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Combined chemical and genetic approach to inhibit proteolysis by the proteasome

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Abstract

Regulated protein destruction by the proteasome is crucial for the maintenance of normal cellular homeostasis. Much of our understanding of proteasome function stems from the use of drugs that inhibit its activity. Curiously, despite the importance of proteasomal proteolysis, previous studies have found that proliferation of the yeast *Saccharomyces cerevisiae* is relatively resistant to the effects of proteasome inhibitors such as MG132, even in the presence of mutations that increase inhibitor levels in cells. We reasoned that part of the resistance of *S. cerevisiae* to proteasome inhibitors stems from the fact that most proteasome inhibitors preferentially target the chymotryptic activity of the proteasome, and that the caspase-like and tryptic sites within the 20S core could compensate for proteasome function under these conditions. To test this hypothesis, we generated a strain of yeast in which the gene encoding the drug efflux pump Pdr5 is deleted, and the tryptic and caspase-like proteasome activities are inactivated by mutation. We find that this strain has dramatically increased sensitivity to the proteasome inhibitor MG132. Under these conditions, treatment of yeast with MG132 blocks progression through the cell cycle, increases the accumulation of polyubiquitylated proteins and decreases the ability to induce transcription of certain genes. These results highlight the contribution of the caspase-like and tryptic activities of the proteasome to its function, and provide a strategy to potently block proteasomal proteolysis in yeast that has practical applications.

Keywords

proteasome; inhibitor; cell-cycle; proteolysis; transcription

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Supporting information on the internet

The following supporting information may be found in the online version of this article:

Figure S1. Growth response to varying doses of MG132

Figure S2. Asynchronous population cytometry profiles

Introduction

In eukaryotes, the proteasome is a critical complex for the regulated destruction of proteins that removes misfolded, damaged and short-lived proteins targeted by ubiquitylation (Goldberg, 2003). The hydrolysis of peptides by the proteasome not only prevents the toxic accumulation of abnormal proteins but also regulates processes such as cell cycle progression (King *et al.*, 1996; Zachariae and Nasmyth, 1999), DNA repair (Daulny and Tansey, 2009; Krogan *et al.*, 2004), transcription (Collins and Tansey, 2006), protein quality control (Goldberg, 2003) and organelle distribution (Campbell *et al.*, 1994). Consequently, nearly all genes encoding proteasome subunits are necessary for the viability of the yeast *Saccharomyces cerevisiae* (Giaever *et al.*, 2002), and defects in the ubiquitin–proteasome system underlie, at least in part, several human diseases (Schwartz and Ciechanover, 1999).

The 26S proteasome is separable into two distinct subcomplexes: the 19S regulatory particle and the 20S core particle (Groll *et al.*, 1997). Within the 20S core reside three distinct proteolytic activities, chymotrptic-, tryptic- and caspase-like, each contained within a separate β-subunit (Pre2, Pup1, and Pre3, respectively) (Arendt and Hochstrasser, 1997; Heinemeyer *et al.*, 1997). This collection of different substrate specificities presumably endows the proteasome with the capacity to efficiently degrade a diverse set of substrates, although it is generally accepted that the chymotryptic activity of the proteasome is most important for its function. Indeed, mutations that inactivate the chymotryptic protease (Pre2) significantly stabilize the degradation of reporter proteins such as α2 or Ub-β gal fusions, whereas mutations that inactivate the trypsin- (Pup1) and caspase-like (Pre3) proteases result in only modest effects on the turnover of these model proteasome substrates (Arendt and Hochstrasser, 1997, 1999; Heinemeyer *et al.*, 1997).

Given the predominant role of the chymotryptic site of the proteasome, much effort has been placed on the development of small molecule inhibitors that block its function. A handful of compounds have been developed (Groll *et al.*, 2009) that typically block the chymotryptic site by forming covalent or pseudocovalent adducts with the catalytic threonine within this subunit. These inhibitors have been extremely valuable in elucidating the biological role of the proteasome in metazoan settings (e.g. Rock *et al.*, 1994) and in the identification of proteasome substrates (Mayor *et al.*, 2007). Consistent with the central importance of proteasomal proteolysis in cellular homeostasis, treatment of mammalian cells with proteasome inhibitors such as MG132 or bortezomib results in rapid cell cycle arrest and the induction of apoptosis, and indeed it is the ability of such compounds to potently kill cycling cells that underlies their utility in cancer therapy (Richardson *et al.*, 2006).

Interestingly, however, despite the sensitivity of mammalian cells to such inhibitors, the yeast *S. cerevisiae* appears relatively resistant to the effects of MG132 or bortezomib (Fleming *et al.*, 2002; Lee and Goldberg, 1998). Part of the resistance of yeast to MG132 is clearly due to poor uptake of these compounds into the cell (Lee and Goldberg, 1998), but even in the presence of mutations that increase the intracellular concentration of the drug (e.g. *ise1* or Δ*pdr5*) (Fleming *et al.*, 2002; Lee and Goldberg, 1998) the physiological response of yeast to these compounds is more subtle than expected. Lee and Goldberg (1998), for example, found that *ise1* yeast continue to grow when exposed to 10 μM MG132, even under conditions in which the chymotryptic activity of the proteasome could be inhibited by up to 95%. Similar results were reported by Fleming *et al.* (2002) in their study of the effects of bortezomib on *pdr5*-null yeast. The modest effects of MG132 and bortezomib on yeast proliferation contrasts strongly with the effects of these inhibitors on mammalian cells and with the effects of a temperature-sensitive mutation in the ubiquitin (Ub)-activating enzyme Uba1 (Ghaboosi and Deshaies, 2007) which, at the restrictive temperature, causes a rapid arrest of yeast growth. Although the basis for these contrasting

behaviours is not known, the lack-lustre response of yeast to compounds such as MG132 raises concern that chemical inhibition of the proteasome in *S. cerevisiae* is incomplete, thereby limiting the utility of current proteasome inhibitors for studies in this species.

We reasoned that part of the resistance of yeast to chemical inhibitors of the chymotryptic activity of the proteasome stems from the fact that, in *S. cerevisiae*, the tryptic- and caspase-like sites can compensate to maintain proteasome function in the presence of inhibitors. We therefore generated a strain of yeast in which *PDR5* is deleted, and the Pup1 and Pre3 subunits of the proteasome are inactivated by mutation. We show here that this strain is exquisitely sensitive to both reversible and irreversible chemical inhibitors of the chymotryptic site of the proteasome. These findings highlight the importance of the tryptic- and caspase-like activities to the physiological role of the proteasome, and provide a system in which to comprehensively inhibit proteasomal proteolysis by chemical inhibition of its chymotryptic function.

Materials and methods

Yeast strains

Strains used in this study are listed in Table 1. Mark Hochstrasser provided the strains of MHY1177 and MHY1178 (Arendt and Hochstrasser, 1999). Within these strains, and the control strain BY4742, *PDR5* was replaced by the *KanMX6* gene (Knop *et al.*, 1999). The resulting strains are GAC201 (*PUP1PRE3pdr5*) and GAC202 (*pup1pre3pdr5*). For α-factor arrest experiments, GAC201 and GAC202 were converted to the **a** mating type by expressing the *HO* endonuclease from a *URA3* selectable vector [Ycp50-HO (Krishnamoorthy *et al.*, 2006), a gift from Shelly Berger] followed by counter-selection with 5-FOA (US Biological) to create the strains GAC201**a** and GAC202**a**. Mating type was verified by failure to prevent the growth of *bar1* yeast [RC634 (Chan and Otte, 1982), a gift from Brehon Laurent] and by growth sensitivity to α-factor (Zymo Research).

Growth assays

Yeast cultures were grown YPAD (1% yeast extract, 2% bacto-peptone, 2% glucose, and 24 mg/l adenine hemisulfate at 30 °C to A_{600} = 0.2 and treated with either 50 μM MG132 (American Peptide) or an equivalent volume of DMSO (Sigma). At the indicated time points, samples were collected and the absorbance measured at 600 nm.

For plating assays with YU101, carfilzomib and bortezomib, GAC201 and GAC202 were grown overnight in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) and diluted to A_{600} = 0.3 in YP (1% yeast extract, 2% bacto-peptone). Serial five-fold dilutions were prepared in YP and spotted onto YPD plates supplemented with various proteasome inhibitor drugs at 10 μM (or 20 μM for carfilzomib). The plates were incubated at 30 °C for 2 days. YU101 and carfilzomib were gifts from Proteolix Inc. Bortezomib was a gift from Millenium Pharmaceuticals.

Cell cycle analyses

GAC201**a** and GAC202**a** were arrested in G_1 using 30 μM α-factor for 2 h at 25 °C. The samples were then treated with an additional 15 mM α-factor with 50 μM MG132 (or DMSO) for another 1 h at 25 °C. One-tenth of the culture was collected for the ‘time zero’ (t_0) sample; the remaining cells were released from arrest by washing twice with YPAD before growing in YPAD with 50 μM MG132 (or DMSO) at 30 °C. One-tenth of the culture was collected at each of the indicated time points.

For G_2–M arrest, GAC201 and GAC202 cultures growing in YPAD were treated with 150 μg nocodazole (Sigma) for 90 min at 30 °C. The samples were then treated with an additional 75 μg nocodazole with 50 μM MG132 (or DMSO) for 60 min. One-tenth of the sample was collected for the 'time zero' (t_0) point. The remaining culture was released from arrest by washing twice with YPAD before incubating in YPAD with 50 μM MG132 (or DMSO) at 30 °C. One-tenth of the culture was collected at each time point.

For flow cytometry, yeast cells were recovered by centrifugation, resuspended in 70% ethanol and stored at 4 °C overnight. These cells were then washed with water, treated for 12 h at 37 °C with DNase-free RNase (Roche), sonicated, treated for 2 h at 42 °C with proteinase K (Roche) and stored in 50 mM Tris–HCl, pH 7.5. One million cells were diluted in 1 ml SYBR gold solution (Invitrogen) and cell cycle profiles analysed by flow cytometry, using an LSR II cell analyser (BD Biosciences).

Anti-ubiquitin Western blot

Cultures of GAC201 and GAC202, growing in YPAD, were treated with 50 μM MG132 for the indicated times. Proteins were extracted in EZ buffer (60 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 2% 2-mercaptoethanol), resolved on an 8% SDS–polyacrylamide gel, and Western blotting used to detect total ubiquitin levels (anti-ubiquitin antibody MAB1510; Millipore). Protein loading was verified by Ponceau S staining.

RNA analysis

INO1 was induced by washing yeast grown in YPAD with water and transferring to complete synthetic medium (CSM) lacking inositol (Foremedium). *ARG1* was induced by transferring yeast to CSM lacking histidine. Induction and treatment with 50 μM MG132 (or equivalent volume of DMSO) lasted 90 min. RNA was extracted using a hot phenol extraction method (Muratani *et al.*, 2005). RNA was treated with DNaseI (Invitrogen) prior to using 1 μg RNA in a SuperScript II reverse transcriptase reaction (Invitrogen). Transcripts were quantified using quantitative PCR (qPCR) normalized to *ACT1* expression.

Results and discussion

Two previous studies (Fleming *et al.*, 2002; Lee and Goldberg, 1998) have reported that, in the presence of mutations that increase intracellular drug concentration, growth of common laboratory strains of *S. cerevisiae* is fairly resistant to drugs that inhibit the chymotryptic activity of the proteasome. Lee and Goldberg (1998) noted, for example, that *ise1* yeast, which are permeable to a variety of drugs (Birse *et al.*, 1998), continue to proliferate for up to 5 h after exposure to 10 μM MG132, despite this treatment resulting in ~95% inhibition of the chymotryptic function of the proteasome. This result is surprising, given the central role of proteasomal proteolysis in cell cycle progression (King *et al.*, 1996; Zachariae and Nasmyth, 1999) and suggests that residual activity of the proteasome is sufficient to allow yeast to proliferate in the presence of drug.

To explore this phenomenon, we deleted the gene encoding the drug efflux pump Pdr5 from the yeast strain BY4742 and repeated the growth analysis in the presence of MG132. Consistent with previous reports (Fleming *et al.*, 2002), we found that *pdr5*-null yeast continue to grow for up to 16 h after exposure to 50 μM MG132 (Figure 1A), albeit at a slightly reduced rate. Although there are numerous explanations for why yeast growth is resistant to MG132, we reasoned that part of the explanation may stem from the fact that MG132 preferentially inhibits the chymotryptic site of the proteasome and that, under these conditions, the tryptic (Pup1)- and caspase (Pre3)-like sites may function to support yeast cell viability. To test this notion, we next examined the behaviour of *pdr5*-null yeast in

which the catalytic threonines of each active site within Pup1 and Pre3 are mutated to alanine (Arendt and Hochstrasser, 1999). In the absence of MG132 (Figure 1B), the *pup1pre3pdr5* yeast grew somewhat more slowly than the congenic *PUP1PRE3pdr5* control strain, congruent with results from Arendt and Hochstrasser (1999), showing that the Pup1 and Pre3 sites within the proteasome contribute slightly to yeast cell viability. In the presence of MG132, however, the behaviours of the two yeast strains were dramatically different. Whereas growth of the 'wild-type' *PUP1PRE3pdr5* strain was unaffected by treatment with 50 μM MG132 (or 100 μM MG132; see Supporting information, Figure S1), the *pup1pre3pdr5* strain responded strongly to the drug, and essentially ceased to proliferate under these conditions (Figure 1B). Similar results were obtained whether we monitored cell growth by optical density (Figure 1) or by cell counting (data not shown). Response of the *pup1pre3pdr5* yeast to MG132 was sensitive to the concentration of MG132 (see Supporting information, Figure S1) and could be reversed by transfer of cells into fresh media, consistent with the reversible nature of MG132 inhibition of proteasome function (Groll *et al.*, 2009). From these data, we conclude that *pup1pre3pdr5* yeast undergo a rapid and reversible arrest of growth upon exposure to MG132. We further conclude that one or both of the compensatory activities of the tryptic- and caspase-like sites of the proteasome help mask the effects of MG132 (and other inhibitors of the proteasome chymotryptic site) on the proliferative capacity of yeast. Similar effects were observed in *pup1pre3PDR5* yeast treated with 0.003% SDS (Liu *et al.*, 2007) to promote MG132 uptake (data not shown).

Because of the pivotal role of the Ub–proteasome system in regulating cell cycle progression (King *et al.*, 1996; Zachariae and Nasmyth, 1999), we next asked whether *pup1pre3pdr5* yeast displayed cell cycle arrest in response to MG132. Analysis of asynchronous cultures of these cells by flow cytometry (see Supporting information, Figure S2) revealed that *pup1pre3pdr5* yeast typically had a lower percentage of cells in S-phase than their *PUP1PRE3pdr5* counterparts, but that the overall ratio of cells with 1n versus 2n DNA content was not significantly affected by treatment with MG132. This result suggested that MG132 restricts yeast growth at multiple points in the cell duplication cycle, consistent with the rapid effect of the drug on yeast proliferation (Figure 1B). To ask specifically whether two major cell cycle transitions are affected by MG132 — exit from G_2–M and G_1 — we repeated the flow cytometry analyses in *pup1pre3pdr5* (and control) cells that had been arrested at either G_2–M (Figure 2A) or G_1 (Figure 2B), using nocodazole or α-factor, respectively. Following release from nocodazole arrest, *PUP1PRE3pdr5* yeast rapidly re-entered the cell cycle (as judged by the appearance of cells with 1n DNA content) and the rate at which this occurred was not significantly affected by treatment with MG132. The *pup1pre3pdr5* strain had a slower rate of re-entry into the cell cycle in the presence of DMSO, and this rate was affected slightly by treatment with MG132; in DMSO-treated cells, 70% of cells remained in G_2–M following release of nocodazole block, whereas this number increased to 80% upon MG132 treatment. We conclude that MG132 treatment of *pup1pre3pdr5* yeast results in a subtle reduction in the ability of these cells to transition out of G_2–M.

A strikingly different result was observed when we probed the ability of *pup1pre3pdr5* yeast to exit from G_1. Following α-factor arrest, *PUP1PRE3 pdr5* yeast rapidly re-entered the cell cycle (as judged by appearance of cells with 2n DNA content), and the rate at which this occurred was not significantly affected by treatment with MG132. The *pup1pre3pdr5* strain, in contrast, re-entered the cell cycle in the presence of the DMSO control, but in the presence of MG132 virtually none of the cells exited G_1. At the 110 min time point, for example, the distribution of cells with 1n and 2n DNA content under these conditions was identical to that observed in α-factor-arrested cells at t_0 (Figure 2B). We conclude that treatment of *pup1pre3pdr5* yeast with MG132 blocks the ability of these cells to transition from G_1 to subsequent phases of the cell cycle.

One of the practical uses of proteasome inhibitors such as MG132 is to promote accumulation of Ub–protein conjugates to allow their subsequent detection by either directed or naive methods, such as proteomic screening analyses (Mayor *et al.*, 2007). If indeed the Pup1 and Pre3 subunits can compensate for chemical inactivation of the chymotryptic site, we would expect that the full spectrum of Ub–protein conjugates would not accumulate in yeast treated with MG132 unless the proteolytic activity of these subunits was inactivated. To address this point, we examined the levels of high molecular weight Ub-conjugates in *pup1pre3pdr5* (and control) cells treated with MG132 (Figure 3). Compared to *PUP1PRE3pdr5* cells, the triple mutant yeast again responded much more robustly to MG132 treatment; high molecular weight Ub conjugates accumulated faster (an increase is apparent at the 15 min time point; cf. lanes 1 and 2 with lanes 6 and 7) and to a significantly higher level (cf. lanes 5 and 10). This result provides molecular support for the notion that one or both of the Pup1 and Pre3 subunits of the proteasome functionally compensate for Pre2-mediated destruction of ubiquitylated proteins in the presence of MG132. Importantly, these results also demonstrate that a combined chemical and genetic strategy to inhibit proteasomal proteolysis can be used significantly enhance detection of ubiquitylated proteins in yeast.

In addition to MG132, we were interested in asking whether *pup1pre3pdr5* yeast also had enhanced sensitivity to other inhibitors of the chymotryptic site of the proteasome. For this analysis, we chose the reversible inhibitor bortezomib, as well as two irreversible inhibitors, YU101, a derivative of epoxomicin (Bo Kim *et al.*, 2005), and carfilzomib, another derivative of epoxomicin that has been taken to clinical trials (Kuhn *et al.*, 2007). We assayed the ability of these compounds to inhibit yeast growth on solid media, as shown in Figure 4. Consistent with our earlier findings, treatment of *PUP1PRE3pdr5* yeast with these compounds had little if any effect on yeast growth under these conditions. Growth of the *pup1pre3pdr5* yeast, in contrast, was strongly inhibited by all three compounds, with the greatest attenuation of growth observed in the presence of 20 μM carfilzomib (cf. growth of *pup1pre3pdr5* yeast on YPD vs medium containing the proteasome inhibitors). These data demonstrate that genetic inactivation of the catalytic activity of Pup1 and Pre3 renders yeast generally susceptible to chemical inhibition of the chymotryptic site of the proteasome.

Finally, we examined the effects of MG132 on gene induction in the *pup1pre3pdr5* strain. A growing body of evidence supports a role for the Ub–proteasome system in the regulation of a diverse set of genes (Lipford and Deshaies, 2003; Muratani and Tansey, 2003), although whether proteasome inhibition via MG132 disrupts gene activation in *S. cerevisiae* is controversial (Collins *et al.*, 2009; Lipford *et al.*, 2005; Nalley *et al.*, 2006). Given the striking effects of MG132 we observe in *pup1pre3pdr5* cells (Figures 1–4), we decided to re-examine this issue under circumstances where we could be confident that we had comprehensively inhibited proteasome function. We were not able to examine *GAL* induction, because this strain background is unable to utilize galactose as a carbon source (not shown). For this analysis, therefore, we selected two strongly induced target genes that are regulated by different stimuli: (a) *ARG1*, which is induced by amino acid starvation via the Gcn4 activator (Crabeel *et al.*, 1995); and (b) *INO1*, which is induced by inositol starvation via the Ino2 and Ino4 transcription factors (Ambroziak and Henry, 1994). We added 50 μM MG132 60 min prior to induction and examined transcript levels from each gene in the congenic *PUP1PRE3pdr5* and *pup1pre3pdr5* strains an additional 60 min after induction. The results of this analysis are shown in Figure 5. For *ARG1*, we consistently observed a small but significant effect of MG132 on induction in the *PUP1PRE3pdr5* cells (Figure 5A) and, as expected, the magnitude of the effect of MG132 was increased in the *pup1pre3pdr5* background. For *INO1*, the effects of the Pup1/Pre3 mutations on response to MG132 were even more dramatic; in the *PUP1PRE3pdr5* setting, *INO1* induction was completely insensitive to MG132 (Figure 5B), whereas in the *pup1pre3pdr5* cells, *INO1*

induction was substantially reduced by MG132 treatment. Thus, as with growth, cell cycle perturbation and accumulation of Ub conjugates, the trypticand/or caspase-like activities of the proteasome appear to mask the full effects of MG132 on gene induction *in vivo*. We conclude that, when the proteasome is effectively inhibited using this combined chemical and genetic approach, transcriptional activation can be significantly compromised.

Concluding remarks

We report here a strategy for comprehensively inhibiting proteasome function in *S. cerevisiae* by combining genetic ablation of the tryptic and caspase-like protease sites with transient chemical inhibition of the chymotryptic site within the proteasome. Using this strategy, we observed robust effects of MG132 and other inhibitors on yeast cell growth, cell cycle progression, accumulation of Ub-conjugates and transcriptional activation. These results highlight the contribution of the tryptic- and caspase-like protease sites to proteasome function in yeast, and provide a setting in which compounds such as MG132 can become potent transient inhibitors of proteasome function. The dramatically enhanced sensitivity of the *pup1pre3pdr5* strain to MG132, bortezomib, YU101 and carfilzomib should prove useful for detection of Ub–protein conjugates, and for probing the role of proteasomal proteolysis in processes such as transcription, DNA repair, protein quality control and organelle dynamics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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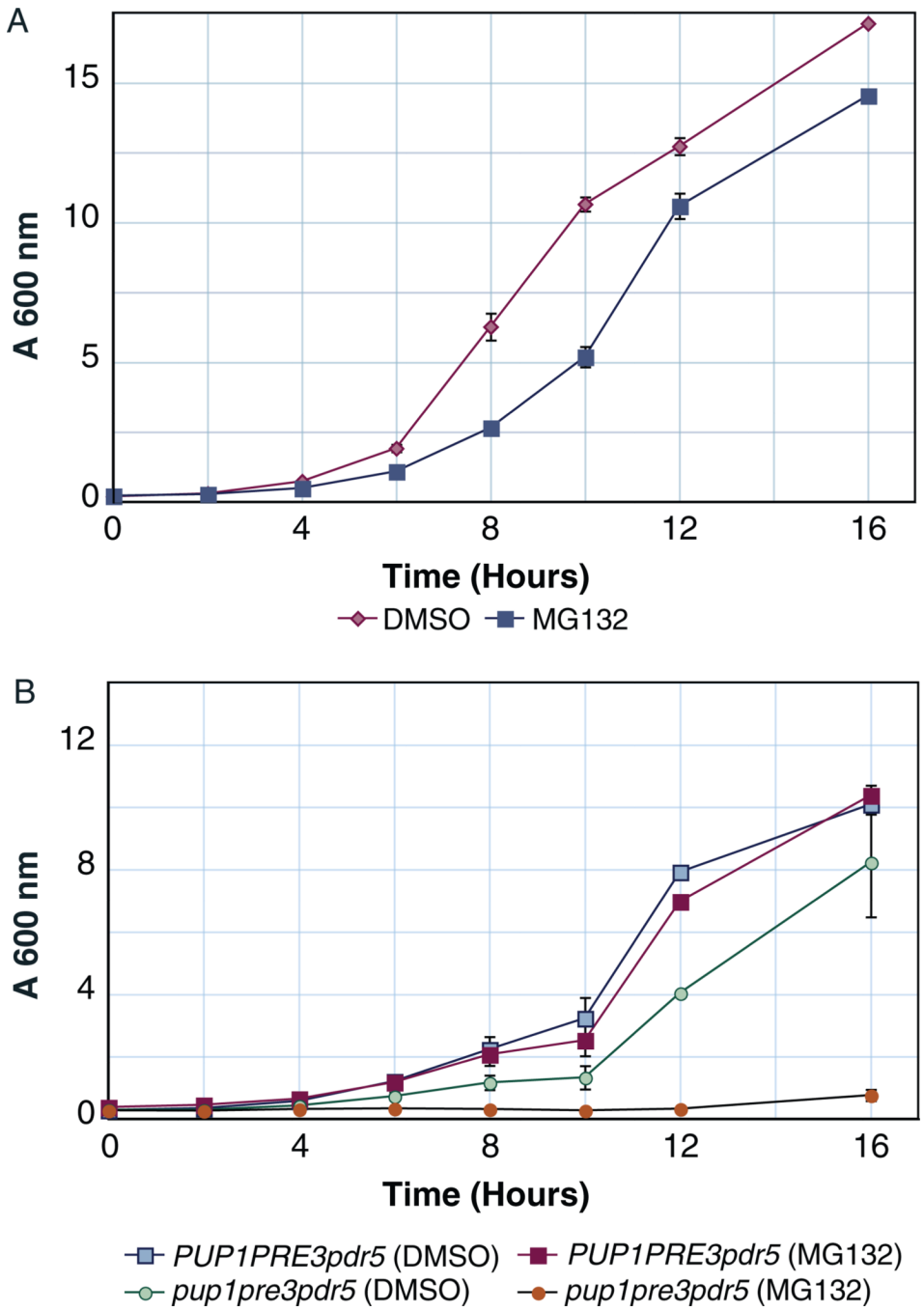

Figure 1.

Effect of MG132 on *S. cerevisiae* growth. (A) BY4742 Δ*pdr5::KanMX6* were treated with either DMSO or 50 μM MG132 and growth measured as increase in the absorbance (A600 nm) of the culture. (B) As in (A) except that strains GAC201 (*PUP1PRE3pdr5*) and GAC202 (*pup1pre3pdr5*) were used. In all cases, data are from three independent experiments with errors representing standard error of the mean (SEM)

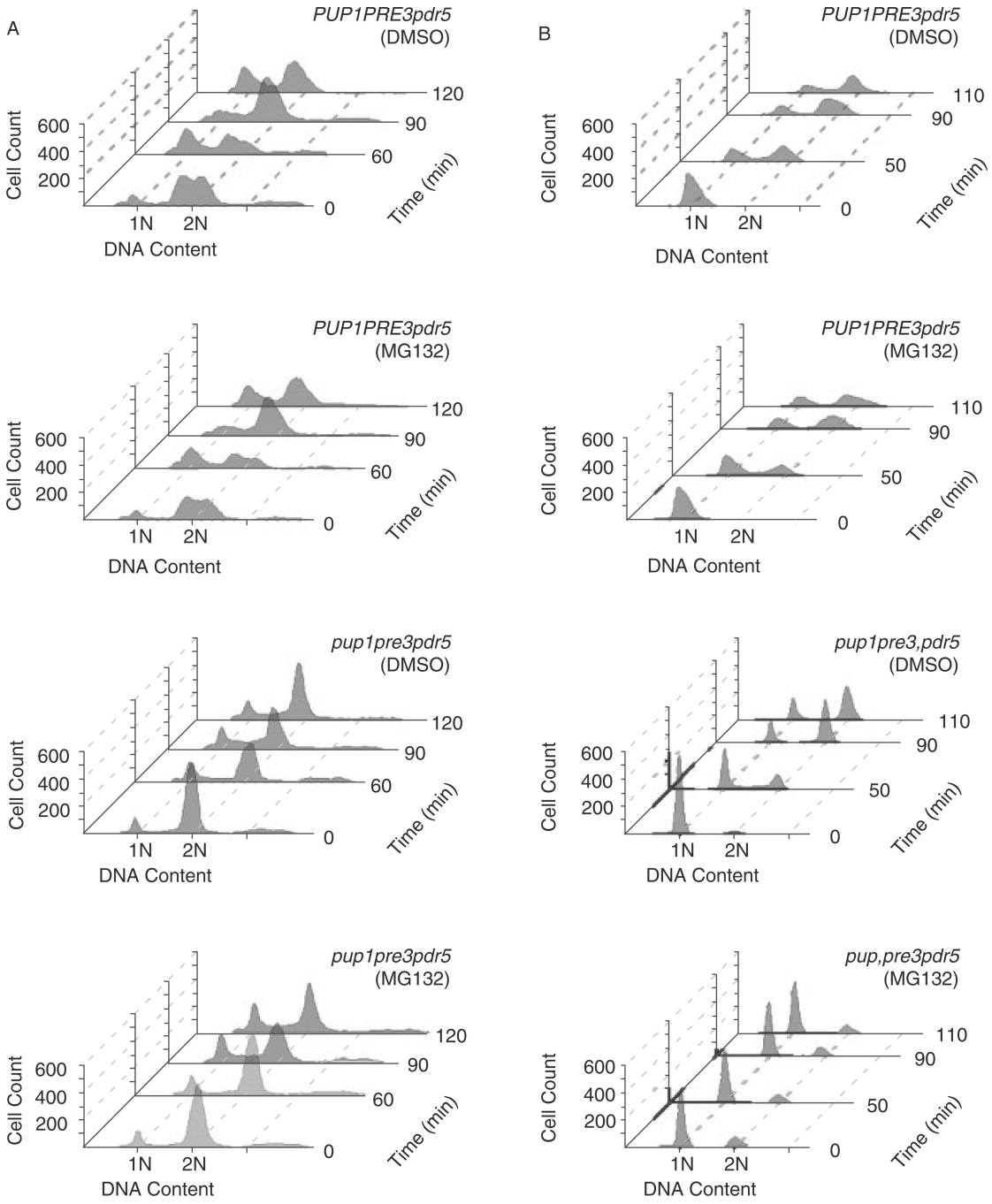

Figure 2.

Effect of MG132 on cell cycle progression in *pup1pre3pdr5* yeast. (A) *PUP1PRE3pdr5* (GAC201) and *pup1pre3pdr5* (GAC202) yeast were arrested at G_2–M by treatment with nocodazole. They were then treated with DMSO or 50 μM MG132 and released from arrest. At the indicated times, samples were collected, and flow cytometry performed to determine the profiles of DNA content. (B) *PUP1PRE3pdr5* (GAC201**a**) and *pup1pre3pdr5* (GAC202**a**) yeast were arrested at G_1 by treatment with α-factor. They were then treated with DMSO or 50 μM MG132 and released from arrest. At the indicated times, samples

were collected and flow cytometry performed to determine the profiles of DNA content. Representative examples of three biologically independent replicates are shown

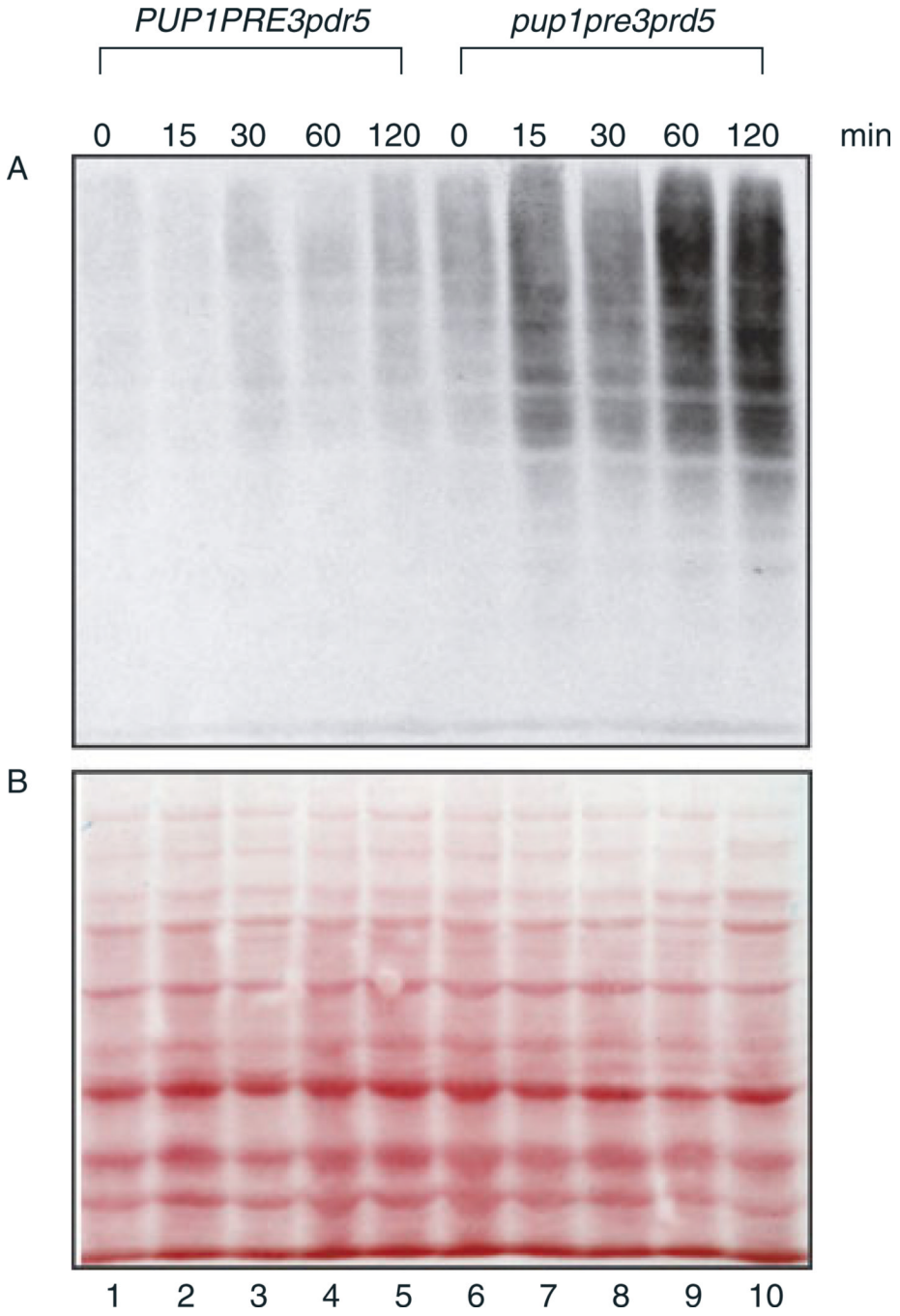

Figure 3.
Analysis of high molecular weight ubiquitin conjugates in *pup1pre3pdr5* yeast upon treatment with MG132. (A) Anti-ubiquitin Western blot of whole cell extracts from *PUP1PRE3pdr5* (GAC201) and *pup1pre3pdr5* (GAC202) yeast treated with 50 μM MG132 for the indicated times. (B) Ponceau S staining of the membrane to demonstrate equivalent loading of whole cell extracts into the gel. A representative of two independent experiments is shown

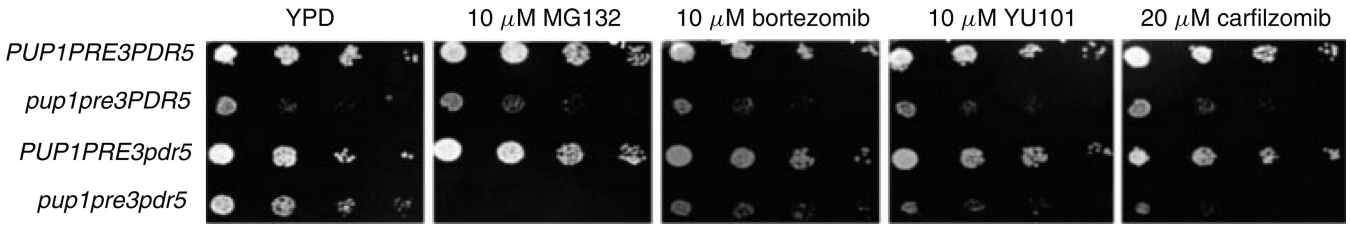

Figure 4.

Sensitivity of pup1pre3pdr5 yeast to multiple proteasome inhibitors. Serial five-fold dilutions of *PUP1PRE3PDR5* (MHY1177), *pup1pre3PDR5* (MHY1178), *PUP1PRE3pdr5* (GAC201), and *pup1pre3pdr5* (GAC202) were spotted onto YPD, YPD + MG132 (10 μM), YPD + bortezomib (10 μM), YPD + Y101 (10 μM) and YPD + carfilzomib (20 μM) plates prior to growth for two days at 30 °C. A representative of two independent experiments is shown

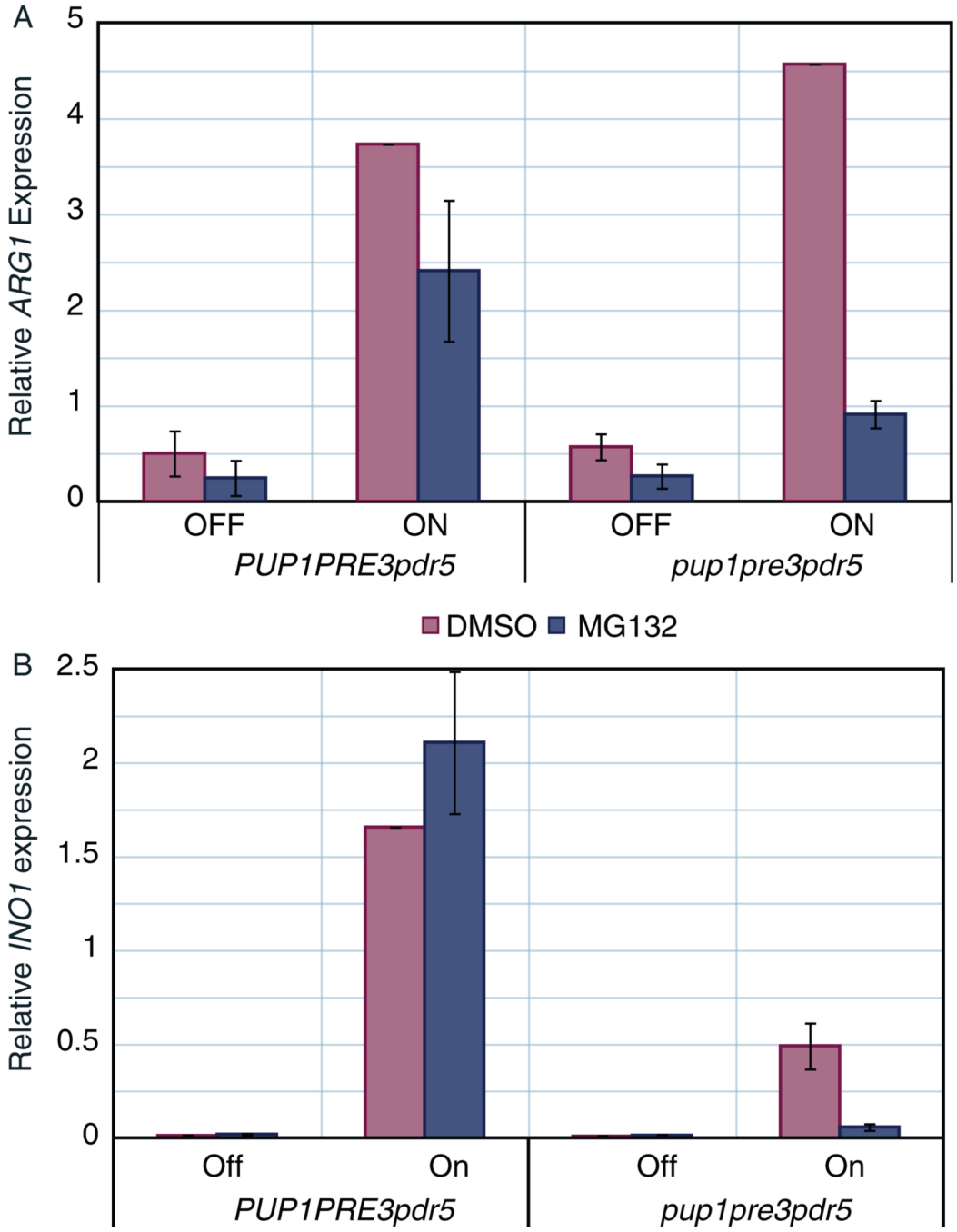

Figure 5.

Effect of MG132 on transcription induction in *pup1pre3pdr5* yeast. (A) *PUP1PRE3pdr5* (GAC201) and *pup1pre3pdr5* (GAC202) cells were treated with 50 μM MG132 (or DMSO) prior to induction by histidine starvation. RNA was harvested and RT–QPCR used to determine ARG1 transcript levels, relative to those of ACT1; $n = 4$, SEM. (B) As in (A) except that induction was by inositol starvation and INO1 RNA levels were monitored; $n = 3$, SEM

Table 1

Strains used in this study

Strain	Genotype	Source
BY4742 Δ*pdr5::KanMX6*	Matα *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 pdr5*Δ*::KanMX6*	This study
MHY1177	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3-*Δ*2::HIS3 pup1*Δ*::leu2-HIS3* [pRS317-PUP1] [YCplac22-PRE3] gal$^-$	Arendt and Hochstrasser, 1999
MHY1178	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3-*Δ*2::HIS3 pup1*Δ*::leu2-HIS3* [pRS317-pup1-T30A] [YCplac22-pre3-T20A] gal$^-$	Arendt and Hochstrasser 1999
GAC201	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pdr5*Δ*::KanMX6 pre3-*Δ*2::HIS3 pup1* Δ*::leu2-HIS3* [pRS317-PUP1] [YCplac22-PRE3] gal$^-$	This study
GAC202	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pdr5*Δ*::KanMX6 pre3-*Δ*2::HIS3 pup1*Δ*::leu2-HIS3* [pRS317-pup1-T30A] [YCplac22-pre3-T20A] gal$^-$	This study
GAC201**a**	Mat**a** *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pdr5*Δ*::KanMX6 pre3-*Δ*2::HIS3 pup1*Δ*::leu2-HIS3* [pRS317-PUP1] [YCplac22-PRE3] gal$^-$	This study
GAC202**a**	Mat**a** *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pdr5*Δ*::KanMX6 pre3-*Δ*2::HIS3 pup1*Δ*::leu2-HIS3* [pRS317-pup1-T30A] [YCplac22-pre3-T20A] gal$^-$	This study
RC634	Mat**a** *rme1 ade2-1 ura1 his6 met1 can1 cyh2 sst1-3*	Chan and Otte, 1982
RJD3269	Mat**a** *can1-100 leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 uba1*Δ*::KanMX6* [pRS313-uba1-204-HIS]	Ghaboosi and Deshaies, 2007