

Reversible 26S Proteasome Disassembly upon Mitochondrial Stress

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SUMMARY

In eukaryotic cells, proteasomes exist primarily as 26S holoenzymes, the most efficient configuration for ubiquitinated protein degradation. Here, we show that acute oxidative stress caused by environmental insults or mitochondrial defects results in rapid disassembly of 26S proteasomes into intact 20S core and 19S regulatory particles. Consequently, polyubiquitinated substrates accumulate, mitochondrial networks fragment, and cellular reactive oxygen species (ROS) levels increase. Oxidation of cysteine residues is sufficient to induce proteasome disassembly, and spontaneous reassembly from existing components is observed both *in vivo* and *in vitro* upon reduction. Ubiquitin-dependent substrate turnover also resumes after treatment with antioxidants. Reversible attenuation of 26S proteasome activity induced by acute mitochondrial or oxidative stress may be a short-term response distinct from adaptation to long-term ROS exposure or changes during aging.

INTRODUCTION

Stress conditions such as UV damage, heat-induced denaturation, peptide-bond cleavage, protein crosslinking, and oxidation of amino-acid side chains can alter protein tertiary structure. Aggregation-prone misfolded proteins, such as those with oxidative damage, require rapid removal, increasing the burden on quality-control and disposal machineries. Reactive oxygen species (ROS) that arise from both environmental and internal sources, such as mitochondrial metabolism, determine the cumulative oxidative stress on cells (Ambrosio et al., 1993). Mitochondrial failure accompanied by ROS production therefore correlates with oxidized protein accumulation and age-related pathologies (de Moura et al., 2010; Friguier et al., 2008; Page et al., 2010; Vendelbo and Nair, 2011). Indeed, damaged mito-

chondria correlate with muscle atrophy, neurodegenerative pathologies, and aging itself (Sheridan and Martin, 2010).

Numerous cellular mechanisms are linked to mitochondrial quality control. The ubiquitin-proteasome system (UPS) is the main eukaryotic apparatus for removing aberrant proteins that may contribute to misregulation of processes and disease pathogenesis (Finley, 2009; Glickman and Ciechanover, 2002; Layfield et al., 2001). Fragmented, malfunctioning, or damaged mitochondria can be removed by mitophagy (Green et al., 2011; Rambold et al., 2011), and degradation of specific mitochondrial outer membrane (MOM) proteins by the UPS can promote proper mitochondrial function and morphology (Livnat-Levanon and Glickman, 2011; Taylor and Rutter, 2011). A UPS-driven, mitochondria-associated degradation (MAD) pathway, like the endoplasmic-reticulum-associated degradation [ERAD] pathway, has been postulated (Cohen et al., 2008; Heo et al., 2010).

A UPS-dependent proteolytic event commences with polyubiquitination of a target, followed by recognition of the conjugate by the 26S proteasome. Proteasomes unfold ubiquitinated substrates, remove ubiquitin (Ub) tags, and hydrolyze the target into short peptides (Finley, 2009). Proteasomes are highly conserved 2.5 MDa molecular machines consisting of a barrel-like 20S proteolytic core particle (CP) and a 19S regulatory particle (RP) that recognize and translocate substrates into the 20S CP. Whether one 19S RP or two attach to a single 20S CP defines the two forms of the 26S proteasome: RP₁CP and RP₂CP, respectively (Beck et al., 2012; Glickman et al., 1998b). Access to 20S CP active sites is restricted by a narrow gated channel that is opened upon 19S RP attachment. Only a limited repertoire of misfolded or damaged polypeptides can be degraded by unaided 20S CP, yet this may explain the removal of damaged proteins by 20S CP during oxidative stress (Bader and Grune, 2006; Davies, 2001). Controlled degradation of globular or structurally stable substrates requires a regulator/activator complex that traps, unfolds, and feeds substrates into the 20S CP chamber. In cells, the majority of proteasomes exist as 26S holoenzymes, the most efficient configuration for ubiquitinated protein degradation (Bajorek et al., 2003; Kruegel et al., 2011). However, in response to certain stress conditions or upon exposure to oxidants, decreased 26S holoenzyme levels

and increased free 20S CP have been reported (Demasi et al., 2003; Reinheckel et al., 1998; Silva et al., 2012; Wang et al., 2010).

To minimize the damaging effects of ROS, aerobic organisms evolved different antioxidant defenses, including the expression of catalases, peroxidases, superoxide dismutases (SODs), and glutathione S-transferases (GSTs) (Hayes et al., 2005; Scandalios, 2005). Ubiquitin and other UPS components are also upregulated upon heat shock or other stresses that induce protein damage (Layfield et al., 2001; Tonoki et al., 2009). Yet, as shown here, 26S proteasome holoenzymes are sensitive to the oxidative conditions under which they disassemble, leading to transient attenuation of ubiquitin-dependent proteolysis. We expand on previous work that described oxidation-driven 26S proteasome dissociation (Wang et al., 2010) by demonstrating comparable shifts in proteasome populations from predominantly 26S holoenzymes to free 20S CP in response to mitochondrial dysfunction or oxidative stress. We find that proteasome disassembly is reversible both in cells and in vitro. Proteolytically active 20S CPs and stable 19S regulatory complexes can rapidly reassociate, leading to resumption of ubiquitin-dependent protein turnover in cells, indicating that proteasomes adapt to environmental conditions and cellular needs.

RESULTS

Proteasomes Dissociate in Response to Oxidative Stress Caused by Mitochondrial Dysfunction

First, we explored whether proteasomes dissociate in response to stimulation of ROS formation due to postdiauxic shift in a respiration-deficient mitochondrial mutant ($\Delta fzo1$) of yeast. Respiration attempts by defective mitochondria induced ROS production in this strain (Figure S1) and proteasome disassembly was accelerated (Figure 1A). This is a remarkable example of a mitochondrial mutant with a proteasome phenotype. A similar phenomenon was observed in a strain in which a proteasome subunit-encoding gene was mutated ($rpn11-m1$; Figure 1A). Fragmented mitochondria networks were previously reported in $rpn11-m1$ (Rinaldi et al., 2002). Extending those observations, we measured high levels of intracellular ROS (versus wild-type [WT]) in both strains ($\Delta fzo1$ and $rpn11-m1$; Figure S1). Cellular damage due to defective mitochondria attempting to respire on low concentrations of nonfermentable carbon may be the root cause of the observed altered proteasome activity (Figure 1).

Next, we explored whether malfunctioning mitochondria can directly induce proteasome disassembly, by testing the effect of a mitochondrial respiration inhibitor (Antimycin A, a Cytochrome C reductase inhibitor; Chen et al., 2003; Turrens, 1997) on proteasomes. We found that treating rapidly dividing yeast cells with Antimycin A resulted in rapid disassembly of 26S proteasomes in vivo (Figure 1B), but had no direct effect on purified proteasomes (Figure 1B). Proteasome disassembly in cells correlated with altered oxidative phosphorylation in mitochondria (i.e., an inability to utilize nonfermentative carbon; Figure 1C), ROS release (Chen et al., 2003; Lin and Beal, 2006; Sedensky and Morgan, 2006), and pervasive protein oxidation (Figure 1C). Attenuated proteasomes were also observed in

cell cultures subjected to direct oxidation. In both yeast and mammalian cells, 26S proteasome activity decreased with exposure to increasing concentrations of external oxidizing reagents (Figure 1D). Concomitantly with diminished 26S proteasome activity, increased 20S CP activity was measured, suggesting that proteasome active sites were not hampered, but rather the proteasome population shifted from predominantly 26S holoenzymes to primarily free 20S CP. 20S CP was detached in its latent form and was chemically activatable (by miniscule concentrations of SDS; Glickman et al., 1998a). Notably, even at these considerably high concentrations of H_2O_2 in the medium, cells were viable for at least a couple of hours, and growth resumed (together with reassociated proteasomes) when the oxidants were removed from the media or the media were neutralized with a reducing agent (Figures 1D, middle panel, and 2F). Thus, our findings demonstrate that proteasome conformation is sensitive to oxidative stress, and despite the essential role of proteasomes in protein turnover, under the tested conditions, cells maintain viability even though the activity of the 26S holoenzyme is attenuated.

Proteasomes Exhibit Reversible Redox-Dependent Dissociation/Reassociation

We evaluated the direct effect of oxidation on the proteasome by exposing purified proteasomes to oxidants and resolving the breakdown products by nondenaturing gels (in order to keep complexes intact and proteolytically active for enzymatic or biochemical analysis). A dose-dependent detachment of active 20S CP from 26S proteasomes was observed upon addition of H_2O_2 (Figure 2A, left panel), and the migration pattern mimicked the behavior of proteasomes in stressed cells (Figure 1). In parallel to increased levels of the 20S CP form, a second proteolytically inactive complex containing 19S RP subunits appeared at the expense of the diminishing 26S (Figure 2A, middle and right panels). In yeast cells too, simultaneous accumulation of complexes containing 19S RP or 20S CP subunits was observed in response to acute oxidative stress concomitantly with diminished 26S proteasome levels (Figure 2A, right). We determined the makeup of these complexes by mass spectrometry (MS)-based, label-free quantitative analysis. All 19S RP subunits in the proteolytically inactive complex were identified at roughly stoichiometric levels (Figure 2B; Table S1). The quantity of intact regulatory 19S complexes in the treated samples relative to the untreated control increased significantly. Released 20S particles were active but latent, and could be reactivated by treatment with a low level of SDS (Figure 2A), similar to 20S CP generated in stressed cells (Figure 1). Thus, these findings demonstrate that in response to oxidative stress, 26S proteasome holoenzymes dissociate into two subcomplexes, which remain stable and intact.

We investigated whether proteasome disassembly is an irreversible consequence of oxidative damage or an environmental-sensing mechanism by testing the effect of a reducing reagent (dithiothreitol [DTT]) on cells after dissociation of proteasome complexes. Proteasome disassembly was partially reversed within minutes of adding DTT to the cell media (Figure 2C). Even disassembly of proteasomes that occurred naturally over time in respiratory-defective cells ($rpn11-m1$) was

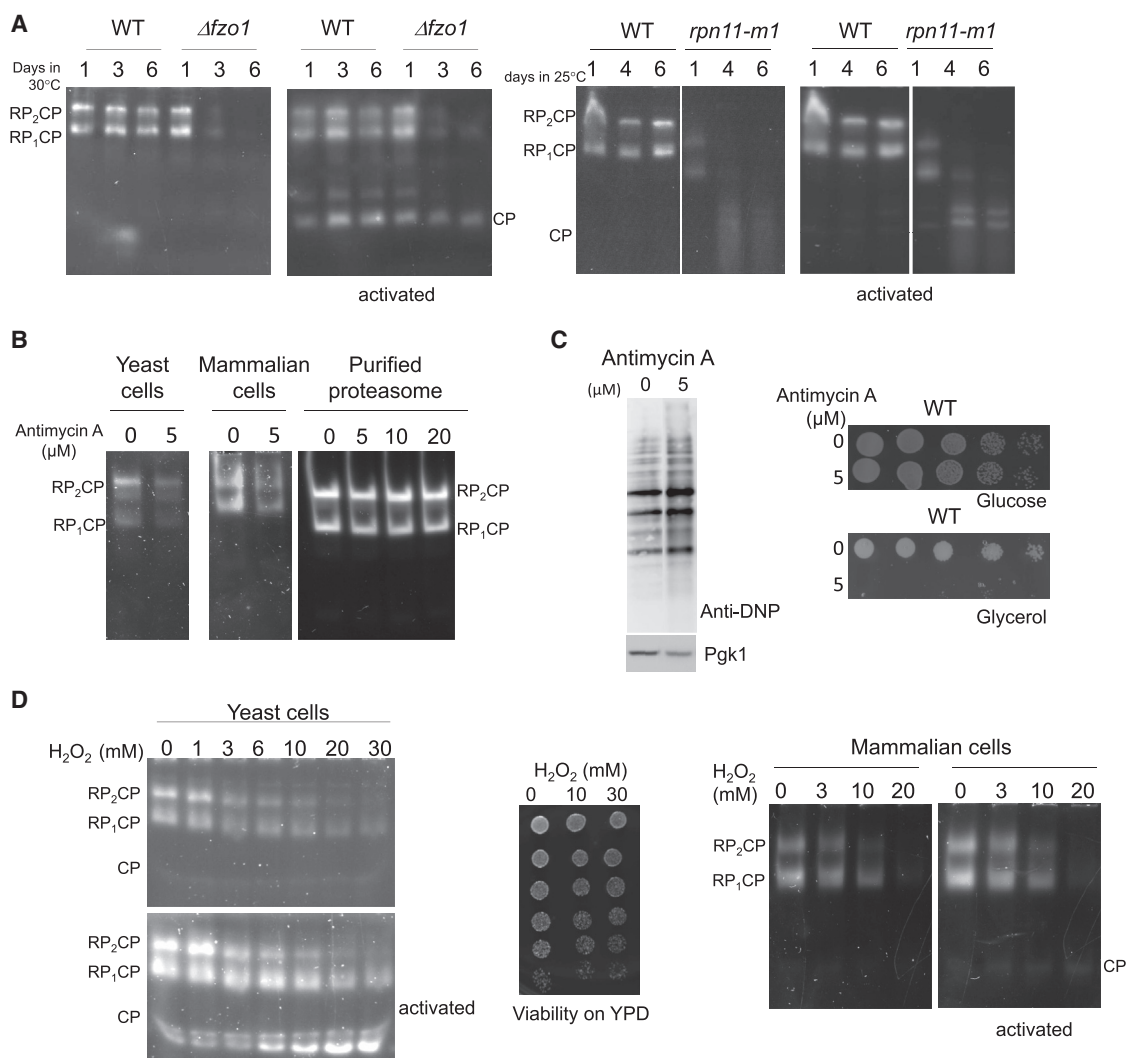


Figure 1. Fragile Proteasomes in Response to Oxidative Stress

(A) Proteasome disassembly following postdiauxic shift. $\Delta fzo1$, $rpn11-m1$, and their isogenic WT strains were shifted from fermentative logarithmic growth to diauxic respiration by glucose starvation. At the indicated days, equal numbers of cells were harvested, lysates were separated by nondenaturing PAGE (native gel), and proteasome activity was detected by in-gel peptidase activity. The main proteasome species are marked (RP₂CP, doubly capped 26S; RP₁CP, singly capped 26S; CP, 20S CPs). Latent 20S CP was activated by infusing 0.02% SDS ("activated").

(B) Proteasome attenuation in response to mitochondria respiration. Yeast (left) or mammalian cells (middle) were incubated with 5 μM Antimycin A for 3 hr and proteasome activity was monitored by native gel. Purified proteasomes were tested with Antimycin A for control (right).

(C) Antimycin A promoted protein oxidation. Cellular extracts following Antimycin A treatment were assayed for oxidized proteins by incubation with DNP, a reagent for oxidized amino-acid side chains (left). Following Antimycin A treatment, cells were also scored as 10-fold dilutions onto glucose-containing (top) or glycerol-containing (bottom) media to evaluate respiration capability.

(D) Direct oxidation led to proteasome dissociation. H₂O₂ was added to logarithmically growing WT yeast or mammalian BHK-21 cells for 30 min prior to harvesting and visualization of proteasome by in-gel peptidase assay.

partially corrected by adding DTT (Figure 2D). Diminished 26S proteasome levels in cells chemically treated for a few hours with a mitochondria inhibitor were also rapidly restored by adding DTT (Figure 2E). Reassembly of oxidized proteasomes in vivo did not require de novo synthesis of subunits, as it occurred spontaneously even when protein synthesis was blocked with cycloheximide (CHX, a ribosome inhibitor; Figure 2F). Consistent with these findings, in vitro, purified 26S proteasomes were rebuilt upon addition of DTT alone (i.e., without chaperones or

other factors) to the buffer (Figure 2G). These findings indicate that reduction-oxidation (redox) of proteasome components directly affects holocomplex stability, and that oxidized sub-complexes remain competent and can reassemble under permissive conditions.

We focused on the role of thiol residues in the dissociation/reassembly process (which was postulated due to the reversible nature of this redox-driven process) by repeating the experiments with diamide, a cysteine-specific reagent that crosslinks

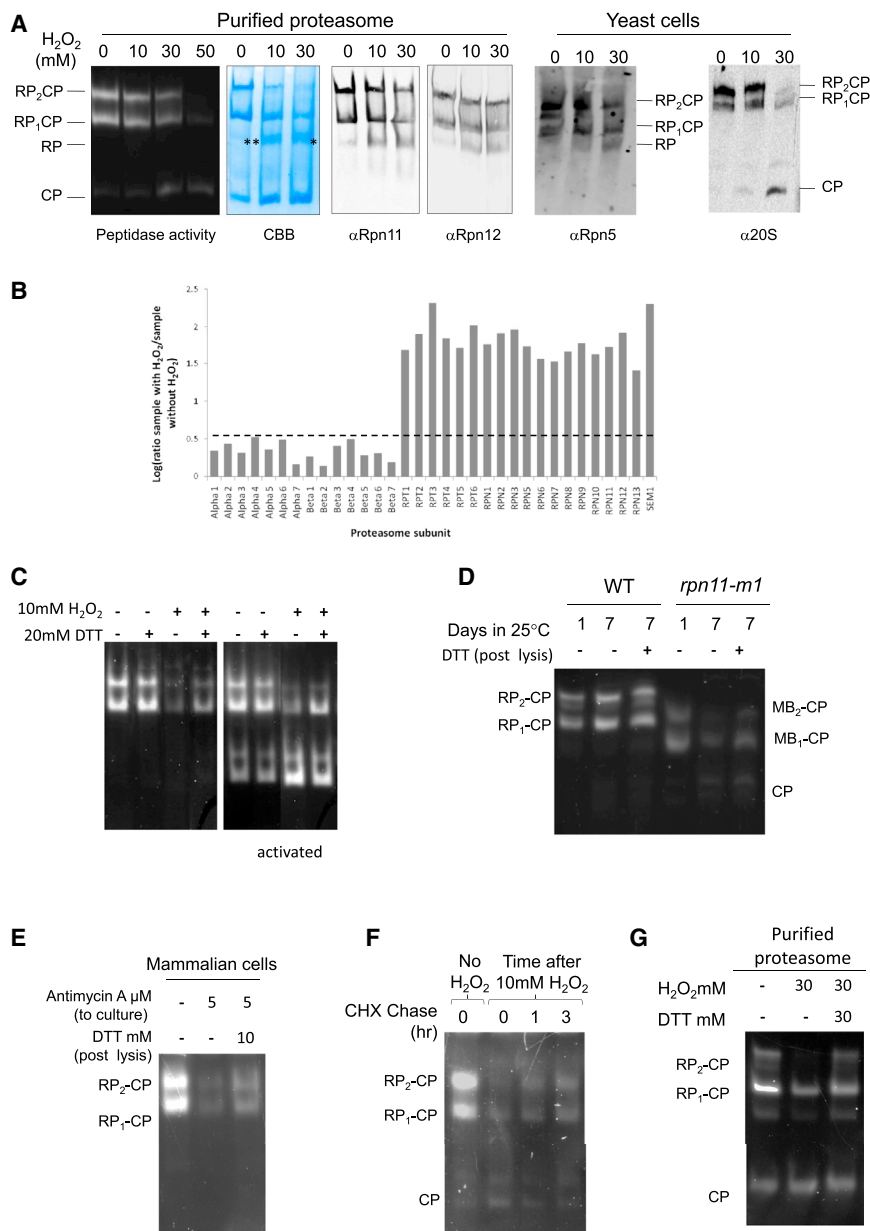


Figure 2. Reversible 26S Proteasome Disassociation in Response to Oxidative Stress

(A) Free 20S CP and 19S RP detached from oxidized proteasomes. Purified 26S proteasomes were exposed to increasing concentrations of H₂O₂ for 30 min and visualized by native gel: peptidase activity (left), protein content, and immunoblots for proteasome subunits Rpn11 or Rpn12 (middle). H₂O₂-treated yeast cells (as in Figure 1D) were immunoblotted for the 19S RP subunit (Rpn5) or the 20S CP subunit (right).

(B) Intact 19S RP subcomplex dissociated from 26S proteasomes. The subunit composition of proteasome species was determined by label-free MS/MS quantification. Proteasome subunits compared before (**) and after (*) H₂O₂ treatment verified the presence of inactive 19S RP (Table S1). (C–G) Proteasome disassembly was reversed by a reducing agent.

(C) Logarithmic-phase yeast was treated with 10 mM H₂O₂ for 30 min and lysed as is or with 20 mM DTT for in-gel peptidase activity.

(D) WT and *rpn11-m1* cells were grown at 25°C and lysed with or without 10 mM DTT.

(E) Mammalian BHK-21 cells were treated with Antimycin A and lysed with or without 10 mM DTT for visualization by native gel.

(F) Reassembly of dissociated proteasomes did not require de novo synthesis. WT cells at logarithmic phase (left lane) were treated with H₂O₂ for 30 min (second lane). H₂O₂ was removed, and cells resumed growth in the presence of 200 μg/ml CHX to block new protein synthesis. Proteasomes in all samples were compared by native gel.

(G) Purified 26S proteasomes (left) were treated with 30 mM H₂O₂ for 30 min, H₂O₂ was removed, and the sample was split (half loaded “as is” [middle] and half supplemented with 10 mM DTT [right]).

proximal thiols (Kosower and Kosower, 1995; Manalo et al., 2002). Diamide alone was sufficient to drive dissociation of 26S proteasomes into intact 20S CP and 19S RP species (Figure 3A), mimicking the experimental results obtained by other means of oxidation (Figures 1 and 2). Adding DTT reversed the diamide effect, thereby demonstrating that redox of thiol groups within the proteasome was sufficient to drive proteasome dissociation/reassembly (Figure 3A). Although crosslinking subunits may stabilize a complex, the 26S holoenzyme was actually split into two subcomplexes upon formation of internal disulfide bonds, suggesting that disulfide formation did not link the two subcomplexes, but rather occurred within each subcomplex. To map the oxidation-prone subunits, we labeled cysteines that were oxidized upon proteasome disassociation with an

epitope-tagged N-ethylmaleimide (NEM; Figure 3B). In this assay, only cysteines that were crosslinked in the free 20S and 19S subcomplexes were labeled by biotin-NEM (see details in legend for Figure 3 and in Supplemental Experimental Procedures). Evidently, multiple subunits in both 19S RP and 20S CP directly participated in disulfide bond formation. MS of oxidized and labeled proteasomes identified the participating cysteines in each subunit, and we compared cysteine modifications between diamide-treated and -untreated proteasomes by differentially labeling cysteines in two proteasome preparations (Table S2). Given that oxidized cysteines (a.k.a. cystines) were identified in dissociated 20S CP and 19S RP subcomplexes, but not in 26S holoenzymes, we conclude that crosslinks formed within each of the two subcomplexes, but not across the 19S RP-20S CP interface. We propose that such cystines (disulfide bonds) probably altered the surface of the interaction and weakened the 19S RP-20S CP association. The formation of disulfide bonds may even protect proteasomes from more severe,

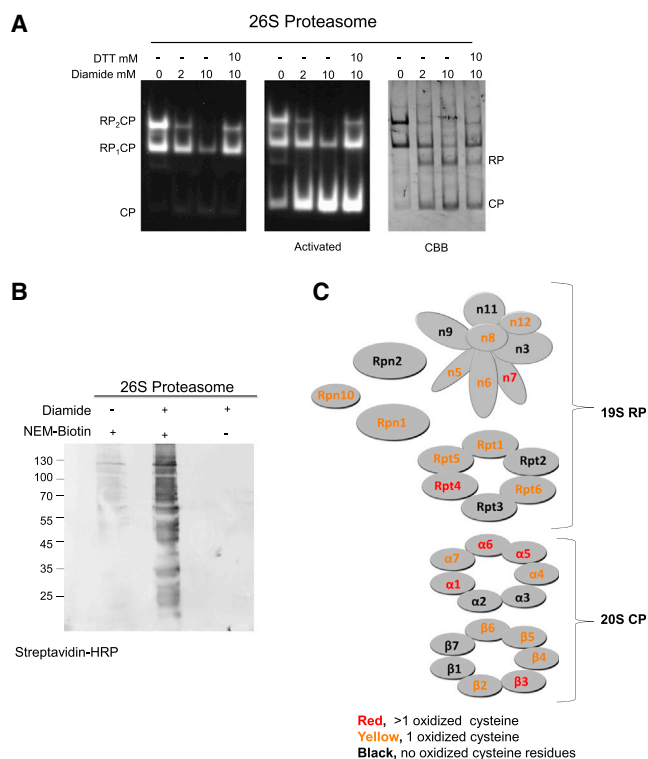


Figure 3. 26S Dissociation by Dicycstine Crosslinking

(A) A specific cysteine modifier, diamide, drives reversible dissociation of 26S proteasomes. Purified 26S proteasomes were incubated with diamide for 30 min at 30°C and resolved by native gel for activity (left) and protein content (right); 10 mM DTT was added to one sample.

(B) Cysteines were crosslinked in dissociated 20S CP and 19S RP. After treatment with 5 mM diamide, proteasomes were denatured with 6 M urea and all remaining reduced cysteines were irreversibly alkylated by 2 mM iodoacetamide. Next, the crosslinked cysteines were reduced by 8 mM DTT, irreversibly labeled with NEM-biotin, and detected by horseradish peroxidase (HRP)-streptavidin.

(C) Illustration of dicycstine bonds in oxidized 19S and 20S subcomplexes identified by MS/MS. Red, single oxidized cysteine; yellow, multiple cysteines; gray, no cysteines. Dicycstine bonds were confined to each subcomplex and did not span the 19S-20S interface.

irreversible thiol oxidation (e.g., due to sulfinic or sulfonic acids) in a manner similar to that observed for glutathione, one of the common antioxidants in cytosols of eukaryotes (Hayes et al., 2005; Scandalios, 2005).

Slowdown of Proteasome-Dependent Substrate Turnover in Response to Acute Oxidative Stress

Next, we assessed the correlation between oxidative stress-induced decrease in the cellular levels of active 26S proteasomes and substrate turnover. Induction of cellular oxidative stress in live *C. elegans* by knocking down mitochondrial respiration components with RNAi (Figure 4A, left) resulted in accumulation of a ubiquitin-fusion degradation model substrate (UFD; Figure 4A, right). Stabilization of UFD substrates is typical of 26S proteasome dysfunction (Bajorek et al., 2003; Rubin et al., 1998; Segref et al., 2011). Supplying worms with an antioxidant (N-acetylcysteine) restored UFD-substrate degradation (Fig-

ure 4B), even when mitochondria were still genetically impaired (Segref et al., 2014), suggesting that the efficiency of UPS was responsive to cellular oxidative conditions. Notably, the effects of mitochondrial mutations (*isp-1*, *nuo-6*, and *gas-1*) on stabilization of a UFD substrate were remarkably similar to those obtained by direct interference with 26S function (*rpn-6* and *rpn-11*). Slower turnover of a natural UPS substrate, Fzo1, was reflected by extended half-life following exposure of yeast to an oxidant (Figure 4C). Accumulation of high-molecular-weight polyubiquitin conjugates in oxidative-stressed cells was a broad phenomenon (Figure 4D; see also Wang et al., 2010) and indicated that bulk protein turnover was generally slowed down. Thus, our findings suggest that UPS activity is malleable and responds to the redox potential of the environment. Notably, proteasome conformation is also modulated by similar conditions, reinforcing the link between the two observations.

Mitochondrial Defects and Antioxidative Stress Response Are Induced by Reduced Proteasome-Dependent Proteolysis

We evaluated the contribution of proteasome capacity to cellular oxidative stress by monitoring the effect of proteasome inhibition on mitochondrial morphology, oxidation damage, and antioxidative stress response. In most tissues during typical growth conditions, mitochondria form an ever-changing branched network driven by fusion of membrane tips and fission into smaller organelles (Okamoto and Shaw, 2005). Such a tubular-reticular network of mitochondria was visualized in live *C. elegans* (in the body-wall muscle cells; Figure 5A, left). A dense interlocked network of mitochondria is also apparent in many mammalian cells (see example in Figure 5B; left) and in WT yeast (Altmann and Westermann, 2005; Merz and Westermann, 2009; Rinaldi et al., 2002). Knocking down individual proteasome subunits by RNAi (20S subunits *pas-1* and *pbs-2* or 19S subunits *rpn-6*, *rpn-11*, and *rpt-1*) led to small fragmented mitochondria in *C. elegans* (Figure 5A). Likewise, within hours of chemical inhibition of proteasomes in mammalian cells, the mitochondrial morphology shifted rapidly from the characteristic tubular network to multiple small and fragmented organelles (Figure 5B). Although in both of these examples proteasome function was significantly decreased, the phenotypic outcome was numerous small, rounded mitochondria. This phenotype is also produced by direct inhibition of oxidative phosphorylation, such as that caused by antimycin A (Sheridan and Martin, 2010). In yeast too, fragmented mitochondria have been characterized in proteasome mutants (Rinaldi et al., 2002). In addition, proteasome mutants accumulated oxidized proteins (Figure 5C), similar to the results observed for mitochondrial malfunction (Figure 1). Thus, these findings suggest that malfunctioning mitochondria appear to be another outcome of insufficient proteasome/UPS capacity.

Fragmented mitochondria (Figures 5A and 5B), elevated ROS levels, and accumulation of damaged proteins (Figures S1 and 5C) all trigger the antioxidative stress response. Interestingly, attenuation of UPS by RNAi knockdown of proteasome subunits did so as well, as reflected by induction of the *gst-4* gene in *C. elegans* (Figure S3). GSTs serve as key enzymes in the antioxidative stress response by maintaining appropriate cellular levels

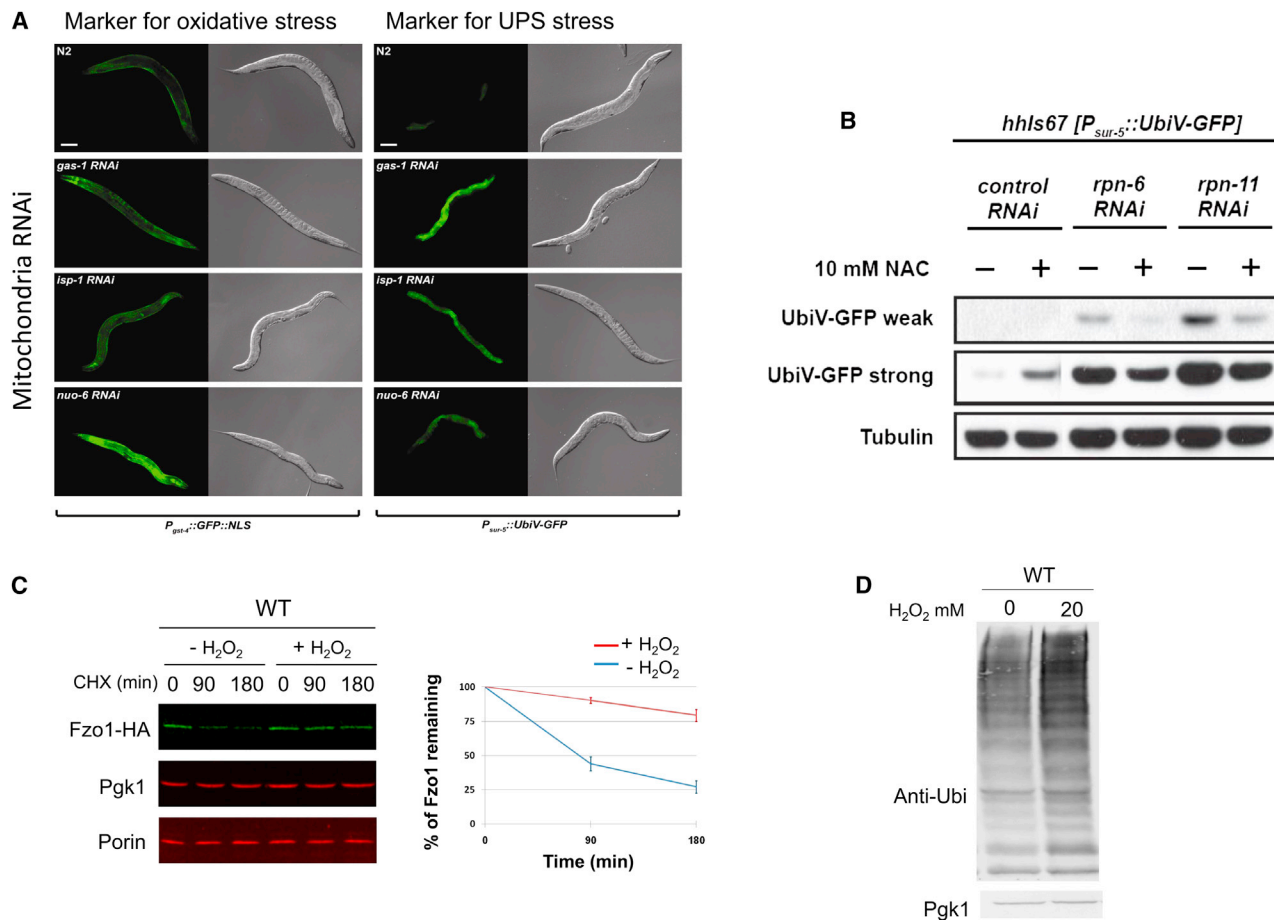


Figure 4. Acute Oxidative Stress Attenuates Ubiquitin-Dependent Proteolysis

(A) Defects in mitochondrial respiration stabilized a UFD substrate. *C. elegans* expressing an oxidative stress marker, GFP::NLS, under the control of the *gst-4* promoter (left), or a model UPS substrate, UbIV-GFP, under the control of the *sur-5* promoter (right), were fed with RNAi-expressing bacteria and imaged 72 hr after L1 stage (fluorescent and differential interference contrast [DIC], right). Scale bar indicates 100 μ m.

(B) Accumulation of UbIV-GFP was reversed by reduction. Nematodes prepared as in (A) were exposed to N-acetylcysteine (NAC) for 24 hr or kept without the antioxidant. Total lysates were immunoblotted by anti-GFP and anti-tubulin. Higher exposure showed that NAC had no effect on the UbIV-GFP substrate in WT nematodes (left), but aided turnover when mitochondrial genes or proteasomal genes were knocked down (Figure S3).

(C) A UPS substrate, Fzo1, accumulated upon oxidative stress. Logarithmically growing WT yeast expressing HA-Fzo1 were exposed to 20 mM H₂O₂ for 30 min, supplemented with 200 μ M CHX to block protein synthesis, and immunoblotted for residual Fzo1 (top), Pgk1 (middle), and mitochondrial Porin (bottom). Quantification of Fzo1-HA is shown in the graph on the right.

(D) Accumulation of polyubiquitin conjugates after acute oxidative stress. Lysates of log-phase WT yeast exposed to H₂O₂ were assayed for ubiquitin conjugates or Pgk1 (normalization control) relative to untreated cells.

of reduced GSH and inactivating oxidized functional groups on cellular macromolecules (Hayes et al., 2005; Scandalios, 2005). Another potent detoxification enzyme, SOD, was similarly elevated in a proteasome mutant (*rpn11-m1*; Figure 5D), emphasizing the antioxidative stress response as a common consequence of decreased proteasome capacity.

In order to grasp the full extent of reduced proteasome activity on cellular response pathways, we compared the *rpn11-m1* proteome in logarithmic phase with that of its congenic WT using stable isotope labeling by amino acids in cell culture (SILAC) followed by a comprehensive MS/MS analysis of total cell lysates (see Table S3). Significant enrichment of proteins related to oxidative stress response, carbohydrate catabolism, or pro-

tein-folding pathways was measured in the *rpn11-m1* proteome relative to the WT (Figure 5E). Notably, induction of GST genes was observed in response to diminished levels of the same proteasome subunit Rpn11 in yeast (*rpn11-m1*; Figure 5E) and in *C. elegans* (*rpn-11*; Figure S3), reflecting an evolutionarily conserved strategy to combat oxidative stress caused by insufficient proteasome capacity. Constitutive induction of reductases, scavenging enzymes, and chaperones (as in Figure 5F) may explain how some strains with proteasome defects sustain viability despite impaired mitochondria, inefficient oxidative phosphorylation (Figures 5A, 5B, and S2), increased ROS production (Figure S1), and misfolded/damaged proteins (Figure 5C).

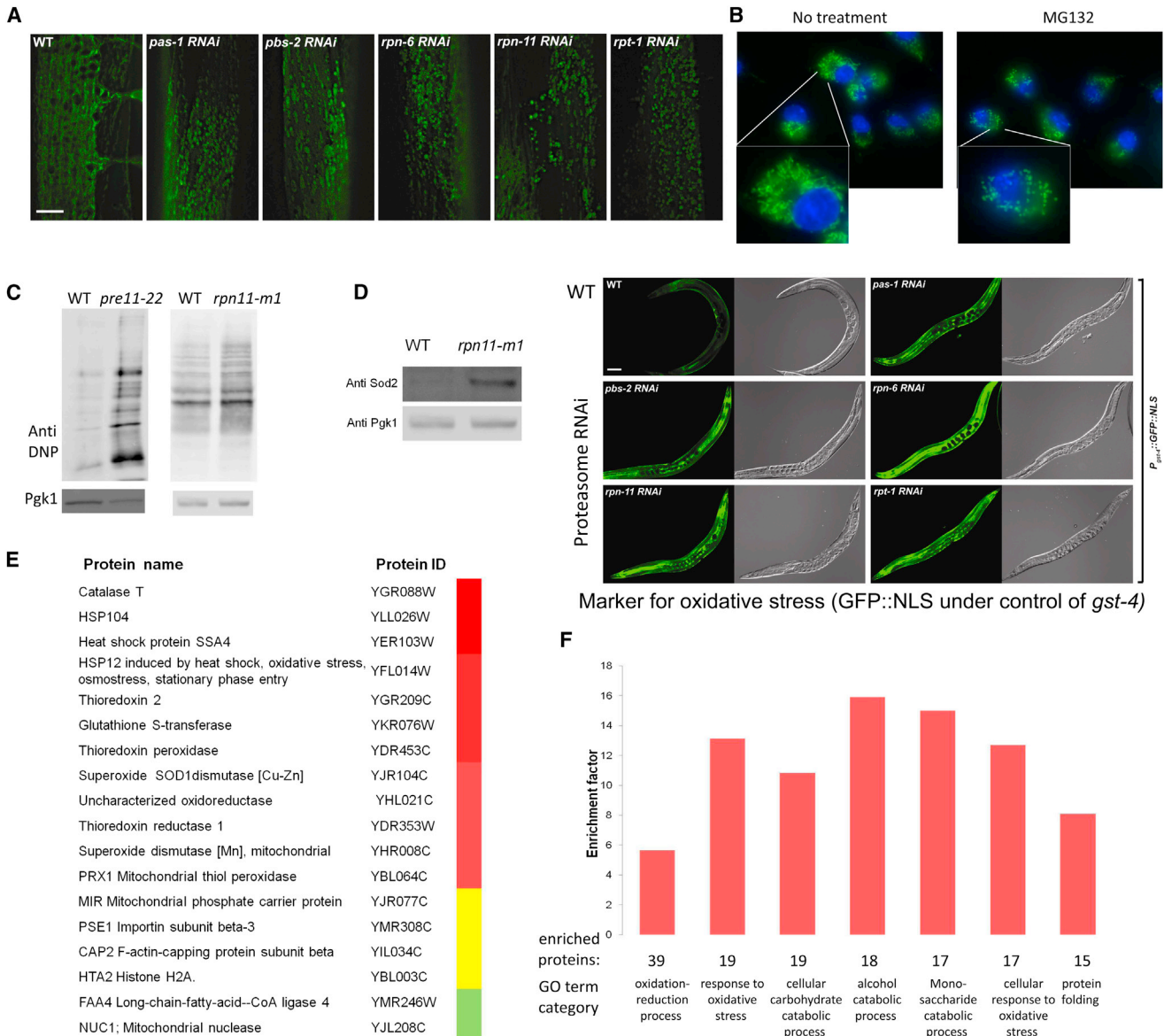


Figure 5. Attenuated Proteasomes and Impaired Mitochondria Display Antioxidative Stress Response Markers

(A) Defective proteasomes cause mitochondrial fragmentation. Fluorescent images of the mitochondrial network in body-wall muscle cells from 2-day-old adult nematodes expressing mitochondria-localized GFP under control of the *myo-3* promoter that were fed with RNAi bacteria from L3 larval stage for 72 hr. Scale bar indicates 8 μ m.

(B) Proteasome inhibition fragments mitochondria in mammalian cells. BHK-21 cells were treated with 10 μ M MG132 for 3 hr, mitochondria were stained with MitoTracker green FM, and nuclei were visualized using Hoechst at 63 \times magnification.

(C) Oxidized proteins correlated with impaired UPS. Total cell extracts from proteasome mutants (*pre11-22* and *rpn11-m1*) and WT yeasts were reacted with DNPH to evaluate oxidized proteins. Pgk1 immunoblot served for normalization.

(D) Proteasome defects induce the oxidative stress response. Nematodes expressing GFP::NLS under the control of the *gst-4* promoter were fed with RNAi-expressing bacteria from L3 larval stage for 48 hr (PAS-1 and PBS-2 are 20S α 1 and β 2 subunits, respectively) and imaged (left: GFP fluorescent; right: DIC). WT and *rpn11-1* strains were immunoblotted to detect SOD levels in whole-cell extract.

(E) Upregulation of the antioxidative stress response in a proteasome mutant, *rpn11-1*. WT and *rpn11-m1* proteomes were compared by SILAC MS/MS analysis of normalized cell lysates. Intensity ratios of identical peptides (heavy-to-light) were obtained by running the entire combined peptide data set through MaxQuant software (Cox and Mann, 2008). More than 100 proteins were significantly enriched in the proteome of *rpn11-m1* relative to WT (Table S3). Representative proteins enriched in *rpn11-m1* (red), unchanged (yellow), or decreased in *rpn11-m1* (green) are listed.

(F) Biological pathways significantly enriched in *rpn11-m1* cells (*rpn11-m1*/WT > 3) were classified using the AmiGO program (right). For each category, the “enrichment factor” is the ratio between the category in highly enriched proteins relative to its proportion of the total proteome (red bar), along with the number of proteins in the enriched sample.

DISCUSSION

Prolonged proteasome inhibition often causes cell death. A decrease in proteolytic capacity may lead to pervasive defects such as protein aggregation, neurodegeneration, certain cancers, aging, and even apoptosis. It was surprising that despite temporary 26S proteasome disassembly and attenuation of ubiquitin-conjugate turnover that followed acute short-term oxidative stress, organisms/cells remained viable. The reversible nature of proteasome disassembly suggests an adaptive response rather than protein damage. "Pausing" the ongoing turnover of ubiquitinated proteins may allow cells to redirect resources elsewhere. Simultaneously, thiol groups on 26S proteasome subunits are blocked (by dicycysteine formation or glutathionylation) and protected from irreversible oxidation or damage. For instance, glutathionylation of cysteine residues on the surface of α -subunits was found to open the 20S channel, thereby activating proteolysis (Silva et al., 2012). In this manner, cysteine modifications may influence ratio of 20S to 26S, as well as their relative activities. Subcomplexes released under stress upon 26S proteasome oxidation may also play new roles. The proteolytic activity of free 20S CP is expected to be less selective than that of 26S holoenzymes and thus more efficient in tackling misfolded/damaged proteins (Tsvetkov et al., 2012). Indeed, free 20S CP is highly efficient in proteolyzing certain loosely folded or unstructured proteins in vitro (Bajorek et al., 2003; Glickman et al., 1998a) and in a ubiquitin-independent, 20S-dependent manner in vivo (Davies, 2001; Mukhopadhyay and Riezman, 2007; Orlowski and Wilk, 2003; Shringarpure et al., 2001, 2003). Thus, CPs may aid the oxidative stress response by degrading oxidized and damaged proteins (Bader and Grune, 2006; Grune et al., 2003; Reinheckel et al., 1998; Sitte et al., 1998). Shifting the proteasome population from a majority of 26S to a greater proportion of 20S CP may be a mechanism for adapting to the proteolytic capacity.

Proteostasis may be preserved by inducing compensatory mechanisms even if the UPS is slowed down. Induction of anti-oxidative stress response, detoxification enzymes, chaperones, heat shock proteins, and mitophagy may alleviate the flux through the UPS, whereas directing ubiquitin molecules toward other protein quality-control systems, such as autophagy and trafficking, may take up some of the slack (Yao, 2010; Ziv et al., 2011). For example, administering a nontoxic low dose of the proteasome inhibitor PS-341 (bortezomib) to rats induced elevation of some of the same antioxidative defense enzymes that we identified in *rpn11-m1* (glutathione reductase, glutathione synthetase, glutathione peroxidase 2, and SOD2) and increased their ability to overcome oxidative stress (Bardag-Gorce et al., 2011). The mutual dependence of proteostasis pathways such as the UPS, autophagy, heat-shock system, and antioxidative stress response may explain their similar importance in staving off protein-based pathologies (Cecarini et al., 2012; de Moura et al., 2010; Green et al., 2011; Ross and Pickart, 2004). Moreover, proteasomal and mitochondrial mutants have each been identified independently as root causes of protein-aggregation and other neurodegenerative diseases (Lin and Beal, 2006). Therefore, studies of metabolic syndromes, mitochondrial disorders, and even aging-related phenomena

may benefit by considering the malleable capacity of UPS-dependent turnover. Apparently, proteasome dissociation/association is a reversible process that responds to cellular needs.

EXPERIMENTAL PROCEDURES

Nondenaturing PAGE and In-Gel Proteasome Activity

Native protein samples were resolved by nondenaturing PAGE and proteasome activity was visualized as previously described in Glickman et al. (1998a) and Bajorek et al. (2003). All gels and buffers used to assay proteasomal stability upon oxidative stress were prepared without DTT.

Trichloroacetic Acid Precipitation

Samples were prepared as previously described (Matiuhin et al., 2008; Ziv et al., 2011) and separated on an 8% SDS polyacrylamide gel to visualize ubiquitin conjugates using a ubiquitin antibody (Dako).

CHX Chase

CHX (Sigma) was added to logarithmic-phase cultures (after removal of oxidative reagents) to a final concentration of 200 μ g/ml. Samples were taken at the indicated time points, lysed, and loaded on native gel for activity assay. In order to detect Fzo1 levels, CHX chase was performed as described previously (Cohen et al., 2008, 2011).

C. elegans Strains

The Bristol N2 strain was used as the WT strain. Strains carrying reporter genes included *dvIs19*[pAF15(Pgst-4::GFP::NLS)], *hhIs67* [unc-119(+); *Psur-5::UbIV-GFP*]; *hhIs57* [unc-119(+); *sur-5::GFP*]; and *unc-119(ed4)*, *zcls14*[Pmyo-3::GFP(mit)].

Other experimental procedures are detailed in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.04.030>.

AUTHOR CONTRIBUTIONS

N.L.-L. designed the project and executed all experiments in mammalian and yeast cells and in vitro. N.L.-L., D.K., and N.R. performed molecular biology and biochemical experiments in yeast. T.R. performed microscopy on yeast. E.K. and A.S. performed experiments with *C. elegans*. O.K. analyzed MS/MS data. Z.E. and M.C. analyzed Fzo1 levels. N.L.-L., T.H., and M.H.G. oversaw the project and wrote the paper. All authors read, commented on, and approved the manuscript.

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