Mutations in Replicative Stress Response Pathways Are Associated with S Phase-specific Defects in Nucleotide Excision Repair*

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Nucleotide excision repair (NER) is a highly conserved pathway that removes helix-distorting DNA lesions induced by a plethora of mutagens, including UV light. Our laboratory previously demonstrated that human cells deficient in either ATM and Rad3-related (ATR) kinase or translesion DNA polymerase η (i.e. key proteins that promote the completion of DNA replication in response to UV-induced replicative stress) are characterized by profound inhibition of NER exclusively during S phase. Toward elucidating the mechanistic basis of this phenomenon, we developed a novel assay to quantify NER kinetics as a function of cell cycle in the model organism Saccharomyces cerevisiae. Using this assay, we demonstrate that in yeast, deficiency of the ATR homologue Mec1 or of any among several other proteins involved in the cellular response to replicative stress significantly abrogates NER uniquely during S phase. Moreover, initiation of DNA replication is required for manifestation of this defect, and S phase NER proficiency is correlated with the capacity of individual mutants to respond to replicative stress. Importantly, we demonstrate that partial depletion of Rfa1 recapitulates defective S phase-specific NER in wild type yeast; moreover, ectopic RPA1–3 overexpression rescues such deficiency in either ATR- or polymerase η-deficient human cells. Our results strongly suggest that reduction of NER capacity during periods of enhanced replicative stress, ostensibly caused by inordinate sequestration of RPA at stalled DNA replication forks, represents a conserved feature of the multifaceted eukaryotic DNA damage response.

Human cells are under constant assault from endogenous and environmental agents that damage DNA, leading to mutagenesis, carcinogenesis, or cell death (1). UV radiation is directly absorbed by DNA to generate helix-distorting dipyrimidine photoproducts (i.e. cyclobutane pyrimidine dimers (CPDs)3 and 6–4 pyrimidine–pyrimidone photoproducts (6–4PPs)) (2). These lesions, particularly CPDs, exhibit strong promutagenic and hence tumorigenic potential; indeed, exposure to natural or artificial UV is a primary cause of skin cancer (3). Nucleotide excision repair (NER) represents the only known mechanism to excise and repair helix-distorting adducts, including CPDs and 6–4PPs. Consistently, inactivating mutations in various NER genes cause the autosomal recessive syndrome xeroderma pigmentosum (XP), which is associated with UV sensitivity and susceptibility to skin cancer development (4).

NER is evolutionarily conserved, and studies using both yeast and human models have been instrumental in elucidating its molecular underpinnings. (For excellent reviews of the human and yeast NER pathways, see Refs. 5 and 6). Two distinct NER subpathways have been identified: global genomic NER (GG-NER) and transcription-coupled NER (TC-NER), which excise UV DNA photoproducts throughout the entire genome and exclusively from the transcribed strands of active genes, respectively. GG-NER is triggered when DDB1-DDB2 (Rad7-Rad16) (yeast homologs in parentheses) and the heterotrimeric XPC-HR23B-CEN2 complex (Rad4-Rad23-Rad33) recognize helical distortions created by UV photoproducts. In contrast, TC-NER is initiated by blockage of elongating RNA polymerase II at photoadducted sites, followed by recruitment of the CSB (Rad26) and CSA (Rad28) proteins. After these initial events, for either GG-NER or TC-NER, the "core NER machinery" is recruited and accomplishes error-free restoration of DNA integrity through (i) strand denaturation surrounding the lesion, mediated by the helicase and ATPase activities of XPD (Rad3) and XPB (Rad25), respectively; (ii) stabilization of the melted structure and lesion verification by heterotrimeric RPA1–3 (RFA1–3) in conjunction with XPA (Rad14); (iii) inci-

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3 The abbreviations used are: CPD, cyclobutane pyrimidine dimer; NER, nucleotide excision repair; DDR, DNA damage response; 6-4PP, 6–4 pyrimidine–pyrimidone; GG-NER, global genomic NER; TC-NER, transcription-coupled NER; XP, xeroderma pigmentosum; XPD, xeroderma pigmentosum variant; RPA, replication protein A; ATR, ATM and Rad3-related; SPR, S phase repair; AID, auxin-induced degron; MMS, methylmethane sulfonate; 4NQO, 4-nitroquinoline 1-oxide; pol λ, DNA polymerase λ; HU, hydroxyurea.
sion of the DNA backbone 10–15 bp on either side of the damage, catalyzed by the XPF-ERCC1 (Rad1-Rad10) and XPG (Rad2) endonucleases; (iv) excision of the resultant 25–30-bp single-stranded DNA segment encompassing the lesion, creating a short gap that is resynthesized using normal DNA replication factors and the opposite undamaged strand as template; and finally (v) sealing of the remaining nick by DNA ligase (Cdc9). It is noteworthy that several essential NER factors (e.g. RPA1–3, proliferating cell nuclear antigen, and DNA ligase) also play independent roles in other critical cellular processes, such as DNA replication and homologous recombination.

Helix-distorting CPDs and 6-4PPs strongly block the progression of DNA polymerases, which causes persistent replication fork stalling and formation of DNA strand breaks, eventually leading to cell death (7). Eukaryotic cells have thus evolved the highly conserved DNA damage response (DDR), a major branch of which (i.e. the S phase checkpoint) acts to slow down DNA synthesis, thereby providing more opportunity to mitigate the genotoxic consequences of replicative stress. Current models propose that blockage of fork progression by DNA adducts uncouples the activity of replicative helicase complexes from that of DNA polymerases, which generates regions of single-stranded DNA (ssDNA) (8, 9). These regions become rapidly coated by the ssDNA-binding protein complex RPA1–3, which triggers activation of the apical DDR kinase, ATM and Rad3-related (ATR; Mec1 in yeast) (10). ATR/Mec1 then phosphorylates a multitude of protein substrates, many of which promote DNA replication completion and hence cell survival (11, 12).

We previously demonstrated that reduced ATR function engenders profound inhibition of NER specifically during S phase in a variety of human cell types (13, 14). We also reported that inactivating mutations in POLH, encoding translesion DNA polymerase η (polη), cause a similarly striking cell cycle-specific repair defect (15). Polη is normally required to forestall mutagenesis in UV-irradiated cells via its capacity to perform relatively error-free replicative bypass of CPDs (16). Interestingly, consistent with our results in polη-deficient human cells, murine embryonic fibroblasts knocked out for translesion DNA polymerase ζ (which is essential for efficient replicative bypass of 6-4PPs) were recently shown to also exhibit defective NER uniquely during S phase (17) (see “Discussion”). It remains unclear whether the requirement for robust ATR and translesion DNA polymerase activities in S phase-specific NER reflects a common underlying mechanism. However, one potential connection relates to the fact that cells derived from polη-deficient skin-cancer-prone xerodermic pigmentosum variant (XPV) patients or from ATR-deficient Seckel syndrome patients each exhibit increased replication fork stalling in response to UV (18, 19). Based on this, we (15) and others (17) have postulated a functional link between levels of replicative stress and NER efficiency.

To further investigate this possibility, we exploited a genetically tractable model organism, the budding yeast Saccharomyces cerevisiae. Specifically, a novel flow cytometry-based assay was developed to quantify NER kinetics as a function of cell cycle in yeast. We demonstrate that deletion of the yeast ATR homolog MEC1 or of any among several other DDR genes involved in the cellular response to replicative stress cripples NER uniquely in S phase. Furthermore, direct evidence is provided that this cell cycle-specific repair defect is triggered by sequestration of RPA1–3 to regions of ssDNA during periods of enhanced replicative stress, ostensibly causing reduced availability of this complex to perform its essential function in NER.

Experimental Procedures

Yeast Strains and Growth Conditions—Unless stated otherwise, deletion mutants were obtained from the BY4741 haploid MATa Yeast Knock-out Collection (Thermo Scientific, YSC1053). Other strains used in this study are described in Table 1. Yeast strains were generated and propagated using standard yeast genetics methods. Expression plasmids for RAD26 and rad26 S27A were kindly provided by Dr. J. Q. Svejstrup (20). For cell synchronization in G1/M, cultures were diluted to a cell density of 0.5 OD and incubated with 15 µg/ml nocodazole (Cedarlane; 1% DMSO final concentration) for 3 h at 30 °C. For S synchronization, cells at 0.1875 OD were incubated with 5 µg/ml α-factor for 90 min at 30 °C, followed by further incubation with a second dose of 5 µg/ml α-factor for 75 min. α-Factor-arrested cells were released toward S phase in medium containing 50 µg/ml Pronase. Genotoxic drugs were purchased from Sigma-Aldrich (methylmethane sulfonate) and Bioshop Canada (hydroxyurea and 4-nitroquinoline 1-oxide). Auxin (indole-3-acetic acid) was purchased from Sigma-Aldrich.

Mammalian Cell Culture—Culture media and supplements used in this study were purchased from Invitrogen. The human melanoma strain WM3248 was obtained from the Coriell Institute (Camden, NJ). The SV40-transformed XPV-skin fibroblast strain XP30R0sv and its derivative expressing wild-type polη (XP30R0sv-polη/c6), were a generous gift of Dr. A. R. Lehmann (University of Sussex). These cells were propagated in Eagle’s minimal essential medium containing 15% FBS, essential and nonessential amino acids, vitamins, l-glutamine, and antibiotics. HeLa and U2OS cells were propagated in DMEM containing 10% FBS, l-glutamine, and antibiotics. Transfections were performed with Lipofectamine 3000 (Invitrogen). The RPA expression vector was generously provided by Dr. J. Lukas (21) (University of Copenhagen). VE-821 was purchased from Cederlane.

UV Irradiation—A monochromatic 254-nm G25T8 germicidal lamp (Philips) was used at a fluence of 1 J/m²/s measured using a Spectroline DRC ×100 digital radiometer equipped with a DIX-254 sensor. Yeast cells were washed with water and irradiated in a thin layer of water at a cell density of 0.5 OD. For yeast grown on agar plates, cells were irradiated immediately after plating. UV irradiation and post-UV incubations were carried out in the dark to prevent CDP reversal by yeast photolyase activity. For mammalian cell lines, monolayers were washed with PBS and irradiated through a thin layer of PBS.

Immunoblotting—Whole-cell lysates of S. cerevisiae were prepared for SDS-polyacrylamide gel electrophoresis by alkaline lysis (22) or standard glass bead/trichloroacetic acid precipitation. Whole-cell lysates of mammalian cells were prepared as described (23). SDS-PAGE and protein transfers were performed using standard molecular biology protocols. Anti-

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bodies used were as follows: mouse monoclonal 12CA5 anti-HA and anti-FLAG M2 (Sigma-Aldrich), rabbit anti-polh (sc-5592, Santa Cruz Biotechnology, Inc.), mouse anti-RPA2 (NA18, Calbiochem), mouse anti-γH2AX (JBW301, Millipore), rat anti-tubulin (ab6161, Abcam), rabbit anti-RPA1 (ab79398, Abcam), and mouse anti-RPA3 (ab6432, Abcam). Detection was performed using a LAS-3000 imaging system (Fuji). Immunoblot signals were quantified with ImageQuant version 5.2 software (GE Healthcare).

Measurement of DNA Content by Flow Cytometry—DNA was stained using SYTOX Green (Invitrogen), as described previously (24). Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences). Results were analyzed with FlowJo version 7.6.5 software (FlowJo, LLC).

Quantification of CDP Removal by Flow Cytometry—For S. cerevisiae, 1 ml (0.5 OD) of cells were fixed overnight with 2.5 ml of 95% ethanol at 4 °C. Cells were washed with 2 ml of PBS, and incubated with 20 μg/mL Zymolyase T20 (BioShop Canada) in PBS at 30 °C for 40 min. This was followed by incubation in 1 ml of 2 N HCl at room temperature for 15 min, followed by neutralization in 3 ml of 0.1 M borax, pH 9. Cells were then washed in PBS and incubated in 1 ml of PBS plus 0.5 mg/ml RNase A (BioShop Canada) for 1 h at 37 °C. Samples were washed with 3 ml of PBS-TB (PBS, 1% BSA, 0.25% Tween 20), followed by incubation in 0.35 ml of PBS-TB + mouse anti-CDP antibody (Kamiya Biomedical, MC-062) at a 1:1000 dilution for 90 min at room temperature. After a wash with PBS-TB, samples were incubated in 0.35 ml of Alexa647-conjugated goat anti-mouse secondary antibody (A21235, Invitrogen) for 1 h at room temperature in the dark. Samples were washed with PBS-TB, resuspended in 1 ml of PBS plus 1 μg/ml SYTOX Green (Invitrogen), and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo version 7.6.5 software (FlowJo). For mammalian cells, the excision of 6-4PPs was quantified in G₀/G₁, S, and G₂/M phase as described in detail previously (13).

Quantification of BrdU Incorporation by Flow Cytometry—Hela cells transfected with vector expressing either RPA or GFP were seeded in 6-cm dishes (10⁶ cells/dish). Cultures were treated for the indicated time with 30 μM BrdU, trypsinned, fixed in ethanol, labeled with an Alexa647-coupled mouse anti-BrdU-antibody (B35133, Thermo Scientific), and analyzed by flow cytometry as described previously (14).

Quantification of CDP Removal by Slot Blot—After UV irradiation, genomic DNA was extracted from yeast using a standard glass bead/phenol/chloroform procedure. DNA concentration was measured with PicoGreen (Invitrogen) using a TBS-380 fluorimeter (Turner Biosystems). 100 ng of DNA samples were bound to Zeta-probe membranes (Bio-Rad) using a Bio-Dot SF slot blotting apparatus (Bio-Rad) according to the manufacturer’s instructions. Membranes were probed with anti-CDP primary antibodies (MC-062, Kamiya Biomedical). Detection was performed using a LAS-3000 imaging system (Fuji). Signals were quantified using ImageQuant version 5.2 software (GE Healthcare).

Fluorescence Microscopy of S. cerevisiae—Cells were fixed using formaldehyde as described (25). Images were acquired at room temperature using a DeltaVision Elite system (GE Healthcare) equipped with a UIS2 ×60/1.42 numerical aperture, plan apo objective (Olympus); a 15-bit EDGE/sCMOS camera (PCO); and SoftWoRx version 6.2.0 software. Images were analyzed using ImageJ version 1.46E (National Institutes of Health). At least 200 cells were evaluated for each sample.

Immunofluorescence Microscopy of RPA2 and BrdU in Human Cells—Cells were seeded in 6-well plates on a microscope glass slide and irradiated or mock-irradiated as described.
above. Post-UV incubations were carried out in medium containing 30 μM BrdU (Sigma-Aldrich). Cells were washed twice with ice-cold PBS and incubated for 3 min on ice with 0.5% Triton X-100 in PBS. Samples were washed with cold PBS and fixed for 15 min on ice with 3% methanol-free paraformaldehyde (Thermo Scientific) in cold PBS. Cells were washed with cold PBS and further incubated with 0.5% Triton X-100 in PBS for 10 min on ice. Samples were denatured with 2 M HCl at room temperature for 10 min and neutralized with 0.1 M borax, pH 9. After washing with PBS-TB, samples were incubated overnight at 4 °C with 3% BSA in PBST (PBS, 0.05% Tween 20). Labeling was performed with mouse anti-RPA32 (NA18, Calbiochem) and rat anti-BrdU (ab6326, Abcam) at a 1:500 dilution in PBST. Secondary antibodies were goat anti-mouse-Alexa488 (A10667, Invitrogen) and chicken anti-rat Alexa594 (A21471, Invitrogen), used at 1:500 dilution in the presence of 20 μg/ml DAPI (Invitrogen). Images were acquired at room temperature using a DeltaVision Elite system (GE Healthcare) equipped with a U1S2 ×60/1.42 numerical aperture, plan apo objective (Olympus); a 15-bit EDGE/sCMOS camera (PCO); and Soft-WoRx version 6.2.0 software. Images were analyzed using ImageJ 1.46E (National Institutes of Health) and custom software to evaluate the intensity of RPA2 signals (see below).

**Automated Evaluation of Nuclear RPA2 Intensity in Mammalian Cells—** An algorithm was created using MATLAB (Mathworks, MA) to process three-channel images based on nuclear segmentation. DAPI channel images (435 nm) were automatically thresholded using the Otsu algorithm to obtain a binary mask, objects smaller than 5 pixels were removed, and holes were filled. A watershed algorithm was applied to the mask in order to separate adjacent nuclei. The average intensity and S.D. of both the 523-nm (RPA2) and 632-nm (BrdU) fluorescence channels were calculated for every nucleus detected in the field. For statistical analysis, at least 400 cells from two independent experiments were used for every condition.

**Results**

**Mec1 Is Required for Efficient NER Uniquely during S Phase in S. cerevisiae—** We developed a novel flow cytometry-based method to quantify NER kinetics as a function of cell cycle in yeast. Toward validating this assay, we initially compared CPD removal in wild type (WT) versus rad4Δ strains synchronized in G2/M using nocodazole. rad4Δ cells are completely defective in NER-mediated CPD removal and exquisitely sensitive to UV irradiation (26). Synchronized populations were irradiated with 100 J/m2 of 254-nm UV (hereafter referred to as UV) and incubated in the dark to prevent CPD reversal by DNA photolyase activity. Cells harvested at regular intervals were treated with Alexa647-conjugated anti-CPD antibody and SYTOX Green, followed by flow cytometry analysis. SYTOX Green was used to label double-stranded DNA in order to analyze Alexa647 signal intensity specifically in G2/M cells using a software gate (Fig. 1A). As expected, initial CPD induction by UV (t = 0 min) increased the Alexa647 signal to an equal extent (~5-fold over background) in both WT and rad4Δ cells (Fig. 1B). During post-UV incubation, the signal decreased gradually for WT cells, reflecting progressive NER-mediated CPD removal (~90% repair by t = 90 min) but remained essentially unchanged in the NER-deficient rad4Δ strain.

We next compared CPD removal in WT, rad4Δ, rad26Δ, and mec1Δ sml1Δ strains synchronized in S phase. Deletion of SML1, encoding an inhibitor of the ribonucleotide reductase enzymatic complex, is required to permit viability of mec1Δ cells (27). Rad26 is the yeast homologue of the human TC-NER-specific lesion recognition factor CSB and was previously shown to be phosphorylated by Mec1 on serine 27 (20). To facilitate comparison between different mutant strains that may traverse the G1-S transition with varying speeds, cells were synchronized in G2/M with nocodazole (Fig. 1F). As expected, rad26Δ mutants were profoundly deficient in this respect (Fig. 1C, bottom). rad26Δ mutants exhibited a reproducible but modest inhibition of CPD removal during S phase. We conclude that yeast Mec1 is required for efficient NER during S phase.

Our use of HU to tightly synchronize cells in early S raised a caveat because this agent is well known to induce replicative stress (28). Therefore, to control for any potential confounding effects of HU treatment, we analyzed repair as above, except each strain was released from G1 in medium lacking HU and irradiated 30 min later, when a majority of cells are in S phase. Essentially the same repair kinetics were observed in both WT and mutant strains either treated or not with HU (Fig. 1, compare D with C). Thus, under the conditions in which we perform our assays, CPD repair during S phase is not significantly influenced by a 1-h exposure to HU.

We next investigated whether Mec1 might be necessary for NER in other cell cycle phases. CPDs were efficiently removed in WT, rad26Δ, and mec1Δ sml1Δ strains maintained in G2 throughout the post-UV incubation using α-factor (Fig. 1E). In addition, no differences in NER kinetics were observed between these strains synchronized and maintained in G2/M using nocodazole (Fig. 1F). As expected, rad4Δ cells synchronized and maintained in either G1 or G2/M exhibited profoundly defective repair. Finally, to further validate our assay and solidify the above results, NER was quantified as a function of cell cycle using another, well established method (i.e. Southwestern (“slot”) blotting using CPD-specific antibody) (29). Very similar results were obtained using this versus our flow cytometry-based approach (Fig. 1, compare G–I with C, E, and F). We emphasize that in our hands, flow cytometry-based evaluation of NER is far less tedious and more reproducible than slot-blotting, which prompted us to use the former method for all subsequent experiments. Overall, our data clearly demonstrate that deletion of MEC1 in yeast causes defective NER uniquely during S phase (i.e. repair during other phases proceeds with much faster kinetics). (Hereafter, NER occurring specifically in S phase will be denoted as S phase repair (SPR).)
Mec1-dependent Phosphorylation of Rad26 Is Not Required for Efficient SPR—Because it has been reported that Mec1 phosphorylates Rad26 on serine 27 after UV irradiation, which in turn influences NER (20) (see “Discussion”), we assessed the possible contribution of Rad26 to SPR in more detail. Specifically, the fraction of CPDs remaining in S phase at 1 h post-UV was quantified in rad26Δ cells containing a plasmid expressing either a WT Rad26 allele or one in which serine 27 is mutated to a non-phosphorylatable alanine residue (S27A). In accord either a WT post-UV compared with WT, whereas expression of either WT or S27A Rad26 alleles improved repair efficiency in S phase to a similar extent as compared with rad26Δ cells (Fig. 2A). We conclude that lack of Mec1-mediated Rad26 phosphorylation cannot explain the striking SPR defect of mec1Δ sml1Δ mutants.

We also evaluated whether Rad53, a key effector kinase downstream of Mec1 during periods of replicative stress (30, 31), might regulate CPD removal during S phase. Quantification of NER kinetics in rad53Δ sml1Δ cells revealed SPR defects similar in magnitude to those observed in mec1Δ sml1Δ mutants (Fig. 2B). However, rad53Δ sml1Δ mutants synchronized in G2/M using nocodazole retained the capacity to remove CPDs with virtually identical kinetics compared with WT or mec1Δ sml1Δ cells (Fig. 2C). Thus, Rad53 is required for UV damage repair exclusively during S phase.

Initiation of DNA Replication Is Necessary for Manifestation of SPR Defects in MEC1-deficient Cells—We considered the possibility that elevated levels of constitutive replicative stress in mec1Δ sml1Δ cells could be linked to defective S phase-specific NER. Such a model predicts that initiation of DNA replication would be required for abrogation of SPR in a Mec1-deficient background. Alternatively, it is possible that certain S phase-specific factors (e.g. cyclin-dependent kinases), could influence SPR in a DNA replication-independent manner in mec1Δ sml1Δ cells. To investigate this, we exploited cells expressing a thermosensitive cdc7-4 mutant allele (32). In conjunction with Dbf4, Cdc7 kinase is required for the initiation of DNA replication by phosphorylating subunits of the MCM helicase complex at the onset of S phase (32–35). Thus, cdc7-4 mutants released from G1 toward S phase at the restrictive temperature of 37 °C become arrested at a very late stage of the G1-S transition (i.e. immediately prior to replication origin firing). However, under these conditions, cells are budded, indicating that G1-S cyclins have been activated (36) and furthermore present S phase-specific cyclin-dependent kinase activity (37).

cdc7-4 cells were released from α-factor-mediated G1 arrest at the restrictive temperature of 37 °C and irradiated with UV. NER-mediated removal of CPDs was evaluated by flow cytometry in cells kept either at 37 °C (to prevent the initiation of DNA replication) or at 25 °C (permitting DNA replication initiation and progression). At 37 °C, cdc7-4 mec1Δ sml1Δ
mutants did not display defective CPD removal relative to cdc7-4 cells (Fig. 3). In contrast, cdc7-4 mec1Δ sml1Δ mutants incubated at 25 °C exhibited a striking delay in CPD removal in comparison with cdc7-4 counterparts. We conclude that the initiation of DNA replication is required for inhibition of SPR in cells lacking Mec1.

Several DDR Pathways Are Required for Efficient SPR—

Mutations in various well defined cellular pathways confer sensitivity to drugs that impede the progression of DNA replication forks (38). For example, defects in Rtt109-mediated acetylation of lysine 56 of newly synthesized histone H3 cause such sensitivity, suggesting that this histone modification is required for efficient repair of damaged replication forks (25, 39). Remarkably, we observed that rtt109Δ mutants exhibit defects in SPR of the same magnitude as mec1Δ sml1Δ cells (Fig. 4A). In contrast, rtt109Δ mutants synthesized in G1/M using nucodazole remove CPDs with kinetics identical to WT cells (Fig. 4B). These results suggest that SPR defects in mec1Δ sml1Δ and rtt109Δ strains may be driven by abnormal responses to replicative stress.

Genome-wide screens have identified several S. cerevisiae genes, which, when deleted, cause (i) elevated levels of spontaneously arising Rad52 foci (40) or (ii) sensitivity to methylmethane sulfonate (MMS), a model DNA-alkylating agent employed widely in yeast to evaluate cellular responses to replication-blocking DNA adducts (38). Several of these genes are known to be directly or indirectly involved in the repair or processing of DNA lesions during S phase. We examined SPR efficiency in a panel of mutants deleted for 26 such genes. These strains were classified into six functional categories: NER (as controls), DNA damage signaling and checkpoint, histone H3 lysine 56 acetylation, homologous recombination repair, post-replication repair/DNA damage tolerance, and a “miscellaneous” group, which included three genes not fitting cleanly into any of the aforementioned categories. Remarkably, we found that 15 of the 26 deletion strains exhibited significant defects in CPD removal at 1 h post-UV, although the magnitude of the defects varied (Fig. 4C).

Five deletion strains (one from each group) exhibiting strong SPR defects were selected for further characterization: mec1Δ sml1Δ, rtt109Δ, rad52Δ, rad6Δ, and dia2Δ. All of these strains display sensitivity to UV, which is consistent with a role for the corresponding genes in modulating NER activity (Fig. 4D). However this UV sensitivity varied; mec1Δ sml1Δ, dia2Δ, and rtt109Δ are more UV-sensitive when irradiated during S phase compared with G1/M, whereas rad52Δ and rad6Δ exhibit equal loss of viability throughout the cell cycle after UV treatment. We further investigated the cell cycle specificity of NER defects observed in these mutants. Cells were synchronized in G1, using α-factor and then, before UV irradiation, either (i) maintained in G1, (ii) released in S phase in the presence or absence of 200 mM HU, or (iii) allowed to progress in G1/M in the presence of nocodazole for 2 or 4 h (Fig. 4E). We observed that for all mutants, CPD removal in G1 was similar to WT (less than 20% remaining at 1 h post-UV). In contrast, mutants released toward S phase displayed strong NER defects. Importantly, this latter result held whether or not HU was added to the medium post-G1 release in order to tightly synchronize cells in early S, bolstering the conclusion derived from Fig. 1 (C and D) that a 1-h exposure to HU per se does not cause SPR deficiency under our experimental conditions. We also observed that mutant cells present a similar SPR defect when released from G1 into nocodazole (without HU) for 2 h prior to UV irradiation. However, when nocodazole incubation is increased to 4 h, each strain showed CPD removal comparable with WT. This suggests that the conditions causing defective SPR are triggered during S phase and persist for some time after cells have replicated the bulk of their genomic DNA. Taken together, our results demonstrate that deletion of genes participating in diverse DDR pathways that play a role in mitigating the genotoxic consequences of replicative stress generates defects in NER exclusively during S phase.

SPR Defects Correlate with Elevated Frequencies of Spontaneous and Genotoxin-induced Rfa1 Foci—RPA-coated ssDNA is known to accumulate at stalled/damaged replication forks and to be a critical factor in activation of the DNA damage checkpoint (7, 11). In yeast, the RPA subunit Rfa1 forms foci at sites of DNA damage (41). We examined spontaneous Rfa1 focus formation in 13 mutant strains expressing Rfa1-GFP by fluorescence microscopy (Fig. 5A, left). In most cases, a fraction of cells presenting large buds (i.e. in G2/M) manifested spontaneous Rfa1 foci. Because such cells are excluded from S phase-specific analysis by flow cytometry gating in our NER experiments, we evaluated Rfa1 focus formation specifically in cells presenting...
small buds (i.e. in early S phase). In general, yeast strains presenting SPR defects (SPR−) harbored foci significantly more frequently in comparison with SPR+ strains (Fig. 5A, right). This difference is even more pronounced when only S phase cells are taken into account, suggesting that SPR− mutants present higher frequencies of spontaneously arising DNA damage during DNA replication compared with SPR+.

HU inhibits the progression of DNA replication forks and activates Mec1-dependent signaling by generating RPA-bound ssDNA (11). We verified whether HU might influence Rfa1-
GFP focus formation under conditions similar to those used herein to evaluate SPR. In both WT and SPR− mutants, the fraction of small-budded cells containing Rfa1-GFP foci was comparable whether or not cells were incubated for 1 h in medium containing HU (Fig. 5B). This is consistent with the fact that HU treatment for 1 h did not enhance existing SPR defects in these mutants (Fig. 4E). However, previous reports in S. cerevisiae showed that HU exposure causes increased ssDNA formation over the course of a few hours; moreover, such an increase is more pronounced in cells lacking intra-S phase checkpoint factors as compared with WT counterparts (42). Similarly, HU exposure was shown to cause a gradual increase in RPA focus formation over a 4-h period in S. pombe checkpoint-defective mrc1Δ cells (43). In accord with these studies, we found that the fraction of WT and mec1Δ smt1Δ S phase cells harboring Rfa1 foci increased gradually over a 4-h time
period in HU-containing medium (Fig. 5C). Importantly, we also observed that HU exposure caused the induction of a time-dependent SPR defect in WT cells, whereas the existing SPR defect in \textit{meclA smllA} mutant was exacerbated (Fig. 5D). This induction was especially apparent after 2- and 4-h incubation in HU (i.e. the efficiency of SPR in both strains after only 1 h was very similar to that documented in Fig. 1 (C and D) for cells either treated or not with HU). The above described correlation between HU-induced Rfa1 focus formation and SPR defects strongly argues that elevated levels of replication stress underlie defective SPR in yeast.

We next compared genotoxin-induced Rfa1 focus formation in SPR+ versus SPR− strains. UV irradiation of cells released toward S phase from α-factor-mediated G1 arrest gave rise to a multitude of small Rfa1 foci, which were difficult to unambiguously distinguish from background fluorescence. The time required to progress from G1 to S was also highly variable among mutant strains in our hands, leading to inconsistencies in the fraction of cells in S at the time of UV irradiation. Furthermore, in most strains, UV irradiation caused G1 arrest in a proportion of the cell population, as reported previously (44, 45). To circumvent these confounding problems associated specifically with UV treatment, we decided to induce replicative stress using 4-nitroquinoline 1-oxide (4NQO), a UV-mimetic agent that generates helix-distorting DNA adducts removed by NER. Cells were synchronized in G1 and released toward S phase in the presence of 0.15 μg/ml 4NQO (Fig. 5E). Under these conditions, all strains synchronously entered S phase within 2 h after release from α-factor and exhibited clearly distinguishable Rfa1-GFP foci. We found that the fraction of cells presenting Rfa1 foci was increased following 4NQO treatment in all strains and that SPR− mutants displayed significantly higher frequencies of foci compared with SPR+. As was the case for spontaneously arising Rfa1 foci, this difference was more striking when only S phase cells were considered, concordant with a model in which 4NQO generates higher levels of replicative stress in SPR− versus SPR+ cells.

Finally, we examined the persistence of MMS-induced Rfa1 foci in WT versus SPR- strains (Fig. 5F). In WT cells, transient exposure to this drug induces Rfa1-GFP foci that are resolved more rapidly than those formed in response to 4NQO, which we expected would facilitate the detection of possible differences in focus persistence between SPR+ and SPR− strains. Cells were synchronized in G1 and released toward S phase in medium containing 0.025% MMS for 1 h. The drug was then inactivated using sodium thiosulfate, and cells were incubated for a further 5 h in MMS-free medium. We observed that after an initial increase in the fraction of WT cells containing Rfa1 foci (0 h time point), these foci rapidly disappeared from the cell population, presumably reflecting resolution of DNA lesions or of structures causing DNA replication forks to stall. In contrast, the proportion of focus-containing cells increased throughout the experiment in SPR− mutant strains. Taken together, our analyses of Rfa1 focus dynamics reveal a significant negative correlation between elevated levels of spontaneous or genotoxin-induced replicative stress and NER capacity in S phase yeast cells.

\textbf{Partial Depletion of Rfa1 Inhibits SPR}—We next speculated (15), and more recently others have presented evidence supporting the notion (17), that abnormal accumulation of RPA1−3 at damaged DNA replication forks could reduce the availability of this complex for NER during S phase. To directly test this model in yeast, we investigated the effect of partial RPA depletion on CPD removal in replicating cells. Rfa1 was epitope-tagged with an auxin-induced degron sequence (AID) in strains expressing the TIR1-Myc ubiquitin ligase (46, 47). In the presence of auxin (indole-3-acetic acid), TIR1 targets AID-tagged proteins for proteasomal degradation. We tested different epitope tags and found that Rfa1-AID-6FLAG produced partial but reproducible depletion of Rfa1 in response to 2 μm auxin (Fig. 6A). As expected, expression of both TIR1-Myc and Rfa1-AID-6FLAG impaired growth on YPD-agar medium containing 0.5 or 2 μm auxin (Fig. 6B). We used this Rfa1-AID-6FLAG strain to test whether partial Rfa1 degradation might mimic a situation in which an inordinate fraction of total RPA is sequestered at damaged/stalled replication forks after UV exposure, thereby reducing its availability for NER. Strains expressing TIR1-Myc with or without Rfa1-AID-6FLAG were synchronized in early S with HU in the presence or absence of 0.5 μm auxin. We observed partial degradation of Rfa1-AID-6FLAG under these conditions, which moreover, remarkably, resulted in a significant decrease in CPD removal in S phase cells but not in nocodazole-synchronized G2/M cells (Fig. 6C, left and right, respectively).

We considered the possibility that AID-mediated RPA depletion could alter the firing of DNA replication origins or the processivity of replication forks, thereby modulating levels of replicative stress experienced by Rfa1-AID-6FLAG-expressing
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Partial depletion of Rfa1 causes S phase-specific defects in CPD removal. A, exponentially growing cells expressing Tir1-Myc alone or in combination with Rfa1-AID-6FLAG were incubated in YPD medium containing 2 mM auxin for 1 h. Samples were then analyzed by immunoblotting. B, cells were spotted on YPD-agar containing the indicated concentrations of auxin. C, cells were synchronized in S phase with HU (left) or G2/M with nocodazole (right) as in Fig. 1, except that 0.5 mM auxin was added to cultures 30 min prior to UV irradiation. Levels of Rfa1-AID-6FLAG were monitored by immunoblotting (top). Removal of CPD following UV irradiation was measured by flow cytometry. D, effect of partial RPA depletion on S phase progression. Tir1-Myc Rfa1-AID-6FLAG cells were arrested in G1, and were then pretreated for 30 min with or without 0.5 mM auxin before release at room temperature with or without auxin. Samples were collected at 5-min intervals for FACS cell cycle analysis. Left, cell cycle profiles for representative time points. Right, the average DNA content in each sample relative to G1 is used as a measure of S phase progression. For each panel in this figure, values represent the mean ± S.E. (error bars) of three independent experiments.

FIGURE 6. Partial depletion of Rfa1 causes S phase-specific defects in CPD removal.

We reasoned that ectopic overexpression of Rfa1–3 could compensate for the postulated sequestration of this complex away from NER in SPR yeast mutants. Unfortunately, in our hands, and as reported previously by others (48), overexpression of Rfa1 either alone or in combination with Rfa2 and Rfa3 was extremely toxic in yeast (data not shown). In contrast, stoichiometric overexpression of RPA subunits has been achieved in human cells using a vector encoding a single polypeptide precursor, where the three RPA subunits are separated by self-cleaving P2A peptide sequences (pAC-GFP-C1-RPA3-P2A-RPA1-P2A-RPA2) (21). The RPA3 subunit encoded by this vector is also conveniently tagged with GFP. We decided to use this construct to test whether SPR defects in ATR-deficient human cells (13) can be rescued by increasing the pool of RPA. HeLa cells were stably transfected with the RPA expression vector or with a control GFP expression plasmid, and stoichiometric overexpression of all three RPA subunits was confirmed by immunoblotting (Fig. 7A). Transfected cells were treated with the well characterized ATR-specific inhibitor VE-821 (49) or mock-treated. As expected, ATR inhibition abrogated UV-induced phosphorylated H2AX (γH2AX), an established marker of DNA damage and major target of ATR phosphorylation in human cells (Fig. 7A). We then used our flow cytometry-based assay to measure the effect of concomitant ATR inhibition and RPA overexpression on NER-mediated removal of 6-4PP as a function of cell cycle, as described previously (13). This assay is similar to the one used here to quantify NER in yeast cells, except there is no requirement for human cells to be synchronized prior to UV irradiation. Indeed, proliferation is completely inhibited for at least 6 h after irradiation of human cells with 20 J/m2 UV, providing a convenient window to quantify 6-4PP removal by NER for populations gated in individual cell cycle phases. We examined 6-4PPs rather than CPDs because the latter are repaired relatively slowly in human cells (i.e. typically only 40–50% removal after 24 h compared with 80–100% by 6 h for 6-4PP). Because of the relatively slow rate of repair, CPD excision is difficult to measure accurately as a function of cell cycle in human cells by flow cytometry. As expected, ATR inhibition severely impaired 6-4PP removal during S phase in HeLa cells expressing a control GFP vector (Fig. 7B). Remarkably, ectopic RPA overexpression significantly improved 6-4PP repair in ATR-inhibited S phase cells, supporting the model that reduced RPA availability accounts for deficient NER in S phase during periods of enhanced replicative stress.

We then used fluorescence microscopy to verify whether RPA recruitment to UV-damaged chromatin is influenced by pharmacological inhibition of ATR. Cells were treated or not with ATR inhibitor, irradiated with UV, and incubated for 2 h in the presence of BrdU to label S phase cells. Soluble RPA was removed by Triton X-100 pre-extraction, before fixing the cells with formaldehyde and staining with anti-RPA2 and anti-BrdU antibodies. Visual inspection of immunofluorescence microscopy images suggested that the nuclear RPA2 signal was strongly increased in BrdU-positive cells after UV irradiation (Fig. 7C). To confirm this in a quantitative manner, we developed software for unbiased evaluation of the intensity of RPA2 nuclear signals in microscopy images (see “Experimental Procedures”). RPA signals were quantified in at least 400 cells for each condition; values are presented as box plots in Fig. 7D.
BrdU-negative cells displayed low RPA2 signals that were not significantly affected by UV or ATR inhibitor. Cells in S phase (BrdU⁺) manifested slightly elevated RPA2 signals in the absence of UV compared with BrdU⁻ cells, consistent with the known roles of RPA in DNA replication. Importantly, RPA2 signals were found to be significantly more intense after UV irradiation in BrdU⁺ VE-821-treated cells as compared with untreated BrdU⁺ cells. These data are consistent with a model in which ATR inhibition causes a larger fraction of the cellular RPA pool to be recruited to UV-damaged replication forks.

Finally, we sought to investigate whether replicative stress-induced sequestration of RPA could explain reduced SPR in human cell lines presenting defects in pathways other than ones directly influenced by ATR. As mentioned earlier, we previously showed that cells deficient in DNA polη are profoundly defective in SPR (15). XP30ROsv is an SV40-transformed fibroblast cell line derived from an XPV polη-deficient patient; moreover, as expected, inhibition of 6-4PP repair during S phase is significantly rescued in this strain when engineered to re-express polη (XP30ROsv-clone 6; Fig. 7E). Remarkably,
also observed significant rescue of defective SPR upon ectopic overexpression of RPA in XP30ROsv. As is the case for ATR-inhibited cells, BrdU+ XP30ROsv cells exhibited increased nuclear RPA2 signals post-UV, as compared with the poly-γ-complemented counterpart (Fig. 7F). In addition to ATR- and poly-γ-deficient cells, we also previously reported that several model human cancer cell lines are deficient in SPR, although the precise basis was not rigorously determined (13, 14). We found that SPR defects in U2OS osteosarcoma and WM3248 malignant melanoma cells are also rescued upon RPA overexpression (Fig. 7G).

As discussed above for yeast, we considered the caveat that ectopic manipulation (in this case overexpression) of RPA in human cells might influence the number of active DNA replication forks or their processivity, thereby modulating levels of UV-induced replicative stress. We therefore monitored DNA synthesis by comparing the rate of BrdU incorporation in S phase HeLa cells transfected with either RPA1–3- or control GFP-expressing vectors (Fig. 7H). We found that increased RPA levels do not influence the rate of BrdU incorporation in S phase cells, arguing against the aforementioned caveat.

Our overall data strongly support a model in which elevated levels of replicative stress caused by diverse mutations in DDR genes promote increased recruitment of RPA1–3 to damaged chromatin in S phase, leading to reduced availability of this complex for NER with severe functional consequences.

Discussion

We previously demonstrated that deficiency in either ATR kinase (required for stabilization and restart of stalled replication forks post-UV) or DNA poly(γ) (required for efficient bypass of replication-blocking UV-induced DNA adducts) generates profound defects in NER exclusively during S phase (13–15). To probe the mechanistic basis for this phenomenon, we exploited the powerful experimental model provided by the budding yeast S. cerevisiae. We initially developed and validated a novel flow cytometry-based approach to evaluate the kinetics of UV damage repair as a function of cell cycle in this organism. Our method, which measures CPD excision across the entire genome, is highly reproducible and quantitative and is unaffected by variations in DNA content between samples. We believe it will prove to be a valuable tool toward convenient and reliable evaluation of CPD excision in yeast.

Using this assay, we show for the first time that yeast mec1Δ mutants, like ATR-deficient human counterparts, exhibit striking inhibition of NER uniquely in S phase. This underscores the strict requirement in both yeasts and humans for a robust DNA damage-induced signaling cascade to promote efficient NER during DNA replication. Importantly, we also now extend our previous findings in human cells with the novel demonstration that in yeast, a host of different mutations crippling independent pathways involved in the cellular response to replicative stress also cause significant SPR deficiency. Such SPR defects would be expected to enhance the deleterious consequences of UV irradiation in the corresponding mutant strains. Indeed, although difficult to assess with precision, defective SPR is likely to promote (i) DNA replication fork stalling, leading to significant loss of cell viability, as well as (ii) increased mutagenesis during translesion synthesis, all due to the persistence of UV-induced DNA photoproducts (i.e. ~50% remaining at 90 min post-UV in the mutants as compared with almost complete removal in WT cells) (Fig. 1).

A prior study on regulation of NER by the DNA damage checkpoint in yeast showed at nucleotide resolution that mec1Δ smn1Δ cells exhibit defective CPD removal along the transcribed and nontranscribed strands of the active RPB2 gene (i.e. reflecting inhibition of both TC-NER and GG-NER, respectively) (20). This investigation did not measure repair as a function of cell cycle, although the extent of the defect as observed in cycling cells presumably indicates, in contrast with our own results, significant inhibition of NER during all phases in Mec1-deficient cells. The apparent discrepancy may be attributable to fundamental differences regarding the approaches used (i.e. quantification of strand-specific repair at a single genetic locus versus quantification of global repair averaged over the entire genome). Of note, the aforementioned study also revealed that the TC-NER-specific DNA damage recognition factor Rad26 (yeast CSB homolog) is phosphorylated on serine 27 by Mec1 in a Rad53-independent manner; moreover, relative to WT cells, the corresponding non-phosphorylatable Rad26 mutant exhibits significantly reduced CPD repair along the transcribed strand but not the nontranscribed strand of RPB2. As alluded to

FIGURE 7. Defects in S phase NER are rescued by RPA overexpression in human cells. RPA was overexpressed in human cells using a polycistronic vector in which each RPA subunit is separated by self-cleaving P2A peptide (pAc-GFP-C1-RPA3-P2A-RPA1-P2A-RPA2). This results in stoichiometric overexpression of subunits of the RPA complex, with RPA3 being fused to GFP. A GFP expression vector was used as negative control (empty vector). A and B, overexpression of RPA in HeLa cells rescues S phase NER defects induced by pharmacological ATR inhibition. Transfected cells were pretreated for 2 h with a 10 μM concentration of the ATR inhibitor VE-821, followed by irradiation with 20 J/m² UV or mock irradiation. A, total protein extracts were prepared 1 h after UV irradiation and analyzed by immunoblot. RPA1 and RPA2 resulting from P2A cleavage (RPA1-P2A and RPA2-P2A) have a slightly higher molecular weight compared with endogenous counterparts. B, HeLa cells were transfected as in A, and removal of UV-induced 6-4PP was measured as a function of cell cycle using a flow cytometry-based assay (see "Experimental Procedures"). C, HeLa cells were treated with VE-821 and mock- or UV-irradiated, as in A and B. Recruitment of RPA2 to chromatin after UV was detected by immunofluorescence microscopy. BrdU labeling was used to identify S phase cells. D, the average intensity of fluorescence for RPA2 signal was quantified for each condition described in C (at least 400 cells/condition). Values are presented as box plots (median, first, and third quartile, with the whiskers representing the first and last 5%). *, p < 0.0001, two-tailed unpaired t test. E, XP30ROsv, an SV40-transformed skin fibroblast derived from an XP2 patient, was transfected with the polycistronic RPA expression vector or control GFP expression vector. Removal of 6-4PP following UV was measured as in B. An isogenic clone expressing poly(γ) (CL6) was used as a control. F, recruitment of RPA to chromatin after UV was measured in XP30ROsv and CL6 as described in D., p < 0.0001, two-tailed unpaired t test. G, U2OS osteosarcoma and WM3248 human melanoma cell lines were transfected with the polycistronic RPA expression vector or control vector. Removal of 6-4PP following UV was measured as in B. H, effect of RPA overexpression on rates of DNA synthesis. HeLa cells were transfected with RPA or control vector and incubated with 30 μM BrdU for 0, 30, 60, and 90 min. Cells were labeled with an anti-BrdU- Alexa647 antibody and PI and analyzed by flow cytometry. Top left, example of bivariate plot after 60-min labeling. Block rectangle, gate used to select S phase cells. Top right, the geometric mean of BrdU signal in S phase cells was used to calculate the incorporation of BrdU in DNA as a function of time. Bottom panels, representative histograms of BrdU signal of gated S phase population. For flow cytometry data in this figure, values represent the mean ± S.E. (error bars) of three independent experiments. Microscopy data combines values from two independent experiments.
above, the flow cytometry-based assay used herein does not discriminate between GG-NER and TC-NER; in any case, our data (Fig. 2) indicate that preventing Mec1-dependent phosphorylation of serine 27 on Rad26 does not significantly influence overall NER efficiency specifically during S phase.

The diversity of function exhibited by genes/pathways conferring SPR defects strongly suggested that they might indirectly modulate UV damage repair through a common mechanism. Consistent with this, we found that the magnitude of SPR defects displayed by the various mutant strains correlates significantly with the frequency of spontaneously arising DNA lesions in S phase cells as assessed by Rfa1-GFP focus formation (Fig. 5A). We interpret these data as suggesting that abrogation of S phase repair in various genetic backgrounds reflects a hitherto unknown indirect effect of elevated levels of spontaneous replicative stress.

Our data in yeast showing that DNA replication initiation is required for manifestation of defective SPR (Fig. 3) prompted us to focus on the possibility that competition for rate-limiting factors between DNA synthesis and repair pathways might represent the root cause of such a defect. Indeed, several proteins are shared between the NER and replication machineries, including RPA1–3, DNA replication clamp proliferating cell nuclear antigen, and DNA polymerase δ (5). As cited earlier, it is well established that cells deficient in either ATR or polη exhibit in common a reduced capacity to overcome replication-blocking DNA damage, which in turn generates abnormally large tracts of RPA-coated ssDNA. Because RPA1–3 is also essential for both the pre-incision and gap-filling steps of NER, we originally hypothesized (15) that inordinate sequestration of this complex on ssDNA during periods of enhanced replicative stress (e.g. in ATR- or polη-deficient human cells) may severely limit its availability to function in NER during S phase. In view of our results, we propose that this hypothesis can also be applied more generally to replicative stress mutants in yeast.

Previous studies emphasized that genomic stability can be negatively impacted due to competition between cellular pathways for limited pools of RPA. Defective repair of Spo11-induced double strand breaks during meiosis generates an overabundance of RPA-bound ssDNA, which in turn causes a deficit in Rfa1 accumulation at, and repair of, other (artificially induced) double strand breaks (50). More recently, it was shown that human U2OS cells co-treated with HU and ATR inhibitor are subject to superfluous firing of replication origins, producing a surplus of ssDNA that limits the pan-nuclear availability of RPA (21). This in turn promotes the collapse of stalled replication forks, leading to cell death, a phenomenon termed “replication catastrophe,” which moreover can be forestalled by ectopic RPA overexpression. In this regard, we note that in yeast, HU exposure also causes greatly increased formation of ssDNA over time in DNA damage checkpoint mutants compared with WT cells (42, 43). Consistently, our data in yeast show a strong correlation between time-dependent increases in HU-induced Rfa1 focus formation and diminished CPD removal during S phase in both WT and mec1Δ smt1Δ cells (Fig. 5, C and D).

Another recent study of particular relevance here showed that Rev3−/− murine embryonic fibroblasts, which lack DNA polymerase ζ activity required for efficient bypass of UV-induced 6-4PPs, exhibit NER deficiency specifically during S phase (17). Rev3−/− murine embryonic fibroblasts (which, like polη-deficient counterparts, are ostensibly characterized by enhanced replicative stress) also exhibited marked reduction in the recruitment of RPA to UV-irradiated nuclear regions. Our data extend these findings by directly demonstrating for the first time that enhancing the availability of RPA by ectopic overexpression rescues SPR deficiency in human strains presenting independent defects in the response to UV-induced replicative stress (i.e. deficiency for either ATR or DNA polη activity). Moreover, this latter result is entirely in accord with our demonstration that degron-mediated depletion of the RPA subunit Rfa1 recapitulates defective SPR in otherwise WT yeast cells.

Of note, the SPR defects of U2OS osteosarcoma and WM3248 melanoma cells, distinct model human cancer cell lines of unclear genetic background, were also rescued by RPA overexpression. This, taken together with other data presented here, highlights the intriguing possibility that a significant proportion of human tumors could exhibit enhanced replicative stress for any among multiple genetic or epigenetic reasons; therefore, in addition, such tumors may also manifest abrogation of SPR due to RPA sequestration. In support of this idea, we previously demonstrated that a majority of human malignant melanoma strains are deficient in NER exclusively during S phase, and moreover, this correlates with reduced levels of ATR signaling and sensitivity to UV-induced genotoxic stress (14).

SPR deficiency may be expected to increase the sensitivity of cancer cells to chemotherapy agents, such as the widely used drug cisplatin, that induce replication-blocking DNA lesions removed by NER. In this respect, future work should aim to investigate whether S phase-specific NER defects are indeed widespread in tumors in situ. If so, this knowledge could be exploited toward predicting chemotherapeutic response and indeed for devising new synthetic lethal approaches for treating cancer.

Author Contributions—F. B. conducted most of the experiments. J.-P. A., E. F., and I. H.-M. generated yeast strains and performed microscopy and immunoblot experiments. F. B., E. D., and H. W. planned the experiments and contributed to the writing of the manuscript. S. C. programmed the algorithm for the quantification of microscopy images. All authors analyzed the results and approved the final version of the manuscript.

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