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Not4 E3 Ligase Contributes to Proteasome Assembly and Functional Integrity in Part through Ecm29▽†

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In this study we determine that the Not4 E3 ligase is important for proteasome integrity. Consequently, deletion of Not4 leads to an accumulation of polyubiquitinated proteins and reduced levels of free ubiquitin. In the absence of Not4, the proteasome regulatory particle (RP) and core particle (CP) form salt-resistant complexes, and all other forms of RPs are unstable. Not4 can associate with RP species present in purified proteasome holoenzyme but not with purified RP. Additionally, Not4 interacts with Ecm29, a protein that stabilizes the proteasome. Interestingly, Ecm29 is identified in RP species that are inactive and not detectable in cells lacking Not4. In the absence of Not4, Ecm29 interacts less well with the proteasome and becomes ubiquitinated and degraded. Our results characterize Ecm29 as a proteasome chaperone whose appropriate interaction with the proteasome requires Not4.

In eukaryotes, short-lived proteins are degraded primarily by the ubiquitin-proteasome system (UPS) (25). The multicatalytic protease, the 26S proteasome, is responsible for this degradation. Most proteasome substrates are modified by polyubiquitin chains that are recognized by the proteasome. The UPS controls a diverse array of biologically important processes, including cell cycle progression, DNA repair, signal transduction, and protein quality control.

The 26S proteasome consists of 2 major subcomplexes: a proteolytically active 20S core particle (CP) bound at one or both ends by a 19S regulatory particle (RP; also called PA700 in mammals). The CP has a hollow cylindrical shape and consists of a stack of 4 heptameric rings. The 2 outer rings contain α-type subunits, and the 2 inner rings contain β-type subunits. The proteolytic sites of the proteasome are located in its central cavity on specific β subunits (19). Free CP exists in an autoinhibited state in which the N termini of α subunits form a gate to block substrate entry. Activation of CP occurs upon opening of this gate by a proteasome activator, 19S. In mammals, two additional activators have been identified: the PA28 (or PA26)/11S regulator and PA200. In *Saccharomyces cerevisiae*, Blm10, which is similar to mammalian PA200, can function as an alternative activator (49).

RP consists of 2 subcomplexes: the base, which binds directly to CP, and a peripheral lid. The base includes 6 ATPase subunits (Rpt1 to -6) that facilitate gate opening, substrate unfolding, and translocation into CP using ATP (47). The lid consists of 9 non-ATPase subunits (Rpn3, -5 to -9, -11, and -12 and Sem1) and is required for the recognition and deubiquitination of substrates (50).

Several recent investigations have focused on how this highly abundant complex of about 2,500 kDa is assembled. CP assembly requires the assistance of CP chaperones (23). Similarly RP assembly is realized by several RP chaperones: Nas2, Hsm3, Nas6, and Rpn14 (36, 46). Once RP is assembled, the base binds to the lid and all chaperones are released prior to, or during, RP-CP association (12, 48). Recent data suggest that lid, base, and CP formation and their association into the 26S proteasome are not discrete events. One subcomplex can facilitate the formation of another. For instance, CP can enhance RP base biogenesis and might be considered an RP assembly factor (22).

The capacity of the proteasome to degrade proteins depends crucially on the RP-CP interaction. There is a wide range of factors affecting this interaction, including metabolites (4), proteasome-associated proteins (35, 49), the metabolic state of the cell (5), and salt concentrations (33). One of these factors is Ecm29, a protein first identified in a screen for yeast displaying cell wall defects (39). Ecm29 was later connected to the proteasome through large-scale proteomic screens in *S. cerevisiae* (14, 24). The association of Ecm29 with proteasomes has been demonstrated for both yeast and mammalian cells (17, 34). It has been proposed that yeast Ecm29 clamps the RP to the CP and stabilizes the 26S proteasome (29). Mammalian Ecm29 was described as an adaptor that recruits the 26S proteasome to specific cellular compartments requiring enhanced rates of protein degradation, such as the endosomal components and molecular motors (18).

Not4 is a RING finger E3 ligase and a subunit of the evolutionarily conserved Ccr4-Not complex. It consists of 9 subunits in yeast (7) and is important for expression of most of the genome (3). Not4's two known substrates are a ribosomal chaperone, the nascent-polypeptide-associated complex (Egd complex in yeast) (43, 44), and the demethylase Jhd2 (40). A synthetic lethal screen first connected Not4 to the ubiquitin pathway (41), and subsequently it was genetically and biochemically shown that Not4 interacts with the proteasome (32). In this study, we determine that Not4 is important for ubiquitin homeostasis and proteasome integrity. Our results define Ecm29 as a proteasome chaperone, show that it associates with

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
BY4741	*MAT***a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	6
sJR287	*MAT*α *trp1-1 ura3-52 his3-Δ200 leu2-3,113 lys2-801 rpn4*::*KanMX4*	D. Finley
SUB61	*MAT*α *lys2-801 leu2-3,2-112 ura3-52 his3-Δ200 trp1-1*	11
SUB62	*MAT***a** lys2-*801 leu2-3,2-112 ura3-52 his3-Δ200 trp1-1*	11
sDL133	*MAT***a** lys2-*801 leu2-3,2-112 ura3-52 his3-Δ200 trp1-1 rpn11*::*RPN11*-TEV-ProtA-*HIS3*	33
sDL135	*MAT***a** lys2-*801 leu2-3,2-112 ura3-52 his3-Δ200 trp1-1 pre1*::*PRE1*-TEV-ProtA-*HIS3*	33
sMK141	*MAT*α *lys2-801 leu2-3,2-112 ura3-52 his3-Δ200 trp1-1 ecm29*::*TRP1*	D. Finley
MY1	*MAT***a** *ura3-52 trp1-1 leu2*::*PET56 gcn4E*	8
MY3593	Isogenic to MY1 except *not4*::*KanMX4*	43
MY3596	Isogenic to MY1 except *MAT*α *his*::*TRP1 not4*::*KanMX4*	This work
MY5558	*MAT***a** *ade2 arg4 leu2-3,112 trp1-289 ura3-52 rpn5*::*RPN5*-TAP tag-*URA3*[a]	Euroscarf; 13
MY5559	*MAT***a** *ade2 arg4 leu2-3,112 trp1-289 ura3-52 rpn11*::*RPN11*-TAP tag-*URA3*	Euroscarf; 13
MY5584	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 doa4*::*KanMX4*	Euroscarf
MY5585	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubp6*::*KanMX4*	Euroscarf
MY5615	Isogenic to BY4741 except *MAT***a** *not4*::*HIS3*	This work
MY5735	Isogenic to MY5585 except *not4*::*HIS3* + pRS316-Not4	This work
MY5739	Isogenic to MY5584 except *not4*::*HIS3* + pRS316-Not4	This work
MY5907	Isogenic to BY4741 except *not4*::*HIS3*	This work
MY6208	Isogenic to MY5615 except *rpn10*::*NatMX6*	This work
MY6467	*MAT*α *not4*::*KanMX4 rpn11*::*RPN11*-TEV-ProtA-*HIS3*	From the sDL133 × MY3593 cross (this work)
MY6494	*MAT*α *not4*::*KanMX4 pre1*::*PRE1*-TEV-ProtA-*HIS3*	From the sDL135 × MY3596 cross (this work)
MY6491	Isogenic to sJR287 except *not4*::*HIS3*	This work
MY6722	*MAT*α *not4*::*HIS3 ecm29*::*TRP1*	From the MY5907 × sMK141 cross (this work)
MY7367	*MAT***a** *rpn11*::*RPN11*-TEV-ProtA-*HIS3 not4*::*HIS3*	From the MY5559 × MY5616 cross (this work)
MY7689	*MAT***a** *ade2 arg4 leu2-3,112 trp1-289 ura3-52 ecm29*::*ECM29*-TAP tag-*URA3*	Euroscarf; 13
MY7695	*MAT*α *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpn10*::*KanMX4*	Euroscarf
MY7738	*MAT***a** *not4*::*KanMX4 ecm29*::*ECM29*-TAP tag-*URA3*	From the MY7689 × MY3596 cross (this work)
MY7820	*MAT***a** *not4*::*HIS3 rpn11*::*RPN11*-TAP tag-*URA3*	From the sMK141 × MY7370 cross (this work)
MY7822	*MAT***a** *not4*::*HIS3 rpn11*::*RPN11*-TAP tag-*URA3 ecm29*::*TRP1*	From the sMK141 × MY7370 cross (this work)
MY7827	*MAT***a** *rpn11*::*RPN11*-TAP tag-*URA3 ecm29*::*TRP1*	From the sMK141 × MY7370 cross (this work)
MY7835	*MAT***a** *not4*::*Myc6-Not4*-TAP tag-*KanMX4*	This work

[a] The TAP (tandem affinity purification) tag contains ProtA-TEV-calmodulin binding protein.

Not4, and suggest that Not4 contributes to proteasome assembly and functional integrity at least partially through Ecm29.

MATERIALS AND METHODS

Media and strains. All media were standard. The strains used in this work derive from MY1 or BY4741 (Table 1). Single-step deletions and/or tagging of genes was performed by PCR. Many strains were created by crossing, which was followed by tetrad analysis.

DNA constructs. The plasmid expressing Myc_6-Not4 was made by cloning the *NOT4* sequence amplified by PCR into the pGREG515 plasmid by homologous recombination in yeast (26), leading to pMAC680 (p*GAL1-Myc*$_6$-NOT4[r]). The *GAL1* promoter was changed to that of *NOT4* (300 nucleotides before the start of the gene) by PCR and homologous recombination in yeast, leading to pMAC684 (p*NOT4-Myc*$_6$-NOT4[r]). Mutagenesis of Not4 was performed on the basis of pMAC684, leading to the plasmids described in Table 2. The plasmid expressing seven-hemagglutinin-tagged Ecm29 (HA_7-Ecm29) was made using the same strategy as before and the pGREG536 plasmid (26), leading to pMAC806 (p*GAL1-HA*$_7$-ECM29[r]) and pMAC809 (p*ECM29-HA*$_7$-ECM29[r]). pMAC565 (p*CUP-His*$_6$-UBI-URA3[r]) was created by replacement of the *LEU2* marker in pE298 (43) with *URA3*. pMAC868 is a high-copy-number plasmid that contains a genomic insert from chromosome VIII, including the *ECM29* gene (clone YGPN5a23 [27]). The sequences of all plasmids were verified.

Proteasome purification and activity. Proteasomes were affinity purified from yeast carrying protein A (ProtA)-tagged Rpn11, Rpn5, Pre1, or Ecm29 as described in reference 33. If not specifically indicated, cells from 3 liters of the

TABLE 2. Plasmids used in this study

Plasmid name	Description	Reference or source
pMAC680	p*GAL1-Myc*$_6$-Not4-*LEU2*	This work
pMAC684	p*NOT4-Myc*$_6$-Not4-*LEU2*	2
pMAC715	p*NOT4-Myc*$_6$-Not4$_{1–78}$-*LEU2*	This work
pMAC716	p*NOT4-Myc*$_6$-Not4$_{1-180}$-*LEU2*	This work
pMAC718	p*NOT4-Myc*$_6$-Not4$_{1-232}$-*LEU2*	This work
pMAC719	p*NOT4-Myc*$_6$-Not4$_{1-330}$-*LEU2*	This work
pMAC721	p*NOT4-Myc*$_6$-Not4$_{1-430}$-*LEU2*	This work
pMAC745	p*NOT4-Myc*$_6$-Not4$_{1-480}$-*LEU2*	This work
pMAC748	p*NOT4-Myc*$_6$-Not4$_{1-530}$-*LEU2*	This work
pMAC749	p*NOT4-Myc*$_6$-Not4$_{I64A}$-*LEU2*	This work
pMAC751	p*NOT4-Myc*$_6$-Not4$_{78-587}$-*LEU2*	This work
pMAC753	p*NOT4-Myc*$_6$-Not4$_{130-587}$-*LEU2*	This work
pMAC754	p*NOT4-Myc*$_6$-Not4$_{180-587}$-*LEU2*	This work
pMAC755	p*NOT4-Myc*$_6$-Not4$_{330-587}$-*LEU2*	This work
pMAC756	p*NOT4-Myc*$_6$-Not4$_{430-587}$-*LEU2*	This work
pMAC757	p*NOT4-Myc*$_6$-Not4$_{78-587}$-*LEU2*	This work
pMAC806	p*GAL1-HA*$_7$-*ECM29-URA3*	This work
pMAC809	p*ECM29-HA*$_7$-*ECM29-URA3*	This work
pMAC868	pGP564$_{39048\ to\ 51123\ VIII}$-*LEU2*	27
pRS316-Not4	pRS316-Not4-*URA3*	This work
pE298	p*CUP-His*$_6$-*UBI-LEU2*	43
pMAC565	p*CUP-His*$_6$-*UBI-URA3*	This work

cultures were collected at an optical density at 600 nm (OD_{600}) of 3.0 and frozen in liquid nitrogen in drops. Cells were ground with an MM 400 CryoMill (Retsch) at 30 pulses/min for 1 min. Cell powder was incubated with buffer 1 (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP) for 10 min at 4°C and centrifuged at 6,000 × *g* for 10 min. Supernatants were clarified at 160,000 × *g* for 30 min. Fifty milliliters of lysate, containing 500 mg of total protein, was incubated with immunoglobulin G-Sepharose (IgG-Sepharose) for 2 h at 4°C. The resin was washed with buffer 2 (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP) to purify holoenzyme, buffer 3 (the same as buffer 2 but with 0.5 M NaCl) to purify RP, or buffer 4a or 4b (the same as buffer 2 but with 0.75 M or 1 M NaCl, respectively) to purify the lid as described in reference 33. Proteasome components were eluted by equilibrating the IgG resin with buffer 5 (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol [DTT]) containing 1.3 U/μl of six-histidine-tagged tobacco etch virus (His_6-TEV) protease (Invitrogen) at 30°C for 1 h. TEV protease was subsequently removed from the eluate by incubation with a Ni-nitrilotriacetic acid (NTA) resin (Qiagen) at 4°C for 20 min. Ecm29-ProtA was purified under the same conditions as the holoenzyme.

Whole cellular extracts (50 to 150 μg of total protein) or purified proteasomes (20 μg) were separated on 3.5% native gels (10) or on 3 to 12% native gradient gels (NativePAGE Novex Bis-Tris, 1.0-mm gel thickness; Invitrogen) (see Fig. S1 in the supplemental material). Gels were analyzed for proteasome activity by staining them with a 100 μM *N*-succinyl–Leu–Leu–Val–Tyr–7-amino-4-methylcoumarin (Suc-LLVY-AMC) (Biomol) without or with 0.02% SDS. For analysis of subcomplex composition, gels were stained with Coomassie blue and analyzed by mass spectrometry or immunoblotted.

In vitro proteasome reconstitution was performed as described in reference 29. Briefly, separately purified RP and CP were mixed in a ratio of 2 to 1, incubated at 30°C for 30 min, and loaded onto a native gel.

Myc-Not4 purification. Myc-Not4 was affinity purified from yeast expressing Myc-Not4-ProtA. Cells from 3 liters of the cultures were collected at an OD_{600} of 3.0, frozen in liquid nitrogen, and ground using the CryoMill. Cell powder was incubated with 2 volumes of buffer D (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], antiprotease cocktail; Roche) for 10 min at 4°C and spun at 6,000 × *g* for 10 min. Supernatants were clarified at 160,000 × *g* for 30 min. Fifty milliliters of lysate, containing 750 mg of total protein, was incubated with IgG-Sepharose for 2 h at 4°C. The resin was washed with 100 ml of buffer D and 35 ml of TEV buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 0.5 mM DTT). Resin was incubated with 1 ml of TEV buffer containing 100 U of His_6-TEV protease at 20°C for 1.5 h. TEV protease was subsequently removed from the eluate by incubation with a Ni-NTA resin at 4°C for 20 min.

***In vitro* association of proteasome and Myc-Not4.** For *in vitro* association of proteasome with Myc-Not4, 75 mg of total protein extract from cells expressing Rpn11-ProtA was incubated with 50 μl of IgG-Sepharose for 2 h and washed with 10 ml of buffer 2 as described earlier. Separately purified Myc-Not4 was added to immobilized holoenzyme in a total reaction volume of 0.5 ml. After incubation at 4°C for 1 h, the resin was washed with 10 ml of buffer 2 and then with 1 ml of buffer 5. The resin was then incubated with 100 μl of buffer 5 containing 20 U of TEV protease at 30°C for 1 h. Eluted proteins were concentrated with trichloroacetic acid (TCA) and resuspended in 50 μl of SDS sample buffer (SB). Twenty-microliter samples were analyzed by SDS-PAGE and immunoblotting.

In the reverse experiments, Myc-Not4-ProtA immobilized on IgG-Sepharose was incubated in buffer 2 with 10 μg of separately purified holoenzyme or the RP from cells expressing Rpn5-ProtA. After washings and TEV cleavage, eluted proteins were concentrated with TCA and analyzed by SDS-PAGE as described above.

Coimmunoprecipitation. One hundred OD_{600} units of cells was broken with 0.5 ml of glass beads in 0.5 ml of immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 10% glycerol, 0.5 mM PMSF) for 15 min at 4°C. Beads were washed with 0.5 ml of IP buffer. After clarification, 0.4 ml of the supernatants containing 2.4 mg of total protein was incubated with 20 μl of magnetic protein G beads (Invitrogen) and with antibodies overnight at 4°C. Then the beads were washed three times with 1 ml of IP buffer and boiled with 40 μl of SB. Total extracts from the strains lacking the immunoprecipitated antigen were used as a control.

Stability assay and *in vivo* ubiquitination assay. These assays were performed as described previously (43).

Mass spectrometry analysis. Matrix-assisted laser desorption ionization–tandem time of flight (MALDI TOF-TOF) analysis was performed as described in reference 3 at the Proteomics Core Facility of the Faculty of Medicine, University of Geneva. Nanoscale liquid chromatography-electrospray ionization-tandem mass spectrometry (NanoLC-ESI-MS/MS) analysis was done with the Mascot program (Matrix Science, London, United Kingdom; version Mascot). Mascot was set up to search the UniProt_sptr_15.10-03-Nov-2009 database (we selected for *Saccharomyces cerevisiae* [34,911 entries], assuming the digestion enzyme trypsin). Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 ppm. An iodoacetamide derivative of cysteine and oxidation of methionine were specified as fixed and variable modifications, respectively. The Scaffold program (version Scaffold 03; Proteome Software Inc., Portland, OR) was used to validate the MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95.0% probability as specified by the PeptideProphet algorithm (28). Protein identifications were accepted if they could be established at >95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the ProteinProphet algorithm (42). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

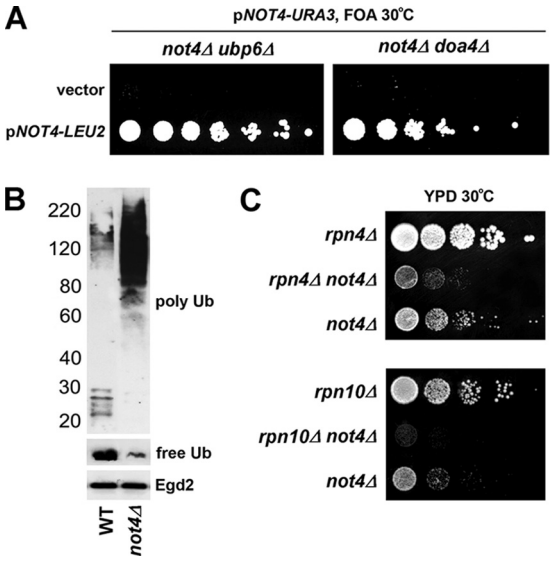

FIG. 1. Not4 is important for ubiquitin homeostasis in the cell. (A) Serial dilutions of the indicated mutants, containing a *NOT4-URA3* plasmid and either a vector alone or a *LEU2* plasmid expressing Not4, were spotted on plates with 5-fluoroorotic acid (FOA) and left to grow for several days. (B) Exponentially growing wild-type (WT) and *not4*Δ cells were collected at an OD_{600} of 1.5. Total protein extracts were prepared as described previously (30), and proteins were separated on a 4 to 12% gel (for polyubiquitinated proteins [poly Ub]) or on a 17% gel (for free ubiquitin [free Ub]), followed by immunoblotting with antiubiquitin antibodies or anti-Egd2 antibodies as a loading control. Numbers at the left are molecular masses (in kilodaltons). (C) The indicated strains were grown exponentially, and serial dilutions were spotted on yeast extract-peptone-dextrose (YPD).

Antibodies. Antibodies against Ecm29, Rpn5, Rpn8, and Rpn12 were kindly provided by Dan Finley. The production of antibodies against Egd2 was described previously (43). Antibodies against ubiquitin, Rpt1, Rpt2, α7, and α1,2,3,5,6,7 (α1–7) were from Biomol. The peroxidase-antiperoxidase (PAP) antibody-soluble complex, which recognizes the protein A tag, were from Sigma. Antibodies against HA and Myc were from Covance.

RESULTS

Not4 is important for ubiquitin homeostasis in the cell. Two deubiquitination enzymes, Ubp6 and Doa4, were identified in a synthetic lethal screen with the *not4*Δ mutant (41). We confirmed that deletion of either *UBP6* or *DOA4* was lethal in the absence of *NOT4* (Fig. 1A). Since both Ubp6 and Doa4 contribute to the maintenance of wild-type levels of free ubiquitin

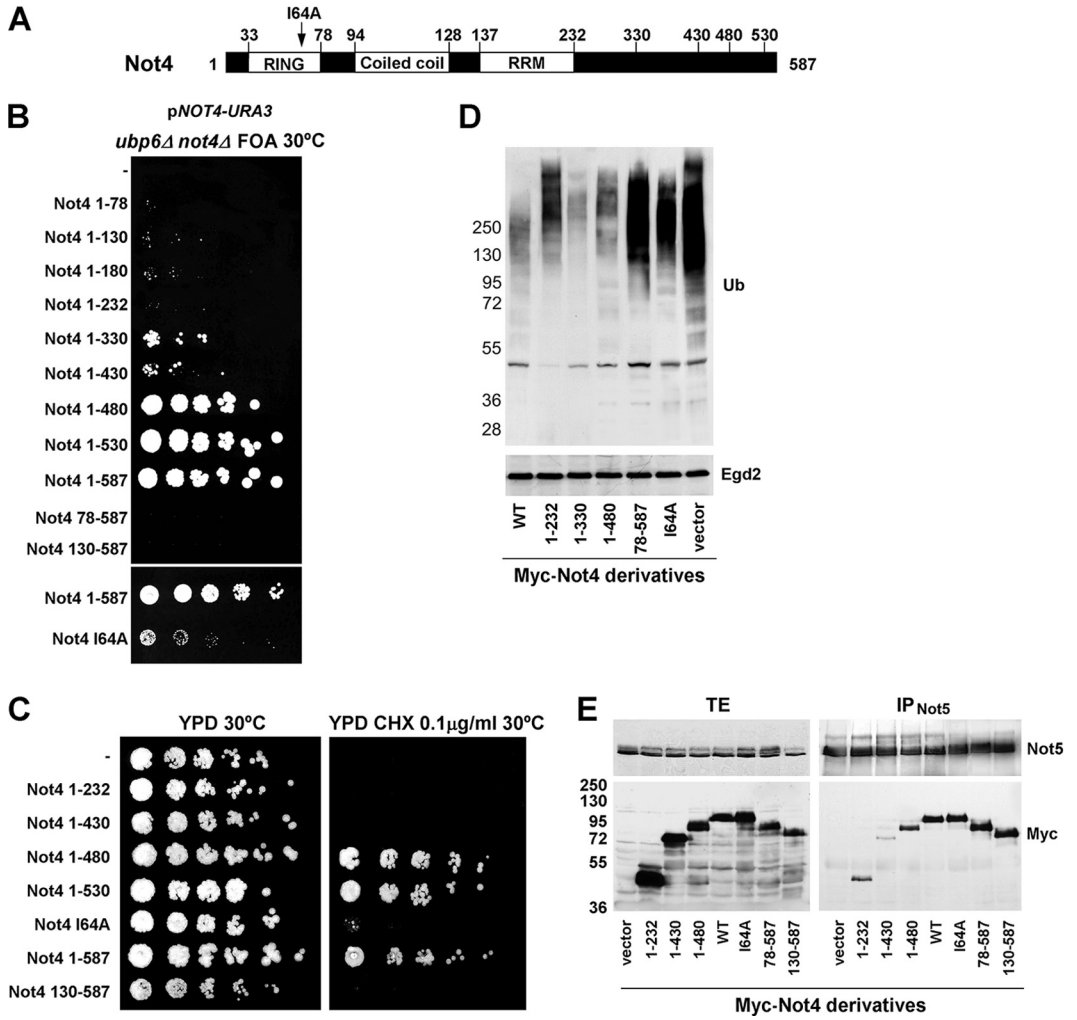

FIG. 2. Structure-function analysis of Not4. (A) Schematic representation of the Not4 protein, with its ring finger domain (RING), putative coiled-coil domain, and RNA recognition motif (RRM). (B) *ubp6*Δ *not4*Δ cells carrying a *NOT4-URA3* plasmid were transformed with *LEU2* plasmids expressing the indicated Myc-Not4 derivatives and analyzed on FOA plates as described for Fig. 1A. (C) Cycloheximide (CHX) sensitivity of *not4*Δ cells expressing the Myc-Not4 derivatives. (D) Total protein extracts were prepared from exponentially growing *not4*Δ cells expressing the Myc-Not4 derivatives and analyzed as described for Fig. 1B. Numbers at the left are molecular masses (in kilodaltons). (E) Not5 was immunoprecipitated (IP_{Not5}) from the total extracts prepared from the same cells as those used for panel D (TE). Samples were analyzed by 4 to 12% SDS-PAGE and immunoblotting for the presence of Not5 or Myc-Not4.

in the cell, we questioned the possible importance of Not4 in ubiquitin homeostasis. We observed reduced levels of free ubiquitin and the accumulation of polyubiquitinated proteins in the *not4*Δ mutant (Fig. 1B), and the synthesis of ubiquitin was increased in the *not4*Δ mutant (see Fig. S2A in the supplemental material).

The proteasome releases polyubiquitin chains from substrates before their degradation. Hence, the phenotype of the *not4*Δ mutant is consistent with a defect in proteasome function. Proteasome mutants generally display a synthetic growth phenotype when combined with a deletion of *RPN4*, which is a transcription factor for proteasome genes (52). The *rpn4*Δ mutant also displayed synthetic slow growth when combined with the *not4*Δ deletion (Fig. 1C). The same was true for a deletion of the proteasome subunit Rpn10, a polyubiquitin receptor that plays a role in maintaining the structural integrity of the RP (15) (Fig. 1C). Synthetic mutant phenotypes were additionally observed when the *not4*Δ mutant was combined with other proteasome mutants (see Fig. S2B in the supplemental material).

Taken together, these findings are compatible with a defect in proteasome function in *not4*Δ cells.

Several domains of Not4 contribute to ubiquitin homeostasis in the cell. Several domains in the Not4 structure have been described (7) (Fig. 2A). A zinc finger domain, specific for RING E3 ligases, is present between amino acids 33 and 78 (21). The function of Not4 as an E3 ligase was confirmed *in vitro* for the human ortholog (1) and both *in vitro* and *in vivo* for yeast Not4 (43). A coiled-coil domain (amino acids 94 to 128) and an RNA recognition motif (amino acids 137 to 228) have been predicted, but the functional importance of these domains is unknown.

To perform a structure-function analysis of Not4, we created plasmids expressing N-terminally Myc-tagged Not4 derivatives

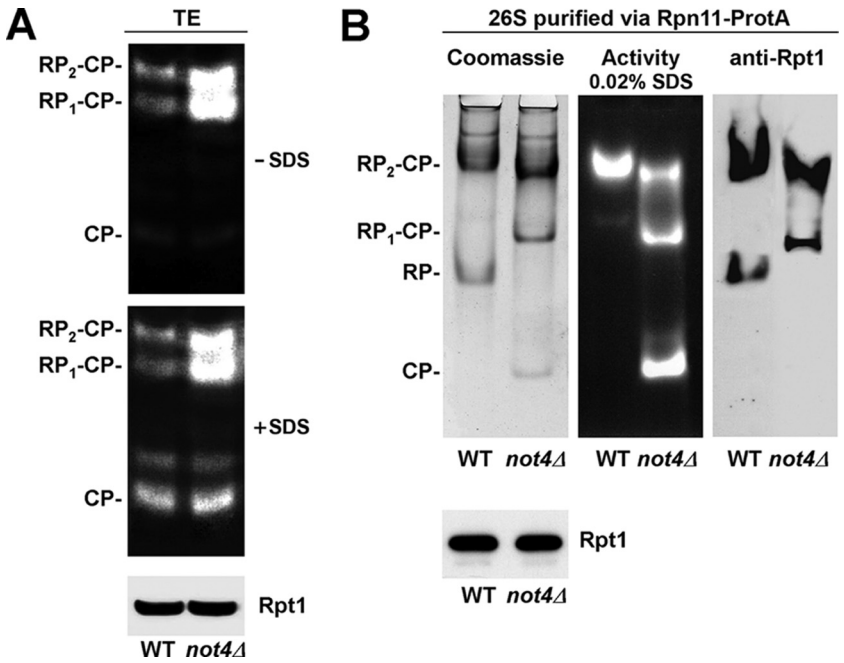

FIG. 3. Proteasome integrity is altered in *not4*Δ cells. (A) Total cellular extracts were prepared from wild-type (MY1) or *not4*Δ mutant (MY3595) cells collected at an OD_{600} of 3.0. One-hundred-microgram samples of total protein extracts (TE) were separated on a 3.5% native gel and analyzed for proteasome activity with Suc-LLVY-AMC without and with SDS. RP_2-CP and RP_1-CP are double- and single-capped proteasomes. Fifty-microgram samples of the same extracts were loaded onto SDS-PAGE gels and immunoblotted with anti-Rpt1 antibodies as a loading control. (B) Twenty-microgram samples of 26S holoenzymes, purified from wild-type (sDL133) or *not4*Δ mutant (MY6467) cells were separated on a 3.5% native gel and either stained with Coomassie blue, tested for activity, or analyzed for the presence of Rpt1 by immunoblotting. The same samples were loaded onto SDS-PAGE gels and immunoblotted with anti-Rpt1 antibodies as a loading control (bottom).

and mutants with N-terminal and C-terminal truncations, as well as a Not4 point mutant in which isoleucine at position 64 was replaced with alanine (Not4 I64A). This mutation disrupts the interaction of Not4 with its partner E2 enzymes, Ubc4 and Ubc5 (41), and results in the reduced ubiquitination of its substrate, Egd2 (see Fig. S3A in the supplemental material). All of the derivatives were stable (Fig. S3C) and expressed at levels comparable to that of endogenous Not4 (data not shown) but led to different growth rates (Fig. S3B and Table S1). Interestingly, for many Not4 derivatives, we noticed an additional slower-migrating form (Fig. S3C). This form was absent in the I64A mutant and in the mutants lacking the RING domain, suggesting that it could be autoubiquitinated Not4.

We determined which domains of Not4 were essential for the viability of *ubp6*Δ cells (Fig. 2B). The mutants with a growth rate comparable to that of the *not4*Δ mutant (from $Not4_{1–78}$ [in which amino acids 1 to 78 are present] to $Not4_{1–232}$ and derivatives with a deletion of the N terminus) had a synthetic phenotype comparable to that of the *not4*Δ mutant. Those with an intermediate growth rate ($Not4_{1-330}$, $Not4_{1-430}$) had an intermediate phenotype. Mutants that grew like a wild type ($Not4_{1-480}$, $Not4_{1-530}$) did not display any synthetic phenotype.

Many proteasome mutants are cycloheximide (CHX) sensitive as a result of ubiquitin depletion (20). Hence, we tested the resistance of cells expressing the different derivatives of Not4 on medium containing CHX. *not4*Δ cells and cells expressing all Not4 derivatives except the longest two ($Not4_{1-480}$, $Not4_{1-530}$), displayed a CHX-sensitive phenotype (Fig. 2C). Even Not4 I64A, which grew relatively well, was CHX sensitive. The accumulation of polyubiquitinated proteins mostly correlated with CHX sensitivity (Fig. 2D).

Next we determined which domains of Not4 were required for its association with the Ccr4-Not complex. We immunoprecipitated Not5 and analyzed the presence of the different Not4 derivatives in the immunoprecipitates (Fig. 2E). Wild-type Not4, all N-terminally truncated derivatives, and Not4 I64A coimmunoprecipitated well with Not5. The coimmunoprecipitation of C-terminally truncated mutants ($Not4_{1-232}$ and $Not4_{1-430}$) was less efficient. The same results were observed after coimmunoprecipitation for other Ccr4-Not subunits (Not3 and Caf40 [data not shown]). These data indicate that the C, but not the N, terminus of Not4 is important for its interaction with the Ccr4-Not complex.

The proteasome is altered in cells lacking Not4. As mentioned above, the deletion of Not4 results in a phenotype typical for mutants of the UPS. Hence, proteasome function might be affected in the *not4*Δ mutant. We compared proteasome activities in total extracts from the wild type and the *not4*Δ mutant. We separated them on a native gel and then incubated the gel with the Suc-LLVY-AMC substrate, without or with SDS treatment to reveal latent activity (Fig. 3A). The amount of proteasome subunits (Rpt1) was the same in total extracts from both strains. However, greater levels of proteasome activity were observed in the *not4*Δ mutant than in the wild type; proteasome activities in the *not4*Δ mutant were comparable to those of complexes that correspond to double-

and single-capped proteasomes (RP_2-CP and RP_1-CP) (Fig. 3A).

To follow up on this observation, we purified the 26S holoenzyme through Rpn11-ProtA from exponentially growing (OD_{600} of 3.0) wild-type and mutant cells (Fig. 3B). The same amount of Rpt1 was isolated from both strains. The holoenzyme purified from the wild type was detected on a native gel as an active double-capped proteasome. Additionally, a substantial amount of free RP was purified, as seen on the Coomassie blue-stained native gel and by immunoblotting of the native gel with anti-Rpt1 antibodies. Purification at later stages of growth (OD_{600} of 13.0) resulted in the isolation of a reduced amount of free RP and the appearance of RP_1-CP and CP species (see Fig. S1 in the supplemental material).

The spectrum of proteasome complexes purified from the *not4*Δ mutant was quite distinct from that from the wild type (Fig. 3B). The holoenzyme from the *not4*Δ mutant migrated on a native gel as active double- and single-capped proteasomes. There was an increase of free CP, compared to that in the wild type, but no free RP could be detected, either by Coomassie blue staining or immunoblotting. These profiles of purification were similar for cells at whatever stage of growth they were collected, even as early as at an OD_{600} of 0.7 (data not shown). These results suggest that the interaction of CP with RP is less stable in the mutant and that either there is less free RP or it is unstable when not associated with CP.

Salt-resistant RP-CP complexes can be purified from the *not4*Δ mutant. The proteasome RP-CP interaction is salt sensitive, and free RP and CP can be obtained by incubation of the holoenzyme with 0.5 M NaCl (33). We used this procedure and cells expressing either tagged RP (Rpn11-ProtA) or CP (Pre1-ProtA) subunits to purify separately RP and CP from the wild type and the *not4*Δ mutant. Purified RP and CP were analyzed first by SDS-PAGE and immunoblotting (Fig. 4A and B). There was the same amount of RP (Rpt1) and CP (α1–7) subunits in total extracts and purified material from both strains (Fig. 4B). For wild-type cells, as expected, there were no α subunits in the RP purification (Fig. 4B, left) or Rpt1 in the CP purification (Fig. 4B, right). In contrast, for the *not4*Δ mutant, we found α7 in isolated RPs and Rpt1 in isolated CPs. These findings suggest that in the *not4*Δ mutant some RP-CP interactions were resistant to salt.

Analysis of purified CPs from the wild type or the *not4*Δ mutant on a native gel did not reveal any noticeable differences in either migration or activity (see below and Fig. 4E). In contrast, remarkable differences were apparent when Rpn11-ProtA was purified in high salt from both strains (Fig. 4C). For wild-type cells, several forms of RP were visible by Coomassie blue staining (Fig. 4C, left). They were all inactive and contained Rpt1 but no α subunits (Fig. 4C, middle and right). The faster-migrating form corresponds to free or "competent" RP (RP_c), the RP species that can reconstitute a proteasome (reference 29 and see below). The complexes of intermediate migration are "noncompetent" RPs (RP_ns) because they are incapable of CP binding (reference 29 and see below). They have a migration similar to that of double-capped proteasomes. The slowest-migrating forms at the top of the native gel were named "high" RPs (RP_hs). Besides these Rpt1-containing complexes, another complex present at low levels and migrating the fastest, likely to be the lid alone, was visible (Fig. 4C, left, and E, bottom). According to the results of mass spectrometry analysis, wild-type RP_c contained all lid and base subunits, Ecm29, and two RP chaperones—Hsm3 and Nas6 (see Fig. S4 and Table S2 in the supplemental material). RP_n complexes contained all lid and base subunits, as well as Caf4, a protein associated with the Ccr4-Not complex (38). In RP_hs, all lid and base subunits, Ecm29, Ubp6, Nas6, and both subunits of fatty acid synthase (Fas1 and Fas2) were identified.

In contrast, when Rpn11-ProtA was purified in high salt from the *not4*Δ mutant (the complex purified in this case will be referred to hereinafter as $RP_{not4\Delta}$ for purposes of simplification), a single proteasome complex migrating at the size of a double-capped proteasome was visible by Coomassie blue staining (Fig. 4C, left). This complex was active and contained both the Rpt1 and α subunits. Mass spectrometry analysis confirmed the presence of all lid, base, and CP subunits but identified no Ecm29, RP chaperones, or Fas in this complex (see Fig. S4 and Table S2 in the supplemental material). We concluded that it was a salt-resistant RP_2-CP form. An additional small amount of RP_c that was positive for Rpt1 by immunoblot analysis at long revelation times (not shown) was detectable on the Coomassie blue-stained gel (Fig. 4C and Fig. S4). By mass spectrometry analysis, we identified only the Rpn2, Rpn3, Rpt1, and Rpt3 subunits in this band (see Table S2 in the supplemental material). This may be due to the lack of sufficient material to detect the remaining RP subunits.

We purified Rpn11-ProtA in high salt (0.5 M) from *not4*Δ cells expressing the different Not4 derivatives. Only the expression of full-length Not4, Not4 I64A, and the longest C-terminally truncated mutant, $Not4_{1–480}$, restored the RP-CP salt sensitivity and normal levels of free RP_c (Fig. 4D).

$RP_{not4\Delta}$ reconstitutes proteasomes with CP more efficiently than wild-type RP. To address the question of why the RP and CP interaction is salt resistant in *not4*Δ cells, we performed *in vitro* proteasome reconstitution experiments using separately purified, in high salt, Rpn11-ProtA (RP) and Pre1-ProtA (CP) from wild-type or *not4*Δ cells. Reconstituted proteasomes were analyzed on a native gel for activity or the presence of the base (Rpt1), lid (Rpn5), or CP (α1–7) subunits by immunoblotting (Fig. 4E).

Proteasomes reconstituted with wild-type RP and either wild-type or *not4*Δ CP were indistinguishable (Fig. 4E, lanes 2 and 3). Both single- and double-capped proteasomes were created. Reconstituted complexes showed the expected electrophoretic mobilities on a native gel and had hydrolytic activities indicating effective opening of the CP gate. Hence, these reconstituted species represent bona fide 26S proteasomes. We noticed that after reconstitution, free RP_c disappeared (Fig. 4E, middle panel, compare lanes 2 and 3 to lane 1), indicating that this is indeed the RP species that was used to reconstitute proteasome. In contrast, the levels of RP_h and the lid subunit were unaltered, indicating that these species were unable to reconstitute proteasomes.

There was no difference in proteasomes reconstituted using $RP_{not4\Delta}$ with either wild-type or *not4*Δ CPs (Fig. 4E, lanes 4 and 6). In both cases, more active double-capped proteasomes were visible after reconstitution, and additional single-capped forms appeared. Interestingly, in both cases, there was a greater incorporation of CPs into single- or double-capped proteasomes than upon reconstitution with wild-type RPs (CP

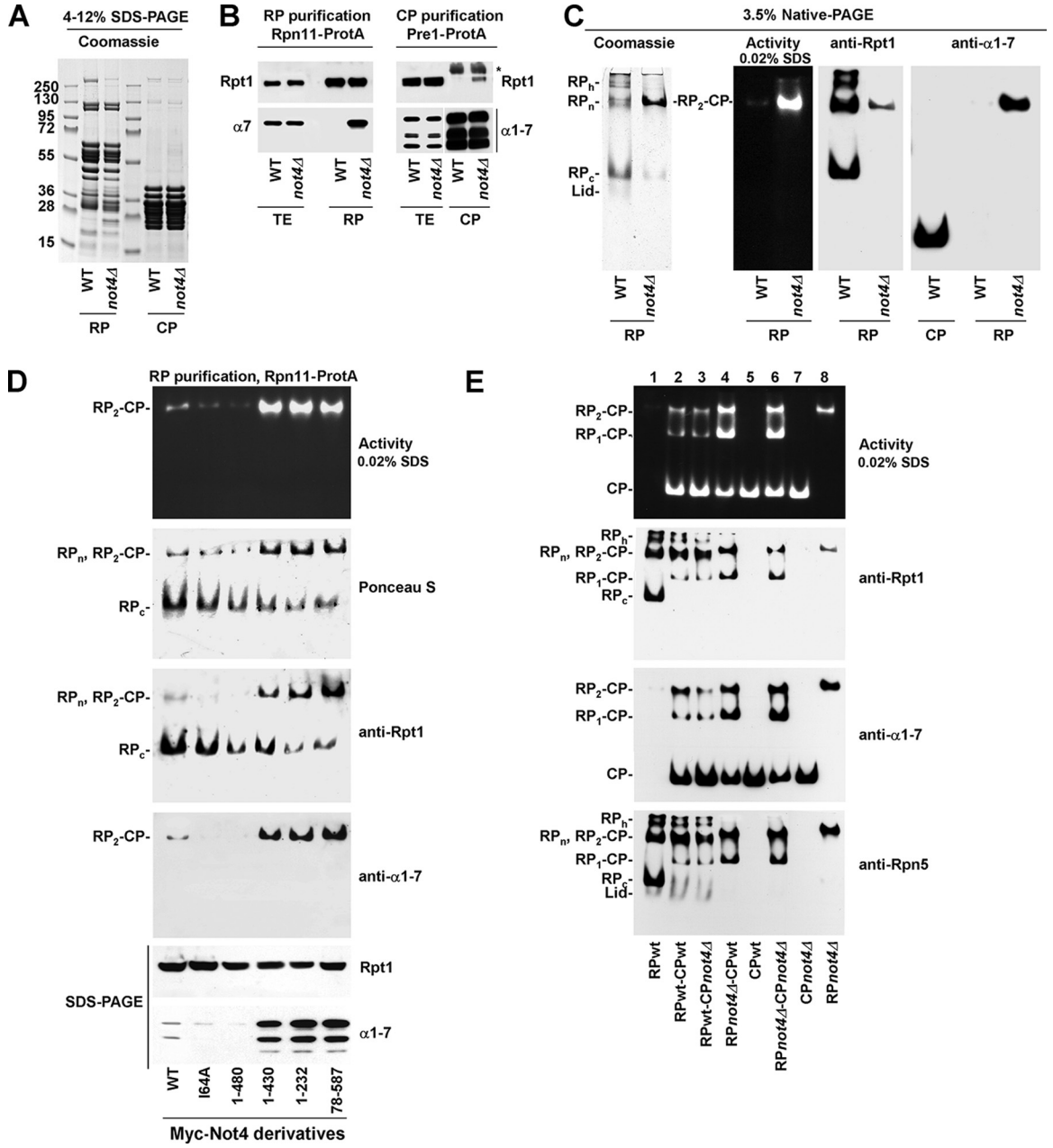

FIG. 4. RP and CP form salt-resistant complexes in *not4*Δ cells. Total cellular extracts from wild-type or *not4*Δ cells expressing Rpn11-ProtA (for RP purification) and Pre1-ProtA (for CP purification) were incubated with IgG beads. After the beads were washed with a high-salt buffer, RP and CP were eluted from the column by TEV cleavage (see Materials and Methods) and analyzed by SDS-PAGE (A) or by immunoblotting (B) for the presence of Rpt1 or CP subunits (α7 or α1–7). Numbers to the left are molecular masses (in kilodaltons). The asterisk in panel B marks a nonspecific band. (C) The same samples were analyzed on a 3.5% native gel and either stained with Coomassie blue (left), tested for activity (middle), or analyzed for the presence of Rpt1 and α1–7 by immunoblotting (two right-most blots). (D) Rpn11-ProtA was affinity purified in a high concentration of salt (0.5 M) from the *not4*Δ mutant expressing the indicated Myc-Not4 derivatives. Purified proteins were then separated on a 3.5% native gel and either tested for activity or transferred to nitrocellulose, probed with Ponceau S, and analyzed for the presence of Rpt1 or α1–7. The same purified proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against Rpt1 or α1–7 (bottom). (E) *In vitro* reconstitution of proteasomes. Separately purified RP and CP from wild-type and *not4*Δ cells (the same as those used for panel A) were mixed in the following combinations: wild-type RP (lane 1), wild-type RP plus wild-type CP (lane 2), wild-type RP plus $CP_{not4\Delta}$ (lane 3), $RP_{not4\Delta}$ plus wild-type CP (lane 4), wild-type CP (lane 5), $RP_{not4\Delta}$ plus $CP_{not4\Delta}$ (lane 6), $CP_{not4\Delta}$ (lane 7), and $RP_{not4\Delta}$ (lane 8). Samples were incubated at 30°C for 30 min, loaded onto a 3.5% native gel, and tested for activity or immunoblotted with anti-Rpt1, α1–7, or Rpn5 antibodies.

levels are reduced in lanes 4 and 6 compared to in lanes 2 and 3). This indicates that $RP_{not4\Delta}$s incorporate better into CPs during reconstitution.

RP species in the *not4*Δ mutant that are not incorporated into RP-CP complexes are unstable. The total amounts of Rpt1 that copurified with Rpn11-ProtA in high salt from wild-type and *not4*Δ cells were the same (Fig. 4B, left). However, after separation of the purified material on a native gel, much less Rpt1 was detectable in the *not4*Δ mutant purification than in that of the wild type (Fig. 4C). Nevertheless, it was possible

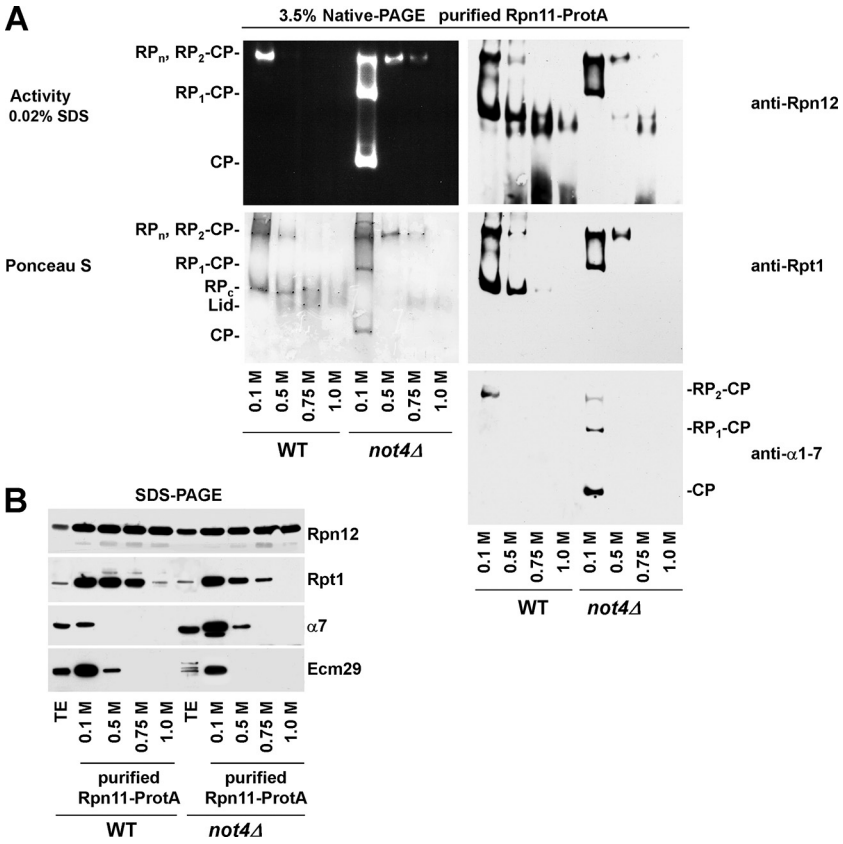

FIG. 5. RP species from the *not4*Δ mutant that are not incorporated into salt-resistant RP-CP complexes are unstable. Total extracts from wild-type or *not4*Δ cells expressing Rpn11-ProtA were split into four portions. The NaCl concentration in each sample was adjusted to allow purification of holoenzyme (0.1 M), RP (0.5 M), or lid (0.75 and 1 M) (33). (A) Purified complexes were analyzed on native gel and either tested for activity or immunoblotted with antibodies against Rpn12, Rpt1, and α1–7. (B) The same purified complexes were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

to reconstitute more RP_2-CP and RP_1-CP complexes using the proteins purified from the *not4*Δ mutant than from the wild type, despite the greater levels of the RP_c in the wild type (Fig. 4E). A possible explanation for these results is that the RP_c purified from the *not4*Δ mutant was unstable and came apart during native gel electrophoresis.

To analyze this in more detail, we prepared extracts from wild-type and *not4*Δ cells expressing Rpn11-ProtA. Proteasomes were purified from fractions that were adjusted to increasing NaCl concentrations (Fig. 5A). For wild-type cells at 0.1 M NaCl, we obtained active RP_2-CP as well as RP_c. Increasing salt concentration led to the isolation of only RP (at 0.5 M NaCl) or the lid (at 0.75 M and 1.0 M NaCl). In the purification from *not4*Δ cells, we obtained RP_2-CP, RP_1-CP, and CP species at 0.1 M NaCl treatment and a salt-resistant RP_2-CP species at 0.5 M NaCl, consistently with our results presented above (Fig. 3B, 4C, and 5A). An increase of salt to 0.75 M led to a low level of lid purification. A further increase of NaCl to 1.0 M led to the absence of any detectable proteasome species on a native gel.

SDS-PAGE and immunoblotting revealed the same amounts of lid, base, and CP subunits in the total extracts used for purification from both strains (Fig. 5B). We isolated more of the α7 subunit at 0.1 M and 0.5 M NaCl from the *not4*Δ mutant than from the wild type. Less Rpt1 was isolated at high salt concentrations (0.5 to 1.0 M) from the *not4*Δ mutant than from the wild type. In contrast, the same amount of lid subunit Rpn12 was found in all purifications. In the wild type, Rpn12 was visible in different complexes on a native gel, namely, in the RP-CP, RP_c, and lid complexes and then in even smaller complexes at high salt (Fig. 5A, upper right). However, in the case of purification from the *not4*Δ mutant, Rpn12 was visible in RP_2-CP complexes isolated in low salt but was hardly detectable at higher salt conditions. Taken together, these results suggest that in the *not4*Δ mutant, RP species, except the RP-CP complex, are not stable. Interestingly the level of Ecm29, a proteasome-associated protein, was reduced in *not4*Δ cells compared to the level in the wild type (see below).

Not4 associates with RP species present in holoenzyme preparations but not with purified RPs. We identified many proteasome subunits in Ccr4-Not purifications, but we did not find Not4 in RP purifications (see Tables S2 and S3 in the supplemental material). Hence, to understand better whether Not4 interacts with the proteasome and, if so, how, we first purified holoenzyme via Rpn11-ProtA from Myc-Not4-expressing cells. Purified proteins were separated by SDS-PAGE and analyzed by immunoblotting. Myc-Not4 was clearly identified in the purification, together with the Rpt1, Rpn8, and α subunits (Fig. 6A).

Next we tried an *in vitro* binding experiment. We fixed ho-

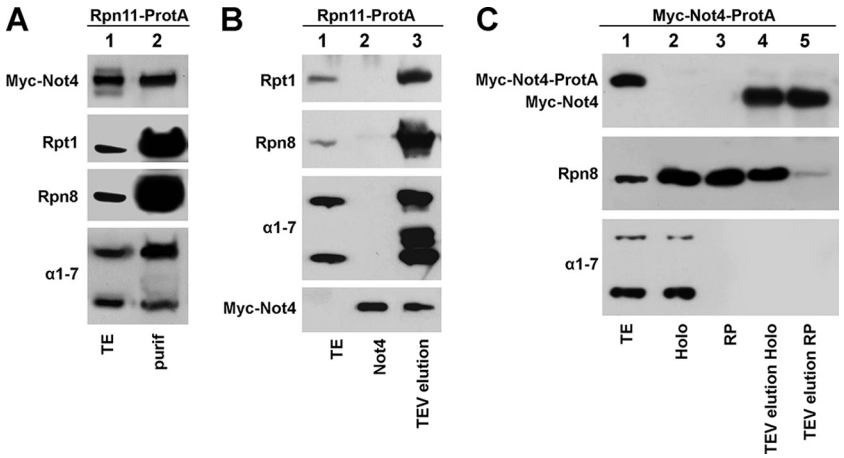

FIG. 6. Not4 associates with RP species present in holoenzyme but not with purified RP. (A) 26S holoenzyme was purified via Rpn11-ProtA from *not4*Δ cells (MY7820) expressing Myc-Not4. Total extracts (TE) (lane 1) and purified proteins (purif) (lane 2) were analyzed by immunoblotting for the presence of Rpt1, Rpn8, α1–7, and Myc-Not4. (B) Holoenzymes from the wild type were immobilized on IgG beads via Rpn11-ProtA and incubated with separately purified Myc-Not4. After the beads were washed, proteasome was eluted by TEV cleavage. Total extracts (lane 1), added Myc-Not4 (Not4) (lane 2), and proteins in the TEV eluate (lane 3) were analyzed by immunoblotting for the presence of Rpt1, Rpn8, α1–7, and Myc-Not4. (C) Myc-Not4-ProtA was immobilized on IgG beads. Separately purified holoenzyme and RP from cells expressing Rpn5-ProtA were added to the beads. After the beads were washed, Myc-Not4 was eluted by TEV cleavage. Total extracts (lane 1), added holoenzyme (Holo) and RP (lanes 2 and 3, respectively), and the proteins in the TEV eluates (lanes 4 and 5, respectively) were analyzed by immunoblotting for the presence of Rpn8, α1–7, and Myc-Not4.

loenzyme from wild-type cells via Rpn11-ProtA on IgG beads and incubated these beads with separately purified Myc-Not4 (Fig. 6B). The IgG beads were washed, and holoenzyme was eluted by TEV cleavage. The presence of Myc-Not4 in the eluate in addition to proteasome subunits was demonstrated by immunoblotting. We also performed the reverse experiment (Fig. 6C). We fixed Myc-Not4-ProtA from total extracts on IgG beads and incubated the beads with purified holoenzyme or RP. After being washed, Myc-Not4 was eluted by TEV cleavage. The presence of the Myc-Not4, Rpn8, or α subunit in the eluates was evaluated by immunoblotting. Rpn8 was found in the eluate from the sample incubated with holoenzyme but not RP. In contrast, no α subunits coeluted with Not4 in either case.

Taken together, these results demonstrate that Not4 can associate, directly or indirectly, with RP species that contain at least the Rpn11 and Rpn8 but not the α subunits and that are present in the holoenzyme but not in RP preparations. Furthermore, Not4 cannot associate with purified RP. This explains why we did not find Not4 in the mass spectrometry analyses discussed above, all performed with RP purifications.

Not4 genetically, physically, and functionally interacts with Ecm29. Our result suggests that, on one hand, Not4 interacts with a proteasome complex or intermediate and that, on the other hand, Not4 is necessary for proteasome integrity. Hence, Not4 might assist proteasome assembly or stability, perhaps through interaction with proteasome assembly or stabilizing factors. One such factor, Ecm29, was identified in affinity-purified Ccr4-Not complexes by mass spectrometry (reference 3 and see Table S3 in the supplemental material). In *not4*Δ cells, Ecm29 levels were decreased, and several forms of Ecm29 accumulated (Fig. 5B). Less Ecm29 copurified with Rpn11-ProtA from the *not4*Δ mutant than from wild-type cells, and Ecm29 was identified in RP species isolated from the wild type but not from the *not4*Δ mutant (Fig. 5B and see Figure S4 and Table S2 in the supplemental material).

Ecm29 has been described to enhance the stability of the proteasome (34). Deletion of Ecm29 results in the same phenotype that we observed for the *not4*Δ mutant: it shifts the equilibrium between RP associated with CPs and free components toward RP-CP complexes and leads to accumulation of polyubiquitinated proteins (37). We hence investigated the relationship between Ecm29 and Not4 in more detail. The mutant with the deletion of *NOT4* displayed a synthetic growth phenotype at 37°C or on CHX when it was combined with the deletion of *ECM29.* However, deletion of *ECM29* suppressed the slow growth of the *not4*Δ mutant at 30°C (Fig. 7A). A reduced level of Ecm29 in the *not4*Δ mutant was confirmed for tagged Ecm29 expressed in wild-type and *not4*Δ cells (Fig. 7B). N-terminally truncated Not4 derivatives or the I64A mutant could complement this reduction, whereas C-terminal derivatives were not effective (Fig. 7C). S1 analysis revealed the same amount of *ECM29* mRNA in wild-type and mutant cells (data not shown). Hence, reduction of Ecm29 levels in the *not4*Δ mutant occurs posttranscriptionally. Therefore, we investigated the stability of Ecm29 in wild-type and *not4*Δ mutant cells. In both the wild type and the *not4*Δ mutant, Ecm29 was rapidly turned over, though in the wild type, a small fraction of Ecm29 seemed relatively stable (Fig. 7D).

Since we previously noted slower-migrating forms of Ecm29 in the *not4*Δ mutant (Fig. 5B), we checked whether Ecm29 might be ubiquitinated. For this, we expressed His ubiquitin in the wild type or in *not4*Δ cells expressing HA-Ecm29 and purified ubiquitinated proteins on a nickel column. After being immunoblotted, ubiquitinated forms of Ecm29 were detectable in the wild type and, more importantly, in the mutant (Fig. 7E).

These results suggest that Not4 has an impact on ubiquitination and steady-state levels of Ecm29. Since we previously

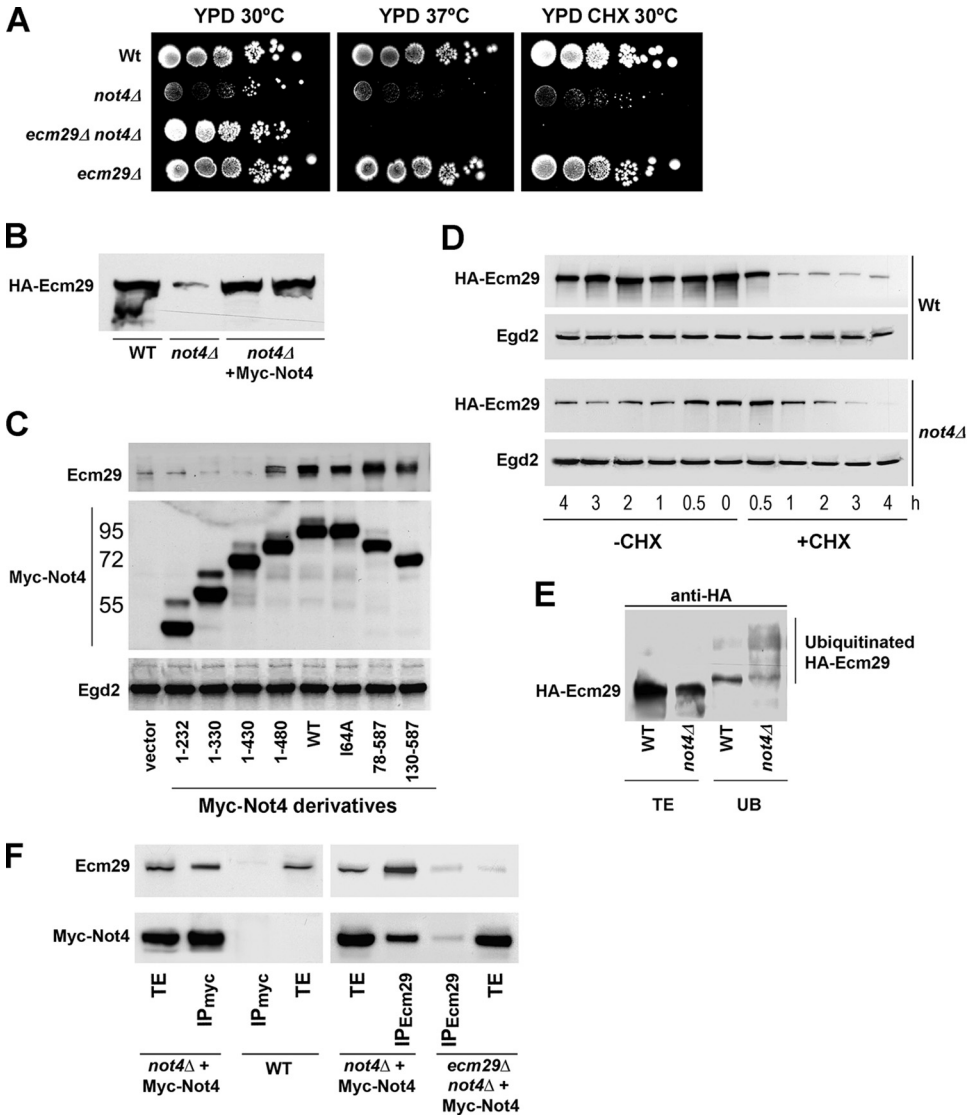

FIG. 7. Not4 genetically, physically, and functionally interacts with Ecm29. (A) The indicated strains were spotted on YPD plates with or without CHX and left to grow at 30 or 37°C. (B) *ecm29*Δ cells expressing HA-Ecm29 from an episome (the wild type, the *not4*Δ mutant, and two different transformants of the *not4*Δ mutant with the Myc_6-Not4 plasmid) were collected at an OD_{600} of 0.8, and total proteins were analyzed by immunoblotting with anti-HA antibodies. (C) Thirty-microgram samples of total protein extracts from *not4*Δ cells expressing the Myc-Not4 derivatives were analyzed by immunoblotting with antibodies against Ecm29, Myc, and Egd2 as a control for loading. Numbers at the left are molecular masses (in kilodaltons). (D) Exponentially growing *ecm29*Δ or *ecm29*Δ *not4*Δ cells expressing HA-Ecm29 were treated or not treated with CHX for the indicated times. Equivalent amounts of total extract were analyzed by immunoblotting with antibodies against HA or Egd2 as a control for loading. (E) Ubiquitinated proteins were purified from *ecm29*Δ or *ecm29*Δ *not4*Δ cells expressing HA-Ecm29 and His_6-ubiquitin (43). Total extract (TE) or ubiquitinated proteins bound to the Ni-resin (UB) were analyzed by immunoblotting with anti-HA antibodies. (F) Total protein extracts (TE) from cells expressing Myc-Not4 were incubated with antibodies against the Myc tag (IP_{Myc}) or against Ecm29 (IP_{Ecm29}). The presence of Ecm29 and Myc-Not4 in the immunoprecipitates was evaluated by immunoblotting. The strains expressing endogenous untagged Not4 (left) and not expressing Ecm29 (right) were used as controls for the specificity of the immunoprecipitation.

detected Ecm29 in affinity-purified Ccr4-Not complexes by mass spectrometry (see Table S3 in the supplemental material), we looked further for an interaction between Ecm29 and Not4. We immunoprecipitated Myc-Not4 from total extracts with anti-Myc antibodies and could identify Ecm29 in the immunoprecipitate (Fig. 7F, left). Then we did the reverse experiment and immunoprecipitated Ecm29 from the total extracts of cells expressing Myc-Not4. We clearly identified Myc-Not4 in the immunoprecipitate (Fig. 7F, right). These results confirm that Ecm29 and Not4 can interact, either directly or indirectly.

Some of Not4's impact on the proteasome is through Ecm29. To answer the question of whether some or all of Not4's impact on the proteasome might be related to its interaction with Ecm29, we purified RPs from the wild type, 2 different *not4*Δ strains, the *ecm29*Δ mutant, the *ecm29*Δ *not4*Δ double mutant, and, finally, the *not4*Δ *ecm29*Δ double mutant trans-

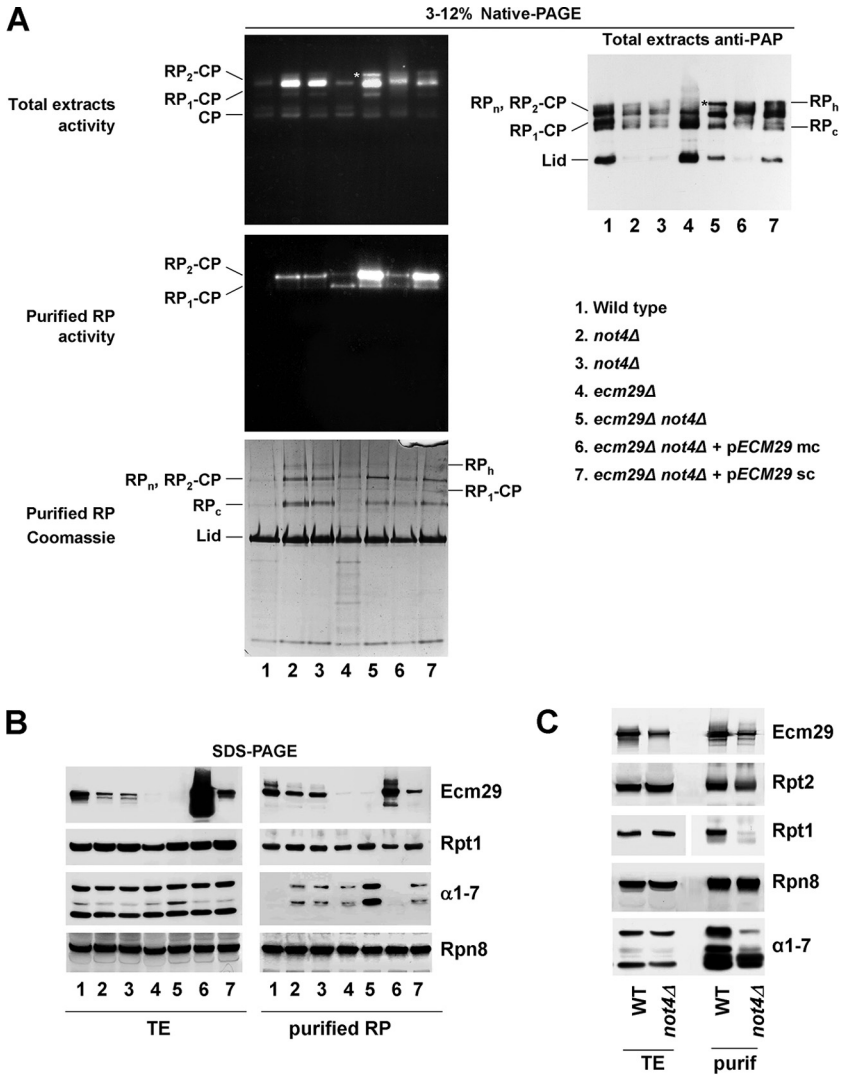

FIG. 8. Some of Not4's impact on the proteasome is through Ecm29. (A) Rpn11-ProtA was purified at 0.5 M NaCl from cells of the wild type (MY5559) (lane 1), 2 different *not4*Δ mutants (MY7367 [lane 2] and MY7820 [lane 3]), the *ecm29*Δ mutant (MY7827) (lane 4), the *ecm29*Δ *not4*Δ mutant (MY7822) (lane 5), and the *ecm29*Δ *not4*Δ mutant expressing Ecm29 from a multicopy plasmid (MY7822 plus p*ECM29* mc) (lane 6) or from a single-copy plasmid (MY7822 plus p*ECM29* sc) (lane 7). One hundred micrograms of total extracts used for purification was loaded on a 3 to 12% native gel and analyzed for activity (top) or by immunoblotting with PAP antibodies (right). Purified RPs were loaded on a 3 to 12% native gel and analyzed for activity (middle) or by Coomassie blue staining (bottom). (B) The same samples were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. (C) Ecm29 was affinity purified from wild-type or *not4*Δ cells. Total extract (TE) and purified proteins (purif) were analyzed by immunoblotting with the indicated antibodies.

formed either with a multicopy plasmid overexpressing Ecm29 or with a centromeric plasmid expressing wild-type levels of HA-Ecm29 (Fig. 8). The same amounts of the Rpt1, Rpn8, and α subunits were found in the total extracts before purification (Fig. 8B, left). As expected, less Ecm29 was found in the total extracts from *not4*Δ cells.

First, we analyzed the proteasome activities in total extracts prepared from the strains indicated in Fig. 8 by native PAGE (Fig. 8A, top). Holoenzyme activity was higher in the *not4*Δ mutant than in the wild type, as expected (Fig. 8A, top, compare lanes 2 and 3 to lane 1). In contrast, activity in the *ecm29*Δ mutant was indistinguishable from that of the wild type (Fig. 8A, top, lanes 1 and 4). Activity in the double mutant was higher than in the *not4*Δ single mutant, and an additional active complex (marked by an asterisk), migrating more slowly than the RP_2-CP complex, was detected (Fig. 8A, top, lane 5). Surprisingly, expression of Ecm29 from either the multicopy or the single-copy plasmid in the *not4*Δ *ecm29*Δ mutant reduced the holoenzyme activity to levels lower than those in the *not4*Δ single mutant (Fig. 8A, top, compare lanes 6 and 7 to lanes 2 and 3).

Immunoblot analysis of the native gel with antibodies to follow Rpn11-ProtA revealed that all active complexes (RP_2-CP and RP_1-CP), as well as RP_n, RP_c, RP_h, and free lid, were present in different amounts in the total extracts (Fig. 8A, top right). The free lid was absent in the *not4*Δ mutant but present in the *ecm29*Δ mutant and in the double mutant. Hence, the deletion of Ecm29 suppressed the instability of

CP-less RP complexes in the *not4*Δ mutant. No free lid was detectable in the double mutant overexpressing Ecm29, suggesting a complementation of the *ECM29* deletion. That did not occur when Ecm29 was expressed only from the centromeric plasmid (Fig. 8A, top right, compare lanes 6 and 7 to lane 5). In this context, it is interesting to note that purification of the proteasome via Rpn11-ProtA from the *ecm29*Δ mutant led to purification of stable lid even under the highest salt conditions (see Fig. S5 in the supplemental material).

Second, we purified Rpn11-ProtA in high salt (0.5 M) from all strains and analyzed the purified proteasomes on a native gel for activity (Fig. 8A, middle) and by SDS-PAGE (Fig. 8B, right). The same amounts of the base (Rpt1) and lid (Rpn8) subunits were purified for all samples (Fig. 8B, right). As expected, there was no activity detected for the purification from wild-type cells (Fig. 8A, middle, lane 1), and consistently, no CP subunits copurified with Rpn11 (Fig. 8B, right, lane 1). For the single mutants, active proteasome forms were observed, mostly RP_2-CP for the *not4*Δ mutant (Fig. 8A, middle, lanes 2 and 3) and RP_1-CP for the *ecm29*Δ mutant (Fig. 8A, middle, lane 4). The presence of CP subunits in both single mutants was confirmed by immunoblotting (Fig. 8B, right, lanes 2 to 4). These results indicate that in the *ecm29*Δ mutant, as in the *not4*Δ mutant, salt-resistant active RP-CP complexes were present. In the *not4*Δ *ecm29*Δ double mutant, the amount of active RP-CP was increased and the presence of CP subunits was more important, revealing an aggravated phenotype (Fig. 8A and B, lane 5). Expression of Ecm29 complemented the increased amount of salt-resistant RP-CP from the *not4*Δ *ecm29*Δ mutant compared to that from the single *not4*Δ mutant (Fig. 8A, middle, and B right, compare lanes 6 and 7 to lanes 2, 3, and 5). Curiously, many faster-migrating RP subcomplexes were detectable by Coomassie blue staining in the purification from the *ecm29*Δ mutant, which were absent in the purification from the double mutant (Fig. 8A, bottom, compare lane 4 to lane 5).

Our results suggest that the impact of Not4 on Ecm29 contributes to the wild type's RP-CP salt sensitivity. Indeed, (i) the salt resistance of the RP-CP interaction was observed in both the *not4*Δ mutant and the *ecm29*Δ mutant, (ii) the levels of Ecm29 were lower in the *not4*Δ mutant than in the wild type, and (iii) the overexpression of Ecm29 in the *ecm29*Δ *not4*Δ double mutant could entirely suppress this RP-CP salt resistance. On the other hand, greater instability of CP-less RP complexes specific for the *not4*Δ mutant was not observed in the *ecm29*Δ mutant (Fig. 8A, right, lane 4) and in fact were partially suppressed by the deletion of *ECM29* in the *not4*Δ mutant (Fig. 8A, right, lane 5). Conversely, the accumulation of small RP complexes in the *ecm29*Δ mutant was suppressed by the *not4*Δ mutant (Fig. 8A, bottom, lanes 4 and 5). Taken together, these results suggest that the functions of Ecm29 and Not4 are partially interconnected and that the phenotype of the *not4*Δ mutant may in part be due to an inappropriate function of Ecm29 in this mutant.

Ecm29 association with proteasome is deficient in the *not4*Δ mutant. To address how Not4 may affect Ecm29 function, we purified Ecm29-ProtA from the wild type and the *not4*Δ mutant. The ProtA tag on Ecm29 stabilized the protein such that its levels in the wild type and in the *not4*Δ mutant were relatively comparable. We analyzed the Ecm29 purification by mass spectrometry (see Table S4 in the supplemental material) and immunoblotting (Fig. 8C). This analysis led to several interesting observations. A number of proteasome subunits, but not all, could be copurified with Ecm29 from the wild type. These are the subunits of the lid, Rpn1 and -2 and Rpt1 to -6 of the base, and the α1 to α4, α6, and α7 as well as the β1, β5, and β6 subunits of the CP. Additionally, Blm10 copurified. This is compatible with the fact that Ecm29 interacts with both RPs and the α ring of CP (34). In the *not4*Δ mutant, neither Rpt1, Rpt3, β1, α3, nor Blm10 was detectable in the purification of Ecm29, and the levels of some other subunits (Rpt2, -4, and -6 and α2, α7, and β5) appeared to be reduced. These results of mass spectrometry were confirmed by immunoblotting (Fig. 8C). Indeed, the same amounts of the Rpt1, Rpt2, Rpn8, and α subunits were detected in total extracts before purification, but slightly less Rpt2 and many fewer α subunits were purified from the *not4*Δ mutant than from the wild type. No Rpt1 at all was purified from the mutant. These results suggest that Ecm29 might make primary contacts with the lid, whereas Not4 contributes to Ecm29 association with the other proteasome components.

Many other proteins were identified in the Ecm29 purifications. These are proteins related to vesicle sorting and transport, cytoskeleton and motors proteins, proteins involved in lipid biogenesis, chaperones, and ribosomal proteins. This is consistent with a recent study that established a link between Ecm29 and both endosomal compartments and molecular motors (18) and suggested that Ecm29 has cellular partners other than the proteasome.

DISCUSSION

Role of Not4 for appropriate and dynamic proteasome assembly. In this work, we report that the Not4 E3 ligase is important for the physical and functional integrity of the proteasome and, consequently, for appropriate ubiquitin turnover in the cell. Indeed, the deletion of Not4 leads to a decrease of free ubiquitin in the cell. Consistently, the synthesis of ubiquitin is increased in the *not4*Δ mutant as it is under stress conditions (11). Our study determined that alteration of ubiquitin turnover in the *not4*Δ mutant correlates with modifications of the proteasome. More proteasome activity can be measured in total extracts from *not4*Δ cells than from wild-type cells, and RP-CP complexes, which are particularly resistant to salt, can be purified. At the same time, polyubiquitinated proteins accumulate. This may seem contradictory, but the same phenotype has recently been described for the *ecm29*Δ mutant (35). Furthermore, it has been reported that RP-CP complexes are stabilized when the active sites of CP are inhibited (29), and polyubiquitinated substrates obviously accumulate.

The proteasome is not a static complex. It is composed of more than 48 subunits, organized in several subcomplexes, which are in a dynamic state. The plasticity of the proteasome is mediated by numerous factors, such as proteasome subunit modifications or subunit exchange (31). Alternative RPs have been isolated, and the assembly of various RPs with CP generates a dynamic repertoire of proteasome complexes exchanging RPs (51). Proteasome subunits can be organized in complexes, distinguishable from the 26S proteasome, that contribute to other cellular functions besides proteolysis, such

as the regulation of gene transcription, DNA repair, chromatin remodeling, mRNA stability, and chaperone function (reviewed in references 9 and 31). 19S, for instance, can dissociate from 20S during proteolysis and ATP hydrolysis (4) and function alone in transcription (16). Hence, for normal function, the proteasome subcomplexes must be in a flexible equilibrium and RP-CP interactions have to be reversible.

From our results, we suggest that Not4 contributes to the dynamic assembly and flexibility of the proteasome. On one hand, in the absence of Not4, RP-CP complexes are in a "stuck" configuration. On the other hand, RP species, which are not present in these complexes, are not stable. Hence, they are probably less able to contribute to CP-independent functions, such as transcription regulation. This is very consistent with previous studies which showed that in the absence of Not4, or its RING domain, RP recruitment to the *PMA1* gene was reduced (32).

We analyzed which part of Not4 is responsible for proteasome integrity (summarized in Table S1 in the supplemental material). Three domains in the Not4 structure have been described (7), but function has been established only for the RING domain (1, 43). We determined that the RING domain, while essential for appropriate ubiquitin homeostasis, proteasome assembly, and growth, is dispensable for Not4's interaction with the Ccr4-Not complex. In contrast, the C terminus of Not4, particularly amino acids 430 to 480, is important for the interaction of Not4 with other Ccr4-Not complex subunits, for ubiquitin homeostasis, for proteasome assembly, and for growth. Interestingly, we can distinguish a difference between the RING domain and Not4's E3 ligase activity. The I64A mutation, which disrupts Not4's interaction with its partner E2 enzymes, does not affect cell growth, proteasome assembly, or Ecm29 levels, but it is sensitive to CHX, results in a synthetic phenotype in the *ubp6*Δ or *doa4*Δ mutant, and allows polyubiquitinated proteins to accumulate. Hence, Not4 probably contributes to ubiquitin homeostasis and proteasome integrity in more than one way, involving its E3 ligase activity, its RING domain, and the interaction of Not4 with the Ccr4-Not complex. Consistently, deletion of other subunits of the Ccr4-Not complex also affects proteasome function and integrity (see Fig. S6 in the supplemental material).

Not4 is important for Ecm29 chaperone activity. The relevant question is obviously how Not4 may contribute to the functional assembly of the proteasome. We show that purified Not4 can associate with subcomplexes of the proteasome present in holoenzyme but not RP preparations. The nature of these subcomplexes is at present unclear, but they probably lack CP α subunits, and we imagine that they include at least Rpn8 and Rpn11. Indeed, our results show that (i) Rpn8, but not α subunits from purified holoenzyme, associates with Not4, (ii) Not4 associates with Rpn11 complexes, and (iii) Not4 copurifies with holoenzyme purified via Rpn11. The fact that Not4 cannot bind *in vitro* to RP or copurify with RP suggests that the proteasome intermediates with which Not4 can associate are unstable in the higher salt conditions used for RP purification. Our suggestion that proteasome intermediates may be binding partners for Not4 is consistent with the results of previous studies that have demonstrated the existence of numerous proteasome intermediates complexed with chaperones (12, 45, 48).

Interactions between Not4 and proteasome intermediates might contribute to dynamic alterations of proteasome composition or assembly. This is compatible with our observation of a pool of "stuck" RP-CP in the *not4*Δ mutant and unstable RP species not associated with CP. Since proteasome chaperones are important for the functional assembly of proteasome complexes, we considered that they could be involved in the mutant phenotype observed in the *not4*Δ mutant. We focused our attention first on Ecm29, because Ecm29 is a protein described to interact with both RP and CP and to contribute to proteasome stability (34). Moreover, we identified Ecm29 copurifying with Ccr4-Not complexes and could confirm its interaction with Not4. In wild-type cells, Ecm29 copurifies with RP species (RP_h and RP_c), and some, but not all, proteasome subunits copurify with Ecm29. Since Ecm29 is important for the stability of the integral enzyme (29), these findings suggest that Ecm29 is a proteasome chaperone rather than a substoichiometric subunit of the intact proteasome. In the *not4*Δ mutant, no Ecm29 copurifies with an active RP-CP complex in high salt, probably because of the absence of free RP species. Furthermore, fewer proteasome subunits copurify with Ecm29 in the *not4*Δ mutant than in the wild type. In particular, some subunits, such as Rpt1, were undetectable, and others, such as α subunits, were present in reduced amounts. Copurification of lid subunits with Ecm29 appeared to be Not4 independent, in contrast to that of the base and CP, suggesting that Not4 may contribute to Ecm29's interaction with proteasome at steps subsequent to lid assembly.

In the absence of Not4, many proteins other than proteasome subunits or previously identified partners copurify with Ecm29, and a major pool of Ecm29 seems to be unstable. Whether these new proteins reflect unspecific contaminants due to reduced specific interactions of Ecm29 or not is unclear. In any event, reduced Ecm29 levels in the *not4*Δ mutant could be a consequence of its improper interaction with its appropriate partner proteins. One can imagine that free Ecm29 or inappropriate Ecm29 complexes are targeted for degradation. In this context, lid species are unstable in the *not4*Δ mutant, but they are stabilized by the Ecm29 deletion. Ecm29 might contribute to lid degradation in the *not4*Δ mutant if, for instance, Ecm29 can associate with lid (supported by our results) but then fails to promote a correct association of lid with additional proteasome subunits (also supported by our results). In the *ecm29*Δ mutant, alternate routes of lid assembly into proteasome may occur, avoiding lid degradation. Indeed, it has been suggested that there is not a unique route for proteasome assembly (12, 45, 46, 48) and that Ecm29 is not an essential protein. These alternate routes nevertheless are probably responsible for the proteasome assemblies that are "stuck," since salt-resistant RP-CP complexes are observed in both the *not4*Δ mutant and the *ecm29*Δ mutant.

At present, it seems likely that the role of Not4 in the UPS extends beyond Ecm29. Besides Ecm29 and proteasome subunits, several other proteins linked to the proteasome were copurified with the Ccr4-Not complex. Both of these are components of fatty acid synthase (Fas1 and -2) (37), Ubp6 (34), and the RP chaperone Hsm3 (36). These proteins, as well as another RP chaperone, Nas6, were also identified in proteasomes purified from the wild type but not from the *not4*Δ mutant. Whether the functions of these proteins are connected to Ecm29 or whether they may

independently play a role in proteasome assembly, which is also linked to the Ccr4-Not complex, remains to be clarified. In addition, Blm10, a CP activator, was found to copurify with the Ccr4-Not complex and with Ecm29 from the wild type but not from the *not4*Δ mutant. It may play an as-yet-unsuspected role in Ecm29's interaction with the proteasome. Obviously, all of these questions are exciting new issues that will have to be addressed in the future to clarify this new role of Not4 and the Ccr4-Not complex in proteasome assembly.

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