Supplementary material

Figures

S1: Cre recombinase mediated deletion of the floxed *Rb1* allele: A: *Lac*Z staining of bone from an F1 cross between *floxed LacZ-Rosa26 reporter mouse x Collagen Type 1 promoter driven Cre*$^{+}$. The X-gal-derived positive blue staining is restricted to the skeleton in *Cre* expressing mice and absent in *Cre*-negative mice. Two biological replicates for each genotype were analysed. B: Alkaline phosphatase (AP) staining to reveal the presence of osteoblastic cells, a) Primary osteoblast cells 24 hours after *in vitro* induction of differentiation showing AP positivity, b) Retinal pigment epithelial cells (negative control), c) ROS 17/2.8 rat osteosarcoma cells (positive control). Determination was performed with 2 biological replicates.

S2: Characterisation of Rb1 expression in the cell lines. A: *Rb1* genotyping of early passage cells (passage 2). The length of the PCR products obtained from the three possible alleles: $Rb1^{floxed\ exon\ 19}$ (700 bp), $Rb1^{wild\ type}$ (650 bp) and $Rb1^{\Delta 19}$ (235 bp) indicates the cellular *Rb1* status. Lane 1: Marker, lane 2: $Rb1^{flox/+}$, lane 3: $Rb1^{flox/flox}$, lane 4: $Rb1^{+/+}$, lane 5: $Rb1^{+/\Delta 19}$.**B:** Genotyping of late passage $Rb1^{+/\Delta 19}$ cells showing retention of the wild type *Rb1* allele after 22 passages. Lane 1 Marker, Lanes 2 and 3 two independent clones of $Rb1^{+/\Delta 19}$ cells. **C:** RT-PCR of $Rb1^{+/+}$ and $Rb1^{+/\Delta 19}$ mRNA levels. The expression of the wild type *Rb1* allele (probe binding exon 19), $Rb1^{\Delta 19}$ allele (probe binding across the rearranged exon 18/20 junction) and pan-*Rb1* (Exon 5 probe binding both wild type and truncated transcripts) were quantified in primary

osteoblast cultures obtained from *Rb1*$^{+/+}$ and *Rb1*$^{+/\Delta 19}$ mice*.* The exon 19-deleted *Rb1* allele was transcribed, and the level of total *Rb1* transcripts (wild type plus deleted allele) was essentially unchanged (supplemental figure S2C). Two biological replicates were used for this experiment.

S3: *Cre* transgene expression in bone and explant cultures of primary osteoblasts. RT-PCR was performed to analyse the *Cre* transgene mRNA levels in bones extracted from *Cre* transgene positive animals and explanted osteoblasts. Expression of the transgene can be seen in the mature osteoblasts in the intact bone but it is absent from the primary osteoblasts in culture due to the undifferentiated phenotype of these cells (16).

S4: *Rb1* mRNA levels after transduction with shRB1-expressing lentivirus. Quantitative PCR amplification of *Rb1* messenger RNA following transduction with either the knockdown lentivirus LV-shRB1 or by the control lentivirus (LV-scr) containing a scrambled RNA sequence.

S5: In vitro *Rb1* excision using lentiviral *Cre*: Upper panel**:** The PCR product of the Cre transgene (727bp) was detectable in all cells 72 hours after transduction with the lentivirus. Lane 1: *Rb1*$^{+/+}$*:CreTg(-)* LV-Cre(-), llane two: *Rb1*$^{+/+}$*:CreTg(-)* LV-Cre(+), lane three: *Rb1*$^{flox/+:}$*CreTg(-)* LV-Cre(-), lane 4: *Rb1*$^{flox/+:}$*CreTg(-)* LV-Cre(+), lane five: marker. Lower panel: Quantification of *Cre* mRNA levels of LV-Cre infected cells determined by RT Q-PCR. TBP was used as the housekeeping gene.

1 **S6: Assessment of telomere length in LV-*Cre* infected cells using genomic PCR**. A
2 global PCR amplification of telomeric sequences was performed using (TTAGGG)n
3 primers. The amplification of an anonymous locus D15mit192 (microsatellite flanking
4 region) was used for normalization of genomic DNA content and data of the infected cells
5 is presented relative to their untransfected counterparts. Data represent mean +/- SD of 2
6 technical replicates.

7 **S7: Transduction with the LVRB1 leads to recovery of retinoblastoma protein**
8 **expression.** Total Rb1 protein levels were determined by western blotting. Upper panel:
9 Western blot of Rb1/RB1 3 weeks after transduction with the LV*RB1* lentivirus, detection
10 using mAB Rb1 (Cell Signalling, Germany) that detects both human and mouse protein. α-
11 tubulin was used as the loading control. Lower panel: quantification of protein bands using
12 ImageQuant software. Data from 2 biological replicates.
13

14 **S8: MDM2 copy number measurement:** PCR quantification of the *Mdm2* gene in $Rb1^{+/+}$
15 and $Rb1^{+/\Delta 19}$ primary osteoblasts showed no amplification at early passage number (p4) or
16 late passages passage number (p13 and p22). Copy number is represented as the ratio of
17 *Mdm2* to D15mit232.
18

19 **S9: p53 expression in primary osteoblasts.** Determined by Western blot: lane 1: $Rb1^{+/+}$,
20 lane 2: $Rb1^{+/\Delta 19}$, lane 3: $Rb1^{+/\Delta 19}$ transformed cells. The p53 protein was not detectable in
21 primary osteoblasts but was detectable in the only foci of transformed cells that appeared

in the cultures. The accumulation of p53 in these cells was found to be due to the acquisition of a mutation in exon 8. α-tubulin served as loading control.

S10: Formation of acentric fragments (micronuclei) after radiation exposure. Affected cells after radiation exposure. The amount of cells harvesting damage after radiation exposure was assayed the same in both cell lines, indicating that both cell lines respond equally to radiation exposure, but is the underlying damage present in the haploinsufficient cultures what increases the final outcome leading to higher absolute damage compared to their wt counterparts.

Primers used in this study

- RB1 PCR amplification primers:

5'NNNNGGATCCTTATTTTTGTAACGGGAGTCGGGAGAGGACG-forward

5'NNGCGGCCGCGTGGCCATAAACAGAACCTGGGAAAG-reverse

- *RB1* Sequencing primers:

RB1-cDNAf1 5'-GGAAGATGATCTGGTGATTTC

RB1-cDNAf2 5'-CTGCAGCAGATATGTATCTTTC

RB1-cDNAf3 5'-GTGCTGAAGGAAGCAACCCTC

RB1-cDNAr1 5'-CTCCCAATACTCCATCCACAG

RB1-cDNAr2 5'-GTATCGCTGTGATCCAATTTC

- Rb1 sequencing:

mRb1c-1760f: CCTTGCATGGCTTTCAGATT

1 mRb1c-2085r: GAAGGCGTGCACAGAGTGTA

2 nRb1c-1763f : TGCATGGCTTTCAGATTCAC

3 mRb1c-2111r: GTGCTCTAGCTCTGGGTGGT

4 mRb1c-996f: GTATCATCTAATGGACTTCCAGAGG

5 mRb1c-1356r: AGATGTGCCCAACATCCTTT

6 mRb1c-1006f: ATGGACTTCCAGAGGTTGAAA

7 mRb1c-1380r: CAGCGTTAGCAAACTTCTCTTT

8 mRb1 -5´ -f1: TGTAACGGGAGTCGGGTGAG

9 mRb1 -5´ -r1: TTCTCCCAAGTTAGCCAAGCTC

10 mRb1 -5´ -f2: TTGTAACGGGAGTCGGGTGA

11 mRb1 -5´ -r2: TCCATCCACGGATGAAACTT

12 Rb1 -f1: CCGATCATGTCAGAGAAAGAGCTTG

13 Rb1 -b1: TCAGGGTTGTTTTTTCGTGGC

14 Rb1 -f10:GAACGCCACGAAAAAACAACC

15 Rb1 -b12: GATGATGTGCTCTAGCTCTGGGTG

16 Rb1 -b13: GAGTCCAGATGATGTGCTCTAGCTC

17 Rb1 -f19: CATTGAAATCTACCTCCCTTGCC

18 Rb1 -b29: GCATTCGTGTTCGAGTGGAAGTC

19 Rb1 -b30 CCTCACTTTTCCTCCTTGTTTGAG

20 Rb1 -f20: GCTAGAGCACATCATCTGGACTCTG

21 Rb1 -b31: CTAACATGAGCAGAACCTGGGAAC

22 Rb1 -b32: GTGGCTTACGAATCACCCACAC

1 Rb1 -f29: ACTCCTGGCTCATGGTTGTGAC

2 Rb1 -b35: GCACTTGGGTTGTACTGTACTAGGG

3 Rb1 -b38: GCAAGTTCAAAAGACCCTGGAAG

4 Rb1 -f31: CATACCTCAGACCCTCTAAGAACCG

5 Rb1 -b41: TGGTAAGCCCTTGACCTAAAACC

6 • Cre genotyping

7 Cre1 (5'ACCAGCCAGCTATCAACTC 3')

8 Cre2 (5'TATACGCGTGCTAGCGAAGATCTCCATCTTCCAGCAG 3')

9 • Cre expression assays

Cre-694f 5'-TCCATATTGGCAGAACGAAA-3'

Cre-804r 5'-CAGCTACACCAGAGACGGAA-3'

Cre-379f 5'-AACATTTGGGCCAGCTAAAC-3'

Cre-446r 5'-AGCATTGCTGTCACTTGGTC-3'

Cre-642f 5'-GCCAGGATCAGGGTTAAAGA-3'

Cre-713r 5'-TTTCGTTCTGCCAATATGGA-3'

10 • R26R genotyping:

R26R forward: 5´AAAGTCGCTCTGAGTTGTTAT-3'

R26R reverse 1: 5´-GCGAAGAGTTTGTCCTCAACC-3'

R26R reverse 2: 5´-GGAGCGGGAGAAATGGATATG-3'

11

12 • ARF (p19)

13 mP19 ARF -f1 TCTCACCTCGCTTGTCACAG

mP19 ARF -r1	CGCTAGCATCGCTAGAAGTG
mP19 ARF -f2	CTCACCTCGCTTGTCACAGT
mP19 ARF -r2	ACGCTAGCATCGCTAGAAGTG

- p53

Trp53_ex.2_f	TGCATCCATACAGTACACAATCTC
Trp53_ex.2_r	TTGTTTCTCTCAGGCAAGGG
Trp53_ex.3+4_f	GCCTGGGATAAGTGAGATTCTG
Trp53_ex.3+4_r	AGGCATTGAAAGGTCACACG
Trp53_ex.5_f	ACACCTGATCGTTACTCGGC
Trp53_ex.5_r	GAATAAGTCAGAAGCCGGGA
Trp53_ex.6_f	GTTAGGACTGGCAGCCTCC
Trp53_ex.6_r	GACGCACAAACCAAAACAAA
Trp53_ex.7_f	GTAGGGAGCGACTTCACCTG
Trp53_ex.7_r	GGGACTCGTGGAACAGAAAC
Trp53_ex.8_f	TGCTGGTCCTTTTCTTGTCC
Trp53_ex.8_r	GAGCAAGAGGTGACTTTGGG

1 Trp53_ex.9_f TTGAGCTTCACCCCAAAGTC

2 Trp53_ex.9_r ATGCGAGAGACAGAGGCAAT

3

4 Trp53_ex.10_f ACCTTGTCCAGTGCTTCCAT

5 Trp53_ex.10_r GGAGGGAGGTCTGGGTAGAG

6 Trp53_ex.11_f GAGGAAAGCCCAAACTGCTA

7 Trp53_ex.11_r TAAGACAGCAAGGAGAGGGG

8

9 - MDM2 primers

10 mMDM2 g QRT- f1 AGAACTGGCTTCCAGACGAT

11 mMDM2 g QRT- R1 CCTCAGCACATGGCTCTTTA

12 mMDM2 g QRT- f2 CTAGCTTCTCCCTGAATGCC

13 mMDM2 g QRT-R2 TTGCACACGTGAAACATGAC

14

15 **Statistical analysis:** Biological replicates used in these studies are cell lines that were
16 established from different animals. Unless specifically stated all studies were performed
17 with two biological replicates. Data represent mean +/- SD. Statistical analysis was
18 performed using t-test unless stated. ANOVA was sued for anaphase bridges before and
19 after radiation. Statistical significance is indicated by ** $P<0.01$ or *** $P<0.001$ in all
20 experiments.

21

1 **Expression assays:** Gene expression assays from Applied Biosystems (CA, USA) were
2 used to quantify human *RB1* mRNA concentration (**Hs01078058_g1)** and mouse *Rb1*
3 mRNA **(Mm00485586_m1).**

4 Expression assay oligos for junction between exon 18 and 20 of *Rb1*:

5 Forward primer: GCCTCTCCAGGGTAACCATACT

6 Reverse primer: TCCGACTAAATACACTCTGTGC

7 Quencher: CTAGACGGTACAATATCTG