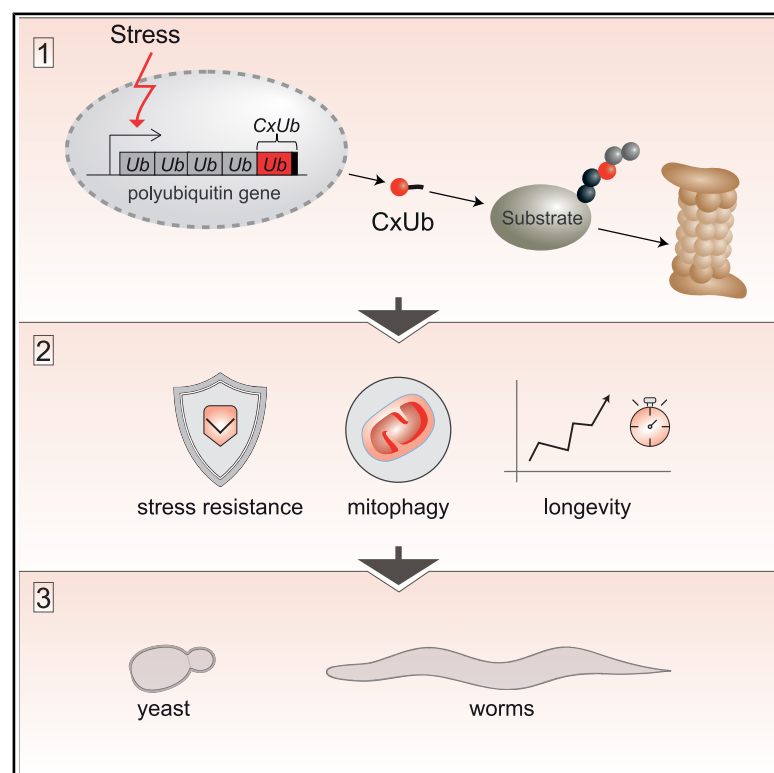


Ubiquitin precursor with C-terminal extension promotes proteostasis and longevity

Graphical abstract



Authors

Selver Altin, Tânia Simões, Christina Behrendt, ..., Thorsten Hoppe, Andreas S. Reichert, Mafalda Escobar-Henriques

Correspondence

reichert@hhu.de (A.S.R.), mafalda.escobar@uni-koeln.de (M.E.-H.)

In brief

Altin et al. identified CxUb, a C-terminally extended ubiquitin molecule dedicated to overcoming stress and present in all eukaryotic genomes. CxUb marks client proteins for efficient degradation by the proteasome, ensuring cellular quality control. CxUb function is critical specifically in stressed cells, safeguarding healthy ones and contributing to healthy aging.

Highlights

- The ubiquitin precursor CxUb represents a distinct and stress-specific ubiquitin entity
- Proteostasis, mitophagy, and longevity require CxUb in yeast and worms
- CxUb binds to the ubiquitin chain-elongating enzyme Ufd2
- Ufd2 conjugates processed CxUb to substrate proteins, boosting their turnover



Article

Ubiquitin precursor with C-terminal extension promotes proteostasis and longevity

Selver Altin,^{1,8} Tânia Simões,^{1,8} Christina Behrendt,^{2,8} Vincent Anton,^{1,6,8} Dennis Domke,^{1,9} Kai Mayor Völtzke,^{2,9} Rajesh Kumar,^{2,9} Hendrik Nolte,^{1,7,9} Thomas Hermanns,^{3,9} Nahal Brocke-Ahmadinejad,² Katja Bendrin,² Marcel Zimmermann,² Reinhard Büttner,⁴ Natascia Ventura,⁵ Marcus Krüger,¹ R. Jürgen Dohmen,³ Kay Hofmann,³ Thorsten Hoppe,¹ Andreas S. Reichert,^{2,*} and Mafalda Escobar-Henriques^{1,10,*}

¹Institute for Genetics, Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), Centre for Molecular Medicine (CMMC), University of Cologne, Cologne, Germany

²Institute of Biochemistry and Molecular Biology I, Medical Faculty and University Hospital Düsseldorf, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

³Institute for Genetics, University of Cologne, Cologne, Germany

⁴Institute of Pathology, Medical Faculty, University Hospital, Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany

⁵Institute for Clinical Chemistry and Laboratory Diagnostics, Medical Faculty and University Hospital Düsseldorf, Leibniz Research Institute for Environmental Medicine (IUF), Heinrich Heine University Düsseldorf, Düsseldorf, Germany

⁶Present address: Medical Research Council Mitochondrial Biology Unit, University of Cambridge, Cambridge, UK

⁷Present address: MPI for Biology of Ageing, Cologne, Germany

⁸These authors contributed equally

⁹These authors contributed equally

¹⁰Lead contact

*Correspondence: reichert@hhu.de (A.S.R.), mafalda.escobar@uni-koeln.de (M.E.-H.)

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SUMMARY

Ubiquitin is a conserved modifier regulating the stability and function of numerous target proteins. In all eukaryotes, polyubiquitin precursors are generated and processed into ubiquitin monomers. The final ubiquitin unit always contains a C-terminal extension, but its physiological significance is unknown. Here, we show that C-terminally extended ubiquitin, termed CxUb, is essential for stress resistance, mitophagy, and longevity in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. CxUb forms ubiquitin chains and binds to a previously undescribed region within the ubiquitin chain-elongating E4 enzyme Ufd2, which also functions during stress and aging. Ufd2 recognizes CxUb and conjugates it to substrate proteins, triggering their degradation. By contrast, CxUb is not required for basal housekeeping functions of the ubiquitin-proteasome system. These data suggest that the CxUb encodes a functionally unique ubiquitin form, specialized for proteostasis defects, expanding the code of post-translational modification processes.

INTRODUCTION

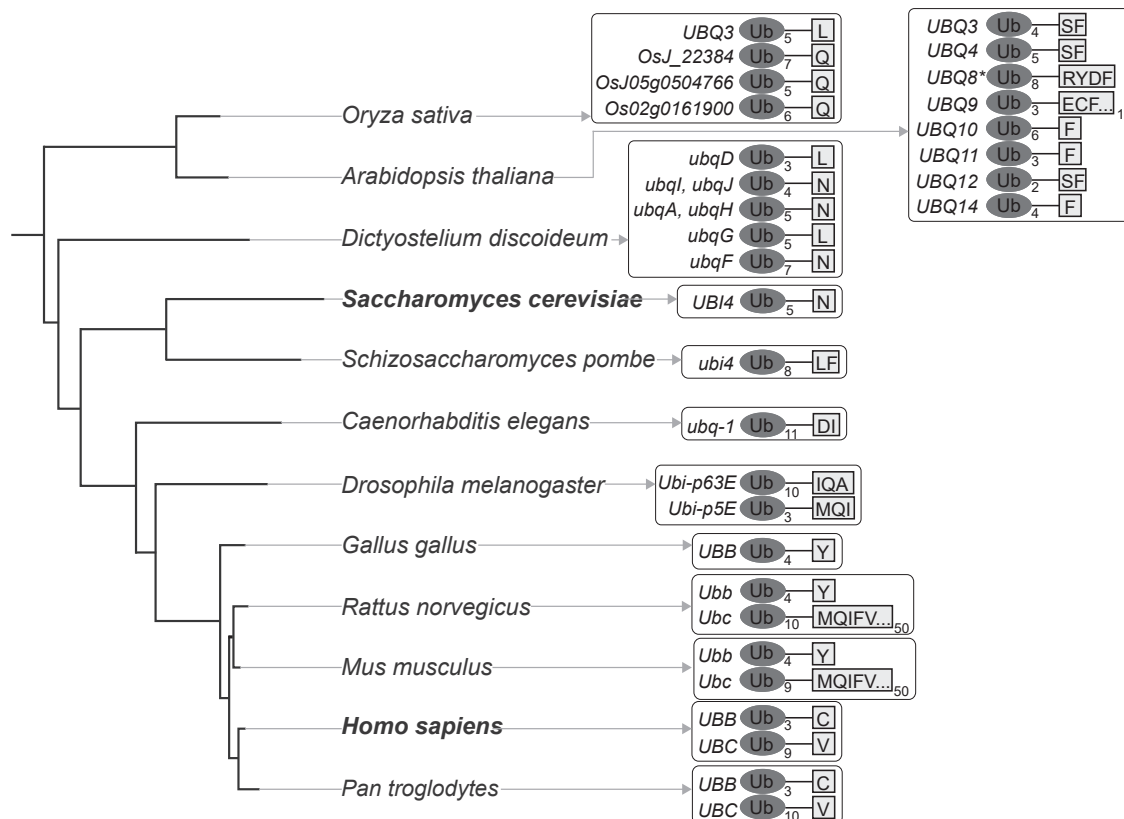
Ubiquitin is a highly conserved, 76-amino-acid protein in eukaryotes that controls many biological processes through its covalent post-translational attachment to diverse substrates.^{1,2} It is critical for housekeeping functions^{1,2} and development.³ Ubiquitin modifications also ensure cellular homeostasis under various stress conditions,^{3,4} in part by orchestrating proteolytic quality control pathways, including the ubiquitin-proteasome system (UPS) and autophagy.⁴ Deficiencies in ubiquitylation lead to birth defects, pediatric diseases, neuropathies, or cancer.³ Ubiquitylation proceeds via the addition of ubiquitin molecules to a single or multiple lysine(s) on the substrate protein. This process is catalyzed by ubiquitin-activating, -conjugating, and -ligation enzymes, called E1, E2, and E3, respectively.^{1,2} Protein substrates

can be modified by monoubiquitylation, multi-monoubiquitylation, and polyubiquitylation.^{1,2} Polyubiquitylation occurs when ubiquitin is added to one of its own amino acids, typically lysine, forming ubiquitin chains.

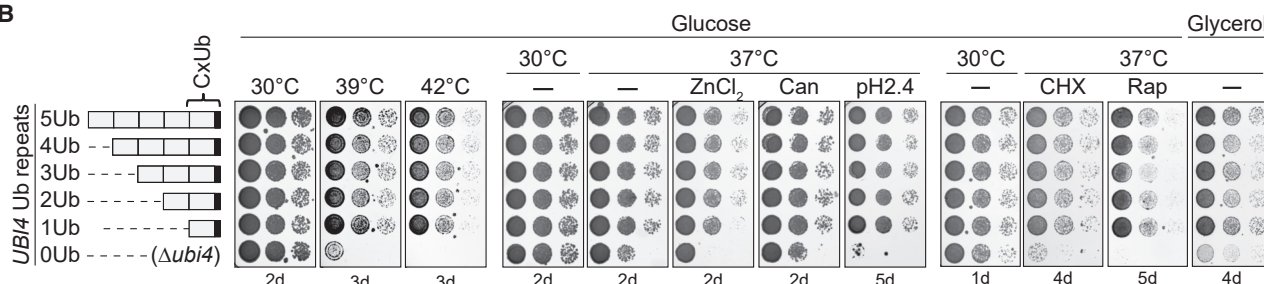
In some cases, an E4 ubiquitin ligase extends this E3-dependent ubiquitin chain.⁵ E4 ligase activity is particularly crucial under stress conditions, where it enhances the UPS-mediated degradation of its target proteins. The first E4 ligase discovered was Ufd2 in *Saccharomyces cerevisiae*. It was initially shown to recognize artificial substrates bearing an N-terminal ubiquitin fusion to a variety of reporter proteins, for example, β -Galactosidase, triggering their degradation via the ubiquitin fusion degradation (UFD) pathway^{6–8} (Figure S1A). The endogenous equivalent of these UFD substrates has not been identified to date. However, under stress, ubiquitin is generated from



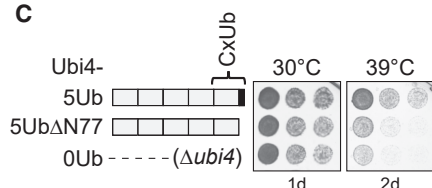
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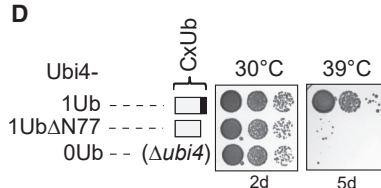
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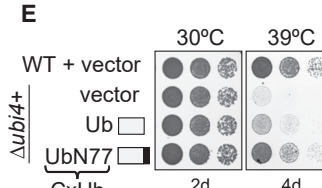


Figure 1. A ubiquitin form extended at its C terminus, CxUb, is required for cellular growth under stress

(A) Phylogenetic tree depicting relationships between the polyubiquitin genes in eukaryotes. Phylogeny was built using iTOL (Interactive Tree Of Life, <https://itol.embl.de/>), to which the number of ubiquitin copies (gray circle subscripts) and the corresponding sequence extensions (light gray box) were added.

(B–E) Cellular growth under stress in different constellations of the polyubiquitin gene: (B) cells from strains with 5 (Ubi4-5Ub, WT) to 0 (Ubi4-0Ub, Δubi4) copies of ubiquitin in the UBI4 gene were spotted in 5-fold serial dilutions on the following media: yeast extract peptone (YP) with glucose or glycerol with or without cycloheximide (CHX, 0.3 μg/mL), ZnCl₂ (10 μM), canavanine (Can, 500 ng/mL), low pH (2.4), and rapamycin (20 ng/mL). Cells were incubated at 30°C, 37°C, 39°C, or (legend continued on next page)

polyubiquitin precursors,^{9–14} and the final ubiquitin moiety invariably harbors a C-terminal extension of one or more amino acids (Figures 1A and S1B). The physiological relevance of this extension is unknown, but, intriguingly, these extended ubiquitin molecules resemble the artificial substrates of the UFD pathway (Figure S1A).

Here, we reveal that C-terminally extended ubiquitin (CxUb) is the endogenous equivalent of the UFD model substrates of E4 ligases in the UFD pathway and represents a variant of ubiquitin with specialized functionality for proteostasis defects in yeast and worms. CxUb directly binds to Ufd2 and is itself ubiquitylated, forming unanchored ubiquitin chains. Upon removal of the C-terminal extension, Ufd2 conjugates the ubiquitin moiety from CxUb to endogenous substrates, signaling their proteasomal turnover and allowing maintenance of cellular proteostasis under stress.

RESULTS

CxUb is essential for stress resistance

Polyubiquitin genes are upregulated in response to various stresses.¹⁵ Although this upregulation boosts ubiquitin pools under stress, overexpressing mature monoubiquitin does not fully rescue the phenotypic defects resulting from deletion of polyubiquitin genes.¹⁶ It is unclear to what extent the beneficial effects of polyubiquitin genes in stress resistance arise from an increase in the monoubiquitin pool, the presence of linear (unprocessed) ubiquitin chains, or the final CxUb.

To investigate the role of polyubiquitin genes in stress resistance, we turned to the budding yeast *Saccharomyces cerevisiae*. Yeast has one polyubiquitin gene, *UBI4*, which encodes five head-to-tail repeats of ubiquitin (Figures 1A and S1A).¹⁰ We analyzed six engineered deletion strains containing 0–5 ubiquitin repeats (designated 0–5Ub; Figure 1B).¹³ The $\Delta ubi4$ strain lacking all five repeats (designated 0Ub) was sensitive to many stress conditions, including heat, metals, amino acid analogues, low pH, cycloheximide (CHX), and rapamycin (Figure 1B). Unexpectedly, the remaining strains containing 1–5 ubiquitin repeats showed no significant differences in stress resistance (Figure 1B). Thus, the number of ubiquitin repeats in the polyubiquitin gene is not critical for stress resistance. These data suggest that unprocessed, linear ubiquitin does not substantially contribute to stress responses.

Yeast harbors three additional ubiquitin genes, *UBI1*, *UBI2*, and *UBI3*, which encode precursors comprising a single ubiquitin moiety fused to ribosomal proteins¹⁷ (Figure S1C). Strains lacking ubiquitin from *UBI1*, *UBI2*, or *UBI3* but expressing the corresponding ribosomal subunits L40 or S31 were much less sensitive to stress than $\Delta ubi4$ strains (Figures S1D–S1F), confirming the unique role of *UBI4* in stress resistance.

Notably, *UBI4* variants 1–5 each contain a copy of CxUb. To evaluate the role of this C-terminal extension, we deleted the final asparagine (N77) codon from CxUb. Strikingly, elimination of just this C-terminal asparagine residue, either in the wild-type (WT) strain with five ubiquitin copies (*UBI4-5Ub Δ N77*) or in the strain with only one ubiquitin copy (*UBI4-1Ub Δ N77*), abolished heat resistance (Figures 1C and 1D). Intriguingly, the UbN77 extension was also required to prevent toxicity caused by α -synuclein^{A53T}, which is associated with protein aggregation and Parkinson's disease (Figure S1G). The heat sensitivity of $\Delta ubi4$ was rescued by ectopic expression of CxUb (UbN77) but not Ub, confirming that the N77 C-terminal extension is required for stress resistance (Figure 1E). We verified the presence of UbN77 in cells by mass spectrometry (MS) (Figure S1H). These strong differences in stress resistance between the strains did not correlate with changes in the cellular ubiquitin profile (Figures S1I–S1L). Taken together, we conclude that *UBI4*-dependent stress resistance in yeast arises mainly from a requirement for CxUb.

Loss of CxUb impairs mitophagy and chronological lifespan in *S. cerevisiae*

Conjugation of ubiquitin to damaged mitochondria promotes their autophagosomal capture and elimination via mitophagy.^{4,18–22} Aberrant mitophagy is associated with various neurodegenerative diseases, including Parkinson's disease, although the causes are incompletely understood.^{23,24} To investigate a role for *UBI4* in mitophagy, we first used the established alkaline phosphatase (ALP)-based assay^{19,25,26} (Figures 2A, left and S2A). In this assay, an inactive proenzyme of ALP targeted to the mitochondrial matrix (mtALP) is transported to the vacuole during mitophagy, cleaved, and activated. We transformed WT and $\Delta ubi4$ cells, lacking the endogenous ALP Pho8, with mtALP and induced mitophagy by treating with rapamycin or by two types of starvation. The activity and proteolytic cleavage of mtALP were impaired in $\Delta ubi4$ cells compared with WT under all three conditions, and these defects were rescued by expression of *UBI4* (Figures 2A and S2B). These data suggest that mitophagy is compromised upon loss of *UBI4*.

As an orthogonal approach, we used the mitophagy reporter mitochondrial-targeted dihydrofolate reductase (mt-DHFR)-GFP.²⁷ Mitophagy induction results in transport of mt-DHFR-GFP to the vacuole, generating proteolysis-resistant GFP. Indeed, GFP was present inside the vacuole in WT cells but not in $\Delta ubi4$ cells (Figure S2C), confirming that *UBI4* is required for autophagic engulfment of mitochondria. By contrast, mitophagy was not dependent on the other three ubiquitin-encoding genes, *UBI1–UBI3* (Figures S2D–S2F). Taken together, we infer that *UBI4* is specifically required for vacuolar turnover of mitochondria by mitophagy.

42°C for the indicated days (represented by "d" in the figure). (C) Growth of WT cells (encoding 5 ubiquitin copies, 5Ub), cells deleted for *UBI4* (0Ub, $\Delta ubi4$), and cells genomically modified to express five ubiquitin copies without the C-terminal asparagine extension (5Ub Δ N77) were assessed by spot tests on YP glucose media, as in (B), and incubated at 30°C or 39°C. (D) Growth by spot tests, as in (C), but of cells genomically modified to encode one ubiquitin copy, either with or without the C-terminal asparagine extension (1Ub or 1Ub Δ N77, respectively), and of cells deleted for *UBI4* (0Ub, $\Delta ubi4$). (E) WT cells expressing an empty vector and $\Delta ubi4$ cells expressing either an empty vector, ubiquitin (Ub), or ubiquitin extended C-terminally by one asparagine residue (UbN77) were analyzed by spot tests as in (C). See also Figure S1.

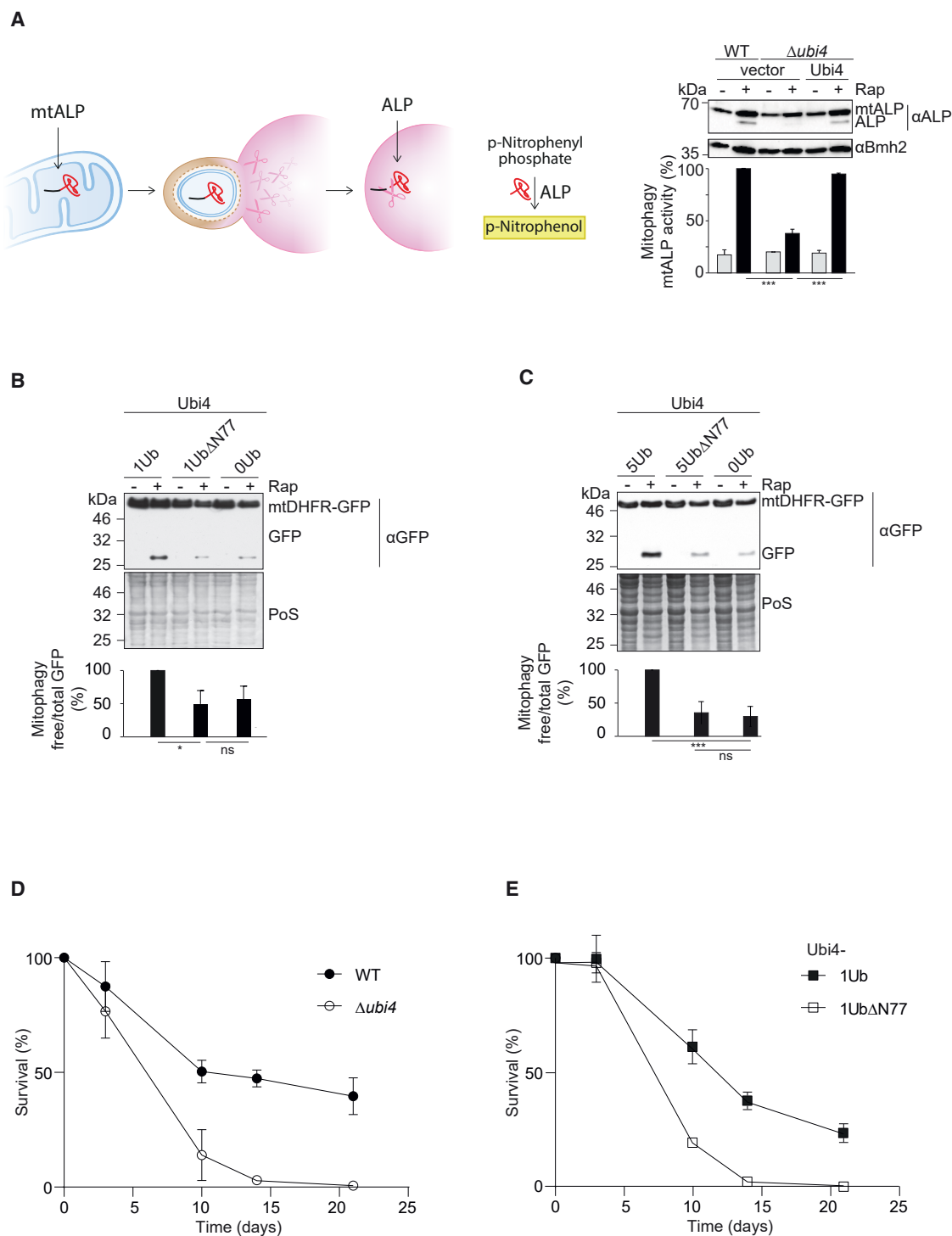


Figure 2. CxUb regulates mitophagy and chronological lifespan

(A) Role of *UBI4* in mitophagy. Left: schematic representation of the ALP assay. Active ALP catalyzes the hydrolysis of p-Nitrophenyl to chromogenic p-nitrophenol. Right: WT and $\Delta ubi4$ cells deleted for endogenous *PHO8* were transformed with the mitophagy reporter mtALP, together with an empty or a *UBI4* coding vector, as indicated. Cells were grown in synthetic minimal media with glycerol (SG) minus leucine (L) (SG-L) media and treated with rapamycin (+) or left untreated (-). ALP proteolytic processing was observed by western blot analysis of total cell lysates and immunoblotting with an ALP-specific antibody (upper). Bmh2 serves as a loading control. ALP activity was determined by absorption spectrophotometry assays (bottom). The quantification below is based on the ALP activity and represents the mean $\pm$ SD ($n = 3$) normalized to the rapamycin-treated WT cells. Statistical significance was determined by Student's *t* test. *** $p \leq 0.001$.

(legend continued on next page)

We next asked whether the role of *UBI4* in mitophagy was dependent on CxUb. Similar to what we observed for cell growth under stress, removal of the C-terminal N77 severely impaired mitophagy, again mimicking $\Delta ubi4$ cells (Figures 2B and 2C). Furthermore, ectopic expression of CxUb (Ub77N) in $\Delta ubi4$ cells was more effective in inducing mitophagy than expression of ubiquitin lacking this single amino acid extension (Ub) (Figure S2G).

To further decipher the role of *UBI4* in mitophagy, we analyzed core autophagy and selective mitophagy components. *UBI4* deletion did neither decrease the level of mitochondrial localization of the outer membrane mitophagy receptor Atg32 (Figures S3A and S3B), the level of its binding partner Atg11 (Figure S3C), nor did it affect Atg8 lipidation (Figure S3D) or the formation of autophagosomes (Figure S3E), which are required for the cytoplasm-to-vacuole targeting (Cvt) pathway.²⁸ Thus, *UBI4* regulates mitophagy independently of core autophagy components.

Cellular and organellar quality control pathways are critical determinants of healthy aging in a variety of organisms.²⁹ Moreover, mitophagy has been found to mediate lifespan extension in response to mitochondrial stress.^{30,31} To test whether CxUb modulates lifespan in *S. cerevisiae*, we determined the chronological lifespan of strains manipulated in the *UBI4* locus. We observed that $\Delta ubi4$ had a reduction of survival upon aging when compared with WT (Figure 2D). Consistently, *UBI4-1Ub Δ N77* induced an equivalent reduction of lifespan when compared with *UBI4-1*, reinforcing the importance of the C-terminal extension (Figure 2E). We conclude that CxUb is required to confer mitophagy and normal lifespan in *S. cerevisiae*.

CxUb regulates mitophagy and lifespan in *C. elegans*

Mitophagy and longevity are also linked in the nematode *Caenorhabditis elegans*.³² We considered that the roles of CxUb in mitophagy and lifespan are conserved in worms. In *C. elegans*, ubiquitin is encoded by two genes, *ubq-1* and *ubq-2*, which are similar to the yeast *UBI4* and *UBI1/UBI2* genes, respectively (Figure S4A).³³ These genes are essential,³⁴ and depletion of both *ubq-1* and *ubq-2* results in meiotic arrest.^{35,36} Due to their high homology, RNAi is expected to target both genes.³⁷ In worms, *ubq-1* encodes 11 tandem ubiquitin moieties followed by the dipeptide aspartate-isoleucine (Figure 3A). To selectively eliminate CxUb in *C. elegans*, we used CRISPR-Cas9³⁸ to replace D-I with two stop codons (Figures 3A and S4B). We generated two independent strains, designated $\Delta CxUb$ 9.6 and 9.11, which completed larval development at the normal rate (Figure S4C) but had reduced progeny compared with WT

worms (Figure 3B). Importantly, similarly to yeast, the $\Delta CxUb$ strains exhibited a reduced lifespan compared with WT worms (Figures 3C and S4D).

To investigate whether CxUb is also important for mitophagy in worms, we used a strain expressing a mitochondrial-targeted version of the Rosella biosensor (translocase of the outer mitochondrial membrane [TOMM20]::Rosella) (Figure 3D). This biosensor combines a pH-insensitive dsRed fused to a pH-sensitive GFP and allows the monitoring of mitophagy by fluorescence microscopy.^{39,40} An increase in the dsRed/GFP ratio indicates engulfment of mitochondria into lysosomes due to mitophagy. We treated WT and $\Delta CxUb$ worms with carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which depolarizes mitochondria and induces mitophagy,^{32,41} or depleted the mitochondrial protein frataxin (*frh-1*) by RNAi, which was previously shown to induce mitophagy.⁴² Strikingly, these treatments did not increase the dsRed/GFP ratio in $\Delta CxUb$ worms, unlike in WT worms, suggesting that mitophagy is impaired in the mutant worms (Figures 3E, S4E, and S4F). As we observed in yeast, these strong differences between the strains did not correlate with changes in the cellular ubiquitin profile (Figure S4G). Together, these results suggest a conserved role for CxUb in mitophagy and lifespan in eukaryotes.

Stress resistance and mitophagy require maturation- and conjugation-competent CxUb

The last two glycine (G) residues in ubiquitin, particularly G76, are critical for the ubiquitylation process, enabling the covalent attachment of ubiquitin to substrate proteins and facilitating the formation of polyubiquitin chains. To determine if conjugation of CxUb is required for its functions, we created a conjugation-deficient variant of CxUb. Specifically, the last two G residues of CxUb were mutated to alanine residues (G75A and G76A). In addition, we created a maturation/deubiquitylase (DUB)-resistant mutant CxUb variant by mutating leucine 73 to proline (L73P), which inhibits cleavage by all ubiquitin-specific processing proteases.⁴³ We genomically introduced these mutations into the last ubiquitin repeat of *UBI4*. Like $\Delta ubi4$, the conjugation- and maturation-deficient variants of CxUb failed to grow at high temperature (Figure 4A). Compared with WT, these mutants also showed reduced mitophagy in the presence of rapamycin (Figure 4B). We infer that the maturation and conjugation of CxUb are required to promote stress resistance and mitophagy.

To explore the maturation properties of CxUb, we tested the DUB specificity of its processing. We incubated purified UbN77 with purified DUBs (Ubp3, Ubp14, and Ubp15) *in vitro* and monitored processing over time by MS. UbN77 was not processed in

(B and C) Role of CxUb in mitophagy. (B) Mitophagy response in cells expressing the reporter mtDHFR-GFP and genomically encoding one ubiquitin copy with the C-terminal asparagine extension (1Ub) or without (1Ub Δ N77), and cells lacking *UBI4* (0Ub). Cells were grown in SG-uracil (SG-U) media and treated with rapamycin (+) or left untreated (–). Total cell extracts, SDS-PAGE, and immunoblotting with a GFP-specific antibody were used to evaluate the levels of mitophagy based on proteolytic processing of the reporter mtDHFR-GFP. The quantification below represents the mean $\pm$ SD ($n = 3$) normalized to the rapamycin-treated WT cells. Statistical significance was determined by one-way ANOVA. (C) Mitophagy of WT (5Ub), $\Delta ubi4$ (0Ub), and *UBI4* mutant cells expressing five ubiquitin copies without the C-terminal asparagine extension (5Ub Δ N77) were analyzed as in (B). The quantification below represents the mean $\pm$ SD ($n = 3$) normalized to the rapamycin-treated WT cells. Statistical significance was determined by one-way ANOVA. ns $p > 0.05$, * $p \leq 0.05$, *** $p \leq 0.001$.

(D and E) *UBI4* and CxUb are needed upon aging in yeast. The chronological lifespan of WT and $\Delta ubi4$ cells (D) or 1Ub and 1Ub Δ N77 cells (E), grown in YP glucose, is shown as the survival percentage for each time point calculated from the fraction of colony-forming units (mean $\pm$ SEM, $n = 3$).

See also Figures S2 and S3.

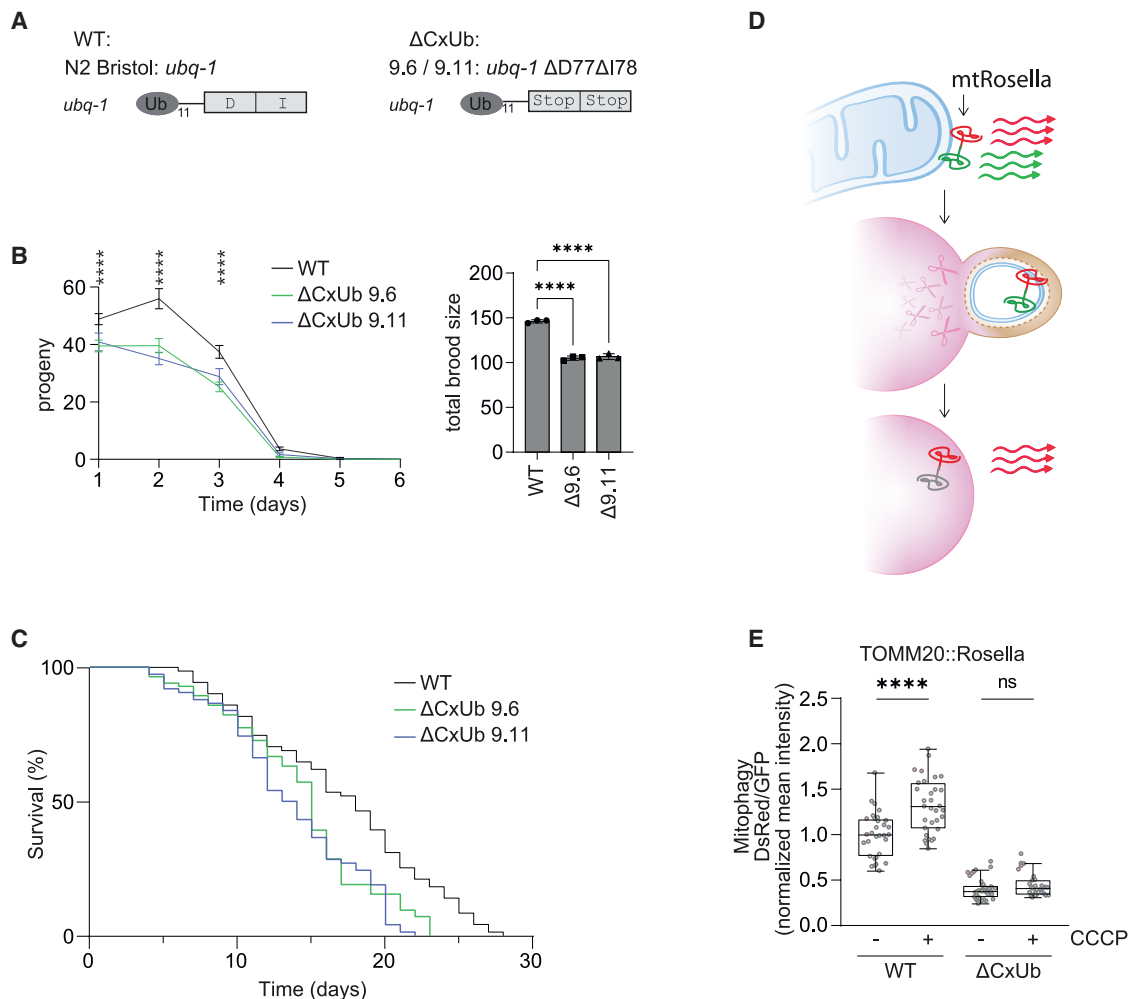


Figure 3. CxUb regulates longevity and mitophagy in *C. elegans*

(A) Representation of the last ubiquitin copy (gray circle) and corresponding sequence extensions (light gray box) encoded by *ubq-1* in WT or Δ CxUb (*ubq-1* Δ D77 Δ I78) worms.

(B) Comparison of mean brood size in WT and Δ CxUb. Number of progeny (eggs laid per day) per worm over time (left) or in total (right). The quantification represents the mean $\pm$ SEM ($n = 3$) of the absolute number of worms. Statistical significance of the biological replicates was determined by one-way ANOVA. **** $p \leq 0.0001$.

(C) Role of CxUb in longevity. Lifespan analysis is represented as the fraction of animals alive after the indicated time in adulthood (in days). Representative graph of WT worms is shown as a black line, and Δ CxUb worms are shown in green (9.6) and blue (9.11) lines. The raw data and statistical analysis from three technical replicates are present in Figure S4D.

(D) Scheme of the mitophagy assay using the Rosella reporter system. Mitochondrial outer membrane targeted TOMM20::Rosella consists of a pH-sensitive GFP and a pH-insensitive dsRed tandem dimer. Upon mitochondrial engulfment by lysosomes, the GFP signal is quenched, and mitophagy can be measured by an increase of the dsRed/GFP fluorescence intensity ratio.

(E) Deletion of CxUb prevents mitophagy. Transgenic animals expressing mtRosella in body wall muscle cells were exposed to 15 μ M CCCP for 24 h. Mitophagy induction was measured by the increase in the ratio between pH-insensitive dsRed (AlexaFluor568) and pH-sensitive GFP (AlexaFluor488) and is shown as Tukey plots. Individual animals from three biological replicates are shown as red, green and blue dots. Statistical significance was determined by one-way ANOVA. ns $p > 0.05$, **** $p \leq 0.0001$.

See also Figure S4.

the absence of DUBs but was promptly cleaved into ubiquitin by all three DUBs (Figure 4C). Importantly, UbN77 was not processed by the catalytically inactive Ubp3 C469A (Figure 4C). Thus, CxUb can be matured to ubiquitin by multiple DUBs.

The sequence and length of the C-terminal extension have diverged among polyubiquitin genes from different species

(Figure 1A). To assess whether the composition of the extension in CxUb is important in yeast, we genomically mutated the endogenous N77 or deleted the stop codon, which elongated the extension to 27 amino acids. These modifications of CxUb did not abolish its function in stress resistance (Figures 4D and 4E), consistent with the divergent

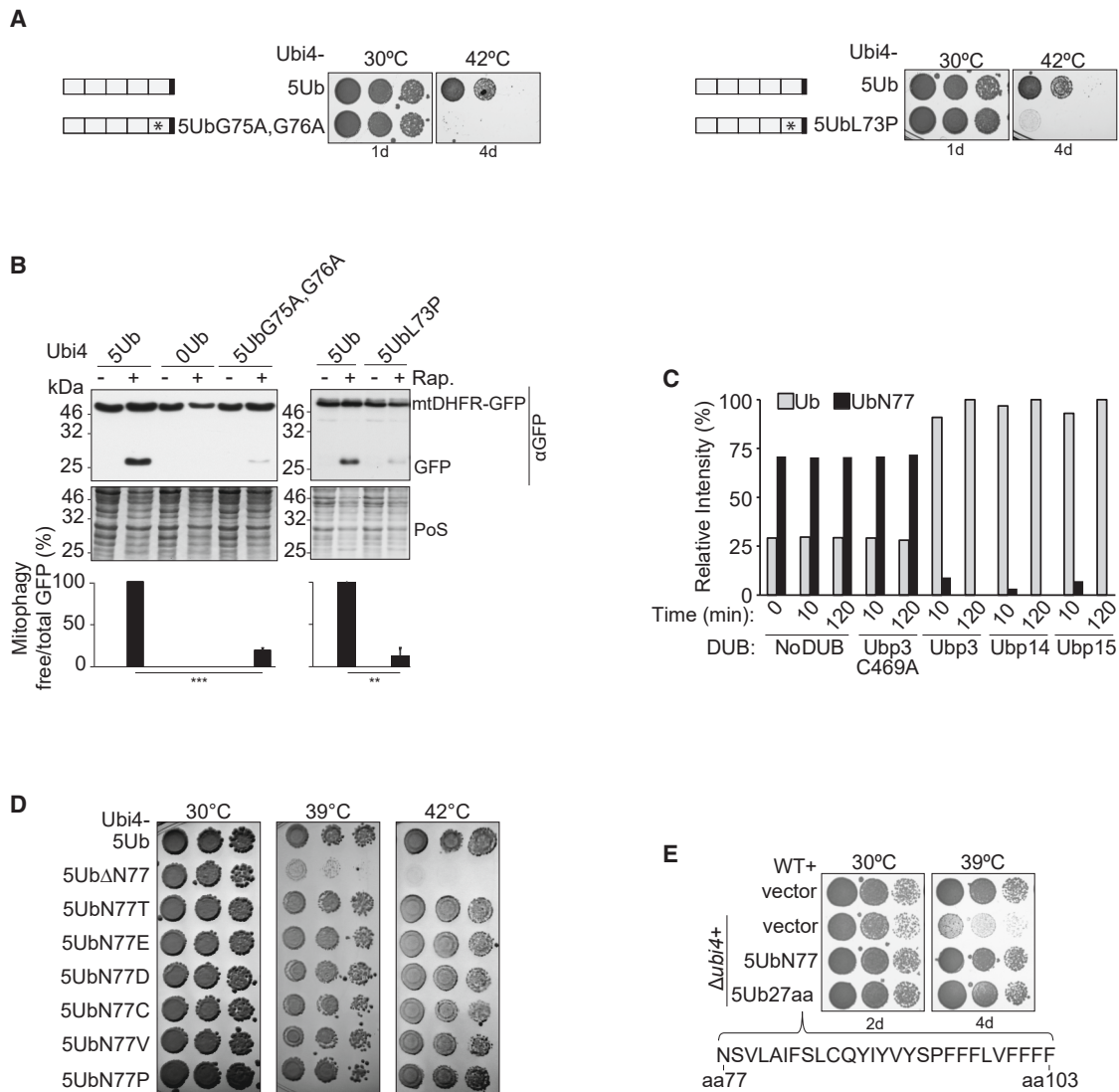


Figure 4. Conjugation and maturation of CxUb are critical for stress responses

(A and B) Cellular growth and mitophagy upon CxUb mutations compromising its conjugation and processing properties. (A) Heat sensitivity, as in Figure 1C, and (B) mitophagy, as in Figure 2C, were assessed in WT cells (5Ub) and in strains genomically mutated on the last ubiquitin repeat of *UBI4*, compromising either its conjugation (5UbG75A and G76A) or DUB processing (5UbL73P). In (B), the quantification represents the mean $\pm$ SD ($n = 3$) normalized to the rapamycin-treated WT cells. Statistical significance was determined by Student's *t* test.

(C) Cleavage of CxUb extension *in vitro*. UbN77, Ubp3 and its catalytically inactive variant Ubp3C469A, Ubp14, and Ubp15 were expressed and purified from *E. coli* cells. Purified UbN77 was incubated at room temperature for the indicated time points without or with the indicated DUBs. Cleavage of the C-terminal extension of UbN77 was assessed by intact MS. The total level of cleaved and uncleaved UbN77 was set to 100%.

(D and E) Cellular growth in dependence of CxUb with different extension variants. (D) Cells genomically expressing ubiquitin extended at its C terminus with either asparagine (5Ub, WT), without extension (5UbΔN77), or with the indicated amino acid substitutions, were spotted on YP glucose media and incubated at 30°C, 39°C, and 42°C. (E) Cellular growth was assessed for WT and Δ*ubi4* cells expressing either the empty vector, Ubi4 (5UbN77), or a Ubi4 variant extended by 26 amino acid residues due to the deletion of the *UBI4* STOP codon (5Ub26AA), as indicated. Cells were spotted on synthetic minimal media (SC) minus uracil (SC-U) glucose media and incubated at 30°C or 39°C for the indicated days.

composition across different species. We infer that the function of CxUb does not depend on the composition of the extension following the G-G motif. In sum, stress resistance and mitophagy require CxUb to be matured and conjugated to substrates, regardless of the composition of the C-terminal extension.

CxUb is required for ubiquitylation and turnover of Ufd2-dependent substrates

UFD substrates are artificial fusion proteins bearing an N-terminal ubiquitin that serves as a degradation signal for the UPS (Figure S1A). CxUb, like UFD substrates, comprises an N-terminal ubiquitin followed by additional amino acids. The ubiquitin

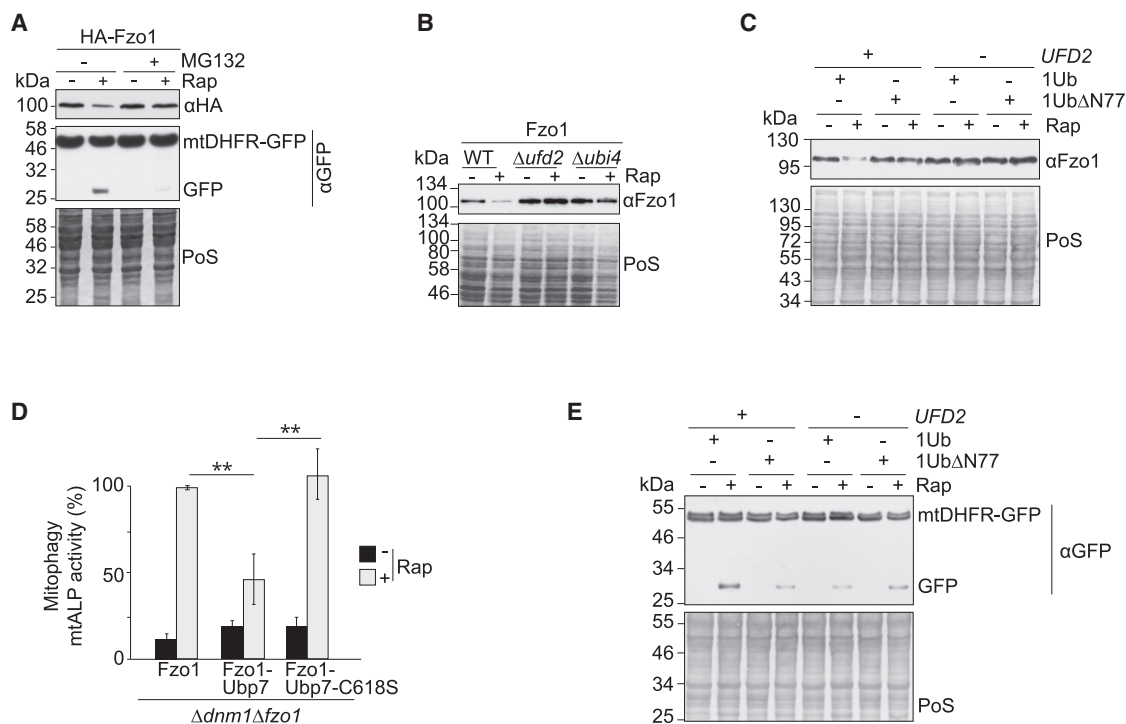


Figure 5. Mitophagy requires Ufd2, Fzo1 ubiquitylation, and proteasomal turnover

(A) Mitophagy and Fzo1 levels upon proteasomal inhibition were simultaneously assessed in $\Delta pdr5\Delta snq2$ cells co-expressing the mitophagy reporter mtDHFR-GFP, as in Figure 2B, and HA-Fzo1. Cells were cultivated in SG-U media and treated with rapamycin (+) to induce mitophagy or left untreated (-), followed by proteasomal inhibition (MG132) or left untreated (DMSO). Total cell extracts, SDS-PAGE, and immunoblotting were performed using HA- and GFP-specific antibodies. Ponceau staining (PoS) was used as a loading control.

(B and C) Assessment of endogenous Fzo1 levels under mitophagy induction. (B) WT, $\Delta ufd2$, and $\Delta ubi4$ cells were grown and analyzed as in (A) but using SG media and Fzo1-specific antibodies. (C) Cells genomically encoding one ubiquitin copy with or without the C-terminal asparagine extension (1Ub or 1Ub Δ N77, respectively), additionally with or without UFD2, as indicated, were grown and analyzed as in (B).

(D) Role of Fzo1 ubiquitylation in mitophagy. Mitophagy was analyzed in $\Delta dnm1\Delta fzo1$ cells expressing the mitophagy reporter mtALP, as in Figure 2A, and complemented either with Fzo1, Fzo1 fused with Ubp7 (Fzo1-Ubp7), or Fzo1 fused with an inactive variant of Ubp7 (Fzo1-Ubp7-C618S). Cells were cultivated in SG-U media and treated with rapamycin (+) or left untreated (-) before measurement of ALP activity. The quantification below represents the mean $\pm$ SD ($n = 3$) normalized to the rapamycin-treated WT cells. Statistical significance was determined by Student's *t* test. ** $p \leq 0.01$.

(E) Role of CxUb and Ufd2 in mitophagy. Cells as in (C), additionally expressing mtDHFR-GFP, were cultivated in SG-U media and analyzed as in 2B.

See also Figure S5.

ligase Ufd2 assembles ubiquitin chains on lysine 48 (K48) in the N-terminal ubiquitin of UFD substrates. Intriguingly, we found that K48 in UbN77 was required to restore heat stress resistance in $\Delta ubi4$ cells (Figure S5A). In addition to modifying UFD model substrates, Ufd2 also assembles K48 chains on various endogenous proteins to promote their proteasomal degradation.^{44–46} Therefore, we considered that UbN77 is a specific, endogenous ubiquitin source for Ufd2-dependent modification and degradation of these endogenous proteins.

We recently showed that mitofusins are Ufd2/UBE4B substrates in yeast and humans under various stress conditions, which targets them for proteasomal degradation.⁴⁴ Notably, the mammalian mitofusins MFN1 and MFN2 are prominent ubiquitylation and proteasomal targets upon mitophagy induction.^{47–50} Similarly, when we induced mitophagy with rapamycin in yeast, we observed proteasome-dependent turnover of the yeast mitofusin Fzo1 (Figures 5A and S5B). Disrupting Ubi4 and Ufd2 prevented Fzo1 turnover during mitophagy

(Figures 5B and S5C). Ectopic expression of Ubi4, but not Ub, restored Fzo1 turnover in $\Delta ubi4$ cells (Figure S5D), whereas successive reduction in the ubiquitin copy number in *UBI4* did not affect Fzo1 degradation (Figure S5E). We tested whether Fzo1 turnover was dependent on CxUb and indeed found that it was abolished by deleting the N77 codon in the WT strain with five ubiquitin copies (5Ub Δ N77) (Figure S5F) or in the strain with only one ubiquitin copy (1Ub Δ N77) (Figure 5C). Moreover, simultaneous deletion of N77 and UFD2 behaved similarly to the single mutants (Figure 5C). By contrast, deletion of genes required for autophagy did not impair Fzo1 turnover (Figure S5G). Overall, these data show that both CxUb and Ufd2, but not autophagy genes, are required for mitofusin turnover during mitophagy.

We then investigated the role of Ufd2 and Fzo1 ubiquitylation for mitophagy. We took advantage of Fzo1-Ubp7 by fusing Fzo1 to the catalytic domain of the DUB Ubp7, which constitutively deubiquitylates Fzo1, and compared it with Fzo1 fused to the respective catalytically inactive mutant (Fzo1-Ubp7-C618S),

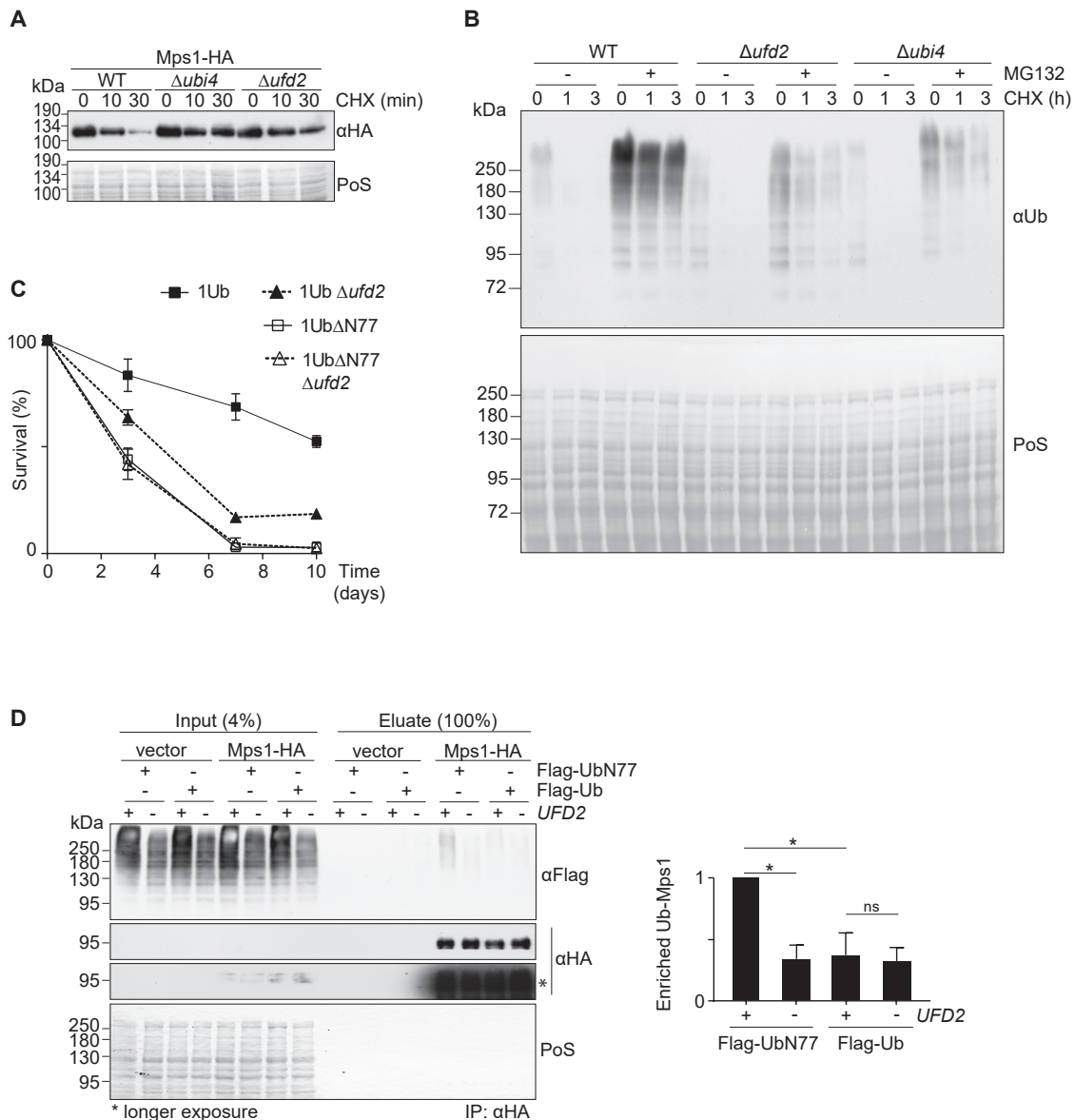


Figure 6. CxUb is required for substrate ubiquitylation by Ufd2

(A) Role of Ubi4 and Ufd2 in Mps1 turnover. Mps1 stability was analyzed in WT, $\Delta ubi4$, and $\Delta ufd2$ cells after translation inhibition using cycloheximide (CHX). Cells expressing Mps1-HA were grown in SC raffinose media to mid-exponential phase and transferred to SC galactose to induce expression of Mps1-HA. Cells were then collected at the indicated time points after inhibition of transcription and translation with 2% glucose and 100 μ M CHX, respectively. Total cell extracts were analyzed by SDS-PAGE and immunoblotting using an HA-specific antibody. Ponceau staining (PoS) was used as a loading control.

(B) Global analysis of the ubiquitylome levels and stability. WT, $\Delta ubi4$, and $\Delta ufd2$ cells additionally deleted for *PDR5* were grown to exponential phase in SC media with raffinose prior to addition of galactose with or without MG132 (50 μ M +, DMSO –, respectively). After 1 h of treatment, the time point 0 h was collected, and CHX (100 μ g/mL) was immediately added to cells, after which the time points 1 h and 3 h were collected. Total cell extracts were analyzed using SDS-PAGE and immunoblotting with a ubiquitin-specific antibody.

(C) Role of CxUb and Ufd2 in yeast aging. The chronological lifespan of cells genomically encoding one ubiquitin copy with or without the C-terminal asparagine extension (1Ub or 1Ub Δ N77, respectively), additionally with or without *UFD2*, as indicated, and growth in synthetic minimal complete media with dextrose (SCD), is shown as the survival percentage for each time point (mean $\pm$ SEM, $n = 3$), calculated from the fraction of colony-forming units.

(D) CxUb and Ufd2 mediate Mps1 ubiquitylation. Mps1-HA ubiquitylation was analyzed in cells expressing FLAG-Ub or FLAG-UbN77 and with (+, $\Delta pdr5$) or without (–, $\Delta ufd2 \Delta pdr5$) *UFD2*. Specific Mps1 ubiquitylation was assessed by comparison to the corresponding empty vector lanes. Mps1-HA expression was induced by shifting mid-exponential cells grown in SC raffinose to SC galactose for 2 h. Detection of Mps1 ubiquitylation was facilitated by treating the cells with

(legend continued on next page)

which maintains Fzo1 ubiquitylation and functionality,⁵¹ and performed the ALP-based mitophagy assay. Our data show that mtALP activity is impaired in Fzo1-Ubp7 compared with Fzo1-Ubp7-C618S (Figure 5D), suggesting that mitophagy depends on Fzo1 ubiquitylation. Consistently, we found that disruption of Ufd2, either in the presence or absence of N77, compromised processing of mtDHFR-GFP upon addition of rapamycin (Figures 5E and S5H). Moreover, proteasomal inhibition also prevented mitophagy (Figure 5A). Overall, our data suggest that Fzo1 ubiquitylation, Ufd2, and the proteasome are important for mitophagy.

Given that mitophagy induction is linked to mitochondrial fragmentation, a possible explanation for the mitophagic dependence on CxUb, Ufd2, and Fzo1 degradation is that, without Fzo1 degradation, mitochondria remain interconnected, which may be less permissive for mitophagy. To assess the connection between mitophagy and mitochondrial morphology, we tested cells lacking the fission factor Dnm1, which cannot fragment mitochondria. We also tested cells expressing Fzo1-T221A, an enzymatically inactive variant of Fzo1 that blocks guanosine triphosphate (GTP) hydrolysis and, consequently, impairs mitochondrial fusion.⁴⁴ However, neither condition interfered with mitophagy. This suggests that Fzo1's role in mitophagy is not linked to its function in membrane fusion or to mitochondrial morphology alterations (Figure S5I).

Next, we investigated whether *UBI4* and CxUb were required for ubiquitylation and turnover of another Ufd2 substrate, namely the kinase Mps1.^{45,46} We expressed hemagglutinin (HA)-tagged Mps1 and monitored its stability after inhibiting translation by CHX treatment. Indeed, like Fzo1, the degradation of Mps1 was compromised in the absence of *UFD2* and *UBI4* (Figure 6A). We then tested the importance of CxUb and Ufd2 in the formation and stability of ubiquitin chains within the whole proteome. Ufd2 functions as an E4 ubiquitin ligase, meaning that it mediates the elongation of ubiquitin chains previously assembled by other ubiquitin E3 ligases via K48 linkages, thus allowing their efficient turnover by the proteasome. We tested the stability of the ubiquitylome over time and dependence on the proteasome for their turnover, especially focusing on the high-molecular weight (HMW) range of ubiquitylated substrates. To this aim, we induced protein translation shut-off by the addition of CHX. Strikingly, we noticed a global impairment in the abundance and turnover of HMW ubiquitylation in the absence of either CxUb, Ufd2, or both (Figures 6B and S6A). As expected, this was especially visible after proteasomal inhibition (Figures 6B and S6A) and depended on K48-linkages (Figure S6B). By contrast, Ubc6, a protein constitutively ubiquitylated and degraded by the proteasome but independently of Ufd2, did not depend on CxUb (Figure S6A, lower). Moreover, neither Ufd4 nor Hul5 was required for the formation and turnover of the HMW global ubiquitylation pattern (Figure S6C). Importantly,

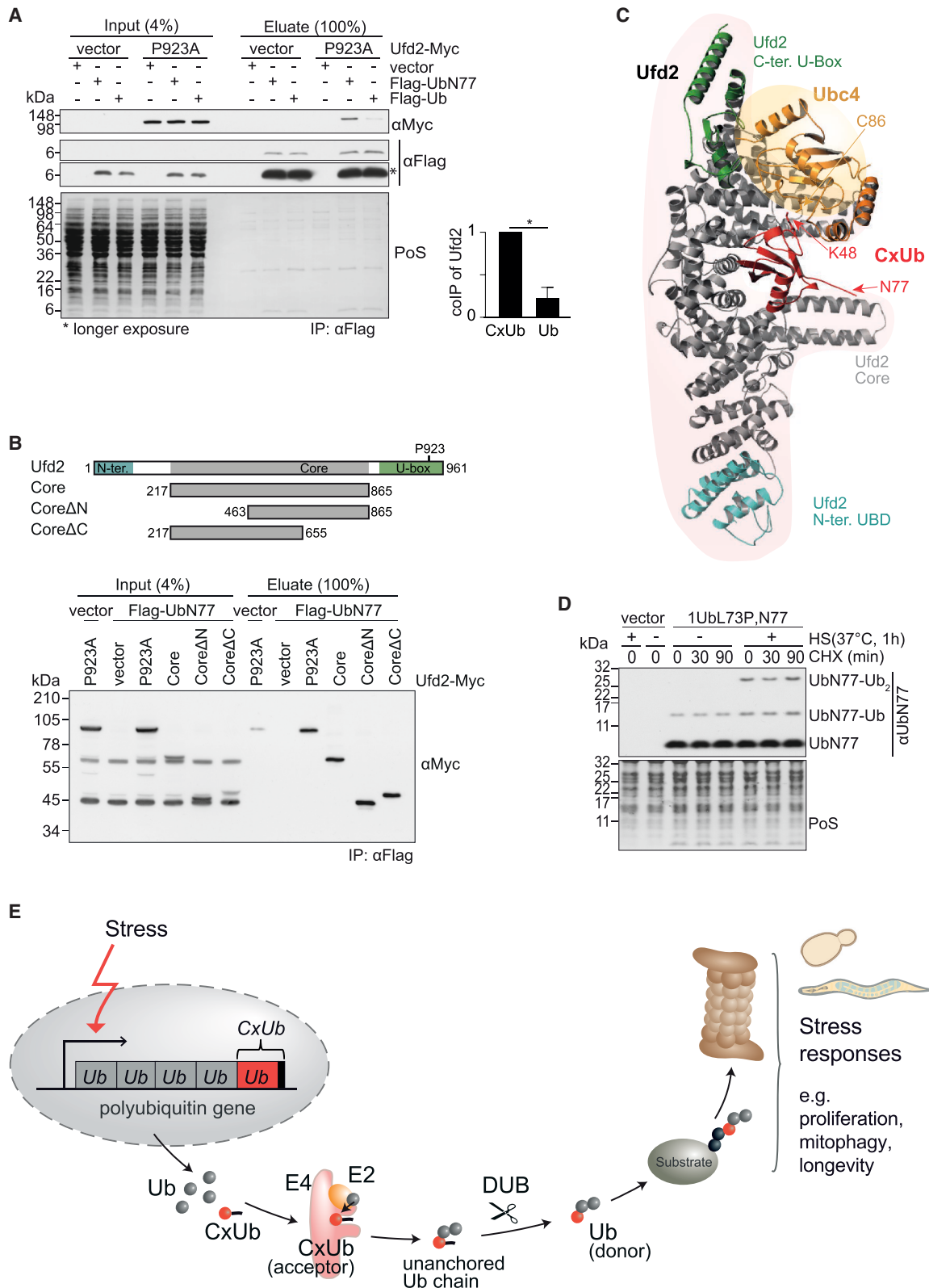
the absence of Ufd2, CxUb, or both reduced yeast lifespan (Figure 6C), consistent with the interdependence between Ufd2 and CxUb. Notably, degradation of the Ufd2 model substrate Ub-P- β gal required *UFD2* but not *UBI4* (Figure S6D), consistent with Ub-P- β gal effectively representing an artificial CxUb. These results confirm the importance of both CxUb and the E4 enzyme Ufd2 for substrate turnover and strengthen the link between CxUb and Ufd2.

We then analyzed whether the ubiquitin generated by CxUb processing is specifically conjugated by Ufd2 to its endogenous substrates. To this end, we examined the efficiency of Mps1-HA ubiquitylation in cells expressing either FLAG-tagged UbN77 or FLAG-tagged ubiquitin, both in the presence and absence of Ufd2. We immunoprecipitated Mps1-HA and investigated the presence of FLAG-Ub conjugated onto Mps1-HA under these different conditions by performing western blotting with an anti-FLAG antibody. Indeed, we detected FLAG-tagged Mps1-HA more efficiently in cells simultaneously expressing Ufd2 and FLAG-UbN77, both under native and denaturing protein extraction conditions (Figures 6D and S6E). This suggests that Ufd2 links processed ubiquitin that originated from UbN77. Taken together, we propose that Ufd2 preferentially conjugates matured CxUb, drives proteasomal turnover of its substrates, and signals stress responses. For example, CxUb- and Ufd2-dependent ubiquitylation of the Fzo1 signal mitophagy.

CxUb forms ubiquitin conjugates and reveals an uncharacterized UBD on Ufd2

To investigate whether CxUb is specifically recognized by the E4 Ufd2, we compared Ufd2 binding with CxUb and with ubiquitin by a co-immunoprecipitation assay. Briefly, FLAG-tagged UbN77 or FLAG-tagged ubiquitin were expressed in yeast cells with catalytically inactive Ufd2-Myc. Strikingly, Ufd2-Myc showed stronger co-immunoprecipitation with FLAG-UbN77 compared with FLAG-Ub (Figure 7A). Ufd2 contains a ubiquitin-binding domain (UBD) at its N terminus and a U-box at its C terminus, which is required for its function as an E4 ubiquitin ligase. Notably, the interaction between UbN77 and Ufd2 did not require the UBD, its interacting partners,⁵² or the U-box (Figures S7A and S7B). We found that the central core domain of Ufd2 was sufficient to bind UbN77 (Figure 7B). These data suggest the presence of a previously unnoticed UBD in Ufd2. Consistently, AlphaFold3⁵³ multimer modeling predicted that UbN77 sits between two arms in the core of Ufd2 (Figure 7C). Moreover, modeling also places the E2 enzyme Ubc4 adjacent to the U-box of Ufd2, in an orientation that positions its active cysteine 86 directly beside the epsilon amino group of K48 of UbN77 (4.3 Å) in the previously unknown UBD of Ufd2. In summary, our data suggest that Ufd2 preferentially binds CxUb over Ub, which depends on a middle domain in Ufd2.

the proteasome inhibitor MG132 for 1 h before cell harvesting and further immunoprecipitation (IP) with HA beads. Immunoblotting was performed with HA- and FLAG-specific antibodies. Ubiquitylated Mps1 (FLAG signal in the immunoprecipitate (IP) fraction) was quantified from 3 biological replicates, normalized to precipitated Mps1 (HA signal in the IP fraction), and represented as the mean $\pm$ SD ($n = 3$) relative to WT cells (encoding *UFD2*) and expressing FLAG-UbN77. Statistical significance was determined by the paired Student's *t* test. ns $p > 0.05$, * $p \leq 0.05$. See also Figure S6.



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Ufd2 assembles ubiquitin species on UFD substrates.⁸ To test if ubiquitin chains can assemble on CxUb, we generated a peptide antibody that specifically detects UbN77 and UbN77 mutants in western blots, but not ubiquitin lacking the C-terminal extension (Figure S7C). The levels of UbN77 variants were higher than WT UbN77, consistent with their reduced maturation by USP DUB enzymes (L37P) and reduced conjugation (G75A and G76A) (Figure S7C). Strikingly, we observed di-ubiquitin chain assembly on the UbN77-L73P variant, and this effect was exacerbated in a strain lacking the ubiquitin C-terminal hydrolase gene *YUH1*, which is also involved in maturation of ubiquitin precursors⁵⁴ (Figures S7C and S7D, labelled as UbN77-Ub). Moreover, ectopic expression of ubiquitin promoted chain assembly and increased the levels of di-, tri-, and even tetra-ubiquitin chains on the processing-resistant variant UbN77-L73P in $\Delta yuh1$ cells (Figure S7E). To investigate if the conjugation capacity of UbN77 affects chain assembly, we expressed UbN77 with all three mutations (L73P, G75A, and G76A) in $\Delta yuh1$ cells. Although simultaneous mutation of three residues in UbN77 was associated with diminished recognition by the antibody, we could detect ubiquitin assembly onto the UbN77-L73P, G75A, G76A variant (Figure S7E). Moreover, chain assembly on CxUb increased upon heat shock, and all forms of UbN77-L73P were stable in the absence of continued translation (Figure 7D). Finally, deletion of *UFD2* impaired chain formation on CxUb (Figure S7F). Taken together, we propose that ubiquitin chains assemble on unprocessed CxUb, particularly under stress, are transferred to Ufd2 substrates, promote their proteasomal degradation, and enable stress responses.

DISCUSSION

Proteostasis and mitochondrial quality control are two hallmarks of healthy aging.²⁹ We uncovered the existence of a

distinct, unique ubiquitin entity that maintains proteostasis and mitochondrial quality control, which we named CxUb. CxUb is a C-terminally extended form of ubiquitin, i.e., a ubiquitin precursor, which after maturation by cleavage of the C-t extension gets conjugated to substrates by the E4 ubiquitin ligase Ufd2, thus promoting their proteasomal turnover (Figure 7E).

Under stress, E4 enzymes extend ubiquitin chains previously assembled by E3 ubiquitin ligases, driving their effective turnover by the proteasome. We now propose that Ufd2 harnesses CxUb to amplify ubiquitin tagging on several substrates and promote their efficient proteasomal turnover, particularly under stress, ensuring cellular quality control (Figure 7E). Thus, we predict that several E3 enzymes will cooperate with Ufd2, or possibly with other E4 enzymes, allowing the multitude of different stress responses that we observed to depend on CxUb. We recently reported on a stress-responsive role of Ufd2 on mitochondria, via ubiquitylation of the mitofusin MFN2 and of Fzo1, the orthologous mitofusin in yeast.⁴⁴ Notably, here we discovered that this process depends on the ubiquitin precursor CxUb. In higher eukaryotes, ubiquitylation and proteasomal turnover of the outer membrane proteins in damaged mitochondria is a well-established signaling event for mitophagy, with MFN1 and MFN2 as prominent targets.^{50,55} We now found that ubiquitylation and proteasomal turnover of Fzo1 by Ufd2 and CxUb drive mitophagy, which demonstrates the physiological importance of CxUb and of Ufd2. Future studies will determine if this involves still unidentified ubiquitin-dependent mitophagy receptors or if it occurs via another mechanism. Importantly, CxUb is conserved in higher eukaryotes and is also required for mitophagy in worms. Besides mitophagy, CxUb controls lifespan in yeast and in worms, a feature also shared by the Ufd2 worm homolog UFD-2.¹⁵ Further investigation is needed to ascertain whether CxUb and UBE4B are also required for mitophagy and

Figure 7. Ufd2 distinguishes CxUb from Ub

(A) Ufd2 binds preferentially to CxUb. IP with anti-FLAG beads followed by western blot analysis with a Myc-specific antibody from $\Delta ufd2$ cells expressing an empty vector, FLAG-UbN77, or FLAG-Ub and additionally expressing catalytically inactive Ufd2 P923A-Myc or the respective empty vector. Cells were grown to mid-exponential phase in selective SD-U-L media, followed by cell disruption, IP, and western blot of the eluates, probed with either FLAG- or Myc-specific antibodies, to assess for co-immunoprecipitation of Ufd2-Myc with either FLAG-UbN77 or FLAG-Ub. Enriched Ufd2 in cells expressing either FLAG-UbN77 (CxUb) or FLAG-Ub (Ub) was quantified and normalized to the enriched FLAG-Ub levels and is represented as the mean $\pm$ SD ($n = 3$) relative to cells expressing FLAG-UbN77. Statistical significance was determined by Student's *t* test. * $p \leq 0.05$.

(B) Ufd2 domains required to bind CxUb. Top: linear representation of Ufd2 structure and truncations. Bottom: IP with anti-FLAG beads, followed by western blot analysis with Myc-specific antibody, as in A, from $\Delta ufd2$ cells expressing FLAG-UbN77 or an empty vector, additionally expressing the indicated Myc-tagged truncation variants of Ufd2 or an empty vector.

(C) AlphaFold2 modeling of Ufd2 (gray)—with its N-terminal UBD in cyan and U-box in green—with UbN77 (red) and Ubc4 (orange),⁵³ which suggests a previously unknown UBD in Ufd2. It also shows the C-terminal N77 of CxUb pointing to the opening of the core region. The model highlights the close proximity of UbN77's K48 to E2 Ubc4's catalytic cysteine 86, i.e., to the thioester with the donor ubiquitin (not represented). The predicted template modeling (pTM) and interface predicted template modeling (ipTM) values are 0.83 and 0.66, respectively.

(D) Formation and stability of UbN77 L73P molecules. $\Delta yuh1$ cells expressing UbN77 L73P or the corresponding empty vector were grown to mid-exponential phase in selective SG-U media and heat shocked for 1 h at 37°C (+) or left at 30°C (–). Cells were incubated with the translation inhibitor cycloheximide (CHX) and harvested at the indicated time points. Total cellular extracts were analyzed by SDS-PAGE and immunoblotting with the UbN77-specific antibody. Mono UbN77, UbN77-Ub (di-Ub), and UbN77-Ub₂ (tri-Ub) chains were observed, as indicated. Ponceau staining (PoS) was used as a loading control.

(E) Model of the stress response regulation by CxUb and Ufd2. Stress induces expression of the yeast polyubiquitin gene *UBI4*, which encodes five ubiquitin copies. The last ubiquitin copy, CxUb, represented in red, is extended at its C terminus by an asparagine residue. The E4 ubiquitin ligase Ufd2 specifically recognizes CxUb, and then ubiquitin chains form on CxUb. First, CxUb serves as an acceptor ubiquitin, which is then converted into an unanchored chain by Ufd2's activity with its E2 enzyme. Subsequently, CxUb, now at the proximal end of a chain, is converted into a donor ubiquitin by DUB activity, which enables the transfer of the preformed chain to a substrate. Thus, Ufd2 acts as a platform that extends the ubiquitin chains of substrates that were previously ubiquitylated (represented schematically by dark gray ubiquitin moieties). This efficiently signals the targeting of substrates for degradation by the proteasome and regulates stress responses, including mitophagy and longevity, in both yeast and worms.

See also Figure S7.

healthy aging in mammals and whether they might constitute valuable disease targets.

Our data show that Ufd2 binds CxUb much more avidly than it binds ubiquitin and that this occurs via the middle domain of Ufd2, which folds into a core cradle domain that was previously unnoticed to bind ubiquitin. Interestingly, although required for proteostasis, the nature of the extension in CxUb is not relevant. This explains how Ufd2 was initially discovered in the UFD pathway. Moreover, we found that the long-studied Ub-fusion proteins are effectively an artificial equivalent of CxUb. Similarly to the UFD substrates, such as Ub- β -Galactosidase,⁶ we detected short chains formed on CxUb. Interestingly, free ubiquitin chains have been observed in several organisms, and a multifaceted picture of the handling and function of unanchored polyubiquitin is emerging, with diverse physiological functions in immune pathways and during cell stress.⁵⁶ For example, in yeast, free ubiquitin chains were upregulated by stress, including heat shock, DNA damage, and oxidative stress,⁵⁷ reminiscent of the induction of CxUb polyubiquitin species observed in our study. In addition, some cell stressors, including heat shock, can lead to a build-up of protein aggregates⁵⁸ or stress granules,⁵⁹ whose formation and disassembly were proposed to depend on untethered polyubiquitin, reminiscent of the role of CxUb in resistance to stress caused by protein aggregation. In fact, unanchored polyubiquitin can be assembled by specialized E2/E3 pairs *in vitro* and in cells⁶⁰ and was very recently suggested to be involved in ribosomal biogenesis.⁶¹ In addition, the transfer en bloc of an intact chain, without its disassembly into monoubiquitin, has been observed *in vitro* and proposed to occur *in vivo*.⁶² Due to Ufd2's role in ubiquitin chain elongation, it is possible that the ubiquitin chain assembled on CxUb remains bound to Ufd2. There, it undergoes maturation of the C-t extension and is activated by E1 and E2 enzymes. Then, it is transferred en bloc to Fzo1 and other Ufd2 substrates. However, we cannot rule out the possibility of release from Ufd2 prior to DUB cleavage and/or activation of the C-terminal G residue. Mechanistic details of how CxUb switches from an acceptor to a donor ubiquitin are interesting topics for follow-up studies.

Notably, a variety of defects were reported upon impairment of the polyubiquitin-encoding genes *UBB* and *UBC* in mammals. First, similarly to Δ *ubi4*, cells lacking *UBC* are sensitive to heat stress and to inhibition of the proteasome and show cell cycle abnormalities.⁶³ Moreover, low levels of *UBB*, observed in aggressive gynecological cancers, rendered these cancer cells sensitive to downregulation of *UBC*.¹⁴ Thus, it would be interesting to analyze the importance of CxUb in cancer cell proliferation. Second, mutations in the polyubiquitin-encoding gene *UBB* occur in Alzheimer's and Down syndrome patients⁶⁴ and are a hallmark of Alzheimer's disease,⁶⁵ suggesting that CxUb also constitutes a valid target in neurodegeneration. Third, disruption of *UBB* in mice was reported to impair meiosis¹¹ and neural stem cell differentiation⁶⁶ and to promote gliosis.⁶⁷ Moreover, mouse embryos lacking the polyubiquitin-encoding gene *UBC* have perturbed hematopoiesis,⁶⁸ confirming a role in cellular differentiation. Importantly, these phenotypes cannot be rescued by overexpression of monoubiquitin, which suggests a need for the ubiquitin precursor CxUb.^{63,68}

In conclusion, substrate ubiquitylation and proteasomal turnover are essential both for housekeeping and for stress responses. Strikingly, the specialized functions of CxUb for substrate ubiquitylation and proteasomal turnover under stress provide an unprecedented approach to specifically target CxUb while leaving ubiquitin and its housekeeping functions intact, thereby potentially bypassing highly toxic side effects from proteasomal inhibitors currently used in oncology treatments. Therefore, the identification of CxUb as an endogenous stress-dedicated ubiquitin entity reveals an additional layer of regulation of ubiquitin signaling and provides further perspectives for understanding the pathomechanisms of various ubiquitin-linked human disorders.

Limitations of the study

One limitation of our study is that we still do not know how and where the transition from acceptor to donor function of CxUb occurs, nor do we know the structural basis for Ufd2's preference for binding CxUb over ubiquitin. Furthermore, the precise regulation of mitophagy by ubiquitylation in yeast remains unclear, as no ubiquitin-dependent mitophagy receptors have been identified to date. Thus, the question of how CxUb/ubiquitin and Atg32/autophagosome-dependent pathways converge in mitophagy activation remains unanswered.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents should be directed to the lead contact, Mafalda Escobar-Henriques (mafalda.escobar@uni-koeln.de).

Materials availability

All unique materials generated in this study are available upon request.

Data and code availability

- All imaging data generated in this study have been deposited to Mendeley Data and are publicly available as of the date of publication. DOI: <https://doi.org/10.17632/mgsw2pcnhc.1>. Accession numbers and DOI are also listed in the [key resources table](#). The proteomics data are deposited to PRIDE under the accession number PRIDE: PXD065783 (token: LBEvS21o5BR9).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

S.A., T.S., and M.E.-H. determined the role of CxUb in stress resistance and mitophagy and identified the mechanistic link to the UFD pathway. A.S.R. initiated the study and together with C.B. determined the role of *UBI4* and CxUb in mitophagy. V.A. analyzed mitochondria morphology and created tools and data in worms, under the guidance of T. Hoppe, with critical input from N.V. D.D. identified the CxUb binding domain in Ufd2 and its role in the global HMW ubiquitylome, with help from T.S. and K.H. K.M.V. determined the role of *UBI4*, CxUb, and Ufd2 for lifespan in *S. cerevisiae* and performed experiments from Figures 4D and S3E. R.K. performed experiments from Figures S2C and S3C. H.N. performed MS experiments. T. Hermanns performed DUB assays. N.B.-A. performed bioinformatic analysis. K.B. and M. Z. generated tools. M.K., N.V., R.J.D., K.H., T. Hoppe, A.S.R., and M.E.-H. discussed experimental designs and results. M.E.-H. and A.S.R. designed and developed the study and wrote the manuscript, with help from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-UbN77	Pineda	N/A
Rabbit polyclonal anti-ATG32	Pineda	N/A
Rabbit polyclonal anti-Fzo1	Simoes et al., 2018 ⁶⁹	N/A
Rabbit polyclonal anti-MBP-Pho8 (mtALP)	Mendl et al., 2011 ²⁶	N/A
Mouse monoclonal anti-GFP	Roche	#11814460001; RRID:AB_390913
anti-Bmh1/2	W. Neupert	N/A
Mouse monoclonal anti-Pgk1	Abcam	#ab197960; RRID:AB_2756444
Mouse monoclonal anti-Ubiquitin (P4D1)	Cell Signaling	#3936; RRID: AB_331292
Rat monoclonal anti-HA	Roche	#11867423001; RRID: AB_390918
Rabbit polyclonal anti-Flag	Merck	F7425; RRID: AB_439687
Rabbit polyclonal anti-Myc	Merck (Sigma)	C3956; RRID: AB_439680
Mouse monoclonal anti-βGalactosidase	Promega	#Z3781; RRID:AB_430877
Mouse monoclonal anti-Atg11	C. Kraft	n/a
Anti-Ape1	C. Kraft	n/a
Rabbit monoclonal anti-Ubc6	T. Sommer	n/a
Rabbit monoclonal anti-Atg8	K. Okamoto	n/a
Bacterial and virus strains		
DH5α competent cells	ThermoFisher Scientific	# 18265017
Rosetta (DE3) pLysS competent cells	Merck	#71137
Chemicals, peptides, and recombinant proteins		
Ponceau S	Serva	#33429.02
Cabonyl cyanide 3-chlorophenylhydrazon (CCCP)	Merck	#C2759
Zinc chloride	Carl Roth	#3533.2
L-Canavanine	Merck	#C1625
BioTracker 640 Red C2(FM4-64)	Merck	#SCT127
MG132 InSolution 10mM in DMSO	Calbiochem	#474791
Cycloheximide In Solution 100mg/ml in DMSO	Merck	#C4859
Copper (II) Sulphate pentahydrate	Merck	#209198
Rapamycin	Merck	#553210
cOmplete, EDTA-free protease inhibitor cocktail	Merck	#11873580001
p-nitrophenylphosphate	Merck	#487663
DTT	MP	#100597
Color Prestained Protein Standard, Broad Range	NEB	#P7719
Lauryl maltose neopentyl glycol (NG310)	Biozol	#ANA-NG310
EZview Red Anti-FLAG M2 Affinity Gel	Merck	#F2426
EZview Red Anti-HA Affinity Gel	Merck	#E6779
ESTLHLVLR*LR*GGN	JPT Peptide Technologies	N/A
Critical commercial assays		
GE Healthcare Amersham™ ECL TM Prime	Amersham	#RPN2232
Deposited data		
Mendeley data	This study	https://doi.org/10.17632/mgsw2pcnhc.1
PRIDE (proteomics)	This study	PRIDE: PXD065783

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: <i>C. elegans</i>		
Bristol (N2) strain as wild-type (wt)	CGC	N2
ΔCxB strain numbers 9.6 & 9.11	This study	N/A
N2;Ex003[pmyo-3::Tomm-20::Rosella]	Palikaras et al., 2015 ³²	N/A
ΔCxB;Ex003[pmyo-3::Tomm-20::Rosella]	This study	N/A
Experimental models: <i>S. cerevisiae</i> strains		
See Table S1 for all yeast strains used in this study.	N/A	N/A
Oligonucleotides		
See Table S2 for all oligonucleotides used in this study.	N/A	N/A
Recombinant DNA		
See Table S3 for all plasmids used in this study.	N/A	N/A
Software and algorithms		
Clustal Omega – Multiple Sequence Alignment	EMBL-EBI	https://www.ebi.ac.uk/Tools/msa/clustalo/
iTasser	Roy et al., 2010 ⁷⁰	https://zhanggroup.org/I-TASSER/about.html
EndNote	Clarivate	https://endnote.com/
MaxQuant	Cox and Mann, 2008 ⁷¹	https://www.maxquant.org/
GraphPad	GraphPad Software, San Diego, California, USA	www.graphpad.com
Adobe Illustrator	Adobe Inc., 2019. <i>Adobe Illustrator</i>	https://adobe.com/products/illustrator
Adobe Photoshop	Adobe Inc., 2019. <i>Adobe Photoshop</i>	https://www.adobe.com/products/photoshop.html
PyMOL	Schrödinger and DeLano, 2020 ⁷²	http://www.pymol.org/pymol
ImageJ (Fiji)	NIH ⁷³	https://imagej.nih.gov/ij/
AlphaFold2 Multimer	Jumper et al., 2021 ⁵³	https://cosmic-cryoem.org/tools/alphafoldmultimer/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All *S. cerevisiae* strains used in this work are listed in Table S1. Single knockout strains were obtained from the EUROSCARF library, derived from a MAT α or a MATa background (BY4742: his3 Δ 1; leu2 Δ 0; lys2 Δ 0; ura3 Δ 0 or BY4741: his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0, respectively). Additional gene manipulations or deletions were created using homologous recombination-based methods as previously described.⁷⁴ Table S2 lists the primers used to amplify selection cassettes, mostly from cassette plasmids but also from the strain UBI3-HIS3, to generate UBI3 Δ Ub. All transformations were carried out using the lithium acetate method and transformants were identified as colonies that grow on selective solid media.

All plasmids used in this study are described in Table S3. Amplification of DNA fragments or site directed mutagenesis by PCR (polymerase chain reaction) was achieved using the Phusion High Fidelity Kit (New England Biolabs) with the primers described in Table S2. Restriction enzymes and T4 DNA ligase were used to cut and ligate DNA fragments into the respective vectors, which were subsequently transformed into *Escherichia coli* (*E. coli*) DH5 α chemical- or electro-competent cells as described elsewhere.⁷⁵

Growth conditions

Culturing of yeast cells was performed using standard methods at the optimal temperature of 30°C, unless indicated otherwise. Strains were incubated in YP (1% yeast extract, 2% peptone) with 2% glucose (D), 2% glycerol plus 0.1% glucose (G), synthetic minimal medium with glycerol (SG, 0.67% yeast nitrogen base with ammonium sulfate, 2% glycerol, 0.1% glucose, amino acids), synthetic minimal medium with lactate (SL, 0.67% yeast nitrogen base with ammonium sulphate, 2% lactate, 0.1% glucose, amino acids) or synthetic minimal medium with glucose and with or without nitrogen (SD or SD-N, respectively; 0.17% yeast nitrogen base with or without ammonium sulfate and amino acids, 2% glucose). For induction of mitophagy, strains were grown on SG and treated with 1 μ M rapamycin (+) for 24 hours, unless indicated otherwise, or left untreated (-).

For nitrogen starvation-induced mitophagy or autophagy, cells were grown in SL media and transferred to SD-N media for 0, 5 or 24 h. Stationary-phase mitophagy was performed by culturing the cells in SL medium for 5, 24, 48 and 72 h.

Cytoplasm-to-vacuole targeting (Cvt) pathway was induced by treating cells grown on SG with 1 μ M rapamycin (+) for 24 hours, or leaving them untreated (-), and was accessed using matured Ape1 levels as a reporter protein.

For galactose-induced protein expression, cells were cultured in synthetic minimal media supplemented with 2% (w/v) raffinose before addition of 2% (w/v) galactose during 2 h. Transcription and translation were shut-down by addition of Glucose to 2% (w/v) and cycloheximide (CHX) to 100 μ g/ml (Sigma, Germany) (from a stock of 10 mg/ml in H₂O), respectively. Analysis of the high molecular weight ubiquitylome was performed under the same conditions, except that galactose was added for 1.5 h and no glucose was added. For proteasomal inhibition cells were incubated for 1 h with MG132 (Calbiochem) at a concentration of 50 μ M (from a stock of 10 mM in DMSO), prior to the addition of CHX.

Caenorhabditis elegans maintenance and transgenic lines

Nematodes were grown at 20 °C (unless stated otherwise) on nematode growth medium (NGM) plates seeded with the bacterial E coli strain OP50 as a food source according to standard protocols and methods.^{76,77} The N2 Bristol strain was used as a wild type. Deletion of the C-terminal amino acids Asp;Ile was performed using CRISPR-Cas9 as previously described.³⁸ Briefly, Cas9 was targeted to 3' region of the ubq-1 coding sequence by using the guide RNA (crRNA) 5'-CTCGTTCTTCGTCTTCGCGG-3'. A repair template was chosen that exchanged the Asp;Ile coding sequence, 5'-GACATT-3', with 5'-TAGTAA-3', encoding two stop codons. crRNA and guides for the ubq-1 5' region and dpy-10 were introduced into wild type animals using microinjections and selected by monitoring dumpy phenotypes and tested by PCR.^{78,79} Deletion of the CxUb coding sequence was validated by Sanger sequencing. Animals were backcrossed with the wild type at least five times. Animals expressing mtRosella in body wall muscle cells were previously described.³⁹

METHOD DETAILS

SDS-PAGE and Western blot analysis

For yeast total cellular protein extracts were obtained by alkaline pH extraction from 3 OD600 cells as described before.⁸⁰ For *C. elegans* whole animal lysates, worms were collected and washed in M9 buffer. Worm pellets were flash frozen in liquid nitrogen, resuspended in an equal volume (ml/g) of 2x sample buffer and lysed by repeated flash freezing, thawing, subsequent sonication (2 times 1 min with 30 sec. break at 70% amplitude) and boiling at 95 °C for 5 min. For ubiquitin profile analysis, proteins were separated by Tris/Tricine (T/T, 11%) SDS-PAGE and transferred to Polyvinylidene difluoride (PVDF) membranes, which were additionally treated with denaturing solution (6 M guanidinium chloride, 20 mM Tris pH 7.5, 1 mM PMSF, 5 mM β -mercaptoethanol) for 30 min and then washed prior to blocking with a 5% (W/V) milk-TBS solution. In all other cases, including the high molecular weight ubiquitin profile analysis, proteins were resolved by Tris/Glycine (T/G, 8% or 10%, depending on the separation required) SDS-PAGE, and transferred into nitrocellulose membranes. For protein detection, several antibodies were used, in 5% milk in TBS, the buffer also used in the washings. For UbN77, Atg32 and Fzo1 detection, we used polyclonal affinity purified antibodies, raised in rabbit, against the peptides NH₂-CGRLRGGN-COOH (anti-UbN77, produced by Pineda, Germany, used at 1:1000), NH₂-QREGKGSSSKSMPPDSSSTTIHTC-COOH (anti-Atg32, produced by Pineda, Germany, used at 1:2000) and NH₂-CHGDRKPDPPYSSS-COOH (anti-Fzo1, produced by GenScript, USA, used at 1:1000). Bmh2 was detected with a rabbit polyclonal antibody, kindly gifted by Walter Neupert (LMU Munich, Germany), and diluted to 1:1000. To detect mtALP, we used a polyclonal antibody raised in rabbit against the fusion protein MBP-Pho8, diluted 1:1000. Moreover, the following commercial antibodies were used: anti-GFP (Roche, clone 7.1/13.1, used at 1:1000), anti-Pgk1 (Abcam, clone 22C5D8, used at 1:2000), anti-ubiquitin (Cell Signaling, clone P4D1-A11, used at 1:1000), anti-HA (Roche, cat no. 11867423001, used at 1:1000), anti-Flag (Sigma, cat no. F1804, used at 1:1000), anti-Myc (Sigma, cat no. C3956, used at 1:1000) and anti- β -Galactosidase (Promega, cat no. Z3781, used at 1:5000). Ape1 was detected with a rabbit polyclonal antibody kindly gifted by Claudine Kraft (University of Freiburg, Germany) and diluted to 1:5000. Atg11 was detected with a mouse monoclonal antibody kindly provided by Claudine Kraft (University of Freiburg, Germany) and diluted to 1:1000. Endogenous Atg8 and Ubc6 were detected with antibodies raised in rabbit at the concentration 1:1000, kindly provided by Koji Okamoto (University of Tokyo, Japan) and Thomas Sommer (Max-Delbrück-Centrum für Molekulare Medizin in der Helmholtz-Gemeinschaft, Berlin, Germany), respectively. Protein levels detected in scanned films (16-bit images) were quantified using Image Quant (GE Healthcare) and protein levels from images acquired in FUSION Chemidoc (Vilber) were quantified using Image J (Fiji). wt values were used as a reference and set to 100. Mean values are shown and the error bars reflect the standard deviation (SD). Statistical tests and replicate numbers are described in figure legends. n.s. > 0.05. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$. **** $p \leq 0.0001$.

Whole proteome ubiquitylation

Total protein extracts of cells grown as described above, in growth conditions, and in the respective figure legends, were analyzed by western blot, on an 8% Tris-Glycine gel, and by protein detection with anti-ubiquitin antibody.

Mps1 ubiquitylation

Mps1 ubiquitylation was analyzed by immunoprecipitation (IP) under native and denaturing conditions. 60 OD₆₀₀ of Δ pd_r5 or Δ ufd2 Δ pd_r5 cells expressing Mps1-HA under the control of the GAL1 inducible promoter, additionally expressing Flag-Ub or Flag-UbN77 under the control of the UBI4 endogenous promoter, were grown to the exponential growth phase in synthetic media

supplemented with 2% (w/v) raffinose. Mps1-HA expression was induced by addition of 2% (w/v) galactose for 45 min, concomitant with 50 μ M MG132 to block proteasomal activity (Calbiochem #474791). Proteins were subsequently extracted, followed by Mps1-HA enrichment by immunoprecipitation, either on TBS buffer for native IP (Figure 6D), or on UREA-containing buffer for denaturing IP (Figure S6D). Native IP was performed as described.⁸¹ Briefly, cells were disrupted with glass beads (0.4–0.6 μ m) in TBS. After centrifugation at 16000 g for 20 min, the cytosolic fraction was used to precipitate Mps1-HA with HA-coupled beads (EZview Red Anti-HA Affinity Gel, Merck #E6779). After 2h of binding reaction with rotation at 4°C, immunoprecipitated proteins were washed 3 times in TBS before elution in sample buffer. The samples were then analyzed by western blot and protein detection with anti-Flag and anti HA-antibodies. For Mps1-HA denaturing IP, cells were disrupted by vortexing with glass beads (0.4–0.6 μ m, 5 cycles 30s ice, 5 cycles 30s vortex) in 300 μ l denaturing buffer (8M Urea buffer containing 0.25% tween 20). Cleared cell extracts obtained by centrifugation (10 mins, 4°C, 1500g) were sonicated for 15 min in a water bath, diluted 6X in TBS and loaded into pre-rinsed (TBS) 30kDa cut-off amicon filter columns (Amicon®Ultra, 0.5ml, ultracel 30K, Millipore). Buffer exchange to TBS was then achieved by centrifugations at 11000g at 4°C, until only 100 μ l were left in the columns. These concentrated protein extracts were submitted to another buffer exchange round, by being diluted one more time via adding 400 μ l TBS and centrifuged again until a final volume of 100 μ l. The concentrated protein extract was mixed with 400 μ l of TBS before its recovery by placing upside down the filter device into a dedicated collection tube and spinning for 2 min at 1000g. The sample was then submitted to HA-coupled beads (EZview Red Anti-HA Affinity Gel, Merck #E6779), and IP was performed as for native IP.

Co-Immunoprecipitations

Co-immunoprecipitation of ubiquitin variants and Ufd2 was performed in exponentially growing cells expressing Flag-Ub or Flag-UbN77 and the indicated variants of Ufd2-Myc. For Figure 7A, Ufd2-P923A-Myc was expressed under its endogenous promoter in Δ ufd2 Δ pdr5 cells. For Figures 7B and S7A, all Ufd2-Myc variants were expressed under the copper inducible promoter of CUP1, also in Δ ufd2 Δ pdr5 cells. Cells expressing N-t truncations were additionally treated with 100 μ M CuSO₄ for 1h i.e the Ufd2-Myc variants Core, Core Δ N or Core Δ C (Figure 7B) and Δ N or Δ N, P923A (Figure S7A). For analysis of Rad23 and Dsk2 in Figure S7B, the indicated variants of Ufd2-Myc under control of its endogenous promoter were co-expressed with Flag-UbN77, in Δ ufd2 or Δ ufd2 Δ rad23 Δ dsk2 cells. 80 OD₆₀₀ of exponentially growing yeast cells in synthetic media were harvested and resuspended in TBS with 0.2% NG310 (Biozol; ANA-NG310-CH210-50-ML), 1mM PMSF and cComplete EDTA-free protease inhibitor cocktail (Merck #11873580001). Cells were broken after the addition of 750mg glass beads (0.4–0.6 μ m) by rigorous vortexing with alternating breaks on ice. Unbroken cells and cell debris were pelleted by centrifugation at 1500g for 2 min. The membrane fraction was subsequently pelleted by centrifugation at 17000g for 15 min. Flag-Ub and Flag-UbN77 were immunoprecipitated from the resulting supernatant using EZview Red Anti-Flag M2 Affinity gel (Merck; F2426) by rotating at 4°C for 2h. The affinity gel was subsequently washed three times using TBS and the precipitated protein was eluted in Laemmli buffer.

In vitro protein expression and purification

UBP3, UBP14 and UBP15 were expressed from pOPIN-S vector with an N-terminal 6His-Smt3-tag. Ubiquitin and UbN77 were expressed from pET17b without a tag. All constructs were transformed into Escherichia coli (Strain: Rosetta (DE3) pLysS). Cultures of 6–12 l were grown in LB media at 37 °C until the OD₆₀₀ of 0.8 was reached. The cultures were cooled down to 18 °C and protein expression was induced by addition of 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). After 16 h, the cultures were harvested by centrifugation at 5000g for 15 min. After freeze thaw, the pellets were resuspended in binding buffer (300 mM NaCl, 20 mM TRIS pH 7, 20 mM imidazole, 2 mM β -mercaptoethanol) containing DNase and Lysozyme, and lysed by sonication. Lysates were clarified by centrifugation at 50000g for 1 h at 4 °C and in case of Ubp3C469A, Ubp3, Ubp14 and Ubp15, the supernatant was used for affinity purification on HisTrap FF columns (GE Healthcare) according to the manufacturer's instructions. Subsequently, Ubp3C469A, Ubp3, Ubp14 and Ubp15 were subjected to size exclusion chromatography (HiLoad 16/600 Superdex 200 pg) in 20 mM TRIS pH 7, 150 mM NaCl, 2 mM dithiothreitol (DTT). In case of Ubiquitin and UbN77, the lysate was acidified with perchloric acid (0,5% f.c.) and precipitated proteins were pelleted and clarified by centrifugation at 50000g for 30 min at 4 °C. The supernatant was dialyzed in 50 mM NaOAc pH 4.5 and subjected to cation exchange chromatography (HiTrap SP FF, GE Healthcare) by using a gradient from 0 to 500 mM NaCl. Subsequently ubiquitin and UbN77 were subjected to size exclusion chromatography (HiLoad 16/600 Superdex 75 pg) in 50 mM TRIS pH 7.5. All proteins were concentrated using VIVASPIN 20 Columns (Sartorius), flash frozen in liquid nitrogen, and stored at –80 °C until further use.

CxUb cleavage assay

DUBs were pre-incubated in 150 mM NaCl, 20 mM TRIS pH 7 and 10 mM DTT for 10 min. The cleavage was performed for the indicated time points with 12,5 μ M DUB and 2,5 mM UbN77 at room temperature. Afterwards the reaction was diluted to 0,1 mg/ml Ubiquitin in 0.1% Formic acid, 4mM NEM and analyzed by intact mass spectrometry.

Intact Mass Spectrometry

A Q Exactive Plus Orbitrap (Thermo Scientific) mass spectrometer that was coupled to an EASY nLC (Thermo Scientific) was used to analyze the reaction products of CxUb cleavage assay. Proteins were diluted in solvent A (0.1% formic acid in water) and loaded onto an in-house packed pulled tip column (18 cm length, 75 μ m I.D., filled with MABPac™ RP polymeric resin, 4 μ m, 1.500 Å, Thermo

Scientific). The column was operated at 50 °C and proteins were separated at a constant flow rate of 600 nL/min using the following gradient: 3–15% solvent B (0.1% formic acid in 80% acetonitrile) within 1.0 min, 15–60% solvent B within 15.0 min, 60–95% solvent B within 1.0 min, followed by washing and column equilibration. Full MS1 scans were acquired from 600 to 2000 m/z combining 2 microscans at a resolution of 140,000. The AGC target was set to 3E6 and the maximum injection time to 200 ms. Combined exact mass spectra of selected scans were exported from Excalibur 3.1 (Thermo Scientific). Annotation of monoisotopic masses and subsequent deconvolution of charge clusters was done in mMass 5.5⁸²

CxUb detection by Mass Spectrometry

Yeast *Δubi4Δyuh1* cells expressing an empty vector (–) and *Δyuh1* cells ectopically expressing UbN77 were grown in SG media to the exponential growth phase and heat-shock treated for 1 h at 39 °C. Cells were disrupted with glass beads (0.4–0.6 μm) in Urea buffer (8M in TBS). After centrifugation at 1500g for 2 min to remove unbroken cells, total cell extracts were stored at 80 °C until further analysis. The protein concentration was determined using the 660 nm Protein Assay (ThermoFisher Scientific, #22660). 20 μg of protein was utilized for Lys-C based digestion. Proteins were reduced (10 mM TCEP) and alkylated (20 mM CAA) in the dark for 45 min at 45 °C. Samples were subjected to SP3-based digestion as described elsewhere.⁸³ Washed SP3 beads (Sera-Mag(TM) Magnetic Carboxylate Modified Particles (Hydrophobic, GE44152105050250), Sera-Mag(TM) Magnetic Carboxylate Modified Particles (Hydrophilic, GE24152105050250) from Sigma Aldrich) were mixed equally, and 3 μL of bead slurry were added to each sample. Acetonitrile was added to a final concentration of 50% and washed twice using 70% ethanol (V=200 μL) on an in-house made magnet. After an additional acetonitrile wash (V=200 μL), 5 μL digestion solution (10 mM HEPES pH = 8.5 containing 0.5 μg LysC (Wako)) was added to each sample and incubated overnight at 37 °C. Peptides were desalted on a magnet using 2 x 200 μL acetonitrile. Peptides were eluted in 10 μL 5% DMSO in LC-MS water (Sigma Aldrich) in an ultrasonic bath for 10 min. Formic acid and acetonitrile were added to a final concentration of 2.5% and 2%, respectively. Peptides were further de-salted using SDB-RP stage tips.⁸⁴ Eluates were stored at -20 °C until subjected to LC-MS/MS analysis. Peptides were separated on an in-house packed 40 cm column (2.7 μm ReproShell beads) using 0.1% formic acid and 80% acetonitrile, 0.1% formic acid. The temperature was controlled using a column oven (PRSO-V2-PF, Sonation) to 50 °C. A 60 min gradient was applied for peptide separation and the column was connected via a nano-spray electronization source to the mass spectrometer QExactive HF-x (Thermo Fisher Scientific). A binary buffer system (A: 0.1% formic acid and B: 0.1% formic acid in 80% acetonitrile) was applied as follows: Linear increase of buffer B from 4% to 27% within 69 min, followed by a linear increase to 45% within 5 min. The buffer B content was further ramped to 65% within 5 min and then to 95% within 6 min. 95% buffer B was kept for further 10 min to wash the column. Prior each sample, the column was washed using 5 μL buffer A and the sample was loaded using 8 μL buffer A. The mass spectrometer operated in a data-dependent mode and subjected the 22 most abundant peaks for MS2 fragmentation. MS1 spectra were acquired at a resolution 60,000 using a max. injection time of 20 ms. MS2 spectra were recorded at a resolution of 15,000 using a max injection time of 22 ms and an AGC target of 5e5. The isolation width was set to 1.4 Th. Dynamic exclusion was enabled for 20 s. Using default settings, the data were analyzed utilizing the MaxQuant (2.0.3) software and the implemented Andromeda search engine⁷¹ generating in-silico spectra of the reference proteome of *Saccharomyces cerevisiae* (strain ATCC 204508 / S288c) (Baker's yeast) including the UbN77 sequence. The intensity for the peptide ESTLHLVLRGGN was extracted from the peptides.txt output file. We added an inclusion list for this particular peptide after examination of elution and detection properties using a heavy labelled peptide (ESTLHLVLR*LR*GGN, whereby R*=Arg U-13C6; U-15N4; JPT Peptide Technologies).

Mitophagy measurements

The alkaline phosphatase (ALP) assay was used to quantify mitophagy in yeast strains expressing the mtALP reporter, as described previously.²⁶ Briefly, ALP activity was determined by measuring the absorption of p-nitrophenyl phosphate at 405 nm after 15 min, using a plate reader. Protein concentrations were determined by standard BCA assay. Specific activities were normalized to the activity of a control strain. All strains were deleted for the *PHO8* gene, which encodes an endogenous alkaline phosphatase. Additional deletion of the *PHO13* gene, together with *PHO8*, was not necessary as the signal-to-noise ratio was sufficiently high (Figure S2A), consistent with an earlier study.⁸⁵ In parallel, cells were harvested and cell lysates analyzed by SDS-PAGE and western blotting with an ALP antibody, to control for ALP processing as another read out of mitophagy. Alternatively, relative levels of mitophagy were monitored in yeast cells expressing the mitochondrial GFP protein mtDHFR-GFP. Total cell extracts were obtained from 3 OD₆₀₀ exponentially growing cells and extracted at alkaline pH,⁸⁰ followed by SDS-PAGE and immunoblotting. A GFP-specific antibody was used to measure the relative levels of free-GFP as a readout of mitophagy.

Chronological Lifespan Assessment

Chronological lifespan was determined as described before⁸⁶ with minor adaptations. The assays presented in Figures 2D and 2E were performed in YPD. Briefly, a single colony of the different yeast strains was picked and grown in YPD (1% yeast extract, 2% peptone, 2% glucose) medium at 30 °C with shaking at 180 rpm. After two days of initial inoculation, cells were in stationary phase, which was considered day 0. This culture was followed for 3 weeks, without changing the medium of the aging cultures. At time points day 0, 3, 10, 14 and 21 cell densities were determined. A fixed number of cells obtained by dilution was plated on YPD agar plates and incubated at 30 °C for 2–3 days to assess the relative number of colony forming units over time (% survival). The assays presented in Figure 6C were performed as described above, but with cells grown on SCD complete media, and plated after 0, 3, 7 and 10 days.

Microscopy analysis

Cells expressing mitochondrial GFP (mtDHFR-GFP) were grown to mid-log phase in respiration medium (SG-U) and were left untreated or treated with rapamycin (final concentration 1 μ M) for 24 h at 200 rpm at 30°C, followed by labeling of the vacuole membrane with the FM4-64 dye as described.⁸⁷ Briefly, 5 ml of cells grown in SG-U and treated or not with rapamycin were spun down at 1000xg for 2 min at room temperature. The cell pellet was washed once with 1 ml of fresh YPD before resuspending in 1 ml of YPD containing 8 μ M FM4-64 in a 2 ml microcentrifuge tube. The tubes were wrapped with aluminum foil and incubated for 30 min at 30°C. Cells were washed twice with YPD and incubated in 1 ml of YPD for 1 h at 30°C. Before being subjected to confocal fluorescence microscopy, cells were harvested at 3000 rpm for 5 min at room temperature, washed twice with 1 $\times$ PBS buffer, and resuspended in 50 μ L 1 $\times$ PBS buffer. Images were acquired using a Spinning Disk Confocal microscope (PerkinElmer, Waltham, MA, USA) 60 $\times$ /1.4 NA oil objective at 561nm (RFP) and 488nm (GFP) excitation filters with z-stacks of 20 slices with a step width of 0.25 μ m using VOLOCITY software, and analyzed in ImageJ (Fiji), being linear brightness and contrast applied equally. Maximum intensity projections were created from a z-stack and representative regions of interest (ROIs) were selected in the corresponding confocal images. Cells accumulating GFP inside the vacuole (FM 4-64, red) represent a readout of mitophagy under live conditions.

Cells co-expressing eRFP-Atg32 and mt-GFP were grown to mid-log phase in YPG medium. Briefly, 1 ml of cells were spun down at 1000 g for 1 min at room temperature. The pellet was gently resuspended in 200 μ L of medium and a drop of 2 μ L was then transferred onto a microscopy slide. Cells were analyzed by epifluorescence microscopy (Axio Imager.M2; Carl Zeiss MicroImaging, Inc.) with a 63x oil-immersion objective⁸⁰ using 568nm (RFP) and 488nm (GFP) excitation filters with z-stacks of 13 slices. All images, including DIC, were acquired with a camera (Axiocam 503 mono; Carl Zeiss MicroImaging, Inc.) and processed with Zen 2 (Carl Zeiss MicroImaging, Inc.). Maximum intensity projections were created with ImageJ (Fiji) from z-stacks and representative regions of interest (ROIs) were selected in the corresponding microscopic images.

Phylogenetic comparison of polyubiquitin genes

Polyubiquitin sequences were extracted by annotation search in UniProtKB (UniProt Knowledge Base, <https://www.uniprot.org/>). A Perl script (www.perl.org) was designed to record all amino acids at the end of the Polyubiquitin by cutting the domains at the conserved pattern [RLRGG] and to count frequencies of the extensions. Phylogeny was built from iTOL (interactive Tree Of Life, <https://itol.embl.de/>) to which the number of Ubiquitin domains and the corresponding sequence extensions were added. The data was manually curated in case of redundant or poor-quality sequence data.

Spot tests

For growth assays, serial 1:5 dilutions of exponentially growing cells using a starting OD₆₀₀ of 0.5 were spotted on YP or SC media containing glucose, glycerol or galactose. For stress resistance analysis, YP was supplemented with or without cycloheximide (CHX, 0.3 μ g/ml), ZnCl₂ (10 μ M), canavanine (500ng/ml, Can), low pH (2.4) and rapamycin (20 ng/ml). Heat stress resistance analysis was assessed in YP or SC media for the indicated days, at 30 °C, 37 °C, 39 °C and 42 °C.

Analysis of *C. elegans* lifespan

For lifespan assays, 100 age-synchronized L4 larvae per strain were transferred to fresh NGM agar plates. The lifespan experiment commenced on the first day after reaching adulthood, designated as Day 0. Animals were placed on new plates daily for the first seven days to separate them from F1 animals and to prevent starvation. After entering the post-reproductive phase, worms were transferred only when necessary. The survival of the worms was monitored on a daily basis by observing their pharyngeal pumping or avoidance behaviour in response to mechanical stimuli. All lifespan experiments were conducted in three technical replicates at a temperature of 20 °C. Statistical analysis was performed using Online Application for Survival Analysis 2 (OASIS2).⁸⁸

Mitophagy measurement in *C. elegans*

For induction of mitophagy, animals expressing TOMM20::Rosella in body-wall muscle cells³⁹ were either grown on NGM plates, seeded with UV-inactivated OP50, coated with CCCP (15 μ M in minimal volume DMSO) for 24 h, or seeded with the *E. coli* strain HT1115 expressing pL4440 empty vector or the corresponding vector expressing *frh-1* dsRNA⁴² for three generations. In order to paralyse the worms for imaging purposes, the animals were treated with 10 mM levamisole (Sigma-Aldrich). Animals were imaged by epifluorescence microscopy (Axio Imager.M2; Carl Zeiss MicroImaging, Inc.) using a 25x objective.⁸⁰ Images were acquired with a camera (Axiocam 503 mono; Carl Zeiss MicroImaging, Inc.) and processed with Zen 2 (Carl Zeiss MicroImaging, Inc.). Fluorescence ratio of Alexa Fluor 568 and Alexa Fluor 488 was measured for individual animals from three biological replicates.

Analysis of brood size and larval development

For brood size analysis, 10 age-synchronized animals at L4 larval stage were grown on NGM and shifted to new plates daily. Plates containing laid eggs were incubated for 48h at 20°C before progeny were counted. Shown is the average of three technical replicates. For larval development analysis, at least 50 age-synchronized animals at L1 larval stage were grown for 48 h at 20 °C on MGN. Larval stages were counted by eye in three biological replicates.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis

Quantification of protein levels was performed using Fiji/ ImageJ.⁷³ Protein levels were normalized to PonceauS intensity and to the appropriate controls as indicated. Quantification of ALP activity was performed with absorption spectrophotometry measurements, using a plate reader. Quantification of mitophagy in *C. elegans* was performed in Fiji, to determine the mean fluorescence per animal. The median lifespan, relative decrease of the median lifespan and the Log Rank P values were determined by using Online Application for Survival Analysis 2 (OASIS2).⁸⁸

Statistical analysis

Quantification was done in ImageJ and GraphPad Prism 9. Sample sizes are annotated with (n), where n represents the number of independent biological replicates, except in Figure S4D, where (n) represents three technical replicates. Statistical details of the experiments can be found in the figure legends, whereby SD stands for standard deviation and SEM stands for standard error of the mean. Statistical values were determined using either Student's t-test or ANOVA and are displayed as ns with $p > 0.05$, * with $p < 0.05$, ** with $p < 0.01$, *** with $p < 0.001$ and **** with $p < 0.0001$.