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Research Communication

The 19S Proteasome Subunit Rpn7 Stabilizes DNA Damage Foci Upon Genotoxic Insult

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Summary

The DNA damage response (DDR) orchestrates the recruitment of repair proteins at sites of damage and arrests cell-cycle progression until completion of repair. Upon irreparable damage, DNA damage foci persist (long-lived foci) and this is believed to induce cellular senescence. The resolution of DNA damage foci has previously been shown to depend on proteasomal degradation and various proteasome subunits have been implicated in the DDR. In this study, we aimed to analyze the possible distinct roles of individual proteasome subunits in the DDR. We show that specific 19S subunits respond to DNA damage by increased protein levels and nuclear translocation. Importantly, two 19S subunits, Rpn7 and Rpn11, colocalize with DNA damage foci over their whole lifespan. Although silencing of Rpn11 does not affect foci stability and lifespan, silencing of Rpn7 promotes faster resolution of DNA damage foci following genotoxic insult. For the first time, we provide evidence that Rpn7 silencing specifically decreases the frequencies of long-lived DNA damage foci without, however, affecting the repair rate of short-lived foci. Therefore, we propose that interaction of Rpn7 with DDR foci *in situ* mediates the protection of DNA damage foci from premature resolution. We suggest that this interaction is involved in enabling cellular senescence following genotoxic insult.

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Abbreviations DDR, DNA damage response; UPS, ubiquitin-proteasome system; NER, nucleotide excision repair; Rpt, regulatory particle ATPase; Rpn, regulatory particle non-ATPase; SA-β-gal, senescence-associated-β-galactosidase; DAPI, 4′,6-diamidino-2-phenylindole; H_2O_2, hydrogen peroxide; siscr, scrambled siRNA; PBS, phosphate buffered saline; ATP, adenosine triphosphate.

INTRODUCTION

Senescence is characterized by a collection of alterations in cellular functionality, which result in significant decline of adequate responses to the changing environment with the ultimate outcome being the loss of homeostasis. The failure of homeostasis is highly linked to macromolecular damage accumulation (mainly on DNA and proteins) caused by spontaneous reactions by numerous endogenous and exogenous reactive agents (*1*). Damage on nuclear DNA that remains unrepaired may have irreversible consequences. Therefore, nature has heavily invested in an intricate genome maintenance apparatus, consisting of several sophisticated DNA damage repair, tolerance, and checkpoint systems; this apparatus enables cell survival or triggers cellular senescence or death upon DNA damage (*2, 3*). The essential feature of a successful DNA damage response (DDR) is the highly conserved recruitment of repair proteins onto DNA damage foci (*4*). Despite extensive recent progress in this field, the factors that determine the kinetics of DNA repair and DNA damage foci resolution still remain elusive (*5*). The impact of DNA injury on the cellular physiology is determined by various parameters, the first of which being the type of damage. Some lesions are primarily mutagenic, greatly promoting cancer, while others, mainly cytotoxic or cytostatic, cause degenerative

changes such as those associated with senescence and aging. The latter type consists of lesions that affect both DNA strands, such as the double-strand breaks induced by irradiation or oxidative insult. Defects in the pathways responsible for the repair of such damage have been strongly linked to numerous syndromes with a characteristic accelerated aging phenotype. These syndromes further support the notion that genomic damage is a major culprit in the aging process (*6*).

Besides damage on the DNA, it is well established that senescence is also accompanied by abnormal and modified protein accumulation. The protein maintenance system is determined by the finely tuned equilibrium between protein synthesis and protein degradation (*7*). Protein degradation is predominantly catalyzed by the ubiquitin proteasome system (UPS). The UPS machinery is composed of ubiquitin, the proteasome, and a multitude of enzymes catalyzing the ubiquitination reaction on a variety of substrates, leading to the final degradation of polyubiquitinated proteins. The major proteasome assembly is the 26S proteasome involved in the regulated degradation of normal as well as abnormal, denatured, or otherwise damaged proteins (*8*). It is constituted of the catalytic 20S core and the 19S regulatory complex. The 20S proteasome is a 700-kDa barrel consisting of four heptameric rings arranged in a stack with two outer α-rings and two central β-rings ($\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$). Three β subunits, namely β1, β2, and β5, are proteolytically active, carrying different substrate specificities. The 19S regulatory subunit has a lid-and-base structure (*9*) that is made up by 17 different, highly conserved proteins, namely six regulatory particle ATPase (Rpt of the AAA superfamily) subunits and 11 regulatory particle non-ATPase (Rpn) subunits (*10*). Whereas the 20S complex confers the proteolytic activities of the proteasome, the documented role of 19S is to recognize, unfold, deubiquitinate, and control the entry of multiubiquitinated substrates into the 20S proteasome (*11*). We and others have shown that damaged protein accumulation upon senescence is caused by a progressive decline in defense mechanisms against damaged proteins (*12*). This decline is accompanied by decreased levels of all proteasome activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing), proteasome content, and proteasome subunit expression (*13, 14*). Additionally, treatment of cells with proteasome inhibitors leads to induction of senescence and shorter cellular lifespan (*13*). Importantly, proteasome activation by overexpression of a catalytic subunit results in increased survival rates following cytotoxic treatments and extends cellular lifespan (*15, 16*).

Recent studies suggest a cross-talk between the different cellular maintenance mechanisms. For instance, a role for the proteasome in the DNA repair response has been proposed (*17, 18*). Several DDR proteins have been recognized as proteasome substrates (e.g., MRN, Rad51, and p53) and proteasome inhibitors affect formation and function of repair foci (*19–22*). Furthermore, several key components of the homologous recombination machinery, such as BRCA1, BARD1, /BACH2, and Rad52, are degraded in a proteasome-dependent manner after DNA damage, and proteasome activity has been shown to favor utilization of homologous recombination over nonhomologous end joining in human cells (*23–25*). Together, these data demonstrate the involvement of the proteasome-dependent degradation in the DDR pathway at multiple levels.

There is also increasing evidence that certain 19S proteasome subunits can interact with DNA damage repair pathways, exerting functions that are not directly related to the 26S proteolytic activity. In studies performed in budding yeast, it was demonstrated that several members of the 19S proteasome regulate the nucleotide excision repair (NER) pathway, independently of proteolysis (*26, 27*). The NER pathway is especially relevant to aging as it is responsible for the removal of various forms of bulky base damage from DNA, such as those known to occur following UV exposure. Other studies demonstrated that DSS1, a well-conserved 19S lid proteasome subunit, binds to the breast cancer susceptibility protein BRCA2 (*28, 29*), which plays an integral role in the repair of double-stranded breaks; this interaction is essential for BRCA2 function in mammalian cells (*30*). Actually, DSS1 deficiency creates a phenotype similar to that seen in BRCA2-deficient cells (*31*). It has also been shown that Rpn7 directly interacts with BRCA2, independently of DSS1 (*23*). Futhermore, the yeast DSS1 homolog (Sem1) has been implicated in the repair of DSB through homologous recombination, via loading the recombination repair protein Rad51 (*24, 30*). Interestingly, Sem1 forms a complex with the proteasome subunits Rpn3 and Rpn7 (*32*).

The aforementioned data suggest an intriguing interplay between the different macromolecular maintenance systems; an interplay that could be important for the manifestation of cellular senescence. To shed light on this interplay, we explored the function of distinct proteasome subunits in the DDR. We focused on stressors such as oxidative exposure and/or irradiation, genotoxic insults especially relevant to aging (*6*). We revealed that two 19S subunits, Rpn7 and Rpn11, respond to the DNA damage by specifically interacting with DDR foci. These interactions appeared immediately following DNA damage and remained until the foci were resolved upon repair. Importantly, knockdown of Rpn7, but not Rpn11, destabilized the persistent DNA damage foci, without affecting the turnover of short-lived foci, thus suggesting a distinct role for Rpn7. We propose that interaction of Rpn7 with DDR foci *in situ* is necessary for long-term maintenance and stability of DNA damage foci, therefore enabling cellular senescence after genotoxic damage.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions

HFL1 and MRC5 human primary embryonic lung fibroblasts were purchased from ECACC. MRC5 cells were lentivirally transduced with the 53BP1 reporter protein AcGFP-53BP1c (*33*). In all experiments, early passage (young) cells of population doubling 20–35 were cultured in Dulbecco's modified

Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (complete medium). Cells were grown in a Binder Incubator at 37 °C, 95% air, 5% CO_2, and cell number was determined in duplicates using a Coulter Z_2 counter (Beckman Coulter, Nyon, Switzerland).

Antibodies and Reagents

Mouse monoclonal anti-γH2AX (05-636; MW: 17 kDa) was from Millipore (Billerica, MA, USA). Rabbit polyclonal anti-γH2AX (9718; MW: 17 kDa) and anti-53BP1 (4937; MW: 450 kDa) were purchased from Cell Signaling (Danvers, MA, USA). Anti-α6 (8100; MW: 33 kDa), anti-β2 (8145; MW: 28 kDa), anti-β3 (8130; MW: 23 kDa), anti-Rpn7 (8225; MW: 46 kDa), anti-Rpn11 (9625; MW: 35 kDa), anti-Rpn12 (8815; MW: 30 kDa), anti-Rpt2 (8160; MW: 60 kDa), and anti-Rpt6 (8215; MW: 48 kDa) were purchased from Enzo Life Sciences (Exeter, UK). Anti-p53 (DO1) (sc-126; MW: 53 kDa), anti-p16 (N20) (sc-467; MW: 16 kDa), anti-GAPDH (25778; MW: 37 kDa), anti-Lamin A/C (H-110) (sc-20681, MW: 69/62 kDa), anti-tubulin (TU-02) (sc-8035, MW: 52 kDa), and secondary antibodies for Western blotting (goat anti-mouse sc2005 and goat anti-rabbit sc2001) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies for confocal microscopy, Alexa Fluor 594 anti-mouse IgG (A11005), AlexaFluor 488 anti-mouse IgG (A11001), Alexa Fluor 594 anti-rabbit IgG (A21207), and AlexaFluor 488 anti-rabbit IgG (A21206), were obtained from Molecular Probes (Invitrogen). LLVY-AMC fluorogenic peptide that serves as proteasome substrate and proteasome inhibitors (MG132, MG262, and epoxomicin) was purchased from Enzo Life Sciences (Exeter, UK).

Senescence-Associated-β-Galactosidase Staining

Staining for senescence-associated-β-galactosidase (SA-β-gal) activity was performed as previously described (*34*). As a minor modification, 4′,6-diamidino-2-phenylindole (DAPI) was used as nuclear stain. Briefly, 0.3–2 $\times$ 10^5 cells were seeded on six-well plates. Twenty-four hours later, cells were treated with 50 μM siRNA against Rpn7, Rpn11, or the relevant scrambled control (siscr), and samples were fixed 2, 4, 5, and 6 days later. Cells were then washed with PBS, fixed in 0.2% glutaraldehyde and 2% formaldehyde for 5 Min, washed again with PBS, and finally stained at 37 °C for 24 H in the absence of CO_2, in staining solution (150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 40 mM citric acid, and 12 mM sodium phosphate, pH 6.0) containing 1 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Cells showing SA-β-gal staining and total number of cells (DAPI staining) were counted in 10 randomly chosen fields.

Treatments

Hydrogen peroxide (H_2O_2) treatment: cells at 50–60% confluence were treated with 400 μM H_2O_2 (Sigma, St Louis, MO, USA) in serum-free medium for 30 Min and then replenished with fresh complete medium for various lengths of time, depending on the individual assays.

Irradiation. X-ray irradiation was performed using an X-Rad 225 irradiator (Precision X-ray, North Branford, CT, USA) fitted with a 2 mm aluminum filter for beam hardening, running at 225 kV, 13.3 mA. Cells were replenished with fresh complete medium immediately after irradiation.

Proteasome Inhibitors. Cells were treated with 10 μM MG132 for the indicated length of time, 500 nM MG262 for 6 H, or 100 nM epoxomicin for 6 H and then replenished with fresh complete medium, depending on the individual assays.

RNA Interference

Predesigned pools of four siRNA oligonucleotides (SMARTpool; Dharmacon, Lafayette, CO, USA) were used for silencing Rpn7, Rpn11, and β1 expression. HFL-1 or MRC5 53BP1 cells at 60–70% confluence were transfected with 50 nM siRNA against Rpn7, Rpn11, and β1 or an equal molar of mismatched siscr and incubated for 48 H. To ensure functional and specific silencing, RT-PCR validation was performed for each experiment. Transfections with siRNAs were performed using Lipofectamine 2000 (Invitrogen) in serum-free Opti-MEM medium (Invitrogen) following the manufacturer's instructions.

Immunoblot Analysis

Cells were harvested at the indicated time points and lyzed in reducing Laemmli buffer. Protein concentrations were determined by the Bradford method with bovine serum albumin as standard (Bio-Rad Laboratories, Hercules, CA, USA). Samples were analyzed by 10–12% SDS–PAGE according to standard procedures and transferred onto nitrocellulose membranes. Membranes were probed with the appropriate antibodies. Secondary antibodies (Santa Cruz Biotechnology) conjugated with horseradish peroxidase and enhanced chemiluminescence were detected using ECL Western blotting substrates (Thermo Fisher Scientific, Rockford, IL, USA). Each immunoblot analysis was performed at least three times and representative blots are shown. Equal protein loading was verified by reprobing each membrane with the antibody against Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunofluorescence

Immunofluorescence experiments were performed as previously described (*35*). Briefly, HFL1 fibroblasts were grown on coverslips until 50–60% confluence, subjected to the described treatments, and fixed in 2% paraformaldehyde. Fixed samples were incubated with primary antibodies of interest and the appropriate secondary antibodies. Coverslips were embedded in

15 μL of Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA, USA) and analyzed on a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). The LAS AF software was used for image acquisition.

Live Cell Microscopy

For live cell microscopy, 1.5×10^5 cells were plated in Iwaki glass-bottomed dishes (Iwaki, Japan) 1 day before utilization and imaged on an inverted Zeiss LSM510 equipped with a Solent incubator (Solent Scientific, Southampton, UK) at 37 °C with humidified 5% CO_2, using a 40 $\times$ 1.3 NA oil objective, and images were acquired as z stacks every 10 Min. Autofocus was performed at each time point before capturing a z stack to ensure the entire cell was captured (1.65 μm pinhole every 1.5 μm over 4.5 μm total z range). Cells and AcGFP–53BP1c foci numbers and times of appearance and disappearance were recorded from projected stacks of deconvolved images (Huygens, SVI). Fluorescence intensity data were extracted using ImageJ (http://rsb.info. nih.gov/ij/).

Statistical Analysis and Quantification

Multigroup comparisons were performed using Analysis Of Variance (ANOVA) and post hoc TukeyHSD test to determine significant differences between treatments. Quantification of band density in blots was performed with Image Quant 5.2 software. All values including quantification data were reported as mean of three independent experiments $\pm$ standard deviation (SD) or standard error of the means (SEM). Statistical significance of $P < 0.05$, $P < 0.01$, or $P < 0.001$ is indicated in the graphs by one, two, or three asterisks, respectively. Foci lifespans were plotted as Kaplan Meier curves, including both censored and uncensored data, and analyzed using Cox regression, Breslow method in R.

RESULTS

Specific 19S Proteasome Subunits Are Recruited to DNA Damage Foci Under Genotoxic Stress

We have explored the role of distinct proteasome subunits in the DDR after genotoxic insult of two human primary cell lines, HFL1 and MRC5, with three different stressors, namely H_2O_2, etoposide, and X-ray irradiation. Young HFL1 human primary fibroblasts were treated with 400 μM H_2O_2 for 30 Min, and the protein levels of 19S and 20S proteasome subunits were analyzed immediately afterward and 2 H post-treatment. This H_2O_2 concentration was chosen because it did not significantly increase apoptosis frequencies in fibroblasts (Supporting Information Fig. S1). None of the α or β subunits of the 20S core proteasome showed any change in protein levels following genotoxic stress (Fig. 1 and data not shown). In contrast, various 19S subunits (Rpt2 and Rpt6 ATPases and Rpn7, Rpn11, and Rpn12 non-ATPases) demonstrated increased protein levels

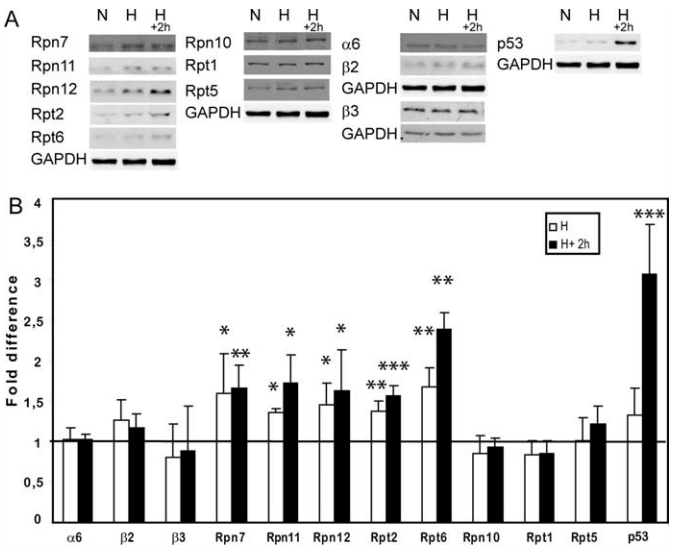

Figure 1. Specific 19S proteasome subunits are upregulated following genotoxic stress. (A) Protein expression levels and (B) quantification of immunoblot data from three independent experiments of 19S and 20S representative subunits in HFL1 primary human fibroblasts treated with 400 μM H_2O_2 for 30 Min (H) and after a 2 H recovery (H + 2 H). N denotes nontreated cells. p53 was used as a marker of DDR. GAPDH was used as a loading control. Expression levels of each subunit in nontreated control cells was arbitrarily set to 1 and shown as a horizontal line. Data are mean $\pm$ SD. Single, double, and triple asterisks denote P values <0.05, <0.01, and <0.001, respectively).

immediately after H_2O_2 treatment, that is, even before a significant stabilization of p53 that was used as a marker of the DDR. Protein levels of Rpt2, Rpt6, Rpn7, Rpn11, and Rpn12 19S subunits remained elevated for at least 2 H (Fig. 1). In contrast, other tested 19S subunits (Rpt1, Rpt5, and Rpn10) did not demonstrate any significant alteration when cells were genotoxically stressed (Figs. 1A and 1B). These data suggest distinct regulation of, and pinpoint potentially distinct roles for, individual 19S subunits.

To further investigate the possible distinct roles of proteasome subunits in the DDR, we examined the subcellular localization patterns of 19S and 20S proteasome subunits. Importantly, all upregulated 19S subunits (Rpn7, Rpn11, Rpn12, Rpt2, and Rpt6) translocated into the nucleus following genotoxic stress (Figs. 2A and 2B). On the contrary, the nonupregulated 19S subunits Rpt1, Rpt5, and Rpn10 or 20S subunits (β2, β3, and α6) did not show any difference in their cellular localization upon H_2O_2 treatment (Figs. 2C-a and 2C-b). Interestingly, Rpn7 and Rpn11 formed discrete foci, which demonstrated a distinct colocalization with γH2AX, a major component of DNA damage foci and a robust marker of DNA damage (*36*) (Fig. 2A). Rpn7 and Rpn11 colocalization with DNA damage foci was also evident when cells were subjected to

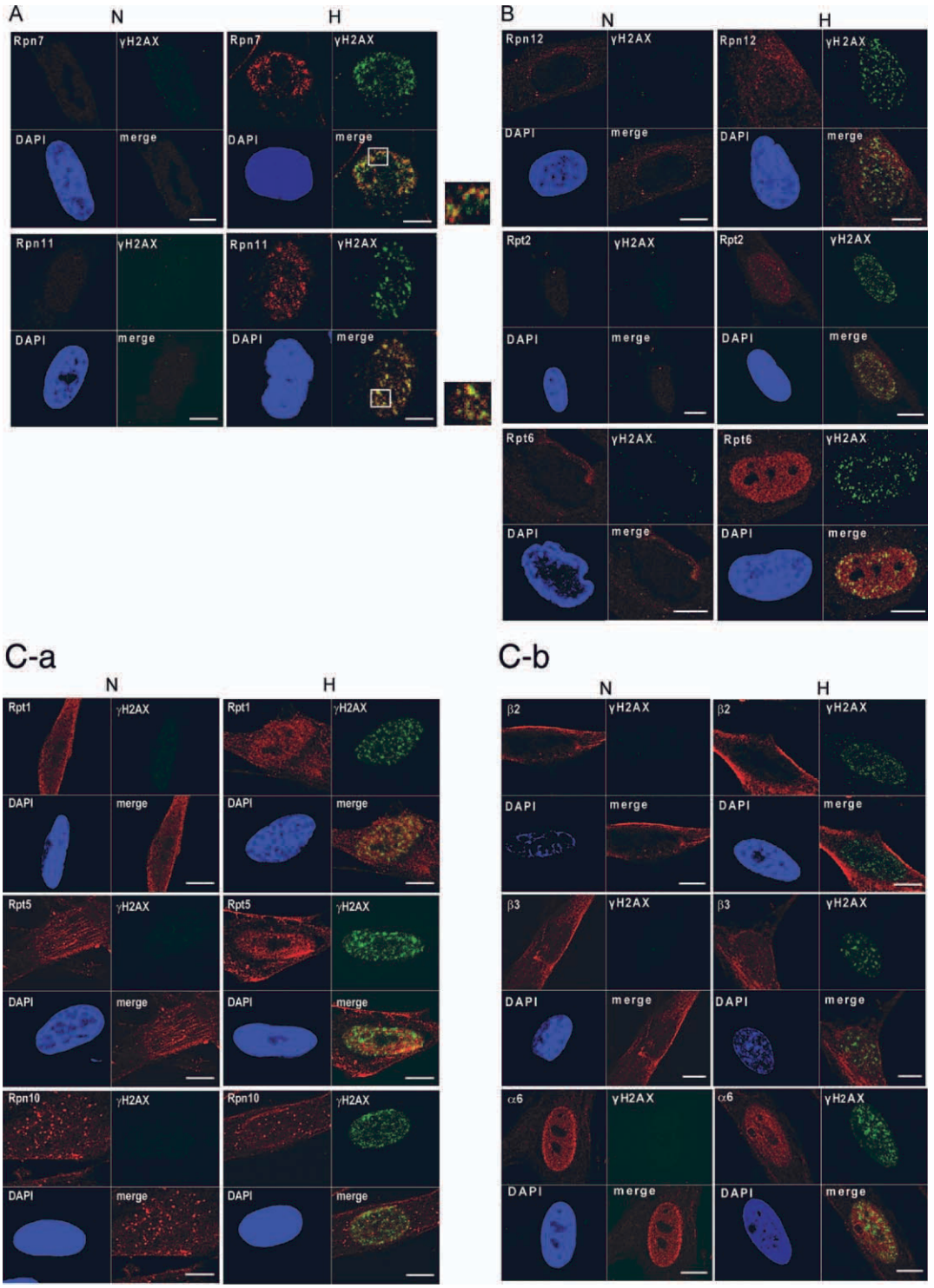

Figure 2. Nuclear translocation of specific 19S proteasome subunits following genotoxic stress. (A, B, and C) Localization of 19S and 20S proteasome subunits in HFL1 primary human fibroblasts treated with 400 μM H_2O_2 for 30 Min (H). N denotes nontreated cells. Samples were stained for γH2AX and the indicated proteasome subunit. Scale bar denotes 10 μm. Representative images of (A) Rpn7 and Rpn11 subunits' nuclear translocation and colocalization with γH2AX, (B) Rpn12, Rpt2, and Rpt6 subunits' nuclear translocation without colocalization with γH2AX and (Ca) Rpt1, Rpt5, and Rpn10, (Cb) β2, β3, and α6 subunits that do not translocate following H_2O_2 treatment. Small panels in (A) represent higher magnification that shows focal colozalization. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

etoposide treatment (Supporting Information Fig. S2A), a drug that causes double-strand breaks (*37*). Moreover, same localization patterns were recorded after exposure of MRC5 human fibroblasts to either etoposide or oxidative stress, verifying that these interactions are not cell line or stressor specific (Supporting Information Fig. S2B).

As validation for our immunofluorescent confocal results, we performed Western blots against nuclear and cytoplasmic lysate fractions of 19S and 20S proteasome subunits. In accordance with our microscopy, Rpn11 nuclear fractions showed a significant increase upon H_2O_2 treatment, whereas its cytoplasmic fraction demonstrated decreased protein levels. The same observation was applied to Rpt2; however, for the 20S subunits tested (beta3 and alpha6), none showed a statistically significant difference in either nuclear or cytoplasmic fractions upon H_2O_2 (Supporting Information Figs. S3A and S3B). Together, these data show that 19S and 20S subunits behave differentially during the DDR. Finally, to validate the colocalization of Rpn7 with DNA damage, we also tested for p53 pull down with Rpn7 and found a significant increase in immunoprecipitation only in treated cells (Supporting Information Fig. S3C).

Rpn7 and Rpn11 Colocalize With DNA Damage Foci Over Their Whole Lifespan

We then analyzed Rpn7 and Rpn11 localization upon DNA damage repair and foci resolution. As shown in Fig. 3A, 48 H after exposure to H_2O_2 (Fig. 3A, t = 48 H), most of the DNA damage was repaired, as depicted by the disappearance of most of γH2AX foci; Rpn7 and Rpn11 foci were also resolved upon DNA damage repair. Interestingly, the few remaining γH2AX foci were still associated with Rpn7 and Rpn11, thus verifying the colocalization of γH2AX with Rpn7/Rpn11 both before and after genotoxic stress (Fig. 3B and data not shown). The proportion of cells positive for either γH2AX or Rpn7/Rpn11 was always similar to one another, before, during and after stress (Fig. 3B). Thus, kinetic changes in the frequencies of Rpn7/Rpn11-positive cells followed DNA damage and repair kinetics.

It is well established that proteasome inhibition suppresses DNA damage repair (*25*). To test whether proteasome inhibition also affects the recruitment of Rpn7 and Rpn11 on DDR foci, HFL1 cells were pretreated with the proteasome inhibitor MG132 (*38*), followed by incubation with 400 μM H_2O_2. Proteasome inhibition did not affect the induction of DDR, as DNA damage foci containing γH2AX and 53BP1 were formed with similar kinetics and frequency as in cells with active proteasomes, and both Rpn7 and Rpn11 were recruited to foci immediately after DNA damage (Fig. 4A). Moreover, proteasome inhibition *per se* (by MG132, MG262, and epoxomicin) did not induce any significant increase in DNA damage foci formation, in the absence of additional genotoxic stress (Fig. 4B). A slight increase can be explained by the stabilization of the few foci present in cells under basal conditions. As expected, continuous proteasome inhibition through MG132 treatment with parallel

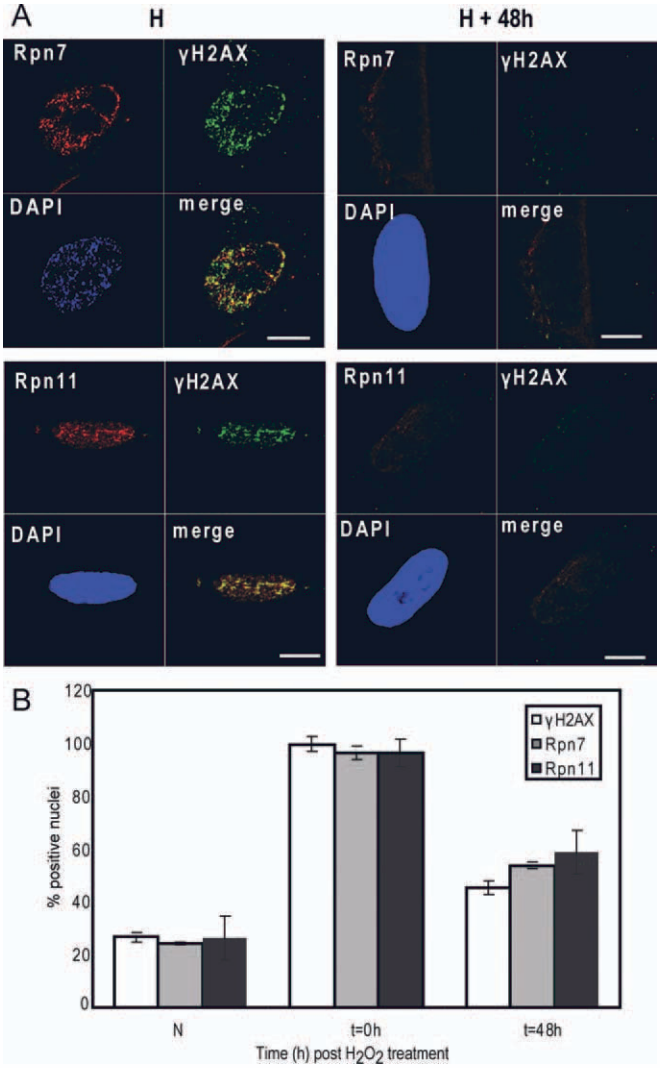

Figure 3. Spatial and temporal correlation of Rpn7 and Rpn11 with DNA damage foci. (A) Representative images of concomitant recruitment and removal of Rpn7 or Rpn11 subunits and γH2AX upon DNA damage and repair in HFL1 primary human fibroblasts treated with 400 μM H_2O_2 for 30 Min (H) and following 48 H recovery (H + 48 H). DAPI was used as nuclear marker. Scale bar denotes 10 μm. (B) Quantification of the percentage of positive nuclei for γH2AX and Rpn7/Rpn11 post-H_2O_2 treatment at the indicated time points from three independent experiments. N denotes nontreated cells. For each treatment, at least 300 cell nuclei were counted in 30–40 randomly chosen fields. Data are mean ± SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

administration of genotoxic stress blocked the resolution of DNA damage foci (Fig. 4C-a); approximately 85% of the nuclei remained positive for γH2AX foci 48 H post-H_2O_2 treatment (Fig. 4C-b). Importantly, both Rpn7 and Rpn11 also persisted on DDR foci under conditions of repair inhibition.

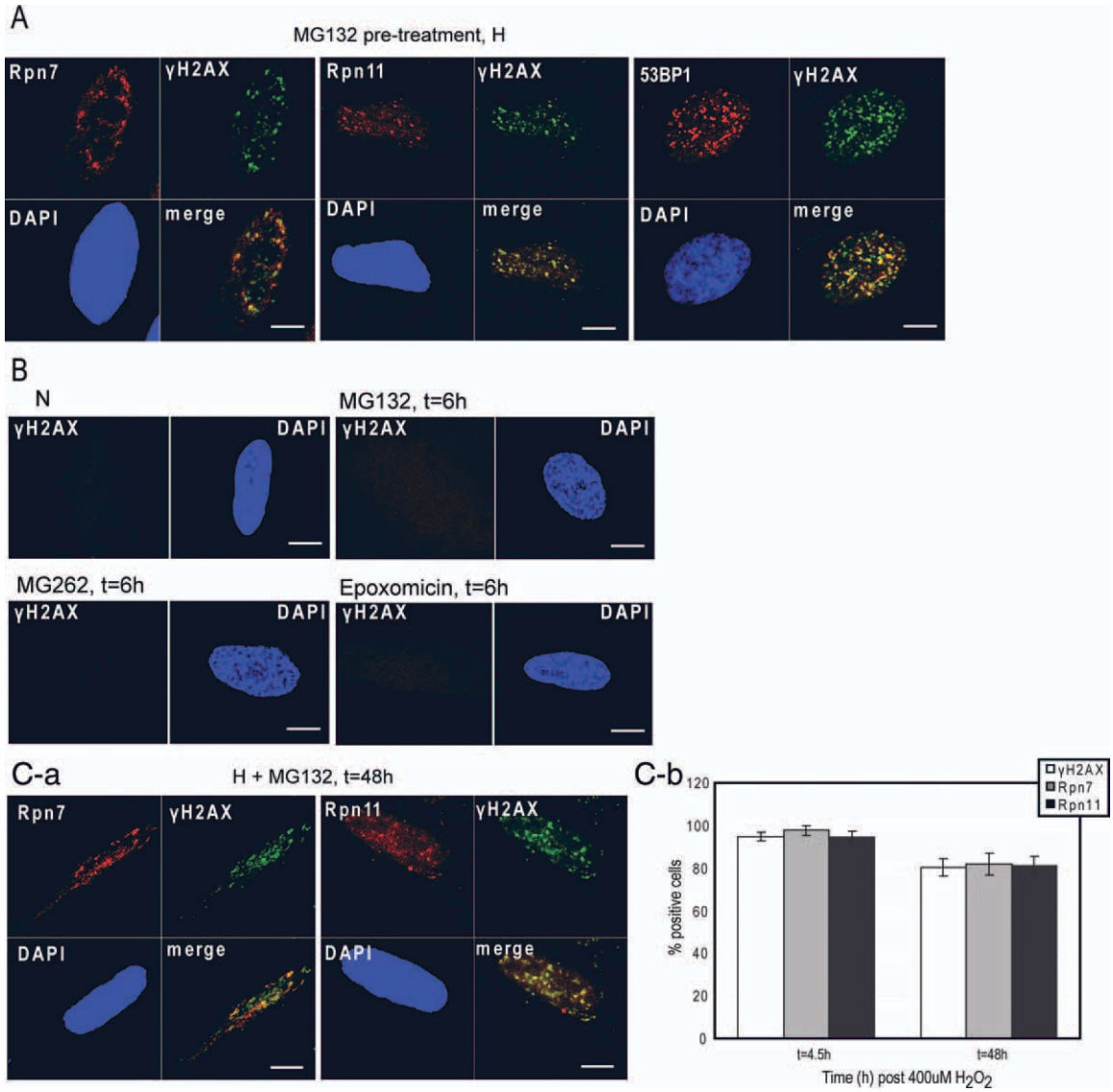

Figure 4. Proteasome inhibition affects DNA damage repair but not DNA damage recognition. (A) Representative images of DNA damage foci containing γH2AX, 53BP1, Rpn7, and Rpn11 in HFL1 fibroblasts pretreated with 10 μM MG132 for 1 H and then subjected to genotoxic stress with 400 μM H_2O_2 for 30 Min (H). (B) Representative images of γH2AX staining in HFL1 fibroblasts treated with the indicated proteasome inhibitors showing that proteasome inhibition does not cause a DDR *per se*. (C-a) Representative images of DNA damage foci containing γH2AX, Rpn7, and Rpn11 in HFL1 fibroblasts pretreated with 10 μM MG132 for 1 H, subjected to genotoxic stress in the presence of the inhibitor with 400 μM H_2O_2 for 30 Min and then maintained in complete medium supplemented with 10 μM MG132 for additional 4.5 H. Cells were fixed and stained 48 H after H_2O_2 treatment. (C-b) Frequencies of immunostained cells for γH2AX, Rpn7, and Rpn11 at the indicated time points after MG132 treatment and genotoxic stress. N denotes nontreated cells. Data are mean ± SEM from three independent experiments. DAPI was used as nuclear marker. Scale bar denotes 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Again, proportions of γH2AX and Rpn7/Rpn11 positive nuclei were similar at the examined time points (Fig. 4C-b). These data show that Rpn7 and Rpn11, but not other proteasome subunits, are specifically recruited to DNA damage foci immediately upon DNA damage induction and remain there until damage is repaired and DDR foci are resolved. This suggests that Rpn7 and Rpn11 exhibit specific roles in DNA repair/foci maintenance on their own, not only as parts of the assembled 26S proteasomes.

Downregulation of Rpn7 Accelerates Turnover of DNA Damage Foci

We then investigated the specific involvement of Rpn7 and Rpn11 19S proteasome subunits on DNA damage foci turnover.

Foci stability was analyzed kinetically using DNA damage reporter MRC5-53BP1 fibroblasts in live cell imaging. These reporter cells stably express a truncated AcGFP-53BP1c fusion protein, which reports quantitatively on the localization and kinetics of DNA damage foci (*33*). MRC5-53BP1 cells responded similarly to HFL1 fibroblasts upon H_2O_2 and X-ray treatment (Supporting Information Fig. S2B and data not shown). To test the effect of each subunit on foci turnover, Rpn7, Rpn11, or the 20S catalytic subunit β1 expression was silenced by specific siRNA transfection (Supporting Information Fig. S4). After 48 H, cells were irradiated with 5 Gy X-ray to induce DNA damage and were continuously visualized for the next 40 H by confocal live cell imaging. Although the kinetics of foci induction and the maximum number of foci/cell were not affected by any of the siRNA treatments, siRNA specifically directed against Rpn7 resulted in significantly faster resolution of 53BP1 foci (Fig. 5A). The time to repair 50% of the maximum induced damage ($t_{1/2}$) varied between 12 and 13 H for controls [no siRNA treatment (N) or siscr] and was hardly reduced to approximately 10.2 and 11.2 H by silencing either β1 or Rpn11 subunits, respectively. On the contrary, $t_{1/2}$ was significantly decreased to ~7 H following Rpn7 silencing (Fig. 5B), leading to significantly lower foci numbers at the end of the experiment (Fig. 5A). The effect of subunit silencing on DNA damage foci turnover was not due to an increase in proteasomal activity (data not shown) but appeared to be a specific effect of Rpn7 interaction with DNA damage foci.

DNA damage foci are highly dynamic structures that get resolved within few hours. If DNA damage cannot be repaired some DNA damage foci become persistent. Persistent foci are larger, associate with PML bodies and possibly additional proteins, and are believed to trigger cellular senescence (*39*). As we reported earlier, live cell imaging allows us to measure the lifespans of individual foci in multiple nuclei (*33*). Here, we generated Kaplan–Meier survival curves for the individual foci, which showed that the main effect of Rpn7 silencing was not simply an acceleration of DNA damage foci turnover (Fig. 6A). Specifically, foci lifespan in irradiated control cells (treated with siscr) was very similar to that described before in senescent cells (*40*); about half of all foci were short-lived with lifespan below 7 H, while the other half became stabilized, persistent foci with lifespan of around 30 H and more (Figs. 6A and 6B). As expected, proteasome inhibition resulted in almost all foci becoming persistent as damage repair was hindered (Fig. 6A). Interestingly, Cox regression analysis of the Kaplan–Meier curves showed that Rpn7 silencing significantly shortened mean and median foci lifespan and did not greatly change the slope of the survival curve for short-lived foci. However, Rpn7 silencing significantly reduced the frequencies of long-lived foci to less than 20% of all foci, whether calculated as total foci frequencies (Fig. 6A) or mean foci frequencies per cell (Fig. 6B). These results suggest that interaction of Rpn7 with DNA damage foci might be necessary to stabilize DNA damage foci at lesions that cannot immediately be repaired. Fewer persistent DNA damage foci are formed in irradiated cells in the absence of Rpn7 than normal, indicating that induction of the senescent phenotype might be compromised in cells lacking Rpn7. In accordance, knocking-down of Rpn7 did not induce SA-β-gal activity or an increase in p16 expression levels, while silencing of Rpn11 induced cellular senescence (Figs. 6C-a and 6C-b), consistent with previously published data (*41*).

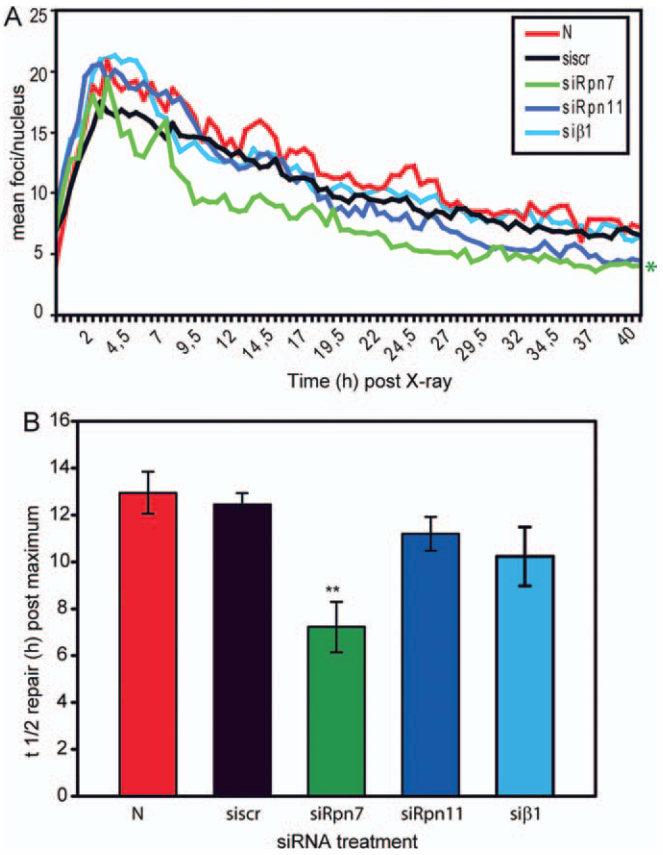

Figure 5. Downregulation of Rpn7 accelerates resolution of 53BP1 foci following genotoxic damage. (A) Average frequencies of 53BP1 foci/nucleus versus time after irradiation in MRC5-53BP1 reporter fibroblasts treated with 50 nM siRNA against Rpn7, Rpn11, β1, or siscr for 48 H before induction of DNA damage with 5 Gy X-ray irradiation. Cells were imaged for 40 consecutive hours after irradiation and 53BP1 foci frequencies were measured every 10 Min. N denotes nontreated cells. Data are means from 30 to 60 nuclei per condition. Asterisk denotes statistically significant difference of siRpn7-treated cells ($P < 0.05$) at the end of the 40 H time course, compared to both untreated and siscr controls (ANOVA with post hoc Tukey). (B) Graph represents time (H) required to repair 50% of the maximum induced damage ($t_{1/2}$) following 5 Gy X-ray in cells pretreated with the indicated siRNAs. Data are mean ± SEM calculated from exponential decay curves fitted to the data shown in (A) from maximal response. Asterisks denote a significant difference ($P < 0.01$) to the siscr and nontreated control (ANOVA, Tukey). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

Despite the high importance of DNA and protein macromolecular maintenance systems for the manifestation of the senescence phenotype, their cross-talk and their possible age-dependent alterations upon the progression of the phenomenon is still elusive. In this study, we aimed to elucidate these interactions by focusing and extensively analyzing the emerging role of specific proteasome components in the DDR. We reveal that Rpn7 proteasome subunit is necessary for the stabilization of DNA damage foci and the establishment of cellular senescence following genotoxic insult.

First, we have demonstrated by confocal immunofluorescence and subcellular fractionation experiments that the components of the two 26S complexes, 19S and 20S are discretely localized upon genotoxic insult. Specifically, as shown by immunofluorescence (γH2AX) and immunoprecipitation (p53; Supporting Information Fig. S3C), particular subunits of the 19S complex appear to colocalize to sites of DNA damage. The differences in the localization patterns of the 19S subunits as compared to the 20S subunits do not support the notion of a proteasomal related function upon DNA damage foci formation. On the contrary, a specific 19S related function is suggested here. In accordance to that, others have shown that DSS1 and Rpn7, two 19S subunits, interact with BRCA2, which is involved in the DNA double-strand break repair (*23*). Rpn11 and Rpn7 are two 19S lid subunits, with the former functioning as a deubiquitinating enzyme (*42*), whereas no functional role has yet been attributed to the latter. Although proteasome inhibition has been shown to completely block foci resolution after DNA damage, in consistence with previous reports (*25*), Rpn7 and Rpn11 silencing have produced totally different effects on foci resolution. Both Rpn7 and Rpn11 were found to associate

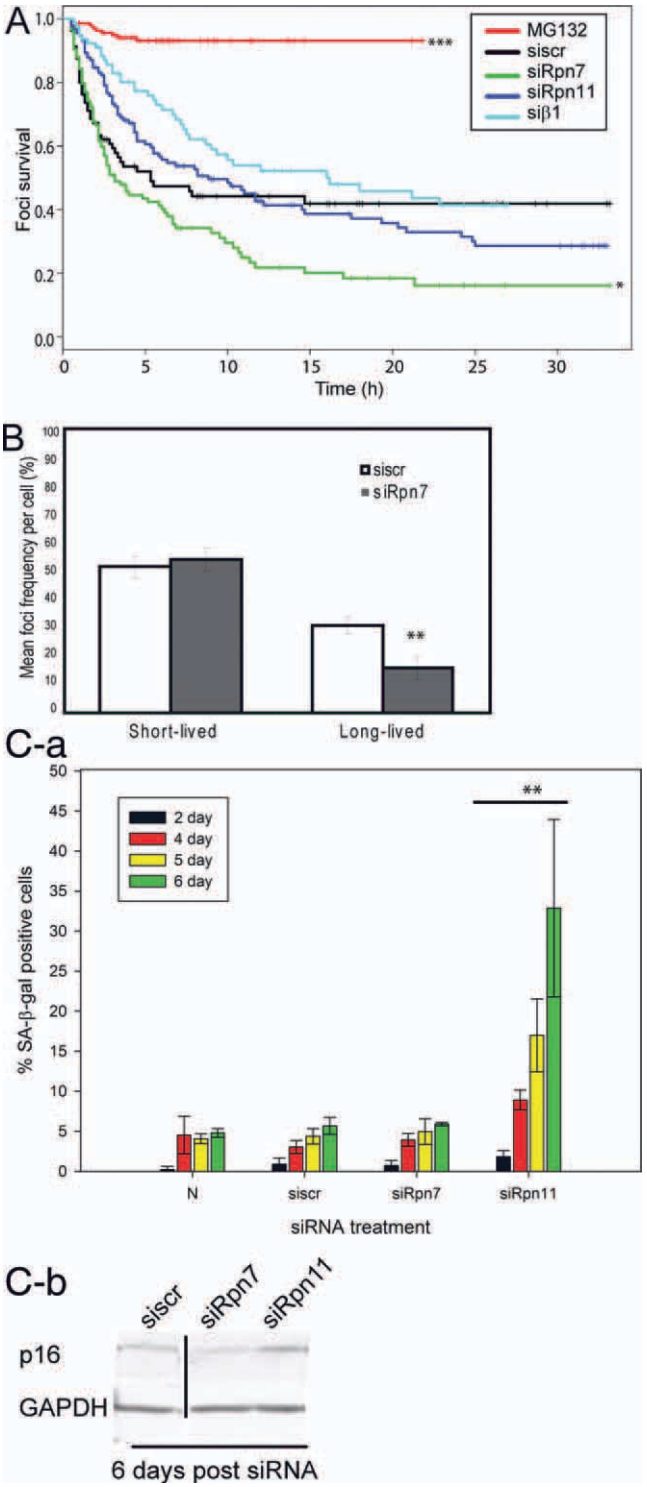

Figure 6. Downregulation of Rpn7 inhibits generation of "persistent" DNA damage foci. (A) Kaplan–Meier curves generated from at least 79 foci per treatment in MRC5-53BP1 cells treated with 50 nM siRNA against Rpn7, Rpn11, β1, or siscr for 48 H, followed by 5 Gy X-ray or treated for 6 H with 10 μM MG132 and immediately imaged. Each individual 53BP1 focus was tracked for up to 37 H, starting 10 H post-irradiation to determine its lifespan. Single, and triple asterisks denote significant differences of P values <0.05 and <0.001, respectively in Cox regression (Breslow method) against siscr control. (B) Numbers of foci per cell were calculated relative to their lifespan over the 37 H. Foci with lifespan less than 15 H and those with lifespan more than 25 H are considered as short-lived and long-lived, respectively. Only long-lived foci were significantly decreased by siRpn7 ($P < 0.01$), denoted by a double asterisk (ANOVA, Tukey). (C-a) Graph demonstrating percentage (%) of SA-β-gal positive cells at various time points post-siRNA treatment with the indicated siRNAs. Data are mean $\pm$ SEM from three independent experiments. Asterisks denote significant differences ($P < 0.01$) of siRpn11 treated cells compared to both nontreated and siscr (two-way ANOVA with post hoc Tukey). (C-b) p16 protein expression levels of the above (C-a) samples 6 days post-siRNA treatment. GAPDH was used as a loading control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with DNA damage foci. However, Rpn11 silencing did not affect foci frequencies or repair kinetics, while Rpn7 silencing reduced the rate of long-lived foci formation, without affecting the repair kinetics of short-lived DNA damage foci. In fact, Rpn7 silencing completely rescued the induction of persistent long-lived foci by genotoxic damage, resulting in levels that were similar to the ones found in young, proliferating cultures (*40*). These results attribute a functional role of Rpn7 on the stabilization of DNA damage foci and its preventive action against premature foci resolution.

The lifespan of DNA damage foci has been linked to cellular senescence (*39, 40*). Using kinetic single cell data combined with stochastic modeling, we have previously demonstrated that the persistence of 4–6 DNA damage foci over more than 8–12 H is necessary to maintain stable proliferation arrest and allow the development of the senescent phenotype in human fibroblasts after genotoxic stress (*40*). This is in accordance with the suggestion that persistent foci are more potent inducers of senescence than short-lived ones (*43*). Our observed differences in foci survival following Rpn7 or Rpn11 silencing led us to further investigate their possible involvement in senescence induction. We found that knockdown of Rpn11 without additional DNA damage was sufficient to induce senescence in human fibroblasts. This was in accordance with previous studies showing that Rpn11 knockdown increased the premature age-dependent accumulation of ubiquitylated proteins, inhibited cell growth, and resulted in cell-cycle arrest and senescence (*41*). Moreover, RNAi-mediated knockdown of Rpn11 in *Drosophila melanogaster* shortened lifespan and enhanced neurodegenerative phenotype (*44*). In our hands, knockdown of Rpn7 did not induce cellular senescence; on the contrary, it promoted the accelerated resolution of long-lived foci, thus preventing the establishment of the senescence phenotype. Therefore, a distinct role of Rpn7 regarding development of senescence is suggested.

As stated, Rpn7 has been previously implicated with the DDR, through its molecular interaction and binding with BRCA2 (*23*). Deficiency of BRCA2 results in a defect in gene conversion, the Rad51-dependent error-free subpathway of homologous recombination and a concomitant increase in the use of alternative, more error-prone repair mechanisms such as single-strand annealing (*31*). Interestingly, inhibition of the proteasome catalytic activity induced a similar shift to error-prone repair mechanisms in human cells (*23*), which might be associated to the induction of premature senescence. However, it has not been shown whether Rpn7 itself affects the choice of DNA double-strand break repair pathways or whether this choice influences the onset of cellular senescence. Recently, it has been shown that persistent foci differ from short-lived ones by the lack of Rad51 (*39*). BRCA2 is also known to be loosely associated with DNA damage foci (*45*), but it is still elusive whether it is differentially associated with short- versus long-lived foci. Our data show that Rpn7 facilitates the formation of persistent foci. An interesting speculation would be that Rpn7 could facilitate the formation of persistent DNA damage foci and, thus, stabilize the senescent response to genotoxic DNA damage, via a direct or indirect interaction with BRCA2 and by possibly affecting the choice of double-strand break repair pathways.

In conclusion, this study provides evidence regarding an additional molecular link between the DDR and the proteasome, thus, unraveling the intriguing interplay between different cellular defense mechanisms. Moreover, it reveals the possible link between this cross-talk and the development and establishment of the senescence phenotype, therefore, paving the way to possible antiaging strategies through manipulation of specific proteasome subunits.

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