Eukaryotic 20S proteasome assembly remains poorly understood. The subunits stack into four heteroheptameric rings; three inner-ring subunits (β1, β2, and β3) bear the protease catalytic residues and are synthesized with N-terminal propeptides. These propeptides are removed autocatalytically late in assembly. In Saccharomyces cerevisiae, β5 (Doa3/Pre2) has a 75-residue propeptide, β5pro, that is essential for proteasome assembly and can work in trans. We show that deletion of the poorly conserved N-terminal half of the β5 propeptide nonetheless causes substantial defects in proteasome maturation. Sequences closer to the cleavage site have critical but redundant roles in both assembly and self-cleavage. A conserved histidine two residues upstream of the autocleavage site strongly promotes processing. Surprisingly, although β5pro is functionally linked to the Ump1 assembly factor, trans-expressed β5pro associates only weakly with Ump1-containing precursors. Several genes were identified as dosage suppressors of trans-expressed β5pro mutants; the strongest encoded the β7 proteasome subunit. Previous data suggested that β7 and β5pro have overlapping roles in bringing together two half-proteasomes, but the timing of β7 addition relative to half-mer joining was unclear. Here we report conditions where dimerization lags behind β7 incorporation into the half-mer. Our results suggest that β7 insertion precedes half-mer dimerization, and the β7 tail and β5 propeptide have unequal roles in half-mer joining.

The degradation rates of intracellular proteins can vary over many orders of magnitude, with half-lives ranging from seconds to years (1–4). In eukaryotes, most non-lysosomal protein degradation occurs through the ubiquitin-proteasome system. The 26S proteasome is a highly conserved and abundant protease complex comprising at least 33 different polypeptides arranged into a 2.5 MDa structure (5–7). For most substrates, their prior modification by ubiquitin polymers targets them to the proteasome, leading to their unfolding, deubiquitylation, and degradation. The proteasome is proving to be an attractive pharmacological target for the treatment of cancer and other disorders (8–10).

Within the 26S proteasome, two major subcomplexes can be resolved: the 19S regulatory particle, which recognizes ubiquitylated substrates and unfolds them, and the 20S proteasome core particle, which is responsible for substrate proteolysis (5, 11). The 20S proteasome is a dyad-symmetric 28-subunit complex made of 14 related but distinct subunits (12, 13). Four heptameric rings of subunits stack coaxially, with an outer pair of α-subunit rings bracketing an inner pair of β-subunit rings. The α rings provide the interface with the 19S particle and other regulators, and they bear narrow central pores that gate substrate entry into a central proteolytic chamber. The central chamber is formed by the two β rings. Of the seven β subunits in each ring, only three bear active sites: β1, β2, and β5. These active subunits are synthesized as N-terminally extended precursors that are processed autocatalytically to yield the mature enzyme. The Thr1 side chain of the mature active subunits serves as the active-site nucleophile, and the N-terminal amino group participates in catalysis as well (13–15). Structural variants of the 20S proteasome have been widely documented, including several with distinct complements of active β subunits; these latter particles are important for MHC class I antigen presentation (16–18).

There are many high resolution crystal structures available for eukaryotic 20S proteasomes (19, 20), but the mechanism of assembly is still far from understood (21). At least three conserved 20S proteasome-specific assembly factors promote its biogenesis (22–24). The first discovered was yeast Ump1 (called hUMP1, POMP, or proteassemblin in humans) (25). In yeast, it is believed that Ump1 associates along with or shortly after the first β subunits (β2, β3, and β4) have been added to a full α-subunit ring (26). This species has been called the 13S or 15S intermediate (26–29); we will use the “13S” name here because the “15S” designation has also been used (30) to refer to a later, nearly complete half-proteasome species that lacks only β7, the “half-mer(−β7) intermediate” (26). Yeast cells lacking Ump1 are viable but accumulate proteasomal particles with partially processed subunits (25). In human cells, depletion of hUmp1 appears to be lethal, and no 20S proteasomal particles
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with unprocessed β subunits accumulate, suggesting an earlier assembly function for hUMP1 not shared with yeast (31).

The other two known 20S proteasome assembly chaperones are the heterodimers Pba1/Pba2 (human PAC1/PAC2) and Pba3/Pba4 (human PAC3/PAC4) (6, 23, 26, 32, 33). These proteins function early in the assembly of the α-subunit ring, and Pba3/Pba4 dissociates before completion of the 13S intermediate. Pba1/Pba2 remains bound to the assembling proteasome all the way to formation of the “pre-holoprotoasome,” in which half-mers have associated but the β-subunit precursors are not yet fully processed. After β-subunit maturation, a conformational change in the α rings is triggered, which leads to both release of Pba1/Pba2 and enhanced 20S affinity for the 19S regulatory particle (30, 32, 34). Degradation of the entrapped Ump1 factor accompanies 20S maturation (25).

In addition to the extrinsic assembly factors that promote 20S proteasome biogenesis, intrinsic elements of the 20S subunits are critical. Subunit-specific appendages that are part of the mature subunits as well as β-subunit propeptides contribute to assembly by supplementing specific subunit-subunit interfaces within the assembling proteasome and possibly by recruiting or positioning extrinsic assembly chaperones (25, 26, 35). The most prominent “intramolecular chaperone” is the β5 propeptide (26, 36). In yeast, this 75-residue propeptide, β5pro,5 is normally essential, and it can function in trans with the truncated mature domain of the β5 subunit (36, 37). Precisely how it promotes assembly is not understood.

Several conditions have been found that allow bypass of the requirement for β5pro. In ump1Δ cells, the β5 propeptide is no longer essential, and the same is true if the propeptide of the β6 subunit is genetically removed (25, 26). The most robust suppression of the β5 propeptide deletion defect occurs through overexpression of the β7 subunit (26). Suppression by excess β7 requires a C-terminal extension of β7 that helps to clamp together the two proteasomal half-mers. This result and additional genetic data strongly suggested that β5pro promotes 20S assembly at least in part by promoting the proper association of two precursor half-mers (26).

In the current work we perform a structure-function analysis of the β5 propeptide to clarify its contributions to 20S proteasome biogenesis in Saccharomyces cerevisiae. We find that β5pro associates only transiently with Ump1-containing precursors even though it is thought to act with Ump1 during assembly (25). Interestingly, large segments of β5pro, particularly in the N-terminal half, are dispensable for viability, although deletions cause substantial maturation defects. Propeptide sequences closer to the autoprocessing site are better conserved and contribute to assembly steps after formation of the 13S intermediate. Sequences immediately adjacent to the processing site are not critical for assembly per se but contribute strongly to autocleavage. In addition, we describe a high copy suppression screen of cells lacking a functional β5pro that yielded β7. Importantly, we found conditions under which β7 incorporates before dimerization of two 15S half-mers (−β7) complexes, and this addition is likely to drive the proper association of half-mers. The results support a model in which the β7 subunit and the β5 propeptide have distinct but overlapping roles in half-proteasome dimerization.

Experimental Procedures

Yeast and Bacterial Media and Methods—Yeast rich (YPD) and minimal (SD) media were prepared as described, and standard methods were used for genetic manipulation of yeast (38). For all phenotypic tests cells were initially grown for several days on rich medium at 30 °C. Cells were tested for growth at 30 °C on SD plates containing 1 μg/ml canavanine and lacking arginine, on SD complete plates containing 30 μM CdCl2, and on either YPD or SD medium at 37 °C. Escherichia coli strains used were MC1061, MC1066, and JM101, and standard methods were employed for recombinant DNA work (26).

Plasmid Constructions—A plasmid expressing N-terminally hemagglutinin (HA)-tagged β5(Doa3)-His6, from the CUP1 promoter (pCIS = YATAG200-CUP1p-HA-DOA3-HIS6) was constructed by amplifying DOA3-His6 from YCplac22-DOA3-His6 (12) with flanking SacI sites and inserting this fragment into the SacI site of the YATAG200 plasmid (GenBankTM U37457). A StyI site was added by silent mutation of β5 codon 73 (GCA to GCC) using QuikChange mutagenesis (Stratagene). The plasmid expressing wild-type (WT) HA-β5 propeptide (β5pro) from the CUP1 promoter (pTRANS = YEpplac112CUP1p-HA-DOA3LS) was prepared as follows. The 5′ primer directed amplification beginning at the 5′ end of the CUP1 promoter of pCIS, whereas the 3′ primer retained the added StyI site and added a stop codon immediately after the Gly75 codon. Flanking XbaI sites were also added by the two primers. After amplification and cloning into pGEM-T EZ, the XbaI fragment was excised and ligated into the corresponding site in YEpplac112 (39), which had its EcoRI site removed by cutting, blunting with Klenow polymerase, and religating (YEpplac112AEcoRI).

Seven charged-to-alanine mutations along the β5pro sequence were introduced into pTRANS and pCIS by two-step PCR. Briefly, in two separate reactions, the 5′ outer primer from the CUP1 promoter region was used together with a long antisense primer containing the desired mutation(s), whereas the other half of the gene was amplified with the 3′ outer primer and a sense primer exactly complementary to the mutant antisense primer, each using pTRANS as template. These two products were annealed to each other and extended in a third PCR reaction, and the mutant fragment was amplified using the same two outer primers. The mutant sequences were subcloned into pCIS and pTRANS using an EcoRI site at the 3′ end of the CUP1 promoter (from YATAG200) and the StyI site introduced into the end of the β5pro sequence and verified by DNA sequencing. The H74A mutation was introduced into pCIS and pTRANS individually as it is 3′ of the StyI site used to subclone the other mutants. The plasmid pCIS-H74A was constructed by two-step PCR and cloning into YATAG200. The pTRANS-H74A plasmid was generated from the HA-β5pro coding sequence by amplification from pTRANS using a 5′ outer primer from the CUP1 promoter that had a flanking XbaI site and a 3′ primer containing the H74A mutation, a stop codon at the 3′ end of β5pro, and a flanking XbaI site. The

5 The abbreviations used are: β5pro, β5 propeptide; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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resulting PCR product was ligated into the XbaI site of YEplac112/AECori. All other site-directed point mutations in β5 were introduced by QuikChange mutagenesis with YCplac22-DOA3-His6 as template. Leaky expression from the CUP1 promoter obviated the need for adding copper to growth media.

N-terminally truncated β5-His6 constructs were produced essentially as described above for pCIS, except that 5′ primers began amplifying from either codon 42 or 56. The resulting plasmids, YATAG200-DOA3Δ2-41 and YATAG200-DOA3Δ2-55, had the N-terminal HA-tag sequences deleted using QuikChange mutagenesis. Internal deletions of codons 42–55 or 56–73 within the N-terminal propeptide coding region of β5 were constructed by a two-stage PCR protocol followed by QuikChange mutagenesis (40) using YCplac22-DOA3-His6 as the template. The resulting alleles were under the control of the natural β5 promoter. The β7 (PRE4) gene was isolated by PCR amplification from genomic DNA and subcloned into yeast- Escherichia coli shuttle vectors YEplac181, YEplac112, and YCplac111. Their functional integrity was verified by complementation of the yeast pre4Δ mutant.

YEplac195-UMP1-HA2 was constructed by subcloning the UMP1-HA2 fragment from pJD-NERF22 (YCplac22-UMP1-HA2; a gift of J. Dohmen) into the SacI sites of YEplac195. Plasmid pRS426-pGPD-UMP1-HA2, which expresses UMP1-HA2 from the strong constitutive GPD promoter (pGPD), was constructed as follows. A DNA fragment containing UMP1-HA2, with a BglII site immediately upstream of the start codon, was PCR amplified from the pJD-NERF22 template. The product was then cloned into pCR-Script Amp (Stratagene), excised with BglII and HindIII, and ligated into the BamHI and HindIII sites of pRS426-pGPD (41). YCplac111-PUP1-HA2 was made by subcloning the EcoRI insert from YCplac22-PUP1-HA2 (42) into YCplac111. pRS317-DOA3pro-His6 and pRS317DOA3His6 were constructed with the BamHI-HindIII inserts from YCplac22doa3Δpro-His6 (36) and YCplac22DOA3-His6 (12), respectively.

Construction of Yeast Strains—Yeast strains used in this study are listed in supplemental Table S1. MHY1030 was isolated by sporulation and tetrad dissection of a heterozygous diploid transformant. MHY1179 was a segregant from a cross between MHY1180 and MHY1326. All CIS strains were made by transforming MHY1030 from a cross between MHY1180 and MHY1326. All CIS strains were made by transforming MHY784 (12) with plasmids bearing the β5 alleles, and TRANS strains were made by cotransforming both pRS317-Doa3Δpro-His6 and pTRANS plasmids into MHY784. Plasmid shuffling was then performed by plating transformants on media containing 5-fluoroorotic acid to identify cells that had lost the URA3-marked YCp50-DOA3 (WT) plasmid (43). All other strains were constructed by plasmid shuffling using the appropriate deletion strains and plasmids. Growth rates of strains with tagged subunits were identical to congenic strains lacking epitope tags.

High-copy Suppressor Screening—TRANS-27/29 and TRANS-69/71 strains were transformed with a yeast high-copy genomic library in the 2μLEU2 YEpl3 vector (44). Transformants were plated on SD Leu− plates and incubated at 35 °C. Temperature-resistant clones were retested. Transformants bearing library plasmids with a full-length β5 gene were weeded out by colony PCR with primers that amplified the β5 gene. The remaining positive library plasmids were recovered in E. coli (MC1060) by selection on M9 (Leu−) minimal plates. Plasmids were transformed back into the original TRANS mutant strain, and those found to continue to show suppression were sequenced. When more than one gene was present within a suppressing genomic fragment, the gene responsible for the suppression was identified by subcloning.

Protein Gel Electrophoresis, Immunoblotting, and Quantitative Analysis of Protein Levels—SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was carried out according to standard procedures (45). For separation of low molecular mass proteins, such as HA-β5pro, 16% Tricine gels were used (46). Otherwise, protein electrophoresis was generally performed using 12% SDS-PAGE gels followed by electrotransfer to Immobilon (Millipore) membranes and immunoblotting. Antibodies used included anti-HA monoclonal 16B12 (Babco), monoclonal MCP231 against 20S proteasome α subunits (Enzo Life Sciences), anti-20S proteasome antisera (gift from K. Tanaka), and an anti-LMP7 (β5-His6) polyclonal antiserum (gift of Y. Yang). Anti-β7 polyclonal antiserum was raised in rabbits using β7-His6 protein purified from E. coli as immunogen or were a gift of W. Heinemeyer. Proteins were detected using ECL reagents (Amersham Biosciences). For protein quantitation, [125I]protein A (PerkinElmer Life Sciences) was used. Pulse-chase analysis was carried out as described (47).

Fractionation of Proteasomes and Peptide Hydrolysis Assays—Proteasomes were resolved by glycerol-gradient ultracentrifugation or FPLC gel filtration as described (26, 36, 48). For chromatography experiments, yeast cell pellets were ground under liquid nitrogen with pestle and mortar, and frozen cell powders were resuspended in ice-cold buffer containing 1 mM ATP and an ATP-regenerating system (49). Peptide hydrolysis activity of 20S proteasomes was measured as described (12). N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methyl-coumarin was used for testing chymotrypsin-like, and N-CBZ-Ala-Arg-Arg-4-methoxy-naphthylamide was used for caspase-like, and N-CBZ-Ala-Arg-Arg-4-methoxy-β-naphthylamide was used for trypsin-like activities (CBZ, carboxyl benzyl). Reactions were carried out at 30 °C for 1 h with 50 μl of gradient fraction, 100 μl of gel filtration fraction, or 6 μg of affinity-purified proteasomes.

Results

Conserved Elements in the β5 Propeptide—Across all eukaryotic species, β5pro is the longest of the active subunit propeptides (~50–85 residues), and studies in different eukaryotes have shown that it has a major role in 20S proteasome assembly (36, 50, 51). Although the sequence of β5pro varies far more among species than does the sequence of the mature domain of the subunit, some common features can be discerned (Fig. 1A). First, the C-terminal-most region of the propeptide is well conserved. This region includes the invariant terminal glycine (at −1 relative to the cleavage site) that is required for autocleavage (36, 52, 53), but also conserved are hydrophobic residues at −4 and −6 and, most strikingly, a histidine residue at the −2 position. A histidine residue at the −2 position is present in precur-
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| A          | Pro-rich          |
|------------|-------------------|
| Sc_Do3     |                   |
| Eg_b5      |                   |
| Sp_P1s     |                   |
| Gc_IMP7    |                   |
| Ms_IMP7    |                   |
| Hs_IMP7    |                   |
| Hs_MBI1    |                   |
| Ce_b3      |                   |
| Dm_b5      |                   |

**TABLE 1**

Growth of cells expressing chimeric B5 subunits

| Rows | Relevant genotype                                      | 30 °C | 37 °C |
|------|--------------------------------------------------------|-------|-------|
| 1    | WT B1                                                  | +++++ | +     |
| 2    | B5pro-B1Δ pro                                          | +++++ | +     |
| 3    | UBI-B1Δ pro                                            | +     | ND    |
| 4    | B1-T20A                                                | +     | ND    |
| 5    | UBI-B5Δ pro B5 pro                                     | +     | ND    |
| 6    | B5pro-B1Δ pro UBI-B5Δ pro                              | +     | ND    |
| 7    | B5pro-B1Δ pro UBI-B5Δ pro UBI-B5 pro                   | +     | ND    |

FIGURE 1. The B5 propeptide. A, sequence comparison of B5 propeptides from a range of eukaryotes. The site of processing (arrowhead) and the first residue of mature B5 are also shown. Sequences were aligned with ClustalW with some manual adjustment. Sc, S. cerevisiae; Eg, Eremothecium goessypii; Sp, Schizosaccharomyces pombe; Gp, Ginglymostoma cirratum; Mm, Mus musculus; Hs, Homo sapiens; Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster. B, the B5 propeptide is not autocatalytically processed when fused to the mature domain of B1. Proteins were visualized by anti-HA immunoblotting. Size markers (kDa) are shown at the right. *Interm* intermediates generated by processing within the proteasome as demonstrated by the increased mass resulting from mutation of the B2 (Pup1) active site Thr to Ala (T30A). Note that the *Interm* band in lanes 3 and 4 migrates more quickly than pre-B1-HA precursor in lane 1.

| Sc_Do3 | 61 D [SIG F P D C N H G T] |
| Eg_b5  | 59 D [S G F P D C N H G T] |
| Sp_P1s | 47 D [P E N N C H G T]    |
| Gc_IMP7| 58 E [G E D E G V N C H G T] |
| Ms_IMP7| 59 G [E R N V C H G T]    |
| Hs_IMP7| 59 G [E R N V C H G T]    |
| Hs_MBI1| 45 G [G A K G Y C H G T]  |
| Ce_b3  | 52 G [N D G K G Y C H G T] |
| Dm_b5  | 59 G [N D G K G Y C H G T] |

Of note, if the only cellular source of B5 propeptide was the B5pro-B1 chimera, cells could grow, albeit poorly (Table 1, row 6). A similar observation had been made for the homologous human chimera B5pro-B1Δ (LMP7 propeptide fused to the LMP2 mature domain) (28). This *trans* effect was not nearly as effective as isolated B5pro expressed in *trans* (Table 1, row 5), and the B5pro-B1 chimera appeared to interfere with the latter (Table 1, compare rows 5 and 7).

**Trans-expressed B5pro Is Not Stably Associated with Later Assembly Intermediates**—The apparent interfering effect of the B5pro-B1 chimera on *trans*-expressed B5pro led us to ask if B5pro associated noncovalently with precursor forms of the 20S proteasome. To examine this we resolved mature proteasomes from proteasomal subparticles by FPLC Superose-12...
fractionation) or the mature proteasome (fractions 15 and 16; marked by mature β2, β5, and β7 subunits). Interestingly, the HA-β5pro peak eluted slightly earlier (fraction 25) than the free β5pro subunit (fraction 26), suggesting that it also was not tightly associated with the mature domain of β5. Because the 9-kDa HA-β5pro is smaller than β5pro-His<sub>6</sub>, its earlier elution implies either that it is associated with itself or another unidentified protein or that it has a highly extended conformation. Roughly half of the propeptide is predicted to be random coil based on secondary structure predictions.

The Ump1 assembly factor becomes entrapped within the 20S proteasome during 20S assembly and is then degraded upon active-site maturation at the end of assembly (25). A similar fate is anticipated for the β5 propeptide. We, therefore, tested if <i>trans</i>-expressed β5pro could be detected in association with mature 20S proteasomes if proteasomal proteolytic activity was impaired. Superose-12 fractionation of extracts from a <i>pre1</i>−1 mutant, which expresses a mutated β4 subunit that causes a significant defect in proteolysis (54), did indeed allow detection of HA-β5pro in association with fully formed proteasome particles (Fig. 2C). Therefore, β5pro likely associates with the proteasomal precursor after 13S formation and is then degraded upon completion of assembly and active-site maturation.

**Deletion Analysis of the β5 Propeptide**—As noted, the β5-propeptide sequence is only weakly conserved outside of a few small regions (Fig. 1A). We used deletion analysis to examine the significance of the different propeptide regions for β5-precursor processing and proteasome assembly (Fig. 3). A deletion leaving only the initiator methionine and the last two residues of the propeptide (Δ2–73) was lethal (Fig. 3A). This result indicates that the remaining highly conserved residues, His-74 and Gly-75, are not sufficient for propeptide function. Deletion of the poorly conserved region upstream of the proline-rich element (Δ2–41) was relatively well tolerated with only a minor impairment to growth under optimal conditions, however, β5Δ2–41-expressing cells were inviable when grown on the amino acid analog canavanine, a condition poorly tolerated by proteasome-deficient cells (Fig. 3B). Similarly, removal of the Pro-rich region (Δ42–55) allowed near-normal growth under most conditions, with the exception of canavanine. In contrast, a deletion encompassing both of the preceding deletions (Δ2–55) caused a much more severe growth defect under all tested conditions, although the cells were still viable. This suggested a partially redundant function shared by regions 2–41 and 42–55. Cells also grew poorly when the region just upstream of the precursor-processing site was deleted (Δ2–41) and 42–55. Therefore, both deletions were extended to include the Pro-rich segment (Δ42–73), cells were no longer viable (Fig. 3, A and B), again suggesting functional redundancy.

The growth defects exhibited by the β5 propeptide deletion mutants could be due to impaired chaperone function or reduced autocleavage at the β5 processing site, which is known to cause a severe growth defect, or both. Propeptide processing in the viable deletion mutants was examined by immunoblotting with an antibody that recognized the C-terminally His<sub>6</sub>-tagged β5 proteins (Fig. 3C). All of the deletions impaired autocleavage compared with WT β5. Interestingly, the Δ2–41 and
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Deletion analysis of the yeast β5 propeptide. A, deletions of the S. cerevisiae β5 propeptide described in the current study. Residues flanking each deletion are indicated. Viability was assessed by a plasmid-shuffle assay. B, growth properties of viable yeast strains with β5 propeptide deletion alleles (all carry a C-terminal His6 tag). Exponentially growing cultures were plated in 1:5 serial dilutions on the indicated medium and incubated for 2 days (30 °C/YPD) or 3 days (other plates). The strains used were (from the top): MHY3543, MHY3544, MHY3545, MHY2970 and MHY2890. C, expression and in vivo processing of β5 that has nonlethal deletions in its propeptide. Extracts from strains expressing the indicated plasmid-borne constructs (same as panel B) were subjected to SDS-PAGE followed by immunoblotting with serum raised against human LMP7-His6. Asterisks to the left of the lanes mark immediately processed precursors. Pgk1 (phosphoglycerate kinase) was used as loading control. D, gel filtration analysis of β5 proteins lacking various propeptide segments. Superose-6 chromatography of whole-cell extracts was followed by anti-LMP7 (β5) immunoblot analysis. All the β5 proteins have a C-terminal His6 tag, which is necessary for anti-LMP7 detection. Yeast strains, from the top: MHY3543, MHY3544, MHY2970, MHY3545, and MHY2890. The WT β5 precursor, which is present at low levels, was not detected at this film exposure. Positions of calibration standards are indicated at the bottom.

FIGURE 3. Deletion analysis of the yeast β5 propeptide. A, deletions of the S. cerevisiae β5 propeptide described in the current study. Residues flanking each deletion are indicated. Viability was assessed by a plasmid-shuffle assay. B, growth properties of viable yeast strains with β5 propeptide deletion alleles (all carry a C-terminal His6 tag). Exponentially growing cultures were plated in 1:5 serial dilutions on the indicated medium and incubated for 2 days (30 °C/YPD) or 3 days (other plates). The strains used were (from the top): MHY3543, MHY3544, MHY3545, MHY2970 and MHY2890. C, expression and in vivo processing of β5 that has nonlethal deletions in its propeptide. Extracts from strains expressing the indicated plasmid-borne constructs (same as panel B) were subjected to SDS-PAGE followed by immunoblotting with serum raised against human LMP7-His6. Asterisks to the left of the lanes mark immediately processed precursors. Pgk1 (phosphoglycerate kinase) was used as loading control. D, gel filtration analysis of β5 proteins lacking various propeptide segments. Superose-6 chromatography of whole-cell extracts was followed by anti-LMP7 (β5) immunoblot analysis. All the β5 proteins have a C-terminal His6 tag, which is necessary for anti-LMP7 detection. Yeast strains, from the top: MHY3543, MHY3544, MHY2970, MHY3545, and MHY2890. The WT β5 precursor, which is present at low levels, was not detected at this film exposure. Positions of calibration standards are indicated at the bottom.
His-74 might function in the β5 precursor, we also changed the residue to lysine or phenylalanine within full-length β5. For these constructs we omitted the N-terminal HA tag because it appeared to interfere slightly with propeptide processing (compare lanes 1 in Fig. 4, B and D). Replacement of His-74 with Lys, a residue found at the –2 position in the β5 propeptides of certain eukaryotic lineages (see above), caused only minimal reduction in propeptide processing efficiency (Fig. 4D). In contrast, mutation of His-74 to Ala or even to Phe, whose aromatic side chain is similar in size to that of histidine, resulted in the accumulation of unprocessed and intermediately processed subunits (Fig. 4D). As we had found previously (36), impaired processing of β5 leads to its overexpression, and levels of mature β5 in the processing-defective strains approached those in WT cells.

Together, these data suggest that autocatalytic cleavage of the β5 precursor utilizes sequences in the propeptide in a way not used in the other active-subunit precursors, which lack a conserved His/Lys residue at the –2 position of their propeptides. This residue is unlikely to function as a general base in the β5 autocatalytic cleavage reaction as processing, although inhibited, is still observed in the Ala and Phe substitution mutants. Recent crystallographic data on the Phe mutant are consistent with this conclusion.6

**Impaired Proteasome Function in Trans-expressed β5 Propeptide Mutants**—Analysis of growth phenotypes for the eight TRANS alanine mutants revealed that all but TRANS-16,17 and TRANS-61,62,64 were defective for growth under at least one of the tested conditions (Fig. 5A). Expression levels of most of the mutated TRANS propeptides were similar to the WT control, although TRANS-61,62,64 and TRANS-74 propeptide levels were slightly reduced (Fig. 5B). This suggests that except for possibly TRANS-74, differences in propeptide expression cannot account for the growth defects. The short-lived model proteasomal substrates MATα2 and Deg1-β-β-galactosidase were stabilized ~2-fold in both the TRANS (WT) and TRANS-27,29 strains (data not shown) relative to their published half-lives in WT cells (45), indicating a modest proteasomal defect when the β5 propeptide is expressed in trans. This might be due to partial acetylation of the β5 Thr-1 α-amino group (42). Pulse-chase analysis of MATα2 at 37 °C, a nonpermissive growth temperature for TRANS-27,29, revealed a further 1.5-fold stabilization in this strain relative to TRANS (WT) (data not shown). This additional defect at high temperature could derive from impairment of particular 20S proteasome active sites or from defective proteasome assembly.

To distinguish between these two possibilities, the two most severe mutants, TRANS-27,29 and TRANS-69,71, were assayed for their relative specific activities based on active site-specific peptidase activity measurements of gradient-purified proteasomes. All of the three diagnostic peptidase activities in proteasomes from these two mutants were similar to or slightly higher than those from the TRANS (WT) strain (Fig. 5C). This result suggests that there are no defects in the active sites in these mutants that could account for the observed proteolytic and growth defects.

To assay potential defects in proteasome assembly, we measured the rate of processing of the β2 subunit in TRANS (WT) and in TRANS-27,29 strains by pulse-chase analysis (Fig. 5D). Normally, preβ2 accumulates in the Ump1-containing 13S assembly intermediate, where it remains unprocessed; once assembly has proceeded to pre-holoproteasomes, preβ2 rapidly self-cleaves (25). The preβ2 processing rate is, therefore, directly related to the rate of conversion of the 13S intermediate into mature proteasomes. The TRANS (WT) strain was already processed fairly slowly, consistent with the in vivo proteolytic defects noted above. Nonetheless, we observed substantially slower processing with the TRANS-27,29 mutant (Fig. 5D), suggesting that TRANS-27,29 is defective for proteasome assembly and/or maturation. Additional evidence for an assembly defect is provided below.

**High-copy Suppressors of Trans-expressed β5 Propeptide Mutants**—The apparent assembly defects of the above TRANS mutants might result from reduced interaction between β5pro and proteasome assembly intermediates or in a general loss of β5pro function. β5pro-interacting proteins or proteins

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6 E. Huber, W. Heinemeyer, X. Li, C. S. Arendt, M. Hochstrasser, and M. Groll, manuscript in preparation.
specific activities were determined by quantitative [125I]protein-A immunodetection. Gradient conditions are described in Ref. 42. Proteasome-containing protein based on Bradford assays were fractionated on 10–40% glycerol gradients.

Spheroplasts of cells grown at 30 °C were lysed, and equal amounts of total cellular protein from the indicated strains in Fig. 6 were separated on a 16% Tricine SDS-PAGE gel followed by immunoblotting using anti-20S antibodies. Each lane was loaded with equal OD at A600 cell equivalents (44). The high-copy suppressors isolated are listed in Table 2.

Not unexpectedly, the WT β5 (DOA3) gene was isolated multiple times and was the strongest suppressor. Also obtained in the screen was a plasmid with an insert bearing the β5 promoter and propeptide coding sequence up to a Sau3AI site, which is predicted to produce a WT version of β5pro through residue 72 followed by a 10-residue missense peptide. This plasmid suppressed the temperature sensitivity of both TRANS-27,29 and TRANS-69,71 strains nearly as well as overexpression of the WT β5pro and also suppressed very strongly the lethality of the β5Δpro mutation (Table 2). These results indicate that the C-terminal three residues of β5pro are not critical for function in trans, although the last two residues are critical in cis for efficient propeptide processing (see Fig. 4 and Ref 36).

The second strongest suppressor, β7 (PRE4), was identified 15 times and strongly suppressed both TRANS-27,29 and TRANS-69,71 defects (Fig. 6A). Moreover, high-copy expression of β7 suppressed a complete deletion of β5pro (Fig. 6B and Ref. 26). Because the lethality of the β5Δpro mutation can also be suppressed by deletion of the LUMP1 gene (25), we asked if there were some link between β5pro expression levels to suppress the growth defects. However, co-overexpression of LUMP1-HA2 with β7 in β5Δpro cells had no effect on growth, suggesting that suppression by overexpression of β7 is independent of Ump1 levels (Fig. 6B). Interestingly, when 20S proteosomes were purified from β5Δpro cells that overproduced β7, the particles were essentially indistinguishable from proteosomes from WT β5 cells (Fig. 6C) as noted earlier (26). However, the efficiency of proteosome maturation appeared slightly impaired, as we could detect the precursor form of the β2 (Pup1) subunit in these affinity-purified particles (arrowhead in Fig. 6C).

The ability of high β7 levels to suppress the growth defects associated with β5 propeptide mutations correlated with enhanced formation of proteosomes. Proteosomes were isolated by glycerol-gradient fractionation of equal amounts of cellular protein from the indicated strains in Fig. 6D. Using the specific-activity values from Fig. 5C and peptidase measure-

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**TABLE 2**
High-copy suppressors of β5 propeptide mutants

| Gene       | Times cloned | Protein function | Strength of suppression | Suppresses β5Δpro? |
|------------|--------------|------------------|-------------------------|--------------------|
| DOA3       | 10           | 20S proteasome β5 subunit | ++++                    | Y                  |
| PRE4       | 15           | 20S proteasome β5 subunit | ++++                    | Y                  |
| DOA3LS5    | 1            | Doa3 β5-subunit propeptide | ++++                    | Y                  |
| FPR3       | 1            | Prolyl isomerase    | +++                     | N                  |
| SLI2       | 1            | Translation initiation factor | +                      | N                  |
| HYP2       | 2            | Translation factor elf-5 | +++                     | N                  |
| OLE1       | 1            | Δ9-Fatty acid desaturase | [N]                    |                    |

* Allele expresses a β5 fragment ending in HRTGVYAMA after the WT propeptide sequence residues 1–72 (residues 73AHG75 of the propeptide missing).

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**FIGURE 5.** Point mutagenesis of the trans-expressed β5 propeptide (TRANS). A, growth of trans-expressed β5 propeptide point mutants in β5Δpro cells. Cells were streaked on the indicated plates and grown for 2 days (YPD) or 4 days (SD + canavanine). Strains used were, counter-clockwise starting from the top, MHY1968 (WT)–MHY1976. B, expression levels of trans-expressed β5 propeptide point mutants. SDS lysates from equal OD at A600 cell equivalents were separated on a 16% Tricine SDS-PAGE gel followed by anti-HA immunoblot analysis. Strains used were, from the left, MHY1968–MHY1976. C, specific activity of 20S proteasomes in selected mutants. Spheroplasts of cells grown at 30 °C were lysed, and equal amounts of total protein based on Bradford assays were fractionated on 10–40% glycerol gradients. Gradient conditions are described in Ref. 42. Proteasome-containing fractions were pooled and analyzed for three peptidase activities. Relative specific activities were determined by quantitative [125I]protein-A immunoblotting using anti-20S antibodies. Each bar represents the mean value calculated from triplicate measurements from three independent extracts. Error bars denote S.D. D, pulse-chase analysis of preβ2-HA2 (Pup1) processing in a strain with a trans-expressed β5pro point mutant. Analysis was done at 30 °C in MHY2006 (TRANS) and MHY2007 (TRANS-27,29). Cells were radiolabeled for 20 min with and chased for the indicated times. β2-HA2 was immunoprecipitated with an anti-HA antibody.
ments, we calculated the relative levels of proteasomes in cells either with or without overexpressed β7. By this measure, ~20–30% decreases in proteasomes were seen in the TRANS-27,29 and TRANS-69,71 mutants relative to the control TRANS (WT) strain. The deficit of proteasomes in these mutants was overcome by increased dosage of β7 (Fig. 6D).

Higher levels of proteasomes were also seen when TRANS (WT) cells expressed excess β7. We observed a similar, but smaller increase in proteasomes in fully WT cells overproducing β7 (not shown), suggesting that β7 levels may normally be at least partially rate-limiting for 20S proteasome assembly in yeast cells.

**FIGURE 6. Suppression of trans-expressed β5 propeptide mutants by overexpressed β7 proteasome subunit and β7 incorporation into half-proteasomes.**

**A,** suppression of high temperature growth defects of β5pro mutants by overexpressed β7. 10-Fold serial dilutions of MHY1968, MHY1972, and MHY1975 strains transformed with empty vector or 2μ vector-borne β7 were spotted on SD Leu− plates and grown as indicated. **B,** high levels of β7 bypass the need for the β5 propeptide by a mechanism not requiring reduced Ump1 levels. The strain MHY2008 was transformed with a high-copy vector that was either empty or expressed UMP1-HA2 from the UMP1 or GPD promoter. 10-Fold serial dilutions were spotted onto selective plates and incubated at 30 °C for 3 days. Lysates from these same strains were used for immunoblotting with anti-HA and anti-Pgk1 antibodies (lower panels). **C,** proteasomes purified from β5 WT (MHY2831) and β5pro (MHY2832) cells overexpressing β7 have similar composition, but the latter appear to mature less efficiently. Proteasomes were purified from yeast by anti-FLAG (β4 tag) affinity chromatography as described (26). The SDS gel was stained with Coomassie Blue. The arrowhead marks the position of the β2 precursor, identified by mass spectrometry of the excised band. The band immediately below it is likely to be mature β2, and its levels are slightly lower than in WT particles, as predicted if β2 maturation is less efficient. **D,** the reduced levels of proteasomes in TRANS-27,29 and TRANS-69,71 β5 propeptide mutants are restored by high-copy β7. Equal amounts of cell protein from each strain were fractionated in parallel on glycerol gradients, and peptidase activities of the gradient-purified proteasomes were measured as in Fig. 5C; specific activities were determined from the data in Fig. 5C were used to calculate relative proteasome levels. **E,** Superose-6 chromatographic fractionation of extracts from cells (left, MHY3541; right, MHY3542) bearing a low-copy (LC) plasmid expressing β7. Immunoblot analysis was performed with anti-β5 and anti-β7 antibodies.
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Four additional genes also isolated in the screen weakly suppressed both TRANS-27,29 and TRANS-69,71 mutants but did not support growth in the complete absence of propeptide (Table 2). Fpr3 is a peptidylprolyl isomerase that concentrates in the nucleolus and has multiple functions (55). SUII2 encodes a subunit of translation initiation factor elf-2 (56). Hyp2, also called elf5A, promotes translation elongation, especially through consecutive proline residues (57). All three of these high-copy suppressors might suppress propeptide defects by promoting increased translation of one or more limiting proteasome subunits or assembly factors. The fourth gene, OLE1, encodes the Δ9 fatty acid desaturase that produces mono-unsaturated fatty acids (58). Although suppression of mutant β5pro by overexpression of OLE1 was not particularly strong, it was the only gene in this group of four that suppressed the full deletion of the β5pro, after loss of a WT β5 plasmid, for enough generations to allow very small colonies to form before growth halted. The proteasome is required for transcription of OLE1 (59). That overexpression of OLE1 can suppress the deletion defect of β5Δpro and, therefore, the cessation of proteasome assembly for more than a few generations supports the suggestion that control of Ole1 levels is an essential function of the proteasome (59).

The β7 Subunit Is Incorporated before Half-mer Joining—Previous results from our laboratory and others indicated that β7 is the final subunit to be added during 20S proteasome assembly (26, 31, 60). Importantly, β7 incorporation is tightly coupled to the joining of two half-proteasomes (half-mers) that form the pre-holoproteasome. This close temporal linkage has prevented the determination of the actual sequence of assembly events at this critical juncture. One possibility is that β7 adds to the half-mer, and this subsequently promotes the joining of completed half-mers. Alternatively, half-mers lacking β7 might transiently associate, creating binding sites for β7 addition and allowing completion of core particle assembly. Further analysis of β7 suppression of the β5Δpro proteasome assembly defect has unexpectedly allowed us to address this question.

As noted above, proteasome formation in β5Δpro cells over-expressing β7 appears to be less efficient than in WT β5 cells (Fig. 6C). We, therefore, separated mature 20S and 26S proteasomes from precursor complexes using Superose-6 FPLC gel filtration followed by immunoblot analysis of column fractions (Fig. 6E). When β7 was expressed from a low-copy plasmid in cells expressing WT β5, virtually all of the detected β7 was in full-size proteasomes, consistent with the tight link between β7 addition and half-mer joining (Fig. 6E, left). The β7 subunit is synthesized as a precursor that is N-terminally processed by a neighboring active site (β2) in the newly matured proteasome (53). No β7 precursor was detected in any proteasomal particles, and no β7 could be detected outside of the proteasome, suggesting that β7 was present in limiting amounts for proteasome assembly. In striking contrast, when the same low-copy β7 plasmid was present in β5Δpro cells, extensive accumulation of free β7 was detected (Fig. 6E, right, fractions 30–32; partially proteolyzed in the extract). Most importantly, a new peak (fraction 21; arrowhead) containing the β7 precursor eluted just ahead of the 13S intermediate peak, consistent with the addition of β7 to the half-mer (−β7) complex before half-mer joining and subunit processing. These data strongly suggest that β7 adds to the half-mer, and this helps drive half-mer joining rather than the reverse sequence.

Discussion

In yeast, β5pro is the only propeptide that is normally essential for proteasome assembly, but before the present study it had not been subject to any systematic mutagenesis analysis. Using both deletion and scanning charged-to-alanine mutagenesis, we find that S. cerevisiae β5pro, despite relatively poor primary sequence conservation, bears elements through much of its length that contribute to both proteasome assembly and processing; sequences in the latter half of the 75-residue propeptide are the most critical. Although propeptide sequences near the β5 precursor cleavage site are crucial for autoprocessing, the trans functions of β5pro remain less well understood. Surprisingly, our data suggest only weak interaction of β5pro with other proteins within proteasome precursors, which include the Ump1 assembly factor. Other results provide support for a role for the propeptide in the joining of half-mer particles, a function partially shared with the β7 subunit, which we now show can incorporate into half-mers before dimerization.

Previously we found that interactions between the tail of the β7 subunit in one β ring and a binding pocket in the opposing β ring allowed overexpressed β7 to suppress the inviability caused by deletion of the β5 propeptide (26). Such overexpression also suppressed a specific β5 mutant allele known to have a destabilized β-β ring interface (12). This led to the inference that β5pro helps guide half-mer joining. A simple way to imagine how β5pro functions in half-mer dimerization would be for it to directly bind specific subunits in the opposing β ring, even potentially the other β5 propeptide, in the juxtaposed half-mer complex. Although computational structure prediction with PHYRE2 (61) failed to yield high confidence predictions of S. cerevisiae β5pro folding, it is intriguing that the strongest similarity found was between the central segment of β5pro (residues 23–44) and the dimerization domain of E. coli EF-Ts (domain d1efu4; 36% sequence identity) (not shown). Potentially, these segments of the β5 propeptides from apposed half-mers could make direct contacts and facilitate dimerization of the two subcomplexes. Although we have not detected β5pro−β5pro interactions by yeast two-hybrid or split-ubiquitin binding assays, weak interactions might suffice, especially because β5pro is normally covalently tethered to the nascent β ring.

One view of the poor sequence conservation of the β5 propeptide is that most of it is of little relevance. Alternatively, there might be functional redundancy within the propeptide or new functions that have evolved rapidly in the less structurally constrained propeptide compared with the mature domain (or both). Our deletion and point mutagenesis argue that even the poorly conserved segments contribute to β5pro activity. For example, removal of the first 41 residues of the propeptide, which show minimal sequence conservation (Fig. 1A), nevertheless leads to a pronounced autoprocessing defect (which might be due to reduced incorporation into assembling protea-

[2] C. S. Arendt, and M. Hochstrasser, unpublished data.
somewhat) and an inability of the mutant cells to grow under proteotoxic stress conditions (Fig. 3). The deletion data also indicate functional redundancy within the propeptide. For example, separate deletions of residues 42–55 and 56–73 yield viable cells capable of proteasome formation, but combining the two (Δ42–73) is lethal (Fig. 3). Moreover, it is likely that β5pro has evolved distinct specializations in different organisms, which would lead to sequence divergence as well. For instance, the propeptides of two β5 paralogs in humans, β5/PSMB5 and β5i/LMP7, have markedly different assembly-promoting activities (62, 63). The propeptide of the third human β5 paralog, β5t, has been shown to allow incorporation of β5t at an earlier assembly stage than the constitutive β5 subunit, again implying functional specialization (64).

A recent chemical cross-linking study of the 15S half-mer (β7) intermediate revealed two cross-links involving the β5 propeptide segment: one cross-linked Lys-69 of the propeptide to β4 and the other was between Lys-16 of β5pro and α6 (30). As noted by the authors, the latter cross-link suggested that the β5 propeptide at this assembly stage pointed toward the α ring rather than toward the future interface with a second half-mer. The former cross-link, however, involved a β4 side chain (Lys-28) that is on the outside surface of the mature 20S proteasome; therefore, in the 15S intermediate or the full half-mer, the β5pro domain might well extend beyond the β-ring surface and facilitate contacts with the other half-mer. Such flexibility and the large distances between these two β5pro cross-links are consistent with an extended conformation of β5pro (Fig. 2) and the prediction of a significant fraction of intrinsic disorder in the propeptide.

A systematic protein interaction study of the proteasome reported a yeast two-hybrid signal between Ump1 and β5, although the segment(s) of β5 responsible for the apparent binding to Ump1, which might be indirect, was not determined (65). Our data indicate that the yeast β5 propeptide is not sufficient for tight association with Ump1 or any other component of the 13S precursor (Fig. 2). By contrast, experiments with human β5 and β5i precursors suggest that the propeptide contributes to association with hUmp1 (17).

The alanine-scanning mutagenesis of the trans-expressed β5pro, which should be minimally disruptive to folding, suggests that two widely separated propeptide surfaces (27ESD29 and 69KIK71) are especially sensitive to mutation (Fig. 5). These data would be consistent with defects in protein-protein interaction, and our high-copy suppression screen of these mutants yielded hits that all could be consistent with an ability to overcome such a defect. Specifically, the screen yielded multiple factors that are involved in protein translation, which by overexpression might enhance levels of limiting factors (such as β7 or mutant β5pro) capable of promoting proteasome biogenesis. The Hyp2/ elf5A translation elongation factor is known to be important for translation through consecutive prolines (57). It could potentially augment expression of β5pro by translation through the proline-rich segment of the propeptide. Another of the translation-related factors, Fpr3, is a peptidylprolyl isomerase known to enhance ribosome biogenesis, among other functions (55, 66). Fpr3 might have a more direct impact on folding of proteasome subunits, including potentially the proline-rich element of β5pro. A conformation switch in the β5 propeptide that repositions Ump1 would be consistent with a previously proposed mechanism for its essential assembly activity (25).

Autocatalytic β5 propeptide cleavage depends strongly on sequences immediately surrounding the cleavage site and the propeptide segment upstream of the cleavage site (Fig. 4 and Ref. 36). The histidine two residues upstream of the cleavage site, although highly conserved, is not absolutely essential, arguing against a direct catalytic role for this residue. It is potentially important for stabilizing a structure of the propeptide necessary for efficient cleavage. In the crystal structure of a 20S mutant with an incompletely processed β1 subunit, the β1 propeptide assumes a γ-turn conformation around the glycine (at −1) residue (67). A His (or Lys) residue preceding this conserved glycine might promote such a conformation in the β5 propeptide. However, additional information beyond the propeptide and flanking residues is required for β5 autocleavage inasmuch as fusion of β5pro to the mature domain of β1 completely blocks proper cleavage even though a substantial fraction, at least, of the chimeric subunit is correctly incorporated into the proteasome (Fig. 1B; Table 1). Misplacement of β5pro in the pre-holoproteasome may prevent its interactions with neighboring subunits or Ump1 that normally place the precur- sor into an autocleavage-competent conformation.

Results with both yeast and mammalian 20S proteasome assembly indicate that β7 is the last subunit to incorporate into precursor complexes, and this is tightly coupled to half-mer dimerization (24). It had been unclear whether β7 adds to the 15S half-mer (β7) complex, which is then rapidly followed by dimerization, or if two 15S half-mers form a metastable complex that is subsequently stabilized by β7 additions. Fortuitously, we have found that low-copy plasmid expression of the β7/Pre4 gene in β5Δpro cells leads to a build-up of the β7 subunit precursor in a complex with an apparent size just slightly greater than the 13S intermediate (Fig. 6E). The peak is substantially below the size of the 20S proteasome (~670 kDa), and the pre-holoproteasome is expected to be even larger than mature 20S (26). Therefore, these data are most consistent with β7 adding to the half-mer before dimerization. It is possible that the full half-mers are nonproductive intermediates, but it is unclear why this would be the case. We also cannot fully exclude the possibility that half-mers associate, bind β7 subunits, and then dissociate again; however, β7 addition should stabilize the dimer, so this idea seems unlikely. We propose that the addition of β7 to the 15S intermediate induces structural changes in the β ring (30) and provides a key additional trans-β-ring interaction through its conserved C-terminal tail to drive rapid dimerization. In cells lacking β5pro but expressing excess β7, a step downstream of β7 addition but before dimerization appears to become limiting, consistent with a distinct role for β5pro in half-mer joining.

The current study provides new insight into the function of the β5 propeptide in 20S proteasome assembly and β5 precursor processing. It also clarifies the entry point of the β7 subunit into the proteasome assembly pathway, reinforcing the hypothesis that β7 and β5pro have overlapping but unequal functions in promoting dimerization of proteasome half-mers. Future structural and biochemical studies of the β5 propeptide in the
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context of various proteasomal precursors will be needed to get a finer-grained understanding of its precise and potentially evolving roles in proteasome biogenesis.

Author Contributions—X. L., Y. L., C. A. S., and M. H. performed all the experiments and analyzed the data. M. H. wrote the paper, which was reviewed and approved by all the authors.

Acknowledgments—We are grateful to Doug DeMarini, Juergen Dohmen, Wolfgang Heinemeyer, Keiji Tanaka, and Young Yang for plasmids and/or antibodies.

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