We report the functional characterization of RPN6, an essential gene from Saccharomyces cerevisiae encoding the proteasomal subunit Rpn6p. For this purpose, conditional mutants that are able to grow on galactose but not on glucose were obtained. When these mutants are shifted to glucose, Rpn6p depletion induces several specific phenotypes. First, multubiquitinated proteins accumulate, indicating a defect in proteasome-mediated protein degradation. Second, mutant yeasts are arrested as large budded cells with a single nucleus and a 2C DNA content; in addition, the spindle pole body is duplicated, indicating a general cell cycle defect related to the turnover of G2-cyclins after DNA synthesis. Clb2p and Pds1p, but not Sic1p, accumulate in the arrested cells. Depletion of Rpn6p affects both the structure and the peptidase activity of proteasomes in the cell. These results implicate Rpn6p function in the specific recognition of a subset of substrates and point to a role in maintaining the correct quaternary structure of the 26S proteasome.

The 26S proteasome is responsible for the ATP-dependent degradation of short-lived regulatory proteins involved in various biological processes such as cell cycle and division, DNA repair, signal transduction, apoptosis, morphogenesis of neuronal networks, metabolic regulation, and antigen presentation (1, 2). Prior to degradation, most substrates of the proteasome are covalently conjugated to ubiquitin (Ub).1 Ubiquitylation requires three enzymatic activities, the first of which activates ubiquitin, which is then transferred to one of the ubiquitin-conjugating enzymes. A third activity conveyed by ubiquitin-protein isopeptide ligase is required for substrate specificity. The multubiquitinated proteins are subsequently recognized and degraded by the 26S proteasome (1, 3, 4).

The 26S proteasome is a 2.4-MDa protein complex composed of two multimeric subcomplexes, the central 20S core particle (CP) and the 19S regulatory particle (RP), also known as PA700. The 670-kDa CP is a cylindrical stack of four heptameric rings whose proteolytically active sites are sequestered within an internal chamber (5, 6). The CP is responsible for polyUb recognition (7, 8), substrate unfolding, and substrate translocation into the CP (9, 10), where it is degraded. At least 17 different subunits have been identified in the CP of yeast proteasomes (11), which is very similar to that of mammals. In vitro the CP can be dissociated in two subcomplexes, the lid, containing eight regulatory subunits, Rpn3p, Rpn5–9, and Rpn11 and 12, and the base, which links the CP to the RP and contains six homologous ATPases, Rpt1–6p, as well as Rpn1p and Rpn2p. Recently, additional proteins have been described as components of affinity-purified proteasomes (12).

The proteasome lid subunits exhibit marked similarities to the COP9-signalosome complex (CSN) (13), a key regulator that has recently been demonstrated to be involved in numerous signaling pathways (reviewed in Ref. 14). The CSN has been found in plants, mammals, Drosophila, and Saccharomyces pombe, but it is absent from S. cerevisiae. The eight subunits of the CSN are each paralogous to one of the eight subunits that form the lid of the 26S proteasome. The CSN and the proteasome are evolutionary conserved particles, but they have unique structures (15). Nevertheless, they might share a common function, because the CSN has been reported recently (16–18) to be involved in protein degradation. Possible cooperation between both complexes in plants has also been reported (19). In addition, a new protein complex related to the RP, also containing Rpn6p, has been found in Arabidopsis seedlings and cauliflower florets (20).

Despite recent advances in the knowledge of the structure and function of the proteasome, the enzymatic activities and specific functions played by most regulatory subunits remain unknown. With the exception of Rpn10p and Rpn11p, all the regulatory subunits are essential. Rpn10p binds polyUb chain both in its free form and when incorporated into proteasomes (21, 22). Thus, Rpn10p presumably functions as a proteasomal polyUb protein receptor. However, other major polyUb receptors must exist in the proteasome, because in Δrpn10 strains only short-lived proteins, degraded by the Ub-fusion degradation pathway, are known to be stabilized (21). Rpn10p is also important for correct association of lid and base (13). The base is sufficient to activate CP proteolysis of certain non-Ub proteins, governing their entry into the CP (11). It is responsible for the unfolding function of the RP (9, 10) and functions as a gating device for the CP channel; this last role played primarily by Rpt2p (23, 24). Recently, a new mediator of ubiquitin chain recognition has been described, Rpt5p, one of the ATPase subunits from the base (8). Another subunit of the base, Rpn1p, acts as a receptor for Rad23p (22), a protein that might deliver proteins to the proteasome for degradation (25). Less is known about the role played by lid subunits during protein degradation. However, the lid is required for the degradation of polyUb-
Table I

| Strain | Genotype | Source |
|--------|----------|--------|
| CEN.PK2 | MATα/a, ura3-52/ura3-52 trp1-889/trp1-889 leu2-3,112/leu2-3,112 his3-Δ1/his3-Δ1 MAL2-8/SUC2 | K.D. Entian, Frankfurt |
| FY1679 | MATα/a, ura3-52/ura3-52 trp1Δ63+/leu2Δ1/+ his3Δ200/+ | Ref. 70 |
| FS6a | MATα, ura3-52/trp1Δ63 his3Δ200 | This study |
| FS64 | MATα, ura3-52/trp1Δ63 his3Δ200 | This study |
| FS6D6 | α/a, ura3-52/ura3-52 trp1Δ63+/leu2Δ1/+ his3Δ200/+ RPN6::kanMX4 | This study |
| FS6D0 | α/a, ura3-52/ura3-52 trp1Δ63+/leu2Δ1/+ his3Δ200/+ RPN6::kanMX6 RPN6::kanMX4 | This study |
| FS6D1 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-G6) | This study |
| FS6D2 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-G6) | This study |
| FS6D3 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-G6) | This study |
| FS6D4 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-G6) (pFC7-66) | This study |
| FS6D11 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-G6) (pFC7-P089) | This study |
| FS6D31 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-G6) (pFC6-689) | This study |
| FS6D20 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC9-G6) | This study |
| FS6D21 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC9-G6) | This study |
| FS6D22 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC9-G6) | This study |
| FS6a2 | MATα, ura3-52/trp1Δ63 his3Δ200 PDS1/1-PDS1/1-HA/URA3 | This study |
| FS6D23 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC9-G6) | This study |
| FS6a3 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC9-G6) | This study |
| FS6D22 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC9-G6) | This study |
| FS6D11 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC9-G6) (pFC6-689) | This study |
| FS6D41 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-GHSis6) | This study |
| FS6D4 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-GHSis6) | This study |
| FS6D24 | MATα, ura3-52/trp1Δ63 RPN6::kanMX4 (pFC8-GHSis6) | This study |

Plasmids

| Plasmid | Characteristics |
|---------|----------------|
| pFC6-66 | ARS-CEN, URA3, RPN6 (pRS416) |
| pFC7-66 | ARS-CEN, HIS3, RPN6 (pFL37) |
| pFC8-G6 | ARS-CEN, URA3, GAL1/RPN6 (pFL38) |
| pFC8-GHSis6 | ARS-CEN, URA3, GAL1/HIS3, RPN6 (pFL38) |
| pFC9-G6 | ARS-CEN, TRP1, GAL1/RPN6 (pFL39) |
| pFC6-659 | ARS-CEN, LEU2, RPN6/S9 (pFL36) |
| pFC7-P089 | ARS-CEN, HIS3, RPN6::S9 (pFL37) |
| pFS-MIC1(HA) | ARS-CEN, LEU2, MBS5/SIC1(HA) (pUG35) |
| pOC52 | ARS-CEN, URA3, PDS1/1-HA |
| pCLB2/HA3 | KANMX CLB3/3-HA |
| YCp-SIC1 | Pr-GAL1/SIC1(HA), URA3 |

* The strains are transformed with plasmids shown in parentheses.

** Plasmid derivatives are shown in parentheses.

Experimental Procedures

**Strains, Media, and Genetic Methods—** *Saccharomyces* strains used in this work are listed in Table I. Cells were grown in synthetic dextrose minimal (SD), synthetic complete (SC), or rich medium with either glucose (YPD) or galactose (YPGal) as a carbon source (37). Hydroxyurea was added to a final concentration of 10 mg/ml and incubated for 4 h. Transformation of *S. cerevisiae* was carried out as described elsewhere (38). For selection of *GAL1* resistance, cells were first grown for 2 h at 30 °C in liquid YPD and then spread on YPD plates containing 200 μg/ml of G418. Plasmids with URA3 as the selection marker were cured by plating the cells onto SC plates containing 5-fluoroorotic acid (37). *Escherichia coli* DH5α was used for cloning steps and was grown in LB medium. Standard DNA cloning and manipulation were performed according to well established techniques (39). PCR amplifications were made using the high fidelity Pfu (Stratagene) or Pwo (Roche Diagnostics) DNA polymerases.

**Gene Replacement—** Disruption of RPN6 was performed using the short flanking homology method (40). Vector pUC6 (41) was used as a source of the geneticin resistance gene. Hemagglutinin (HA) tagging of *PDS1* (strains FS6a2 and FSD622) and *CLB2* (strains FS6a3 and FSD63) was done by transforming either FS6a (for FSD62) and FSD620 (for FSD622) with plasmid pOC52 linearized with SacI and ClaI or FS6a (for FS6a3) and FSD621 (for FSD623) with pCLB2/HA3 linearized with BglII. Strains used for proteasome purification were obtained from FS6a and FSD641 by replacing the PRE1 locus with a protein A-tagged PRE1 (12), thus yielding the strains FS6a4 and FSD642, respectively.

**Plasmid Construction—** Relevant features of the plasmids used in this work are shown in Table I. These were constructed as follows. For pRC-66 and pFC7-66, the 2.1-kbp fragment containing the Rpn6p encoding region plus 450 and 330 nucleotides from the 5' and 3' ends, respectively, was cloned into plasmid pRS416 (42), yielding plasmid...
pRC66. The same fragment was cloned into pFL37 (43), thus yielding pFC7-66. For pFC8-G6 and pFC9-G6, for conditional expression of Rpn6p, the DNA encoding fragment was fused to the GAL1 promoter in centromeric plasmids pFL38 and pFL39 (44). From plasmid pRC66 a 1.7-kbp fragment was digested and cloned into pFLUraGp0 (45). The obtained plasmid was termed pFC8-G6. The GAL1-RPN6 fragment was also cloned in pFL39, yielding plasmid pFC9-G6. For pFC8-GHi6, a PCR-amplified fragment of RPN6 was cloned in plasmid pSETC (D- vitrogen), which places in the N terminus the hexahistidine and T7 tags, yielding pSET6. The tagged RPN6 thus generated was subcloned into pFLUraGp0 (45) to obtain pFC8-GHi6, which harbors His6–RPN6 under the control of the GAL1 promoter. For pC68-659 and pFC7-P0S9, a 1.3-kbp PCR fragment containing the S9 coding region amplified from a HeLa cell cDNA library was cloned in plasmid pBP0S9 (46), yielding plasmid pBP0S9. The 3-kbp fragment containing the S9 open reading frame flanked by the 5′ and 3′ untranslated regions of RPP0 was cloned into pFL37 (43), thus yielding pFC7-P0S9. S9 open reading frame was also placed under the control of the RPN6 promoter by overlapping PCR fragments and cloned in pFL36 plasmid (44), yielding pC68–659. The p6S-RPN6(HA) construct was obtained by cloning SIC1/HA from YCp-SIC1 together with the MET25 promoter, obtained from pUG35 (kindly provided by Dr. Hegemann, Heinrich-Heine-Universität, Düsseldorf, Germany) in plasmid pFL46S (44).

**Flow Cytometry Analysis**—DNA content was quantified with a fluorescence-activated cell sorter (FACScalibur; BD Biosciences). Samples containing 10,000 cells were fixed in 70% ethanol, resuspended in 0.5 mM sodium citrate supplemented with 0.2 mM/ml RNase, incubated for 2 h at 37 °C, and treated with 5 mg/ml pepsin before staining with propidium iodide and sonication.

**Immunological Methods**—Standard procedures were used for immunoblotting. Tubulin was detected by using rat monoclonal antibody YOL1/34 (Harlan Sera-Lab), polyUb-conjugated proteins were detected with a commercially available rabbit polyclonal antibody (Affiniti), T7-His6-tagged Rpn6p was detected with a commercially available monoclonal antibody against the T7 tag (Novagen), and HA-tagged proteins were detected using the monoclonal antibody 12CA5. As a loading control in SDS-PAGE, antibodies recognizing the ribosomal protein P0 were used (45).

Indirect immunofluorescence and 4′,6-diamindine-2′-phenylindole (DAPI) staining were performed as described elsewhere (47). Rhodamine-conjugated mouse anti-rat IgG (Biomedia Corp.) was used as a second antibody for tubulin detection.

For protein extraction cells were resuspended in Buffer 1 (100 mM Tris-HCl, pH 7.4, 20 mM KCl, 12.5 mM MgCl₂, 5 mM 2-mercaptoethanol) plus protease inhibitors. Glass beads were added, and cells were broken with a FastPrep FP120 (Bio 101 Inc.). A total extract of 50 to 100 μg was routinely resolved by SDS-PAGE and electroblotted onto polyvinylidene fluoride filters (Immobilon-P; Millipore).

**Scanning Electron Microscopy**—As a part of the yeast genome sequencing project our group described an open reading frame named YDL097c (D2381) (33), which was later identified as a component of the proteasome RP named Rpn6p (11). To further characterize this proteasome subunit, a cassette was constructed containing the kan gene flanked by 40 nucleotides from the 5′ and 3′ ends of RPN6, respectively, to direct the disruption of the gene in diploid strains FY1679 and CEN.PK2. After transformation, geneticin-resistant colonies were selected, and the correct integration of the cassette into the genome was confirmed by PCR (data not shown). Transformants from both genetic backgrounds were chosen for sporulation and tetrad analysis. In all tetrads dissected, the four spores segregated in a 2:2 ratio of viable versus non-viable spores (Fig. 1A).

**RESULTS**

RPN6 Is an Essential Gene Necessary for Protein Degradation—As a part of the yeast genome sequencing project our group described an open reading frame named YDL097c (D2381) (33), which was later identified as a component of the proteasome RP named Rpn6p (11). To further characterize this proteasome subunit, a cassette was constructed containing the kan gene flanked by 40 nucleotides from the 5′ and 3′ ends of RPN6, respectively, to direct the disruption of the gene in diploid strains FY1679 and CEN.PK2. After transformation, geneticin-resistant colonies were selected, and the correct integration of the cassette into the genome was confirmed by PCR (data not shown). Transformants from both genetic backgrounds were chosen for sporulation and tetrad analysis. In all tetrads dissected, the four spores segregated in a 2:2 ratio of viable versus non-viable spores (Fig. 1A). As expected, all the viable spores were sensitive to geneticin, indicating that they did not carry the disruption marker. This result indicates that
Rpn6p function is essential for either vegetative growth or spore germination.

Heterozygous RPN6-disrupted diploids (FS6D6) were transformed with the centromeric plasmid pPC8-G6, which directs the expression of RPN6 under the control of the GAL1 promoter, and allowed to sporulate and then tetrads were dissected. As expected from the previous results, only two colonies per tetrad grew on glucose whereas all four spores formed colonies on galactose plates (data not shown). Geneticin-resistant haploid cells rpn6-Δ1 from strain FS6D6 stopped cell division after prolonged incubation in the restrictive glucose-containing medium (Fig. 1B), allowing further study of the function of Rpn6p. Initially, the depletion of Rpn6p on glucose in these mutants was studied. A His₆-tagged version of Rpn6p was cloned in the same centromeric plasmid under the control of the GAL1 promoter as explained above, and the same procedure was followed to obtain strain FSD641, which conditionally expresses His₆-Rpn6p on galactose. The His₆-tagged Rpn6p fully complemented the absence of the endogenous Rpn6p on galactose, as no differences in the growth rate were observed (Fig. 1B). After transferring the cells to restrictive conditions, growth was monitored, and cell samples were collected and analyzed by immunoblotting. As shown in Fig. 1C, His₆-Rpn6p progressively disappears once the cells are transferred to glucose, being undetectable after 9 to 12 h. In parallel, polyUb proteins began to accumulate after 6 h under these restrictive conditions, reaching the maximal amount after 9 to 12 h (Fig. 1D).

Human Proteasomal Protein S9 Partially Rescues the Effect of Rpn6p Depletion—Rpn6p was first suggested to be a component of the yeast proteasome based on a 42% identity to human proteasome subunit S9 (34). To determine whether this sequence similarity extends to its function, the ability of the human S9-encoding cDNA to complement the rpn6-Δ1 mutation in S. cerevisiae was tested. Centromeric plasmids pFC8–6S9 and pFC7–P0S9, expressing, respectively, S9 under the control of either the RPN6 or the ribosomal protein P0 promoters, were used to transform rpn6-Δ1 mutant cells, and their ability to grow on glucose was tested. Fig. 2A shows that S9 gene expressed under the control of the RPN6 promoter only partially rescues growth on glucose (FSD631). In contrast, when overexpressed under the control of a stronger promoter, such as that of the RPP0 gene (FSD611), the growth defect was suppressed almost completely. However, as shown in Fig. 2B, even when highly expressed, human S9 did not completely abolish the accumulation of polyUb proteins.

Cell Cycle-related Defects of Conditional Null Mutants rpn6-Δ1—We characterized the terminal phenotypes of diploid FSD60 and haploid FSD61 cells after shifting from galactose to glucose. The morphology of the cells was followed by Nomarski optics, scanning electron microscopy, and fluorescence microscopy. After 9 h on glucose, a homogeneous culture of large budded cells was observed (Fig. 3, A–D). Mother and daughter cells failed to separate (Fig. 3E), and they contained only one nucleus, which was located close to or in the bud neck in most cases, as shown by DAPI staining (Fig. 3F). Moreover, tubulin staining revealed the duplication of the spindle pole body, with the presence of a short intranuclear mitotic spindle in the arrested cells (Fig. 3G). To further analyze whether DNA duplication proceeded normally in rpn6-Δ1 cells, the DNA content of mutant arrested cells was measured. Fig. 4, A and B shows the cytometry profiles of asynchronous wild type and rpn6-Δ1 mutant cultures growing on glucose. On galactose the two cultures showed a similar profile at all time points, with 60% of the cells having a 2C content when growing in mid-log phase. A 2C DNA content was observed in 80% of the mutant cells after 6 h on glucose and in almost 100% after 9 h. However, treatment with hydroxyurea revealed that only 30% of the cells had actually completed DNA replication after 6 h in the restrictive condition, whereas all of them were arrested after 9 h (Fig. 4C).

These results demonstrated clearly that cells lacking Rpn6p were arrested in the cell cycle after DNA synthesis, in G₂/M. As progression of the cell cycle from metaphase is directly related to the inhibition of the Cdc28 kinase activity (48), a preliminary study of the variations in the level of this activity was approached. For this purpose, whole cell extracts from FSD60 mutant strain maintained on glucose were assayed to measure their histone H1 phosphorylating activity. As shown in Fig. 4D, this activity progressively increased, reaching a maximum after 9 to 12 h.

The results aforementioned suggested that Rpn6p is essential for progression from S/M to G₁. This transition is driven by the anaphase-promoting complex/cyclosome (APC/C), which is switched on at the metaphase/anaphase boundary to trigger the ubiquitination and proteolysis of sister chromosome cohesion factors and mitotic cyclins (49). The stability of two critical APC targets, Cbl2p and Pds1p, was therefore examined. Wild type and rpn6-Δ1 conditional strains, both tagged with a triple HA epitope at their CLB2 or PDS1 loci, were shifted from galactose to glucose and harvested at different time points. The abundance of Cbl2p or Pds1p was estimated by immunoblotting against HA. As shown in Fig. 5A, Cbl2p was almost undetectable in the wild type cells after 9 to 12 h under restrictive conditions, whereas there was a striking accumulation of this cyclin in the mutant cells, reflecting a significant stabilization. As shown in Fig. 5B, Pds1(HA)₃ levels similarly increased in the mutant cells under the same conditions. Accumulation of Pds1p, however, was not as obvious as that of Cbl2p. To further confirm this observation, PDS1 was deleted in an rpn6-Δ1 conditional strain. Because it is well established that Pds1p is not essential for growth at 25 °C (50), a pds1-Δ1 rpn6-Δ1 conditional strain behaved as wild type under permissive condi-
This result clearly confirmed that the metaphase arrest phenotype in the rpn6Δ1 mutant cells is because of its inability to degrade Pds1p.

The homogeneous phenotype described in the experiments shown above implicated Rpn6p in the degradation of two substrates ubiquitinated by APC/C. To study the role of Rpn6p in the degradation of substrates ubiquitinated by a different ubiquitin ligase, variations in the level of Sic1p were measured. Degradation of Sic1p, an inhibitor of Cdc28p kinase activity, is necessary for cells to enter S phase (48). Ubiquitination ligase, variations in the level of Sic1p were measured. Degradation of Sic1p, an inhibitor of Cdc28p kinase activity, is necessary for cells to enter S phase (48). Ubiquitination of Sic1p by the ubiquitin-protein isopeptide ligase SCP3-1 (Skp-cullin-F-box protein) during S/M triggers its degradation by the 26 S proteasome (31, 52). To determine whether Sic1p proteolysis depends on the presence of Rpn6p in the proteasome, we examined the stability of Sic1p in rpn6Δ1 cells. Wild type and rpn6Δ1 mutant cells were transformed with a plasmid carrying an HA-tagged copy of SIC1 under the control of the MET25 promoter, yielding strains FS6d1 and FS632, respectively. These strains were grown under conditions allowing the expression of HA-tagged Sic1p and harvested for further analysis (Fig. 5C). Immuno blot analysis using an anti-HA-specific antibody revealed that Sic1p levels were comparable in wild type and mutant cells, indicating that Sic1p turnover is not compromised in rpn6Δ1 cells and suggesting that Rpn6p is not involved in G1 degradation.

Structure and Peptidase Activity of Rpn6p-depleted Proteasomes—Rpn6p harbors a structural motif, the PCI domain (53), also present in other five proteasome subunits and in components of distinct multiprotein complexes such as COP9 and eIF3 (13). It has been suggested that the PCI domain could serve as a structural scaffold helping the subunits to assemble in these multimeric complexes (54, 55). If this was the case, rpn6Δ1 mutants should be unable to properly assemble 26 S proteasome particles. To study the assembly of the 26 S proteasomes, whole cell lysates from wild type and mutant strains, expressing the untagged proteins was used as a negative control.

Fig. 3. Cytological analysis of rpn6Δ1 cells under restrictive conditions. A–D: microscopic analysis with Nomarski optics of cells maintained for 9 h in glucose. A, FS6d1 (rpn6Δ1) haploid; B, FS6a (wild type RPN6 haploid); C, FS6d0 (rpn6Δ1 diploid); D, FY1679 (wild type RPN6 diploid). E, scanning electron micrographs (SEM) of FS6d0 cells after 12 h under restrictive conditions. F and G, FS6d0 cells were stained with DAPI (F) and with anti-tubulin antibodies using rhodamine-conjugated secondary antibodies (IF) (G). The scale bar in all panels is 2 μm.

Fig. 4. rpn6Δ1 mutants arrest in G1/M and accumulate Cdc28 kinase activity. Wild type FS6a (A) or mutant rpn6Δ1 FS6d1 (B) strains were grown on galactose to mid-exponential phase, and samples were withdrawn at the indicated times after being transferred to glucose, fixed and stained with propidium iodide, and analyzed by flow cytometry. C, duplicated samples from FS6d1 (B) were incubated for 4 h in the presence of hydroxyurea prior to fixation and propidium iodide staining. D, histone H1 kinase activity was assayed in vitro from 60 µg of total protein extracts obtained from strain FS6d1 at the indicated times after transfer to glucose.

Fig. 5. rpn6Δ1 cells are defective in APC-dependent degradation. Wild type (RPN6) and rpn6Δ1 mutant cells, expressing either HA-tagged Clb2p (FS6a3 and FS6d23), Pds1p (FS6a2 and FS6d22), or Sic1p (FS6d1 and FS6d32), were grown on galactose and then transferred to glucose medium. Cells growing on galactose (GAL), or after transferring to glucose (at the indicated times), were recovered, and 50 µg of whole lysates were analyzed by immunoblot with an anti-HA antibody. Ribosomal protein P0, immunodetected with a polyclonal antibody, was used as a loading control. Extract from a wild type strain expressing the untagged proteins was used as a negative control.
density gradient centrifugation. 50/H9262

As shown in Fig. 6 against Rpn12p and Rpt6p, which are, respectively, located in the gradient, the fractions were subjected to immunoblot analysis to test their ability to hydrolyze Suc-LLVY-AMC. Equal amounts of proteasomes were assayed for their hydrolyzing activity against the fluorogenic substrate Suc-LLVY-AMC. Equal amounts of proteasomes were incubated in the presence (+ATP) or absence (−ATP) of ATP to dissociate CP and RP. B, peptide activity of wild type and mutant CP before (−SDS) and after (+SDS) the activation of the basal activity with SDS. The CPs from wild type and rpn6Δ1 mutant proteasomes were released upon incubation of the proteasome samples in Buffer A without ATP and 500 mM NaCl for 0 min at 30 °C.

Regarding the defect in peptidase activity, in vitro activity assays were performed to test the ability of mutant proteasomes to hydrolyze the fluorogenic peptide Suc-LLVY-AMC. Confirming the results described above, the in vitro peptidase activity of proteasomes lacking Rpn6p was drastically reduced compared with wild type (Fig. 7A). As it was one possibility that this lower peptidase activity was because of a lesser amount of proteasomes produced when Rpn6p is depleted, the purified proteasomes were pre-incubated in the absence of ATP to release their CPs. As shown in Fig. 7B, under this condition the basal peptidase activity of wild type and mutant proteasomes was similar, thus indicating that the absence of Rpn6p does not affect the CP. Furthermore, under dissociation conditions, together with a mild SDS treatment, which also stimulates the peptidase activity of the yeast CP (56), there is a 50-fold stimulation of the mutant CP activity (Fig. 7B). This stimulation, exceeding that of wild type CP itself, confirmed that the presence of incomplete RPs in rpn6Δ1 strain has an inhibitory effect on the peptidase activity, highlighting the Rpn6p relevance to maintain both the proper assembly of 26 S proteasomes and their full peptidase activity.

Composition of Proteasomes from rpn6Δ1 Strain—The results shown above indicated that Rpn6p depletion was responsible for proteasome disassembly in the mutated strain, suggesting also that proteasomes assembled in the absence of Rpn6p might lack other subunits. To study the composition of Rpn6p-depleted proteasomes, the holoenzymes were purified from a wild type and an rpn6Δ1 strain, both expressing the CP subunit Pre1p fused to the TeV-ProA tag. As the main differences were expected in the composition of RP, these complexes were also purified separately to avoid CP subunits. Holoenzymes and RP purified from the mutant strain grown under restrictive conditions were subjected to SDS-PAGE analysis. As there are not commercially available antibodies to detect the distinct RPs, comparison of subunit composition was based on the published pattern of migration of CP and RP subunits in an SDS-PAGE gel (11, 12). Analysis of the subunits present in wild type and mutant strains revealed the lack of most RP

![Image](https://example.com/image.png)
requires p100 ubiquitination and mediates its processing, of them components of the proteasomal base. In Rpn6p-depleted proteasomes, together with the ATPases, all subunits (Fig. 8) except for Rpn1p and Rpn2p, clearly present in Rpn6p-depleted proteasomes, together with the ATPases, all of them components of the proteasomal base.

**DISCUSSION**

In this report an analysis of the function of Rpn6p, a proteasome RP subunit (11), is presented. **RPN6** gene disruption in two different genetic backgrounds demonstrated that this protein is essential for vegetative growth, in agreement with results reported previously (35). As expected for a proteasome subunit, ubiquitin-dependent degradation is impaired in a conditional *rpn6-Δ1* mutant, which accumulates mult ubiquitinated proteins under restrictive conditions.

Proteasome subunits are well conserved in all eukaryotic organisms, and the overall composition of the holocomplex is nearly the same from yeast to human (57). However, when comparing individual human and yeast proteins, differences among them can be observed. Although the identity among the homologous ATPases is about 70%, that of most of the non-ATPase components is decreased to about 40% (11). Rpn6p shows a 42% identity to human S9 subunit. Complementation of the *rpn6-Δ1* mutation with the homologous human S9 cDNA was only possible when overexpressed under the control of the strong ribosomal protein P0 promoter but not under the endogenous **RPN6** promoter. Upon overexpression of S9, the growth inhibition because of Rpn6p absence was relieved completely. However, some mult ubiquitinated proteins remained accumulated. A possible explanation is that, despite the conservation of the sequence of this subunit, human S9 is not able to recognize certain yeast substrates. In fact, whereas hS13 can complement Rpn11p absence (27), hS14 does not complement the Rpn12p absence (28), and hS7 only does so when overexpressed in an Rpt1p mutant strain (58), even though they have a 75% identity. This led us to hypothesize that the function of the RP subunits is species-specific, reflecting a specific recognition of certain substrates restricted to each organism. Supporting this idea, it has been described recently (59) that S9 interacts specifically with NF-kB2 precursor protein p100. This interaction requires p100 ubiquitination and mediates its processing, which is required for generating the p52 NF-kB subunit.

To further analyze the function of Rpn6p, galactose-dependent conditional null *rpn6-Δ1* mutants were constructed. After prolonged incubation of the conditional strain under restrictive conditions (9–12 h on glucose), when no Rpn6p is present, *rpn6-Δ1* cells are blocked in metaphase. They arrest with a uniform terminal phenotype, large budded cells with a 2C DNA content and an undivided nucleus located near the neck. In addition, tubulin staining reveals that the arrested cells have duplicated the spindle pole body but were arrested with a short intranuclear spindle, which correlates with the absence of chromosome segregation. This metaphase block was confirmed by the inability of *rpn6-Δ1* cells to degrade two APC/C substrates, Clb2p and Pds1p, as a consequence of a failure in the APC/C-dependent degradation by the 26 S proteasome. Pds1p (securin) is a key regulatory protein responsible not only for the onset of anaphase but also implicated in the exit from mitosis (60, 61). Pds1p is bound to Esp1 (separase), and this association helps to correctly locate Esp1 in nucleus and spindle (62). At the onset of anaphase, the Ub ligase APC/C targets Pds1p for 26 S-dependent degradation, and separase is thus released, resulting in the elimination of cohesion among sister chromatids and in spindle movement (60). It is known that Δpds1 mutant cells are viable (50). Moreover, they do not liberate chromatid sisters prematurely, and separase still localizes properly and cleaves cohesin in a regulated fashion (63) suggesting that Pds1p degradation is not the sole requirement for progression through mitosis, and an alternate mechanism to regulate Esp1 activity should exist. Indeed, *rpn6-Δ1* mutants accumulate polyUb proteins, indicating a failure to degrade other substrate proteins and probably among them those accounting for the mechanism that cooperatively with Pds1p regulate faithful chromosome segregation during cell division.

Interestingly, *rpn6-Δ1* mutants retain the ability to degrade other substrates, such as Sic1p, which means that Rpn6p-depleted proteasomes maintain partial activity. This feature is not unique for *rpn6-Δ1* mutants but has been also described for other proteasome subunits. Among the RP proteins, it has been shown that depletion of ATPases Rpt1p and Rpt6p (58), Rpt4p (64) and non-ATPases Rpn3p (26), Rpn9p (65), Rpn11p (27), and Rpn12p (28) produce similar phenotypes. However, even among the proteasome mutants that are arrested in G2/M, differences in the above-mentioned phenotypes are observed. Available data in the literature report that although *rpn1*/*rp6*, *rpn3*, and *rpn9* mutants duplicate the spindle pole body and have a short intranuclear spindle (26, 58, 65), *rpt4* mutants fail to duplicate it (64). With respect to Cdc28/Cln2 kinase activity, it is absent in *rpn12* mutants (28) but present in *rpt1*, *rpt6*, and *rpn3* (26, 58). The phenotype displayed by *rpn6-Δ1* mutants appears to match more closely that of the ATPases *rpt1* and *rpt6* and the non-ATPases *rpn3* and *rpn9*. This suggests that among proteasome regulatory subunits a specialization in substrate recognition exists that might represent an additional mechanism of the spatial- and temporal-regulated degradation performed by the ubiquitin-proteasome pathway. A physical association among these subunits might explain the parallels in their substrate recognition properties.

Proteasomes assembled in the *rpn6-Δ1* strain are clearly different in size and subunit composition from wild type 26 S proteasomes. Moreover, most of the lid RP subunits, if not completely absent, are not incorporated in mutant proteasomes as they are in the wild type. We may then conclude that Rpn6p is important to maintain the proteasomal lid bound to the base *in vivo*. A similar role has been described for Rpn10p, as in the absence of this protein purified proteasomes do not contain the lid subunits (13). However, contrary to *rpn6-Δ1* mutants,

![Fig. 8. Subunit composition of Rpn6p-depleted proteasomes.](image-url)
Assembly and Activity of Rpn6p-depleted Proteasomes

$\Delta$rp10 cells are viable. As most lid subunits are essential, the composition of purified proteasomes from $\Delta$rp10 cells cannot reflect the situation in vivo, indicating that, in the absence of Rpn10p, lid subunits can interact with the base, assembling functional proteasomes, as revealed by native gel electrophoresis (13). This is not the case for rpn6$\Delta$1 cells, as demonstrated by density gradient sedimentation and native gel electrophoresis analysis, highlighting the importance of Rpn6p for the correct assembly of the lid into the 26 S proteasome. While this manuscript was under revision, it was reported that proteasomes purified from an mprr1 strain with a frameshift in RPN11 also lack lid subunits (31). Taken together with our results, it can be expected that the deletion of additional RP genes will also render proteasomes inactive as a consequence of the improper assembly of the RP. Within this context, the presence of a PCI motif in the C terminus of six of the eight lid subunits may be significant. This PCI domain has been described as a structural scaffold that may help the assembly of subunits into multimeric complexes (54, 55). In fact, the presence of an intact PCI domain is essential for Rpn6p function as the expression of C-terminally truncated Rpn6p derivatives is not able to restore the growth of rpn6$\Delta$1 mutants under nonpermissive conditions.2

It has been reported recently (23) that enhancement of the peptidase activity of yeast CP observed upon RP binding is caused in the RP by the depletion of Rpn6p subunit also hindering its ability to properly open the CP channel.

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2 P. G. Santamaria, J. P. G. Ballesta, and Miguel Remacha, unpublished results.

The above data are limited to Rpn6p function in 26 S-dependent degradation. However, it would be interesting to find out whether Rpn6p is implicated in the novel activities described recently for the RP as transcription elongation (67) or nucleotide excision repair (68, 69) and its relevance in a distinct function in the lid context, which would account for the similarities found with the COP9-signalosome complex.

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