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

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Background: Isoaspartyl damage is a common spontaneous protein modification repaired by the protein isoaspartyl methyltransferase (PCMT).
Results: Although *Saccharomyces cerevisiae* is one of the few organisms that lack PCMT, isoaspartyl-damaged polypeptides slowly accumulate except in extracts incubated with EDTA.
Conclusion: Isoaspartyl-containing polypeptides are degraded by a metalloprotease.
Significance: The mechanism of isoaspartyl control in *S. cerevisiae* may also be used in higher organisms.

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 quality 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 *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.

Protein function can be impaired by spontaneous posttranslational modifications as a result of exposure to oxygen- and nitrogen-reactive species, sugars, aldehydes, and even water (1). 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 pharmaceutical 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 undegraded (25).

The protein repair enzyme protein-L-isoaspartyl (D-aspartyl) *O*-methyltransferase (PCMT[2]; 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-

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[2] The abbreviations used are: PCMT, protein-L-isoaspartyl (D-aspartyl) *O*-methyltransferase; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; [^{14}C]AdoMet, *S*-adenosyl-L-[*methyl*-^{14}C]methionine; CSM, complete synthetic medium; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

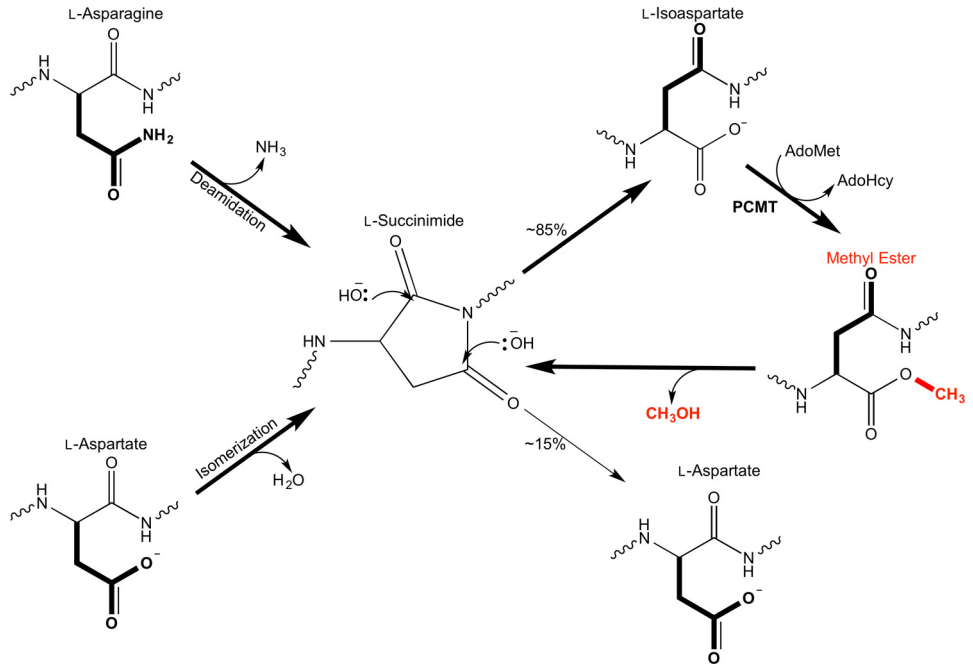

FIGURE 1. **Pathways for the formation and metabolism of isoaspartyl residues in peptides and proteins.** The deamidation or isomerization of L-asparaginyl or L-aspartyl residues, respectively, results in the formation of an L-succinimidyl intermediate. Although ~15–40% of this intermediate will non-enzymatically hydrolyze into the normal L-aspartyl residue, the majority product is the abnormal L-isoaspartyl residue that reroutes the polypeptide backbone through the side chain. To repair this protein damage, most organisms rely on PCMT, which uses the cofactor *S*-adenosylmethionine (*AdoMet*) to methylate the L-isoaspartyl residue side chain and form a methyl ester. This methyl ester is non-enzymatically readily hydrolyzed to reform the succinimidyl intermediate, which once again can convert either into the L-isoaspartyl or L-aspartyl residue. The PCMT repair cycle continues until the L-isoaspartyl residue is fully converted to the normal L-aspartyl residue. Additionally, the succinimide is racemization-prone and can also form D-isoaspartyl and D-aspartyl products, the latter of which is also a substrate for PCMT methylation (9, 11). *AdoHcy*, *S-adenosylhomocysteine*.

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

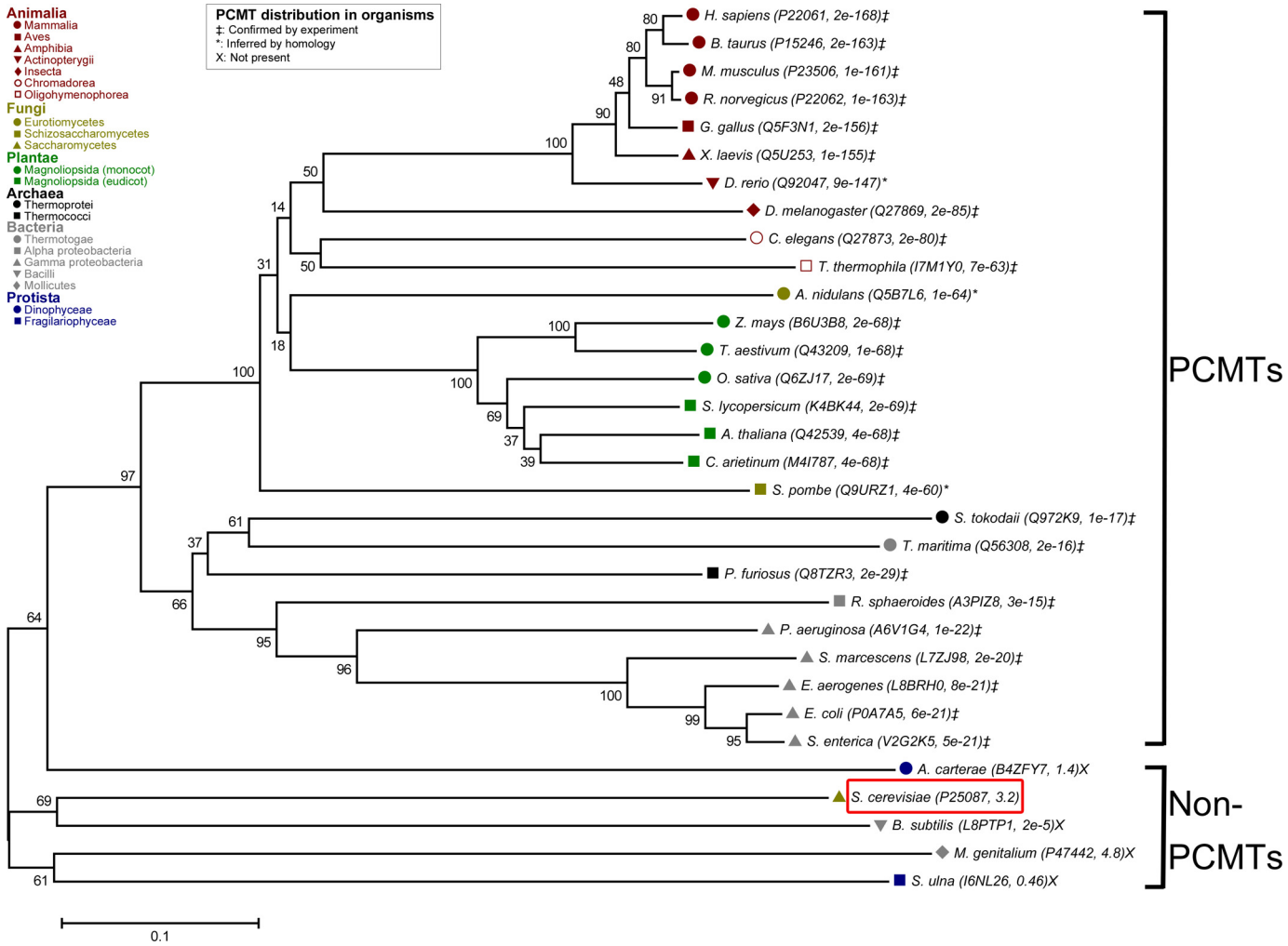

FIGURE 2. **PCMT is highly conserved in many organisms except for some Gram-positive bacteria and a few fungal species, including *S. cerevisiae*.** A protein-protein BLAST search was performed comparing various organisms with human PCMT (UniProt P22061). The UniProt ID of the top ranking alignment for 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. ‡, PCMT has been previously detected in the organism; *, 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).

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 Na_2HPO_4, and 0.1 mM KH_2PO_4, 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, *pdr5*Δ yeast overnight cultures in 5 ml of YPD were used to inoculate 30-ml flasks of YPD to a starting A_{600} 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*Δ and *pup1pre3pdr5*Δ 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 cells were cultured for 8 (to 0.6–0.8 A_{600}) or 24 h (to >5 A_{600}) 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-

TABLE 1
***S. cerevisiae* strains used in this study**

Strain	Genotype	Biological function	Source
BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Wild type	Open Biosystems
BY4742	MAT*α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Wild type	Open Biosystems
LWY7235	MAT**a** *leu2-3,112 ura3–52 his3Δ200 trp1Δ901 lys2-801 suc2Δ9*	Wild type	Lois S. Weisman (92)
*pdr5*Δ	BY4742 background	Drug transport	Open Biosystems
*atg8*Δ	BY4742 background	Autophagy and cytoplasm-to-vacuole targeting pathway	Open Biosystems
*atg18*Δ	BY4742 background	Autophagy and cytoplasm-to-vacuole targeting pathway	Open Biosystems
*mon1*Δ	BY4742 background	Autophagy and cytoplasm-to-vacuole targeting pathway	Open Biosystems
*doa4*Δ	BY4742 background	Recycling ubiquitin from proteasome-bound ubiquitinated intermediates	Open Biosystems
*rpn4*Δ	BY4742 background	Transcription factor that stimulates expression of proteasome genes	Open Biosystems
*ubr2*Δ	BY4742 background	Cytoplasmic ubiquitin-protein ligase (E3); required for ubiquitylation of Rpn4p	Open Biosystems
*ubc4*Δ	BY4742 background	Ubiquitin-conjugating enzyme (E2); mediates degradation of abnormal or excess proteins	Open Biosystems
GAC201	MAT*α his3-11 leu2-3,112*; *ura3–52 lys2-801 trp1-1 pdr5*Δ*::KanMX6 pre3*Δ*2::HIS3 pup1*Δ*::leu2-HIS3* [pRS317-PUP1][YCplac22-PRE3] gal$^-$	Wild type	William Tansey (48)
GAC202	MAT*α his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pdr5*Δ*::KanMX6*; *pre3*Δ*2::HIS3 pup1*Δ*::leu2-HIS3* [pRS317-pup1-T30A][YCplac22-pre3-T20A] gal$^-$	*Pre3*, β1 subunit of the 20 S proteasome; cleavage after acidic residues in peptides; *Pup1*, β2 subunit of the 20 S proteasome; endopeptidase with trypsin-like activity that cleaves after basic residues	William Tansey (48)

tures were used to inoculate 5-ml YPD cultures supplemented with 10 nM or 1 μM concanamycin A (Sigma, catalog number C9705) or 10, 100, or 200 mM ammonium chloride to a starting A_{600} of 0.1. Cells not incubated with the inhibitors served as the control. After culturing for 7 days at 30 °C and 250 rpm, the cells were spun down and washed as described above before the pellets were stored at −80 °C until lysis.

Cytosolic Protein Preparation—Yeast pellets were resuspended in 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.7 mM phenylmethylsulfonyl fluoride (PMSF), and one Roche Applied Science Complete protease inhibitor tablet with EDTA (catalog number 11836145001)/25 ml of buffer) and ~1 ml of 0.5-mm diameter glass beads (BioSpec, catalog number 11079105). The cells were vortexed at room temperature for 1 min on setting 10 (Fisher Scientific analog Vortex mixer, catalog number 02-215-365) and subsequently placed on ice for 1 min. After repeating this cycle seven times, the extract was removed from the glass beads and centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was removed and centrifuged at 18,000 × *g* for 10 min at 4 °C. Finally, the supernatant was transferred to a new microcentrifuge tube and stored at −20 °C until analysis.

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 KH_2PO_4, 6 g/liter Na_2HPO_4, 5 g/liter NaCl, and 1 ml/liter 1 M $MgSO_4$), 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 lysed 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 L-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 μg 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-[*methyl*-^{14}C]methionine ([^{14}C]AdoMet) (PerkinElmer Life Sciences; 47 mCi/mmol, 20 μCi/ml in 10 mM H_2SO_4: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 [^{14}C]AdoMet as described above and was subtracted from the value obtained in experimental samples.

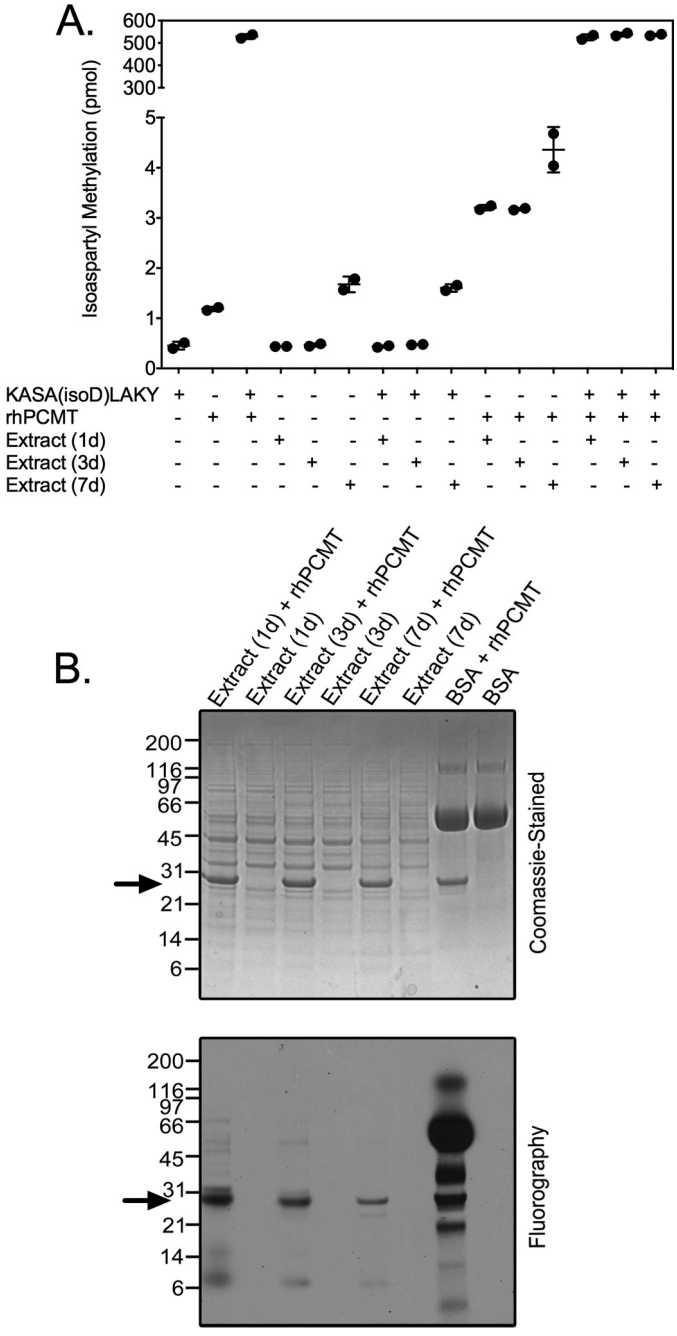

FIGURE 3. ***S. cerevisiae* does not have PCMT activity.** Strain BY4742 was cultured in YPD for 1, 3, or 7 days (*d*) at 30 °C, and extracts were prepared as described under "Experimental Procedures." *A*, *in vitro* vapor diffusion assays to measure the formation of methyl esters were performed as 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 [^{14}C]AdoMet for 2 h at 37 °C. Technical duplicates for each condition were analyzed, and each point represents the average of three radioactivity measurements. The *horizontal bars* indicate the average, and the *error bars* represent S.D. *B*, *in vitro* SDS-PAGE/fluorography to visualize isoaspartyl-containing species in reaction mixtures prepared as in *A*. 25 μg of bovine serum albumin incubated with or without 5 μg of recombinant human PCMT served as the positive and negative controls, respectively. The reactions were quenched by the addition of SDS-PAGE loading buffer and analyzed as described under "Experimental Procedures." Polypeptide molecular mass markers (Bio-Rad broad range, catalog number 161-0317) were electrophoresed in a parallel lane and include myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14 kDa), and aprotinin (6 kDa). Fluorography was performed by treating the gel with EN^{3}HANCE 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.

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*-^{3}H] methionine ([^{3}H]AdoMet) (PerkinElmer Life Sciences; 75–85 Ci/mmol, 0.55 mCi/ml in 10 mM H_2SO_4: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) bromphenol 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 50% (v/v) methanol) for 1 h and destained with 10% (v/v) 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 EN^{3}HANCE (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 μM 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 sam-

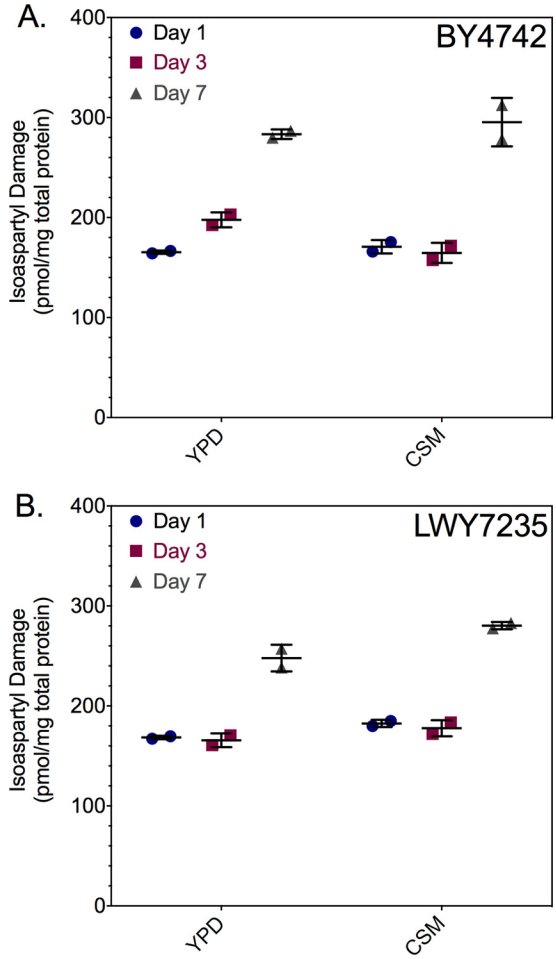

FIGURE 4. ***S. cerevisiae* has low isoaspartyl damage.** Strain BY4742 (*A*) or strain LWY7235 (*B*) parent cells were cultured in 30 ml of YPD or CSM medium for 1, 3, or 7 days at 30 °C and 250 rpm, and extracts were prepared as described under "Experimental Procedures." Isoaspartyl damage was quantified by vapor diffusion assay using 25 μg of extract, 5 μg of recombinant human PCMT, and 10 μM [^{14}C]AdoMet as described under "Experimental Procedures." The individual extracts were measured in duplicate; each data point is the average of three radioactivity measurements. The *horizontal lines* represent the average, and the *error bars* represent S.D.

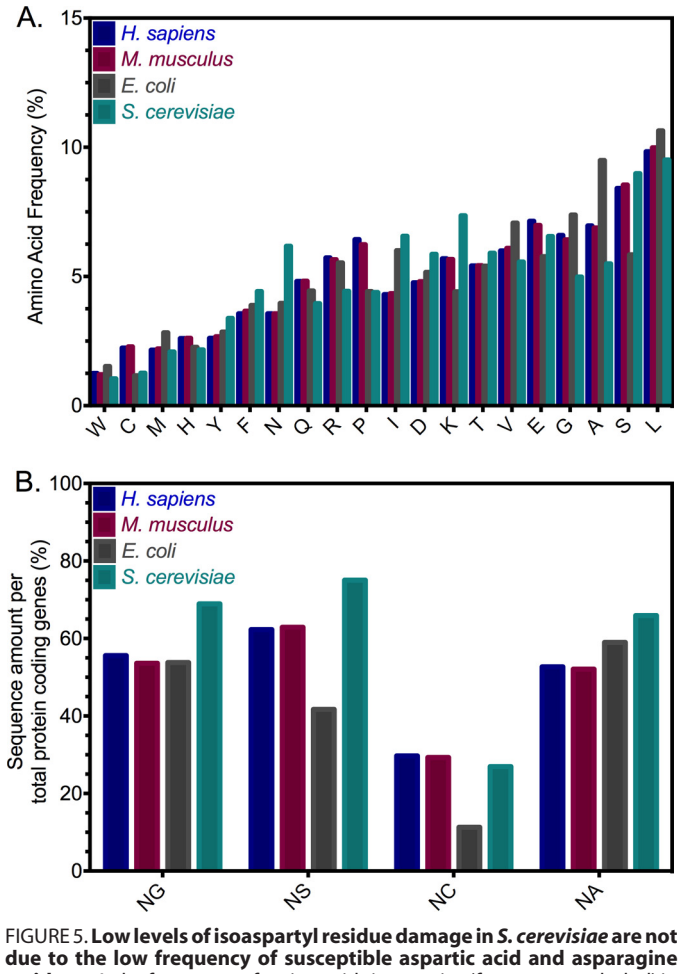

FIGURE 5. **Low levels of isoaspartyl residue damage in *S. cerevisiae* are not due to the low frequency of susceptible aspartic acid and asparagine residues.** *A*, the frequency of amino acids in proteins (fragments excluded) in *S. cerevisiae* are compared with *H. sapiens*, *M. musculus*, and *E. coli* using the Integr8 server, which was recently closed and archived. *B*, particularly susceptible asparagine-glycine, asparagine-serine, asparagine-cysteine, and asparagine-alanine sequences are compared in the proteins of *H.* sapiens, *M. musculus*, *E. coli*, and *S. cerevisiae*. The number of proteins containing at least one of the above sequences as determined by the Motif Search at GenomeNet is compared with the total number of protein-coding genes from the *Kyoto Encyclopedia of Genes and Genomes* genome statistics.

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 C_{18} 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 C_{18} resin with 300-Å pores (AcuTech, San Diego, CA)) was used for peptide chromatography. Electrospray 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 dissocia-

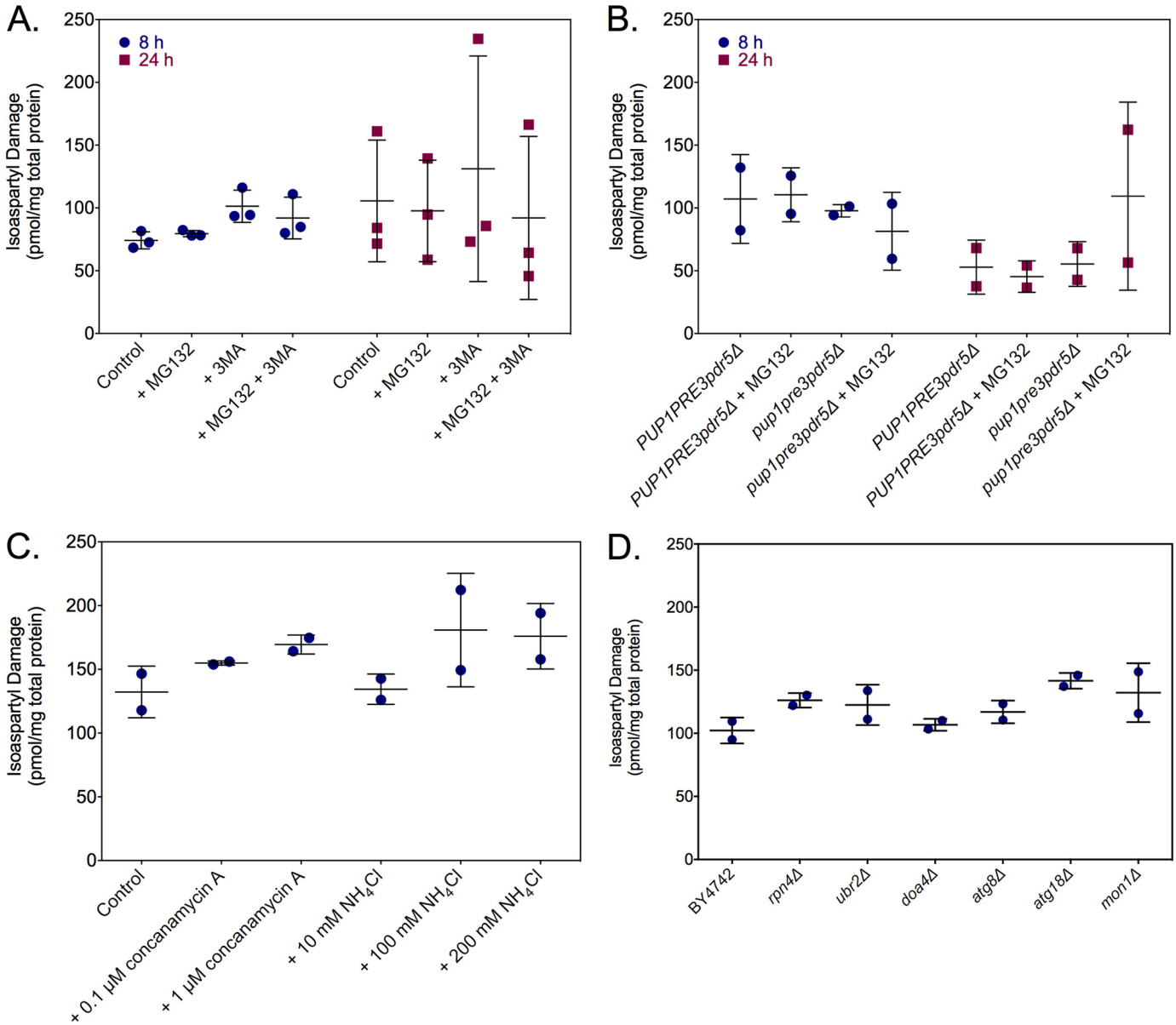

FIGURE 6. **The proteasome and autophagy pathways are not significantly involved in limiting isoaspartyl residue damage in *S. cerevisiae*.** Isoaspartyl residue quantification by vapor diffusion assays of *S. cerevisiae* strains cultured with inhibitors or in yeast mutated in the proteasome and autophagy pathways was performed as described under "Experimental Procedures." *A*, overnight cultures of *pdr5*Δ cells were used to inoculate 30 ml of YPD. After 8 or 24 h at 30 °C and 250 rpm, the medium was removed, and the cells were washed. The cells were resuspended in 30 ml of water with or without 20 μM MG132 and/or 5 mM 3-methyladenine (*3MA*). Control cultures were not incubated with the inhibitors. After 24 h of incubation, extracts were prepared as described under "Experimental Procedures." Isoaspartyl quantification was determined by incubating 15–75 μg of extract, 5 μg of recombinant human PCMT, and 10 μM [^{14}C]AdoMet. Three independent experiments were performed for each condition with each data point representing a technical duplicate of the sample. The *horizontal lines* denote the average, and the *error bars* represent S.D. *B*, to determine whether combining proteasome mutations with chemical inhibition would increase isoaspartyl damage in *S. cerevisiae*, overnight cultures of congenic strains *PUP1PRE3pdr5*Δ and *pup1pre3pdr5*Δ in YPAD medium were used to inoculate 30 ml of YPAD medium with or without 50 μM MG132. The cells were cultured for 8 or 24 h at 30 °C and 250 rpm and lysed as described under "Experimental Procedures." Isoaspartyl residues were quantified by incubating 4–89 μg of extract with 5 μg of recombinant human PCMT and 10 μM [^{14}C]AdoMet. The data represent two individual experiments with each data point representing an average of two technical duplicates. The *horizontal lines* denote the average, and the *error bars* represent S.D. *C*, to determine whether other autophagy inhibitors could increase isoaspartyl damage, BY4741 or BY4742 overnight cultures were used to inoculate 5-ml YPD cultures supplemented with 10 nM or 1 μM concanamycin A or 10, 100, or 200 mM ammonium chloride. BY4741 or BY4742 cells not incubated with inhibitors served as the control. After culturing for 7 days at 30 °C and 250 rpm, the cells were spun down and lysed as described under "Experimental Procedures." Isoaspartyl residues were quantified by incubating 35–120 μg of extract with 5 μg of recombinant human PCMT and 10 μM [^{14}C]AdoMet for 2 h at 37 °C. The data represent two individual experiments with each data point representing the average of technical duplicates. The *horizontal lines* denotes the average, and the *error bars* represent S.D. *D*, other proteasome and autophagy components were tested by incubating various strains for 7 days in 30 ml of YPD medium at 30 °C and 250 rpm. The cells were spun down and lysed as described under "Experimental Procedures." Isoaspartyl residues were quantified by incubating 25–130 μg of extract with 5 μg of recombinant human PCMT and 10 μM [^{14}C]AdoMet as described under "Experimental Procedures." The data represent two individual experiments with each data point representing the average of two technical replicates. The *horizontal lines* denote the average, and the *error bars* represent S.D.

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 NCBInr) 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. Peptides were analyzed for asparagine deamidation if they had an ion score of at least 20 and ppm error no greater than 13. The N and C termini of both non-isoaspartyl 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.

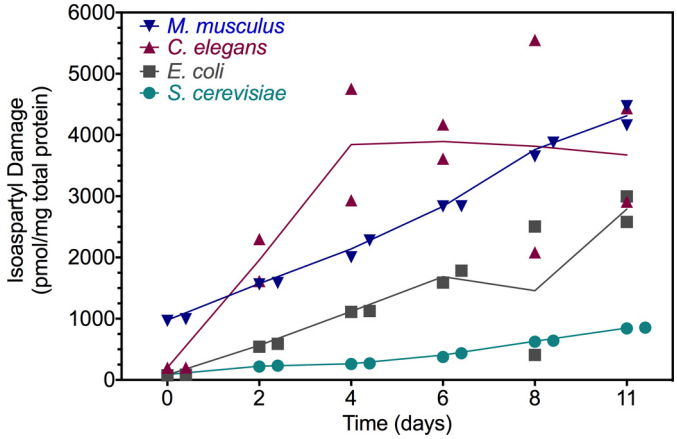

FIGURE 7. **Comparison of *in vitro* aged *S. cerevisiae* cytosolic extracts with other organisms.** To determine whether isoaspartyl residues accumulate over time in *S. cerevisiae* extracts, we incubated 25 μg of extract in Tris, pH 7.5 buffer as described under "Experimental Procedures." 25 μg of *M. musculus* PCMT−/− brain cytosolic extract, 25 μg of cytosolic extract from a mixed population of N2 *C. elegans*, and 25 μg of OP50 *E. coli* cytosolic extract were prepared as described under "Experimental Procedures" and aged identically to *S. cerevisiae* to serve as a comparison. Isoaspartyl residues were quantified via vapor diffusion assay by incubating 12.5 μg of *in vitro* aged extract with 5 μg of recombinant human PCMT and 10 μM [^{14}C]AdoMet as described under "Experimental Procedures." Results are given for two independent experiments with each data point representing the average of three radioactivity measurements.

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 *Amphidinium 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)-LAKY (52), with *S. cerevisiae* extract 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. 3*A*). 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. 3*A*). When *S. cerevisiae* extracts were incubated with [^{3}H]AdoMet, separated by SDS-PAGE, and analyzed by fluorography, no radioactive bands were found (Fig. 3*B*). 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. 3*B*).

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

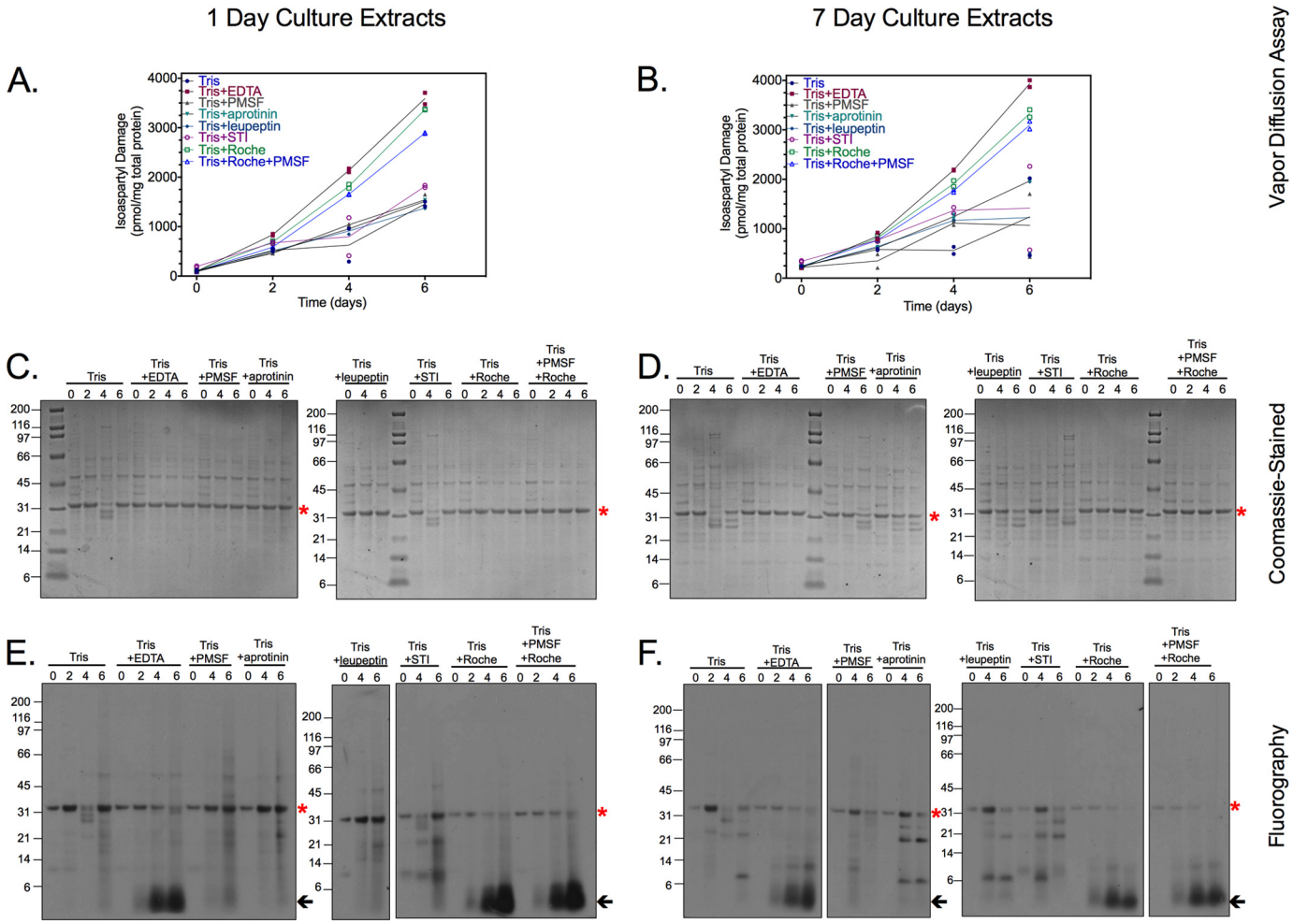

FIGURE 8. ***S. cerevisiae* cytosolic extracts incubated with EDTA accumulate isoaspartyl residues over time.** Cytosolic protein extracts of BY4742 *S. cerevisiae* cultured in YPD for 1 or 7 days were prepared as described under "Experimental Procedures." 25 μg of protein was incubated in either Tris-HCl, pH 7.5 buffer alone or Tris-HCl with a final concentration of 2 mM EDTA, 0.7 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml soybean trypsin inhibitor (*STI*), Roche Applied Science inhibitor solution (*Roche*), or Roche Applied Science inhibitor solution and 0.7 mM PMSF for 0, 2, 4, or 6 days at 37 °C. *A* and *B*, isoaspartyl residues were quantified via vapor diffusion assay by incubating 12.5 μg of *in vitro* aged extract with 5 μg of recombinant human PCMT and 10 μM [^{14}C]AdoMet as described under "Experimental Procedures. The *in vitro* aging was performed in duplicate with each data point representing a single technical measurement. *C* and *D*, to determine the molecular weights of the isoaspartyl-damaged species, SDS-PAGE was performed on the aged extracts. 5 μg of *in vitro* aged extract was incubated with 1 μg of recombinant human PCMT and 0.3 μM [^{3}H]AdoMet for 2 h at 37 °C. SDS-PAGE and fluorography (*E* and *F*; 5-day exposure) were performed as in Fig. 3*B*. The *red asterisks* denote the positions of recombinant human PCMT and its automethylation at ~30 kDa, and the *black arrows* indicate the region where an increase in isoaspartyl residues is observed with samples incubated in EDTA.

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. 5*A*). 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. 5*B*).

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) in addition to the regulation of proteins (56, 57) 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 *pdr5*Δ *S. cerevisiae*, which are deficient

in an ATP-binding cassette multidrug transporter involved in removing drugs from inside the cell (65, 66). *pdr5*Δ 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. 6*A*). Recent studies have shown that although the inhibition of proteasome activity by MG132 targets mainly the chymotryptic activity of the proteasome the tryptic and caspase-like activities imparted by the Pup1 and Pre3 proteases are still present (48). Therefore, we treated *pup1pre3pdr5*Δ *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. 6*B*). 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. 6*C*). 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. 6*C*). Finally, we tested mutants associated with the proteasome and autophagy pathways to further verify our inhibition studies (Fig. 6*D*). For the proteasome, we tested cells deficient in Rpn4, a transcription factor responsible for the expression of proteasome genes (70, 71); Ubr2, an E3 ligase that regulates Rpn4 (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. 6*D*).

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. 8*A*). 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. 8*A*). Analysis of these isoaspartyl species using SDS-PAGE (Fig. 8*C*) and fluorography (Fig. 8*E*) 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.

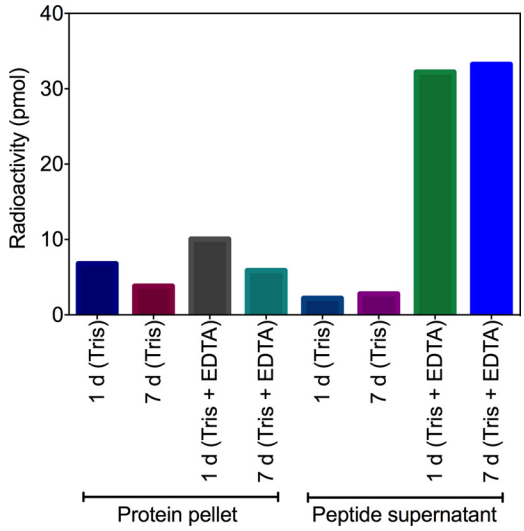

FIGURE 9. ***In vitro* aged extracts incubated with EDTA have an increased amount of isoaspartyl-containing peptides.** To determine the amount of isoaspartyl damage in the peptides and proteins of *in vitro* aged *S. cerevisiae* samples, we aged 15 μg of BY4742 cytosolic extract derived from 1- or 7-day (*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 μM [^{14}C]AdoMet as described under "Experimental Procedures." Each sample was analyzed once with each bar representing the average of three radioactivity measurements.

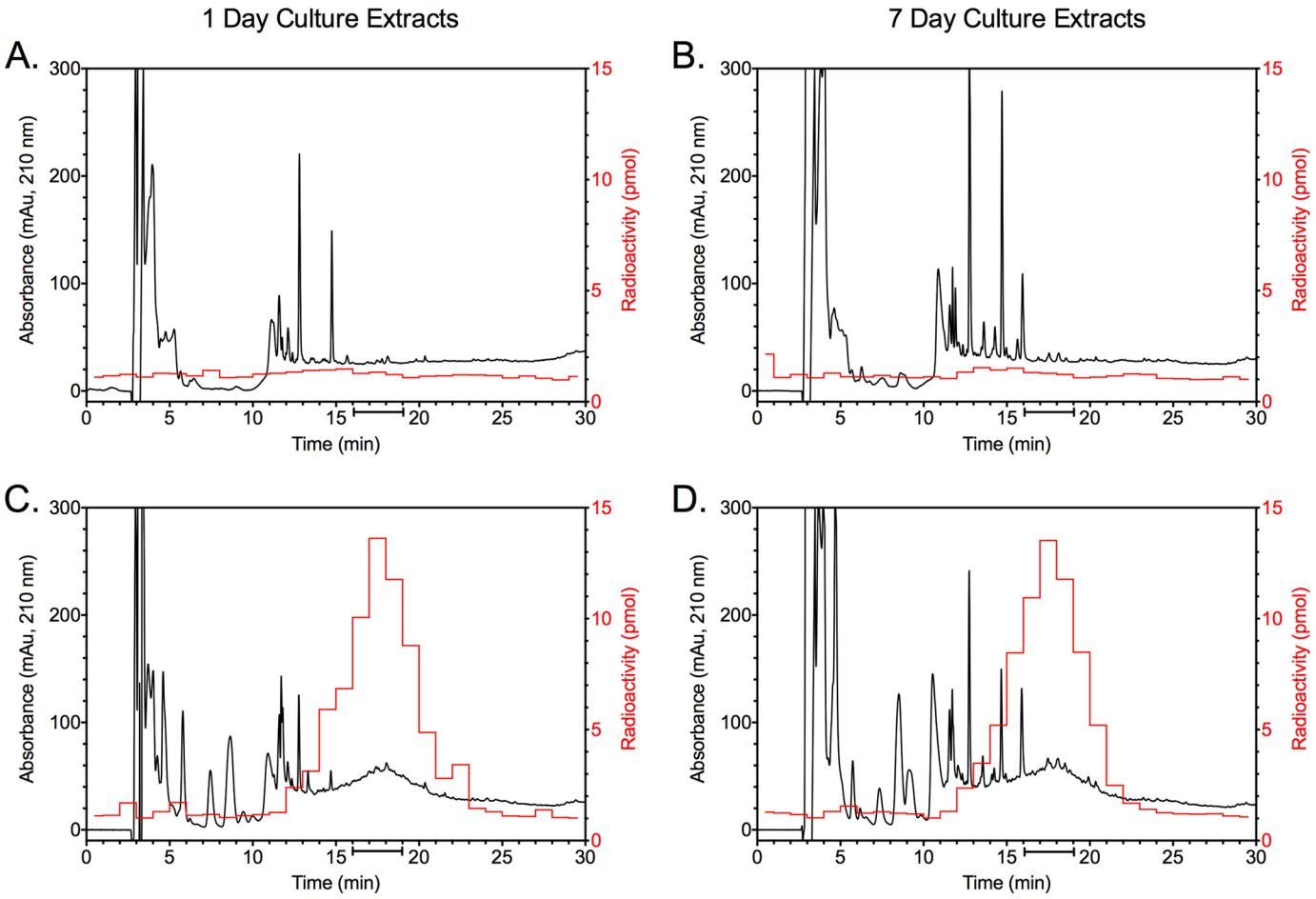

FIGURE 10. **HPLC analysis confirms an increase in isoaspartyl-containing peptides in *in vitro* aged *S. cerevisiae* extract incubated with EDTA.** To confirm an increase in isoaspartyl-containing peptides in *S. cerevisiae* extracts treated with EDTA, 100 μg of *S. cerevisiae* extracts cultured for 1 (*A* and *C*) or 7 days (*B* and *D*) were *in vitro* aged without (*A* and *B*) or with (*C* and *D*) EDTA. After ~5 days, the samples were precipitated with 5% trifluoroacetic acid, and an equivalent of 47 μg of extract was separated by reverse-phase HPLC as described under "Experimental Procedures." The absorbance (milliabsorbance units (*mAu*)) is represented on the *left y axis* by the *black* line. 1-ml fractions were collected, dried under vacuum centrifugation, and resuspended in 25 μl of 0.2 M Bis-Tris-HCl, pH 6.4 buffer. Isoaspartyl residues were quantified by vapor diffusion assay using 5 μg of recombinant human PCMT and 10 μM [^{14}C]AdoMet as described under "Experimental Procedures," and the radioactivity is indicated on the *right y axis* by the *red* trace. The *brackets* at the *bottom* indicate fractions 17, 18, and 19 of each experiment that were taken for mass spectrometry analysis.

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. 10*A*) and 7-day (Fig. 10*B*) 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. 10*C*) and 7-day (Fig. 10*D*) 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 1α-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

TABLE 2
Deamidation sites in *in vitro* aged 1-day extracts incubated with EDTA

		Rank[a]	Abundance[a]	$t_{1/2}$[b]	Deamidation sites[c]
			ppm	*min*	
Glycolysis, glyoxylate cycle, gluconeogenesis, tricarboxylic acid cycle, and electron transport					
Adh1/Adh2	Alcohol dehydrogenase	11/247	8,179/1,010	ND/41	
Cdc19	Pyruvate kinase	9	12,228	ND	NGVHMV NFSHGSYEY
Eno1/Eno2	Enolase	16/1	7,491/24,563	ND/27	NVLNGGSHAGGALA NGGSHAGGALALQE
Fba1	Fructose-1,6-bisphosphate aldolase	3	21,368	104	
Gpm1	Phosphoglycerate mutase	18	6,668	Stable	
Hxk1	Hexokinase	410/32	437/3,413	49/ND	NLRVVLVKLGGNH
Pgi1	Phosphoglucose isomerase	26	4,112	540	
Pgk1	3-Phosphoglycerate kinase	5	16,232	199	
Qcr2	Subunit 2 of ubiquinol cytochrome-*c* reductase	591	259	352	
Tdh2/Tdh3	Glyceraldehyde-3-phosphate dehydrogenase	15/2	7,519/22,369	ND/ND	INSTGVFKEL AINSTGVFKELDTA
Alcoholic fermentation					
Pdc1/Pdc5/Pdc6	Pyruvate decarboxylase	4	18,671	29	NATFPGVQMK HHTLGNGDFTV NGIAGSYAEHVGVL FVLNNNGYTIE[d] PVFDAPQNLVKQAKLT
Ribosomal proteins and translation					
Egd1/Egd2	Subunit of the nascent polypeptide-associated complex	111/161	1,925/1,552	Stable/263	
Efb1	Translation elongation factor 1β	61	2,542	ND	
Eft1/Eft2	Elongation factor 2	128/40	1,767/3,015	155/stable	
Rpl13B	Ribosomal 60 S subunit protein L13B	76	2,327	67	
Rpp0	Ribosomal protein P0 of the ribosomal stalk	117	1,872	ND	
Rps7A	Ribosomal 40 S subunit protein S7A	85	2,184	ND	
Rps12	Ribosomal 40 S subunit protein S12	142	1,673	ND	
Rps19A	Ribosomal 40 S subunit protein S19A	176	1,430	Stable	
Ssb1/Ssb2	Ribosome-associated molecular chaperone	77/49	2,320/2,791	149/712	
Tef2	Translation elongation factor EF-1α	8	12,435	30	
Tif2	Translation initiation factor eIF4A	153	1,590	ND	
Yef3	Subunit of translational elongation factor eEF1B	17	6,951	191	
Cofactor, purine, pyrimidine, amino acid, and glycerol biosynthesis					
Gln1	Glutamine synthetase	71	2,389	73	
Hom6	Homoserine dehydrogenase	270	885	56	
Shm2	Serine hydroxymethyltransferase	187	1,306	ND	
Ura1	Dihydroorotate dehydrogenase	118	1,871	203	
Rhr2	DL-Glycerol-3-phosphate phosphatase	38	3,125	113	
Protein folding, sorting, and translocation					
Hsp82	Hsp90 chaperone	90	2,117	ND	
Ssa1/Ssa2/Ssa3	ATPase involved in protein folding; HSP70 family	12/13/547	8,178/7,986/290	77/ND/59	
Ssc1	Constituent of the TIM23 complex; HSP70 family	306	709	ND	
Sse1	ATPase component of heat shock protein Hsp90 chaperone complex	106	1,959	101	
Vacuolar acidification and proteases					
Vma1	Subunit of vacuolar H^+-ATPase	180	1,406	109	
Stress adaptation and viability					
Ahp1	Thiol-specific peroxiredoxin	10	9,384	Stable	
Ipp1	Cytoplasmic inorganic pyrophosphatase	221	1,125	883	
Sod1	Cytosolic copper-zinc superoxide dismutase	73	2,358	Stable	
Trx1	Cytoplasmic thioredoxin	305	715	ND	
Transcription factors, DNA replication, nuclear localization, cell cycle, spore development					
Gna1	Glucosamine-6-phosphate acetyltransferase	3,007	11.9	25	
Gsp1	Ran GTPase	403	446	ND	SAPAANGEVPTFKL
Mbf1	Transcriptional coactivator and suppressor of frameshift mutations	206	1,206	106	
Rnr4	Ribonucleotide-diphosphate reductase small subunit	140	1,681	372	

[a] PaxDb integrated data set for protein abundance, rank out of 6,153 proteins (77).
[b] Protein half-life data from Belle *et al.* (78). ND, no data.
[c] Deamidation sites are underlined.
[d] It is unclear whether this peptide represents the unmodified peptide of Pdc1 or the deamidated form of Pdc5.

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

		Rank[a]	Abundance[a]	$t_{1/2}$[b]	Deamidation sites[c]
			ppm	*min*	
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	11/247	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	NVVAGSDLRRTSIIGTIGPK
Eno1	Enolase	16/1	7,491/24,563	ND/27	NIDVKDQKAVDDFL VLNGGSHAGGALALKEF
Fba1	Fructose -1,6-bisphosphate aldolase	3	21,368	104	
Gpm1	Phosphoglycerate mutase	18	6,668	Stable	
Hxk1/Hxk2	Hexokinase	410/32	437/3,413	49/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* reductase	591	259	352	
Tdh1/Tdh2/Tdh3	Glyceraldehyde-3-phosphate dehydrogenase	48/15/2	2,792/7,519/22,369	85/ND/ND	NKETTYDEIKKVVKA NGFGRIGRL
Alcoholic fermentation					
Pdc1/Pdc5/Pdc6	Pyruvate decarboxylase	4	18,671	29	HHTLGNGDFT FVLNNNGYTIE[d] PVFDAPQNLVKQAKLT
Ribosomal proteins and translation					
Efb1	Translation elongation factor 1β	61	2,542	ND	
Grs1	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 S5	30	3,476	ND	
Ssb1/Ssb2	Ribosome-associated molecular chaperone	77/49	2,320/2,791	149/712	
Tef2	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	
Hsp82	Hsp90 chaperone	90	2,117	ND	
Hsp104	Disaggregase	304	721	61	
Ssa1/Ssa2/Ssa3	ATPase involved in protein folding; HSP70 family	12/13/547	8,178/7,986/290	77/ND/59	
Ssc1	Constituent of the TIM23 complex; HSP70 family	306	709	ND	
Sse1	ATPase component of heat shock protein Hsp90 chaperone complex	106	1,959	101	
Vacuolar acidification and proteases					
Ape3	Vacuolar aminopeptidase Y	512	309	9	
Pep4	Vacuolar aspartyl protease	652	225	ND	
Vma2	Subunit of vacuolar H^+-ATPase	238	1,045	29	
Stress adaptation and viability					
Cpr1	Cytoplasmic peptidyl-prolyl cis-trans isomerase	55	2,661	Stable	
Ctt1	Cytosolic catalase T	2,401	22.8	33	
Dak1	Dihydroxyacetone kinase	508	312	42	
Gnd1	6-Phosphogluconate dehydrogenase	27	3,865	ND	
Pst2	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, spore development					
Gsp1	Ran GTPase	403	446	ND	SAPAANGEVPTFKL
Pol1	Subunit of the DNA polymerase Iα-primase complex	3,102	10.3	Stable	NVLQGTLLANPP
Vik1	Forms a kinesin-14 heterodimeric motor with Kar3	5,569	0.54	ND	

[a] PaxDb integrated data set for protein abundance, rank out of 6,153 proteins (77).
[b] Protein half-life data from Belle *et al.* (78). ND, no data.
[c] Deamidation sites are underlined.
[d] It is unclear whether this peptide represents the unmodified peptide of Pdc1 or the deamidated form of Pdc5.

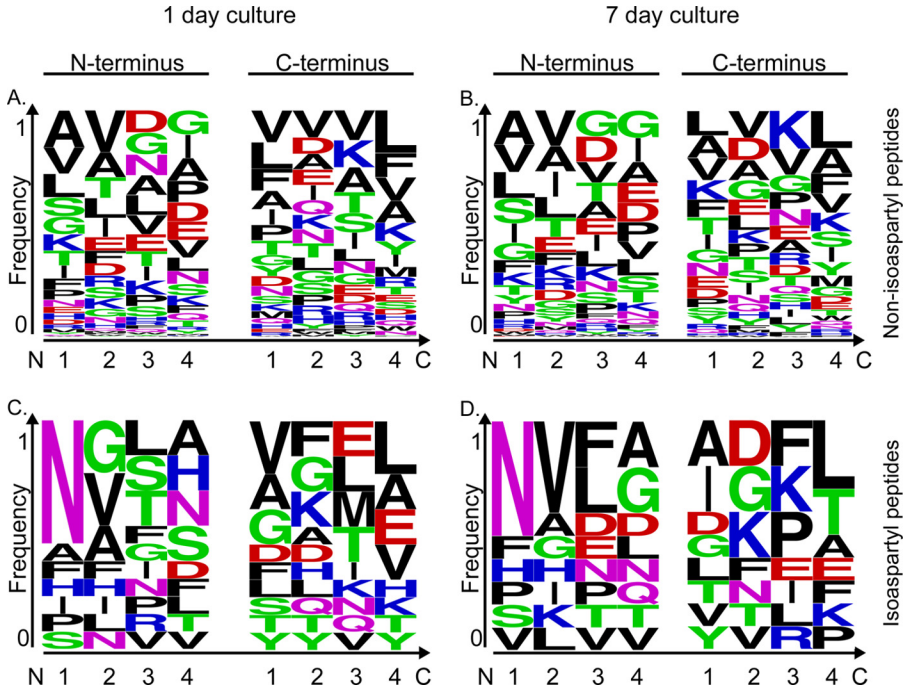

FIGURE 11. **Isoaspartyl-containing peptides have a distinctive N terminus.** The four amino acids present at the N and C termini of the peptides identified by mass spectrometry were analyzed. The amino acid frequencies of both non-isoaspartyl (*A* and *B*) and isoaspartyl-containing (*C* and *D*) peptides from 1- (*A* and *C*) and 7-day (*B* and *D*) cultures *in vitro* aged for 5 days were inputted into WebLogo. We analyzed only peptides with an ion score greater than 20 and ppm error less than 13.

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 metallopeptidases (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. 11*C*). 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. 11*D*). Combined, these data suggest that metallopeptidase 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 metallopeptidases 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 deamidation 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 of the asparagine residues in these sequences deamidating to isoaspartyl residues in 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

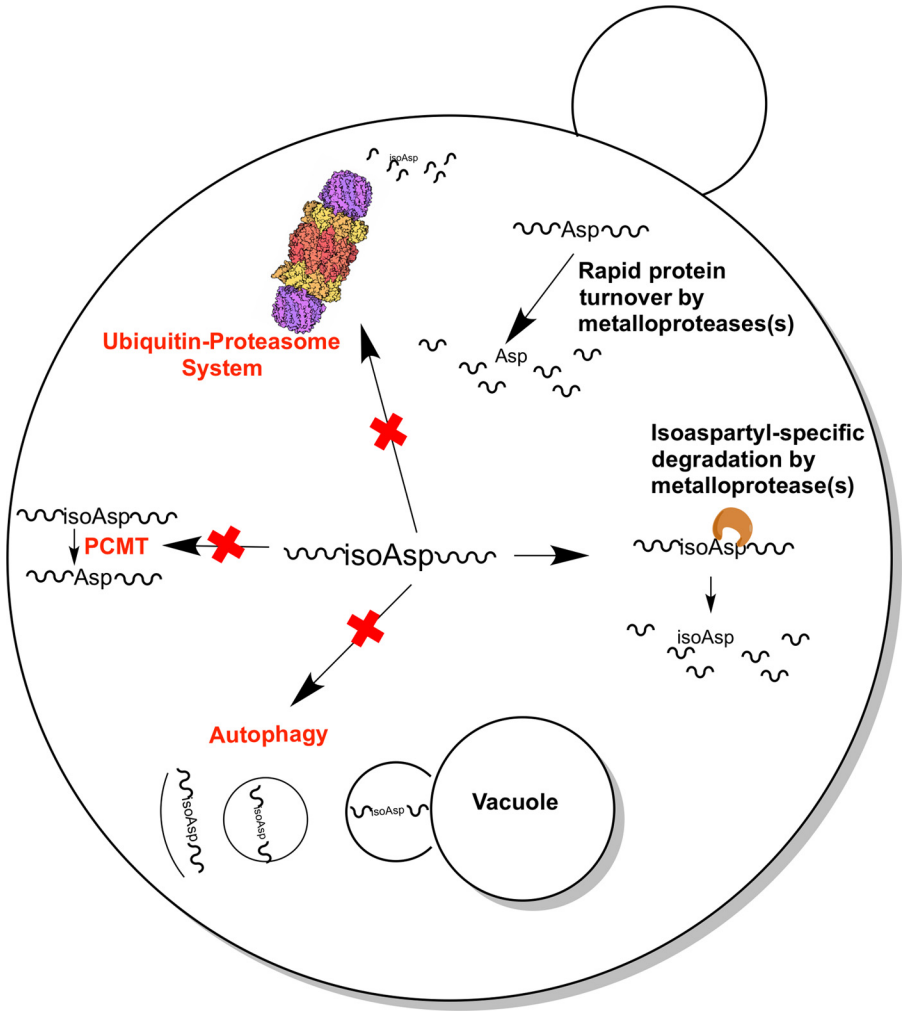

FIGURE 12. **Model of isoaspartyl damage removal in *S. cerevisiae*.** Although *S. cerevisiae* lacks the PCMT enzyme, isoaspartyl damage is low compared with other organisms with PCMT, suggesting an active mechanism to remove isoaspartyl damage. The proteasome and autophagy pathways do not appear to be significantly involved in isoaspartyl control. However, one or more metallopeptidases may be involved in preventing isoaspartyl residue accumulation. These enzymes may be a part of the normal protein turnover or may be specific in recognizing isoaspartyl residues.

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 residues in *S. cerevisiae*. We note that N-terminal isoaspartyl residues can be cleaved in peptides by the IaaA isoaspartyl aminopeptidase present in a variety of organisms (44–47), but BLAST searches against this non-metallopeptidase did not identify any homolog in yeast or other fungi.

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 MATα strain of *S. cerevisiae*, and no change was observed in the MAT**a** 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 *S. cerevisiae* was performed by DeLuna *et al.* (84), who showed that a specific asparagine-glycine sequence in Gdh3 was deamidated *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 *C. elegans*, mice, and humans.

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