The Proteasome Ubiquitin Receptor hRpn13 and Its Interacting Deubiquitinating Enzyme Uch37 Are Required for Proper Cell Cycle Progression*

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Recently, we reported that bisbenzylidine piperidone RA190 adds to Cys-88 of the proteasome ubiquitin receptor hRpn13, triggering accumulation of ubiquitinated proteins and endoplasmic reticulum stress-related apoptosis in various cancer cell lines. hRpn13 contains an N-terminal pleckstrin-like receptor for ubiquitin domain that binds ubiquitin and docks it into the proteasome as well as a C-terminal deubiquitinase adaptor (DEUBAD) domain that binds the deubiquitinating enzyme Uch37. Here we report that hRpn13 and Uch37 are required for proper cell cycle progression and that their protein knockdown leads to stalling at G0/G1. Moreover, serum-starved cells display reduced hRpn13 and Uch37 protein levels with hallmarks of G0/G1 stalling and recovery to their steady-state protein levels following release from nutrient deprivation. Interestingly, loss of hRpn13 correlates with a small but statistically significant reduction in Uch37 protein levels, suggesting that hRpn13 interaction may stabilize this deubiquitinating enzyme in human cells. We also find that RA190 treatment leads to a loss of S phase, suggesting a block of DNA replication, and G2 arrest by using fluorescence-activated cell sorting. Uch37 knockdown leads to stalling at G0/G1. Moreover, serum-starved cells display reduced hRpn13 and Uch37 protein levels with hallmarks of G0/G1 stalling and recovery to their steady-state protein levels following release from nutrient deprivation. Overall, this work implicates hRpn13 and Uch37 in cell cycle progression, providing a rationale for their function in cellular proliferation and for the apoptotic effect of the hRpn13-targeting molecule RA190.

The ubiquitin-proteasome pathway is the major route for regulated proteolysis in eukaryotes, as reviewed in Refs. 1, 2. Substrates are ubiquitinated by an enzymatic cascade and ultimately delivered to the proteasome for triage, partial proteolysis, or release (3). Dysfunction in this pathway is associated with human diseases, and it has emerged as a major pharmaceutical target for cancer and neurodegenerative diseases (4). The proteasome inhibitors bortezomib (Velcade®), carfilzomib (KyprolisTM), and ixazomib (Ninlaro®) are approved to treat certain hematological cancers, and other inhibitors are in clinical trials (5–8). Bortezomib resistance and the toxicity of approved proteasome inhibitors motivates efforts to find alternative approaches to target the ubiquitin-proteasome pathway (5, 6). We recently discovered a bisbenzylidine piperidone class of molecules that restricts mouse xenograft models of multiple myeloma and ovarian cancer and covalently attaches to the proteasome ubiquitin receptor hRpn13 (9). Another group independently also identified an hRpn13-targeting molecule to restrict multiple myeloma cells (10).

hRpn13 is part of the proteasome regulatory particle (RP), which abuts the catalytic core particle (CP) at either end, as reviewed in Refs. 1, 11. Bortezomib, carfilzomib, and ixazomib target the CP. The RP houses substrate receptors, ubiquitin processing enzymes, and an ATPase ring that recognize substrates and prepares them for entry into the CP for proteolysis (reviewed in Refs. 1, 11). There are three substrate receptors in the RP that can bind to ubiquitinated substrates directly or indirectly by interacting with shuttle factors that deliver them to the proteasome (1, 12–22). The proteasome ubiquitin receptors hRpn13 (14, 15) and Rpn10 (20) recognize ubiquitinated substrates through a pleckstrin-like receptor for ubiquitin domain (14, 15) and ubiquitin-interacting motifs (18, 19), respectively. More recently, the proteasome/cyclosome repeat protein Rpn1 was found to recognize ubiquitin fold proteins with a receptor site for ubiquitin and ubiquitin-binding shuttle factors and a nearby second site for the ubiquitin-like domain of the deubiquitinating enzyme Ubp6/Usp14 (22). The intrinsically disordered protein Dss1/Sem1 is a proteasome RP component that has also been found to bind ubiquitin (23), although whether it does so at the proteasome remains to be established (22).

* This work was supported by the Intramural Research Program of the NCI, National Institutes of Health Center for Cancer Research (to K. J. W.), NCI, National Institutes of Health Grant P50 CA098252 (to R. B. S. R.), and the Alleghany Health Network-Johns Hopkins Cancer Research Fund (to R. B. S. R.). R. K. A. and R. B. S. R. are inventors of intellectual property described in this article. The terms of this arrangement are being managed by the University on an invention described in this article. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. To whom correspondence should be addressed: Protein Processing Section, Structural Biophysics Laboratory, Center for Cancer Research, NCI, Frederick, MD 21702. Tel.: 301-228-4374; Fax: 301-846-6844; E-mail: kylie.walters@nih.gov.

1 The abbreviations used are: RP, regulatory particle; CP, core particle; DEUBAD, deubiquitinase adaptor; NFRKB, nuclear factor-related k B-binding protein; SUMO, small ubiquitin-like modifier; DMSO, dimethyl sulfoxide; EdU, 5-ethynyl-2′-deoxyuridine; Bis-Tris, 2-(bis(2-hydroxyethyl) amino)-2-(hydroxymethyl)propane-1,3-diol.

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Prior to translocation into the proteasome CP, substrates are deubiquitinated by proteasome deubiquitinating enzymes, which include Rpn11 (24–26), the aforementioned Ubp6/Usp14 (27), and Uch37/UCHL5 (28), which is contributed to the proteasome by hRpn13 (29–31). When free of the proteasome, the hRpn13 DEUBAD and pleckstrin-like receptor for ubiquitin domains interact, reducing the hRpn13 affinity for ubiquitin (32). Docking of hRpn13 into the proteasome releases this intramolecular interaction, activating it for ubiquitinated substrates (32). The hRpn13 DEUBAD domain binds to a unique region of Uch37 that is C-terminal to its catalytic domain (29–31), referred to as the Uch37-like domain. This interaction activates Uch37 activity (30, 31), most likely by increasing accessibility to the Uch37 active site (33–35). Interestingly, the hRpn13 DEUBAD domain in its free form adopts an eight-helical compact structure (32) but fragments to engulf the Uch37-like domain (33–35). The Uch37-like domain also binds to the DEUBAD domain of nuclear factor-related N B-binding protein (NFRKB) from the chromatin-remodeling Ino80 complex, an interaction that sterically occludes ubiquitin accessibility to the Uch37 catalytic domain (33–35). The role of Uch37 at the Ino80 complex remains elusive, however (1, 36).

Cryo-electron microscopy studies in yeast map Rpn13 to an apical location in the proteasome (37, 38), and immunodepletion analyses suggest that not all proteasomes contain hRpn13 (29), which is reported to be at only one RP of the double-capped 26S proteasome (39) and potentially released from the RP by PSMD1 SUMOylation (40). A more recent cryo-electron microscopy study of the 26S proteasome in intact rat neurons found hRpn13 to populate only ~58% of proteasomes analyzed (41).

The gene expressing hRpn13 is amplified in ovarian (42), colorectal (43), acute leukemia (44), and metastatic gastric cancers (45), with the latter study implicating hRpn13 as promoting gastric epithelial cell proliferation. High levels of Uch37 have been found in lung, breast, ovarian, vulva, and parathyroid cancers (reviewed in Ref. 46) as well as esophageal squamous cell carcinoma (47). Uch37 has also been shown to be more highly expressed in multiple myeloma cells and, therefore, has become a therapeutic target in its own right (48, 49).

p27^{kip1} and Wee1 regulate cell cycle progression and are degraded in a phosphorylation- and ubiquitination-dependent manner (reviewed in Refs. 50–52). p27^{kip1} is a cyclin-dependent kinase inhibitor that stalls passage into S phase from G1 until its ubiquitin-mediated degradation enables cell cycle progression (53–56). Wee1 is a tyrosine kinase that inhibits mitotic entry and is degraded for advancement into M phase (57–60). Cell cycle dysregulation is a hallmark of cancer (61), and p27^{kip1} dysregulation has been postulated to be a prognostic marker of poor outcome for many human epithelial cancers (reviewed in Refs. 62, 63), including colorectal, ovarian, and breast cancer (64). We report here that hRpn13 and Uch37 are essential for proper cell cycle progression and provide insights into the cellular effects of the hRpn13-targeting bisbenzylidene piperidone RA190. We find that loss of either hRpn13 or Uch37 leads to a stabilization of the G_0/G_1 to S phase negative regulator p27^{kip1} with no change in the G_2 to M regulator Wee1. Consistent with this finding, knockdown of Uch37 by siRNA induces a significant G_0/G_1 arrest, as assayed by flow cytometry. RA190 treatment results in a block in DNA replication and G_2 arrest as well as a 2-fold increase in apoptosis. Altogether, this study demonstrates an important role for hRpn13 and Uch37 in cell cycle progression that provides mechanistic insights into their association with cellular proliferation and cancer.

Experimental Procedures

Cell Culture—HeLa cells were purchased from the American Tissue Culture Collection (ATCC) and grown at 37 °C in Eagle’s minimum essential medium supplemented with 10% FBS in a humidified atmosphere of 5% CO₂. To harvest cells, 0.25% trypsin-EDTA (Life Technologies) was used, and after washing in PBS (Life Technologies), cell pellets were either immediately lysed for subsequent experiments or stored at −80 °C.

siRNA—hRpn13 and Uch37 siRNA experiments were performed on HeLa cells using Lipofectamine® RNAiMAX transfection reagent (Life Technologies) with Opti-MEM® reduced serum medium (Life Technologies). Cells were incubated with either 5 nM (Uch37) or 10 nM (hRpn13) siRNA (Life Technologies) with incubation times of either 48 h (Uch37) or 72 h (hRpn13). NFRKB siRNA experiments were performed in a manner identical to that used in Ref. 36. Briefly, 293T cells were transfected with 100 nM ON-TARGETplus SMARTpool human NFRKB siRNA using DharmaFECT1 transfection reagent (Dharmacon/GE Healthcare) and incubated for 48 h. After 48 h, the cells were split and transfected with an additional 100 nM NFRKB siRNA and incubated for another 48 h, at which point cells were harvested and used in immunoblotting experiments. The mock control for all siRNA experiments consisted of the respective transfection reagent with no siRNA.

Plasmid DNA Transfection—Empty, siRNA-safe full-length wild-type Uch37 and siRNA-safe full-length Uch37 C88A p3xFLAG-CMV™-7.1 vectors were transfected into HeLa cells using Lipofectamine® LTX transfection reagent (Life Technologies). Cells were transfected with plasmid DNA for 5 h, after which the medium was changed and treated with Uch37 siRNA.

Immunoblotting—HeLa cells were lysed and cleared, and total protein concentration was determined via Bradford (Sigma-Aldrich) or BCA (Pierce) assay. Protein lysate (10–20 μg) was loaded onto 4–12% Bis-Tris polyacrylamide gels (Life Technologies) and subjected to SDS-PAGE. Following electrophoresis, the contents of the gel were transferred to an Invitrolon™ PVDF membrane (Life Technologies). The membrane was then blocked in 5% skim milk in Tris-buffered Saline with 0.1% Tween 20 (TBST) for 1 h, followed by primary antibody incubation. Following primary antibody incubation, the membrane was washed with TBST, incubated with HRP-conjugated secondary antibody, and washed again with TBST. Antibody signals were detected using ECL reagent (GE Life Sciences).

Cycloheximide Treatment—HeLa cells were treated with cycloheximide (Sigma-Aldrich) at 30 μg/ml and harvested at the indicated time points. Cycloheximide was dissolved in DMSO.

Serum Starvation—HeLa cells were starved for 72 h by incubating in Eagle’s minimum essential medium lacking 10% FBS.
Cells were subsequently released from starvation with the addition of Eagle's minimum essential medium containing 10% FBS and harvested at the indicated time points.

**RA190 Treatment**—Bisbenzylidine piperidone RA190 powder was resuspended in DMSO and used to treat HeLa cells at 1 μM. An equal volume of DMSO was used as the negative control.

**Cell Cycle Assay**—HeLa cells were treated as indicated and subsequently incubated with 5-ethyl-2′-deoxyuridine (EdU, Invitrogen) at 10 μM for 1 h (37 °C, 5% CO2). Cells were then harvested, fixed, and incubated with Pacific Blue™ azide according to the instructions of the manufacturer (Click-IT® EdU flow cytometry assay kit, C10418, Invitrogen) and counterstained with FxCycle™ propidium iodide/RNase staining solution (F10797, Invitrogen). Cells were analyzed by flow cytometry using a BD FACSCanto II flow cytometer (BD Biosciences). A total of four independent experiments were performed.

**Apoptosis Assay**—HeLa cells were treated as indicated, and apoptosis was measured by Annexin V-Alexa Fluor® 488 and SYTOX® AADvanced™ staining (A13201 and S10349, respectively, Invitrogen) and analyzed by flow cytometry on a BD FACSCanto II flow cytometer (BD Biosciences). A total of four independent experiments were performed.

**Statistical Analysis**—Statistical analyses were performed in Excel using either a two-tailed, two-sample equal variance Student’s t test or a paired two-tailed Student’s t test, with p values at or below 0.05 being considered significant.

**Antibodies**—The antibodies used in this study included anti-hRpn13 (PW8895, Enzo); anti-p27kip1 (04-240, Millipore); anti-PSMD2/S2 (PA-964, Pierce); anti-Uch37 and anti-Cdc25c (ab124931 and ab3244, respectively, Abcam); anti-Wee1, anti-p21Cip1, anti-S5a, and anti-β-actin (4936, 2947, 12441, and 4970, respectively, Cell Signaling Technology), anti-FLAG (F1804, Sigma); and anti-NFRAKB (A301-459A, Bethesda Laboratories Inc.).

**Results**

**RA190 Treatment Leads to a Block in DNA Replication and Cell Cycle Arrest in G2**—RA190 selectively adducts to hRpn13 Cys-88 and causes rapid accumulation of ubiquitinated proteins, unfolded protein response, and apoptosis (9). We tested whether RA190 treatment impacts the cell cycle as well as certain cell cycle regulators, including p27kip1 and Wee1. HeLa cells were treated with 1 μM RA190 or DMSO (at equal volume as a control) for 12 h and subjected to cell cycle profiling by using EdU incorporation and counterstaining with propidium iodide. Significant changes in all phases of the cell cycle were detected by FACS analysis when comparing RA190- with DMSO-treated HeLa cells (Fig. 1A). RA190 treatment shifted the cellular distribution so that the average percentage of cells in G0/G1 decreased by 6% (p = 0.045) from 54.5% to 48.5%, whereas those in G2/M increased by 7.4% (p = 0.004) from 12.3% to 19.6% over four independent experiments (Fig. 1A, right panel), suggesting a cell cycle arrest in G2. Strikingly, RA190 treatment decreased the average percentage of cells in S phase by 15.2% (p = 0.0064), reducing this population from 26.7% to 11.5% (Fig. 1A, right panel). Taken together, these results suggest that RA190 treatment induces a cell cycle arrest in G2 and an inhibition of DNA replication.

We next tested the effect of RA190 treatment on apoptosis by Annexin V staining and FACS analysis (Fig. 1B). HeLa cells were incubated with Annexin V and SYTOX® dead cell stains after a 12-h treatment with RA190 or DMSO (as a control). As expected (9), RA190 treatment induced apoptosis with an average 2-fold increase in apoptotic cells, 3.3% versus 1.5% for RA190 and DMSO, respectively (p = 0.078) (Fig. 1B, right panel).

To further evaluate the cell cycle and apoptotic effects measured by flow cytometry, we treated HeLa cells with either RA190 (1 μM) or DMSO for 12 h to assess the protein level of certain cell cycle regulators and indicators of apoptosis (Fig. 1C). Following treatment, cell lysates were resolved and immunoprobed for the cell cycle regulators p27kip1, Cdc25c, Wee1, and p21Cip1 (Fig. 1C, left panel). A noticeable reduction in p27kip1, a negative regulator of G0/G1 to S phase, supported the reduction of G0/G1 cells detected by FACS analysis (Fig. 1, A and C). In addition, a significant loss of the critical G2 to M phase phosphatase Cdc25c (p = 0.00024) provided a strong indication of cell cycle arrest in G2 (Fig. 1C). Cdc25c must dephosphorylate Cdc2/Cdk1 for mitotic entry (reviewed in Ref. 65). CDK inhibitor p21Cip1 was also significantly increased following RA190 treatment (p = 0.00005, Fig. 1C, right panel), further supporting cell cycle arrest in G2. p21Cip1 protein levels are high during G2 but must be reduced for M phase progression (66, 67). Finally, a significant loss of full-length Wee1 (molecular mass, ~71.5 kDa) with RA190 treatment (p = 0.0033) (Fig. 1C, right panel) to its cleavage product at ~64 kDa supported the induction of apoptosis (68) (Fig. 1C, left panel) observed by FACS (Fig. 1B).

**hRpn13 Knockdown Disrupts p27kip1 Turnover, Suggesting Cell Cycle Stalling in G0/G1**—hRpn13 appears to play a role in cellular proliferation, with overexpression in colorectal (43), ovarian (42), and gastric (45) cancers. We therefore hypothesized that hRpn13 may play a role in cell cycle progression and used p27kip1 and Wee1 as cell cycle markers, as described above for RA190-treated cells. We found it difficult to knock down hRpn13 protein levels in any attempted cell line, including the human papillomavirus-positive HeLa, human embryonic kidney 293T, and human lung carcinoma A549 cell lines (data not shown). The best results were obtained in HeLa cells by siRNA treatment for 72 h, resulting in an appreciable reduction of hRpn13 protein levels, as assessed by immunoprobing whole cell lysates with anti-hRpn13 antibody (Fig. 2A, left panel). The cell lysates from such cells were immunoprobed for the cell cycle regulators p27kip1 and Wee1 and compared with a mock siRNA control. p27kip1 was found to be stabilized by hRpn13 knockdown, whereas Wee1 was not affected (Fig. 2A, left panel). This experiment was conducted independently four times to yield a p value of 0.039 for p27kip1 stabilization following hRpn13 knockdown by two-tailed, two-sample equal variance Student’s t test analysis (Fig. 2A, right panel).

To further investigate the effect of hRpn13 loss on p27kip1 stability, we performed three independent 3-h cycloheximide chase experiments with and without hRpn13 knockdown by siRNA treatment for 72 h, as described above (Fig. 2B). Whole
cell lysates over the course of the chase were separated by SDS-PAGE and immunoblotted for p27\(^{kip1}\) and Wee1 with normalization to \(\beta\)-actin (Fig. 2B). Averaged values and the standard error of the mean across the three independent experiments were plotted for p27\(^{kip1}\) (Fig. 2C, top panel) and Wee1 (Fig. 2C, bottom panel) to reveal p27\(^{kip1}\) stabilization and only a slight effect for Wee1.

These effects of hRpn13 reduction on the cell cycle markers p27\(^{kip1}\) and Wee1 suggest that it plays a role in cell cycle progression from G\(_0\)/G\(_1\). Cell cycle profiling by FACS was
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FIGURE 2. hRpn13 knockdown leads to G0/G1 stalling, as indicated by stabilization of p27Kip1 and not Wee1. A, lysate from HeLa cells treated with mock or siRNA against hRpn13 for 72 h was resolved and immunoblotted as indicated. Quantitation of protein levels normalized to β-actin is displayed for four independent experiments. ImageJ and Excel were used for these analyses (right panel). Error bars indicate the standard error of the mean between experiments. **, p < 0.05, Student’s t test (two tails, two-sample equal variance). B, HeLa cells were treated with mock or siRNA against hRpn13 for 72 h, followed by cycloheximide (CHX) treatment. Cells were harvested and washed in PBS at the indicated time points. Lysates were resolved and immunoblotted as indicated. C, Quantitation of cycloheximide chase experiments as shown in B, for p27Kip1 (top panel) and Wee1 (bottom panel) normalized to β-actin. ImageJ and Excel were used for quantitation and analyses. Each point depicts the average of three separate experiments, with error bars indicating the standard error of the mean between experiments.

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Loss of hRpn13 Reduces Uch37 Protein Levels, whereas Loss of Uch37 Has No Detectable Effect on hRpn13 Protein Levels—Because the change of hRpn13 and Uch37 protein levels appeared to follow the same trend during and following nutrient deprivation (Fig. 3), we tested whether hRpn13 loss affects Uch37 protein levels and vice versa. hRpn13 was reduced by siRNA for 72 h in HeLa cells, and total cell lysates were immunoblotted with antibodies against Uch37 (Fig. 4A) or S5a (as a control, Fig. 4C). The Uch37 protein level was visibly reduced with the loss of hRpn13 (Fig. 4A), and three independent experiments revealed a statistically significant effect (p = 0.05) based on a paired two-tailed Student’s t test (Fig. 4B). In contrast, S5a protein levels did not noticeably change upon hRpn13 knockdown (Fig. 4D), as validated by quantification of three independent experiments (p = 0.83) (Fig. 4D).

Uch37 also binds the NFRKB subunit of the Ino80 chromatin-remodeling complex (33–36), and we attempted to reduce NFRKB protein levels in HeLa cells to investigate whether it also contributes to Uch37 stability. In our hands however, using the published protocol (36) and reagents (ON-TARGETplus human NFRKB siRNA-SMARTpool, Dharmacon, catalog no. L-015648-00, lot no. 15116), NFRKB protein levels were unchanged (Fig. 4E). The knockdown was first attempted in 293T cells as published, but the identical protocol was also tried in HeLa cells and led to cell death.

We next tested whether Uch37 knockdown affects the hRpn13 protein level. We reduced Uch37 levels with siRNA for 48 h and compared the hRpn13 steady-state protein levels in whole cell lysate to a mock control to find negligible effects (Fig. 4F). This experiment was repeated in triplicate and quantified to yield no statistically significant change in the hRpn13 protein level (p = 0.48) (Fig. 4G).

Uch37 Loss Leads to Cell Cycle Arrest in G0/G1, and Reduced DNA Replication—Given that the loss of hRpn13 leads to reduced levels of Uch37, we tested whether Uch37 reduction alone impacts p27Kip1 stability. HeLa cells were treated with
siRNA against Uch37 for 48 h, and efficacy was tested by immunoprobing whole cell lysates with anti-Uch37 antibody (Fig. 5, A and B). Uch37 knockdown was significantly more effective than hRpn13 reduction (Fig. 5A, left panel, versus Fig. 2A, left panel). The lysates of treated cells were thus immunoprobed for p27Kip1 and Wee1 and compared with a mock siRNA control (Fig. 5A, left panel). p27Kip1 was found to be stabilized by Uch37 knockdown, whereas Wee1 was not affected (Fig. 5A). This experiment was performed independently three times to yield a p value of 0.001 for p27Kip1 stabilization upon Uch37 knockdown by a two-tailed, two-sample, equal variance Student’s t test analysis (Fig. 5A, right panel). We found that the effect on p27Kip1 stability is greater with Uch37 knockdown than with hRpn13 knockdown (Figs. 5A and 2A). However, as discussed above, we were able to achieve better protein reduction of Uch37, which was not observed by immunoblotting after siRNA treatment (Fig. 5A, left panel).

The effect of Uch37 loss on p27Kip1 stability was further tested by three independent 3-h cycloheximide chase experiments with and without Uch37 knockdown. Uch37 protein levels were reduced in HeLa cells by siRNA (Fig. 5B), and the p27Kip1 (Fig. 5C, top panel) and Wee1 (Fig. 5C, bottom panel) protein levels were quantified, normalized to β-actin, and plotted over the course of the cycloheximide chase. This analysis indicated that Uch37 loss stabilizes p27Kip1 but not Wee1.

Given the increased efficiency of Uch37 siRNA compared with hRpn13 siRNA, we next tested whether loss of Uch37 in HeLa cells affects the cell cycle profile. In four independent experiments, HeLa cells were treated with siRNA against Uch37 for 48 h, followed by incorporation of EdU and subsequent counterstaining with propidium iodide for FACS analysis (Fig. 5D). Loss of Uch37 significantly arrested the cell cycle in G0/G1 phase with an average percentage increase of cells in G0/G1 with Uch37 knockdown of 7.2% (p = 0.0025) from 54.2% to 61.4% (Fig. 5D, right panel). We also saw a significant reduction of cells in S phase upon Uch37 loss with an average decrease of cells in S phase with Uch37 knockdown of 9.3% (p = 0.0065) from 29.2% to 19.9%. No significant change was observed for G2/M (p = 0.54) (Fig. 5D, right panel). This result supports the finding that p27Kip1 is stabilized with loss of Uch37 because p27Kip1 negatively regulates the G0/G1 to S transition (Fig. 5, A–C).

We attempted to establish the importance of Uch37 catalytic activity in cell cycle progression by using a Uch37 construct in which the catalytic cysteine (Cys-88) is replaced with alanine (C88A), as used in previous studies (31). In our experiments, however, we found that this amino acid substitution results in low protein levels for Uch37. Cell lysates from HeLa cells treated with Uch37 siRNA and expressing FLAG-tagged Uch37 wild-type or C88A protein were resolved and immunoprobed with anti-Uch37 antibody to reveal a robust reduction in the Uch37 C88A protein level compared with wild-type Uch37 (Fig. 5E). We next expressed FLAG-tagged Uch37 C88A protein in HeLa cells and subjected the whole cell lysate to immunodetection with anti-FLAG antibody, which indicated a ladder of Uch37 species of molecular weight difference expected for ubiquitination (Fig. 5F).

We next assessed whether Uch37 reduction triggers apoptosis by using Annexin V staining and FACS analysis. HeLa cells were treated with Uch37 siRNA as described above, followed by
Annexin V with SYTOX® dead cell staining and FACS analysis (Fig. 5G). There was a slight increase in apoptotic cells, with the average percentage of Annexin V only-staining cells for Uch37 siRNA being 3.2% compared with a value of 2.8% for the mock control-treated cells. This increase was not statistically significant, however \( p > 0.77 \) (Fig. 5G, right panel). Overall, these results indicate that loss of Uch37 inhibits cellular proliferation in HeLa cells by G0/G1 stalling.

**Discussion**

Two independently discovered hRpn13-targeting molecules are reported to restrict multiple myeloma cells (9, 10). We have found that one such molecule, RA190, interferes with DNA replication and G2 progression in HeLa cells (Fig. 1A). It is possible that RA190-induced inhibition of DNA replication leads to an activation of the DNA damage response pathway and, in turn, cellular arrest in G2 (reviewed in Ref. 69). Future experiments are needed to define the underlying mechanisms linking RA190 treatment to its effect on the cell cycle, including whether there are additional targets for RA190 that may contribute to this effect.

Nonetheless, here we provide evidence for a direct role of the RA190 target hRpn13 and its interacting deubiquitinating enzyme Uch37 in cell cycle progression. Loss of hRpn13 or Uch37 induced stalling of the cell cycle in G0/G1 (Figs. 2 and 5), and it is interesting that these two proteins are reduced during nutrient deprivation (Fig. 3, A and B). These findings implicate this protein pair in cell cycle progression to S phase and are consistent with the reported role of hRpn13 in cellular proliferation (42, 43, 45). It is not clear why RA190 arrests cells in G2, whereas hRpn13 knockdown does so in G0/G1. A technical detail to these experiments, however, is that we were unable to achieve complete knockdown of hRpn13 (Fig. 2A). It may be noteworthy that knockdown of hRpn13 in nutrient-starved HeLa cells leads to cellular detachment from the plates because this effect may reflect stalling in M phase (70). Future experiments that take advantage of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology (71, 72) may be required to fully address the role of hRpn13 in cell cycle progression.

It is notable that we were able to readily knockdown Uch37 (Fig. 5A) but not hRpn13 (Fig. 2A). We propose that this effect is derived from hRpn13 stability in human cell lines. We attempted to perform protein stability assays by cycloheximide but found hRpn13 to be stable with no protein loss over 12 h (data not shown). As a proteasome component, hRpn13 most likely evolved signatures that prevent its degradation at the pro-
teasome. Between its two functional domains, hRpn13 has a long, flexible linker region. However, this segment has low sequence complexity (32), most likely protecting it from becoming a proteasome substrate (73). Moreover, when docked at its apical position on the proteasome (38), Rpn13 is remote from the ATPase ring and proteolytic CP, as reviewed in Ref. 1.

FIGURE 5. Uch37 knockdown interferes with DNA replication and stalls cells in G0/G1. A, lysate from HeLa cells treated with mock or siRNA against Uch37 for 48 h was resolved and immunoprobed as indicated. Quantification of each protein normalized to β-actin for three sets of experiments, with error bars depicting the standard error of the mean, was performed with ImageJ and Excel (right panel). **, p < 0.05 using Student’s t test (two tails, two-sample equal variance). B, HeLa cells were treated with siRNA against Uch37 for 48 h, followed by cycloheximide (CHX) treatment for the indicated time. The lysates were resolved and immunoprobed as indicated. C, quantitation of cycloheximide chase experiments as in B for p27kip1 (top panel) and Wee1 (bottom panel) normalized to β-actin. ImageJ and Excel were used for quantitation and analysis for three sets of experiments, with the standard error of the mean between experiments depicted by error bars. D, HeLa cells were treated with siRNA against Uch37 for 48 h, followed by labeling with EdU and propidium iodide, and analyzed by FACS. The right panel depicts the average change of Uch37 RNAi-treated cells versus mock control cells for four sets of experiments, with the standard error of the mean depicted by error bars. **, p < 0.05 as determined by Student’s t test (two tails, two-sample equal variance). E, HeLa cells treated with Uch37 siRNA were transfected with either empty (E), full-length Uch37 wild-type (WT), or full-length Uch37 C88A mutant (C88A) p3xFLAG-CMV™-7.1 vectors. Lysates were then resolved and immunoprobed as indicated. IB, immunoblot. F, HeLa cells were transfected with the full-length Uch37 C88A p3xFLAG-CMV™-7.1 vector, and lysates were resolved and immunoprobed as indicated (the numbers at the right indicate molecular mass in kilodaltons). G, apoptotic induction of HeLa cells with mock or Uch37 RNAi treatment as measured by Annexin V staining and FACS analysis. The right panel displays the average Q4 value (Annexin V-positive only) of Uch37 RNAi-treated cells compared with mock-treated cells from four independent experiments.

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hRpn13 is reported to be ubiquitinated in human cells (74–76), and we found hRpn13 and Uch37 protein levels to be correlated in nutrient-deprived HeLa cells (Fig. 3B). We thus speculated that Uch37 may affect hRpn13 protein levels by deubiquitination. We found no such effect but, rather, that this question. Nonetheless, this study is the first that implicates Rpn10 and Dsk2 as a polyubiquitin chain-length sensor. Mol. Cell 36, 1018–1033

We thank Dr. Fen Liu for helpful discussions and Kathleen Noer (Frederick Center for Cancer Research Flow Cytometry Core) for help with the flow cytometry experiments.

Acknowledgments—We thank Dr. Fen Liu for helpful discussions and Kathleen Noer (Frederick Center for Cancer Research Flow Cytometry Core) for help with the flow cytometry experiments.

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