Supplementary figure legends

Figure S1 Heat-shock (HS) induces protein misfolding and poly-ubiquitylation. (**a**) BY4741 cells were subjected to heat-shock (45°C, HS), 1 mM H_2O_2, 5 mM paraquat, 10 mM $CoCl_2$ and 2 M NaCl for 30 min or no stress. Experimental triplicates were analyzed by dot blot assay with anti-ubiquitin and anti-Pgk1 antibodies (left). The whole spotted signals in the dot blot were quantified (right). Ubiquitylation signals were normalized to Pgk1 levels and averaged (with standard deviations). (**b**) Schematic diagram of two possible mechanisms to account for the increase of poly-ubiquitylation following heat shock stress: increased ubiquitylation is due to misfolding (1), or accumulation of ubiquitylated proteins is due to proteasome inhibition (2). (**c**) MG132 experimental procedure to examine whether HS only causes dysfunction of proteasome (left). BY4741 cells were exposed to HS (15 min at 45°C) or no HS with or without the presence of 20 μM MG132. Experimental triplicates were analyzed by dot blots with anti-ubiquitin and anti-Pgk1 antibodies (middle). Ubiquitylation signals were normalized to Pgk1 levels and averaged (with standard deviations). One group of samples was also subjected to analysis by Western blot (right). The region above 60 kDa in the Western blot was quantified, and the relative levels, normalized to Pgk1, are indicated above (grey). There is no major difference in the two quantification approaches indicating that the dot-blot is adequate to quantify the heat shock ubiquitylation response. If proteasome inhibition was the sole cause of the increased ubiquitylation, there should not be any difference between samples 2 and 3. In this experiment, HS induced a higher increase of ubiquitylation in MG132-treated cells (compare sample 3 with samples 2 and 6). In this case, the increase of ubiquitylation is due to a combination of misfolding due to the stress and absence of proteasome activity (due to MG132). Regardless of the exact contribution of each phenomenon, the increased ubiquitylation after heat-shock cannot be solely accounted for by the inhibition of the proteasome. Hence, an increase in misfolding (and not proteasome inhibition) plays a major role in inducing poly-ubiquitylation after heat-shock.

Figure S2 Identification of HS affected proteins by quantitative mass-spectrometry analysis. (**a and c**) Two experiments were conducted to validate the purification method

for identifying ubiquitylated proteins using His_8-ubiquitin (H_8-Ubi) and metabolic labeling. The percentage of proteins above the corresponding $\log_2$ values of the $^{14}N/^{15}N$ ratios in each experiment is shown. Analysis of proteins in the total cell lysate (grey; 481 proteins in a, 479 proteins in c) and of IMAC enriched ubiquitylated proteins (green; 253 proteins in a, 259 proteins in c) in each experiment are shown. Note, that the few $\log_2(^{14}N/^{15}N)$ values greater than five were converted to the fixed value of five, as large ratio differences are often inaccurate due to the background signal noise, as well as to accommodate near "infinite" ratio values. In one experiment (a), unstressed H_8-Ubi expressing cells (YTM434) were differentially labeled. IMAC purified ubiquitylated proteins were expected to be equally enriched in both cell populations to confirm that no bias was introduced due to the labeling or the IMAC. Indeed, most ratios were close to one (0 in $\log_2$), with the exception of a small tail (corresponding to false positives that are also present in the total cell lysate analysis). In the other experiment (c), we compared cells expressing H_8-Ubi (YTM434; ^{14}N labeled) to cells expressing untagged ubiquitin (YTM419; ^{15}N labeled). We expected that truly ubiquitylated proteins would be further enriched in ^{14}N labeled cells ($\log_2(\text{ratio}) \geq 0.5$). The few proteins that were not specifically enriched ($\log_2(\text{ratio}) \sim 0$) mainly consisted of proteins containing short histidine stretches. In this particular data set, less then 4% of proteins quantified in the total cell lysate were considered enriched using the cut off threshold ($\log_2(\text{ratio}) \geq 0.5$). We similarly reported for all the other IMAC analyses the portion of proteins quantified above the threshold in the corresponding total cell lysate sample (see estFPR in Table S1). Note that we specifically selected a lower ratio threshold value to account for the fixed ubiquitin concentration in the cell, which precludes high enrichment of all conjugated proteins during the global heat-shock stress. (**b**) Venn diagram representing all 387 proteins identified as more ubiquitylated ($\log_2(\text{ratio}) \geq 0.5$) in three independent heat-shock experiments (experiments I to III in Figure 2b). There were 155 proteins that were found enriched in at least two of three experiments, which were then further analyzed for their localization (Figure 2d). (**d**) Two MS datasets are plotted in a scatter diagram. Y axis is the ratio of proteins ($\log_2(^{14}N/^{15}N)$) identified in HS vs. noHS experiment with H_8-Ubi cells (same data as experiment I in Figure 2b). X axis is the ratio of proteins ($\log_2(^{14}N/^{15}N)$) in H_8-Ubi vs. untagged cells that were both subjected to HS

(20 min at 45°C; 342 proteins). A majority of proteins that were found ubiquitylated (X axis) were further ubiquitylated after HS (Y axis). Proteins that were not further ubiquitylated after HS, de-ubiquitylated after HS and unspecific are circled in green, blue and red, respectively. (**e**) Full MS scan spectra (MS1) of representative peptides from two HS affected proteins Rps7B (top) and Pin3 (bottom) in experiment I (Figure 2b) are shown. The quantification of peptides was done by comparing peaks in MS1 scans using MSQuant. $^{14}N/^{15}N$ ratios of these two peptides at the selected retention times are also indicated.

Figure S3 *HUL5* is required for the full ubiquitylation response and cell fitness after heat-shock. (**a**) Wild-type, *hul5*Δ and strains with single deletion of E3s that are known or suspected to be involved in protein quality control were subjected to HS (15 min at 45°C) or no HS. Biological triplicates were analyzed by dot blots with anti-ubiquitin and anti-Pgk1 antibodies. Quantified increase of ubiquitylation levels of each strain (HS - noHS) is shown with standard deviations. Student's *t*-test was used to assess the significance of differences between each deletion and wild-type strain. While deletion of *SAN1* or *RKR1/LTN1* led to a decrease of the response, it was not significant. We also tested strains carrying a double deletion of *HUL5* and either *SAN1* or *RKR1/LTN1*, and found no significant differences (of heat-shock ubiquitylation response) compared to cells carrying the single *HUL5* deletion (data not shown), confirming that both *SAN1* and *RKR1/LTN1* are unlikely to be involved in this stress response pathway. (**b**) MYC pull-down experiments with cells expressing or not Ubc4^{TAP} at endogenous levels with or without a plasmid expressing ^{13Myc}Hul5 were analyzed by Western blot using 9E10 anti-MYC and anti-TAP antibodies. Inputs (1%) are shown below. Pull-down of ^{13Myc}Hul5 was performed using 9E10 antibody bound to protein-G agarose (Roche) similar to TAP pull downs. We were not able to co-immunoprecipate Ubc5^{TAP} with ^{13Myc}Hul5 presumably due to the low expression levels of Ubc5^{TAP}. All the pull-down experiments were also positive when using cells not treated by heat-shock (Figure S7; see noHS lanes). We also verified that ^{13Myc}Hul5 does not interact with the TAP tag, as it does not co-immunoprecipitate with Sik1^{TAP}, which is expressed at slightly higher levels than Ubc4 in our conditions (data not shown). (**c**) Time course HS experiment (45°C) was used to compare *hul5*Δ to

the BY4741 wild-type strain. The region above 45 kDa in the Western blot was quantified (left). Ubiquitylation signals were normalized to Pgk1 levels and the increase in ubiquitylation at each time point from both strains is shown (right). (**d**) Cell viability before and after HS was analyzed for wild-type, *hul5*Δ, *hul5*Δ + *hul5*-C878A (BPM310) and *hul5*Δ + *HUL5* (BPM309) strains. Cells were spotted in a 3 fold dilution series on YPD plates after or without HS (30 min at 45°C) and were incubated at 25°C for 3 days. (**e**) Average growth delays caused by heat-shock with standard deviations (n=3) are indicated using data derived from Figure 3c.

Figure S4 Heat-shock causes a re-localization of Hul5. (**a**) Representative images of $Hul5^{GFP}$ cells that were used for quantification in Figure 4b are shown for unstressed (25°C) and heat-shocked (30 min at 42°C) cells. Merge (left), $Hul5^{GFP}$ (middle) and $Nic96^{RFP}$ (right) panels are shown. Untagged wild-type cells (marked with asterisks) are shown. The scale bar is 5 µM. For quantification, single-stack images were taken with a 40X oil EC Plan-Neofluar objective, and 100 cells in each condition were quantified in two separate image fields. For each cell in focus (based on $Nic96^{RFP}$ signal), the averaged GFP signal intensity was measured in a 0.7 μm^2 area in the nucleus (defined by $Nic96^{RFP}$ signal) and in the cytosol. Average background signal was also measured from untagged cells (n=25/image), which were mixed and imaged with the tagged cells, then subtracted from the GFP signal intensities measured in tagged cells. (**b**) The average delay in growth of the indicated strains caused by heat-shock is indicated (with standard deviations, n=3) using data derived from Figure 4f. For comparison, the growth delay for *HUL5* and *hul5*Δ cells carrying an empty pRS316 plasmid is also shown.

Figure S5 *HUL5* is required for ubiquitylation of misfolded proteins under both heat-shock and physiological (no stress) conditions. (**a**) A schematic diagram of the workflow to identify, by quantitative mass spectrometry, Hul5 misfolded substrates. (**b**) ^{14}N and ^{15}N metabolic labeling was performed in wild-type and *hul5*Δ cells, respectively. IMAC was directly performed with the whole cell lysate (instead of the low solubility cell fraction) derived from unstressed cells. The graph represents the percentage of proteins above the corresponding $\log_2$ values of the $^{14}N/^{15}N$ ratios. Analysis of proteins in the total cell

lysate (grey; 457 proteins) and of IMAC enriched ubiquitylated proteins (green; 179 proteins) are shown. In contrast to Figure 6a, here deletion of *HUL5* does not abrogate the ubiquitylation of a significant fraction of the ubiquitin proteome. In unstressed cells, most ubiquitylated polypeptides are unlikely to correspond to low solubility proteins, as most conjugates remain soluble after centrifugation (Figure 1b). Therefore deletion of *HUL5* does not affect the overall ubiquitylation in the cell, but only perturbs the conjugation of a subpopulation that corresponds to low solubility proteins. (**c**) Venn diagram representing 155 proteins that are more ubiquitylated after heat-shock (Figure 2b; light green) and 95 proteins identified as Hul5 candidate substrates in unstressed cells in Figure 6a (dark green). (**d**) ^{14}N and ^{15}N metabolic labeling was performed for wild-type and *hul5*Δ cells, respectively. Cells were treated with heat-shock (20 min at 45°C) before equal mixing and lysis. IMAC was performed from low solubility cell fraction. The graph represents the percentage of proteins above the corresponding $\log_2$ values of the $^{14}N/^{15}N$ ratios. Analysis of proteins in the total cell lysate (grey; 374 proteins) and of IMAC enriched ubiquitylated proteins (green; 490 proteins) are shown. 99 proteins with ratios that are higher than the cut-off ($\log_2(^{14}N/^{15}N) \geq 0.5$) are considered candidate Hul5 substrates after heat-shock induced misfolding (dotted box). (**d**) Venn diagram representing 99 Hul5 substrate candidates that are misfolded after heat-shock in Figure S5c (light green) and 95 Hul5 substrate candidates that are enriched in the low solubility fraction in unstressed cells in Figure 6a (dark green). Five proteins were enriched in both approaches such as the prion-like protein Pin3, which may be susceptible to misfolding and therefore readily detected in both unstressed and heat-shock stressed cells.

Figure S6 Hul5 is required for the ubiquitylation of several low solubility proteins in unstressed cells. (**a, b, c**) Validation of the Hul5 candidate substrates Tsa2, Fbp26, and Slh1 using TAP-tagged strains expressing H_8-Ubi. IMAC was performed to pull down ubiquitylated proteins and anti-TAP antibody was used for Western blots; corresponding signal intensities for poly- and mono-ubiquitin were measured by subtracting the background signal in control cells (a and b). Heat-shock (45°C, 20 min) was also performed on $Slh1^{TAP}$ expressing cells prior to cell lysis (c). High contrast of the $Tsa2^{TAP}$ image is also presented to show the high molecular weight bands in cells with HUL5 (a,

right). The asterisks denote unspecific signals. (**d**) Solubility of ubiquitylated $Slh1^{TAP}$ was assessed by comparing both soluble and pellet fractions (after 16,000 g centrifugation) subjected to IMAC and analyzed by anti-TAP. (**e**) Turnover of $Slh1^{TAP}$ was assessed in exponentially growing cells maintained at 25°C or shifted to 38°C after the addition of 100 µg/ml cycloheximide. Samples were collected at the indicated times. Cells were lysed in 1x SDS-PAGE Laemmli sample buffer and $Slh1^{TAP}$ relative averaged signal intensities (with standard errors) were quantified after Western blotting and normalized to Pgk1 levels in three independent experiments. (**f**) Schematic representation of a possible model for the Hul5 quality control pathway.

Figure S7 The uncropped images shown in the figures. Area quantified in Figure 7 and S6 to estimate levels of mono- (green) and poly-ubiquitylated (blue) species are also shown.

Table S1 Lists of proteins quantified from the IMAC mass spectrometry analyses. The table provides the lists of quantified proteins and their verified $\log_2$ ($^{14}N/^{15}N$) ratios obtained by MSQuant for each IMAC mass spectrometry dataset presented in this study. Each dataset is in a separate sheet. The front sheet provides a summary of each experiment with additional information, including experimental description and figure index.

Table S2 Lists of proteins ubiquitylated after heat-shock and potential Hul5 substrates in unstressed cells. This table provides two lists and a front information sheet that contains a brief description. List A is a list of 155 proteins with $\log_2$ ($^{14}N/^{15}N$) ratio above threshold in 2 out of 3 biological replicates in Figure 2b, which are considered to be ubiquitylated after heat-shock. List B is a lists of 95 proteins with $\log_2$ ($^{14}N/^{15}N$) ratio above threshold in 1 out of 3 biological replicates presented in Figure 6a. These proteins are considered potential quality control substrates of Hul5. Proteins identified in at least two analyses are highlighted in light grey.

Table S3 Lists of strains and plasmids used in this study. This table includes an information sheet and three separate sheets with a list of 82 known or putative ubiquitin ligases assessed in the heat-shock ubiquitylation assay; all yeast strains used in this study (with genotype and figure index); and a list of plasmids used in this study (with short description and figure index).