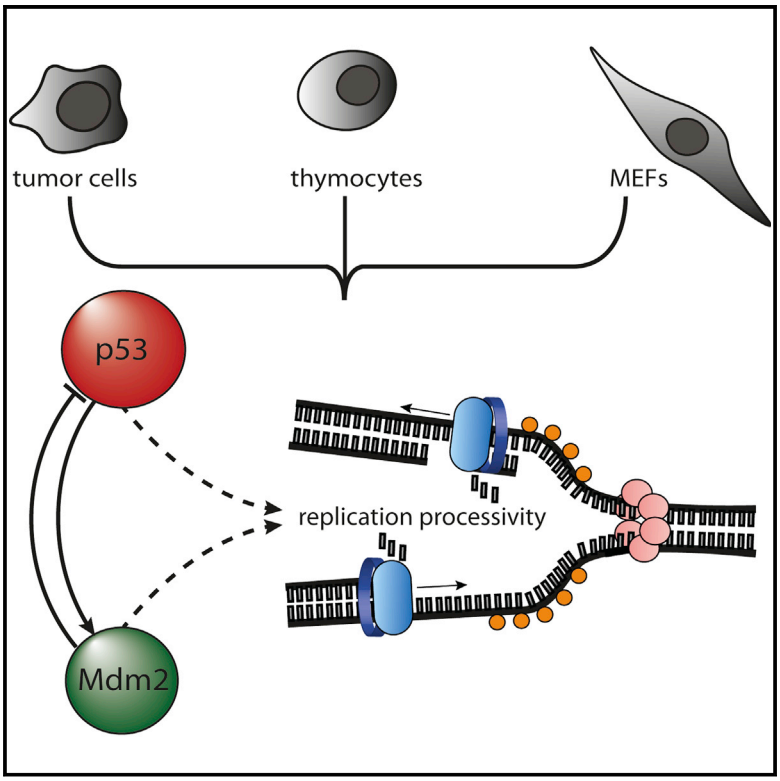
Article

Cell Reports

p53 Activity Results in DNA Replication Fork Processivity

Graphical Abstract



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In Brief

p53 eliminates cells upon genotoxic stress, acting after damage occurred. Klusmann et al. now report that p53 can act to preserve DNA integrity before damage occurred. p53 supports DNA replication processivity, and the p53 target gene product Mdm2 helps to avoid replicative stress.p53, DNA replication, gemcitabine, Mdm2, Nutlin-3a, DNA fiber assays, DNA damage response, murine embryonic fibroblasts, thymocytes

Highlights

- p53 supports DNA replication by increasing its processivity
- Mdm2, a p53 target gene product, similarly supports DNA replication
- p53 prevents replicative stress in primary cells (e.g., thymocytes)

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p53 Activity Results in DNA Replication Fork Processivity

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SUMMARY

p53 induces cell death upon DNA damage, but this may not confer all of its tumor suppressor activity. We report that p53 activation enhances the processivity of DNA replication, as monitored by multi-label fiber assays, whereas removal of p53 reduces fork progression. This is observed in tumor-derived U2OS cells but also in murine embryonic fibroblasts with heterozygous or homozygous p53 deletion and in freshly isolated thymocytes from mice with differential p53 status. Mdm2, a p53-inducible gene product, similarly supports DNA replication even in p53-deficient cells, suggesting that sustained Mdm2-expression is at least one of the mechanisms allowing p53 to prevent replicative stress. Thus, p53 helps to protect the genome during S phase, by preventing the occurrence of stalled or collapsed replication forks. These results expand p53's tumor-suppressive functions, adding to the ex-post model (elimination of damaged cells) an ex-ante activity; i.e., the prevention of DNA damage during replication.

INTRODUCTION

No other gene is as frequently mutated across most tumor species as TP53. Thus, p53 must prevent tumor initiation and/or progression. Current concepts summarize the function of p53 largely as a mediator of cell death or permanent cell-cycle arrest whenever cells suffer intolerable stresses, most notably when DNA damage occurs. DNA damage induces the activation of p53 as a transcription factor. Many of the p53-inducible genes mediate apoptosis. This ensures the elimination of cells that had suffered extensive DNA damage, conceivably avoiding the accumulation of cells with heavily mutated DNA that might otherwise give rise to malignant growth. Thus, the initial designation of p53 as a "guardian of the genome" (Lane, 1992) only applies to a whole organism, when damaged cells are eliminated to avoid danger to the whole body. From the perspective of a single cell, the "guardian" has a destructive role. According to currently accepted models, p53 is acting largely "ex post" by destroying damaged cells, but not "ex ante" to avoid DNA damage in the first place. Although DNA repair has now become another well-acknowledged activity promoted by p53 (Bieging et al., 2014), this does still not imply that p53 actually prevents DNA damage, rather than merely reacting to it.

However, some observations at least argue that p53 not only eliminates cells with damaged DNA but exerts some of its tumor suppressive activity by precluding such damage. Mice with a constitutive deletion of p53 develop largely normally. At 4–6 months of age, however, tumors occur (Donehower et al., 1992). In mice with switchable p53 alleles, p53 function can be turned on and off at will, allowing the establishment of time windows during which p53 is essential or dispensable for tumor suppression (Martins et al., 2006). Using this system, established lung tumors were only temporarily halted by reintroducing wild-type p53 (Junttila et al., 2010), arguing that p53 may have a more important role in preventing the formation of tumor cells rather than destroying them ex post. Investigating mouse strains with targeted deletions of key p53 target genes further challenged our current concept of p53-mediated tumor suppression. Even when the major mediator of cell-cycle arrest, CDKN1A/p21, and the key proapoptotic gene product, BBC3/Puma, were both eliminated, p53 was still capable of suppressing T cell lymphomas that otherwise occur almost without exception when p53 itself is deleted (Valente et al., 2013). Similarly, an acetylation-deficient p53 mutant that is largely unable to induce cell-cycle arrest or apoptosis can still suppress T cell lymphomas in mice (Li et al., 2012). Thus, neither the proapoptotic nor the cell-cycle regulatory function of p53 may be key to its tumor suppressive activity.

In further support of a protective function of p53 toward individual cells, the elimination of p53 does not always enhance cell survival. Rather, removing p53 in the colon cancer-derived cell line HCT116 increases the sensitivity of cells toward certain chemotherapeutics, most notably doxorubicin and cisplatin (Bunz et al., 1999). The sensitivity of p53-deficient cells toward topoisomerase inhibitors was recently characterized in depth by a drug screen and mechanistic analysis (Yeo et al., 2016). Small interfering RNA (siRNA) screens revealed that the depletion of some gene products decreases the viability of $p53^{-/-}$ cells to a higher degree than their p53-proficient counterparts. These genes are involved in nucleotide synthesis (e.g., UMPS) (Bartz et al., 2006), DNA replication (e.g., Geminin) (Krastev

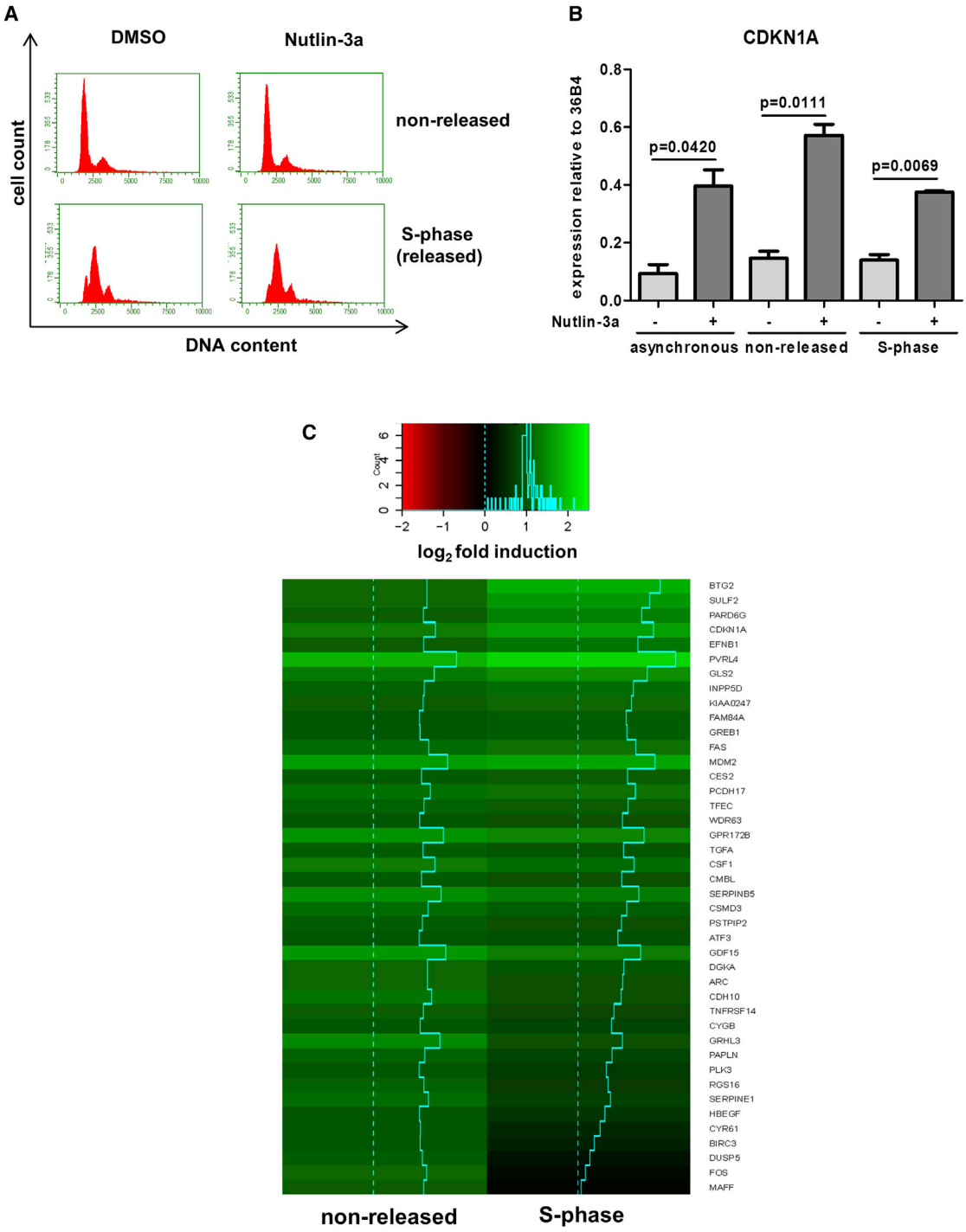

Figure 1. p53-Mediated Induction of Genes during S Phase

(A) Thymidine block and release result in comparable cell-cycle distribution, independent of Nutlin pretreatment. U2OS cells were subjected to a double thymidine block. Four hours before release from the block (or maintaining the block), 10 μM Nutlin (control: DMSO alone) was added. When the block was removed, the previous concentration of Nutlin was maintained, followed by further incubation for 4 hr. The cellular DNA content was determined by propidium iodide staining and flow cytometry.

(B) Nutlin induces comparable CDKN1A/p21 mRNA levels in proliferating cells, thymidine-arrested cells, and during S phase. U2OS cells were treated as in (A) or grown asynchronously. Subsequently, CDKN1A/p21 mRNA (RT-PCR) and protein (Figure S1A; immunoblot analysis) levels were determined in triplicate (n = 2).

(C) RNA deep sequencing analysis reveals comparable induction of genes by Nutlin, in thymidine-block as well as during S phase. U2OS cells were treated as in (A), followed by reverse transcription and next generation sequencing (Illumina). The heatmap reflects fold induction of the indicated genes by Nutlin according to

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et al., 2011), or DNA repair by homologous recombination (e.g., BRCA1 and RAD51) (Xie et al., 2012). Thus, p53 may support cell survival by preventing the accumulation of DNA damage during challenges to DNA replication.

Our previous results indicate that p53 can protect cells toward chemotherapeutics. These drugs still represent the mainstay of cancer treatment (Dobbelstein and Moll, 2014), and the induction of replicative stress is a key mechanism of many chemotherapies (Dobbelstein and Sørensen, 2015). When we activated p53 using the pharmacological Mdm2 inhibitor Nutlin-3a (Nutlin) (Vassilev et al., 2004), we observed decreased cytotoxicity of gemcitabine (Kranz and Dobbelstein, 2006), UV-irradiation (Kranz et al., 2008), and Wee1 inhibitors (Li et al., 2015). Initially, we ascribed this mostly to the cell-cycle arrest function of p53, keeping the cells out of S phase.

Here, we investigated whether p53 can influence the accumulation of DNA damage and replicative stress during S phase. However, previous reports suggested that p53 activity might be attenuated during DNA replication. Most of these experiments used hydroxyurea, an inhibitor of ribonucleotide reductase, to arrest the cells in S phase. Under such circumstances, the induction of CDKN1A by p53 appeared reduced (Gottifredi et al., 2001; Mattia et al., 2007). However, this does not exclude an activity of p53 when cells proceed through S phase rather than being arrested in it.

We show that p53 induces most of its target genes during S phase and increases the processivity of DNA replication. The absence of p53 causes replicative stress. This was observed not only in tumor-derived cell lines, but in fibroblasts and thymocytes from mice, comparing p53-proficient and p53-deficient genotypes. Thus, p53 protects the genome by ensuring undisturbed progression of DNA replication forks.

RESULTS

p53 Is Capable of Inducing the Majority of Its Target Genes during S Phase

Previous reports have suggested that p53 activity might be attenuated while cells are replicating their DNA, but this was mostly studied in the context of exogenous replicative stress (Gottifredi et al., 2001; Mattia et al., 2007). To address this during ongoing, unperturbed S phase, we synchronized U2OS cells using a double thymidine block (Bootsma et al., 1964; Xeros, 1962). We then compared the expression of a bona fide p53 target gene, CDKN1A/p21, between asynchronously proliferating cells, cells that were arrested by a thymidine block, and cells that had been released from the block to enter S phase. In each condition, the cells were treated with the Mdm2-inhibitor Nutlin-3a (Nutlin) to induce p53. Nutlin did not preclude the onset of S phase upon release from the thymidine block (Figure 1A). We found that CDKN1A/p21 mRNA levels were enhanced by Nutlin under all three conditions but did not grossly differ between asynchronous, arrested, and released cells (Figure 1B). When analyzing p21 protein levels by immunoblot, we observed the induction by Nutlin under all three conditions again; p21 was even more abundant in the cells that were released to enter S phase (Figure S1A). This argues against the view that p53 activity might be impaired during DNA replication. To broaden this analysis, we performed next-generation RNA sequencing to identify Nutlin-inducible genes in thymidine-blocked versus released cells. The induction of most p53-responsive genes was largely unchanged regardless of the thymidine block. Less than ten genes were no longer found induced by Nutlin when the cells were allowed to proceed in S phase (Figures 1C and S1B; Table S1). Thus, most capabilities of p53 to activate transcription are preserved while cells replicate their DNA. Previous investigations have mostly used hydroxyurea to arrest cells in S phase. Then, the expression of p53-responsive genes was indeed found attenuated (Gottifredi et al., 2001; Mattia et al., 2007). We propose that unperturbed S phase, but not an intra S phase arrest, permit full p53 activity.

p53 Activation Enhances DNA Replication Processivity

Next, we asked whether p53 might exert a genome-protective function during S phase by affecting DNA replication. U2OS cells were first treated by Nutlin to induce p53 activity, as confirmed by accumulation of p53 and its target gene products (Figure 2A). Subsequently, the characteristics of DNA replication were assessed by DNA fiber assays. The cells were sequentially incubated with two different nucleoside analogs. Upon spreading of the DNA on glass slides, we determined the length of DNA tracks that were detected by antibodies due to incorporation of the labels (Figure 2B).

Strikingly, the replication fork rate, indicating the distance that a replication fork moves within a given amount of time, consistently increased when the cells had been treated with Nutlin before adding the labeling nucleosides (Figures 2C and 2D). Notably, increased fork rate was only observed when Nutlin treatment was long enough to fully induce its target genes p21 and Mdm2, while DNA replication still continued (Figures S2A–S2F). We cannot exclude, however, that Nutlin may have shifted a majority of DNA-replicating cells toward the late S phase. Shortening the incubation time with the second label IdU still allowed the observation of an increased fork rate upon Nutlin treatment (Figures S2G–S2I). Conversely, the rate of origin firing, as determined by a stretch of first label flanked by two stretches of second label, was reduced upon Nutlin treatment (Figure 2E). This is in agreement with the frequent observation that replication fork progression rate and origin firing are inversely correlated (Petermann et al., 2010). However, interfering with origin firing by an inhibitor of Cdc7, as described previously (Montagnoli et al., 2008), did not compromise the increased fork progression rate in the presence of Nutlin, whereas Cdc7 inhibition was itself sufficient to induce p53 and to enhance the fork rate (Figures S3A–S3C). Thus, the increased fork rate by p53 does not strongly depend on origin firing. Counterstaining of the non-labeled fibers confirmed the

the color scheme (color and blue line, log 2). Genes displaying an induction of >2-fold and a p value <0.05 (based on at least two independent samples for each condition) in thymidine-blocked cells (Table S1) were included in the analysis. Most of these genes were induced by p53 to a similar degree when the cells had been released to enter S phase. The genes were sorted according to their p value identified in released cells. Only a small proportion of genes (bottom of the heatmap) were inducible only in non-released cells but not in S phase.

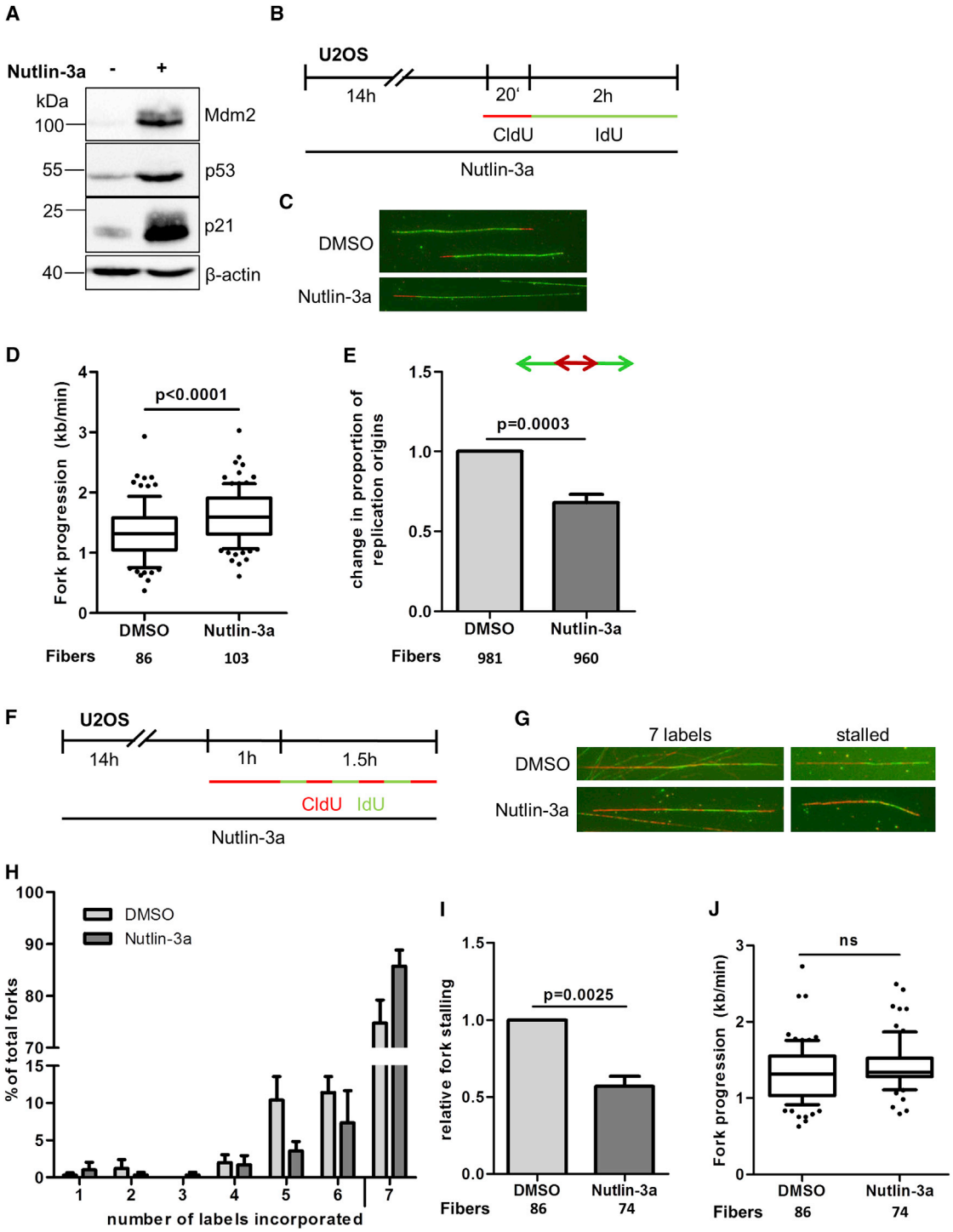

Figure 2. Enhanced Replication Fork Progression upon p53 Activation

(A) U2OS cells were treated with 8 μM Nutlin or the DMSO solvent for 14 hr and subjected to immunoblot analysis, detecting Mdm2, p53, p21, and β-actin to reveal the induction of p53 and its target gene products. A representative result of three biological replicates is shown.

(B U2OS cells were treated as described in (A) and further incubated with 5-chloro-2′deoxy-uridine (CldU, 25 μM, 20 min) followed by iodo-deoxy-uridine (IdU, 250 μM, 120 min) as indicated, to label newly synthesized DNA.

(C) Tracks of newly synthesized DNA were visualized by immunostaining of CldU (red) and IdU (green).

(D) Fork progression was determined through the length of the second label (IdU; kb/min). Nutlin pretreatment caused a highly significant increase in the fork rate. Note that fork progression is not identical to fork velocity, since shortening the observed track length can be a result of either slower replication or otherwise of sudden fork stalling. In addition to the experiment shown here, two replicates are shown in Figure S2D.

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notion that the labeled track is part of a larger, intact DNA fiber (Figure S3D). Taken together, the activation of p53 enhances the ability of replication forks to move along the template DNA.

In principle, two phenomena can affect the movement of a replication fork: (1) the speed by which the polymerases move along the template might vary, and (2) the frequency that replication stalls can affect the outcome of a fiber assay when stalling occurs during the period of DNA labeling (Figure S3E). To distinguish between these two possibilities, we modified the fiber assay. We incubated the cells with two different nucleosides, with repeated changes every 15 min (Figures 2F and 2G). In this way, we distinguished the reasons for increased track length upon p53 activation. Longer "stripes" containing one label would indicate faster polymerization, whereas a higher rate of fibers that contain all stripes with the two colors would indicate less fork stalling (i.e., higher processivity). Upon induction of p53 activity by Nutlin, we found a higher percentage of fibers with the maximum number of labels (Figures 2H, 2I, and S3F; Tables S2 and S3). In contrast, the length of the individual labels 2–5 did not significantly differ between the treatments with Nutlin or DMSO alone (Figure 2J; Tables S2 and S3). Thus, activation of p53 reduces the frequency of fork stalling and increases the processivity of DNA replication.

p53 Depletion Hampers Replication Fork Progression and So Does Mdm2 Depletion in a p53-Deficient Background

We then sought to determine whether the depletion of p53 might also modulate the ability of cells to replicate their DNA. First, we depleted p53 from U2OS cells by siRNA. One set of cells was also treated with gemcitabine, a nucleoside analog that induces replicative stress by false incorporation and by inhibition of ribonucleotide reductase. To assess the DNA damage response, we determined the levels of phospho-H2AX by immunoblot analysis. As expected, gemcitabine induced the accumulation of phospho-H2AX and also increased the levels of p53. Strikingly, however, phospho-H2AX was further increased when p53 had been knocked down (Figure 3A). We conclude that p53 depletion augments the DNA damage response upon treatment with an inducer of replicative stress.

To directly determine the impact of p53 depletion on DNA replication, we performed fiber assays in the presence or absence of gemcitabine (Figure 3B). In both cases, we observed a significant decrease in replication fork progression (Figures 3C, 3D, and S4A–S4F). Thus, baseline levels of p53 carry out a protective function against replicative stress in U2OS cells.

The depletion of p53 did not detectably alter the cell-cycle distribution of U2OS cells (Figures S3G and S3H), and in contrast with a previous report (Sablina et al., 2005), we did not observe changes in the levels of mitochondrial reactive oxygen species (ROS) upon depletion of p53 (Figure S4I). We then determined whether p53 might affect fork regression upon stalling, as has been reported in BRCA1-deficient cells (Ray Chaudhuri et al., 2016). However, the interruption of DNA synthesis by transient treatment with hydroxyurea (HU) (Figure 3E) after the first label did not decrease the proportion of replication forks that proceeded to incorporate the second label, regardless of p53 depletion (Figures S5A–S5C), nor did it shorten the track that was labeled before HU addition (Figures S5D–S5H), as would have been seen in the case of fork regression (Ray Chaudhuri et al., 2016). However, the depletion of p53 decreased the speed of fork progression after release from the HU block (Figures 3F, 3G, and S5I).

One of the most well-studied p53-responsive genes is Mdm2, encoding a p53 antagonist. Indeed, even the baseline expression levels of Mdm2 were found to depend on p53 (Figure 3A). We have recently reported an unexpected function of Mdm2 as a chromatin modifier (Wienken et al., 2016), raising the possibility that Mdm2 might affect DNA replication as well. To test this, we depleted Mdm2 from cells and determined the DNA replication fork progression rate. However, knocking down Mdm2 in p53-proficient cells would inevitably have led to robust and long-term activation of p53. Therefore, we subjected a cell line with a targeted deletion of p53 (Bunz et al., 1998) to siRNA transfection, thus knocking down Mdm2 in a p53-deficient background (Figure S5J). Strikingly, the depletion of Mdm2 reduced the fork rate of HCT116 $p53^{-/-}$ cells and exacerbated gemcitabine-mediated replicative stress (Figures 3H–3J). The transcription of known replicative stress response genes was not affected by Mdm2 depletion (Figure S5K).This suggests that Mdm2 may act as at least one mediator that allows p53 to protect DNA replication, perhaps through its ability to modify histones and chromatin structure (Wienken et al., 2016).

p53 Promotes the Progression of DNA Replication Forks in Murine Embryonic Fibroblasts

To validate a possible influence of p53 on DNA replication in non-transformed cells, we employed murine embryonic fibroblasts

(E) The proportion of first pulse origins, characterized by first label in the middle flanked by second label at both sides, among all labeled structures was determined. Nutlin-pretreated cells had a lower proportion of firing origins, in agreement with lower replicative stress. The number of evaluated fibers with labeled tracks from five independent experiments is indicated below each box.

(F) Fiber assay to distinguish the speed of fork progression from the frequency of fork discontinuation (processivity; Figure S3E). After treatment as in (A), the cells were subjected to one long first CldU label and then repeatedly incubated with IdU and CldU for 15 min each.

(G) As a result of fiber staining, a long red track is followed by "stripes" that reflect CldU and IdU incorporation during the subsequent 15 min pulses. Stalled forks are reflected by fibers with less than seven labels.

(H) The number of forks that proceeded through n labels is displayed for DMSO-treated and Nutlin-treated cells. Seven labels reflect full progression of the fork throughout the entire labeling time. Numbers lower than seven indicate that the replication fork had discontinued during the labeling time.

(I) The proportion of prematurely stalled forks (less than seven labels) indicates that Nutlin-treatment reduced fork stalling and enhanced the processivity of DNA replication. Results from three independent experiments are summarized by the mean and the SEM. In each experiment, the rate of prematurely stalled forks was determined for DMSO-treated or Nutlin-treated cells, and the relative decrease in this rate upon Nutlin treatment was calculated.

(J) Fork speed was determined through the length of labels two to five (kb/min). Nutlin treatment did not change the fork speed significantly, in contrast to its impact on fork processivity (H and I).

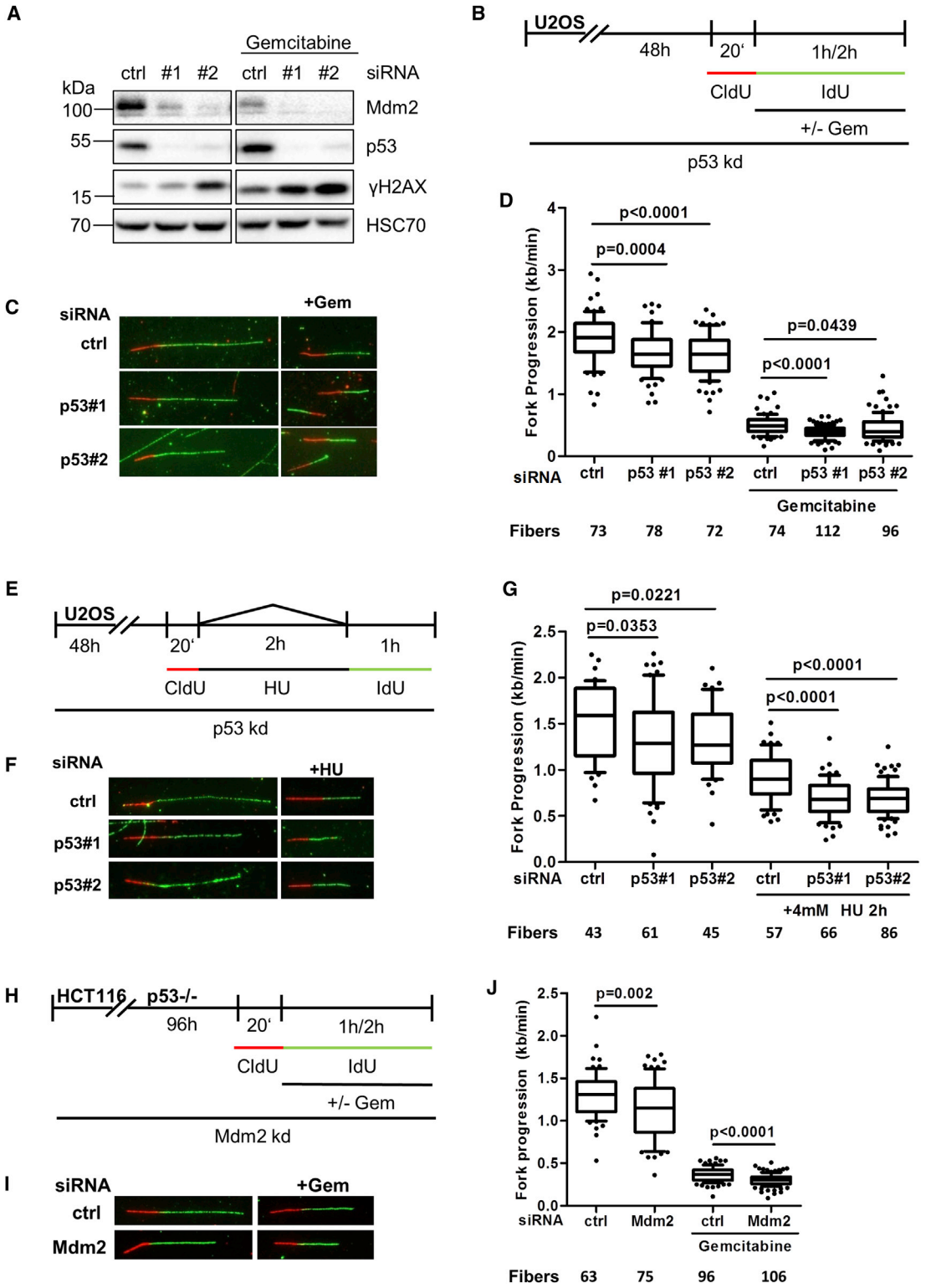

Figure 3. Increased Replicative Stress upon p53 Depletion and upon Depletion of Mdm2 from p53-Deficient Cells

(A) U2OS cells were treated with siRNA to knock down p53 or control siRNA for 48 hr, followed by treatment with 400 nM gemcitabine for 2 hr and immunoblot analysis. Upon p53 knockdown, decreased amounts of Mdm2 and increased levels of phosphoH2AX (γH2AX) were observed. A representative result out of greater than five replicates is shown.

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(MEFs). Wild-type MEFs were treated with Nutlin to induce p53 activity, followed by DNA fiber assays (Figure 4A). Strikingly, the median DNA replication fork rate was increased 1.5-fold by Nutlin treatment, indicating that p53 is capable of strongly augmenting the movement of DNA replication forks in these cells (Figures 4B and 4C). To test whether baseline p53 levels also affect fork progression, we employed MEFs from mice with a conditional p53 knockout (Jonkers et al., 2001). In these cells, the cre-recombinase can be induced by 4-hydroxy-tamoxifen (4-OHT). We were comparing cells that contained wild-type p53 with cells that had had been modified (''floxed'') by LoxP-insertions on both p53 alleles, as well as heterozygous cells, in the presence or absence of gemcitabine When cells had been treated with 4-OHT to delete the floxed alleles, we observed that replication fork progression was severely impaired in MEFs with a homozygous p53 deletion (Figures 4D–4F, S6A, and S6B). Similarly, the impact of gemcitabine on fork progression was aggravated by loss of p53, even in MEFs where only one allele was lost by recombination (Figures 4G–4I and S6C). Some increase in the proportion of cells in S phase was also observed in cells that lack one or two p53 alleles (Figures S6D and S6E). In sum, p53 supports DNA replication in primary cells, and for this task, haploinsufficiency of the p53 gene was observed.

To assess the role of Mdm2 in DNA replication, we again used a p53-deficient cell system to avoid p53 induction by loss of mdm2. When comparing MEFs with a targeted deletion of p53 alone (single knockout) with cells that lack both p53 and mdm2 (double knockout) (Montes de Oca Luna et al., 1995), we observed a marked decrease in DNA replication fork rate in mdm2-deficient cells (Figures 4J–4L). Genes with relevance to replicative stress were not found to be transcribed differentially upon Mdm2 deletion (Figure S6F), based on our previous analyses (Wienken et al., 2016). In conclusion, and similar to HCT116 $p53^{-/-}$ cells (Figures 3H–3J), Mdm2 is required for efficient DNA replication fork progression and may thus serve as a mediator allowing p53 to enhance replication processivity.

p53 Increases DNA Replication Fork Processivity in Murine Thymocytes before the Occurrence of T Cell Lymphoma

Finally, we sought to test the impact of p53 on replication fork progression under conditions that are as close as possible to a living organism and in cells that are prone to cancer formation in animals that lack p53. To this end, we employed thymocytes freshly isolated from $p53^{-/-}$ mice or their p53-proficient littermates, at 4 weeks of age (i.e., at a time point prior to the occurrence of pre-cancerous cell clones) (Dudgeon et al., 2014). Thymocytes from $p53^{-/-}$ mice had a higher level of phosphorylated H2AX than the corresponding $p53^{+/+}$ cells (Figure 5A), at least compatible with the view that the deletion of p53 leads to enhanced replicative stress in vivo. This was not accompanied by changes in mitochondrial ROS (Figure S7A). Of note, $p53^{-/-}$ thymocytes showed a markedly impaired progression of DNA replication forks, in the presence or absence of gemcitabine, (Figures 5B–5E and S7B–S7G). We also determined the processivity of replication forks by frequent changes in the label (Figures 5F–5J), an assay that we had first performed in U2OS cells (Figures 2F–2J). When $p53^{-/-}$ thymocytes were repeatedly incubated with different labels of newly synthesized DNA, it turned out that the number of stalled replication forks increased in comparison to p53-proficient cells (Figure 5H, summarized in Figure 5I). In contrast, the replication speed observed in continuous tracks was not detectably affected by the p53 status (Figure 5J), nor did we observe gross changes in cell-cycle distribution (Figures S7H and S7I). Thus, p53 increases the processivity of DNA replication in cells from the thymus. We propose that the resulting replicative stress in response to p53 loss might contribute to the occurrence of chromosomal damage in these cells (Dudgeon et al., 2014), in accordance with the formation of T cell lymphomas, observed later in the lives of more than 90% of $p53^{-/-}$ mice (Donehower et al., 1992).

DISCUSSION

P53 is active during S phase, and it strengthens the processivity of DNA replication. This was not only observed in p53-proficient cancer cells but also in primary cells. Even thymocytes, the precursors of the most frequently found tumor species in $p53^{-/-}$ mice, displayed impaired DNA replication. These observations all support the view that p53 is not only acting ex post, by eliminating or repairing cells that suffered DNA damage. Rather, p53 appears to avoid replicative stress and associated damage ex ante.

(B) Upon depletion of p53 (or control siRNA transfection), newly synthesized DNA was labeled with CldU and IdU. Gemcitabine was added during the IdU label only.
(C) DNA fibers were immunostained under the conditions described in (A) and (B).
(D) Fork progression was determined through the length of the second label (IdU; kb/min). p53-depleted cells showed a highly significant reduction in fork progression. This experiment was repeated as shown in Figure S4C.
(E) U2OS cells were treated with control and p53 siRNA for 48 hr, after which they were labeled with 25 μM CldU for 20 min. Subsequently, cells were treated with 4 mM hydroxyurea (HU) for 2 hr to arrest replication forks. Upon release from the HU block, the cells were labeled with 25 μM IdU for another hour. Control cells were labeled with CldU and IdU without interrupting replication by HU.
(F) Labeled replication tracks were immunostained to visualize CldU (red) and IdU (green).
(G) Fork progression was determined through the track length corresponding to the second label (IdU). P53 depletion significantly reduced the progression of replication forks. This effect was even more pronounced after an HU block. Similar results to this experiment were found independently in Figure S5I.
(H) HCT116 cells with a targeted deletion of p53 were transfected with siRNA to Mdm2 or control siRNA. Subsequently, fork progression was determined in the presence or absence of gemcitabine. Similar results were obtained with independent siRNAs to Mdm2 (not shown).
(I) Representative labeled tracks are shown as in in (C) and (F).
(J) Boxplot analysis of the fork rates, as determined by the tracks derived from the second label, revealed reduced replication fork progression in response to Mdm2 depletion.

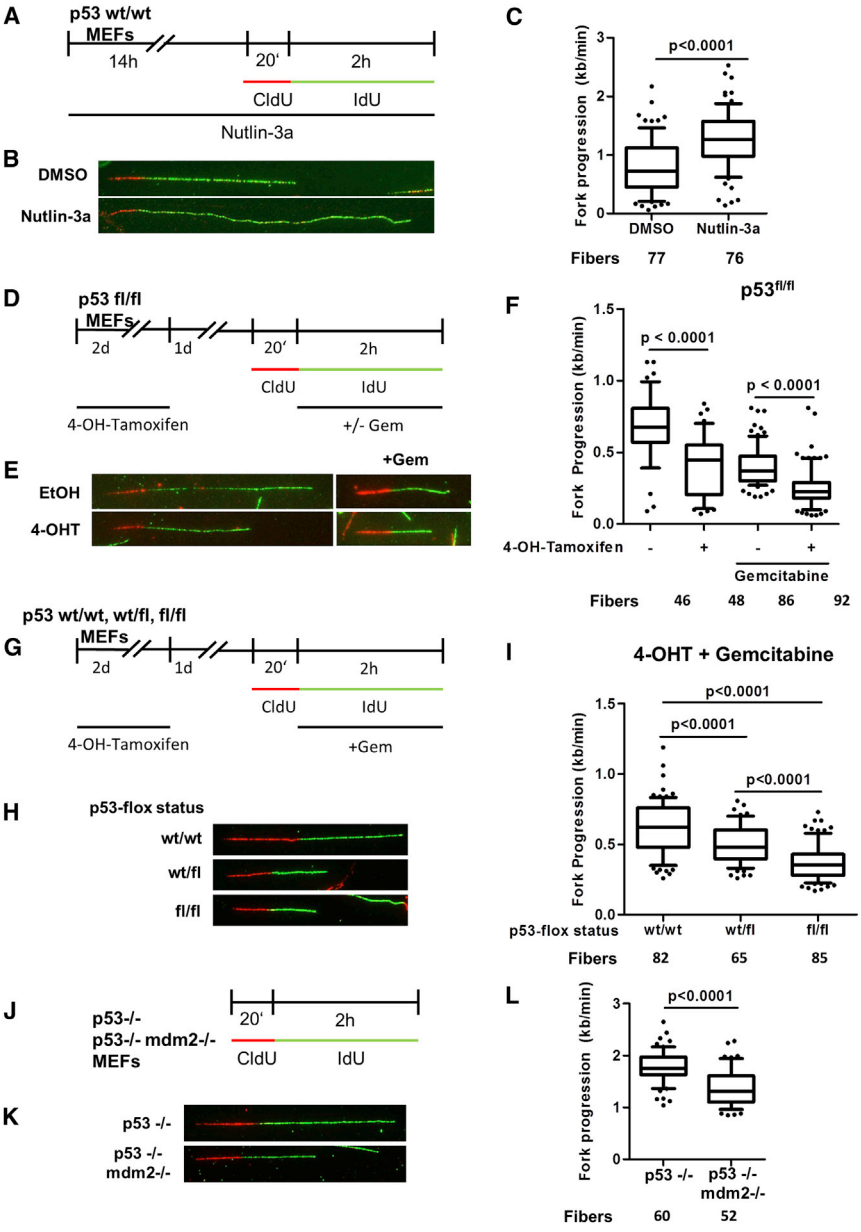

Figure 4. p53-Supported DNA Replication in Murine Embryonic Fibroblasts

(A) Wild-type murine embryonic fibroblasts (MEFs) were treated with Nutlin or the DMSO control for 14 hr, followed by two-label fiber assays as indicated.

(B) Fork progression was determined by immunostaining of CldU (red) and IdU (green).

(C) The degree of fork progression was increased by Nutlin pretreatment, on a highly significant level. The indicated numbers of labeled fibers were evaluated independently by two independent evaluators.

(D) MEFs with floxed p53 and a Cre-ERT2 fusion gene driven by the ubiquitously active Rosa26 promoter were first treated with 4-hydroxytamoxifen (4-OHT; 200 nM, 48 hr) to excise p53 exons 2–10 (Jonkers et al., 2001). Moreover, ethanol was used as a solvent control to 4-OHT. Subsequently, the cells were labeled with CldU (25 μM, 20 min) and IdU (250 μM, 2 hr). In the indicated experiments, 400 nM gemcitabine was added to the second label.

(E) Labeled DNA tracks were immunostained to detect CldU (red) and IdU (green).

(F) When comparing MEFs with two floxed p53 alleles before and after treatment with 4-OHT to excise p53, a strong reduction in fork progression was observed. This was true in otherwise untreated cells and also upon treatment with gemcitabine. A replicate of this experiment is shown in Figure S6B.

(G) Cells with two wild-type p53 alleles (wt/wt) and with a mixed genotype (wt/fl) were investigated in comparison with the fl/fl p53 genotype. 4-OHT treatment was applied to excise p53 exons 2–10, followed by labeling with CldU (25 μM, 20 mins) and IdU (25 μM), in the presence of gemcitabine, for 2 hr.

(H) Labeled tracks of newly synthesized DNA were stained with antibodies against CldU and IdU.

(I) Labeled track lengths were significantly reduced upon removal of one or both p53 alleles. The result was confirmed in Figure S6C.

(J) MEFs with a $p53^{-/-}$ (single knockout) or a $p53^{-/-}$;$mdm2^{-/-}$ (double knockout) genotype were incubated with the CldU and IdU labels as indicated, followed by analysis of DNA fork progression. Note that these MEFs are not directly comparable to the preparations used in (A)–(I), due to differences in passage numbers and genetic background, as specified in the Experimental Procedures.

(K) Representative tracks are shown for each genotype.

(L) Quantification of fiber lengths revealed that the absence of mdm2, on the $p53^{-/-}$ background, led to a marked loss in replication fork progression.

Our previous studies had already indicated that p53 activation by Nutlin can protect p53-proficient cells from the cytotoxic effects of gemcitabine (Kranz and Dobbelstein, 2006) and also from the combined effects of Wee1 inhibitors and gemcitabine (Li et al., 2015). However, in those experiments, we had pretreated the cells with Nutlin for an extended period of time, largely avoiding their entry into S phase. It appears obvious that cells are resistant to replicative stressors when not in S. Here, however, we show that p53 activity reaches far beyond cell-cycle arrest to protect the cell against nucleoside analogs. Even under conditions where cells still undergo DNA replication, p53 still exerts protection against replication fork stalling.

The vast majority of mice that lack p53 will develop T cell lymphoma at ~6 months of age (Donehower et al., 1992). Deep sequencing analysis revealed that the first clones with rearranged DNA become detectable at 9 weeks after birth (Dudgeon et al., 2014). Our results show that replicative stress occurs in the bulk of thymocytes even earlier, i.e., at 4 weeks of age. This strongly argues that replicative stress precedes the occurrence of detectable premalignant cell clones in $p53^{-/-}$ mice. It is

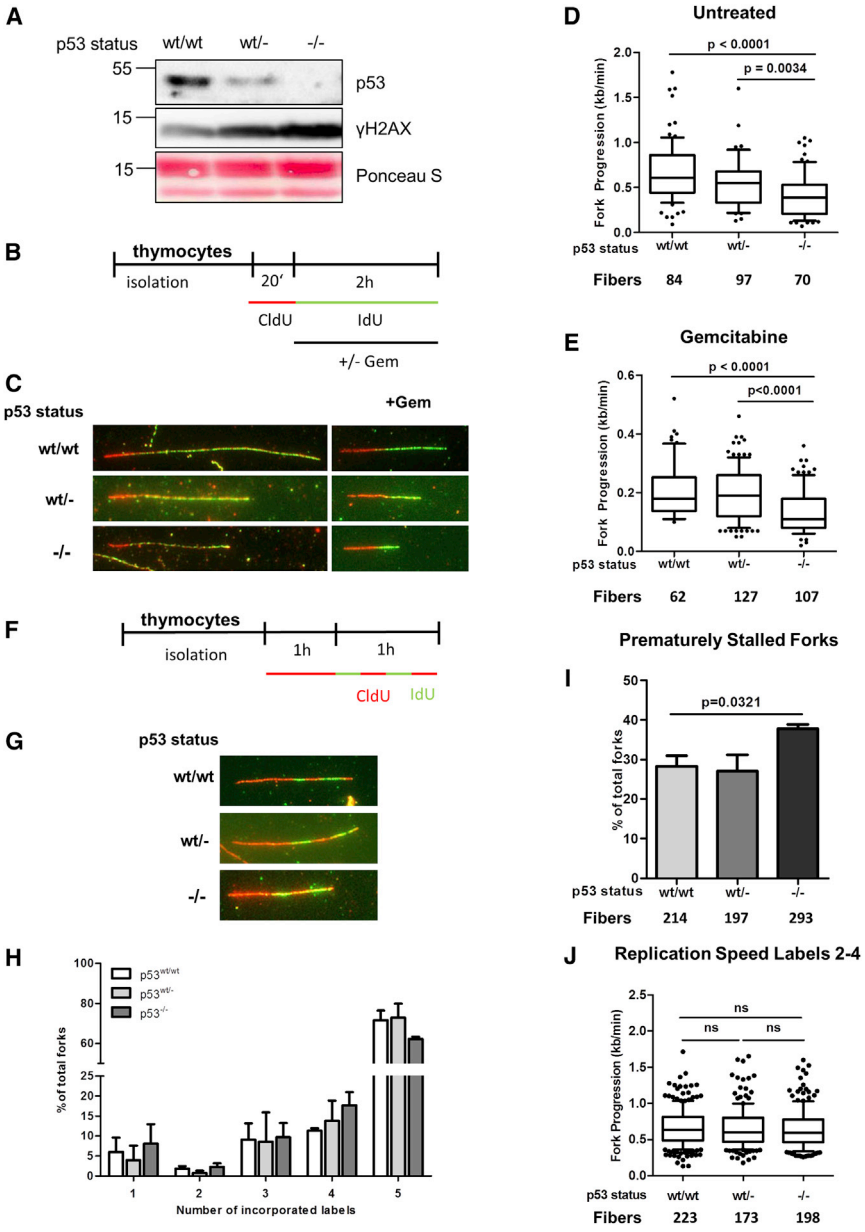

Figure 5. Replicative Stress in Murine Thymocytes Lacking p53

(A) Immunoblot analysis revealing enhanced H2AX phosphorylation in $p53^{-/-}$ thymocytes. Freshly isolated thymocytes from mice with the indicated genotype were subjected to immunoblot analysis to detect p53 and phospho-Ser139 histone 2A (γH2AX). Total protein staining by Ponceau S served as a loading control.

(B) Thymocytes were subjected to fiber assays immediately after their isolation from 4-week-old mice with different p53 status (homozygous knockout, heterozygous, or wild-type). The labeling (CldU and IdU, 25 μM each) was performed in the presence or absence of 100 nM gemcitabine.

(C) Labeled DNA tracks were immunostained to visualize incorporated CldU (red) and IdU (green).

(D and E) Fork progression was assessed by the length of the IdU fibers and displayed by boxplots. Decreased fork progression was observed in homozygous knockout cells, at a highly significant level, in untreated cells (D) and in gemcitabine-treated thymocytes (E) (confirmation in Figures S7C and S7D).

(F) To determine fork processivity, multiple labels were performed as in Figures 2F–2J, with a total of five labels.

(G) Immunostaining allowed the clear distinction of up to five CldU/IdU-labeled tracks.

(H) The number of prematurely stalled replication forks (less than five labels) was found increased in thymocytes from $p53^{-/-}$ mice, compared to cells from p53-proficient mice.

(I) The proportion of prematurely stalled replication forks was significantly increased in p53-deficient thymocytes. Results from three independent experiments are summarized by the mean and the SEM. In each experiment, the rate of prematurely stalled forks was determined for cells from mice with a $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ genotype, and the relative decrease in this rate depending on the deletion of the p53 alleles was calculated.

(J) As observed in U2OS cells (Figure 2J), the replication speeds observed in single tracks, here determined by labels two through four, were unchanged.

at least conceivable that replicative stress in thymocytes plays a causal role for tumor formation. In such a scenario, the attenuation of replicative stress would represent an essential part of the tumor suppressive activity of p53. Accordingly, it is noteworthy that even the triple deletion of the p53 target genes cdkn1a, pmaip1/noxa, and bbc3/puma does not recapitulate the phenotype of p53 null mice. The triple deletions do not give rise to T cell lymphoma or other cancers (Valente et al., 2013). Rather, p53 target genes that support DNA replication (e.g., mdm2) might represent mediators of tumor suppression in vivo.

It would be tempting to speculate that p53 can support DNA replication by physical association with the replication fork. It has long been noticed that p53 is capable of interacting with insertion/deletion mismatches that may form during replication (Lee et al., 1995) and with the "chickenfoot" structure at retracted replication forks (Subramanian and Griffith, 2005) in vitro. However, in our hands, no visible fork regression was found when depleting p53 (Figures S5D and S5H), and the accumulation of p53 only affected DNA replication fork progression if it had lasted for half a day (Figures 2A–2D and S2A–S2F), an amount of time required to enhance the mRNA levels corresponding to most p53 target genes (Zhao et al., 2000). While not excluding transcription-independent contributions of p53 (e.g., by suppressing excessive homologous recombination) (Arias-Lopez et al., 2006; Bertrand et al., 2004; Janz and Wiesmüller, 2002), we propose that p53-target genes are at least necessary for the impact of p53 on DNA replication. Indeed, one

of the genes with the strongest p53-inducibility, Mdm2, was found to facilitate DNA replication independent of its ability to antagonize p53 (Figures 3H–3J and 4J–4L). Interestingly, as we have shown recently (Wienken et al., 2016), Mdm2 acts as a chromatin modifier, and chromatin modifications are capable of interfering with DNA replication in multiple ways (Alabert and Groth, 2012). However, Mdm2 is also capable of binding Nbs1 (Bouska et al., 2008), raising the possibility that it might support the functions of the MRN complex in resolving replication intermediates (Bruhn et al., 2014) or in activating ATR-Chk1-signaling (Duursma et al., 2013). The anti-oxidative activity of Mdm2 (Riscal et al., 2016) may also contribute to smooth DNA replication, although we have not observed increased ROS upon removal of p53 (Figures S4I and S7A). Curiously, Mdm2 was also reported to enhance replicative stress when overexpressed (Frum et al., 2014). However, transgenic overexpression of Mdm2 has long been known to yield paradoxical growth arrest rather than its physiological oncogenic function (Brown et al., 1998), and similar effects might be responsible for the observed replication stress in overexpression systems. In our study, we thus avoided Mdm2 transgenes, and all observations were made by exploring Mdm2 from an endogenous source.

Perhaps similarly, a recent report described the deceleration of replication fork progression as a response to p53 activity (Hampp et al., 2016), in seeming contrast with our observations and with another report on a role for p53 in avoiding conflicts of transcription and replication (Yeo et al., 2016). However, the majority of the experiments reported by Hampp et al. (2016) were carried out using transgenic overexpression of p53, which might well have led to non-physiological situations, or in cell systems other than those described in our study. In contrast, we have performed our assays in the very same cells from p53-deficient animals that give rise to cancer later in life (i.e., thymocytes) (Figure 5). There, endogenous wild-type p53 contributed to DNA replication with greater processivity than in p53-deficient thymocytes. Therefore, we propose that in a cancer-relevant system, p53 acts to protect DNA replication forks against stalling and collapse, thus preserving the genome during this highly vulnerable phase.

On top of Mdm2, additional p53 target genes might be involved in the p53-driven support of DNA replication. Candidates include the ribonucleotide reductase p53R2, capable of increasing the available dNTP pool (Nakano et al., 2000; Tanaka et al., 2000). Similarly, the p53-inducible lncRNA NEAT1 dampens replicative stress through paraspeckle formation and ATR activation (Adriaens et al., 2016). On the other hand, the p53-inducible gene product p21/CDKN1A was reported to enhance rather than inhibit replicative stress, at least under circumstances that involve UV irradiation (Mansilla et al., 2013) or senescence (Galanos et al., 2016). Thus, the impact of p53 on DNA replication may also depend on the exogenous stressors and the cell-specific set of p53-responsive genes.

The relatively long time periods of labeling replicating DNA used in many of our experiments might conceivably have led to the collision of forks, considering average replication origin distances (Conti et al., 2007; Lebofsky et al., 2006). However, we rarely observed such collisions, perhaps pointing to different replication origin distances between cell species and also to the slower progression of replication forks between closely situated origins (Conti et al., 2007).

How could replicative stress lead to cancer? In addition to nucleotide misincorporations, cells with incompletely replicated DNA might enter irregular mitosis, giving rise to chromosomal instability. In agreement, sites of replicative stress often coincide with common fragile sites (i.e., breakpoints of chromosome rearrangements) (Glover et al., 2005). The removal of p53 strongly compromises the G2-M checkpoint that normally prevents the onset of mitosis when DNA is damaged or incompletely replicated (Taylor et al., 1999). Thus, p53 may prevent the accumulation of chromosomal rearrangements by avoiding fork stalling and also premature mitosis. In support of this model, the thymocytes in $p53^{-/-}$ mice accumulate chromosomal rearrangements, and even chromotrypsis, at ages before full-blown T cell lymphomas arise (Dudgeon et al., 2014).

At least in MEFs, even a heterozygous p53 deletion was capable of increasing replicative stress (Figure 4). This is similar to the situation in patients suffering from Li-Fraumeni syndrome. There, all somatic cells carry one allele with a p53 mutation and another allele with an intact TP53 gene. So far, the occurrence of cancer in these patients was mostly ascribed to a loss of heterozygosity. In contrast, our results suggest that even the heterozygous situation may increase replicative stress at least in some cell species and thus enhance the probability of mutations or chromosomal rearrangements. Thus, a subset of cells in such patients may be cancer-prone even before the intact allele of p53 is lost.

Approximately 50% of all human malignant tumors carry a p53 mutation. However, tumor progression studies such as those on colorectal cancer revealed that, in general, p53 undergoes mutations late during tumor development (Fearon and Vogelstein, 1990). One reason for this phenomenon might be that the presence of p53, through its ability to ensure unperturbed DNA replication, can support the initial survival of cancer cells. Only at late stages, the tumor cell might be able to tolerate the loss of p53 and the concomitant replicative stress. It might then take advantage of the enhanced genomic variability and the consequent ability of adaptation (e.g., during invasion and metastasis).

Our results also point to a selective vulnerability of p53-deficient cells toward chemotherapeutics that enhance replicative stress. Indeed, enhanced sensitivity of $p53^{-/-}$ cells toward selected chemotherapeutics was described more than 15 years ago (Bunz et al., 1999). Recently, this phenomenon was ascribed to an ability of p53 to prevent interference between transcription and DNA replication that would otherwise require topoisomerase activity to be resolved (Yeo et al., 2016). Here, we identify p53 as a supporter of DNA replication processivity in primary cells that undergo tumor formation when p53 is deleted.

P53 has long been known as a guardian of the genome. However, most of these protective functions appeared retroactive from the point of a single cell, as if a guardian killed his injured master after an attack. Here, however, we identify a proactive way of how p53 prevents DNA damage even before it occurs.

EXPERIMENTAL PROCEDURES

The Supplemental Experimental Procedures are available online.

Cell Culture, siRNA Transfections, and Treatment

U2OS (Osteosarcoma, p53 wild-type) cells were transfected with Lipofectamine 2000, synchronized with a double 2 mM thymidine block, and/or treated with Nutlin-3a (Sigma, N6287), gemcitabine (Eli Lilly), and hydroxyurea (Sigma-Aldrich, H8627) as indicated.

Mouse embryonic fibroblasts (MEFs) were isolated from $p53^{loxP}$ mice (Jonkers et al., 2001) (B6.129P2-Trp53tm1Brn/J, Jackson Laboratories) at E13.5. Littermates were used to prepare MEFs from a C57Bl6N/129SV background (50%–50%, N2 Backcross). MEFs from $p53^{-/-}$ and from $p53^{-/-}$;$mdm2^{-/-}$ mice were obtained from Y. Zhang, University of North Carolina (Itahana et al., 2007; Wienken et al., 2016).

Thymocytes were isolated from 4-week-old Trp53tm1Tyj mice (Jackson Laboratories), carrying a deletion of the p53-encoding gene (Jacks et al., 1994). The thymus was isolated and strained through a 40 μM mesh. Cells were used for experiments immediately thereafter.

Fiber Assays

DNA fiber assays to analyze replication fork progression and origin firing were essentially carried out as described previously (Köpper et al., 2013). For two-label assays, the cells were incubated with 5-chloro-2′-deoxyuridine (CldU) for 20 min, followed by 5-iodo-2′-deoxyuridine (IdU; both from Sigma-Aldrich) for 20 min, 1, or 2 hr, accompanied by treatment with gemcitabine or inhibitors as indicated. For multiple label assays, cells were pulse labeled with CldU for 1 hr, followed by alternate labels of IdU and CldU.

DNA fibers were spread on glass slides as described (Köpper et al., 2013). After acid treatment, CldU- and IdU-labeled tracts were detected by 1 hr incubation at 20°C with rat anti-BrdU antibody (dilution 1:500 detects BrdU and CldU; AbD Serotec) and mouse anti-BrdU antibody (1:200–1:500, detects BrdU and IdU; Becton Dickinson). Slides were fixed in 4% paraformaldehyde/PBS and incubated for 2 hr at 20°C with Alexa Fluor 555-conjugated goat anti-rat antibody (dilution 1:150–250) or Alexa Fluor 488-conjugated goat anti-mouse antibody (dilution 1:100–1:250; both from Molecular Probes/Thermo Fisher). In some cases, counterstaining was performed with an antibody to single-stranded DNA (Millipore, #MAB 3034) and an Alexa647-coupled secondary antibody. Fiber images were acquired by fluorescence microscopy.

Statistical Testing

Statistical testing was performed using Graph Pad Prism 6. A two-sided unpaired t test was calculated with an assumed significance for p values $\leq$ 5%. The term "n.s." indicates that no significant difference was found.

ACCESSION NUMBERS

The accession number for the gene expression data reported in Figures 1C and S1B and Table S1 is NCBI/GEO: GSE87668.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.036.

AUTHOR CONTRIBUTIONS

M.D. conceived the project and drafted the manuscript. All authors revised and approved the manuscript. I.K., S.R., M.F., and L.M. performed the experiments. M.W. provided guidance with the RNA-seq analysis, Y.L. with the fiber assays, and R.S.-H. with mouse experiments.

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