Using the Ubiquitin-modified Proteome to Monitor Distinct and Spatially Restricted Protein Homeostasis Dysfunction*

Joshua M. Gendron‡§, Kristofer Webb‡¶, Bing Yang‡, Lisa Rising‡, Nathan Zuzow‡, and Eric J. Bennett‡**

Protein homeostasis dysfunction has been implicated in the development and progression of aging related human pathologies. There is a need for the establishment of quantitative methods to evaluate global protein homeostasis function. As the ubiquitin (ub) proteasome system plays a key role in regulating protein homeostasis, we applied quantitative proteomic methods to evaluate the sensitivity of site-specific ubiquitylation events as markers for protein homeostasis dysfunction. Here, we demonstrate that the ub-modified proteome can exceed the sensitivity of engineered fluorescent reporters as a marker for proteasome dysfunction and can provide unique signatures for distinct proteome challenges which is not possible with engineered reporters. We demonstrate that combining ub-proteomics with subcellular fractionation can effectively separate degradative and regulatory ubiquitylation events on distinct protein populations. Using a recently developed potent inhibitor of the critical protein homeostasis factor p97/VCP, we demonstrate that distinct insults to protein homeostasis function can elicit robust and largely unique alterations to the ub-modified proteome. Taken together, we demonstrate that proteomic approaches to monitor the ub-modified proteome can be used to evaluate global protein homeostasis and can be used to monitor distinct functional outcomes for spatially separated protein populations. Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.058420, 2576–2593, 2016.

Maintenance of the collective proteome is achieved through the careful balance of protein synthesis and degradation (1, 2). As the primary protein degradation system within the cell, the ubiquitin proteasome system (UPS) plays a key role in maintaining proper protein homeostasis and responding to internal and external insults to proteome fidelity (3). Separate from overseeing the regulated degradation of critical cell signaling factors, the UPS plays an essential quality-control role by identifying and destroying misfolded or otherwise nonfunctional proteins (4).

Defects in protein quality control function have been widely hypothesized to contribute to the pathology of a wide array of human aging-associated disorders (5, 6). For instance, cells containing visible protein aggregates that are associated with neurodegeneration display impaired protein homeostasis function and manipulating protein homeostasis pathways modulates neurodegenerative phenotypes in mouse models (7–12). Further, protein homeostasis dysfunction occurs in various cancer models and tumor cells have been hypothesized to experience chronically elevated levels of protein homeostasis stress (13). This observation suggests that the acquisition of an elevated protein homeostasis capacity may be a key event during tumorigenesis (14, 15). Thus, there is a need to precisely monitor protein homeostasis function to both evaluate the contribution of protein homeostasis dysfunction to disease progression, and to detect the distinct protein homeostasis impairment that accompanies human aging-associated disorders.

One approach to monitor protein homeostasis is to use engineered fluorescent sensors as protein homeostasis reporters (16–18). These optical UPS sensors report on protein homeostasis function in live cells and have been widely utilized to examine protein homeostasis impairment in cell culture and mouse models upon expression of aggregation prone proteins (7, 8, 19–21). However, these engineered reporters require exogenous expression and the abundance of engineered reporters require exogenous expression and the abundance of a single fluorescent reporter protein is often governed by a relatively small number of protein degradation factors which limits the ability of a single reporter protein to examine global protein homeostasis function.

The development of quantitative proteomic approaches to interrogate the ubiquitin (ub)1-modified proteome provides an

1 The abbreviations used are: ub, ubiquitin; UPS, ubiquitin-proteome system; diGLY, diGlycine; PCF, purified cytosolic fraction; UPR, unfolded protein response; ERAD, ER associated degradation.
opportunity to globally monitor protein homeostasis function without exogenous expression of reporter proteins (22, 23). One advantage of the ub-proteomics approach is the ability to interrogate a wide-array of endogenous ubiquitylation events that either target proteins for degradation or regulate protein function without proteasomal targeting. This approach has been employed to examine alterations to the ub-modified proteome upon exposure to cell stressors (23–28), during differentiation (29), and to identify substrates that are targeted by specific ubiquitin ligases (30–34). Interestingly, a large fraction of the ub-modified proteome that is stabilized upon by specific ubiquitin ligases (30–34). An interesting observation is that the ubiquitin ligase activity without proteasomal targeting. This approach has demonstrated that VCP inhibition, despite similarly impacting global ubiquitin homeostasis, results in largely distinct alterations of the ub-modified proteome compared with proteasome inhibition. Taken together, we demonstrate that the ub-modified proteome can serve as a sensitive reporter for protein homeostasis and can be used to monitor distinct functional outcomes for spatially separated protein populations.

**Experimental Procedures**

**Reagents**—Epoxomicin (EMD Millipore, Billerica, MA) and MG132 (Enzo, Farmingdale, NY) were resuspended in DMSO and used at 1 \( \mu \text{M} \) or 10 \( \mu \text{M} \), respectively unless otherwise noted. Dithiothreitol (DTT, ACROS Organics, Geel, Belgium) was resuspended in water and used at 5 mM. The following antibodies were utilized in this study. CB-5083 (Cleave, Burlingame, CA) was resuspended in DMSO and used at 1 \( \mu \text{M} \). Antibodies for Nrf2 (ab262352) were from Abcam Inc., Cambridge, MA. Antibodies for \( \alpha \)-tubulin (3873), HDAC2 (5113P), Ubiquityl-Histone H2B (5548), COX IV (4850), Caienex (2433), E-Cadherin (3195), were from Cell Signaling Technology, Danvers, MA. Ubiquitin antibody (MAB1510) was from EMD Millipore/Chemicon. Antibodies for c-Myc (sc-40) were from Santa Cruz Biotechnology Inc., Dallas, TX. Antibodies for GFP were from Roche, Indianapolis, IN (1181446001).

**Cell Culture**—HCT116 cell lines were both purchased from American Type Culture Collection (ATCC) and grown in complete DMEM media (Gibco, Waltham, MA) containing 10%FBS (Omega Scientific, Farmingdale, NY), penicillin (50 I.U./ml), and streptomycin (50 \( \mu \text{g/ml} \)) (Mediatech). All cell lines were grown at 37 °C in the presence of 5% CO2.

**Cell Line Generation**—Plasmids containing the coding sequences for GFPu, Ub-M-GFP, Ub-R-GFP, and UFD-GFP (7, 16, 20) were amplified by PCR and cloned into pDONR223 using recombination-based gateway™ cloning (Invitrogen, Carlsbad, CA). The alpha S2 ORF (aATCC) and grown in complete DMEM media (Gibco, Waltham, MA) containing 10%FBS (Omega Scientific, Farmingdale, NY), penicillin (50 I.U./ml), and streptomycin (50 \( \mu \text{g/ml} \)) (Mediatech), L-Arginine hydrochloride (85 \( \mu \text{g/ml} \)) Sigma, St. Louis, MO) and either “light” L-lysine hydrochloride (50 \( \mu \text{g/ml} \)) Sigma) or heavy 13C6,15N2-L-Lysine-hydrochloride (50 \( \mu \text{g/ml} \)) Sigma) or heavy 13C6,15N2-L-Lysine-hydrochloride (50 \( \mu \text{g/ml} \)) Sigma) or heavy 13C6,15N2-L-Lysine-hydrochloride (Sigma, Cambridge, MA) and 292 \( \mu \text{g/ml} \) L-glutamine (Mediatech). All cell lines were grown at 37 °C in the presence of 5% CO2.

**Sample Processing**—Samples to be analyzed for protein or ubiquitylated peptide abundance changes were processed as follows. HCT116 cells were grown in media containing either light (K0) lysine or 13C15N-labeled (K8) lysine. Cell pellets containing a 1:1 mixture of heavy and light cells were lysed in 4 ml of denaturing lysis buffer (8 mM urea, 75 mM NaCl, 50 mM Tris-Cl pH 8.2, Roche complete protease inhibitor, 1 mg NaF, 1 mg \( \beta \)-glycerophosphate, 1 mg sodium orthovanadate, 1 mg PMSF, 5 mM NEM). Lysates were sonicated twice for 5s with 30s rest on ice between cycles. Clarified lysates were digested with Lys-C (Wako, Richmond, VA) at final concentration of 10 ng/\( \mu \text{L} \) for 4 h at 37 °C. Lysates were then diluted to 2 \( \mu \text{L} \) 50 mM Tris-Cl pH 7.2. Trypsin (Sigma) was added at a ratio of 1:100 enzyme/substrate and allowed to incubate overnight at 37 °C. Trypsin digestion was halted by addition of Trifluoroacetic acid (TFA, Sigma) to a final concentration of 0.4%. Digested samples were clarified by
centrifugation (2500 × g for 15 min) at room temperature and the supernatant was collected. Digested peptides were desalted with C18 solid-phase extraction cartridges (Waters). Eluted peptides were flash frozen with liquid nitrogen dried down to completion in a lyophilizer. For protein level analysis, peptides were resuspended in 5% formic acid, 5% CAN. For diGLY-modified peptide enrichment, dried peptides were resuspended in 1.3 ml of 2 × IAP buffer (50 mM MOPS-NaOH pH 7.5, 10 mM NaHPO4, 50 mM NaCl). Resuspended peptides were incubated with α-diGly antibody (Cell Signaling Technologies) preconjugated to Protein-A (Thermo) beads at 4 °C for 2 h with rotating. Beads were then washed 4× with 1 ml IAP buffer with rotating for 10 min between each wash. Peptides were eluted with 5% formic acid. The resulting peptides were desalted with in-house prepared C18 stage-tips and dried in a vacuum centrifuge. Samples were resuspended in 10 μl 5% formic acid, 5% ACN and transferred to autosampler vials.

LC-MS-MS Parameters—Samples were analyzed in triplicate by LC-MS/MS using a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA) with the following conditions. The following is a generalized nHPLC and instrument method that is representative of individual analyses. Peptides were first separated by reverse-phase chromatography using a fused silica microcapillary column (100 μm ID, 20 cm) packed with C18 reverse-phase resin (XSELECT CSH 130 C18 2.5 μm, Waters Co., Milford, MA) using an in-line nano-flow EASY-nLC 1000 UHPLC (Thermo Scientific). Peptides were eluted over a 2 min 0–5% ACN gradient, followed by a 158 min 5–30% ACN gradient, a 15 min 30–45% ACN gradient, a 1 min 45–98% gradient, with a final 14 min isocratic step at 98% ACN for a total run time of 190 min at a flow rate of 250 nl/min. All gradient mobile phases contained 0.1% formic acid. MS/MS data were collected in a data-dependent fashion using a top 10 method with a full MS mass range 300–1750 m/z, 70,000 resolution, and an AGC target of 3e6. MS2 scans were triggered when an ion threshold intensity of 1e5 was reached with a maximum injection time of 250 ms. Peptides were fragmented using a normalized collision energy setting of 22. A dynamic exclusion time of 40 s was used and the peptide match setting was disabled. Singly charged ions, charge states above 8 and unassigned charge states were excluded. All mass spectrometry data files are available through the Massive archive (massive.ucsd.edu) ID: MSV000079454. Annotated MS/MS spectra for all ubiquitin-modified sites were filtered and quantified as described previously (23). For label-free data, spectral counts for ub-modified peptides from triplicate runs for each epoxomicin concentration was first normalized to the CUL5_K724 peptide. Spectral counts from triplicate runs were then summed and made relative to spectral counts in the untreated sample. p value determination for the volcano plots was determined using a two-tailed t test compared with the untreated sample.

Proteasome Activity Assay—Proteasome activity assays were performed as described (41). The chymotryptic activity was measured using the fluorogenic peptide substrate Suc-LLVY-AMC (Boston Biochem, Cambridge, MA). AMC fluorescence was measured over a 60 min time course in a SpectraMAX plate reader. Slopes of the progress curves was used to determine percent proteasome inhibition with the slope of the progress curve from untreated cell lysates set to 0% and the same lysates with exogenous addition of 1 μM epoxomicin set to 100% inhibition.

Cell Fractionation—Cells were lysed in hypotonic lysis buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM HEPES-KOH, pH 7.5) with a prechilled Dounce homogenizer and homogenized in batches with tight-fitting pestle (1 set of 10 strokes). Subsequent fractions were collected through a series of differential centrifugation steps. Each collected fraction was mixed 1:1 with urea lysis buffer (8 M urea, 75 mM NaCl, 50 mM Tris pH 8.2, Roche complete protease inhibitor, 1 mM NaF, 1 mM β-glycerophosphate, 1 mM Na orthovanadate, 1 mM PMFS, 5 mM N-ethylmaleimide (NEM) prior to Lys-C and trypsin digestion, peptide clean-up, diGly immuno-affinity enrichment and subsequent LC-MS/MS analysis.

See Supplemental material for detailed supplemental methods.

Experimental Design and Statistical Rationale—For the label-free experiments depicted in Figure 2, each sample (epoxomicin dose) represents three technical replicates. Error in the normalized spectral counts was determined across the technical replicates. The SILAC-based diGly-peptide quantification was performed with two biological duplicates at each epoxomicin concentration. Each replicate sample comprises three technical replicates. Error bars for individual peptide measurements represent standard deviations from replicate experiments. SILAC-based quantitative data for ub-modified peptide enrichment experiments with subcellular fractionation was performed with three technical replicates for each sample. Error bars for individual peptide measurements represent S.E. from all peptide SILAC ratios quantified for each sample. Each experimental data set with SILAC quantification was done with a control untreated sample in both the heavy and light population to determine the population-level standard deviation. Peptides with a SILAC ratio outside this standard deviation for all treatment samples were considered as altered in abundance.

RESULTS

Substantial Proteasome Inhibition is Required to Block Degradation of Model Optical UPS Reporters—Impaired ubiquitin-mediated protein degradation has been implicated in a variety of human pathologies (42). These observations have driven the development of tools and methods to measure ubiquitin-proteasome system (UPS) function in cells and tissues. Model unstable UPS reporter proteins generated by fusing degron sequences to GFP are utilized as optical probes to measure...
cellular UPS function (16, 43). As such, we employed cell lines stably expressing three different optical UPS reporters to determine the sensitivity of each reporter to proteasome inhibition. These reporters were chosen because they have been widely utilized as optical UPS sensors and they use largely distinct ubiquitylation components to facilitate their degradation (supplemental Fig. S1). As such, they report on three independent paths to proteasomal degradation. To evaluate the sensitivity of each of the optical UPS reporters to proteasome inhibition, we treated each reporter cell line with increasing amounts of the irreversible proteasome inhibitor epoxomicin. As expected, epoxomicin treatment did not result in the accumulation of untagged GFP as determined from a cell line expressing Ub-M-GFP which generates an untagged GFP protein upon proteolytic cleavage of the ubiquitin moiety by cellular deubiquitylating enzymes (supplemental Fig. S1). All unstable reporter proteins accumulated upon treatment with the highest concentration of epoxomicin compared with loading controls (Fig. 1A). Epoxomicin concentrations below 10 nM were insufficient to block degradation of the reporter proteins and 25 nM epoxomicin was the minimum dose required to observe reporter accumulation in all three cell lines (Fig. 1A). To correlate the level of enzymatic proteasome inhibition observed at similar epoxomicin treatments, we performed peptide-based proteasome activity assays from cell lysates treated with the same range of epoxomicin concentrations. More than 60% of proteasome activity was inhibited at the minimum epoxomicin concentration required to observe reporter accumulation across each cell line (Fig. 1B). Consistent with previous studies (7, 20), these observations suggest that substantial proteasome inhibition is required to observe any accumulation of commonly utilized optical UPS reporter proteins.

**Sensitivity Determination of the Ubiquitin-modified Proteome to Proteasome Inhibition Using Label-free Proteomics**—One of the limitations of using the abundance of a single engineered or endogenous reporter protein as a proxy for cellular UPS activity is their limited ability to report on global UPS activity. This limitation stems from the small number of UPS components that are utilized to catalyze the degradation of an individual reporter protein. Proteomic approaches can interrogate the abundance of the endogenous ubiquitin-modified proteome and offer a more unbiased and comprehensive solution to the limitations of using single model substrates to report on UPS activity. Immunoaffinity isolation of the diGlycine (diGLY) ubiquitin remnant remaining after tryptic digestion of ubiquitylated proteins can be utilized to identify and quantify the ub-modified proteome (22, 44). Even though this approach does not unambiguously identify ubiquitylated proteins, we will refer to the resulting data as the ub-modified proteome because of the observation that over 94% of all diGLY-modified proteins arise from ubiquitylation events (23). Despite wide application of the diGLY-enrichment proteomics approach, the sensitivity of the ub-modified proteome to proteasome inhibition has yet to be determined.

We first utilized a label-free proteomics approach to evaluate the response of the ub-modified proteome to proteasome inhibition as metabolic labeling techniques would not be available for clinically relevant human or mouse samples. In a manner similar to what was used for cell lines expressing

---

**Fig. 1. Substantial levels of proteasome inhibition are required to accumulate exogenous UPS reporter substrates.** A, HCT116 cells with stable expression of GFPu, Ub-M-GFP, Ub-R-GFP, and UFD-GFP were treated with the indicated concentration of epoxomicin for 8 h. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. (s) and (l) denote short and long exposures, respectively. B, HCT116 cells were treated with the indicated concentration of epoxomicin for 8 h and the chymotryptic activity of the proteasome was measured in whole cell extracts. Error bars represent S.E. of triplicate measurements. See also supplemental Fig. S1.
Spatial Separation of Functionally Distinct Ubiquitylation Events

optical UPS reporter proteins, unlabeled HCT116 cells were treated with increasing concentrations of epoxomicin for 8 h. Western blotting revealed that a minimum concentration of 10 nm epoxomicin was required to visualize subtle abundance changes in total ubiquitylation levels as well as the well-characterized ub-modified proteins NRF2 and Histone H2b (Fig. 2A). We utilized diGLY-remnant immunoaffinity approaches followed by mass spectrometry to determine how the ub-modified proteome was altered across the same range of epoxomicin concentrations. Over 4500 unique ubiquitylated sites across 1800 proteins were identified using a label-free approach (supplemental Tables S1–S3). Although ~200 ub-modified peptides displayed statistically significant alterations at epoxomicin concentrations below 10 nm, large alterations in the ub-modified proteome were only visible at concentrations of 10 nm and higher (Fig. 2B, 2C supplemental Figs. S2A, S2B).

The ub-modified proteome displayed switch-like behavior across the range of epoxomicin concentrations with a clear transition at 10 nm that is not visible at 5 nm (Fig. 2B, 2C supplemental Table S2). This suggests that a minimum level of proteasome inhibition is required to observe any changes in substrate levels and that proteasome capacity is buffered against inhibition below this minimum level. The same switch-like behavior was apparent for a ub-modified peptide within the well-characterized UPS substrate β-catenin where its levels are undetectable at low epoxomicin concentrations and then dramatically accumulates (more than 100-fold) upon treatment with 10 nm and higher concentrations of epoxomicin (Fig. 2D). Similar observations were made for multiple ubiquitylated peptides of a G-protein complex subunit, GNAS, suggesting it to be a previously uncharacterized highly unstable protein (Fig. 2D). Overall ubiquitylation, as judged by ub-linkage peptides, accumulated at 10 nm epoxomicin but to a lesser overall extent compared with GNAS or β-catenin (supplemental Fig. S2C). Unlike β-catenin which is ubiquitylated for canonical proteasomal targeting (45), histones and a subset of recently described 40S ribosomal proteins undergo regulatory, nondegradative ubiquitylation are reduced upon proteasome inhibition (25). Additional regulatory ubiquitylation events are also substantially reduced upon treatment with 10 nm epoxomicin (Fig. 2D). The reduction of histone monoubiquitylation in response to proteasome inhibition has been hypothesized to arise from the depletion of free ubiquitin levels observed upon proteasome inhibition (46). Thus, we apply this characteristic reduction of ubiquitylation in response to proteasome inhibition to indicate putative regulatory, nondegradative, ubiquitylation events. However, it is possible that existing, low-priority ubiquitylation events that mark proteins for degradation are also reduced upon proteasome inhibition to recycle ubiquitin to be used for more high-priority substrates. Careful examination of individual ubiquitylation events will be necessary to distinguish between these possibilities (25). Taken together, these results suggest that quantitative monitoring of the ub-modified proteome is as much as twofold more sensitive than model optical substrates to report UPS function.

A Narrow Range of Proteasome Inhibition is Required to Observe Quantitative Alterations in the Ubiquitin-modified Proteome—The label-free proteomic approach, although useful in determining the minimum level of proteasome inhibition required to elicit substantial alterations in the ub-modified proteome, lacks the quantitative sensitivity of SILAC-based approaches to observe more subtle but significant changes in ub-modified peptide levels. To obtain this level of quantitative information and to assess the reproducibility of the measurements, we repeated the epoxomicin sensitivity experiment with a narrower range of critical epoxomicin concentrations using SILAC-based proteomics in biological replicate samples. As with the previous label-free experiment, total levels of ubiquitylated proteins and NRF2 accumulated at the highest concentration of epoxomicin (Fig. 3A). However, NRF2 accumulation was only visible at 25 nm epoxomicin and was unaffected at 10 nm (Fig. 3A). This reveals inherent biological noise that likely stems from subtle differences in the cell state at the time of the experiment or in differences between the SILAC growth media versus standard media. To confirm that the proteasome was inhibited at 10 nm epoxomicin, direct measurement of proteasome activity in the same cell lysates demonstrated more than 50% proteasome inhibition upon treatment with 10 nm epoxomicin, which rose to near complete inhibition with 25 nm epoxomicin (Fig. 3B). These results are similar to what we observed previously and suggest that cells are buffered against substantial levels of proteasome inhibition and individual reporters can vary in their response to proteasome treatment lessening their impact in highly variable clinical settings.

Using SILAC-based proteomics coupled with diGLY-affinity enrichment of ub-modified peptides over the same range of epoxomicin concentrations resulted in the identification and quantification of more than 9000 individual ubiquitylation events across more than 3000 proteins (supplemental Tables S4–S6). Similar to what we observed using label-free approaches, examination of population-level dynamics of the ub-modified proteome over a range of epoxomicin doses demonstrated a switch-like behavior. Unlike the previous experiment, substantial alteration of the global ub-modified proteome is only apparent at 25 nm epoxomicin but not at lower concentrations (Fig. 3C–3E). However, a small but consistent set of ub-modified peptides were altered at 10 and 25 nm epoxomicin across all experiments (supplemental Fig. S3C). These results suggest that there is increased confidence in measuring protein homeostasis function when a diverse set of ubiquitylation targets are measured rather than individual reporters that are subject to environmental variability. Quantitative proteomics measurement of total protein abundance did not reveal substantial alterations at any epoxomicin concentration (supplemental Fig. S3A supplemental Table S5).
Spatial Separation of Functionally Distinct Ubiquitylation Events

A

IB: Ub
IB: NRF2
IB: Ub-H2B
IB: Tubulin

B

1 Relative Normalized diGLY SCs 30

C

sites that are 2-fold altered by SC

sites with pvalue<0.05 compared to untreated

D

CTNNB1-K508

GNAS

RPS10

HIST1H2BC

RPS2

GNB2L1

K981

K950

K981

K859

K138

K275

K106

K121

K106

K121
Further, the gross alterations in the abundance of the ubiquitylated GNAS protein was undetectable upon induction of expression and validate our findings using ub-proteomics and establish epitope-tagged versions of GNAS. Exogenous GNAS experiments (Fig. 4A). This reduction was mirrored by the known monoubiquitylated protein histone H2A which suggests the ribosomal proteins RPS10, RPS2, and GNB2L1 (RACK1) are similarly monoubiquitylated or otherwise modified in a regulatory fashion. Our recent characterization of 40S ribosome regulatory ubiquitylation (RRub) validated that RPS2 is indeed monoubiquitylated in a site-specific manner (25). Conversely, the unstable proteins CTNNB1 (β-catenin), DDIT4, and GADD45A (DDIT1) displayed increased ubiquitylation upon 10 nm epoxomicin treatment (Fig. 4A). Taken together, these results suggest that the ubiquitylation of known unstable proteins can be detected at a level of proteasome inhibition that is insufficient to cause global changes in ubiquitin dynamics that is reflected by the demodification of regulatory ubiquitylation events (46). The G-protein GNAS, which functions downstream of G-protein coupled receptors, also contained multiple ubiquitylated peptides that increased in abundance at lower epoxomicin concentrations in all experiments (Fig. 4B). As GNAS was not previously demonstrated to be a particularly unstable protein, we sought to validate this finding by generating inducible cell lines that express epitope-tagged versions of GNAS. Exogenous GNAS protein was undetectable upon induction of expression and could only be visualized upon proteasome inhibition (Fig. 4C). Epoxomicin concentrations of 10 nm were sufficient to observe GNAS accumulation with higher concentrations resulting in more robust GNAS accumulation that was not observed in control GFP-expressing cell lines (Fig. 4C). These results validate our findings using ub-proteomics and establish GNAS as a highly unstable protein whose abundance is sensitive to proteasome function. Overall, our observations demonstrate that the abundance of a small subset of ubiquitylated proteins is reproducibly altered at levels of proteasome inhibition that does not result in global UPS dysfunction. Further, these results suggest that the development of targeted mass-spectrometry based assays for this suite of ubiquitylated peptides may be employed to detect proteasome dysfunction prior to overt proteasome-level alterations.

Spatial Separation of Functionally Distinct Ubiquitylation Events

Site-specificity of Ub-modification Suggests Diverse Functional Outputs—We noted that proteasome inhibition resulted in both increased and decreased abundance of individual ubiquitylated peptides that mapped to the same protein. This site-specificity may indicate that individual ubiquitylation events on a single protein may represent distinct functional outputs (35). To begin to examine this, we simply asked if specific cellular functions were associated with the most highly ubiquitylated proteins in cells. We performed gene ontology enrichment analysis on the 300 most abundant ubiquitylated proteins in this study. This analysis revealed that specific cellular functions like anion transport and translation were indeed enriched among the most abundantly ubiquitylated proteins (Fig. 5A). Examination of the individual ubiquitylated peptides among these two classes of proteins revealed a striking divergence in the behavior of specific ubiquitylated peptides in response to proteasome inhibition. For example, the transmembrane domain-containing proteins SLC29A1, SLC3A2, and SLC6A8 which are involved in transport functions across the plasma membrane all contained multiple ubiquitylated peptides whose abundance was reproducibly altered upon epoxomicin treatment (Fig. 5B). However, distinct ubiquitylated peptides increased in abundance upon epoxomicin treatment whereas others decreased in abundance within the same protein. Interestingly, nearly all lysine residues observed to be ubiquitylated in predicted extracellular domains of these proteins increased in abundance upon proteasome inhibition whereas those present within intracellular domains decreased in abundance (Fig. 5B). This divergent behavior is particularly apparent within SLC3A2. All intracellular ubiquitylated lysines detected on SLC3A2 decrease in abundance upon proteasome inhibition and the extent of this decrease abates in lysine residues that are more proximal to the transmembrane...
**Fig. 3.** Alterations in the abundance of the diGLY-modified proteome are a sensitive indicator of proteasome inhibition. 

A, HCT116 cells grown in media containing $^{13}$C$^{15}$N lysine (K8) were treated with the indicated concentration of epoxomicin for 8 h. Replicate experiments performed on different days are shown. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. 

B, SILAC labeled HCT116 cells were treated with the indicated concentration of epoxomicin for 8 h and the chymotryptic activity of the proteasome was measured in whole cell extracts. Error bars represent S.E. of triplicate measurements. 

C, Distributions of the SILAC ratios (Log2 transformed) for all quantified diGLY-modified peptides from untreated, light (K0) labeled cells mixed with heavy (K8) labeled cells treated with the indicated epoxomicin concentration for 8 h. The data for biological replicate experiments is shown with the total number of quantified diGLY-modified sites indicated. 

D, Total number (top) or fraction (bottom) of diGLY-modified sites whose abundance changed more than 1 standard deviation (S.D.) as measured by the SILAC ratio for each indicated concentration of epoxomicin. The standard deviation of the SILAC ratio from untreated cells was used to determine cutoffs. 

E, Log2 SILAC peptide ratios for all diGLY-modified peptides that were quantified in both replicate experiments at the indicated epoxomicin treatment is depicted. The best-fit line from linear regression analysis is shown as the coefficient of determination. See also supplemental Fig. S3 and supplemental Tables S4–S6.
Transmembrane-domain containing proteins are known to be monoubiquitylated in a regulatory manner on intracellular facing lysines to stimulate endocytosis of receptor proteins (38). Our observation that intracellular facing lysine residues decrease in abundance upon proteasome inhibition is consistent with the hypothesis that these ubiquitylation events are regulatory in nature and likely occurs on the plasma-membrane localized portion of these proteins. In stark contrast, ub-modified lysine residues found within the extracellular domain all increase in abundance upon proteasome inhibition (Fig. 5B). Components of the translation machinery were also highly enriched when examining the most abundantly ubiquitylated proteins in the cell (Fig. 5A, 5C). Ubiquitylation of core ribosomal proteins as well as various translation elongation and initiation factors can be detected in untreated cells as well as upon proteasome inhibition. These proteins also represent some of the most abundant proteins within the cell and their ubiquitylation may simply reflect that a portion of these highly abundant proteins are defective translation products that require ubiquitin-dependent degradation. If this explanation was valid we would predict that the abundance of those ubiquitylation events would increase upon proteasome inhibition. Although this is the case for a subset of ubiquitylation events on translation associated proteins, the majority of ubiquitylation events on these highly abundant proteins decreased upon proteasome inhibition (Fig. 5C, supplemental Table S7). These ubiquitylation events are site-specific as individual ribosomal proteins contain distinct lysine residues that are used for proteasomal targeting and others that are

Spatial Separation of Functionally Distinct Ubiquitylation Events

Fig. 4. Accumulation of unstable ubiquitin-modified proteins occurs at levels of proteasome inhibition that do not display overt alterations in ubiquitin homeostasis. A, Log2 SILAC peptide ratios for individual diGLY-modified peptides from RPS10, RPS2, GNB2L1, Histone H2A, and CUL5 (left), β-catenin (middle), or DDIT4 and GADD45A are depicted for the indicated epoxomicin concentration. Error bars represent S.D. from replicate experiments. The individual diGLY-modified lysine for each protein is indicated. B, Log2 SILAC peptide ratios for individual diGLY-modified peptides from GNAS. Error bars represent S.D. from replicate experiments. The individual diGLY-modified lysine for GNAS is indicated. C, 293T cells with stable expression of His-Myc (HM) tagged GFP or GNAS were treated with increasing concentrations of epoxomicin for 8 h (left) or with 500 nM epoxomicin for the indicated times (right). Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. (s) and (l) denote short and long exposures, respectively.
likely regulatory in nature (Fig. 5D). We have previously demonstrated that ubiquitylation of some of these 40S ribosomal proteins (K58 and K275 on RPS2) is regulatory in nature and can be stimulated by translation elongation inhibition and activation of the unfolded protein response (25). Taken together, these results suggest the regulatory ubiquitylation of the translation machinery is pervasive and that site-specific ubiquitylation events signify distinct functional outputs.

Quantitative Characterization of the Subcellular Ub-modified Proteome—Ubiquitylation of proteins can vary in both

---

**Fig. 5.** Distinct alterations in abundance of individual diGLY-modified peptides within a protein imply alternative functional outputs. A, Selected enriched gene ontology biological process categories from proteins comprising the 300 most abundant diGLY-modified peptides as judged by spectral counting. The -Log10 of the p values of enrichment for depicted categories is shown. B, Log2 SILAC peptide ratios (top) and diGLY-peptide spectral counts (bottom) for individual diGLY-modified peptides from SLC29A1 (left), SLC3A2 (middle), or SLC6A8 (right) are depicted for the indicated epoxomicin concentration. Error bars represent S.E. from replicate peptide measurements. The individual diGLY-modified lysine for each protein is indicated. The predicted topology of each transmembrane protein is indicated as predicted by topcons (63). C, The SILAC ratio (H-500 nM Epox: L-untreated) for diGLY-modified peptides from all proteins with direct roles in translation initiation or elongation is depicted. Only diGLY-modified peptides with greater than 10 spectral counts are depicted. D, Log2 SILAC peptide ratios for individual diGLY-modified peptides from RPS2, RPS3, RPS20, and RPS10 are depicted for the indicated epoxomicin concentration. Error bars represent S.E. from replicate peptide measurements. The individual diGLY-modified lysine for each protein is indicated. See also supplemental Table S7.
Spatial Separation of Functionally Distinct Ubiquitylation Events

subcellular location and outcome. Such variation could account for our observation that a single ribosomal protein, such as RPS3, contains ub-modified lysines that are either ubiquitylated or deubiquitylated in response to proteasome inhibition. To identify and separate ubiquitylation events that take place in distinct subcellular compartments and may have distinct functional outputs, we carried out extensive subcellular fractionation of native cell lysates prior to quantitative analysis of the ub-modified population of each sample by mass spectrometry. Specifically, we fractionated whole cell lysates into six subcellular fractions using a differential centrifugation scheme (Fig. 6A).

We first validated the fidelity of our fractionation scheme using both immunoblotting and mass spectrometry (Fig. 6B, supplemental Fig. S4A, S4B, supplemental Table S8). Effective separation was apparent by the observation that known nucleolar localized preribosomal assembly factors were contained within the chromatin bound fraction (CBF) and mitochondrial ribosomes were located within the heavy mitochondrial fraction (HMF) (Fig. 6C). As expected, ribosomes were broadly distributed across many fractions (Fig. 6D–6H). Interestingly, spectral counting and immunoblotting provided evidence that the purified cytoplasmic fraction (PCF) of unperurbed cells contained relatively small quantities of ribosomal proteins; these likely represent ribosomal components that are not assembled with the larger ribosomal subunits (Fig. 6B, 6D).

To quantify individual ub-modified peptides in specific subcellular fractions that putatively report on functionally distinct ubiquitylation events, we combined our SILAC and immuno-affinity approaches with subcellular fractionation. We quantitatively monitored changes in abundance of ub-modified peptides upon proteasome inhibition or activation of the unfolded protein response (UPR) because we have previously demonstrated that a subset of regulatory 40S ribosomal ubiquitylation (RRub) events is stimulated by cell stressors that activate the UPR (25). The majority of ub-modified peptides were identified in the crude microsomal and purified cytoplasmic fractions (CMF, PCF) with few ub-modified peptides in mitochondria-enriched fractions (HMF, CMF) (Fig. S4C, Tables S8–S10). Examination of well-established ub-modified proteins validated our fractionation approach (supplemental Fig. S4D). For example, ub-modified peptides from HIF1α and DDIT4, known targets of ub-mediated proteasomal degradation, were almost exclusively identified in the PCF and increased in abundance more than 16 fold in response to proteasome inhibition (supplemental Fig. S4D). Thus, subcellular fractionation coupled with SILAC-based quantitative ub proteomics effectively interrogates alterations in ub-modification in specific subcellular fractions.

Regulatory and Degradative Ubiquitylation Events on Ribosomal Proteins Occur in Distinct Subcellular Fractions—As ubiquitylation of ribosomal proteins plays a role during ribosome biogenesis, some of the observed ubiquitylation of ribosomal proteins might be part of the ribosome assembly cascade (47). Further, ubiquitylation of ribosomal proteins could take place on unassembled ribosomal proteins rather than on assembled ribosomes to target unassembled proteins for degradation. To evaluate these possibilities, we utilized our proteomic data generated from subcellular fractionation followed by ub-modified peptide enrichment and analysis by mass spectrometry to examine ubiquitylation events on ribosomal proteins. In addition to treatment with epoxomicin, we treated cells with DTT, a stressor of the endoplasmic reticulum that causes changes in the ub-modified proteome that are distinct from proteasome inhibition (25). Our analysis identified ub-modified ribosomal proteins in three fractions of note. Although ribosomal proteins were found in many subcellular fractions, greater than 50% of all ub-modified ribosomal proteins were identified in the crude microsomal fraction in DTT treated cells (Fig. 6D, 6E). We also detected ub-modified ribosomal peptides in the chromatin bound fraction, presumably reflective of ubiquitylation events within the ribosome assembly pathway. Furthermore, whereas fewer than 5% of all ribosomal protein spectral counts were found in the purified cytoplasmic fraction, more than 10% of all ub-modified ribosomal peptides were observed in the purified cytoplasmic fraction upon DTT treatment (Fig. 6D, 6E). Our observation that the bulk of ubiquitylated ribosomal peptides could be found in the crude microsomal fraction upon DTT treatment suggests that the majority of ribosomal ubiquitylation takes place on assembled ribosomal subunits (supplemental Tables S9–S10). Interestingly, this fraction of ubiquitylated ribosomal peptides found in the purified cytoplasmic fraction rose to more than 70% upon epoxomicin treatment, indicating a dramatic effect of proteasome inhibition on the accumulation of ub-modified, unassembled ribosomal proteins likely resulting from misfolded translation products (Fig. 6E).

If site-specific ubiquitylation of 40S ribosomal proteins regulates protein biogenesis, we would expect to find the corresponding ub-modified peptides in the same subcellular fractions as assembled ribosomal complexes. We first reexamined the abundance of individual ub-modified peptides from 40S proteins upon proteasome inhibition, distinguishing those that are regulatory in nature versus those that likely trigger degradation (Fig. 6F). Individual ub-modified peptides from RPS20, RPS10, and RPS2 that decrease in abundance in response to proteasome inhibition resided exclusively in the crude microsomal fraction and were similarly decreased in abundance upon proteasome inhibition (Fig. 6F). In contrast, the majority of ub-modified peptides from RPS3 were found exclusively in the purified cytoplasmic fraction and those peptides increased in abundance upon proteasome inhibition (Fig. 6F). The sole exception was the ub-modified peptide comprising K214 on RPS3, which was only found in the crude microsomal fraction and which decreased in abundance upon epoxomicin treatment (Fig. 6F). These observations indicate
**Fig. 6.** Distinct ubiquitylation events on ribosomal proteins occur on spatially separated protein populations. A, Schematic of the subcellular fractionation method. B, Subcellular fractions were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. C, The total proteome from each subcellular fraction was analyzed by mass spectrometry. The percentage of total spectral counts (TSCs) that correspond to known preribosome assembly factors (red bars) and mitochondrial ribosomal proteins (blue bars) is shown for each subcellular fraction. D, The percentage of total spectral counts (TSCs) that correspond to 40S (red bars) and 60S proteins (blue bars) is shown for each subcellular fraction analyzed by mass spectrometry.

E, Heavy (K8) lysine labeled HCT116 cells were treated with DTT or Epox and mixed with light-labeled control cells prior to subcellular fractionation. Histogram indicates the percentage of all diGLY-modified peptide spectral counts that correspond to cytosolic ribosomal proteins across the fractions in each experimental condition. F–G, Heavy labeled cells were treated with Epox (F) or DTT (G) and fractioned prior to quantitative analysis of the diGLY-modified proteome from each fraction. The diGLY-peptide spectral counts (SCs) (top) or the Log2 SILAC ratio (bottom) for individual diGLY-modified peptides from the 40S proteins RPS10 (10), RPS2 (2), RPS20 (20) or RPS3 (3) in either the crude microsomal fraction (CMF, blue bars) or the purified cytosolic fraction (PCF, red bars) is depicted. The position of the diGLY-modified lysine residue is indicated. See also supplemental Fig. S4 and supplemental Tables S8–S10.
that most of the ubiquitylation events on RPS3 occur on unassembled RPS3 proteins and likely target the protein for destruction whereas ubiquitylation of RPS3 on lysine 214 is functionally distinct and occurs within the context of the assembled ribosome. This confirms our previous observations that regulatory monoubiquitylation of RPS3 on K214 is stimulated by UPR activation and translation elongation inhibition and occurs on assembled ribosomal particles as judged by density centrifugation analysis (25). Our demonstration that ~10–20% of total RPS2 and RPS3 undergoes regulatory ubiquitylation in response to UPR activation suggests that other ribosomal regulatory modifications may be considerably higher stoichiometry events compared with degradation-based ubiquitylation events on the same proteins. The results with DTT treatment were markedly different from those with proteasome inhibition. Nearly all ub-modified peptides from the same set of 40S ribosomal proteins were found in the crude microsomal fraction upon DTT treatment. However, only those ub-modified peptides that decreased in abundance in response to proteasome inhibition, marking putative regulatory ubiquitylation events, increased in abundance upon DTT treatment (Fig. 6G). Thus subcellular fractionation prior to ub-modified peptide enrichment effectively distinguishes likely regulatory ubiquitylation of ribosomal proteins from ubiquitylation events that trigger degradation. Further, these results are consistent with previous studies showing that DTT-induced ubiquitylation of the 40S proteins RPS2 and RPS3 occurs on fully assembled ribosomal particles (25).

**VCP Inhibition Induces Distinct Alterations to the Ub-modified Proteome Compared with Proteasome Inhibition**—Since the initial demonstration that proteasome inhibitors could be used to treat multiple myeloma there has been increased interest in developing inhibitors targeting distinct components of the ubiquitin proteasome system (48). Valosin-containing protein (VCP, also known as p97) is an abundant and essential AAA ATPase that has pleiotropic functions within the ubiquitin proteasome system (49). The ability of VCP to facilitate the retro-translocation of ER-associated degradation (ERAD) substrates prior to proteasome-mediated degradation is one of the more well-characterized roles for VCP (50). Several VCP inhibitors have been developed with more recent VCP ATPase inhibitors showing antitumor activity in xenograft tumor models (39, 51, 52). Motivated by our results demonstrating that the ub-modified proteome can be used to monitor protein homeostasis dysfunction upon proteasome inhibition or distinct challenge with an ER stressor, we employed our quantitative ub-proteomics strategy to interrogate the impact of pharmacological VCP inhibition on the ub-modified proteome. We utilized a recently described potent and specific VCP inhibitor that targets the second AAA-ATPase domain (D2) within VCP (CB-5083) (39). CB-5083 treatment of HCT116 cells resulted in the accumulation of poly-ubiquitylated material over an 8 h time course albeit to a lesser extent than proteasome inhibition (Fig. 7A). SILAC-based quantitative proteomics of the ub-modified proteome demonstrated that VCP inhibition resulted in time-dependent alterations in the abundance of the ub-modified proteome with more than 35% of all ub-modified peptides displaying significant abundance changes after 8 h of VCP inhibition (Fig. 7B, 7C supplemental Table S11). Monitoring of the total proteome did not reveal substantial changes upon VCP inhibition (supplemental Fig. S5A, supplemental Table S12). Many of the same ub-modified peptides whose abundance was altered by VCP inhibition were also altered by proteasome inhibition (Fig. 7D). However, direct comparison of abundance changes of individual ub-modified peptides between VCP and proteasome inhibition revealed little correlation suggesting that VCP inhibition induces largely unique perturbations to the ub-modified proteome compared with proteasome inhibition (Fig. 7E, supplemental Fig. S5B, supplemental Table S13).

Similar to proteasome inhibition, VCP inhibition results in substantial changes to overall ubiquitin dynamics. VCP inhibition resulted in the broad accumulation of polyubiquitin chains as well as a decrease in the abundance of monoubiquitylated histones (Fig. 7F). This response is indicative of a loss of ubiquitin homeostasis that also occurs upon proteasome inhibition. However, the extent of histone deubiquitylation was substantially lower upon VCP inhibition compared with proteasome inhibition suggesting that VCP inhibition induces a less severe ubiquitin starvation effect. Previous studies have demonstrated that VCP inhibition induces the UPR which likely stems from an inability to remove misfolded proteins from the ER upon VCP inhibition (39). Consistent with this, we observed an accumulation of ubiquitylated peptides corresponding to several transmembrane domain containing proteins upon VCP inhibition (supplemental Tables S12, S15). These same ub-modified peptides were also demonstrated to accumulate upon canonical UPR induction using DTT. Direct comparison of alterations in the total ub-modified proteome upon UPR activation or VCP inhibition did not reveal a correlation (supplemental Fig. S5C). However, comparison of the alterations in only ubiquitylation of SLC-family proteins, which are largely transmembrane proteins targeted plasma membrane, revealed a modest correlation between DTT and CB-5083 treatment that was not observed when comparing VCP inhibition with proteasome inhibition (supplemental Fig. S5D, supplemental Table S15). Taken together, these observations suggest that individual ub-modification events on these plasma-membrane localized proteins are reversed upon proteasome inhibition, indicative of regulatory modification, but stimulated upon UPR activation and VCP inhibition. Interestingly, even though VCP inhibition induces the UPR it does not result in elevated RRub on RPS2 and RPS20 observed upon DTT treatment (Fig. 7F). This suggests that alterations in ubiquitin homeostasis observed upon VCP inhibition may limit the ability to modify the highly abundant ribosomal proteins despite UPR induction. VCP inhibition induced similar distinct effects on the ub-modified population of DDI2, EIF3I, and PPP2R2A.
Spatial Separation of Functionally Distinct Ubiquitylation Events

Fig. 7. 

VCP inhibition alters the ubiquitylation patterns in a manner largely distinct from proteasome inhibition. 

A, HCT116 cells were treated with either 1 μM VCP inhibitor (CB-5083) or 10 μM MG132 for the indicated times. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. B, Distributions of the SILAC ratios (Log2 H:L) for all quantified diGLY-modified peptides from untreated, light (K0) labeled cells mixed with heavy (K8) labeled cells treated with 1 μM CB-5083 for the indicated times (hr). C, The fraction of unique diGLY-modified sites whose abundance changed more than 1 standard deviation (S.D.) as measured by the SILAC ratio for each indicated 1 μM CB-5083 treatment. The standard deviation of the SILAC ratio from untreated cells was used to determine cutoffs. D, All diGLY-modified peptides that increased or decreased in abundance upon either treatment with epoxomicin (500 nM) or CB-5083(1 μM) for 8 h and were quantified in both experiments are compared. The overlap represents diGLY-modified peptides whose abundance was altered by 2-fold upon both treatments regardless of direction of change. E, Log2 SILAC peptide ratios for individual diGLY-modified peptides from ubiquitin (top, left), histone proteins (top, center), RPS2 or RPS20 (top, right), DDI2 (bottom, left), and EIF3I or PPP2R2A (bottom, right) are depicted for the indicated drug treatment. Error bars represent S.E. from replicate peptide measurements. The individual diGLY-modified lysine for each protein is indicated. See also supplemental Fig. S5 and supplemental Tables S11–S15.
Spatial Separation of Functionally Distinct Ubiquitylation Events

(Fig. 7G). In total, our results demonstrate that VCP inhibition induces global and significant alterations to the ub-modified proteome that are largely unique to those induced by direct proteasome inhibition. These results also establish the validity of our ub-proteomics approach to precisely determine how individual protein ubiquitylation events are impacted by distinct protein homeostasis insults.

DISCUSSION

Protein homeostasis imbalance is a central feature of protein misfolding disorders that include several neurodegenerative diseases (6). There is growing evidence that UPS dysfunction occurs during the development of neurodegenerative disease in both mice and man (5, 53), but whether UPS dysfunction is a driver of disease progression or merely a result of a collapsing protein homeostasis system is unknown. Regardless, the ability to directly monitor global protein homeostasis function would assist in determining the relative timing of UPS dysfunction to disease onset and may identify key points of intervention. Our development and utilization of quantitative proteomics approaches to monitor the ubiquitin-modified proteome allows for the global characterization of endogenous ubiquitylation events in any system (23, 25). The ability to directly monitor these key degradation intermediates allows access to monitoring the fidelity of protein production and destruction. We demonstrate that large scale alterations to the ub-modified proteome occur at similar levels of proteasome inhibition that are required to accumulate well-characterized exogenously expressed optical UPS reporter proteins. This demonstrates the efficacy of our approach and suggests that utilization of similar approaches will illuminate the impact and timing of protein homeostasis dysfunction during the progression of neurodegenerative disease development across model systems.

Surprisingly, we find that global disruption of the ub-modified proteome only occurs after substantial enzymatic proteasome inhibition. This result suggests that cells have a large “overhead” of protein degradation capacity that can buffer against moderate to large fluctuations in proteasome activity. Previous characterization of a subset of optical reporters demonstrated a similar robust cellular capacity to tolerate a large degree of proteasome inhibition before substrate accumulation could be detected (7, 20). However, similar determinations have not been done in neuronal cell populations which are the most vulnerable cell type to protein homeostasis dysfunction. It remains possible that neurons have a lower proteasomal “overhead,” which make them more sensitive to protein homeostasis perturbations. Further, it remains unclear if UPS capacity can be elevated through pharmacological or genetic methods. Similar quantitative proteomic methods to evaluate the sensitivity of the ub-modified proteome to stress, as demonstrated in this study with nonneuronal cells, will be necessary to evaluate the efficacy of alerting protein homeostasis capacity in neuronal populations.

Protein ubiquitylation has been extensively studied for decades and the enzymatic machinery that participates in ubiquitylation has been amazingly well-characterized both functionally and structurally (35, 36, 54). Despite this extensive knowledgebase, the details regarding the function of individual ubiquitylation events for specific substrates are restricted to a small subset of highly regulated targets. The recent implementation of quantitative ubiquitin-proteomics approaches like the ones utilized in this study has led to an explosion of the number of endogenous ubiquitylation events that have been identified (22). However, the function of these ubiquitylation events and where in the cell they occur is unknown for ~99% of demonstrated ubiquitylated proteins. It is likely that ~60% of ubiquitylation events target proteins for degradation given our observation that these ubiquitylation events accumulate upon proteasome inhibition. Interestingly, nearly all of these ubiquitylation events increase in abundance without any evidence of a corresponding increase in overall protein levels. This observation is consistent with the hypothesis that only a small subpopulation of the total protein population is targeted for degradation for the vast majority of UPS substrates (23, 28). This subpopulation likely originates from terminally defective translation products that are targeted for degradation either co-translationally or shortly after release from the ribosome (4). Previous studies have demonstrated that ~10% of nascent chains are co-translationally ubiquitylated and this number can be elevated upon treatment with proteasome inhibitors (55–57). Consistent with this idea is the observation that ~70% of ubiquitylation events that increase upon proteasome inhibition required ongoing protein synthesis (23). By combining subcellular fractionation and ub-proteomics we were able to demonstrate that, upon proteasome inhibition, ~70% of all ubiquitylation events occur in the purified cytoplasmic fraction which is where we would expect to find ubiquitylated newly synthesized proteins. This localization profile is in stark contrast to what was observed in untreated or DTT treated cells as most ubiquitylation events were found in heavier crude microsomal fractions. These findings suggest that proteasome inhibition results in substantial alteration of both the identity and the location of the ubiquitylated proteome and that quantitative ub-proteomics can detect these informative defective translation products.

Although the function of most ubiquitylation events appears to be canonical proteasomal targeted, it is clear that a substantial fraction of protein ubiquitylation impacts substrate function separate from regulating protein turnover. Indeed, a simple investigation into the most prevalent ubiquitylation targets revealed that the majority of the 300 most abundant ubiquitylation events in the cell decrease in abundance upon proteasome inhibition which we interpret to infer a regulatory, nondegradative function although we cannot rule out that the modification is a low-priority degradative event (Fig. 5C). The ribosome and proteasome represent two of the most abundant complexes within the cell and each contains many ub-
modified protein constituents that undergo regulatory ubiquitylation (Fig. 5C). Our utilization of spatial proteomics clearly separated ubiquitylation events on the ribosome that occurred within the context of the larger ribosomal complex versus those that target individual proteins for degradation (Fig. 6). We have demonstrated that a subset of these ubiquitylation events on 40S proteins is stimulated by activation of the unfolded protein response and translation elongation inhibition and loss of these ubiquitylation events leads to enhanced cell death upon UPR stimulation (25). However, determination of the precise molecular functions imparted by individual regulatory ubiquitylation will require in-depth mechanistic studies. The reversal of regulatory ubiquitylation upon proteasome inhibition suggests that the development of more targeted mass spectrometry methods to track these events may be useful for monitoring global ubiquitin dynamics upon protein homeostasis dysfunction. Recent advances in targeted mass spectrometry methods may now accommodate the development of assays to track some of the more relevant and sensitive ubiquitylation events described in this study (58). Such PTM-centric assays would allow for detailed and quantitative evaluations of protein homeostasis function across a wider range of clinically relevant samples.

It is clear from cancer sequencing efforts that most cancers that afflict people later in life accumulate thousands of genomic alterations (59). The impact of the majority of these mutations on the resulting protein is often unknown. It has been hypothesized that cancer proteomes are more unstable because of this accumulation of presumably destabilizing mutations and that targeting the protein homeostasis machinery may be an effective anticancer strategy (13). The successful development of proteasome inhibitors to treat multiple myeloma provides support for this strategy and has fueled the development of small molecular inhibitors for other UPS components (48, 60, 61). Indeed, the recent development of potent small molecular inhibitors for the essential AAA ATPase VCP and the demonstration that these inhibitors can reduce tumorigenesis in mouse xenograft models further validates this approach (39). Both proteasome and VCP inhibition result in potent activation of the unfolded protein response which may explain why proteasome inhibitors work well in the context of multiple myeloma (39, 62). Despite these commonalities, our results demonstrate that VCP inhibition and proteasome inhibition results in largely distinct alterations to the ub-modified proteome and suggests that VCP inhibitors may elicit antitumor effects in a unique manner compared with proteasome inhibition. Utilization of ub-proteomics will be an invaluable tool to investigate how future chemical modulators of the ubiquitin-proteasome system impact global protein homeostasis fidelity mechanisms.

Acknowledgments—We thank D. Anderson and M. Rolfe (Cleave Biosciences) for providing the VCP inhibitor CB-5083 and S. Beausoleil (Cell Signaling Technologies) for diGLY antibodies. We thank W. Partlo (UCSD) for initial subcellular fractionation methods development.

* This work was supported by New Scholar awards from the Sidney Kimmel Foundation for Cancer Research and the Ellison Medical Foundation, a Hellman Fellowship, and funding from the NIH (PGM085764, GM119132) (E.J.B.). N.Z. is supported by the UCSD Cell and Molecular Genetics Training Program (T32 GM007240).

This article contains supplemental material.

** To whom correspondence should be addressed: Cell and Developmental Biology, UCSD, 9500 Gilman Dr. MC 0377 NSB Room 5316, La Jolla, CA 92093. Tel.: 858-822-7900; E-mail: e1bennett@ucsd.edu.

† Present address: Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT.

¶ This article contains supplemental material.

This article contains supplemental material.

†† Present address: Department of Microbiology Immunology and Pathology, Colorado State University, Fort Collins, CO.

These authors contributed equally to this work.

The authors declare that they have no conflict of interest.

REFERENCES

1. Rodrigo-Brenni, M. C., and Hegde, R. S. (2012) Design principles of protein biosynthesis-coupled quality control. Dev. Cell 23, 896–907

2. Wolf, S., Weissman, J. S., and Dilin, A. (2014) Differential scales of protein quality control. Cell 157, 52–64

3. Lykke-Andersen, J., and Bennett, E. J. (2014) Protecting the proteome: Eukaryotic cotranslational quality control pathways. J. Cell Biol. 204, 467–476

4. Comyn, S. A., Chan, G. T., and Mayor, T. (2014) False start: cotranslational protein ubiquitination and cytosolic protein quality control. J. Proteomics 100, 92–101

5. Koga, H., Kaushik, S., and Cuervo, A. M. (2011) Protein homeostasis and aging: The importance of exquisite quality control. Aging Res. Rev. 10, 205–215

6. Labbadia, J., and Morimoto, R. I. (2015) The biology of proteostasis in aging and disease. Annu. Rev. Biochem. 84, 435–464

7. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. Science 292, 1552–1555

8. Bennett, E. J., Bence, N. F., Jayakumar, R., and Kopito, R. R. (2005) Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. Mol. Cell 17, 351–365

9. Chu, J., Hong, N. A., Masuda, C. A., Jenkins, B. V., Nelms, K. A., Goodnow, C. C., Glyne, R. J., Wu, H., Masilah, E. J., loweiro, C. A., and Kay, S. A. (2009) A mouse forward genetics screen identifies LISTERIN as an E3 ubiquitin ligase involved in neurodegeneration. Proc. Natl. Acad. Sci. U S A 106, 2097–2103

10. Das, I., Krzyzosiak, A., Schneider, K., Wrabetz, L., D’Antonio, M., Barry, N., Sigurdardottir, A., and Bertolotti, A. (2015) Preventing proteostasis diseases by selective inhibition of a phosphatase regulatory subunit. Science 348, 239–242

11. Lee, J. W., Bebe, K., Nangle, L. A., Jang, J., Longo-Guess, C. M., Cook, S. A., Davison, M. T., Sundberg, J. P., Schimmel, P., and Ackerman, S. L. Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. Nature 443, 50–55

12. Ishimura, R., Nagy, G., Dotu, I., Zhou, H., Yang, X. L., Schimmel, P., Senju, S., Nishimura, Y., Chuang, J. H., and Ackerman, S. L. (2014) RNA splicing deficiency in a mouse forward genetics screen identifies LISTERIN as an E3 ubiquitin ligase involved in neurodegeneration. Science 345, 455–459

13. Deshaies, R. J. (2014) Proteotoxic crisis, the ubiquitin-proteasome system, and cancer therapy. BMC Biol. 12, 94

14. Bhat, M., Robichaud, N., Hulea, L., Sonenberg, N., Pelletier, J., and Topisirovic, I. (2015) Targeting the translation machinery in cancer. Nat. Rev. Drug Discov. 14, 261–278

15. Silvera, D., Forment, S. C., and Schneider, R. J. (2010) Translational control in cancer. Nat. Rev. Cancer 10, 254–266

16. Bence, N. F., Bennett, E. J., and Kopito, R. R. (2005) Application and analysis of the GFPu family of ubiquitin-proteasome system reporters. Methods Enzymol. 399, 481–490

17. Salomons, F. A., Acs, K., and Dantuma, N. P. (2010) Illuminating the ubiquitin/proteasome system. Exp. Cell. Res. 316, 1289–1295
ubiquitination pathway for quality control of misfolded proteins. Mol. Cell 50, 368–378
58. Lesur, A., and Domon, B. (2015) Advances in high-resolution accurate mass spectrometry application to targeted proteomics. Proteomics 15, 880–890
59. Garraway, L. A., and Lander, E. S. (2013) Lessons from the cancer genome. Cell 153, 17–37
60. Wang, M., Martin, T. Bensinger, W. Alsina, M. Siegel, D.S. Kavalerchik, E. Huang, M. Orlowski, R.Z. and Niesvizky, R. (2013) Phase 2 dose-expansion study (PX-171–006) of carfilzomib, lenalidomide, and low-dose dexamethasone in relapsed or progressive multiple myeloma. Blood 122, 3122–3128
61. Richardson, P.G., Sonneveld, P., Schuster, M. W., Irwin, D., Stadtmauer, E. A., Facon, T., Harousseau, J. L., Ben-Yehuda, D., Lonial, S., Goldschmidt, H., Reece, D., San-Miguel, J. F., Blade, J., Boccadoro, M., Cavenagh, J., Dalton, W. S., Boral, A. L., Esseltine, D. L., Porter, J. B., Schenkein, D., and Anderson, K. C., (2005) Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N. Engl. J. Med. 352, 2487–2498
62. Obeng, E. A., Carlson, L. M., Gutman, D. M., Harrington, W. J., Jr., Lee, K. P., and Boise, L. H. (2006) Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. Blood 107, 4907–4916