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Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence

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Summary

The RB protein family (RB, p107, p130) has overlapping and compensatory functions in cell cycle control. However, cancer-associated mutations are almost exclusively found in RB, implying that RB has a non-redundant role in tumor suppression. We demonstrate that RB preferentially associates with E2F target genes involved in DNA replication and is uniquely required to repress these genes during senescence but not other growth states. Consequently, RB loss leads to inappropriate DNA synthesis following a senescence trigger and, together with disruption of a p21-mediated cell cycle checkpoint, enables extensive proliferation and rampant genomic instability. Our results identify a non-redundant RB effector function that may contribute to tumor suppression and reveal how loss of RB and p53 cooperate to bypass senescence.

Introduction

Loss-of-function mutations in the retinoblastoma gene product (RB) or its signaling network are considered requisite for cancer development; hence, the roles and regulation of RB have been intensively studied [reviewed in (Burkhart and Sage, 2008; Rowland and Bernards, 2006)]. The best-characterized RB activity relates to its ability to control the G1-S transition, where it negatively regulates the E2F family of transcription factors. Cyclin-dependent kinases (CDKs) activated in response to mitogenic stimuli phosphorylate and inactivate RB, allowing the released E2F to transcriptionally activate genes required for cell cycle progression. Certain viral oncoproteins bind RB and release E2F, leading to forced S phase entry. Since spontaneous mutations in *RB* may produce similar effects, the ability of RB to halt cell cycle transitions is considered central to its tumor suppressor function. Nevertheless, RB binds other proteins besides E2F and can regulate processes such as apoptosis, quiescence, differentiation, and senescence. How these proteins and processes contribute to the tumor suppressor activities of RB is poorly understood.

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All the expression profiling data and genome binding data are available from the GEO repository (accession number GSE19899).

RB is a member of a multigene family consisting also of *RBL1* (p107) and *RBL2* (p130) (Burkhart and Sage, 2008). Studies using both biochemical and genetic approaches have identified distinct and overlapping functions of each family member (Classon and Harlow, 2002). Like RB, both p107 and p130 bind E2F proteins and are substrates for phosphorylation by active cyclin/CDKs (Classon and Harlow, 2002). Furthermore, p107 and p130 also associate with DNA tumor virus oncoproteins and can induce cell cycle arrest when over-expressed (Mulligan and Jacks, 1998). Yet, despite the similarities among the RB proteins in structure and function, somatic mutations affecting p107 or p130 are rare in human cancers (Burkhart and Sage, 2008).

In contrast to their action in cell cycle control, less is known about how RB proteins influence cellular senescence. Senescent cells exit the cycle irreversibly, acquire a large and flat morphology, accumulate a senescence-associated β-galactosidase (SA-β-gal) and undergo changes in gene expression linked to cell cycle inhibition and inflammation (Campisi and d'Adda di Fagagna, 2007). In cultured cells, senescence can be triggered by replicative exhaustion, or in response to activated oncogenes, DNA damage, or oxidative stress (Courtois-Cox et al., 2008)., Accordingly, the senescence program acts as a general anti-proliferative stress response and is considered a potent tumor suppressive mechanism in vivo [reviewed in (Narita and Lowe, 2005) (Prieur and Peeper, 2008)]. Indeed, senescent cells accumulate in benign tumors in mice expressing activated oncogenes and in these settings co-disruption of genes controlling senescence regulators lead to malignant progression. Moreover, certain DNA damaging chemotherapeutic agents can induce senescence in tumors, and the integrity of the senescence program contributes to the anti-tumor effect of these agents.

The regulation of cellular senescence involves interplay between the p53 and RB tumor suppressor networks (Courtois-Cox et al., 2008). For example, DNA tumor virus oncoproteins that target p53 and RB bypass senescence in cultured cells (Shay et al., 1991). Although these oncoproteins bind all three RB family members, acute inactivation of RB is sufficient to promote proliferation in senescent mouse embryo fibroblasts (MEFs) (Sage et al., 2003) and prevents SAHF accumulation and cooperates with p53 loss to bypass senescence in human diploid fibroblasts (Narita et al., 2003; Voorhoeve and Agami, 2003). Based on these observations, we hypothesized that RB must have targets in senescence that differ from those controlled by p107 and p130, and that these targets might highlight processes that mediate its tumor suppressive effects.

Results

RB has a non-redundant role in oncogene-induced senescence

To understand the relative contribution of individual RB family members to various proliferative states, we generated multiple short hairpin RNAs (shRNAs) targeting each family member using the mir-30 design (Silva et al., 2005). These shRNAs were transduced into IMR90 human diploid fibroblasts (HDF), a well-characterized normal human cell strain that has an intact RB pathway, is widely used to study both replicative and oncogene-induced senescence (Narita et al., 2003; Shay et al., 1991), and can rapidly and completely transition between various growth states by simple cell culture manipulations. As shown in Figure 1A, each shRNA efficiently and specifically represses its target RB family protein. To elucidate the contribution of the individual RB family members to ras-induced senescence, we transduced IMR90 cells with shRNAs targeting each RB family protein together with a retrovirus expressing oncogenic ras and the populations were selected for cells harboring both constructs. Nine days post-infection, cell populations were examined for markers of senescence. For comparison, IMR90 cells expressing each shRNA were examined in normal growth conditions, or following induction of quiescence by serum withdrawal or contact inhibition (confluence).

Although each shRNA suppressed its corresponding family member (Figure 1B), only those targeting RB reduced SA-β-gal accumulation (Figure 1C) and SAHF formation (Figure 1D and 1E) in cells triggered to senesce. None of the shRNAs were able to completely bypass senescence; however, a small but reproducible percentage of shRB expressing cells (but not shp107 or shp130-expressing cells) continued to incorporate BrdU at the 9 day time point (Figure 1E, see below). Identical results were obtained using two different shRNAs targeting each gene, suggesting no RNAi off-target effects. Therefore, most subsequent experiments were performed using the most potent shRNAs. Consistent with the overlapping functions of the RB family, none of the shRNAs had any notable impact on proliferation of growing or quiescent cells (Figure 1E and 5B; data not shown). Similar results were also observed in WI38 cells, another normal human fibroblast strain (data not shown). These observations confirm that, in normal human fibroblasts, RB plays a crucial role in cellular senescence that is not shared with other family members.

Impact of individual RB family members on gene expression in different growth conditions

The RB family proteins function primarily as transcriptional co-repressors by binding and redundantly modulating the activity of the E2F transcription factor family (Burkhart and Sage, 2008). We therefore reasoned that RB might have transcriptional targets not shared by p107 or p130 during senescence (but not during quiescence), and that these targets might contribute to the impaired program observed in cells lacking RB. Previous attempts to identify specific targets of individual RB family of proteins have examined growing or quiescent mouse embryo fibroblasts from the corresponding knockout mice that may have been susceptible to developmental compensation and were not comprehensive (Markey et al., 2007). Here, we examined the impact of acutely inhibiting different pocket proteins on genome-wide gene expression patterns in normal human cells.

Transcriptional profiling was performed on IMR90 cells in growing, low serum quiescent (0.1% FBS for 4 days), contact inhibited quiescent (5 days post-confluency) or senescent conditions expressing shRNAs targeting RB, p107, p130 or control. RNA from two independent experiments was hybridized to the Affymetrix U133 Plus 2.0 microarray and the generated data was processed as described in the experimental procedures. Average-linkage hierarchical clustering analysis was performed to aggregate arrays and genes based on similarities of gene expression (Eisen et al., 1998). This analysis clustered the different growth conditions over the effect of the different shRNAs, indicating that each shRNA has a smaller effect on global gene expression than the particular growth condition (Figure S1A).

Senescent cells displayed a global up-regulation of anti-proliferative genes, down-regulation of growth promoting genes, and a pattern of gene expression known as the senescence-associated secretory phenotype (Coppe et al., 2008). Accordingly, we observed up-regulation of cyclin-dependent kinase inhibitors (CDKis), protein metalloproteinase (MMPs) as well as many cytokines and chemokines (CXCs) in senescent cells (Figure S1A).

A non-redundant role for RB in repressing some E2F target genes during senescence

To understand how each RB family member influences gene expression in different growth conditions, we identified differentially expressed genes in the presence of each shRNA. Unique and reproducible shRNA-dependent changes in gene expression were observed under all conditions (Figure S1B). Interestingly, senescent cells expressing shRB underwent the most substantial changes in gene expression as evident by the number of probe sets that were up or down regulated (Figure S1B). Gene ontology (GO) analysis (Dennis et al., 2003) showed little overlap in the processes affected by repressing each RB proteins under different conditions (Table S1). However we observed a differential but overlapping effect of RB on gene expression categories depending on the growth state. Whereas genes upregulated in cells

undergoing senescence in the absence of RB were enriched with "DNA replication" factors (Table S1, $P<9.2\times10^{-14}$), those upregulated in growing cells lacking RB were enriched with "cell cycle" genes (Table S1, $P<6.12\times10^{-8}$). Using a non-biased bioinformatics analysis, we identified the E2F motif, with the consensus sequence TTTSSCGC (where S represents C or G), as the most enriched motif in genes upregulated in cells undergoing senescence in the absence of RB ($P<2.1\times10^{-7}$ with Bonferroni correction) (Figure S1C). Surprisingly, the E2F motif was only moderately enriched in the set of genes upregulated in growing cells lacking RB ($P< 0.09$ with Bonferroni correction), and not enriched in gene sets impacted by other conditions or shRNAs (data not shown). These observations suggest that RB has a unique specificity in regulating a subset of E2F targets in senescent cells.

RB represses distinct genes depending on cellular context

To better characterize the genes and processes controlled by RB, we grouped genes that were significantly upregulated by RB loss in senescent, low serum, confluent and growing conditions (741, 120, 784, and 291 respectively; Table S2) and subjected the corresponding probe sets to hierarchical clustering (Figure 2A). Consistent with our more global analyses, these genes often contained E2F binding sites and were highly expressed in growing but not in quiescent or senescent cells (Figure 2A; Table S2). Interestingly, most predicted E2F target genes were contained within two distinct adjacent clusters that were only divided by the influence of RB in senescent cells. Whereas the genes in clusters C3 (Figure 2D) were highly expressed in RB-deficient senescent cells, the genes in cluster C4 (Figure 2E) remained repressed. Gene ontology analysis revealed that genes in cluster C3 (Table S3; Replication cluster) were enriched for DNA replication factors ($P<3.0\times10^{-29}$ with Benjamini correction), particularly components of the pre-replication complex, while genes in cluster C4 (Table S3; Cell cycle cluster) were enriched for mitotic cell cycle factors ($P<1.0\times10^{-21}$ with Benjamini correction). We have confirmed these results with additional biological replicates (Figure S1D). Thus RB is uniquely required to repress the transcription of replication genes during cellular senescence.

Clustering analysis also revealed two other distinct clusters. One cluster contains a group of genes that were highly expressed only in senescent cells lacking RB (Figure 2C). The genes in this cluster (Table S3; Cyclin E cluster) are poorly characterized, except for cyclin E1, a *bona fide* E2F target gene known to activate several cyclin-dependent kinases. The other cluster is enriched in many cytokines and chemokines (CXCs) that are part of the senescence-associated secretory phenotype (Figure 2B). However, these genes, some of which contribute to senescence, were still induced in the absence of each RB family member and, in some cases, their expression further increased in the absence of RB (data not shown). Thus, while RB is required for some aspects of the senescence program, the vast majority of changes are RB-independent.

Selective high-affinity binding of RB to the "replication factor" set of E2F targets

The expression analysis described above suggests that RB discriminates between different E2F target genes and growth states. Previous attempts to detect RB bound to specific E2F target promoters in growing or quiescent cells have failed or yielded contrasting results (Cam et al., 2004; Rayman et al., 2002; Wells et al., 2000), perhaps because RB binds weakly if at all to these promoters under these conditions. To investigate whether the selectivity for DNA replication factors in senescent cells was due to preferential binding of RB to the promoters of these genes, we identified RB binding sites by combining chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-Seq) (Robertson et al., 2007). This procedure provides a completely non-biased view of where proteins bind chromatin and can be more sensitive than conventional ChIP or ChIP-chip analysis (Park, 2009).

Libraries made following chromatin immunoprecipitation with an anti-RB antibody from growing, quiescent or senescent cells produced a total of 14.4, 13.5 and 12 million reads, of which 40%, 37% and 33% uniquely aligned to the human genome, respectively (Table S4). We used MACs (model-based analysis for ChIP-seq) (Zhang et al., 2008) to find ChIP-seq peak regions (RB binding sites) with a significance threshold (p-value $\leq 10^{-5}$) and false discovery rate (FDR) threshold of 1%. We identified 4525, 4214 and 8336 RB binding sites in growing, quiescent and senescent cells, respectively, with an overlap of 1281 peaks (Table S4; Figure S2A). About 80% of the peaks can be mapped within 5kb upstream of predicted transcriptional start sites of known refseq genes and about half are located near promoters (data not shown). Of note, the ChIP-seq experiments were reproducible (Pearson correlation coefficient 0.84) (Figure S2B), and about 89.7% and 76.5% of the top 1000 and top 2000 RB binding sites were identified in biological replicates, respectively (Table S4; Senescence #2). Saturation analysis indicated that the most high affinity RB binding sites should have been identified at the sequencing depth we achieved (Figure S2C). Importantly, the signal intensity from these sites was substantially reduced in cells expressing shRNAs targeting RB, confirming the specificity of the antibody (Figure S2D). Thus ChIP-seq analysis allowed us to identify and validate thousands of RB binding sites under different growth conditions in a manner that has not been previously possible.

Gene ontology (GO) analysis revealed that genes targeted by RB were highly enriched in DNA replication factors (p<7.1E-12), irrespective of growth condition (Table S5; Common targets). Using the non-biased de novo motif search algorithm DME (Smith et al., 2006) we found that the most highly enriched motif in all conditions is similar to the known E2F motif (TTTCCCGC). We also showed that the majority of RB ChIP-seq peaks were centered at the E2F motif and that RB binding intensity correlated with the quality of E2F motif (Data not shown and Figure S2E). For example, we detected RB specifically bound to the promoter region of the MCM3 (Figure 3A), an E2F target genes de-repressed in the absence of RB. Importantly, no significant peaks were observed outside the promoter region or in the control (beads-only) precipitation (Figure 3A). Together, these global and non-biased analyses indicate that the primary targets of RB mediated repression are E2F target genes.

To identify those RB targets that required RB for repression, we integrated the RB ChiP-seq and transcriptional profiling data. We observed that 2.9%, 1% and 4.5% of genes bound by RB in growing, quiescence and senescence conditions, respectively, were significantly de-repressed in the absence of RB (Figure S2F). This analysis revealed that the affinity of RB was greatest for those genes that required RB for repression (Figure 3C). Interestingly, gene ontology analysis revealed that this class of genes was highly enriched for DNA replication factors ($P<3.0\times10^{-20}$)(Figure 2D). To confirm the preference of RB for DNA replication genes, we also compared the average affinity of RB for genes in either cluster C3 (those that are depressed by RB suppression and are enriched in replication factors) or simply RB target genes defined by the GO term “DNA replication factor” to all other direct RB target genes. Remarkably, RB showed an average 3-fold (P<1E-9) or 1.7-fold (p <1E-6) higher affinity for the genes in cluster C3 or “DNA replication factor” genes, respectively, under all three conditions (Figure 3B; data not shown) and a higher affinity for these factors in senescence that in growing cells (Figure S2G-H). Together, these data indicate that, although RB can bind to genes involved in a variety of processes, DNA replication genes are uniquely dependent on RB in senescent cells.

Redundancy of RB family of proteins in quiescent cells

We were surprised by the nominal impact of suppressing individual RB proteins on E2F target gene expression during quiescence (Figures 4A-C and 2D-E), a cell cycle state thought to be influenced by the RB family (Burkhart and Sage, 2008). There is ample evidence that the RB

proteins have redundant and/or compensatory functions (Sage et al., 2000). To test whether the RB proteins had an overlapping ability to repress E2F targets in quiescent HDFs, we developed retroviral vectors that express combination of two or three shRNAs targeting RB, p107 and p130 in tandem (Figure 4D) and introduced these into IMR90 cells. Cells expressing each vector efficiently suppressed the targeted proteins, although two or more shRNAs slightly reduced the efficiency of each individual shRNA (Figure 4E). Despite this, only cells in which all three RB family proteins were suppressed showed up-regulation of E2F target genes in quiescent cells (Figure 4E). Therefore, in stark contrast to the situation in senescence, the RB family shares the capacity to suppress E2F target genes during quiescence.

We tested whether the ability of the other RB family proteins to compensate for loss of RB in quiescent HDFs reflected the ability of these proteins to replace RB at E2F target gene promoters. Since p130 is the most prominent RB family member bound to E2F target genes in quiescent cells (Balciunaite et al., 2005), we compared the binding of p130 in the presence and absence of RB in quiescent and senescent cells by ChIP-seq. We found that like RB, the main targets of p130 are genes containing E2F binding sites (Figure S3). Importantly, we found that whereas binding of p130 to many promoters increased in quiescent cells lacking RB, no effect was observed in senescent cells (Figure 4F). For example, p130 bound the MCM5 gene promoter with a 1.8 Fold higher intensity in quiescent cells lacking RB than those expressing RB, while the binding of p130 in senescent cells was not affected by RB suppression (Figure 4G). Thus p130 can compensate for RB loss in quiescent cells by enhancing its binding to E2F target gene promoters. Together these data suggest that there is a binding equilibrium between RB and p130 such that at a given moment either one or the other is bound to particular E2F sites. Loss of RB shifts the equilibrium towards p130 perhaps explaining why loss of individual RB proteins does not effect expression of E2F targets in quiescent cells.

RB represses the DNA replication machinery in cells undergoing senescence

Among the genes de-repressed in the absence of RB are key components of the pre-replication complex (pre-RC), pointing to a possible defect in the proper shut down of DNA synthesis in cells undergoing senescence. Assembly of the pre-RC on origins of replication is a crucial and limiting step in the synthesis of DNA occurring only once per cell cycle and during a short window of time prior to S phase (Stillman, 1996). We therefore tested whether RB-deficient cells inappropriately retained an assembled pre-RC by immunoblotting the chromatin-bound fraction of ras-transduced cells for specific replication factors (Mendez and Stillman, 2000). While components of the pre-RC were bound to chromatin in growing cells under all conditions, they were not detected in quiescent cells or in senescent cells lacking p107 or p130 or expressing a vector control. In contrast, pre-RC components were bound tightly to chromatin in RB-suppressed cells triggered to senesce (Figure 5A).

Consistent with the above results, experiments examining BrdU incorporation and DNA content revealed that cells lacking RB show unscheduled DNA synthesis when entering senescence. Specifically, shRB-expressing cells continued to incorporate BrdU for at least 4 days longer than control, shp107- and shp130-expressing cells upon ras transduction (Figure 5B). These cells also showed a higher percentage of cells with 4N and 8N DNA content compared to the other cell populations (>2-fold and >7-fold, respectively, Figure 5C). Despite ongoing DNA replication, shRB-expressing cells triggered to senesce did not expand after post selection day 7 (PS7) (Figure 7B), indicating that they activated an additional barrier to proliferation (see Figure 7). Nevertheless, these results demonstrate that RB is required to halt DNA synthesis during cell cycle exit into senescence, such that cells lacking RB undergo unscheduled DNA replication and, in some cases, endoreplication. Interestingly, quiescent cells lacking RB did not display these defects (Figure 5B-5C), further supporting the unique role of RB in senescent cells.

RB repression of cyclin E1 is required to prevent replication of senescent cells

Our results argue for a unique role for RB in repressing the replication machinery, particularly during senescence. However, in addition to the pre-RC components, loss of RB also led to de-repression of the cyclin E1 gene (Figure 2C, 4C, 5A), a key regulator of the cell cycle engine (Sherr and Roberts, 2004). Although less appreciated, cyclin E1 has also been directly linked to DNA replication and endoreplication (Zhang, 2007)– phenotypes observed in senescent cells lacking RB. We therefore hypothesized that the aberrant cyclin E expression could contribute to the unscheduled DNA replication leading to polyploid cells. To test this hypothesis, we introduced tandem shRNAs capable of repressing cyclin E1 and RB (Figure 6A) into IMR90 cells together with oncogenic ras. Remarkably, despite a nominal impact on normal growing cells [data not shown, see also (Geng et al., 2003)], suppression of cyclin E1 was sufficient to prevent the aberrant DNA replication and endoreplication, otherwise observed in cells undergoing senescence in the absence of RB (Figure 6B). Furthermore, we rescued the phenotype by reintroducing a shRNA-resistant cDNAs encoding either wild type or a kinase-deficient cyclin E1 (cyclin E1 KD-E) (Sheaff et al., 1997) (Figure S4).

Distinct from its cell cycle activity, cyclin E1 can directly influence DNA replication by recruiting MCMs to the pre-RC in a kinase-independent manner (Geng et al., 2007). To investigate the requirement for cyclin E1 in promoting assembly of the pre-RC in cells lacking RB, we used the association between MCM2 and chromatin as a surrogate marker (Geng et al., 2007). Immunofluorescence on pre-extracted samples showed that suppression of cyclin E1 caused a significant reduction in MCM2-positive cells (Figure 6C), indicating inefficient loading of MCM2 onto chromatin. Similarly, immunoblotting of chromatin-bound proteins showed less chromatin-bound MCM2 in cells lacking cyclin E1 (Figure 6D). These results were not due to a reduction in MCM2 expression, as total MCM2 protein levels were unchanged (Figure 6E). The levels of MCM2 and MCM3 RNA also remained unchanged in cells lacking cyclin E1 indicating that cyclin E is not required for the up-regulation of these genes in RB-deficient cells (data not shown). Thus, in senescent cells, RB is required to repress both replication factors as well as cyclin E1, which would otherwise facilitate loading these factors into the pre-RC leading to aberrant replication and endoreplication.

RB loss triggers a p53/p21 dependent checkpoint that prevents escape from senescence

Although loss of RB prevents the appropriate shut down of DNA replication in cells triggered to senesce, these cells eventually arrest, suggesting the activation of a second proliferation barrier that prevents unrestrained proliferation. In addition to the RB pathway, the p53 tumor suppressor also contributes to senescence (Courtois-Cox et al., 2008). Interestingly, p53 can respond to replicative stress and (Marusyk and DeGregori, 2007), accordingly, we noted an increase in p53 ser15 phosphorylation (Figure S5A) and a concomitant increase on its target gene p21 in RB-deficient cells triggered to senesce (Figure 7A).

To test whether p21 was required for this second proliferation barrier, we introduced shRNAs targeting both RB and p21 into IMR90 cells together with oncogenic ras. Strikingly, these cells efficiently bypassed senescence, as assessed by cell proliferation, BrdU incorporation, and colony formation relative to controls in both IMR90 and WI38 cells (Figure 7B-Cand data not shown). Furthermore, consistent with their ongoing proliferation, cells expressing the RB/p21 shRNA derepressed both the DNA replication E2F targets (MCM3, Cyclin E1) and mitotic E2F targets (cdc2, Cyclin A, cyclin B) (Figure 7E, Figure S5B). Similarly, cells co-expressing shRNAs targeting RB and p53, or p16 and p21, bypass senescence (data not shown), which is associated with sustained expression of cell cycle/mitotic E2F targets (e.g.cyclin A, B, cdc2), albeit to varying degrees (Figure 7E). Interestingly, cells co-suppressing p16 and p21 did not hyperinduce cyclin E, perhaps owing to incomplete knockdown or a subtly different mechanism of senescence escape. Regardless, in the absence of this proliferation block, cells

co-suppressing RB together with p21 accumulate more polyploid cells compared to those lacking RB alone (Figure 7D, Figure S5C-E). Therefore, in human fibroblasts, p21 mediates a checkpoint that is activated in response to RB loss and serves to limit the consequences of unrestrained DNA replication. In the absence of this checkpoint, proliferation continues unabated, thus providing an explanation for how RB and p53 loss cooperate to bypass senescence.

Discussion

The *RB* gene family encodes important regulators of cell proliferation with overlapping and redundant functions, yet only *RB* is commonly mutated in human tumors. By performing a series of genome wide analyses, we see that RB has a non-redundant role in binding and repressing E2F target genes that are directly involved in DNA replication, particularly in senescent cells. Consequently, in cells triggered to senesce, RB loss leads to sustained loading of pre-replication complexes, aberrant DNA replication and – in the absence of a second cell cycle checkpoint – proliferation and genomic instability. By contrast, RB is dispensable for proper cell cycle exit during quiescence because other RB family members act redundantly to control key E2F targets. Consistent with our observations, cells harboring a transcriptionally compromised RB mutant fail to repress replication targets during oncogene-induced senescence but not cell cycle exits, including those required for normal development (Talluri et al., 2009).

Implications for senescence control

A hallmark of senescence is its stable cell cycle arrest coordinated by interplay between the RB and p53 tumor suppressor networks (Courtois-Cox et al., 2008). Whereas p53 promotes senescence by transactivating growth inhibitory genes, RB represses growth-promoting genes, which, in some cases, may involve heterochromatization of E2F target genes (Narita et al., 2003; Zhang et al., 2005). Here we see that RB is dispensable for many of the gene expression changes that accompany senescence but, instead, uniquely and specifically represses a subset of E2F target genes required for DNA replication. Whether these effects involve the same processes that influence SAHF formation remains to be determined; nevertheless, they highlight the importance of repressing DNA replication for proper execution of the senescence program.

Consistent with a role for p53 in senescence, cells lacking RB eventually arrest because they engage a second proliferation barrier mediated by the p21 cyclin-dependent kinase inhibitor. In response to certain mitogenic oncogenes, p53 activates p21 and triggers senescence in response to replication stress (Halazonetis et al., 2008), and thus it seems likely that the aberrant DNA synthesis produced by RB loss exacerbates this failsafe mechanism. Regardless, p21 loss reveals the full capacity of RB deficient cells to aberrantly replicate DNA, thus explaining how RB and p53 cooperate to promote senescence.

It remains to be determined whether our observations in fibroblasts can be extended to other cells types, though it is interesting that, human vulvar intraepithelial neoplesias (VIN) that have bypassed senescence through inactivation of the RB family and p53 (Santegoets et al., 2007) also show hyperinduction of the replication factors studied here (Figure S1E). Furthermore, the same genetic principle that control senescence in fibroblasts also apply to at least some cultured epithelial cells (e.g. RB and p53 cooperate to bypass senescence in HMECs and keratinocytes) and spontaneous loss of p16 due to DNA methylation in HMECs does not completely bypass senescence but is associated with the accumulation of polyploidy cells (Romanov et al., 2001).

Insights into RB action in proliferation control

By combining RNAi with genome-wide gene expression and chromatin binding analyses, we identified genes that are direct RB targets *and* whose regulation is strictly RB-dependent. This comprehensive analysis revealed that the specific targets of RB can vary between growth states. For example, only a specific subset of E2F target genes, particularly those directly involved in DNA replication, were both bound by RB and de-repressed in RB-deficient cells entering senescence. Thus our results not only reveal the unique specificity of RB for E2F replication targets but a strict concordance between the nature of genes regulated by RB and the specific phenotype associated with RB loss – i.e. unscheduled DNA replication during senescence but not quiescence. Although previous studies hinted that RB family proteins can regulate different E2F targets (Hurford et al., 1997) and that loss of RB can ultimately lead to increased loading of replications complexes on chromatin (Srinivasan et al., 2007), the selectivity of RB towards repressing replication genes was not appreciated.

By identifying thousands of RB binding sites throughout the genome, our analyses decisively show that the primary targets of RB mediated repression are genes harboring E2F binding sites and, moreover, reveal that RB preferentially associates with a subset of E2F targets. By integrating these data with transcriptional profiling, we further demonstrate that RB mediates repression through physical association with these promoters. Still, only a fraction of RB target genes are de-repressed in the absence of RB and only in senescence. Such a situation has also been observed for similar studies on the androgen receptor and may suggest compensation or redundancy in transcriptional control (Massie et al., 2007). Nevertheless, to our satisfaction, the genes that are sensitive to RB loss are those genes that show the higher affinity for RB. The binding of RB to replication factor genes during senescence has not been examined, and this unique combination of genes and circumstances may explain why RB binding to chromatin has previously been difficult to detect (Rayman et al., 2002).

In human cells, we see that RB is dispensable for cell cycle exit into quiescence – instead, any RB family member could repress E2F target genes in this setting [see also (Rayman et al., 2002)]; here we show that this redundancy can be explained, in part, by an increase in p130 to replication genes in cells lacking RB. In contrast, studies using mouse embryo fibroblasts show that acute ablation of *Rb* in quiescent cells leads to S phase entry and proliferation (Sage et al., 2003). Likewise, cre-mediated deletions of *Rb* in small intestine enterocytes allows for cell cycle reentry of quiescent villus cells (Guo et al., 2009). The apparent discrepancy in our results may relate to cell type or species differences or the status of the helix-loop-helix protein HES1, which may play a crucial role in determining the reversibility of the quiescent state (Sang et al., 2008). Nevertheless, why other RB family members cannot always compensate for RB loss remains to be determined, though we suspect that the stable silencing of RB-target genes during senescence may require recruitment of an RB-specific chromatin modifying activity to these promoter of these genes. Indeed, an RB mutant that is unable to associate with chromatin modifying enzymes fails to repress DNA replication during oncogene-induced senescence but not during quiescence or differentiation (Talluri et al., 2009)

One key RB target in senescence was cyclin E, which was expressed at low levels in growing cells but strongly upregulated in RB-suppressed cells triggered to senesce. Since cyclin E promotes cell cycle progression by activating CDKs (Zhang, 2007), its selective control by RB stands out from other RB targets directly involved in DNA replication. However, cyclin E has additional activities and can directly stimulate DNA replication by facilitating recruitment of MCM proteins to the pre-RC (Geng et al., 2007). Similarly, we see that the inappropriate expression of cyclin E1 in RB-deficient cells leads to sustained loading of MCM proteins onto chromatin in a CDK-independent manner, unscheduled DNA replication, and endoreplication. These results establish a connection between the kinase-independent function of cyclin E1,

loading of MCM complexes onto chromatin, and endoreplication, and highlight how RB acts in a coordinated way to shut down DNA synthesis as cells enter senescence.

RB action in tumor suppression

The importance of RB as a tumor suppressor gene is evident by the frequency of inactivating mutations that target RB or its pathway in human cancer; indeed, the RB pathway is thought to be disabled in virtually all tumor cells (Burkhart and Sage, 2008). Although RB can modulate the G1-S transition, cellular differentiation, DNA replication, mitosis, apoptosis, chromosomal stability and cellular senescence, it is not clear which of these functions are crucial for its tumor suppressor activity. Our results reveal a unique and non-redundant role for RB in preventing DNA replication in cells undergoing senescence. Hence, when RB is lost, cells undergo inappropriate DNA replication, aberrant proliferation, and display genomic instability – processes that are exacerbated by other checkpoint defects and contribute to the development of malignant tumors. In this regard, it is interesting that a key target of RB-mediated repression in senescence is cyclin E1 – a *bona fide* oncogene whose over-expression occurs in many human tumor types and is often associated with poor prognosis (Butt et al., 2008). Since cellular senescence has been established as a general anti-proliferative, stress-response program that acts as a potent barrier to tumorigenesis, our findings suggest that selective targeting of DNA replication genes during senescence represents one key activity of RB in tumor suppression.

Experimental Procedures

A more detailed description of the reagents and experimental procedures used in this study can be found in the Supplemental Experimental Procedures.

Vectors

The following retroviral vectors were used in this study: pWZL-Hygro (H-rasV12) (Serrano et al., 1997), pWZL-Blasticidin (H-rasV12) (Narita et al., 2006). MiR30 design shRNAs targeting, Rb, p107, p130 and cyclin E1 were subcloned from the pSM2 RNAi codex library vector into MSCV-derived LMP vector. The polycistronic shRNA vectors were cloned in two steps (see Supplementary materials). The cyclin E1 cDNA and cyclin E1 kinase deficient mutant were obtained from Bruce Clurman (Geng et al., 2007). Cyclin E1 and cyclin E1 mutant cDNAs were made resistant to shRNA inactivation by modifying the targeted sequence to include 7 mismatches that did not alter the amino acid sequence, using a QuickChange II Site-Directed Mutagenesis Kit (Stratagene).

Cell Culture and Gene Transfer

Human diploid IMR90 fibroblasts and WI38 (ATCC) were cultured in DMEM supplemented with 10% FBS and antibiotics. Retroviruses were packed using Phoenix cells (G. Nolan, Stanford University, CA) and infections were performed as described (Narita et al., 2003). The infected population was selected using either 2 μg/ml puromycin (Sigma) for 2 days, 100 μg/ml hygromycin B (Roche) for 3 days or Blastacidin (10 ug/ml for 4 days). For co-infection, cells were sequentially selected with Puromycin, Hygromycin and then Blastacidin. Post-selection day 7 refers to 7 days after the puromycin selection.

Senescence Assays

Ras-induced senescent IMR90 cells (post-selection day 7) were plated on coverslips coated with 0.1% gelatin and fixed in 4% paraformaldehyde. For cell cycle arrest, the cells were labeled with 5-Bromo-2′-deoxyuridine (BrdU, 100 μg/ml, Sigma) and 5-fluor-2′-deoxyuridine (FdU, 10 mg/ml, Sigma) for 4 hr. Nuclei incorporating BrdU were visualized by immunolabeling using a anti-BrdU antibody (Pharmingen, 1:400) as described (Narita et al., 2003).

Immunoassays

Immunoblotting was carried out as described (Narita et al., 2003). See Supplemental Experimental Procedures for a list of antibodies used. For immunofluorescence of chromatin-bound MCM2, cells were plated as described above and pre-extracted in CSK buffer with 0.3-mg/ml digitonin (Sigma) followed by fixation in 4% paraformaldehyde. The MCM2 polyclonal antibody was a gift from Bruce Stillman (CSHL) and was used at 1/1000 dilution. Alexa Fluor Conjugates (Molecular Probes) were used as the secondary antibodies, and DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/ml). Isolation of chromatin-bound proteins was performed as described (Mendez and Stillman, 2000) with minor modifications (see Supplementary materials). For flow cytometry, cells were collected, wash with PBS, resuspended in 100ul PBS plus 900ul cold methanol and stored at +4 overnight. Cells were washed in PBS and resuspended in 500ul of PI/RNAse Staining buffer (BD pharmingen). Data was collected on LSRII flow cytometer (BD Bioscience) and analyzed with Flowjo software (TreeStar, Ashland, OR).

Gene expression analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and was converted to cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems) and used for qPCR or utilized for cRNA preparation [Message AmpII (Ambion)] and hybridized to U133 Plus 2.0 microarray (Affymetrix) according to the manufacturer's instructions. Gene-specific primer sets were designed using Primer Express 1.5 (sequences are available from the authors upon request). Real-time PCR was carried out in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) on the Roche's IQ5 ICycler. GAPDH or β-actin served as an endogenous normalization control. The quantification was done using an external standard curve made with a serial dilution of one of the RT reactions. Details on the microarray data analysis are found on Supplemental Experimental Procedures.

Chip-seq analysis

The chromatin immunoprecipitation (ChIP) experiments were done as previously described (Orlando et al., 1997) with some modifications (see Supplemental Experimental Procedures). Immunoprecipated DNA was prepared for 1G sequencing as described (Robertson et al., 2007) with some modification (see Supplemental Experimental Procedures). Further details of the analysis are found in Supplemental Experimental Procedures.

Significance

The action of RB as a tumor suppressor has been difficult to define, in part, due to the redundancy of the related proteins p107 and p130. By coupling RNAi technology with a genome-wide analysis of gene expression and RB chromatin binding, we identified a unique and specific activity of RB in repressing DNA replication as cells exit the cell cycle into senescence. We further show how failure of this activity, when coupled to loss of a failsafe checkpoint, leads to both senescence bypass and genomic instability. Our study provides a comprehensive dataset of the genes controlled by RB family members in distinct proliferative states and insights into the regulation of cellular senescence and RB action in tumor suppression.

Highlights

A non-redundant role for RB in repressing DNA replication genes during senescence

Selective high-affinity binding of RB to the DNA replication E2F target genes

RB repression of cyclin E1 is required to prevent replication of senescent cells

RB loss triggers a p53/p21 dependent checkpoint that prevents escape from senescence

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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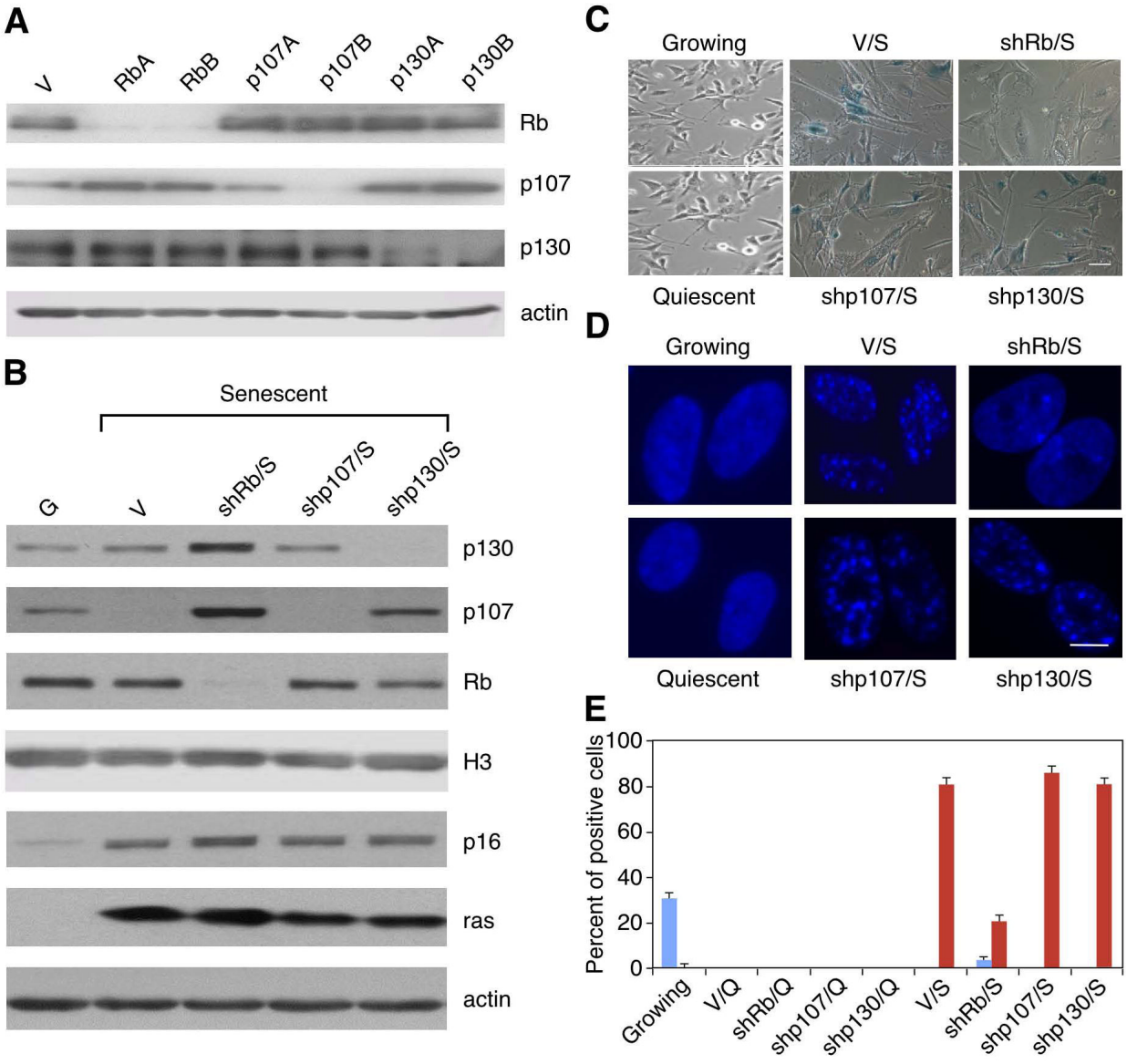

Figure 1. RNAi-mediated knockdown of RB but not p107 or p130 impairs ras-induced senescence

(A) Immunoblots of growing IMR90 cells infected with the indicated shRNAs probed for RB, p107 or p130. Actin was used as loading control. (B) Immunoblots of ras-senescent IMR90 cells. Chromatin-bound fractions were used for the RB, p107 and p130 blots and Histone H3 was used as loading control. Whole cell lysates were used for the p16 and ras blots and actin was used as loading control. (C) SA-β-galactosidase staining. Scale bar=100uM (D) DAPI staining to visualize SAHF. Scale bar=10uM. (E) Quantification of SAHF (red bars), BrdU incorporation (blue bars). Values represent the mean +/− standard error of at least three independent experiments. (V) is empty vector, (S) is senescent, (G) is growing, and (Q) is quiescent.

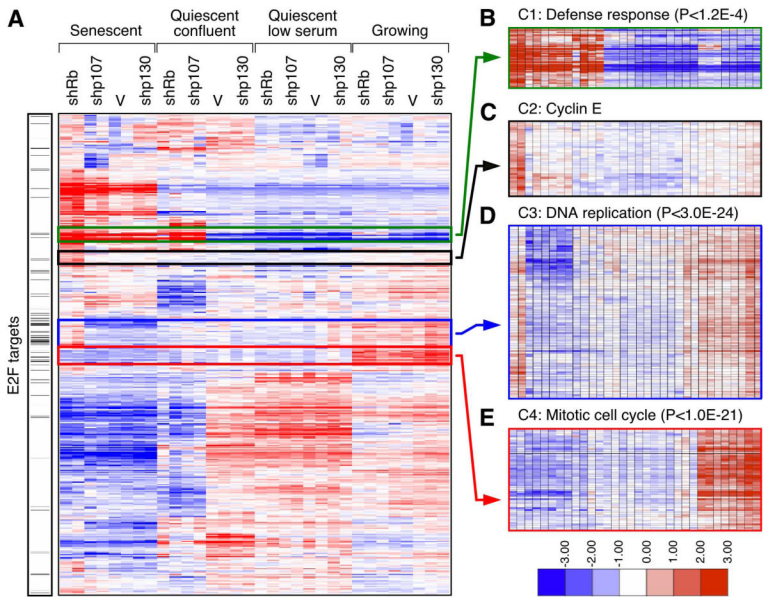

Figure 2. RB represses components of the replication machinery during cellular senescence

(A) Heatmap of expression patterns derived from hierarchical clustering of RB responsive genes (1826 probes) highlighting the E2F target genes. (B-E) Magnification of various gene clusters. (B) Immuno-surveillance cluster (C) Cyclin E cluster (D) DNA replication factors clusters. (E) Mitotic cell cycle cluster. See also Figure S1 and Tables S1-S3.

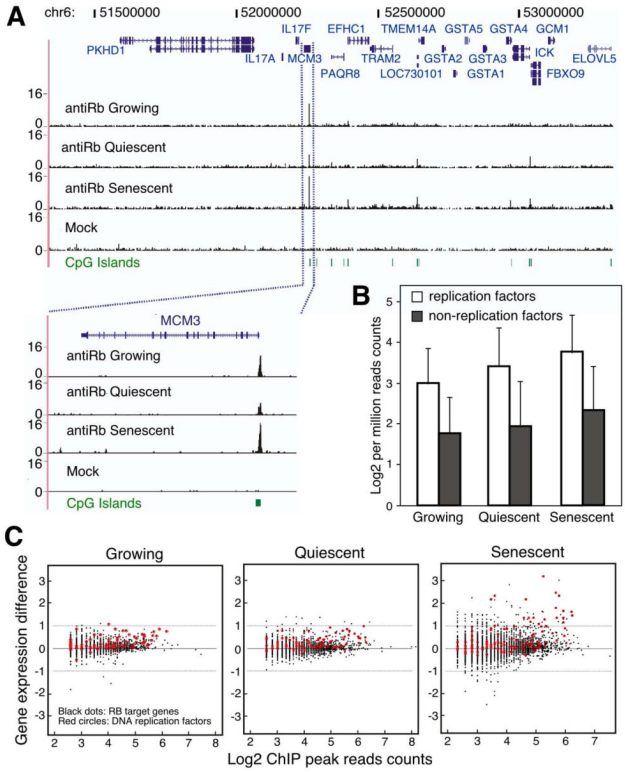

Figure 3. Selective binding of RB to the promoters of DNA replication factors

(A) Binding patterns of RB to the MCM3 gene shown as custom tracks on the UCSC genome browser. (B) Histogram comparing binding of RB-as measured by read counts-to DNA replication factors vs other RB targets. Error bars represent the standard deviation of the read counts. (C) Correlation of RB binding to gene promoters and expression of those genes in RB-deficient cells under different growth conditions. See also Figure S2 and Table S4-S5.

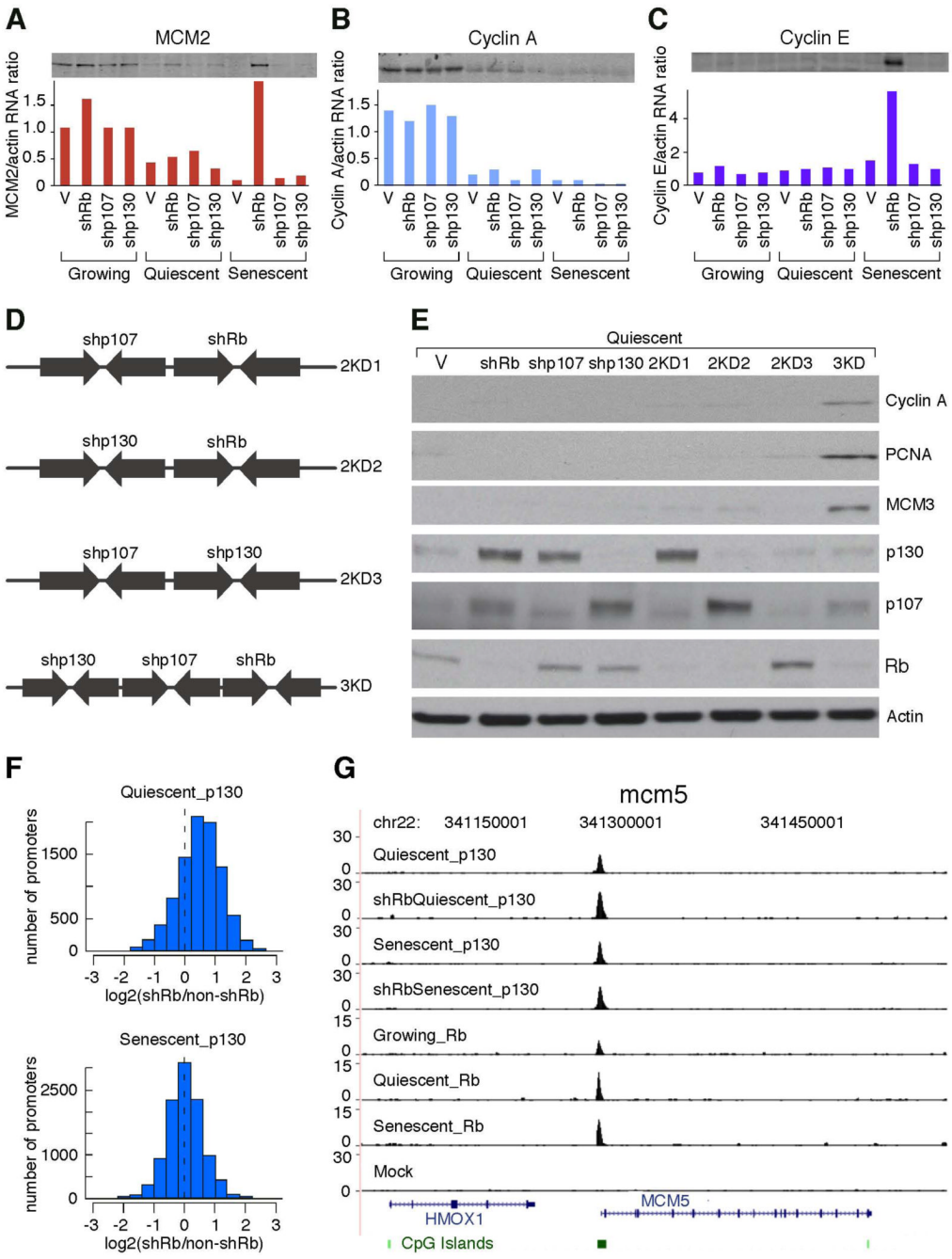

Figure 4. Repression of E2F target genes is unique for RB in senescence but is redundant in quiescence

Immunoblots and qPCR to measure expression of (A) MCM2, (B) Cyclin A, and (C) Cyclin E1 in growing, quiescent or senescent cells expressing the indicated shRNA. qPCR values are averages of representative experiments done in triplicates. (D) Schematic diagram of the polycistronic shRNAs used to knockdown the indicated members of the RB family. (E) Immunoblots probed for the indicated protein from quiescent IMR90 cells lysates infected with the indicated shRNA. (F) Histograms comparing the binding intensity of p130 at gene promoters before (non-shRB) and after RNAi-mediated suppression of RB (shRB) in quiescent (top) and senescent (bottom) cells. The shift to the right of zero indicates that the p130-specific

antibody co-immunoprecipitates more promoter DNA in the absence of RB. (G) Binding patterns of p130 and RB to the MCM5 gene shown as custom tracks on the UCSC genome browser. p130 binding to the MCM5 gene promoter increases 1.8 fold in RB-deficient quiescent cells while binding in senescent cells remain unchanged. See also Figure S3 and Table S6

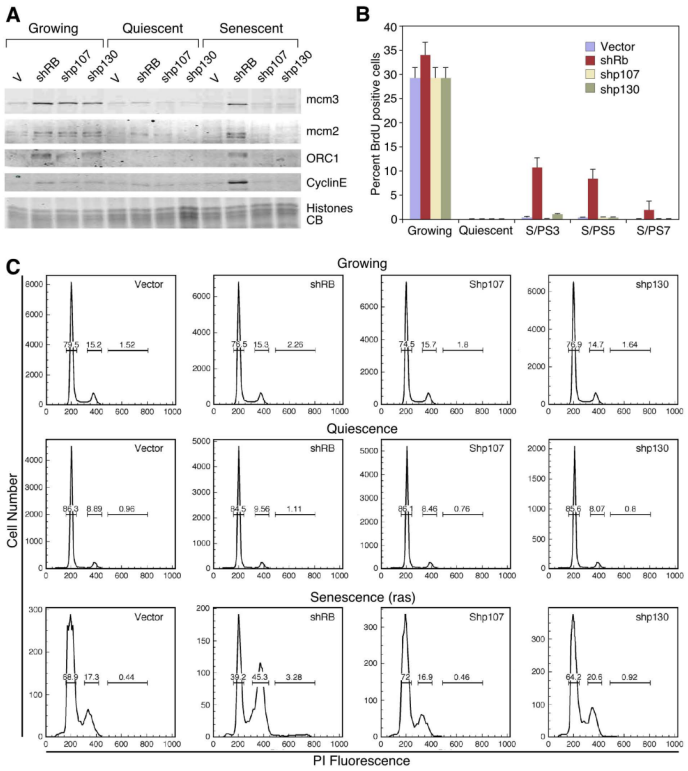

Figure 5. RB is required to prevent inappropriate DNA replication in cells undergoing senescence

(A) Immunoblots of chromatin-bound proteins probed for MCM2, MCM3, ORC1, and Cyclin E1. Blots were normalized by Coomassie Blue staining for histones. (B) Time course BrdU incorporation assays of cells undergoing senescence in the absence of RB, p107 or p130 compared to vector control. (S/PS3, S/PS5, S/PS7 stand for post-selection day 3, 5, or 7 respectively). Values represent the mean +/− standard error of a least three independent experiments. (C) Cells cycle profiles of growing (top), quiescent (middle) or senescent (bottom) IMR90 cells expressing the indicated shRNA. .

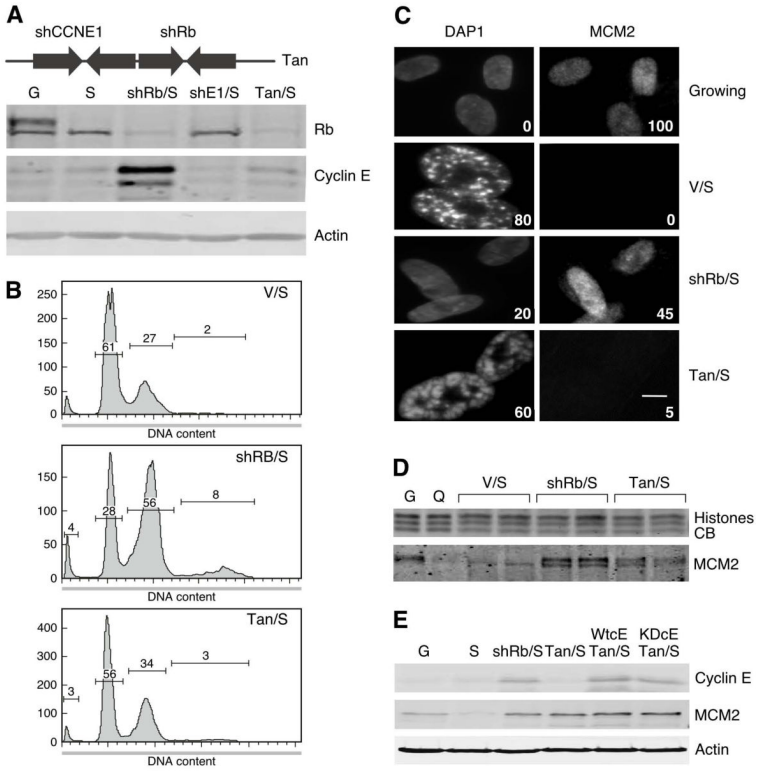

Figure 6. RB repression of Cyclin E1 is required to prevent replication of senescent cells

(A) Immunoblots of lysates from senescent cells expressing a polycistronic shRNA targeting RB and cyclin E1 (Tan depicted on the top of the figure) probed for RB and Cyclin E1. Growing (G), Senescent (S), senescent cells expressing shRNA targeting only RB (shRB/S) or only cyclin E1 (shE1/S) are used as controls. Actin is used as loading control. (B) Cell cycle profiles of vector control senescent cells (V/S), senescent cells expressing shRB (shRB/S) or the tandem shRB/cE1 shRNA (Tan/S). (C) Immunofluorescence of pre-extracted cells to measure proper association of MCM2 with chromatin. Percentage of MCM2 positive cells is indicated in the bottom right corner of the images. DAPI staining was used to visualize the nuclei. At least 200 cells were counted per experiment. Scale bar =100um. (D) Immunoblots of chromatin-bound extracts from growing (G), quiescent (Q), vector control senescent cells (V/S), senescent cells expressing shRB (shRB/S) or the tandem shRB/cE1 shRNA (Tan/S): two independent samples are shown. (E) Immunoblots of lysates from senescent cells expressing the tandem shRB/cE1 shRNA (Tan/S) and shRNA-resistant cyclin E1 cDNA encoding either wild type (WtcE) or kinase-deficient (KDcE) cyclin E1. Lysates from growing (G), vector control senescent cells (V/S), senescent cells expressing shRB (shRB/S) or the tandem shRB/cE1 shRNA (Tan/S) alone are used as controls. See also Figure S4.

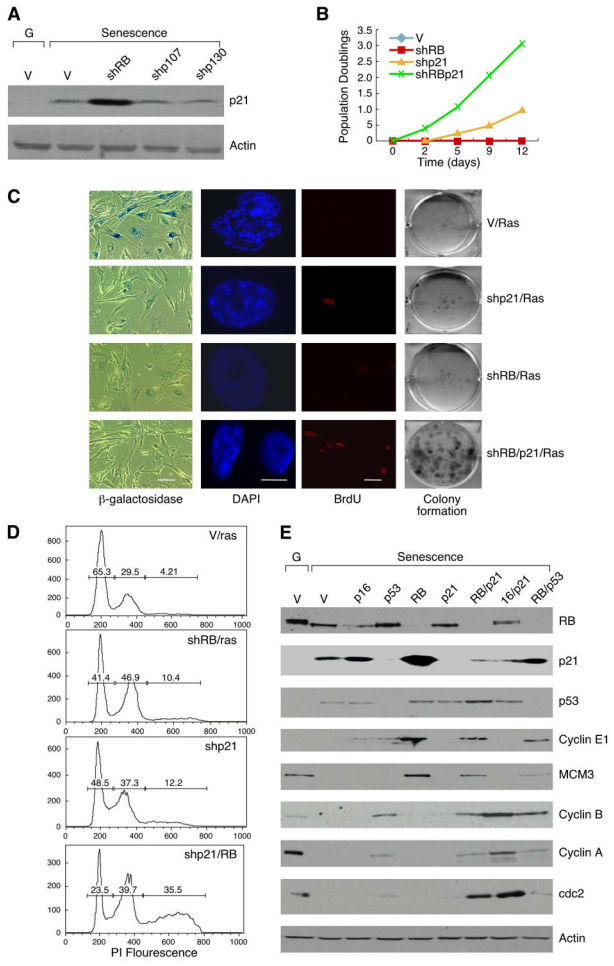

Figure 7. RB loss triggers a p53/p21 dependent checkpoint that prevents escape from senescence

(A) Immunoblots of lysates from IMR90 senescent cells expressing the indicated shRNA probed for p21 or actin. (B) Growth curve of ras-infected cells expressing the indicated shRNAs. Counting was initiated at PS7 (post-selection day 7) at which time the vector control cells are fully growth arrested as measure by BrdU incorporation. (C) Analysis of PS7 cells for different proliferation and senescence markers and for the ability to form colonies at low density. Unlike vector control, RB or p21 suppression, suppression of both RB and p21 leads to increase in cell proliferation as measured by BrdU incorporation (20% positive compared to 2% for either shRB or shp21 alone) and colony formation. Scale bars for the micrographs showing SA-β-gal staining and BrdU immunoflourescence=100uM and for DAPI staining=10uM. (D) Cell cycle profiles of ras-infected cells expressing the indicated shRNA. (E) Immunoblots of lysates from ras-infected cells expressing the indicated shRNA probed for the indicated proteins. See also figure S5