

Early recovery of proteasome activity in cells pulse-treated with proteasome inhibitors is independent of DDI2

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Abstract

Rapid recovery of proteasome activity may contribute to intrinsic and acquired resistance to FDA-approved proteasome inhibitors. Previous studies have demonstrated that the expression of proteasome genes in cells treated with sub-lethal concentrations of proteasome inhibitors is upregulated by the transcription factor Nrf1 (NFE2L1), which is activated by a DDI2 protease. Here, we demonstrate that the recovery of proteasome activity is DDI2-independent and occurs before transcription of proteasomal genes is upregulated but requires protein translation. Thus, mammalian cells possess an additional DDI2 and transcription-independent pathway for the rapid recovery of proteasome activity after proteasome inhibition.

eLife assessment

The study presents **important** findings regarding a transcription-independent component of the early recovery of proteasome activity from a short pulse of proteasome inhibitor treatment, which has not been appreciated before and which is independent of the DDI2-NRF2 axis. While the evidence is in principle **solid**, with recapitulation in several cell line models, the proposed alternative underlying mechanism, namely regulation at the level of proteasome assembly, lacks experimental support, and at this point remain speculative.

Introduction

The ubiquitin-proteasome system is the primary protein quality control pathway in every eukaryotic cell. By degrading numerous regulatory proteins, this pathway also plays a pivotal role in regulating many cellular functions such as cell cycle and gene expression. Malignant cells are more dependent on proteasome function than non-transformed cells because they divide rapidly and produce abnormal proteins at a higher rate than normal cells [1, 2]. Proteasome inhibitors (PIs) bortezomib (Btz), carfilzomib (Cfz), and ixazomib are approved for the treatment of multiple myeloma (MM). Btz is also approved for the treatment of mantle cell lymphoma (MCL).

MM cells are exquisitely sensitive to PIs because the production of immunoglobulins by these malignant plasma cells places an enormous load on the proteasome and other components of the protein quality control machinery [3–6].

Clinically, Btz and Cfz are administered once or twice weekly as a subcutaneous (Btz) or intravenous bolus. They cause rapid inhibition of proteasome activity in the patients' blood but are metabolized rapidly [7]. Within an hour after the administration, PIs concentrations in the blood drop below the levels needed to kill tumor cells *in vitro* [8, 9]. Although Btz has a very slow off-rate and Cfz is an irreversible inhibitor, proteasome activity recovers within 24 hours [6, 8, 10, 11]. This activity recovery may explain discrepancies between robust activity against cell lines derived from various cancers, continuously treated with Btz (www.carcerrxgene.org) [6], and a lack of clinical efficacy except in MM and MCL. In addition, recovery of activity has recently been implicated in PI resistance in MM [12].

In cells treated with PIs, a transcription factor Nrf1 (also known as TCF11, encoded by the *NFE2L1* gene) upregulates transcription of genes encoding all proteasome subunits [13, 14]. When the proteasome is fully functional, Nrf1 is constitutively degraded in a ubiquitin-dependent manner [13]. When the proteasome is partially inhibited, the ubiquitylated Nrf1 is recognized by DDI2 (DNA-Damage-Inducible I Homolog 2), a ubiquitin-dependent aspartic protease that activates Nrf1 by a site-specific cleavage [15, 16]. Although knockdown of DDI2 blocks the PI-induced transcription of proteasome genes [15, 16], initial studies implicating DDI2 in the activation of Nrf1 did not determine whether DDI2/Nrf1-dependent transcription leads to the recovery of activity after clinically relevant pulse treatment with PIs. In this work, we asked whether DDI2 is involved in activity recovery after such treatment. Unexpectedly, we found that proteasome activity recovered in the absence of DDI2, and activity recovery preceded the upregulation of proteasome genes. This data demonstrates the existence of a novel, DDI2-independent pathway for the recovery of proteasome activity in PI-treated cells.

Results

To analyze DDI2 involvement in the recovery of proteasome activity after treatment with PIs, we used commercially available clones of HAP1 cells, in which DDI2 was knocked out by CRISPR, and a clone with an unaltered DDI2, which we will refer to as a wild type (wt, **Fig. 1a**). We analyzed three different clones that were generated by using two different gRNAs (Table S1). We treated cells for 1 hr with a range of concentrations of Cfz and Btz and then cultured them in drug-free media (**Fig. 1b**). We measured inhibition of the proteasome's $\beta 5$ site, which is the prime target of Cfz and Btz [2], immediately after the one-hour treatment, and 12 or 24 hours thereafter (**Fig. 1c**), which is when recovery plateaued (not shown). In a parallel experiment, we used a CellTiter-Glo assay, which measures intracellular ATP levels, to determine cell viability 12 and 24 hours after treatments (**Fig. 1c**). Initial inhibition of proteasome was observed at sub-lethal concentrations, and proteasome activity recovered in cells treated with such concentrations. Surprisingly, no differences in the recovery between wt and DDI2-KO clones were observed (**Fig. 1c**). Deletion of DDI2 did not affect recovery, despite inhibition of Btz-induced proteolytic activation of Nrf1 (**Fig. 1d** and S1c). These findings confirm that DDI2 activates Nrf1, but indicate that it is not involved in the recovery of proteasome activity in Btz and Cfz-treated HAP1 and DDI2 KO cells.

Next, we knocked down DDI2 by two different highly efficient siRNAs in two PI-sensitive triple-negative breast cancer cell lines, SUM149 and MDA-MB-231 (**Fig. 1e**). Proteasome activity in these cells and their sensitivity to PIs were similar to HAP1 cells (**Fig. S2**). The knockdown did not significantly affect the recovery of proteasome activity in cells treated with 100 nM Btz (**Fig. 1f**). Finally, we found that inactivation DDI2 by the D252N mutation of the catalytic aspartic acid

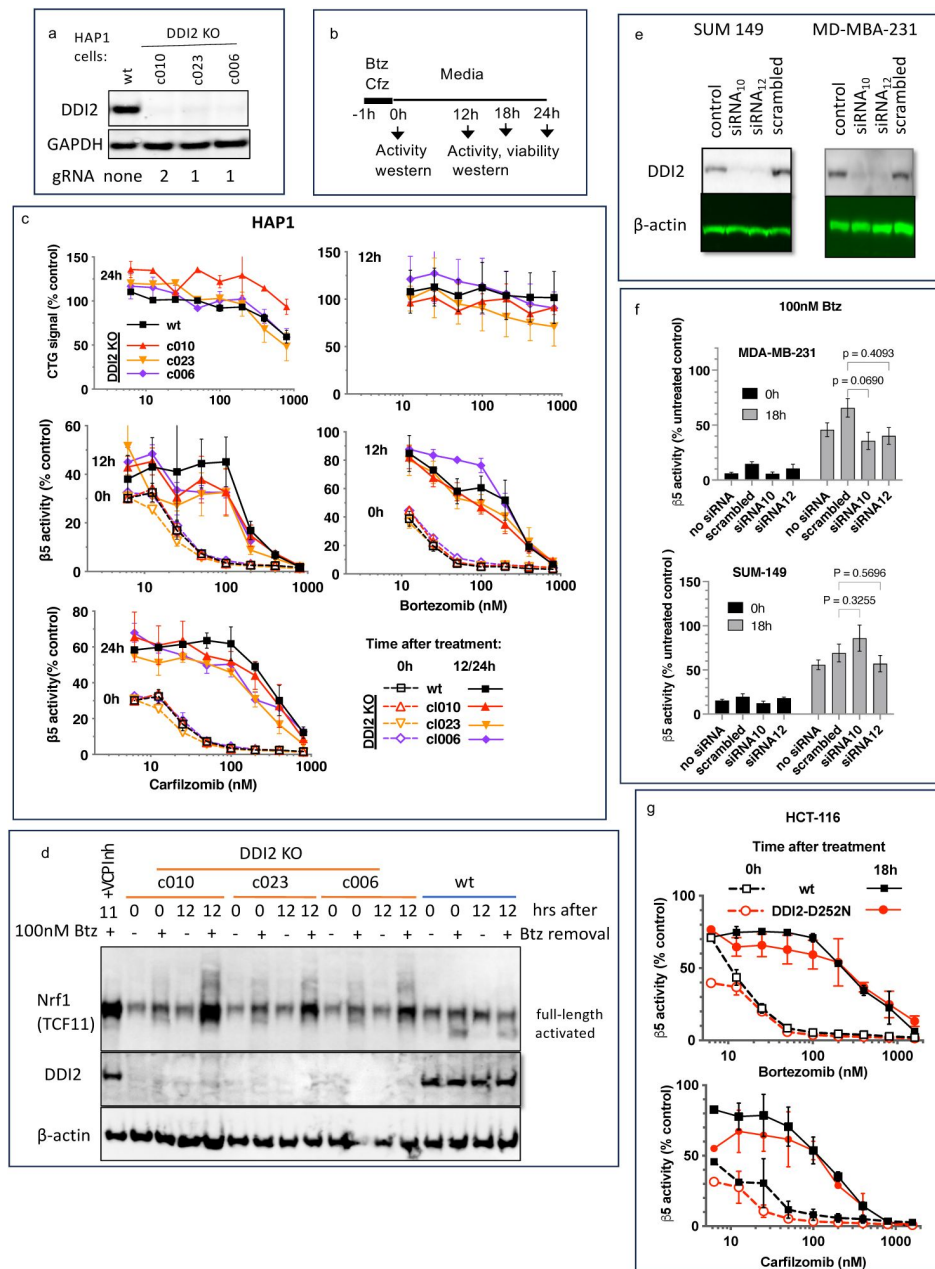


Figure 1.

Recovery of proteasome activity is DDI2 independent.

a. Expression of DDI2 in the CRISPR-generated clones of HAP1 cells used in this work was analyzed by western blot. The full-size membrane is presented in Fig. S1a, and gRNAs sequences are in Table S1. **b.** The experimental setup used in this study. Cells were pulse treated with Btz or Cfz for 1h, then cultured in drug-free media for times indicated and analyzed as described. **c.** The viability of wt- and DDI2 KO clones of HAP1 cells was measured using CellTiter-Glo, and the inhibition of $\beta 5$ sites was measured with the Proteasome-Glo assay at times indicated; $n=2-5$. **d.** Knockout of DDI2 inhibits the Nrf1 processing. Western blots of Btz-treated HAP1 cells. The sample in the first lane is wt cells treated with VCP/p97 inhibitor CB-5083 immediately after removal of Btz. VCP inhibitors blocks Nrf1 processing [17–19]. The full-size membranes and proteasome activity measurements from this experiment are presented in Fig. S1b and S1c. **e.** MDA-MB-231 and SUM149 cells were analyzed by western blot 72h after transfection with DDI2 siRNAs (Table S3). The full-size membranes are presented in Fig. S3. **f.** The $\beta 5$ activity in siRNA-transfected SUM149 and MDA-MB-231 was measured using Suc-LLVY-AMC immediately and 18h after treatment with 100 nM Btz; $n=3$. **g.** $\beta 5$ activity was measured in HCT-116 cells with the Proteasome-Glo assay immediately and 18h after treatment with PIs; $n=2$.

residue [15] did not block the recovery of activity after pulse treatment of HCT-116 cells with Btz and Cfz (Fig. 1g). Thus, the recovery of proteasome activity after pulse treatment with sub-toxic concentrations of PIs is DDI2-independent.

If inhibitor-induced transcription of proteasome genes is responsible for the recovery of proteasome activity, the upregulation of proteasome gene expression should precede the activity recovery. However, we found that the recovery of activity started immediately after one-hour pulse treatment and approached a plateau after 8 hours (Fig. 2a), but the first significant increase in the expression of proteasomal mRNAs occurred only 8 hours after the removal of the inhibitor (Fig. 2b). These results suggest that the early recovery of the proteasome activity is not a transcriptional response.

Ruling out transcriptional response does not rule out the production of new proteasomes because protein synthesis can be regulated at the translational level. To determine whether the activity recovery involves the biosynthesis of new proteasomes, we studied the effects of cycloheximide (CHX), an inhibitor of protein biosynthesis, on the recovery. Except for the first hour, the recovery was completely blocked by CHX, independent of DDI2 expression status (Fig. 3a). Thus, the recovery of proteasome activity involves protein synthesis.

Activation of proteasomal mRNA translation could explain transcription-independent production of new proteasomes if a significant fraction of proteasomal mRNAs is untranslated in the absence of PI treatment. We used polysome profiling to determine the distribution of proteasomal mRNA between translated and untranslated fractions. We found that 90% of proteasomal mRNAs are ribosome or polysome bound in untreated cells (Fig. 3b), and treatment with inhibitors did not increase this fraction (Fig. S4). This result agrees with a published result that the amount of proteasome mRNA in the polysomal fraction does not increase when proteasome is inhibited in MM1.S cells [20]. Thus, the biosynthesis of active new proteasomes immediately after treatment with sub-lethal concentrations of PIs appears to occur without upregulation of translation of mRNAs encoding proteasome subunits.

Discussion

The most important conclusion of this work is that, in addition to Nrf1/DDI2 pathway, mammalian cells possess at least one additional pathway to restore proteasome activity after treatment with PIs, and this DDI2-independent pathway is responsible for the rapid synthesis of new proteasomes immediately after treatment with PI. While this study was underway, two other laboratories found that knockout of DDI2 reduced recovery of proteasome activity in multiple myeloma and NIH-3T3 cells pulse-treated with PIs by ~30% [21, 22]. Similarly, Nrf1 knockdown did not completely block the recovery of proteasome activity in mouse embryonal fibroblasts [14]. The clinical impact of our study and these studies in the literature is somewhat limited because we all conducted a single pulse treatment and did not explore whether Nrf1/DDI2 plays a more prominent role in the recovery of proteasome activity after repeated treatment with PIs. These limitations, however, do not question the existence of the DDI2-independent recovery pathway.

Our findings necessitate reconsidering the role of the DDI2/Nrf1 pathway in basal and inhibitor-induced proteasome expression. Previous studies have also reported that DDI2/Nrf1 contributes to the maintenance of basal levels of proteasomes [21, 23, 24] and that Nrf1 is essential for the basal proteasome expression in the brain [25], liver [26], and retina [27]; yet, in our experiments, the effects of DDI2 KO on the basal proteasome activity was not significant (Fig. 1f and S1c). These differences may reflect that heavy secretory MM cells, embryonic cells, and certain specific tissues require higher levels of proteasome activity and use the DDI2/Nrf1 pathway to

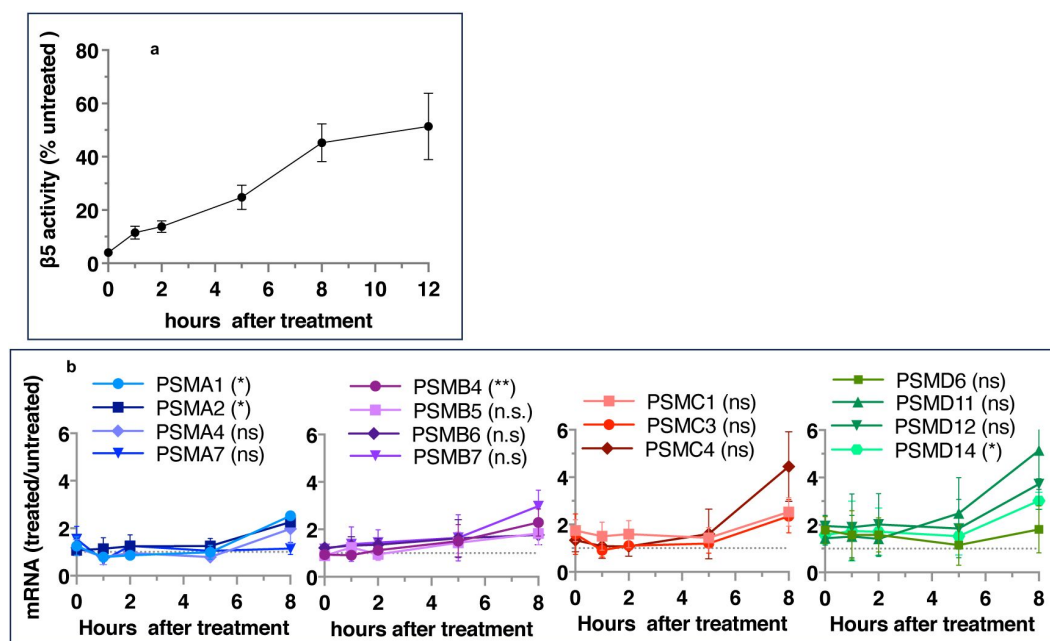


Figure 2.

Proteasome activity recovers before upregulation of proteasome gene expression.

Wt-HAP1 cells were pulse-treated with Btz (100 nM), cultured in a drug-free medium, and analyzed at indicated times. **a.** $\beta 5$ activity was measured using Proteasome-Glo and normalized first to CellTiter-Glo viability data and then to proteasome activity in the mock-treated samples; $n=2-5$. **b.** In a parallel experiment, the mRNA was isolated, and the expression of proteasome genes was quantified using quantitative RT-PCR; $n=3$. Results of the t-test at h are in parenthesis.

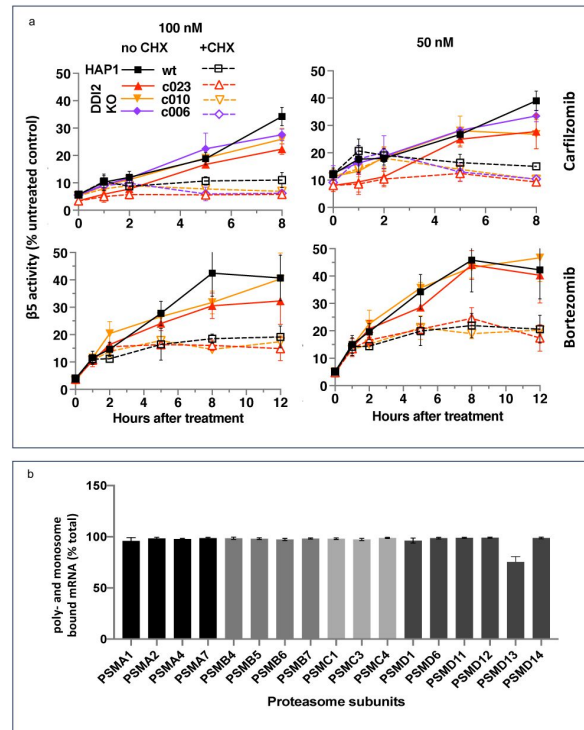


Figure 3.

The recovery of proteasome activity requires protein synthesis.

a. Wt-HAP1 and DDI2 KO cells were treated for 1h at indicated concentrations of Btz and Cfz and then cultured in a drug-free media in the absence (solid lines) or presence (dashed lines) of CHX. The $\beta 5$ activity was measured using Proteasome-Glo and normalized first to cell viability, which was determined in a parallel experiment using CellTiter-Glo, and then to untreated controls; $n=3-4$. **b.** All proteasome mRNAs are actively translated. mRNA isolated from untreated wt-HAP1 cells were analyzed by polysome profiling. The combined mRNAs in the 80S and polysomal fractions as a % of total is shown; $n=2$.

supplement other pathways responsible for proteasome expression [28]. Other studies demonstrated the importance of Nrf1-dependent proteasome expression during cardiac regeneration [29] and thermogenic adaptation of the brown fat [30].

Several studies found that the knockout of DDI2 sensitizes cells to proteasome inhibitors [11, 12, 21, 22, 31]. This was further interpreted as supportive of a role for DDI2-dependent recovery in the de-sensitization of cells to PI-induced apoptosis. Although we confirmed this observation in HAP1 cells (not shown), the present findings raise a possibility that DDI2 desensitizes cells to PI by a different mechanism. Activation of non-proteasomal Nrf1-dependent oxidative stress response genes [32, 33] may help overcome the deleterious consequences of PI-induced overproduction of reactive oxygen species (ROS) [34]. Alternatively, the ability of DDI2 to bind and participate in the degradation of large ubiquitin conjugates [31, 35] may help alleviate the stress associated with proteasome inhibition. DDI2 and proteasome are involved in DNA repair [36–40], and impairment of the proteasome in the absence of DDI2 can lead to excessive spontaneous DNA damage, even without DNA-damaging agents. Finally, the proteolytic activation of another yet-to-be-identified DDI2 substrate cannot be ruled out. In summary, our study provides strong evidence for a novel pathway responsible for the recovery of proteasome activity in inhibitor-treated cells. It should stimulate research on additional biological roles of DDI2, which can explain the embryonic lethality of DDI2 deletion [23] and DDI2's role in tumorigenesis [41].

Ideas and Speculations

We want to propose a model explaining the upregulated biogenesis of proteasomes without an increase in the efficiency of proteasomal mRNA translation. To gain activity, the catalytic subunits must assemble into mature particles in a complex process involving multiple dedicated chaperones [42, 43]. The efficiency of nascent subunits incorporation into the mature proteasomes is not known. One study found that proteasomes degrade a significant fraction of nascent proteasome subunits within 2 – 4 hours after synthesis [44], after which the remaining fraction is highly stable (Fig. 4a). We hypothesize that nascent proteasome subunits are partitioned between immediate degradation and assembly, and the inhibition of the proteasome blocks degradation and increases the efficiency of proteasome assembly (Fig. 4b). Increased expression of proteasome assembly chaperone POMP and an increase in proteasome assembly intermediates after treatment with PIs has been previously reported (see Fig. 6 in [45]). If nascent polypeptides take 1 – 2 hours to assemble into proteasomes, this model explains translation-independent recovery of proteasome activity in the first hour after the removal of PIs (Fig. 3a). The fraction of nascent polypeptides degraded may be much larger than in Fig. 4 because that experiment used one-hour pulse labeling and was therefore unable to detect nascent proteins that are degraded within minutes after synthesis. Thus, partitioning proteasome nascent polypeptides between degradation and assembly allows cells to instantaneously upregulate proteasome biogenesis immediately after proteasome inhibition. This model will be tested in future experiments.

Materials and Methods

Source of materials

HAP1 cells (wt-clone 631, DDI2 KO clones 006, 023, and 010, Table S1) were obtained from Horizon Discovery. MDA-MB-231 cells were purchased from ATCC® (Cat. #HTB-26™), and SUM149 cells (CVCL_3422) were obtained from Asterand [11]. A CRISPR-generated clone of HCT-116 cells, in which catalytic Asp-252 residue of the DDI2 gene was mutated into an asparagine (D252N) [15], and a control clone carrying wt-DDI2 allele were kindly provided by Dr. Shigeo Murata. Sources of inhibitors and other chemicals are listed in Table S2.

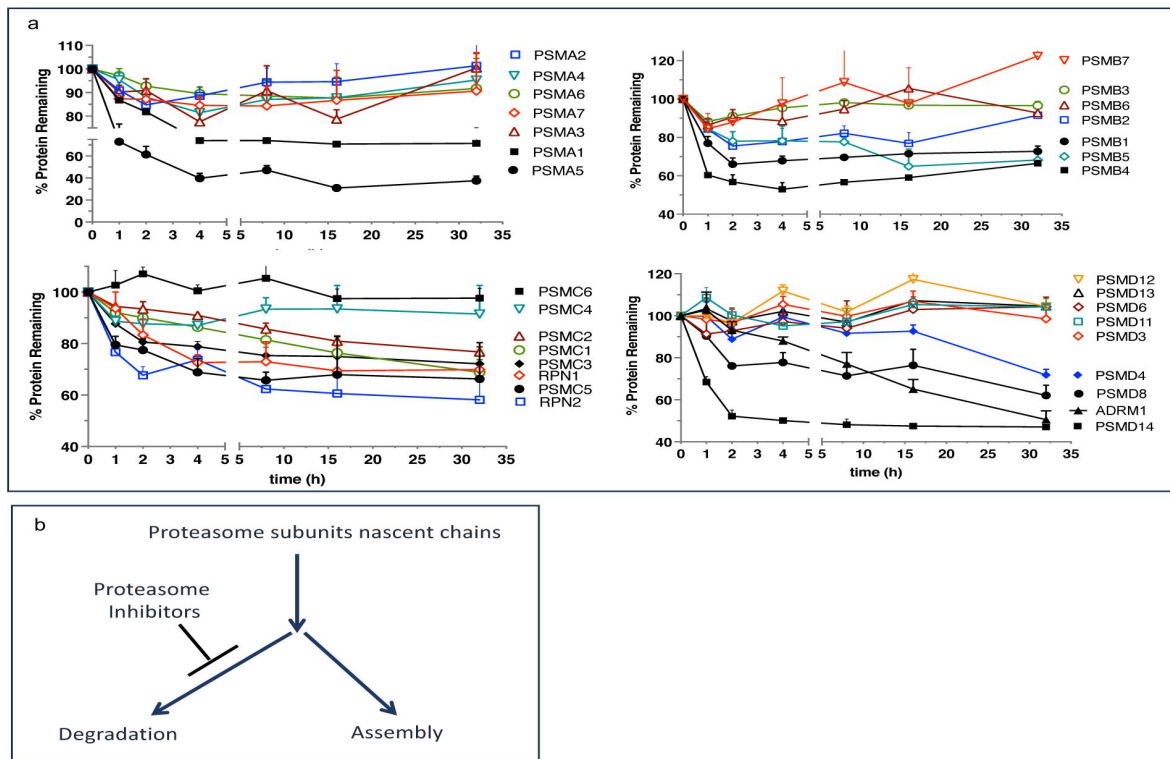


Figure 4.

Escape from rapid degradation of nascent subunits can explain rapid recovery of proteasome activity.

a. Turnover of proteasome subunit in human RPE-1 cells was measured by quantitative mass-spectrometry following 1h labeling with heavy isotopes. Data from Table S4 in [44]. **b.** Proposed model of how nascent proteasome subunits are partitioned between assembly and degradation.

Cell culture

All cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. HAP1 cells were cultured in Iscove's medium supplemented with 10% Fetal Bovine Serum (FBS). MDA-MB-231 and SUM149 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Hams F-12 50/50 Mix supplemented with 5% FBS. SUM149 cell media were also supplemented with 4.8 µg/mL insulin, 10 mM HEPES, pH 7.3, and 1 µg/mL hydrocortisone. HCT-116 cells were cultured in McCoy medium supplemented with 10% FBS. In addition, all media were supplemented with 100 µg/mL Penicillin-streptomycin, 0.2 µg/mL ciprofloxacin, and 0.25 µg/mL amphotericin B. Cells were plated overnight before treatment, then treated with inhibitors for 1 hour in a fresh medium. The inhibitor-containing medium was aspirated, except for the experiments in Fig. S2a, where it was shaken off, and the cells were cultured in a drug-free medium for times indicated when they were harvested and analyzed as described in the figure captions. siRNAs were transfected 72 hours before treatments. The MDA-MB-231 or SUM149 cells were seeded in 6-well plates at 2×10^5 cells/well the day before the transfections. The cells were transfected with 25 nM DDI2 siRNAs (Table S3) by using 0.3% DharmaFECT 1 (Cat. #T-2001-03, Dharmacon) in Gibco™ Opti-MEM 1X Reduced Serum Medium and Corning® DMEM:F-12(1:1) without antibiotics and amphotericin B. Cell viability was assayed with CellTiter-Glo® (Promega) or Alamar Blue (resazurin).

Proteasome activity assays

The activity of the proteasome's $\beta 5$ sites was determined either by Succinyl(Suc)-LLVY-AMC (7-amido-4-methylcoumarin) fluorogenic substrate or by the Proteasome-Glo™ assay (Promega), a luciferase coupled assay, which uses Suc-LLVY-aminoluciferin as a substrate [46, 47]. In the Proteasome-Glo assays, the cells in 96-well plates were washed with PBS and lysed by one cycle of freezing and thawing in 25 µL of cold PBS containing 0.05% digitonin. 25 µL of Suc-LLVY-aminoluciferin containing Proteasome-Glo reagent was added, and plates were preincubated on a shaker for ~10 minutes at room temperature before luminescence measuring using a mixture of PBS and Proteasome-Glo reagent as a blank. Each sample contained three technical replicates.

To determine proteasome's $\beta 5$ activity in the cell extracts, cells were lysed in ice-cold 50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5% CHAPS, 5 mM MgCl₂, 1 mM EDTA, 100 µM ATP, 1 mM DTT, and 1x PhosSTOP. The cells were incubated for 15 min on ice, centrifuged at $20,000 \times g$ for 15 – 20 min at 4°C, and the supernatants were used for the experiments. Protein concentrations were determined using Pierce™ Coomassie Plus (Bradford) Assay reagent (Cat. #23238) with bovine serum albumin as a standard. An aliquot of cell lysate containing 1 µg of protein was spiked into a 100 µL per well of the 26S assay buffer (50 mM Tris-HCl pH 7.5, 40 mM KCl, 2 mM EDTA, 1 mM DTT, and 100 µM ATP) containing 100 µM of Suc-LLVY-AMC. The mixture was thoroughly mixed and preincubated at 37°C for 10 minutes. An increase in fluorescence was monitored continuously at 37°C at the excitation wavelength of 380 nm and emission of 460 nm. The slopes of the reaction progress curves for three technical replicates were averaged, and the inhibition was calculated as a percentage by dividing the slopes of the inhibitor-treated samples by the slope of mock-treated controls. Assays were calibrated with AMC standards [48].

Western blotting

Lysates were prepared, and total protein was quantified as described above for the fluorescent proteasome assays. Samples were mixed with lithium dodecyl sulfate loading buffer and heated before fractionation on either NuPAGE™ Bis-Tris 8% Midi Gel (Invitrogen™, Cat. #WG1003BOX) or SurePAGE™ Bis-Tris 8% mini gel (GenScript, Cat. #M00662), using MES-SDS running buffer (GenScript Cat. #M00677). The proteins were transferred on 0.2 µm pore-diameter Immobilon-pSQ PVDF membrane (Cat. #ISEQ00010) using Invitrogen™ Power Blotter 1-Step™ Transfer Buffer (Thermo Cat. #PB7300). The membrane was blocked with 5% Milk in TBST and probed with antibodies listed in Table S4.

RNA isolation and qPCR

The mRNA was isolated from cells using TRIzol™ Reagent (ThermoFisher Scientific Cat. #15596018) according to the manufacturer's protocol. Then, cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems cat. #4368814). Before the qPCR run, the RNA and cDNA were quantified by UV absorbance using NanoDrop2000 (Thermo Scientific™). The Real-time qPCR was performed using 2x SYBR Green Bimake™ qPCR Master Mix on a Bio-Rad C1000 thermal cycler CFX96™ Real-Time System. The primers are listed in Table S5.

Polysome profiling

was conducted according to a published procedure [49, 50]. Cells were washed in a cold PBS containing 100 µg/mL CHX and were resuspended in the hypotonic buffer containing 5 mM Tris-HCl pH 7.5, 2.5 mM MgCl₂, 1.5 mM KCl, 1x complete mini protease inhibitor EDTA free, 100 µg/mL CHX, 1 mM DTT, and 0.2 units/mL RNasin Plus. Triton X-100 and sodium deoxycholate were added to the cell suspension to a final concentration of 0.5%, followed by centrifugation at 20,000g for 15 – 20 min 4°C. Extracts were loaded on 5 – 55% gradients of sucrose in 20 mM HEPES-KOH, pH 7.6, 0.1 M KCl, 5 mM MgCl₂, 100 µg/mL cycloheximide, 1x complete EDTA free protease inhibitor cocktail, and 100 units/mL RNasin®. Following the centrifugation at 35,000 rpm for 2.5 hours at 4°C, the gradients were manually fractionated into 200 µL fractions. Fractions containing the non-translated mRNA, 80S ribosomes, and polysomes were pooled. The RNA was isolated and quantified by quantitative RT-PCR.

Statistical analysis

Data points on all figures are averages+/-S.E.M. of n biological replicates, and n is provided in figure captions. Statistical analysis was carried out in GraphPad PRISM and used mixed-effect multiple comparisons on Fig. 1f and a t-test on Fig. 2b. p-values <0.05 were considered significant.

Acknowledgements

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Conflict of interest statement

AFK is a Co-founder and Chief Scientific Officer of InhiProt, LLC.

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Reviewer #1 (Public Review):**Summary:**

There has been substantial prior work trying to understand the transcriptional control of proteasome expression as an adaptive response to proteasome inhibition. This field has been mired by fierce debates over the role of the protease Ddi2 in activating the transcription factor Nrf1/NFE2L1. As the authors of this manuscript point out, most of the previous research centers on the continuous treatment of cells with proteasome inhibitors rather than a brief pulse of inhibition that better models the situation when these drugs are used clinically. The authors find that the initial recovery of proteasome activity is independent of Ddi2 and involves a mechanism distinct from transcription. The authors intriguingly point to a model in which the assembly of proteasomes is regulated. If true, this would be a significant finding, but for now, this model remains more speculative.

Strengths:

The pulsed treatment of proteasome inhibitors is a strength of this lab that few others use. It better mimics the clinical use of these inhibitors and allows for a more detailed analysis of the initial response to inhibition. The authors have used multiple different clones of Ddi2 knockouts and siRNA against Ddi2 to rule out the necessity of Ddi2 in the early production of proteasomes when cells are inhibited with proteasomes, establishing a thorough knockout approach while also avoiding compensatory mutations. These experiments are well controlled, showing both the levels of Ddi2 upon knockout or knockdown and demonstrating that the cleavage of Nrf1, one of two known targets of Ddi2, is impaired. However, it should be noted that faint bands for Ddi2 mysteriously remain even in the knockout.

This article sensitively monitors the recovery of proteasome function with the $\beta 5$ activity assay and for the production of new proteasome transcripts by qPCR. This precision and a detailed analysis of the timing are strengths that pointed to a more rapid recovery than transcription alone.

Weaknesses:

This paper's major weakness is the difficulty in establishing the authors' model that assembly is regulating this process. They do a convincing job demonstrating that activity recovers before transcription. The evidence that translation is unaffected depends entirely on the polysome RNA profiling from two replicates. Clearer and orthogonal data would help establish this finding. The stability of subunits is interesting and important in its own right.

In short, the authors establish that Ddi2 is unnecessary for the initial, non-transcriptional recovery of proteasome activity after a pulse of proteasome inhibition.

It is not clear what clinical impact this work will have. Although it models the pulse of proteasome inhibition more perfectly, it only looks at a single pulse rather than multiple treatments. Thus, ruling out Ddi2's importance for clinical benefit may be premature. More significantly, this work suggests that assembling proteasomes might be a regulated process worth substantial follow-up that will be interesting to follow.

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Reviewer #2 (Public Review):**Summary:**

In this work Ibtisam and Kisselev explore the role of DDI2 in the proteasome function recovery after a clinically relevant pulse dosing using different proteasome inhibitors and their corresponding PK properties. The authors report that despite lack of NRF1 activation by DDI2 there was no difference in recovery from pulsed proteasome inhibition observed in DDI2 KO cells as compared to WT controls suggesting DDI2 is not required for recovery in this system. They further show that transcription of the proteasome subunits is initiated only after partial recovery of proteasome activity is already observed suggesting that non-transcriptional mechanisms might be also involved. The authors further show that translation inhibition blocked the recovery from proteasome inhibitors.

Strengths:

Overall, it is very important and informative to use a pulse treatment type approach (mimicking the PK properties of the drugs) to explore the biology of PIs as used in this study. The authors also provide convincing data that DDI2 is not required for proteasome activity recovery post-PI pulse treatment in the systems they explored.

Weaknesses:

The authors show that the recovery of one specific catalytic activity of the proteasome post-PI treatment is transcription independent. However, in this work they do not explore the other catalytic activities of the proteasome, the protein levels of the individual subunits and most importantly the level of the different assembled proteasome complexes and how they change over time. Without this data the proposed mechanism is still speculative, in particular the conclusion on the role of translation, and ignores other findings in the field that suggest that alternative mechanisms (such as proteasome complex assembly regulation for instance) might be just as plausible.

<https://doi.org/10.7554/eLife.91678.2.sa0>

Author Response

The following is the authors' response to the original reviews.

We are grateful to the reviewers for recognizing the importance of our work on transcription-independent early recovery of proteasome activity. We also thank them for their thoughtful criticisms and suggested improvements, which we addressed in the revised version as described below.

The reviewers and editors asked for data to support the model that early recovery of proteasome activity is due to accelerated proteasome assembly. This model is backed by published data that proteasome assembly intermediates increase dramatically in cells treated with proteasome inhibitors (Fig. 6 in Ref. 46 of the revised manuscript). We expanded the discussion of this paper in a paragraph that describes our model. Another key experiment to confirm this model would be to determine what fraction of nascent polypeptides is degraded within minutes after synthesis, which is not trivial, and Ibtisam ran out of time to conduct these experiments because she had to graduate in spring before the expiration of her visa. This type of experiment usually uses metabolic labeling by a heavy or radioactive amino acid that always includes a prior depletion of a non-labeled amino acid. However, the fundamental flaw of this approach, which is not recognized by the scientific community, is that depletion of an amino acid stresses cells and reduces the rate of protein synthesis, especially if this amino acid is methionine. Thus, this model is not easy to test, and should be considered a speculation. We therefore moved the description of this model, together with Fig. 4, into a separate "Ideas and Speculations" section and removed this model's description from the abstract.

Reviewer 1 raised the possibility that a background band detected on the western blot of DDI2 KO cells could be a highly homologous protease DDI1. This is highly unlikely because, according to Protein Atlas, DDI1 is selectively expressed in the testis and is not expressed in the cell lines we used. Reviewer 1 also suggested that we should base our conclusion on Nrf1 KD, which we de-facto did because we confirmed that DDI2 KD blocks Nrf1 activation (Fig. 1d).

In response to Reviewer 1 critiques regarding the presentation of proteasome subunits stability data in Fig. 4 (Ref. 45 of the revised manuscript), we removed PSMB8 and replaced chaperons with the subunits of the 26S base. We changed color palettes, symbols, and axis scales to improve clarity.

We acknowledged in the discussion that our work did not exclude DDI2 role in the recovery of proteasome after repeated pulse treatments, as suggested by Reviewer 1.

We agree with Reviewer 2 that using “proteasome levels” is inaccurate when describing our activity measurement data. However, in the manuscript, we use “levels” only when discussing data in the literature. We believe measuring activity and not the total levels is more important because not all proteasomes are active, e.g., latent 20S proteasome core particles.

Reviewer 3 expressed concern that our conclusions were based on data in HAP1 cells, which are haploid, and appear not very sensitive to proteasome inhibitors. This is why we used DDI2 KD in MDA-MB-231 and SUM149 cells, which are highly sensitive to proteasome inhibitors (Weyburne et al., Ref. 11). In our experience, full extent of proteasome inhibitor cytotoxicity is not revealed until 48hr after treatments, and viability determined at 12hr and 24hr as on Fig. 1c should not be used to determine sensitivity (it was used for activity assay normalization). We added a new supplementary figure showing that HAP1 cells are as sensitive to proteasome inhibitors as MDA-MB-231 cells when cell viability is assayed 48hr after treatment (new Fig. S2). Another panel on this new figure demonstrates that the baseline proteasome activity is very similar in HAP1, MD-MB-231 and SUM149 cells. We also added data demonstrating that inactivation of DDI2 by mutation does not change the recovery of proteasome activity in HCT-116 cells (new Fig. 1g). Recovery in MDA-MB-231, SUM149, and HCT-116 cells was measured at 18hr, which is still within the 12 – 24hr window when other investigators observed partially DDI2-dependent recovery.

We have conducted an experiment in which we followed activity recovery for up to 72hr. We found that activity plateaued at 24hr and opted against the repeat because there were no changes. We feel that the manuscript should not include one biological replicate data. The fact that the recovery is incomplete and that cells seem to survive with lower levels of proteasome activity is interesting; however, investigating the molecular basis for this phenomenon is beyond the scope of the current project.

We were not disputing the conclusions of previous studies that DDI2/Nrf1 is responsible for enhanced expression of proteasomal mRNA in cells continuously treated with proteasome inhibitors. In fact, we confirmed that pulse-treatment causes similar increase (Fig. 2b). As for papers that measured activity recovery after pulse treatment, we objectively discuss our results in the context of these papers. In response to Reviewers' recommendations and minor points:

- We reviewed the revised version carefully to eliminate spelling and grammatical errors and typos.
- We no longer refer to DDI2 as a novel protease, as suggested by Reviewer 1.

- We agree with Reviewer 2 that our CHX results do not necessarily mean that recovery involves translation of proteasomal mRNAs, and we now conclude that proteasome recovery requires protein synthesis.
- We revised Fig. 1c, 3a and 4a to improve clarity.
- We have stated in the caption that data in Fig. 4a comes from Table S4 in Ref. 45.
- We accepted an excellent suggestion of Reviewer 3 to change "recovery" to "early recovery" in the title.
- Regarding Reviewer 3 request to assay activity recovery at additional time points before 12h, this was done in the cycloheximide experiment in Fig. 3A.
- Even if we assume that the differences in the observed recovery activity in MDA-MB-231 cells (Fig. 1f) are statistically significant, which may implicate DDI2 involvement in the activity recovery, the percentage is still small, suggesting that most activity recovery is DDI2-independent.
- We toned down the statement "the present findings suggest that DDI2 desensitizes cells to PI by a different mechanism," replacing "suggest" with "raise a possibility".
- We indicated that only Bortezomib is approved for mantle cell lymphoma.
- We changed the description of clinical dosing as suggested by Reviewer 3. We added a reference on PK of subcutaneous bortezomib (Ref. 9), even though the review we quoted (Ref. 7) discussed subcutaneous dosing.