Identification of Candidate Substrates for the Golgi Tul1 E3 Ligase Using Quantitative diGly Proteomics in Yeast*

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Maintenance of protein homeostasis is essential for cellular survival. Central to this regulation are mechanisms of protein quality control in which misfolded proteins are recognized and degraded by the ubiquitin-proteasome system. One well-studied protein quality control pathway requires endoplasmic reticulum (ER)-resident, multi-subunit E3 ubiquitin ligases that function in ER-associated degradation. Using fission yeast, our lab identified the Golgi Dsc E3 ligase as required for proteolytic activation of fungal sterol regulatory element-binding protein transcription factors. The Dsc E3 ligase contains five integral membrane subunits and structurally resembles ER-associated degradation E3 ligases. Saccharomyces cerevisiae codes for homologs of Dsc E3 ligase subunits, including the Dsc1 E3 ligase homolog Tul1 that functions in Golgi protein quality control. Interestingly, S. cerevisiae lacks sterol regulatory element-binding protein homologs, indicating that novel Tul1 E3 ligase substrates exist.

Here, we show that the S. cerevisiae Tul1 E3 ligase consists of Tul1, Dsc2, Dsc3, and Ubx3 and define Tul1 complex architecture. Tul1 E3 ligase function required each subunit as judged by vacuolar sorting of the artificial substrate Pep12D. Genetic studies demonstrated that Tul1 E3 ligase was required in cells lacking the multivesicular body pathway and under conditions of ubiquitin depletion. To identify candidate substrates, we performed quantitative diGly proteomics using stable isotope labeling by amino acids in cell culture (SILAC) methodology to obtain a robust platform for screening for stress conditions that require Tul1 E3 ligase activity. Molecular & Cellular Proteomics 13: 10.1074/mcp.M114.040774, 2871–2882, 2014.

Control of protein homeostasis or proteostasis is key for cell function and survival (1). An important aspect of proteostasis is protein quality control in which misfolded proteins are recognized and degraded by the ubiquitin-proteasome pathway (2). Complex mechanisms regulate whether proteins are targeted for degradation, but ultimately misfolded proteins are recognized and ubiquitylated by specific E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases (3). One well-studied protein quality control pathway is ER-associated degradation (ERAD) (4–6). ER luminal and membrane proteins are targeted for cytosolic proteasomal degradation by a set of multi-subunit E3 ligases integral to the ER membrane, such as Hrd1 and Doa10 in Saccharomyces cerevisiae and Hrd1 and gp78 in mammals. Key open questions in the protein quality control field are (i) what are the physiological substrates of protein quality control pathways and (ii) how do these E3 ligases recognize proteins for degradation.

The sterol regulatory element–binding protein (SREBP) family of transcription factors regulates lipid homeostasis in mammals and fungi (7). These ER membrane-bound proteins are proteolytically activated in the Golgi to release the transcription factor domain from the membrane, allowing it to

1 The abbreviations used are: ERAD, endoplasmic reticulum-associated degradation; ER, endoplasmic reticulum; SREBP, sterol regulatory element–binding protein; Dsc, defective for SREBP cleavage; SNARE, soluble NSF attachment protein receptor; MVB, multivesicular body; MS, mass spectrometry; SILAC, stable isotope labeling by amino acids in cell culture; Ni-NTA, nickel-nitrilotriacetic acid; TFA, trifluoroacetic acid; ACN, acetoniitrile; FA, formic acid; ESCRT, endosomal sorting complex required for transport; H/L, heavy-to-light.
activate gene expression. Studies of the SREBP pathway in the fission yeast Schizosaccharomyces pombe led to the discovery of the Golgi Dsc E3 ligase that is required for the cleavage of yeast SREBPps (8, 9). The Golgi Dsc E3 ligase contains five subunits, Dsc1–Dsc5, that form a stable complex with a defined architecture (10). Dsc1 is a really interesting new gene (RING) domain-containing E3 ubiquitin ligase, and its RING domain function is required for SREBP cleavage (8, 11). Collectively, data are consistent with SREBPps being substrates for the Dsc E3 ligase, but ubiquitylation has not been demonstrated. Interestingly, the subunits and organization of the Dsc E3 ligase resemble the Hrd1 and gp78 E3 ligases that function in ERAD (10, 12). This similarity to ERAD E3 ligases, along with the Golgi localization, suggests a broader role for the Dsc E3 ligase in Golgi protein quality control and degradation.

The budding yeast S. cerevisiae encodes sequence homologs of Dsc E3 ligase subunits, but S. cerevisiae lacks SREBPps, suggesting that additional substrates exist for this Golgi enzyme. We reasoned that characterizing the function of the Dsc E3 ligase in S. cerevisiae would advance our understanding of its role in Golgi protein degradation outside of the SREBP pathway. To date, only S. cerevisiae Tul1, the homolog of S. pombe Dsc1, has been characterized in detail. Tul1 is an integral Golgi membrane protein with a carboxy-terminal RING domain that binds and functions with the E2 ubiquitin-conjugating enzyme Ubc4 (13). Tul1 functions in Golgi protein quality control inasmuch as Tul1 ubiquitylates Pep12D, a mutant endosomal SNARE protein with a transmembrane domain containing a charged residue (13). As a consequence of ubiquitylation, Pep12D is sorted into the multivesicular body (MVB) pathway that results in vacuolar degradation rather than localizing to the limiting membrane of the vacuole. In addition, Tul1 recognizes unpalmitoylated SNARE Tlg1 and targets this mutant protein to the MVB pathway (14). Although Tul1 acts on these engineered mutant proteins, no endogenous physiological substrates have been identified.

Stable isotope labeling by amino acids in cell culture (SILAC) is a well-established method for labeling the cellular proteome to allow precise mass spectrometry (MS)-based protein quantitation (15–17). Recent technical advances enable enrichment of ubiquitylated peptides and greatly improve detection of this post-translational modification (18). Antibodies that recognize the diGly remnant on ubiquitin-conjugated lysine residues after trypsin digestion now allow the identification of thousands of ubiquitylated peptides via MS (19–21). Combining these tools permits quantitative analysis of ubiquitylation sites under different experimental conditions (20–26).

In this study, we characterized the S. cerevisiae Tul1 E3 ligase complex. Here we define its subunit architecture and demonstrate that all four subunits are required for Tul1 function in Golgi protein quality control. Genetic studies indicate that Tul1 function is required in the absence of the MVB degradation pathway and under conditions of ubiquitin depletion. We used quantitative diGly proteomics to identify 3116 ubiquitylation sites in S. cerevisiae and found 10 candidate Tul1 E3 ligase substrates. Quantitative proteomics in wild-type and tu1 ∆ cells revealed that Tul1 functions in proteostasis under non-stress conditions. Collectively, these data are consistent with the proposed function for the Tul1 E3 ligase in Golgi protein quality control.

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**EXPERIMENTAL PROCEDURES**

** Yeast Strains, Plasmids, and Media — S. cerevisiae BY4741-derived strains (S288C background, [**supplemental Table S1**](#)) were generated using homologous recombination and standard molecular biology and genetic techniques (27, 28). Yeast strains were cultured in rich yeast extract peptone dextrose medium and standard molecular biology and genetic techniques (27, 28). Yeast strains were cultured in rich yeast extract peptone dextrose medium in exponential phase unless otherwise noted. Geneticin (100 μg/l, Invitrogen) and ClonNAT (100 μg/l, Werner, BioAgents, Jena, Germany) were used to select for the kanMX and natMX marker genes, respectively. Plasmids pUB221 [pCUP1-6HIS-myc-ubiquitin, 2 μg], pUB100 [ubi1-tail, CEN, HIS3], and GFP-Pep12D [P_\text{TRP1}}-\text{GFP-Pep12D, CEN, UR2A] were described previously (13, 29, 30).

**Antibody Preparation and Immunoblotting —** Hexahistidine-tagged recombinant protein antisera, Tul1 (amino acids 31–300), Dsc2 (amino acids 241–322), Dsc3 (amino acids 35–185), and Ubx3 (amino acids 265–455), were purified from Escherichia coli using Ni-NTA (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Tul1, Dsc3, Dsc3, and Ubx3 antisera were generated by Covance Inc. (New York, NY) using a standard protocol. Affinity-purified antibodies (anti-Dsc IgG) were isolated from rabbit antisera via affinity chromatography using Ubc4 (amino acids 241–322), Dsc3 (amino acids 35–185), and Ubx3 (amino acids 265–455), were purified from Escherichia coli using Ni-NTA (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Tul1, Dsc2, Dsc3, and Ubx3 antigens and the AminoLink Plus Immobilization Kit (Pierce) according to the manufacturer’s instructions. Affinity-purified anti-Tul1-HRP, anti-Dsc2-HRP, anti-Dsc3-HRP, and anti-Ubx3-HRP were generated using an EZ-Link Plus Activated Peroxidase kit (Pierce). Microsome preparation and immunoblotting was performed as described previously (8). PNGaseF treatment of microsomal extracts was performed according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA).

**Co-immunoprecipitation —** Exponentially growing cells (5 × 10⁸ cells) were washed with water and resuspended in 500 μl of immunoprecipitation buffer with 1% digitonin and supplemented with 1× Complete Protease Inhibitor - EDTA (Roche). Cell lysates were prepared by bead-beating (0.5 mm, Sigma) at 4 °C followed by the addition of 500 μl of immunoprecipitation buffer with 1.9% digitonin to raise the final digitonin concentration to 1%. Membrane proteins were solubilized by rotating lysates at 4 °C for 40 min. Non-solubilized material was removed by centrifugation at 100,000g for 10 min, and an equal amount of clarified lysates (1 mg of total protein in a 1-ml volume) was incubated with affinity-purified anti-Dsc2, anti-Dsc3, or anti-Ubx3 IgG (30 μg) for 2 h. Incubation was followed by the addition of 20 μl of recombinant protein A affinity resin (Repligen, Waltham, MA) and overnight incubation. Protein A affinity resin was then washed four times with 1 ml of immunoprecipitation buffer containing 0.1% digitonin, and bound proteins were eluted by boiling with 100 μl of SDS lysis buffer (31). Equal amounts of total and unbound fractions were immunoblotted along with 5× bound fractions.

**Real-time PCR —** Total RNA was isolated, and first-strand cDNA was synthesized as described previously (32). cDNA was quantified via real-time PCR using GoTaq qPCR Master Mix (Promega, Madison WI) and a MyiQ single-color-detection thermal cycler (Bio-Rad). Each sample was analyzed in triplicate using gene-specific primers chosen.
using Primer3 software to amplify a ~100-bp fragment upstream of the stop codon.

**Fluorescence Microscopy**—Cells were observed using a Zeiss Axioskop microscope equipped with fluorescence and Nomarski optics (Zeiss, Thornwood, NY). Images were captured with a Photometrics Cool Snap EZ CCD camera and IP Lab Spectrum software (Biovision Technologies, Inc., Exton, PA). GFP images were processed using Image J software (NIH).

**Quantitative Proteomic Analysis by SILAC**—Wild-type (JMP001) and tua1Δ strain (PEY1619) were cultivated for approximately eight generations to reach A600 of 1.0 in 500 ml of Synthetic Complete medium lacking lysine (33) that was supplemented with l-lysine (−8.0142 Da, Cambridge Isotope Laboratories, Tewksbury, MA) for light and heavy media, respectively. Differentially labeled cells were lysed separately as part of the PTMScan ubiquitin remnant motif (K-9, GlyGly) beads were washed with 1 ml of 80% ACN (0.1% FA) and eluted with 2 ml of 0.1% FA before the peptide solution was loaded. Peptides were vacuum-dried and kept at −20 °C until LC-MS/MS analysis.

**LC-MS/MS Analysis**—Dry peptides were resuspended in 9 μl of 0.1% FA, out of which 8 μl of peptide solution was loaded onto a trap column (2 cm long × 360 μm outer diameter × 75 μm inner diameter) and separated by an analytical column (20 cm long × 360 μm outer diameter × 75 μm inner diameter) packed in-house with C18 packing material (Magic C18 AQ, 3-μm size, 100-Å pore). Solvent A was 0.1% FA, and solvent B was 90% ACN (0.1% FA). Peptides were resolved with a solvent gradient generated by a Proxeon Easy-nLC system coupled online to an LTQ-Orbitrap Elite mass spectrometer. Peptides eluted were ionized by the nano-electrospray ionization at 1.8 kV, and peptide ions were introduced directly into the mass spectrometer at a flow rate of 300 nl/min of solvent with a gradient of 8% B for 1 min, 35% B for 115 min, 95% for 14 min, and 95% for 20 min with a total running time of 150 min. The mass spectrometer was operated in data-dependent acquisition mode acquiring higher-energy collisional dissociation MS/MS scans (γ = 30,000) after each precursor scan (γ = 120,000) on the 15 most abundant ions using an MS1 target of 1 × 106 ions and an MS/MS target of 5 × 104 ions. The maximum ion time used for MS/MS scans was 300 ms, the higher-energy collisional dissociation normalized collision energy was set at 32%, and the dynamic exclusion time was set at 60 s. For the whole proteome analysis, all the parameters were identical except for the nano-electrospray ionization at 2.0 kV and a total running time of 100 min.

**RESULTS**

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Mass spectrometry data were analyzed using the MaxQuant platform (1.3.0.5) (37) and searched against the NCBI Yeast Reference Protein Sequence database (downloaded July 2012) containing 6026 entries including 115 common laboratory contaminants provided by GPM using the Andromeda database search algorithm (38). The enzyme specificity was set to trypsin with up to two missed cleavages allowed. The “Lysine8” option was selected for a SILAC heavy label with a maximum of three labeled amino acids in a given peptide. The precursor mass tolerance was set to 20 ppm for the first search and 10 ppm for the main search. Variable modifications were set as follows: oxidation at methionine, GlyGly addition to lysine, and acetylation at protein N terminus, with a fixed modification set to carboxamidomethylation at cysteine. A maximum of five modifications were allowed in a given peptide. The false discovery rates at peptide, protein, and site identification levels were set at 0.01. Ubiquitylation sites with wild-type: tua1Δ ratios ≥ 2.0 were manually confirmed (Table I). Site identification and quantification values were obtained from the MaxQuant GlyGly site table. Peptide ratios were normalized such that the median equaled zero. Data analysis from the whole proteome was carried out using MaxQuant (1.4.1.2) with the same protein database used in the ubiquitylated peptide analysis. The enzyme specificity was set to LysC/P with a maximum of two missed cleavages allowed. Mass errors for precursor and fragment ions were set to 7 ppm and 20 ppm, respectively. Variable modifications were set as follows: pyro-glu at glutamine, oxidation at methionine, and acetylation at protein N terminus, with a fixed modification set to carboxamidomethylation at cysteine. The false discovery rates at both peptide spectrum match and protein levels were set at 0.01.

**Data Availability**—Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD000918 (39).

**RESULTS**

*S. cerevisiae* Tul1 E3 Ligase—Bioinformatic analysis of the *S. cerevisiae* genome revealed *S. cerevisiae* homologs of Dsc...
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A. S. cerevisiae DSC Homologs

| Gene | Tul1 | Dsc2 | Dsc3 | Ubx3 |
|------|------|------|------|------|
| Tul1 | 1    | 1    | 1    | -    |
| Dsc2 | 1    | 1    | 1    | 1    |
| Dsc3 | 1    | -    | -    | -    |
| Ubx3 | 1    | 1    | 1    | 1    |

B. DRYGIN Database Correlation Coefficients

| Gene | Tul1 | Dsc2 | Dsc3 | Ubx3 |
|------|------|------|------|------|
| Tul1 | 0.332 | 0.145 | -    | -    |
| Dsc2 | -    | 0.247 | -    | -    |
| Dsc3 | -    | -    | 0.217 | 0.145 |
| Ubx3 | 0.217 | 0.247 | 0.145 | -    |

Fig. 1. S. cerevisiae Dsc homologs. A, schematics of Dsc homologs in which black boxes represent predicted transmembrane domains. Predicted signal sequence (SS) and conserved domains are indicated. Underlining denotes amino acids used as antigens for antibody production. B, Pearson correlation coefficients between genetic signatures of S. cerevisiae DSC homologs from DRYGIN database (45). Genetic interactions were tested in a pairwise fashion such that each gene yielded two datasets. The database default setting for significance is CC > 0.1, and dashes denote the absence of a correlation coefficient in database.

Subunits. S. pombe dsc1, dsc2, dsc3, and dsc5 correspond to Tul1, YOL073C, YOR223W, and Ubx3 in S. cerevisiae (Fig. 1A). We did not identify an S. cerevisiae homolog for dsc4. We named the two uncharacterized genes YOL073C and YOR223W, Dsc2 and Dsc3, respectively (40). Tul1 is an integral membrane RING E3 ubiquitin ligase that localizes to the Golgi (13). Dsc2 is a multi-transmembrane protein with sequence similarity to Der1 and UBAC2, rhomboid pseudo-proteases that function in ERAD (12, 41–43). A conserved domain search using Dsc3 identified an N-terminal ubiquitin-like domain (Fig. 1A). Ubx3 is predicted to contain both a UAS like domain (Fig. 1A). Ubx3 is predicted to contain both a UAS domain and a C-terminal UBX domain that is known to interact with the AAA-ATPase Cdc48 (44). The DRYGIN database revealed strong genetic correlations among Tul1, Dsc2, Dsc3, and Ubx3, suggesting that these genes function in a related process (Fig. 1B) (45). In addition, comparisons of genetic interactions between S. pombe and S. cerevisiae suggested that these four proteins form a complex, and we confirmed this biochemically (40). Collectively, these data demonstrate that Tul1, Dsc2, Dsc3, and Ubx3 are subunits of the Tul1 E3 ligase in S. cerevisiae that is homologous to the S. pombe Dsc E3 ligase.

To further characterize the Tul1 E3 ligase, we analyzed protein levels in the absence of individual subunits. Tul1 was reduced in dsc2Δ, dsc3Δ, and ubx3Δ cells (Fig. 2A). Dsc3 was slightly reduced in dsc2Δ and ubx3Δ cells, but not in tul1Δ cells. Expression of Dsc2 and Ubx3 was unchanged in the mutants. Treatment of microsomal extracts with the glycosidase PNGaseF increased the mobility of Tul1, demonstrating that Tul1 is N-glycosylated (Fig. 2B). In line with this, the N terminus of Tul1 contains eight potential N-linked glycosylation sites and is predicted to localize to the secretory pathway lumen. Parallel analysis of Dsc2, Dsc3, and Ubx3 showed no change in protein mobility (data not shown). Treatment of microsomal extracts with phosphatase had no effect on the mobility of Tul1, Dsc2, Dsc3, and Ubx3, indicating that these proteins are not highly phosphorylated (data not shown). Thus, Tul1 is a glycoprotein whose expression in wild-type cells requires each subunit of the Tul1 E3 ligase.

Quantitative real-time PCR analysis showed no significant change in Tul1 mRNA between wild-type and mutant cells, indicating that Tul1 transcription was not altered (supplemental Fig. S1). Treatment of wild-type and mutant cells with the proteasome inhibitor MG-132 had no effect on Tul1 expression (Fig. 2C) but increased expression of Dsc3 in dsc2Δ and ubx3Δ cells, suggesting that Dsc3 is degraded by the proteasome in the absence of Dsc2 or Ubx3. The yeast vacuole is another principal site of protein degradation, so we assayed Tul1 levels in vacuolar protease-deficient pep4Δ cells. Deletion of PEP4 in dsc2Δ, dsc3Δ, and ubx3Δ cells restored Tul1 to wild-type levels but did not increase Dsc3 (Fig. 2D). Thus in the absence of any one E3 ligase subunit, Tul1 is degraded in a vacuole-dependent manner, whereas Dsc3 is degraded in a proteasome-dependent manner in dsc2Δ and ubx3Δ cells.

Tul1 E3 Ligase Consists of Subcomplexes—Dsc2 binds to Tul1, Dsc3, and Ubx3 when purified from detergent cell extracts (40). To confirm that these proteins constitute a single complex and to probe its architecture, we performed co-immunoprecipitation analysis for each subunit and tested the requirement of individual subunits for complex assembly. We pulled down endogenous Dsc2, Dsc3, and Ubx3 from digitonin-solubilized cell extracts and subjected them to immunoblot analysis with HRP-conjugated antibodies against Tul1, Dsc2, Dsc3, and Ubx3. We were unable to functionally epitope-tag Tul1, and Tul1 antiserum did not function for immunoprecipitation. Protein antigens are described in Fig. 1. Antibodies to Dsc2, Dsc3, and Ubx3 recovered a complex from wild-type cells that contained the four subunits (Figs. 3A–3C, lane 6), demonstrating that Tul1, Dsc2, Dsc3, and Ubx3 form a multi-subunit E3 ligase complex.

To probe complex architecture, we performed co-immunoprecipitation in subunit mutants. Dsc2 and Ubx3 formed a subcomplex in the absence of either Tul1 or Dsc3 (Figs. 3A and 3C, lanes 7 and 9). Dsc3 formed a subcomplex with Dsc2-Ubx3 that did not require Tul1 (Figs. 3A–3C, lane 7), but deletion of either Dsc2 or Ubx3 disrupted Dsc3 binding to the other subunits (Fig. 3B, lanes 8 and 10). Binding of Tul1 to any subunit required the presence of all three other subunits (Figs. 3A–3C, lanes 6–10). These data demonstrate that the Tul1 E3 ligase consists of two subcomplexes, Dsc2-Ubx3 and...
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Fig. 2. Tul1 and Dsc3 expression requires E3 ligase subunits. A, microsomal extracts from wild-type, tul1Δ, dsc2Δ, dsc3Δ, and ubx3Δ cells immunoblotted with anti-Tul1, anti-Dsc2, anti-Dsc3, and anti-Ubx3 IgG. B, microsomal extracts from wild-type, tul1Δ, dsc2Δ, dsc3Δ, and ubx3Δ cells were treated in the presence or absence of PNGase F and immunoblotted with anti-Tul1 IgG. C, indicated strains were treated with proteasome inhibitor MG-132 (100 μM) or dimethyl sulfoxide for 2 h. Microsomal extracts were prepared and immunoblotted with anti-Tul1 and anti-Dsc3 IgG. D, microsomal extracts from the indicated strains were prepared and immunoblotted with anti-Tul1 and anti-Dsc3 IgG.

Dsc2-Ubx3-Dsc3 (Fig. 3D), and that Tul1 binding to the complex occurs only once the Dsc2-Ubx3-Dsc3 subcomplex has assembled. These data are completely consistent with the genetic requirements for protein expression in Fig. 2, suggesting that the reduced expression of Tul1 and Dsc3 resulted from failure to assemble Tul1 E3 ligase subcomplexes.

Pep12D Vacuolar Sorting Requires the Tul1 E3 Ligase—The endosomal SNARE Pep12 localizes primarily to late endo-
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somes, but when overexpressed it localizes to the limiting membrane of the vacuole (46, 47). Mutant Pep12 that contains a single aspartic acid residue in its transmembrane domain (GFP-Pep12D) is sorted through the MVB pathway to the vacuolar lumen where the GFP tag accumulates (48). Sorting to the vacuolar lumen requires Tul1-dependent ubiquitylation of GFP-Pep12D (13). To test whether other subunits of the Tul1 E3 ligase are required for sorting Pep12D into the vacuolar lumen, we examined the localization of GFP-Pep12D in mutant strains. As expected, GFP-Pep12D localized to the vacuolar lumen in wild-type cells (Fig. 4A). Efficient luminal sorting required Tul1 as GFP-Pep12D accumulated on the limiting membrane of the vacuole in tul1Δ cells (Fig. 4A). Deletion of DSC2, DSC3, or UBX3 also caused GFP-Pep12D to accumulate on the vacuolar rim, indicating that these genes are also required for GFP-Pep12D ubiquitylation and proper targeting to the vacuolar lumen (Fig. 4A). Dsc2, Dsc3, and Ubx3 might not function directly in GFP-Pep12D ubiquitylation, as Tul1 expression was reduced in these mutants (Fig. 2A). These data demonstrate that Tul1, Dsc2, Dsc3, and Ubx3 form a functional E3 ligase complex required for Pep12D ubiquitylation in cells.

**Tul1 E3 Ligase Subunit Genes Show Genetic Interactions with MVB Pathway Genes**—The MVB pathway delivers cargo to the vacuole for degradation, and this process requires the sequential function of endosomal sorting complex required for transport (ESCRT) complexes 0, I, II, and III (49). Genetic interaction mapping in *S. pombe* revealed that genes showing

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**Fig. 4. Tul1 E3 ligase complex displays genetic interactions with the multivesicular body pathway.** A, indicated strains expressing GFP-tagged mutant form of Pep12 (Pep12D) were imaged using fluorescence microscopy. Staining of vacuolar lumen or limiting membrane is visible. B, indicated single and double mutant yeast strains were spotted on YPD rich medium at 37 °C and grown for 2 days. C, 5-fold serial dilutions of indicated single and double mutants carrying empty vector (pRS426) or expressing 6HIS-myc-ubiquitin (pUB221) were spotted on YPD rich medium with no added copper at 30 °C or 37 °C and grown for 2 days.
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Identification of Candidate Tul1 E3 Ligase Substrates—To date, Tul1 has been shown to ubiquitylate and target Pep12D to the MVB pathway (13). In addition, Tul1 targets the unpalmitoylated endosomal SNARE Tlg1 to the MVB pathway (14). The ability of Tul1 to recognize these engineered mutant proteins suggests that Tul1 functions in a protein quality control pathway, but whether the Tul1 E3 ligase ubiquitylates endogenous proteins is unknown. To identify Tul1 substrates, we employed a methodology to detect and quantify endogenous ubiquitylated peptides using mass spectrometry and SILAC with anti-K-GG remnant antibody enrichment (19, 20). We quantified ubiquitylated peptides from wild-type and tul1Δ cells expressing 6xHis-Myc tagged ubiquitin from the sole source of ubiquitin (Fig. 5). Wild-type and tul1Δ cells expressing 6xHis-Myc-tagged ubiquitin were grown in heavy and light lysine, respectively. Extracted proteins were mixed equally, and ubiquitylated proteins were then affinity purified using Ni-NTA under denaturing conditions. The resulting proteins were trypsin digested and incubated with an anti-K-GG remnant antibody that recognizes the diGly remnant of ubiquitin on modified lysine residues following trypsin digestion (20). Recovered peptides were quantified using LC-MS/MS.

We performed two biological replicates using cells grown exponentially in synthetic complete medium. In total, we identified 3116 distinct K-GG peptides in 1111 proteins (supplementary Table S2) and obtained a heavy-to-light (H/L) ratio in at least one experiment for 2896 ubiquitin-modified peptides (93% of the total). Multiple studies have identified ubiquitylated peptides in S. cerevisiae, with the recent application of quantitative diGly proteomics yielding thousands of ubiquitylated peptides per study (18, 22, 23). To evaluate our methodology, we compared our results to a published study that combined ubiquitylated protein enrichment followed by diGly proteomics without SILAC (22). We identified 25% more ubiquitylation sites (3116 compared with 2499) (Fig. 6A, supplementary Table S3). Interestingly, Beltrao et al. identified only 1221 of the sites identified in this study (39%), suggesting that many S. cerevisiae ubiquitylation sites remain unidentified.
Fig. 6. Identification of candidate Tu1 substrates using quantitative mass spectrometry. A. Venn diagram comparing ubiquitylation sites found in this study and by Beltrao et al. (22). B. scatter plot of ubiquitylated peptide ratios (wild-type/tul1Δ) from two replicate experiments (n = 2289). C. expression of YKL033W-A mRNA in wild-type and tul1Δ cells. cDNA synthesized from total RNA (1 μg) was quantified via real-time PCR. YKL033W-A mRNA expression was normalized to ACT1 and shown as fold change relative to wild-type cells. Data are the average of three biological replicates, and error bars show standard error among biological replicates.

Our use of SILAC allowed the quantification of peptide ubiquitylation between wild-type and tul1Δ cells. Importantly, the individual values contributing to the mean H/L ratios were highly correlated between the two replicate experiments (Fig. 6B). These results show that our methodology yielded reproducible quantitative data for thousands of ubiquitylated peptides. Among these data, we identified 173 peptides with differential ubiquitylation in wild-type versus tul1Δ cells (H/L ≥ 2.0 or H/L ≤ 0.5) (supplemental Table S4). Gene ontology searches failed to identify enrichment of particular terms (p < 0.01) for peptides with either increased or decreased ubiquitylation. To identify candidate Tu1 E3 ligase substrates, we reasoned that peptides from substrates would have reduced ubiquitylation in tul1Δ cells (H/L ≥ 2.0). Data for 10 peptides from candidate Tu1 E3 ligase substrates were manually validated and fit this criterion (Table I). Among the 10 peptides, one site was found in YKL033W-A, whose gene promoter is located immediately downstream of TUL1 (YKL034W). We hypothesized that deletion of TUL1 might affect expression of YKL033W-A, resulting in fewer YKL033W-A ubiquitylated peptides. To test this, we measured YKL033W-A mRNA expression in wild-type and tul1Δ cells via quantitative PCR. Deletion of Tu1 decreased transcription of YKL033W-A to 60% of that in wild-type cells (Fig. 6C), consistent with the reduced YKL033W-A ubiquitylation in tul1Δ cells. Although YKL033W-A is likely not a Tu1 E3 ligase substrate, this result validates the quality of our mass spectrometry dataset and demonstrates our ability to quantify small changes in protein ubiquitylation.

Among the other candidate substrates was Bul2, an arrestin-like adaptor and component of the Rsp5 ubiquitin ligase complex. Bul2 is itself ubiquitylated by Rsp5 (51, 52). Many yeast plasma membrane transporters and receptors are endocytosed and ultimately degraded through the MVB pathway in response to nutrient levels or signaling. Down-regulation of these proteins frequently requires arrestin ubiquitylation (53). We identified decreased ubiquitylation on Bul2 K540 in tul1Δ cells (Table I). In addition, we observed decreased ubiquitylation on a second residue in Bul2 K563 (H/L = 1.2, supplemental Table S5). Interestingly, we identified the corresponding residues in the homologous Bul1, but ubiquitylation was only slightly decreased in tul1Δ cells (H/L = 1.1 for each). Notably, Beltrao et al. identified these same four sites (22). These data confirm the existence of ubiquitylation sites on Bul1 and Bul2 and suggest that these residues may play a role in Rsp5-dependent regulation of plasma membrane proteins. Notably, two other arrestins, Ecm21/Art2 and Art10, are also ubiquitylated by Rsp5 (54, 55), and we detected decreased ubiquitylation of specific residues in tul1Δ cells (Ecm21/Art2 K95, H/L = 1.5; Art10 K118, H/L = 1.6). However, analysis of ubiquitylation for all 10 Art proteins revealed that Tu1-dependent ubiquitylation of arrestins was limited to Ecm21/Art2 and Art10 (supplemental Table S5).

To determine the role of the Tu1 E3 ligase in cellular proteostasis, we performed quantitative proteomics of whole cell lysates using SILAC. Wild-type and tul1Δ cells were grown in heavy and light lysine-containing media, respectively. Cell lysates were mixed, and duplicate samples were separated
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**Table I**

| Protein | Functional description | Average peptide ratio (wild type/tul1Δ) | Amino acid position (K) | Modified sequence |
|---------|------------------------|------------------------------------------|-------------------------|------------------|
| Bul2    | Component of the Rsp5p E3-ubiquitin ligase complex | 2.86 | 540 | K(gl)LDQLHINNR |
| YLR225C | Putative protein of unknown function | 2.58 | 22 | EVLSEK(gl)DHANYTK |
| Aki1    | Ser-Thr protein kinase of the Ark family | 2.25 | 940 | SSDASK(gl)SNOFK |
| YKL033W-A | Putative protein of unknown function | 2.22 | 117 | NIPIALC(TS)N(gl)K |
| Aro4    | 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase | 2.20 | 136 | GLINDPVDN(TF)N(gl)GLQSAR |
| Zeo1    | Peripheral membrane protein of the plasma membrane | 2.19 | 81 | TEAANKVEETK(gl)K |
| YPR084W | Putative protein of unknown function | 2.17 | 4 | (ac)SEK(gl)ASEEPIR |
| Rdi1    | Rho GDP dissociation inhibitor | 2.02 | 21 | (ac)AEESTDFSCFEERNNDOYK(gl)VS |
| Pal1    | Protein of unknown function thought to be involved in endocytosis | 2.02 | 291 | AAPVLAFPDGPNSTIGGASTK(gl)K |
| Bna1    | 3-hydroxyanthranilic acid dioxygenase | 2.01 | 60 | TGYHINPTEFWYQK(gl)K |

via SDS-PAGE. Proteins in gel slices were digested using LysC, and eluted peptides were quantified via LC-MS/MS. In total, we identified 1373 proteins, of which 1172 were quantified (supplemental Table S6). Of the 1373 proteins, 608 were found to be ubiquitylated proteins based on the diGly immunoprecipitation experiment (supplemental Table S7). A total of 53 proteins were found to be 2-fold differentially expressed in wild-type versus tul1Δ cells (40 up-regulated and 13 down-regulated; supplemental Table S8). Gene ontology searches failed to identify term enrichment (p < 0.01) for proteins with either increased or decreased levels between wild-type and tul1Δ cells. Using these data, we corrected the quantitative ubiquitylation data for differences in protein levels between the two strains (corrected values for 1823 sites; supplemental Table S9). Using this approach, we identified 196 peptides with differential ubiquitylation in wild-type versus tul1Δ cells (H/L ≥ 2.0 or H/L ≤ 0.5) (supplemental Table S10). Using the corrected values, we identified 16 additional Tul1-dependent ubiquitylation sites with H/L ≥ 2.0 in 11 different proteins (supplemental Table S10). Overall, correcting the quantitative ubiquitylation site data for protein expression increased the number of candidate Tul1 substrates identified.

**DISCUSSION**

In this study, we performed a detailed characterization of the *S. cerevisiae* Golgi Tul1 E3 ligase complex. Several lines of evidence support the conclusion that Tul1, Dsc2, Dsc3, and Ubx3 form a multi-subunit E3 ligase complex that is the budding yeast equivalent of the *S. pombe* Golgi Dsc E3 ligase required for activation of SREBP transcription factors (8, 9). First, each of these proteins shows sequence homology to the fission yeast proteins, and we were able to co-purify the four subunits using antibody to Dsc2, Dsc3, and Ubx3 (Fig. 3). Second, although *S. cerevisiae* lacks a homolog of *S. pombe* Dsc4, the subunit architecture of the Tul1 E3 ligase mirrors that of the Dsc E3 ligase with Dsc2-Ubx3 forming a minimal subcomplex to which Dsc3 and Tul1 bind (10). It is possible that additional Tul1 E3 ligase subunits exist and that one of these performs the functions of Dsc4 in *S. cerevisiae*. Third, Tul1 expression requires each of the other subunits (Fig. 2A). Fourth, like Tul1, vacuolar targeting of the Pep12D mutant requires DSC2, DSC3, and UBX3 (Fig. 4A). Given that Tul1 ubiquitylates Pep12D to target it to the vacuole (13), DSC2, DSC3, and UBX3 are also likely required for Tul1 E3 ligase activity. Finally, published genetic interaction data indicate that these four genes function in the same pathway (40, 45).

Tul1 localizes to the Golgi apparatus (13), and the biochemical stability of the Tul1 E3 ligase complex suggests that the other subunits will co-localize. Tul1 is degraded in a vacuole-dependent manner in the absence of complex subunits (Fig. 2D). Tul1 binds to a subcomplex of Dsc2-Dsc3-Ubx3, and degradation may result from improper trafficking to the vacuole or through an autophagic pathway in the absence of complex assembly. Dsc3 binds to the Dsc2-Ubx3 subcomplex, and its protein expression requires both of these subunits (Fig. 2A). However, Ubx3 is degraded through a proteasome-dependent pathway (Fig. 2C), possibly in the endoplasmic reticulum through ERAD. These data demonstrate that the Golgi Tul1 E3 ligase consists of subcomplexes whose proper assembly is monitored by cellular quality control mechanisms.

Tul1 was identified through its ability to ubiquitylate and target the mutant Pep12D protein to the MVB pathway (13), but the physiological function of the Tul1 E3 ligase is unknown. To investigate Tul1 E3 ligase function, we sought to identify candidate substrates. We combined enrichment of ubiquitylated proteins and diGly antibody immunopurification with SILAC to perform quantitative proteomics of ubiquitin modifications in wild-type and tul1Δ cells (Fig. 5). We identified 3116 ubiquitylation sites and quantified 2896 of these, with results similar to those of other yeast studies employing the diGly remnant antibody (22, 23). Our methodology was sensitive, and we were able to detect a 2-fold change in the ubiquitylation of Ykl033w-a that resulted from reduced ex-
pression of this gene in *tul1Δ* cells (Fig. 6C). This result validates our methodology and demonstrates its ability to detect small changes in ubiquitination.

To identify candidate Tul1 E3 ligase substrates, we focused on ubiquitination sites whose levels changed 2-fold between wild-type and *tul1Δ* cells. Using this criterion, we identified 173 peptides with differential ubiquitination in wild-type versus *tul1Δ* cells (supplemental Table S4). The majority of these (161 peptides) increased in *tul1Δ* cells, suggesting that they were not direct substrates of the Tul1 E3 ligase and resulted from altered physiology in *tul1Δ* cells. We expected direct Tul1 substrates to show reduced ubiquitination in *tul1Δ* cells and validated 10 peptides as having H/L ≥ 2.0 (Table I). Excluding Ykl033w-a, our analysis yielded nine candidate Tul1 substrates. Among these was Bul2, an arrestin-like adapter that is a component of the Rsp5 E3 ligase and a known substrate of Rsp5 itself (51, 52). Two other known Rsp5 substrates, the arrestins Ecm21/Art2 and Art10, also showed reduced ubiquitination in *tul1Δ* cells, suggesting that Tul1 is required for Rsp5 E3 ligase activity. This hypothesis will be tested in future studies.

The analysis rested on the assumption that individual protein levels were equivalent in wild-type and *tul1Δ* cells. To test this assumption, we performed quantitative proteomics using SILAC and compared protein expression in wild-type and *tul1Δ* cells. We found that 4.5% (53/1172) of quantified proteins were differentially expressed between wild-type and *tul1Δ* cells (H/L ≥ 2.0 or H/L ≤ 0.5, supplemental Tables S6 and S8), indicating that Tul1 is required to maintain cellular protein homeostasis. Given this finding, we corrected the ubiquitination site data, taking into account differences in total protein levels (supplemental Table S9). Using this approach, we identified 16 additional Tul1-dependent ubiquitination sites in 11 different proteins (supplemental Table S10). Thus, correcting for total protein expression increased the number of candidate Tul1 substrates identified. Overall, our quantitative diGly proteomics methodology identified multiple candidate substrates for the Golgi Tul1 E3 ligase. Future *in vitro* validation studies will be required in order to demonstrate that these are direct substrates.

The Dsc and Tul1 E3 ligases have been proposed to function in Golgi protein quality control and degradation (8, 13). Consistent with this idea, we found that Tul1 E3 ligase subunits showed negative genetic interactions with ESCRT genes in the MVB pathway that targets proteins for degradation in the vacuole (Fig. 4B). In addition, genes coding for the Tul1 E3 ligase are required under conditions of limiting ubiquitin caused by deletion of the deubiquitinating enzyme Doa4 (Fig. 4C). Interestingly, we identified several Tul1 substrates with functional links to the secretory pathway: Bul2, Ak1, Rdi1, Pal1, Ktr1, and Gga2 (Table I, supplemental Table S10), making these proteins promising substrates.

Despite employing a proteomic approach, we identified a limited number of candidate Tul1 substrates (Table I, supplemental Table S10). One possible explanation is that Tul1 substrates are rapidly degraded, and we quantified ubiquitination in the absence of proteasome inhibitors. During method optimization, we observed that proteasome inhibition caused the accumulation of polyubiquitin chains, and the resultant high level of ubiquitination sites on ubiquitin prevented the detection of novel sites (data not shown). Alternatively, if the Tul1 E3 ligase functions in protein quality control, we would expect only a small fraction of protein molecules to be ubiquitinated and degraded at any given moment. Thus, signals for these proteins may be low, and total protein levels may change little in *tul1Δ* cells. Indeed, only 13 proteins increased >2-fold in *tul1Δ* cells relative to wild-type (supplemental Table S8). Lastly, we performed our analysis under non-stress conditions; cells were cultured in synthetic complete medium and harvested in exponential growth phase. Under these conditions, few substrates may be channeled to Tul1 E3 ligase for degradation. Future studies will use this methodology to screen stress conditions under which Tul1 E3 ligase is required for growth, such as in ESCRT mutants.

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