A High Throughput Screen to Identify Substrates for the Ubiquitin Ligase Rsp5†§

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Ubiquitin-protein ligases (E3s) are implicated in various human disorders and are attractive targets for therapeutic intervention. Although most cellular proteins are ubiquitinated, ubiquitination cannot be linked directly to a specific E3 for a large fraction of these proteins, and the substrates of most E3 enzymes are unknown. We have developed a luminescent assay to detect ubiquitination in vitro, which is more quantitative, effective, and sensitive than conventional ubiquitination assays. By taking advantage of the abundance of purified proteins made available by genomic efforts, we screened hundreds of purified yeast proteins for ubiquitination, and we identified previously reported and novel substrates of the yeast E3 ligase Rsp5. The relevance of these substrates was confirmed in vivo by showing that a number of them interact genetically with Rsp5, and some were ubiquitinated by Rsp5 in vivo. The combination of this sensitive assay and the availability of purified substrates will enable the identification of substrates for any purified E3 enzyme.

The ubiquitin pathway is conserved throughout eukaryotic evolution and is implicated in numerous cellular processes (1). Proteins modified by the ubiquitin pathway are processed for degradation, endocytosis, protein sorting, and subnuclear trafficking (2, 3). Ubiquitination is catalyzed by three enzymes termed E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin protein ligase). E3 regulates the specificity of the reaction by binding directly to substrates (1, 3). The E3-substrate interaction is implicated in an increasing number of diseases, including neurodegeneration, immunological disorders, hypertension, and cancers. For example, numerous E3 enzymes such as Fbw7, Skp2, Mdm2, and VHL and their respective substrates, cyclin E, p27, p53, and HIF, have been linked to tumor progression (4, 5). The therapeutic importance of understanding ubiquitination has been underscored recently by the success of anticancer strategies that affect the ubiquitin pathway (6).

Most, if not all, proteins are regulated by the ubiquitin pathway. A recent proteomic approach identified over a thousand proteins that are ubiquitinated in yeast under normal conditions (7). This study, which likely did not detect many non-abundant proteins or proteins that are ubiquitinated under specific conditions (e.g. stress and nutrition), underlines the breadth of the ubiquitin system. Current estimates also predict that there are hundreds of E3 enzymes in eukaryotic genomes (8) whose role is to ubiquitinate these proteins. Despite the biomedical importance of E3 enzymes and great advances in understanding the mechanics of the ubiquitin system, a very small fraction of E3 enzymes has been linked to specific substrates, and currently, the scarcity of identified E3-substrate pairs in the literature is a major bottleneck in the ubiquitin field.

Rsp5 is a yeast E3 enzyme, and many of its substrates have not yet been characterized. It belongs to the Nedd4 family of E3 ligases (9), which contain a C2 domain, WW domains, and a catalytic HECT domain (Fig. 1A). WW domains are protein-protein interaction modules that usually bind substrates directly by recognizing (L/P)XXY sequences (PY motifs) (10–13). The best characterized role of this E3 family is to ubiquitinate transmembrane proteins and to regulate their endocytosis (9). In addition, Rsp5 has been implicated in various other biological functions, such as mini-chromosome maintenance, mitochondrial inheritance, actin cytoskeleton maintenance, drug resistance, regulation of intracellular pH, biosynthesis of fatty acids, and protein sorting at the trans-Golgi network (2, 14, 15).

Rsp5 also regulates the activity of RNA polymerase II in response to DNA damage by ubiquitinating Rpb1, the large subunit of the holoenzyme (16, 17). The mammalian Rsp5 homolog, Nedd4 (or Nedd4-2), binds to the PY motifs of the plasma membrane-localized epithelial sodium channel and regulates its endocytosis (10, 18–20). Mutations in these PY motifs cause Liddle syndrome, a genetic form of hypertension (21).

In this study, the yeast Rsp5 E3 ligase was used as a model to develop an in vitro ubiquitination assay in order to discover substrates for Rsp5 from among large numbers of purified GST-tagged proteins. Traditional immunoblotting (Western blot) assays used to monitor ubiquitination were unsuitable for this purpose because they are difficult to standardize, not quantitative, and not amenable to genome-scale analysis. We elected to develop a ubiquitination assay based on luminescence (AlphaScreen™ technology) in order to achieve higher.

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The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; Ub, ubiquitinylated ubiquitin; GST, glutathione S-transferase; TCEP, tris(2-chloroethyl) phosphate; CTD, C-terminal domain.
sensitivity, greater throughput, and the potential for automation (22, 23). The assay measures the proximity of biotinylated ubiquitin (b-Ub) and GST-tagged substrate proteins (22, 23) (Fig. 1B).

The luminescent assay was used to screen 188 purified yeast proteins for ubiquitination by Rsp5. We identified novel substrates as well as proteins already known to interact with Rsp5. The relevance of the novel substrates was confirmed in vivo by showing that their overexpression compromised the viability of strains deficient in Rsp5 function and by experiments demonstrating that a novel protein that produced a very strong signal in the luminescence assay was also ubiquitinated in vivo.

**EXPERIMENTAL PROCEDURES**

*Purification of Yeast E2 Enzymes—* Yeast E2 genes UBC1 and UBC4 were cloned into pET15b plasmids as described (24). All proteins were expressed in *Escherichia coli* strain BL21 (DE3). Transformed cells were grown at 37 °C to an *A*_{opt} of 0.6 in 2 liters of Luria broth, and expression was induced by addition of 1 mM isopropyl-β-D-galactopyranoside. After 12 h of induction at 16 °C, the cells were harvested and lysed by sonication in binding buffer (20 mM HEPES, pH 7.4, 25 mM MgOAc, 2.5 mM TCEP, 500 mM NaCl, and 50% glycerol), 1 μg of biotinylated ubiquitin (b-Ub), 0.16 μg of yeast E1, 3.8 μg of UbcE2, 4 μg of UbcE1 E2, 1.2 μg of Rsp5 E3, 8 μmol of GST-tagged substrate, and 3.3 μM ATP (Sigma). E1 and b-Ub were purchased from Boston Biochem. Where indicated, Rsp5 was substituted with an equal amount of Rsp5 C777A or 0.52 μg of HECT domain. Water was added to each of the reactions to bring the final volume of all reactions to 15 μl. ATP was either omitted or added last in order to minimize autocatalytic ubiquitination reactions by the ubiquitination enzymes. Reactions were allowed to proceed for 4 h at room temperature and stopped by boiling in 5 μl of SDS-PAGE sample buffer containing 3 μl urea, or by diluting the samples 100–1000-fold with AlphaScreen assay buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% Tween 20, and 1 mM dithiothreitol). To prepare the ubiquitination reactions for analysis using AlphaScreen, the samples were diluted 270-fold with AlphaScreen assay buffer (unless otherwise indicated) and transferred to 384-well OptiPlate-NEW microplates containing 5 μl of AlphaScreen anti-GST acceptor and streptavidin donor beads (both at a final concentration of 20 μg/ml). This dilution step brings the concentration of b-Ub and GST proteins into the nanomolar range, which corresponds to the binding capacities of AlphaScreen beads. Plates were incubated at 23 °C for 1 h and analyzed on an AlphaQuest HTS analyzer. A homogenous assay format was also developed in which similar conditions were used (not shown). For AlphaScreen detection of GST fusion proteins, microplates were purchased from PerkinElmer Life Sciences.

*Detection of Ubiquitination by Immunoblotting—* Complete ubiquitination reactions (15 μl) were boiled in 3× SDS-PAGE sample buffer and resolved by gel electrophoresis on 4–12% gradient SDS–polyacrylamide gels or 12% polyacrylamide gels. GST-tagged substrate proteins were detected using a 1:2000 dilution of the α-GST antibody (B-14, Santa Cruz Biotechnology) or mouse monoclonal α-His antibodies (Amersham Biosciences). Protein yield was measured by Bradford assay (Bio-Rad) followed by gel densitometry using the Genius Gel Imaging System, GeneSnap, and GeneTools software (all from SynGene). Purified proteins were frozen in liquid nitrogen and stored at −80 °C.

**In Vitro Ubiquitination (Luminescence) Assays—** Standard ubiquitination reactions contained 3 μl of 5× assay buffer (250 mM HEPES, pH 7.4, 25 mM MgOAc, 2.5 mM TCEP, 500 mM NaCl, and 50% glycerol), 1 μg of biotinylated ubiquitin (b-Ub), 0.16 μg of yeast E1, 3.8 μg of UbcE2, 4 μg of UbcE1 E2, 1.2 μg of Rsp5 E3, 8 μmol of GST-tagged substrate, and 3.3 μM ATP (Sigma). E1 and b-Ub were purchased from Boston Biochem. Where indicated, Rsp5 was substituted with an equal amount of Rsp5 C777A or 0.52 μg of HECT domain. Water was added to each of the reactions to bring the final volume of all reactions to 15 μl. ATP was either omitted or added last in order to minimize autocatalytic ubiquitination reactions by the ubiquitination enzymes. Reactions were allowed to proceed for 4 h at room temperature and stopped by boiling in 5 μl of SDS-PAGE sample buffer containing 3 μl urea, or by diluting the samples 100–1000-fold with AlphaScreen assay buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% Tween 20, and 1 mM dithiothreitol). To prepare the ubiquitination reactions for analysis using AlphaScreen, the samples were diluted 270-fold with AlphaScreen assay buffer (unless otherwise indicated) and transferred to 384-well OptiPlate-NEW microplates containing 5 μl of AlphaScreen anti-GST acceptor and streptavidin donor beads (both at a final concentration of 20 μg/ml). This dilution step brings the concentration of b-Ub and GST proteins into the nanomolar range, which corresponds to the binding capacities of AlphaScreen beads. Plates were incubated at 23 °C for 1 h and analyzed on an AlphaQuest HTS analyzer. A homogenous assay format was also developed in which similar conditions were used (not shown). For AlphaScreen detection of GST fusion proteins, microplates were purchased from PerkinElmer Life Sciences.

*In Vivo Overexpression Yeast Experiments—* The expression library. To assay lethality at restrictive and permissive temperatures, strains were grown in 1-ml 96-well plates (Ulidint) in liquid **Ura**-synthetic drop-out media containing 2% glucose and then spotted onto synthetic drop-out plates containing 2% glucose or 2% galactose using a 96-well pin (Ulidint). Plates were grown at the indicated temperatures for 40 h.

In **Vivo Ubiquitination Experiments—** The rop-1 and the corresponding wild-type strains expressing the desired GST proteins were grown to log phase in **Ura**-synthetic drop-out media containing 2% raffinose. The temperature was then shifted to 37 °C, the expression of the GST proteins was induced by the addition of galactose to 2%, and growth was continued at the restrictive temperature for 2 h. The cells were then harvested, and the GST proteins were purified from the cells as described except that 50 μM LLLN (Sigma), 1 μM chloroquine (Sigma), and 1 μM MG-132 (Boston Biochem) was added to the lysis buffers in addition to benzamidine and phenylmethylsulfonyl fluoride. The proteins extracted from the mutant and wild-type strains were analyzed using SDS-PAGE and probed with α-GST and α-ubiquitin (Covance) antibodies.

### RESULTS

**Development of the Luminescent Ubiquitination Assay—** The new luminescent assay was designed to monitor the ubiquitination of GST fusion proteins in reactions containing purified E1, E2, and E3 (Rsp5) enzymes, b-Ub, and ATP (Fig. 1B). As a starting point for assay development, we measured Rsp5-dependent ubiquitination of the C-terminal domain (CTD) of Rpb1, a known Rsp5 substrate, by using Western blots. Once these assay conditions were established, we developed the initial luminescent assay conditions using CTD as the substrate. Furthermore, the ubiquitination of four other in vitro GST-tagged substrates of Rsp5 (Ybr196c, Ypr084w, Ynl136w, and Ydr203c, which were discovered in the course of the study) and five proteins not ubiquitinated by Rsp5 in vitro (GST alone, 6.8 KDa). 

2 N. Fong, unpublished data.
Yer177w, Ycl040w, Yor057w, and Yar015w) was also reconstituted and monitored using both Western blot and luminescent assays. Fig. 2A shows the detection of ubiquitination using the luminescent assay, and Fig. 2B shows the ubiquitination of these same proteins as detected by Western blot. Ubiquitination of the Rsp5 substrates was 6–11-fold greater in the presence of Rsp5 because they contained a PY motif, which is a potential target for Rsp5. Several of the substrates identified in our screens, e.g., CTD, Bul1, and Hpr1. To test whether a novel in vitro substrate identified in our screen, Ydl203c, was also a substrate of Rsp5, we investigated in vivo ubiquitination of Ydl203c in Rsp5 and rsp5-1 mutant strains, and we compared it to a protein that gave a very weak signal in the luminescence assay, GST. We opted to focus on in vivo ubiquitination rather than on protein stability because not all ubiquitinated proteins generated a low background signal (0.01 units, Fig. 5 and supplemental Table I). As expected, most of the proteins that generated strong luminescent signal contain a PY motif (Fig. 5), and on average, these proteins produced higher signal ($p > 0.01$, $t$ test). The WW domains of Rsp5 therefore appeared to retain specificity for PY motifs in the in vitro assays.

The nine proteins generating signal higher than CTD were designated as potential substrates of Rsp5. To provide evidence that Rsp5 recognizes substrates through its WW domain in the screen, we selected six of these substrates and tested their ubiquitination by a construct of Rsp5 harboring the HECT domain alone, and lacking the C2 and WW domains, or with a mutant Rsp5 in which the catalytic cysteine was mutated to alanine (Rsp5-C777A) to render it catalytically inactive. The full-length Rsp5 was able to ubiquitinate the six substrates much more efficiently than did either the HECT domain alone or the Rsp5 mutant (Rsp5-C777A) (26) (Fig. 6). The HECT domain alone and the wild-type Rsp5 possessed equal specific activity as monitored by their auto-ubiquitination, a property of some E3 enzymes (not shown). These results suggest that substrate recognition (likely via the Rsp5-WW domains), as well as catalytic activity of Rsp5, is required for efficient substrate ubiquitination in these assays.

Monitoring Poly- or Mono-ubiquitination Using a Western Blot Assay—In order to provide further evidence for covalent attachment of ubiquitin to the novel substrates, and to determine the ubiquitin chain topology, we used a Western blot assay. Fig. 2B demonstrates that all the proteins whose ubiquitination was detected in the luminescent assay were visibly ubiquitinated on the Western blot. Most of the proteins were polyubiquitinated or ubiquitinated on multiple lysines, except for Ybr196c, and possibly Ydr404c, which appeared to be either mono- or di-ubiquitinated. Proteins whose ubiquitination was not detected by the luminescent assay did not appear to be ubiquitinated on the Western blot (Fig. 2B).

Genetic Interactions between Novel Substrates and Rsp5 in Vivo—To provide genetic evidence for the relevance of the substrates discovered in the biochemical screens, we tested the genetic interaction of strains overexpressing the putative substrates in a strain harboring a temperature-sensitive mutant of Rsp5 (rsp5-1) (27). 34 wild-type and rsp5-1 strains were transformed with GST fusion protein expression plasmids. All of the transformed rsp5-1 strains grew normally at 30°C, and none were able to grow at 37°C (not shown). Colony growth was monitored at the weakly permissive temperature (35°C) at which overexpression of the Rsp5 substrates would be predicted to be lethal. 11 of the overexpressed proteins caused lethality in the rsp5-1 strains (Fig. 7). 8 of these 11 toxic proteins were from the group representing highly ubiquitinated Rsp5 substrates; 2 were from the moderately ubiquitinated proteins, and 1 was from the weakly ubiquitinated proteins, revealing a positive correlation between the extent of substrate ubiquitination in vitro and toxicity in yeast cells.
uitinated proteins in the cell are degraded. In addition, ubiquitination of a small fraction of a cellular pool of a protein can be detected, even if its total cellular level may not be altered significantly. We thus monitored the in vivo ubiquitination of GST-Ydl203c or GST alone in rsp5-1 and wild-type yeast cells at the restrictive temperature. These proteins (and any attached ubiquitins) were purified in the presence of proteasome and lysosome inhibitors, and equal amounts were analyzed by SDS-PAGE and Western blotting with anti-ubiquitin antibodies. As seen in Fig. 8, GST-Ydl203c, but not GST alone, was strongly ubiquitinated in the Rsp5 but not rsp5-1 mutant cells, suggesting Ydl203c is an in vivo substrate for Rsp5.

**DISCUSSION**

Six lines of evidence support the hypothesis that some or all of the Rsp5 substrates identified in our screen have physiological relevance. (i) In addition to Rpb1, other known substrates of Rsp5 were identified in the screen, including Bul1 and Hpr1. Both of these proteins were among the most strongly ubiquitinated proteins that scored as positive in the screen and have been validated in vivo as Rsp5 substrates. Bul1 interacts with Rsp5 in vivo through its PY motif and modifies its activity (28). Hpr1 is a member of the transcription-export complex and couples mRNA transcription to nuclear export. It was discov-
ered recently that Hpr1 is also a substrate of Rsp5 and is targeted for ubiquitination by this E3 at high temperatures (29). The fact that known substrates of Rsp5 were uncovered in our screen provides strong evidence that our approach is capable of discovering biologically relevant substrates for this E3 enzyme. (ii) Three other potential substrates that we identified (Ydl203c, Ybr196c, and Ypr084w) have been described as Rsp5 substrates in large scale protein-protein interaction screens (30, 31). (iii) A greater proportion of heavily ubiquitinated proteins in our screen contained PY motifs; eight of the nine novel substrates have PY motifs, and proteins containing these motifs produced, on average, higher luminescent signal in the assay. This is consistent with the accepted model of Rsp5 substrate recognition and suggests that this mechanism is retained in vitro. (iv) A mutant of Rsp5 lacking its protein-protein interaction (WW) domains failed to ubiquitinate at least six of the new substrates. (v) Seven of the new substrates interacted genetically with Rsp5. (vi) In vivo ubiquitination assays suggest that a highly ubiquitinated novel substrate in the luminescence assay, Ydl203c, is also ubiquitinated in vivo. Collectively, these data suggest that the application of this screen on a larger scale could identify many new Rsp5 as well as other E3 ligase substrates. Evidence that the nine in vitro substrates may be physiological substrates of Rsp5 is summarized in Table I.

Genetic Evidence of Substrate-Rsp5 Interactions—The genetic experiments in this study provide evidence for the physiological interaction of some of the substrates with Rsp5. Two models can explain why specific overexpressed proteins cause lethality in the temperature-sensitive rsp5-1 strain. One possibility is that at 35 °C, the overexpressed proteins, which are normally degraded by Rsp5, are toxic to the cell because they are stabilized as a result of low Rsp5 activity. We have shown experimentally, however, that stability itself does not account for the toxicity of at least three proteins and that these proteins appear to be stable when overexpressed in both rsp5-1 and wild-type cells at restrictive temperatures (data not shown). Perhaps a more plausible explanation is that overexpressed proteins with affinity for Rsp5 act as a “molecular sink” and sequester active Rsp5 away from its essential functions of ubiquitinating cellular substrates. Although cells can survive this added stress at permissive temperatures, at 35 °C, Rsp5 activity is already compromised and results in cell death.

One interesting candidate protein, Rpb7 (Ydr404c), interacted with Rsp5 in both genetic and biochemical assays and merits further attention. Both Rpb1, which is ubiquitinated and degraded in the presence of DNA damage (16, 17),3 and Rpb7 are members of the RNA polymerase II complex. The crystal structure of RNA polymerase II predicts that Rpb1 is adjacent to Rpb7 and that the PY motif of Rpb7 is exposed (32). Recent evidence suggests that in humans both Rpb1 and Rpb7 subunits are targeted for ubiquitination by a single E3 ligase, the VHL complex (33, 34). Thus, it is possible that in yeast, both subunits are also ubiquitinated by a single E3 ligase.

3 B. Kus, A. Gajadhar, K. Stanger, R. Cho, W. Sun, N. Rouleau, T. Lee, D. Chan, C. Wolting, A. Edwards, R. Bosse, and D. Rotin, unpublished data.
Rsp5. Although Rpb7 is stable in the presence of DNA damage, Rpb7 may be ubiquitinated under other conditions, and Rpb7 may play a role in Rpb1 ubiquitination because it mediates DNA damage responses (35). Finally, the interaction between Rpb7 and Rsp5 was predicted in data base searches for proteins containing optimal Rsp5-binding sequences (36).

**In Vivo Evidence for Ubiquitination of Substrates by Rsp5**—The physiological roles of most substrates discovered in this study are not well characterized and have yet to be placed in Rsp5-dependent pathways in the cell. Upon the completion of the in vitro luminescent screen, however, we realized that it would be difficult to validate all the in vitro substrates using simple in vivo ubiquitination (or stability) assays because: (i) ubiquitination and degradation of many Rsp5 substrates likely require specific environmental cues (14), such as DNA damage, temperature changes, nutrient availability, or other stresses. For example, two known in vivo substrates of Rsp5 that were also identified in our screen, Rpb1 and Hpr1, require specific environmental cues to be ubiquitinated in vivo. (ii) In vivo, subcellular localization of Rsp5 may prevent it from accessing...
its substrates (until the appropriate environmental signal is provided). (iii) Cellular inhibitors absent in vitro may negatively regulate Rsp5-substrate interactions in vivo. (iv) Our in vitro luminescent assay is much more sensitive than the in vivo ubiquitination assay, rendering the latter more difficult to detect. Nevertheless, we were able to provide evidence for physiological interactions between some of the novel substrates and Rsp5, including data suggesting genetic interactions, as well as Rsp5-dependent in vivo ubiquitination of one of the novel substrates Ydl203c.

Ydl203c, a Novel Rsp5 Substrate—Ydl203c is an interesting candidate for further study as a potential physiological substrate of Rsp5. Evidence for its role in Rsp5 pathways includes the following: (i) the presence of a PY motif in the N-terminal portion of the protein; (ii) failure of the HECT domain alone construct to ubiquitinate Ydl203c; (iii) a genetic interaction between Rsp5 and Ydl203c in the overexpression screen; (iv) Rsp5-dependent ubiquitination of Ydl203c in vivo (Fig. 8); and finally (v) the identification of Ydl203c as a substrate of Rsp5 in a large scale yeast two-hybrid screen (30).

Ydl203c is an otherwise uncharacterized protein with seven C-terminal Sel1 domains downstream of its PY motif. This domain is repeated in the C terminus of a number of other proteins, including Hdr1, which is a yeast ubiquitin ligase, and Sel1, a Caenorhabditis elegans protein whose role is to negatively regulate Notch. A human protein, Sel1L, also contains C-terminal Sel1 repeats that are necessary for its anti-tumorigenic activity (37). The precise function of this domain has not

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**Fig. 6.** Validation of biochemical screen. A, six of the substrates generating high luminescent signal in the screen were titrated in increasing concentrations into reactions containing either full-length Rsp5 or just the HECT domain itself (HECT domain alone). Absolute luminescent values were normalized to the signal produced by Ybr196c (when incubated at 0.53 μM). B, the same six substrates were ubiquitinated in standard conditions in the presence/absence of Rsp5, catalytically inactive Rsp5 C777A, or the HECT domain alone.

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**Fig. 7.** Genetic validation of the biochemical screen. Wild type (WT) and rsp5-1 mutant strains transformed with plasmids coding for GST fusion proteins were grown at 35 °C on glucose (to repress overexpression of proteins) and galactose (to induce protein overexpression). rsp5-1 strains unable to form colonies when grown with galactose are marked with a box. The strains are placed into one of three groups based on whether they produced high, moderate, or weak signal in the biochemical (luminescent) screen.
This table summarizes properties of the in vitro substrates discovered in this study. Yellow color highlights data that are consistent with a physiological interaction of the substrate with Rsp5. GST alone, which is not ubiquitinated by Rsp5, is also shown for comparison.

| systematic name | in vitro ub | type of ub | PY motif | needs full length Rsp5 for ub/n | lethal to rps5-1 cell | interacts w/ Rsp5 in large-scale interaction screens | evidence for interaction with Rsp5 in vivo |
|-----------------|-------------|------------|----------|--------------------------------|----------------------|-----------------------------------------------|-------------------------------------------|
| YMR272C         | End1        | 1.54       | Yes      | Yes                            | Yes                  | binding (ref 28)                               |                                           |
| YVL024W         | poly        | 1.24       | Yes      | Yes                            | Yes                  | ref (30)                                       | ubiquitination (this study)               |
| YDL203C         | poly        | 1.23       | Yes      | Yes                            | Yes                  | ref (31)                                       |                                           |
| YNL139W         | mono        | 1.06       | Yes      | Yes                            | Yes                  | part of RNP (Rsp5-interacting complex) (ref 32) |
| YDR404C         | Rbi1        | 0.97       | Yes      | Yes                            | Yes                  | binding, stability (ref 29)                    |
| YBR196C         | mono        | 0.97       | No       | No                             | No                   | ref (31)                                       |                                           |
| YPK084W         | poly        | 0.90       | Yes      | Yes                            | Yes                  | binding, stability (ref 29)                    |
| YDR139W         | Hpr1        | 0.96       | Yes      | Yes                            | Yes                  | binding, stability, ubiquitination (ref 17)    |
| YLP141C         | poly        | 0.74       | Yes      | Yes                            | Yes                  | binding, stability (ref 29)                    |
| GST             | GST         | 0.01       | No       | No                             | No                   | binding, stability (ref 29)                    |

†The level of ubiquitination as measured by the biochemical assay, see Fig. 5 and supplemental Table S1.
‡Polyubiquitination or monoubiquitination as measured by Western blot, see Fig. 6C.
§Based on the inability of the HECT domain alone mutant to ubiquitinate the novel substrates, see Fig. 6A and B.
*Genetic interaction, see Fig. 7.
††Rsp5-substrate interaction detected in large scale proteome-wide screens.
*Evidence (from this study and other studies) supporting that the putative substrate is part of Rsp5-dependent pathways (also see Fig. 8).

In summary, we developed and used a luminescent platform to test 188 purified GST-tagged yeast proteins as potential substrates of Rsp5, and we identified 7 new proteins that were specifically ubiquitinated and that showed a genetic interaction with Rsp5. As might have been predicted, a high proportion of the heavily ubiquitinated substrates contained PY motifs, in keeping with the known specificity of Rsp5. More importantly, this assay may also allow for the identification of novel Rsp5-interacting sequences in substrates lacking PY motifs. Taken together, the highly sensitive luminescence assay we developed to detect and quantify ubiquitination will serve a means to identify novel E3 substrates and perhaps as a platform to screen for inhibitors of E3-dependent ubiquitination.

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REFERENCES
1. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
2. Hicke, L., and Dunn, R. (2003) Annu. Rev. Cell Dev. Biol. 19, 141–172
3. Pickart, C. M. (2001) Annu. Rev. Biochem. 70, 503–533
4. Wang, B. R., Parlati, F., Qu, K., Demo, S., Pray, T., Huang, J., Payan, D. G., and Bennett, M. K. (2003) Drug Discov. Today 8, 746–754
5. Burger, A. M., and Seth, A. K. (2004) Eur. J. Cancer 40, 2217–2229
6. Orlovski, R. Z. (2004) Expert Rev. Anticancer Ther. 4, 171–179
7. Peng, J., Schwartz, D., Elia, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Reelods, J., Finley, D., and Gygi, S. P. (2003) Nat. Biotechnol. 21, 921–926
8. Willems, A. R., Schwarb, M., and Tyers, M. (2004) Biochim. Biophys. Acta 1695, 133–170
9. Rotin, D., Staub, O., and Haguenauer-Tsapis, R. (2000) J. Membr. Biol. 176, 1–17
10. Kanelis, V., Rotin, D., and Forman-Kay, J. D. (2001) Nat. Struct. Biol. 8, 407–412
11. Kasanov, J., Prouzi, G., Uveges, A. J., and Kay, B. K. (2001) Chem. Biol. 8, 231–241
12. Hu, H., Columbus, J., Zhang, Y., Wu, D., Lian, L., Yang, S., Goodwin, J., Lueck, C., Carter, M., Chen, L., James, M., Davis, R., Stul, M., Rodwell, J., and Herrero, J. J. (2004) Proteomics 4, 643–655
13. Scherbik, N., Kee, Y., Lyon, N., Huibregtse, J. M., and Haines, D. S. (2004) J. Biol. Chem. 279, 53892–53998
14. Hein, C., Springael, J. Y., Volland, C., Haguenauer-Tsapis, R., and Andre, B. (1999) Mol. Microbiol. 18, 77–87
15. Horak, J. (2003) Biochim. Biophys. Acta 1614, 139–155
16. Huibregtse, J. M., Yang, J. C., and Beaudenon, S. L. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 3656–3661
17. Beaudenon, S. L., Huscay, M. R., Wang, G., McDonnell, D. P., and Huibregtse, J. M. (1999) Mol. Cell. Biol. 19, 6972–6979
18. Staufb, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996) EMBO J. 15, 2371–2380
19. Abriel, H., Loffing, J., Rebhun, J. F., Pratt, J. H., Schild, L., Horischerger, J. D., Rotin, D., and Staub, O. (1999) J. Clin. Invest. 103, 667–673
20. Kamynina, E., Debonenville, C., Bens, M., Vandewalle, A., and Staub, O. (2001) FASEB J. 15, 204–214
21. Li, P. R., Gharavi, A. G., and Geller, D. S. (2001) Cell 104, 545–556
22. Warner, G., Illy, C., Pedro, L., Roby, P., and Bosse, R. (2004) Curr. Med. Chem. 11, 721–730
23. Rouleau, N., Turcotte, S., Mondou, M. H., Roby, P., and Bosse, R. (2003) J. Biolom. Screen. 8, 191–197
24. Kus, B. M., Caldon, C. E., Andorn-Broza, R., and Edwards, A. M. (2004) Proteins 54, 455–467
25. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Heufel, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., and Snyder, M. (2001) Science 293, 2101–2105
26. Dunn, R., and Hicke, L. (2001) Mol. Biol. Cell 12, 421–435
27. Wang, G., Yang, J., and Huibregtse, J. M. (1999) Mol. Cell. Biol. 19, 342–352
28. Andoh, T., Hirata, Y., and Kikuchi, A. (2000) Mol. Cell. Biol. 20, 6712–6720
29. Gwizdek, C., Hobeika, M., Kus, B., Ossareh-Nazari, B., Dargemont, C., and Rodriguez, M. S. (2005) J. Biol. Chem. 280, 13401–13405
30. Ito, T., Chiha, T., Ozawa, R., Yoshiha, M., Hattori, M., and Sakaki, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4569–4574
31. Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutelier, K., Yang, L., Wolding, C., Donaldson, I., Schandorff, S., Shewnarane, J., Vo, M., Taggart, J., Goudreault, M., Muskat, B., Altarano, C., Dewar, D., Lin, Z., Michalickova, K., Willems, A. R., Sassi, H., Nielsen, P. A., Rasmussen, K. J., Andersen, J. R., Johansen, L. E., Hansen, L. H., Jespersen, H., Podtelejnikov, A., Nielsen, E., Crawford, J., Poulsen, V., Sorensen, B. D., Matthiesen, J., Hendrickson, R. C., Gleeson, F., Pawson, T., Moran, M. F., Duruch, D., Mann, M., Hogue, C. W., Figyea, D., and Tyers, M. (2002) Nature 415, 180–183
32. Fu, J., Gnatt, A. L., Bushnell, D. A., Jensen, G. J., Thompson, N. E., Burgess, R. R., David, P. R., and Kornberg, R. D. (1999) Cell 98, 799–810
33. Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, J. W., Conaway, R. C., and Czyzyk-Krzeska, M. F. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2706–2711
34. Na, X., Duan, H. O., Messing, E. M., Schoen, S. R., Ryan, C. K., di Sant'Agnese, P. A., Golemis, E. A., and Wu, G. (2003) EMBO J. 22, 4249–4259
35. He, C. H., and Ramotar, D. (1999) Biochem. Cell Biol. 77, 375–382
36. Chang, A., Cheang, S., Espanel, X., and Sudol, M. (2000) J. Biol. Chem. 275, 20562–20571
37. Cattaneo, M., Canton, C., Albertini, A., and Biunno, I. (2004) Gene (Amst.) 326, 149–156