Pathogenesis of Human Mitochondrial Diseases Is Modulated by Reduced Activity of the Ubiquitin/Proteasome System

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SUMMARY

Mitochondria maintain cellular homeostasis by coordinating ATP synthesis with metabolic activity, redox signaling, and apoptosis. Excessive levels of mitochondria-derived reactive oxygen species (ROS) promote mitochondrial dysfunction, triggering numerous metabolic disorders. However, the molecular basis for the harmful effects of excessive ROS formation is largely unknown. Here, we identify a link between mitochondrial stress and ubiquitin-dependent proteolysis, which supports cellular surveillance both in Caenorhabditis elegans and humans. Worms defective in respiration with elevated ROS levels are limited in turnover of a GFP-based substrate protein, demonstrating that mitochondrial stress affects the ubiquitin/proteasome system (UPS). Intriguingly, we observed similar proteolytic defects for disease-causing mutations associated with mitochondrial failure in humans. Together, these results identify a conserved link between mitochondrial metabolism and ubiquitin-dependent proteolysis. Reduced UPS activity during pathological conditions might potentiate disease progression and thus provides a valuable target for therapeutic intervention.

INTRODUCTION

In eukaryotic cells mitochondria are central for various cellular processes including the generation of energy and the orchestration of anabolic and catabolic metabolism. Defects in mitochondrial homeostasis are closely linked to the development of human pathologies such as cancer, neurodegenerative diseases, type 2 diabetes, and additional metabolic disorders (Wallace, 2005). While limited amounts of reactive oxygen species (ROS) appear to exert health-promoting functions in diverse species (Ristow and Zarse, 2010), mitochondrial dysfunction and protein damage are mainly caused by excessive formation of ROS, which are byproducts of oxidative phosphorylation and energy production in the form of ATP (Livnat-Levanon and Glickman, 2011; Tatsuta, 2009). Oxidative stress provoked by respiration defects usually results in misfolding and aggregation of mitochondrial proteins and finally leads to irreversible damage of mitochondria (Tatsuta, 2009). Additionally, metabolic failure in catabolic pathways can cause accumulation of intermediates and biochemical stress within mitochondria (Sack and Finkel, 2012).

Several quality-control mechanisms have evolved to maintain functional mitochondria by eliminating damaged proteins or even the entire organelle. Besides autophagic consumption of damaged mitochondria (called mitophagy) (Atken and Schwarz, 2013), these mechanisms include different proteolytic quality control pathways in all mitochondrial compartments. One major system for protein degradation within mitochondria is based on the AAA-proteases Oma1 and Yme1, which mediate the turnover of inner mitochondrial membrane (IMM) proteins (Tatsuta, 2009). The mitochondrial matrix harbors Lon and ClpP proteases, and the latter is also discussed to be part of the mitochondrial unfolded protein response (UPRmt) (Matsushima and Kaguni, 2012). In contrast, selective turnover of at least some outer mitochondrial membrane proteins involves the UPS (Livnat-Levanon and Glickman, 2011). Damaged proteins are marked with a polyubiquitin chain formed by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin protein ligases (E3) (Kerscher et al., 2006). In fact, the recently described mitochondria-associated degradation (MAD) pathway involves the ubiquitin-selective chaperone Cdc48/p97 in retrotranslocation of substrates from mitochondria for degradation by the 26S proteasome (Cohen et al., 2008; Heo et al., 2010; Karbowski and Youle, 2011; Taylor and Rutter, 2011). Upon high oxidative stress conditions, Cdc48 and UPS components including the E3 ligase HUWE1 are recruited to the mitochondrial outer membrane (Cohen et al., 2008; Heo et al., 2010; Taylor and Rutter, 2011). This network of different mitochondrial quality control systems is transcriptionally controlled by the UPRmt (Haynes and Ron, 2010;
Cell Metabolism
Mitochondrial Stress Affects UPS Activity

RESULTS

A Genetic Screen for Proteolytic Defects Identifies the E3 Ligase HECD-1

The UPS is central for the elimination of damaged proteins and the maintenance of cellular proteostasis; however, the physiological requirements for tissue function and organismal growth remained unclear. To identify factors regulating protein turnover in multicellular organisms, we used a well-established in vivo degradation assay that monitors even minor defects in proteostasis and stress response in *C. elegans* (Segref and Hoppe, 2012; Segref et al., 2011). This system is based on short-lived ubiquitin-fusion proteins (called UFD substrates), and was extensively applied to define fundamental mechanisms and, importantly, physiologically relevant degradation pathways of the UPS in single-celled studies (Johnson et al., 1995; Richly et al., 2005). Worms expressing a GFP-based UFD substrate Ubv-GFP (Ub-GFP) under control of the ubiquitous sur-5 promoter show no green fluorescence due to low steady-state levels of the fusion protein enforced by polyubiquitylation of the N-terminal ubiquitin moiety and subsequent rapid proteasomal degradation. We used this reporter strain to monitor proteolytic defects in a forward genetic screen, where similar coexpression of the stable mCherry protein served as control to exclude transcriptional and translational changes (Figure 1A). Ten thousand worms were mutagenized using ethyl methane-sulfonate (EMS) and screened in the F2 generation for viable, GFP-positive mutants with increased Ub-GFP substrate levels (Figure 1A). Only nonlethal mutations were examined in our study, precluding disruption of essential functions such as proteasomal integrity. Among others, we first focused on four mutants with very strong and similar stabilization of GFP for further characterization (hecd-1(hh2-4), hh9; see Table S1 available online). A noncomplementation analysis revealed that these mutants were not able to complement each other (Table S2), and whole-genome sequencing (WGS) of three mutants revealed independent stop mutations in the same gene, called *hecd-1* (Figure S1A). Additionally, a *hecd-1(tm2371)* deletion mutant caused Ub-GFP stabilization in all tissues, which confirmed the phenotype of the *hecd-1* mutants identified in our screen (Figures 1B and 1C). Moreover, these mutants did not complement the *hecd-1* deletion mutant (Table S2). Thus, our forward genetic screen identified HECD-1 as an important factor for ubiquitin-dependent substrate turnover. In fact, HECD-1 is a HECT domain-containing E3 ligase related to human HECTD1 and HUWE (Leboucher et al., 2012).

Loss of Functional IVD-1 and ACS-19 Limits Ubiquitin-Dependent Protein Degradation

In addition, our screen identified two other mutants with strong stabilization of the Ub-GFP substrate, which are independent of *hecd-1* defects (ivd-1(hh6), acs-19(hh5); Table S1 and Table S2). SNP mapping and WGS placed the mutations into a 3.96 and 3.6 Mbp region on chromosomes III and IV, respectively (Figures S1B and S1C). RNAi against potential candidates revealed that *ivd-1* (RNAi) and *acs-19* (RNAi) generate similar GFP stabilization in the intestine compared to the *ivd-1(hh6)* and *acs-19(hh5)* mutations found in the screen (Figures 2A–2C). The mutation in *ivd-1(hh6)* causes a G-to-A splice donor mutation Pellegrino et al., 2013); however, the coordination between mitochondrial and cellular proteostasis pathways has not been addressed so far.

Here, we identified a link between mitochondrial metabolism and the UPS, which supports cellular surveillance. Inactivation of gene products engaged in oxidative phosphorylation or mitochondrial metabolism results in stabilization of a GFP-based substrate in the worm intestine, suggesting a yet-unidentified influence emanating from mitochondria on ubiquitin-dependent proteolysis in the cytosol. These defects in degradation are linked to enhanced oxidative stress, since substrate stabilization can be suppressed by antioxidants. Moreover, an increase in ROS levels accumulating with acute depletion of mitochondrial gene transcripts strictly correlates with reduced UPS activity. The failure in ubiquitin-dependent proteolysis appears to be downstream of the ubiquitin conjugation process, because increased proteasomal capacity is able to restore substrate turnover. Since we show similar results in patient cell lines defective in mitochondrial metabolism, our findings support the idea that ROS-induced changes in ubiquitin-mediated proteolysis contribute to human mitochondrial pathology and might foster disease progression.

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of the second intron, creating an aberrantly spliced mRNA (Figure S1C). Consequently, sequencing of the ivd-1(hh6) cDNA confirmed a deletion of 45 nucleotides of the second exon, causing an in-frame deletion of 15 amino acids that are highly conserved between different species including human IVD (Vockley and Ensenuer, 2006). Thus, loss of important residues in the ivd-1 or acs-19 mutants together with the recessive nature in genetic complementation studies suggest reduced protein functions. Accordingly, transgenic expression of the respective wild-type genes could rescue the proteolytic defect of ivd-1(hh6) and acs-19(hh5) worms (Figure 2D and Figures S2A–S2C), indicating that IVD-1 and ACS-19 activity are linked to ubiquitin-dependent protein turnover. IVD-1 is the ortholog of human isovaleryl-CoA dehydrogenase (IVD) (Mohsen et al., 2001), a mitochondrial enzyme with a defined role in the leucine catabolism pathway. During leucine degradation, IVD catalyzes the conversion of isovaleryl-CoA into 3-methylcrotonyl-CoA (Figure S2E) (Vockley and Ensenuer, 2006). We therefore asked if this particular step during leucine degradation might be important for turnover of the Ub-GFP substrate. RNAi against predicted ortholog genes of human BCKDHB and MCCC1 expected to act upstream or downstream, respectively, of ivd-1 (Marcotte et al., 2000), revealed an accumulation of Ub-GFP (Figures 2E and S2E), indicating that perturbation of the mitochondrial leucine degradation pathway per se affects ubiquitin-dependent protein turnover. ACS-19 is defined as acetyl-CoA synthetase, the predicted ortholog to human ACSS2, a cytosolic enzyme that catalyzes the conversion of acetate to...
acetyl-CoA required for fatty acid cycle in the mitochondrial matrix (Starai and Escalante-Semerena, 2004). In this regard it is interesting that the human HECT E3 ligase HUWE1 is also required for mitochondrial integrity, because it targets mitofusin 2 for proteasomal degradation (Leboucher et al., 2012). Therefore, it is very likely that HECD-1 identified in our screen is also involved in mitochondrial maintenance in worms. These data indicate that our screen for UPS activity defects predominantly identified mutants with diminished mitochondrial function.

### Increased Proteasomal Activity Restores Substrate Turnover in ivd-1, acs-19, or hecd-1 Mutants

To address the correlation between mitochondrial defects and decreased UPS activity, we further evaluated the role of ivd-1, acs-19, and hecd-1 in ubiquitin-dependent protein turnover. Importantly, RNAi-mediated depletion of ivd-1, acs-19, and hecd-1 had no effect on the amount of ^29,48RUB-GFP, a stable version of the substrate that cannot be polyubiquitylated, or of GFP alone (Figure S2D). In contrast to loss of the E3 ligase HECED-1, the UFD substrate accumulates as polyubiquitylated forms in worms lacking IVD-1 or ACS-19 (Figure 2F). This comparison confirmed that the effects of ivd-1, acs-19, and hecd-1 on steady-state levels of Ub-GFP were due to diminished UPS function. In contrast to proteolysis of Ub-GFP in the cytosol, ER-associated protein degradation (ERAD) is not affected (Figure 3A). The ERAD substrate CPL-W32A,Y35A (Miedel et al., 2012) is only stabilized upon depletion of the Hrd3 ligase homolog sel-1 (Grant and Greenwald, 1997), suggesting that IVD-1, ACS-19, and HECED-1 do not participate in ERAD (Figure 3A). Indeed, the ER chaperone hsp-4 was also not induced after ivd-1, acs-19, or hecd-1 RNAi (Figure S3A). The expression level of HSP-4 directly monitors defects in degradation of misfolded proteins accumulating in the ER lumen (Urano et al., 2002). It would thus appear that IVD-1, ACS-19, and HECED-1 are not relevant for ER function. To investigate whether the mutants have an overall defect in protein turnover, we analyzed the levels of polyubiquitylated substrates and found them to be unchanged compared to wild-type worms (Figure 3B). This is consistent with the fact that these mutants are viable, whereas severe defects in the UPS induced by codepletion of cdc-48.1 and cdc-48.2 lead to strong stabilization of polyubiquitylated substrates and lethality (Figure 3B) (Kuhlbrodt et al., 2011).

Since IVD-1, ACS-19, and HECED-1 seem to be linked to mitochondrial function, one explanation for the reduction in UPS activity could be a decrease in ATP production, which would affect E1-dependent ubiquitin activation and proteasomal degradation. However, measurement of the ATP levels in the ivd-1, acs-19, and hecd-1 mutants revealed no decrease in the general ATP levels (Figure 3C). Thus, the defects in ubiquitin-dependent protein turnover are not due to changes in ATP availability. Similarly, the function of the 20S proteasome core particle is not reduced, as revealed by measurements of chymotrypsin-, trypsin-, and caspase-like activities (Figure 3D). We therefore...
examined if overexpression of the rpn-6 subunit, a component of the 19S lid complex that was previously shown to increase assembly and activity of the 26S proteasome, could suppress the degradation defects of our mutants (Vilchez et al., 2012). Interestingly, overexpression of rpn-6 could indeed suppress the degradation defects of ivd-1 and acs-19 but not hecd-1 RNAi-depleted worms (Figure 3E). This is consistent with the proposed role of HECD-1 acting as an E3 ligase in substrate recognition and suggests that the defects seen upon ivd-1 and acs-19 inactivation occur downstream of substrate recognition.

**Enhanced Oxidative Stress Correlates with Impaired UPS Activity in Worms Lacking IVD-1, ACS-19, or HECD-1**

To evaluate the induction of stress responses, we analyzed the levels of heat shock protein 70 (hsp70) homologs in the ivd-1, acs-19, and hecd-1 mutants. Hsp-70 supports protein folding in the cytosol, whereas HSP-6 facilitates mitochondrial import, biogenesis, and energy generation (Kimura et al., 2007). However, we could not detect any increase in cytosolic HSP-70 or mitochondrial HSP-6 levels (Figures S3B and S3C). Therefore, we depleted key regulators of general stress pathways such as SKN-1, DAF-16, or HIF-1 by RNAi and examined proteolytic changes in ROS strictly correlate with reduced UPS activity. Indeed, similar to the elevated ROS level, NAC also attenuated the protein turnover defects in ivd-1, acs-19, and hecd-1.
Inhibition of Oxidative Phosphorylation Affects Ubiquitin-Dependent Degradation

Since our observations link ROS production with ubiquitin-dependent protein turnover defects, we next wondered whether mitochondrial dysfunction might be the underlying cause or contributes to this inhibition. Therefore we analyzed the stabilization of the Ub-GFP substrate upon RNAi depletion of critical components important for oxidative phosphorylation. Indeed, inhibition of respiratory chain function by downregulation of complex I (gas-1, nuo-1, nuo-6), complex II (mev-1), complex III (isp-1), and complex IV (cco-1) (Dingley et al., 2010; Tsang et al., 2001) resulted in proteolytic failure in the cytosol (Figure 5A). Similarly, disruption of mitochondrial fission by drp-1(RNAi) (Labrousse et al., 1999) and mitochondrial UPR by hsp-60(RNAi) result in strong stabilization of the GFP-based reporter substrate (Figure 5A). In contrast, RNAi against hsp-4 and hsp-12.6, major chaperons involved in ER stress and heat-shock response, respectively, had no effect, which indicates a specific crosstalk between mitochondrial stress response and the UPS. As shown for ivd-1 and acs-19 depletion, the degradation defect of mitochondrial respiration mutants can be restored by rpn-6 overexpression or NAC treatment (Figures 3E, 4E, 5B, and 5C). Moreover, treatment with antimycin A and sodium azide, two drugs known to inhibit respiratory complex III and IV function of mitochondria (Chen et al., 1999; Rotsaert et al., 2008), respectively, also resulted in strong stabilization of Ub-GFP (Figures 5D and 5E). In contrast, rotenone generating ROS at complex I exclusively in the mitochondrial matrix (Sena and Chandel, 2012) did not cause defects in UFD substrate degradation. Thus, oxidative and metabolic stress in mitochondria generally affect ubiquitin-dependent protein turnover in the cytosol.

Reduced UPS Activity Is Linked to Human Mitochondrial Disease Pathology

Our data strongly suggest that mitochondrial stress impairs UPS activity in C. elegans. Therefore, we asked whether this observation reflects a conserved link between mitochondrial metabolism and cellular ubiquitin-dependent proteostasis. To address this question we used MelJuSo cell lines constitutively expressing the UFD or N-end rule substrate (Figure 5A). In contrast, RNAi against hsp-4 and hsp-12.6, major chaperons involved in ER stress and heat-shock response, respectively, had no effect, which indicates a specific crosstalk between mitochondrial stress response and the UPS. As shown for ivd-1 and acs-19 depletion, the degradation defect of mitochondrial respiration mutants can be restored by rpn-6 overexpression or NAC treatment (Figures 3E, 4E, 5B, and 5C). Moreover, treatment with antimycin A and sodium azide, two drugs known to inhibit respiratory complex III and IV function of mitochondria (Chen et al., 1999; Rotsaert et al., 2008), respectively, also resulted in strong stabilization of Ub-GFP (Figures 5D and 5E). In contrast, rotenone generating ROS at complex I exclusively in the mitochondrial matrix (Sena and Chandel, 2012) did not cause defects in UFD substrate degradation. Thus, oxidative and metabolic stress in mitochondria generally affect ubiquitin-dependent protein turnover in the cytosol.
2004) has normal IVD protein levels (Figure S4C; Table S3). Western blot analysis of cell lysates showed that the overall polyubiquitin levels are unchanged in severe and mild IVA patient cells (Figure S4D). Interestingly, transfection of the IVA fibroblasts with the N-end rule substrate revealed a strong stabilization of UbR-GFP, especially in severe IVA patient cells (c.IVS4+2T > C) (Figure 6C). Moreover, the UFD substrate accumulated exclusively in c.IVS4+2T > C fibroblasts as compared to control (c) or c.932C > T patient cells. The stabilization seen in fibroblasts with the severe IVA phenotype (c.IVS4+2T > C) is similar to treatment of control fibroblasts with the proteasomal inhibitor bortezomib (Figure 6D). These turnover defects correlate with increased ROS levels in the patient cells, with c.IVS4+2T > C exhibiting higher oxidative stress (Figure 6E). We tested whether the stabilization of the UFD and N-end rule substrate reflects defects in MAD. However, the levels of the OMM proteins mitofusin 2 (Mfn2) and Mcl1, important for mitochondrial fusion and apoptosis (Chan et al., 2011; Tanaka et al., 2010; Xu et al., 2011), did not increase in c.IVS4+2T > C patient cells (Figure 6F).

Thus, in addition to the previously identified accumulation of metabolites (Table S3), patients suffering from IVA also display defects in ubiquitin-dependent protein turnover, which correlates with the degree of enzymatic impairment and disease pathology. Similarly, substrate degradation was reduced in cybrid cells from patients with a defect in the COX1 gene affecting respiratory complex I (G6930A) (Bruno et al., 1999) (Figures S4E and S4F). Thus, it appears that, comparable to C. elegans, mitochondrial dysfunction also affects UPS activity in humans.

**DISCUSSION**

As defects in mitochondrial respiration are known to cause protein damage and mitochondrial dysfunction, the cytotoxic consequences of ROS remained enigmatic. With regard to human metabolic diseases it is particularly interesting how oxidative stress affects mitochondrial homeostasis and cellular protein quality control mechanisms. Here, we discovered a conserved influence of respiration defects and mitochondrial stress conditions on the efficiency of ubiquitin-dependent degradation pathways in the cytosol both in C. elegans and humans. Acute depletion of ivd-1 and acs-19 by RNAi caused a mild but significant increase in ROS, which correlates with defects in UFD substrate turnover. Whereas the ROS levels of the originally identified ivd-1 and acs-19 mutants are comparable to wild-type worms or even decreased, the mutants are still limited in substrate degradation. This discrepancy suggests that the adaptation against an initial increase in ROS is not able to restore protein turnover defects, probably by long-term changes of respiratory and metabolic processes, and might support the manifestation of chronic disorders (Figures 4C, 4D, and S3D).
defects in mitochondrial metabolism are closely linked to chronic human diseases including cancer and neurodegeneration (Wallace, 2005). So far, changes in UPS function have not been specifically linked to mitochondrial diseases. Analyzing different human metabolic disorders, we noticed a strict correlation between mitochondrial failure and proteolytic defects similar to the observations in C. elegans (Figures 6C, 6D, S4E, and S4F).

According to our model, the inhibition of cytosolic UPS pathways might contribute to survival on a cellular level but does not compensate the organismal consequences of the different mitochondrial defects (Figure 7B). In this regard, sustained maintenance of defective mitochondria could actually be the reason for disease pathology of otherwise harmful mutations. Patients with IVA specifically suffer from an accumulation of isovaleryl-CoA derivatives based on a defect in IVD, resulting in acidification of blood and urine (Table S3). The accumulation of both UFD and N-end rule substrates is closely correlated with increased ROS level and the severity of the IVA disease pathology (Figures 6C–6E). It is intriguing to speculate that changed UPS activity potentiates development and progression of metabolic diseases through a conserved mitochondrial surveillance response (MSR) (Figure 7B). Thus, the response mechanisms that limit ubiquitin-dependent protein turnover provide a target for therapeutic intervention of chronic metabolic diseases caused by mitochondrial failure.

EXPERIMENTAL PROCEDURES

Strains

Worms were handled according to standard procedures and grown at 15 °C or 20 °C unless otherwise stated. Mutations and transgenes used in this study are listed as chromosomes as follows: cdc-48;tm5044II, unc-119(ed4)III, sur-5::UbV-GFPIII, cdc-19(tm4853III), hecd-1(tm327)IV, sur-5::mCherry, sur-5::GFPIII, ub-GFP, sur-5::GFP. The Bristol strain N2 was used as wild-type and CB4856 Hawaii for mutant mapping studies. The strains AGD614 N2, uhhEx33 [Pmyo-3::GFP] and AGD639 N2, uhhEx39 [Psur-5::rpn-6.1, Pmyo-3::GFP], VK1879 [Ppmyo-7.1::pmyo-7::GFP], CL2166 [PfAT5(gsp-4::GFP;::NLS)], SJ4005 zcs1 [Psur-4::GFPV, SJ4100 zcs13 [Psur-6::GFPIV, BC10060 dpy-5(e907); sEx884] in pCeH361] were kind gifts from A. Dillin, C.J. Luke, C. Link, and D. Ron, respectively.

Mutagenesis

The strain PNP68 hhIs64 [unc-119(+)], sur-5::UbV-GFP;III, hhIs73 [unc-119(+)], sur-5::mCherry was grown at 15 °C. A total of 10,000 L4-stage hemaphrodites were treated with 50 mM EMS for 4 hr at room temperature. Worms were grown to day 1 of adulthood and bleached in sodium hypochlorite solution, and 86,000 F1 worms obtained were cultured at 15 °C. Worms were handled according to standard procedures and grown at 15 °C or 20 °C unless otherwise stated. Mutations and transgenes used in this study are listed as chromosomes as follows: cdc-48;tm5044II, unc-119(ed4)III, sur-5::UbV-GFPIII, cdc-19(tm4853III), hecd-1(tm327)IV, sur-5::mCherry, sur-5::GFPIII, ub-GFP, sur-5::GFP. The Bristol strain N2 was used as wild-type and CB4856 Hawaii for mutant mapping studies. The strains AGD614 N2, uhhEx33 [Pmyo-3::GFP] and AGD639 N2, uhhEx39 [Psur-5::rpn-6.1, Pmyo-3::GFP], VK1879 [Ppmyo-7.1::pmyo-7::GFP], CL2166 [PfAT5(gsp-4::GFP;::NLS)], SJ4005 zcs1 [Psur-4::GFPV, SJ4100 zcs13 [Psur-6::GFPIV, BC10060 dpy-5(e907); sEx884] in pCeH361] were kind gifts from A. Dillin, C.J. Luke, C. Link, and D. Ron, respectively.

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day 1 and day 2 of adulthood for stabilized Ub-GFP signal. Mutant worms were selected to new plates and cultured at 15°C. For further experiments ivd-1(h66) and acs-19(h55) were outcrossed six times against wild-type worms.

**Mutant Identification**

Mutants obtained by EMS mutagenesis were analyzed for chromosome linkage (ivd-1, acs-19 and hecd-1) and interval mapping (ivd-1, acs-19) according to Davis et al. (2003). Here, mutant worms were crossed with CB4856, and Ub-GFP stabilization served as marker for the mutant phenotype. The mutants were sequenced by WGS as described recently (Doitsidou et al., 2010; Sarin et al., 2008). For acs-19 and ivd-1 the mutations were verified after six times outcrossing against wild-type worms, selection of mutant phenotype, and Sanger sequencing of the mutation.

**RNAi**

RNA interference was performed using the feeding method (Segref and Hoppe, 2012). Typically, worms were treated with RNAi from L1 stage and the phenotype observed during day 1 of adulthood unless otherwise stated. For ivd-1, acs-19, and hecd-1 RNAi, stabilization of Ub-GFP served as an internal control for RNAi efficiency.

**Generation of Transgenic C. elegans**

The original mutant strains obtained after EMS mutagenesis were injected with 10 ng/μL of the fosmosids (WRM062aH08 [for acs-19] or WRM061bH04 [for ivd-1]) and 100 ng/μL of pRF4 (rol-6[su1006 dm]). Rescued F1 animals were analyzed at day 1 of adulthood for expression of GFP.

**In Vivo Imaging**

In vivo imaging was performed as described recently (Segref and Hoppe, 2012).

**Immunoblotting**

Worms were lysed in SDS sample buffer and equal volumes applied to SDS-PAGE as described recently (Segref and Hoppe, 2012). Western blotting was performed using antibodies against GFP (Clontech), ubiquitin (Upstate), mCherry (Abcam), tubulin (Sigma), and IVD (R. Ensenauer).

**Polyubiquitylation**

To investigate the polyubiquitylation status, worms were grown at 20°C till day 1 of adulthood. Worms were washed off the plates with 1 x M9 buffer, settled on ice, and the worm pellet lysed in SDS sample buffer containing 10 mM NEM at 95°C for 4 min. Worms were analyzed by western blotting using an antibody against ubiquitin and tubulin as loading control. For polyubiquitylation of tissue culture cells, cells grown at similar densities were collected in SDS sample buffer and subjected to western blotting.

**Quantification of ROS in Nematodes by MitoTracker Red Fluorescence**

Nematodes were synchronized and either grown to day 1 adult stage or L1 larvae and treated with RNAi against, ivd-1, acs-19, or the empty control vector for 48 hr. Worms were washed off the plates with S-basal buffer (pH 6) and allowed to settle by centrifugation (200 × g, 1 min). The worm pellet was transferred to plates spotted with 300 μM heat-inactivated OP50 (65°C, 30 min) mixed with 100 μl MitoTracker Red CM-H2X stock solution (100 μM; freshly prepared) for 2 hr at 20°C. To remove excess dye from the gut, the nematodes were washed with S-basal buffer three times and transferred to new plates with either OP50 or RNAi for 1 hr at 20°C. A total of 100 μl worm suspension aliquots in S-basal buffer were distributed into eight wells/condition of a 96-well FLUOTRAC plate (Greiner Bio-One). Fluorescence (ex, 570 nm; em, 610 nm) was measured in a plate reader (FLUOstar Optima, BMG Labtech). To normalize the fluorescence signal, the protein content in the remaining worm suspension was determined using the Bradford assay.

**Quantification of Hydrogen Peroxide Production of Nematodes by AmplexRed Fluorescence**

Worms were treated with RNAi against ivd-1, acs-19, or control vector with and without NAC (final 10 mM) for 48 hr beginning at L1 stage. As internal positive control, we incubated one part of the control worms on plates containing anti-mycin A (final 1 μM) for 2 hr right before the measurement. Worms were removed from plates with sodium-phosphate buffer (50 mM, pH 7.4) and washed twice. Amplifu (Sigma-Aldrich) solution (final concentration 10 μM) and horse-radish peroxidase (Sigma-Aldrich, final concentration 10 μM/l) were added to the nematodes. Measurement was carried out in 96-well Fluorotrac plates (Bio Greiner One) using a microplate reader (FLUOstar Optima, BMG Labtech) and filters for excitation at 544 nm and emission at 590 nm for 20 min at 20°C.

**Measurement of Mitochondrial ROS in Primary Human Fibroblasts**

Primary human fibroblast cells were incubated with 5 μM MitoSOX Red (Molecular Probes) for 30 min and ROS levels determined by imaging the cells with a Zeiss Meta 510 Confocal Laser Scanning Microscope (Abs., 514 nm; Em., 561 nm). Fluorescent images of MitoSOX-stained cells were taken with a 20× objective. Images were processed with ImageJ 1.48e software, and mean fluorescence intensities of single cells are shown on the graph.

**Stress Tests**

NGM plates containing 3 μM rotenone (Sigma, 100 mM stock in DMSO), 2 μM antimycin A (Sigma, 10 mM stock in 100% ethanol), or 0.2 mM sodium azide (Sigma, 200 mM stock in water, (only DMSO, ethanol, or water was added to the respective control plates) were prepared freshly before the start of the experiment. The strain P5P63 hts64 [unc-119(+); sur-5::UbV-GFPIII] was grown from L4 stage for 12 hr at 20°C and analyzed for expression of Ub-GFP by microscopy and western blotting. To analyze development, staged young adult worms were placed on NGM plates with ethanol as control or with 2 μM antimycin A and allowed to lay eggs for 4 hr. The adult worms were removed from the plates and the offspring grown at 15°C for 5 days. Worms were examined for their developmental stage and grouped into adult worms or worms that had reached only a larval stage. For treatment with antioxidants, NGM plates containing NAC (Sigma) (500 mM stock in water) were prepared freshly. L1-staged mutants worms expressing sur-5::UbV-GFP were added to the plates and grown at 15°C till day 1 of adulthood. Worms were collected in SDS sample buffer and analyzed by western blotting for expression of Ub-GFP.

**ATP Measurements**

Two hundred day 1 adult hermaphrodite worms grown at 15°C were collected, washed four times in 1 x M9 buffer, and frozen at –80°C before extract preparation. ATP levels were measured using the ATP bioluminescence kit HSll (Roche) according to the manufacturer’s instruction. In brief, worms were lysed in 500 μl lysis buffer provided with the kit, kept on ice for 10 min with brief vortexing every 2 min. The lysate was incubated at 95°C for 5 min, sonicated with a microsonicator, and incubated at 95°C for 5 min. The lysates were centrifuged at 13,000 rpm for 2 min at room temperature. Of the lysate, 50 μl was mixed with 50 μl of the Luminescence reagent provided with the kit using the Tecan infinite 200 microplate reader. Analysis was performed with a readout time of 10 s. An ATP curve in the extract was performed to ensure linearity of the measurement.

**Proteasome Activity**

Proteasome activity was measured essentially as described previously (Vilchez et al., 2012).

**Lifespan Analysis**

Lifespan analysis was performed as described recently (Kuhlbrodt et al., 2011).

**Cell Culture and Transfections**

WT and G8930A cells were cultivated in DMEM with Glutamax supplemented with 5% FCS, 50 μg/ml uridine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C and 5% CO2. After informed consent was obtained, primary human iVD fibroblast cell lines were derived from skin of two children with isovaleric acidemia diagnosed by a disease-related metabolic pattern and homozygous IVD gene mutations, and of a healthy child undergoing inguinal hernia repair that served as a control cell line, with approval of the Institutional Review Board of the Ludwig-Maximilians-Universität München (Table S3). MeJuSo cells were cultivated in DMEM and fibroblasts in
DMEM with Glutamax supplemented with 10% FCS, 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C and 5% CO2. Transfection was performed in 6 cm dishes using 10 μg of UbG76V-GFP or UbR-GFP (Dantuma et al., 2000) obtained from N. Dantuma and 5 μg pmCherry-C1 with 10 μl Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instruction. Cells were collected in SDS sample buffer after 24 hr and analyzed by SDS-PAGE and western blotting. In the case of Bortezomib treatment, 20 nM Bortezomib or as control DMSO was added to the culture 6 hr after transfection.

Statistics
The mean ± SD is reported unless otherwise indicated. Statistical comparisons among groups were determined using two-tailed Student’s t test with a significance value of p ≤ 0.05.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and five tables and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2014.01.016.

AUTHOR CONTRIBUTIONS
A.S. and T.H. designed the study and analyzed data. A.S. contributed most of the results; E.K. analyzed mitochondrial stress response; W.P. performed turnover assays; E.K., J.M., K.S., and M.R. provided ROS; N.L.-L. and M.H.G. provided advice and reagents; R.E. provided human material; A.S. and T.H. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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REFERENCES
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