Non-repair Pathways for Minimizing Protein Isoaspartyl Damage in the Yeast Saccharomyces cerevisiae*

Received for publication, March 13, 2014, and in revised form, April 14, 2014. Published, JBC Papers in Press, April 24, 2014, DOI 10.1074/jbc.M114.564385

Alexander N. Patananan‡, Joseph Capri§, Julian P. Whitelegg§, and Steven G. Clarke†1

From the ‡Department of Chemistry and Biochemistry and the Molecular Biology Institute and the §Pasarow Mass Spectrometry Laboratory, Neuropsychiatric Institute-Semel Institute for Neuroscience and Human Behavior, UCLA, Los Angeles, California 90095

The spontaneous degradation of asparaginyl and aspartyl residues to isoaspartyl residues is a common type of protein damage in aging organisms. Although the protein-L-isoaspartyl (D-aspartyl) O-methyltransferase (EC 2.1.1.77) can initiate the repair of L-isoaspartyl residues to L-aspartyl residues in most organisms, no gene homolog or enzymatic activity is present in the budding yeast Saccharomyces cerevisiae. Therefore, we used biochemical approaches to elucidate how proteins containing isoaspartyl residues are metabolized in this organism. Surprisingly, the level of isoaspartyl residues in yeast proteins (50–300 pmol of isoaspartyl residues/mg of protein extract) is comparable with organisms with protein-L-isoaspartyl (D-aspartyl) O-methyltransferase, suggesting a novel regulatory pathway. Interfering with common protein quantity control mechanisms by mutating and inhibiting the proteasomal and autophagic pathways in vivo did not increase isoaspartyl residue levels compared with wild type or uninhibited cells. However, the inhibition of metalloproteases in vitro aging experiments by EDTA resulted in an ~3-fold increase in the level of isoaspartyl-containing peptides. Characterization by mass spectrometry of these peptides identified several proteins involved in metabolism as targets of isoaspartyl damage. Further analysis of these peptides revealed that many have an N-terminal isoaspartyl site and originate from proteins with short half-lives. These results suggest that one or more metalloproteases participate in limiting isoaspartyl formation by robust proteolysis.

Because many of these modifications irreversibly lead to nonfunctional and aggregated proteins, biological pathways can be disrupted, resulting in disease (2–5). To prevent the accumulation of modified proteins, organisms have developed repair mechanisms such as the reductase and isomerase repair enzymes associated with methionine sulfoxide- and cis-proline-containing damaged proteins, respectively (1, 4, 6). We have been particularly interested in the widespread isomerization damage to aspartyl and asparaginyl residues that is also associated with a repair enzyme (2, 6, 7).

Isoaspartyl-damaged polypeptides result from the spontaneous deamidation and isomerization of asparaginyl and aspartyl amino acids, respectively, and is one of the most common types of protein damage under physiological conditions (8). Asparaginyl and aspartyl residues are hot spots for damage due to the favorable nucleophilic attack on the side chain carbonyl group by the peptide backbone nitrogen atom of the following residue (see Fig. 1). This reaction leads to the formation of an unstable succinimidyl intermediate that non-enzymatically hydrolyzes into either L-aspartyl or, to a greater extent, the abnormal L-isoaspartyl residue (8–11). The latter kinks the protein by rerouting the polypeptide backbone through the β-carbonyl instead of the α-carbonyl moiety (12). Altered proteins containing isoaspartyl residues have been linked to changes in the pharmacological efficacy of antibodies (13), p53 tumor suppressor regulation (14), autoimmunity disorders (15–19), Alzheimer disease (20, 21), and cataract formation (22–24). Because no known protease cleaves isoaspartyl linkages, aging cells can accumulate these dysfunctional proteins to unfavorable levels if left unrepaired or degraded (25).

The protein repair enzyme protein-L-isoaspartyl (D-aspartyl) O-methyltransferase (PCMT; EC 2.1.1.77) prevents the accumulation of isoaspartyl-damaged polypeptides in cells. Using the cofactor S-adenosylmethionine, PCMT methylates abnormal isoaspartyl residues and initiates a process that results in the formation of normal L-aspartyl residues as the major prod-

* This work was supported, in whole or in part, by National Institutes of Health Grants AG023203 and GM026020 (to S. G. C.) and GM007185, a Ruth L. Kirschstein National Research Service Award (to A. N. P.). This work was also supported by the Elizabeth and Thomas Plott Chair in Gerontology of the UCLA Longevity Center (to S. G. C.) and a Senior Scholar in Aging Award from the Ellison Medical Foundation (to S. G. C.).

† To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry and the Molecular Biology Instit., University of California, Los Angeles, 607 Charles E. Young Dr. East, Los Angeles, CA 90095. Tel.: 310-825-8754; Fax: 310-825-1968; E-mail: clarke@chem.ucla.edu.

‡ The abbreviations used are: PCMT, protein-L-isoaspartyl (D-aspartyl) O-methyltransferase; [3H]AdoMet, S-adenosyl-L-[methyl-3H]methionine; [14C]AdoMet, S-adenosyl-L-[methyl-14C]methionine; CSM, complete synthetic medium; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
uct (Fig. 1 and Refs. 26 and 27). This repair of isoaspartyl-containing polypeptides by PCMT is important for a diverse array of organisms. In Escherichia coli, strains deficient in PCMT are more susceptible to oxidative stress, high salt, and elevated temperature in stationary phase (28, 29). Overexpressing PCMT in E. coli (30) or Drosophila melanogaster (31) results in an increase in longevity under stress conditions. Similarly in Caenorhabditis elegans, PCMT mutant L1 larvae have decreased survival in minimal medium (32) and developmental defects with oxidative stress (33), whereas PCMT-overexpressing animals up-regulate Daf-16-dependent stress response genes and have increased heat stress survival (34, 35). Finally and most striking, homozygous PCMT knock-out mice rapidly accumulate isoaspartyl-damaged polypeptides in the brain, heart, testis, and erythrocyte cells and have fatal tonic-clonic seizures at ~42 days of age (7, 36–40). Despite being well characterized in the organisms described above and less thoroughly characterized in other animals, fungi, plants, bacteria, and archaea, PCMT appears to be absent in a few organisms, including the budding yeast Saccharomyces cerevisiae (Fig. 2).

Although PCMT is important for the repair of isoaspartyl-damaged polypeptides, there is also evidence supporting a compensatory proteolytic degradation pathway. In C. elegans, proteins of aged dauer stage animals have increased isoaspartyl content at normal and elevated temperatures (41). However, when non-viable animals are removed and only living nematodes are analyzed, no difference in isoaspartyl damage is observed, suggesting that an alternative regulation pathway is keeping damage low in mutant strains. When the isoaspartyl-containing species were characterized, the majority of damage was found in peptides rather than proteins (41). Similarly in mice, PCMT knock-out animals exhibit an increase in isoaspartyl damage in urinary peptides over time (36, 42). These data suggest that one or more proteolytic pathways can participate in the degradation and excretion of isoaspartyl-containing species in parallel with the methylation repair pathway. Thus far, only the isoaspartyl dipeptidase (IadA) in bacteria (43) and the isoaspartyl aminopeptidase (IaaA) in bacteria, cyanobacteria, plants, and animals (44–47) have been associated with the proteolytic degradation of isoaspartyl-containing di- and tripeptides. Although further evidence is lacking from other organisms, data from C. elegans and mice suggest that an isoaspartyl-specific protease targeting large polypeptides and proteins and not simply short peptides may also be responsible for removing isoaspartyl damage.

Here we show that even though a PCMT homolog or equivalent activity is not present in S. cerevisiae isoaspartyl damage is low in this organism. We show that the proteasome and autophagy pathways are not significantly involved in limiting damage. In this work, we characterize the isoaspartyl-containing polypeptides found in S. cerevisiae and provide evidence for a metalloprotease that limits isoaspartyl damage.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—The S. cerevisiae strains used in this study are listed in Table 1. Overnight cultures (5 ml) were grown in YPD (Difco, catalog number 242810; 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose) or complete synthetic medium (CSM; 0.07% (w/v) CSM powder (MP Biomedicals, catalog number 114500012), 0.17% (w/v) yeast extract...
nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, and 2% (w/v) dextrose) and used to inoculate 30-ml flasks of YPD or CSM. After culturing cells at 30 °C in a shaker at 250 rpm, the cultures were transferred to 50-ml polypropylene tubes and centrifuged at 2,000 g for 5 min at 4 °C. The supernatant was removed, the cell pellet was resuspended in phosphate-buffered saline (PBS; 13.7 mM NaCl, 0.3 mM KCl, 0.4 mM Na2HPO4, and 0.1 mM KH2PO4, pH 7.4), and the cells were centrifuged at 2,000 g for 5 min at 4 °C. After repeating the wash three times, the cells were transferred to 1.5-ml polypropylene microcentrifuge tubes and centrifuged at 2,000 g for 5 min at 4 °C. The supernatant was aspirated, and the pellet was stored at −80 °C until lysis.

**Proteasome and Autophagy Inhibition**—To determine the relationship between protein isoaspartyl content and the proteasome and autophagy pathways, pdr5Δ yeast overnight cultures in 5 ml of YPD were used to inoculate 30-ml flasks of YPD to a starting A600 of 0.1. After culturing for 8 or 24 h, cells were washed three times in PBS as described above and resuspended in 30 ml of water with 20 μM MG132 (Calbiochem, catalog number 80053-196) and/or 5 mM 3-methyladenine (Sigma, catalog number M9281). The cultures were incubated for an additional 24 h at 30 °C and 250 rpm before the cultures were centrifuged, and the pellet was washed three times with PBS. Cell pellets were stored at −80 °C until lysis. To analyze the effect of combining proteasome mutations with chemical inhibition, 5-ml overnight cultures of PUP1PRE3pdr5 and pup1pre3pdr5 cells (obtained as a gift from the William Tansey laboratory at Vanderbilt University) (48) were prepared in YPAD (YPD with 0.002% adenine hemisulfate (Sigma, catalog number A9126)) and used to inoculate 30 ml of YPAD with or without 50 μM MG132. The cultures were incubated for 8 (to 0.6–0.8 A600) or 24 h (to >5 A600) before centrifugation at 2,000 × g for 5 min at 4 °C. After washing three times with PBS, the pellets were stored at −80 °C until lysis. Finally, to test the effect of other autophagy inhibitors, BY4741 and BY4742 overnight cul-

---

**FIGURE 2.** PCMT is highly conserved in many organisms except for some Gram-positive bacteria and a few fungal species, including *S. cerevisiae*. A protein-protein BLAST search was performed comparing various organisms with human PCMT (UniProt P22061). The UniProt ID of the top ranking alignment for each organism is indicated along with their respective E-values. To construct a phylogenetic tree, a blastp protein-protein search was performed using the human PCMT protein (UniProt P22061), and the tree was constructed in MEGA5. The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the p-distance method and are in units of number of amino acid differences/site. All positions containing gaps and missing data were eliminated. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. †, the enzyme has been inferred by BLAST analysis; X, no PCMT homolog is found in the organism by BLAST analysis or by experimental evidence (49–51).
TABLE 1

S. cerevisiae strains used in this study

| Strain       | Genotype       | Biological function                          | Source                |
|--------------|----------------|---------------------------------------------|-----------------------|
| BY4741       | MATαhis3Δ1 leu2Δ0 met15Δ0 ura3Δ0             | Wild type                      | Open Biosystems     |
| BY4742       | MATαhis3Δ1 leu2Δ0 lys2Δ0 ura3Δ0             | Wild type                      | Open Biosystems     |
| LW7235       | MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ001 lys2-801 sac2Δ9 | Wild type                      | Lois S. Weisman     |
| pdr5Δ        | BY4742 background                             | Drug transport                 | Open Biosystems     |
| atg8Δ        | BY4742 background                             | Autophagy and cytoplasm-to-vacuole targeting pathway | Open Biosystems     |
| mon1A        | BY4742 background                             | Autophagy and cytoplasm-to-vacuole targeting pathway | Open Biosystems     |
| doa4Δ        | BY4742 background                             | Recycling ubiquitin from proteasome-bound ubiquitinated intermediates | Open Biosystems     |
| rpm4Δ        | BY4742 background                             | Transcription factor that stimulates expression of proteasome genes | Open Biosystems     |
| ubr2Δ        | BY4742 background                             | Cytoplasmic ubiquitin-protein ligase (E3); required for ubiquitylation of Rpn4p | Open Biosystems     |
| ubc4Δ        | BY4742 background                             | Ubiquitin-conjugating enzyme (E2); mediates degradation of abnormal or excess proteins | Open Biosystems     |
| GAC201       | MATαhis3-11 leu2-3,112; ura3-52 lys2-801 trp1-1 | Wild type                      | William Tansey      |
|              | pdr5Δ;kanMX6 pre3Δ2::HIS3 pup1Δ::leu2-HIS3 [pRS317-Pup1][YCplac22::Pre3] gal |                      | (48)                |
| GAC202       | MATαhis3-11 leu2-3,112; ura3-52 lys2-801 trp1-1 | Pre3, β1 subunit of the 20S proteasome; cleavage after acidic residues in peptides; Pup1, β2 subunit of the 20S proteasome; endopeptidase with trypsin-like activity that cleaves after basic residues | William Tansey      |

Protein extracts were also prepared from OP50 E. coli, N2 C. elegans, and PCMT-deficient mice. Briefly, overnight cultures of E. coli grown in Luria broth (Difco, catalog number 241410) were centrifuged at 5,000 × g for 5 min at 4 °C, and the pellet was washed five times with PBS as described above. C. elegans samples were obtained by washing plates of mixed populations of worms with M9 medium (3 g/liter KH2PO4, 6 g/liter Na2HPO4, 5 g/liter NaCl, and 1 ml/liter 1 M MgSO4) performing a sucrose float to separate the worms from E. coli, and finally washing the worms three times with M9 medium. Both the E. coli and C. elegans pellets were resuspended in the lysis buffer described above, freeze-thawed with liquid nitrogen, and lyed for a total of 5 min with a Sonifier cell disrupter W-350 (Branson Sonic Power Co.; 5-s sonication and 25 s on ice, continuous, 50% output, setting 4). The extracts were centrifuged at 20,000 × g for 10 min at 4 °C, and the supernatant was stored at −20 °C until analysis. Protein extracts from mouse brains deficient of PCMT were obtained as described previously (36).

Protein concentrations were determined by Lowry assays after precipitation with 10% trichloroacetic acid. The assays were performed in duplicate with 2–5 μl of extract, and bovine serum albumin served as the standard.

Quantification of Isoaspartyl Protein Damage—We used the human recombinant PCMT protein as an analytical reagent to quantitate isoaspartyl residue damage in yeast proteins and peptides. This enzyme has a high activity with peptides and proteins containing 1-isoaspartyl residues and a lower activity for those containing d-aspartyl residues (9). In a volume of 100 μl, 4–130 μg of cytosolic S. cerevisiae extract was incubated in 135 mM Bis-Tris-HCl, pH 6.4, 5 μM of recombinant human PCMT (purified from E. coli as described in Dai et al. (42), approximate concentration of 1 mg/ml, specific activity of 9,682 pmol of methyl esters formed on ovalbumin at 37 °C/min/mg of protein), and 10 μM S-adenosyl-L-[14C]methionine ([14C]AdoMet) (PerkinElmer Life Sciences; 47 mCi/ml in 10 mM H2SO4:ethanol (9:1, v/v)) for 2 h at 37 °C. After the incubation, 10 μl of 2 M sodium hydroxide was added to the reaction, and 100 μl of the sample was immediately transferred to a 9 × 2.5-cm piece of thick filter paper (Bio-Rad, catalog number 1650962) suspended above 5 ml of scintillation reagent (Safety Solve, Research Products International, catalog number 111177) in a sealed 20-ml scintillation vial at room temperature (Research Products International, catalog number 121000). After a 2-h incubation, the filter paper was removed, and each individual sample was counted three times for 3 min each in a Beckman LS6500 scintillation counter to determine the average radioactivity. Each sample was assayed in duplicate, and the reported data represent an average of the two radioactive measurements. Background radioactivity was determined by incubating recombinant human PCMT with lysis buffer, 135 mM Bis-Tris-HCl buffer, and 10 μM [14C]AdoMet as described above and was subtracted from the value obtained in experimental samples.
Processing of L-Isoaspartyl-containing Proteins in Yeast

Characterization of Isoaspartyl-damaged S. cerevisiae Proteins by SDS-PAGE—In a volume of 30 µl, 5 µg of protein extract was incubated with 1 µg of recombinant human PCMT, 0.3 µM S-adenosyl-L-[methyl-3H] methionine ([3H]AdoMet) (PerkinElmer Life Sciences; 75–85 Ci/mmol, 0.55 mCi/ml in 10 mM H2SO4-ethanol (9:1, v/v)), and 80 mM Bis-Tris-HCl buffer, pH 6.4 for 2 h at 37 °C. The reaction was quenched by adding 5 µl of SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.05% (w/v) bromophenol blue). The samples were heated at 100 °C for 3 min and separated on a 4–12% Bis-Tris NuPAGE gel (Invitrogen, catalog number NP0323BOX) with NuPAGE MES SDS running buffer (Invitrogen, catalog number NP0002) at 200 V for 1 h. The gels were stained with Coomassie (0.1% (w/v) Brilliant Blue R-250, 10% (v/v) glacial acetic acid, and 5% (v/v) methanol) and the protein bands were imaged using an Alpha Imager 2200 (Alpha Innotech Corp.). To visualize the location of isoaspartyl-damaged proteins by fluorography, the gels were treated with EN3HANCE (PerkinElmer Life Sciences, catalog number 6NE9701) for 1 h, incubated in water for 30 min, and dried before the gels were exposed to film (Denville Scientific, 8 × 10-inch Hyblot Cl) for 5–7 days at −80 °C.

In Vitro Aging of S. cerevisiae Extracts—Extracts of BY4742 yeast cultured for 1 and 7 days in YPD were prepared as described above. Yeast extract (25 µg of protein) was diluted with 50 mM Tris-HCl, pH 7.5 to a total volume of 10 µl. To this, 40 µl of one of the following buffers was added: 1) 50 mM Tris-HCl, pH 7.5; 2) 50 mM Tris-HCl, pH 7.5 and 2 mM sodium EDTA, pH 7.5; 3) 50 mM Tris-HCl, pH 7.5 and 0.7 mM PMSF; 4) 50 mM Tris-HCl, pH 7.5 and 2 µg/ml aprotinin from bovine lung (Sigma, catalog number A1153, 10.7 trypsin inhibitor units/mg of solid); 5) 50 mM Tris-HCl, pH 7.5 and 21 µg leupeptin hemisulfate salt (Sigma, catalog number L2884); 6) 50 mM Tris-HCl, pH 7.5 and 100 µg/ml soybean trypsin inhibitor (Calbiochem, catalog number 65035); 7) 50 mM Tris-HCl, pH 7.5 and a Roche Applied Science protease inhibitor tablet (one tablet/25 ml); and 8) 50 mM Tris-HCl, pH 7.5, a Roche Applied Science protease inhibitor tablet (one tablet/25 ml), and 0.7 mM PMSF. The samples were placed in a 37 °C incubator for specific periods of time. The reactions were stopped by storing the samples at −80 °C until use.

Trifluoroacetic Acid Precipitation of S. cerevisiae Extracts—To compare isoaspartyl damage in the peptides and proteins, 5-day in vitro aged S. cerevisiae extracts obtained from 1- or 7-day cultures were incubated with a final concentration of 5% trifluoroacetic acid for 20 min at room temperature. The samples were centrifuged at 20,000 × g for 10 min, and the supernatant was transferred to a new vial. Both the supernatant and the pellet fractions were dried under vacuum centrifugation. 25 µl of 0.2 M Bis-Tris-HCl, pH 6.4 buffer was added to each sample. lysozyme (14 kDa), and aprotinin (6 kDa). Fluorography was performed by treating the gel with EN3HANCE and exposing the dried gel to film for 7 days at −80 °C. The arrow denotes the position of recombinant human PCMT and its automethylation band at −30 kDa.

FIGURE 3. S. cerevisiae does not have PCMT activity. Strain BY4742 was cultured in YPD for 1, 3, or 7 days (d) at 30 °C, and extracts were prepared as described under “Experimental Procedures.” Briefly, 25 µg of extract protein with or without 5 µg of recombinant human PCMT (rhPCMT) was incubated with or without 50 µM isoaspartyl-containing peptide KASA(isoD)LAKY in the presence of 10 µM [3H]AdoMet for 2 h at 37 °C. Technical duplicates for each condition were analyzed, and each point represents the average of three independent experiments. The horizontal bars indicate the average, and the error bars represent S.D. of three experiments. The fluorographs were performed as described in “Experimental Procedures.”

In Vitro Aging of S. cerevisiae Extracts—Extracts of BY4742 yeast cultured for 1 and 7 days in YPD were prepared as described above. Yeast extract (25 µg of protein) was diluted with 50 mM Tris-HCl, pH 7.5 to a total volume of 10 µl. To this, 40 µl of one of the following buffers was added: 1) 50 mM Tris-HCl, pH 7.5; 2) 50 mM Tris-HCl, pH 7.5 and 2 mM sodium EDTA, pH 7.5; 3) 50 mM Tris-HCl, pH 7.5 and 0.7 mM PMSF; 4) 50 mM Tris-HCl, pH 7.5 and 2 µg/ml aprotinin from bovine lung (Sigma, catalog number A1153, 10.7 trypsin inhibitor units/mg of solid); 5) 50 mM Tris-HCl, pH 7.5 and 21 µg leupeptin hemisulfate salt (Sigma, catalog number L2884); 6) 50 mM Tris-HCl, pH 7.5 and 100 µg/ml soybean trypsin inhibitor (Calbiochem, catalog number 65035); 7) 50 mM Tris-HCl, pH 7.5 and a Roche Applied Science protease inhibitor tablet (one tablet/25 ml); and 8) 50 mM Tris-HCl, pH 7.5, a Roche Applied Science protease inhibitor tablet (one tablet/25 ml), and 0.7 mM PMSF. The samples were placed in a 37 °C incubator for specific periods of time. The reactions were stopped by storing the samples at −80 °C until use.

Trifluoroacetic Acid Precipitation of S. cerevisiae Extracts—To compare isoaspartyl damage in the peptides and proteins, 5-day in vitro aged S. cerevisiae extracts obtained from 1- or 7-day cultures were incubated with a final concentration of 5% trifluoroacetic acid for 20 min at room temperature. The samples were centrifuged at 20,000 × g for 10 min, and the supernatant was transferred to a new vial. Both the supernatant and the pellet fractions were dried under vacuum centrifugation. 25 µl of 0.2 M Bis-Tris-HCl, pH 6.4 buffer was added to each sample. lysozyme (14 kDa), and aprotinin (6 kDa). Fluorography was performed by treating the gel with EN3HANCE and exposing the dried gel to film for 7 days at −80 °C. The arrow denotes the position of recombinant human PCMT and its automethylation band at −30 kDa.
ple, and isoaspartyl content was quantified by the vapor diffusion assay described above.

**HPLC Analysis of in Vitro Aged S. cerevisiae Extracts**—1- and 7-day in vitro aged extracts were prepared and precipitated with trifluoroacetic acid as described previously except that instead of vacuum centrifugation 100 μl of the supernatant fractions was transferred to HPLC vials and separated using an HP 1090 II liquid chromatograph and a Grace Vydac 218 MS polymeric C18 analytical HPLC column (5 μm, 4.6-mm inner diameter, 250-mm length) at room temperature and a flow rate of 1 ml/min. Solvent A consisted of 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The HPLC gradient was as follows: isocratic for 5 min at 100% A, a linear gradient for 30 min from 100% A to 5% A, 5 min at 5% A, a linear gradient for 1 min from 5% A to 100% A, and isocratic for 19 min at 100% A. 1-ml fractions were collected throughout the run, and 500 μl of each fraction was dried by vacuum centrifugation. These samples were resuspended in 25 μl of 0.2 M Bis-Tris-HCl, pH 6.4 and analyzed for isoaspartyl content by the vapor diffusion assay as described above.

**Nano-liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Analysis**—Nano-LC-MS/MS with collision-induced dissociation was performed on an Orbitrap XL (Thermo Fisher, Waltham, MA) integrated with an Eksigent nano-LC instrument. A reverse-phase column (75 μm × 200 mm containing 5-μm C18 resin with 300-Å pores (AcuTech, San Diego, CA)) was used for peptide chromatography. Electro-spray ionization conditions using the nanospray source (Thermo Fisher) for the Orbitrap were set as follows: capillary temperature at 220 °C, tube lens at 110 V, and spray voltage at 2.3 kV. The flow rate for reverse-phase chromatography was 500 nl/min for loading and analytical separation (buffer A, 0.1% formic acid and 3% acetonitrile; buffer B, 0.1% formic acid and 100% acetonitrile). Peptides were loaded onto the column for 30 min and resolved by a gradient of 0–40% buffer B over 180 min. The Orbitrap was operated in data-dependent mode with a full precursor scan at high resolution (60,000 at m/z 400) and 10 MS/MS experiments at low resolution in the linear trap with a scan range of 350–2,000 m/z. For collision-induced disso-
tion, the intensity threshold was set to 500 counts, and a collision energy of 40% was applied. Dynamic exclusion was set with a repeat count of 1 and exclusion duration of 30 s.

Database searches of the acquired spectra were analyzed with Mascot (v2.4; Matrix Science). The NCBI non-redundant database (December 26, 2013; 35,149,712 sequences;
12,374,887,350 residues) was used and limited to S. cerevisiae. The search parameters used were as follows: no enzyme cleavage, no missed cleavages, no fixed modifications, deamidated (Asn and Gln) and acetyl (N-terminal) variable modifications, monoisotopic mass values, unrestricted protein mass, ±50 ppm peptide mass tolerance, and ±0.8-Da fragment mass tolerance. With these parameters, an overall peptide false discovery rate of 9.35% (or 10 decoy peptide matches above identity threshold versus 107 matches in the NCBI nr) was obtained against a decoy database.

Proteins were confirmed from the Mascot entries if they had at least one peptide designated in bold and red with an ion score of at least 20 and ppm error no greater than 13. The N and C termini of both non-isooaspartyl and isoaspartyl-containing peptides were compared using the criteria established for peptides. Four amino acids from both the N and C termini were inputted into the University of California, Berkeley WebLogo server for each unique peptide, and a frequency plot was obtained.

RESULTS

S. cerevisiae Does Not Have a PCMT Homolog or Equivalent Activity—To determine whether alternative mechanisms exist to remove isoaspartyl protein damage other than by PCMT methylation repair, we first wanted to identify an organism that lacked PCMT. We identified the closest protein match to human PCMT in a diverse array of organisms by BLAST searching and phylogenetic tree analysis (Fig. 2). Although PCMT activity and homologous proteins have been previously observed in many animals, plants, fungi, archaea, and bacteria, PCMT was found to be absent in Amphisidinium carterae (dinoflagellate) (49), Bacillus subtilis (Gram-positive bacteria) (50), Mycoplasma genitalium (Gram-positive bacteria) (51), Synedra ulna (diatom) (49), and S. cerevisiae (Fig. 2). In these organisms, the closest match to PCMT is not a protein repair methyltransferase but rather an unrelated protein such as elongation factor 2 (A. carterae), 2-heptaprenyl-1,4-naphthoquinone methyltransferase (B. subtilis), DNAJ-like protein (M. genitalium), photosystem II reaction center protein (S. ulna), and the sterol 24-C-methyltransferase (S. cerevisiae). These data confirm previous studies that also found a lack of PCMT homolog and/or enzymatic activity in these organisms (6, 49–51).

Because S. cerevisiae is a frequently used model organism and knowledge of isoaspartyl damage in fungi is lacking, we were interested in determining whether S. cerevisiae had endogenous PCMT activity despite lacking a homologous enzyme. We incubated an isoaspartyl-containing peptide, KASA(L-isoD)PKRR, with S. cerevisiae extracts to determine whether it would be methylated by an enzyme with PCMT activity. When incubating the peptide with extracts derived from S. cerevisiae cultured for 1, 3, or 7 days, representing young, middle-aged, and old stage cells, respectively, no isoaspartyl methylation was observed compared with controls (Fig. 3A). However, methylation was observed in reactions of these extracts with recombinant human PCMT, confirming the presence of isoaspartyl residues in S. cerevisiae polypeptides and indicating the absence of inhibitory factors (Fig. 3A). When S. cerevisiae extracts were incubated with [3H]AdoMet, separated by SDS-PAGE, and analyzed by fluorography, no radioactive bands were found (Fig. 3B). However, when these extracts were incubated with recombinant human PCMT, a limited number of protein bands were present, confirming again the existence of isoaspartyl-damaged species (Fig. 3B).

S. cerevisiae Has Low Isoaspartyl Damage Levels Despite Lacking PCMT—To quantify the amount of isoaspartyl residues in cells, we cultured S. cerevisiae for 1, 3, or 7 days in either YPD or CSM medium and used recombinant human PCMT as an analytical probe to determine the number of methyl-accepting isoaspartyl substrates. The amount of damage observed ranged from 165 to 295 pmol of isoaspartyl residues/mg of total protein extract for the 1- and 7-day cultures, respectively, with similar levels for cells cultured in either YPD or CSM medium (Fig. 4). Other rich and minimal medium conditions were also tested, including medium based on lactate or lacking ammonium salts, but no significant differences were observed in isoaspartyl levels (data not shown). When compared with extracts of other organisms that contain PCMT such as E. coli, C. elegans, and Mus musculus, the levels of isoaspartyl damage observed in S. cerevisiae are strikingly low. For example, E. coli cultured for 1 or 11 days in Luria-Bertani broth have ~450 and 2,000 pmol of isoaspartyl residues/mg of protein, respectively, and the removal of PCMT and its cotranscribed surE gene results in an increase to 3,500 pmol of isoaspartyl residues/mg of protein in 11-day cultures (28). In C. elegans, 14- and 31-day-old dauer cultures have 453 and 1,115 pmol of isoaspartyl residues/mg of extract, respectively, and animals deficient in PCMT have 511 and 1,890 pmol of isoaspartyl residues/mg.
protein, respectively (53). Finally, in mice, isoaspartyl damage in the brain is 200 and 1,100 pmol/mg of protein in wild type and PCMT-deficient animals, respectively (36).

We hypothesized that isoaspartyl damage could be limited in *S. cerevisiae* due to a lower content of deamidation/isomerization-susceptible asparagine and aspartate residues. Thus, we analyzed the frequency of each amino acid in *S. cerevisiae*. We found that the frequency of these two amino acids in *S. cerevisiae* is comparable, if not greater, than that seen in *Homo sapiens*, *M. musculus*, and *E. coli* (Fig. 5A). Next, we asked whether there was a decrease in the amount of four sequences known to rapidly convert to isoaspartyl residues: asparagine-glycine (NG), asparagine-serine (NS), asparagine-cysteine (NC), and asparagine-alanine (NA) (10). In *S. cerevisiae*, the percentage of proteins containing at least one NG, NS, NC, or NA was 68.9, 75.0, 26.9, and 65.9%, respectively, and was comparable with other organisms (Fig. 5B).

**The Proteasome and Autophagy Pathways Do Not Control Isoaspartyl Damage in *S. cerevisiae***—The proteasome and the autophagy pathways are two of the most important protein quality control mechanisms in the cell. The proteasome, which is responsible for 80–90% of protein degradation in mammals (54), is associated with the removal of 40–60% of short-lived proteins (55) and removal of denatured and oxidatively damaged proteins (58–60). Conversely, the autophagy pathway largely degrades long-lived proteins (61, 62) and protein aggregates (63, 64). Because isoaspartyl damage kinks proteins and may result in protein aggregation, we hypothesized that the proteasome and/or autophagy pathways could remove this type of damage in *S. cerevisiae*, and reducing the activity of these pathways would result in an increase in isoaspartyl-damaged species. To test this hypothesis, we analyzed the effects of proteasome and autophagy inhibitors on pdr5Δ *S. cerevisiae*, which are deficient
in an ATP-binding cassette multidrug transporter involved in removing drugs from inside the cell (65, 66). pdrΔ cells treated with the proteasome peptide aldehyde inhibitor MG132 (10) and/or the autophagy inhibitor 3-methyladenine (67) showed no significant increase in isoaspartyl damage when compared with the controls (Fig. 6A). Recent studies have shown that although the inhibition of proteasome activity by MG132 targets mainly the chymotryptic activity of the proteasome the trypsin and caspase-like activities imparted by the Pup1 and Pre3 proteases are still present (48). Therefore, we treated pup1pre3pdrΔ S. cerevisiae with MG132 to see whether there was an increase in isoaspartyl damage. Consistent with our previous results, there was no statistically significant increase in isoaspartyl residue levels when incubating the cells for 8 or 24 h with the inhibitor (Fig. 6B). Likewise, although 3-methyladenine is a commonly used autophagy inhibitor, recent research has shown that it can also promote autophagy when cells are incubated in rich conditions (68). Therefore, we tested two other autophagy inhibitors, concanamycin A and ammonium chloride (69), to see whether these inhibitors would increase isoaspartyl levels (Fig. 6C). Once again, no significant increase in isoaspartyl damage was observed when compared with the controls even when the cells were incubated with the inhibitors for 7 days (Fig. 6C). Finally, we tested mutants associated with the proteasome and autophagy pathways to further verify our inhibition studies (Fig. 6D). For the proteasome, we tested cells deficient in Rp4, a transcription factor responsible for the expression of proteasome genes (70, 71); Ubr2, an E3 ligase that regulates Rp4 (72, 73); and Doa4, a deubiquitinating enzyme associated with recycling the ubiquitin pool in cells (74). We also analyzed cells deficient in Atg8, Atg18, and Mon1, proteins necessary for autophagy (75, 76). Once again, comparing the mutant strains with the control BY4742 cells indicated no increase in isoaspartyl damage after 7 days of culture (Fig. 6D).

In Vitro Aged S. cerevisiae Extracts Accumulate Isoaspartyl Residues at Lower Rates than Those of Other Organisms—We next investigated in vitro formation of isoaspartyl residues in S. cerevisiae extracts. We incubated extracts derived from cells cultured 7 days for various periods of time and found that isoaspartyl residue content increased linearly from ~89 to 849 pmol of isoaspartyl residues/mg of protein over the course of 11 days (Fig. 7). Although the initial amount of isoaspartyl damage is similar to that observed in extracts of a mixed population of C. elegans and in stationary phase E. coli, the rate of isoaspartyl residue accumulation in stationary phase extracts of S. cerevisiae is lower than that found for the worm and bacterial extracts. We also found a lower rate of accumulation in brain extracts derived from PCMT-deficient mice (Fig. 7). These results suggest that an active mechanism such as a proteolytic pathway may be limiting the amount of isoaspartyl residues in S. cerevisiae.

In Vitro Aged S. cerevisiae Extracts Incubated with EDTA Rapidly Accumulate Isoaspartyl Residues in Low Molecular Weight Polypeptides—To test whether proteolysis limits isoaspartyl damage in S. cerevisiae, both 1- and 7-day culture extracts were in vitro aged in Tris buffer alone or Tris buffer with protease inhibitors of different types, including cysteine (leupeptin), serine (PMSF, aprotinin, leupeptin, and soybean trypsin inhibitor), and metallo- (EDTA) protease inhibitors. Additionally, samples were incubated with a commercial protease inhibitor mixture from Roche Applied Science. When we quantified the level of isoaspartyl residues in extracts of 1-day cultures in vitro aged over the course of 6 days, no difference in the rate of accumulation was observed when comparing the control buffer sample with those incubated with PMSF, aprotinin, leupeptin, and soybean trypsin inhibitor (Fig. 8A). However, extracts aged in the presence of EDTA or the Roche Applied Science inhibitor mixture resulted in an increased accumulation of isoaspartyl-containing species (Fig. 8A). Analysis of these isoaspartyl species using SDS-PAGE (Fig. 8C) and fluorography (Fig. 8E) showed an increase in isoaspartyl residue damage in polypeptides of about 6 kDa and smaller for only the samples aged with EDTA and the Roche Applied Science inhibitor mixture. These results were reproduced using S. cerevisiae cultured for 7 days and in vitro aged under the same conditions as for the 1-day cultures (Fig. 8, B, D, and F).

Interestingly, the Roche Applied Science inhibitor tablets contain EDTA, and only extracts in vitro aged in the presence of EDTA had an increase in isoaspartyl residues. Because EDTA is a metalloprotease inhibitor, we hypothesized either that a metalloprotease is specifically degrading isoaspartyl sites in damaged polypeptides or that a nonspecific metalloprotease is degrading polypeptides before isoaspartyl residues have a chance to form. Inhibiting the metalloprotease, therefore, would result in an increase in damaged peptides. To confirm the presence of an increased amount of isoaspartyl-damaged peptides, protein extracts derived from 1- and 7-day S. cerevisiae cultures were in vitro aged for 5 days, and trifluoroacetic acid precipitation was used to separate peptides from proteins.

FIGURE 9. In vitro aged extracts incubated with EDTA have an increased amount of isoaspartyl-containing peptides. To determine the amount of isoaspartyl damage in the peptides and proteins of in vitro aged S. cerevisiae samples, we aged 15 μg of BY4742 cytosolic extract derived from 1- or 7-day (d) cultures with or without EDTA as described under “Experimental Procedures.” After aging for 5 days, trifluoroacetic acid precipitation was used to separate protein and peptide fractions as described under “Experimental Procedures.” Isoaspartyl residues were quantified for isoaspartyl residues via vapor diffusion assay by incubating 25 μl of sample with 5 μg of recombinant human PCMT and 10 μl [14C]AdoMet as described under “Experimental Procedures.” Each sample was analyzed once with each bar representing the average of three radioactivity measurements.
Although there was a slight increase in isoaspartyl-damaged proteins in the presence of EDTA for both the 1- and 7-day extracts (less than 2-fold), a large increase (about 15-fold) in isoaspartyl species was observed in the peptide fraction of samples incubated with the metalloprotease inhibitor (Fig. 9).

Isoaspartyl Damage in S. cerevisiae Is Ubiquitous, and N-terminal Isoaspartyl-containing Peptides Accumulate in the Presence of EDTA—To isolate isoaspartyl-damaged peptides, the trifluoroacetic acid supernatants of 1- and 7-day S. cerevisiae extracts in vitro aged for 5 days were separated by reverse-phase HPLC. In the absence of EDTA, both the extracts from the 1- (Fig. 10A) and 7-day (Fig. 10B) cultures did not show a significant amount of isoaspartyl damage in the HPLC fractions. However, in the presence of EDTA, an increase in damaged polypeptides eluting between ~16 and 20 min was observed for the 1- (Fig. 10C) and 7-day (Fig. 10D) culture extracts. To identify the isoaspartyl species isolated by HPLC, the material in fractions 17, 18, and 19 was analyzed by tandem mass spectrometry. In the samples not treated with EDTA, no peptides were found that matched yeast proteins under our stringent criteria. However, for the samples incubated with EDTA, a number of peptides derived from yeast proteins were detected (Tables 2 and 3). In the aged extract from the 1-day culture, peptides derived from pyruvate kinase, enolase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate decarboxylase, and the Ran GTPase were found to have deamidated asparagine sites consistent with the presence of isoaspartyl residues (Table 2). In the aged extract from the 7-day culture, a similar set of proteins was found to contain deamidated asparagine residues with the addition of Pol1, a subunit of the DNA polymerase 1–primase complex (Table 3). With the exception of Pol1, all the deamidated proteins observed were in the top 10% of abundant proteins in S. cerevisiae as calculated by the comprehensive protein abundance database PaxDb (77). Additionally, many of the deamidated peptides observed were from proteins with relatively short half-lives such as enolase, hexokinase, pyruvate decarboxylase, and glyceraldehyde-3-phosphate dehydrogenase, which have half-lives of 27, 42, 29, and 85 min, respectively, as determined previously by Belle et al. (78) (Tables 2 and 3).

To determine whether there is a metal ion-dependent isoaspartyl-specific protease activity in yeast, we analyzed the amino acids.
acid sequences of the peptides identified by mass spectrometry in the extracts aged in the presence of EDTA (Fig. 11). Peptides showing evidence for deamidation at coded asparagine residues are expected to contain largely isoaspartyl residues at that site, although aspartyl residues may also be found in lower amounts. For peptides with no deamidation sites, non-polar aliphatic
### TABLE 3
Deamidation sites in in vitro aged 7-day extracts incubated with EDTA

| Deamidation sites in in vitro aged 7-day extracts incubated with EDTA |
|-----------------|-----------------|----------------|-----------------|
| Glycolysis, glyoxylate cycle, gluconeogenesis, tricarboxylic acid cycle, and electron transport |
| Aco1 Aconitase | 340 | 595 | 72 |
| Acs1 Acetyl-CoA synthetase | 2,944 | 12.8 | 29 |
| Adh1/Adh2 Alcohol dehydrogenase | 11/124 | 8,179/1,010 | ND/41 |
| Atp3 Subunit of the F1 sector of mitochondrial F1F0 ATP synthase | 443 | 384 | Stable |
| Cdc19 Pyruvate kinase | 9 | 12,228 | ND |
| Eno1 Enolase | 16/1 | 7,491/24,563 | ND/27 |
| Fba1 Fructose -1,6-bisphosphate aldolase | 3 | 21,368 | 104 |
| Gpm1 Phosphoglycerate mutase | 18 | 6,668 | Stable |
| Hxk1/Hxk2 Hexokinase | 9 | 12,228 | ND |
| Icl1 Isocitrate lyase | 3,353 | 7.55 | ND |
| Idh1 Isocitrate dehydrogenase | 499 | 320 | 29 |
| Mls1 Malate synthase | 1,675 | 46.2 | ND |
| Pck1 Phosphoenolpyruvate carboxykinase | 2,059 | 31.6 | ND |
| Pgk1 3-Phosphoglycerate kinase | 5 | 16,232 | 199 |
| Qcr2 Subunit 2 of ubiquinol cytochrome-c oxidase | 591 | 259 | ND |
| Tdh1/Tdh2/Tdh3 Glyceraldehyde-3-phosphate dehydrogenase | 48/15/2 | 2,792/7,519/22,369 | 85/ND/ND |
| Alcoholic fermentation |
| Fdc1/Pdc5/Pdc6 Pyruvate decarboxylase | 4 | 18,671 | 29 |
| Ribosomal proteins and translation |
| Efb1 Translation elongation factor 1β | 61 | 2,542 | ND |
| Gru1 Cytoplasmic and mitochondrial glycyl-tRNA synthase | 273 | 878 | 44 |
| Hyp2 Translation elongation factor eIF5A | 167 | 1,484 | ND |
| Rpp0 Ribosomal protein P0 of the ribosomal stalk | 117 | 1,872 | ND |
| Rps5 Ribosomal 40 S subunit protein 5S | 30 | 3,476 | ND |
| Sbi6/Sbi2 Ribosome-associated molecular chaperone | 77/49 | 2,320/2,791 | 149/712 |
| Te72 Translation elongation factor EF-1α | 8 | 12,435 | 30 |
| Tma19 Associates with ribosomes | 98 | 2,078 | 166 |
| Yef3 Subunit of translational elongation factor eEF1B | 17 | 6,951 | 191 |
| Cofactor, purine, pyrimidine, and amino acid biosynthesis |
| Adk1 Adenylate kinase | 97 | 2,078 | 217 |
| Gln1 Glutamine synthetase | 71 | 2,389 | 73 |
| Hom6 Homoserine dehydrogenase | 270 | 885 | 56 |
| Rnr4 Ribonucleotide-diphosphate reductase small subunit | 140 | 1,681 | 372 |
| Ura1 Dihydroorotate dehydrogenase | 118 | 1,871 | 203 |
| Ura2 Carbamoylphosphate synthetase-aspartate transcarbamylase | 383 | 494 | 22 |
| Protein folding, sorting, and translocation |
| Hsp26 Small heat shock protein with chaperone activity | 366 | 530 | Stable |
| Hsp90 chaperone | 90 | 2,117 | ND |
| Disaggregase | 304 | 721 | 61 |
| Snr1/Snr2/Snr3 ATPase involved in protein folding; Hsp70 family | 12/13/547 | 8,178/7,986/290 | 77/ND/59 |
| Sce1 Constituent of the TIM23 complex; Hsp70 family | 306 | 709 | ND |
| Sce2 ATPase component of heat shock protein Hsp90 chaperone complex | 106 | 1,959 | 101 |
| Vacular acidification and proteases |
| Apc3 Vacular aminopeptidase Y | 512 | 309 | 9 |
| Pep4 Vacular aspartyl protease | 652 | 225 | ND |
| Vma3 Subunit of vacuolar H+-ATPase | 238 | 1,045 | 29 |
| Stress adaptation and viability |
| Cpr1 Cytoplasmic peptidyl-prolyl cis-trans isomerase | 55 | 2,661 | Stable |
| Cpr2 Cytoplasmic peptidyl-prolyl cis-trans isomerase | 2,401 | 22.8 | 33 |
| Dak1 Dihydroxyacetone kinase | 508 | 312 | 42 |
| Gnd1 6-Phosphogluconate dehydrogenase | 27 | 3,865 | ND |
| Pat2 Similar to flavodoxin-like proteins | 427 | 411 | 40 |
| Sub2 Component of the transcription export (TREX) complex required for nuclear mRNA export | 462 | 361 | 17 |
| Trx1 Cytoplasmic thioredoxin | 305 | 715 | ND |
| Uga1 γ-Aminobutyrate transaminase | 1,006 | 110 | 67 |
| Transcription factors, DNA replication, nuclear localization, cell cycle, spor development |
| Gsp1 Ran GTase | 403 | 446 | ND |
| Pol1 Subunit of the DNA polymerase Iα-primase complex | 3,102 | 10.3 | Stable |
| Vka2 Forms a kinesin-14 heterodimeric motor with Kar3 | 5,569 | 0.54 | ND |

---

* PaxDb integrated data set for protein abundance, rank out of 6,153 proteins (77).

* Protein half-life data from Belle et al. (78). ND, no data.

* Deamidation sites are underlined.

* It is unclear whether this peptide represents the unmodified peptide of Pdc1 or the deamidated form of Pdc5.
amino acids were predominant at the N and C termini (Fig. 11, A and B). For peptides with evidence for deamidation of asparagine residues, the C-terminal amino acid sequences were similar to those of the non-deamidated peptides. However, we found that the their N-terminal residues contained a disproportionate frequency of deamidated asparagine residues compared with the non-damaged proteins, suggesting that these may represent isoaspartyl residues that are not cleaved in the absence of one or more metalloproteases (Fig. 11, C and D). Of the 13 deamidated peptides identified from the 1-day culture, six peptides demonstrated deamidation at the N-terminal position (Table 2 and Fig. 11C). Likewise, of the 10 deamidated peptides identified from the 7-day culture, five peptides were deamidated at the N-terminal position (Table 3 and Fig. 11D). Combined, these data suggest that metalloprotease activity is required to fully degrade peptides containing isoaspartyl damage. Thus, such activity represents a crucial part of the proteolytic pathway to limit the accumulation of isoaspartyl residues in yeast cells that do not have the repair pathway.

**DISCUSSION**

Previous work investigating the importance of isoaspartyl repair focused on several key model organisms, including *E. coli* (28–30), *Arabidopsis thaliana* (79–81), *C. elegans* (32–35, 41, 53), *D. melanogaster* (31, 82), and mice (7, 36–39). However, little is known about the role of PCMT in fungi other than in the observation of PCMT activity in mushroom (83) and the presence of deamidated asparagine in glutamate dehydrogenase isoenzymes (84). This study is the first to quantify isoaspartyl residues in a fungus, confirm the lack of PCMT in *S. cerevisiae*, and shed light on how isoaspartyl residues could potentially be removed in an organism lacking repair methyltransferase activity. Because isoaspartyl levels are generally lower in *S. cerevisiae* compared with other organisms with PCMT, we hypothesized that alternative mechanisms to limit this ubiquitous damage must exist. In *C. elegans* and mice, the presence of isoaspartyl-containing peptides supports the hypothesis that isoaspartyl damage may not only be repaired but also degraded (36, 41, 42). However, no biochemical study has further searched for such proteolytic activity in other organisms. In this work, we showed that the proteasome and autophagy pathways, two common mechanisms to remove altered proteins from cells, do not appear to be involved in limiting isoaspartyl damage. We now provide evidence for the participation of one or more metalloproteases with the ability to cleave peptides containing N-terminal isoaspartyl residues. These latter enzymes can ensure the full hydrolysis of isoaspartyl-containing proteins to free amino acids.

In *S. cerevisiae*, there are ~47 enzymatically active metalloproteases (85). Because EDTA is a broad spectrum inhibitor of metalloprotease activity, more than one metalloenzyme may play a role in reducing the accumulation of isoaspartyl residue damage, including both enzymes that ensure the rapid overall degradation of proteins and those that specifically recognize isoaspartyl sites (Fig. 12). The deamination and isomerization of asparaginyl and aspartyl residues to isoaspartyl damage are relatively slow processes that depend on protein sequence. Asparagine-glycine and asparagine-serine form isoaspartyl residues most rapidly with half-lives of 1.2 and 6.8 days after formation, respectively (10). Additionally, sites in sequences such as asparagine-valine and aspartate-histidine are some of the slowest to form isoaspartyl residues with half-lives...
of 107 and 266 days, respectively (10). In comparison with these formation rates, most proteins in *S. cerevisiae* are robustly turned over and have a half-life of ~43 min (78). In the presence of EDTA, many of the peptides we identified with isoaspartyl residues were derived from proteins with turnover rates ranging between 27 and 85 min (Tables 2 and 3). Consequently, repair mechanisms may not be needed by *S. cerevisiae* because proteins are typically degraded before they are allowed to age a sufficient time to generate isoaspartyl damage.

A comparison of peptides identified by mass spectrometry in our *in vitro* aging experiments showed that peptides with and without isoaspartyl residues have similar C-terminal sequences, but the N terminus of isoaspartyl peptides contain mostly deamidated asparagine in the first position. We hypothesize that this N-terminal isoaspartyl site blocks degradation of the peptide by typical proteases. In the presence of EDTA, the degradation of isoaspartyl species by an isoaspartyl-specific metalloprotease is inhibited. Future studies will look further into these 47 metalloproteases to see whether one or a combination of proteases is required to limit isoaspartyl residue accumulation. These enzymes may be a part of the normal protein turnover or may be specific in recognizing isoaspartyl residues.

Protein oxidation and isoaspartyl formation are comparable types of protein damage, and it is reasonable to hypothesize that both modifications are limited by similar mechanisms. As with PCMT and isoaspartyl residues, repair enzymes restore methionine and cysteine oxidation sites in addition to protein thiols (86). In non-repairable protein oxidative damage involving methionine sulfones and carbonyl derivatives, the proteasome and vacuole systems have increased activity to degrade damaged proteins (86–90). The present study shows that neither the proteasome or autophagy pathway regulates isoaspartyl damage significantly. However, we were interested in seeing whether the vacuole was important for isoaspartyl control and tested *S. cerevisiae* strains deficient in the key vacuole proteases Pep4 and Prb1. In both cases, the amount of isoaspartyl residues was no different when compared with controls (data not shown). Interestingly, when we analyzed other vacuole mutants, the Fab1 protein was consistently found to have at least a 2-fold
increase in isoaspartyl residues (data not shown). Fab1 is a 1-phosphatidylinositol-3-phosphate 5-kinase involved in vacuole sorting and homeostasis, and Fab1-deficient cells have deacidified vacuoles (91). However, this increase in isoaspartyl damage was only observed in the MATa strain of \textit{S. cerevisiae}, and no change was observed in the MATα and diploid mutant strains (data not shown). Nevertheless, this result brings attention to the fact that at least eight metalloproteases are located in the vacuole and could participate in isoaspartyl degradation (85).

The only previous study that investigated isoaspartyl residues in \textit{S. cerevisiae} was performed by Deluna et al. (84), who showed that a specific asparagine-glycine sequence in Gdh3 was deamidated \textit{in vitro}, resulting in protein instability. We did not observe the presence of Gdh3 in our mass spectrometry data, which may be due to the relative low abundance of the protein (PaxDb abundance of 32.2 ppm) or because it is not deamidated under physiological conditions. It is important to note that our mass spectrometry analysis only analyzed isoaspartyl formation due to asparagine deamidation. A proteomic approach using electron transfer dissociation mass spectrometry techniques would provide a more thorough characterization of not only asparagine deamidation but also isoaspartyl formation due to aspartyl isomerization (42). Nevertheless, our analysis provided the first in-depth analysis of isoaspartyl damage in fungi. Of the proteins identified by tandem mass spectrometry, many of the isoaspartyl-damaged species were found to originate from abundant proteins involved in metabolism and gene regulation (Tables 2 and 3). The present study also provides evidence for metalloproteases that may limit the amount of isoaspartyl damage in other organisms such as \textit{C. elegans}, mice, and humans.

Acknowledgments—We thank Dr. Jonathan Lowenson for providing mouse tissue extracts and Drs. Lois S. Weisman and William Tansey for providing yeast strains.

REFERENCES

1. Chondrogianni, N., Petropoulos, I., Grimm, S., Georgila, K., Catalgol, B., Friguet, B., Grune, T., and Gonos, E. S. (2014) Protein damage, repair and proteolysis. \textit{Mol. Aspects Med.} 35, 1–71

2. Aswad, D. W., Paranandi, M. V., and Schurter, B. T. (2000) Isoaspartate in peptides and proteins: formation, significance, and analysis. \textit{J. Pharm. Biomed. Anal.} 21, 1129–1136

3. Cloos, P. A., and Christgau, S. (2002) Non-enzymatic covalent modifications of proteins: mechanisms, physiological consequences and clinical applications. \textit{Matrix Biol.} 21, 39–52

4. Friguet, B. (2006) Oxidized protein degradation and repair in ageing and oxidative stress. \textit{FEBS Lett.} 580, 2910–2916

5. Hipskind, A. R. (2006) Accumulation of altered proteins and ageing: causes and effects. \textit{Exp. Gerontol.} 41, 464–473

6. Clarke, S. (2003) Aging as war between chemical and biochemical processes: protein methylation and the recognition of age-damaged proteins for repair. \textit{Ageing Res. Rev.} 2, 263–285

7. Kim, E., Lowenson, J. D., MacLaren, D. C., Clarke, S., and Young, S. G. (1997) Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice. \textit{Proc. Natl. Acad. Sci. U.S.A.} 94, 6132–6137

8. Geiger, T., and Clarke, S. (1987) Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides: succinimide-linked reactions that contribute to protein degradation. \textit{J. Biol. Chem.} 262, 782–794

9. Lowenson, J. D., and Clarke, S. (1992) Recognition of D-aspartyl residues in polyepptides by the erythrococyte l-isoaspartyl/o-aspartyl protein methyltransferase. Implications for the repair hypothesis. \textit{J. Biol. Chem.} 267, 5985–5995

10. Radkiewicz, J. L., Zipse, H., Clarke, S., and Houk, K. N. (2001) Neighboring side chain effects on asparaginyl and aspartyl degradation: an \textit{ab initio} study of the relationship between peptide conformation and backbone NH acidity. \textit{J. Am. Chem. Soc.} 123, 3499–3506

11. Radkiewicz, J. L., Zipse, H., Clarke, S., and Houk, K. N. (1996) Accelerated racemization of aspartic acid and asparagine residues via succinimide intermediates: an \textit{ab initio} theoretical exploration of mechanism. \textit{J. Am. Chem. Soc.} 118, 9148–9155

12. Noguchi, S. (2010) Structural changes induced by the deamidation and isomerization of asparagine revealed by the crystal structure of \textit{Listiago sphaerogena} ribonuclease U2B. \textit{Biopolymers} 93, 1003–1010

13. Cacia, J., Keck, R., Presta, L. G., and Frenz, J. (1996) Isomerization of an aspartic acid residue in the complementarity-determining regions of a recombinant antibody to human IgE: identification and effect on binding affinity. \textit{Biochemistry} 35, 1897–1903

14. Lee, J. C., Kang, S. U., Jeon, Y., Park, J. W., You, J. S., Ha, S. W., Bae, N., Lubec, G., Kwon, S. H., Lee, J. S., Cho, E. J., and Han, J. W. (2012) Protein L-isoaspartyl methyltransferase regulates p53 activity. \textit{Nat. Commun.} 3, 927

15. Manuela, M. J., Gee, R. J., Elliott, J. L., Sette, A., Southwood, S., Jones, P. J., and Blier, P. R. (1999) Isoaspartyl post-translational modification triggers autoimmune responses to self-proteins. \textit{J. Biol. Chem.} 274, 22321–22327

16. Doyle, H. A., Gee, R. J., and Mamula, M. J. (2003) A failure to repair self-proteins leads to T cell hyperproliferation and autoantibody production. \textit{J. Immunol.} 171, 2840–2847

17. Doyle, H. A., Zhou, J., Wolff, M. J., Harvey, B. P., Roman, R. M., Gee, R. J., Koski, R. A., and Mamula, M. J. (2006) Isoaspartyl post-translational modification triggers anti-tumor T and B lymphocyte immunity. \textit{J. Biol. Chem.} 281, 32676–32683

18. Yang, M. L., Doyle, H. A., Gee, R. J., Lowenson, J. D., Clarke, S., Lawson, B. R., Aswad, D. W., and Mamula, M. J. (2006) Intracellular protein modification associated with altered T cell functions in autoimmune disease. \textit{J. Immunol.} 177, 4541–4549

19. Doyle, H. A., Gee, R. J., and Mamula, M. J. (2007) Altered immunogenicity of isoaaspartate containing proteins. \textit{Autoimmunity} 40, 131–137

20. Yang, H., Luytvinisky, Y., Soininen, H., and Zubarev, R. A. (2011) Alzheimer’s disease and mild cognitive impairment are associated with elevated levels of isoaspartyl residues in blood plasma proteins. \textit{J. Alzheimers Dis.} 27, 113–118

21. Shimizu, T., Watanabe, A., Ogawara, M., Mori, H., and Shirasawa, T. (2000) Isoaspartate formation and neurodegeneration in Alzheimer’s disease. \textit{Arch. Biochem. Biophys.} 381, 225–234

22. Kodama, T., Mizobuchi, M., Takeda, R., Torikai, H., Shinomiya, H., and Ohashi, Y. (1995) Hampered expression of isoaspartyl protein carboxyl methyltransferase gene in the human cataractous lens. \textit{Biochim. Biophys. Acta} 1245, 269–272

23. Hooi, M. Y., Raftery, M. J., and Truscott, R. J. (2012) Racemization of two proteins over our lifespan: deamidation of asparagine 76 in yS crystallin is greater in cataract than in normal lenses across the age range. \textit{Invest. Ophthalmol. Vis. Sci.} 53, 3554–3561

24. Hooi, M. Y., Raftery, M. J., and Truscott, R. J. (2013) Accelerated aging of Asp58 in nA crystallin and human cataract formation. \textit{Exp. Eye Res.} 106, 34–39

25. Desrosiers, R. R., and Fanélus, I. (2011) Damaged proteins bearing \textit{l}-isoaspartyl residues and aging: a dynamic equilibrium between generation of isomerized forms and repair by PIMT. \textit{Curr. Aging Sci.} 4, 8–18

26. McFadden, P. N., and Clarke, S. (1987) Conversion of isoaspartyl peptides to normal peptides: implications for the cellular repair of damaged proteins. \textit{Proc. Natl. Acad. Sci. U.S.A.} 84, 2595–2599

27. Johnson, B. A., Murray, E. D., Jr., Clarke, S., Glass, D. B., and Aswad, D. W. (1987) Protein carboxyl methyltransferase facilitates conversion of atypical \textit{l}-isoaspartyl peptides to normal l-aspartyl peptides. \textit{J. Biol. Chem.} 262, 5622–5629
28. Visick, J. E., Ichikawa, J. K., and Clarke, S. (1998) Mutations in the Escherichia coli surE gene increase isoaspartyl accumulation in a strain lacking the pcn repair methyltransferase but suppress stress-survival phenotypes. *FEMS Microbiol. Lett.* **167**, 19–25

29. Visick, J. E., Cai, H., and Clarke, S. (1998) The L-isoaspartyl protein repair methyltransferase enhances survival of aging *Escherichia coli* subjected to secondary environmental stresses. *J. Bacteriol.* **180**, 2623–2629

30. Kindrachuk, J., Parent, J., Davies, G. F., Dinsmore, M., Attaah-Poku, S., and Napper, S. (2003) Overexpression of L-isoaspartate O-methyltransferase in *Escherichia coli* increases heat shock survival by a mechanism independent of methyltransferase activity. *J. Biol. Chem.* **278**, 50880–50886

31. Chavous, D. A., Jackson, F. R., and O’Connor, C. M. (2001) Extension of the *Drosophila* lifespan by overexpression of a protein repair methyltransferase. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14814–14818

32. Gomez, T. A., Banfield, K. L., Troger, D. M., and Clarke, S. G. (2007) The L-isoaspartyl-O-methyltransferase in *Caenorhabditis elegans* larval longevity and autophagy. *Dev. Biol.* **303**, 493–500

33. Khare, S., Gomez, T., Linster, C. L., and Clarke, S. G. (2009) Defective protein L-isoaspartyl methyltransferase activity and insulin-like signaling to extend lifespan in *Caenorhabditis elegans*. *PLoS One* **6**, e20850

34. Banfield, K. L., Gomez, T. A., Lee, W., Clarke, S., and Larsen, P. L. (2008) Protein-repair and hormone-signalling pathways specify dauer and adult longevity and dauer development in *Caenorhabditis elegans*. *J. Gerontol. A Biol. Sci. Med. Sci.* **63**, 798–808

35. Lowenson, J. D., Kim, E., Young, S. G., and Clarke, S. (2001) Limited accumulation of damaged proteins in L-isoaspartyl (O-isoaspartyl) O-methyltransferase-deficient mice. *J. Biol. Chem.* **276**, 20695–20702

36. Farrar, C., and Clarke, S. (2002) Altered levels of S-adenosylmethionine and S-adenosylhomocysteine in the brains of L-isoaspartyl (O-isoaspartyl) O-methyltransferase-deficient mice. *J. Biol. Chem.* **277**, 27856–27863

37. Kim, E., Lowenson, J. D., Clarke, S., and Young, S. G. (1999) Phenotypic analysis of seizure-prone mice lacking L-isoaspartate (O-isoaspartate) O-methyltransferase. *J. Biol. Chem.* **274**, 20671–20678

38. Yamamoto, A., Takagi, H., Kitamura, D., Tatsuoka, H., Nakano, H., Kawano, H., Kuroyanagi, H., Yahagi, Y., Kobayashi, S., Koizumi, K., Sakai, T., Saito, K., Chiba, T., Kawamura, K., Suzuki, K., Watanabe, T., Mori, H., and Shirasawa, T. (1998) Deficiency in protein L-isoaspartyl methyltransferase results in a fatal progressive epilepsy. *J. Neurosci.* **18**, 2063–2074

39. Qin, Z., Yang, J., Klassen, H. I., and Aswad, D. W. (2014) Isoaspartyl protein damage and repair in mouse retina. *Invest. Ophthalmol. Vis. Sci.* **55**, 1572–1579

40. Niewmierzyczka, A., and Clarke, S. (1999) Do damaged proteins accumulate in *Caenorhabditis elegans* L-isoaspartate methyltransferase (pcm-1) deletion mutants? *Arch. Biochem. Biophys.* **364**, 209–218

41. Dai, S., Ni, W., Patananan, A. N., Clarke, S. G., Karger, B. L., and Zhou, Z. S. (2013) Integrated proteomic analysis of major isoaspartyl-containing proteins in the urine of wild type and protein L-isoaspartate O-methyltransferase-deficient mice. *Anal. Chem.* **85**, 2423–2430

42. Patananan, A. N., and Clarke, S. G. (2013) in *Handbook of Proteolytic Enzymes* (Rawlings, N. D., and Salvesen, G. G., eds) 3rd Ed., pp. 1654–1663, Academic Press, Oxford

43. Larsen, R. A., Knox, T. M., and Miller, C. G. (2001) Aspartic peptide hydrolases in *Salmonella enterica* serovar typhimurium. *J. Bacteriol.* **183**, 3089–3097

44. Borek, D., Michalska, K., Brzezinski, K., Kesiel, A., Podkowski, J., Bonchon, D. T., Krowarsch, D., Otlewski, J., and Jaksol, M. (2004) Expression, purification and catalytic activity of *Lupinus luteus* asparagine β-amidohydrolase and its *Escherichia coli* homolog. *Eur. J. Biochem.* **271**, 3215–3226

45. Hejazi, M., Pioutk, K., Mattow, J., Deutzmann, R., Volkmann-Engert, R., and Lockau, W. (2002) Isoaspartyl dipeptidase activity of plant-type asparaginases. *Biochem. J.* **364**, 129–136

46. Cantor, J. R., Stone, E. M., Chantranupong, L., and Georgiou, G. (2009) The human asparaginase-like protein 1 hASRGL1 is an Ntn hydrolase with β-aspartyl peptidase activity. *Biochemistry* **48**, 11026–11031

47. Collins, G. A., Gomez, T. A., Deshaies, R. J., and Tansey, W. P. (2010) Combined chemical and genetic approach to inhibit proteolysis by the proteasome. *Yeast* **27**, 965–974

48. Mudgett, M. B., Lowenson, J. D., and Clarke, S. (1997) Protein repair L-isoaspartyl methyltransferase in plants. Phylogenetic distribution and the accumulation of substrate proteins in aged barley seeds. *Plant Physiol.* **115**, 1481–1489

49. Li, C., and Clarke, S. (1992) Distribution of an L-isoaspartyl protein methyltransferase. Implications for the repair hypothesis. *J. Biol. Chem.* **266**, 16291–16298

50. Li, C., and Clarke, S. (1998) A highly active protein repair enzyme from an extreme thermophile: the L-isoaspartyl methyltransferase from *Thermotoga maritima*. *Arch. Biochem. Biophys.* **358**, 222–231

51. Balzi, E., Wang, M., Leterme, S., Van Dyck, L., and Goffeau, A. (1994) The human asparaginase-like protein 1 hASRGL1 is an Ntn hydrolase with β-aspartyl peptidase activity. *Biochemistry* **33**, 5155–5164

52. Collins, G. A., Gomez, T. A., Deshaies, R. J., and Tansey, W. P. (2010) Combined chemical and genetic approach to inhibit proteolysis by the proteasome. *Yeast* **27**, 965–974
Processing of L-Isoaspartyl-containing Proteins in Yeast

69. Mizushima, N., Yoshimori, T., and Levine, B. (2010) Methods in mammalian autophagy research. *Cell* **140**, 313–326

70. Xie, Y., and Varshavsky, A. (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3056–3061

71. Ju, D., Wang, L., Mao, X., and Xie, Y. (2004) Homeostatic regulation of the proteasome via an Rpn4-dependent feedback circuit. *Biochem. Biophys. Res. Commun.* **321**, 51–57

72. Wang, L., Mao, X., Ju, D., and Xie, Y. (2004) Rpn4 is a physiological substrate of the Ubc2 ubiquitin ligase. *J. Biol. Chem.* **279**, 55218–55223

73. Kruegel, U., Robison, B., Dange, T., Kahlert, G., Delaney, J. R., Kotireddy, S., Tsuchiya, M., Tsuchiyama, S., Murakami, C. J., Schleit, J., Sutphin, G., Carr, D., Tar, K., Dittmar, G., Kaeberlein, M., Kennedy, B. K., and Schmidt, M. (2011) Elevated proteasome capacity extends replicative lifespan in *Saccharomyces cerevisiae.* *PLoS Genet.* **7**, e1002253

74. Swaminathan, S., Amerik, A. Y., and Hochstrasser, M. (1999) The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. *Mol. Biol. Cell* **10**, 2583–2594

75. Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999) Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J. Cell Biol.* **147**, 435–446

76. Meiling-Wesse, K., Barth, H., Voss, C., Barmark, G., Murén, E., Ronne, H., and Thumm, M. (2002) Yeast Mon1p/Aut12p functions in vacuolar fusion of autophagosomes and ctn-vesicles. *FEBS Lett.* **530**, 174–180

77. Wang, M., Weiss, M., Simonovic, M., Haertinger, G., Schrimpf, S. P., Hengartner, M. O., and von Mering, C. (2012) PaxDb, a database of protein abundance averages across all three domains of life. *Mol. Cell. Proteomics* **11**, 492–500

78. Belle, A., Tanay, A., Bitincka, L., Shamir, R., and O’Shea, E. K. (2006) Quantification of protein half-lives in the budding yeast proteome. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 13004–13009

79. Villa, S. T., Xu, Q., Downie, A. B., and Clarke, S. G. (2006) Arabidopsis protein repair L-isoaspartyl methyltransferases: predominant activities at both seed longevity and germination vigor in *Arabidopsis.* *Plant Cell* **20**, 3022–3037

80. Nayak, N. R., Putnam, A. A., Addepalli, B., Lowenson, J. D., Chen, T., Jankowsky, E., Perry, S. E., Dinkins, R. D., Limbach, P. A., Clarke, S. G., and Downie, A. B. (2013) An *Arabidopsis* ATP-dependent, DEAD-box RNA helicase loses activity upon IsoAsp formation but is restored by PROTEIN ISOASPARTYL METHYLTRANSFERASE. *Plant Cell* **25**, 2573–2586

81. O’Connor, M. B., Galus, A., Hartenstine, M., Magee, M., Jackson, F. R., and O’Connor, C. M. (1997) Structural organization and developmental expression of the protein isoaspartyl methyltransferase gene from *Drosophila melanogaster.* *Insect Biochem. Mol. Biol.* **27**, 49–54

82. Johnson, B. A., Ngo, S. Q., and Aswad, D. W. (1991) Widespread phylogenetic distribution of a protein methyltransferase that modifies L-isoaspartyl residues. *Biochem. Int.* **24**, 841–847

83. DeLuna, A., Quezada, H., Gómez-Puyou, A., and González, A. (2005) Asparaginyl deamidation in two glutamate dehydrogenase isoenzymes from *Saccharomyces cerevisiae.* *Biochem. Biophys. Res. Commun.* **328**, 1083–1090

84. Hecht, K. A., Wytiaze, V. A., Ast, T., Schuldiner, M., and Brodsky, J. L. (2013) Characterization of an M28 metalloprotease family member residing in the yeast vacuole. *FEMS Yeast Res.* **13**, 471–484

85. Costa, V., Quintanilha, A., and Moradas-Ferreira, P. (2007) Protein oxidation, repair mechanisms and proteolysis in *Saccharomyces cerevisiae.* *IUBMB Life* **59**, 293–298

86. Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., and Toldano, M. B. (1999) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J. Biol. Chem.* **274**, 16040–16046

87. Marques, M., Mozjita, D., Amorim, M. A., Almeida, T., Hohmann, S., Moradas-Ferreira, P., and Costa, V. (2006) The Pep4p vacuolar protease contributes to the turnover of oxidized proteins but PEP4 overexpression is not sufficient to increase chronological lifespan in *Saccharomyces cerevisiae.* *Microbiology* **152**, 3595–3605

88. Thorpe, G. W., Fong, C. S., Alic, N., Higgins, V. J., and Dawes, I. W. (2004) Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes. *Proc. Natl Acad. Sci. U.S.A.* **101**, 6564–6569

89. Chen, Q., Thorpe, J., Dohmen, J. R., Li, F., and Keller, J. N. (2006) Ump1 extends yeast lifespan and enhances viability during oxidative stress: central role for the proteasome? *Free Radic. Biol. Med.* **40**, 120–126

90. Gary, J. D., Wurmser, A. E., Bonangelino, C. J., Weisman, L. S., and Emr, S. D. (1999) Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. *J. Cell Biol.* **143**, 65–79

91. Bonangelino, C. J., Catlett, N. L., and Weisman, L. S. (1997) Vacc7p, a novel vacuolar protein, is required for normal vacuole inheritance and morphology. *Mol. Cell. Biol.* **17**, 6847–6858