Rb1 haploinsufficiency promotes telomere attrition and radiation-induced genomic instability

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Abstract

Germ-line mutations of the retinoblastoma gene (RB1) predispose to both sporadic and radiation-induced osteosarcoma, tumors characterized by high levels of genomic instability and activation of ALT (alternative lengthening of telomeres). Mice with haploinsufficiency of the Rb1 gene in the osteoblastic lineage reiterate the radiation-susceptibility to osteosarcoma seen in patients with germ-line RB1 mutations. We demonstrate that the susceptibility is accompanied by an increase in genomic instability resulting from Rb1-dependent telomere erosion. Radiation exposure did not accelerate the rate of telomere loss but amplified the genomic instability resulting from the dysfunctional telomeres. These findings suggest that telomere maintenance is a non-canonical caretaker function of the retinoblastoma protein, such that its deficiency in cancer may potentiate DNA damage-induced carcinogenesis by promoting formation of chromosomal aberrations, rather than simply by affecting cell cycle control.
Introduction

The Fukushima nuclear accident has reawakened concerns about the long-term health consequences of radiation exposure, especially the risk of cancer and the contribution of individual genetic susceptibility to that risk. However, little is known about the cellular events that follow radiation exposure and lead to the development of cancer in a susceptible individual (1).

Osteosarcoma is a tumor that is readily induced by exposure to ionizing radiation, in particular through the deposition of alpha-particle emitting radionuclides in the mineralizing skeleton. This is reflected by the high osteosarcoma incidences in luminescent dial painters who ingested large quantities of radium salts (2), and in children treated with preparations of thorium (3). Exposure of the skeleton to external photon irradiation is also osteosarcomagenic, with this tumor being a frequent secondary cancer arising in radiation therapy fields (4).

Germ-line mutation of the RB1 tumor suppressor gene increases sensitivity to both sporadic and radiation-induced osteosarcoma (5-7). A study of the genetic determinants that predispose inbred mouse strains to radiation-induced osteosarcoma following injection of bone-seeking $^{227}\text{Th}$ revealed a major modifying gene mapping to the Rb1 locus (8). Analysis of the increased susceptibility to radiation that is associated with Rb1 is hindered by the high sporadic rate of cancer in animals engineered to lack one copy of Rb1 in the germ line (9). However, the increased sensitivity to radiation could be reiterated in mice that had been rendered Rb1 haploinsufficient through the conditional deletion of one Rb1 allele in the osteoblastic lineage (6).

Osteosarcomas are characterized by the frequent activation of the ALT pathway of telomere maintenance (10) and a high degree of genomic instability. As the loss of telomeric DNA may lead to karyotypic instability (11), and as RB1 haploinsufficiency has been associated with increased genomic instability in pre-malignant retinoma cells (12), we postulate that the Rb1 gene may be the common denominator linking susceptibility, genomic instability and telomeric integrity. We report here that the loss of a single copy of the Rb1 gene is sufficient to cause
sustained telomeric attrition and spontaneous genomic instability in cells of the osteoblastic lineage. The extent of the genomic instability is increased following exposure to radiation.

Materials and methods

Animal breeding and conditional mutagenesis

FVB/N Rb1loxP/loxP mice (13) were obtained from the NIH Mouse Models of Human cancer Consortium, whilst the FVB/N COL1A1-Cre-Tg mice (14) were obtained from the NIH Mutant Mouse Regional Resource Centre at UC Davis, California. The ROSA26R reporter mice B6:129-Gtrosa26sm1Sor (15) were purchased from the Jackson Laboratory, Maine. All animals were housed in facilities approved by the animal welfare committee of the State of Bavaria. The correct targeting of the Cre expression to the osteoblastic lineage of in vivo was confirmed using (FVB/N COL1A1-Cre-Tg x B6:129-Gtrosa26sm1Sor) mice (Supplemental figure S1).

Inactivation of one allele of the Rb1 gene in the osteoblastic lineage was achieved by mating FVB-COL1A1-Cre-Tg and FVB-Rb1loxP/loxP mice.

Primary osteoblast explant cultures

Long bones of 2-4 week old offspring of FVB-COL1A1-Cre-Tg x FVB-Rb1loxP/loxP mice were isolated as described previously (16). In brief, long bones were excised under sterile conditions and scraped free of adherent tissue. After removal of the epiphyses the bone marrow was flushed using 1ml PBS. Fragments of the cleaned bone were placed into 6 well dishes and cultured in 1 ml DMEM supplemented with 10% FCS. After one week the emergent osteoblastic cells were re-plated to give passage 1 (P1).

All cells were genotyped for Cre, Rb1 and the excised Rb1+/Δ19 locus (supplemental figure S2A). For all studies the wild type Rb1+/+ and Rb1+/Δ19 cells used were Cre positive, unless specifically stated otherwise. As expected no Cre mRNA transcripts were detectable in cultured osteoblasts (supplemental figure S3), due to the repression of the collagen gene expression in the absence of differentiation-promoting medium supplements (16).
Senescence-associated β-galactosidase assay
The assay was performed using the β-gal staining kit (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's instructions.

Micronuclei assay: The presence of DNA micronuclei fragments was quantified using a standard protocol (17). 250,000 cells were seeded onto glass microscope slides and 24h later were irradiated with either 2Gy or 4Gy using an Isovolt 200 X-ray device operating at 230kVp and 20mA, with a 1mm Al and 0.5mm Cu-filter. Sham-irradiated cells were handled in the identical manner without exposure to the X-irradiation. Cytochalasin B (0.5μg/ml) was added to the cultures for 18 hours at time 0 after irradiation to block the next cytokinesis. Cells were subsequently fixed in 80% ethanol for 15 minutes. DNA was visualized using DAPI staining (150 ng/ml) for 5 minutes. Slides were washed in distilled H₂O, air-dried and cover slip mounted using one drop of Vectashield. Scoring of micronuclei in binucleate cells was performed according to the HUMN project protocols.

Quantification of anaphase bridges: The fidelity of chromosomal segregation was determined by visualization of the chromatin in post-mitotic cells. 250,000 cells per glass slide were irradiated 24 hours after seeding at 0Gy, 2Gy and 4Gy (see above). Cells were grown for a further 48 hours to allow at least 4 cell divisions (cycle time of approximately 12h). After fixation in 80% methanol for 15 minutes the slides were stained with Hoechst 33342 for 5 minutes, washed twice with PBS and cover slip mounted with one drop of Vectashield. Scoring was performed visually by counting the number of bridges per 250 cells. Each cell revealing a spontaneous or radiation induced anaphase bridge was scored once, even if multiple bridges existed within that cell. Chromatin bridges between two post-mitotic daughter cells were also scored and were considered to belong to one parental cell.

Propidium Iodide labeling for FACS analysis of the cell cycle: Cells were trypsinised, washed with 5 mL PBS and spun down at 1400 rpm for 5 min 5 mL of 70% Ethanol (cold) was added while gently vortexing and left in Ethanol at 4°C for 4 hours. The fixed cells were spun down at 1400 rpm for 5 min. and resuspended 475 μl PBS, plus 3.3 μl RNAs (30 mg/ml) and...
25 µl Propidium Iodide (1 mg/ml). Cell cycle analysis was performed using a FACSCoulter (Becton Dickinson, San Jose, CA, USA).

**Telomeric staining by in-situ FISH:** Telomere length was determined by in situ FISH labeling of genomic DNA (18). Chromosome spreads or interphase nuclei were prepared by incubating cells with 0.05 µg/ml Colcemid for 6h. Cells were harvested, incubated in 0.056 M KCl at 37°C for 10 min and fixed in methanol/acetic acid (3:1). Metaphase chromosomes were spread on glass slides, air dried overnight and fixed in 4% formaldehyde in 1xPBS for 4 min. To perform the hybridization, the samples were treated with 100 µg/ml RNase A in 2xSSC at 37°C for 1h, dehydrated through an ethanol series (70%, 85% and 100% ethanol for 4 minutes each) at room temperature and air-dried. A hybridization mixture (70% formamide, 0.5% BSA, 10 mM Tris-HCl (pH 7.2) and 0.5 µg/ml FITC conjugated (C₃TA₂)₃ peptide nucleic acid (PNA) probe (PANAGENE, Korea) was applied onto the slides. DNA was denatured at 75°C for 5 min. Hybridizations were carried out at room temperature for 4 h. Slides were washed 2 x 15 min in 70% formamide, 10 mM Tris-HCl (pH 7.2) and 3 x 5 min in 0.1 M Tris-HCl, 0.15 M NaCl, 0.08% Tween-20 (pH 7.5) at room temperature. Slides were covered with one drop of Vectashield (containing 100 ng/ml DAPI for counterstaining) and mounted with a cover slip.

**Telomere length measurement by flow-cytometry (Flow-FISH):** FISH labeled telomeres were quantified as previously described (19). Primary cells in culture were washed in phosphate buffered saline (PBS) and centrifuged, the supernatant was discarded and 500,000 cells resuspended in 500 µl of hybridization mixture containing 70% deionized formamide, 20mM Tris buffer, pH 7, 1% bovine serum albumin (BSA), and 0.3 µg/ml of the FITC conjugated PNA probe. Samples were denatured for 10 min at 80°C and left to hybridize for 3 h in the dark at room temperature. Samples without PNA probe were used as negative control. Excess probe was removed in a wash solution containing 70% formamide, 10 mM Tris buffer (pH 7), 0.1% BSA, and 0.1% Tween-20 followed by two washes in PBS, 0.1% BSA, and 0.1% Tween-20. Cells were then incubated in 500 µl PBS, 0.1% BSA, 10 µg/ml RNase A, and 0.1 µg/ml propidium iodide for 1 h at 4°C before analysis. All samples were stored on ice until analysis by flow-cytometry.
Analysis was performed on fresh samples using a FACSCoulter (Becton Dickinson, San Jose, CA, USA). The FITC signal was detected in the green channel and the propidium iodide fluorescence in the red channel with no compensation set on the instrument.

**Lentiviral expression vectors:** RB1 expressing lentivirus (LV-RB1) was produced by PCR amplification of the RB1 cDNA insert of cDNA clone MGC 29887 (Life Technologies, Darmstadt, Germany). The insert was cloned into the lentiviral vector (pCDH-EF1-MCS-T2A-copGFP) (Biocat, Heidelberg, Germany). The Rb1 shRNA-expressing lentivirus (LV-shRb1) was generated by insertion a short hairpin sequence targeting Rb1 (GCTTAATCAGAAGAAGAA) into the pFUGW plasmid (20). Control shRNA lentivirus (LV-scr) was generated using a scrambled sequence (Dharmacon research, Chicago). Constructs were sequenced to confirm error-free amplification prior to transduction. Cre-expressing lentivirus under the control of the CMV promoter with a GFP expression cassette (LV526) for transduction control was purchased from GenTarget, San Diego, CA. In this case the control lentivirus expressed only the GFP mRNA (LV-GFP).

Production of lentivirus was performed as recently described (21). For all lentiviral transductions we used an MOI of 2 (2x10^5 cells per well and 4x10^5 TU/ml). Transduction efficiency was determined 72 hours after infection by flow-cytometric analysis of GFP-positive cells. Three or four days after infection cells were analyzed by RT-PCR and western blotting to ensure efficacy of the expression.

**Analysis of status of genes involved in osteosarcomagenesis in ex-vivo cultured osteoblast cells**

The coding sequences of Rb1, Cdkn2a/Arf and p53 were amplified from cDNA and subjected to cycle sequencing using the BigDye 3.1 kit (Applied Biosystems, Foster City CA, USA). Mdm2 gene copy number was determined by real-time quantitative PCR of genomic DNA (SybrGreen, Applied Biosystems, Foster City CA, USA). Relative copy numbers of Mdm2 were calculated by the ΔΔCt method, using normal mouse tissue as the diploid reference. The primers used for amplification and sequencing are listed in supplemental material.
Results

Haploinsufficiency of Rb1 leads to dysfunctional cell cycle arrest and senescence following radiation exposure

To confirm the conditional deletion of one Rb1 allele and the expected haploinsufficiency we used exon-specific PCR to amplify both wild-type and exon 19-deleted copies of the Rb1 gene. The content of the wild type Rb1 allele was almost halved in cells where the exon 19 deleted allele was present (supplemental figure 2A). The same change was evident at both the transcriptional (supplemental figure S2C) and translational levels (Figure 1A and B) confirming haploinsufficiency. No reduction of the remaining wild type Rb1 allele was evident, even after 22 continual passages (supplemental figure S2B).

As expected from the known tumour suppressor role of Rb1 in cell cycle regulation the reduced level of Rb1 altered the cell cycle kinetics, with a reduced number of cells present in both the G1 and S Phases assayed by flow cytometry (Figure 1C). The presence of a large fraction of polyploid cells may account for this shift as the population doubling time remained unaffected. A functional deficit in the cell cycle checkpoints was evident following a challenge with 2Gy acute irradiation. In wild type cells the expected rapid G2/M arrest after irradiation was evident, but was severely abrogated in haploinsufficient Rb1+/Δ19 cells (Figure 1C). This was associated with a further increase in the polyploid fraction but not the appearance of a subG₀ apoptotic fraction. Cell stress induced by the exposure to ionizing radiation effectively induced senescence of wild type cells in a dose-dependent manner. However, the induction of senescence was diminished in haploinsufficient cells, even following a 4Gy exposure (Figure 2A).

Rb1 haploinsufficiency is associated with sporadic increases in micronuclei formation and the polyploid cell fraction

Possible alterations in the stability of the genome in Rb1 haploinsufficient cells were studied by examining micronuclei and polyploidy. The number of spontaneous micronuclei (acentric fragments of genomic DNA) (Figure 2B) was elevated in Rb1+/Δ19 cells when compared to Rb1 proficient wild type cells. As with the polyploid fraction, the number of cells showing
micronuclei rose with increasing passage number. Polyploid cells were also present in wild type osteoblasts, where they also showed an increase in numbers at higher passages (Figure 2C). In contrast, the size of the polyploid cell fraction in haploinsufficient $Rb1^{+/Δ19}$ cells increased dramatically with increasing passage numbers, comprising almost 25% of the population by passage 12 (Figure 2C). Even though we are unable to definitively determine the origin of the polyploid cells a dot plot of cell size vs. DNA content of the haploinsufficient cells indicates that a small percentage of the measured G2/M population are possibly an uncertain overlap of tetraploid G1 cells.

The spontaneous genomic instability of the $Rb1$ haploinsufficient cells is accompanied by increased telomere attrition

The stability of telomeric regions is associated with loss of genome stability. We therefore quantified telomeric integrity after loss of one Rb1 allele. Labeling of telomeric DNA sequences with the fluorescent Cy3-PNA telomeric DNA probe revealed the presence of telomeric DNA within the micronuclei in both wild type and $Rb1^{+/Δ19}$ cells at passage 11. The Rb1 haploinsufficient cells exhibited more than two times the number of telomere-positive micronuclei than the wild type cells (Figure 2D). An examination of interphase cells indicated an overall reduction in the level of PNA-labeled telomeric sequences in $Rb1^{+/Δ19}$ cells, compared to their wild type counterparts. Labeling of metaphase spreads revealed that the decrease in telomeric signal strength was due to a general reduction in signal intensity and to the development of considerable heterogeneity in the labeling intensity of different chromosomes in $Rb1^{+/Δ19}$ primary osteoblastic cells (Figure 3A).

Assay of telomere length using quantitative genomic PCR amplification (Figure 3B and 3C) established that there was a global reduction in telomere repeats in the $Rb1^{+/Δ19}$ cells that coincided with the reduced PNA labeling. As with the parameters describing genomic instability the reduction in the average telomere lengths of $Rb1^{+/Δ19}$ cells grew larger with subsequent passages compared to wild type cells. The mean number of 102 telomeres detected in wild type cell at passage 8 approached the theoretical mean value of 108 telomeres that
would be expected, taking into account the relative numbers of diploid and tetraploid cells we observed in asynchronous cultures. The number of distinct PNA-labeled telomeres in $Rb1^{+/Δ19}$ cells was reduced by more than half (Figure 3D).

**Telomeric loss is induced by shRNA mediated knock-down of $Rb1$ expression**

To confirm that the engineered $Rb1$ haploinsufficiency is indeed responsible for the observed changes we studied the effect of a direct knock-down of $Rb1$. Wild type and haploinsufficient $Rb1^{+/Δ19}$ cells were transduced with the LV-$shRb1$ lentivirus, This lead to the expected reduction in $Rb1$ expression (supplemental figure S4) and was associated with a reduction in telomere length as early as the third passage post-transduction in both wild type and haploinsufficient cells, compared to the same cells transduced instead with the LV-scr control lentivirus (Figure 3E). The decrease in telomere length was more pronounced when LV-$shRb1$ was used to further deplete the already reduced $Rb1$ expression of the $Rb1^{+/Δ19}$ cells (Figure 3E).

Transduction of Cre negative wild type ($Rb1^{+/+}$) cells and of Cre negative cells containing a non-deleted floxed $Rb1$ allele ($Rb1^{+/fl}$) with the Cre-expressing lentivirus resulted in in vitro Cre expression (supplemental figure S5). Telomere shortening was observed in the Cre-expressing primary osteoblasts containing the non-deleted floxed $Rb1$ allele ($Rb1^{+/fl}$) but not in the Cre-expressing wild type ($Rb1^{+/+}$) cells three passages after transduction (supplemental figure S6).

**Telomere loss and genomic instability in $Rb1^{+/Δ19}$ haploinsufficient cells are prevented by expression of $RB1$**

As a final proof of the role of $Rb1$ in telomere maintenance we followed telomeric status after restoration of $Rb1$ expression. The accelerated rate of telomere loss in $Rb1^{+/Δ19}$ cells could be prevented by transduction with LV-$RB1$ lentivirus, whilst transduction with control LV-GFP lentivirus had no effect, with telomere shortening continuing unabated (Figure 4A). The expression of $RB1$ in the haploinsufficient $Rb1^{+/Δ19}$ cells rescued the increased level of sporadic
genomic instability, as measured by the number of anaphase bridges per metaphase plate (Figure 4B). The expression of RB1 following transduction was equivalent to that of endogenous Rb1 in wild type cells (supplemental figure S7) and did not cause cell cycle arrest. Thus, the proportion of cells in G1, S and G2/M -Phase were comparable to those of wild type cells at the same passage (38.5%, 21.5% and 40% vs. 39.6%; 22.8% and 37.6%).

Telomere attrition is not a consequence of changed oncogene / suppressor gene activity
The direct sequencing of the p53, Cdkn2a/Arf, and the remaining wild type Rb1 allele in Rb1+/Δ19 cells at the 12th passage did not reveal any alterations in the coding regions of these genes. In addition, the copy number of the Mdm2 oncogene was shown to be the same as that found in wild type tissue (supplemental figure S8). P53 expression was undetectable in wild type and haploinsufficient Rb1+/Δ19 cells, but in a subclone of apparently spontaneously transformed cells detected in long-term cultures of Rb1+/Δ19 cells we were able to detect a point mutation in exon 8 of the p53 gene, leading to accumulation of immunoreactive p53 protein (supplemental figure S9).

Radiation exposure increases genomic instability of Rb1 haploinsufficient cells
The double strand breaks induced by ionizing radiation may promote genomic instability in cells already rendered susceptible by lack of telomeres. Consequently we exposed haploinsufficient Rb1+/Δ19 cells to an acute dose of radiation. The exposure to ionizing radiation caused cytogenetic abnormalities (micronuclei) in equal numbers of wild type and haploinsufficient Rb1+/Δ19 cells 24h after exposure, attesting to the uniformity of the radiation field (supplemental figure S10, Figure 5A). However, the number of micronuclei induced in 2Gy irradiated haploinsufficient Rb1+/Δ19 cells greatly exceeded that induced by the same exposure in wild type cells (Figures 5B) from 1.45 +/-0.13 in the wild-type to 2.2 +/- 0.26 in the haploinsufficient cultures. The number of radiation-induced anaphase bridges in Rb1+/Δ19 cells was more than four-fold higher than that in irradiated wild type cells (Figure 5C). The size of the polyploid cell fraction was also increased by radiation exposure in both wild type and Rb1+/Δ19 cells. The number of polyploid cells arising in the irradiated haploinsufficient
*Rb1+/Δ19* cells was considerably greater (29.6%) than that seen in the irradiated wild type cells (2.8%) (Figure 5D). Importantly, the radiation exposure did not influence the telomere length in either the wild type or haploinsufficient cells (Figure 5E). Telomeric DNA was present in both acentric fragments (micronuclei) and anaphase bridges (Figure 5F) indicating involvement of the truncated telomeres in the radiation induced increase in genomic instability.

**Discussion**

Loss of one copy of the retinoblastoma gene (*RB1*) increases predisposition to the development of both sporadic and radiation-induced osteosarcoma (4, 22). The increase in sensitivity to radiation-induced osteosarcoma is reiterated in a mouse model upon conditional inactivation of the *Rb1* gene in the osteoblastic lineage (6). We now show that loss of only one *Rb1* allele in non-transformed primary osteoblast cell cultures is accompanied by a number of hallmarks of genomic instability. Thus, in *Rb1+/Δ19* cells, as well as in cells where the endogenous Rb1 expression was suppressed by shRNA, we observe the appearance of a polyploid cell fraction, the presence of sporadic micronuclei (acentric DNA fragments), and extreme shortening of the telomeric DNA. Restoration of the *Rb1* status by lentiviral expression of the human *RB1* gene was sufficient to prevent the continued increase in instability. These observations are consistent with a role for Rb1 in maintaining telomere integrity and ultimately genomic stability.

Reduced telomere length is closely associated with increasing numbers of completed cell cycles. This raises the formal possibility that the shortened telomeres in haploinsufficient *Rb1+/Δ19* cells may have arisen through accelerated cell division. However, no proliferation-promoting mutations were detected in the tumor suppressor genes *Cdkn2a/Arf* and *p53*, nor alterations of copy number changes in the oncogene Mdm2 or in the remaining wild type *Rb1* allele. Thus there is only a small likelihood of expansion of a rapidly proliferating clone of mutant cells. Moreover, the *in vitro* deletion of the floxed *Rb1* allele by lentiviral-mediated expression of the Cre-recombinase resulted in telomere loss within three passages. Wild type cells would require more than 12 passages to reduce telomere length to that induced three passages after the loss of the floxed *Rb1* allele, making it unlikely that telomeric losses can be explained simply by increased proliferation.
A direct action of the Cre recombinase on telomere length can also be excluded as the collagen transgene driving Cre expression is not active in the undifferentiated osteoblast cells studied in vitro, leading to undetectable levels of Cre in these cells (16). The presence of lentiviral expressed Cre recombinase also had no effect on the telomere length of wild type cells, indicating that the recombinase plays no part in the telomere shortening. The telomere reduction showed a consistent relationship to the copy number of the Rb1 gene, with attrition increasing further when the remaining wild type allele was targeted by shRNA knockdown, and reduced attrition when the deficiency in retinoblastoma was reversed by expression of RB1.

Exposure of haploinsufficient Rb1+/Δ19 cells to ionizing radiation raised the level of instability, as indicated by increases in the polyploid fraction, in failed chromosomal segregation, and the appearance of acentric DNA fragments. All three parameters reveal levels of instability that are far above those that are induced by the exposure of wild type cells to radiation. The presence of telomeric DNA within both acrocentric fragments and anaphase bridges in irradiated cells suggests involvement of the shortened telomeres. This is consistent with the formation and breakage of end-to-end fusions between chromosomes capped by truncated telomeres or between truncated telomeres and radiation-induced double strand breaks (23, 24). Such fused chromosomes would be subject to physical tension at the next cytokinesis, causing random breakage with the potential to form chromatin bridges and micronuclear fragments during the next cell cycle (25). Such repeated cycles of breakage-fusion-breakage (BFB) would continually increase the level of instability, as we have observed in the haploinsufficient Rb1+/Δ19 cells. In support of this we observed that telomere length was unaffected by radiation exposure. This indicates that the increased instability arises due to the combination of the induced DNA damage and the pre-existing truncated telomeres.

The loss of one copy of the Rb1 gene, and the resultant haploinsufficiency, impaired the ability of the cells to handle radiation-induced stress by either G2/M arrest or senescence. Impairment of both of these Rb1-dependent DNA damage restoring functions that follow cell stress (23, 26, 27) may explain the persistence polyploid cells generated by the increased genomic instability.
The concomitant loss of the canonical cell cycle regulatory function of \textit{Rb1} would prevent cells with chromosomal damage from entering G1/S arrest and subsequently entering the apoptosis or senescence pathways. This sequence of events is consistent with the existence of a mutator phenotype originally proposed by Loeb (28) to explain the appearance of mutations in tumor cells at a rate above that predicted from the sporadic mutation rate alone. The duality of action (cell cycle and telomere length) that we ascribe to \textit{Rb1} can explain the increases susceptibility to both sporadic and radiation-induced osteosarcoma that is associated with retinoblastoma gene mutations (4, 6). This may also give an explanation for the high levels of chromosomal rearrangements that is frequently observed in osteosarcoma, and more recently in retinoma prior to the loss of the second \textit{RB1} allele (12).

Another mayor component of the \textit{Rb} pathway, \textit{P16INK4A} has been shown to promote telomere erosion, formation of anaphase bridges and genomic instability in tumors of epithelial origin (29). The function of the retinoblastoma gene product in maintaining genomic instability has been suggested to involve chromosome condensation (30), centromere function and mitosis-related genomic instability (31, 32). However, these previous studies indicate an apparently paradoxical situation, where loss of the retinoblastoma family members \textit{p107} and \textit{Rb2/p130}, but not of \textit{Rb1} itself, were seen to promote telomere lengthening (33, 34). This prompts us to speculate that there is a balance between mutually antagonistic interactions of the retinoblastoma family of proteins that serve to regulate telomeric length.
**Figure legends**

**Figure 1:** A: Detection of Rb1 protein expression. Upper panel: Rb1 western blot analysis performed with anti-Rb1 (Becton and Dickinson, Germany). Lane 1 and Lane 2 are biological replicates of wild type cells, lanes 4 and 5 are biological replicates of Rb1<sup>+/Δ19</sup> cells. Lower panel: Blot labelled with anti-Tubulin as loading control. B: Rb1 protein quantification of Rb1 in primary osteoblasts from 3 biological replicates with 2 technical replicated each. A reduction of the Rb1 protein levels of 50% is observed in Rb1<sup>+/Δ19</sup> cultures (t-test: * p \leq 0.01). C: Cell cycle analysis of Rb1<sup>+/+</sup> and Rb1<sup>+/Δ19</sup> cells. A representative FACS-based cell cycle analysis of sham-and 2Gy irradiated cells at passage 6 that were harvested 6 hours after radiation exposure. Cell cycle distribution analysis was performed by setting a fixed gate for each phase of the cell cycle. Impairment of the ability of irradiated Rb1<sup>+/Δ19</sup> osteoblasts to block cell cycle progression in G1/S and to arrest in G2/M were detected.

**Figure 2:** Genomic instability in Rb1<sup>+/Δ19</sup> osteoblasts. A: Entry of irradiated cells into senescence. Radiation exposure was associated with the appearance of the senescence marker SA-βGal in wild type cells but no increase in senescent cells was observed in irradiated Rb1<sup>+/Δ19</sup> osteoblasts. Three biological replicates with 3 technical replicates each were used. B: Quantification of acentric DNA fragments per cell after increasing passages. Data are from two biological replicates. Note that at passage 2 no fragments were detectable in wild type cells. C: Changes in the polyploid population of Rb1<sup>+/+</sup> and Rb1<sup>+/Δ19</sup> cells: Changes in the polyploid fraction at increasing passage numbers. Data are from two biological replicates. D: Telomeric fragments (TFs) present in micronuclei (acentric fragments). The presence of PNA-positive telomeres signals per 200 acentric DNA fragments in wild type and Rb1<sup>+/Δ19</sup> cells. Two biological replicates at passage 11.

**Figure 3:** Increased telomere attrition in Rb1 haploinsufficient primary mouse osteoblasts. A: Representative images (40x) from cell lines that were established from two animals. Panels a) and b) show wild type cells at passage 8. Panels c), d) and e) show haploinsufficient cells (Rb1<sup>+/Δ19</sup>) at passage 8. DAPI nuclear staining is shown in panels a) and c), whilst PNA-Cy3 telomeric labeling is depicted in panels b) and d). Representative metaphase spreads of haploinsufficient cells (Rb1<sup>+/Δ19</sup>) at passage 8 are shown in panel e). Heterogeneous telomeric signal intensity was detected by PNA-Cy3 labeling counterstained with DAPI. Solid arrowheads indicate examples of chromatids with different signal intensities. B: Quantitative genomic PCR measurement of average telomeric repeats. A global PCR amplification of telomeric sequences was performed using (TTAGGG)n primers. The anonymous locus D14-192 (microsatellite flanking region) was used for normalization of genomic DNA content. Rb1<sup>+/Δ19</sup> cells showed a decrease in telomere length of 30% at passage 10, which correlated with the results using Flow-FISH. Data represent mean +/- SD of 3 biological replicates. C: The telomeric length of Rb1<sup>+/Δ19</sup> cells was measured by Flow-FISH counting of 20 000 cells. The length relative to wild type cells of the same passage was followed over consecutive passages. Data are from two biological replicates. D: Average of
PNA signals in interphase nuclei for *Rb1*+/− and *Rb1*+/Δ19 counted in 100 interphase nuclei. Data from 2 biological replicates. **E: Rb1 Knock down.** Telomeric length assayed by Flow-FISH (20 000 cells) at the third passage of cells infected at passage 5 with either control lentivirus (LV-scr) or shRB1 expressing lentivirus (LV-shRB1). Data from two biological replicates.

**Figure 4: Rescue of the Rb1 genotype leads to buffered telomeric loss. A: RB1 expression.** Telomere length was quantified by Flow-FISH at 0, 3 and 6 passages after transfection of passage eight cells using the LV-RB1 lentivirus. Hatched columns are *Rb1*+/Δ19 cells infected with control lentivirus (LV-GFP) and vertical barred columns are *Rb1*+/Δ19 cells transfected with LV-RB1. Data from 2 biological replicates. **B: Anaphase bridges quantification.** Bridges per 100 anaphase plates measured six passages after infection with LV-RB1. Data from 2 biological replicates at passage 6.

**Figure 5: Radiation induced genomic instability in *Rb1*+/Δ19 osteoblasts. A: Compromised divisions: Left panels: Anaphase bridges (AB) and chromatin bridges (CB) detected in DAPI-labeled nuclei of unirradiated *Rb1*+/Δ19 osteoblasts. Right panels: Upper left: Anaphase bridges (AB) and chromatin bridges (CB) detected in DAPI-labeled nuclei of irradiated *Rb1*+/Δ19 osteoblasts. Arrowheads depict acentric fragments (micronuclei). **B: Radiation induced acentric DNA (micronucleus) fragments induced 24h after a 2Gy acute exposure.** Data from three biological replicates. **C: Quantification of anaphase bridges and chromatin bridges induced 24h after a 2Gy acute exposure.** Data from three biological replicates. **D: Polyploidy induced by radiation exposure:** Increase in the polyploid population in *Rb1*+/Δ19 cells 24 hours after exposure to 2Gy, data arising from cell cycle Flow analysis at passage 8. 2 biological replicates. Cells with a DNA content that was higher than 2N were considered to be aneuploid. **E: Telomeric length measured by Flow-FISH 48 hours after an acute 2Gy radiation exposure.** Data from 3 biological replicates of cells grown at passage 8 were used for this experiment. **F: PNA stained chromatin bridges:** Two representative images revealing chromatin bridges (CB) and telomere-containing acentric fragments (TF) 48h after exposure to 2Gy in *Rb1*+/Δ19 osteoblasts at passage 12.
References:


Figure 1

A

1 2 3 4

- pRb1
- Rb1

- α-Tubulin

B

Relative expression ratios (E-Ras)

Rb1+/+

Rb1+Δ19

C

Sham irradiated 6h

Rb1+/+

G1 46.3
S 35.7
G2/M 15.4
Polyploid 2.4

2Gy 6h

G1 33.7
S 12.2
G2/M 51.3
Polyploid 2.8

Rb1+Δ19

G1 39.6
S 14.3
G2/M 23.6
Polyploid 22.4

(Chart data)
Figure 2

A. Bar graph showing percentage of senescence with different doses of radiation. 

B. Bar graph showing acentric fragments per cell with different passage numbers.

C. Bar graph showing percentage of polyploid cells with aging.

D. Bar graph showing telomere fragments per micronuclei with different genotypes.
Rb1 haploinsufficiency promotes telomere attrition and radiation-induced genomic instability


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