix Fig S5A). MCM4, a DNA replication
licensing factor, and PDX1, a transcription factor required for β-cell
maturation, were also elevated in pancreatic RbK7 islets (Fig 5I;
Appendix Fig S5B).

In contrast to E2F1−/− mice (Annicotte et al, 2009) but in accor-
dance with normal insulin secretion in young RbK7 mice (Fig 5C),
no significant difference in expression of Kir6-2, an inward-rectifier
potassium ion channel, was observed by microarray profiling or
immunohistochemistry analysis between RbK7 and control islets
(Appendix Fig S5C). There was also no significant difference in
expression of p16INK4a or its transcriptional regulator, Enhancer
of zeste homologue 2 (Ezh2; Table EV1).

To identify signaling pathways that are deregulated in RbK7
islets, we performed Gene Set Enrichment Analysis (GSEA) with the
entire set of differentially regulated genes. Relative to control, RbK7
islets exhibited elevated expression of pathways implicated in
integrin/extra-cellular matrix, adaptive immune response, wound
healing, cell motility, migration, cytokine–cytokine receptor interac-
tion, and cell adhesion (Fig 6A). Induction of similar pathways was
observed by gProfiler using only the 241 genes that were differenti-
tially expressed over 2 fold (P < 0.05) in RbK7 versus control islets
(Fig 6B). These pathways represent hallmarks of senescence-
associated secretory phenotype (SASP) in which senescent cells
secrete inflammatory cytokines, other immune modulators and
extracellular matrix-degrading enzymes that impact both cancer and
aging (Campisi, 2005; Coppe et al, 2008; Kuilman et al, 2008; Kirk-
land & Tchkonia, 2017).

In senescing mouse epithelial cells, SASP includes 37 proteins,
14 of which are regulated at the mRNA level (Coppe et al, 2010). Of
these 37 SASP genes, 24 were present on our microarray chip
including 6 of the 14 transcriptionally regulated mRNAs. Impor-
tantly, the majority of pathways that were significantly enriched in
RbK7 islets were connected to 19 of the 24 SASP genes (Fisher’s
exact test P = 0.026; connections are marked by gray lines in Fig 6A
and B). Moreover, of the 6 transcriptionally regulated SASP genes, 5
(Mmp3, Pgf/Cxcl-4, Cxcl-1, Ccl-2, and Ccl-11) were upregulated in
RbK7 islets (Fig 6C). Four additional SASP genes (Vcam, Selp,
Lgfbp3, and Csf2rb), which are not transcriptionally regulated in ep-
thelial SASP, as well as Cxcl12, were also upregulated in RbK7
islets. To corroborate the SASP phenotype, we performed immuno-
fluorescent staining using antibodies for IL-6 and MMP-2, hallmarks
of SASP that are also induced in mouse models of T1D and T2D
(Aguayo-Mazzucato et al, 2019; Thompson et al, 2019), as well as
for Cxcl12, which is transcriptionally induced in RbK7 islets
(Fig 6C). In contrast to control wild-type mice, many RbK7 islet
cells strongly expressed IL-6, MMP2, and Cxcl12, demonstrating
bona fide SASP (Figs 6D and EV2B).

Induction of SASP in RbK7 islets was suggestive of DDR, which
activates Ataxia telangiectasia mutated (ATM) and ATR (ATM and
Rad3-related) (Ciccia & Elledge, 2010; Schumacher et al, 2021).

Figure 4. RbK7 adult mice display signs of premature aging and diabetes.

A Reduced weight (in grams) of adult RbK7 (n = 11) vs control littermate (n = 17) males. Results are presented as mean ± SD; P values calculated by two-tailed
unpaired student’s t-test.
B-D Insulin in RbK7 males: (B) number of litters from breeding of homozygous RbK7 (n = 4) or control wild-type (n = 5) males with control wild-type females. Bars
represent mean ± SD; P values calculated by two-tailed unpaired Student’s t-test. (C) Representative images of testes from two RbK7 mice compared with a
control littermate. (D) Sections through seminiferous tubules. Arrows point to mature spermatids in control testses that are lacking in RbK7 adult testes. Scale bar,
20 μm.
E Kyphosis in RbK7 mice. Top, images of an eight-month-old RbK7 mouse with kyphosis (right) and control wild-type littermate (left). Bottom, radiographs of these
mice under anesthesia showing abnormal cervical curvature (arrows) in the RbK7 knock-in mouse.
F-I Delayed hair re-growth in eight-month-old RbK7 mice two weeks post-hair plucking (f). (G) Abnormal skin histology in RbK7 mice relative to control. FCL,
subcutaneous fat cell layer, T, telogen; A, anagen. (H and I) Representative immunostaining for keratin 14 and PCNA showing reduced expression in RbK7 hair
follicles vs. controls. Scale bar, 50 μm.
J Hyperglycemia in RbK7 but not RbX4 mice. Blood glucose in 10- to 12-month-old fasting RbK7 (left, n = 11) or 18-month-old fasting RbX4 (right, n = 15) mice
versus control littersmates (n = 6 and n = 8, respectively). Mean ± SD; P values by two-tailed unpaired student’s t-test.
Figure 5.
Consistent with this, the ATM substrate, histone H2AX, was robustly phosphorylated (γ-H2A) in Rb<sup>AK7</sup> β-cells compared with control littermates (Fig 6E). Consistent with senescence, double staining for γH2A and BrdU revealed that the γH2A<sup>−</sup> β-cells were non-proliferating (i.e., BrdU-negative; Appendix Fig S6A).

A pan-anti-phospho-ATM/ATR-substrate antibody, which detects phosphorylated ATM/ATR targets, revealed punctate staining in control cell nuclei in Rb<sup>AK7</sup> mice compared with control littermates (n = 5-6 each per group); Scale bar, 100 μm.

Figure 6. Diabetes in Rb<sup>AK7</sup> mice is associated with failure of pancreatic β-cells to re-enter the cell cycle.

A Insulin tolerance test (ITT) demonstrating that hyperglycemic Rb<sup>AK7</sup> mice are insulin-sensitive. Blood glucose in fasting Rb<sup>AK7</sup> mice (n = 6) vs. control littermates (n = 6) following insulin injection. Error bars represent SD.

B Glucose tolerance test (GTT) demonstrating progressive defect in clearance of blood glucose in Rb<sup>AK7</sup> mice. 6-7 week (left) or 6-month (right) old fasting Rb<sup>AK7</sup> mice and control littermates (n = 5-6 each per group) were injected with glucose, and blood glucose was determined at indicated intervals. Error bars represent SD.

C Glucose-stimulated insulin secretion (GSIS) analysis demonstrating near-normal insulin secretion in response to glucose injection in young Rb<sup>AK7</sup> mice. Serum insulin levels in 5- to 6-week-old fasting Rb<sup>AK7</sup> vs. control mice (n = 4 each) after glucose injection determined by ELISA. P values were calculated by two-tailed Student’s t-test. Bars represent mean ± SD.

D Rb<sup>AK7</sup> mice exhibit a progressive loss of pancreatic islet mass. Left to right, representative images of pancreatic islets of 1-year-old Rb<sup>AK7</sup> vs. control mice stained for insulin (green). Note the enlarged, irregular shape, and reduced number of pancreatic insulin<sup>−</sup> cells in Rb<sup>AK7</sup> islets. Ratio of β-cell area to pancreatic area in 1-month and 1-year-old Rb<sup>AK7</sup> vs. control mice determined by immunostaining for insulin followed by ImageJ analysis; relative length of pancreatic insulin<sup>−</sup> β-cells in 9-month-old Rb<sup>AK7</sup> islets (n = 5) vs. control (n = 4) using > 8 determination per biological replicate, normalized for control length. P values calculated by two-tailed Student’s t-test. Scale bar, 100 μm.

E Reduced levels of circulating insulin in fasting 6- to 10-month-old diabetic Rb<sup>AK7</sup> mice compared with control wild-type littermates (0.13 vs. 0.2 ng/ml; n = 8 for each group). P value was calculated by one-tailed Student’s t-test. Bars represent mean ± SE. Average levels of blood glucose are indicated (17 mM in Rb<sup>AK7</sup> compared with 3.2 in control mice; P = 0.0001 by two-tailed Student’s t-test).

F Loss of pancreatic islet area in Rb<sup>AK7</sup> mice correlates with reduced proliferation and H3K36me3 histone marks, but not with increased cell death. TUNEL analysis showing apoptosis in 10-day-old Rb<sup>AK7</sup> islets is even lower than in control littermates. Arrow points to a rare apoptotic cell in control islet. Serum800/804 phosphorylation of pRb in β-cells from 10-day-old control but not Rb<sup>AK7</sup> islets with a Ser-to-Asa substitution at this site. BrdU incorporation and PH3 staining showing reduced 5-phase and mitotic cells, respectively, in 2- and 3-week-old Rb<sup>AK7</sup> vs. control islets. Reduced H3K36me3 staining in pancreatic islets from 3-week old Rb<sup>AK7</sup> mice compared to control littermates (n ≥ 3 for each experiment); Scale bar, 100 μm.

G Quantification of BrdU incorporation, PH3 and H3K36me3 staining shown in panel (F). Mean ± SD, P values calculated by two-tailed Student’s t-test.

H Reduced ability of Rb<sup>AK7</sup> β-cell to re-enter the cell cycle in response to pregnancy (top) or mitogenic (bottom) signals. Top, cell proliferation in P<sub>14</sub>5 pregnant Rb<sup>AK7</sup> islet β-cell vs. control was determined by double immunostaining with BrdU and insulin (left); quantification (right). Results were normalized for the number of BrdU/Insulin-positive cells in control islets. An independent experiment using Ki67 to quantify cell cycle re-entry is shown in Fig EV2A. Bottom, cell proliferation in Rb<sup>AK7</sup> β-cell vs. control islets following Exendin4 treatment was determined by immunostaining for Ki67; results were normalized for the number of positive cells in control islets. Mean ± SD, P values by two-tailed Student’s t-test. Scale bar, 100 μm.

I Representative immunostaining showing induction of PDX1 (islet), SOX9 (pancreatic epithelium), and MCM4 (islet) expression in Rb<sup>AK7</sup> vs. control mice. See also Appendix Fig S5. Scale bar, 100 μm.

Vitamin C attenuates diabetes in Rb<sup>AK7</sup> mice

The aforementioned results suggest that constitutive expression of pRb<sub>AK7</sub> induces an irreversible terminal differentiation state, which, upon mitogenic signals, elicits DDR (γ-H2A) and senescence (SASP markers) of pancreatic Rb<sup>AK7</sup> β-cells. We therefore asked whether the epigenetic modulator, vitamin C, which facilitates induction of pluripotent stem cells (iPS) from adult cells, prevents p53-induced replicative senescence, and exerts anti-aging effects (Kim et al, 2008; Esteban et al, 2010), would ameliorate this defect.

To determine whether vitamin C would prevent diabetes when given before β-cells permanently exit the cell cycle, heterozygote Rb<sup>K7/+</sup> breeders and their Rb<sup>−/−</sup> and Rb<sup>AK7</sup> progenies were continuously fed on vitamin C-supplemented diet (1%; Fig 6F). Under these conditions, Rb<sup>AK7</sup> mice fed on regular chow exhibited age-dependent diabetes (Fig 6G, left). Remarkably and in stark contrast, Rb<sup>AK7</sup> mice exposed in utero and thereafter to vitamin C diet maintained near-normal glucose level (n = 8 each; P < 0.0001 at 9 months by ANOVA). This was confirmed with a larger cohort, showing a dramatic reduction in serum glucose in ~9-month Rb<sup>AK7</sup> mice continuously fed on vitamin C diet compared with same age Rb<sup>AK7</sup> mice fed on regular food (four groups, n ≥ 30 per group; P < 0.0001 by ANOVA; Fig 6G, right). Notably, no therapeutic benefits were observed when weaned Rb<sup>AK7</sup> mice, in which pancreatic β-cells are already post-mitotic, were fed on vitamin C-rich diet, indicating that vitamin C prevents but does not reverse diabetes.

One mechanism by which vitamin C diet could rescue diabetes in Rb<sup>AK7</sup> mice could involve the induction of phosphorylation of upstream sites such as Thr350, which are completely blocked in Rb<sup>AK7</sup> thymocytes (Fig 2E). To test for this, we immunoblotted lysates from Rb<sup>AK7</sup> and control thymocytes fed on normal vs. vitamin C diet and found that Thr350-pRb<sup>AK7</sup> remained unphosphorylated under both conditions (Appendix Fig S6F). This suggests vitamin C does not attenuate diabetes by inactivating pRb<sup>AK7</sup> through phosphorylation of upstream sites. Alternatively, vitamin C could prevent diabetes through its activity as a cofactor for Fe(II) 2-oxoglutarate dioxygenases such as Ten-Eleven Translocation (TET) enzymes that demethylate DNA by converting 5-methyl-cytosine (5mC; repressive mark) to 5-hydroxymethylcytosine (5hmC; transcriptional active mark) (Blaschke et al, 2013; Chen et al, 2013; Gimmeso et al, 2018). In accordance, pancreatic islets of vitamin C-fed Rb<sup>AK7</sup> and control mice exhibited increased 5hmC expression compared with mice fed on regular diet (Fig 6H, left), demonstrating DNA demethylation in vivo.

Importantly, vitamin C-fed Rb<sup>AK7</sup> mice had larger islets with an improved, normal-like morphology of insulin-positive β-cells
Figure 6.
Figure 6. Diabetes in Rb\textsuperscript{AKT7} mice involves a DDR and senescence/SASP and is attenuated by vitamin C diet.

A Induction of SASP pathways in Rb\textsuperscript{AKT7} islet cells. Pathway analysis by GSEA comparing Rb\textsuperscript{AKT7} and control islets using expression data for all genes. Nodes (circles) represent pathways significance at FDR ≤ 0.01 with normalized enrichment scores of > 1.4; red indicates pathways enriched in up-regulated genes in Rb\textsuperscript{AKT7} and blue indicates pathways enriched in control for array 1 (node center) and array 2 (node border). Green line width between nodes corresponds to the number of shared genes. Murine SASP genes (yellow triangle) are connected to enriched-pathways (yellow node center) by gray lines, line thickness indicates significance level (proportional to log(P value), P < 0.05). Red font corresponds to pathways in common with the gProfiler analysis in panel B.

B gProfiler analysis showing significantly enriched pathways (FDR < 0.05) using 228 genes that are significantly regulated in Rb\textsuperscript{AKT7} vs. control islets (>2 fold; P < 0.05; see panel A for details). In panels (A) and (B), murine SASP genes (yellow triangle) are connected to enriched pathways (yellow node center) by gray lines when the gene overlap size estimated by a Fisher's exact test is significant at P ≤ 0.05; line thickness is proportional to significance level. Red label font corresponds to pathways in common between the GSEA analysis (panel A) and the gProfiler analysis (panel B).

C Fold induction (log) of SASP genes in Rb\textsuperscript{AKT7} vs. control littermates. Genes that overlap between the GSEA and gProfiler are highlighted in green. Yellow indicates genes that are transcriptionally regulated in epithelial SASP. Cxcl12, chemokine (C-X-C motif) ligand 12, has not been previously implicated in SASP in epithelial cells or fibroblasts, but is induced in Rb\textsuperscript{AKT7} islets. Log ratio is the difference between the mean of Rb\textsuperscript{AKT7} samples and the mean of control samples of the log2 normalized values.

D Representative image of a pancreatic islet from a 9-month-old Rb\textsuperscript{AKT7} mouse stained for the SASP marker IL-6 (red) and insulin (green). DAPI was used to mark nuclei (blue). Arrows point to IL-6\textsuperscript{+} insulin\textsuperscript{-} pancreatic β-cells. Scale bar, 20 μm. See Fig EV2B for wild-type control, MMP-2, and CXCL12 staining.

E Representative immunostaining for the DDR marker γ-H2A in islets from 3-week-old Rb\textsuperscript{AKT7} and control mice (left); statistical analysis (right; n = 5 each). Arrows point to pancreatic γ-H2A-positive Rb\textsuperscript{AKT7} islet cells. Error bars represent SD, P value calculated by one-way ANOVA, Tukey's multiple comparisons test. Scale bar, 100 μm.

F A schematic outline for the vitamin C experiment in vivo. Rb\textsuperscript{AKT7} heterozygous mice fed on regular or vitamin C diet were intercrossed. Progenies were genotyped to identify Rb\textsuperscript{+/-} and Rb\textsuperscript{+/+} male mice, continuously maintained on the same diet, and monitored for blood glucose.

G Vitamin C-supplemental diet (1%) significantly delays diabetes in Rb\textsuperscript{AKT7} mice. Shown are two independent experiments with 8 mice per group at indicated time points (left) and 28 and 34 Rb\textsuperscript{AKT7} mice or 37 and 20 control littermates in each group fed on regular or vitamin C diet, respectively (right). Blood glucose was determined in fasting mice at indicated months (left) or at -9 months (right). Bars denote mean ± SD. Adjusted P values were calculated using one-way ANOVA, Tukey's multiple comparisons test.

H Vitamin C diet increases Shh\textsuperscript{+}C expression and improves β-cell morphology and islet size in Rb\textsuperscript{AKT7} mice. Scale bar, 100 μm.

I Vitamin C diet reduces the difference in γ-H2A\textsuperscript{+} β-cells in Rb\textsuperscript{AKT7} vs. control pancreatic islets relative to regular diet by 3.74 folds. Bars indicate mean ± SD. Adjusted P values by one-way ANOVA, Tukey's multiple comparisons test.

J Vitamin C diet increases pancreatic islet cell cycle re-entry during pregnancy of Rb\textsuperscript{AKT7} mice relative to control pregnant mice (no statistical difference, n.s) or relative to pregnant Rb\textsuperscript{AKT7} vs. control mice fed on regular diet (P < 0.0001) by 38 folds. Bars represent mean ± SD. Adjusted P values by one-way ANOVA, Tukey's multiple comparisons test.

Ser243/Thr246- or Ser605-phosphorylated pRb\textsuperscript{AKT7} is not bound to E2F1

To understand the mechanistic basis for its differential effect on different tissues, we next asked whether pRb\textsuperscript{AKT7} is constitutively bound to or dissociated from E2F1 when phosphorylated on upstream sites in proliferating cells (Fig 2E). Immuno-precipitation of pRb or pRb\textsuperscript{AKT7} from thymocytes using anti-pRb antibody followed by immunoblotting for E2F1 revealed strong interaction of both proteins with E2F1, confirming the functional activity of pRb\textsuperscript{AKT7} (Fig 7A, lanes 2–3, 11). Next, phosphorylated pRb species were immuno-precipitated from proliferating Rb\textsuperscript{AKT7} or control thymocytes using phospho-Ser243/Thr246-pRb or phospho-Ser605-pRb antibodies, followed by immunoblotting for E2F1. In contrast to anti-pRb antibodies, both pSer243/Thr246- and pSer605-pRb antibodies failed to co-precipitate E2F1 from either control or pRb\textsuperscript{AKT7} lysates (Fig 7A, lanes 7–10). Ser243/Thr246 and Ser605 phosphorylated pRb\textsuperscript{AKT7} are not bound to E2F1 likely because pRb\textsuperscript{AKT7} phosphorylation on these and other sites disrupts its interaction with E2F1, providing a plausible explanation for the apparent defect in G0-to-G1 transition in pancreatic and other differentiated cells, yet near-normal proliferation and development of most tissues in Rb\textsuperscript{AKT7} embryos and newborn mice (Discussion).

Rb\textsuperscript{AK11} blocks proliferation in vivo and induces senescence in vitro that can be attenuated by vitamin C

To determine the effect of Thr246 and Ser605 within pRb\textsuperscript{AK11} in vitro and in vivo, we used Rb\textsuperscript{AK11} (Jiang & Zacksenhaus, 2002), with Ser/Thr-to-Ala substitutions in (i) all the 7 sites in exon 23; (ii) T350 and S601, which are not phosphorylated in Rb\textsuperscript{AK7} mice; and (iii) T246 and S605, which are still phosphorylated in proliferating Rb\textsuperscript{AK7} cells. Transduction of Rb\textsuperscript{AK11} with a retrovirus vector encoding Rb\textsuperscript{AK11}, led to a strong inhibition of cell proliferation and induction of senescence as determined by SA-BGAL staining (Fig 7B).

Next, we transfected retinal cells from P0 neonates or from E15.5 explants with GFP-tagged Rb-WT or Rb\textsuperscript{AK11} vectors and determined the effect on cell proliferation two days later (Fig 7C). Over-expression of Rb\textsuperscript{AK11} had no effect. In contrast, over-expression of Rb\textsuperscript{AK11} significantly reduced BrdU incorporation both in vivo and ex vivo to the same extent as over-expression the CDK4/6 inhibitor p16\textsuperscript{INK4A} (Fig 7D and E). As a positive control, transduction of cyclin D1 induced cell cycle re-entry. Thus, while endogenous/physiological levels of Rb\textsuperscript{AK7} and over-expression of wild-type pRb are well tolerated in certain cell types, over-expression of Rb\textsuperscript{AK11} with
Discussion

Aging is instigated by diverse processes that promote cellular senescence and local inflammation, resulting in exhaustion of resident stem cells, tissue degeneration, and systemic organ failure (Rando & Chang, 2012; Lopez-Otin et al., 2013; Imai & Guarente, 2014; Johnson & Imai, 2018). Inhibition of pRB phosphorylation in response to increased expression of CDK inhibitors such as p16ink4a and p21cip1 is viewed as a major landmark in the onset of tissue degeneration and aging (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). Indeed, pRb is depicted at the center of biological processes that dictate whether a cell undergoes proliferation, quiescence, or differentiation on the one hand, or cellular senescence on the other hand (Lopez-Otin et al., 2013; Signer & Morrison, 2013; Gorgoulis et al., 2019; Amaya-Montoya et al., 2020). However, so far, no experimental evidence directly links pRb phosphorylation to the control of homeostasis and aging in vivo. Here, we show for the first time that inhibition of pRb phosphorylation in knock-in mice induces diabetes and other hallmarks of accelerated aging, thus establishing pRb phosphorylation as a central node that governs tissue regeneration and aging.

Using knock-in mice with Ser/Thr-to-Ala substitutions in conserved CDK sites, we show that blocking endogenous pRb phosphorylation on all seven C-terminal sites in exon 23 does not interfere with embryogenesis or early neonatal development in homozygous RbK7 mice. However, in accordance with its tumor suppressor activity, RbK7-derived MEFs show reduced cell proliferation and enhanced replicative senescence in culture. As RbK7 mice mature, they develop defects associated with reduced tissue regeneration: Males are infertile and both genders exhibit short telomeres, reduced hair regeneration, kyphosis, and diabetes. Diabetes in RbK7 mice is insulin-sensitive and associated with increased DDR and senescence, abnormal islet morphology, cellular disorganization, gradual loss of pancreatic β-cells, and reduced islet mass. Interestingly, PDG-1, which interferes with β-cell function and insulin secretion (Nasteska et al., 2021), is intensely expressed in adult pancreatic RbK7 β-cells. Although insulin secretion is not the primary defect in young RbK7 mice, high PDG-1 expression and senescence of pancreatic islet cells in older RbK7 mice point to a gradual defect not only in islet size and organization but also in β-cell function. Indeed, diabetic RbK7 mice exhibit reduced circulating insulin levels. We further show that pancreatic islet degeneration in these mice is due to failure of RbK7 β-cells to transition through the quiescent/G0-to-G1 phase and re-enter the cell cycle in response to distinct mitogenic signals (pregnancy; exendin4).

Remarkably, we found that the epigenetic regulator vitamin C, provided in diet before pancreatic β-cells exit the cell cycle, reduced the DDR, increased cell-cycle re-entry during pregnancy, and ameliorated diabetes in RbK7 mice. Vitamin C also attenuated senescence induced by over-expression of under-phosphorylatable RbAK1 allele in cell culture. We propose that constitutive expression of pRBK7 establishes a stable pancreatic β-cell differentiation that impedes re-entry into the cell cycle in response to mitogenic cues (Fig 7G). When encountering mitogens, pRBK7 in β-cells cannot be inactivated by phosphorylation, triggering a DDR that culminates in cellular senescence, SASP, and tissue aging. Vitamin C, in this view, preempts the permanent cell cycle exit imposed by pRBK7 and thereby the DDR, hypo-proliferation, and cellular senescence induced by mitogenic signals in RbK7 mice.

Tissue degeneration was exclusively observed in RbK7 mice, not in RbK4 mice. In both mouse strains, Thr350 phosphorylation is completely blocked in proliferating thymocytes. In contrast, Ser601 phosphorylation is completely suppressed in proliferating RbK7 thymocytes but slightly phosphorylated in RbK4 cells. Additional differences, yet to be elucidated, in phosphorylation status and activity of these phospho-pRB alleles likely account for the phenotypic differences in these two mouse strains. Interestingly, phosphorylation of Ser243/The246 and Ser605 (Ser249/The252 and Ser612 in humans) persists in proliferating RbK7 thymocytes. These sites are phosphorylated by CKD4 and CKD2 (Zarkowska & Mittnacht, 1997), as well as by other kinases in response to stress signals. Ser249/The252 can be phosphorylated by p38 stress-activated protein kinase (SAPK) (Gubern et al., 2016), whereas Ser612 by Chk1/2 (Inoue et al., 2007). We found no significant change in sensitivity of RbK7 MEFs to p38α plus p38β inhibitors, but additional analysis is needed to define the role of this kinase family in Ser249/The252 phosphorylation in RbK7 mice. pRb phosphorylation involves the docking of CyclinD-CDK4/6 to an alpha-Helix in its C terminus (downstream of exon 23) and mutation in this Helix blocks pRb phosphorylation (Topacio et al., 2019). Whether RbK7 substitutions affect interaction with CyclinD-CDK4/6 and which exact kinase (p38-SAPK; Chk1/2, others) phosphorylates pRBK7 on Ser243/The246 and Ser605 await further investigation.

Insulin−β-cells are larger in RbK7 compared to control mice, and increased cell size is observed in all pancreatic β-cells, not only in IL-6− or MMP-2− senescent cells, pointing to an intrinsic role for pRb phosphorylation in cell size control. Both CDK4/6 and p38-MAPK have been implicated in cell size homeostasis in vitro (Tan et al., 2021); RbK7 mice provide a powerful tool to analyze cell size regulation in vivo.

The diabetic phenotype in RbK7 mice resembles mouse models of some but not all upstream regulators of pRB phosphorylation. Thus, while CyclinD1 is essential for retinal and pregnancy-induced mammary alveolar development (Sicinski et al., 1995) and CyclinD3 is needed for lymphocyte development (Sicinski et al., 2003), CyclinD2 is uniquely required for pancreatic β-cell proliferation (Sicinski et al., 1996; Kushner et al., 2005). Likewise, whereas CDK2 is essential for meiosis but not mitosis (Berthet et al., 2003) and CDK6 is non-essential, CDK4 is distinctively required for pancreatic β-cell duplication (Rane et al., 1999). These results point to CyclinD2...
Figure 7.
and CDK4 as chief regulators of pRb phosphorylation in pancreatic β-cells. However, the CyclinD2-CDK4 complex controls G1-to-S progression, whereas our results implicate pRb phosphorylation in the transition of pancreatic β-cells from a quiescent/differentiated state (G0) back to cycle (G1). Further analysis is therefore needed to determine the stage (G0-to-G1 or G-to-S transition) in which pRb phosphorylation is critical for pancreatic β-cell regeneration, and the specific kinase that phosphorylates and inactivates pRb during β-cell reduplication.

The cell cycle re-entry defect and diabetes in RbΔK7 adult mice are in sharp contrast to the apparent normal proliferation and development of RbΔK7 embryos, suggesting that while certain adult quiescent/differentiated cells fail to phosphorylate and inactivate pRbΔK7, embryonic cells/stem cells can readily divide despite persistent pRbΔK7 expression. These differences may reflect the high CDK activity in embryonic stem cells as opposed to the low CDK activity in differentiated cells (Kareta et al., 2015), and/or the fact that in dividing cells, active pRb never establishes a stable, epigenetically repressed chromatin characteristic of differentiated cells.

We previously demonstrated that targeted deletion of Rb in islet progenitors leads to expansion of β-cell mass (Cai et al., 2013). Together with results presented herein, β-cells appear particularly sensitive to both the level of pRb and its phosphorylation status. Additional tissues including testis and skin exhibit specific defects in RbΔK7 mice. Other tissues in RbΔK7 mice may show regeneration defects only if directly challenged (e.g., by hepatectomy, muscle injury, or mammary epithelial transplantation) or if followed up for a longer period by suppressing the early onset of death from diabetes, for example by targeting the expression of an Rb-shRNA transgene to islet progenitors. Notably, tumors were not detected in 1-year-old RbΔK7 mice. This is in contrast to the paradoxical development of mammary tumors in MMTV-RbΔK7 transgenic mice at ~1.5 years of age (Jiang & Zacksenhaus, 2002), possibly as a result of inhibition of apoptosis or induction of pro-tumorigenic senescence/SASP by constitutively active pRbΔK. Genetic rescuing of the diabetic defect or sequential transplantation assays may reveal pro-tumorigenic effects of persistent pRbΔK7 expression on tumorigenesis.

Our observation that systemic inhibition of pRb phosphorylation leads to telomere attrition indicates that the impact of pRbΔK7 on tissue regeneration may be more complex than its negative regulation of cell proliferation. Telomere maintenance is crucial for aging; hyper-long telomeres extend life span in mice (Munoz-Lorente et al., 2019), while short telomeres predict mortality and aging-related diseases (Blackburn et al., 2015), including pancreatic β-cell destruction in late-stage T2D (Guo et al., 2011). Thus, the effect of pRbΔK7 on telomere length alone may suffice to trigger replicative stress, senescence, and aging. However, short telomeres were observed in pRbΔK mice, which do not exhibit any aging defect or diabetes, indicating that telomere erosion alone may not account for the homeostasis defects in pRbΔK7 mice. Loss of all Rb family members (Rb, p107, and p130) has previously been shown to increase telomere length (Garcia-Cao et al., 2002). Thus, both Rb family loss (Garcia-Cao et al., 2002) and pRb activation (this study) impact telomere length, highlighting the importance of this tumor suppressor in controlling chromosomal ends. We attributed short telomeres in RbΔK7 mice to its constitutive interaction with SUV4-20H1 and SUV4-20H2, which regulate telomere replication (Benetti et al., 2007). In addition, we previously demonstrated that pRb directly interacts with the RNA polymerase II CTD phosphatase SSU72 (St-Pierre et al., 2005). SSU72 has recently been shown to function as a telomere replication terminator (Escandell et al., 2019), suggesting another mechanism by which pRbΔK7 may control telomere maintenance.

Vitamin C is an epigenetic regulator that can reprogram the chromatin by demethylating the DNA via TET enzymes (Blaschke et al., 2013; Chen et al., 2013; Cimmino et al., 2018). Vitamin C diet was shown to increase survival in mice, which synthesizes this vitamin (Massei et al., 1984). Humans are completely dependent on external sources of vitamin C, and maintaining healthy vitamin C levels can
protect against age-related cognitive decline and Alzheimer’s disease (Harrison, 2012), mortality (Khw et al, 2001), T1D (Ceriello et al, 2009, 2013), and T2D (Cunningham, 1998; Afkhami-Ardekan & Shojaoddiny-Ardekan, 2007; Mason et al, 2019). The underlying mechanism by which vitamin C exerts its effects is likely complex, involving epigenetic reprogramming as well as other processes (oxidative stress, senescence, inflammation, etc.). RbΔK7 mice provide a novel platform to assess potential new combinations of pro-longevity supplements. Several agents such as senolytics that selectively eliminate senescent cells (Chang et al, 2016; Xu et al, 2018; Thompson et al, 2019), NAD+ precursors (Zhang et al, 2016), PI3K inhibitors (Johnson et al, 2013; Bitto et al, 2016), and aspirin (Pietrocola et al, 2018) have been demonstrated to reverse aging and/or extend life span in animal models. In particular, the senolitics ABT-199 and ABT-263, specific inhibitors BC-L2 protein family, were shown to clear senescent cells and ameliorate T1D- and T2D-like diseases in mice (Aguayo-Mazzucato et al, 2019; Thompson et al, 2019). Future experiments will determine whether vitamin C, which prevents senescence, would synergize with ABT-199, ABT-263, or other senolitics, which eliminate senescent cells, to cure diabetes and extend life span of RbΔK7 mice and other models of diabe-
tes and aging.

The complex effect of blocking pRb phosphorylation, uncovered here, should be taken into account whenever its activity is manipulated. CDK4/6 inhibitors, which block pRb phosphorylation, show efficacious clinical results for the treatment of RB proficient tumors such as ER+ breast cancer. Side effects associated with anti-CDK4/6 therapy are not severe, yet, high doses of palbociclib induce pancreatic β-cell failure in experimental young rats (Sacaan et al, 2017). Given our observation that vitamin C attenuates diabetes in RbΔK7 mice, it would be important to determine whether concurrent vitamin C diet would diminish such adverse effects in conjunction with high-dose anti-CDK4/6 cancer therapy.

In summary, our results demonstrate that complete phosphorylation of endogenous pRb is not required for normal embryogenesis but is crucial for telomere maintenance, male fertility, and regeneration of several tissues, particularly pancreatic β-cells. Thus, persistent expression of hypo-phosphorylated, active pRB blocks cancer cell proliferation but also suppresses normal tissue regeneration, leading to accelerated aging. We show that diabetes in RbΔK7 mice can be mitigated at least in part by vitamin C diet, suggesting that the deleterious effects of stress/aging-induced inhibition of pRB phosphorylation may be amenable to prophylactic treatment.

Materials and Methods

Mouse analysis

Mouse experiments were conducted with approval of the University Health Network (UHN) Animal Care Committee. Heterozygous RbK4/- and RbK7/- knock-in mice were generated by homologous recombination (Appendix) and identified initially by Southern blot hybridization and then genotyped by PCR of tail DNA. Homozygo-
gous RbΔK4 and RbΔK7 were generated by crossing heterozygote mice. Mice were fed on normal diet (Harlan Laboratories Teklad, 7912) or 1% vitamin C-supplemented pellet.

Telomere length analysis

Primary mouse splenocytes were isolated and activated with anti-CD3 antibody (BD Biosciences) as described (Snow et al, 2007). The following day, the cultures were treated with 0.1 μg/ml Colcemid (Roche) for 5–6 h, swollen in 0.075 M KCl hypotonic buffer, fixed in a 3:1 volume of methanol-acetic acid, and dropped onto slides. Quantitative fluorescence in situ hybridization (Q-FISH) of activated splenocytes was performed as described previously (Blasco et al, 1997; Zijlmans et al, 1997). A Cy3-labeled (CCCTAA)3 peptide nucleic acid (Applied Biosystems) was used as a probe. Chromosomes were counterstained with DAPI (4',6'-diamidino-2-phenylindole). Telomere fluorescence signal intensities were quantified using TFL-Telo Q-FISH software (version 2.1.04.1217; BC Cancer Agency; Steven S. Poon and Peter M. Lansdorp). Telomere signal intensities were compiled from at least 10 metaphases (1,600 telo-

Histology, pathology, TUNEL, and immunohistochemistry

Adult mice were immersed in 10% paraffin and indicated tissues were processed for pathological analysis. For timed-pregnancy, the morning of vaginal plug observation was considered E0.5. Embryos were fixed in fresh 4% paraformaldehyde in PBS at 4°C overnight, dehydrated, paraffin-embedded, and sectioned at 4μ. TUNEL analysis was performed as described (Jiang et al, 2000). Antibodies for IHC were used at 1:200 dilution: phospho-pRb-Ser807/811 anti-
bodies (Cell signaling, #9308), phospho-Rb-Ser612 (Affinity Bioreagents #OPA1-03981), RbpSer/Thr-249/252 (BioSource #44-584G), phospho-Rb-Ser773 (human Ser780; Cell Signaling), phospho-Rb-Ser788 (Cell Signaling, #9307), phospho-Rb-Ser795 (Cell Signaling, #9301), phospho-Rb-Ser605 (human Ser612; Cyclex, clone 3C11 Cat# CY-M1013, and ABR Affinity Bioreagents Cat # OPAL-03981), phospho-Rb-Ser601 (Cell signaling; #2181), phospho(Ser/Thr)-ATM/ATR substrate (Cell Signaling #2851), phospho-ATM (Rock-
lane, #200-301-500), mouse E2F1 (Invitrogen #32-1400), PDX1 (Millipore #AB3503), SOX9 (SantaCruz, sc-166505), p53BP1 (Cell Signaling #4937), phospho-Histone H2A.X(Ser139) (Cell Signaling #9718), phospho-p53(Ser15) (Cell Signaling #9284), H3K9m3e3 (Milli-
pore #07-442), PH3(Ser10) (Cell Signaling #9701), H3P(Ser10) (Cell Signaling #9701), HP1g (Abcam #ab10480), BrdU (Abcam #ab6326), monoclonal PCNA (Sigma), CyclinD1 (MedCorp. #RB-9041), Ki67 (Biocare medical #CRM325); MCM4 (Cell signaling #12973), Kir6.2 (Abcam; ab79171 IL-6 Novus Biological (clone 1270; # NBP2-44953, 1:80 dilution), CXCL12/SDF-1 (clone 79018; # MAB350-SP; 1:120); Insulin (Sigma, #I2018) was used at 1:1,000 dilution.

Kinase inhibitor screens

Robotic screens were performed at the SMART facility, Toronto, using a Biomek FX liquid handler equipped with a pintool for auto-
mated compound dispensing. Passage 4 MEFs were plated at 1,000 cells per well in 384 plates. Compounds, resuspended in DMSO as
1mM stock solutions, were added at a final concentration of 4uM. As a reference for 100% activity, each plate included 32 wells with cells treated with vehicle only; background was measured with media in the absence of cells. Cell viability for screens was determined by alamar blue and confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays in 96-well plates, read at 570 nm.

**Blood glucose and insulin analysis**

Glucose tolerance, insulin tolerance and glucose-stimulated insulin secretion tests were performed on overnight-fasted (14–16 h) RbΔK7, RbΔK4 or control wild-type male mice exactly as described (Liadis et al, 2005; Choi et al, 2010; Cai et al, 2013, 2014). For GTT and GSIS, mice were injected with a single dose of 1g glucose/kg bodyweight. For the ITT, mice received insulin at a dose of 1.5mL/kg body weight.

**Retrovirus preparation, infection, and β-galactosidase assay**

1.5 × 10⁶ to 2.0 × 10⁶ phoenix cells were plated on 10 cm plates containing 10 ml of DMEM (+ 10% FBS and 1× PEST) and incubated overnight at 37°C and 5% CO₂. The following day, 3 plasmids (pHelper, pSVS-g, Rb pMXIE-empty, or pMXIE-RbΔK7) were mixed with TransIT-LT1 in OPTI-MEM, incubated at room temperature for 30 min and added to phoenix cells. The next day, media was replaced with 10 ml of virus-harvesting media (DMEM + 30% FBS + 1× PEST). After 24 h, medium was collected in polypropylene tubes. 1.5 × 10⁵ tumor epithelial cells seeded onto 10 cm plates in 10 ml DMEM with 10% FBS and 1× PEST were cultured overnight and then infected with pMXIE-empty or pMXIE-RbΔK7 virus-containing media (5 ml virus + 6 ml DMEM + polybrene, final conc. 8 ng/ml). For β-galactosidase assay, 20,000 GFP-positive cells were plated onto 6-well plates containing DMEM (+ 10% FBS and 1× PEST) and allowed to grow for 4 days. After 4 days, media was removed, and cells were washed twice with PBS, fixed in 3% formaldehyde at room temperature for 4 min, and washed with PBS 3 times. Senescence-associated β-galactosidase assay solution was added to the plates and incubated at 37°C (without CO₂) overnight in the dark.

**Retrovirus injection and retinal analysis**

RbΔK7 or littermate mice were briefly anesthetized on ice and (1–2 µl) plasmid DNA (1.5 µg/µl) was injected into the sub-retinal space through a small corneal incision as previously described (Chen et al, 2009). With tweezer electrodes placed over the eyes, the mice were immediately electroporated using a NEPA21 system (NEPAGENE, Chiba, Japan) with the following settings: voltage, 80 V; pulse length, 50 ms; pulse interval, 950 ms; number of pulses: 5. The eyes were harvested 2 days later (P2). For in vitro electroporation, the retinas were dissected from E15.5 mouse embryos and electroporated in a drop of plasmid DNA, placing the electrodes near the tissue. The embryonic retinas were cultured ex vivo on filters for 2 days. Prior to collecting all the retinas, mice were injected with BrdU (50 mg/kg) or culture media were supplemented with BrdU (1 µg/ml) for 2 h. Retinal tissue was fixed in 4% PFA overnight, dehydrated in 30% sucrose, frozen, cryo-sectioned, and immunostained with the following antibodies: BrdU (1:500; Abcam Ab1893-125), Ki67 (1:300; BD Pharmingen 550609), PH3 (1:500; Upstate 06-570), GFP (1:1,000; Abcam Ab 6662) and counterstained with DAPI (200 ng/ml; Sigma), as described (Pacal & Bremner, 2012). Three E16.5 and P0 control/ RbΔK7 retinas from three different litters were quantified and at least three different electroporated retinas. At least 400 GFP+ cells were scored for each indicated electroporated plasmid. Plasmids either contained GFP (retroviral control pMXIE-IRE5-GFP and pMXIE-IRE5-wt (mouse) Rb and RbΔK7 vectors) or they were mixed with a control pMXIE-IRE5-GFP vector in 4:1 ratio (pECE-p16INK4a, pECE-Ccnd1). Statistical analysis was based on one-way ANOVA followed by Tukey’s multiple comparison tests.

**Pancreatic islet preparation and microarray analysis**

Islets were isolated from mice as previously described (Liadis et al, 2005; Choi et al, 2010), using 3 ml of 3 mg/ml collagenase (Sigma-Aldrich) injected into the common bile duct for pancreatic digestion, and islets were picked up under dissecting microscope in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, and frozen immediately. RNA was extracted using NucleoSpin® RNA II, Macherey-Nagel and underwent PC and microarray analysis by Miltenyi Biotec. Briefly, 15 ng of each total RNA sample was used to produce Cy3(control samples)- and Cy5(experimental samples)-labeled cRNA, the RNA samples were amplified and labeled using Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies). Next, 300 ng of the corresponding Cy3- and Cy5-labeled fragmented cRNA were combined and hybridized overnight (65°C) to Agilent Whole Mouse Genome Oligo Microarrays 8 × 60 K. Fluorescence signals of the hybridized Agilent Oligo Microarrays were detected using Agilent’s DNA microarray scanner (Agilent Technologies).

**Bioinformatics analysis**

Gene expression data were analyzed using GSEA with parameters set to 2,000 gene-set permutations and gene-sets size between 15 and 500. Genes were ranked using the normalized Cy5/Cy3 log10 ratios between RbΔK7 (KI; Cy5) and wild-type control (WT; Cy3). The gene-sets included in the GSEA analyses were obtained from KEGG, MsigDB-c2, NCi, Biocarta, IOB, Netpath, HumanCyc, Reactome and the Gene Ontology (GO) databases, updated September 2011 (http://baderlab.org/GeneSets). GSEA results were visualized by enrichment map (Merico et al, 2010) (Cytoscape, version 1.2 of Enrichment Map software) from array 1 and array 2 using enriched gene sets with a nominal P-value < 0.001, FDR < 1% and overlap coefficient set of 0.5. Gene set enrichment analysis was also performed using a list of 228 genes that were up-regulated more than 2 fold and with a P-value equal or less than 0.05 (g:Profiler software; Reimand et al, 2011), using the options significant only, ordered query and Generic Enrichment Map output type). An enrichment map was generated using enriched gene sets with a P-value < 0.05 and overlap coefficient set of 0.5. The “log ratio” represents the difference in expression between RbAK7 and control gene expression. Thus, the array data were first normalized and then log2 transformed. Log ratio is the difference between the mean of RbAK7 samples and the mean of control samples of the log2 normalized values for any given gene.
Data quantification

All studies were performed in three or more biological replicates as indicated. Results are presented as mean ± standard deviation (SD) and statistical tests (two-tailed Mann–Whitney U-test, two-tailed unpaired Student’s t-test, or one-way ANOVA) used to generate error bars and P values are stated in each panel.

Additional Methods are available in the Appendix.

Data availability

Microarray data deposited in NCBI GEO Datasets: accession GSE180507.

Expanded View for this article is available online.

Acknowledgements

This work was supported by the Ontario Institute for Cancer Research through funding provided by the Government of Ontario and US NIH grants P41 RR031228 and GM103504 to GDB, by Canadian Institute for Health Research and the Krembil Foundation to RB, by Canadian Institute for Health Research and GM and the Cancer Research Society and Canadian Institute for Health Research to EZ.

Author contributions

Zhe Jiang: Conceptualization; Formal analysis; Supervision; Validation; Investigation; Visualization; Methodology; Project administration; Co-corresponding author. Huiqin Li: Data curation; Methodology. Stephanie A Schroer: Data curation; Methodology. Veronique Voisin: Software; Investigation. Youngjun Ju: Investigation. Marek Pacal: Investigation. Natalie Erdmann: Investigation. Wei Shi: Investigation. Philip E D Chung: Investigation. Tao Deng: Investigation. Nien-jung Chen: Investigation. Giovanni Ciavarra: Investigation. Alessandro Datti: Supervision; Investigation. Tak W Mak: Supervision. Lea Harrington: Supervision. Frederick A Dick: Resources; Supervision. Gary D Bader: Resources; Supervision. Rod Bremer: Supervision. Minna Woo: Supervision. Eldad Zackshenhaus: Conceptualization; Formal analysis; Supervision; Funding acquisition; Investigation; Project administration.

In addition to the CRediT author contributions listed above, the contributions in detail are:

ZJ and EZ conceived the project and wrote the manuscript with input from all the authors; ZJ, HL, SAS, VW, YJ, MP, NE, WS, PEDC, TD, N-JC, GC, and EZ performed experiments/bioinformatic analysis and made the figures; ZJ, AD, TWM, LH, FAD, GDB, RB, MW, and EZ supervised the analysis and obtained research fund.

Disclosure statement and competing interests

The authors declare that they have no conflict of interest.

References

Afkhami-Ardekani M, Shoaoddiny-Ardekani A (2007) Effect of vitamin C on blood glucose, serum lipids & serum insulin in type 2 diabetes patients. Indian J Med Res 126: 471 – 474

Aguayo-Mazzucato C, van Haaren M, Mruk M, Lee Jr TB, Crawford C, Hollister-Lock J, Sullivan BA, Johnson JW, Ebrahimi A, Dreyfuss JM et al (2017) beta cell aging markers have heterogeneous distribution and are induced by insulin resistance. Cell Metab 25: 898 – 910

Aguayo-Mazzucato C, Andie J, Lee Jr TB, Midha A, Talemal L, Chipashvili V, Hollister-Lock J, van Deursen J, Weir G, Bonner-Weir S (2019) Acceleration of beta cell aging determines diabetes and senolysis improves disease outcomes. Cell Metab 30: 129 – 142

Ait-Si-Ali S, Guasconi V, Fritsch L, Yahi H, Sekhri R, Naguibneva I, Robin P, Cabon F, Polesskaya A, Harel-Bellan A (2004) A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. EMBO J 23: 605 – 615

Amaya-Montoya M, Perez-Londono A, Guatibonza-Garcia V, Vargas-Villanueva A, Mendivil CO (2020) Cellular senescence as a therapeutic target for age-related diseases: a review. Adv Ther 37: 1407 – 1424

Anniccotte JS, Blanchet E, Chavey C, Iankova I, Costes S, Assou S, Teyssier J, Dalle S, Sardet C, Fajas L (2009) The CDK4-pRB-E2F1 pathway controls insulin secretion. Nat Cell Biol 11: 1017 – 1023

Ashcroft FM, Rorsman P (2012) Diabetes mellitus and the beta cell: the last ten years. Cell 148: 1160 – 1171

Benetti R, Gonzalo S, Jaco I, Schotta G, Klatt P, Jenuwein T, Blasco MA (2007) Suv4–20h deficiency results in telomere elongation and derepression of telomere recombination. J Cell Biol 178: 925 – 936

Berthe C, Alem E, Coppola V, Tessarollo L, Kaldis P (2003) Cdk2 knockout mice are viable. Curr Biol 13: 1775 – 1785

Bitto A, Ito TK, Pineda VW, LeTexier NJ, Huang HZ, Sutlief E, Tung H, Vizzini N, Chen B, Smith K et al (2016) Transient rapamycin treatment can increase lifespan and healthspan in middle-aged mice. elife 5: e16351

Blackburn EH, Epel ES, Lin J (2015) Human telomere biology: a contributor and interactive factor in aging, disease risks, and protection. Science 350: 1193 – 1198

Blaschke K, Ebata KT, Jancower T, Blasco MA (2007) Amplification of cyclin genes in human breast cancer. Proc Natl Acad Sci USA 104: 17082 – 17087

Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW (1999) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 91: 25 – 34

Bremmer R, Cohen BL, Sopta M, Hamel PA, Ingles CJ, Gallie BL, Phillips RA (1995) Direct transcriptional repression by pRb and its reversal by specific cyclins. Mol Cell Biol 15: 3256 – 3265

Brown JD, Phillips RA, Gallie BL (1999) Cumulative effect of phosphorylation of pRb on regulation of E2F activity. Mol Cell Biol 19: 3246 – 3256

Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CK, Musgrove EA, Sutherland RL (1993) Expression and amplification of cyclin genes in human breast cancer. Oncogene 8: 2127 – 2133

Burkhardt DL, Sage J (2008) Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer 8: 671 – 682

Byun TS, Pacek M, Yee MC, Walter JC, Cimprich KA (2005) Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes Dev 19: 1040 – 1052

Cai EP, Wu X, Schroer SA, Elia AJ, Nostro MC, Zackshenhaus E, Woo M (2013) Retinoblastoma tumour suppressor protein in pancreatic progenitors controls alpha- and beta-cell fate. Proc Natl Acad Sci USA 110: 14723 – 14728

Cai EP, Luk CT, Wu X, Schroer SA, Shi SY, Sivasubramaniyam T, Brunt JJ, Zackshenhaus E, Woo M (2014) Rb and p107 are required for alpha cell survival, beta cell cycle control and glucagon-like peptide-1 action. Diabetologia 57: 2555 – 2565
Campisi J (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 120: S13 – S22
Cerillo A, Esposito K, Ihnat M, Thorpe J, Giugliano D (2009) Long-term glycemic control influences the long-lasting effect of hyperglycemia on endothelial function in type 1 diabetes. J Clin Endocrinol Metab 94: 2751 – 2756
Cerillo A, Novials A, Ortega E, Canivell S, La Sala L, Pujadas G, Bucciarelli L, Rondinelli M, Genovese S (2013) Vitamin C further improves the protective effect of glucagon-like peptide-1 on acute hyperglycemia-induced oxidative stress, inflammation, and endothelial dysfunction in type 1 diabetes. Diabetes Care 36: 4104 – 4108
Chang J, Wang Y, Shao L, Laberge R-M, Demaria M, Campisi J, Janakiram K, Sharpless NE, Ding S, Feng W et al (2016) Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. Nat Med 22: 78 – 83
Chen D, Pacal M, Wenzel P, Kneepfier PS, Leone G, Bremner R (2009) Division and apoptosis of Er2f-deficient retinoid progenitors. Nature 462: 925 – 929
Chen J, Guo L, Zhang M, Liu HE, Wang X, Hu G, Gu T, Zhou Z et al (2013) Vitamin C modulates TET1 function during somatic cell reprogramming. Nat Genet 45: 1504 – 1509
Chicas A, Wang X, Zhang C, McCurrah M, Zhao Z, Mert O, Dickins RA, Narita M, Zhang M, Lowe SW (2010) Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. Cancer Cell 17: 376 – 387
Chien Y, Scapulo C, Wang X, Fang X, Baigley B, Bolden JF, Premkumar P, Luo W, Chicas A, Lee CS et al (2011) Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. Genes Dev 25: 2125 – 2136
Chkhotua AB, Gabusi E, Altman A, D’Errico A, Yakubovich M, Vienken J, Stefoni S, Chieco P, Grigioni WF et al (2022) The telomere replication terminator. Nature 592: 870 – 877
Ciccia A, Elledge SJ (2018) Senescence: a two-faced genome guardian. Trends Cell Biol 28: 471 – 481
Dor Y, Brown J, Martinez Ol, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429: 41 – 46
Drissi R, Dubois ML, Douziech M, Boisvert FM (2015) Quantitative proteomics reveals dynamic interactions of the minichromosome maintenance complex (MCM) in the cellular response to etoposide induced DNA damage. Mol Cell Proteomics 14: 2002 – 2013
Dyson N (2016) RB1: a prototype tumor suppressor and an enigma. Genes Dev 30: 1492 – 1502
Enge M, Arda HE, Migardini M, Beausang J, Bottino R, Kim SK, Quake SR (2017) Single-cell analysis of human pancreas reveals transcriptional signatures of aging and somatic mutation patterns. Cell 171: 321 – 330
Escandell JM, Canavalho ES, Gallo-Fernandez M, Reis CC, Matmati S, Luis IM, Abreu IA, Coulon S, Ferreira MG (2019) Ssu72 phosphatase is a conserved telomere replication terminator. EMBO J 38: e100476
Esteban MA, Wang T, Qin B, Yang J, Qin D, Cai J, Li W, Weng Z, Chen J, Ni SU et al (2010) Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. Cell Stem Cell 6: 71 – 79
Fu M, Wang C, Li Z, Sakamaki T, Pestell RG (2004) Minireview: Cyclin D1: normal and abnormal functions. Endocrinology 145: 5439 – 5447
Garcia-Cao M, Gonzalez S, Dean D, Blasco MA (2002) A role for the Rb family of proteins in controlling telomere length. Nat Genet 32: 415 – 419
Georgakilas AG, Martin OA, Bonner WM (2017) p21: a two-faced genome guardian. Trends Mol Med 23: 310 – 319
Goodrich DW, Wang NP, Qian YW, Lee EYHP, Lee WH (1991) The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell 67: 293 – 302
Gorgoulis V, Adams PD, Alimonti A, Bennett DC, Bischof O, Bishop C, Campisi J, Collado M, Evangelou K, Ferbeyre G et al (2019) Cellular senescence: defining a path forward. Cell 179: 813 – 827
Gubern A, Joaquim M, Marquès P, Maseser P, García-García J, Amat R, González-Nuñez D, Oliva B, Reau F, de Nadal E et al (2016) The N-terminal phosphorylation of RB by p38 bypasses its inactivation by CDKs and prevents proliferation in cancer cells. Mol Cell 64: 25 – 36
Guo N, Parry EM, Li KS, Kembou F, Lauder N, Hussain MA, Berggren PO, Armanios M (2011) Short telomeres compromize beta-cell signaling and survival. PLoS One 6: e17858
Harbour JW, Dean DC (2000) Chromatin remodeling and Rb activity. Curr Opin Cell Biol 12: 685 – 689
Harrison FE (2012) A critical review of vitamin C for the prevention of age-related cognitive decline and Alzheimer’s disease. J Alzheimers Dis 29: 711 – 726
Imai S, Guarente L (2014) NAD+ and sirtuins in aging and disease. Trends Cell Biol 24: 464 – 471
Inoue Y, Kitagawa M, Taya Y (2001) Phosphorylation of pRB at Ser612 by Chk1 leads to a complex between pRB and E2F-1 after DNA damage. EMBO J 26: 2083 – 2093
Janzén V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT (2006) Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443: 421 – 426
Jiang J, Liang P, Leng R, Guo Z, Liu Y, Liu X, Bubnic S, Keating A, Murray D, Goss P et al (2009) E2F1 and p53 are dispensable whereas p21Waf1/Cip1 cooperates with Rb to restrict endoreduplication and apoptosis during skeletal myogenesis. Dev Biol 227: 28 – 41
Jiang Z, Zacksenhaus E (2002) Activation of retinoblastoma protein in mammary gland leads to ductal growth suppression, precocious differentiation, and adenocarcinoma. J Cell Biol 156: 185 – 198
Jiang Z, Deng T, Jones R, Li H, Herschkowitz JI, Liu JC, Weigman VJ, Tsao M-S, Lane TF, Perou CM et al (2010) Rib deletion in mouse mammary progenitors induces luminal-B or basal-like/EMT tumor subtypes depending on p53 status. *J Clin Invest* 120: 3296–3309

Johnson SC, Yanos ME, Kayser E-B, Quintana A, Sangesland M, Castanza A, Uhde L, Hui J, Wall VZ, Gagnidze A et al (2013) mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. *Science* 342: 1524–1528

Johnson S, Imai S (2018) NAD (+) biosynthesis, aging, and disease. *F1000Res* 7: 132

Kanavaran P, Stefanaki K, Rontogianni D, Papalazarou D, Gorgoulis V, Siatitsas I, Agnantis NJ (2013) Vitamin C inhibits p53-induced replicative senescence through suppression of ROS production and p38 MAPK activity. *Int J Mol Med* 29: 651–655

Kim JE, Jin DH, Lee SD, Hong SW, Shin JS, Lee SK, Jung DJ, Kang JS, Lee WJ (2008) Vitamin C inhibits p53-induced replicative senescence through suppression of ROS production and p38 MAPK activity. *Int J Mol Med* 22: 651–655

Kirkland JL, Tchkonia T (2017) The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA1. *Science* 349: aaa5612

Kareta MS, Sage J, Wernig M (2015) Crossstalk between stem cell and cell cycle machineries. *Curr Opin Cell Biol* 37: 68–74

Kent LN, Leone G (2019) The broken cycle: E2F dysfunction in cancer. *Nat Rev Cancer* 19: 326–338

Khall T, Bingham S, Welch A, Ruben R, Wareham N, Oakes S, Day N (2001) Relation between plasma ascorbic acid and mortality in men and women in EPIC-Norfolk prospective study: a prospective population study. *European Prospective Investigation into Cancer and Nutrition. Lancet* 357: 565–663

Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Rageay K, Su L, Sharpless NE (2004) Ink4a/Arf expression is a biomarker of aging. *J Clin Invest* 114: 1299–1307

Kushner JA, Ciernyson MA, Sicinska E, Wartchow LM, Teta M, Long SY, Sicinska P, White MF (2005) Cyclins D2 and D1 are essential for postnatal pancreatic beta-cell growth. *Mol Cell Biol* 25: 3752–3762

Lees KA, Buchkovich KJ, Marshall DR, Anderson CW, Harlow E (1991) The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. *EMBO J* 10: 4279–4290

Liedt N, Murakami K, Eweida M, Elford AR, Sheu L, Gaisano HY, Hakem R, Ohashi PS, Woo M (2005) Caspase-3-dependent beta-cell apoptosis in the initiation of autoimmune diabetes mellitus. *Mol Cell Biol* 25: 3620–3629

Liu Y, Johnson SM, Fedoriy W, Rogers AB, Yuan H, Krishnamurthy J, Sharpless NE (2011) Expression of p16(INK4a) prevents cancer and promotes aging in lymphocytes. *Blood* 117: 3257–3267

Massie HR, Aliero VR, Doherty TJ (1984) Dietary vitamin C improves the survival of mice. *Gerontology* 30: 371–375

Merc ro D, Isserlin R, Stueker O, Emili A, Bader GD (2010) Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS One* 5: e13984

Mittnacht S (1998) Control of pRB phosphorylation. *Curr Opin Genet Dev* 8: 21–27

Molosky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, Sharpless NE, Morrison SJ (2006) Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during aging. *Nature* 443: 448–452

Munoz-Lorente MA, Cano-Martin AC, Blasco MA (2019) Mice with hyper-long telomeres show less metabolic aging and longer lifespan. *Nat Commun* 10: 4723

Naray-sima AM, Kaulich M, Shapiro GS, Choi YJ, Sicinska P, Dowdy SF (2014) Cyclin D activates the RB tumor suppressor by mono-phosphorylation. *eLife* 3: e02872

Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 113: 703–716

Nasteskas D, Fine NHF, Ashford FB, Cuozzo F, Viloria K, Smith G, Dahir A, Dawson PWJ, Lai YC, Bastidas-Ponce A et al (2021) PDX1(Low) MAFA(Low) beta-cells contribute to islet function and insulin release. *Nat Commun* 12: 746

Nelsen GP, Stemmer-Rachamimov AO, Shaw J, Roy JE, Koh J, Louis DN (1999) Immunohistochemical survey of p16INK4a expression in normal human adult and infant tissues. *Lab Invest* 79: 1137–1143
Nik-Zainal S, Davies H, Staaf J, Ramakrishna M, Glodzik D, Zou X, Martincorena I, Alexandrov LB, Martin S, Wedge DC et al (2016) Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature 534: 47 – 54

Nir T, Melton DA, Dor Y (2007) Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 117: 2553 – 2561

Otto T, Sicinski P (2017) Cell cycle proteins as promising targets in cancer therapy. Nat Rev Cancer 17: 93 – 115

Pacak M, Bremner R (2012) Mapping differential detection in the mouse retina reveals an extensive period of cell cycle protein expression in post-mitotic newborn neurons. Dev Dyn 241: 1525 – 1544

Pai C-C, Kishkevich A, Deegan RS, Keszthelyi A, Folkes L, Kearsey SE, De León N, Soriano I, de Bruin RAM, Carr AM et al (2017) Set2 methylationtransferase facilitates DNA replication and promotes genotoxic stress responses through MIF-dependent transcription. Cell Rep 20: 2693 – 2705

Pernas S, Tolaney SM, Winer EP, Gol C (2018) CDK4/6 inhibition in breast cancer: current practice and future directions. Ther Adv Med Oncol 10: 175885918786451

Pietrocola F, Castoldi F, Markaki M, Lachkar S, Chen G, Enot DP, Durand S, Sanidas I, Morris R, Fella KA, Rumde PH, Boukhali M, Tai EC, Ting DT, Sacaan AI, Thibault S, Hong M, Kondegowda NG, Nichols T, Li R, Rosselot C, Pacal M, Bremner R, Rubin SM, Nir T, Melton DA, Dor Y, Rieck S, Kaestner KH (2020) Set2 methylationtransferase facilitates DNA replication and promotes genotoxic stress responses through MIF-dependent transcription. Cell Rep 20: 2693 – 2705

Pirrotta V, Castaldi F, Markaki M, Lachkar S, Chen G, Enot DP, Durand S, Bossut N, Tong M, Malik SA et al (2018) Aspirin recapitulates features of caloric restriction. Cell Rep 22: 2395 – 2407

Pu M, Ni Z, Wang M, Yang X, Wood JC, Helfand SL, Yu H, Lee SS (2015) Trimethylation of Lys36 on H3 restricts gene expression change during aging and impacts life span. Genes Dev 29: 718 – 731

Rando TA, Chang HY (2012) Aging, rejuvenation, and epigenetic reprogramming: resetting the aging clock. Cell 148: 46 – 57

Rane SG, Dubus P, Mettus RV, Galbreath EJ, Boden G, Reddy EP, Barbacid M (1999) Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. Nat Genet 22: 44 – 55

Reimand J, Arak T, Vilo J (2011) gProfiler—a web server for functional interpretation of gene lists (2011 update). Nucleic Acids Res 39: W307 – W315

Rieck S, Kaestner KH (2010) Expansion of beta-cell mass in response to caloric restriction. Cell 142: 2395 – 2407

Roberts PJ, Kumarasamy V, Witzkiewicz AK, Knudsen ES (2020) Chemotherapy and CDK4/6 inhibitors: unexpected bedfellows. Mol Cancer Ther 19: 1575 – 1588

Rubin SM (2013) Deciphering the retinoblastoma protein phosphorylation code. Trends Biochem Sci 38: 12 – 19

Sacaan AI, Li Y, Harrington L, Soriano I, de Bruin RAM, Carr AM et al (2017) Set2 methylationtransferase facilitates DNA replication and promotes genotoxic stress responses through MIF-dependent transcription. Cell Rep 20: 2693 – 2705

Talchai C, Xuan S, Lin HV, Sussel L, Accili D (2012) Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell 150: 1223 – 1234

Tan C, Ginzman MB, Webster R, Iyengar S, Liu S, Papadopoli D, Concordon J, Wang Y, Auld DS, Jenkins JL et al (2021) Cell size homeostasis is maintained by CDK4-dependent activation of p38 MAPK. Dev Cell 56: 1756 – 1769

Teta M, Rankin MM, Long SY, Stein GM, Kushner JA (2007) Growth and regeneration of adult beta cells does not involve specialized progenitors. Dev Cell 12: 817 – 826

Thompson PJ, Shah A, Ntranos V, Van Gool F, Atkinson M, Bhushan A (2019) Targeted elimination of senescent beta cells prevents type 1 diabetes. Cell Metab 29: 1045 – 1060

Topacio BR, Zatulovskiy E, Cristea S, Xie S, Tambo CS, Rubin SM, Sage J, Koivomagi M, Skotheim JM (2019) Cyclin D–Cdk 4/6 drives cell-cycle progression via the retinoblastoma protein’s C-terminal helix. Mol Cell 74: 758 – 770

Turner NC, Slamon DJ, Ro J, Bondarenko I, Im S-A, Masuda N, Colleoni M, DeMichele A, Loi S, Verma S et al (2018) Overall survival with palbociclib and fulvestrant in advanced breast cancer. N Engl J Med 379: 1926 – 1936

Uchida C (2016) Roles of pRB in the regulation of nucleosomes and chromatin structures. Biol Res 49: 5599721

Xiong W, Wang X, Li B, Chowdhury S, Lu Y, Srikant CB, Ning G, Liu JI (2011) Pancreatic islet-specific overexpression of Reg3g beta protein induced the expression of pro-islet genes and protected the mice against streptozotocin-induced diabetes mellitus. Am J Physiol Endocrinol Metab 300: E669 – E680

Xu G, Stoffers DA, Habener JF, Bonner-Weir S (1999) Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48: 2270 – 2276

Xu M, Pirtskhalava T, Farr JN, Weigand BM, Palmer AK, Weivoda MM, Inman CL, Ogrodnik MB, Hachfeld CM, Fraser DG et al (2018) Senolytics improve physical function and increase lifespan in old age. Nat Med 24: 1246 – 1256

Zacksenhaus E, Shrestha M, Liu JC, Vorobieva I, Chung PED, Ju Y, Nir U, Jiang Z (2017) Mitochondrial OXPHOS induction by R81 deficiency in breast cancer: implications for anabolic metabolism, stemness, and metastasis. Trends Cancer 3: 768 – 779

Zarkowska T, Mittnacht S (1997) Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J Biol Chem 272: 12738 – 12746
Zhang H, Ryu D, Wu Y, Gariani K, Wang X, Luan P, D’Amico D, Ropelle ER, Lutolf MP, Aebersold R et al (2016) NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice. Science 352: 1436 – 1443
Zhang J, Bu X, Wang H, Zhu Y, Geng Y, Nihira NT, Tan Y, Ci Y, Wu F, Dai X et al (2018) Cyclin D-CDK4 kinase destabilizes PD-L1 via cullin 3-SPOP to control cancer immune surveillance. Nature 553: 91 – 95
Zijlmans JM, Martens UM, Poon SS, Raap AK, Tanke HJ, Ward RK, Lansdorp PM (1997) Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. Proc Natl Acad Sci USA 94: 7423 – 7428

License: This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.