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YPL260W, a high-copy suppressor of a copper-sensitive phenotype in yeast, is linked to DNA repair and proteasome function

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ABSTRACT

The ubiquitin–proteasome system directly impacts the metabolism of heavy metals and yeast has become an important model in understanding this interplay. We demonstrate that yeast mutants with defects in proteasome function are able to tolerate elevated levels of copper. In the course of our analysis, we isolate a yeast mutant that not only negates this copper tolerance in proteasome mutants, but renders yeast exquisitely sensitive to this metal. To better understand the nature of the defect, we carry out a plasmid-based genetic screen to identify high-copy suppressors of this strong copper sensitivity. We identify four genes not previously known to be associated with copper metabolism: *CDC53*, *PSP1*, *YNL200C*, and *YPL260W*. The latter is a highly conserved fungal gene of no known function. Here, we undertake the first characterization of *YPL260W*. We demonstrate *YPL260W* to have a role in bleomycin tolerance with links to DNA repair and proteasome function.

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1. Introduction

Copper is an essential yet highly toxic nutrient. It is required to maintain the structure and function of a number of key metalloproteins (Festa and Thiele, 2011; Palm-Espling et al., 2012). Copper insufficiency and copper excess manifest in a number of adverse conditions, both genetic and acquired (Keen et al., 1998; Kodama et al., 1999; Kumar et al., 2004; Pfeiffer, 2011; Eskici and Axelsen, 2012). Consequently, strict regulation of copper intake, delivery, storage, and removal is carried out by all organisms. Yeast has been a key model organism for the study of copper homeostasis owing to the conservation of many key aspects of the copper metabolism machinery among eukarya (Nevitt et al., 2012).

Recently, we demonstrated that certain mutations that affect the assembly of the proteasome core particle (CP[1]) in yeast result in increased tolerance of the heavy metal cadmium (Kusmierczyk et al., 2008). The proteasome is at the heart of the ubiquitin–proteasome system (UPS); it is a large (~2.5 MDa) multi-subunit protease that consists of the 20S proteasome, or core particle, which can be capped on one or both ends by the 19S regulatory particle (RP) (Tomko and Hochstrasser, 2013). The CP provides the proteolytic function of the proteasome and comprises a stack of four seven-membered rings; the outer rings contain 7 distinct α subunits (α1–α7) and the inner rings contain 7 distinct β subunits (β1–β7), three of which possess catalytic activity in eukaryotes (Groll et al., 1997; Unno et al., 2002). The UPS is responsible for the degradation of the majority of intracellular proteins in eukaryotes (Finley et al., 2012) and UPS function impacts virtually every cellular process, from the cell cycle, to DNA replication, to apoptosis, to differentiation, etc. Since exposure to cadmium can affect normal copper homeostasis (Heo et al., 2010), we reasoned that proteasome mutants may exhibit altered response to copper as well. This would be relevant to increasing our understanding of the role of the UPS in copper metabolism (Ooi et al., 1996; Bertinato and L'Abbe, 2003; Burstein et al., 2004; Liu et al., 2007; Brady et al., 2010). We demonstrate that yeast proteasome mutants exhibit increased resistance to copper. However, in the course of this analysis, we isolate an unknown mutation that renders yeast cells uniquely sensitive to copper. To better understand the nature of this mutation, we carry out a plasmid-based genetic screen to identify high-copy suppressors of this phenotype. We identify four genes not previously known to have links to copper metabolism (*CDC53*, *PSP1*,

Abbreviations: BCA, bicinchoninic acid; CP, core particle; DNase I, deoxyribonuclease I; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; HU, hydroxyurea; ICAR, immobilized cobalt affinity resin; kDa, kilodalton(s); MDa, megadalton(s); *mut*, unknown mutation in this study conferring sensitivity to copper; NAD, nicotinamide-adenine dinucleotide; NADH, reduced form of NAD; NADHX, hydrated form of NAD; NADP, nicotinamide-adenine dinucleotide phosphate; NADPH, reduced form of NADP; NADPHX, hydrated form of NADP; OD_{600}, optical density measured at 600 nm; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; RP, regulatory particle; SCF complex, Skp-Cullin-F-box containing complex; SD, synthetic defined yeast media; SD-Leu, synthetic defined yeast media lacking leucine; SDS, sodium dodecyl sulfate; UPS, ubiquitin–proteasome system.

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YNL200C, and *YPL260W*). We speculate on the relationship of these suppressors to copper homeostasis and provide the first characterization of *YPL260W*, a highly-conserved fungal gene of no known function.

2. Materials and methods

2.1. Yeast strains, plasmids, and media

All relevant yeast strains are listed in Table S1 and were generated in this study unless otherwise noted (Chen and Hochstrasser, 1995; Fu et al., 1998; Ramos et al., 1998; Velichutina et al., 2004). All plasmids are listed in Table S2. Yeast manipulations were carried out according to established protocols (Guthrie and Fink, 1991). For serial dilutions, yeast strains were grown overnight in minimal (SD or SD-Leu) media and diluted to an OD_{600} of 0.2. Six-fold dilutions were prepared in water and spotted onto various media as indicated in the figure legends. Bleomycin (bleocin) was purchased from EMD Millipore and used at indicated concentrations.

2.2. High-copy suppressor screen

Strain AKY604 (*pre9*Δ::*HIS3*, containing the unknown mutation rendering cells sensitive to copper) was transformed with a genomic DNA library on plasmid YEp13 purchased from ATCC. Several aliquots of the transformation mixture were plated on minimal media lacking leucine (SD-Leu) in order to determine an optimal dilution that would result in roughly 100 to 500 colonies per plate. The remainder of the transformation mixture was diluted accordingly and spread directly onto SD-Leu plates supplemented with 1.5 mM $CuCl_2$. In this manner, it was estimated that a sufficient volume of transformation mixture was plated onto copper containing media to screen 27,000 transformants. Plates were incubated at 30 °C and colonies appearing up to nine days following plating were streaked to fresh copper containing plates to verify that they were copper resistant. Plasmids were rescued from these copper resistant colonies and retransformed into mutant cells (AKY604 and others) to confirm which of these were capable of imparting the copper-resistant phenotype and to eliminate false positives. In this manner, six plasmids were verified as bona fide suppressors. Verification experiments and subsequent dilution series were carried out at 1.2 mM $CuCl_2$ in order to enable faster colony growth. The lower copper concentration did not affect interpretation of the results because the unknown mutation still results in a considerable growth defect even at 1.2 mM $CuCl_2$. Both ends of the genomic DNA inserts present on each of the six suppressing plasmids were sequenced to determine the identity of the insert and the genes located therein. Fragments of the genomic DNA inserts were generated by restriction digest and subcloned into YEp13 or pRS425 plasmids. The subclone-containing plasmids were tested for their ability to suppress the copper sensitivity of the unknown mutation. Additionally, the *YPL260W* open-reading frame was cloned into p425CYC1, placing this ORF under the control of the heterologous *CYC1* promoter. In this manner we identified 4 unique open reading frames, not previously known to impart copper resistance when present in high copy.

2.3. Bacterial expression

A C-terminally hexahistidine (his) tagged version of the *YPL260W* ORF was generated by PCR and subcloned into the pET42 vector. This plasmid was transformed into *Escherichia coli* BL21 cells; protein induction and bacterial cell harvesting were carried out as described (Kusmierczyk et al., 2011). Frozen cell pellets were thawed on ice and resuspended in 0.6 ml of Buffer A (50 mM HEPES-NaOH, pH 7.5, 0.3 M NaCl, and 5 mM $MgCl_2$) supplemented with 2 mM Pefabloc, 0.3 mg ml^{-1} lysozyme, 10 μg ml^{-1} DNase I and 0.1% (v/v) Triton X-100. The suspensions were lysed by shaking at 30 °C for 30 min then centrifuged at 10,000 ×*g* for 10 min at room temperature to separate soluble and pellet fractions. The soluble fraction was applied to 50 μl of equilibrated immobilized cobalt affinity resin (ICAR) (Talon resin; Clontech) and incubated for 1 h. Resin was collected by centrifugation at 700 ×*g* for 5 min and washed 2 times with 1 ml of Buffer A, 2 times with 1 ml of Buffer B (Buffer A supplemented with 5 mM imidazole), and 1 time with 1 ml of Buffer C (Buffer A supplemented with 10 mM imidazole). The washes were carried out with gentle rocking for 5 min at 4 °C. The purified Ypl260w protein was eluted in 600 μl of Buffer E (Buffer A supplemented with 200 mM imidazole) and desalted by serial centrifugation as described (Kusmierczyk et al., 2011). Prior to gel electrophoresis or size exclusion chromatography, protein concentrations were measured using the BCA Assay (ThermoScientific).

2.4. Electrophoresis and Western blotting

For nondenaturing PAGE, equal amounts of protein (10 μg) were mixed with 5× nondenaturing sample buffer (0.5 M Tris–HCl, pH 8.8, 50% (v/v) glycerol, traces of bromophenol blue) and loaded onto 4–15% Mini-PROTEAN TGX gradient gels (BioRad). Native high molecular weight marker mix (GE Healthcare) was combined with 5× nondenaturing sample buffer and loaded along with the protein samples. Electrophoresis was carried out at 55 V and 4 °C until the dye front ran off the gel. Where indicated, samples were analyzed by 12% SDS-PAGE following mixing with 5× SDS sample buffer (Laemmli, 1970). Gels were stained with Gelcode blue or Pierce Silver Stain Kit (ThermoScientific). For Western blot analysis, transfer to Immobilon-P membrane (EMD Millipore) and detection was carried out as described (Li et al., 2007) using Penta-his HRP Conjugate antibody (Qiagen). Bands excised from native gels were cut into small pieces, and incubated overnight at 4 °C in 1× SDS sample buffer to allow proteins to elute. The supernatants containing the eluted proteins were analyzed by 12% SDS-PAGE.

2.5. Gel filtration chromatography

A HiPrep Sephacryl S-300 HR column (GE Healthcare) was coupled to an AKTA Prime Plus chromatography system connected to a PC running Prime View evaluation software (GE Healthcare). The column was equilibrated with Buffer D (25 mM Tris–HCl, pH 7, 150 mM NaCl) and set to a flow rate of 0.8 ml min^{-1}. The column was calibrated with 360 μg of each of six molecular weight standards (Serva). Following calibration, ICAR-purified Ypl260w-his protein (780 μg) was loaded onto the column and 3 ml fractions were collected. Aliquots (15 μl) of every other fraction were mixed with 5× SDS sample buffer and analyzed by 12% SDS-PAGE followed by staining with Imperial stain (ThermoScientific).

3. Results

3.1. Increased copper tolerance in proteasome mutants

Certain proteasome mutants exhibit enhanced growth in the presence of the toxic heavy metal cadmium (Kusmierczyk et al., 2008). Since some of cadmium's negative effects can be mediated through its effects on copper homeostasis in yeast (Heo et al., 2010), we reasoned that proteasome mutants may display alterations in copper metabolism. Elevated levels of copper inhibit the growth of common laboratory wild-type yeast strains (Pearce and Sherman, 1999). The growth of our laboratory wild-type strain was strongly impaired at 1.5 mM $CuCl_2$ (Fig. 1A). We evaluated a panel of proteasome mutants, with varying degrees of proteasome function impairment, in the presence of copper. Strains lacking *PBA2* or *PBA4*, encoding subunits of two 20S core particle (CP) assembly chaperones (Hirano et al., 2005; Le Tallec et al., 2007; Li et al., 2007; Kusmierczyk et al., 2008; Yashiroda et al., 2008), showed no difference from wild-type in growth on copper-containing media (Fig. 1A). This is likely due to the mild nature of

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