Chapter 4
Reduction of p53 in Mitogen-Deprived Rb Protein Deficient Cells Overcomes Replication Associated DNA Damage and Alleviates G2 Arrest

Tanja van Harn
Marleen Dekker
Elly Delzenne-Goete
Anja van der Wal
Floris Foijer
Hein te Riele

Submitted in adapted form

1: Division of Biological Stress Response; The Netherlands Cancer Institute; Amsterdam; The Netherlands
2: Present Address: Eriba; University Center Groningen; Groningen; The Netherlands
Abstract

Mouse embryonic fibroblasts lacking all three pocket proteins, pRB, p107 and p130 (TKO MEFs) have lost G1/S control and can enter S-phase in mitogen-deprived conditions. This resulted in the accumulation of replication-associated DNA DSBs. In this study, we show that mitogen-deprived TKO-p53 RNAi MEFs also experienced replication stress as evidenced by the appearance of γ-H2AX foci. However, aberrant replication structures were not turned into DNA DSBs. We propose that this was due to extra origin firing initiated from adjacent replication origins. In the absence of p53, the levels of p21Cip1 and p27Kip1 were sufficiently low for CDK activity to reach levels promoting origin firing. A similar mechanism may operate in vivo: in a murine retinoblastoma model early dysplastic lesions and full-blown tumors showed γ-H2AX positivity, but only in the latter we found evidence for alterations in the p53 pathway. These results unmask a novel rationale for loss of p53, i.e. alleviating the deleterious effects of replication stress in tumor cells that have lost G1/S control.
Introduction

During tumorigenesis various biological processes are abrogated to permit proliferation and cell survival (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Two major tumor suppressor pathways, the p14ARF-MDM2-p53 and p16INK4A-Rb-E2F pathways, are frequently altered in tumors (Sherr and McCormick 2002; Polager and Ginsberg 2009). The importance of the p14ARF-MDM2-p53 pathway is emphasized by the observation that the p53 gene is mutated in approximately 50% of all human tumors (Hollstein et al. 1991; Hainaut and Hollstein 2000). Furthermore, p53 function can be diminished indirectly by overexpression of MDM2 or inactivation of p14ARF, resulting in increased MDM2 dependent ubiquitination of p53 (Weber et al. 1999). Tumors also have evolved diverse ways to target the p16INK4A-Rb-E2F pathway, for example by mutations in RB1, loss of p16INK4A or overexpression of Cyclin D1 (Malumbres and Barbacid 2001). Viral oncoproteins expressed by small DNA tumor viruses like simian virus 40 (SV40), adenovirus and human papillomaviruses (HPVs) target both pathways simultaneously to permit unrestricted proliferation, illustrating that both pathways need to be inactivated (Levine 2009).

One of the functions of the p16INK4A-Rb-E2F pathway is to inhibit cell cycle progression in response to anti-proliferative signals by sequestering and inactivation of E2F transcription factors (Burkhart and Sage 2008). Prevention of E2F activity reduces the transcription of genes required for S-phase and hence cells halt in the G1 phase of the cell cycle. More recently, we and others showed that ablation of Rb protein activity resulted in DNA double strand breaks (DNA DSBs) and promoted genomic instability (Zheng et al. 2002; Gonzalo et al. 2005; Mayhew et al. 2005; Iovino et al. 2006; Srinivasan et al. 2007; Amato et al. 2009; van Harn et al. 2010). These defects could, in addition to or as a consequence of deregulated E2F activity, be linked to functions of the Rb protein family beyond G1/S control, like chromosomal condensation, sister chromatid cohesion and regulation of the nucleotide pool (Coschi et al. 2010; Manning et al. 2010; van Harn et al. 2010; Bester et al. 2011).

The p53 pathway is essential for the cellular response to various stress signals, including DNA damage, oncogene activation, DNA replication stress and hypoxia (Levine and Oren 2009). In response to DNA damage and replication stress, p53 is phosphorylated at Ser15 by ATM, ATR, Chk1 and/or Chk2 and hence stabilized since ubiquitination by the E3 ligase MDM2 is prevented (Banin et al. 1998; Lakin et al. 1999; Shieh et al. 2000). Stabilized p53 regulates the transcription of genes involved in cell cycle arrest, apoptosis, senescence, DNA repair and prevention of angiogenesis (Vogelstein et al. 2000). The exact transcriptional programme that is turned on depends on the amount of DNA damage, cell type and other stress related signals that, in addition to p53, regulate transcription (Vousden and Prives 2009).

Previously, we showed that mouse embryonic fibroblasts (MEFs) that lack Rb protein function by disruption of the genes encoding for pRB, p107 and p130, and were made resistant to apoptotic cell death (hereafter referred to as TKO-Bcl2 MEFs), were not able to proliferate under non-permissive conditions (Foijer et al. 2005). Mitogen deprivation did not prohibit these cells from entering S-phase, but they experienced severe replication problems due to low CDK activity and an unbalanced nucleotide pool (Chapter 3). Subsequently, they arrested in G2 with numerous DNA DSBs and high levels of the cyclin dependent kinase inhibitors (CKIs) p21Cip1 and p27Kip1, which inhibited Cyclin B-CDK1 kinase activity (Foijer et al. 2005; van Harn et al. 2010). RNAi mediated reduction of p53 levels alleviated this G2 arrest and cells were able to continue
proliferation albeit at slow pace (Foijer et al. 2005).

In this study, we examined why reduction of p53 levels in mitogen-deprived TKO MEFs resulted in bypass of G2 arrest. Most logically this was due to an inability to sustain the DNA damage response since p53 has an essential function in the maintenance of the G2 arrest by preventing Cyclin B-CDK1 activity. p53 achieves this effect by transcriptional activation of downstream targets that can inhibit Cyclin B-CDK1 kinase activity (p21<sup>Cip1</sup>), sequester Cyclin B-CDK1 in the cytoplasm (14-3-3-<i>α</i>) or dismantle Cyclin B-CDK1 complexes (GADD45) (Taylor and Stark 2001). Now, we show that this is not the sole reason why mitogen-deprived TKO-p53 RNAi MEFs continue proliferating. We demonstrate that RNAi-mediated knockdown of p53 in mitogen-deprived TKO MEFs rescued replication defects by restoring new origin firing. As a result, mitogen-deprived TKO-p53 RNAi MEFs contained less DNA DSBs.

By studying early and late stages of retinoblastoma development in Rb<sup>−/−</sup>p130<sup>−/−</sup> chimeric mice, we provide evidence that this mechanism also operates in vivo.

**Results**

**A**

![Cell cycle distribution](image)

**B**

![Protein levels](image)

**Figure 1: Reduction of p53 abolishes G2 arrest in mitogen-deprived TKO MEFs**

(A) Cell cycle distribution in TKO-Bcl2 MEFs (upper graph) and TKO-p53 RNAi MEFs (lower graph) in the presence or absence of 10% FCS for the indicated days. (B) p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p53 protein levels in TKO-Bcl2 and TKO-p53 RNAi MEFs cultured in the presence or absence of 10% FCS for 7 days. Anti-γ-tubulin was used a loading control.

**Reduction of p53 alleviates the G2 arrest**

Mitogen starvation in TKO-Bcl2 MEFs resulted in the accumulation of cells in the G2 phase of the cell cycle due to up-regulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> (Figure 1A, upper panel and...
p53 Knockdown Reduces DNA Damage

Figure 1B). As previously shown (Foirier et al. 2005), down-regulation of p53 in mitogen-deprived TKO MEFs alleviated the G2 arrest (Figure 1A, lower panel), although the cells were not able to proliferate at the same rate as cells cultured in the presence of mitogens (data not shown). Consistent with the inability of mitogen-deprived TKO-p53 RNAi to arrest in G2, the levels of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were reduced compared to TKO-Bcl2 MEFs (Figure 1B), resulting in increased Cyclin B-CDK1 activity and mitotic entry (Foirier et al. 2005).

**Mitogen starvation in TKO-p53 RNAi MEFs does not induce DNA DSBs**

TKO-Bcl2 MEFs cultured in the absence of mitogens contain numerous DNA DSBs that activate the p53 pathway and as a consequence p21<sup>Cip1</sup> levels are induced (van Harn et al. 2010). In contrast, TKO-p53 RNAi cells were not able to induce p21<sup>Cip1</sup> likely explaining their inability to sustain a G2 arrest. As a result, these cells might have no time to repair their DNA DSBs before mitotic entry. In TKO-Bcl2 MEFs the amount of DNA DSBs was comparable to irradiation with 20 Gy (van Harn et al. 2010), which we think would severely impair mitosis and result in cell death (Zachos et al. 2003). However, TKO-p53 RNAi MEFs continued to proliferate in the absence of mitogens (Foirier et al. 2005). Therefore, we wondered whether mitogen starvation would still induce DNA DSBs in TKO-p53 RNAi MEFs.
<Figure 2: Reduced DNA DSB induction in TKO-p53 RNAi MEFs

(A) Representative comets of nuclei of TKO-Bcl2 and TKO-p53 RNAi MEFs stained with propidium iodide in the presence or absence of 10% FCS (7 days). (B) Tail moments obtained from TKO-Bcl2 and TKO-p53 RNAi MEFs cultured in the presence or absence of 10% FCS (7 days). Box plots represent interquartile ranges, horizontal bars denote the median, + indicate the mean value and points indicate outliers. For each condition, 50 cells were analyzed using the CASP software. (C) Immunofluorescence images of TKO-Bcl2 and TKO-p53 RNAi MEFs cultured in the presence or absence of 10% FCS (7 days) to detect γ-H2AX and Rad51 foci. In the merge picture DNA is blue (labeled with To-Pro3), γ-H2AX is green, Rad51 is red and colocalization of γ-H2AX and Rad51 is seen as yellow foci. (D) Quantification of γ-H2AX and γ-H2AX/Rad51 foci formation in TKO-Bcl2 and TKO-p53 RNAi MEFs in the absence of 10% FCS (7 days). The number of counted cells is indicated above each bar.

To directly assess the presence of broken DNA we performed a neutral comet assay (Olive and Banath 2006). Mitogenic restriction of TKO-Bcl2 MEFs caused a clear increase in tail moment (van Harn et al. 2010), a quantitative measurement of the amount of DNA DSBs that takes into account the length of the comet tail as well as the percentage of DNA present in the comet tail. In contrast, the tail moments in TKO-p53 RNAi MEFs cultured in the presence or absence of mitogens were not significantly different (Figure 2A and 2B). However, the basal level of DNA DSBs was increased in TKO-p53 RNAi MEFs compared to TKO-Bcl2 MEFs. This might be explained by the function of p53 as an essential regulator of DNA damage repair (Levine and Oren 2009).

Another way to look at the formation of DNA DSBs is to assess Rad51 focus formation. Rad51 recruitment to DNA DSBs is dependent on the BRCA pathway and is involved in homologous recombination (HR) by promoting strand invasion (Branzei and Foiani 2008; Evers et al. 2008). In mitogen-deprived TKO-Bcl2 MEFs, Rad51 focus formation was detected at chromatin wherein the histone variant H2A.X was phosphorylated resulting in γ-H2AX foci (van Harn et al. 2010). γ-H2AX foci form at regions of ssDNA, which are exposed due to replication stress, or at DNA DSBs by the action of the ATR and ATM kinases, respectively (Rogakou et al. 1998; Gagou et al. 2010). In mitogen-deprived TKO-p53 RNAi MEFs, γ-H2AX foci were present (Figure 2C and D), however, the number of co-localizing Rad51 foci was clearly reduced (Figure 2C and 2D). Altogether, these results indicate that TKO-p53 RNAi MEFs still experienced replication stress upon mitogen deprivation, however, the induction of DNA DSBs was prevented.

Replication stress in mitogen-deprived TKO-p53 RNAi MEFs

To investigate whether in mitogen-deprived TKO-p53 RNAi MEFs γ-H2AX foci (Figure 2C and 2D) were linked to perturbed DNA replication, we pulse-labeled TKO-p53 RNAi MEFs with chloro-deoxyuridine (CldU) for 30 minutes. CldU is a thymidine analogue that is incorporated in newly synthesized DNA during DNA replication. Previously, we showed that mitogenic restriction reduced the number of CldU foci per cell in TKO-Bcl2 MEFs, but the CldU foci that were present showed an overlay with γ-H2AX foci (Chapter 3). In mitogen-deprived TKO-p53 RNAi MEFs we observed a reduction of CldU foci per cell as well, although this was less pronounced (Figure 3). In addition, the CldU foci co-localized with γ-H2AX foci indicative for a DNA damage response at sites of DNA replication (Figure 3). Thus, also in TKO-p53 RNAi MEFs mitogen withdrawal resulted in replication stress.
Figure 3: Replication stress in mitogen-deprived TKO-p53 RNAi MEFs
Immunofluorescence images of CldU-treated TKO-p53 RNAi MEFs cultured in the presence or absence of 10% FCS for 7 days to detect CldU and γ-H2AX foci. DNA was labeled with To-Pro3. In the merge picture DNA is blue, γ-H2AX is green, CldU is red and colocalization of γ-H2AX and CldU is seen as yellow foci.

Mitogen starvation in TKO-p53 RNAi MEFs does not reduce origin firing
Mitogenic restriction in TKO-Bcl2 MEFs resulted in reduced replication progression and less origin firing, as determined by DNA fiber analysis (Chapter 3). We similarly assessed the replication dynamics in TKO-p53 RNAi MEFs by pulse labeling the cells for 20 minutes with CldU, followed by a 20 minutes pulse with iodo-deoxyuridine (IdU), that were labeled red and green, respectively after spreading. We measured the length of ongoing forks (red – green tracks) and calculated the replication speed. In mitogen-deprived TKO-p53 RNAi MEFs we observed a significant reduction in replication speed after mitogen withdrawal, which was comparable to that in mitogen-deprived TKO-Bcl2 MEFs (Figure 4A). In addition, we quantified the level of origin firing by looking at 1st label origins (green - red - green tracks) that were origins initiated during the labeling with CldU and 2nd label origins (green only tracks) that were initiated during the labeling with IdU as a percentage of all ongoing forks. After one day of mitogen withdrawal the amount of initiated origins was clearly reduced in TKO-Bcl2 MEFs, whereas in TKO-p53 RNAi MEFs origin firing was not perturbed (Figure 4B). Thus, knockdown of p53 restored origin firing.
Figure 4: Reduced replication speed, but normal origin firing in mitogen-deprived TKO-p53 RNAi MEFs

(A) Average replication fork speeds in TKO-Bcl2 and TKO-p53 RNAi MEFs cultured in the presence or absence of 10% FCS for 1 and 2 days. Box plots represent interquartile ranges, horizontal bars denote the median, whiskers indicate 5-95 percentile and points are outliers. At least 350 track lengths of ongoing forks were measured (from 2 independent experiments) with ImageJ. Significant values are indicated with asterisks (nonparametric Kruskal-Wallis test, p < 0.05). (D) Quantification of origin firing in TKO-Bcl2 and TKO-p53 RNAi MEFs cultured in the presence or absence of 10% FCS for 1 day. 1st label and 2nd label origins are shown as percentage of all labeled tracks.

Replication stress and p53 status in murine dysplastic lesions and retinoblastomas

In contrast to humans where loss of RB1 function results in retinoblastoma (Knudson 1984), retinoblastoma development in mice also requires loss of p107 or p130 function in addition to ablation of RB1 (Robanus-Maandag et al. 1998; Chen et al. 2004; Dannenberg et al. 2004; MacPherson et al. 2004). In order to study retinoblastoma development in vivo, we generated chimeric mice by injecting Rb\(^{-/-}\)p130\(^{-/-}\)embryonic stem (ES) cells into wild-type blastocysts. Within a few months, these chimeric mice developed retinoblastomas displaying characteristics of the amacrine cells within the inner nuclear layer (Dannenberg et al. 2004).

Strikingly, these tumors all contained γ-H2AX positive nuclei, indicative for DNA replication stress and/or DNA DSBs (Figure 5A and B), whereas normal tissue was negative for γ-H2AX. We also detected pronounced staining for p53 in full-blown retinoblastomas, but again, not in normal tissue (Figure 5A and B). p53 stabilization could either be due to a DNA damage response (DDR) resulting in decreased MDM2 dependent ubiquitination of wildtype p53 or be indicative for mutant p53 which could be detected immunohistochemically in murine tumors (Olive et al. 2004; Terzian et al. 2008). The later explanation seems more likely since abrogation of the MDM2-p53 pathway was frequently detected in retinoblastoma.

In early dysplastic lesions we did not detect elevated p53 levels, indicating that abrogation of the MDM2-p53 pathway was a late step during retinoblastoma development. In contrast, half of these lesions (3 out of 6) did contain γ-H2AX positive cells, indicative for DNA replication stress or DNA DSBs (Figure 5A and 5B). We envisage that these observations mirror the in vitro behaviour of Rb protein deficient MEFs: the outgrowth of early dysplastic lesions is accompanied by DNA replication stress and DNA DSB formation; the later can, surprisingly, be suppressed by inactivation of p53, allowing the development of full-blown retinoblastomas.

Figure 5: murine retinoblastomas contain γ-H2AX positive cells and stabilized p53

(A) Quantification of the number of dysplastic lesions in the retina and small and large retinoblastomas that are positive for γ-H2AX and p53. (B) Examples of HE pictures and immunohistochemical stainings for γ-H2AX and p53 (10x magnification) of dysplastic cells in the retina (upper panel: γ-H2AX negative, lower panel: γ-H2AX positive; both p53 negative). Scale bar represents 100 μm. (C) Examples of HE pictures and immunohistochemical stainings for γ-H2AX and p53 (5x magnification) of a small (upper panel) and large (lower panel) retinoblastoma. Scale bar represents 100 μm.
Previously, we have shown that mitogen-deprived TKO-Bcl2 MEFs experienced replication stress due to inappropriate S-phase entry (Chapter 3) and arrested in the G2 phase of the cell cycle with a high level of DNA DSBs (van Harn et al. 2010). Both, replication speed and origin firing were reduced. Reduced fork speeds likely resulted form unbalanced nucleotide pools. We envisage that new origin firing was insufficient to rescue stalled replication resulting in the formation of DNA DSBs. The firing of new origins requires Cyclin-CDK activity. Together with Dbf4/Drf1-dependent CDC7 kinase (DDK), CDKs phosphorylate several components of the preRC resulting in recruitment of Cdc45 and the replicative polymerases (Jones and Petermann 2012).

Our observations in mitogen-deprived TKO-p53 RNAi MEFs lend support to this scenario. In these cells CDK activity was less offended since the levels of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were reduced compared to mitogen-deprived TKO-Bcl2 MEFs (Foijer et al. 2005). We therefore expected that mitogenic restriction in TKO-p53 RNAi MEFs would only marginally affect origin firing. This turned
out to be the case: in mitogen-deprived TKO-p53 RNAi MEFs fork speeds were still reduced, but origin firing was as high as in the presence of mitogens (Figure 4). Strikingly, mitogenic restriction of TKO-p53 RNAi MEFs did not induce DNA DSBs (Figure 2), indicating that the reduced origin firing in mitogen-deprived TKO-Bcl2 MEFs was causal to the DNA DSB formation.

Based on these results, we hypothesize that in mitogen-deprived TKO-Bcl2 MEFs fork stalling could not be rescued by replication initiated from activated neighbouring replication origins, due to the high p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} levels. In the end, stalled replication forks may be turned into DNA DSBs by the action of endonucleases, like Mus81/Eme1 (Hanada et al. 2007; Forment et al. 2011). In contrast, in the absence of p53, replication fork stalling was still present, but CDK levels were maintained at sufficiently high levels to support new origin firing, preventing the collapse of stalled forks and DNA DSB formation.

Loss of the p16\textsuperscript{INK4A}-Rb-E2F and the p14\textsuperscript{ARF}-MDM2-p53 pathways frequently co-occur in human tumors (Polager and Ginsberg 2009). Apart from abrogating the G1/S checkpoint, hyperactivated E2F1 induces the transcription of genes that promote apoptosis, like caspases, Apaf1, BH3-only and p73 (Polager and Ginsberg 2008). Loss of the MDM-p53 pathway counteracts this response enabling indefinite proliferation. In TKO MEFs, we abrogated the apoptotic response by overexpression of Bcl2 (Foijer et al. 2005). This enabled us to unravel another feature of p53 loss in cells that have lost proper G1/S control. Under mitogen-deprived conditions it turned out to be important to have a certain level of CDK activity to be able to accomplish DNA replication and prevent the formation of numerous DNA DSBs. In this study, we show that this could be achieved by reducing p53 levels.

To investigate whether this mechanism also operates in vivo, we have used a mouse model for retinoblastoma. This tumor type is particularly interesting as it was suggested that the apoptotic response does not need to be suppressed since murine retinoblastomas originated from an intrinsically death-resistant cell of origin (Chen et al. 2004). In our chimeric Rb\textsuperscript{-/-}p130\textsuperscript{-/-} murine retinoblastoma model, we found full-blown tumors to encounter DNA replication stress and/or DNA breakage since they invariably contained \( \gamma \)-H2AX positive cells. Moreover, we immunohistochemically observed stabilized p53, indicative for mutation within the p53 gene (Hall and Lane 1994). In contrast, early dysplastic lesions were always negative for p53, whereas half of the cases contained \( \gamma \)-H2AX positive cells. We therefore envisage that the outgrowth of incipient tumor cells outside their normal tissue context leads to replication stress, DNA DSBs and cell cycle arrest. Loss of the p53 pathway prevents DNA DSB formation and cell cycle arrest, permitting outgrowth of tumor cells despite the continuing replication stress they still encounter. Assessment of DNA breaks in early and late retinoblastoma lesions is required to find support for this hypothesis.

In human retinoblastomas, p53 mutations were not frequently observed (Kato et al. 1996; Nork et al. 1997). More recently, it was shown that human RB1 deficient retinal cells undergo strong selection for amplification and hence overexpression of MDMX during tumorigenesis, indicating that the MDM2-p53 pathway is indeed abrogated in retinoblastomas (Laurie et al. 2006). Whether this is required for alleviation of the apoptotic response or for induction of CDK activity in stress conditions remains to be determined. The requirement for induction of CDK activity is supported by data showing that lack of p27\textsuperscript{kip1} or overexpression of Skp2, the E3 ubiquitin ligase for p27\textsuperscript{kip1}, accelerated tumorigenesis in pocket protein deficient
cells (Park et al. 1999; Foijer et al. 2007; Wang et al. 2010).

**Material and Methods**

**Cell culture**

MEFs were isolated from chimeric embryos as described previously (Dannenberg et al. 2000) and cultured in GMEM (Invitrogen – GIBCO), supplemented with 10% fetal calf serum, 0.1mM nonessential amino acids (Invitrogen-GIBCO), 1mM sodium pyruvate (Invitrogen-GIBCO), 100μl/ml penicillin, 100μg/ml streptomycin (Invitrogen-GIBCO) and 0.1mM β-mercaptoethanol (Merck). Bcl2 expressing MEFs were generated as described previously (Foijer et al. 2005). For serum starvation experiments, cells were trypsinized and allowed to attach in the presence of serum for 4 hours. Subsequently, cells were washed with PBS and supplemented with serum free medium.

**Constructs, transfection and lentiviral infections**

The 19-mer p53 targeting sequence in pRetroSuper-p53 is GTACATGTGTAATAGCTCC. Ecotropic viral supernatants were produced by transfecting Phoenix cells by calcium-phosphate coprecipitation. Thirty-six hours post transfection retroviral supernatants were filtered through a 0.45 μm filter and used to infect MEFs in the presence of 4 μg/ml polybrene.

**Immunoblots and antibodies**

Cells were harvested and subsequently lysed for 30 min in ELB (150mM NaCl; 50mM Hepes pH7.5; 5mM EDTA; 0.1% NP-40) containing protease inhibitors (Complete, Roche). Protein concentrations were measured using the BCA protein assay kit (Pierce).

The primary antibodies used were rabbit polyclonal p21 (C19; Santa Cruz), mouse monoclonal p27 (BD Transduction Laboratory), mouse monoclonal p53 (IMX25; monosan) and γ-tubulin (GTU-88; Sigma). Secondary antibodies used were HRP-conjugated Goat anti-Mouse and HRP conjugated Goat anti-Rabbit (Dako).

**Immunofluorescence**

For Rad51 and γ-H2AX immunofluorescence stainings, cells were cultured on cover slides, washed with PBS and fixed for 5 min using 4% paraformaldehyde (Merck). Cells were permeabilized by 0.1% Triton-X100 (Sigma) in PBS for 5 min. Subsequently, cells were washed three times using staining buffer (0.15% glycine (Merck), 0.5% Bovine Serum Albumine (BSA, Sigma) in PBS) and incubated for 60 min at room temperature in staining buffer. Cells were incubated for 4h and 1h with primary and secondary antibodies, respectively.

For CldU and γ-H2AX immunofluorescence stainings, cells were cultured on cover slides, incubated with CldU (100mM) for 30 min, washed with PBS and fixed for 10 min using 70% EtOH. Cells were treated with MeOH for 5 min and incubated with 1.5M HCl for 20 min. Subsequently, cells were blocked using PBS, 0.5% Tween, 0.25% BSA, 5% FCS for 30 min. Cells were incubated with primary and secondary antibodies for 2h and 1h, respectively in PBS, 0.5%Tween, 025% BSA. Bleaching was prevented by Vectashield (Vector Laboratories). The primary antibodies used were rat-anti-BrdU (Clone BU1/75, Novus Biologicals), rabbit polyclonal Rad51 (a gift from Prof. dr. Roland Kanaar) and mouse monoclonal phosphorylated H2AX (Upstate) in 1:20, 1:2500 and 1:100 dilutions, respectively. Secondary antibodies used were Alexa 488-labeled Chicken-anti-
Mouse, Alexa 568-labeled Goat-anti-Rabbit and Alexa 568-labeled Goat-anti-Rat antibodies (Molecular probes) and these were used in a 1:100 dilution. DNA was stained using To-Pro3 dye (Molecular probes).

**Comet assay**
Neutral comet assays were performed as described by Olive et al (Olive and Banath 2006). Briefly, $8 \times 10^3$ cells were diluted in 0.4ml PBS and added to 1.2 ml 1% low-gelling-temperature agarose (Sigma). Subsequently, the cell suspension was transferred onto pre-coated slides (Menzel-Gläser). Cell lysis was performed in neutral lysis solution (2% sarkosyl, 0.5M Na$_2$EDTA, 0.5 mg/ml proteinase K) pH 8.0 overnight at 37°C. Slides were washed three times with neutral rinse and electrophoresis buffer (90mM Tris, 90mM boric acid, 2mM Na$_2$EDTA) pH 8.5, and electrophoresis was performed in neutral rinse and electrophoresis buffer for 25 min at 20V. Nuclei were stained with 2.5μg/ml Propidium Iodide (Invitrogen) in distilled water for 20 min. Pictures of individual cells were taken with a Zeiss AxioObserver Z1 inverted microscope equipped with a cooled Hamamatsu ORCA AG Black and White CCD camera and analyzed with CASP software (http://www.casp.of.pl). The p-value was measured using 1-way ANOVA (nonparametric Kruskal-Wallis test).

**DNA Fiber analysis**
Cells were pulse-labeled with 25μM CldU followed by 250μM IdU, for 20 minutes each. Labeled cells were trypsinized and lysed in spreading buffer (200mM Tris-Hcl pH 7.4, 50mM EDTA and 0.5% SDS) before spreading on a microscope slide. Slides were fixed in methanol:acetic acid 3:1. Before immunodetection, slides were treated with 2.5M HCl for 75 min. To detect CldU and IdU labeled tracts slides were incubated for 1h with rat-anti-BrdU (Clone BU1/75, Novus Biologicals; 1:500) and mouse-anti-BrdU (clone B44, Becton Dickinson; 1:750), respectively. Subsequently, slides were fixed with 4% paraformaldehyde for 10 min and incubated with Alexa 488-labeled goat-anti-mouse and Alexa 555-labeled goat-anti-rat (Molecular probes; 1:500) for 1.5h. Pictures were taken with a Zeiss AxioObserver Z1 inverted microscope using a 63x lens equipped with a cooled Hamamatsu ORCA AG Black and White CCD camera and tract lengths were analyzed with ImageJ software. μm values were converted into kb using the conversion factor 1μm = 2.59 kb (Jackson and Pombo 1998). The 1-way ANOVA (nonparametric Kruskal-Wallis test) was used for statistical analyses.

**Flow cytometry**
After fixation in ice-cold 70% ethanol, cells were counterstained with Propidium Iodide (PI) diluted in PBS containing RNase. Finally, data acquisition was performed on a FACSCalibur, using CellQuestPro software (BD Biosciences).

**Generation of chimeric mice**
*Rb$^+/p130^+*$ ES were generated previously (Dannenberg et al. 2004). These cells were injected into C57Bl/6 blastocysts (6 cells per blastocyst) to generate chimeric mice, which were monitored weekly for retinoblastoma development.

**Histological analysis**
Eyes were removed immediately after euthanasia and fixed in 4% formaldehyde for at least 24
hours. For histological analysis, formaldehyde fixed eyes were embedded in paraffin, cut into 5 μm sections and stained with Hematoxilin and Eosin. The antibodies used were α-γ-H2AX (Cell signaling) and α-p53 (VectorLabs).

Acknowledgements

We thank L. Oomen and L. Brocks for help with the microscopical visualization of the DNA fibers. We are grateful to S. Bakker for fruitful discussions. This work was supported by the Dutch Cancer Society (NKI 2007–3790) and an EMBO short term fellowship to TvH (194-2011).
References


Srinivasan SV, Mayhew CN, Schwemmer S, Zagorski W, Knudsen ES. 2007. RB loss promotes aber-


p53 Knockdown Reduces DNA Damage