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Excess ribonucleotide reductase R2 subunits coordinate the S phase checkpoint to facilitate DNA damage repair and recovery from replication stress

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ABSTRACT

Ribonucleotide reductase (RNR), which consists of R1 and R2 subunits, catalyzes a key step of deoxyribonucleoside triphosphate (dNTP) synthesis for DNA replication and repair. The R2 subunit is controlled in a cell cycle-specific manner for timely DNA synthesis and is negatively regulated by p53 in response to DNA damage. Herein we demonstrate that the presence of excess R2 subunits in *p53*(−/−) HCT-116 human colon cancer cells protects against DNA damage and replication stress. siRNA-mediated stable knockdown (>80%) of excess R2 subunits has no effect on proliferative growth but results in enhanced accumulation of γ-H2Ax and delayed recovery from DNA lesions inflicted by exposure to cisplatin and Triapine. This accentuated induction of γ-H2Ax in R2-knockdown cells is attributed to reduced ability to repair damaged DNA and overcome replication blockage. The lack of excess R2 subunits consequently augments chk1 activation and cdc25A degradation, causing impeded cell progression through the S phase and enhanced apoptosis in response to DNA damage and replication stress. In contrast, the level of R1 subunits appears to be limiting, since depletion of the R1 subunit directly activates the S phase checkpoint due to replication stress associated with impaired RNR activity. These findings suggest that excess R2 subunits facilitate DNA damage repair and recovery from replication stress through coordination with the S phase checkpoint in the absence of functional p53. Thus, the level of the R2 subunit constitutes an important determinant of the chemosensitivity of cancer cells and serves as a potential target for enhancement of DNA-damage based therapy.

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Abbreviations: RNR, ribonucleotide reductase; ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related; DSBs, DNA double-stranded breaks; IR, ionizing radiation; chk1, checkpoint kinase 1; γ-H2Ax, phosphorylated-histone 2Ax; sh/siRNA, short hairpin/short interference RNA; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; MDC1, mediator of DNA damage checkpoint protein 1; 53BP, p53 binding protein 1; BRCA1, breast cancer 1 early onset; cdk2, cyclin dependent kinase 2; cdc25, cell division cycle 25; NBS1, Nijmegen breakage syndrome 1

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1. Introduction

Ribonucleotide reductase (RNR) catalyzes a critical step in the enzymatic reactions involved in the conversion of ribonucleoside diphosphates (NDPs) to their corresponding deoxyribonucleoside diphosphates (dNDPs) by reduction of the hydroxyl group at the C-2′ position of the ribose moiety [1]. The active form of RNR is believed to be a heterotetramer consisting of two R2 (44 kDa) and two R1 (88 kDa) subunits [1]. The R2 subunit contains a tyrosyl free radical and an iron center required for the enzymatic reaction [2,3]. The R1 subunit contains a pair of sulfhydryl groups at the catalytic site essential for the reduction of the NDPs, as well as two independent allosteric sites that regulate the catalytic activity and substrate specificity [1].

In mammalian cells, the cell cycle-specific variation of the R2 subunit plays an important role in the regulation of overall RNR activity for timely DNA synthesis. The level of the R2 subunit of RNR begins to rise in late G1 and reaches the highest level during the S phase, while the level of the R1 subunit of RNR remains relatively constant throughout the cell cycle [4–6]. The fluctuation of the R2 protein level is attributed to transcriptional up-regulation during S phase, as well as proteasome-mediated degradation of the protein when cells enter mitosis [7,8]. A marked increase in RNR activity during the S phase coincides with the S-phase specific elevation in the R2 subunit [4,6,9]. Thus, the R2 subunit has been considered to be limiting for RNR activity.

In contrast, evidence from a variety of studies has demonstrated that the R2 subunit is not limiting for RNR activity. The addition of exogenous purified R1 subunits, but not R2 subunits, to cell-free extracts causes a marked stimulation of measurable RNR activity [10–13]. Various hydroxyurea-resistant cell lines containing a high level of overproduced R2 subunits exhibit only a modest increase in RNR activity and dNTP pools, with the same S phase-specific regulation of the R2 subunit as parental cells [6,7,10]. Therefore, the elevated level of R2 subunits, which can be excessively produced during the S phase, is not strictly coordinated with an increase in RNR activity required for DNA synthesis. It has been reported that overexpression of the R2 subunit in cooperation with activated oncogenes causes malignant transformation [14,15]. Overexpression of the R2 subunit has also been demonstrated to result in reduced radiosensitivity by enhancing repair of IR-induced DNA damage [16].

The integrity of the genome and the progress of DNA replication are constantly monitored by a set of checkpoint kinase cascades that respond to DNA damage and stalled DNA replication. Activation of checkpoint pathways promotes the delay of cell cycle progression, presumably allowing any required repair to take place before commencement of DNA replication and mitosis [17]. DNA double-stranded breaks (DSBs) and arrested DNA replication forks prompt the activation of members of the phosphatidylinositol kinase-related protein family, ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad3-related) [18,19]. ATM and ATR mediate the phosphorylation at serine 139 near the C-terminal of the histone H2A variant H2Ax in chromatin flanking DSBs [20,21]. This process is essential for the recruitment of MDC1, 53BP1, and BRCA1 to sites of DNA damage to facilitate repair [17,22].

In addition, activated ATM and ATR act directly to propagate checkpoint signals through the phosphorylated activation of their downstream kinases, chk1 and chk2 [23,24]. Chk1 and chk2 block the G2/M transition by phosphorylating and inhibiting cdc25C, a phosphatase promoting the activation of cdc2/cyclin B for mitotic entry [23,25]. In response to DNA damage or replication stress, chk1 and chk2 also mediate the S phase checkpoint through phosphorylation and degradation of cdc25A, which prevents the activation of cdk2/cyclin E [26,27].

The involvement of RNR in DNA damage repair has been elucidated relatively recently by the discovery of p53R2, a homologue of the R2 subunit. p53R2 is targeted by the p53-dependent checkpoint pathway through transcriptional activation in response to DNA damage [28,29]. Concomitantly, the level of R2 subunits is progressively repressed in a p53-dependent manner [28,30,31], which serves to stop replicative DNA synthesis. Hence, p53R2 is able to form RNR with the R1 subunit to supply dNTPs for DNA repair during p53-dependent G1 arrest [32]. However, considering that about one-half of all human cancers harbor mutant p53, the ability of these malignant cells to support DNA damage repair may be compromised due to a deficiency in p53R2 induction. Whether alternative sources of dNTPs or R2 subunits are utilized to support DNA repair in this scenario remains unclear.

We have previously demonstrated that, in response to DNA damage, HCT-116 human colon cancer cells lacking p53 do not exhibit the degradation of the R2 protein as manifested by their wild-type p53 counterparts [31]. Furthermore, short hairpin/short interference RNA (sh/siRNA)-mediated stable knockdown of the R2 subunit leads to increased sensitivity of these cells to both DNA damaging agents and RNR inhibitors. These findings suggest that the R2 subunit of RNR is also involved in the supply of dNTPs for DNA damage repair especially in the absence of p53R2 induction. In the present study, we examined the functional importance of RNR subunits and further investigated the role of the R2 subunit in the ability of p53-deficient cancer cells to support DNA damage repair. The relationship between the abundance of the R2 subunit and the S phase checkpoint was also elucidated. We have found that the presence of excess R2 subunits functions coordinately with the S phase checkpoint to contend with DNA damage and to alleviate replication stress.

2. Materials and methods

2.1. Cell culture and reagents

p53-null, *p53*(−/−) HCT-116 human colon cancer cells was a gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Vector (pooled total population) and individual R2-knockdown *p53*(−/−) HCT-116 cell clones were established by stable transfection with the vector (pZZeomU6) and R2-sh/siRNA expressing (pZZeomU6-R2-siRNA) constructs, respectively, and maintained in the selective marker Zeocin as described previously [31]. All cell lines were cultured

in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin–streptomycin antibiotics. To determine growth kinetics, cells were trypsinized and counted using a Multisizer II/Coulter Counter (Beckman-Coulter). Cisplatin was purchased from EMD Biosciences (San Diego, CA). Triapine was synthesized by methodology previously described by Liu et al. [33].

2.2. Generation of sh/siRNA and transient transfections

Human R1 shRNA was derived from the 19 base sequence of human R1 cDNA GCTGCAACCTTGACTACTA (349–367, GenBank accession number BC006498). The control shRNA was a non-targeting sequence shRNA derived from the 19 base non-mammalian sequence of *Euglena gracilis* chloroplast genome GCGCGCTTTGTAGGATTCG (88–106, GeneBank accession number X70810). shRNA was generated by in vitro transcription of a DNA oligonucleotide consisting sequentially of an 8 base sequence complementary to the T7 promoter, a 19 base sense sequence of targeted cDNA/mRNA, a 9 base spacer TTCTAGAGA, and a 19 base inverted antisense sequence. The oligonucleotide was annealed with a T7 promoter oligo and filled-in with a Klenow fragment to form the DNA template that was transcribed using a MessageMuter shRNAi production kit (Epicentre, Madison, WI). The shRNA produced was further purified by phenol/chloroform extraction/ethanol precipitation and by Micro Bio-spin 6 columns (Bio-Rad, Hercules, CA). For transient transfections with shRNA, cells were plated in 6-well plates, incubated for 24 h, and transfected with 100 nM of shRNA using an Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfected cells were incubated for appropriate times before assays.

2.3. Cytidine 5′-diphosphate (CDP) reductase assay

The activity of ribonucleotide reductase was assayed by determining the reduction of [^{14}C]-CDP as described [11,12]. One millilitre of packed cells was suspended in 1 ml of 0.05 M HEPES (pH 7.2) containing 1.0 mM dithiothreitol, sonicated, and centrifuged at 21,000 × *g* for 1 h. The cell-free supernatant was collected and quick frozen in an acetone–dry ice bath. CDP reductase activity was measured in an assay mixture (0.10 μCi, 0.05 mM [^{14}C]-CDP, 4 mM magnesium acetate, 1 mM dithiothreitol, 16 mM sodium phosphate, 1 mM ATP) with 25 μl of cell-free supernatant at 35 °C for 30 min in triplicate. The reactions were terminated by heating and treating with snake venom. [^{14}C]-dCDP was separated from [^{14}C]-CDP on Dowex-1-borate columns, and aliquots of eluant were collected for measurement of radioactivity by liquid scintillation spectrometry.

2.4. Western blot analysis and immunoprecipitation

Both procedures were performed as described previously [31], with a modification in which 0.5 mM Na_3VO_4 was added to the cell lysis buffer to minimize the loss of phosphorylated protein. Gel images were quantified by densitometry with ImageQuaNT software (Molecular Dynamics). Anti-R2 (E-16), chk1 (G-4), cdc25A (F-6), and PCNA (C-20) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti-R1 (AD203) antibody was from Chemicon (Temecila, CA). Anti-actin (20–33) antibody was from Sigma–Aldrich (Saint Louis, MO). Anti-γ-H2Ax (Ser-139; JBW301) and H2Ax antibodies were obtained from Upstate (Lake Placid, NY). Anti-phospho-chk1 (Ser-345) antibody was from Cell Signaling (Beverly, CA). Anti-PARP antibody was from Roche (Indianapolis, IN).

2.5. Flow cytometry for cell cycle analysis, apoptosis assay, and γ-H2Ax staining

To determine the cell cycle distribution, 1×10^6 cells were collected and fixed/permeabilized with 70% ethanol for at least 1 h. Subsequently, samples were incubated with 1 mg/ml of RNase I at 37 °C for 30 min, stained with 50 μg/ml of propidium iodide (Sigma–Aldrich) for 1 h, and analyzed by flow cytometry. For apoptosis assays, 2×10^5 cells were suspended in Annexin-binding buffer and stained with Alexa Flour 488 annexin V (Vybrant Apoptosis Assay kit #2; Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Samples were immediately assayed by flow cytometry for levels of annexin V-positive apoptotic cells. To detect the cell populations positive for γ-H2Ax, 5×10^5 cells were fixed, permeabilized, stained with FITC-conjugated anti-phospho-H2Ax (Ser139) antibody using an H2Ax phosphorylation kit (Upstate) following the manufacturer's protocol. Cells were then washed and resuspended in PBS containing 50 μg/ml of RNase I and 10 μg/ml of propidium iodide for 30 min prior to analysis by flow cytometry.

2.6. Measurement of cisplatin-induced DNA lesions

Cells were plated and incubated for 24 h prior to treatment with various concentrations of cisplatin for 6 h. Cells were then harvested and total genomic DNA isolated using a DNA isolation kit (Roche) according to the manufacturer's instructions. Purified genomic DNA was quantified by spectrophotometry. PCR amplification of a 13.5 kb human *β-globin* gene [34] was conducted using an Expand long template PCR system kit (Roche). Fifty microlitres of the PCR reaction contained 150 ng of genomic DNA, 500 μM of each dNTP, 300 nM of each β-globin gene primer (forward 5′-GCACTGGCTTAGGAGTTGGACT-3′ and reverse 5′-CGAGTAAGAGACCATTGTGGCAG-3′), 1% DMSO, 1 × PCR buffer containing 2.75 mM $MgCl_2$, and 3.75 units of Expand enzyme mix. The PCR conditions consisted of an initial denaturation at 93 °C for 3 min, 10 cycles of 93 °C for 15 s, 65 °C for 30 s, 68 °C for 11 min, and 18 cycles of 93 °C for 15 s, 65 °C for 30 s, and 68 °C for 11 min plus 20 s increments after each cycle, followed by a final extension at 65 °C for 7 min. The PCR products were resolved on a 0.8% agarose gel stained with ethidium bromide. The bands were quantified using a Bio-Rad gel documentation system with QuantityOne software (Bio-Rad). The frequency of DNA lesions was calculated by a Poisson distribution [35]: DNA lesions/13.5 kb segment of *β-globin* gene = $-\ln(A_D/A_C)$, where A_D is the amount of PCR product amplified from the cisplatin-damaged DNA template and A_C is the amount of PCR product amplified from the vehicle-treated control DNA template. The frequency was converted and expressed as the number of lesions/10 kb of genomic DNA.

2.7. DNA synthesis assay

Cells were plated into 6-well plates and incubated for 24 h prior to transfection with shRNA as described above. Subsequently, cells were labeled with 20 nCi/ml of [^{14}C]-thymidine (Moravek, Brea, CA) for 24 h. The medium was then replaced with non-radioactive-containing McCoy's 5A complete medium in which cells were incubated for various times. Prior to harvesting, cells were pulse-labeled with 1 μCi/ml of [^{3}H]-thymidine (Moravek) for 1 h. Following washing twice with ice-cold PBS, once with ice-cold 10% trichloroacetic acid, and twice with ice-cold PBS, cell monolayers were solubilized with 400 μl of 0.2 N NaOH. One hundred microlitres of lysate were applied onto a Whatman GF/A filter disk and air-dried. DNA synthesis was determined by the ratio of [^{3}H]/[^{14}C] measured by scintillation spectrometry, and expressed as a percentage of the value of a non-targeted shRNA-transfected control at each time point.

2.8. Quantification of dNTP levels

Cellular deoxyribonucleoside triphosphates were quantified using an enzymatic assay based upon the DNA polymerase-catalyzed incorporation of deoxyribonucleotide into DNA as described previously [31]. Cells were transiently transfected with shRNA and incubated for 48 h. Approximately 1×10^6 cells were collected for the assay.

3. Results

3.1. p53(−/−) HCT-116 cells contain an excessive level of the R2 subunit of RNR for proliferative growth and RNR activity

p53(−/−) HCT-116 human colon cancer cells were transfected with the vector and R2-knockdown constructs [31] followed by selection with Zeocin to generate stable clones expressing R2-siRNA. Fourteen clones were expanded into stable cell lines. Clones 1, 4, 5, and 9 displayed pronounced reductions (>80%) in R2 protein levels compared to that of vector-transfected cells (Fig. 1A). The growth rates of R2-knockdown cells were determined by monitoring the increase in cell number over a 6-day period under normal growth conditions. Clone 4 R2-knockdown cells, selected as a representive clone, exhibited growth kinetics comparable to that of vector transfected cells (Fig. 1B). Vector and all R2-knockdown clones exhibited a doubling time of approximately 22 h during the logarithmic phase of cell growth.

Because stable knockdown of the R2 subunit resulted in enhanced sensitivity to cisplatin [31], R2-knockdown clones 1, 4, and 9 were examined for the level of H2Ax phosphorylation at Ser-139 (γ-H2Ax) as a measure of DSBs (DNA double-stranded breaks) which are generated when replication forks encounter cisplatin–DNA crosslinks [36,37]. Following 24 h of exposure to cisplatin, clones 1, 4, and 9 all exhibited an enhanced level of γ-H2Ax compared to vector transfected cells (Fig. 1C). The levels of PCNA (proliferating cell nuclear antigen), a marker of cell proliferation, remained unchanged in these clones. These findings indicate that stable knockdown of the R2 subunit does not affect normal proliferative growth but results in augmented DNA damage inflicted by exposure to cisplatin.

To determine whether R2-knockdown influenced RNR enzymatic activity, an in vitro cell-free assay of CDP reductase activity was performed (Fig. 1D). The addition of exogenous R1 subunit, but not of exogenous R2 subunit, to the cell-free extract has been shown to significantly augment RNR activity [11,12]. The lysate of R2-knockdown cells exhibited a modest decrease in RNR activity compared with that of vector cells (Fig. 1D). A significant increase in RNR activity ($p < 0.01$) was observed in the lysate of vector cells when an excess amount of the R1 subunit was added to the assay. In contrast, considerably less of an increase in RNR activity was detected in the lysate of R2-knockdown cells following the addition of excess R1 subunit. These findings suggest that the level of the R2 subunit is in excess for RNR activity in *p53*(−/−) HCT-116 cells.

The effects of R2-knockdown on the composition of RNR subunits were also investigated through analysis of co-immunoprecipitation of RNR subunits. Western blot analyses of total protein showed that the level of the R2 protein was lower in R2-knockdown cells than in vector cells; whereas, R1 protein levels were comparable in both cell lines (Fig. 1E). Using an antibody against the R1 subunit to immunoprecipitate the RNR complex, a reduced amount of the R2 subunit was pulled down with the R1 subunit in R2-knockdown cells. In the reciprocal immunoprecipitation with an antibody against the R2 subunit, the amount of R1 subunits co-precipitated with R2 subunits was minimally reduced in R2-knockdown cells despite a marked reduction in R2 subunits being pulled down. These results suggest that a functional RNR complex is constituted possibly with a reduced amount of the R2 subunit when the level of R2 subunit is substantially abrogated.

3.2. Abrogation of excess R2 subunit leads to enhancement of H2Ax phosphorylation and the S phase checkpoint in response to DNA damage and replication blockage

To explore the relationship between the level of the R2 subunit and the DNA damage/replication stress response, the time course of γ-H2Ax accumulation and chk1 activation was examined following exposure of cells to cisplatin and Triapine. DNA damage and replication stress elicit the activation of chk1 as evidenced by ATM or ATR-mediated phosphorylation of serine-317 and 345 [38]. Chk1 is required to activate the S phase checkpoint through the phosphorylation and degradation of cdc25A in response to DNA damage [26,27]. The increase in chk1 phosphorylation at serine-345 was detected by western blot analysis. Both vector and R2-knockdown cell lines exhibited a time-dependent increase in H2Ax and chk1 phosphorylation following cisplatin treatment (Fig. 2A). The induction became very prominent at 24 h, with the response of R2-knockdown cells being the most pronounced. Replication stress also promotes the phosphorylation of H2Ax in an ATR-dependent manner [21]. Following exposure to the RNR inhibitor Triapine [31,39], a relatively faster increase in H2Ax and chk1 phosphorylation was observed (Fig. 2B). R2-knockdown cells also displayed a greater increase in these responses than vector cells.

The levels of γ-H2Ax accumulation were also corroborated quantitatively using flow cytometry. After 24 h of exposure to cisplatin, the population of γ-H2Ax-positive R2-knockdown cells was significantly greater than that of vector cells (Fig. 2C and D). The level and kinetics of γ-H2Ax accumulation were consistent with those of chk1 phosphorylation, indicating that cisplatin-induced DNA damage was responsible for the checkpoint activation.

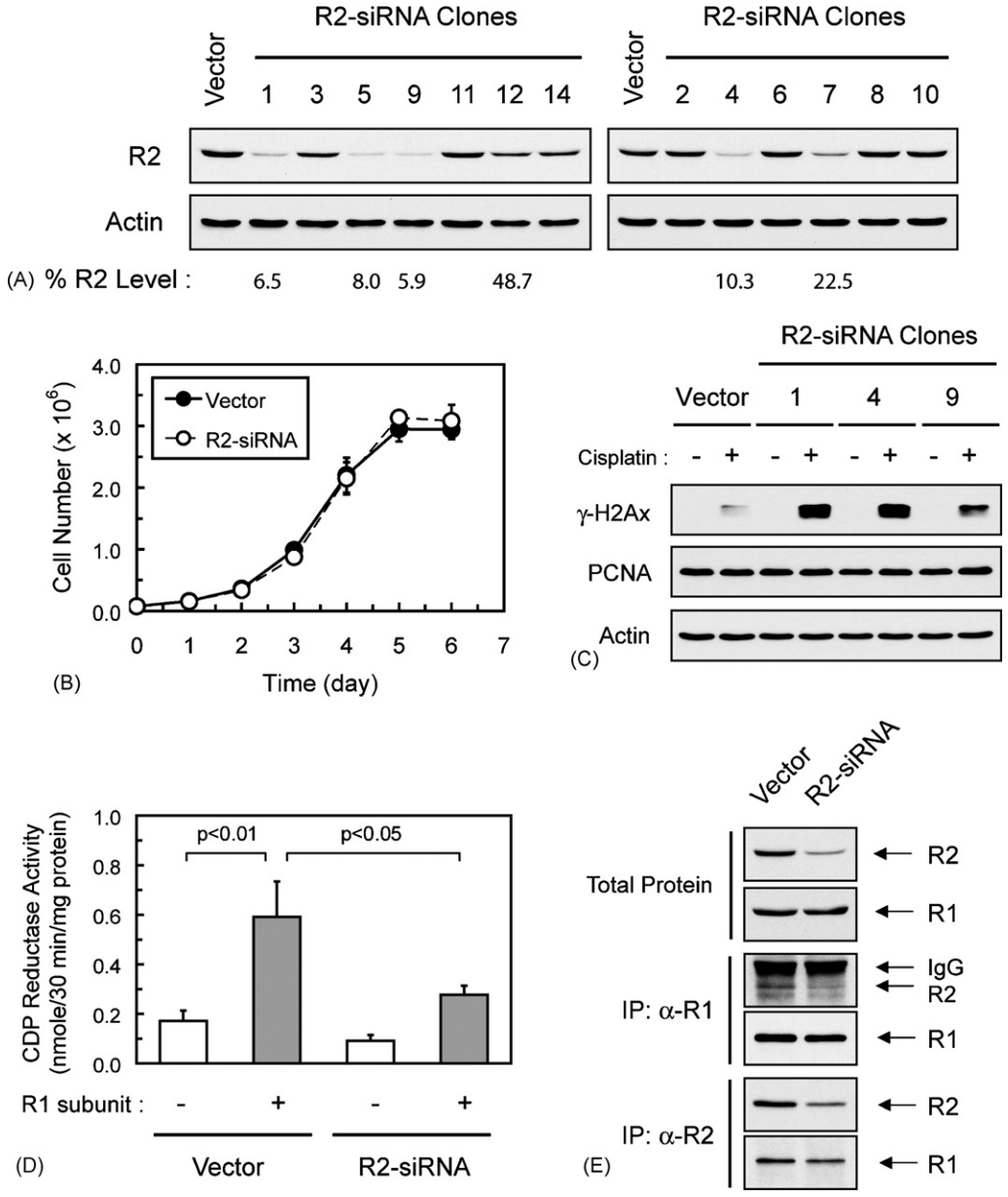

Fig. 1 – The R2 subunit of RNR is in excess for proliferative growth and RNR activity in *p53*(−/−) HCT-116 cells. (A) Cells were transfected with the control and the R2-siRNA vectors and selected for stable clones. Fourteen stable cloned lines were obtained and the levels of the R2 subunit were assessed by western blot analysis. Actin protein levels were used to demonstrate approximately equal loadings. A vector control and 13 single cell clones are shown. The R2 protein level in each clone is normalized against its respective actin protein level and expressed as a percentage of vector control cells. Only the R2 levels below 50% of vector control cells are shown. (B) Cells (clone 4) were cultured for up to 6 days under normal growth conditions. The numbers of cells were counted from aliquots at various time points as indicated. Data are the means ± S.E. from at least three independent experiments. (C) Stable clones 1, 4, and 9 were treated with 20 μM cisplatin for 24 h and analyzed for the levels of γ-H2Ax, PCNA, and actin by western blotting. (D) CDP reductase activity was measured in cell-free extracts with and without the addition of exogenous R1 subunit. Partially purified R1 subunit alone had no activity. Data are the means ± S.E. from three independent experiments. The *p* values are shown for the significant differences between samples using the Student–Newman–Keuls multiple comparison test. (E) Total protein (upper), immunoprecipitates with an anti-R1 antibody (middle), and immunoprecipitates with an anti-R2 antibody (lower) from native cell lysates were assessed for the levels of R1 and R2 protein by western blotting.

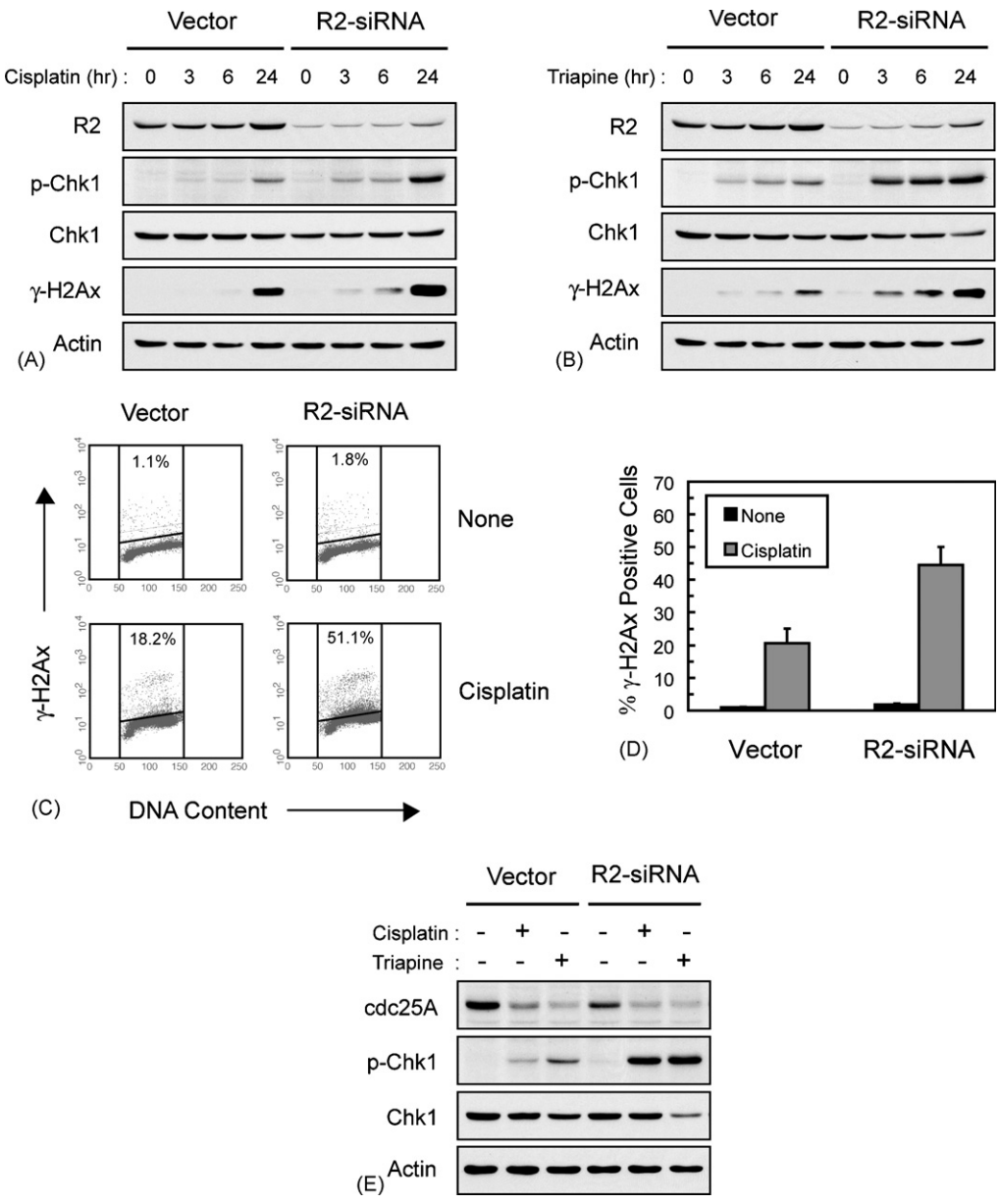

Fig. 2 – Stable knockdown of excess R2 subunits augments phosphorylation of chk1 and H2Ax, and degradation of cdc25A in response to DNA damage and replication blockage. (A) Cells (clone 4) were treated with 20 μM cisplatin, collected at the indicated time points, and analyzed for the levels of R2, phospho-chk1 (Ser345), chk1, γ-H2Ax (phospho-Ser139) and actin by western blotting. (B) Cells were treated with 1 μM Triapine and at the indicated time points assayed for protein levels as described in A. (C) Cells were treated with 20 μM cisplatin for 24 h, then fixed and stained with anti-γ-H2Ax antibody and propidium iodide for analysis by flow cytometry. A window was set to determine the percentage of the γ-H2Ax-positive population in a total of 200,000 cells analyzed. (D) A bar graph is presented to demonstrate the comparative results shown in C. Data are the means ± S.E. from three independent experiments. (E) Cells were exposed to 20 μM cisplatin or 1 μM Triapine for 24 h and assayed for the levels of cdc25A, phospho-chk1, chk1, and actin by western blotting. Similar results were obtained with clones 1 and 9.

The degradation of cdc25A subsequent to chk1 activation was also examined to confirm the activation of the S phase checkpoint. Both vector and R2-knockdown cells exhibited a decrease in the level of cdc25A corresponding to an increase in chk1 phosphorylation when treated with cisplatin or Triapine for 24 h (Fig. 2E). These results suggest that the presence of excess R2 subunits protects against cisplatin-inflicted DNA damage and Triapine-induced replication blockage, thereby mitigating the S phase checkpoint activation.

3.3. *Excess R2 subunits alleviate cisplatin-induced γ-H2Ax formation by facilitating DNA damage repair*

To determine whether excess R2 subunits contributed to the repair of cisplatin-elicited DNA damage, the accumulation of

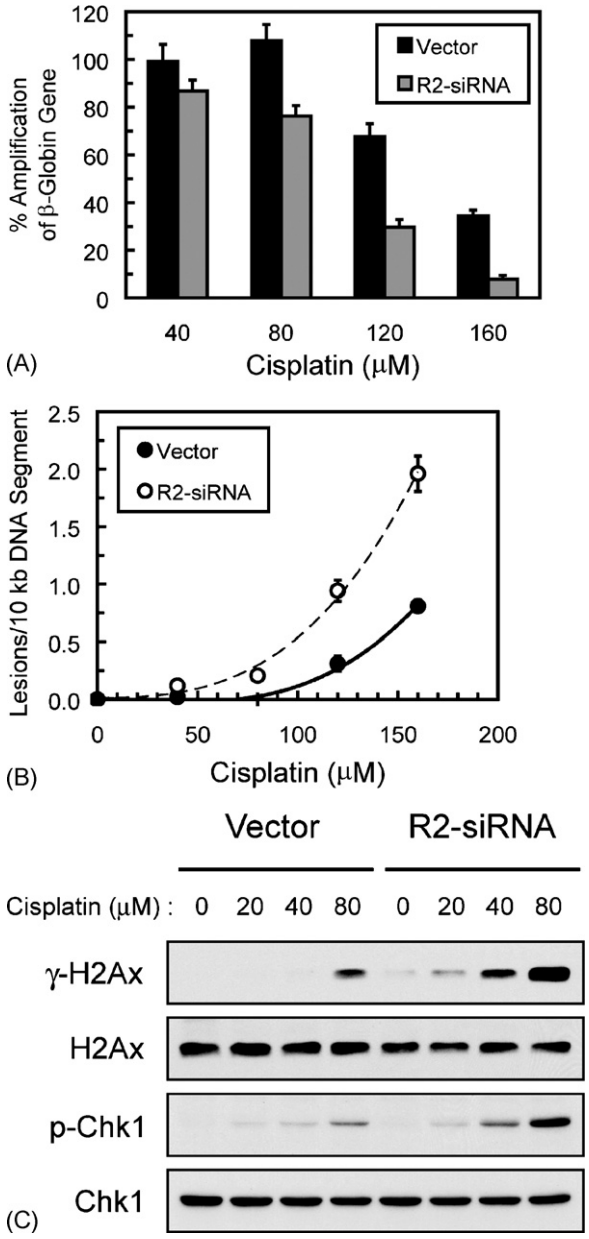

Fig. 3 – Excessive R2 subunit attenuates the accumulation of cisplatin-inflicted DNA damage and facilitates recovery from cisplatin-induced DNA lesions. (A) Cells (clone 4) were exposed to the indicated concentrations of cisplatin for 6 h and harvested. DNA was then isolated for PCR amplification of the 13.5 kb *β-globin* gene. The levels of PCR products were quantified by densitometry following agarose gel electrophoresis. The amplification efficiency was expressed as a percentage of vehicle-treated controls. The data are the means ± S.E. from 9 to 12 independent experiments. (B) Cisplatin-induced DNA lesions are presented as the frequency per 10 kb DNA region. The data were calculated using a Poisson distribution by converting the percentage of amplification shown in A into the number of DNA lesions. (C) Cells were treated with the indicated concentrations of cisplatin for 2 h and then allowed to recover in cisplatin-free medium for 24 h. Cells were then collected for analysis of the levels of γ-H2Ax, H2Ax, phospho-chk1, and chk1 by western blotting. Similar results were obtained with clones 1 and 9.

DNA adducts in cells exposed to cisplatin was measured. Exposure of cells to cisplatin predominantly causes the formation of 1,2-intrastrand crosslinks, as well as some minor adducts, including interstrand and 1,3-intrastrand crosslinks of DNA [40]. The presence of these DNA lesions hinders the DNA polymerase from replicating the damaged DNA template during PCR amplification [35,41]. To assess the extent of cisplatin-induced damage to DNA, PCR amplification over a 13.5 kb segment of the *β-globin* gene was conducted using genomic DNA obtained from cells exposed to cisplatin for 6 h. A progressive decrease in the amplification of the *β-globin* gene occurred with increasing concentrations of cisplatin using DNA isolated from vector and R2-knockdown cells (Fig. 3A). The degree of amplification of the DNA from R2-knockdown cells was significantly lower than that from vector cells at concentrations of cisplatin of 120 and 160 μM. In addition, the number of DNA lesions produced by cisplatin in R2-knockdown cells was at least two-fold greater than that occurring in vector cells (Fig. 3B). The results are consistent with the finding of elevated γ-H2Ax levels, indicating that a substantial increase in accumulation of cisplatin-induced lesions occurs when excess R2 subunits are abrogated.

To further ascertain whether R2-knockdown hampered the repair of DNA damage, cells were pulsed with a range of concentrations of cisplatin for 2 h and then allowed to recover for 24 h in the absence of cisplatin. The levels of γ-H2Ax and chk1 phosphorylation were determined by western blot analysis to monitor the outcome of recovery from cisplatin-induced DNA lesions. R2-knockdown cells displayed a pronounced increase in γ-H2Ax in a concentration-dependent manner (Fig. 3C). In contrast, vector cells almost completely recovered from cisplatin-induced DNA damage, except for a residual level of γ-H2Ax detected at the highest concentration of cisplatin employed. The increase in chk1 phosphorylation corresponded closely with that of γ-H2Ax, indicating that the degree of checkpoint activation paralleled the extent of unrepaired DNA damage. These findings confirm the expectation that ablation of excess R2 subunits decreases the ability of cells to repair cisplatin-induced lesions, thereby delaying recovery from DNA damage.

3.4. *R2 knockdown prolongs the S phase block in response to DNA damage and replication stress*

To relate the above findings to alterations in the cell cycle distribution, the DNA content of cells challenged with DNA damage and replication stress was analyzed by flow cytometry. R2-knockdown cells exhibited a cell cycle distribution comparable to that of vector cells under normal growth conditions (Fig. 4A). After exposure to cisplatin for 24 h, both cell lines exhibited a loss of the G1 peak and an increase in the number of cells in S phase. At 48 and 72 h of exposure to cisplatin, an increased proportion of vector cells progressed to the G2/M phase, while a substantial fraction of R2-knockdown cells remained arrested in S phase over the course of cisplatin treatment (Fig. 4B). Treatment with Triapine caused both cell lines to arrest at very early S phase, with vector cells

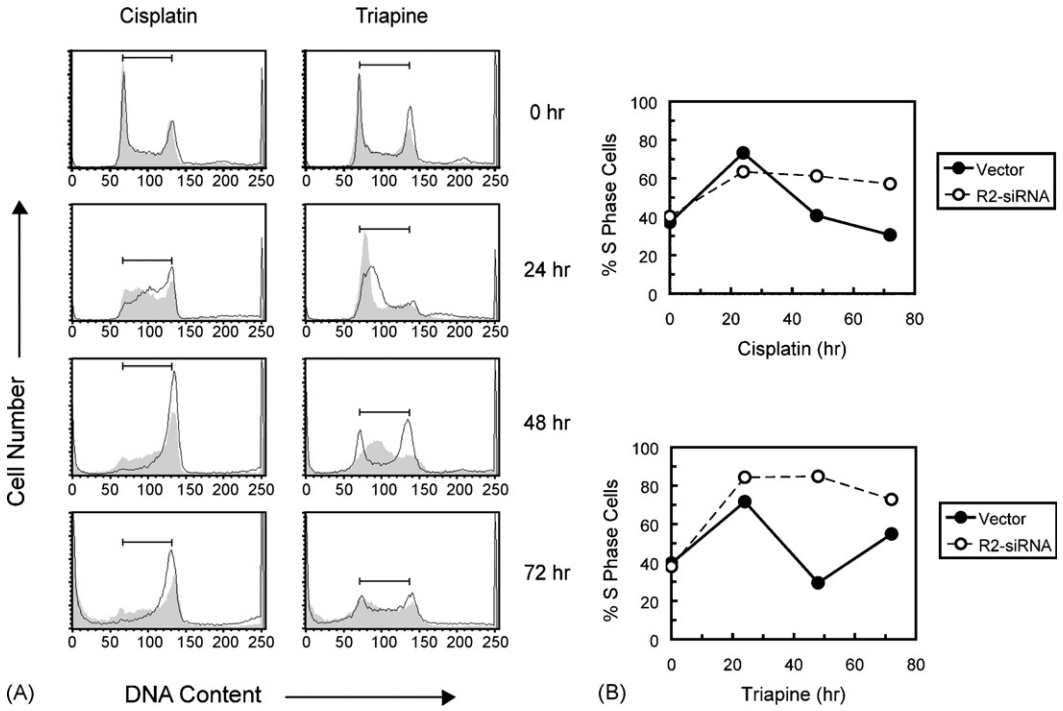

Fig. 4 – Abrogation of excessive R2 subunit prolongs the S phase blockage caused by DNA damage and stalled replication. (A) Cells (clone 4) were exposed to 30 μM cisplatin or 1 μM Triapine, collected at the indicated time points, fixed, and stained with propidium iodide for analysis of DNA content by flow cytometry. A representative result is shown. The area under the solid lines represents vector cells and the grey-shaded area represents R2-knockdown cells. (B) The cell cycle distribution of the S phase population is presented for the results in A. Data are expressed as a percentage of the total number of cells analyzed by flow cytometry at the indicated time points. Similar results were obtained with clones 1 and 9.

progressing slightly faster than R2-knockdown cells. At 48 h of exposure to Triapine, most vector cells overcame the S-phase block and returned to a somewhat normal cell cycle distribution. In contrast, a majority of R2-knockdown cells remained arrested in S phase. A rebounding increase in the proportion of S phase vector cells at 72 h may result from the overall reduction in both G1 and G2/M populations caused by Triapine (Fig. 4B). These results agree with the finding of exacerbated checkpoint responses in R2-knockdown cells treated with cisplatin and Triapine. Abrogation of excess R2 subunits causes a prolonged blockage at the S phase checkpoint in response to DNA damage and replication stress.

3.5. *R2-knockdown cells exhibit enhanced apoptosis following DNA damage and replication blockage*

We have demonstrated that abrogation of excess R2 subunit caused an increase in sensitivity to DNA damaging and RNR inhibitory agents [31]. In addition, cisplatin- and Triapine-induced S phase blockage was accompanied by an increase in the sub-G1 population beginning at 48 h (Fig. 4A). Therefore, the effects of R2-knockdown on the apoptotic response to DNA damage and replication stress were investigated. Poly(ADP-ribose) polymerase (PARP) cleavage was assessed following treatment with cisplatin and Triapine. After exposure to cisplatin for 72 h, an 89 kDa apoptotic fragment resulting from the cleavage of PARP [42] was detected in both vector and R2-knockdown cells (Fig. 5A). R2-knockdown cells displayed a greater level of PARP cleavage than vector cells. Treatment with Triapine for 72 h also caused a pronounced increase in the 89 kDa apoptotic fragment of PARP in R2-knockdown cells, while a slight increase in PARP cleavage occurred in vector cells (Fig. 5B).

Progression of an increase in apoptosis was monitored by annexin V binding to phosphatidylserine (PS) translocated to the outer leaflet of the plasma membrane [43]. A time-dependent increase in annexin V-binding to both vector and R2-knockdown cells occurred following exposure to cisplatin and Triapine. R2-knockdown cells exhibited a greater increase in apoptosis than vector cells after 48 and 72 h exposure to cisplatin (Fig. 5C). Likewise, Triapine produced a pronounced increase in the apoptotic fraction of R2-knockdown cells compared to that of vector cells at these time points (Fig. 5D). These findings support the notion that prolonged blockage at S phase due to R2-knockdown leads to augmented apoptosis in response to DNA damage and replication stress.

3.6. *Depletion of the R1 subunit elicits replication stress and checkpoint responses*

Depletion of the R1 subunit was initially carried out by stable transfection with an R1-knockdown construct. However, none of selected surviving clones exhibited a reduction in the R1 protein (data not shown). Thus, transient transfection of cells with an R1-targeted shRNA was employed to circumvent this problem. Following transfection, cells were monitored for

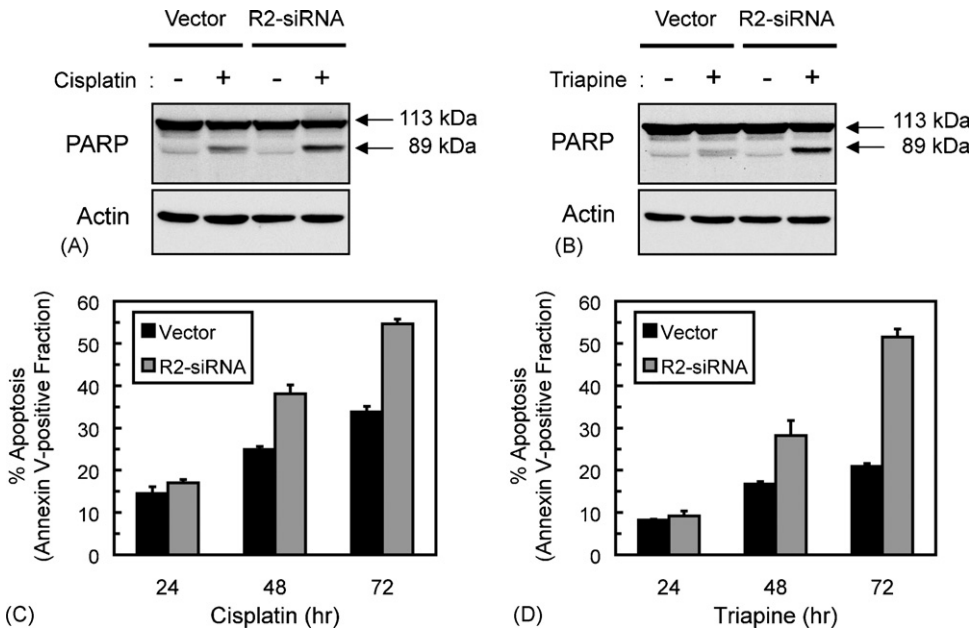

Fig. 5 – Stable knockdown of excessive R2 subunit enhances cisplatin- and Triapine-induced apoptosis. (A) Cells (clone 4) were treated with 30 μM cisplatin for 72 h, collected, and assayed for PARP (113 kDa) cleavage into an apoptotic PARP fragment (89 kDa) by western blotting. The antibody used recognizes both full length PARP and the cleaved fragment. (B) Cells were treated with 1 μM Triapine for 72 h and collected for analysis of PARP cleavage as described in A. (C) Cells were exposed to 30 μM cisplatin and collected at the indicated time points for analysis of annexin V binding by flow cytometry. The percent apoptosis/annexin V-positivity is presented as the difference between drug-treated cells and vehicle-treated controls at each time point. Data are the means ± S.E. from three independent experiments. (D) Cells were exposed to 1 μM Triapine and collected at the indicated time points for analysis of annexin V binding by flow cytometry as described in C. Similar results were obtained with clones 1 and 9.

checkpoint activation as a diminution in the R1 protein progressed (Fig. 6A). At 24 h post-transfection, phosphorylation of chk1 rose slightly when the levels of R1 began to decline. When R1 levels were further diminished at 48 h, the increases in chk1 phosphorylation became evident in vector cells and markedly enhanced in R2-knockdown cells. The levels of γ-H2Ax at 48 h post-transfection were also examined by flow cytometry (Fig. 6B). Both vector and R2-knockdown cells exhibited an increase in an γ-H2Ax-positive population corresponding to the increase in chk1 phosphorylation caused by R1 knockdown.

To determine whether R1 knockdown induced the S phase checkpoint, the rate of DNA synthesis following transfection with R1-shRNA was measured (Fig. 6C). Transfection with R1-shRNA led to a reduction in DNA synthesis of both vector and R2-knockdown cells as a function of time. The reduction in DNA synthesis of R2-knockdown cells was greater than that of vector cells over 72 h of measurement following transfection. Vector cells responded initially with an increase in DNA synthesis at 24 h followed by a decline at 48 and 72 h.

The effects of R1 knockdown on RNR activity were evaluated through determination of changes in dNTP pools at 48 h when a substantial level of R1 knockdown occurred (Fig. 6D). Among all of the dNTPs, the levels of dATP were the most responsive to R1 knockdown. Stable R2-knockdown cells exhibited a low dATP level compared to vector cells. Transient R1 knockdown also produced a marked decrease in the dATP level in vector cells and a further reduction in the dATP level in R2-knockdown cells. In addition, R1 knockdown caused an increase in dCTP and dTTP levels in R2-knockdown cells in a fashion opposite to the response of dATP levels. We have found that the dATP level is the only dNTP remaining persistently depressed following inhibition of RNR activity in HCT-116 cells (unpublished observations). Thus, the results collectively indicate that depletion of the R1 subunit induces replication stress and the S phase checkpoint as a result of impaired RNR activity. Abrogation of excess R2 subunits renders RNR more susceptible to R1 knockdown and therefore exacerbates checkpoint responses.

4. Discussion

The level of R2 subunits, which fluctuates in a cell cycle-dependent fashion, is thought to be limiting for the enzymatic activity of RNR [4,6,9]. The level of RNR activity has been shown to correlate strongly with the rate of proliferation of tumor cells [44,45]. Our findings in *p53*(−/−) HCT-116 cells, however, favor the concept that the increasing amount of R2 subunits which occurs during S phase can exceed the level of RNR activity required for proliferative cell growth. Thus, the cellular growth rate of *p53*(−/−) HCT-116

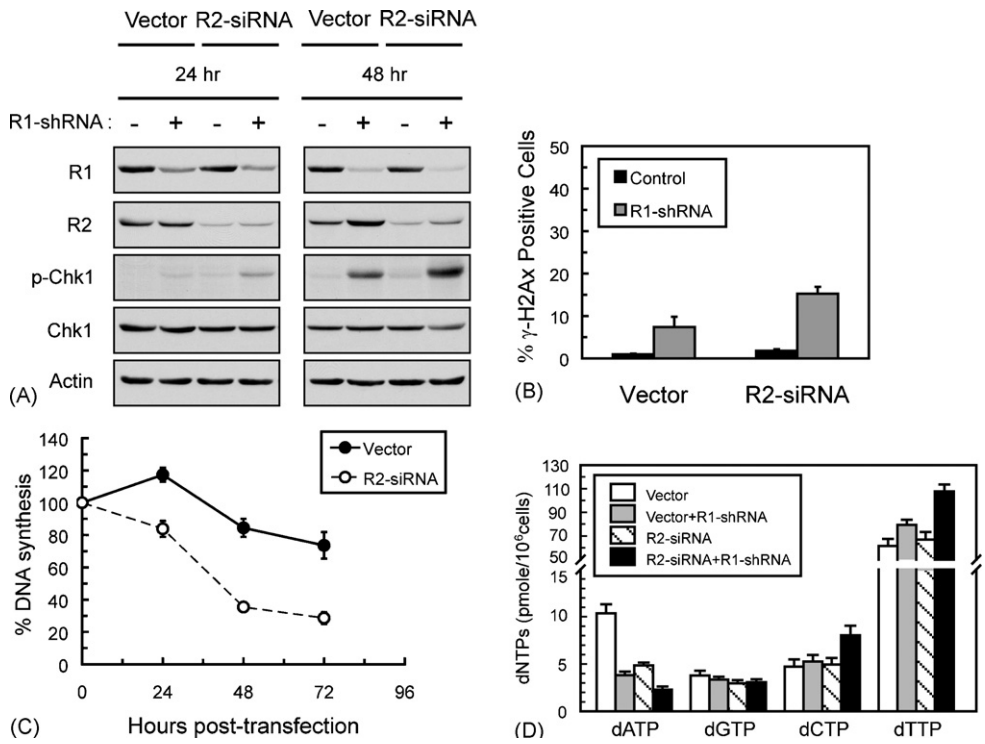

Fig. 6 – Transient knockdown of the R1 subunit causes replication stress and activates the S phase checkpoint. (A) Cells (clone 4) were transiently transfected with 100 nM non-targeted shRNA or R1-shRNA, collected at the indicated time points, and analyzed for the levels of R1, R2, phospho-chk1, chk1, and actin by western blotting. (B) Cells were transiently transfected with 100 nM non-targeted shRNA or R1-shRNA for 48 h. Cells were then collected, fixed, and stained with anti-γ-H2Ax antibody and propidium iodide for analysis by flow cytometry as described in Fig. 2C and D. Data are the means ± S.E. from three independent experiments. (C) Cells were transiently transfected with 100 nM non-targeted shRNA or R1-shRNA and then incubated with [^{14}C]-thymidine for 24 h. Cells were pulsed for 1 h with [^{3}H]-thymidine and thereafter assayed for thymidine incorporation at the indicated time points. DNA synthesis following R1-shRNA transfection is expressed as a percentage of the [^{3}H]/[^{14}C] ratio from non-targeted shRNA transfected cells at each time point. Data are the means ± S.E. from three independent experiments. (D) Cells were transfected with 100 nM non-targeted shRNA or R1-shRNA and harvested at 48 h post-transfection. The individual levels of dNTPs were measured as described in Section 2. Data are the means ± S.E. from three independent experiments. Similar results were obtained with clones 1 and 9.

cells is not affected by a substantial level of knockdown of the R2 subunit (>80%). We speculate that the excess R2 subunits may be functionally dormant when existing RNR activity is sufficient to support cell proliferation under normal growth conditions.

The existence of excess R2 subunits is also demonstrated by an in vitro assay in which RNR activity of the cell lysate can be significantly increased by the addition of exogenous R1, but not of R2 protein [11,12] (Fig. 1D). Stable knockdown of the R2 subunit attenuates the increase in RNR activity caused by the addition of the R1 subunit. Other lines of evidence are also available to support our finding of an abundance of the R2 subunit. The studies of Shao et al. [46] have demonstrated that reconstituted RNR activity in vitro is dependent on the concentration of the R1 subunit. Maximum activity of RNR is achieved when the molar ratio of the R2 subunit is five times more than that of the R1 subunit. Another recent report also shows that almost two times more R2 subunit than R1 subunit is detected in S-phase-enriched cells [47].

The active form of RNR has long been regarded as a heterotetramer consisting of two R1 and two R2 subunits. However, our immunoprecipitation study demonstrates a possibly reduced ratio of R2 to R1 subunits in the RNR complex of R2-knockdown cells compared to that of vector cells. Kashlan and Cooperman [48,49] have proposed active forms of RNR existing as R1 dimer, tetramer, and hexamer complexed with altered stoichiometry of R2 subunits. We speculate that excess R2 subunits promote the formation of $R2_6R1_6$ for optimal enzymatic functions and activity. Abrogation of excess R2 subunits possibly leads to an increase in the formation of $R2_2R1_6$, an active form of RNR still capable of supporting proliferative DNA synthesis. Recently, Rofougaran et al. [50] have independently arrived at a similar conclusion using gas-phase electrophoretic mobility macromolecular analysis.

The excessive level of R2 subunits also has a physiological relevance in DNA damage repair during S phase progression. The S phase checkpoint has recently been delineated as an important safeguard against genotoxic insults during the most vulnerable period of the cell cycle [51]. In response to DNA damage and stalled replication, at least two branches of signaling pathways, the ATM/ATR-mediated cdc25A

degradation cascade and ATM-mediated NBS1 phosphorylation, jointly initiate the S phase checkpoint by transiently inhibiting the firing of replication origins [26,27,52,53]. It has been suggested that these pathways also promote the recovery from stalled replication by coordination with the DNA repair machinery [54]. Our results indicate that excess R2 subunits are required to support the process of DNA repair, such as nucleotide excision repair and homologous recombination, during the progression of S phase. Hence, R2-knockdown cells sustain accumulating cisplatin-induced DNA lesions, thereby exhibiting accentuated and prolonged S phase checkpoint responses. These consequences are strongly associated with enhanced sensitivity [31] and apoptosis in R2-knockdown cells exposed to cisplatin. Further investigation is needed to define the mechanism employed for the involvement of excess R2 subunits in DNA repair.

In contrast to the R2 subunit, the R1 subunit appears to be limiting for RNR activity and proliferative cell growth. We have conducted stable transfection of *p53*(−/−) HCT-116 cells with an R1-siRNA construct and observed a 94% reduction in clonogenic survival. Cells stably transfected with an R2-siRNA, however, exhibited only a 34% decrease in clonogenic survival (data not shown). These results suggest that stable knockdown of R1 subunits is potentially lethal to cells. Furthermore, our findings demonstrate that transient knockdown of the R1 subunit elicits the S phase checkpoint, an indicator of replication stress, due to a direct impact on the RNR activity required for the support of replicative DNA synthesis. The R1 subunit contains the catalytic mechanism and the critical regulatory properties to control overall enzymatic activity for balanced dNTP pools [1]. Thus, R1 subunits are indispensable for a functional RNR complex that generates the necessary RNR activity to maintain DNA synthesis for proliferative growth.

Our findings not only elucidate the involvement of the R2 subunit in DNA repair but also have important implications in the response of human malignancies to DNA damage-based therapeutic interventions. Thus, cancer cells lacking wild-type p53 functions can only be arrested at S and G2/M checkpoints to repair damaged DNA with dNTPs supplied predominately by RNR containing the R2 subunit. In contrast, following DNA damage, normal tissues undergo p53-dependent G1 arrest and degradation of the R2 subunit to permit induced p53R2 to engage in DNA repair. Thus, abrogation of excess R2 subunits after DNA damage may produce preferential cytotoxicity to p53-deficient or mutant cancer cells with relatively less impact on normal tissues. However, clinically used hydroxyurea and the newly developed, more potent agents that target the R2 subunit, such as Triapine and Trimidox, indiscrimately inhibit both the R2 and p53R2 subunits [46]. Pharmacological and molecular strategies that target the R2 subunit rather than p53R2 appear to have greater promise in preferentially destroying p53-mutant cancer cells. Therefore, it would be useful to continue searching for small molecules that preferentially inhibit the R2 subunit. In addition, molecular approaches such as the use of antisense agents, which are currently undergoing clinical evaluation, and of the in vivo delivery of siRNA against the R2 subunit may prove useful in combination with DNA damage-based therapies.

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REFERENCES

[1] Jordan A, Reichard P. Ribonucleotide reductases. Annu Rev Biochem 1998;67:71–98.
[2] Sjoberg BM, Reichard P, Graslund A, Ehrenberg A. The tyrosine free radical in ribonucleotide reductase from *Escherichia coli*. J Biol Chem 1978;253:6863–5.
[3] Graslund A, Ehrenberg A, Thelander L. Characterization of the free radical of mammalian ribonucleotide reductase. J Biol Chem 1982;257:5711–5.
[4] Engstrom Y, Eriksson S, Jildevik I, Skog S, Thelander L, Tribukait B. Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. J Biol Chem 1985;260:9114–6.
[5] Mann GJ, Musgrove EA, Fox RM, Thelander L. Ribonucleotide reductase M1 subunit in cellular proliferation, quiescence, and differentiation. Cancer Res 1988;48:5151–6.
[6] Bjorklund S, Skog S, Tribukait B, Thelander L. S-phase-specific expression of mammalian ribonucleotide reductase R1 and R2 subunit mRNAs. Biochemistry 1990;29:5452–8.
[7] Chabes A, Thelander L. Controlled protein degradation regulates ribonucleotide reductase activity in proliferating mammalian cells during the normal cell cycle and in response to DNA damage and replication blocks. J Biol Chem 2000;275:17747–53.
[8] Chabes AL, Pfleger CM, Kirschner MW, Thelander L. Mouse ribonucleotide reductase R2 protein: a new target for anaphase-promoting complex-Cdh1-mediated proteolysis. Proc Natl Acad Sci USA 2003;100:3925–9.
[9] Eriksson S, Martin Jr DW. Ribonucleotide reductase in cultured mouse lymphoma cells. Cell cycle-dependent variation in the activity of subunit protein M2. J Biol Chem 1981;256:9436–40.
[10] Akerblom L, Ehrenberg A, Graslund A, Lankinen H, Reichard P, Thelander L. Overproduction of the free radical of ribonucleotide reductase in hydroxyurea-resistant mouse fibroblast 3T6 cells. Proc Natl Acad Sci USA 1981;78:2159–63.
[11] Cory JG, Fleischer AE. Noncoordinate changes in the components of ribonucleotide reductase in mammalian cells. J Biol Chem 1982;257:1263–6.
[12] Rubin EH, Cory JG. Differential turnover of the subunits of ribonucleotide reductase in synchronized leukemia L1210 cells. Cancer Res 1986;46:6165–8.
[13] Carter GL, Cory JG. Cross-resistance patterns in hydroxyurea-resistant leukemia L1210 cells. Cancer Res 1988;48:5796–9.
[14] Fan H, Villegas C, Wright JA. Ribonucleotide reductase R2 component is a novel malignancy determinant that cooperates with activated oncogenes to determine transformation and malignant potential. Proc Natl Acad Sci USA 1996;93:14036–40.
[15] Fan H, Villegas C, Huang A, Wright JA. The mammalian ribonucleotide reductase R2 component cooperates with a variety of oncogenes in mechanisms of cellular transformation. Cancer Res 1998;58:1650–3.
[16] Kuo ML, Hwang HS, Sosnay PR, Kunugi KA, Kinsella TJ. Overexpression of the R2 subunit of ribonucleotide

reductase in human nasopharyngeal cancer cells reduces radiosensitivity. Cancer J 2003;9:277–85.
[17] Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature 2004;432:316–23.
[18] Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 1998;281:1677–9.
[19] Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL, et al. Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J 1998;17: 159–69.
[20] Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem 2001;276:42462–7.
[21] Ward IM, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J Biol Chem 2001;276:47759–62.
[22] Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, et al. Genomic instability in mice lacking histone H2AX. Science 2002;296:922–7 [Epub April 4, 2002].
[23] Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science 1998;282:1893–7.
[24] Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. Genes Dev 2000;14:1448–59.
[25] Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, et al. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through cdc25. Science 1997;277:1497–501.
[26] Zhao H, Watkins JL, Piwnica-Worms H. Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. Proc Natl Acad Sci USA 2002;99:14795–800.
[27] Sorensen CS, Syljuasen RG, Falck J, Schroeder T, Ronnstrand L, Khanna KK, et al. Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. Cancer Cell 2003;3:247–58.
[28] Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, et al. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature 2000;404:42–9.
[29] Nakano K, Balint E, Ashcroft M, Vousden KH. A ribonucleotide reductase gene is a transcriptional target of p53 and p73. Oncogene 2000;19:4283–9.
[30] Yamaguchi T, Matsuda K, Sagiya Y, Iwadate M, Fujino MA, Nakamura Y, et al. p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint. Cancer Res 2001;61:8256–62.
[31] Lin ZP, Belcourt MF, Cory JG, Sartorelli AC. Stable suppression of the R2 subunit of ribonucleotide reductase by R2-targeted short interference RNA sensitizes p53(−/−) HCT-116 colon cancer cells to DNA-damaging agents and ribonucleotide reductase inhibitors. J Biol Chem 2004;279:27030–8 [Epub April 19, 2004].
[32] Guittet O, Hakansson P, Voevodskaya N, Fridd S, Graslund A, Arakawa H, et al. Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. J Biol Chem 2001;276:40647–51 [Epub August 21, 2001].
[33] Liu MC, Lin TS, Sartorelli AC. Synthesis and antitumor activity of amino derivatives of pyridine-2-carboxaldehyde thiosemicarbazone. J Med Chem 1992;35:3672–7.
[34] Mukai M, Kanzaki A, Chen ZS, Miyashita H, Sumizawa T, Furukawa T, et al. Enhanced nucleotide excision repair in cisplatin resistant human KB carcinoma cells. Oncol Rep 2002;9:839–44.
[35] Kalinowski DP, Illenye S, Van Houten B. Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay. Nucleic Acids Res 1992;20:3485–94.
[36] Bessho T. Induction of DNA replication-mediated double strand breaks by psoralen DNA interstrand cross-links. J Biol Chem 2003;278:5250–4 [Epub December 8, 2002].
[37] Bosco EE, Mayhew CN, Hennigan RF, Sage J, Jacks T, Knudsen ES. RB signaling prevents replication-dependent DNA double-strand breaks following genotoxic insult. Nucleic Acids Res 2004;32:25–34.
[38] Zhao H, Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol 2001;21:4129–39.
[39] Finch RA, Liu M, Grill SP, Rose WC, Loomis R, Vasquez KM, et al. Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone): a potent inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity. Biochem Pharmacol 2000;59:983–91.
[40] Fichtinger-Schepman AM, van der Veer JL, den Hartog JH, Lohman PH, Reedijk J. Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. Biochemistry 1985;24: 707–13.
[41] Ponti M, Forrow SM, Souhami RL, D'Incalci M, Hartley JA. Measurement of the sequence specificity of covalent DNA modification by antineoplastic agents using Taq DNA polymerase. Nucleic Acids Res 1991;19: 2929–33.
[42] Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res 1993;53:3976–85.
[43] van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998;31:1–9.
[44] Elford HL, Freese M, Passamani E, Morris HP. Ribonucleotide reductase and cell proliferation. I: Variations of ribonucleotide reductase activity with tumor growth rate in a series of rat hepatomas. J Biol Chem 1970;245:5228–33.
[45] Takeda E, Weber G. Role of ribonucleotide reductase in expression in the neoplastic program. Life Sci 1981;28: 1007–14.
[46] Shao J, Zhou B, Zhu L, Qiu W, Yuan YC, Xi B, et al. In vitro characterization of enzymatic properties and inhibition of the p53R2 subunit of human ribonucleotide reductase. Cancer Res 2004;64:1–6.
[47] Hakansson P, Hofer A, Thelander L. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. J Biol Chem 2006;281:7834–41 [Epub January 24, 2006].
[48] Kashlan OB, Scott CP, Lear JD, Cooperman BS. A comprehensive model for the allosteric regulation of mammalian ribonucleotide reductase. Functional consequences of ATP- and dATP-induced oligomerization of the large subunit. Biochemistry 2002;41:462–74.
[49] Kashlan OB, Cooperman BS. Comprehensive model for allosteric regulation of mammalian ribonucleotide reductase: refinements and consequences. Biochemistry 2003;42:1696–706.
[50] Rofougaran R, Vodnala M, Hofer A. Enzymatically active mammalian ribonucleotide reductase exists primarily as

an α6β2 octamer. J Biol Chem 2006;281:27705–11 [Epub July 22, 2006].
[51] Bartek J, Lukas C, Lukas J. Checking on DNA damage in S phase. Nat Rev Mol Cell Biol 2004;5:792–804.
[52] Lim DS, Kim ST, Xu B, Maser RS, Lin J, Petrini JH, et al. ATM phosphorylates p95/NBS1 in an S-phase checkpoint pathway. Nature 2000;404:613–7.
[53] Falck J, Petrini JH, Williams BR, Lukas J, Bartek J. The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. Nat Genet 2002;30:290–4.
[54] Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 2004;73: 39–85.