**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 novel DDI2 protease. Here we demonstrate that the recovery of proteasome activity is DDI2-independent and occurs before the upregulation of proteasome gene expression. The recovery requires protein translation, but the efficiency of translation of proteasomal mRNA does not increase upon proteasome inhibition. Based on this data, we propose that the increased efficiency of proteasome assembly is responsible for the recovery of proteasome activity.

## Introduction

The ubiquitin-proteasome pathway 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) and 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].

In the clinic, Btz and Cfz are administered twice weekly as a bolus IV where 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 blood drops below the levels needed to kill tumor cells *in vitro* [8]. Although Btz has a very slow off-rate and Cfz is an irreversible inhibitor, proteasome activity recovers within 24hrs [6, 8-10]. This activity recovery may explain discrepancies between robust activity against cell lines derived from various cancers, continuously treated with Btz [11], and lack of clinical efficacy except for MM and MCL. In addition, recovery of activity has recently been implicated in PI resistance in MM [12].

In cells treated with proteasome inhibitors, transcription factor Nrf1 (also known as TCF11, encoded by *NFE2L1* gene) upregulates transcription of genes encoding all proteasome subunits [13, 14]. When the proteasome is fully functional, Nrf1 is constitutively degraded in an

ubiquitin-dependent manner [13]. When the proteasome is partially inhibited, the ubiquitylated Nrf1 is recognized by DDI2 (Damaged DNA-Inducible 1 Homolog 2), a novel ubiquitin-dependent 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/Nfr1-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. Surprisingly, we found that the proteasome activity recovered in the absence of DDI2 when the transcription of proteasome genes was not upregulated. Activity recovery preceded the upregulation of proteasomal genes. This data demonstrates the existence of a novel, DDI2-indendent pathway for the recovery of proteasome activity in PI-treated cells.

**Results and Discussion**

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 (Fig.1a). We have analyzed three different clones that were generated by using two different gRNAs (Table S1). We treated cells for 1hr with a range of concentrations of Cfz and Btz, and then cultured them in drug-free media (Fig. 1b). We measured inhibition of proteasome β5 site, which is the prime target of Cfz and Btz [2], immediately after the 1hr treatment, and 12hr or 24hr thereafter (Fig. 1c). In a parallel experiment, we determined cell viability 24hr after treatment using the CellTiter-Glo assay (Fig. 1c), which measures intracellular ATP levels. 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 Nrf1in DDI2-KO cells (Fig. 1d). Thus, while confirming that DDI2 carries proteolytic activation of Nrf1, these findings indicate that DDI2 is not involved in the recovery of proteasome activity in Btz and Cfz-treated HAP1 and DDI2 KO cells.

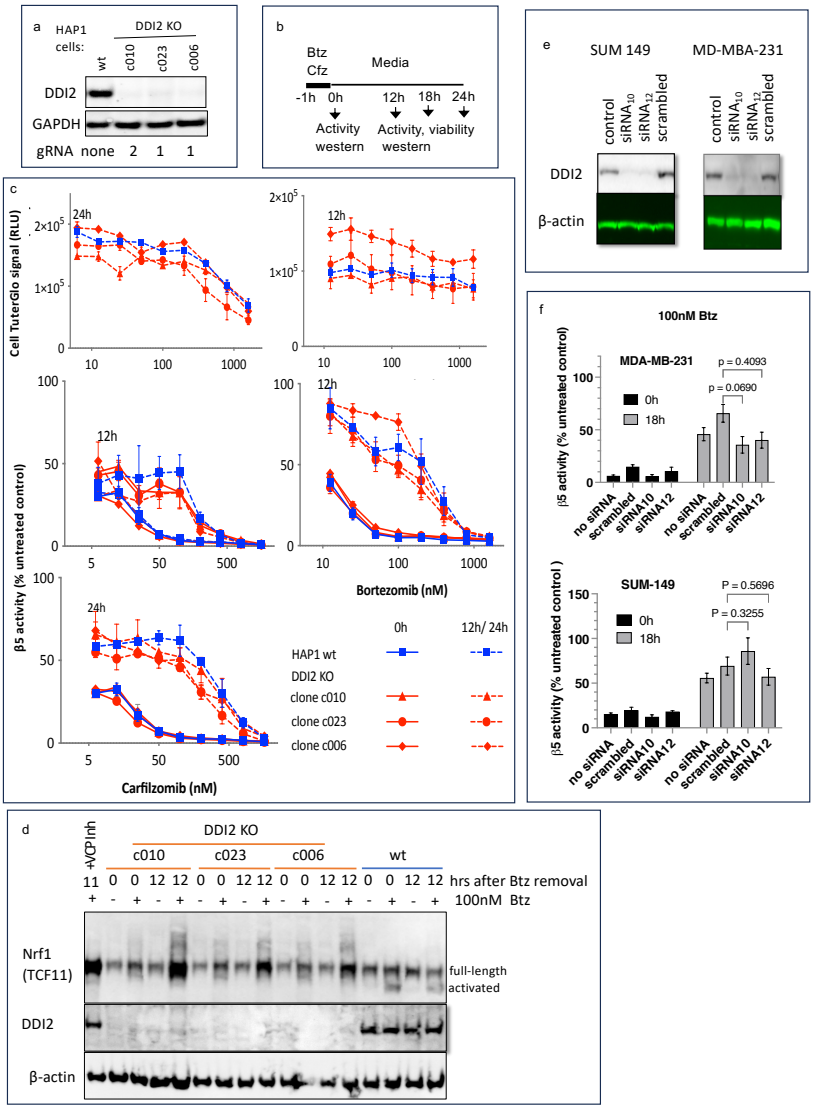






Figure 1. **Recovery of proteasome activity is DDI2 independent. a.** Expression of DDI2 in the CRISPR-generated clones of HAP1 cells used in this work was analyzed by western blot. The full-size membrane is presented in Fig. S1a. **b.** The experimental set up in this study. Cells were pulse treated with Btz and Cfz for 1hr, then cultured in a drug-free media, and analyzed at times indicated. **c.** Wt- and DDI2 KO clones of HAP1 cells were treated for 1hr. The cell viability was measured using CellTiter-Glo, and the inhibition of β5 sites was measured with the Proteasome-Glo assay at times indicated. Data are averages+/-S.E.M. of two to five biological replicates **d.** Knockout of DDI2 inhibits the Nrf1 processing. Western blots of Btz-treated HAP1 cells. Sample in the first lane are wt cells treated fwith VCP/p97 inhibitor CB-5083, which blocks Nrf1 processing [17-19], immediately after removal of Btz. The full-size membranes and proteasome activity measurements from this experiment are presented in Figs. S1b and S1c. **e.** MDA-MB-231 and SUM149 cells were analyzed by western blot 72hr after transfection with DDI2 siRNA*s*. The full-size membranes are presented in Fig. S2. **f.** The β5 activity in siRNA-transfected SUM149 and MDA-MB-231 treated with 100 nM Btz for 1hr. The β5 activity was measured using Suc-LLVY-AMC immediately and 18 hrs after treatment. Data are averages+/-S.E.M. of three to five biological replicates. Statistical analysis was conducted using mixed-effect multiple comparisons; p-values ≤ 0.05 were considered significant.

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). The knockdowns did not significantly affect the recovery of proteasome activity in cells treated with 100 nM Btz

(Fig. 1f). Thus, the recovery of proteasome activity after pulse treatment with sub-toxic concentrations of inhibitors 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 treatment and approached a plateau after 8hr (Fig. 2a), but first significant increase in the expression of proteasomal mRNAs occurred only 8hr after the removal of the inhibitor (Fig. 2b). These results suggest that the early recovery of the proteasome activity is not a transcriptional response.

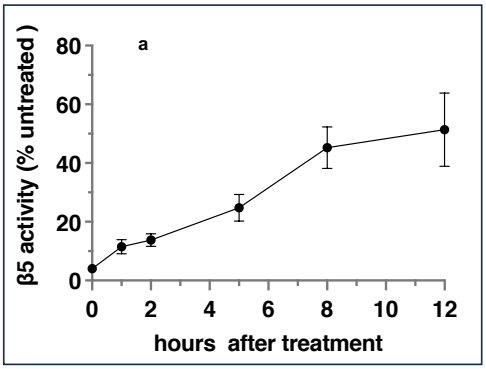
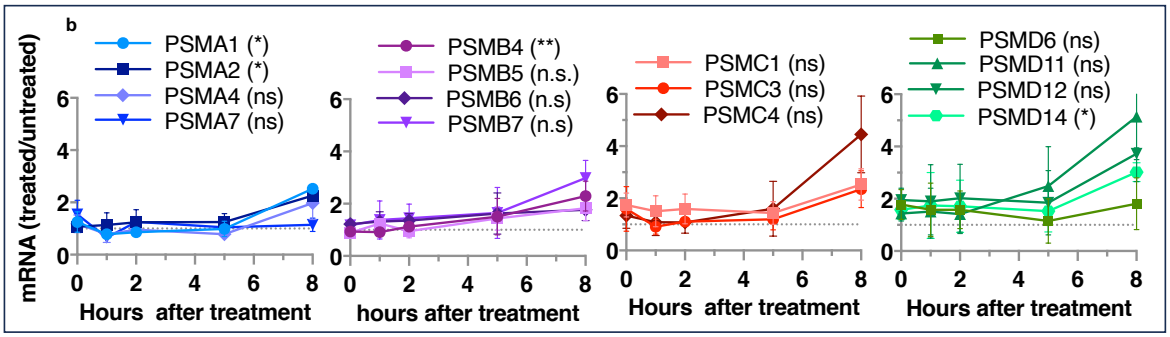
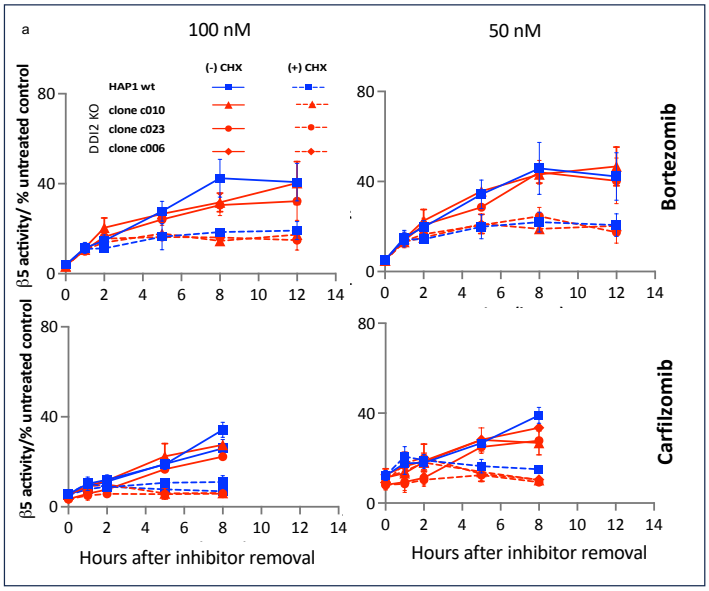



**Figure 2. Proteasome activity recovers before up-regulation of proteasome gene expression.** Wt-HAP1 cells were pulse-treated with Btz (100 nM), cultured in drug-free medium, and analyzed at indicated times. **a.** β5 activity was measured using Proteasome-Glo and normalized first to CellTiter-Glo viability data, and then to proteasome activity in the mock-treated samples. The data are averages±S.E.M of two to five biological replicates. b. In a parallel experiment, the mRNA was isolated, and the expression of proteasome genes was quantified using quantitative RT-PCR. Data are averages±S.E.M. of three biological replicates. The number in parenthesis indicates the t-test results at an 8hr time point.




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 biosynthesis of new proteasomes, we studied effects of the treatment with 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, recovery of proteasome activity involves transcription-independent proteasome biogenesis.



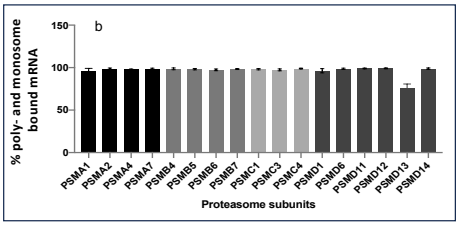




Figure 3. **The recovery of proteasome activity required the synthesis of new proteasomes. a.** Wt (blue) and DDI2 KO (red) HAP1 cells were treated for 1hr at indicated concentrations of Btz and Cfz and then cultured in a drug-free media in the absence (solid line) or presencence of CHX (dashed line). The β5 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. Data are averages+/-S.E.M of two to five biological replicates. **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 fraction relative to the cumulative amount of mRNA in all fractions are shown. Data are averages+/-S.E.M of two biological replicates.

Activation of mRNA translation could explain transcription-independent production of new proteasomes if a significant fraction of proteasomal mRNAs are 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 that treatment with inhibitors did not increase this fraction (Fig. S3). 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 synthesis of new proteasomes in cells treated with sub-lethal

concentrations of proteasome inhibitors occurs without upregulation of translation of mRNA encoding proteasome subunits.

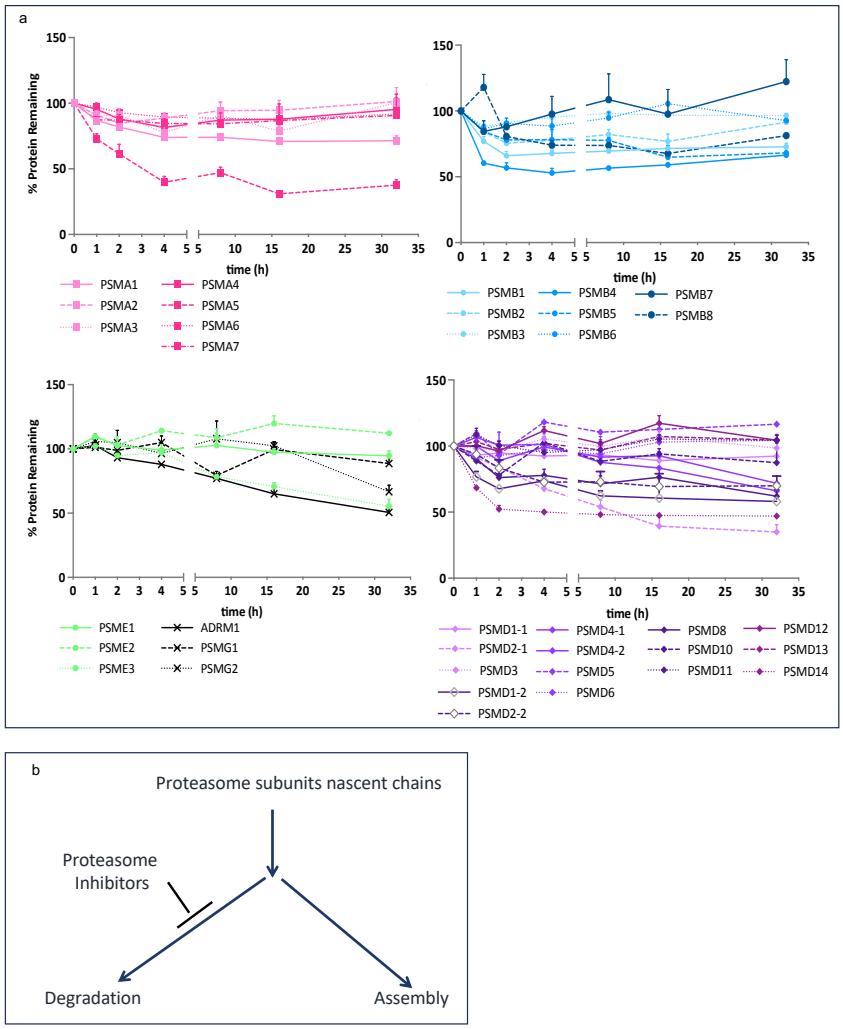






Figure 4. **Rapid degradatio of nascent proteasome polypeptides can explain the rapid recovery of activity**. **a**. Turnover of proteasome subunit in human RPE cells was measured by quantitative mass-spectrometry following 1hr labeling with heavy isotopes. Data from Table S4 in [23]. **b**. Proposed model of how nascent proteasome subunits are partitioned between assembly and degradation.

How can the biogenesis of proteasomes be upregulated without an increase in the efficiency of mRNA translation? To gain activity, the catalytic subunits must assemble into the mature particles in a complex process involving multiple chaperones [21, 22]. It is unknown what fractions of nascent subunits are incorporated into the mature proteasomes. One study found that proteasomes degrade a significant fraction of nascent proteasome subunits within 2 – 4hr following the synthesis [23], 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 proteasome assembly after treatment with proteasome inhibitors has been previously reported [24]. If nascent polypeptides take ~1 – 2h to assemble into proteasomes, this model explains translation-independent recovery of proteasome activity in the first hour after removal of PIs (Fig. 3a). The fraction of nascent polypeptides that is degraded may be much larger than on Fig. 4 because that experiment used 1hr pulse labeling, and was thus 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.

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 that 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% [25, 26]. Similarly, knockdown of Nrf1 did not completely block recovery of proteasome activity [14]. These findings are consistent with the existence of the DDI2-independent pathway of recovery.

These 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 [25, 27, 28], and that Nrf1 is essential for the basal proteasome expression in the brain [29], liver [30], and retina [31]; yet, in our experiments, the effect of DDI2 KO at the basal proteasome activity was not significant (Figs. 1f and S1c). These differences may reflect the fact that heavy secretory MM cells,

embryonic cells, and certain sepcifc tissues require higher levels of proteasome activity and use DDI2/Nrf1 pathway to supplement other pathways responsible for proteasome expression [32]. Other studies demonstrated importance of Nrf1-depenedent proteasome expression during cardiac regeneratin [33] and brown fat thermogenic adaptation [34].

Several groups have previously found that knockout of DDI2 sensitizes cells to proteasome inhibitors [10, 12, 25, 26, 35]. This was further interpreted as supportive of a role for DDI2-dependent recovery in de-sensitization of cells to PI-induced apoptosis. Although we confirmed this observation in HAP1 cells (not shown), the present finding suggests that DDI2 desensitizes cells to PI by a different mechanism. Activation of non-proteasomal Nrf1-dependent oxidative stress response genes [36, 37] may help overcome the deleterious consequences of PI-induced overproduction of reactive oxygen species (ROS) [38]. Alternatively, the ability of DDI2 to bind and participate in the degradation of large ubiquitin conjugates [35, 39] may help alleviate the stress associated with proteasome inhibition. DDI2 and proteasome are both involved in DNA repair [40-44] and impairment of proteasome in the absence of DDI2 can lead to excessive spontaneous DNA damage even in the absence of DNA-damaging agents. Finally, the proteolytic activation of another yet-to-be-identified DDI2 substrate cannot be ruled out. Thus, our study provides strong evidence for the existence of a novel pathway responsible for the recovery of proteasome activity in the inhibitor-treated cells, and, in combination with the published literature, should stimulate research on novel biological roles of DDI2, which can explain the embryonic lethality of DDI2 deletion [27] and DDI2 role in tumorigenesis [45].

## Materials and Methods

**Source of materials.** HAP1 (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™), SUM149 cells were obtained from Asterand [10]. Sources of inhibitors and other chemicals are listed in Table S2.

**Cell culture.** All cells were cultured at 37°C in a humidified atmosphere with 5% $CO_2$. 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. 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 1hr in fresh medium. The inhibitor-containing medium was aspirated, and the cells were re-cultured in drug-free medium for times indicated when they were harvested and analyzed as described in figure captions. In siRNA knockdown experiments, the cells were transfected for 72hr before the treatments. The MDA-MB-231 or SUM149 cells were seeded in 6-well plates at $2x10^5$ cells/well the day prior to the transfections. The cells were transfected with DDI2 siRNAs (Table S3) at a final concentration of 25 nM using 0.3% DharmaFECT 1 (Cat. #T-2001-03, Dharmacon) in Gibco™ Opti-MEM 1X Reduced Serum Medium and Corning® DMEM/Ham's F-12 50/50 Mix without antibiotics and anti-mycotic.

**Proteasome activity assays**. The activity of proteasome β5 sites was determined either by 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]. For the Proteasome-Glo assay experiments, the cells in 96-well plates were washed with PBS and lysed by one cycle of freezing and thawing in 25µL of cold PBS containing Digitonin 0.05%. 25µL Suc-LLVY-aminoluciferin and luciferase containing Proteasome-Glo reagent were added, thoroughly mixed, and preincubated on a shaker for ~10 minutes at room temperature (RT) before luminescence measuring using a mixture of PBS and Proteasome-Glo reagent as a blank. Each sample contained three technical replicates.

To determine the proteasome activity in the cell extracts, cells were lysed in ice-cold 50 mM Tris-HCl, pH 7.5, 25% sucrose, 0.05% digitonin, 2 mM EDTA, 100 µM ATP, 1 mM DTT, and 1x PhosSTOP. The cells were incubated for 15 min on ice, centrifuged at 20,000 × *g* for 15 – 20 min at +4ºC, 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 lysates 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 with the slope of mock-treated controls.

**Western blotting.** Cells were lysed in an ice-cold lysis buffer containing 50 mM Tris-HCl pH 7.5, 0.5% CHAPS, 10% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 100 µM ATP, and 1x

PhosSTOP™, and were centrifuged at 20,000 × *g* for 15 – 20 min at +4ºC. The protein concentration in the supernatants was determined using Pierce™ Coomassie Plus (Bradford) Assay reagent and used to normalize the sample loading. A mixture of samples with lithium dodecyl sulfate loading buffer was heated before fractionated 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 0.2µ 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 S4**.**

**Polysome profiling** was conducted according to a published procedure [48, 49]. 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 $MgCl_2$, 1.5 mM KCl, 1x complete mini protease inhibitor EDTA free, 100 µg/mL CHX, 1 mM DTT, and 100 units 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 $MgCl_2$, 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.** All statistical analyses were carried out in GraphPad PRISM.

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**Conflict of interest statement**

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

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