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.

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 1hr 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 β5 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.

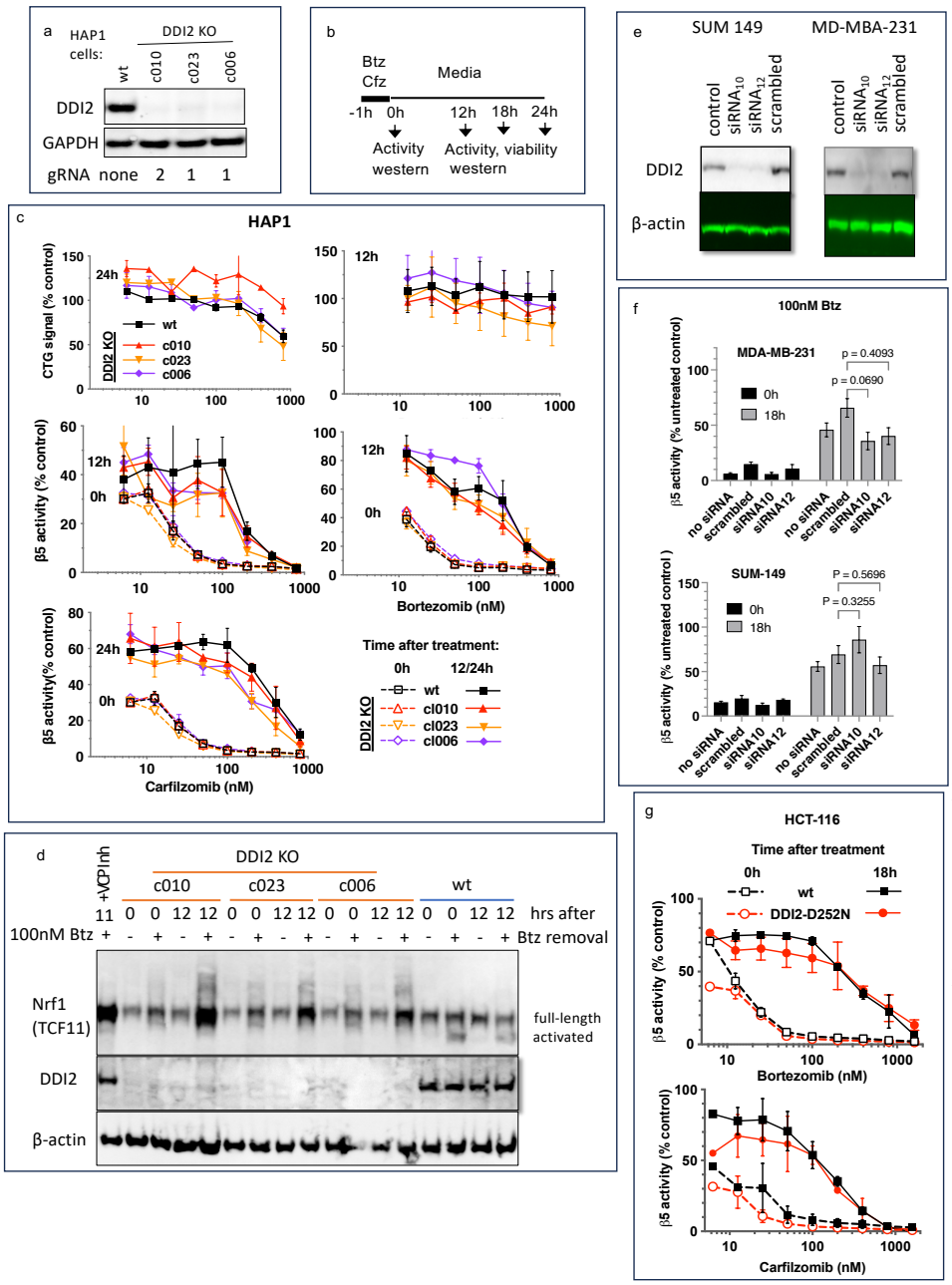


Figure 1. Recovery of proteasome activity is DDI2 independent. a. Expression of DDI2 in the CRISPR-generated clones of HAP1 cells used in this work was analyzed by western blot. The full-size membrane is presented in Fig. S1a, and gRNAs sequences are in Table S1. **b.** The experimental setup used in 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 β5 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 β5 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.** β5 activity was measured in HCT-116 cells with the Proteasome-Glo assay immediately and 18h after treatment with PIs; n=2.

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 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.

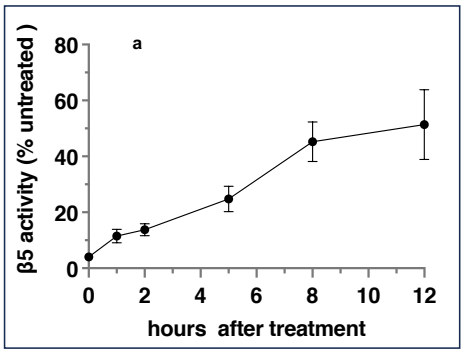
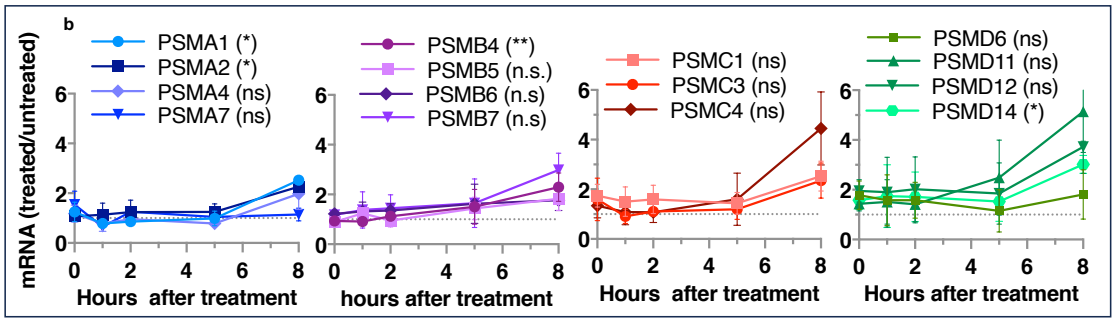


Figure 2. Proteasome activity recovers before upregulation of proteasome gene expression. Wt-HAP1 cells were pulse-treated with Btz (100 nM), cultured in a drug-free medium, and analyzed at indicated times. **a.** β5 activity was measured using Proteasome-Glo and normalized first to CellTiter-Glo viability data and then to proteasome activity in the mock-treated samples; 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.



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.

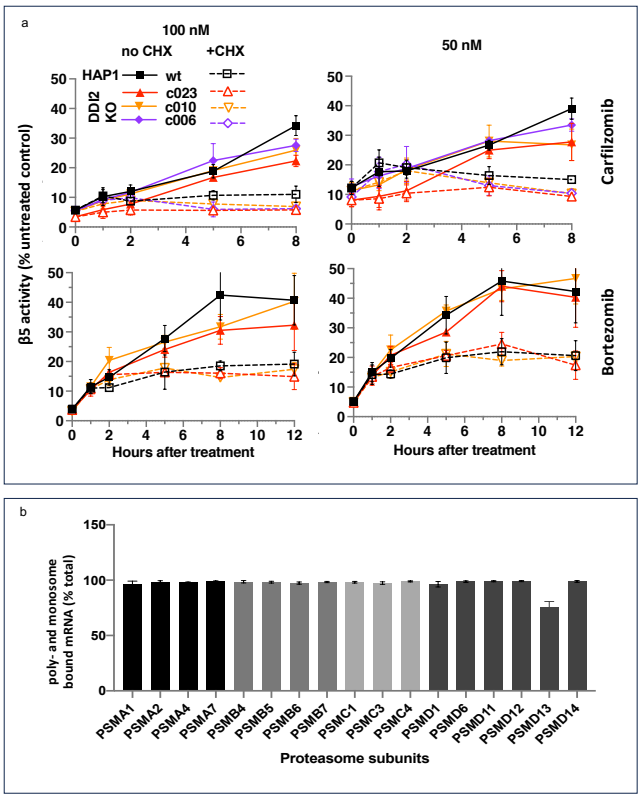


Figure 3. The recovery of proteasome activity requires protein synthesis. a. Wt-HAP1 and DDI2 KO cells were treated for 1h at indicated concentrations of Btz and Cfz and then cultured in a drug-free media in the absence (solid lines) or presence (dashed lines) of CHX. The β5 activity was measured using Proteasome-Glo and normalized first to cell viability, which was determined in a parallel experiment using CellTiter-Glo, and then to untreated controls; 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.

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.

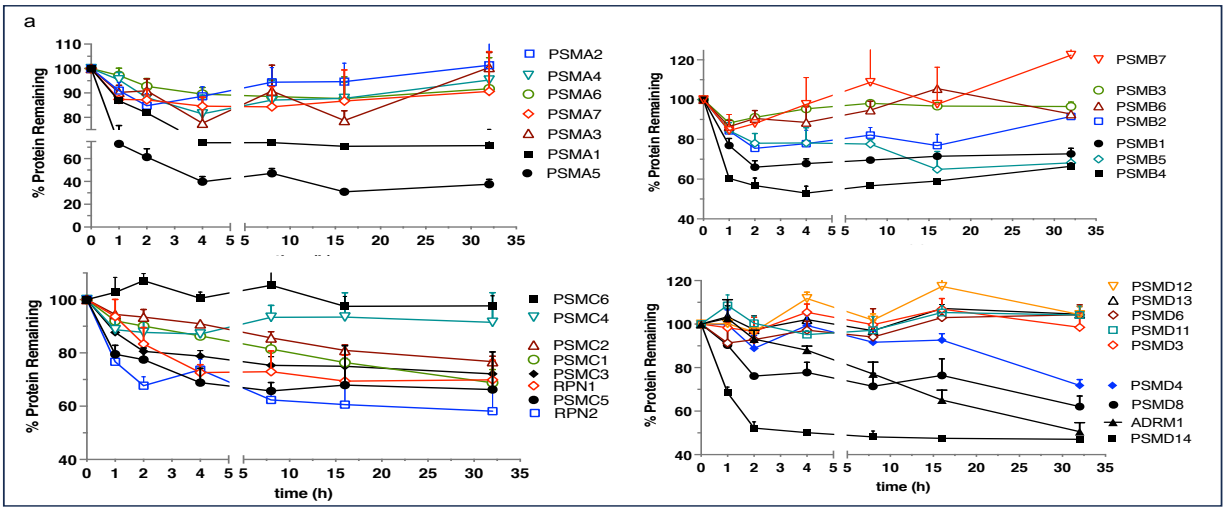
Discussion

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 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].



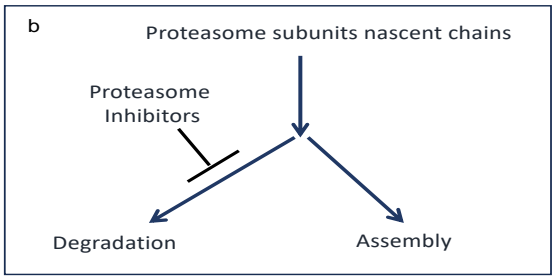


Figure 4. Escape from rapid degradation of nascent subunits can explain rapid recovery of proteasome activity. **a**. Turnover of proteasome subunit in human RPE-1 cells was measured by quantitative mass-spectrometry following 1h labeling with heavy isotopes. Data from Table S4 in [44]. **b**. Proposed model of how nascent proteasome subunits are partitioned between assembly and degradation.

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.

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. 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 $2x10^5$ 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 β5 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 25μL of cold PBS containing 0.05% digitonin. 25μL 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 β5 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 $MgCl_2$, 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 × *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 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 $MgCl_2$, 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 $MgCl_2$, 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. 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.

Acknowledgments

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CRISPR-engineered HCT-116 cells that express the D252N-DDI2 mutant, to Dr. Wade Harper and late Dr. Alfred L. Goldberg for advice, and to Tyler Jenkins and Sriraja Srinivasa for the critical reading of the manuscript.

Conflict of interest statement

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

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