The Rb/E2F pathway and Ras activation regulate RecQ helicase gene expression

Yongqing LIU†, Shahenda EL-NAGGAR†, Brian CLEM*, Jason CHESNEY* and Douglas C. DEAN*††

*Molecular Targets Program, James Graham Brown Cancer Center, Louisville, KY 40202, U.S.A., and †Department of Ophthalmology and Visual Sciences, University of Louisville Health Sciences Center, Louisville, KY 40202, U.S.A.

Disruption of the Rb (retinoblastoma protein)/E2F cell-cycle pathway and Ras activation are two of the most frequent events in cancer, and both of these mutations place oncogenic stress on cells to increase DNA replication. In the present study, we demonstrate that these mutations have an additive effect on induction of members of the RecQ DNA helicase family. RecQ activity is important for genomic stability, initiation of DNA replication and telomere maintenance, and mutation of the BLM (Bloom’s syndrome gene), WRN (Werner’s syndrome gene) or RECQL4 (Rothmund–Thomson syndrome gene) family members leads to premature aging syndromes characterized by genetic instability and telomere loss. RecQ family members are frequently overexpressed in cancers, and overexpression of BLM has been shown to cause telomere elongation. Concomitant with induction of RecQ genes in response to Rb family mutation and Ras activation, we show an increase in the number of telomeric repeats. We suggest that this induction of RecQ genes in response to common oncogenic mutations may explain the up-regulation of the genes seen in cancers, and it may provide a means for transformed cells to respond to an increased demand for DNA replication.

Key words: Ras activation, Rb/E2F pathway, RecQ family, telomere elongation.

INTRODUCTION

The RecQ family of DNA helicases is conserved from prokaryotes to vertebrates [1]. Mutations in three family members, BLM (Bloom’s syndrome gene), WRN (Werner’s syndrome gene) and RECQL4 (Rothmund–Thomson syndrome gene) lead to autosomal-recessive diseases characterized by premature aging, genonomic instability and predisposition to cancers [2]. There is mounting evidence that RecQ family members are crucial for telomere maintenance, for genomic stability, and for proper loading of proteins at origins of DNA replication [1–4]. BLM interacts with Top3 and can functionally substitute for Sgs1 in yeast telomerase-independent telomere maintenance, and WRN has also been shown to at least partially substitute [5,6]. Furthermore, both BLM and WRN are associated with telomeric repeats located in PML (promyelocytic leukaemia) bodies in cells undergoing recombination-based telomerase-independent alternative lengthening of telomeres [7], BLM and WRN associate with TRF1 and TRF2 (telomere repeat factors 1 and 2), and overexpression of BLM leads to lengthening of telomeres [8–10]. By contrast, mutation of BLM or WRN leads to premature loss of telomeric repeats [2,11], suggesting that RecQ proteins are also important for normal replication of telomeric repeats.

BLM is expressed in S-phase of proliferating cells and is a marker of cell proliferation in vivo [12]. Accordingly, its expression is up-regulated in tumours compared with normal tissues [13]. Oncogenes such as myc and Epstein–Barr virus, or treatment with the tumour-promoting agent PMA, induces expression of BLM, WRN and RECQL4; WRN expression is associated with Burkitts lymphoma, where expression of c-Myc is classically induced [12–14], and BLM is induced by BCR/ABL (breakpoint cluster region/v-abl Abelson murine leukaemia viral oncogene homologue 1) in leukaemia [15]. Knockdown of WRN in Burkitts lymphoma cells led to growth arrest and increased apoptosis [16], indicating a role for WRN in maintaining viable proliferating cancer cells.

The BLM (retinoblastoma protein) family cell-cycle regulatory pathway, which is disrupted in cancer [17,18], is also linked to telomere maintenance; mutations in the Rb family genes RBL1 (p107) and RBL2 (p130) led to an increase in telomere length [19]. Although mutation of RB1 itself was not sufficient to trigger this lengthening, telomeres were further lengthened when all three family members were mutated [19]. Similarly a recent study has demonstrated that knockdown of RBL2 is sufficient to trigger telomere lengthening, and that this effect appears to be mediated at least in part through association of RBL2 with the Rad50/Mre11 recombination complex at telomeres [20].

Previously it was found using ChIP (chromatin immunoprecipitation) ChIP-on-ChIP assays that E2F4 can bind to the BLM promoter [21], potentially linking RecQ expression to the Rb/E2F pathway and thus the cell cycle. In the present study, we provide evidence that RecQ family members WRN, BLM and RECQL4 are each repressed by the Rb family pathway, however, in distinct fashions. Furthermore, we demonstrate that oncogenic Ras activation also induces each of the genes. Mutation of the Rb pathway and Ras activation appear to act additively to induce the RecQ genes. In addition to mutation of the Rb family, we show that Ras activation also leads to telomere elongation, and we correlate telomere length to the level of induction of RecQ genes.

Abbreviations used: BLM, Bloom’s syndrome gene; cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; DB-E2F, E2F1 DNA-binding domain; HDAC, histone deacetylase; IPTG, isopropyl β-D-thiogalactoside; MEF, murine embryonic fibroblast; mER, modified (tamoxifen-dependent) oestrogen receptor; Rb, retinoblastoma protein; RECQL4, Rothmund–Thomson syndrome gene; TKO, triple knockout; WRN, Werner’s syndrome gene.

† To whom correspondence should be addressed (email dcdean01@louisville.edu).
**EXPERIMENTAL**

**Ras expression and activity**

TKOs (triple knockouts) were infected with V12 Ha-Ras retroviral vector as described previously [39], and Ras activity was carried out using the EZ-Detect Ras activation kit (Pierce Biotechnology).

**Cells and cell culture**

Rb family TKO MEFs (murine embryonic fibroblasts) and littermate control wild-type fibroblasts have been described previously [30] and were a gift from T. Jacks and J. Sage. Three independent TKO and wild-type isolates were used with similar results. U2OS cells expressing IPTG (isopropyl β-D-thiogalactoside)-inducible p16INK4a have been described previously [22], as have the U2OS cells expressing both IPTG-inducible p16INK4a and mER-DB-E2F [mER is modified (tamoxifen-dependent) oestrogen receptor; DB-E2F is E2F1 DNA-binding domain] [24,25]. U2OS cells were cultured with 1 mM IPTG in DMEM (Dulbecco’s modified Eagle’s medium) for either one or three days to induce p16INK4a, or with 100 nM tamoxifen for one day to induce mER-DB-E2F expression. For combined treatments, cells were treated with IPTG for one day, and then tamoxifen was added along with the IPTG for an additional one day.

**Quantification of telomere repeats**

Real-time PCR quantitation of telomere repeats was performed as described previously [40]. This assay was linear over a range 2.5–80 ng of MEF genomic DNA. The standard curve for telomeric repeats was constructed along with two other different single-copy genes, 36B4 (also known as acidic ribosomal phosphoprotein P0) and β-actin. Each assay was performed in triplicate using 5 and 20 ng of genomic DNA, with similar results.

**RNA extraction and real-time PCR**

Total cellular RNA was extracted using TRIzol® solution (Invitrogen). SYBR Green real-time quantitative PCR was performed in a Mx3000P Real-Time PCR system (Stratagene) according to the manufacturer’s instructions. Primer sequence, Tm (melting temperature) and PCR product sizes are listed in Supplementary Tables 1 and 2 (at http://www.BiochemJ.org/bj/412/bj4120299add.htm). Real-time quantitative PCR was performed in 25 μl reaction volumes containing 0.25 μl aliquots of cDNA, gene-specific primer pairs, and SYBR Green 1 fluorescent dye (Molecular Probes), in an Mx3000P Real-Time PCR system (Stratagene), according to the manufacturer’s instructions.

**mRNA stability assays**

Wild-type MEFs, TKOs and TKO-Ras cells were split equally into 5 cm plates, and the following day 1 μg/ml actinomycin D was added to the medium to block transcription. mRNA was then isolated at different time points, cDNA was generated and RNA levels were quantified by real-time PCR as described above.

**ChIP assay**

ChIP assays were based on the Upstate protocol (http://www.upstate.com/misc/protocol) using formaldehyde to cross-link genomic DNA. Antibodies for Rb (sc-50; Santa Cruz Biotechnology), E2F1 (sc-193; Santa Cruz Biotechnology), E2F4 (sc-866; Santa Cruz Biotechnology) and histone modifications (Abcam) were used for immunoprecipitation. Equal amounts of anti-IgG or pre-immune serum were used as controls. ChIP PCR reactions were similar to those described above for real-time PCR, but with an additional 1 % BSA, 1 % DMSO and a higher annealing temperature (e.g. 60–68°C) and a longer extension time (e.g. 1 min).

**Cellular senescence assays**

Senescent β-galactosidase activity was analysed using the x-Gal-based Sigma cell senescence staining kit using the manufacturer’s protocol (Sigma).

**RESULTS**

**BLM and RECQL4 mRNA expression is repressed in response to p16INK4a**

To examine potential regulation of RecQ genes by the Rb family/E2F pathway, we initially used a human Rb family(+) p16INK4a(−) osteosarcoma cell line, U2OS, which expresses the cdk (cyclin-dependent kinase) inhibitor, p16INK4a, under control of an IPTG-inducible promoter. We have described these cells previously [22]; Figure 1A). The Rb family is hyperphosphorylated and inactivated by cdk4 and 6 (preventing its interaction with E2F, and thus its recruitment to target genes), and p16INK4a binds and inhibits these cdks, leading to accumulation of hypophosphorylated, active Rb family members [17]. Thus these cells accumulate hypophosphorylated (active) Rb and arrest in response to p16INK4a expression, whereas in the absence of p16INK4a, Rb is hyperphosphorylated and inactive. We used real-time PCR to analyse the effect of p16INK4a on RecQ family mRNA expression. As a positive control, treatment with IPTG to induce p16INK4a led to repression of mRNAs for two known p16INK4a/Rb family/E2F target genes, RRM2 and NTF3.
E2F has distinct roles in regulation of RecQ family members

The DB-E2F has been used previously to effectively displace E2Fs from their binding sites on promoters [22–25]. We reasoned that using DB-E2F to displace E2Fs should allow us to determine a role for E2Fs in expression of these RecQ genes. DB-E2F was fused to a mER and stably co-expressed into the U2OS cells along with the IPTG-inducible p16INK4A vector. This cell system has been described previously [24,25]. Upon treatment with tamoxifen, the mER-DB-E2F fusion protein translocates to the nucleus, where it displaces E2Fs from target genes, based on ChIP assays ([25]; Figure 1A). As a control, treatment of parent cells (cells not expressing mER-DB-E2F) with tamoxifen did not lead to significant changes in gene expression, as determined by microarray analysis [24].

Treatment of cells with tamoxifen had relatively little effect on expression of mRNAs for the cell-cycle control genes, RRM2 and NTF3, or on mRNAs for RECLQ4 and BLM (Figure 1B). These results suggest that E2F is not contributing a significant transactivation function to expression of these genes in normally proliferating U2OS cells (in the absence of p16INK4a, and where Rb is hyperphosphorylated and does not bind E2F). Next, we asked whether tamoxifen treatment to activate mER-DB-E2F would reverse the repression triggered by IPTG-mediated induction of p16INK4a. Indeed, repression of the genes was reversed (Figure 1B), implying that mER-DB-E2F may be displacing an Rb-family–E2F repressor complex assembled at the promoters in response to p16INK4a expression. Even though WRN expression was not affected by IPTG to induce expression of p16INK4a, its expression was nevertheless significantly induced following tamoxifen treatment to activate mER-DB-E2F (Figure 1B).

Taken together, the above results suggest that RECLQ4 and BLM are repressed in a cell-cycle-dependent fashion by a p16INK4a-dependent Rb-family–E2F repressor complex (they are repressed under conditions where p16INK4a is expressed to activate Rb, triggering Rb–E2F assembly and cell-cycle arrest). However, it appears that WRN may be under constitutive repression by an E2F complex which does not require p16INK4a for assembly.

Induction of p16INK4a and senescence of MEFs coincides with down-regulation of mRNAs for BLM and RECLQ4, but not WRN

p16INK4a is induced in stem cell populations during aging, leading to decreased proliferative capacity in progenitor cells in the brain, bone marrow and pancreas [26]. Induction of the INK4a locus (which encodes p16INK4a and ARF) with passage in culture is responsible for replicative senescence of cultured MEFs [27]. Therefore as an alternative way of correlating p16INK4a with passage of MEFs in culture with real-time PCR (Figures 2A and 2B). Consistent with previous reports [28], we found that expression of p16INK4a mRNA increased with passage number until cells began to undergo senescent arrest at passage 6 (Figure 2B), as evidenced by expression of senescence-associated β-galactosidase (results not shown). There was a corresponding decrease in expression of RECLQ4 and BLM mRNAs with this induction of p16INK4a mRNA and senescence (Figure 2A). But consistent with our findings above in U2OS cells, there was little or no change in WRN mRNA expression with induction of p16INK4a mRNA and senescence. Thus each of the RecQ family mRNAs is expressed in proliferating MEFs, but only WRN mRNA expression is maintained as the cells express p16INK4a and undergo senescence.

Mutation of the Rb family leads to induction of RecQ family mRNAs

To further examine the role of the Rb family in RecQ gene expression, we used another model system; MEFs where all three Rb family member genes were mutated (TKOs) [29–30]. A knockout of all three family members is important to fully test a role for the family, because remaining family members expand their activities to compensate when a family member(s) is lost [18]. When compared with β-actin mRNA as a control, the level of the three RecQ mRNAs was similar in wild-type MEFs; however, we found that mRNAs for each of these RecQ genes were induced in the TKOs compared with wild-type MEFs (Figures 3A and 3B). Thus expression of each of the three RecQ genes appeared to be under constitutive repression by Rb family members in the wild-type MEFs.

E2Fs and Rb bind RecQ gene promoters in vivo

We examined the sequence of the RecQ gene promoters for consensus E2F-binding sites (Figure 4A). Each of the promoters has similar sequence characteristics in that the gene was immediately flanked on the 5′ end by a GC-rich sequence which in turn was followed by one or more consensus E2F-binding sites. Additional consensus E2F-binding sites were present (Figure 1B). Similarly, expression of RECLQ4 and BLM mRNAs was also repressed; however, expression of WRN mRNA was unaffected (Figure 1B).
Figure 4 Binding of E2Fs and Rb to RecQ genes *in vivo*

(A) Sequences of the mouse RecQ gene promoters are shown with consensus E2F sites boxed. `+1` indicates the 5′-end of the genes. (B) ChIP assays showing binding of E2F1, E2F4 and Rb to RecQ promoters in MEFs *in vivo*. (B and C) Control ChIP assays showing no binding of E2Fs or Rb to the GAPDH promoter. Pan H3 and H3 antibodies are positive controls which recognize both modified and unmodified histones. ‘IgG’ indicates non-specific antibody controls. ‘Input’ indicates 10% of the DNA used for immunoprecipitation.

Further upstream, and multiple consensus-binding sites were also evident in the human promoters, which shared similar characteristics with the mouse promoters (results not shown). Next, ChIP assays were performed to determine whether E2F and Rb bind to these RecQ gene promoters *in vivo*. E2F1 and E2F4 were examined as well-studied members of the E2F family [18]. E2F1 contains a transactivation domain and, in the absence of Rb family members, it can contribute to transcriptional activation. By contrast, E2F4 lacks a transactivation domain, and thus its primary role is repressive (e.g. recruiting an Rb family repressor complex to target genes) [17,18]. We found that both E2Fs as well as Rb appeared to bind to the promoters in wild-type MEFs (Figures 4B). As a negative control, neither E2F nor Rb was detected at the *Gapdh* promoter (Figure 4C). Although it is important to present gels of PCR products to ensure that single products of the correct size are seen, it is of note that these gels are performed on products after PCR amplification is complete, thus they are not quantitative. Therefore we then compared binding of these E2Fs in wild-type and TKO MEFs, using real-time PCR to quantify the PCR products. Although binding of E2F1 was similar in MEFs and TKOs, E2F4 binding to the promoters increased in TKOs (Figures 5A–5C). E2F4 lacks a nuclear localization signal, and thus it was thought to be dependent on Rb family members for nuclear localization [31]. However, more recent studies have demonstrated E2F4 at numerous promoters in the absence of Rb family members [21]. The finding that E2F4 is bound to the promoters in TKO cells in place of E2F1 (which correlates with transcriptional activation), implies that E2F is functioning primarily in recruitment of an Rb family repressor complex to these promoters, as opposed to providing a transactivation function (which is absent in E2F4).

**Rb-family-dependent repressive epigenetic modifications at RecQ gene promoters**

Next, we used ChIP assays to examine epigenetic marks at the RecQ gene promoters in wild-type compared with TKO MEFs. Acetylation of histones H3 and H4 disrupts nucleosome assembly, and thus is associated with a transcriptionally active chromatin conformation [32]. The Rb family has been shown to bind HDAC (histone deacetylase) [17] and to be important for deacetylation of histone H3 [33]. Using real-time PCR to quantify PCR products, we found recruitment of HDAC2 to each of the promoters in MEFs (this recruitment was less pronounced with the *BLM* promoter) (Figures 5A–5C). HDAC2 binding was diminished in TKO cells, which lack Rb family members. Correspondingly, there was a significant increase in acetylation of histone H3 at the *RECQL4* and *WRN* promoters in TKO cells (Figures 5A and 5B). Histone H3 can be trimethylated on Lys9 (H3-K9 trimethylation) and histone H4 can be trimethylated on Lys14 (H4-K20 trimethylation), which prevent inhibitory acetylation and are marks of repressive heterochromatin; the Rb family has been shown be associated with both of these repressive marks [32–35]. Although we found evidence of H3-K9 trimethylation at the promoters,
Figure 5 Epigenetic changes and alterations in protein recruitment at RecQ gene promoters when the Rb family is mutated

ChIP assays were used to compare RecQ gene promoters in wild-type MEFs and TKOs. Real-time PCR was used to quantify the results. Values were normalized to input DNA, and ratios from three independent experiments are shown +/− S.D. Ac, acetylated histones; H3-K9, trimethylated H3-K9; H4-K20, trimethylated H4-K20; pan antibodies recognize modified and unmodified histones.

this methylation did not change with Rb family mutation in the TKOs (Figures 5A–5C). We also found H4-K20 trimethylation at the WRN promoter in MEFs and this methylation was diminished in TKOs (Figure 5B). Less H4-K20 trimethylation was seen at the RECQL4 and BLM promoters, and there was little or no significant change in this methylation at these promoters in TKOs (Figures 5A and 5C).

Induction of RecQ mRNAs in response to oncogenic Ras

Previous studies have shown that RecQ genes are induced by oncogenes such as c-Myc [14]. Perhaps the most common oncogenic mutations in cancer are those leading to constitutive activation of a Ras gene. Activated Ras is not sufficient to transform wild-type MEFs (it triggers oncogenic stress and Rb-family-dependent senescence), but it is sufficient to transform TKO MEFs, leading to their ability to form tumors in immunodeficient mice [30]. We then asked whether expression of activated Ras in the TKOs would affect expression of RecQ genes. TKOs were infected with a Ha-Ras V12-expressing retrovirus, and this led to increased Ras expression and activity in the cells (Figures 6A and 6B). As reported previously [30], this activated Ras expression also allowed the cells to grow efficiently in soft agar and form tumors in immunodeficient mice (results not shown).

Using real-time PCR, we found that expression of mRNAs for RECQL4, WRN and BLM was induced by activated Ras (Figure 6C). This induction was beyond that seen with Rb family mutation, indicating an additive effect of Rb family mutation and Ras activation in RecQ gene induction. Thus the RecQ genes are induced by both of the oncogenic mutations leading to a tumorigenic phenotype in the MEFs (mutation of the Rb family and Ras activation).

Neither Rb family mutation nor activated Ras increases the half-life of the RecQ mRNAs

The studies above suggest that the Rb family and E2F bind to regulate transcription of the RecQ gene promoters. However, these findings do not rule out an effect of the Rb family and E2F on RecQ mRNA stability. Additionally, the mechanism through which activated Ras increases RecQ mRNA levels is unknown. Therefore we analysed RecQ mRNA stability in wild-type MEFs, TKO and TKO-Ras cells. As a control, we compared stability of β-actin in the three cell types. β-Actin mRNA stability was similar in the three cell types (Figure 7). RECQL4 mRNA appeared generally less stable than mRNA for BLM and WRN (Figure 7), but importantly stability of the three mRNAs in wild-type MEFs was greater than or equal to that seen in TKO or TKO-Ras cells. Therefore neither Rb family mutation nor expression of activated Ras leads to an increase in RecQ mRNA stability. Thus we conclude that the effects of Rb family mutation and Ras activation on RecQ mRNA levels must be at the level of transcription.

Ras activation and Rb family mutation have an additive effect on telomere length

Previous studies have demonstrated that overexpression of BLM triggers telomere elongation [8]. In a collaborative study, we previously found that overall telomere length was increased approx. 2-fold with Rb family mutation [19]. Although only correlative, this telomere length increase occurred in conjunction with induction of the RecQ genes. Because activated Ras causes further induction of RecQ genes, we wondered whether Ras activation might also affect telomere length. To quantify the relative number of telomeric repeats, we used a real-time PCR-based assay [33]. As previously, we found an approx. 2-fold increase in the number of telomeric repeats in the TKOs, and there was an additional 5-fold increase in the TKO-Ras cells (Figures 8A and 8B). We found previously that neither expression...
Figure 7 RecQ mRNA stability in wild-type MEFs, TKOs and TKO-Ras cells

Actinomycin D was added to block transcription, and mRNA levels were quantified by real-time PCR at different time points. Starting mRNA levels were normalized to 1. An average of three independent assays ± S.D. are shown. ACTB, β-actin.

Figure 8 Induction of RecQ genes by Rb family mutation and activated Ras correlates with lengthening of telomeres in the absence of induction of telomerase

(A) Real-time PCR was used to compare the relative number of telomere repeats in TKOs and TKO-Ras cells. 36B4 (also know as acidic ribosomal phosphoprotein P0) is a single-copy gene used as a DNA-loading control. Similar results were seen using the β-actin gene as a control. (B) Standard curve showing the fold-change quantified by real-time PCR in telomeric repeats and a control single-copy gene (36B4) compared with genomic DNA input. (C) Real-time PCR showing the level of telomerase mRNA in the indicated cells. ‘ES’ indicates D3 mouse embryonic stem cells.

The results of our present study link two of the most common oncogenic mutations (Rb pathway disruption and Ras activation) to induction of RecQ genes. These mutations both stress cells to increase DNA replication, and the induction of RecQ genes in the mutant cells may facilitate this increased DNA replication as well as telomere maintenance.

Interestingly, there were differences in regulation of the RecQ genes by Rb family/E2F, and there were also differences in the two cell types that we used to examine expression. For example using mER-DB-E2F to acutely displace E2F complexes from target genes in U2OS cells, did not affect expression of BLM or RECQL4 mRNAs, but it induced expression of WRN mRNA. However, mutation of Rb family members in MEFs led to induction of each of the RecQ genes, indicating that the genes are under constitutive repression by Rb family members in proliferating MEFs. Although it was originally presumed that repression of genes by Rb family members was cell-cycle-dependent, more recent studies have found three classes of genes regulated by the Rb family: (i) genes such as cell-cycle regulators that are repressed only when the Rb family is activated (e.g. via expression of p16INK4a) and cells arrest [21]; (ii) genes which are constitutively repressed by the Rb family in a cell-cycle-independent fashion, and Rb family activation does not lead to further repression; and (iii) genes under mixed regulation, they are under repression by the Rb family but are further repressed when the Rb family becomes activated. It appears that WRN falls into category (ii), whereas BLM and RECQL4 fall into category (iii). Although mutation of RecQ genes can lead to genomic instability and cancer predisposition, there is mounting evidence that overexpression of RecQ genes in cancer can facilitate the increased need for DNA synthesis and telomere maintenance in transformed cells.

Taken together, these findings suggest that careful modulation of RecQ gene expression is important. We propose that the partial repression imposed constitutively on the genes by the Rb family is important for maintaining their level.

Overexpression of oncogenes such as Myc and Ras are frequent in tumours. As protection against such oncogenes, cells normally undergo senescence in response to such overexpression. It has...
been presumed that this senescence is the direct result of induction of cell-cycle inhibitors such as p16INK4a. However, the finding that Myc induction of WRN can overcome senescence instead implies that induction of cell-cycle inhibitors may be a secondary event which occurs because overexpression of oncogenes such as Myc or Ras places stress on the cell to increase DNA synthesis (e.g. to keep up with increased cell cycling) and maintain telomere length. A failure to increase DNA synthesis transmits a DNA damage signal, which in turn leads to elaboration of cell-cycle inhibitors. In the present study we provide evidence that a second oncogene, Ras, which like Myc classically triggers senescence, induces RecQ genes. We show that this occurs in the absence of functional Rb family members (which is a hallmark of most cancers), but this scenario apparently does not occur in wild-type cells which undergo senescence under these conditions. Although the mechanism of transcriptional activation of these genes by Ras is still unknown, it is of note that there is an established link between Myc and the Rb family. Classically Myc and Ras collaborate to transform MEFs; however, Myc is no longer required when the Rb family is mutated [30]. Indeed, Myc has been shown to up-regulate E2F1-3, which has transactivation domains [36], and whose overexpression would be expected to induce transcription of the RecQ promoters. But why would this not occur in wild-type cells? It has been demonstrated that overexpression of E2F1 in cells with functional Rb family members can actually cause assembly of additional Rb/E2F1 repressor complexes at target genes [25]. Thus expression of E2F1-3 may only effectively transactivate the genes in tumours where the Rb family is mutated or inactivated (e.g. via constitutive hyperphosphorylation by cdk5). Indeed, we show in the present study that while there is induction of the RecQ genes with mutation of the Rb family of repressors (analogous to loss of Rb family function in tumours), the predominant E2F species at the RecQ promoters in TKO cells is E2F4, which lacks a transactivation domain. Thus Myc induction of E2F1-3, and the resulting potential replacement of E2F4 at the promoters with these transactivating E2Fs, would be expected to lead to further transactivation of the RecQ genes.

It is also of note that WRN expression is not cell-cycle-dependent, and it was the only one of the three RecQ genes whose expression was maintained in senescent cells. It has been demonstrated that WRN is required to prevent oxidative DNA damage in non-proliferating cells [37]. We suggest that this role is dependent upon WRN because of its expression pattern. Furthermore it is of note that clinical symptoms in Werner syndrome appear later than in patients with mutations in BLM or RECQL4, and that many of the effects can be characterized as premature aging. Senescence of non-proliferating stem cells triggered by cumulative oxidative DNA damage has been proposed as an aging mechanism [38]. Thus it is possible that the premature aging effects seen in Werner syndrome reflect its important role in preventing accumulation of oxidative damage in quiescent stem cells, and that the relatively late onset of symptoms reflects the point when the accumulation of such damage finally begins to trigger senescence.

It is of note that we observed H4-K40 trimethylation at the WRN promoter. This histone modification mediates stable formation of heterochromatin, for example in the pericentri of chromosomes [33,34]. Interestingly, as with WRN expression, this heterochromatin assembly in pericentrichromatin is dependent upon the Rb family, but is not cell-cycle regulated. We propose that H4-K20 trimethylation at the WRN promoter is responsible for establishing a stable heterochromatin-like complex that limits WRN expression. This complex is Rb-family dependent for assembly, but once established is not further regulated by Rb family activation. However, the complex is not established or is lost when Rb-family function is disrupted. By contrast, there was less evidence of stable marks of histone trimethylation of H3-K9 or H4-K20 at the other two RecQ promoters. However, there was Rb-family-dependent recruitment of HDAC2 to these promoters, implying that their activity may be regulated primarily by a more reversible process of histone acetylation–deacetylation at sites on the promoters.

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REFERENCES
Y. Liu and others
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