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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

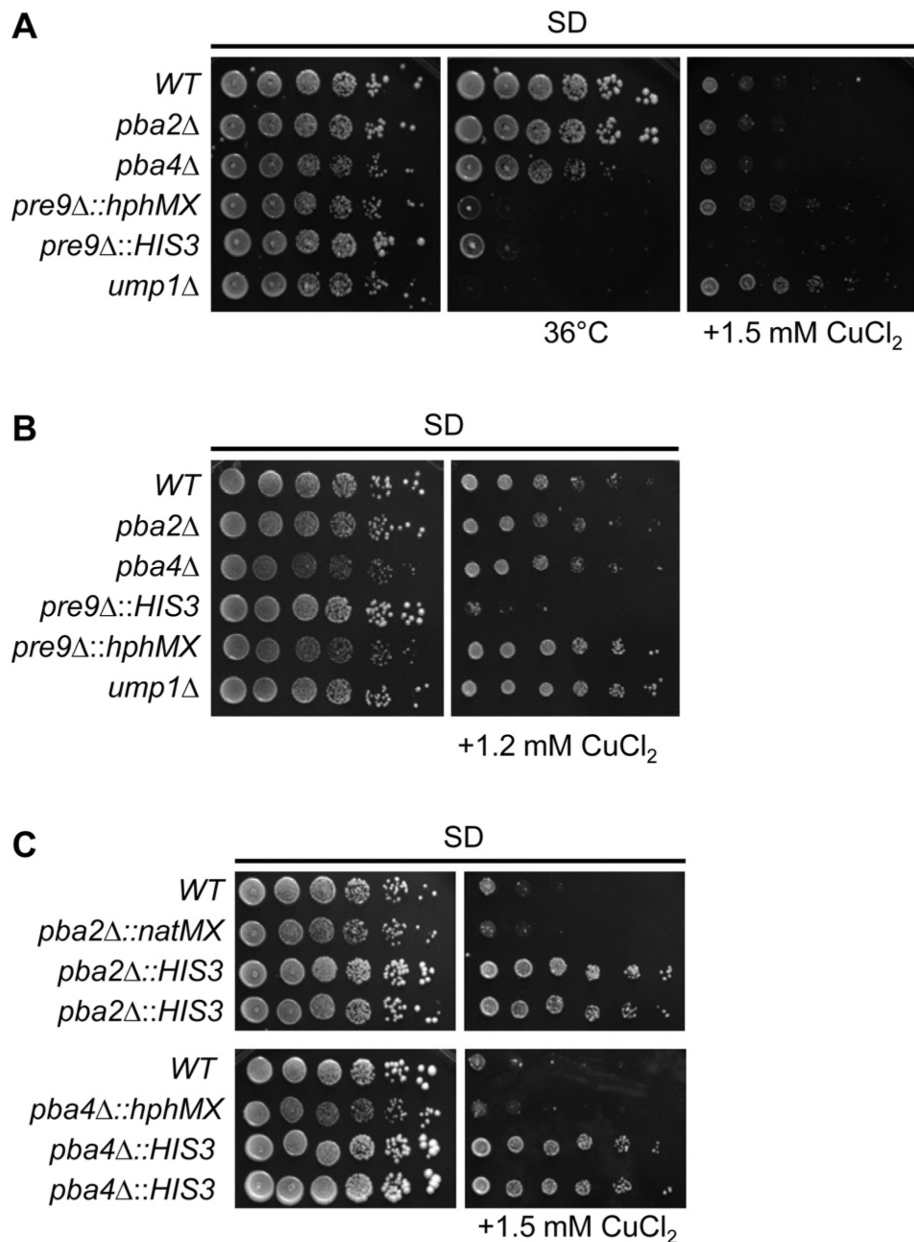

Fig. 1. Copper-tolerance of various yeast proteasome mutants. All yeast strains were spotted onto SD plates supplemented with 1.2 mM or 1.5 mM $CuCl_2$ where indicated and incubated for 3 days at 30 °C, or at the stated temperature. (A, B) Comparison of 20S proteasome mutant strains. (C) A His^+ phenotype imparts copper tolerance to yeast mutant strains. Two independent isolates of newly generated *pba2*Δ::HIS3 and *pba4*Δ::HIS mutants are shown.

these deletions on proteasome function, demonstrated here by their relatively robust growth at elevated temperature (Fig. 1A, 36 °C), and by previous work showing these mutations become deleterious only when proteasome function is already compromised (Le Tallec et al., 2007; Li et al., 2007; Kusmierczyk et al., 2008). Deletion of the α3 subunit, encoded by *PRE9*, and of a third CP assembly factor, *UMP1*, result in progressively greater defects in proteasome function (Fig. 1A, 36 °C and references (Ramos et al., 1998; Velichutina et al., 2004; Kusmierczyk et al., 2008)). Both of these mutants (*pre9*Δ::*hphMX* and *ump1*Δ) exhibited better growth in the presence of copper than wild-type (Fig. 1A). Improved growth in the presence of copper was also observed in a 19S regulatory particle (RP) mutant (*rpn12-234*Δ; Fig. S1) that appreciably impairs proteasome function (Tomko and Hochstrasser, 2011). Taken together, the results suggest that when proteasome function is strongly impaired, there is an alteration of copper metabolism leading to increased copper tolerance.

3.2. Isolation of a copper-sensitive mutant

Despite the increased copper tolerance exhibited by proteasome mutants, one of our mutant strains (*pre9*Δ::*HIS3*) exhibited copper sensitivity (Fig. 1A). This sensitivity was quite striking, even under copper conditions where growth of the wild-type yeast strain is only mildly impaired (1.2 mM $CuCl_2$; Fig. 1B). This sensitivity was unexpected because our two *pre9*Δ strains otherwise behaved similarly (see growth at 36 °C, Fig. 1A). The only difference between the *pre9*Δ::*hphMX* mutant and the *pre9*Δ::*HIS3* mutant was the identity of the marker cassette used to disrupt the *PRE9* gene. In the former, the cassette contained a gene that confers hygromycin resistance (Goldstein and McCusker, 1999); in the latter, the marker cassette is the yeast *HIS3* gene (Fu et al., 1998). Like many laboratory strains, our strain is a histidine auxotroph (His^- phenotype), and the *HIS3* marker restores histidine prototrophy (His^+ phenotype). His^+ strains are normally more resistant to copper

(Pearce and Sherman, 1999), a finding that we recapitulate here (Fig. 1C). This made the copper sensitivity of the *pre9*Δ::*HIS3* strain even more odd. We considered the possibility that a second mutation at an unknown locus (henceforth referred to as *mut*) in the background of the *pre9*Δ::*HIS3* strain was responsible for its unique phenotype.

To test this, we backcrossed the *pre9*Δ::*HIS3* strain to our laboratory wild-type strain and sporulated the resulting diploid. We analyzed the tetrads on media containing copper and made a number of observations (Fig. 2A). First, the sensitivity to copper can segregate away from histidine prototrophy (sector 3), arguing that an unknown mutation (*mut*) unlinked to *pre9*Δ::*HIS3* was responsible for the copper sensitivity. Second, the *pre9*Δ::*HIS3* progeny that no longer possessed *mut* were now strongly resistant to copper (sector 5), as expected for a His^+ strain (Pearce and Sherman, 1999). Third, the copper sensitive phenotype segregated in a 2:2 fashion, arguing for *mut* being the result of a change (or changes) at a single locus. Next, we mated one of the copper sensitive *mut* progeny from this cross to copper tolerant *pre9*Δ::*hphMX* and *ump1*Δ strains. Surprisingly, all *mut*-containing double mutants were now strongly sensitive to copper (Fig. 2B). This argues that *mut* is sufficient to affect a yeast cell's growth in the presence of copper and that *mut* negates the copper tolerance conferred by proteasome dysfunction.

3.3. High-copy suppressors of mut

We initiated a high-copy suppressor screen on the rationale that identifying suppressors of the copper sensitivity of *mut* strains could shed light on the nature of the defect, if not identify the locus responsible for *mut*. We transformed copper sensitive *pre9*Δ::*HIS3* yeast cells containing *mut* with a genomic library based on the YEp13 episomal plasmid (Nasmyth and Reed, 1980). We chose to conduct the screen in this strain, as opposed to a strain that only contained *mut*, because there was a much bigger difference in the growth on copper between *pre9*Δ::*HIS3* cells and *pre9*Δ::*HIS3 mut* cells than there was between wild-type and *mut* cells (Fig. 2A). This broader range should allow for increased sensitivity in selecting for copper resistant transformants. We analyzed approximately 27,000 transformants for the ability to support growth in the presence of 1.5 mM $CuCl_2$ and, following additional measures to screen out false positives, isolated six candidate suppressor plasmids of the copper sensitive phenotype in *pre9*Δ::*HIS3* cells that also contained *mut* (Fig. 3A).

We retransformed the six candidate suppressors into *mut* cells that did not have *PRE9* deleted to verify that they could suppress the *mut* defect itself (Fig. 3B). The contents of the genomic DNA fragments present in each of the six verified suppressors were identified by sequencing the ends of the fragments; they are listed in Table 1. The genomic DNA

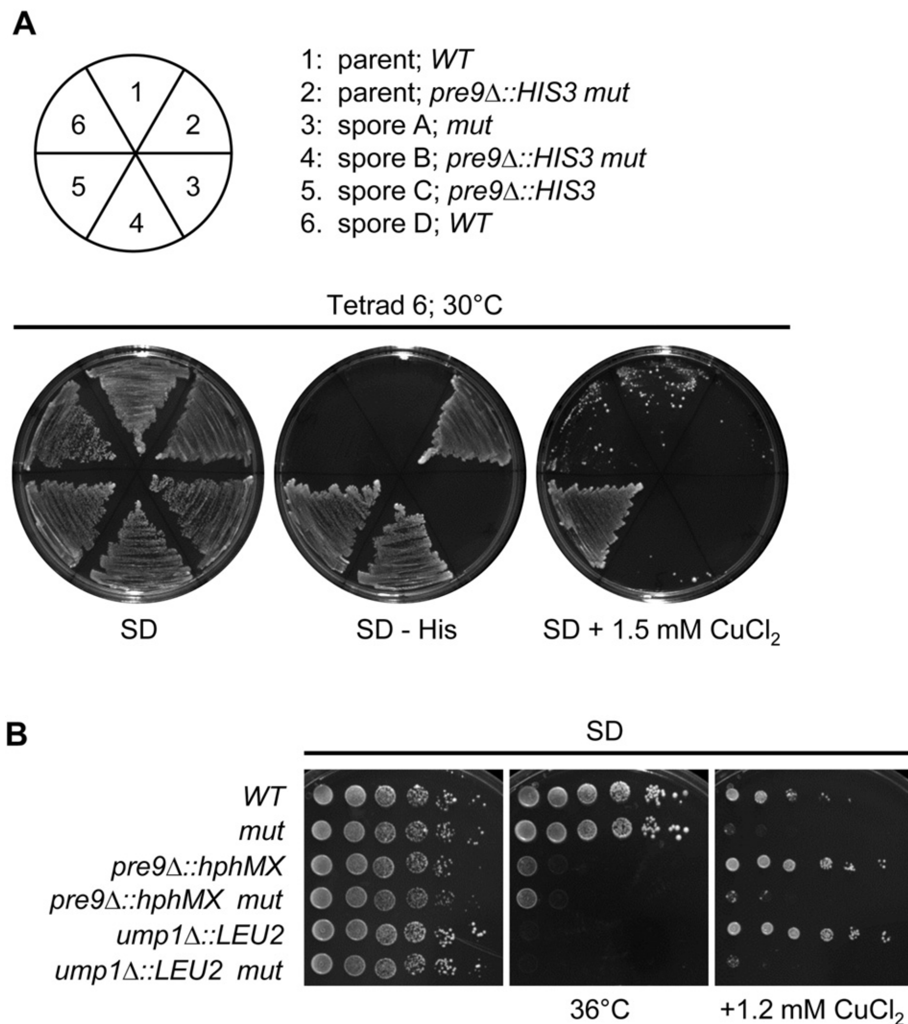

Fig. 2. An unknown mutation is responsible for copper sensitivity of the *pre9*Δ::*HIS3* strain. Copper-sensitive *pre9*Δ::*HIS3* yeast cells were crossed with wild-type cells and the resulting diploids sporulated. (A) Spores from a representative tetrad were struck out to the indicated media and incubated for 3 days at 30 °C. (B) Unknown mutation is sufficient to negate the copper-tolerance conferred by proteasome mutants. Six-fold dilution series of yeast cells were plated to the indicated media and incubated for 3 days at 30 °C unless otherwise noted.

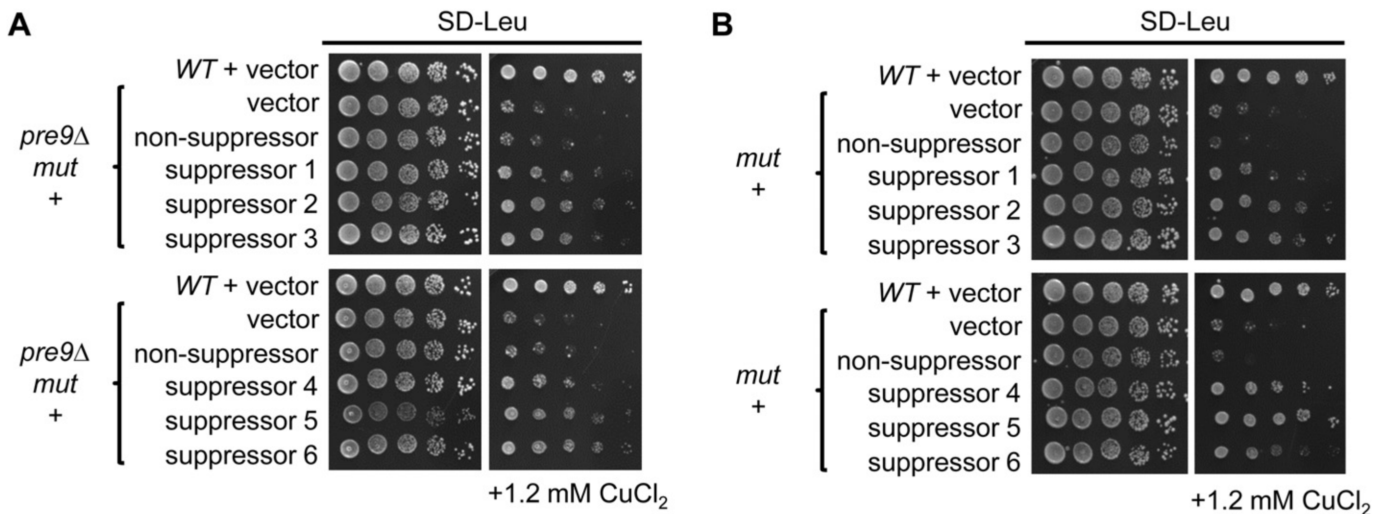

Fig. 3. High copy suppressor screen identifies six unique suppressors. (A) Dilution series comparing growth of the copper-sensitive *pre9*Δ::*HIS3 mut* yeast strain (AKY604) transformed with the six suppressor plasmids, or with vector only and non-suppressing plasmid controls. (B) The same plasmids as in (A) were transformed into *mut* yeast cells (AKY786) and dilution series carried out to verify that the plasmids are suppressing the copper-sensitive phenotype caused by the unknown (*mut*) mutation. In all cases, plates were photographed after 3 days at 30 °C.

fragments of suppressors 5 and 6 contain genes (*CUP1-2* and *SLF1*, respectively) previously shown to suppress copper sensitivity when present in high copy (Ecker et al., 1986; Jeyaprakash et al., 1991; Yu et al., 1996). *CUP1-2* encodes a yeast copper metallothionein that binds to copper in vivo and protects yeast from copper toxicity (Winge et al., 1985; Jeyaprakash et al., 1991). *SLF1* is an RNA-binding protein whose targets include mRNAs of genes important in copper homeostasis, including *CUP1* (Schenk et al., 2012). Recovery of *CUP1-2* and *SLF1* validated our screening method as capable of isolating bona fide suppressors of copper sensitivity. The suppressor plasmids containing these two genes were not analyzed further.

Fragments of the genomic DNA inserts from suppressor plasmids 1 to 4 (Fig. S2) were subcloned into a high-copy yeast vector in order to determine which ORF was responsible for the suppression (Fig. S3 and not shown). The generation of overlapping fragments was necessary to discriminate between two closely spaced ORFs. As an example, subclone F derived from suppressor plasmid 3 was capable of suppressing the copper sensitivity of *mut* cells (Figs. S2 and S3). This subclone contained two ORFs, *PSP1* and *SPG3*, with the latter being immediately downstream of the former. However, subclone E, containing the complete *SPG3* ORF but only half of the upstream *PSP1* ORF, was unable to suppress (not shown). We concluded that *PSP1* (and not *SPG3*) was responsible for the suppression observed with subclone F. The fragment analysis enabled us to determine that *CDC53*, *PSP1*, *YNL200C* and *YPL260W* were the suppressors of copper sensitivity.

In the case of *YPL260W* (found on subclone D; Fig. S2), we carried out one additional verification experiment. The open reading frame of *YPL260W* overlaps minimally (2 nucleotides) with *YPL261C*, a divergent but dubious ORF. This places the *YPL261C* ORF in the promoter region of the *YPL260W* gene. Even though *YPL261C* has been annotated as dubious, there is evidence that a polypeptide is expressed from this ORF (Gelperin et al., 2005). Therefore, it was imperative to rule out the very remote possibility that the suppression observed with subclone D was due to *YPL261C*. To this end, we cloned the *YPL260W* ORF downstream of a heterologous promoter, effectively removing the *YPL261C* ORF from consideration, and confirmed that this construct could also suppress the copper sensitivity of *mut* cells (Fig. 4A). This verified the *YPL260W* ORF as the bona fide suppressor.

DNA sequencing of the open reading frames of *CDC53*, *PSP1*, *YNL200C* and *YPL260W*, as well as regions immediately up and downstream of their start and stop codons, respectively, did not reveal any mutations in the *mut* strain (not shown). This ruled out the genomic loci of these four suppressors as the source of the defect in *mut* cells. Identification of the locus responsible for the copper sensitive phenotype of *mut* cells will be pursued elsewhere; here we focus on the identified suppressors.

Table 1
Suppressor plasmids isolated from screen.

Suppressor 1	Complete ORFs:	*YPL260W*; *YPL261C* (dubious)
	Partial ORFs:	*AMP1* (nt. 593–1428); *FUM1* (nt. 1320–1467)
Suppressor 2	Complete ORFs:	*YNL200C*; *PSY2*; *SPS19*; *YNL203C* (dubious); *SPS18*; *YNL205C* (dubious)
	Partial ORFs:	*GCR2* (nt. 1192–1605); *RTT106* (nt. 1–715 out of 1368)
Suppressor 3	Complete ORFs:	*PSP1*; *SPG3*; *LPP1*
	Partial ORFs:	*GMC1* (nt. 722–1827)
Suppressor 4	Complete ORFs:	*CDC53*
	Partial ORFs:	*LYS21* (nt. 1–526 out of 1323); *SRF1* (nt. 884–1314)
Suppressor 5	Complete ORFs:	*YHR054W-A* (dubious); *CUP1-2**
	Partial ORFs:	*YHR054C* (nt. 1–388 out of 1065); *RSC30* (nt. 475–2652)
	Other:	*RUF5-2*; *ARS811*
Suppressor 6	Complete ORFs:	*SLF1**; *YDR514C*; *GRX2*; *EMI1*; *ACN9*; *YDR510C-A* (dubious); *SMT3*
	Partial ORFs:	*EMI2* (nt. 1066–1503)

nt. denotes nucleotides; * denotes ORFs known to impart copper resistance when present in high copy.

3.4. YPL260W, DNA integrity, and the proteasome

Of the identified high copy suppressors, we focused on the two ORFs without an annotated standard name in the Saccharomyces Genome Database, *YPL260W* and *YNL200C*. Deletion of either gene did not result in sensitivity to copper (Fig. 4B) or other divalent cations tested, cobalt and nickel (not shown). The protein product of the *YNL200C* ORF has been characterized as an NAD(P)HX epimerase involved in repair of nicotinamide nucleotides (Marbaix et al., 2011), and thus not directly linked to the metabolism of copper or other divalent heavy metals. Its ability to suppress the copper sensitivity of a *mut* cells could be due to an indirect effect. This is consistent with a lack of genetic interaction observed between *mut* and deletion of *YNL200C* on copper-containing media (Fig. 4C, left panel). In contrast to *YNL200C*, *YPL260W* is a completely uncharacterized gene. The lack of phenotype of *ypl260w*Δ cells on copper-containing media might mean that the ability of

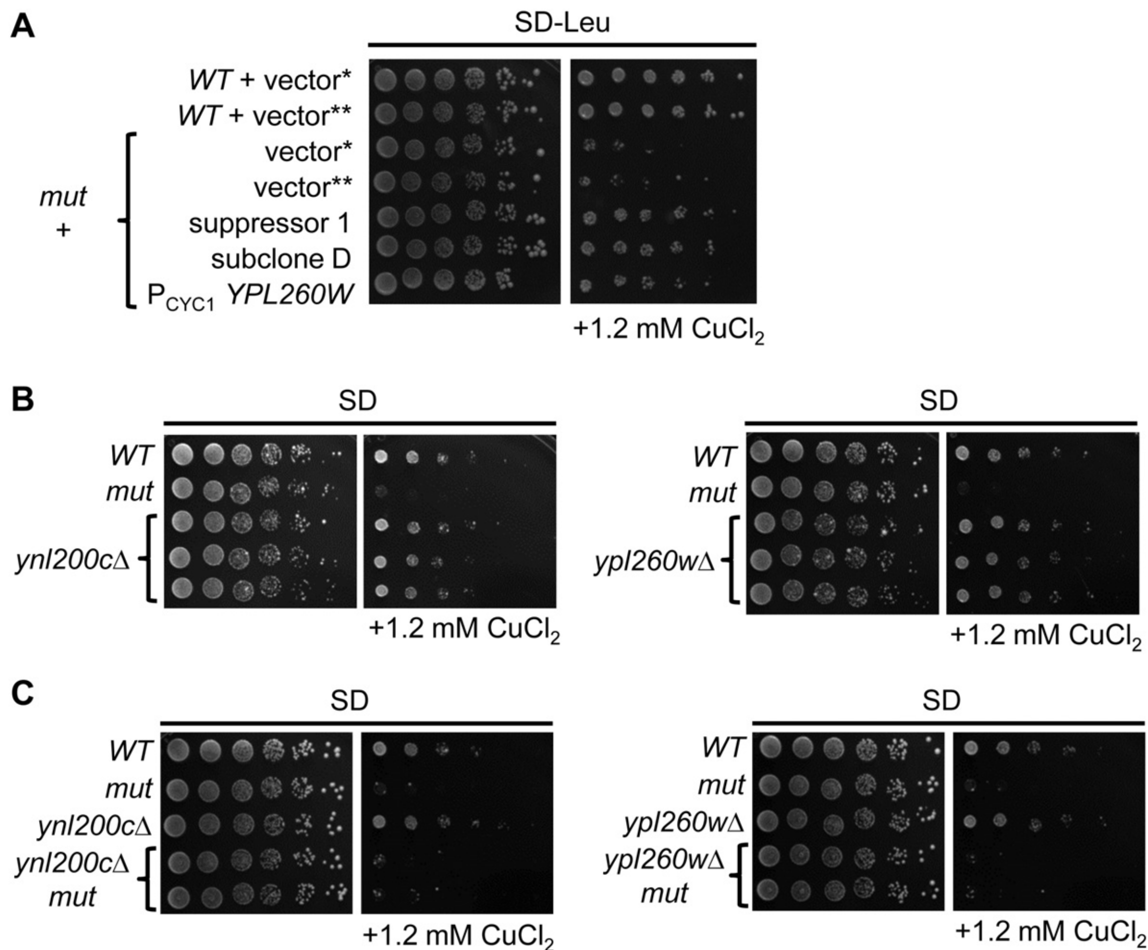

Fig. 4. Phenotype analysis of *ypl260w*Δ and *ynl200c*Δ strains. All yeast strains were spotted onto SD or SD-Leu media and, where required, supplemented with the indicated additions. All plates were incubated at 30 °C for 3 days, unless noted otherwise. (A) *YPL260W* is a high copy suppressor of the copper sensitive phenotype of the unknown (*mut*) mutation. Asterisks denote empty vector controls (*AKB736; **AKB826). P_{CYC1} *YPL260W* denotes the *YPL260W* ORF whose expression is driven by the *CYC1* promoter found in the p425 CYC1 plasmid (AKB828). Plates were photographed after 5 days at 30 °C. (B) Deletion of *YNL200C* or *YPL260W* does not result in copper sensitivity. Three separate isolates of each mutant are shown. (C) Deletion of *YNL200C* or *YPL260W* does not impact the copper sensitivity of the yeast strain harboring the unknown (*mut*) mutation. Two separate isolates of each double mutant are shown.

YPL260W to suppress the copper sensitivity of *mut* cells is also due to an indirect effect on copper homeostasis (Fig. 4C, right panel). However, we cannot rule out a direct role in copper metabolism that is compensated for by some redundant functionality in *ypl260w*Δ cells.

YPL260W is a highly conserved fungal gene (Fig. S4) and we sought to characterize it and its protein product. We observed that *ypl260w*Δ strains were sensitive to the glycopeptide antibiotic bleomycin (Fig. 5A,B). Bleomycin exerts its antibiotic effects primarily through binding and cleaving DNA (Burger, 1998; Hecht, 2000). Proteasome function is required for effective DNA repair and proteasome mutants are also sensitive to bleomycin (Krogan et al., 2004; Ben-Aroya et al., 2010). The severity of the bleomycin-sensitive phenotype of the *ypl260w*Δ strain was comparable in magnitude to the bleomycin sensitivity of proteasome mutants (Fig. 5C). By contrast, *mut* cells were not sensitive to bleomycin and combining the deletion of *YPL260W* with *mut* did not alter the sensitivity of *ypl260w*Δ cells to this antibiotic (Fig. 5C). However, combining the deletion of *YPL260W* with mutations that affect proteasome function (*pre9*Δ or *ump1*Δ) resulted in increased sensitivity to bleomycin (Fig. 6A). This result suggested that *YPL260W* was functionally linked to the proteasome and may also play a role in DNA repair. To provide support for this hypothesis, we crossed the *ypl260w*Δ yeast strain to a *rad6*Δ yeast strain. *RAD6* encodes a ubiquitin-conjugating enzyme (E2) with important functions in DNA repair (Jentsch et al., 1987; Hoege et al., 2002; Game et al., 2006). Cells lacking Rad6 protein are strongly sensitive to bleomycin (Fig. 6A). Hence, we carried out experiments involving *rad6*Δ at lower bleomycin concentrations. Under these conditions, the doubly-deleted *rad6*Δ *ypl260w*Δ cells were visibly more sensitive to bleomycin than either mutant alone (Fig. 6B). Cells lacking Rad6 protein are also strongly sensitive to hydroxyurea, but deletion of *YPL260W* did not further alter this sensitivity (Fig. 6C). Taken together, the results suggest that *YPL260W* function is genetically linked to DNA repair.

3.5. Ypl260w protein

We subcloned *YPL260W* into a bacterial expression vector and expressed the protein with a C-terminal hexahistidine tag (his tag). The protein was not soluble when expressed at 37 °C (not shown). By contrast it was partially soluble when expressed at 30 °C (Fig. 7A) and could be isolated using immobilized cobalt-affinity resin (ICAR; Fig. 7B). The purified Ypl260w-his protein migrated near its expected molecular mass of 64 kDa on SDS-PAGE, though several smaller species were sometimes also apparent, likely the result of non-specific proteolysis during purification. On nondenaturing PAGE the full length Ypl260w-his protein migrated as a single species between the 67 kDa and 140 kDa size standards (Fig. 7B, native). This was verified by cutting this band out of the nondenaturing gel, eluting the proteins within, and separating the eluted protein by SDS-PAGE followed by silver staining. In this manner, full length Ypl260W-his protein was recovered (Fig. 7C).

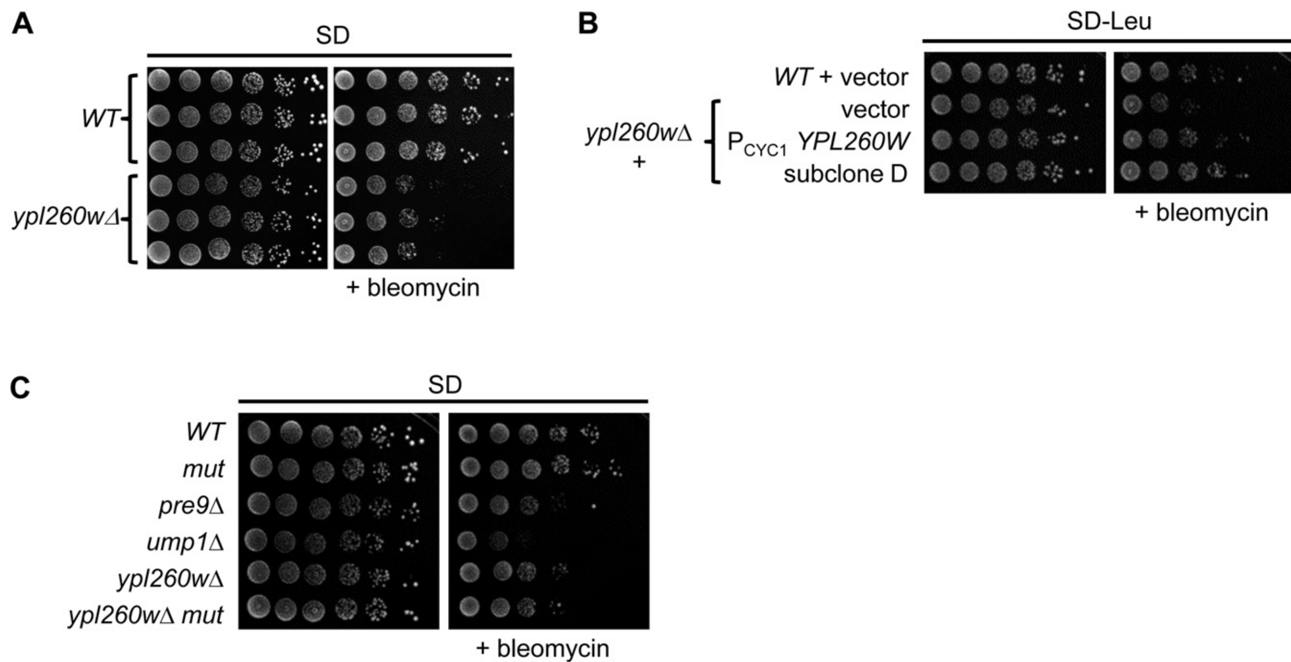

Fig. 5. Bleomycin sensitivity of *ypl260w*Δ yeast cells. Yeast strains were spotted onto the indicated media and all plates were photographed after 3 days at 30 °C. (A) Deletion of *YPL260W* results in bleomycin sensitivity. Dilution series carried out in triplicate with independent isolates of *ypl260w*Δ. Bleomycin concentration was 1.25 μg/ml. (B) The bleomycin sensitivity of *ypl260w*Δ yeast cells can be complemented by plasmids bearing *YPL260W*. Subclone D refers to plasmid AKB833. P_{CYC1} *YPL260W* refers to plasmid AKB828. Bleomycin concentration was 2.5 μg/ml. (C) The bleomycin sensitivity of *ypl260w*Δ yeast cells is not affected by the presence of the unknown (*mut*) mutation and is comparable in magnitude to the bleomycin sensitivity of proteasome mutants (AKY408, *pre9*Δ; AKY605, *ump1*Δ). Bleomycin concentration was 1.25 μg/ml.

Since nondenaturing PAGE does not predict mass, we subjected the purified Ypl260w-his protein to gel filtration chromatography on a Sephacryl S-300 column pre-calibrated with a set of protein standards. The full length Ypl260w-his protein eluted with an estimated molecular mass of 77 kDa (Fig. 7D). The peak fraction (fraction 23) was subjected to Western blotting with anti-his antibody (Fig. 7E, left panel) and to nondenaturing PAGE (Fig. 7E, right panel). This confirmed that: the ~64 kDa species was full length Ypl260w-his protein; the smaller species that are sometimes observed were non-specific proteolytic products; and that full length Ypl260w-his protein exists as a single species with electrophoretic mobility between the 67 kDa and 140 kDa size standards on nondenaturing PAGE. The biochemical analysis suggests that recombinant Ypl260w-his protein is a monomer.

4. Discussion

Building on previous results showing that certain mutations in proteasome function render yeast cells more tolerant of cadmium, we demonstrate that mutations in proteasome function also confer copper tolerance. In the course of our studies, we isolate an unknown mutant (here referred to as *mut*) that not only negates this copper tolerant phenotype of proteasome mutants but generally renders yeast cells very sensitive to this heavy metal. To understand the nature of the defect in *mut* cells, we carried out a high-copy suppressor screen and identified four *ORF*s capable of suppressing the copper sensitive phenotype: *CDC53*, *PSP1*, *YNL200C* and *YPL260W*. The screen did not identify the ORF responsible for the defect in *mut* cells itself. This could be due to several reasons. Although we screened more than 27,000 transformants, it is possible that the screen was not exhaustive. It is also possible that a plasmid containing the wild-type locus responsible for the *mut* phenotype is not present in the library; this can happen if the relevant DNA insert would be toxic to *E. coli* where the library is propagated. Finally, our most recent evidence suggests that the *mut* phenotype is dominant (Firestone, K and Kusmierczyk, A.R., unpublished). Consequently, depending on the mechanism of dominance (Wilkie, 1994), a suppressor screen may not be capable of identifying the responsible locus. Efforts to identify the locus responsible for the *mut* phenotype will be described elsewhere.

The four suppressors identified here have not been previously associated with copper metabolism and represent potential new links in the copper homeostasis chain. *CDC53* is an essential gene encoding a yeast cullin, the scaffolding component of large modular ubiquitin ligases, called SCF complexes. SCF complexes are best known as regulators of cell cycle progression (Mathias et al., 1996; Willems et al., 1996). However, they also regulate sulfur metabolism and cellular responses to arsenic and cadmium stress (Kaiser et al., 2006) via the activity of SCF towards the Met4 transcription factor (Kaiser et al., 2000; Rouillon et al., 2000; Barbey et al., 2005; Yen et al., 2005). It has been suggested that Met4 may also co-regulate copper and iron metabolism (Moler et al., 2000) and since Met4 is regulated by Cdc53-containing SCF complexes, the recovery of *CDC53* in this screen is consistent with this possibility. *PSP1* has been identified as a high copy suppressor of DNA polymerase mutations in yeast, though the function of Psp1 protein remains unknown (Formosa and Nittis, 1998).

YNL200C encodes an NAD(P)HX epimerase involved in repair of nicotinamide nucleotides (Marbaix et al., 2011). NADHX and NADPHX are hydrated forms of NAD and NADP whose formation is catalyzed as a side-reaction by cellular dehydrogenases (Prabhakar et al., 1998). These hydrated nucleotides can, in turn, act as powerful inhibitors of the same dehydrogenases. Not surprisingly, repair systems for these 'damaged' nucleotides are highly conserved across all taxa (Marbaix et al., 2011). Bearing this in mind, there are two possible explanations for the recovery of *YNL200C* in this screen; they need not be mutually exclusive. First, nicotinamide nucleotide-dependent enzymes feature prominently in many aspects of copper metabolism (Nevitt et al., 2012), and Cu(II) can oxidize NADH either in solution or when bound to an enzyme (Chan and Kesner, 1980; Jiang et al., 2006; Jiang et al., 2008). Second, it has been shown that oxidative stress results in the inactivation of key glycolytic enzymes (Cabiscol et al., 2000; Costa et al., 2002); this is also true of copper-induced oxidative stress (Shanmuganathan et al., 2004). Carbon flux is therefore shifted from glycolysis to the pentose phosphate pathway, resulting in the

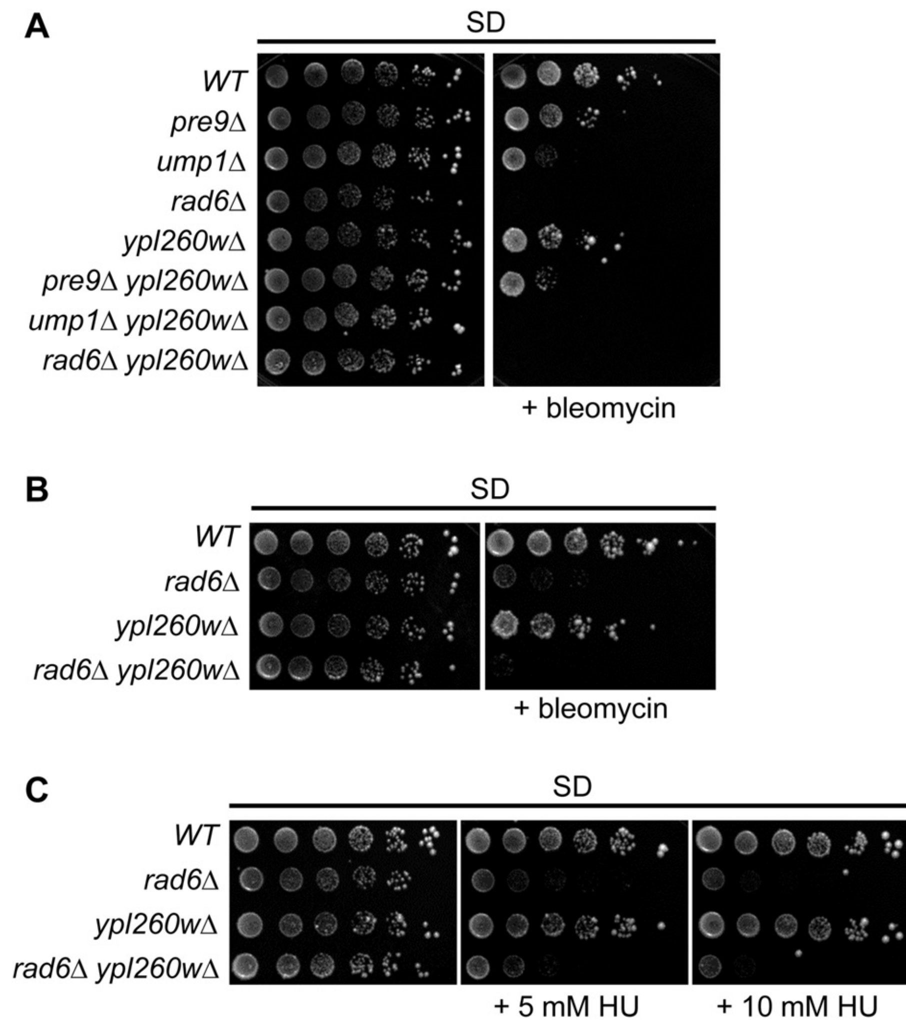

Fig. 6. *YPL260W* is linked to proteasome function and DNA repair. Yeast strains were spotted onto the indicated media and all plates were photographed after 5 days at 30 °C. (A) Genetic interaction between proteasome mutants (*pre9Δ* or *ump1Δ*) and *ypl260wΔ*. Bleomycin concentration was 1.25 μg/ml. (B) Genetic interaction between *rad6Δ* and *ypl260wΔ*. Bleomycin concentration was 0.6 μg/ml. (C) Deletion of *YPL260W* does not exacerbate the hydroxyurea (HU) sensitivity of *rad6Δ* cells.

generation of more NADPH (Godon et al., 1998; Shenton and Grant, 2003). Increased NADPH levels are helpful to antioxidant enzymes (e.g. glutaredoxins and others) in dealing with the effects of oxidative stress. Thus, it is not surprising that an enzymatic activity which helps maintain normal NAD(P)H levels would have a protective effect from the oxidizing effects of excess copper. Deletion of *YNL200C* did not result in copper sensitivity, but this could simply reflect the ability of de novo nucleotide synthesis to compensate for lack of Ynl200c protein. In eukaryotes, the NAD(P)HX epimerase functions with a partner protein, an NAD(P)HX dehydratase, encoded by the *YKL151C* locus in yeast (Marbaix et al., 2011). The bacterial homolog YjeF is a bifunctional protein that possesses both epimerase and dehydratase activities, and the name "*nnr*" (for nicotinamide nucleotide repair) has been suggested as the name for the bacterial gene that encodes YjeF (Marbaix et al., 2011). Accordingly, we propose that *YNL200C* be renamed *NNR1* and the dehydratase-encoding *YKL151C* be renamed *NNR2*.

YPL260W is a highly conserved fungal gene (Fig. S4) whose function is not known. Deletion of *YPL260W* did not result in sensitivity to copper, cobalt, or nickel. *YPL260W*'s ability to suppress the copper sensitivity of *mut* cells could be due to an indirect effect on copper metabolism, though it is also possible that a redundant function masks copper sensitivity in a *ypl260wΔ* strain. Deletion of *YPL260W* resulted in sensitivity to the glycoside antibiotic bleomycin and initial biochemical characterization of recombinant Ypl260w protein suggests it forms a monomer. We propose the name *CUB1* (*Cu*$^{2+}$ suppressing and *b*leomycin sensitive) for *YPL260W*.

There are no readily identifiable sequence features to hint at a possible function for the Cub1 protein. High-throughput localization studies in yeast place the GFP-tagged version of Cub1p in the nucleus and cytoplasm (Huh et al., 2003). Its strong conservation among fungi suggests it plays a key role in these organisms. It is notable that *CUB1*-containing fungi and the *Streptomyces verticillus* bacteria that produce bleomycin are both found in soil (Umezawa et al., 1966). Bleomycin refers to a group of related compounds that differ in their amine moiety (Umezawa, 1971). Bleomycin binds metals (Ramotar and Wang, 2003), including copper ions (Sugiyama et al., 2002; Lehmann, 2004) and in this study we used bleocin, the trademarked name for bleomycin A5 complexed with copper. Although it is an iron-bleomycin complex that is responsible for the DNA cleaving properties of this antibiotic (Sausville et al., 1978a, 1978b; Burger et al., 1981), the affinity of bleomycin for copper is higher than that of iron (Oppenheimer et al., 1981), and it is isolated from *S. verticillus* as a copper complex (Umezawa et al., 1966). This makes it more likely that fungi would encounter bleomycin as a copper-complex in the soil, and transport it as such; the switch to an iron-bound bleomycin would then occur following internalization. Hence, *CUB1* function could lie at the crossroads

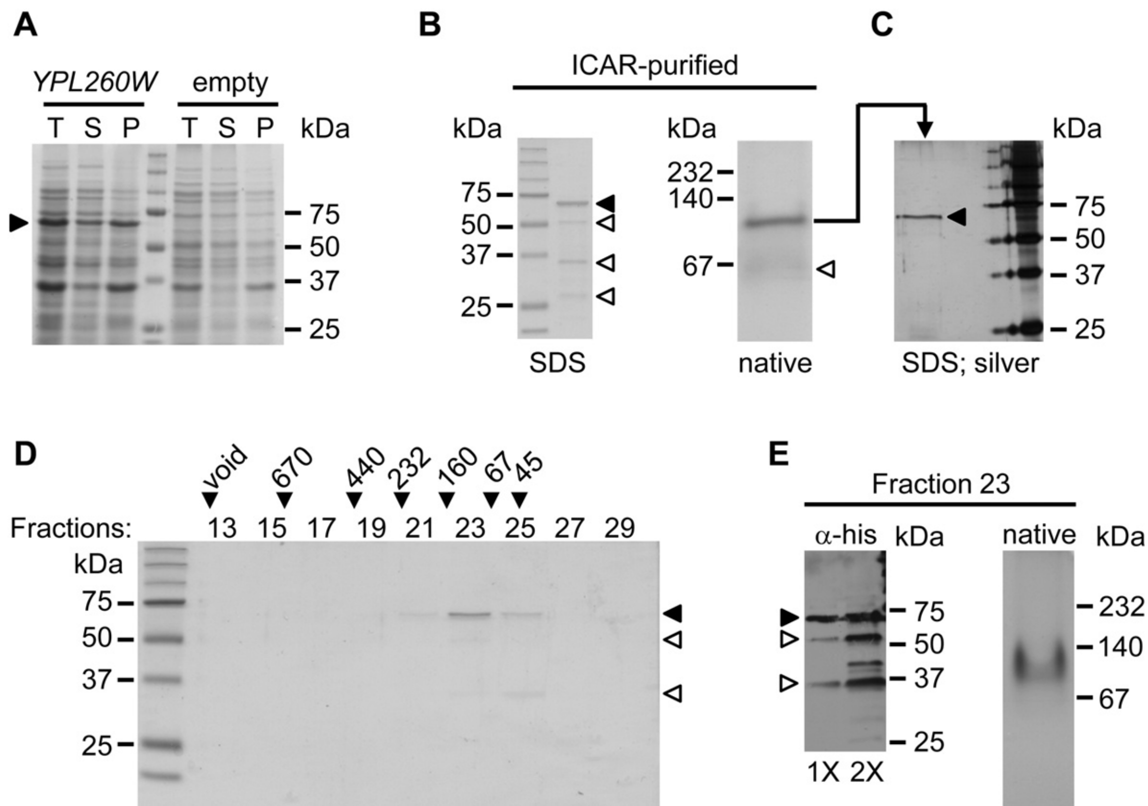

Fig. 7. Biochemical analysis of recombinant Ypl260w protein. For all gels, the migration of various molecular size standards (kDa) is indicated. Black arrow denotes full length Ypl260w-his protein. White arrows denote non-specific proteolytic products of Ypl260w-his. (A) Recombinant Ypl260w protein is partially soluble at 30 °C. Total (T), soluble (S), and insoluble/pellet (P), fractions of *E. coli* lysates expressing C-terminally hexahistidine-tagged Ypl260w protein (Ypl260w-his) are fractionated by SDS-PAGE and stained with Gelcode blue. (B) Ypl260w-his purified by immobilized cobalt affinity resin (ICAR) was electrophoresed by SDS-PAGE (SDS) and nondenaturing PAGE (native) followed by staining with Gelcode blue. (C) The indicated band from the nondenaturing gel in (B) was excised. The proteins therein were eluted and electrophoresed by SDS-PAGE followed by silver staining. (D) ICAR-purified Ypl260w-his was fractionated by gel filtration chromatography on a Sephacryl S-300 column. Aliquots of alternating fractions were electrophoresed by SDS-PAGE followed by staining with Imperial stain. The location of the void volume (void), and of the elution peaks of several molecular size standards, is indicated above the gel. (E) Aliquots of the peak Ypl260w-his fraction from (D), fraction 23, were analyzed by SDS-PAGE and Western blotting using anti-his antibodies (α-his) or by nondenaturing PAGE followed by silver staining (native). 2× denotes twice as much sample loaded onto the SDS-PAGE gel as 1×.

of copper and bleomycin metabolism, part of a larger framework that specifically allows fungi, which share the soil niche with bleomycin producing *Streptomycetes*, to tolerate this antibiotic.

An alternate, though not mutually exclusive explanation for the bleomycin phenotype places *CUB1* function in pathways related to DNA repair. Several findings support this hypothesis. First, proteasome function is required for DNA repair in yeast and we observe synthetic phenotypes between *cub1*Δ and several proteasome mutants in the presence of bleomycin. Second, we observe genetic interaction between *CUB1* and *RAD6*, a key component of the post-replicative DNA repair machinery. Deletion of *CUB1* is synthetic sick with deletion of *RAD6* on media containing bleomycin, but not hydroxyurea (HU). Bleomycin directly cleaves DNA (Sausville et al., 1978a, 1978b; Burger et al., 1981) while HU depletes deoxyribonucleotide pools resulting in stalled replication forks (Bianchi et al., 1986). Stalled forks need not progress to double strand breaks prior to being repaired (Petermann et al., 2010). Consequently, the effects of bleomycin and HU on DNA are only partially overlapping, and the genetic interaction of *cub1*Δ and *rad6*Δ on bleomycin but not HU suggests that *CUB1* may be involved in a specific DNA repair process. Third, levels of Cub1p reportedly increase in response to DNA replication stress (Tkach et al., 2012). Finally, the yeast copper response is linked to DNA damage repair (Dong et al., 2013); this could explain our recovery of *CUB1* in the genetic screen looking for high-copy suppressors of the copper sensitive phenotype of *mut* yeast cells. Going forward, it will be interesting to determine precisely how *CUB1* function relates to DNA repair and/or proteasome function.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plgene.2015.11.002.

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