RNF8-dependent and RNF8-independent Regulation of 53BP1 in Response to DNA Damage

Ryo Sakasai and Randal Tibbetts
From the Department of Pharmacology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin 53706

The DNA damage surveillance network orchestrates cellular responses to DNA damage through the recruitment of DNA damage-signaling molecules to DNA damage sites and the concomitant activation of protein phosphorylation cascades controlled by the ATM (ataxia-telangiectasia-mutated) and ATR (ATM-Rad3-related) kinases. Activation of ATM/ATR triggers cell cycle checkpoint activation and adaptive responses to DNA damage. Recent studies suggest that protein ubiquitylation or degradation plays an important role in the DNA damage response. In this study, we examined the potential role of the proteasome in checkpoint activation and ATM/ATR signaling in response to UV light-induced DNA damage. HeLa cells treated with the proteasome inhibitor MG-132 showed delayed phosphorylation of ATM substrates in response to UV light. UV light-induced phosphorylation of 53BP1, as well as its recruitment to DNA damage foci, was strongly suppressed by proteasome inhibition, whereas the recruitment of upstream regulators of 53BP1, including MDC1 and H2AX, was unaffected. The ubiquitin-protein isopeptide ligase RNF8 was critical for 53BP1 focus targeting and phosphorylation in ionizing radiation-damaged cells, whereas UV light-induced 53BP1 phosphorylation and targeting exhibited partial dependence on RNF8 and the ubiquitin-conjugating enzyme UBC13. Suppression of RNF8 or UBC13 also led to subtle defects in UV light-induced G2/M checkpoint activation. These findings are consistent with a model in which RNF8 ubiquitylation pathways are essential for 53BP1 regulation in response to ionizing radiation, whereas RNF8-independent pathways contribute to 53BP1 targeting and phosphorylation in response to UV light and potentially other forms of DNA replication stress.

DNA-damaging stimuli elicit highly conserved responses in eukaryotic cells that are required for the maintenance of genomic integrity and organismal viability. Protein kinase cascades are central to the DNA damage-signaling paradigm, and members of the phosphoinositide 3-kinase-related kinase gene superfamily play particularly important roles as apical DNA damage response regulators. Two functionally related members of the phosphoinositide 3-kinase-related kinase family, the ATM (ataxia-telangiectasia-mutated) and ATR (ATM-Rad3-related) kinases, initiate overlapping signaling pathways in response to distinct types of genetic lesions. ATM is principally activated by ionizing radiation (IR) and other agents that induce DNA double-strand breaks, whereas ATR responds to pauses in DNA replication that transiently expose single-strand DNA. Once activated, ATM and ATR phosphorylate and activate the CHK2 and CHK1 protein kinases, respectively, which in turn promote cell cycle checkpoint arrest through inhibition of CDC25 family phosphatases (2–4). ATM and ATR phosphorylate numerous other substrates, including p53 and BRCA1, which participate in DNA metabolism. The regulatory reach of ATM and ATR extends to many other physiologic processes, and recent studies suggest that the true number of ATM substrates may number several hundred (5).

The checkpoint-signaling activities of ATM and ATR are strongly influenced by a group of proteins collectively referred to as checkpoint “mediators,” including MDC1 (mediator of DNA damage checkpoint 1) and 53BP1 (p53-binding protein 1), that are recruited to DNA damage sites and may function in a scaffolding capacity (6). More recently, an amplification loop involving ATM, histone H2AX, and MDC1 has been described that sustains ATM activity in response to a transient damage insult. In this model, ATM phosphorylates the carboxyl terminus of the variant histone H2AX, which binds to the BRCT domain of MDC1 (7–9). H2AX-bound MDC1 recruits additional ATM molecules, which stimulate H2AX phosphorylation, establishing a feed-forward activation cycle in which activated ATM proteins spread from the initial break site.

53BP1 is a BRCT domain-containing protein that is rapidly recruited to DNA damage sites (10). The recruitment of 53BP1 requires a Tudor fold that associates with methylated histones H3 and H4 (11, 12). In addition, 53BP1 associates with phosphorylated Ser-139 of H2AX at DNA damage sites (13, 14). 53BP1-deficient cells exhibit S- and G2/M-phase checkpoint defects (15) and defects in double-strand break repair through non-homologous end joining (16, 17), whereas 53BP1-deficient mice exhibit radiation sensitivity, tumor predisposition, and defects in immunoglobulin heavy chain class-switch recombination (18–20). 53BP1 is also phosphorylated by ATM and ATR (21, 22), which may stimulate 53BP1-mediated repair (23). The picture that has emerged from these findings is that 53BP1 functions in a scaffold capacity to concentrate DNA damage response regulators at DNA damage sites.
Proteasome Regulation of 53BP1

In this study, we set out to define the early events that contribute to the DNA damage response, with emphasis on the potential contributions of the proteasome. We show that proteasome activity is critically important for phosphorylation and targeting of 53BP1 to damage sites in response to DNA replication stress and that participation of the UBC13-RNF8 ubiquitylation pathway partially accounts for the antagonistic effects of proteasome inhibitors on 53BP1-dependent cellular events.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatment—HELa and U-2 OS cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. A JL Shepherd Model JL-109 irradiator with a 137Cs source was used for irradiation. For UV irradiation, cells were irradiated without medium using a UVP CL-1000 ultraviolet cross-linker. Hydroxyurea (HUR) was dissolved in water at 1 m and used at a final concentration of 2 mM. Camptothecin (CPT) was dissolved in DMSO at 10 mM and used at a final concentration of 2 μM. MG-132 and N-acetyl-Leu-Leu-Nle-CHO dissolved in DMSO at 10 mM (Calbiochem) were added to the medium at a final concentration of 50 μM 1 h prior to DNA damage. Bortezomib (commercially obtained from Millennium Pharmaceuticals, Inc. (Cambridge, MA) for experimental purposes) was dissolved in water at a concentration of 100 μM and used at a final concentration of 100 μM. KU-55933 (Sigma) was dissolved in DMSO at 10 mM and used at a concentration of 10 μM. For UBC13 knockdown, siRNA against UBC13 (24) was transfected as described (25). For RNF8 knockdown, pooled siRNAs against RNF8 (Dharmacon) were used.

Reverse Transcription-PCR—Total RNA was extracted from siRNA-transfected cells with RNeasy (Qiagen) and reverse-transcribed using an iScript™ cDNA synthesis kit (Bio-Rad). PCR was performed using GoTaq polymerase (Promega) with the following primers: UBC13, 5'-ATG GCC GGG CTG CCC-3’ and 5'-GGC ATA TAG CCT AGT CCA TGC-3’; RNF8, 5’-GAA GTG GCC AGT ACA CCC-3’ and 5’-AGG AGC AGA AAC TGT GGG C-3’; and β-actin, 5’-TAC CAC TGG CAT CGT GAT GG-3’ and 5’-TCC TTC TGC ATC CTG TCG G-3’ (26).

Immunoblotting—Cells were lysed with SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 5% 2-mercaptoethanol, and bromphenol blue) and separated by SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membrane (Millipore), and the membrane was blocked with 5% nonfat dry milk in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 supplemented with 0.05% sodium azide. The membrane was incubated with primary antibodies against phospho-ATM (S1981, catalog no. AF-1655, R&D Systems), ATM (catalog no. GTX70103, GeneTex), phospho-53BP1 (S25, catalog no. A300-652A, Bethyl Laboratories, Inc.), 53BP1 (catalog no. A300-272A, Bethyl Laboratories, Inc.), phospho-CHK2 (E68, catalog no. AF-1626, R&D Systems), CHK2 (ab8108, Abcam), phospho-CHK1 (S317, catalog no. AF-2054, R&D Systems), CHK1 (G-4, Santa Cruz Biotechnology), p53 (DO-1, Santa Cruz Biotechnology), RPA2 (Ab-3, Oncogene), and UBC13 (E11, Zymed Laboratories Inc.). After primary antibody incubation, the membranes were incubated with secondary antibodies and visualized by chemiluminescence as described (25).

Immunofluorescence Microscopy—Cells grown on 15-mm coverslips were fixed with 3.65% formaldehyde or 4% paraformaldehyde in PBS for 15 min and then permeabilized with PBS containing 0.2% Triton X-100 for 5 min. The fixed cells were incubated with primary antibodies (diluted in PBS containing 5% bovine serum albumin) specific for 53BP1 and MDC1 (kindly provided by Dr. Junjie Chen, Yale University) (27) or γH2AX (JBW301, Upstate). For bromodeoxyuridine (BrdUrd) staining, cells were treated with 20 μM BrdUrd for 30 min before fixation and treated with 2 N HCl for 20 min at 37 °C. Cells were stained with anti-BrdUrd (Ab-2, Oncogene) and anti-53BP1 antibodies, followed by secondary antibodies conjugated to fluorescein isothiocyanate or Cy3. Anti-c-Myc (9E10, Santa Cruz Biotechnology) was used for Myc-53BP1 Tudor staining as a primary antibody. The cells were stained with 4’,6-diamidino-2-phenylindole in PBS (2 μg/ml) and mounted using Vectashield (Vector Laboratories). A Carl Zeiss Axiovert 200 fluorescence microscope was used to visualize all samples. For focus quantification experiments, cells with ≥10 foci were counted as 53BP1 focus-positive cells, and the percentage was calculated among at least 200 cells by dividing the number of 53BP1 focus-positive cells by the number of 4’,6-diamidino-2-phenylindole-stained cells. The error bars represent S.E. in three independent experiments.

Flow Cytometry—For staining phosphorylated histone H3, cells were fixed with 70% ethanol 2 or 4 h following IR or UV irradiation, respectively. The fixed cells were treated with PBS containing 0.2% Triton X-100 for 5 min. Cells were then incubated with anti-phospho-histone H3 antibody (Ser-10; catalog no. 06-570, Upstate) and incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody. After propidium iodide staining, cells were analyzed using a FACSCalibur (BD Biosciences).

RESULTS AND DISCUSSION

Effects of Proteasome Inhibition on DNA Damage Signaling—To examine the effect of proteasome inhibition on the DNA damage response, we first examined the impact of proteasome inhibitors on indices of ATM and ATR signaling. HeLa cells were pretreated with the proteasome inhibitor MG-132 for 1 h before treatment with DNA-damaging agents, and then the phosphorylation of ATM/ATR downstream factors was analyzed by Western blotting. MG-132 pretreatment caused a slight reduction in the accumulation of Ser-1981-phosphorylated ATM 2 h after exposure to IR, UV light, and HU (Fig. 1A), but it did not substantially inhibit CHK2 phosphorylation of Thr-68 or CHK1 phosphorylation of Ser-317 at the 2-h time point. Although MG-132 marginally inhibited UV light-induced signaling at the 2-h time point, it had a more noticeable inhibitory effect at earlier times after UV irradiation. CHK2 phosphorylation and ATM autophosphorylation were reduced by >2-fold in UV light-irradiated, MG-132-treated cells relative to UV light-irradiated controls (Fig. 1B). However, both phospho-CHK2 and phospho-ATM levels recovered to near-normal levels 2 h after UV treatment.

13550 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 283 • NUMBER 20 • MAY 16, 2008
In contrast to the attenuating effects of MG-132 on ATM auto-
phosphorylation and CHK2 on Thr-68 phosphorylation, the
UV light- and HU-induced phosphorylation of 53BP1 at Ser-25
was almost completely abolished by MG-132 treatment (Fig. 1,
A and B). 53BP1 phosphorylation at Ser-25 caused by UV irra-
diation was dependent on ATR (supplemental Fig. S1B). How-
ever, UV light-induced CHK1 phosphorylation was not affected
by MG-132, indicating that the strong inhibitory effect of
MG-132 on 53BP1 phosphorylation is not a secondary conse-
quence of impaired S-phase progression or ATR activation.
Finally, we also showed that other proteasome inhibitors,
including bortezomib and N-acetyl-Leu-Leu-Nle-CHO, inhi-
bit UV light-induced 53BP1 phosphorylation in HeLa cells
(Fig. 1C). These results suggest that proteasome inhibitors sup-
press the activation of ATM/ATR-regulated pathways in UV
light-damaged cells and that 53BP1 phosphorylation is partic-
ularly sensitive to proteasome inhibition.

53BP1 targets to nuclear foci corresponding to DNA damage
sites in mammalian cells (22). To test the effect of MG-132 on
53BP1 focus formation, HeLa cells were pretreated with
MG-132 or vehicle for 1 h, exposed to 5 Gy of IR or 20 J/m² UV
light, and then immunostained with anti-53BP1 antibody.
MG-132 abolished UV light-induced 53BP1 focus formation
and caused a qualitative reduction in the size and intensity of
IR-induced 53BP1 foci, although the number of cells displaying
foci was reduced by only 30% (Fig. 2, A and B). 53BP1 foci
induced by HU, which causes replication fork arrest, were also
completely suppressed by MG-132 (data not shown). The ATM
inhibitor KU-55933 had no discernible effect on 53BP1 focus
formation in response to either stimulus (Fig. 2C). Thus, 53BP1
phosphorylation and targeting to nuclear foci are suppressed by
proteasome inhibition, which is most pronounced in response
to DNA replication stress.

Because UV light-induced checkpoint activation through
ATR is enhanced during S-phase (28–30), we wished to deter-
mine whether MG-132 inhibition of 53BP1 focus formation is
restricted to S-phase cells. HeLa cells were labeled with BrdUrd,
exposed to UV light, and then fixed and stained with antibodies
against 53BP1 and BrdUrd. Approximately equal numbers of
BrdUrd-positive and BrdUrd-negative cells scored positive for
53BP1 foci after UV irradiation, indicating that active DNA
replication is not required for 53BP1 focus targeting (supple-
mental Fig. S2). On the other hand, MG-132 pretreatment
caused a more pronounced reduction in 53BP1 focus formation
in BrdUrd-positive cells following IR treatment. These results
suggest that the strong inhibitory effect of MG-132 on 53BP1
focus formation in response to UV exposure is not restricted to
S-phase cells.

**MG-132 Suppresses 53BP1 Focus Formation Downstream of
H2AX and MDC1**—The focal accumulation of 53BP1 is
dependent on phosphorylated histone H2AX (γH2AX) and
another DNA damage mediator, MDC1 (7, 14). However, UV
light-induced focal targeting of both γH2AX and MDC1 was
qualitatively normal in MG-132-treated cells, suggesting that
the defect in 53BP1 focus targeting was not secondary to defects
in the focal accumulation of these DNA damage mediators (Fig.
3, A and B). We also examined the potential relationship
between MG-132-dependent inhibition of 53BP1 phosphoryl-
ation and p53, a labile protein that accumulates in response to
proteasome inhibition (31). Similar to results obtained using

**53BP1 Regulation by DNA Damage Is Impaired by MG-132**—
In contrast to the attenuating effects of MG-132 on ATM auto-
phosphorylation and CHK2 on Thr-68 phosphorylation, the

---

**FIGURE 1. DNA damage-induced phosphorylation of 53BP1 is suppressed by proteasome inhibitors.** A, HeLa cells were treated with IR (5 Gy, 1 h), UV light (20 J/m², 2 h), or HU (2 mM, 2 h) following MG-132 (50 μM, 1 h) or DMSO treatment, and cell extracts were immunoblotted with the indicated antibodies. B, HeLa cells were irradiated with UV light (20 J/m²) following MG-132 (50 μM, 1 h) or DMSO treatment, collected at the indicated time points, and ana-
yzed by Western blotting with the indicated antibodies. C, HeLa cells were pretreated with MG-132 (50 μM), N-acetyl-Leu-Leu-Nle-CHO (ALLN, 50 μM), or bortezomib (100 nm) for 1 h, 1 h, or 3 h, respectively, and incubated for 2 h after UV (20 J/m²) irradiation. Protein phosphorylation was analyzed by West-
ern blotting using appropriate phospho-specific (P) antibodies.
Proteasome Regulation of 53BP1

HeLa cells, which are functionally null for p53, 53BP1 phosphorylation (Fig. 3C) and focus targeting (supplemental Fig. S3) were inhibited in wild-type p53 U-2 OS cells in response to IR, UV light, or CPT treatment. We also observed that CPT-induced phosphorylation of the 32-kDa subunit of replication protein A (RPA2) was completely abolished in MG-132-treated cells (Fig. 3C). Amino-terminal phosphorylation of RPA2 in response to CPT is both ATR- and DNA-protein kinase-dependent (32–34) and is required for optimal S-phase checkpoint activation in mammalian cells (34). Finally, overexpression of p53 had no effect on the suppression of 53BP1 phosphorylation by MG-132 (Fig. 3D). Thus, inhibition of 53BP1 phosphorylation and inhibition of RPA2 phosphorylation represent two downstream consequences of proteasome inhibition in mammalian cells.

To gain insight into potential mechanisms of proteasome-dependent 53BP1 focus targeting, we examined the effects of MG-132 on the localization of a minimal focus-targeting fragment of 53BP1 spanning amino acids 1220–1703 (Fig. 4A) (35). This fragment of 53BP1 spans the Tudor domain, which targets 53BP1 to foci through interaction with dimethylated histone H4 (11). The focus targeting of 53BP1Tudor in UV light- and IR-treated cells was inhibited by MG-132 treatment (Fig. 4B). This finding suggests that MG-132 inhibits 53BP1 focus targeting by blocking an upstream event required for Tudor domain recruitment. Dimethylation of histone H4 at Lys-20, which recruits 53BP1 to DNA damage foci through the Tudor domain (11), was unaffected by MG-132 treatment (data not shown), indicating that proteasome inhibition does not suppress this step in the 53BP1 recruitment pathway.

UBC13 and RNF8 Are Required for Optimal 53BP1 Regulation—The MG-132 sensitivity of 53BP1 phosphorylation and targeting to nuclear foci suggested that regulated protein degradation by the ubiquitin-proteasome pathway may be a key step in the 53BP1 activation process. The ubiquitin-conjugating enzyme UBC13 participates in DNA post-replication repair of DNA damage in conjunction with Rad6 and Rad18 (36). UBC13 activity is also required for efficient homologous recombination repair of double-strand breaks in vertebrate cells (37). Most recently, it has been shown that UBC13 and the E3 ligase RNF8 are required for 53BP1 focus targeting in response to IR (24, 38–40). Thus, we tested whether UBC13 and/or RNF8 was required for 53BP1 focus targeting in response to UV light. We found that

![Image](https://example.com/image.png)

**FIGURE 2.** 53BP1 focus formation is suppressed by MG-132 treatment. A, HeLa cells were treated with MG-132 (50 μM) or DMSO for 1 h, irradiated with IR (5 Gy) or UV light (20 J/m², 2 h), and incubated for 1 or 2 h, respectively. The cells were stained with anti-53BP1 antibody. B, HeLa cells were irradiated with IR (5 Gy, 1 h) or UV light (20 J/m², 2 h) with or without MG-132 pretreatment (50 μM, 1 h). Cells were immunostained with anti-53BP1 antibody, and the percentages of 53BP1 focus-positive cells (see “Experimental Procedures”) were determined by immunofluorescence microscopy (n = 3). C, HeLa cells were treated with KU-55933 (KU; 10 μM, 1 h) or DMSO before IR or UV irradiation and incubated for 1 or 2 h, respectively. The cells were stained with anti-53BP1 antibody.
Proteasome Regulation of 53BP1

FIGURE 3. MG-132 suppresses 53BP1 focus formation downstream of H2AX and MDC1. A and B, MG-132 does not inhibit γH2AX or MDC1 focus formation. HeLa cells were irradiated with UV light (20 J/m²) with or without MG-132 (50 μM, 1 h) pretreatment and then incubated for 2 h before immunostaining with γH2AX-specific (A) or MDC1-specific (B) antibodies. C, MG-132 inhibits 53BP1 and RPA2 phosphorylation (P) in wild-type p53 U-2 OS cells. U-2 OS cells were treated with IR (5 Gy, 1 h), UV light (20 J/m², 2 h), or HU (2 mM, 2 h), or CPT (2 μM, 2 h) following MG-132 (50 μM, 1 h) or DMSO treatment. Cell extracts were immunoblotted with the indicated antibodies. The arrow indicates the phosphorylated form of RPA2. D, MG-132 inhibits 53BP1 phosphorylation in the presence of overexpressed p53. pCEP4-p53 was transfected into U-2 OS cells. Transfected or untransfected U-2 OS cells were irradiated with UV light after MG-132 (50 μM, 1 h) or DMSO treatment and incubated for 2 h. 53BP1 phosphorylation and p53 expression levels were analyzed by Western blotting.

FIGURE 4. MG-132 suppresses focus targeting of the 53BP1 Tudor domain. A, schematic diagram of human 53BP1. Bold bar depicts the minimum fragment of 53BP1, designated Myc-53BP1Tudor, that targets to DNA damage foci (41). B, HeLa cells were transfected with the Myc-53BP1Tudor expression plasmid and then irradiated in the presence or absence of MG-132 as described in the legend to Fig. 2A. After fixation and permeabilization, the cells were stained with anti-Myc antibody. Unt., untreated; DAPI, 4′,6-diamidino-2-phenylindole.

FIGURE 5. UBC13 and RNF8 are required for optimal 53BP1 regulation in response to genotoxic stress. HeLa cells were transfected with control siRNA (siCTR) or with siRNA targeted to UBC13 (siUBC13) or RNF8 (siRNF8). A, at 48 h after siRNA transfection, total mRNA was extracted, and mRNA levels of UBC13 and RNF8 were measured by reverse transcription-PCR. B and C, at 48 h following siRNA transfection, cells were exposed to UV light (20 J/m², 2 h) or mock-irradiated. Representative 53BP1 immunostaining images are shown in B. Arrowheads indicate 53BP1 focus-positive cells. The data from three separate experiments are presented in C. Unt., untreated; D, IR- or UV light-induced 53BP1 and CHK1 phosphorylation (P) was analyzed by Western blotting of extracts from cells transfected with control, UBC13, or RNF8 siRNA.

Requirements for RNF8 and UBC13 for G2/M Checkpoint Activation—We also investigated the contribution of UBC13 and RNF8 to G2/M checkpoint activation using a phospho-H3 staining assay. Transfection of HeLa cells with siRNA against RNF8 led to a modest but reproducible inhibition of IR-induced G2/M checkpoint activation, most evident at a low dose of 1.5 Gy (Fig. 6). RNF8 knockdown also caused a small defect in the UV light-induced G2/M checkpoint, and similar results were obtained using UBC13 knockdown cells (Fig. 6). On the other hand, knockdown of UBC13 did not inhibit IR-induced G2/M checkpoint activation in our hands (Fig. 6), irrespective of the dose used. Taken together, these results suggest that UBC13 and RNF8 play a modulatory role in UV light-induced G2/M checkpoint activation and that RNF8, but not UBC13, is...
Proteasome Regulation of 53BP1

**FIGURE 6. UBC13 and RNF8 modulate the G2/M checkpoint.** HeLa cells were transfected with the indicated siRNAs and incubated for 48 h. Cells were then fixed and stained with anti-phospho-histone H3 antibody. The percentage of phospho-H3-positive cells (% total) was determined using a FACSCalibur instrument. The averaged results of at least three independent experiments are shown. CTR, control.

required for the IR-induced G2/M checkpoint. Suppression of G2/M checkpoint activation by RNF8 knockdown is consistent with the phenotype of 53BP1-deficient cells, which display impaired G2/M checkpoint activation in response to a low dose of IR (13).

We have shown that two parameters of 53BP1 regulation by DNA damage, phosphorylation and focus targeting, are suppressed by inhibitors of the proteasome in mammalian cells. The inhibitory effect of MG-132 on 53BP1 phosphorylation and targeting was most pronounced in cells exposed to the DNA replication stress agents UV light and HU. In addition, the damage-induced phosphorylation of RPA2 is almost completely suppressed by proteasome inhibition, whereas the phosphorylation of CHK2, MCM3, and other damage-regulated substrates is less severely affected (Fig. 2 and data not shown).

The phosphorylation of RPA2 in response to CPT is suppressed in 53BP1-depleted cells (41), suggesting that the RPA2 phosphorylation defect in MG-132-treated cells could be a consequence of defective 53BP1 regulation. The amino-terminal phosphorylation of RPA2 at multiple sites by ATR is implicated in the down-regulation of DNA synthesis after UV light exposure (42), suggesting that the gross defect in RPA2 phosphorylation in MG-132-treated cells may lead to checkpoint deregulation. Our findings are partially congruent with a study by Jacquemont and Taniguchi (43) reporting that proteasome inhibition inhibits FANCDD2 monoubiquitination and focus formation of BRCA1 and 53BP1 after IR. In our hands, MG-132 partially inhibited 53BP1 foci in response to IR, whereas a stronger effect was seen in cells exposed to the canonical ATR stimuli UV light and HU (Figs. 1 and 2 and data not shown). Focal targeting of γH2AX and MDC1 in response to UV light was not grossly affected by MG-132 in our hands.

Our data implicate the ubiquitin-conjugating enzyme UBC13 and E3 ligase RNF8 as components of the 53BP1 activation pathway in DNA-damaged cells (Fig. 5). UBC13 mediates Rad6/Rad18-dependent post-replication repair in *Saccharomyces cerevisiae* and human (36, 44). UBC13-deficient cells are also defective in homologous recombination-mediated repair of double-strand breaks (37). In addition, Kolas et al. (38) and three other studies (24, 39, 40) have shown that UBC13 and RNF8 are essential regulators of IR-induced 53BP1 focus formation. We confirmed the absolute requirement of RNF8 for 53BP1 focus targeting and additionally showed that IR-induced 53BP1 phosphorylation depends on RNF8. Defective 53BP1 phosphorylation and targeting in RNF8-depleted cells correlated with reduced G2/M checkpoint activation. The dependence of both 53BP1 phosphorylation and targeting on RNF8 is interesting given the fact that the two events are thought to occur independently (14). RNF8 interacts with phosphorylated MDC1 at DNA damage foci through the RNF8 forkhead-associated domain and promotes the ubiquitylation of histones H2A and H2AX (24, 39), which may mediate the recruitment of 53BP1. Although we can only speculate, it is possible that inhibition of RNF8 blocks a distinct ubiquitylation step required for 53BP1 phosphorylation.

The UV light-induced phosphorylation and targeting of 53BP1 occurs by a mechanism that is at least partially independent of RNF8. In side-by-side experiments, transfection with RNF8 siRNA nearly abolished IR-induced 53BP1 foci but caused only a 2-fold reduction in the number of HeLa cells displaying ≥10 foci in response to UV light. On the other hand, MG-132 abolished UV light-induced 53BP1 foci, but not IR-induced 53BP1 foci, and the inhibition of UV light-induced foci by MG-132 occurred irrespective of whether or not the cells were in S-phase at the time of irradiation (supplemental Fig. S2). A similar discrepancy between MG-132 and RNF8 was observed when measuring UV light-induced 53BP1 phosphorylation. Although there is a number of potential explanations for these complex results, the findings are consistent with a model in which protein degradation, as opposed to non-degradative protein ubiquitylation, is important for the UV light-induced 53BP1 response. In this scenario, RNF8 and potentially other E3 ligases may contribute to UV light-induced 53BP1 activation through proteasome-dependent degradation of specific factors. RNA interference experiments showed that UBC13 also contributes to UV light-induced 53BP1 phosphorylation and targeting (Fig. 5). Thus, it appears that UBC13 and RNF8 work together to promote UV light-induced 53BP1 phosphorylation and targeting, albeit to a lesser extent than observed under IR conditions.

Proteasome inhibitors have been studied as effective reagents for cancer therapy and are now approved for second-line treatment of mantle cell lymphoma and multiple myeloma (45–47). The finding that pathways leading to 53BP1 and RPA2 phosphorylation are antagonized by proteasome inhibitors could be relevant for understanding the mechanisms of cell killing by bortezomib and other proteasome inhibitors of clinical utility.

**REFERENCES**

1. Abraham, R. T. (2001) *Genes Dev.* **15**, 2177–2196
2. Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L. A., and Elledge, S. J. (2000) *Genes Dev.* **14**, 1448–1459

| pH3-positive cells (% total) | siRNA | UV | IR |
|-----------------------------|-------|----|----|
| CTR | UBC13 | 0 | 1 | 2 |
| RNF8 | 0 | 1 | 2 |

| 20 J/m² | 5 J/m² | 5 Gy | 1.5 Gy |
|---------|--------|------|--------|
| CTR | UBC13 | RNF8 | UBC13 | RNF8 |
