

A multimeric assembly factor controls the formation of alternative 20S proteasomes

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The proteasome is the central regulatory protease of eukaryotic cells. Heteroheptameric α -subunit and β -subunit rings stack to form the 20S proteasome, which associates with a 19S regulatory particle (RP). Here we show that two yeast proteins, Pba3 and Pba4, form a previously unidentified 20S proteasome-assembly chaperone. Pba3-Pba4 interacts genetically and physically with specific proteasomal α subunits, and loss of Pba3-Pba4 causes both a reduction and a remodeling of cellular proteasomes. Notably, mutant cells accumulate proteasomes in which a second copy of the $\alpha 4$ subunit replaces $\alpha 3$. 20S proteasome-assembly defects also are associated with altered RP assembly; this unexpected result suggests that the 20S proteasome can function as an RP-assembly factor *in vivo*. Our data demonstrate that Pba3-Pba4 orchestrates formation of a specific type of proteasome, the first example of a *trans*-acting factor that controls assembly of alternative proteasomal complexes.

In eukaryotes, much of the intracellular degradation of proteins occurs through the ubiquitin-proteasome system^{1,2}. Most substrates are first polyubiquitinated and then degraded by the 26S proteasome, which is composed of a proteolytically active 20S proteasome core bound at one or both ends by a 19S RP^{3,4}. The RP, which can be divided into lid and base subcomplexes, confers energy- and ubiquitin-dependence on proteasome-mediated proteolysis. Although we understand a good deal about the composition and enzymatic mechanisms of the 26S proteasome, we are only beginning to investigate how such an intricate machine of ~2,500 kDa and more than 32 different subunits is assembled *in vivo*.

The 20S proteasome (also called the core particle or CP) is a barrel of four coaxially stacked rings of seven subunits each⁴. Two structurally related classes of subunits make up the rings. The outermost rings are composed of α -type subunits and the inner rings of β -type subunits, each the product of a different gene in eukaryotes. There are three distinct protease centers in the proteasome interior, formed by specific β subunits⁵⁻⁷.

Assembly of eukaryotic 20S proteasomes is believed to begin with the formation of a heteroheptameric α -ring^{4,8,9}. β -subunits then enter the complex in a defined order, with propeptides of some of the β subunits having a crucial role in their incorporation^{5,10}. Once all β subunits are in place, the resulting half-proteasomes dimerize and the β -subunit propeptides are removed, leading to the mature 20S proteasome⁵. Several proteins have been identified that carry out various chaperone-like functions during 20S assembly. These include the yeast *Saccharomyces cerevisiae* Ump1 protein and its orthologs; the Pba1-Pba2 heterodimer, which is the yeast counterpart of mammalian PAC1-PAC2; and mammalian PAC3 (refs. 9,11-13). Ump1 functions, in part, as a checkpoint factor that helps to limit half-proteasome

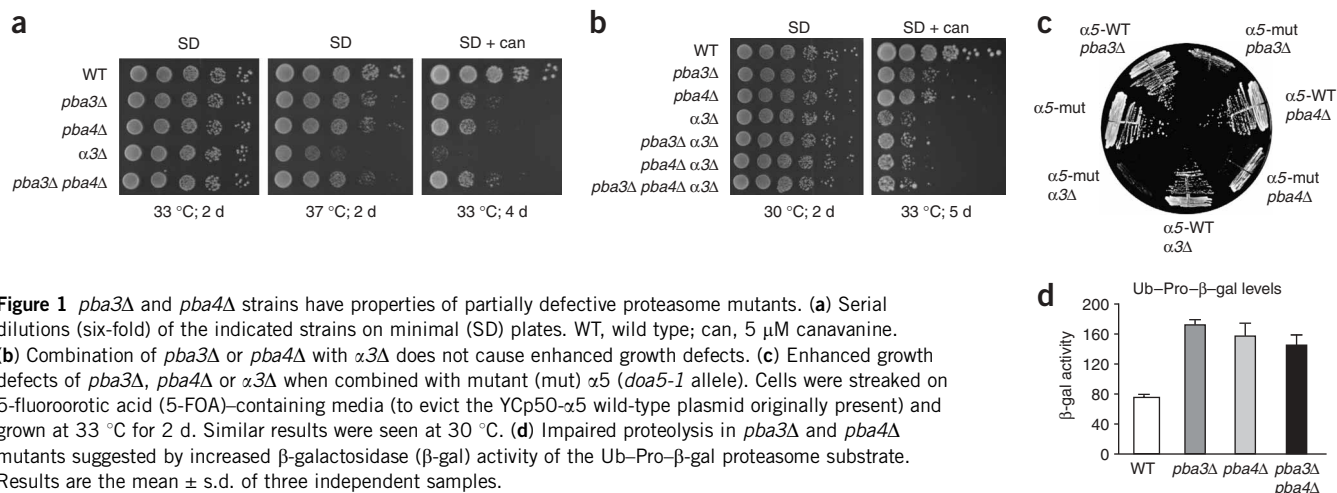
dimerization until all β subunits are inserted¹³. The PAC1-PAC2 heterodimer, and by extension the yeast Pba1-Pba2 complex, has been proposed to facilitate α -ring assembly^{9,13}, whereas PAC3 has a poorly defined role early in 20S biogenesis¹².

Generally, eukaryotic proteasome assembly has been assumed to generate a 'universal' 20S proteasome core, with a dyad-symmetric $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ architecture. However, some species have several closely related isoforms of specific 20S subunits, potentially enabling subtle modulation of proteasome composition and activity¹⁴. Organisms with an adaptive immune system also express three alternative β -subunit precursors in response to γ -interferon stimulation; these substitute for their constitutive counterparts, thereby enhancing the processing of antigens for major histocompatibility complex (MHC) class I presentation. The selective incorporation of this trio of subunits is driven by their unique propeptides¹⁵. Positional substitution of a much more divergent subunit occurs in *S. cerevisiae* cells that lack $\alpha 3$ (Pre9), the only 20S proteasome subunit that is not required for viability¹⁶. In $\alpha 3\Delta$ cells, a second copy of $\alpha 4$ (Pre6), with a sequence only ~33% identical to $\alpha 3$, takes the position normally occupied by $\alpha 3$. In wild-type cells, the $\alpha 3$ subunit is incorporated to the virtual exclusion of $\alpha 4$ at this position, at least under standard growth conditions. No mechanism for the alternative assembly of α -subunit rings has been identified to date.

Here we describe Pba3-Pba4, a previously unknown protein complex that regulates early events in *S. cerevisiae* 20S proteasome biogenesis. Sequence analysis indicates that the Pba3 and Pba4 proteins are widely conserved, including in humans. Under many circumstances loss of this assembly factor is deleterious to growth, but under certain conditions this loss confers a growth advantage. We show that Pba3-Pba4 binds directly to specific α subunits. *In vivo*, the

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Pba3–Pba4 chaperone acts to ensure the exclusive incorporation of $\alpha 3$ between the $\alpha 2$ and $\alpha 4$ subunits in the α ring. Mutation of Pba3–Pba4 leads to cells accumulating distinct subpopulations of 20S proteasomes. Unexpectedly, assembly of the RP is also perturbed in 20S proteasome-assembly mutants, suggesting that the 20S proteasome functions as an assembly factor for RP biogenesis. These data have broad implications for our understanding of proteasome assembly and evolution.

RESULTS

Bioinformatic search identifies potential proteasome regulators

To identify novel factors involved in yeast proteasome function, we undertook a targeted bioinformatic analysis of published genomic-scale functional studies. One consistently observed defect of proteasome mutants is impaired sporulation and meiosis⁴. Consistent with this, a genomic screen for sporulation defects in diploid mutants from the *S. cerevisiae* gene-deletion collection identified mutants lacking $\alpha 3$, Rpn10 or Ump1 (ref. 17). These proteasome-defective mutants all had a reduced number of four-spore asci. In the same study, deletions of 11 uncharacterized open reading frames (ORFs) resulted in the same specific sporulation defect.

Another characteristic of proteasomal mutations is that they cause a severe growth defect when combined with a deletion of the *RPN4* gene. Rpn4 is a transcription factor that positively regulates most proteasome subunit genes, and it is a short-lived proteasome substrate¹⁸. Deletions of 2 of the 11 newly identified genes in the aforementioned sporulation study are lethal in combination with *rpn4Δ*¹⁹. These genes are *YLR021W* and *YPL144W*. As we show below, the corresponding gene products function together as a previously unknown proteasome-assembly factor. Following previous nomenclature¹³, we propose the names proteasome biogenesis-associated 3 and 4 (*PBA3* and *PBA4*) for *YLR021W* and *YPL144W*, respectively.

Proteasomal defects in *pba3Δ* and *pba4Δ* mutants

We deleted the *PBA3* and *PBA4* genes in our laboratory-strain background. Both *pba3Δ* and *pba4Δ* mutants were hypersensitive to the amino acid analog canavanine and showed mild temperature sensitivity (Fig. 1a,b). These are traits commonly associated with proteasomal defects, as shown by the control $\alpha 3\Delta$ strain. The *pba3Δ pba4Δ* double mutant showed growth deficiencies comparable to the single mutants. Notably, no enhancement of $\alpha 3\Delta$ growth defects was observed when combined with either *pba3Δ* or *pba4Δ* (Fig. 1b), but

when we combined the *pba3Δ* or *pba4Δ* allele with a partial loss-of-function point mutation in the gene encoding $\alpha 5$ —*doa5-1* (ref. 20)—the double mutants grew much more poorly than the single mutants (Fig. 1c). An $\alpha 3\Delta \alpha 5$ (*doa5-1*) double mutant was synthetically lethal. The lack of synthetic growth defects when *pba3Δ* or *pba4Δ* was combined with $\alpha 3\Delta$ suggested that loss of Pba3, Pba4 or $\alpha 3$ might affect a common aspect of proteasome assembly or function.

To test *pba3Δ* and *pba4Δ* cells for a possible deficiency in proteasomal proteolytic activity, we measured the relative levels of the short-lived proteasome test substrate ubiquitin–proline– β -galactosidase (Ub–Pro– β -gal)²¹. The mutants showed an approximately two-fold increase in β -gal activity relative to wild-type cells (Fig. 1d). A comparable defect had been seen with the $\alpha 3\Delta$ mutant¹⁶. Increased β -gal activity is consistent with a deficiency in proteasome-mediated degradation of this model substrate. Moreover, we note that the *pba4Δ* mutant was previously identified in our laboratory in a genetic screen that revealed weakly impaired degradation of another proteasome substrate; the mutant was not characterized further²².

Pba3 and Pba4 directly bind in a stoichiometric complex

The similarity of the *pba3Δ*, *pba4Δ* and *pba3Δ pba4Δ* phenotypes suggested that the functions of Pba3 and Pba4 are closely linked. Notably, a proteomic analysis of yeast protein complexes suggested that Pba3 and Pba4 could associate *in vivo*²³. We expressed the two proteins in *Escherichia coli* to ascertain whether they interact directly. Expressed individually, Pba3 and Pba4 were completely insoluble or nearly so (Fig. 2a); however, their coexpression resulted in a mutual increase in solubility, suggesting that they might interact. Indeed, hexahistidine-tagged Pba3 (Pba3-his) and untagged Pba4 coeluted from a nickel–nitrilotriacetic acid (Ni-NTA) resin in a ratio of approximately 1:1 (Fig. 2a, last lane).

When eluates from the Ni-NTA column were resolved by non-denaturing PAGE followed by protein staining, a single strong band with an apparent size of 75 kDa was observed in the eluate derived from bacteria expressing both Pba3 and Pba4 (Fig. 2b). The band was excised, and the proteins in it were eluted and subjected to SDS-PAGE and protein staining. Both Pba3 and Pba4 were present at comparable levels (Fig. 2c). Given their predicted monomer masses, the 75-kDa size of the complex is most consistent with a heterotetramer containing two copies each of Pba3 and Pba4. It is possible that a tetramer forms only at high concentrations or that the 75 kDa species represents an aberrantly migrating complex of different stoichiometry,

