Rsp5/Nedd4 is the main ubiquitin ligase that targets cytosolic misfolded proteins following heat stress

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The heat-shock response is a complex cellular program that induces major changes in protein translation, folding and degradation to alleviate toxicity caused by protein misfolding. Although heat shock has been widely used to study proteostasis, it remained unclear how misfolded proteins are targeted for proteolysis in these conditions. We found that Rsp5 and its mammalian homologue Nedd4 are important E3 ligases responsible for the increased ubiquitylation induced by heat stress. We determined that Rsp5 ubiquilates mainly cytosolic misfolded proteins upon heat shock for proteasome degradation. We found that ubiquitylation of heat-induced substrates requires the Hsp40 co-chaperone Ydj1 that is further associated with Rsp5 upon heat shock. In addition, ubiquitylation is also promoted by PY Rsp5-binding motifs found primarily in the structured regions of stress-induced substrates, which can act as heat-induced degrons. Our results support a bipartite recognition mechanism combining direct and chaperone-dependent ubiquitylation of misfolded cytosolic proteins by Rsp5.

In eukaryotic cells, protein quality control (PQC) either promotes folding or eliminates misfolded polypeptides that threaten cell integrity owing to their high propensity to aggregate. The importance of PQC is underscored by the numerous conformational pathologies associated with protein misfolding or aggregation such as Huntington’s disease. Several compartmentalized degradation PQC pathways have been identified in which E3 ubiquitin ligases selectively target misfolded proteins for degradation by the proteasome, often with the help of chaperones to mediate substrate recognition. These degradation pathways, especially in the cytosol, are often directly competing with other components of the folding machinery, and it is unclear how misfolded proteins are generally sorted by PQC.

The heat-shock (HS) response is a major system that protects the cell from perturbations caused by protein misfolding. Pioneering work showed that, in addition to upregulating HS proteins (Hsp), HS also causes higher ubiquitylation levels and increased proteasome degradation in eukaryotic cells. Although the Hul5 proteasome-associated ubiquitin ligase contributes to this response, the main PQC pathway has remained elusive. More importantly, it is unclear how misfolded proteins that are destined for proteolysis are recognized and triaged under stress conditions, as most chaperone proteins are presumably sequestered by the large bulk of misfolded polypeptides.

In this study, we found that the yeast Rsp5 and its mammalian homologue Nedd4 are important ubiquitin ligases responsible for the increased ubiquitylation on heat stress. We found that Rsp5 targets mainly cytosolic misfolded proteins for proteasome degradation upon HS. We also provide insight into the mechanism of how misfolded proteins are recognized.

RESULTS

The RSP5 E3 ligase is required for the HS-induced ubiquitylation response

We sought to identify the main ubiquitin ligase that mediates the ubiquitylation of cytosolic misfolded proteins on HS. In addition to its roles in endocytosis, transcription and unsaturated fatty acid and sterol synthesis, the yeast Rsp5 ubiquitin ligase targets misfolded plasma membrane proteins for lysosomal degradation and its overexpression increases the thermotolerance of Saccharomyces cerevisiae cells. We therefore investigated whether Rsp5 could also target cytosolic misfolded proteins upon HS. We first assessed the rps5-1 thermo-sensitive (ts) mutant allele, which has been widely used in the past, by comparing levels of total ubiquitin conjugates before and after HS (45°C, 15 min). We found that the HS-induced ubiquitylation response was markedly reduced in rps5-1 cells in comparison with wild-type.
Rsp5 is a major E3 that mediates the increased ubiquitylation upon HS. (a) Ubiquitylation levels in both WT and rsp5-1 cells before and after HS (open circle) and after a direct HS (black) analysed by western blots and quantified by dot blot (right; see also Supplementary Fig. 1a). Free mono-ubiquitin detected on the lower portion of the gel and Pgk1 loading control are also shown. (b) Normalized ubiquitylation levels quantified by dot blot in Tetp::RSP5 cells before and after HS (see also Supplementary Fig. 1b). (c) Normalized ubiquitylation levels before and after HS in WT and rsp5-1 cells containing the indicated plasmids (— denotes the presence of a control empty plasmid). Both RSP5 and rsp5-C777A were expressed under a Gal-promoter induced for 60 min with 2% galactose at 37°C. In a–c, a.u. denotes arbitrary units (each value is relative to the averaged value of the reference sample), n = 3 (average ± s.d.; source values are listed in Supplementary Table 5). (d) In vitro ubiquitylation performed with the indicated (black circles) recombinant proteins in WT and rsp5-1 cell extracts incubated at 25°C (open circle) or 42°C for 10 min (HS; black) and analysed by western blot. Mono-MYC–ubiquitin, Rsp5 (with relative quantified revels) and Pgk1 loading control are also shown. The asterisk denotes a nonspecific band. All uncropped images are in Supplementary Fig. 8.

We next sought to demonstrate that Rsp5 directly ubiquitylates proteins upon HS. One concern is that Rsp5 may indirectly affect ubiquitylation levels, as it regulates the nuclear export of Hsf1 and Msn2/4 messenger RNAs, two major transcription factors of the HS response.21,22 We therefore developed an in vitro HS type (WT) cells when analysed by western blot, by a quantitative dot blot assay (Fig. 1a and Supplementary Fig. 1a), and when assessed at various temperatures or time points (Supplementary Fig. 1b,c). We confirmed these data using two additional ts alleles of the essential RSP5 gene (rsp5-sm1 and rsp5-3), for which the HS-induced ubiquitylation response was also significantly decreased (Supplementary Fig. 1d).

To further verify that RSP5 is involved in the HS ubiquitylation response, we repeated our experiments by reducing RSP5 expression in vivo using a doxycycline-titratable promoter. As RSP5 is essential for its regulatory role in unsaturated fatty acid synthesis,14 we supplemented cells with the oleic acid precursor Tween-80 to maintain cell viability during the RSP5 downregulation. In these conditions, the increased ubiquitylation level upon HS was also largely impaired in the absence of Rsp5 (Fig. 1b and Supplementary Fig. 1e).

To determine whether the ubiquitin ligase activity of Rsp5 is important, we performed an add-back experiment with WT RSP5 and the catalytically inactive mutant rsp5-C777A. Whereas expression of RSP5 from a plasmid rescued the HS ubiquitylation response in rsp5-1 cells, expression of rsp5-C777A did not (Fig. 1c).

These results indicate that a functional RSP5 ubiquitin ligase is required for the ubiquitylation of proteins upon HS. As ubiquitin levels can be affected by the absence of Rsp5 in stressed cells,20 we also verified that levels of free mono-ubiquitin were not significantly altered in rsp5-1 cells in our conditions (Fig. 1a). In addition, overexpression of ubiquitin did not restore the increased ubiquitylation in rsp5-1 cells (Supplementary Fig. 1f), indicating that the observed impairment was unlikely due to reduced levels of free mono-ubiquitin.

Rsp5 directly ubiquitylates heat-induced misfolded proteins

We next sought to demonstrate that Rsp5 directly ubiquitylates proteins upon HS. One concern is that Rsp5 may indirectly affect ubiquitylation levels, as it regulates the nuclear export of Hsf1 and Msn2/4 messenger RNAs, two major transcription factors of the HS response.21,22 We therefore developed an in vitro HS...
ubiquitylation assay in cell extracts to monitor newly catalysed ubiquitylation events. In these conditions, we found that there was an RSP5-dependent increase in polyubiquitylation upon a short HS (10 min; Fig. 1d and Supplementary Fig. 1g). Importantly, the addition of WT recombinant Rsp5, but not the ligase-inactive mutant Rsp5-C777A, restored the increased polyubiquitylation upon HS in extracts derived from rsp5-1 cells (Fig. 1d). Rsp5 possesses three WW domains of approximately 35 amino acids each, which include two conserved tryptophan residues that bind predominately to substrates or substrate-adaptor proteins containing PY motifs. Addition of the triple-WW-domain mutant Rsp5-WW1,2,3 failed to complement the lack of RSP5 activity in rsp5-1 cell extracts (Fig. 1d). Our results indicate that Rsp5 ubiquitylates heat-induced misfolded proteins and that the recognition of these misfolded proteins is mediated by Rsp5-WW domains.

**Nedd4 is also required for the HS-induced ubiquitylation in mammalian cells**

To determine whether the role of Rsp5 in HS-mediated ubiquitylation is conserved in higher eukaryotes, we assessed its homologue Nedd4 in tissue culture cells. We first measured ubiquitylation levels in two HeLa cell lines that were each stably transfected with a different short hairpin RNA (shRNA) targeting NEDD4. We found that the increased ubiquitylation upon HS (45 °C, 30 min) was significantly reduced in these cells in comparison with the cells stably transfected with control shRNA (Fig. 2a), and that the amplitude of the impairment correlated with Nedd4 levels in those cells (Supplementary Fig. 2a), while levels of free mono-ubiquitin were not significantly altered in those same unstressed cells (Supplementary Fig. 2b). The same results were obtained when we assessed ubiquitylation levels after HS (45 °C, 15 min) in mouse embryonic fibroblast (MEF) cells derived from WT or nedd4−/− knockout mice (Fig. 2b). Importantly, no reduced cell viability that could compromise the response was observed shortly after HS (Supplementary Fig. 2c) and a similar increase in ubiquitylation levels was obtained when cells were incubated at a lower temperature (42 °C; Supplementary Fig. 2d). To verify that Nedd4 (and its ligase activity) is required for the HS ubiquitylation response, we performed add-back experiments in HeLa cells with NEDD4 stably knocked-down (targeting a non-coding region of the gene), choosing the clone with the lowest Nedd4 levels (clone B). We found that, following transient transfection, the expression of WT, but not the ligase-inactive Nedd4 (C867S), restored the increased ubiquitylation levels upon HS (45 °C, 30 min; Fig. 2c). These results show that the role of Rsp5/Nedd4 in mediating the increased ubiquitylation upon HS is conserved from yeast to mammalian cells.
Rsp5 ubiquitylates mainly heat-induced cytosolic misfolded proteins

To then identify which proteins are ubiquitylated by Rsp5 upon HS, we used a proteomic approach in which we combined triple-SILAC (stable isotope labelling with amino acids in cell culture) analysis with antibody-based enrichment of diGly peptides (that correspond to ubiquitylated peptides)\(^{24,25}\). In this experiment, we compared ubiquitylated proteins in WT cells that were subjected or not to a HS treatment (light and medium SILAC-labelled, respectively) and HS-treated \(\text{rsp5-1}\) cells (heavy-labelled) to unequivocally distinguish which proteins are ubiquitylated upon HS in an \(\text{RSP5}\)-dependent manner (Fig. 3a). We found that about 80% (148/182 sites; Supplementary Table 1) of the diGly sites that were further enriched upon HS were affected by the \(\text{rsp5}\) mutation (≥2-fold; Fig. 3b and Supplementary Fig. 3a). Analysis of the whole-cell lysate also confirmed that the observed decrease in ubiquitylation was not due to changes in corresponding proteins levels, except in one case (Supplementary Fig. 3b). These results confirm that Rsp5 plays a major role in the heat-induced ubiquitylation of proteins. To validate the proteomic analysis, we selected three Rsp5-candidate substrates: the Cdc19 pyruvate kinase, the Pdc1 pyruvate decarboxylase and the Sup45 translation release factor 1. We used an orthogonal approach, in which conjugated proteins were enriched from cells expressing the octo-histidine (H\(_8\))-tagged ubiquitin by immobilized-metal affinity chromatography (IMAC) for western blot analysis. We found that for all three substrates tested there was a marked increase in ubiquitylation upon HS in WT cells, which was readily reduced in \(\text{rsp5-1}\) cells (Fig. 3c). We also confirmed that these proteins were polyubiquitylated in an \(\text{RSP5}\)-dependent manner upon HS by immunoprecipitation followed by ubiquitin immunoblotting (Supplementary Fig. 3c; see also Fig. 4d,e). When assessing the proteomic data, we found that most of the 82 proteins ubiquitylated upon HS in an \(\text{RSP5}\)-dependent
Increased ubiquitylation
(Ubi 45 °C–Ubi 25 °C)
WT hul5Δ
Δ

Figure 4 Both Rsp5 and Hul5 mostly target their substrates independently. (a) Increased ubiquitylation levels quantified by dot blot after a 15 min
HS at 45 °C in the indicated cells. Data shows three biological replicates
(average ± s.d.; source values are listed in Supplementary Table 5) and a.u.
denotes arbitrary units. (b) Western blot analysis of the indicated cells with
a plasmid that expressed the indicated N-terminally MYC-tagged ubiquitin
constructs (Lys 48 and Lys 63 designate ubiquitin variants that contain only
Lys 48 and Lys 63, respectively, whereas all other lysines are mutated to
arginines) that were subjected or not to HS (45 °C, 15 min). (c,d) C-terminally
3xHA-tagged Cdc19 (c) and Pdc1 (d) expressed from their endogenous
promoters on a plasmid were immunoprecipitated (IP) from the indicated
cells (hul5Δ and rsp5-1 designated by green circles) expressing the indicated
N-terminally MYC-tagged ubiquitin constructs. Cells were subjected to HS
or not for 20 min at 45 °C. WT MYC-tagged ubiquitin (MYCUb) was also
expressed in the control cells used in lanes 1. All uncropped images are in
Supplementary Fig. 8.

manner were cytosolic (Fig. 3d). We confirmed this trend in a
second independent experiment (Supplementary Fig. 3a,d). Our
results indicate that Rsp5 is a major E3 ligase that ubiquitylates mainly
cytosolic misfolded proteins upon HS.

We found that, consistent with a role in targeting cytosolic proteins,
the addition of a nuclear localization signal to Rsp5 impaired the
increased ubiquitylation in cells upon HS (Supplementary Fig. 3e).
Furthermore, we found that the rapid HS ubiquitylation response
mediated by Rsp5 is distinct from the plasma membrane surveillance
system that relies on a network of arrestin-related tracking adaptor
(ART) proteins to mediate the ubiquitin-dependent endocytosis and
lysosome degradation of misfolded plasma membrane proteins15.
Deletion of all yeast ART proteins (art1-10Δ) does not inhibit the
increase in levels of ubiquitin conjugates upon HS (Supplementary
Fig. 3f). Furthermore, deletion of the amino-terminal C2 domain of
Rsp5, which is required for the function of Rsp5 in sorting cargo
into multivesicular body vesicles26, does not impair RSP5 function in
the HS ubiquitylation response (Supplementary Fig. 3g). These data
dicate that the role of Rsp5 in the increased ubiquitylation induced
by HS is a previously unknown function of this ubiquitin ligase distinct
from its role at the plasma membrane.

Rsp5 and Hul5 ubiquitin ligase mainly target their
heat-induced substrates independently

We previously found that the HECT ligase Hul5 also ubiquitylates a
fraction of cytosolic misfolded proteins11. It was proposed that Hul5
is an E4-elongating enzyme that further extends ubiquitin chains on
conjugated substrates27,28. One possibility is that the main role of Rsp5
is to prime misfolded proteins that would then be further processed by
Hul5. In this case, both ligases would mainly target the same pool of
substrates. However, we found that the defect of the HS response in the
double hul5 and rsp5 mutant (hul5Δ, rsp5-1) was additive compared
with the single mutants and, strikingly, caused a complete abolition
of the accumulation of ubiquitin conjugates upon HS (Fig. 4a and
Supplementary Fig. 4a). These results suggest that a subset of Hul5 substrates is not targeted by Rsp5. For instance, ubiquitylation of Lsm7, a previously identified substrate of Hul5 (ref. 11), was not impaired in rsp5-1 cells (Supplementary Fig. 4b). In addition, we found that the accumulation of Lys-48-linked chains upon HS was impaired in rsp5-1 but not in hul5Δ cells (Fig. 4b). In contrast, the HS-induced increase of Lys-63-linked chains was mostly impaired in hul5Δ but not in rsp5-1 cells (Fig. 4b). If Hul5 was mostly further conjugating substrates mono-ubiquitylated by Rsp5, then a greater decrease of Lys-48-linked chains should have also been observed in hul5Δ cells. To confirm these data, we assessed the ubiquitylation levels of the three Rsp5 substrates in hul5Δ cells. Whereas ubiquitylation of Pdc1 was also HUL5 dependent, the ubiquitylation of both Cdc19 and Sup45 was not affected by HUL5 deletion (Fig. 4c,d and Supplementary Fig. 4c). Importantly, although there was a loss of Lys-48-linked chains conjugated on Cdc19 and Pdc1 in rsp5-1 cells, deletion of HUL5 affected only the conjugation of Lys-63 chains on Pdc1 that was affected to a much lesser extent in rsp5-1 cells (Fig. 4c,d). These results indicate that Rsp5 and Hul5 are the two main ubiquitin ligases responsible for the accumulation of ubiquitin conjugates upon HS, and imply that both ubiquitin ligases most likely target their substrates independently of each other.

**Figure 5** Rsp5 is required for the degradation of cytosolic misfolded proteins. (a-d) Degradation of 35S-labelled proteins in WT (grey) and rsp5-1 (green) cells at 25 °C (dotted lines) or during HS (solid lines; 38 °C in a, c and d and 45 °C in b). The portion of proteins degraded after the chase was measured at the indicated times for short-lived (a,b) and long-lived (c) proteins. The portion of proteins that mainly correspond to cytosolic proteins degraded after the chase was measured at the indicated times in d. All data were collected in three independent experiments (average ± s.d.; source values are listed in Supplementary Table 5).

**Rsp5 is required for the degradation of cytosolic misfolded proteins**

We next sought to identify the role of Rsp5-mediated ubiquitylation of cytosolic misfolded proteins. Degradation of short-lived proteins by the proteasome is markedly augmented on increased misfolding due to a mild HS (ref. 10). We found that the increased degradation rate of short-lived proteins induced in these mild HS conditions was abolished in rsp5-1 cells (Fig. 5a). We confirmed that, in our experimental conditions, the increased degradation of 35S pulsed-labelled proteins during HS was proteasome-dependent in RSP5 cells and not affected by the deletion of two major lysosome proteases (Supplementary Fig. 5a,b). We also verified that RSP5 is required for the heat-induced degradation of short-lived proteins when shifting the cells to a higher temperature (45 °C; Fig. 5b). Cell viability was not affected by a 30 min HS at 45 °C in both WT and rsp5-1 cells, whereas longer incubations reduced viability (data not shown). We therefore performed subsequent turnover experiments at lower temperatures. In addition to short-lived proteins, we also observed that the increased degradation of long-lived proteins (90 min labelling) upon HS was abrogated in rsp5-1 cells (Fig. 5c). Consistent with a role in targeting long-lived proteins, the increased ubiquitylation upon HS in cells pre-treated...
with cycloheximide (which blocks translation, thereby allowing the depletion of short-lived proteins) was also affected in *rsp5-1* cells (Supplementary Fig. 5c). To further demonstrate a role for Rsp5 in targeting cytosolic misfolded proteins for degradation, we isolated a cytosolic fraction before quantification of radiolabelled proteins using a lysis buffer lacking detergent to deplete membrane-associated proteins (Supplementary Fig. 5d). In these conditions, the degradation of these mostly cytosolic pulse-labelled proteins was abrogated in *rsp5-1* cells (Fig. 5d). These results indicate that *Rsp5* is essential for the HS-induced proteasome degradation of cytosolic proteins.

**Association of the Hsp40 Ydj1 with Rsp5 mediates the ubiquitylation of Rsp5 substrates upon HS**

We next sought to determine how Rsp5 recognizes cytosolic misfolded proteins to target them for proteasome degradation. We first reasoned that a chaperone could mediate the recognition of a wide range of heat-induced misfolded proteins. Similarly to other Rsp5 substrate-adaptor proteins, several chaperone proteins contain PY or PY-like motifs including Ydj1, which contains a putative PY sequence (PIPKY) in its carboxy-terminal region. Ydj1 is a type 1 Hsp40 co-chaperone of the DnaJ family, which both stimulates Hsp70 ATPase activity and mediates selectivity of Hsp70 client proteins[30]. Interestingly, *YDJ1* was also shown to be required for the ubiquitylation and degradation of certain misfolded proteins in earlier studies[30,31]. However, the putative role of Ydj1 in the proteinolysis of cytosolic proteins remained unclear. We posit that Ydj1 may associate with Rsp5 to facilitate substrate recognition. Indeed, we found that there was a significant enrichment of proteins containing short sequences predicted to mediate binding with Ydj1 upon misfolding among HS-induced Rsp5 substrates (Supplementary Fig. 6a).

We first assessed the overall role of Ydj1 in the increase of ubiquitylation upon HS and found that the deletion of *YDJ1* impaired the ubiquitylation response (Fig. 6a and Supplementary Fig. 6b). Correspondingly, the absence of *YDJ1* also prevented the degradation of 35S-labelled short-lived proteins upon HS (Fig. 6b). We next determined whether Ydj1 and Rsp5 interact in HS conditions by performing co-immunoprecipitations in extracts derived from cells crosslinked before lysis (to preserve a possible heat-induced interaction during immunoprecipitation). Under these conditions, we observed an increased interaction between Rsp5 and Ydj1 upon HS (Fig. 6c). Importantly, the HS-induced Rsp5 interaction was mostly abrogated on the expression of WT *YDJ1* (Supplementary Fig. 6d). Accordingly, the increased ubiquitylation was restored on the expression of WT *YDJ1* but not after the expression of the *ydi1* (PP/GG) mutant (Fig. 6a and Supplementary Fig. 6b). We verified this result by assessing the ubiquitylation levels of Sup45, a heat-induced substrate of Rsp5. We found that the deletion of *YDJ1* led to the abolition of Sup45 ubiquitylation in HS conditions, which can be fully restored on the expression of WT *YDJ1* but not of the *ydi1* (PP/GG) mutant (Fig. 6e). Similarly, expression of the *ydi1* (PP/GG) mutant failed to restore degradation of short-lived proteins upon HS (Fig. 6b). Our results indicate that the association of Ydj1 with Rsp5 plays a major role in the HS-induced ubiquitylation and degradation of Rsp5 substrates.

**Heat-induced Rsp5 substrates contain PY motifs preferably embedded in structured regions**

We reasoned that, in addition to Ydj1-binding, substrates themselves could contain elements that directly promote their recognition by Rsp5. Indeed, we observed that the heat-induced ubiquitylation of Cdc19, another Rsp5 substrate upon HS, was not fully abrogated in *ydi1A* cells (Supplementary Fig. 6c); a similar observation was obtained for Pdc1 (see also Supplementary Fig. 7). These results suggest that, in some cases, a bipartite mechanism could mediate the recognition of misfolded proteins by Rsp5. Accordingly, we found a large portion of HS-induced substrates with PY or PY-like motifs (Fig. 7a and Supplementary Table 2). Note that not all Rsp5 candidate substrates contain obvious PY-like motifs (for example, Sup45, which was entirely ubiquitylated in a *YDJ1*-dependent manner in Fig. 6e). Interestingly, the Rsp5 canonical PPxY motif was more prevalent among candidate Rsp5 substrates identified using a protein array[32], whereas slight variations of the PxY motif such as SPxF and VPxF (referred to as PY-like) were significantly enriched among HS-induced candidate substrates (Supplementary Table 2). We also found that PY and PY-like motifs among HS-induced Rsp5 candidate substrates had a significantly lower frequency of locating in regions predicted to be disordered in comparison with other Rsp5 candidate substrates identified in the protein array (Fig. 7a). The higher prevalence of PY motifs in disordered regions among substrates identified in non-HS conditions is consistent with the required accessibility of the motif to mediate binding with one of the Rsp5-WW domains under normal conditions (that is, no HS). One possibility is that the HS-induced substrates are mainly recognized by Rsp5 upon misfolding, which exposes otherwise shielded motifs. In agreement with this idea, PY and PY-like motifs of Rsp5 candidate substrates induced by HS were predicted to be less accessible in comparison with the other candidate substrates, when considering motifs in structured regions (Supplementary Fig. 7a).

To assess the role of these PY and PY-like motifs in mediating substrate recognition by Rsp5 upon HS, we mutated the putative Rsp5-binding motif of Cdc19 (LPNY to LANA), which we previously identified as an Rsp5 substrate. This putative motif is located, on the basis of a crystal structure, close to the interaction interface between two monomers (Supplementary Fig. 7b) and is thereby not readily accessible for Rsp5 binding. We found that the ubiquitylation of the Cdc19 (PY/AA) mutant upon HS was reduced in comparison with the WT Cdc19 (Fig. 7b). When we repeated the same analysis in *ydi1Δ* cells, we found that the Cdc19 (PY/AA) mutant was no longer ubiquitylated upon HS (Fig. 7c). Similarly, mutation of either the LPTF motif (to LATA) or LPVF motif (to LAVA) of Pdc1 reduced ubiquitylation of this Rsp5 substrate in *ydi1Δ* cells (Supplementary Fig. 7c) but not in *ydi1* cells (Supplementary Fig. 7d). In addition, crosslinked Rsp5 was specifically co-immunoprecipitated with Cdc19 in *ydi1Δ* cells after HS, but it was not with Cdc19(PY/AA) (Fig. 7d). These results suggest that, in addition to Ydj1-mediated recognition, PY or PY-like motifs on substrates also promote HS-induced Rsp5 interaction and ubiquitylation. To further test this idea, we determined whether the addition of a PY motif would be sufficient to mediate the Rsp5-dependent ubiquitylation upon HS. Pyk2 is a Cdc19 parologue that arose from the whole-genome duplication. Despite good conservation between both proteins (71%
Figure 6 A Ydj1 adaptor mediates ubiquitylation of misfolded proteins upon HS. (a) Increased ubiquitylation levels quantified by dot blot after HS (45 °C, 15 min) in the indicated cells that carried a LEU2 plasmid that was empty (−) or with YDJ1 or ydj1 (PP/GG) expressed from the YDJ1 promoter. Three biological replicates were assessed (average ± s.d.; source values are listed in Supplementary Table 5). (b) Degradation of short-lived 35S-labelled (5 min) proteins in ydj1 cells that carried a plasmid expressing YDJ1 (grey) or the ydj1 (PP/GG) mutant (light green) from the YDJ1 promoter or an empty plasmid (dark green) at 25 °C (dotted lines) or 38 °C (solid lines). Three independent experiments were performed (average ± s.d.; source values are listed in Supplementary Table 5). (c) TAP immunoprecipitation (IP) was performed after in vivo crosslinking with 1% formaldehyde in cells grown at 25 °C (open circle) or during a HS (45 °C, 20 min; black). The endogenous YDJ1 was C-terminally TAP-tagged in the indicated lanes. The immunoprecipitated samples were analysed by western blot (the — TAP control was analysed on the same membrane but not adjacent to the other two lanes, black vertical line). (d) HA immunoprecipitation was performed after in vivo crosslinking with 1% formaldehyde in ydj1 cells subjected to a HS (45 °C, 20 min) and that carried a LEU2 plasmid that was empty or contained C-terminally tagged (3xHA) YDJ1 or ydj1 (PP/GG) expressed from the YDJ1 promoter. (e) IMAC from YDJ1 or ydj1 cells expressing when indicated H8–ubiquitin from a first URA3 plasmid, Sup45 fused N-terminally to GFP or GFP alone from a second HIS3 plasmid, and YDJ1 or ydj1 (PP/GG) under the YDJ1 promoter from a third LEU2 plasmid. All uncropped images are in Supplementary Fig. 8.
**Figure 7** PY motifs on substrates promote RSP5-dependent ubiquitylation upon HS. (a) The pie chart (left) indicates which portion of HS-induced Rsp5 candidate substrates identified in the two mass spectrometry experiments contains a proline-containing motif ([PLSV]Px[YL], or PPPP; dark green), additional PxY motifs (light green) or no obvious PY motif (grey). x denotes any residue. The histogram (right) indicates whether [PLSV]Px[YL] or PPPP motifs are located in regions predicted to be disordered or not among Rsp5 candidate substrates induced by HS or identified in a protein array (Array). Assigned PY motifs are listed in Supplementary Table 5. (b,c) IMAC analysed by western blots. Samples were derived from cells expressing H8–ubiquitin (from a URA3 plasmid) and the indicated WT or mutants Cdc19 (fused C-terminally with 13xMYC and expressed from a HIS3 plasmid). A 20 min HS at 45 °C was applied to RSP5 (black) or rsp5-1 (green) cells in b, and ydj1Δ cells in c. (d) MYC immunoprecipitation (IP) was performed after in vivo crosslinking with 1% formaldehyde (10 min) in cells grown at 25 °C (empty circle) or during a HS (40 °C, 20 min; black). The indicated Cdc19 (fused C-terminally with 13×MYC) was expressed (GDP promoter) in ydj1Δ cells. The immunoprecipitated and input samples were analysed by western blots as indicated. All uncropped images are in Supplementary Fig. 8. (e,f) IMAC analysed by western blots as in b. Py+ designates the A365P/L366N mutations in Pyk2. When indicated (black), cells were subjected to a 45 °C HS for 20 min in e and a 38 °C HS for 30 min in f. (g) Levels of Cdc19(D367R) C-terminally tagged with 13×MYC were determined by quantitative western blotting after incubating cells (WT in grey and rsp5-1 in green) in the presence of 100 μg ml⁻¹ cycloheximide at 25 °C (dotted lines) or 42 °C (solid lines) at the indicated times in three independent experiments (average ± s.e.m.; source values are listed in Supplementary Table 5; representative images are shown in Supplementary Fig. 7f).
Here, we discovered a conserved PQC degradation pathway in which Rsp5/Nedd4 are the main ubiquitin ligases that target misfolded proteins upon HS. Using both genetic and biochemical approaches, we show that Rsp5 ubiquitylates mainly cytosolic misfolded proteins upon HS to target them for proteasome degradation. We previously found that the Hul5 ubiquitin ligase, which has been proposed to be an E4 enzyme, also conjugates cytosolic misfolded protein upon HS (ref. 11). The absence of Hul5 impaired the accumulation of Lys-63-linked ubiquitin chains on HS, consistent with previous data.\(^3\) In contrast, mainly the accumulation of Lys-48-linked chains was affected in rsp5-1 mutant cells, indicating that each ligase conjugates its substrates independently. As Rsp5 preferentially catalyses Lys-63-linked ubiquitin chains, it will be important to determine whether the RSP5-dependent accumulation of Lys-48 chains on HS is directly mediated by Rsp5 or with the help of another ligase and/or deubiquitinase.

We propose that Ydj1 acts as a substrate-adaptor protein to promote the ubiquitylation of its misfolded client proteins either alone or in conjunction with exposed PY motifs on the substrate (Fig. 8). Buried PY motifs in proteins could signal misfolding by mediating Rsp5 recognition when exposed. Consistent with this misfolding-inducible degron model, PY motifs are widely distributed in the proteome and primarily found in structured regions (Supplementary Fig. 7i).

Interestingly, the Hsp40 co-chaperone Sis1, but not Ydj1, is sequestered in protein aggregates.\(^3\) Sis1 can cooperate with the Ubr1 ubiquitin ligase to target a misfolded model substrate for degradation,\(^36\) and Sis1 sequestration in protein aggregates inhibits the degradation of misfolded proteins.\(^35\) One possibility is that Ydj1 and Rsp5 participate in a major PQC pathway that is activated on major misfolding stresses when other PQC components such as Sis1 are sequestered by the abundant load of misfolded proteins.

Nedd4/Rsp5 were recently shown to potentially play a major role in α-synuclein proteopathies. The mammalian Nedd4 targets α-synuclein for degradation,\(^37\) and the NAB2 compound that reduces α-synuclein toxicity in mammalian and yeast cells targets Rsp5 (ref. 38). More recently, Rsp5 was also shown to be required for the degradation of aggregation-prone proteins by macro-autophagy.\(^39\) It will be important to determine the interplay between these pathways and whether a similar mechanism for the recognition of misfolded proteins upon HS is used in other instances.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

N.N.F designed most of the experiments through discussions with T.M. and additional inputs from J.G., R.I.D. and D.R.; N.N.F carried out most experiments; G.T.C. carried out the computational analyses with additional participation from J.G.; M.Z. and S.A.C. prepared several plasmids and strains; A.P. and N.N.F carried out the mammalian cells experiments together. D.R. provided reagents. Development of the conjugate assay was initiated in R.I.D’s laboratory. T.M. supervised the study. N.N.F. and T.M. wrote the paper and all other authors edited or commented on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Strains, plasmids and reagents. All Saccharomyces cerevisiae strains (S288C background) and plasmids are listed in Supplementary Tables 3 and 4, respectively. Temperature-sensitive (ts) mutant strains 8p5-1, 8p5-891, 8p5-3 and 8p5-20 were provided by C. Boone (University of Toronto, Canada) from the yeast to conditional mutants collection. The strain in which Rsp5 expression is controlled by the Tet promoter (Tetp::RSP5) was purchased from the Yeast Tet-Protomer Hughes Collection distributed by Open Biosystems (Thermo Scientific; ref. 41). All of the single-deletion strains were from the Yeast Knockout (YKO) Collection (Open Biosystems, Thermo Scientific) and provided by C. Loewen and M. Roberge (University of British Colombia, Canada). The art1-105 and art1-8,101 deletion strains in the BY4741 background were provided by H. Pelham (MRC Laboratory of Molecular Biology, UK). All of the double-mutant strains were generated by mating and tetrads dissection. All SILAC mass spectrometry experiments were done in BY4742 background cells. YDJ1–TAP was provided by E. Conibear (University of British Colombia, Canada) from the Yeast TAP-Tagged ORFs Collection (Open Biosystems, Thermo Scientific). CDC19–3HA, PDC1–3HA and SUP45–3HA were generated by inserting the 3xHA tag at the 3’ end of the endogenous gene by homologous recombination.

Plasmids expressing RSP5 (BPM587) and rps5-C777A (BPM588) were obtained by subcloning the GALp into pRS426 (URA3, 2μ) using EcoRI and Nal, and HA–RSP5 and HA–rps5-C777A that were provided by J. Hubrengste (University of Texas at Austin, USA) using Nal and NotI. The GST–RSP5 (BPM598) in pGEX-6P-1 was previously described44. GST–rps5-C777A (BPM514) was generated by subcloning p-rps5-C777A to replace a C-terminal fragment of RSP5 in BPM96 using NotI and AflI restriction sites. rps5-WW1,2,3* (in which all three WW domains are mutated)46 was provided by T. Zodalek (Polish Academy of Sciences, Poland) and subcloned with primers containing EcoRI and SalI restriction sites to replace RSP5 in BPM98 to generate GST–RSP5-WW1,2,3* (BPM518). The human Nedd4 plasmids in pcDNA3.1-N5-V5 (Invitrogen) were previously described45. The MYC-tagged (1 epitope) ubiquitin-expressing constructs were generating by PCR amplification of the WT or K0 ubiquitin that were subcloned after a GFP promoter and before the PGK1 terminator sequence in pRS313 (BPM590 for WT). Lys-48-only (BPM591) and Lys-63-only (BPM592) were then generated by site-directed mutagenesis. The YDJ1 plasmid (BPM390) was obtained from E. Craig (University of Wisconsin-Madison, USA), and was then modified by site-directed mutagenesis to mutate Pro 317 and Pro 319 to glycine residues to generate ydj1-PY/PG (BPM569). The megaepimer method used to generate HA-tagged YDJ1 and ydj1-PY/PG was adapted from a previously described method46. Briefly, the 3xHA sequence was PCR amplified using pFA6a-3xHA-His3MX6 to generate the megaepimer, which was then used to PCR amplify BPM390, BPM569 before DpnI digestion and bacteria transformation. The LSMT–TAP, His–ubiquitin plasmid (BPM297) was generated by PCR amplifying LSMT with its endogenous promoter (+750 base pairs) and C-terminal TAP sequence and inserted into pRS316 using NotI and His-ubiquitin-PGK–PGB (BPM30) using the NotI site. The GFP–SUP45 (BPM580) was obtained by PCR amplifying SUP45 from genomic DNA and inserted into pRS313–GFP–EGFP–PGK1 (BPM519). The CDC19–3HA (BPM635) and PDC1–3HA (BPM636) were PCR amplified and inserted into pRS314–C-terminal tagged genes (3xHA; YTM1308 and 1295) with their own promoters (400 base pairs) into pRS316. The CDC19–13MYC (BPM653), PYK2–13MYC (BPM603) and PDC113HA (BPM636) plasmids were used for endogenous reporter insertions. 8p5-1, 8p5-20 and 8p5-36 were subcloned into pRS424 (URA3, 2μ), using EcoRI and NalI.

In vitro HS ubiquitylation assay in yeast cell extracts. Recombinant Rsp5, Rsp5-C777A and Rsp5–ww1,2,3* were purified from BL21 (DE3) bacteria. After a three-hour induction with 1 mM IPTG at 25°C, cells were lysed in cold lysis buffer (1 μg/μl PMSF pH 7.3, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT), 0.5% Triton X-100 (TX-100)) by sonication. Purification procedures were performed according to the manufacturer’s protocol using glutathione Sepharose 4B resin (GE Healthcare). Rsp5, Rsp5-C777A or Rsp5–ww1,2,3* was cleaved from the GST tag with the PGK1 signal. When reporting the normalized ubiquitylation levels (for example, 25°C and 45°C), the signals were normalized to the averaged ubiquitylation level in the reference sample (typically WT at 25°C) that was set to the arbitrary value of 1 and then averaged. When reporting the increase in ubiquitylation, the difference of normalized signals between two temperatures for each sample (for example, 45°C–25°C) was calculated, averaged across the three replicates and normalized to the averaged difference in the reference sample (set to the arbitrary value of 1).

In vitro HS ubiquitylation assay in yeast cell extracts. Recombinant Rsp5, Rsp5-C777A and Rsp5–ww1,2,3* were purified from BL21 (DE3) bacteria. After a three-hour induction with 1 mM IPTG at 25°C, cells were lysed in cold lysis buffer (1 μg/μl PMSF pH 7.3, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT), 0.5% Triton X-100 (TX-100)) by sonication. Purification procedures were performed according to the manufacturer’s protocol using glutathione Sepharose 4B resin (GE Healthcare). Rsp5, Rsp5-C777A or Rsp5–ww1,2,3* was cleaved from the GST tag with the PGK1 signal. When reporting the normalized ubiquitylation levels (for example, 25°C and 45°C), the signals were normalized to the averaged ubiquitylation level in the reference sample (typically WT at 25°C) that was set to the arbitrary value of 1 and then averaged. When reporting the increase in ubiquitylation, the difference of normalized signals between two temperatures for each sample (for example, 45°C–25°C) was calculated, averaged across the three replicates and normalized to the averaged difference in the reference sample (set to the arbitrary value of 1).
further supplemented with L-glutamine. All cell media were purchased from Life Technologies. The generation of stably transfected HeLa cells, in which Nedd4 (also called Nedd4-1) was stably knocked-down, is described elsewhere. As a control an empty shRNA (pGIPZ, Open Biosystems) was used and two different sequences complementary to Nedd4 were designed. Generation of the mouse embryonic fibroblast cells was previously described, in which β-gal cDNA was inserted between exons 6 and 7, and embryonic (13.5–14.5 days) stages were immortalized by serial passages. Cells at approximately 90% confluency in 6-cm-diameter plates were replenished with fresh media (4 ml) 2–3 h before HS, and then were transferred to an incubator pre-warmed at 45 °C for the indicated times. For add-back experiments, the indicated DNA was transfected using PolyJet (SignaGen) according to the manufacturer’s instructions 24 h before HS. Mock transfection without DNA was performed for control cells. After HS, cells were washed twice with cold 1× PBS and lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSE, 1× Protease inhibitor cocktail by cell scraping. Protein concentrations were measured using a Bradford assay (BioRad) before mixing with SDS sample buffer (final 2% SDS). All samples were boiled at 96 °C for 15–20 min with constant vortexing to reduce the viscosity before western blot analysis. Ubiquitylation levels were normalized to α-tubulin levels. For the add-back experiments, ubiquitylation levels were first compared on each membrane between HS and unstressed cells for each replicate and then averaged between the three experiments. This method was preferred owing to variability of the ubiquitin signals from one membrane to another.

35S-labelling and protein turnover assays. Quantification of the degradation of S-labelling proteins was performed as previously described. Yeast cells were grown in SC medium with additional tryptophan (20 μg ml⁻¹) to a D₆₅₀ of 0.8–1, washed and incubated in SD-Met media with additional tryptophan (20μg ml⁻¹) for 50 min (ref. 51). EXPRES35S35S protein labelling mix (50 μl cm⁻², PerkinElmer) was used for 5 min or 90 min before washing cells with ice-cold SC- chase media containing cytochrome (0.5 mg ml⁻¹), methionine (6 mg ml⁻¹) and cycloheximide (0.5 mg ml⁻¹). Cells were collected at the indicated times to be then mixed to a final concentration of 10% TCA. After an overnight incubation at 4 °C, radioactivity in both TCA-soluble and -insoluble fractions was measured in a MicroBeta2 radiometric detector (PerkinElmer). The percentage of protein degradation was calculated by subtracting the signal from TCA-soluble at time 0 from the indicated time that was then divided by the signal in the TCA-insoluble fraction at time 0. To assess turnover of soluble proteins, an equivalent of 5 D₆₅₀ of radiolabelled cells were lysed in 100 μl native buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 1 mM PMSE, 10 mM chloroacacetamide, 1 mM phenanthroline, and 1× protease inhibitor cocktail) by glass beating. After a 15 min centrifugation at 16,000g in a microfuge, samples were normalized to the same concentration in 90 μl. One fraction of soluble proteins (70 μl) was precipitated by TCA, while the other fraction (20 μl) was analysed by SDS–PAGE. Radioactivity for each time point was measured in the TCA-soluble fraction and normalized to the amount of proteins detected by Coomassie on the protein gel. All experiments were performed in three independent replicates.

Microscopy. Cells were grown to a D₆₅₀ of 1 at 25 °C and subject or not to a 40 °C HS for 2 h. In the last 30 min of incubation, 2.5 μg ml⁻¹ of Hoechst 33342 (Invitrogen) was added to each culture for DNA staining. Live cells were mounted on a slide with 1× PBS and covered with a coverslip labeled with an inverted Zeiss Axio Observer microscope. One image plane per field (225 μm²) was acquired with a ×63 oil objective and processed with Zeiss Zen software.

DIGeFP peptide enrichment for triple SILAC mass spectrometry analysis. Enrichment of DIGeFP-containing peptides was performed using the PTMScan kit (Cell Signaling) with minor modifications from a previous description. Only lysine (K) labelling was used for the DIGeFP SILAC experiments. RPS25 cells labelled with light-lysine (K0) and rps-5:1 cells labelled with heavy-lysine (K8) were subjected to 20 min HS at 45 °C before lysis, while medium-labelled RPS25 cells (K4) were kept at 25 °C for control. Equal amounts of lysate from differentially labelled cells were mixed together to obtain ~30 mg of proteins in total. The PTMScan Ubiquitin Remnant Motif Kit (Cell Signaling Technology) was used to immunoprecipitate unmodified peptides according to the manufacturer’s instructions. Generation of the except beads was crosslinked before the immunoprecipitation and 1/8 of the recomended amount of beads was used per immunoprecipitation instead. Bound peptides were eluted with 50 or 0.15% (vol/vol) trifluoroacetic acid at 25 °C for 5 min before being cleaned up by C18 stage tips without fractionation. An aliquot of the whole-cell lysate (WCL) was also analysed after C18 stage tipping. For the first experiment, WCL peptides were also separated into 6 fractions by strong cationic exchange (SCX).

Mass spectrometry analysis. The instrument methods below are adapted from a previous study. Purified peptides were analysed using a LTQ-Orbitrap Velos (ThermoFisher Scientific) coupled to an Agilent 1290 Series HPLC using a nanospray ionization source (ThermoFisher Scientific). The LTQ-Orbitrap Velos was set with the following parameters: full-range scan from 350,000 resolution from 350 to 1600 thomson in the Orbitrap, fragmentation of top 5 ions by HCD in each cycle in the LTQ (minimum intensity 1,000 counts), exclusion of charge ±1 for 30 s. The Orbitrap was continuously recalibrated using the lock-mass function. Mass accuracy: error of mass measurement is typically within 5 ppm and is not allowed to exceed 10 ppm.

Centroided fragment peak lists were processed to the Mascot generic format using Proteome Discoverer (PD, 1.2). Fragment spectra were searched using the Mascot algorithm (2.3.0) against the Saccharomyces Genome Database (SGD-02Jan2012 with 6,147 protein sequences and 6,147 randomized sequences). The cuto FDR of GlyGly peptides was set below 1% (0.01). As in previous studies, we removed peptides with a C-terminal GlyGly and considered peptides with log, (ratios) ≥ 1 (refs 24,25). For the peptide quantification, missing values were replaced with default minimum ion intensity. For all data set, peptide ratios were normalized to the median protein ratio obtained from an aliquot of the input whole-cell lysate sample. The ratios of GlyGly peptides independently quantified several times were averaged.

MAC—purification of ubiquitylated proteins. In all experiments, about 1 μl of MAGeHa (Promega) was used per 200 μg of protein extract. For HA-tagged substrate validation, 150 ml of cells carrying the His₆-ubiquitin plasmid (BPM30; ref. 57) or the corresponding empty plasmid for control were grown in SD-URA media at 28 °C. Cells with or without 20 min HS treatment at 45 °C were washed twice with ice-cold 1× TBS and cell pellets snap frozen in liquid nitrogen. Thawed cells were lysed in HUBuffer (8 M urea, 100 mM HEPES at pH 8, 0.05% SDS, 10 mM chloroacetamide, 1 mM PMSE, 10 mM imidazole and protease inhibitor cocktail) by glass beads. Following 90 min incubation with cell extracts at ambient temperature, nickel beads were washed three times in HU buffer with 1% SDS. Bound proteins were eluted by incubating the beads in one volume of 8 M HU and one volume of 2 M imidazole for 10 min at ambient temperature with shaking. One volume of 3× SDS–PAGE Laemmli sample buffer was added and samples were heated at 70 °C for 10 min before western blot analysis.

In vivo crosslinking and co-immunoprecipitation experiments. Cells were grown at 25 °C to a D₆₅₀ of 1 and then heated shocked at 40 °C or 45 °C for 10 min before adding 1% formamide and incubating for a further 10 min. The crosslinking reaction was quenched with an excess of glycine (250 mM) for five minutes at 4 °C. The samples were centrifuged, washed twice with 1× TBS, and then frozen in liquid nitrogen. Cells were lysed with modified RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1 mM PMSE, 10 mM chloroacetamide, 1 mM phenanthroline, and 1× protease inhibitor cocktail) using glass beads. For the TAP-tagged protein immunoprecipitation, Tris-HCl was replaced by 50 mM HEPES at pH 8. For MYC-tagged proteins, 25 mM Tris-HCl pH 7.6, 1% NP40 was used instead of 50 mM and 1% Triton X-100, respectively. Lysates were then slowly diluted to 0.1% SDS final for immunoprecipitation. Cell pellets were also resolubilized in lysis buffer containing 0.1% SDS at 4 °C for 30 min and mixed with corresponding lysates, as some additional precaptions occurred in the presence of 0.5% SDS. Sample concentrations were measured by Bradford assay. The samples were incubated with an inverted Zeiss Axio Observer microscope. One image plane per field (225 μm²) was acquired with a ×63 oil objective and processed with Zeiss Zen software.

Localizations of Rsp5 candidates. Localization of Rsp5 candidates was assigned according to a previous system-wide localization analysis. Candidate Rsp5 substrates induced by HS (combining both proteomic experiments; 112 proteins) and high-confidence Rsp5 substrates identified in a protein array (41 proteins; ref. 32) were used to search for MYC motifs (PLSV[P][X][Y], and PPPP) or PXYS using an in-house python script. Enrichment of a given motif among Rsp5 candidates relative to its prevalence in the whole proteome was tested using Fisher’s exact test. We determined whether MYC motifs (PLSV[P][X][Y], and PPPP) are located in the disordered regions using Disopred2 (refs 59,60). Solvent accessibility of motifs was predicted using Sable for the four individual residues in the MYC motif and was then averaged over all possible single amino acid changes as GX[LMQ][P][X][CIMPW] (ref. 63). Fisher’s exact test was used to determine whether proteins containing at least one, two or three Ydi1 motifs were enriched in the HS data set compared with the genome. To identify which Cdc19 residue
mutation may enhance ubiquitylation, X-ray crystallography structure (1A3W; ref. 64) was repaired using the repairPDB function of FoldX. Using the BuildModel function of FoldX, residue Asp 367 was mutated to arginine. The interaction energies of the WT structure and of the mutant were measured using the AnalyseComplex function of FoldX. A similar approach was used to assess other residues located in the binding interface; the D367R mutation was selected as it is near the PY motif and was predicted to more strongly affect the homodimer interaction compared with other mutations (ranked fifth among 45 mutations assessed). We verified that the addition of a PY motif on Pyk2 and the mutation destabilizing the Cdc19 dimer were not predicted to destabilize the structure of Pyk2 and Cdc19, respectively, by using the Stability function of Foldx. For Pyk2, the Modbase-generated homology model9 was used.

**Data sets.** All raw proteomics data have been deposited to the ProteomeXchange Consortium through the PRIDE partner repository with the identifier PXD001214.

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**Supplementary Figure 1** Rsp5 is required for the increased ubiquitination induced by HS.  

**a.** Dot-blot images used for the quantification in Figure 1a. 
**b.** Normalized ubiquitination levels quantified by dot-blot in WT and rsp5-1 cells incubated at the indicated temperatures for 15 minutes. Increases in ubiquitination from 25°C to the HS conditions in rsp5-1 cells were each compared to WT with a two-tailed student t-test (**: p<0.01; ***: p<0.001). 
**c.** Ubiquitination levels in WT and rsp5-1 cells incubated at 45°C for the indicated times were assessed by western-blot. 
**d.** Normalized ubiquitination levels quantified by dot-blot in WT, rsp5-1, rsp5-sm1 and rsp5-3 cells under the indicated conditions. Increase in ubiquitination from 37°C to 45°C was compared to WT with a two-tailed student t-test (**: p<0.01; *: p<0.05). 
**e.** Ubiquitination levels of Tetp::Rsp5 cells treated with (OFF) or without (ON) 100µg/ml doxycycline before and after heat-shock were assessed by both dot-blot assay (bottom; quantified in Figure 1b) and western-blot (top). Experiments in a, b, d and e were done with three biological replicates and when shown the averaged values are shown with standard deviations. a.u.: denotes arbitrary units (each value is relative to the reference sample). The source values are listed in Table S4. 
**f.** Ubiquitination levels in WT and rsp5-1 cells with an empty plasmid (-) or a plasmid that expressed His8-tagged ubiquitin from the GPD1 promoter were assessed by western-blot. Prior to analysis cells were subjected or not to a HS (45°C for 15 min). 
**g.** In vitro ubiquitination assay performed with MYC-ubiquitin in WT and rsp5-1 cell extracts incubated at 25°C, 42°C or 45°C for 10min and analyzed by western blot using anti-MYC antibodies. Pgk1 loading control is also shown. Lower increased in poly-ubiquitination was observed at 45°C, presumably due to the observed increase in protein precipitation in these conditions (data not shown).
Supplementary Figure 2 Lower levels of Nedd4 affect the increased ubiquitination but not cell viability upon HS. a. Levels of Nedd4 were visualized by western blot after loading the same protein amounts derived from stably transfected HeLa cells with the indicated shRNA (- stands for scrambled control, #A and #B express two different hairpins that target Nedd4). b. The lysates in Figure 2a, were re-analyzed by western blot on a 15% SDS-PAGE in order to visualize free mono-ubiquitin. c. Viability of the indicated cells was assessed by mixing cells with 0.4% trypan blue stain and then counting with a Countess™ Automated Cell Counter (Invitrogen) after the indicated heat-stress in three independent HS assays. d. Nedd4 (+/+) or (-/-) MEFs were incubated at the indicated temperatures at the noted times and ubiquitination levels were analyzed by immunoblotting. Upon quantification, ubiquitination levels were higher at 45°C (data not shown), and hence that temperature was used for the subsequent experiments.
Supplementary Figure 3 Rsp5 ubiquitates mainly cytosolic proteins upon HS without the help of arrestin-containing proteins. a, Summary table of ubiquitination sites (GG peptides) and proteins in two independent SILAC experiments. Experiment 1 (Exp1) analyses are also shown in Figure 3b and 3d. b, Comparison of SILAC ratios for proteins identified in both the whole cell lysate (WCL) and after the GG peptide enrichment. 523 proteins with at least two unique peptides were identified and 446 proteins quantified in WCL (only lysine was SILAC-labelled in this experiment). Protein levels were quantified in WCL for 137 ubiquitinated peptides. The log_2 ratio values of light (WT, HS) versus medium (WT, no HS) SILAC-labeled proteins are compared in the top graph, and light versus heavy (rsp5-J, HS) in the lower graph. The ratio values of the ubiquitinated peptides are shown in the x-axis, while the ratio values of the corresponding proteins in the WCL are shown in the y-axis. There was one Rsp5 candidate substrate protein (Sec27) that was more abundant in WT cells in comparison to the observed decrease in ubiquitination in mutant cells may be attributed to lower protein levels. A few more Rsp5 candidate substrates were also slightly more abundant in WCL (New1, Pbp1, Gt1, Gln1 and Cdc48), but their GG ratios were lower. The changes in ubiquitination levels cannot be fully attributed to changes in protein levels and correspond to a decrease in ubiquitination levels. c, Localization (exp 2) of Rsp5 NLS or an empty plasmid CDC19 control lanes (Figure 4c, d). d, Localization of HS-induced Rsp5 candidate substrates identified in the second SILAC-diglycine mass spectrometry experiment shown in a. Distribution of proteins in each compartment is shown in percentage. e, Tet::RSP5 cells expressing N-terminal GFP tagged Rsp5 with or without a NLS or an empty HIS plasmid Lsm7 was also included. Ubiquitination of Cdc19 and Pdc1 was also assessed using a MYC tagged ubiquitin, for which there was lower background in control lanes (Figure 4c, d). e, Tet::RSP5 cells expressing N-terminal GFP tagged Rsp5 with or without a NLS or an empty HIS plasmid Lsm7 was also included. Ubiquitination of Cdc19 and Pdc1 was also assessed using a MYC tagged ubiquitin, for which there was lower background in control lanes (Figure 4c, d). f, Increased Ub. (Ubi 45°C - Ubi 25°C) signals were quantified in the molecular weight regions indicated by grey dotted lines. Signals were normalized to Pgk1 levels after background subtraction from the control lane. For comparison the HuI5 substrate Lsm7 was also included. Ubiquitination of Cdc19 and Pdc1 was also assessed using a MYC tagged ubiquitin, for which there was lower background in control lanes (Figure 4c, d). g, Ubiquitination Levels in WT, art1-10∆, and art1-10∆Δ cells from 25°C to 45°C were assessed by dot-blot. Ubiquitination levels were normalized to Pgk1 levels. g, Normalized ubiquitination levels before and after heat-shock in WT and rsp5-J cells containing the indicated plasmids (- denotes presence of a control empty plasmid) were quantified by dot-blot. Both RSP5 and rsp5-C23 were expressed under a Gal-promoter induced for 60min with 2% Galactose at 37°C. Experiments in e-g were done with three biological replicates and the averaged values are shown with standard deviations; a.u. denotes arbitrary units (each value is relative to the reference sample). The source values are listed in Supplementary Table S5.
Supplementary Figure 4 Rsp5 and Hul5 are mainly required for the increased ubiquitination of different proteins. a. Increased ubiquitination levels quantified by dot-blot after a 15min HS at 45°C in the indicated cells. Cells were pre-incubated at 37°C for 30 min prior to the HS. Experiments were done with three biological replicates and the averaged values are shown with standard deviations; a.u. denotes arbitrary units (each value is relative to the reference sample). The source values are listed in Table S4. b. IMAC of samples from WT, hul5Δ and rsp5-1 cells that expressed His8-ubiquitin and Lsm7TAP from the same plasmid after a HS at 45°C for 20 min were analyzed by western blot using anti-TAP antibodies. Relative levels of the poly-ubiquitin signal are indicated below. c. IMAC-enriched ubiquitin conjugates were analyzed by western blots. Samples were derived from indicated cells (rsp5-1 designated by green circles) expressing endogenously tagged candidates (3xHA) and H8-Ubiquitin. HS was applied to all samples in b and c.
Supplementary Figure 5 The heat-induced degradation of short-lived proteins requires functional proteasome. **a-b.** Degradation of 35S labeled proteins in WT (grey) and indicated mutant or deletion strains (green) cells at 25°C (dotted lines) or 38°C (solid lines). The portion of proteins degraded at the indicated times was measured for short-lived proteins (5 min labeling). The same values for WT samples are shown in a and b as all samples were analyzed together. Experiments were done in three independent experiments and averaged values are shown with standard deviations. The source values are listed in Supplementary Table 5. **c.** Increased ubiquitination levels from 25°C to 45°C (15 min) in WT and rsp5-1 cells treated with or without 100 μg/ml cycloheximide were analyzed by dot-blot assay. Three biological replicates were analyzed, averaged values are shown with standard deviation and source values are listed in Table S4. a.u. denotes arbitrary units (each value is relative to the reference sample). **d.** Protein localization of the cytoplasmic fraction sample obtained by native lysis and analyzed by mass spectrometry. 348 proteins were identified in this experiment. The same lysis method was used for samples analyzed in Figure 5d. C: Cytoplasm; N: Nuclear; M: Membrane; Mit: Mitochondria.
Supplementary Figure 6 The Ydj1 Hsp40 is required for the increased ubiquitination induced by HS.  

**a.** Histograms of proteins with at least 1, 2 or 3 Ydj1 binding motifs among HS-induced Rsp5 candidate substrates and in the proteome. The Ydj1 binding motif GX[LMQ][EP][CIMPVW] was previously identified, p-values calculated using a Fishers exact test are shown, and assigned motifs are listed in Table S4.  

**b.** Western blot analysis of one of three lysates quantified by dot-blot in Figure 5a. Ubiquitination levels were assessed after HS (45°C, 15min) in the indicated cells that carried a LEU2 plasmid that was empty or with YDJ1 or ydj1 (PP/GG) expressed from the YDJ1 promoter. Ubiquitination levels normalized to Pgk1 levels are indicated in grey and were quantified in the region indicated by the dotted line and.  

**c.** IMACs from samples derived from WT, ydj1Δ or rps5-1 cells expressing His8-ubiquitin from a first plasmid and CDC19 (with C-terminal 13xMYC) in a second plasmid. Cells were HS (45°C, 20 min) or not prior to lysis and IMACs were analyzed by western blot using anti-MYC antibodies. The relative level of poly-ubiquitination is shown for each lane. Inputs and Pgk1 are also shown.
Supplementary Figure 7  A destabilizing mutation near a PY-motif of Cdc19 induces proteasome degradation. a. The predicted percentage of relative solvent accessibility was plotted for the PY motifs within structured regions of Rsp5 candidate substrates that are HS-induced (grey) or identified in a protein array (green). Solvent accessibility of motifs was predicted using Sable (http://sable.cchmc.org/) by averaging relative accessibility for the four PY residues (source data are listed in Table S4). The median solvent accessibility was plotted for the PY motifs within structured regions that are located in regions predicted to be disordered (IDP) or not (presumably accessible) is marked with a dotted line for comparison. A box plot of the data is also shown above the plot. b. Surface contour (upper left) of the crystal structure of the Cdc19 homo-dimer (1A3W); chains are depicted in green and cyan, and D367 in orange. Enlargement (lower right) of the crystal structure of the Cdc19 homo-dimer (1A3W): chains are depicted in green and cyan, and D367 in orange. Enlargement (upper left) of the crystal structure of the Cdc19 homo-dimer interface near D367 is showed. Backbones are depicted in cyan and pale green, sidechains in bright green and blue, and D367 in red. The predicted change caused by the D367R mutation in the interaction energy of the homo-dimer is also indicated. c-d. IMACs from samples derived from ydj1Δ (c) or YDJ1 (d) cells expressing His₆-ubiquitin from a first plasmid and the indicated Pdc1 constructs (with C-terminal 13xMYC) in a second plasmid. Cells were HS (45°C, 20 min) prior to lysis and IMACs were analyzed by western blot using anti-MYC antibodies. Inputs and Pgk1 are also shown. Note that Pdc1 may still be slightly ubiquitinated in rsps5-1 cells, presumably by Hul5 (Figure 3f). e. Western blots of samples derived from wild-type cells (BY4741) expressing the indicated C-terminally 13xMYC tagged Cdc19 and that were shifted to 42°C in the presence of 100µg/ml cycloheximide (CHX) at the indicated time. The MYC band signals, normalized to the corresponding Pgk1 signals, are indicated below in grey. f. Representative western blots used for quantification in Figure 7g. g-h. Western blots of samples derived from indicated cells that expressed the C-terminally 13xMYC tagged Cdc19(D367R) and that were shifted to 42°C in the presence of 100µg/ml cycloheximide (CHX) at the indicated time. The MYC band signals, normalized to the corresponding Pgk1 signals, are indicated below in grey. i. The proportion of PY and PY-like motifs ([PLV]PxY, [PSV]PxF, PPPP or PPR) in proteins encoded in the yeast genome that are located in regions predicted to be disordered (IDP) or not (i.e., structured) are indicated.
Supplementary Figure 8 Uncropped images of Western blots of main figures.
Supplementary Table Legends

Supplementary Table 1. List of ubiquitinated peptides and proteins quantified in the Rsp5 mass spectrometry analyses.

Supplementary Table 2. Number of indicated PY motifs identified among Rsp5 substrate candidates identified by mass spectrometry in the HS-conditions and by protein array.

Supplementary Table 3. List of yeast strains used in this study.

Supplementary Table 4. List of plasmids used in this study.

Supplementary Table 5. Graphs and statistics source data. Source data for each figure panel are listed on a separate spreadsheet.