Chapter 3
INAPPROPRIATE S-PHASE ENTRY IN MITOGEN-DEPRIVED pRB-DEFICIENT CELLS RESULTS IN DNA REPLICATION STRESS

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Submitted in adapted form

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Abstract

Unfavorable growth conditions, like mitogen deprivation, halt cell cycle progression in normal cells. However, cells lacking the retinoblastoma genes $RB1$, $p107$ and $p130$ (TKO MEFs) are unable to prevent S-phase entry under these conditions and start replicating their DNA. Here, we show that DNA synthesis in mitogen-deprived TKO MEFs is severely perturbed. Replication stress manifested as reduced fork speeds and reduced origin activation, resulting in delayed progression through S-phase. Part of this phenotype could be explained by decreased expression of enzymes involved in nucleotide synthesis. Depletion of one of these enzymes, PPAT, by shRNA reduced fork speeds and resulted in replication stress in TKO MEFs in the presence of mitogens, whereas exogenous supply of nucleosides in mitogen-deprived TKO MEFs partly rescued the fork speed. These collective results show that replication fork speed is dependent on the nucleotide pool. However, rescue of replication fork speed in mitogen-deprived TKO-Bcl2 MEFs could not prevent the DNA Damage Response and the accumulation of these cells in G2.
Introduction

The classical role of the retinoblastoma (Rb) proteins pRB, p107 and p130, also called pocket proteins, is the repression of E2F-mediated transcription to prevent entry into S-phase under growth-inhibitory conditions. Lack of growth factors, oncogene activation or reduced oxygen levels reduces the transcription of D-type cyclins and the activity of the associated G1 Cyclin Dependent Kinases (CDKs), CDK4 and CDK6. This results in hypo-phosphorylated Rb proteins that can bind E2F transcription factors and, by recruitment of chromatin modifiers like HDACs, DNMT1, HP1A and Suv39H1, inhibit the transcription of E2F target genes required for S-phase entry (Burkhart and Sage 2008). This G1/S control mechanism is an important barrier to tumorigenesis and is defective in virtually all human tumors (Malumbres and Barbacid 2001).

More recently, it has become clear that lack of pocket protein function interferes with the maintenance of genomic stability. pRB-deficient cells contain diverse genomic alterations, like polyploidy, aberrations in chromosome number or micronuclei formation (Zheng et al. 2002; Hernando et al. 2004; Gonzalo et al. 2005; Mayhew et al. 2005; Iovino et al. 2006; Srinivasan et al. 2007; Amato et al. 2009). This might be attributed to the classical role of pRB in G1/S regulation, but also to pRB functions in S phase (Manning and Dyson 2012). Deregulation of E2F target genes results in elevated levels of various proteins functioning in DNA replication, DNA damage response and mitosis (Iovino et al. 2006; Knudsen and Knudsen 2008). Some of these targets could be linked to chromosome instability upon pRB loss, like MAD2 (Schvartzman et al. 2011). MAD2 functions in the Spindle Assembly Checkpoint (SAC) and overexpression of MAD2 can induce chromosome instability (Sotillo et al. 2010). During S-phase, pRB is important for the establishment of cohesion between sister chromatids (Manning et al. 2010; van Harn et al. 2010) and at mitotic entrance for chromosome condensation (Longworth et al. 2008; Coschi et al. 2010). Both these defects might compromise chromosome segregation during mitosis.

Previously, it was shown that pocket protein deficiency results in the formation of DNA Double Strand Breaks (DNA DSBs) (Duensing and Munger 2002; Pickering and Kowalik 2006) that contribute to genomic instability when not properly repaired (van Harn et al. 2010). However, how DNA DSBs arise in Rb-deficient cells remains to be determined. An important feature of cells that lack a proper G1/S control mechanism is their capability to enter S phase under non-permissive conditions. The uncoupling of S phase entry and metabolic state may affect the accuracy of DNA replication (Sidorova and Breeden 2003). In G1 phase, cells assemble pre-replicative complexes (pre-RCs) to be able to trigger the initiation of DNA replication efficiently (Bell and Dutta 2002). In addition, cells have to make sure that they accumulate sufficient resources to be able to replicate their genome. E.g., in *Saccharomyces cerevisiae* precocious entry into S-phase with insufficient dNTP levels by overexpression of the G1 cyclins Cln1 or Cln2 resulted in lethality in mec1 strains (Vallen and Cross 1999).

To address DNA replication defects in cells that fail to arrest in G1 in response to unfavorable growth inhibitory conditions, we studied TKO-Bcl2 Mouse Embryonic Fibroblasts (MEFs) under mitogen-deprived conditions. TKO-Bcl2 MEFs lack all three pocket proteins and are therefore unable to arrest at the G1/S boundary (Dannenberg et al. 2000; Sage et al. 2000) and overexpress Bcl2 to prevent apoptosis (Fojier et al. 2005). Interestingly, mitogen-deprived TKO-Bcl2 MEFs arrested in G2 with numerous DNA DSBs and cohesion defects (van Harn et al. 2010). In contrast, WT MEFs arrested in G1 in the absence of mitogens and did not accumulate...
DNA DSBs and cohesion defects suggesting that these alterations arose due to S-phase entry under non-permissive conditions, i.e. lack of mitogens. Now, we show that S-phase progression is disturbed in mitogen-deprived TKO-Bcl2 MEFs, eventually resulting in a G2 arrest after one or two DNA replication cycles.

Recent work from Bester and colleagues showed that aberrant activation of the Rb-E2F pathway by HPV-16 E6/E7 decreased the cellular nucleotide level in primary keratinocytes (Bester et al. 2011). Here, we show that mitogen withdrawal resulted in decreased levels of various nucleotide synthesis enzymes, presumably causing a low-nucleotide pool. Importantly, we were able to rescue fork speed by exogenous supply of nucleosides. In addition, we show that down-regulation of phosphoribosyl pyrophosphate amidotransferase (PPAT), an enzyme involved in the synthesis of purines, impaired DNA replication in TKO-Bcl2 MEFs under normal mitogenic conditions consistent with an important role of tightly regulated nucleotide levels for normal S-phase progression.

Altogether, we show that precocious entry into S-phase under non-permissive conditions results in various DNA replication defects, partly due to insufficient resources for DNA replication. This eventually results in DNA damage and genomic instability in pRB protein deficient MEFs. This phenomenon could explain how additional mutations occur in pRB protein deficient tumors.

Results

Cell cycle dynamics in mitogen-deprived TKO-Bcl2 MEFs

To examine delays in cell cycle progression, we transduced TKO-Bcl2 MEFs with the so-called FUCCI (fluorescent ubiquitin-based cell cycle indicator) constructs mKO-hCdt1 and mAG-hGem that label individual cell nuclei red in G1 and green in S/G2/M phase, respectively (Sakaue-Sawano et al. 2008). In the beginning of S-phase both markers are expressed, resulting in yellow nuclei. This allowed us to follow individual cells by time-lapse microscopy and determine their fate after mitogen withdrawal.

TKO-Bcl2 MEFs cultured in the presence of 10% fetal calf serum (FCS) generally showed a normal cell cycle profile encompassing 15 to 20 hours (Figure 1A). In contrast, in the absence of mitogens, the majority of TKO-Bcl2 MEFs appeared to be delayed in S/G2. However, the relatively high density of these cells may have prohibited further cell cycle progression. Indeed, when plated at lower density, different cell fates became apparent. Figure 1B shows an example of a mitogen-deprived TKO-Bcl2 MEF (marked with *) expressing mAG-hGem and hence being in S/G2 phase. This cell divided and formed two daughter cells (1 and 2) both expressing mKO-hCdt1 and mAG-hGem, indicative for early S-phase (21 h). These cells progressed through S-phase since they started again to express exclusively mAG-hGem (30 h). One of these daughter cells underwent mitosis (44h) to form two daughter G1 cells (1A and 1B, 62 h) that eventually arrested expressing mAG-hGem (91 h). We tracked over 50 mitogen-deprived TKO-Bcl2 MEFs and could distinguish 5 different fates, as illustrated in Figure 1C. Most of the cells arrested in S/G2 (i.e. expressing mAG-hGem) either immediately (30.4%; Type 1) or after one additional cell cycle (23.2%; Type 2) (Figure 1D). The other cells died after mitosis (17.9%; Type 3), started to express mKO-hCdt1 without going through mitosis (19.6%; Type 4) or passed mitosis, but were unable to perform cytokinesis and formed multinucleated cells (8.9%; Type 5).

These tracking experiments suggest that mitogen-deprived TKO-Bcl2 MEFs immediately
experienced S-phase problems since most of the cells arrested before entering mitosis (type 1 and 4). In addition, half of the cells that did progress into mitosis had problems (type 3 and 5), presumably due to disturbed S-phase progression.

Figure 1: Cell cycle dynamics of mitogen-deprived TKO-Bcl2 MEFs

(A) Timing of cell cycle phases in TKO-Bcl2 MEFs expressing mKO-hCdt1 and mAG-hGem cultured in the presence (left diagram) or absence (right diagram) of 10% FCS. Cells expressing only mKO-hCdt1 (G1 phase) are marked red, cells expressing only mAG-hGem (S/G2/M phase) are marked green and cells expressing both mKO-hCdt1 and mAG-hGem (early S phase) are marked yellow. (B) Live cell imaging of mitogen-deprived TKO-Bcl2 MEFs expressing mKO-hCdt1 (red) and mAG-hGem (green). For details see text.

(C) Schematic representation of cell fates of TKO-Bcl2 MEFs after mitogen withdrawal. Type 1 cells arrested

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(C) Schematic representation of cell fates of TKO-Bcl2 MEFs after mitogen withdrawal. Type 1 cells arrested...
in G2 after one DNA replication cycle; Type 2 cells arrested in G2 after two DNA replication cycles; Type 3 cells died after mitosis; Type 4 cells became red (G1 phase) without going through mitosis; Type 5 cells represent cells that were multinucleated after mitosis. (D) Quantification of cell fates in TKO-Bcl2 MEFs after mitogen withdrawal. The number of cells counted for each cell fate is indicated above the bars.

**Delayed S-phase progression in mitogen-deprived TKO-Bcl2 MEFs**

To monitor DNA synthesis in mitogen-deprived TKO-Bcl2 MEFs, we briefly labeled newly synthesized DNA with the thymidine analogue chloro-deoxyuridine (CldU). DNA synthesis can be visualized as distinct foci by confocal microscopy with antibodies directed against CldU. (Bugler et al. 2010) (Figure 2A). We quantified the occurrence of CldU foci in TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS. 38.3% of TKO-Bcl2 MEFs cultured in the presence of FCS contained more than 5 foci (Figure 2B). This percentage corresponded to flow cytometry data where 42.5% of TKO-Bcl2 MEFs cultured in the presence of FCS incorporated the thymidine analogue bromo-deoxyuridine (BrdU) (Supplementary Figure 1). Most of the CldU-positive TKO-Bcl2 MEFs cultured in the presence of FCS showed high numbers of CldU foci (>50) (Figure 2B). In contrast, cells cultured without FCS had fewer CldU foci per cell indicative for reduced DNA synthesis. In addition, the number of cells containing more than 5 CldU positive foci was reduced to 10.8% in TKO-Bcl2 MEFs cultured in the absence of FCS for 7 days.
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Figure 2: Delayed S-phase progression in mitogen-deprived TKO-Bcl2 MEFs

(A) Immunofluorescent images of CldU-treated TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for indicated days to detect CldU foci (red). DNA was labeled with To-Pro3 (blue). Nuclei are outlined with a white dashed line. (B) Quantification of the number of CldU foci in TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for the indicated days. The number of cells counted for each condition is indicated above the bars. (C) Propidium Iodide (PI) distribution of BrdU-labeled TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for the indicated days. TKO-Bcl2 MEFs were pulse labeled with BrdU and released into medium containing nocodazole for 0, 3 or 6h before fixation.

To follow S-phase progression, we pulse-labeled TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS with BrdU for 1 hour. After the pulse, we fixed the cells or released the cells in BrdU-free medium for 3 or 6 hours before fixation. We analyzed the DNA content of BrdU-labeled cells by flow cytometry to measure the rate at which these cells progressed through S-phase (Figure 2C). To prevent entry into the next cell cycle we treated the released cells with nocodazole, which traps cells in mitosis (4N DNA content). TKO-Bcl2 MEFs cultured in the presence of FCS rapidly progressed through S-phase: the BrdU-labeled TKO-Bcl2 MEFs with a 2N DNA content after the 1 hour BrdU pulse had reached the end of S-phase after 6 hours. Strikingly, in mitogen-deprived TKO-Bcl2 MEFs, the intensity of BrdU staining in 2N cells was as high as in serum-stimulated cells indicating normal S-phase entry (Figure S1). However, at 3 and 6 hours after the BrdU pulse, the DNA profiles in mitogen-deprived cells had not changed significantly. In addition, the number of cells entering S phase decreased the longer cells were cultured in the absence of FCS and BrdU became predominantly incorporated in cells with a 4N DNA content, i.e. in cells that had nearly finished DNA replication. Also, the level of BrdU incorporation in these 4N cells gradually decreased (Figure S1). Thus, after an initial wave of DNA synthesis upon entry into S-phase, serum-starved TKO cells were severely impaired in DNA synthesis. After prolonged starvation, cells arrested with a near 4N DNA content presumably with persistent regions of unreplicated DNA.

Mitogen deprivation reduces fork speed and inhibits origin activation in TKO-Bcl2 MEFs

To study why S-phase progression in mitogen-deprived TKO-Bcl2 MEFs was impaired, we assessed the dynamics of individual replication forks by the DNA fiber technique (Tuduri et al. 2010). We pulse labeled TKO-Bcl2 MEFs with the thymidine analogues chloro-deoxyuridine (CldU) and iodo-deoxyuridine (IdU) for 20 minutes each. DNA fibers were spread on glass slides and visualized with specific antibodies against CldU (red) and IdU (green). With the double labeling protocol used, we could define five types of replication structures (Figure 3A and 3B): type 1 – ongoing forks (red-green tracks); type 2 – origin firing during the 1st labeling (green – red – green tracks); type 3 – origin firing during the 2nd labeling (green only tracks); type 4 – termination during the 1st labeling (red only tracks); type 5 – termination of two converging forks during the 2nd labeling (red – green – red tracks). To determine fork speeds, we measured the length of the ongoing forks (type 1) since these forks were initiated before the labeling with CldU and still elongating during the second labeling with IdU. TKO-Bcl2 MEFs cultured in the presence of FCS replicated their DNA with an average fork speed of 1.38 kb/min (Figure 3C), whereas cells cultured in the absence of FCS for 1 day had a significantly reduced fork speed of 1.10 kb/min. Culturing without FCS for 2 days reduced the fork speed almost twofold to 0.71 kb/min. After more than two days of culturing in the absence of FCS, we could not obtain enough DNA fibers to analyze, indicative for severely impaired DNA synthesis.
Figure 3: Mitogen-deprived TKO-Bcl2 MEFs have a reduced fork speed and origin firing

(A) Schematic representation of replication tracts observed after pulse labeling with CldU (red) and IdU (green) for 20 minutes each. Ongoing forks were used to determine fork speeds (kb/min). 1\textsuperscript{st} label and 2\textsuperscript{nd} label origins are origins of replication (Ori) initiated during the labeling period with CldU and IdU, respectively. 1\textsuperscript{st} label and 2\textsuperscript{nd} label terminations stopped DNA replication during the labeling period with CldU and IdU, respectively. (B) Representative images of replication tracks observed in TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for 1 and 2 days. CldU tracks are labeled red and IdU tracks are labeled green. (C) Average replication fork speeds in TKO-Bcl2 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 100 track lengths of ongoing forks were measured. All values are significantly different (nonparametric Kruskal-Wallis test, p < 0.05). (D) Quantification of origin firing in TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for 1 day. 1\textsuperscript{st} label and 2\textsuperscript{nd} label origins are shown as percentage of all red (CldU) labeled tracks. SEM (bars) of five independent experiments is shown. Values were significantly different (p value: 0.0421, student’s t-test).

Besides reduced fork speed, also reduced origin activation could contribute to impaired S-phase progression. Origins are licenced in late M-phase and G1 by recruitment of the minichromosome maintenance (Mcm) complex (Mcm 2-7) to origins by Cdt1 and Cdc6 to form pre-replicative complexes (pre-RCs). These complexes are converted into origins of replication by Cyclin Dependent Kinases (CDKs) together with Cdc7 through recruitment of Cdc45 and the loading of the replicative polymerases (Mendez and Stillman 2003). Mitogen withdrawal in TKO-
Bcl2 MEFs resulted in a reduction in CDK kinase activity due to increased levels of p27\(^{kip1}\) and p21\(^{CIP1}\) (Folger et al. 2005). Since mitogen-deprived TKO-Bcl2 MEFs are not able to arrest in G1, they start DNA replication with reduced CDK activity and this might affect origin firing. We tested this hypothesis by determining the number of 1\(^{st}\) and 2\(^{nd}\) label origins (Figure 3A) as percentage of the number of CldU (red) containing tracks. After 1 day of culturing without mitogens the percentage of newly fired origins was reduced to 10.8% compared to 17.6% in TKO-Bcl2 MEFs cultured in the presence of mitogens (P value = 0.0421) (Figure 3D).

Thus, the reduction in S-phase progression in mitogen-deprived TKO-Bcl2 MEFs could be attributed to reduced fork speed and compromised origin firing.

\textit{Mitogen-deprived TKO-Bcl2 MEFs experience replication stress}

Disturbed S-phase progression might activate the S-phase checkpoint to slow progression through S-phase and facilitate DNA repair (Harper and Elledge 2007; Jones and Petermann 2012). This checkpoint is mainly mediated by ATR that is activated by regions of RPA-coated single-stranded DNA at stalled replication forks (Zou and Elledge 2003). ATR phosphorylates various downstream targets, among which the effector kinase Chk1 on Ser345 and Ser317 (Guo et al. 2000; Liu et al. 2000; Zhao and Piwnica-Worms 2001). We determined whether this checkpoint is activated in TKO-Bcl2 MEFs cultured without FCS by looking at phosphorylated Chk1 (pChk1). Upon mitogen withdrawal, pChk1 levels gradually increased in TKO-Bcl2 MEFs (Figure 4A), whereas Chk1 levels were unchanged. These results show that TKO-Bcl2 MEFs activate the S-phase checkpoint indicative for replication stress.

We previously showed that mitogen-deprived TKO-Bcl2 MEFs contain DNA DSBs (van Harn et al. 2010). Stalled replication forks that are not properly stabilized and processed can eventually result in the formation of DNA DSBs (Hellday et al. 2008). To determine whether the DNA DSBs occurred at sites of DNA replication, we assessed the co-localization of CldU (as marker for DNA replication) and γ-H2AX (as marker for DNA DSBs) (Seiler et al. 2007). Although the number of CldU positive cells decreased after prolonged culturing without mitogens (Figure 2B), virtually all CldU foci that were present after 4 or 7 days of culturing in the absence of mitogens co-localized with γ-H2AX foci (Figure 4B and 4C). These results strongly suggest that the DNA DSBs observed in mitogen-deprived TKO-Bcl2 MEFs were formed at replication forks.

\textit{S-phase defects in mitogen-deprived TKO-Bcl2 MEFs is partly caused by nucleotide deficiency}

Reduced fork speed and origin firing in S-phase cells could be caused by a reduction in CDK activity, a reduced number of licensed origins, the presence of replication blocks resulting in activation of the S-phase checkpoint and/or a shortage in resources for replication, among which nucleotides (Krude et al. 1997; Vallen and Cross 1999; Sidorova and Breeden 2003; Petermann et al. 2010b; Bester et al. 2011). Previously, it was shown that aberrant activation of the Rb-E2F pathway by HPV-16 E6/E7 significantly reduced the nucleotide pool resulting in DNA replication stress. This effect was attributed to reduced transcription of genes required for nucleotide synthesis (Bester et al. 2011). The transcription of nucleotide synthesis genes is under control of E2F and Myc transcription factors (Dang and Lewis 1997). Although in TKO-Bcl2 MEFs E2F-mediated transcription is increased (Burkhart and Sage 2008), the absence of growth factors is a strong anti-proliferative signal that inhibits Myc transcription factors and therefore might still repress genes involved in nucleotide synthesis (Gassmann et al. 1999; Perna et al. 2012).
Figure 4: Replication stress in mitogen-deprived TKO-Bcl2 MEFs

(A) pChk1 (phosphorylated on Ser317, indicated by an arrow) and Chk1 protein levels in TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for the indicated days. Anti-CDK4 was used as loading control.

(B) Immunofluorescent images of CldU-treated TKO-Bcl2 MEFs cultured in the absence of 10% FCS for 2 and 4 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 co-localization of γ-H2AX and CldU is seen as yellow foci.

(C) Quantification of CldU positive TKO-Bcl2 MEFs cultured in the absence of FCS for the indicated days that contained five or more superimposed γ-H2AX and CldU foci. At least 100 cells were counted per condition.

To assess whether the transcription of nucleotide synthesis genes was reduced upon mitogen starvation in TKO-Bcl2 MEFs, we determined the expression level of various genes involved in purine metabolism (Figure 5A) (Liu et al. 2008). The transcript levels of phosphoribosyl pyrophosphate amidotransferase (PPAT), inosine monophosphate dehydrogenase 1 and 2 (IMPDH1 and IMPDH2) were reduced 2-fold upon mitogen withdrawal for 7 days. However, transcription of another gene involved in nucleotide biosynthesis, phosphoribosylaminimidazole carboxylase (PAICS), was not significantly altered.

Reduced levels of nucleotide synthesis enzymes could impair DNA replication by disturbing the balance in the dNTP pool. Since PPAT expression was immediately and most strongly down regulated after mitogen withdrawal and the product of this gene, ATase
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(amidophosphoribosyltransferase), is involved in the rate-limiting step of de novo purine synthesis (Gassmann et al. 1999), we tested whether reduction in PPAT expression affected DNA synthesis in TKO-Bcl2 MEFs in the presence of mitogens. We used two independent shRNAs (#1 and #2) that reduced the PPAT mRNA levels to 33 and 47% compared to TKO-Bcl2 MEFs transduced with a non-targeting shRNA (EV), respectively (Figure 5B). To study DNA replication, we performed DNA fiber analysis two days after transduction. Low PPAT mRNA levels reduced replication fork speeds significantly to 0.84 (#1) and 0.86 (#2) kb/min compared to 1.25 kb/min in TKO-Bcl2 MEFs transduced with EV (Figure 5C). These results indicate that imbalances in the nucleotide pool due to reduced expression of PPAT interferes with fork progression.

Figure 5: Reduced expression of genes involved in nucleotide synthesis disturbs DNA replication

(A) Transcript level of PPAT, IMPDH1, IMPDH2 and PAICS (corrected for HPRT transcript levels) in TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for the indicated days. The expression in the presence of 10% FCS is set as 100%. Standard deviation (bars) of two independent experiments is shown. (B) Transcript level of PPAT (corrected for HPRT transcript level) in TKO-Bcl2 MEFs 2 days after transduction with shRNAs against PPAT (#1 and #2) or non-targeting shRNA (EV, set as 100%). Standard deviation (bars) of three independent experiments is shown. (C) Average replication fork speeds in TKO-Bcl2 MEFs 2 days after transduction with shRNAs against PPAT (#1 and #2) or non-targeting shRNA (EV). Box plots represent interquartile ranges, horizontal bars denote the median, whiskers indicate 5-95 percentile and points are outliers. At least 75 track lengths of ongoing forks were measured (Values are significantly different (nonparametric Kruskal-Wallis test, p < 0.05).

Reduced fork speed two days after transduction with the PPAT shRNAs (#1 and #2), was accompanied by Chk1 phosphorylation, indicative for replication stress. Also, p21<sup>Cip1</sup> levels were increased, indicative for DNA damage (Supplementary Figure 2A). At this time point, two
days after transduction, we did not detect significant changes in the cell cycle profile (data not shown). However, after three days, many PPAT shRNAs (#2) cells had accumulated in late S-phase (Supplementary Figure 2B). This effect was not seen with PPAT shRNAs (#1) cells, but here we detected massive cell death (data not shown).
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Figure 6: Exogenous supply of nucleosides rescues fork speed in mitogen-deprived TKO-Bcl2 MEFs.
(A) Average replication fork speeds in TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for 1 day with or without the exogenous supply of nucleosides. Box plots represent interquartile ranges, horizontal bars denote the median, whiskers indicate 5-95 percentile and points are outliers. At least 100 track lengths of ongoing forks were measured. Values marked with asterisk are significantly different (nonparametric Kruskal-Wallis test, p < 0.05). (B) Cell cycle distribution of TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for the indicated days with or without the exogenous supply of nucleosides each day. (C) pChk1 (Ser317), Chk1, p21Cip1 and p27Kip1 protein levels in TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for the indicated days either in the presence or absence of nucleosides. Anti-CDK4 was used as loading control.

Thus, a modest twofold decrease of PPAT expression led to replication stress and, with some delay, to accumulation of cells in late S-phase. This delay in cell cycle effect was also observed in mitogen-deprived TKO-Bcl2 MEFs. During the first two days they showed major difficulties replicating their DNA and they accumulated clearly in late S/G2 phase only 3-4 days after mitogen withdrawal (Supplementary Fig. 1).

Our observations predict that fork speeds in mitogen-deprived TKO-Bcl2 MEFs may be rescued by the addition of nucleosides. To test this, we supplemented serum-free medium with the four nucleosides (Adenosine, Thymidine, Cytidine and Guanosine) and determined the fork speeds after 1 day of culturing. Mitogen deprivation of TKO-Bcl2 MEFs without exogenous supply of nucleosides significantly decreased fork speeds from 0.94 kb/min to 0.65 kb/min. In contrast, in cells supplemented with nucleosides fork speeds only modestly decreased (from 1.03 kb/min to 0.83 kb/min) (Figure 6A).

Finally, we studied the effect of exogenous nucleoside supply in mitogen-deprived TKO-Bcl2 MEFs on cell proliferation and cell cycle distribution. Surprisingly, the addition of exogenous nucleosides did not alleviate the proliferation defect of TKO-Bcl2 MEFs cultured in the absence of FCS (data not shown). Cell cycle profiles showed that G2 accumulation in mitogen-deprived TKO-Bcl2 MEFs was hardly affected by nucleosides supplementation (Figure 6B). Also Chk1 phosphorylation and p21Cip1 induction were not reduced upon nucleosides supplementation (Figure 6C). Thus, partial restoration of fork speeds was not sufficient to alleviate replication stress and DNA damage, indicative for other factors to interfere with replication in mitogen-deprived pRB protein defective cells.

Discussion

In this study, we demonstrate that mitogen deprivation in pRB protein deficient MEFs resulted in DNA replication defects. TKO MEFs were not capable to arrest in G1 upon mitogen withdrawal and started replicating their DNA. However, after an initial wave of origin firing, fork speeds were reduced twofold and subsequent origin firing was reduced delaying progression through S-phase. Previously, we have shown that TKO-Bcl2 MEFs cultured in the absence of FCS accumulated numerous DNA DSBs and cohesion defects (van Harn et al. 2010). Now, we show that these DNA DSBs co-localized with replication foci, indicating that the DNA damage was caused by aberrantly replicating DNA (Figure 4C and 4D).

Disturbed DNA replication results in activation of the S-phase checkpoint that delays cell cycle progression, stabilizes stalled or damaged forks to prevent fork collapse (Lopes
et al. 2001; Tercero and Diffley 2001; Sogo et al. 2002) and induces repair of damaged DNA (Harper and Elledge 2007; Jones and Petermann 2012). This checkpoint functioned properly in mitogen-deprived conditions since Chk1 became phosphorylated on Ser317 (Figure 3B) and Rad51 was loaded onto the DNA (van Harn et al. 2010). However, why were the stalled forks not properly stabilized to prevent the formation of DNA DSBs? The FA/BRCA tumor suppressor pathway was shown to protect stalled replication forks, possibly by preventing fork reversal and the accumulation of branched DNA structures (Schlacher et al. 2012). These branched DNA structures can undergo nucleolytic processing by Mus81/Eme1 endonucleases, eventually turning them into DNA DSBs (Hanada et al. 2007; Forment et al. 2011). However, transcript levels of various genes in the FA/BRCA pathway remained unaltered upon mitogen withdrawal in TKO-Bcl2 MEFs (data not shown), but this was apparently not sufficient prevent the formation of DNA DSBs. A reasonable explanation might be that stalled DNA replication forks in mitogen-deprived TKO-Bcl2 MEFs cannot be stabilized for a long period of time. We observed DNA replication defects, i.e., prolonged S-phase and reduced fork speeds, immediately after mitogen withdrawal, nevertheless, DNA DSBs arose only after four days mitogenic restriction (van Harn et al. 2010). This corresponds to data showing that treatment with either aphidicolin or hydroxyurea resulted in the accumulation of DNA DSBs more than a day after the onset of treatment (Hanada et al. 2007; Petermann et al. 2010a).

Upon fork stalling and/or collapse, global replication can be rescued by dormant origin firing (Ge et al. 2007; Ibarra et al. 2008). In mitogen-deprived TKO-Bcl2 MEFs, we observed a decrease in origin firing (Figure 3D) and the number of replication factories per cells was also significantly reduced in mitogen-deprived TKO-Bcl2 MEFs (Figure 2A and 2B). This observation could be due to defective replication licensing during G1 which in budding yeast was shown to result in reduced origin firing (Lengronne and Schwob 2002). However, our results suggest that early origin firing was normal (Figure S1). We therefore speculate that the gradually diminishing CDK activity in mitogen-deprived cells particularly affects late origin firing (Petermann et al. 2010b).

Ultimately, the combined effect of increased fork stalling and the lack to compensate this by increased origin activation might result in incomplete DNA replication. After 7 days of mitogen deprivation, TKO-Bcl2 MEFs arrested at a near 4N stage (G2), but still incorporated low amounts thymidine analogs (BrdU and CldU) indicating that there is still a low level of DNA synthesis (Figure 1A and 1B and Supplementary Figure 1). Given that some parts of the genome are more difficult to replicate, it would be interesting to see which parts of the DNA are still not properly duplicated. Previously, it has been shown that fork stalling in the presence of replication stress due to oncogene activation is more prevalent in repetitive regions of genome, including fragile sites (Di Micco et al. 2006; Bester et al. 2011; Ozeri-Galai et al. 2011).

Apart from the DNA DSBs, mitogen-deprived TKO-Bcl2 MEFs showed severe loss of centromeric sister chromatid cohesion (van Harn et al. 2010). Sister chromatid cohesion is established by cohesion complex that is loaded onto the DNA during S-phase (Peters et al. 2008). Perturbations in S-phase progression might interfere with this process resulting in reduced tethering of sister chromatids.

Previously, the importance of a balanced nucleotide pool in oncogene-expressing cells was demonstrated (Bester et al. 2011). Indeed, we were able to partly rescue fork speed in mitogen-deprived TKO-Bcl2 MEFs by the exogenous supply of nucleosides (Figure 6A). However,
this could not prevent the lack of proliferation since most of these cells still arrested efficiently with a 4N DNA content (Figure 6B). We hypothesize that the reduced levels of nucleotides is not the only difficulty mitogen-deprived TKO-Bcl2 MEFs are facing when they enter S-phase under non-permissive conditions. For example, p27<sup>kip1</sup> protein levels remain elevated and could inhibit CyclinB/CDK1 activity and therefore prevent mitotic entry. However, we were able to demonstrate that a balanced pool of nucleotides is essential for a proper replication by reducing the levels of PPAT with shRNAs. In the presence of mitogens this clearly impaired replication since fork speeds were reduced significantly (Figure 5C and Supplementary Figure 2B). Furthermore, these cells were also delayed at late S/G2 border (Supplementary Figure 2C), indicating that these cells were also not able to finish replication completely and probably also contain numerous DNA DSBs.

Our results shed new lights on the cause of DNA DSBs and cohesion defects in Rb protein deficient cells showing that unscheduled entry into S-phase under growth restricted conditions results in various replication defects. This might be a way to acquire additional mutations in pRB protein deficient cells to, in the end, become tumorigenic. This feature of pRB protein deficient cells might be exploited in the development of strategies to tackle pRB protein deficient tumors.

**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) in the absence or presence of nucleosides (200µM of Cytidine, Guanosine, Adenosine and Thymidine). 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 FUCCI constructs CSII-EF-MCS-mKO-hCdt1 (30/120) and CSII-EF-MCS-mAG-hGem (1/110) were kindly provided by A. Miyawaki (Sakaue-Sawano et al. 2008). The 21-mer oligos in pLKO.1 targeting PPAT are: #1: CCACATGCTTATGTATGTATA and #2: CCGGAGAAATTGTAGAAATAT. Corresponding empty vector (EV) was used as control. The plasmids were cotransfected with the helper plasmids pMDLgpRRE, VSV-G and pRSV-Rev into 293T cells by calcium-phosphate co-precipitation. Thirty-six hours post transfection lentiviral supernatants were filtered through 0.45 µm filter and used to infect MEFs in the presence of 4 µg/ml polybrene for 24h.

**Immunoblots and antibodies**

Cells were harvested and subsequently lysed for 30 min in RIPA (25mM Tris-HCl pH 7.6; 150mM NaCl; 1% NP-40; 1% Sodiumdeoxycholate and 0.1% SDS) containing protease inhibitors (Complete, Roche). Protein concentrations were measured using the BCA protein assay kit (Pierce). The used primary antibodies were rabbit polyclonal phospho-Chk1 Ser317 (Bethyl), mouse monoclonal Chk1 (G4; Santa Cruz), goat polyclonal CDK4 (C22; Santa Cruz), rabbit polyclonal p21
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(C19; Santa Cruz), mouse monoclonal p27 (BD Transduction Laboratory). Secondary antibodies used were IR Dye 800CW Goat anti Mouse IgG, goat anti rabbit IgG and Donkey anti Goat IgG (Licor).

Immunofluorescence
For 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, 0.25% BSA. Bleaching was prevented by Vectashield (Vector Laboratories). The primary antibodies used were rat-anti-BrdU (Clone BU1/75, Novus Biologicals) and mouse monoclonal phosphorylated H2AX (Upstate) in 1:20 and 1:100 dilutions, respectively. Secondary antibodies used were Alexa 488-labeled Chicken-anti-Mouse 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).

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 1h and 15 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 tracts 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.

Time-lapse microscopy
For live imaging, dishes were transferred to a heated stage (37°C) on a Zeiss Axiovert 200M inverted microscope. PhC (Phase Contrast) images (59ms exposure) and fluorescent images (red: 250ms and green 300ms exposure) were captured with a 10x/0.25 Ph1 Achromplan objective in combination with 1.6 optovar every 60 min using a cooled Hamamatsu ORCA R2 Black and White CCD-camera and appropriate filter blocks to select specific fluorescence. Images were taken in 2x2 binning mode (672x512 pixels) and processed using AxioVision Rel. 4.7.2. software.

Flow cytometry
TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS were labeled with BrdU (10µM) for 1h and fixed in 70% EtOH (0h) or washed with PBS twice and supplemented with serum-free or serum containing medium in the presence of nocodazole (250ng/ml) and fixed 3h or 6h later. After o/n fixation, cells were incubated with 5M HCl + 0.5% Triton for 20 min. After inactivation with 1M Tris-HCl pH 7.5 and wash with PBS + 0.5% Tween, nuclei were incubated
with mouse-anti-BrdU (DAKO; 1:40) for 1h, washed in PBS + 0.5% Tween and incubated with anti-mouse FITC goat F (clone ab2, DAKO; 1:20) for 1h. Subsequently, 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) and data analysis (cell cycle) was performed using FlowJo software version 7.6.1 (Tree Star, Ashland, OR, USA).

**RNA isolation, generation for cDNA and quantitative PCR**
Total RNA was isolated from MEFs using the High Pure RNA isolation kit (Roche). To prepare cDNA by reverse transcriptase using random primers 1 μg of total RNA was used. Subsequently, cDNA was used as template for qPCR in the presence of SYBR-green (Applied Biosystems) to label the product. Fluorescence detection was performed using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The relative amounts of cDNA were compared to HPRT as a reference for total cDNA. Subsequently, TKO-Bcl2 +10% FCS or TKO-Bcl2 + EV was used for normalization. For qPCR of PPAT we used 5’-CCAGAGTCTGCTACGCTGC-3’ as forward and 5’-CAGCCCAACACTTTGTTGCAT-3’ as reverse primer, for PAICS we used 5’-GATGTAACCACAAAGAGATTGTT-3’ as forward and 5’-ATCTGGAGTCTTCTGGAGTTACTT-3’ as reverse primer, for IMPDH1 we used 5’-ATGGATAACGTACAGAGGCTGAT-3’ as forward and 5’-ACTTCTTGACCTCCGTACTTCATT-3’ as reverse primer, for IMPDH2 we used 5’-TACATCAAGGAGAATTCCTCCAGTC-3’ as forward and 5’-AAGCATCTACACTCTCAGAC-3’ as reverse primer, for HPRT we used 5’- CTGTGAAAAGGACCTCTCG-3’ as forward and 5’-TGAAGTACTCATTATAGGCGCA-3’ as reverse primer.

**Acknowledgements**
We thank A. Miyawaki for CSII-EF-MCS-mKO-hCdt1 (30/120) and CSII-EF-MCS-mAG-hGem (1/110). We thank L. Oomen and L. Brocks for help with the microscopical visualization of the DNA fibers and help with time-lapse microscopy and T. Harmsen for technical support. We are grateful to S. Bakker en N. Wit 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


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Supplementary Information

Supplementary Figure 1: TKO-Bcl2 MEFs cultured in the absence of 10% FCS incorporate less BrdU
Density plot of TKO-Bcl2 MEFs cultured in the presence or absence of 10% FCS for the indicated days labeled with BrdU for 1h. On the x-axis the Propidium Iodide distribution is plotted and on the y-axis the BrdU signal. Red indicates G1 cells, orange indicates S-phase cells and blue indicates G2 cells, analyzed with FlowJow software (cell cycle).

Supplementary Figure 2: PPAT depleted TKO-Bcl2 MEFs have replication stress
(A) pChk1 (Ser317), Chk1, p21\textsuperscript{CIP1} and p27\textsuperscript{KP1} protein levels in TKO-Bcl2 MEFs 2 days after transduction with shRNAs against PPAT (#1 and #2) or non-targeting shRNA (EV). Anti-CDK4 was used as loading control. (B) Propidium Iodide (PI) distribution of TKO-Bcl2 MEFs three days after transduction with shRNAs against PPAT (#1 and #2) or non-targeting shRNA (EV). Profiles were analysed with FlowJow cell cycle platform using the Watson Model andFreq. G1, Freq. S and Freq. G2 are indicated.