Reversible phosphorylation of Rpn1 regulates 26S proteasome assembly and function

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The fundamental importance of the 26S proteasome in health and disease suggests that its function must be finely controlled, and yet our knowledge about proteasome regulation remains limited. Posttranslational modifications, especially phosphorylation, of proteasome subunits have been shown to impact proteasome function through different mechanisms, although the vast majority of proteasome phosphorylation events have not been studied. Here, we have characterized 1 of the most frequently detected proteasome phosphosites, namely Ser361 of Rpn1, a base subunit of the 19S regulatory particle. Using a variety of approaches including CRISPR/Cas9-mediated gene editing and quantitative mass spectrometry, we found that loss of Rpn1-S361 phosphorylation reduces proteasome activity, impairs cell proliferation, and causes oxidative stress as well as mitochondrial dysfunction. A screen of the human kinome identified several kinases including PIM1/2/3 that catalyze S361 phosphorylation, while its level is reversibly controlled by the proteasome-resident phosphatase, UBLCP1. Mechanistically, Rpn1-S361 phosphorylation is required for proper assembly of the 26S proteasome, and we have utilized a genetic code expansion system to directly demonstrate that S361-phosphorylated Rpn1 more readily forms a precursor complex with Rpt2, 1 of the first steps of 19S base assembly. These findings have revealed a prevalent and biologically important mechanism governing proteasome formation and function.

proteasome | phosphorylation | UBLCP1 | PIM | genetic code expansion

The ubiquitin-proteasome system (UPS) is responsible for selective degradation of the majority of cellular proteins in eukaryotes (1). Protein substrates to be degraded are often polyubiquitinated and then captured, unfolded, and digested by the 26S proteasome, an ATP-driven machinery that is essential for cell viability (2, 3). In addition to its well-established roles in cell cycle regulation and signal transduction, the proteasome is also important for regulating the dynamics and fitness of organelles such as mitochondria and the endoplasmic reticulum (ER), which are key to the maintenance of cellular homeostasis (4–7). However, proteasome dysregulation under a variety of stress conditions has been considered an underlying mechanism of aging and certain diseases (8–12). Therefore, uncovering and understanding the mechanisms of proteasome regulation is of both biological importance and clinical relevance.

Proteasomal degradation of substrates takes place within the 20S core particle (CP) of the proteasome complex, which houses caspase-like, trypsin-like, and chymotrypsin-like peptidase activities. The free 20S CP is a closed cylindrical structure with low or no activity toward protein substrates. Binding of the 19S regulatory particle (RP) at 1 or both ends of the 20S CP leads to CP activation with the formation of singly capped (26S) or doubly capped (30S) proteasome holoenzyme competent for efficient protein degradation. The 19S RP consists of a "lid" and a "base," and the latter is formed by 6 AAA^+ type ATPases (Rpt1–6) plus 3 non-ATPase subunits Rpn1/2/13. Base assembly starts with 3 modules, each containing a pair of Rpt subunits, namely Rpt1-Rpt2-Rpn1-S5b, Rpt3-Rpt6-p28-PAAF1, and Rpt4-Rpt5-p27, with S5b, p28, PAAF1, and p27 being specific chaperone proteins (13–16). Joining of these precursor complexes as well as sequential leaving of the chaperones leads to formation of the base, and subsequent incorporation of the remaining Rpn subunits completes 19S RP assembly (2, 17–19).

Rpn1 of the 19S base is 1 of the 3 proteasome subunits (the other 2 being Rpn10 and Rpn13) that function as receptors of ubiquitin and proteins with ubiquitin-like (UBL) domains (2). Rpn1 was recently found to possess 2 adjacent regions designated as T1 and T2 (for toroid 1 and 2, respectively). T1 is the receptor site for ubiquitin and certain UBL domain proteins such as Rad23, while T2 is the binding site of USP14, a well-studied proteasome-associated deubiquitinating enzyme with a UBL domain (20). Despite the critical requirement of Rpn1 for proteasome formation and function, little is known about its regulation, and its structural details have been vague in currently available cryo-electron microscopy models (21–24).

The proteasome has been shown to be regulated by multiple mechanisms (19, 25, 26), 1 of which is reversible protein

Significance

The 26S proteasome is responsible for the degradation of the majority of cellular proteins in eukaryotes. In this study, we characterized a common and basic mechanism for proteasome regulation through reversible phosphorylation of a key proteasome subunit, Rpn1, at Ser361. Mutation of this single phosphosite impairs proteasome complex formation and perturbs cellular homeostasis. Using the genetic code expansion system, we obtained phosphorylated Rpn1 protein and provided a clear biochemical explanation of how Rpn1-S361 phosphorylation promotes proteasome assembly. We further identified the corresponding kinases and phosphatases of this site, which may lead to new approaches of manipulating proteasome activity for therapeutic purposes.

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phosphorylation (27, 28). Several proteasome kinases have been identified, such CaMKIIα, PKA, and DYRK2 (29–32). Considering that the vast majority of the over 400 known phosphosites of the human 26S proteasome have not been characterized, it is highly likely that many more proteasome kinases await discovery. Proteasome phosphorylation is also reversibly controlled by phosphatases such as UBLCP1 (ubiquitin-like domain containing CTD phosphatase 1, ref. 33). As the only member of the human phosphatome that harbors a UBL domain (34), UBLCP1 is directly and specifically recognized by Rpn1 (33, 35). UBLCP1-mediated dephosphorylation down-regulates proteasome activity by interfering with proteasome assembly (33, 36). However, the exact proteasome phosphosite(s) targeted by UBLCP1 has been unknown.

Here, we provide evidence that Rpn1-Ser361 is a prevalent phosphosite that is required for proper assembly and activity of the 26S proteasome. We have identified multiple kinases, including PIM1/2/3, that can phosphorylate Rpn1-S361 and have demonstrated that UBLCP1 is a physiologically relevant phosphatase of this site. Blocking phosphorylation of Rpn1-S361 leads to slowed proliferation and mitochondrial dysfunction. Our data have thus expanded our current knowledge of proteasome regulators and the biological meanings of proteasome phosphoregulation.

Results

Rpn1-Ser361 Is a Conserved and Prevalent Proteasome Phosphosite. A survey of the PhosphoSitePlus database reveals that Rpn1-Ser361 is among the top 10 most frequently detected human proteasome pS/pT sites by mass spectrometry that are conserved in vertebrates (Fig. 1 *A* and *B* and ref. 27). We purified proteasomes from cells stably expressing the Rpn11 subunit with a

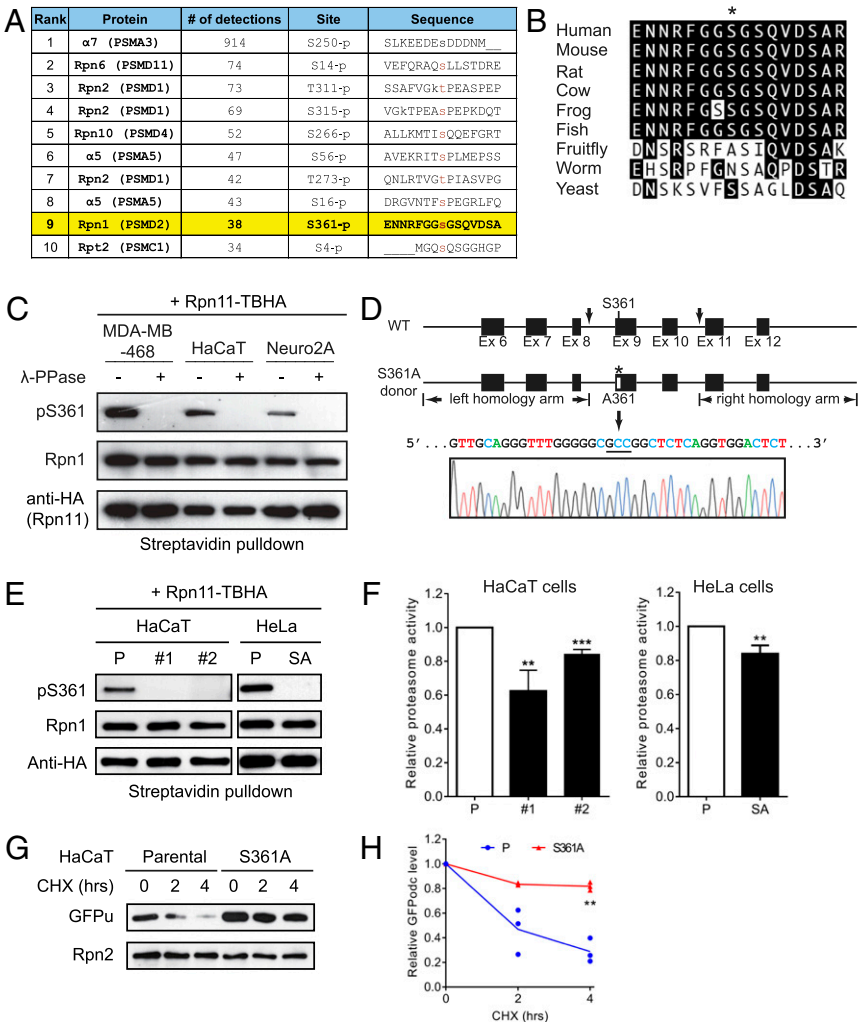

Fig. 1. Rpn1-S361 is a conserved phosphosite regulating proteasome activity. (*A*) Top 10 pS/pT sites of human 26S proteasome that are most frequently detected by mass spectrometry. Rpn1-S361 is highlighted. (*B*) Alignment and conservation of Rpn1 sequences flanking the S361 site (indicated by an asterisk). Accession numbers of the sequences are NP_002799 (*Homo sapiens*), NP_598862 (*Mus musculus*), NP_001026809 (*Rattus norvegicus*), NP_001094667 (*Bos taurus*), NP_001084631 (*Xenopus laevis*), NP_956840 (*Danio rerio*), NP_649158 (*Drosophila melanogaster*), NP_501064 (*C. elegans*), and NP_011892 (*Saccharomyces cerevisiae*). (*C*) Proteasomes were isolated by streptavidin pulldown from the indicated cell lines stably expressing Rpn11-TBHA. Samples were treated with or without λ-phosphatase, and endogenous Rpn1-pS361 was determined by Western blot. (*D*) For generating Rpn1-S361A knock-in, 2 target sequences (downward arrows) flanking exons 9 and 10 of the *PSMD2* gene (encoding Rpn1) were chosen for CRISPR/Cas9-mediated gene editing. (*E*) Rpn1-S361A mutation in 2 independent clones of HaCaT cells (#1 and #2) and in a representative clone of HeLa cells was confirmed by Western blot as in *C*. P, parental cells. SA, S361A knock-in. (*F*) Proteasome activity from the same HaCaT and HeLa cells in *E* was measured with the fluorogenic peptide substrate Suc-LLVY-AMC. Results are presented as mean ± SD $^{**}P < 0.01$; $^{***}P < 0.001$ from 2-tailed paired Student's *t* test (3 independent experiments for each cell type). (*G* and *H*) Parental and knock-in cells were transfected with the GFPu (*G*) or GFPodc (*H*) reporters and treated with cycloheximide (CHX, 50 μg/mL) for the indicated time course. GFP levels from whole-cell extracts were determined by Western blot and quantified (*H*). $^{**}P < 0.01$ from 2-tailed paired Student's *t* test ($n = 3$).

TBHA tag (TEV site-biotinylation sequence-HA tag, ref. 31), and S361 phosphorylation of copurified endogenous Rpn1 was readily detected from multiple human and mouse cell lines using a rabbit monoclonal phospho-specific antibody generated toward this site (Fig. 1*C* and *SI Appendix*, Fig. S1 *A–C*). Rpn1-S361 phosphorylation was also found in mouse brain homogenates (see Fig. 4*C*), consistent with its wide distribution in various mouse organs (ref. 37 and *SI Appendix*, Fig. S1*D*). Moreover, the pS361 level in human embryonic stem cells (ESCs) dropped considerably after differentiation (*SI Appendix*, Fig. S1*E*). These initial observations indicate that Rpn1-S361 phosphorylation is present in a broad range of cell types and can be dynamically regulated.

To characterize the biochemical and biological functions of Rpn1-S361 phosphorylation, we introduced the S361A mutation in HeLa and HaCaT cells (immortalized human keratinocytes) using CRISPR/Cas9-mediated gene editing (Fig. 1*D*). S361 phosphorylation was abrogated in these cells while the Rpn1 protein level was unchanged (Fig. 1*E*). Loss of S361 phosphorylation significantly reduced all 3 proteasome peptidase activities in cell extracts as determined by the fluorogenic peptide substrates Suc-LLVY-AMC (for chymotrypsin-like activity, Fig. 1*F*), Z-LLE-AMC, and Boc-LRR-AMC (for caspase-like and trypsin-like activities, respectively, *SI Appendix*, Fig. S1*F*). Proteasomal degradation of folded protein substrates such as GFPu (38) and GFPodc (39) was also impeded in S361A cells (Fig. 1 *G* and *H*). Similar results were seen in different cell types (*SI Appendix*, Fig. S1 *G* and *H*). Moreover, we noted prominent accumulation of proteins modified by K48-linked polyubiquitin chains in 293A cells harboring heterozygous S361A knock-in (*SI Appendix*, Fig. S1*I*). These results suggest that Rpn1-S361 phosphorylation is necessary for proper proteasome function in cells.

Loss of Rpn1-Ser361 Phosphorylation Impairs Cellular Fitness. To systematically assess the biological significance of Rpn1-S361 phosphorylation, we performed quantitative proteome analysis on HaCaT parental and S361A cells using the SILAC (stable isotope labeling by amino acids in cell culture) label-swap approach, which is generally considered as the most accurate technology for relative protein quantification (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). A total of 5,068 proteins were confidently quantified in 4 biological samples (Dataset S1). Proteins with significant changes in expression were selected according to fold change > 1.5 and P value <0.05 determined with 2-tailed Student's t test. Based on these criteria, 212 proteins were up-regulated and 162 proteins were down-regulated in the S361A cells (Fig. 2*B* and Datasets S2 and S3). The increased levels of some proteins were further validated through Western blotting, highly consistent with the proteome results (Fig. 2*C*). Gene Ontology (GO) analysis of the altered proteins showed their broad involvement in cell cycle regulation, apoptosis, adhesion, signal transduction, metabolism, and protein degradation, reflecting the vast activities governed by the 26S proteasome. Almost all of the up-regulated proteins in HaCaT-S361A cells can be ubiquitinated (https://www.phosphosite.org), and their accumulation might have been a direct result of the overall weakening of proteasomal degradation. Notably, according to GO analysis, the up-regulated proteins were significantly enriched in cellular components such as mitochondria and ER and in processes such as oxidation-reduction and lipid metabolism (Fig. 2*D*). This suggests that loss of Rpn1-S361 phosphorylation could lead to perturbed mitochondrial/ER functions in these cells. Indeed, the mitochondria in S361A cells appeared more fragmented with impaired bioenergetic capacity compared to those in WT cells (Fig. 2*E* and *SI Appendix*, Fig. S2 *B* and *C*). Consistently, the maximal oxygen consumption rate (OCR) was drastically reduced in the knock-in cells (Fig. 2*F*), while the amount of glutathionylated proteins increased, indicative of oxidative stress (Fig. 2*G*). In addition, the mutant cells also exhibited reduced glycolysis (*SI Appendix*, Fig. S2*D*). These findings underscore the importance of Rpn1-S361 phosphorylation in supporting cellular metabolism and redox homeostasis. Moreover, S361A cells exhibited prolonged cell cycle with evident accumulation/stabilization of cell cycle inhibitors such as $p21^{Cip1}$ and $p27^{Kip1}$ (Fig. 2 *C* and *H* and *SI Appendix*, Fig. S2 *E–I*). All these changes led to a marked reduction in cell proliferation and viability (Fig. 2*I*), further highlighting the importance of Rpn1-S361 phosphorylation for cellular fitness.

Multiple Kinases Including PIM1/2/3 Regulate Rpn1-S361 Phosphorylation. The functional importance of Rpn1-S361 phosphorylation indicates the necessity for maintaining its proper level in cells. We therefore screened a human kinome cDNA library (40) to identify the Rpn1-S361 kinase(s). 293T cells stably overexpressing Rpn1-TBHA were transfected with individual kinase cDNAs, and an ELISA-based system was devised to capture and detect pS361 from each cell extract (Fig. 3*A*). More than a dozen kinases, when transiently overexpressed, were found to markedly increase S361 phosphorylation (Fig. 3 *B* and *C*). Seven of these kinases (PIM1/2/3, MAP4K1/2, PKA, and NEK6) directly and robustly phosphorylated recombinant GST-Rpn1 at S361 in vitro (Fig. 3*D* and *SI Appendix*, Fig. S3 *A* and *B*). Loss-of-function studies showed that simultaneous disruption of PIM1, 2, and 3 (PIM triple KO or tKO) in 293T cells decreased, although did not abolish, endogenous S361 phosphorylation, whereas knockout or inhibition of the other candidate kinases alone or in combination had no discernable effect on pS361 level (Fig. 3*E* and *SI Appendix*, Fig. S3 *C–G*). Importantly, PIM1/2/3 tKO cells exhibited lowered proteasome activity as seen in S361A knock-in cells (Fig. 3 *F* and *G*), while overexpression of WT PIM1, but not the kinase-deficient mutant (K67R), enhanced proteasomal degradation of the GFPu reporter (Fig. 3*H*). These results are in agreement with the role of Rpn1-S361 phosphorylation and support PIM1/2/3 as the major (albeit not the only) S361 kinases.

UBLCP1 Dephosphorylates Rpn1-pS361. We next searched for the pS361 phosphatase and UBLCP1 naturally became our top candidate, not only because it is the only known proteasome-resident phosphatase but also because Rpn1 is the very proteasome subunit that UBLCP1 directly binds (33). Four lines of evidence substantiated the role of UBLCP1 in regulating pS361. First, in in vitro phosphatase assays, bacterially expressed WT UBLCP1, but not the catalytically dead mutant (D143A), effectively dephosphorylated pS361 of Rpn1 affinity-purified from 293T cells (Fig. 4*A* and *SI Appendix*, Fig. S4*A*). UBLCP1 was also capable of dephosphorylating a Rpn1-derived phosphopeptide harboring pS361 (*SI Appendix*, Fig. S4*B*), demonstrating that Rpn1-pS361 is a direct substrate of UBLCP1.

Second, we generated *Ublcp1* KO mice and evaluated the level of endogenous Rpn1-pS361. To facilitate detection of endogenous pS361, we crossed $Ublcp1^{-/-}$ mice with Rpn11-TBHA knock-in mice ($Psmd14^{TBHA}$, also generated in our laboratory) to obtain $Ublcp1^{+/+}$;$Psmd14^{+/TBHA}$ and $Ublcp1^{-/-}$; $Psmd14^{+/TBHA}$ animals (Fig. 4*B*). Endogenous proteasomes were then affinity purified from lysates of cerebellar tissues of littermates. Rpn1-S361 phosphorylation was readily detectable from the brain proteasomes, with a 3-fold increase in UBLCP1-null mice compared to WT control (Fig. 4*C*). A similar increase in pS361 was observed when UBLCP1 was disrupted in 293T and Py8119 cells (a mouse breast cancer cell line) by CRISPR/Cas9 (Fig. 4*D* and *SI Appendix*, Fig. S4 *C–E*). Moreover, a specific UBLCP1 inhibitor, Compound 13 (41), as well as the generic UBLCP1 inhibitor $BeCl_2$ also led to enhanced S361 phosphorylation in control cells but failed to further augment the phosphorylation in UBLCP1 KO cells (*SI Appendix*, Fig. S4*E*). Together, these loss-of-function data demonstrate

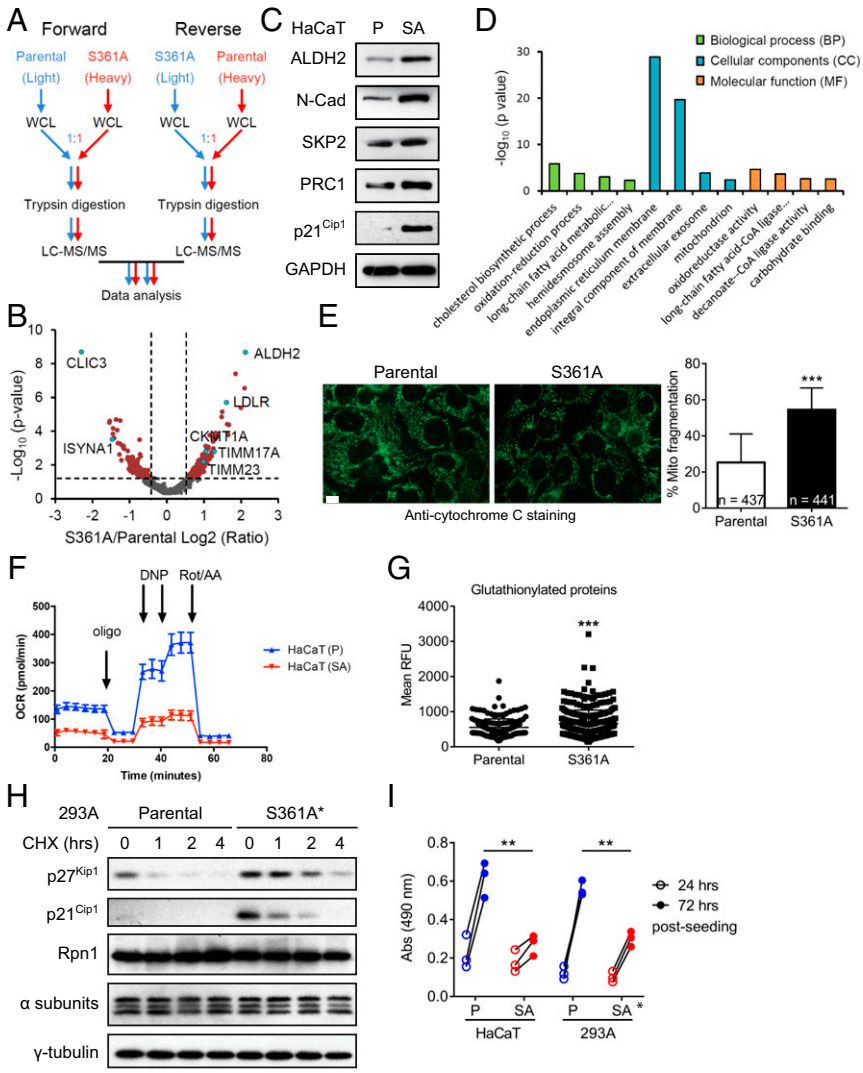

Fig. 2. Loss of Rpn1-S361 phosphorylation impairs cell homeostasis and viability. (*A*) Outline of the SILAC method for comparing whole cell lysates (WCL) of HaCaT parental and S361A cells. (*B*) Volcano plot of the proteome results. Proteins significantly up-regulated or down-regulated ($\log_2$[S361A/Parental] > 0.585 or < −0.585) with *P* value <0.05 are highlighted in dark red. (*C*) Equal amounts of whole-cell lysates (20 μg) from HaCaT parental and S361A cells were probed with the indicated antibodies. ALDH2, aldehyde dehydrogenase (mitochondrial); N-Cad, N-Cadherin; PRC1, protein regulator of cytokinesis 1; SKP2; S-phase kinase-associated protein 2. (*D*) GO categories of the 212 up-regulated proteins in S361A cells. (*E*) The fragmentation status of mitochondria was evaluated by anticytochrome *c* immunostaining. (Scale bar, 10 μm.) The number of cells analyzed are shown. ***$P < 0.001$ from 2-tailed unpaired Student's *t* test. (*F*) Seahorse measurement of oxygen consumption rates (OCR) of parental and S361A HaCaT cells. AA, antimycin A; DNP, dinitrophenol; Oligo, oligomycin; Rot, rotenone. (*G*) Quantification of antiglutathionylated protein immunostaining of parental and S361A HaCaT cells. The number of cells analyzed are shown. ***$P < 0.001$ from 2-tailed unpaired Student's *t* test. (*H*) 293A cells were treated with CHX (50 μg/mL), and the indicated endogenous proteins were examined by Western blot. The asterisk designates heterozygous mutation (WT/S361A) in those cells. (*I*) MTS cell viability assays on HaCaT and 293A cells. Measurement was made at 24 h (open circles) and 72 h (filled circles) postseeding. Data are presented as mean ± SD from 3 independent experiments. **$P < 0.01$ from 2-tailed paired Student's *t* test (72-h results).

that UBLCP1 is a physiologically relevant phosphatase that dephosphorylates Rpn1-pS361 in vivo.

Third, mutation of the Rpn1-T2 region essentially abrogated USP14 interaction as reported (20) and also greatly reduced UBLCP1 binding (*SI Appendix*, Fig. S4 *F* and *G*), suggesting a very similar mode employed by Rpn1 for binding these 2 proteins with highly homologous UBL domains (*SI Appendix*, Fig. S4*H* and ref. 33). Consistent with its weakened interaction with UBLCP1, the Rpn1-T2 mutant showed elevated S361 phosphorylation compared to control (Fig. 4*D*). Importantly, such a difference in pS361 was not observed when the Rpn1 variants were expressed in UBLCP1 KO cells (Fig. 4*D*). These data again strongly support UBLCP1 as a Rpn1-pS361 phosphatase.

Fourth, we confirmed that knockdown of UBLCP1 enhanced nuclear proteasome peptidase activity in control cells as shown before (Fig. 4*E* and ref. 33). However, the S361A cells no longer responded to UBLCP1 depletion (Fig. 4*E*), suggesting that Rpn1-S361 is a major phosphosite through which UBLCP1 regulates proteasome function.

Rpn1-S361 Phosphorylation Facilitates Rpn1-Rpt2 Subcomplex Formation. UBLCP1 negatively regulates proteasome activity by restricting 26S proteasome assembly (33, 36), and the above data suggest that this function of UBLCP1 might be achieved through controlling Rpn1-S361 phosphorylation. To test this hypothesis and define how S361 phosphorylation regulates proteasome function, we examined the proteasome complexes in parental

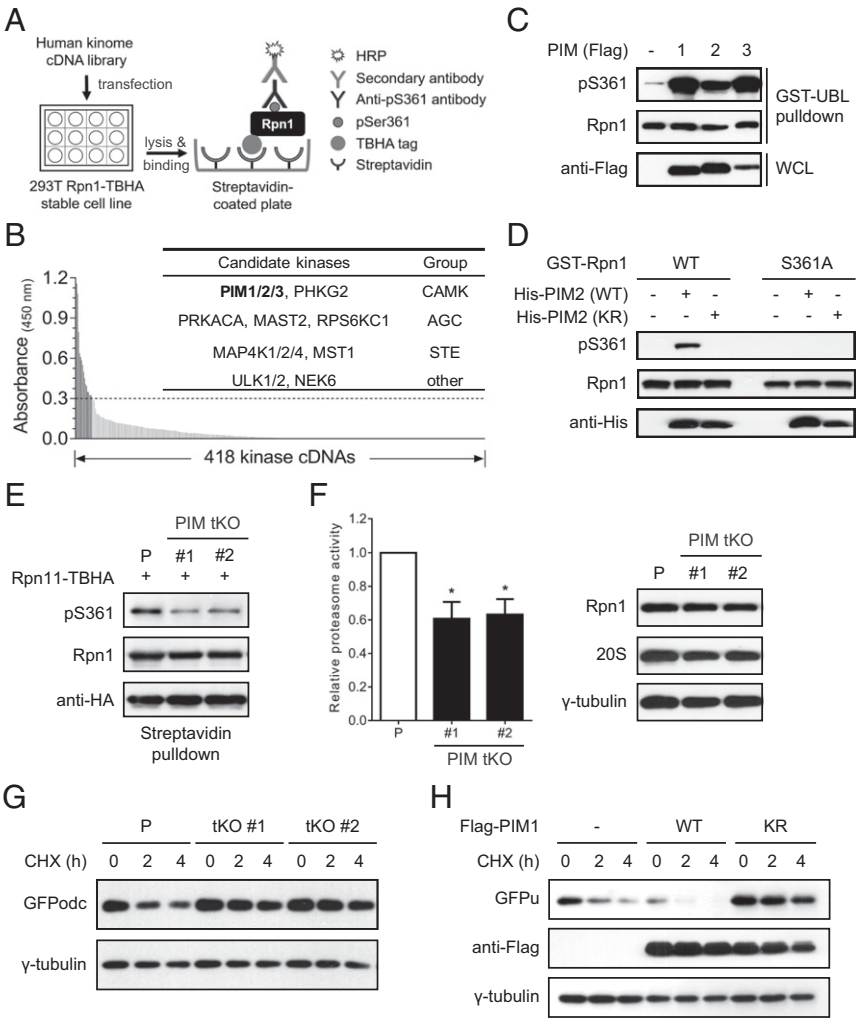

Fig. 3. Identification of Rpn1-S361 kinases. (*A*) Schematic of the ELISA-based kinome screen. (*B*) Summary of kinase screen results. Each kinase is represented by a vertical line. Candidate kinases that caused strong S361 phosphorylation (above the cutoff of $OD_{490} = 0.3$, dotted line) are shown as thick lines and listed in *Inset*. (*C*) 293T cells transfected with vector control ("−"), Flag-tagged PIM1, 2, or 3 were used for GST-UBL pulldown. Endogenous Rpn1-pSer361 was probed. (*D*) In vitro kinase assay with purified GST-Rpn1 (WT or S361A) and His-PIM2 (WT or the dead mutant K61R). Samples were blotted with the indicated antibodies. (*E*) 293T parental cells and 2 clones with PIM1/2/3 triple knockout (tKO) were stably transduced with Rpn11-TBHA. Proteasomes isolated from these cells were probed with the indicated antibodies. (*F*) Proteasome activity from the same cells in *E* was measured with Suc-LLVY-AMC (*Left*). $*P < 0.05$ (2-tailed paired Student's *t* test from 3 independent experiments). PIM KO did not alter the total amounts of proteasome subunits as shown by the Western blot (*Right*). (*G*) GFPodc was expressed in the same 293T cells as in *E*, and its degradation following CHX treatment (50 μg/mL) was shown by anti-GFP blotting. (*H*) GFPu was coexpressed with vector control ("−"), PIM1-WT, or PIM1-K61R (KR) in 293T cells. CHX treatment and anti-GFP blotting were performed as in *G*.

and S361A cells by native polyacrylamide gel electrophoresis (PAGE) (Fig. 5*A*). Loss of Rpn1-S361 phosphorylation caused a considerable decrease in 26S/30S proteasome contents and a corresponding increase in the level of free 20S proteasomes, indicating defective proteasome assembly in the knock-in cells. Gel filtration analysis also revealed a similar impairment of proteasome formation and markedly lower peptidase activity from fully assembled proteasomes in the mutant cells (Fig. 5*B* and *SI Appendix*, Fig. S5*A*), consistent with their overall reduction in proteasome activity (Fig. 1*F*). These results support the idea that Rpn1-S361 phosphorylation is required for proper proteasome assembly, echoing the reported function of UBLCP1.

Rpn1 could be phosphorylated at S361 both before and after its incorporation into the 26S proteasome (*SI Appendix*, Fig. S5*B*). The native PAGE results showed that certain smaller protein complexes containing Rpn1 and Rpt2 (likely RP precursor complexes) were also much depleted in the S361A cells (Fig. 5*A*). Improper formation of the Rpn1-Rpt1-Rpt2-S5b subcomplex can cause defects in the assembly of 19S and eventually 26S proteasomes (13–16). Therefore, we set up an in vitro binding assay using recombinant proteins of Rpn1 and Rpt2 to directly assess the role of S361 phosphorylation in precursor complex formation. Here, we utilized the genetic codon expansion technique (42) and changed the codon of S361 (AGT) to the Amber codon (TAG, designated as TAG361). When Rpn1(TAG361) was expressed in an engineered *Escherichia coli* strain C321 (43), phospho-serine ("Sep") instead of serine was site-specifically incorporated at the 361 position of Rpn1, allowing us to purify the phospho-Rpn1 protein (*SI Appendix*, Fig. S5 *C* and *D*). Immunodepletion assays with our anti-pS361 antibody indicated that more than 80% of the Rpn1 protein obtained this way was phosphorylated at S361 (*SI Appendix*, Fig. S5*E*). For in vitro binding, equal amounts of purified phosphorylated Rpn1(TAG361) and unphosphorylated WT Rpn1 proteins were individually incubated with recombinant Rpt2. Unphosphorylated Rpn1 interacted weakly with Rpt2 in vitro as reported before (44). However, S361 phosphorylation greatly increased Rpn1-Rpt2 binding, while this difference could be completely nullified by

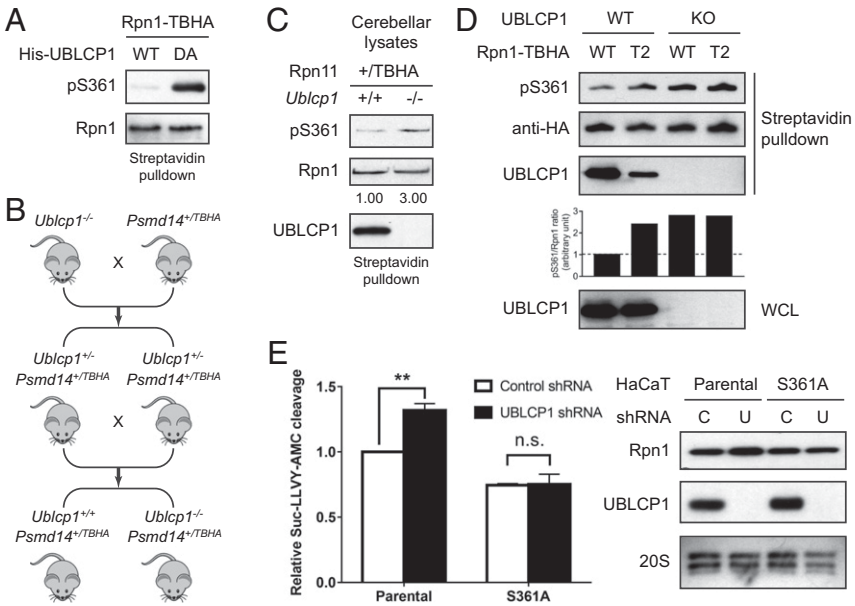

Fig. 4. UBLCP1 is a physiological phosphatase of Rpn1-pS361. (*A*) In vitro dephosphorylation of affinity-purified Rpn1 by recombinant UBLCP1. DA, the inactive D143A mutant. (*B*) The breeding process for generating *Ublcp1* WT and KO mice with Rpn11-TBHA knock-in. (*C*) Proteasomes in cerebellar homogenates from P12 littermates of the indicated genotypes were isolated by streptavidin pulldown, and the pS361 level of endogenous Rpn1 was determined by Western blot. The relative ratios of pS361 over total Rpn1 are shown. (*D*) TBHA-tagged Rpn1 variants stably expressed in 293T parental or UBLCP1-null cells were purified by streptavidin pulldown. Levels of pS361 and associated endogenous UBLCP1 were determined. The relative ratios of pS361 over total Rpn1-TBHA (represented by the anti-HA blot) are shown for each pulldown sample. (*E*) HaCaT parental and S361A cells were stably transduced with control or UBLCP1-specific shRNA. After cell fractionation, nuclear proteasome activity of the indicated cells was assayed with Suc-LLVY-AMC (*Left*). $^{**}P < 0.01$ from 2-tailed paired Student's *t* test ($n = 3$). n.s., not significant. UBLCP1 knockdown efficiency was confirmed by Western blot (*Right*). C, control shRNA. U, UBLCP1 shRNA.

pretreating phospho-Rpn1 with alkaline phosphatase (Fig. 5*C* and *SI Appendix*, Fig. S5*F*), demonstrating that it was indeed phosphorylation of S361 that markedly strengthened the Rpn1–Rpt2 interaction.

To gain insights into the molecular details of Rpn1–Rpt2 interaction, we examined 1 of the latest Cryo-EM structures of the human 26S proteasome (Protein Data Bank [PDB] ID code: 6MSJ, ref. 22). In this structure, the side chain of Rpn1-S361 seems to point to the unresolved N-terminal region of Rpt2 (Fig. 5*D*). Interestingly, this region of Rpt2 contains a stretch of 7 lysine residues (K15/16/19/21/22/23/24) that are conserved in different species (*SI Appendix*, Fig. S5*G*). Mutation of these lysine residues to alanine (7KA) did not affect the binding between Rpt2 and unphosphorylated Rpn1, but completely canceled the effect of S361 phosphorylation on promoting the Rpt2–Rpn1 interaction (Fig. 5*E*). Consistently, when expressed in 293T cells, the Rpt2-7KA mutant was incorporated into the 26S proteasome at a lesser amount than WT Rpt2 (Fig. 5*F*). Thus, by forming a charge–charge interaction with the N-terminal poly-basic region of Rpt2, phosphorylation of Rpn1-S361 facilitates 1 of the first steps of base assembly, hence allowing for more efficient formation of 19S and 26S proteasomes subsequently (Fig. 5*G*). These data provide a mechanistic explanation and unequivocal proof for the role of Rpn1-S361 phosphorylation in regulating 26S proteasome assembly and function.

Discussion

Proteasome phosphorylation has emerged as an important and versatile mechanism for regulating protein turnover by the UPS (27, 28). Previous studies have demonstrated that phosphorylations of Rpt6-Ser120 (by CaMKIIα), Rpn6-Ser14 (by PKA), and Rpt3-Thr25 (by DYRK2) all result in proteasome activation and are functionally involved in neuronal synapse formation, clearance of misfolded proteins, and tumorigenesis, respectively (29–32, 45). In this study, we characterized a different proteasome phosphorylation event that also positively affects proteasome activity and safeguards mitochondrial homeostasis. Loss of Rpn1-S361 phosphorylation in HaCaT cells led to a major metabolic shift as a result of defective proteasome assembly and chronic mitochondrial impairment. This phenomenon should be distinct from the transient and reversible 26S proteasome disassembly induced by acute mitochondrial/oxidative stress (46, 47). Reciprocal regulations between the UPS and mitochondria are known to be important but also intricate (48–52), making it very challenging to pinpoint the primary proteasome substrate(s) that caused the mitochondrial defects in the first place. Nevertheless, our work demonstrates that mutation of a single proteasome phosphosite is sufficient to cause mitochondrial dysfunction and oxidative stress, highlighting the functional importance of proteasome phosphorylation.

The impact of S361 phosphorylation extends beyond mitochondrial regulation as suggested by our mass spectrometry results, while a different proteomic signature and different cellular responses might be expected with the same S361A substitution in a different cell type. Earlier proteomic studies have documented changes in pS361 in certain cell types during immune responses or upon oncogene expression (53, 54); the biological meanings of these have not been investigated. We also observed a prominent decrease in pS361 level when human ES cells differentiated into pancreatic precursor cells, and it correlated with a concurrent reduction in proteasome activity (*SI Appendix*, Fig. S1*E*). Exactly how this happened remains unknown, but the finding is in agreement with the idea that a higher proteasome activity is required to maintain the undifferentiated status of ES cells (55, 56). It further raises the possibility that proteasome modifications are also important in regulating ES cell self-renewal, a mechanism alternative or in addition to up-regulation of proteasome expression in ES cells (56). Further studies are needed

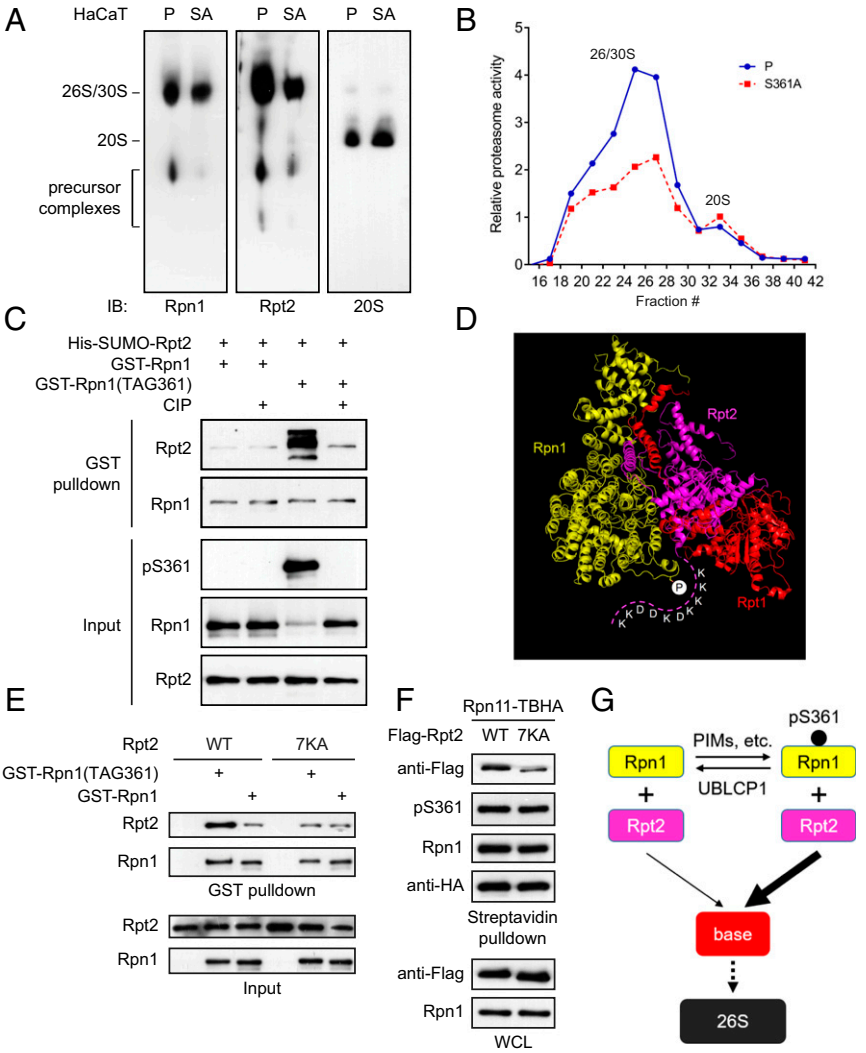

Fig. 5. Rpn1-S361 phosphorylation regulates proteasome assembly. (*A*) Native gel analysis of proteasome complexes in HaCaT parental and S361A cells. Equal amounts of whole-cell lysates (25 μg) were loaded and probed with the indicated antibodies. (*B*) Cell extracts of HaCaT parental and S361A cells were fractionated by gel filtration and proteasome activity of the indicated fractions was measured with Suc-LLVY-AMC. (*C*) In vitro binding assay with purified Rpt2 and Rpn1 proteins. Anti-pS361 blot is shown to demonstrate the successful production of S361-phosphorylated Rpn1 protein and its complete dephosphorylation by pretreatment with the CIP phosphatase. (*D*) Cyro-EM structures of Rpn1 (yellow), Rpt2 (magenta), and Rpt1 (red) within the 26S proteasome (adapted from PDB ID code 6MSJ). Phospho-S361 (white circle) is predicted to face an unresolved region of the Rpt2 N terminus (dotted line), which contains multiple lysine residues. (*E*) A similar in vitro binding assay as in *C* was performed with untagged Rpt2-WT and Rpt2-7KA proteins. The 7KA mutant had all 7 lysine residues shown in *D* mutated to alanine. (*F*) Flag-tagged Rpt2 (WT or 7KA) was expressed in 293T Rpn11-TBHA cells. Proteasomes were isolated by streptavidin pulldown and probed with the indicated antibodies. (*G*) Schematic depicting how Rpn1-S361 phosphorylation, which is reversibly controlled by multiple kinases including PIM1/2/3 and the phosphatase UBLCP1, promotes base assembly and the ultimate formation of 26S proteasome holoenzyme.

to fully understand the regulation and function of Rpn1-S361 phosphorylation in vivo.

Several previous reports showed that proteasome assembly can be regulated by phosphorylations of different subunits, but the molecular mechanisms have been unclear (32, 33, 36, 57, 58). We took advantage of the unnatural amino acid (UAA, in this case pSer/Sep) incorporation technology and provided biochemical evidence that the interaction between phosphorylated Rpn1-S361 and lysine residues of the Rpt2 N terminus may serve as an anchor to facilitate the binding between these 2 subunits. This model agrees well with the cryo-EM structures of the proteasome and with the role of UBLCP1 as the pS361 phosphatase in controlling proteasome assembly. It should be pointed out that Rpn1-S361 phosphorylation promotes, but is not required for, proteasome formation as the S361A mutant cells are still viable. Such phosphoregulation must also be absent in yeast and *Caenorhabditis elegans* given the lack of S361 and its flanking sequences in their Rpn1 proteins (Fig. 1*B*). Interestingly, there is no UBLCP1 homolog in these species, either (33). Despite that UBLCP1 may dephosphorylate other proteasome phosphosites (33, 36), we argue that Rpn1-S361 is the primary target through which UBLCP1 controls proteasome assembly in higher organisms.

Our work identified multiple kinases that can phosphorylate Rpn1-S361. It is quite possible that different kinases may be at work in different tissues or under different conditions to maintain a sufficient level of pS361. This may explain its wide presence in many mouse organs (37) and in all types of cells we have examined. Detection of endogenous S361 phosphorylation by Western blot or mass spectrometry was relatively easy with no need for treatment or stimulation of the cell. These features distinguish Rpn1-S361 from the other

proteasome phosphosites previously reported, suggesting that phosphorylation of this site is a basic and common mechanism for proteasome regulation. However, our efforts in generating homozygous Rpn1-S361D mutant cells to mimic constitutive S361 phosphorylation have been unsuccessful, suggesting that dephosphorylation of Rpn1-S361 may also be necessary for optimal functioning of cells.

Pharmacological targeting of proteasome phosphorylation has proven to be an effective approach for manipulating proteasome function with therapeutic potentials, as exemplified by studies on PKA and DYRK2 (45, 59). Cancer cells often highly rely on the proteasome for survival (10, 60). The PIM kinases that phosphorylate Rpn1-S361 are well-known oncogenes overexpressed in a variety of hematological and epithelial cancers, and PIM inhibitors are currently tested in clinical trials for treating several malignancies including multiple myeloma (61, 62), where proteasome inhibitors are used as first-line drugs. Our finding of PIM-mediated Rpn1-S361 phosphorylation may provide an important clue for proteasome hyperactivation in certain cancers and a molecular basis for new combinatorial therapies with PIM inhibitors and proteasome inhibitors.

Materials and Methods

Details on the general methods, antibodies, reagents, plasmids, cell lines, gene editing, protein purification, proteasome assays, quantitative mass spectrometry, cell respirometry, and in vitro binding assay are presented in *SI Appendix*.

Data Availability Statement. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository with the dataset identifier PXD016643.

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