Distinct proteostasis circuits cooperate in nuclear and cytoplasmic protein quality control

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Protein misfolding is linked to a wide array of human disorders, including Alzheimer’s disease, Parkinson’s disease and type II diabetes1,2. Protective cellular protein quality control (PQC) mechanisms have evolved to selectively recognize misfolded proteins and limit their toxic effects3–9, thus contributing to the maintenance of the proteome (proteostasis). Here we examine how molecular chaperones and the ubiquitin–proteasome system cooperate to recognize and promote the clearance of soluble misfolded proteins. Using a panel of PQC substrates with distinct characteristics and localizations, we define distinct chaperone and ubiquitination circuitries that execute quality control in the cytoplasm and nucleus. In the cytoplasm, proteasomal degradation of misfolded proteins requires tagging with mixed lysine 48 (K48)- and lysine 11 (K11)-linked ubiquitin chains. A distinct combination of E3 ubiquitin ligases and specific chaperones is required to achieve each type of linkage-specific ubiquitination. In the nucleus, however, proteasomal degradation of misfolded proteins requires only K48-linked ubiquitin chains, and is thus independent of K11-specific ligases and chaperones. The distinct ubiquitin codes for nuclear and cytoplasmic PQC appear to be linked to the function of the ubiquilin protein Dsk2, which is specifically required to clear nuclear misfolded proteins. Our work defines the principles of cytoplasmic and nuclear PQC as distinct, involving combinatorial recognition by defined sets of cooperating chaperones and E3 ligases. A better understanding of how these organelle-specific PQC requirements implement proteome integrity has implications for our understanding of diseases linked to impaired protein clearance and proteostasis dysfunction.

Misfolded proteins, arising during biogenesis or through proteotoxic damage, are highly toxic; they accumulate in distinct regions (puncta) within cells6–9 and form aggregates that are associated with neurodegenerative diseases1. Misfolded proteins must be cleared: the process involves the cooperation of chaperones and components of the ubiquitin–proteasome system (UPS)3–5 (Fig. 1a), but is poorly understood.

To better understand the PQC of soluble proteins, we used a panel of substrates that reflects different types of misfolding, including two temperature-sensitive proteins (Ubc95 and luciferase8), a protein (the Von Hippel–Lindau tumour suppressor, VHL) that cannot fold without its oligomeric partners elongin B and C, and translocation-defective carboxypeptidase yyc (CPY+) that lacks its signal sequence (CPY+)9,10. We found that terminally misfolded VHL or CPY+ conjugated to green fluorescent protein (GFP) was cleared within 1 hour, with only around 10% of cells containing GFP-positive puncta (Fig. 1b, c). Blocking proteasomal degradation with the proteasome inhibitor bortezomib led to the accumulation of these proteins in GFP-positive puncta. Temperature-sensitive proteins, such as Ubc95–GFP, are diffuse when folded at 30 °C, but upon misfolding at 37 °C are also degraded through the proteasome or accumulate in puncta (Extended Data Fig. 1a)6,7. Of note, native wild-type Ubc9 is soluble at either temperature.

To identify the E3 ubiquitin (Ub) ligases involved in PQC, we expressed GFP–VHL in a library of 41 Saccharomyces cerevisiae single-deletion strains comprising most non-essential E3 ubiquitin ligases (Extended Data Fig. 1b), including E3s that have previously been implicated in PQC (Fig. 1d). No single deletion caused a notable increase in puncta formation compared with wild-type cells, suggesting that no single E3 is essential for PQC of this substrate.

Given that E3 ligases can have redundant functions11, we next deleted pairs of E3s previously implicated in PQC. The cytoplasmic E3 Ubr1 and the nuclear E3 San1 cooperate in the clearance of some cytoplasmic proteins12,13. The E3s Hrd1, which is anchored in the endoplasmic reticulum (ER) membrane, and Doa10, also localized to the ER and to the inner nuclear membrane13, trigger ER-associated protein degradation (ERAD)14. We found that both Δubr1Δsan1 and Δdoa10Δhrd1 strains abrogated the degradation of all PQC substrates to the same extent as proteasome inhibition (see, for example, the results of cycloheximide chase in Fig. 1e, f and Extended Data Fig. 1c). Notably, all PQC substrates accumulated in the same puncta in these strains (Fig. 1g and Extended Data Fig. 1d). Therefore, multiple misfolded proteins use the same E3 systems for proteasomal degradation, and are sequestered together in the same PQC compartments in the absence of these E3 systems.

To determine why the deletion of either E3 pair stabilized our PQC substrates similarly, we tested the effect of doubly deleting all possible combinations of these four E3 ligases—Ubr1, San1, Doa10 and Hrd1 (Fig. 1h and Extended Data Fig. 1e). Puncta formation and cycloheximide chase assays showed that only specific combinations of deletions abrogate clearance. Strong stabilization was observed in Δubr1Δsan1 and Δdoa10Δhrd1 strains. A moderate effect was found with Δubr1Δdoa10Δhrd1 and Δhrd1Δsan1. Strikingly, Δdoa10Δsan1 and Δhrd1Δubr1 had no effect on PQC, suggesting that these pairs of ligases—Doa10/San1 and Hrd1/Ubr1—provide parallel, optimal combinations for PQC clearance. This E3 circuit logic appeared to be general, as it operated for all substrates tested at 30 °C and 37 °C. Thus PQC clearance requires parallel pathways of specific pairs of E3 ligases, combining one of the soluble E3s (either Ubr1 or San1; blue in Fig. 1i) and one of the membrane-bound E3s (either Doa10 or Hrd1; red). Overexpressing an E3 in any of the double-deletion strains rescued clearance only in those strains deleted for that particular E3 (Extended Data Fig. 1f). Therefore, E3 function is not interchangeable, even at higher expression levels, and PQC requires specific E3 combinations. The functional cooperation between E3 ligases may involve a physical complex, as immunoprecipitation experiments show that San1 associates with Doa10 but not with Hrd1 (Extended Data Fig. 2). We were unable to co-immunoprecipitate Ubr1 with Hrd1 (or Doa10), so these ligases may bind transiently, or cooperate functionally through separate complexes.

To examine whether the PQC defects observed above were linked to impaired ubiquitination, we expressed a terminally misfolded Flag-tagged VHL mutant (L158F; ref. 10) in either wild-type cells or the panel of double E3 deletions. VHL was immunoprecipitated under harsh denaturing conditions, followed by anti-ubiquitin immunoblotting.
Consistent with our data implicating ER-resident E3 ligases in K11–Ub ubiquitination were not generally required for proteasomal clearance, given that there therefore unable to form specific linkages (Fig. 2d). Ub K48R is lethal,19,20,21 reacted with misfolded VHL in both immunofluorescence and immunoprecipitation experiments (Extended Data Fig. 5c–e). Notably, reactivity with the branched K1/K48 antibody required the presence of both K48- and K11-specific E3 ligases (Extended Data Fig. 5e). We conclude that a dual ubiquitin code involving both K11 and K48 is required for clearance of soluble PQC substrates. K11 ubiquitination is mediated by either of the membrane-bound E3s, Doa10 or Hrd1, and K48 ubiquitination is mediated by either of the soluble E3s, Ubr1 or San1, thus explaining the requirement for pairs of E3 ligases for PQC clearance. Given that deletion of one E3 pair did not abrogate the action of the other, addition of K11- or K48-linked chains does not require a particular sequential order. It is unclear how chaperone proteins—known to be key mediators of PQC,3,4—facilitate ubiquitination and clearance. Chaperones could simply maintain the solubility of misfolded proteins, or they could specifically direct them along an E3 clearance pathway (Fig. 3a). We used the ubiquitin-linkage-specific ELISA assay to identify whether chaperones implicated in PQC are required to tag misfolded VHL with either K11–Ub or K48–Ub (Fig. 3b). We found that cells lacking Ssa1 and Ssa2—the major cytosolic proteins of the heat-shock protein (Hsp)70 family required for PQC clearance7,10,20,21—were strongly impaired in ubiquitination with either linkage. By contrast, the
with Hsp70 in promoting Doa10/Hrd1-mediated K11 ubiquitination, whereas the Ydj1/Sse1/Sti1 chaperones cooperate with Hsp70 and Hsp90 for Ubr1/San1-mediated K48 ubiquitination. Requiring two distinct E3 ligases to communicate with distinct chaperone pathways may provide a checkpoint in the triage decision to refold or degrade. That cytoplasmic misfolded proteins are degraded via a nuclear E3 ligase—San1—was puzzling. In principle, misfolded cytoplasmic proteins might be actively imported into the nucleus for degradation11,12,20,21. Alternatively, they might passively diffuse through the nuclear pores, owing to their small size21. To directly investigate cytoplasmic-specific and nuclear-specific PQC degradation pathways, we spatially restricted two PQC substrates—Hsp90 and luciferase21—to the nucleus by using a nuclear localization signal (NLS), or to the cytoplasm by using a nuclear export signal (NES, Fig. 4a). Similar results were obtained for both substrates, corroborating the generality of our conclusions. Treatment with bortezomib showed that both nuclear and cytoplasmic variants are degraded by the UPS (Fig. 4b, c and Extended Data Fig. 6a). NES–GFP–VHL accumulated in cytoplasmic perinuclear puncta, whereas NLS–GFP–VHL accumulated in intranuclear puncta (Fig. 4b). Thus, misfolded proteins are either degraded or form inclusions in the cellular compartment where misfolding occurs.

Degradation of cytoplasmic NES–VHL or NES–luciferase21 required the K11 ubiquitin ligases Doa10 and Hrd1, but only the cytoplasmic K48 ligase Ubr1 (Fig. 4d and Extended Data Fig. 6b). Therefore, San1 is dispensable for UPS degradation of strictly cytoplasmic PQC substrates. Surprisingly, the nuclear NES–GFP–VHL or luciferase21 required only the nuclear K48 ligase, San1, for clearance.

Consistent with their E3 requirements, cytoplasmic-conjugated misfolded NES-labelled proteins were conjugated to both K11- and K48-linked chains at levels similar to those of their unmodified counterparts. However, nuclear-restricted misfolded NES-labelled proteins were ubiquitinated only with K48 chains, with the K11 signal reduced to baseline levels (Fig. 4e and Extended Data Fig. 6c). Notably, nuclear misfolded proteins tagged with K48–Ub were efficiently cleared by the proteasome (Fig. 4c, d and Extended Data Fig. 6a). Confirming the distinct role of K11 ubiquitination in nuclear versus cytoplasmic PQC, clearance of NES–tagged misfolded proteins was impaired in UbK11R

ribosome-associated Hsp70s Ssb1 and Ssb2 were dispensable for degradation10 and ubiquitination.10 Binding of Hsp70s to substrates relies on many J-domain proteins, themselves often chaperones that ferry substrates for Hsp70 binding1,3. We examined VHL ubiquitination in cells lacking two J-domain proteins, Ydj1 and Sis1, that have been implicated in PQC7,9,11,20,22. Strikingly, each J-domain protein reduced ubiquitination, but in a linkage-specific manner. We found that Ydj1 cells showed reduced K48 ubiquitination, albeit to a modest degree, probably because Ydj1 is partially redundant with other J-domain co-factors7. Depleting cells of the essential Sis1 left K48 ubiquitination unaffected but caused a dramatic loss of K11 ubiquitination. Of note, three other chaperones important for PQC—the Hsp70 nucleotide-exchange factor Sse1 (an Hsp110 chaperone), Hsp90, and Sti1/HOP (which bridges the interaction of Hsp70 with Hsp90)7,10,11,23—were all required for K48 ubiquitination but were dispensable for K11 ubiquitination. We conclude that specific chaperone pathways direct PQC substrates to distinct E3 pathways to promote mixed linkage ubiquitination (Fig. 3c). Sis1 cooperates
cytoplasmic PQC—mediated by distinct E3 ligases and chaperones—
aggregation—perhaps leading to relaxed ubiquitination requirements.
For example, the importance of the UPS in chromatin regulation
for the triage decision between (re-)folding and targeting for degrada-
tion. For instance, the interaction between nuclear and cytoplasmic
aggregates. Nuclear ubiquilin Dsk2 shuttles K48-linked sub-
K11 linkages, can act as a sorting factor, and is sequestered in protein
misfolding. It is also possible that K11-linked ubiquitin chains facilitate recogni-
tion by another cytoplasmic PQC factor. Intriguingly, Sis1, required for K11
linkages, can act as a sorting factor, and is sequestered in protein
aggregates. Nuclear ubiquilin Dsk2 shuttles K48-linked sub-
strates to nuclear proteasomes, whereas in the cytoplasm, mixed K11/
K48 linkages are cleaved by the proteasome. Cycloheximide chase and immunoblot-
were used to assess the stability of these proteins in WT cells treated with
(Bz) or without (Ctrl) 50 μM Bz. Immunoblots represent three biologically independent
experiments. d, Confining VHL to the nucleus or cytoplasm alters
PQC requirements. Confining misfolded proteins to the nucleus or cytoplasm
alter the flow of unfolded proteins 26 . Strikingly, cells lacking Dsk2 were
protected from proteasomal degradation. Cycloheximide chase performed as in
Fig. 4c, but in WT or Δdsk2 cells. The densitometric quantification in panel b
shows relative to t = 0 (mean ± s.e.m. from three biologically independent experiments). I.
Nuclear and cytoplasmic misfolded proteins have distinct clearance requirements. Cytoplasmic
misfolded proteins require tagging with both K11–Ub and K48–Ub by chaperones and E3 ligases for proteasomal degradation. In the nucleus,
tagging with K48–Ub is sufficient for recognition by Dsk2 and subsequent protein degradation.
I-nuclear and cytoplasmic protein degradation. In the nucleus,
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tagging with K48–Ub is sufficient for recognition by Dsk2 and subsequent proteasomal degradation. In the nucleus,
E3 ligases identified here are associated with a host of human diseases (Extended Table 2). Dissection of these circuits in normal and diseased states might provide mechanistic clues and open up therapeutic opportunities.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0678-x.

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METHODS

 Yeast media, plasmids and strains. Preparation of yeast media, growth, transformations and manipulations were performed according to standard protocols. All E3- and chaperone-deletion yeast strains were derived from the BY4742 wild-type strain. Single deletions were generated by homologous recombination using the NAT gene. The Sis1–DamP strain was also generated in this way. Double deletions were generated with both NAT and hygromycin. All strains were checked by poly- merase chain reaction (PCR) using at least two sets of primers (see Supplementary Table 1).

 All ubiquitin K-to-R mutant strains—expressing a single, galactose-inducible ubiquitin gene—were gifts from D. Finley (Harvard Univ, MA, USA)35. Yeast strains expressing GFP-tagged Dna10 or Hrd1 from their endogenous loci were derived from the Yeast-GFP Clone Collection (Thermo Fisher Scientific). The Add2 strain was obtained from the Saccharomyces Genome Deletion Project36. We acknowledge gifts of pGAL–CPY–GFP (R. Hampton, Univ. California San Diego, CA, USA), pADH–Flag–Ub1 and pGAL–San1–V5His6 (D. Wolf, Univ. Stuttgart, Germany), pFLUC–DM–YFP (E. U. Hartl, Martinsried, Germany), and Ub–M–GFP, Ub–R–GFP, UbCG27–GFP and GFP–CL1 (N. Dantuma, Karolinska Institute, Stockholm, Sweden)34. All other plasmids were constructed using the Gateway cloning technology as described37.

Galactose shut-off protein expression. Yeast strains transformed with plasmids encoding the galactose-inducible protein of interest were grown overnight in raffinose synthetic medium at 30 °C before dilution to an optical density at 600 nm (OD600) of between 0.05 and 0.1 in galactose synthetic medium. The cells were grown for 4–6 h (OD600 0.6–0.8) to induce expression of the galactose-inducible protein. Expression was shut off by switching the cells to glucose synthetic medium, and the fate of the label was monitored in the absence of galactose (negative control) or in the presence of 0.2% (w/v) galactose (positive control).

 Counting puncta-containing cells. Cells were grown as described for galactose shut-off protein expression. Following shut-off, cells were allowed to grow at 30 °C or 37 °C for 1 h in glucose synthetic medium. Note that for WT plus bortezomib conditions, 50 μM bortezomib (LC Laboratories) was dissolved in the glucose synthetic medium before addition. Cells were then fixed for 15 min in 4% paraformaldehyde before mounting on concanavalin-A-coated coverslips using Prolong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Fluorescence was visualized using a Zeiss LSM780 confocal microscope with a 63 oil-immersion lens. Image analysis was performed by ImageJ software (http://imagej.nih.gov/ij/).

 Immunofluorescence. Cells were grown and paraformaldehyde fixed as described above. Fixed cells were spheroplasted by incubating for 20–40 min at 30 °C with Zymolyase 100T (Zymo Research) in potassium phosphate buffer (0.1 M potassium phosphate pH 7.5, 1.2 M sorbitol) supplemented with 25 mM DTT and 5 mM EDTA. Spheroplasts were collected by centrifugation (10,000g, 5 min), resuspended in spheroplast buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 1 mM PMSF, and a complete cocktail of protease inhibitors) (Sigma), 50 mM 2-chloroacetamide, 10 mM β-mercaptoethanol and 5 μg/mL BSA. Antibodies used were against K48–Ub covalently linked to Alexa Fluor 680 (1/500, Jackson ImmunoResearch catalogue number 715-035-150), donkey anti-rabbit (1/5,000, Jackson ImmunoResearch catalogue number 711-035-152) or donkey anti-mouse (1/5,000, Jackson ImmunoResearch catalogue number 709-035-149). The HRP signal was detected with Pierce ECL Western blotting substrate (Thermo Fisher Scientific) and exposure to GenEx Mate Blue Ultra Film (BioExpress). Immunoblots are shown as representative of three independent experiments.

 Immunoprecipitation of E3 ligases. To test for co-immunoprecipitation of E3 ligases, we transfected pADH–Flag–Ubr1 or pGAL–San1–V5His6 plasmids into yeast strains from the yeast-GFP collection (expressing GFP-tagged Dna10 or Hrd1 from the endogenous loci). Each strain was grown overnight in raffinose synthetic medium at 30 °C before dilution to an OD600 of between 0.05 and 0.1 in galactose synthetic medium. The cells were grown for 24 h to induce expression of the galactose-inducible San1–V5His6, diluted back to OD600 = 0.1, and then grown for another 4–6 h (OD600 0.6–0.8). For consistency between experimental conditions, the same protocol was followed for cells expressing Flag–Ubr1 from the alcohol dehydrogenase (ADH) promoter, even though this does not require galactose for expression. Cells were pelleted and washed once with 15 mM sodium azide supplemented with 1× Roche CompleteTM EDTA-free protease-inhibitor tablet (Sigma) and 50 mM 2-chloroacetamide, to inhibit proteases and deubiquitinases, respectively. Pellets were resuspended in an equal volume of lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethane sulfonyl fluoride (PMSF) Roche CompleteTM EDTA-free protease-inhibitor tablet (Sigma), 50 mM 2-chloroacetamide, 10 μM PR-619 (Sigma)) and frozen drop-wise in liquid nitrogen by passing through a 20.5-gauge syringe. Frozen samples were lysed by cryogrinding in a Retsch MM-301 (five cycles, 30 Hz, for 3 min per cycle) and proteins solubilized by adding Triton X-100 (1% w/v final concentration). Lysates were clarified (16,000g for 30 min at 4 °C) and quantified for total protein by bicinchoninic acid (BCA) assay. We incubated 2 mg of lysate with anti-GFP rabbit IgG conjugated to protein G dynabeads (Thermo Fisher Scientific) for 2 h at 4 °C to immunoprecipitate the GFP-tagged protein complexes, which were then eluted from the beads by heating for 30 min at 70 °C in non-reducing LDS sample buffer (Thermo Fisher Scientific). The bead-free samples were reduced with DTT (50 mM final concentration, 10 min at 70°C) before SDS–PAGE analysis.

 Immunoprecipitation of ubiquitinylated VHL. For immunoprecipitation of ubiquitinylated Flag–VHL, yeast strains were grown as described for galactose shut-off protein expression. Following shut-off in glucose synthetic medium supplemented with 30 μM Bz for 1 h at 30°C, cells were pelleted and snap frozen in liquid nitrogen. All subsequent steps were performed at 4°C or on ice. Pellets were resuspended in an equal volume of urea lysis buffer (50 mM Tris–HCl pH 7.5, 8 M urea, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1 mM phenylmethane sulfonyl fluoride (PMSF) Roche CompleteTM EDTA-free protease-inhibitor tablet (Sigma), 50 mM 2-chloroacetamide, 10 μM PR-619 (Sigma)) and lysed by bead beating (five cycles at 1 min each, with 1 min on ice in between cycles). Following dilution tenfold in Triton immunoprecipitation buffer (same composition as urea lysis buffer, but with 1% w/v Triton X-100 instead of 8 M urea), lysates were clarified (16,000g for 30 min at 4 °C) and quantified for total protein by BCA assay. We incubated 2 mg of lysates with Flag–M2 magnetic beads (Sigma) for 2 h at 4°C to immunoprecipitate the Flag-tagged protein, which was then eluted from the beads by heating for 30 min at 70°C in non-reducing LDS sample buffer (Thermo Fisher Scientific), to avoid cross-contamination. The bead-free samples were reduced with DTT (50 mM final concentration, 10 min at 70°C before SDS–PAGE analysis).

 The same protocol was used for denaturing immunoprecipitation of ubiquitinylated GFP–VHL, but with addition of 1% w/v SDS in lysis and immunoprecipitation

SDS–PAGE and immunoblotting. Protein samples from cell lysates or immunoprecipitates were denatured in SDS sample buffer (95°C for 5 min) or LDS sample buffer (70°C for 10 min) before separation by SDS–PAGE. Precision Plus prestained protein standards (Bio-Rad) were used to estimate protein weight. Proteins were transferred onto polyvinylidene fluoride (PVDF) or nitrocellulose membranes (Bio-Rad) and immunoblotted with primary antibodies against GFP (1/1,000, Roche catalogue number 1181446001) or Santa Cruz Biotechnology catalogue number sc-9906, peroxidase anti-peroxidase complex for detection of Protein A in the tandem affinity purification tag (TAP; 1/2,000 to 1/75,000; Sigma catalogue number P1291), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1/5,000, Abcam catalogue number ab9485; or 1/10,000, Genetex catalogue number GTX100118), α-tubulin (1/2,500; DSHB Hyridoma Product 12G10; deposited by J. Frankel and E.M. Nelson), Flag (1/1,000; Cell Signalling Technology catalogue number 2368), pan-ubiquitin (1/1,000; Life Sensors catalogue number VU101), K11–Ub (1/1,000; EMD Millipore catalogue number MAB5107-F), K48–Ub (1/1,000; Cell Signalling Technology catalogue number 12805) or K11/K48–Ub bispecific antibody (1/500; Genetech). Specific primary antibodies used are indicated next to the uncropped immunoblots in Supplementary Fig. 1. For immunoblotting of ubiquitin, samples were separated by SDS–PAGE, transferred to a PVDF membrane and denatured by boiling for 10 min at 95°C before antibody incubation. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated donkey anti-mouse (1/5,000; Jackson ImmunoResearch catalogue number 715-035-150), donkey anti-rabbit (1/5,000; Jackson ImmunoResearch catalogue number 711-035-152) or donkey anti-human (1/5,000; Jackson ImmunoResearch catalogue number 709-035-149). The HRP signal was detected with Pierce ECL Western blotting substrate (Thermo Fisher Scientific) and exposure to GenEx Mate Blue Ultra Film (BioExpress). Immunoblots are shown as representative of three independent experiments.
buffers, and incubation with GFP-TRAP_MA magnetic beads (ChromoTek) instead of Flag-M2 magnetic beads. For double-immunoprecipitation experiments, 10 mg of cell lysate was incubated with Flag-M2 magnetic beads for 2 h at 4 °C and eluted from the beads by competition with 3× Flag peptide (Apx Bio). The resultant eluate was subsequently incubated with an antibody against K48–Ub (1/500; Cell Signaling Technologies catalogue number 4289) or K11–Ub (1/500; EMD Millipore catalogue number MAB107–D) covalently conjugated using bis sulfosuccinimidyl) suberate (BS5) to protein G dynabeads (Thermo Fisher Scientific) overnight at 4 °C. The bead-bound fraction (‘eluate’) was eluted by heating for 10 min at 70 °C in non-reducing LDS sample buffer, and analysed alongside the unbound fraction (‘flow-through’) by SDS–PAGE.

Ubiquitin linkage ELISA. For quantification of K11–Ub or K48–Ub linkages on Flag–VHL, cell lysates were prepared as described for immunoprecipitation of ubiquitinated Flag–VHL. We added 200 μg of lysate protein to each well of an anti-Flag-M2-coated 96-well plate and then incubated the plate for 2 h. All incubation steps were performed at room temperature with gentle shaking. Four wells were used for each technical replicate (two replicates per strain per experiment). After washing four times with Triton immunoprecipitation buffer to remove unbound protein, each well was incubated for 1 h with rabbit antibodies against one of GFP (1/1,000; Cell Signaling Technology catalogue number 2956), Flag (1/1,000; Cell Signaling Technology catalogue number 2368), K11–Ub (1/50; EMD Millipore catalogue number MAB107–I), or K48–Ub (1/50; Cell Signaling Technology catalogue number 4289) diluted in 100 μl tris-buffered saline/Tween-20 (TBS-T) buffer with 0.1% BSA. After another four washes, each well was incubated for 1 h with HRP-conjugated donkey anti-rabbit antibody (1/2,000; Jackson ImmunoResearch catalogue number 711–035–152) diluted in 100 μl TBS-T with 0.1% BSA, washed another four times with Tween immunoprecipitation buffer, and incubated for 30 min with 100 μl Pierce tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific) followed by 100 μl 0.16 M sulfuric acid to stop the reaction. Absorbance was measured at 450 nm.

To calculate the K11–Ub or K48–Ub signal for each strain, we subtracted the raw absorbance readings of the negative control (GFP) signal, and then divided by the Flag signal to account for variations in total Flag–VHL. These K11–Ub or K48–Ub signals were then expressed as a proportion of the K11–Ub or K48–Ub signal in the WT strain to allow direct comparison between strains. Bars represent means ± s.e.m. from three individual experiments.

The same protocol was followed for quantification of linkages on GFP-tagged proteins, except with the use of GFP-multiTrap 96-well plates (ChromoTek) instead of Flag-M2-coated plates, and using the GFP and Flag signals as positive and negative controls, respectively.

**SILAC mass spectrometry of VHL immunoprecipitates.** WT yeast cells transformed with one of NLS–GFP–VHL, NES–GFP–VHL or Flag–VHL were grown overnight in raffinose-synthetic media supplemented with light Lys0 (Cambridge Isotope Laboratories catalogue number ULM-8766-PK), heavy Lys8 was shut off by switching the cells to glucose synthetic medium supplemented (OD 600 0.6–0.8) to induce expression of the galactose-inducible protein. Expression was enabled.

Raw data from four biological replicates were processed using MaxQuant37 (http://www.maxquant.org/doku.php?id=maxquant-start, version 1.6.2.3) and searched against the Saccharomyces Genome Database (https://download.yeast-genome.org/sequence/SC288C_reference/orf_protein; downloaded in January 2015) with common contaminant entries. The default MaxQuant parameters for a triple SILAC experiment were used, with the exception of ‘Re-quantify’, which was enabled.

The proteinGroups.txt file was filtered to exclude contaminants, reverse hits, hits ‘only identified by site’, and hits for which only one peptide was identified. The normalized SILAC ratios were used to generate median fold-change values per protein. Proteins with a log2(light/medium) or log2(heavy/medium) value of more than 0.5 were considered ‘enriched’ in NLS–VHL or NES–VHL interactomes, respectively. Enriched proteins from each interactome were subjected to pathway analysis to search for enriched Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and PFAM protein domains in either interactome using the STRING database36 (http://string-db.org, version 10.5).

**Data availability.** The data sets generated and/or analysed during this study are available from the corresponding author on reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomexchange.org) via the PRIDE partner repository with the data identifier PXD010660. Uncropped images of all immunoblots shown in this study are in Supplementary Fig. 1.
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Extended Data Fig. 1 | E3 ligases work in optimal combinations to clear misfolded proteins. a, Assay for puncta formation distinguishes between misfolded versus natively folded proteins. WT cells expressing natively folded Ubc9WT–GFP or temperature-sensitive Ubc9ts–GFP from a galactose-inducible promoter for 4–6 h at 30 °C were shifted to glucose-containing medium for 1 h at 30 °C or 37 °C to shut off expression. Cells were fixed and imaged by fluorescence microscopy. 300 cells were counted per condition, and the percentage of cells with GFP-positive puncta is shown (means ± s.e.m. from three biologically independent experiments). Only cells expressing Ubc9 ts–GFP showed a statistically significant change in the percentage of puncta-positive cells compared with WT (two-tailed Student’s t-test, ****P < 0.0001; ns, not significant).

b, Deletion of individual E3 ligases does not increase puncta formation. Experiment performed as in panel a, but using strains with endogenous deletions of the genes shown on the x-axis. E3 ligases that have previously been implicated in PQC (as shown in Fig. 1d) are grouped to the right (QC, quality control). Bars represent mean ± s.e.m. from three biologically independent experiments, with the exception of rad5, hel1, etp1, in20, hel2, apc11, hrt1, sbf3, cdc24, pep19, upf1, upf3, itt1 and rad18, where bars represent the mean from two biologically independent experiments, as well as WT, where bars represent the mean ± s.e.m. from seven biologically independent experiments. No strains showed statistically significant differences compared with WT by one-way ANOVA followed by Dunnett’s multiple comparisons test compared with WT are indicated with the adjusted P value or with **** for P < 0.0001. Overexpressing a single E3 ligase does not compensate for the loss of others. Ubr1, San1 or Hrd1 were overexpressed alongside GFP–VHL in the indicated strains. The rest of the experiment was performed as in a. Bars represent means ± s.e.m. from three biologically independent experiments.
Extended Data Fig. 2 | San1 forms a complex with Doa10 but not with Hrd1. a, b, San1–V5His6 co-immunoprecipitates with Doa10–GFP but not with Hrd1–GFP. Yeast cells co-expressing Doa10–GFP (a) or Hrd1–GFP (b) from their endogenous promoters with San1–V5His6 from a galactose-inducible promoter for 16 h were shifted to 37 °C for 1 h, and immediately lysed by cryo-grinding. Native complexes were immunoprecipitated with GFP-Trap-MA nanobodies before immunoblotting with the indicated antibodies. Immunoblots are representative of three biologically independent experiments. c, d, Flag–Ubr1 does not co-immunoprecipitate with Doa10–GFP or Hrd1–GFP. The experiment was performed as in panel a and b, but with cells expressing Flag–Ubr1 (from the constitutive ADH promoter) instead of San1–V5His6. Immunoblots are representative of three biologically independent experiments.
Extended Data Fig. 3 | K48–Ub and K11–Ub linkages are reduced in \( \Delta_{ubr1} \Delta_{san1} \) and \( \Delta_{doa10}\Delta_{hrd1} \) strains, respectively. a, Diagram showing the Ub-linkage ELISA used to quantify Ub linkages. Flag–VHL from a yeast lysate was immunoprecipitated in an anti-Flag-conjugated 96-well plate (using four wells per sample), and incubated with antibodies against GFP (negative control), Flag, K11–Ub, or K48–Ub. Following incubation with a secondary antibody (anti-rabbit-HRP), the strength of each signal was detected by electrochemiluminescence at 450 nm. To quantify the K11–Ub or K48–Ub linkages on Flag–VHL, we subtracted the anti-K11 or anti-K48 signal from the negative control (anti-GFP) and normalized to the total Flag–VHL signal for each sample. b, Ub-linkage ELISA confirms that K48–Ub and K11–Ub linkages are reduced on Flag–VHL in \( \Delta_{ubr1}\Delta_{san1} \) and \( \Delta_{doa10}\Delta_{hrd1} \) strains, respectively. WT or E3 double-deletion strains expressing Flag-VHL at 30 °C for 4–6 h were lysed after 1 h Bz treatment, also at 30 °C. Ub-linkage ELISA was then performed as described in a. Bars represent Flag-normalized values from each strain (mean ± s.e.m. from three biologically independent experiments), expressed as a proportion of the Flag-normalized WT values. Strains with statistically significant differences compared with WT by one-way ANOVA followed by Dunnett’s multiple comparisons test are indicated (****P < 0.001). c, GFP–VHL denaturing immunoprecipitation (1% SDS + 8 M urea) followed by immunoblot for K48–Ub or K11–Ub in WT or E3 double-deletion strains. Immunoblots are representative of three independent experiments. d, Relative amounts of K11–Ub and K48–Ub linkages present on GFP–VHL in \( \Delta_{ubr1}\Delta_{san1} \) or \( \Delta_{doa10}\Delta_{hrd1} \) strains compared with WT. WT or E3 double-deletion strains expressing GFP–VHL at 30 °C for 5–6 h were lysed in denaturing conditions (1% SDS + 8 M urea) after 1 h Bz treatment, also at 30 °C. Ub-linkage ELISA was then performed using GFP-multiTrap plates. Bars represent GFP-normalized values from each strain (means ± s.e.m. from three biologically independent experiments) expressed as a proportion of the GFP-normalized WT values. Strains for which statistically significant differences were observed by one-way ANOVA followed by Dunnett’s multiple comparisons test compared with WT are indicated with the adjusted P value, or with **** for P < 0.0001.
Extended Data Fig. 4 | K11–Ub linkages are not necessary for proteasomal degradation of all cytoplasmic substrates. a–d, WT or UbK11R cells expressing stable Ub-M-GFP (a), the N-end-rule substrate Ub-R-GFP (b), the ubiquitin fusion degradation (UFD) substrate UbG76V–GFP (c) or GFP fused to the artificial degron CL1 (d) from galactose-inducible promoters for 4–6 h at 30 °C were shifted to glucose-containing medium for 1 h at 30 °C or 37 °C to shut off expression. Cells were fixed and imaged by fluorescence microscopy. 300 cells were counted per condition, and the percentage of cells with GFP-positive puncta is shown (mean ± s.e.m. from three biologically independent experiments). There was a statistically significant increase in puncta compared with WT when GFP-CL1 (which contains a short amphipathic CL1 helix that could mimic a partially unfolded protein) was expressed in UbK11R cells, as judged by two-tailed Student’s t-test (P = 0.0127). The differences for all other substrates were not significant (ns, P > 0.05). DUB, deubiquitinating enzyme, which cleaves Ub from Ub-M-GFP or Ub-R-GFP.
Extended Data Fig. 5 | Misfolded VHL is modified with branched K11/K48 ubiquitin chains. a, b, Both K11–Ub and K48–Ub linkages are present on the same VHL molecule. a, This experiment was designed to determine whether both K48–Ub and K11–Ub linkages are present in the same VHL population. Sequential immunoprecipitation was carried out, first with anti-Flag antibody, then with an anti-K11–Ub or anti-K48–Ub antibody. The resulting negative control (‘no Flag’, with mock Flag plus K11 or K48 immunoprecipitation with lysate from cells expressing GFP–VHL instead of Flag–VHL), bead control (‘Control’, with no K11–Ub or K48–Ub antibody), ‘Bound’ and ‘Flow-through’, in addition to samples with just the first Flag immunoprecipitation (Input), were subjected to SDS–PAGE and immunoblotted for the presence of the other Ub linkage (b). Immunoblots representative of three biologically independent experiments are shown. The arrow indicates the size of un-ubiquitinated Flag–VHL. The asterisks indicate proteins in the stacking gel that did not enter the resolving gel. c, This bispecific anti-K11/K48–Ub antibody was designed to bind ubiquitin chains with K11 and K48 linkages branching off the same ubiquitin moiety. d, Misfolded VHL co-localizes with K11/K48–Ub chains. WT cells expressing GFP–VHL from a galactose-inducible promoter for 4–6 h at 30 °C were shifted to glucose-containing medium with 50 μM bortezomib for 1 h to shut off expression. Cells were fixed, spheroplasted and detergent permeabilized before immunostaining with an antibody designed to recognize ubiquitin that had K11 and K48 linkages emanating from the same moiety (K11/K48). Confocal fluorescence microscopy images are representative of at least 100 cells from each of three biologically independent experiments. Scale bars represent 2 μm. e, VHL is modified with branched K11/K48–Ub chains. GFP–VHL denaturing immunoprecipitation was followed by immunoblot for K11/K48–Ub or GFP (VHL) in WT or E3 double-deletion strains. Immunoblots representative of three biologically independent experiments are shown.
Extended Data Fig. 6 | Nuclear and cytoplasmic proteins require different PQC pathways for clearance. a, NLS–GFP–VHL and NES–GFP–VHL form a single punctum in the nucleus or cytoplasm, respectively, upon proteasome inhibition. WT cells expressing NLS–GFP–VHL or NES–GFP–VHL from a galactose-inducible promoter for 4–6 h at 30 °C were shifted to glucose-containing medium with 50 μM Bz for 1 h at 30 °C to shut off GFP–VHL expression. Fixed and spheroplasted cells were immunostained for the nuclear pore complex protein Nsp1 (red) before imaging by fluorescence microscopy. Representative cells from three biologically independent repeats are shown. b–d, Misfolded luciferase ts (Lucts) confined to the nucleus can be cleared by San1-mediated K48-linked ubiquitination. b, The increase in the percentage of cells containing puncta of NLS–GFP–Luc ts and NES–GFP–Lucts across the E3 single- and double-deletion strains is similar to the pattern observed with NLS–GFP–VHL and NES–GFP–VHL in Fig. 4. Shown is the percentage of cells (mean ± s.e.m. from three biologically independent experiments, each with n = 300) containing NLS–GFP–Luc ts or NES–GFP–Lucts puncta in WT, single- or double-deletion strains after 4–6 h expression of the protein at 30 °C followed by 1 h shut-off at 37 °C. Strains for which statistically significant differences were observed by one-way ANOVA followed by Dunnett’s multiple comparisons test compared with WT are indicated with the adjusted P value, or with **** for P < 0.0001. c, Misfolded nuclear Luc ts has severely reduced K11–Ub linkages (****P < 0.0001 by one-way ANOVA followed by Dunnett’s multiple comparisons test). Ubiquitin-linkage ELISA was performed on lysates of WT yeast expressing NLS-, NES- or unaltered GFP–Luc ts at 37 °C as described in Extended Data Fig. 2c, but in GFP-multitrapping 96-well plates instead of anti-Flag-conjugated 96-well plates. Anti-Flag was used instead of anti-GFP as the ELISA negative control. Bars represent means ± s.e.m. from three biologically independent experiments. d, Misfolded luciferase ts confined to the nucleus does not require K11–Ub linkages for clearance. The experiment was performed as in panel b, but with yeast strains expressing WT or mutant K11R–Ub as their sole source of ubiquitin. 300 cells were counted per condition, and the percentages of cells with GFP-positive puncta are shown in (means ± s.e.m. from three biologically independent experiments). Only NES–GFP–Luc ts had a statistically significant change in puncta-positive cells in the K11R strain when compared with WT (one-way ANOVA followed by Dunnett’s multiple comparisons test; ****P < 0.0001; ns = P > 0.05). e, VHL confined to the nucleus (NLS) or cytoplasm (NES) requires different chaperones for clearance. The experiment was performed as in panel b, but with the indicated chaperone-deletion strains. Bars represent means ± s.e.m. from three biologically independent experiments. Strains for which statistically significant differences were observed compared with WT by one-way ANOVA followed by Dunnett’s multiple comparisons test are indicated with the adjusted P value, or with **** for P < 0.0001.
Extended Data Fig. 7 | See next page for caption.
Mass spectrometry of the VHL interactome identifies distinct PQC circuitries for nuclear and cytoplasmic VHL.

a, Triple SILAC-base mass spectrometry of VHL immunoprecipitates. WT yeast cells transfected with one of NLS–GFP–VHL, NES–GFP–VHL or Flag–VHL were grown overnight at 30 °C in raffinose-synthetic media supplemented with light Lys0, heavy Lys8 or medium Lys4, respectively. Growth of VHL was induced in galactose for 4–5 h before shut off in glucose for 90 min. Next, 1.5 mg of protein from each of the three lysed samples were mixed before immunoprecipitation using GFP-TRAP_MA magnetic bead on-bead restriction digestion and peptide clean-up. Peptides were identified using liquid-chromatography/mass-spectrometry analysis before analysis using MaxQuant.

b, Strong correlation between the four biological repeats (R1–R4). Raw intensities for light (NLS–GFP–VHL; top), heavy (NES–GFP–VHL; middle) and medium (VHL–Flag control, bottom) were log10-transformed and plotted as scatterplot matrices. The Pearson correlation coefficient for each pairwise comparison is indicated, and the density distribution of intensities within each repeat is shown in the diagonal axis of the matrices.

c, Enriched PQC proteins in NLS–GFP–VHL and NES–GFP–VHL interactomes. Normalized median light/medium (NLS–GFP–VHL) and heavy/medium (NES–GFP–VHL) SILAC ratios were log2-transformed. Proteins with log2(SILAC ratio) of greater than 0.5 were considered as enriched, yielding 49 and 56 proteins for the NLS and NES interactomes, respectively. Enriched proteins known to play a role in PQC are shown. Both nuclear and cytoplasmic VHL share enrichments in proteasomal subunits, the Hsp70 chaperones Ssa1, Ssa2, Ssa4 and Ssb2, and the thioredoxins Trx1, Trx2 and Tsa1 (previously implicated in misfolded-protein management). All enriched proteins are shown in Extended Data Table 1.

d, Enriched PQC pathways in NLS–GFP–VHL and NES–GFP–VHL interactomes. The enriched proteins from each interactome (median values from four biologically independent experiments) were subjected to pathway analysis to search for enriched GO terms, KEGG pathways and PFAM protein domains in either interactome using the STRING database. Selected enriched PQC pathways are shown (P < 0.05 using Fisher’s exact test followed by Benjamini–Hochberg multiple testing correction).
## Extended Data Table 1 | Protein and pathways enriched in nuclear and cytoplasmic interactomes

### Enriched proteins [$\log_2\text{(NLS-VHL/Control)} > 0.5$]

| Fasta headers | log2(Median NLS/Control) |
|---------------|--------------------------|
| URA3          | 2.077                    |
| CCT5          | 1.550                    |
| SSA2          | 1.334                    |
| SSA4          | 1.239                    |
| MKT1          | 1.208                    |
| TIA1          | 1.203                    |
| TRX2          | 1.035                    |
| CCT8          | 0.952                    |
| ARO1          | 0.923                    |
| MDN1          | 0.902                    |
| RPT6          | 0.879                    |
| CCT7          | 0.873                    |
| TUB1          | 0.867                    |
| CCT3          | 0.841                    |
| GCD6          | 0.837                    |
| TRX1          | 0.808                    |
| BGL2          | 0.790                    |
| VMA2          | 0.775                    |
| AAIH1         | 0.773                    |
| NEW1          | 0.759                    |
| RPA135        | 0.745                    |
| SSA1          | 0.745                    |
| YNL134C       | 0.728                    |
| URA7          | 0.710                    |
| PRE10         | 0.699                    |
| EFT1;EFT2     | 0.688                    |
| URA2          | 0.684                    |
| RPT1          | 0.670                    |
| NUL1          | 0.657                    |
| HTS1          | 0.649                    |
| SSB2          | 0.642                    |
| SAM1          | 0.630                    |
| RPB2          | 0.615                    |
| KRE33         | 0.605                    |
| RPN2          | 0.578                    |
| FAS2          | 0.561                    |
| ADE6          | 0.552                    |
| MIS1          | 0.550                    |
| YEF3          | 0.545                    |
| CCT6          | 0.540                    |
| CPA2          | 0.524                    |
| CCT4          | 0.520                    |
| ADE3          | 0.516                    |
| RRP5          | 0.514                    |
| LEU1          | 0.510                    |
| CRM1          | 0.508                    |
| GCN1          | 0.507                    |
| HIS1          | 0.507                    |
| HIS4          | 0.504                    |

### Enriched proteins [$\log_2\text{(NES-VHL/Control)} > 0.5$]

| Fasta headers | log2(Median NES/Control) |
|---------------|--------------------------|
| YNL134C       | 3.229                    |
| DBP5          | 2.501                    |
| RK11          | 2.494                    |
| URA3          | 2.319                    |
| SSA4          | 2.166                    |
| SHM1          | 1.835                    |
| SEC14         | 1.727                    |
| TIA1          | 1.234                    |
| SSA2          | 1.234                    |
| IMD3:IMD2     | 1.213                    |
| SSA1          | 1.199                    |
| TRX2          | 1.054                    |
| GDH1          | 0.984                    |
| BGL2          | 0.964                    |
| TP1           | 0.880                    |
| RPN3          | 0.876                    |
| CCT5          | 0.820                    |
| CIT1          | 0.803                    |
| TIF4631       | 0.792                    |
| CPA2          | 0.776                    |
| RRP5          | 0.771                    |
| PRE5          | 0.731                    |
| IPP1          | 0.731                    |
| MET17         | 0.730                    |
| DYS1          | 0.702                    |
| SIS1          | 0.695                    |
| TRX1          | 0.675                    |
| MMF1          | 0.672                    |
| ENO2          | 0.672                    |
| PG1           | 0.670                    |
| RPT6          | 0.665                    |
| PRE9          | 0.658                    |
| HOM6          | 0.645                    |
| HKX2          | 0.628                    |
| ERG10         | 0.625                    |
| BMH1          | 0.625                    |
| UBA1          | 0.616                    |
| PGK1          | 0.612                    |
| HTS1          | 0.608                    |
| YPTS2         | 0.607                    |
| SSB2          | 0.607                    |
| URA2          | 0.598                    |
| RPN6          | 0.597                    |
| FPR4          | 0.592                    |
| TKL1          | 0.578                    |
| PRE6          | 0.571                    |
| RNR4          | 0.561                    |
| CDC48         | 0.558                    |
| SDH1          | 0.535                    |
| HSP82         | 0.526                    |
| TUB1          | 0.520                    |
| YPL225W       | 0.515                    |
| TAL1          | 0.508                    |
| LEU1          | 0.506                    |
| GPM1          | 0.503                    |

Enriched proteins in the NLS-GFP-VHL (left) and NES-GFP-VHL (right) interactomes. Normalized median light/medium (NLS-GFP-VHL) and heavy/medium (NES-GFP-VHL) SILAC ratios from four biologically independent experiments were log2-transformed. Proteins with log2(SILAC ratio) greater than 0.5 were considered as enriched, yielding 49 and 56 proteins for the NLS and NES interactomes, respectively.
Extended Data Table 2 | Human homologues of the ubiquitination machinery characterized here are associated with a range of diseases

| Yeast gene | Human orthologue | Associated Diseases | PQC in disease pathology? |
|------------|------------------|---------------------|--------------------------|
| Doa10      | MARCH6/TEB4      | Cri-du-chat syndrome (same chromosomal region) | -                        |
|            |                  | Lipidogenesis imbalance | -                        |
| Hrd1       | HRD1             | Alzheimer’s Disease  | 43 (Review)              |
|            |                  | Parkinson’s Disease  |                          |
| Gp78/AMFR  |                  | Cancer               | 44 (Review)              |
|            |                  | Cystic fibrosis      |                          |
|            |                  | ALS                  |                          |
|            |                  | Parkinson’s Disease  |                          |
|            |                  | Huntington’s Disease |                          |
|            |                  | Prion disorders      |                          |
| Ubr1       | UBR1             | Johnson-Blizzard Syndrome | 45                      |
| UBR4       |                  | Episodic Ataxia Type 8 | Unclear (yes for related Types 1 & 2) |
| UBR5       |                  | Adult Myoclonal Epilepsy | Unclear (yes for related Lafora Myoclonal Epilepsy) |
| UBR7       |                  | Autism Spectrum Disorder | 50-58                   |
| San1       |                  | No clear human orthologue | -                       |
| Dsk2       | UBQLN1           | Alzheimer’s Disease  | 57,58 (Reviews)          |
|            |                  | Huntington’s Disease |                          |
| UBQLN2     |                  | ALS                  | 61-65                    |
|            |                  | Frontotemporal Dementia |                        |
|            |                  | Huntington’s Disease |                          |

Literature-based evidence for disease pathology being directly related to PQC is indicated in the last column, where applicable. References cited are 39–45.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. \( F, t, r \)) with confidence intervals, effect sizes, degrees of freedom and \( P \) value noted
- Give \( P \) values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's \( d \), Pearson's \( r \)), indicating how they were calculated
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

All fluorescence microscopy images were collected using ZEN 2 Blue Edition v 1.0 en (Carl Zeiss Microscopy GmbH, Germany).

Data analysis

Fluorescence microscopy image analysis was performed on ImageJ software (http://rsbwe.d.nih.gov/ij). Statistical analysis was performed using the R statistics package or GraphPad Prism 7.0d for Mac OS X (GraphPad Software, La Jolla California USA).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data set generated and/or analysed during this study are available from the corresponding author on reasonable request. The mass spectrometry proteomics...
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Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
For experiments involving cell counting, the number of cells counted were representative of 3 full fluorescence micrographs (at 63x magnification) from different parts of the sample. No statistical methods were used to pre-determine sample size; however, we ensured we counted at least 300 cells--from different parts of the sample--to minimise the risk of observing differences due to local variations on the slide.

**Data exclusions**
No data was excluded from the analysis.

**Replication**
Experiments depicted in Fig. 1 and Extended Fig. 1a-b were performed independently (several years apart) by both R.S.S. and C.M.L. All experiments shown in this study are representative of at least 3 experiments performed separately (with the exception of 14 single E3 deletion strains in Fig. 1e (n = 2)). These are indicated with 2 data points (instead of 3) and no error bars on the bar chart in Extended Fig. 1b. For experiments involving microscopy, no data was excluded from the analysis (provided the positive and negative controls worked as expected). For experiments involving immunoblots, all cases where the antibody detected the protein of interest in the control (wild-type condition, and/or 0 hour time-point) were included for densitometry.

**Randomization**
Experiments involved comparing differences between yeast strains. Multiple colonies per strain were picked at random at the start of each experiment.

**Blinding**
For all puncta-counting assays, samples were randomized and blinded to ensure no bias was introduced during the counting step.

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a | Involved in the study |
| ☐ ☒ Unique biological materials | ☒ ChiP-seq |
| ☒ Antibodies | ☒ Flow cytometry |
| ☒ Eukaryotic cell lines | ☒ MRI-based neuroimaging |
| ☒ Palaeontology | |
| ☒ Animals and other organisms | |
| ☒ Human research participants | |

**Unique biological materials**

Policy information about availability of materials

Obtaining unique materials
All plasmids and strains generated for this study are freely available from the corresponding author.

**Antibodies**

Antibodies used
The K11/K48 anti-human antibody was obtained form Genentech (South San Francisco, California USA) (1:500 dilution). All other antibodies used are commercially available. For Immunoblotting, primary antibodies use were against GFP (1:1,000, Roche catalogue number 11814460001 or Santa Cruz Biotechnology catalogue number sc-9996), TAP (1:2,000-1:7,500, Sigma catalogue number P1291), GAPDH (1:5,000, Abcam catalogue number ab9485 or 1:10,000 Genetex catalogue number GTX100118), alpha-tubulin (1:2,500, DSHB Hybridoma Product 12G10, deposited by J. Frankel & E.M. Nelson), FLAG (1:1,000, Cell Signaling Technology catalogue number #2368), pan-Ubiquitin (1:1,000, Life Sensors catalogue number VU101), K11-Ub (1:100, EMD Millipore catalogue number MAB5107-I), and K48-Ub (1:1,000, Cell Signaling Technology catalogue number #12805). Primary antibodies used for immunofluorescence were K48-Ub-Alexa568 (1:100, Abcam catalogue number ab208136),
K11-Ub (1:50, EMD Millipore catalogue number MABS107-I) covalently linked to Alexa FluorTM 647 NHS Ester (Thermo Fisher Scientific), and Nsp1 (1:500, EnCor catalogue number MCA-32D6). Secondary antibodies used were HRP-conjugated donkey anti-mouse (1:5,000, Jackson ImmunoResearch catalogue number 715-035-150), donkey anti-rabbit (1:5,000, Jackson ImmunoResearch catalogue number 711-035-152), Cy5 donkey anti-human (1:1,000, Jackson ImmunoResearch catalogue number 709-175-149), and Alexa 568 donkey anti-mouse (1:1,000, Thermo Fisher Scientific catalogue number A10037).

**Validation**

The K11/K48 anti-human antibody has been validated by Genentech (published in Yau et al., Cell 2017). All other antibodies are commercially available and validated by the manufacturer.