

## ORIGINAL PAPER

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*PWP2*, a member of the WD-repeat family of proteins, is an essential *Saccharomyces cerevisiae* gene involved in cell separation

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**Abstract** WD-repeat proteins contain four to eight copies of a conserved motif that usually ends with a tryptophan-aspartate (WD) dipeptide. The *Saccharomyces cerevisiae PWP2* gene, identified by sequencing of chromosome III, is predicted to contain eight so-called WD-repeats, flanked by nonhomologous extensions. This gene is expressed as a 3.2-kb mRNA in all cell types and encodes a protein of 104 kDa. The *PWP2* gene is essential for growth because spores carrying the *pwp2Δ1::HIS3* disruption germinate before arresting growth with one or two large buds. The growth defect of *pwp2Δ1::HIS3* cells was rescued by expression of *PWP2* or epitope-tagged *HA-PWP2* using the galactose-inducible *GAL1* promoter. In the absence of galactose, depletion of Pwp2p resulted in multibudded cells with defects in bud site selection, cytokinesis, and hydrolysis of the septal junction between mother and daughter cells. In cell fractionation studies, HA-Pwp2p was localized in the particulate component of cell lysates, from which it would be solubilized by high salt and alkaline buffer but not by nonionic detergents or urea. Indirect immunofluorescence microscopy indicated that HA-Pwp2p was clustered at multiple points in the cytoplasm. These results suggest that Pwp2p exists in a proteinaceous complex, possibly associated with the cytoskeleton, where it functions in control of cell growth and separation.

**Key words** *PWP2* · WD-repeat ·  $\beta$ -Transducin · Essential gene · Yeast

## Introduction

A conserved amino acid motif is repeated several times in members of an ancient and diverse family of proteins. First named the  $\beta$ -transducin repeat (Fong et al. 1986), it has also been designated the periodic tryptophan protein (PWP) repeat (Durino et al. 1992) the GH-WD repeat (Neer et al. 1993), and the WD-repeat (Voorn and Ploegh 1992). It is a loosely conserved sequence of approximately forty amino acids, bracketed by glycine-histidine and tryptophan-aspartate (WD) dipeptides, repeated four to eight times within each polypeptide (Voorn and Ploegh 1992). More than three dozen WD-repeat proteins are now known. Some are composed almost entirely of WD segments whereas others contain nonhomologous extensions at the N- and C-termini as well as insertions between repeats. The function of the motif is undefined, but the observation that proteins containing WD-repeats often exist in multiprotein complexes suggests they may have a general regulatory role either in facilitating macromolecular assembly or in controlling protein-protein interactions (Neer et al. 1994). The proteins are found in various cellular locations, including the plasma membrane, nucleus, cytoskeleton, and peroxisomes, and they have diverse cellular functions including cell division, signal transduction, gene transcription, RNA processing, vesicle fusion and cell-fate determination.

*PWP2*, the period tryptophan protein described here, is one of the few WD-repeat proteins with an essential role in *S. cerevisiae*. We have identified and characterized the *PWP2* gene and constructed gene disruptions to study its function. Expression of *PWP2* under control of the regulated *GAL1* promoter, was used to characterize defects in morphology resulting from depletion of the Pwp2 protein. Information about its cellular distribution was obtained by indirect immunofluorescence and subcellular fractionation.

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## Materials and methods

### Strains, media and microbiological techniques

The *S. cerevisiae* strains used in this study are described in Table 1. Yeast strains were grown on YPD rich medium (1% yeast extract, 2% peptone, 2% glucose), YPGal (1% yeast extract, 2% peptone, 2% galactose) or SD (0.67% yeast nitrogen base without amino acids, 2% glucose) (Guthrie and Fink 1991). Growth medium was supplemented with amino acids as required and solid medium contained 2% agar. Standard methods of yeast genetics, sporulation of diploids and dissection of tetrads were performed as described (Guthrie and Fink 1991). Yeast transformations were performed using the lithium acetate method (Geitz et al. 1992).

### Biochemistry and molecular biology

Standard methods of molecular biology were performed as described (Sambrook et al. 1989), except where indicated. For Southern analysis total yeast DNA prepared from saturated cultures was digested with restriction enzymes for 10–12 h and electrophoresed on 0.8% agarose gels. DNA was transferred to a Hybond membrane and hybridized with radiolabelled probes for 18–24 h at 42°C in 6 × SSC, 5 × Denhardt's solution, 0.1% SDS, and 200 mg of denatured salmon sperm DNA per ml (Sambrook et al. 1989). After hybridization, filters were washed one for 30 min in 1 × SSC, 0.1% SDS at 23°C and then twice for 30 min at 55°C. After washing, filters were subjected to autoradiography. For Northern analysis, total yeast RNA was isolated and poly(A)<sup>+</sup> mRNA, selected on Dynabeads Oligo (dT), was fractionated by electrophoresis, and transferred to filters. Random primer labeling of probes with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) was carried out using Klenow polymerase. To map the 5' end of *PWP2* mRNA, a synthetic oligonucleotide primer (5'-CGGTGAGAGTAGTTGCTTGCC-3') was employed in a primer extension reaction involving reverse transcriptase. The oligonucleotide was labeled with [ $\gamma$ -<sup>32</sup>P]ATP using polynucleotide kinase, annealed to poly(A)<sup>+</sup> RNA, and extended using reverse transcriptase; from avian myeloblastosis virus. The cDNA products were fractionated on an 8% polyacrylamide gel in the presence of 7 M urea and compared with the products of a standard

nucleotide sequencing reaction. For in vitro translation, plasmids pRS26 and pRS5 were transcribed and translated in the presence of [<sup>35</sup>S]methionine using the coupled transcription/translation system from Promega. The resulting <sup>35</sup>S-labeled polypeptides were analyzed by SDS-polyacrylamide gel (12%) electrophoresis and visualized by an autoradiography. For in vivo labeling, 10 ml of cells grown in YPGal, were harvested at an optical density (OD) at 600 nm of 1, washed with water and resuspended in an equal volume of Wickerham's minimal medium (WiMP) supplemented with appropriate amino acids and galactose. After incubation for 30 min at 30°C the cells were harvested, resuspended in 1 ml of supplemented WiMP containing 150  $\mu$ Ci Trans-[<sup>35</sup>S]-label and incubated for 15 min at 30°C. The reaction was terminated by adding trichloroacetic acid to a final concentration of 5%. For immunoprecipitation from yeast extracts (Paravicini et al. 1992) the Pwp2 protein, tagged at the N-terminus with an epitope from the influenza virus hemagglutinin protein (HA), was selected with the 12CA5 antibody (BABC0).

### DNA and protein sequence analysis

The 6.7-kb *Eco*RI fragment of chromosome III (from positions 218770–225540) cloned in pUC19-6.7 was a gift from R. Planta. Following subcloning of the 3.2-kb *Xba*I fragment into pBluescript II KS (Stratagene), to generate pRS26, the *PWP2* gene was sequenced on both strands using the manual dideoxy chain-termination method with [<sup>35</sup>S]ATP (Amersham) and the automated fluorescent dye terminator method with the Taq polymerase-DyeDeoxy Cycle sequencing kit (ABI). The synthetic oligonucleotide primers used in DNA sequence determination were as follows: SEGB3-1: 5'-TTAATCAAGATAGGTTCACTGCC-3'; SEGB3-2: 5'-CTGATTTTCGCCTGCATCG-3'; SEGB3-3: 5'-GATGTAGTCATGGAAATCTGACGACGAGG-3'; SEGB3-4: 5'-GGAATCATTTT-CATTAGACAAGGG-3'; SEGB3-5: 5'-GGCTTTGAATGGTATCTCTCG-3'; SEGB3-6: 5'-TTTGATCTGGATGTGGACGTC-3'; SEGB3-7: 5'-GCCTTATATATAAAGAAAA TAGCC-3'; SEGB3-8: 5'-CTTGTCAATTTCAAAGCCCCGTAACGTG-3'; SEGB3-9: 5'-GTCTGTCGTCATCGGATGGCC-3, SEGB3-10: 5'-CAAAATTGTCCAGGGACCCGGC-3'. Sequences were compared with the contents of the Genbank, EMBL, PIR and Swissprot data bases, using the appropriate programs in the University of Wisconsin Genetics Computer Group package (Derereux et al. 1984). Alignment

**Table 1** Yeast strains used in this study

Strain	Genotype
SP1	<i>MATa his3 leu2 trp1 ade8 can1</i>
JRY181 <sup>a</sup>	<i>MAT<math>\alpha</math> his3 leu2 trp1 ade8 can1</i>
JRY182 <sup>b</sup>	<i>MATa/MAT<math>\alpha</math> isogenic diploid of SP1</i>
RSY12	<i>MATa/MAT<math>\alpha</math> JRY182 pwp2-1::HIS3/PWP2</i>
RSY15	<i>MATa/MAT<math>\alpha</math> JRY182 pwp2<math>\Delta</math>-1::HIS3/PWP2</i>
RSY18	<i>MATa pwp2<math>\Delta</math>1::HIS3 [pRS10 (CEN4, PWP2, TRP1)]</i>
RSY24	<i>MATa pwp2<math>\Delta</math>1::HIS3 [pRS18 (GAL1-PWP2, URA3)]</i>
RSY38	<i>MATa pwp2<math>\Delta</math>1::HIS3 [pRS35 (GAL1-HA-PWP2, URA3)]</i>
RSY39	<i>MATa [pRS18 (GAL1-PWP2, URA3)]</i>
RAY3A-D	<i>MATa/MAT<math>\alpha</math> ura3/ura3 leu2/leu2 trp1/trp1 his3/his3</i>
RSY17	<i>MATa/MAT<math>\alpha</math> pwp2<math>\Delta</math>1::HIS3/PWP2</i>
RSY41	<i>MATa pwp2<math>\Delta</math>1::HIS3 [pRS18 (GAL1-PWP2-URA3)]</i>
RSY43	<i>MAT<math>\alpha</math> pwp2<math>\Delta</math>1::HIS3 [pRS18 (GAL1-PWP2, URA3)]</i>
RSY50	<i>MATa/MAT<math>\alpha</math> pwp2<math>\Delta</math>1::HIS3 [pRS18 (GAL1-PWP2, URA3)]</i>
RSY45	<i>MATa [pRS18 (GAL1-PWP2, URA3)]</i>
RSY54	<i>MATa pwp2<math>\Delta</math>1::HIS3 [pRS42 (CEN4, HA-PWP2, LEU2)]</i>
RSY55	<i>MATa pwp2<math>\Delta</math>1::HIS3 [pRS41 (2 <math>\mu</math>m, HA-PWP2, LEU2)]</i>
RSY59	<i>MATa pwp2<math>\Delta</math>1::HIS3 [URA3::GAL1-PWP2]</i>

<sup>a</sup> Constructed by transformation of the haploid strain with a plasmid containing the *HO* gene

<sup>b</sup> Obtained by isolation of zygotes after mating SP1 and JRY181

of the predicted amino acid sequences of Pwp2p and human G $\beta$  transducin involved the insertion of gaps to obtain an optimal alignment. Graphic matrix analyses (Maizel and Lenk 1981) of internal repeats was performed with a 40-residue sliding window (segment size = 40) at a matching criteria of 45% (stringency = 18). Statistical analysis of the Pwp2 protein sequence by SAPS (Brendel et al. 1992) was kindly performed by S. Karlin (Stanford University).

#### Disruption of the *PWP2* gene

The plasmids pRS6 and pRS8 were constructed for one-step gene disruption. The 1.4-kb fragment containing the predicted YCR57c open reading frame flanked by *XbaI* and *KpnI* sites was obtained by PCR of *S. cerevisiae* genomic DNA using the following pair of primers: 5'-CTTAAGCTCTAGAATATGGTCCGTAGATTGAGG-3' and 5'-ATCAAACTAAGGTACCTGCTTCAGTCATTTC-3'. The resulting fragment was subsequently cloned into *XbaI* + *KpnI*-digested pBluescript II KS to obtain pRS5. Plasmid pRS5 was digested with *BamHI* and ligated with the 1.8-kb *BamHI* fragment of *HIS3*, from a plasmid obtained from E. Phizicky, to yield pRS6. In parallel, pRS5 was digested with *EcoRI*, blunt-ended, digested with *BamHI* and finally ligated with the *XhoI*-*BamHI* *HIS3* fragment in which the *XhoI* site had been blunt-ended to generate pRS8. *PvuII*-cleaved pRS6 or pRS8 were introduced into a diploid strain (JRY182) by transformation followed by selection for His<sup>+</sup> prototrophy. Restriction mapping and Southern hybridization analysis of genomic DNA from the resulting transformants was conducted to confirm that transplacement had occurred at the *PWP2* locus. The diploid transformants designated RSY12 and RSY15, carried the insertion (*pwp2-1::HIS3/PWP2*) and the deletion (*pwp2 $\Delta$ 1::HIS3/PWP2*), respectively.

#### Plasmid constructions

YCplac22 (*CEN4*, *ARS1*, *TRP1*), YCplac111 (*CEN4*, *ARS1*, *LEU2*), YEplac181 (2 $\mu$ m, *LEU2*) and YIplac211 (*URA3*) were obtained from R. Gietz (Gietz and Sugino 1988). Plasmids used in complementation analysis were derived by subcloning the 4.6-kb *XbaI* fragment containing *PWP2* from pUC19-6.7 into YCplac22 to create pRS10. The vector pGT5 (*GAL1/10*, *CEN4*, *ARS1*, *URA3*), obtained from I. Miyajima, was used to generate pRS9, pRS12, pRS18 and pRS25. Plasmid pRS9 was constructed by subcloning the 1.4-kb *XbaI*-*KpnI* fragment containing YCR57c from pRS5 in pGT5. The 2.6-kb *AflIII*-*XbaI* fragment from pRS10 (indicated in Fig. 2A) was blunt-ended and cloned into pGT5 to form pRS12. The 3.2-kb *XbaI* fragment containing the entire *PWP2* gene from pRS10 was cloned in *XbaI*-digested pGT5 to obtain pRS18. Construction of pRS25 was achieved by digesting plasmid pRS10 with *XbaI*-*NcoI* to obtain the 2.6-kb fragment, blunting ends with Klenow enzyme, and inserting into pGT5. The construction of *HA-PWP2* first involved site-directed mutagenesis to introduce a unique *NotI* site after the first ATG of *PWP2* in pRS18. Next, the *NotI* fragment from pSM491 (from B. Futcher, Cold Spring Harbor), which encodes three copies of the HA epitope, was introduced in-frame at the *NotI* site of *PWP2*. Finally the *HA-PWP2* *XbaI* fragment was cloned in YCplac111, into which the *GAL1/10* promoter had been inserted, to generate pRS35. For expression of *HA-PWP2* using the *PWP2* promoter, the *PstI*-*XbaI* fragment containing the 5' region of *PWP2* and the *XbaI* fragment containing *HA-PWP2* from pRS35 were cloned in YEplac181 and YCplac111 to generate pRS41 and pRS42.

#### Expression of *PWP2* under control of the *GAL1* promoter

Site-directed mutagenesis was performed on the 4.6-kb *XbaI* fragment from pUC19-6.7 subcloned in the pSelect (Promega) derivative

pRS15 to create an *XbaI* recognition site 4 bp upstream of the ATG initiation codon of *PWP2*. The mutagenic oligonucleotide was 5'-GAACCGCATCTAGATGAAATCCG-3'. Nucleotides that were changed from the wild-type sequence are underlined. The resulting 3.2-kb *XbaI* fragment containing the *PWP2* gene was subcloned in pGT5. The resulting plasmid, pRS18, was transformed into yeast strain RSY15 and Ura<sup>+</sup> transformants were sporulated and dissected. Segregants were germinated on YPGal and tested for histidine (*pwp2 $\Delta$ 1::HIS3*), and Ura<sup>+</sup> (pRS18) prototrophy. Haploid *PWP2/pRS18*, and *pwp2 $\Delta$ 1::HIS3/pRS18* segregants were grown at 30°C on media containing galactose (YPGal). To test the phenotype associated with Pwp2 protein depletion, expression of the plasmid-borne *PWP2* gene was reduced by transferring the cells to YPD or SD (glucose containing) solid medium and incubating at 30°C; cells were monitored several times over 3 days after transfer. Growth of segregants containing the *pwp2 $\Delta$ 1::HIS3* (RSY24) arrested after transfer to glucose medium, whereas cells containing a wild-type *PWP2* gene continued to grow.

#### Analysis of morphology and bud site selection

Methods for interference-contrast and fluorescence microscopy were performed as described (Pringle et al. 1991). Microscopy of single cells was done with a Zeiss Axiophot microscope using DIC optics or fluorescence and a 100 $\times$  objective. Fluorescent staining with Calcofluor, DAPI and rhodamine phalloidin was performed as described (Pringle et al. 1991). The assay for completion of cytokinesis (Healy et al. 1991) involved formaldehyde fixation and removal of the cell wall with Zymolyase 100-T (Seikahaku). For chitinase treatment, RSY24 cells and control cells cultured in YPD or YPGal for 12–18 h were washed, resuspended at an OD of 1 in 10 mM phosphate buffer (pH 6.3), 0.1% sodiumazide containing either 1 unit *Streptomyces griseus* chitinase (Sigma C1525) or an equal volume of buffer. After incubation at 30°C for 4 h, followed by vigorous vortexing, the cells were counted and chains or clumps of 3 or more cells were scored as clusters. Sites of bud formation were quantitated by the method described by Flescher et al. (1993) which involves staining fixed cells with Calcofluor and grouping cells with one bud and a single bud scar into three classes: those with a bud adjacent to the bud scar (axial pattern), those with a bud at the opposite pole to the bud scar (polar pattern) and those with a bud at an intermediate distance from the bud scar (central pattern).

#### Fractionation and Western blot analysis of HA-Pwp2p

The *pwp2 $\Delta$ 1::HIS3* strains RSY54 and RSY55 carrying the *HA-PWP2* gene on low- and high-copy-number plasmids respectively, were used for the fractionation analysis. Lysis of exponentially growing cells and fractionation was by the method of Espenshade et al. (1995) except where indicated. Briefly, approximately 1  $\times$  10<sup>8</sup> cells were harvested by centrifugation and washed once in breakage buffer (50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 15 mM MgCl<sub>2</sub>) and resuspended in 500 ml of the same ice-cold buffer. An equal volume of acid-washed glass beads was added to the cell suspension and lysis was achieved by vigorous vortex mixing for 2 min, four times with 1 min intervals on ice. The resulting homogenates were collected and unbroken cells, glass beads, and large debris were removed twice by centrifugation for 5 min at 450  $\times$  g. Aliquots (0.4 ml) of this fraction were adjusted to 0.5 ml with breakage buffer or with one of the following reagents, to the indicated final concentrations: 2 M urea, 1% Triton X-100, 0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH 11), 1 M NaCl. The samples were incubated on ice for 20 min and subsequently centrifuged at 10 000  $\times$  g for 40 min. The resulting soluble fraction (S10) was further centrifuged at 100 000  $\times$  g for 60 min at 4°C. The particulate fractions were rinsed with the breakage buffer and resuspended in the same buffer. Samples from the total cell

lysate (T), the soluble fractions (S10, S100) and insoluble fractions (P10, P100) were subjected to SDS-PAGE and separated proteins were electrophoretically transferred to a nitrocellulose membrane. The filter was processed to detect HA-Pwp2p with 10 ng/ml of 12CA5 mAb as a primary antibody and using the ECL Western blotting system (Amersham).

#### Indirect immunofluorescence

Strains RSY55 and RSY54 were processed for immunofluorescence microscopy by the method of Pringle et al. (1991). Cells from early log phase cultures were fixed in 4% formaldehyde at 25°C for 30 min. Spheroplasts were prepared by using Glusulase (DuPont) and Zymolyase 20T (Seikahaku) at a final concentration of 0.1 mg/ml. HA-Pwp2 was detected with affinity-purified 12CA5 mAb and FITC-labeled goat-anti-mouse IgG. The cells were co-stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) at a final concentration of 0.1 mg/ml for 15 min after washing three times with PBS-1% BSA.

#### Results

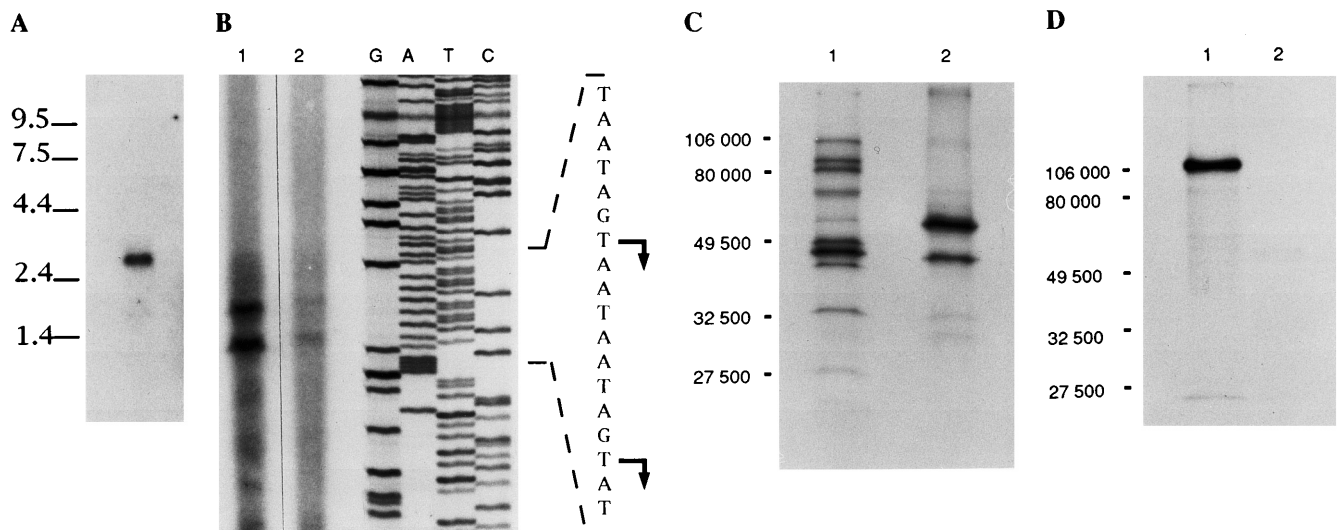
The YCR57c mRNA is 3.2 kb long and includes YCR58c and YCR55c

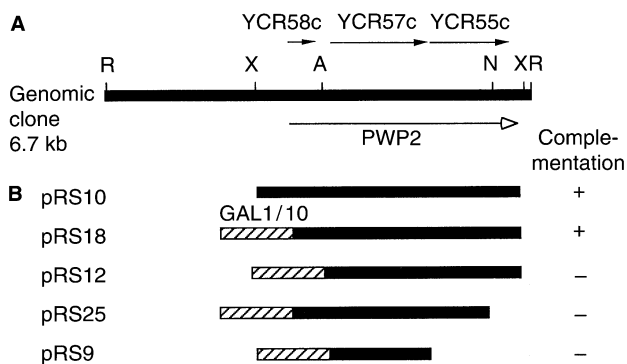
The sequence of *S. cerevisiae* chromosome III revealed three open reading frames with homology to G protein  $\beta$ -transducin: YCR84c, previously identified as *TUP1* (Genbank accession number P16649) YCR57c, and YCR72c (Bork et al. 1992; Oliver et al. 1992). In order to investigate the function of YCR57c, we first examined the pattern of its mRNA expression in vivo. Northern analysis of poly(A)<sup>+</sup> RNA isolated from exponentially growing wild-type cells using a YCR57c-specific probe revealed a single 3.2-kb mRNA band (Fig. 1A). The size of this band was consistent with a previous report that the primary transcript from this region was 3.1 kb long (Akikazu and Isono 1990). As judged by the relative intensity of the signal obtained

after hybridization of the same membrane with an *ACT1* probe, the expression level of the 3.2 kb mRNA was approximately 10% that of actin. Analysis of total RNA from isogenic *MATa*, *MAT $\alpha$*  and *MATa/ $\alpha$*  strains also showed a single band of 3.2 kb which hybridized with the YCR57c probe (data not shown), indicating that the 3.2 kb species was of the same length and equally abundant in all cell types.

YCR57c is an open reading frame of 1317 bp (Oliver et al. 1992). Since the YCR57c mRNA was surprisingly large, additional mapping of the 3.2-kb transcript was performed using probes specific for sequences flanking YCR57c (Fig. 2). Probes corresponding to YCR58c and

**Fig. 1A–D** Expression of *PWP2* mRNA and protein. **A** Poly(A)<sup>+</sup> RNA (5  $\mu$ g) from strain SP1 was fractionated on agarose, hybridized with the radioactive YCR57c probe, and visualized by autoradiography. Numbers indicate marker sizes in kb. Markers were from the 0.24–9.15 kb RNA ladder (BRL). **B** Transcription initiation sites of the *PWP2* RNA was mapped by primer extension with reverse transcriptase and a synthetic oligonucleotide complementary to a region near the ATG start codon of YCR58c. Lanes 1 and 2 contain total RNA and poly(A)<sup>+</sup> RNA, respectively, from strain JRY182. The same primer was used in standard dideoxy sequencing reactions (G, A, T, and C) with pRS10 and the products were subjected to electrophoresis in the same gel as the cDNA extension products to permit direct comparison. The DNA sequence presented is that corresponding to the transcript and hence is complementary to the sequencing ladder. The sites of transcription initiation are indicated by the arrows. **C** In vitro translation. *PWP2* (pRS26, lane 1) and YCR57c (pRS5 lane 2) were transcribed and translated in vitro in the presence of [<sup>35</sup>S]methionine. The translation products were analyzed by SDS-polyacrylamide gel electrophoresis (12%) and visualized by autoradiography. Numbers indicate the sizes (Da) of marker proteins. **D** Immunoprecipitation of HA-tagged Pwp2 protein. The immunoprecipitates of lysed cells from <sup>35</sup>S-labeled cultures of RSY38 (*HA-PWP2*, lane 1), and RSY24 (*PWP2*, lane 2) were immunoprecipitated, analyzed by SDS-polyacrylamide gel electrophoresis (12%) and visualized by autoradiography. Numbers indicate the sizes (Da) of marker proteins





**Fig. 2A, B** Gene map and complementation analysis of *PWP2*. **A** Partial restriction map of the 6.7-kb fragment of chromosome III containing the *PWP2* gene. Restriction site abbreviations: B, *Bam*HI; R, *Eco*RI; X, *Xba*I; A, *Afl*III; N, *Nco*I. The arrows indicate the position and orientation of the previously predicted ORFs of YCR55c, YCR57c and YCR58c. The open arrow indicates the position and orientation of the predicted *PWP2* ORF. **B** Complementation analysis. Horizontal rectangles indicate the DNA sequences which were tested for complementation of the chromosomal *pwp2* disruption (*pwp2Δ1::HIS3*). Successful complementation is indicated by plus

**Fig. 3** Nucleotide and predicted amino acid sequence of the *PWP2* gene. The DNA sequence of the 3.2-kb DNA fragment that complemented the *pwp2Δ1::HIS3* mutation was determined on both strands. Positions in the DNA sequence that diverge from the previously published sequence are in bold and starred. The putative TATA box is underlined. Arrowheads indicate the mRNA start sites determined by primer extension analysis. The eight amino acid sequences that contain the WD-repeats are underlined. The EMBL database accession number for *PWP2* is X78964

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1  TATAATATCAATAAGTAAATAGTATCGTTTTCACAGGTAGACAGGAGCAGACCCGAGATTGAACCCGATCCCAATGAAATCCGATTTCAAGTT  100
                                     >
101 CTCTAACCTTTTAGTACGGTCTACAGGCAAGTAAACATCACCTTTCCGATGATGGCAAGCAACTACTCTCACCGGTGGGAATAGGCTCAGCCTGTT  200
    S N L L G T V Y R Q G N I T F S D D G K Q L L S P V G N R V S V F
201 GACTTAATCAACAACAATCGTTACGCTTTGAATACGAGCATCCGAAAAAATTCCTGCCATGATCTGAACAAACAAGCCACATTCCTGATTTCTATTG  300
    D L I N N K S F T F E Y E H R K N I A A I D L N K G T G L L I S I D
301 ACGAGACCGTCCGCGCACTCTTGCAATTTCAAAGCCCTAACGTGCTTCCACATTTCCAAAGAAAAATKCTCCGCTGTGAAGTTCCGCGCTGA  400
    E D G R A I L V N F K A R N V L H F N F K E K C * S A V K F S P D
401 TGGGAGACTCTTTCGATAGCTCAGCGAGTGTTCACAGATTTGGAAGACTCCAGATCTAATAAAGACAGACAGATTTGCTCCCTTCCTCCGCAATAGG  500
    G R L F A L A S G R F L Q I W K T P D V N K D R Q P A F V R H R
501 CTGCATCGGGACACTTCAAGACATAACGCTTTGACGTGGTCAACAAGATTCAGATTTATCTTACGACTTCCAAAGACTTAAGCCGAAATAATGTT  600
    V H A G H F O D I T S L T W S O D S R F I L T T S K R D L S A K I I W S
601 CCCTAGATTCCAGAGAAAAGAACCTTCGGCGGACACATTTAATGGGCACAGACTACGTTATGGTGGCTTCTTCAGTCATGATCAGGAAAAATCTA  700
    _V_D_S_E_E_K_N*_L_A_A_T_T_E_N_G_H_R_D_Y_V_M_G_A_F_S_H_D_O_E_K_I_Y
701 CACTCTAAGCAAAGCGTGTCTTTGTCTGGGAATTTACCAAGAGGCCATCCGATGACGACGACAAATGAAGTGGAGGACGACGACCAAGCAAGAA  800
    T_V_S_K_D_G_A_V_F_Y_W_E_F_T_T_K_R_P_S_D_D_D_N_E_S_D_D_G_K_D_Q_D
801 GTAGATTTCCGAATACAGCTGGAGATCAAGAAAACATTTTTCAGCAACCAAGCAAAAGTGTGTCCCTTCATCCGACCAACCAAGGC  900
    V_D_I_T_S_K_Y_S_W_R_I_T_T_K_H_F_F_Y_A_N_Q_A_R_V_K_A_V_S_T_F_H_P_A_T_R_L
901 TTTTACGCTCCGATTTACTAGTGGGGAATTCGCTCTTACGATTTGCCGTGATTTCACTTTGATTTCAACAGCTTCTATGGGGCAAAACCCAGTCAAC  1000
    L A V G F T S G E F R L Y D L P D F T L I Q Q L S M G Q N P V N T
1001 CGTTAGCCTCAACCAACCGCGAATGGCTGGCGTTGGTTCAGCAAACTGGGCCAATTTACTAGTTTACGATTTAGCAATCGGAATCGTATCTTTGAAG  1100
    V S V N O T G E W L A F G S S K L G Q L L V Y E W Q S E S Y I L K
1101 CACCAAGCCCATTTTCGATTTCAACAATAGTCTTCATCACTCCCGGATGGTTCACGTGTAGTACAGCATCCGAAGATGGGAAAAATCAAATTTGGGACA  1200
    O O G H F D S T N S L A Y S P D G S R V V T A S E D G K I K V W D I
1201 TTACATCAGGTTTTGTTGGCCACTTTGAGAGAACACACCTTTCAGTTACTGCTGTACAGTTTGGCAAAAGGGGTCAAGTCTTCTCATCATCGTT  1300
    T S G F C L A T F E E H T S S V T A V O F A K R G O V M F S S S L
1301 AGATGGTACGGTACAGCGTGGGACTTAATCAGTATCCTAATTTAGAACATCTACTGGTACIAGAAGCAATCCAATTTCAATGTTTAGCGGTGATCCA  1400
    D_G_T_V_R_A_W_D_L_I_R_Y_R_N_F_R_T_F_T_G_T_E_R_I_Q_F_N_C_L_A_V_D_P
1401 TCAGGTGAAGTGGTTTGTCCGGTCCCTGGACAAATTTGACATTCATGTTTGGTCCGTCGTAACCTGGTCAATTTAGATGCTTTGTCCGACATAG  1500
    S G E V V C A G S L D N F D I H Y W S V O T G Q L L D A L S G H E G C
1501 GCCCTGTTTCGTGCTTTCATTTAGTCAAGAGAACAGTGTCTTACCTTCGATCATGGTAAANCAATAGAACTGCTGCTGATTTCTGATAGCC  1600
    _P_V_S_C_L_S_F_S_O_E_N_S_V_L_A_S_A_S_W_D_K_T_I_R_I_W_S_I_F_G_R_S_Q
1601 ACAAGTAGAACCTATAGAAGTTTATCCGATTTTACCTTATCAATGACAGCAGATGGTAAAGAAATTCAGTATCTACCTTAAAGGCTCAAAATATCC  1700
    Q V E P I E V Y S F V L A L S M R P D G K E V A V S T L K G Q I S
1701 ATTTTCAACATAGAAGATCCCAAGCAGGTGGCCAACTGACTGTAGAAGGATATAATCTCTGTAGGTTTATCAAGATAGGTCCTCCGCAAAAAT  1800
    I F N I E D A K Q V G N I D C R K D I I S G R F N D R F T A K N * S
1801 CTGAAGCATCCAAATTTTTACTACAATACATTCAGATTTGATGGTATGGCTATTTGGCTGGTGGTAAATAAATACCTCAATTTGCTATATGATGTTCC  1900
    E R S K P F T T I H Y S F D G M A I V A G G N N S I C L Y D V P
1901 AAATGAAGTCTGTTAAAAAGATTCATTTGCTCCAGAACATGGCTTGAATGGTACTCTCGAATTTTAAACAGTAAAGAAATGACTGAAGCAGTTCA  2000
    N E V L L K R F I V S R N M A L N G T L E F L N S K K M T E A G S
2001 TTAGATTTGATTCAGCATGCAGCCAAAATTCAGATTTGGAGGATCCTATTCAGATTTCTTACCAGGGTCTCAAGAGGTGGCGACTGTCACCAAGAA  2100
    L D L I D D A G E N S D L E D R I D N S L P G S Q R G G D L S T R K
2101 AAATGAGACAGAGGTTAGAGTACTTCGGTGCATTTCCCCCAACCGCGAATGCATTTGCCGCTCTCAACGGAAGGTTATTTGATATATCCACCAA  2200
    M R P E V R V T S V Q F S P T A N A F A A S T E G H D L I Y S T N
2201 TGACAGATATATTTGATCCCTTTGATCTGGATGTCAGCTCACCCTCCATCTACTGAGAGCGCTACGAGAAAGCAGITTTAAATGATAGTA  2300
    D T I L F D P F D L D V D V T P H S T V E A L R E K Q F L A L V
2301 ATGGCGTTCAGGTTAAATGAAGAAATTTGATCAATAAAGTCTATGAAGCCATACCTATTAAGAAATCCCTTGGTTCAGAAATATTTCTGCAAT  2400
    M A F R L N E E Y L I N K V Y E A I P I K E I P L V A S N I P A I Y
2401 ATTTACCGAGGATCTGAAGTTCATCGGTGATTTGCCATTGAATCCCAACACATTCAGTTTAACTAAITTTGGATCAAAGCTTATTCTCGGACGGG  2500
    L P R I L K F I G D P A I E S Q H I E F N L I W I K A L L S A S G
2501 TGTTACATAAATGAACAAATATCTCTTCGACGGCTATGAGTTCGATACAAGATTTATTTGTTAGAGGCTAAGGAAGTAGTCAATACCCTACT  2600
    G Y I N E H K Y L F S T A M R S I Q R F I V A G A E V V N T T
2601 GATACAAATACACCTATAGATTTTGGTATCAACTGATGGTCCATGGAAGATGGCGGGCTGATGATGACGAGGTTCTATTAAGAAATGACGCAGAT  2700
    D N K Y T Y R F L V S T D G S M E D G A A D E G V L L K D D A D E
2701 AAGATAACGAGAGAACGAGCAACGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT  2800
    D N E E N E N D V M E S D D E G W I G F N G K D N K L P L S
2801 TAATGAAATGATTCAGTGTAGAGAGAAATGAGAAAGAGCTTCTTGAATAATTTCACTGCCCTTATTCACATTCATGATTTCC  2900
    N E N D S S D E E E N E K E L P e n d
2901 CAAGTATAGACTATATAAGCAATACATTTGATTTAATATCAACAAAAAAAATATCATCGCCAAATTCGATATACATCATGATGAGAGCCCTTA  3000
    TAACTGAGCTACCATTCGGGAGTCTCCCTTGACTGACGAGGCTATTTCTTATATATAAGGACTTCACTGATCAAAAAAAAATATGAGAAG  3100
    GAAAAATGAGGATTTGATGGGATGAGAAAAATTTGACCAAAATTTATCGACTCCGTTTACTACTATCATCGGAGAACCAATACACTAAGCCTT  3200
    AAAATTTTGTATTCARAAAAAGAGATACTAATAAACAATGCCATGA  3251
    
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YCR55c also hybridized to the 3.2-kb mRNA. The 5' end of the transcript was mapped by primer extension analysis to bases located at positions -567 and -576 distal to the predicted initiator ATG of YCR57c but only -54 and -63 bases upstream of YCR58c (Fig. 1B). These results indicated that the 3.2-kb RNA transcript extended for several hundred bases on either side of the predicted YCR57c coding sequence and included sequences from YCR55c and YCR58c.

Sequences from YCR58c, YCR57c and YCR55c are part of a single open reading frame, *PWP2*

Resequencing of chromosome III between positions 218778 and 222139 revealed four deviations from the previously published sequence (Oliver et al. 1992): insertions of G, C and A, respectively, at positions 221662, 221416 and 220247 as well as a single substitution of C for T at position 219175. These changes altered the predicted reading frame between YCR58c,

YCR57c, and YCR55c, resulting in a single, continuous ORF flanked by typical control elements (Fig. 3). This ORF will be referred to as *PWP2*. A putative TATA element (5'-TATAAT-3'), resembling the consensus sequence, was found at position -80 from the predicted ATG start codon. Immediately downstream of the TATA box, the sequence 5'-AATAATAGTA-3' is present as a tandem repeat. Transcription start sites were detected at the penultimate T in each element (Fig. 1B). The ATG at position 1 is the first ATG sequence after the TATA box and the flanking residues resemble the consensus sequence for the initiation of translation for eukaryotic ribosomes (Kozak 1986). At the opposite end of the ORF, potential pre-mRNA polyadenylation sequences are present as 5'-TATTTAT-3', 5'-TAG . . . TTTGT TTT-3', and 5'-TATATA-3' (Heidmann et al. 1992; Russo et al. 1991).

### Identification of the *PWP2* gene product

To characterize the polypeptides encoded by the 3.2-kb mRNA, the fragment of chromosome III from position 218778 to 221957 was subcloned in an appropriate vector (pRS26) and subjected to coupled *in vitro* transcription and translation. As shown in Fig. 1C, the largest of the translation products had an estimated mass of 104 kDa, which would correspond to an ORF of approximately 3 kb.

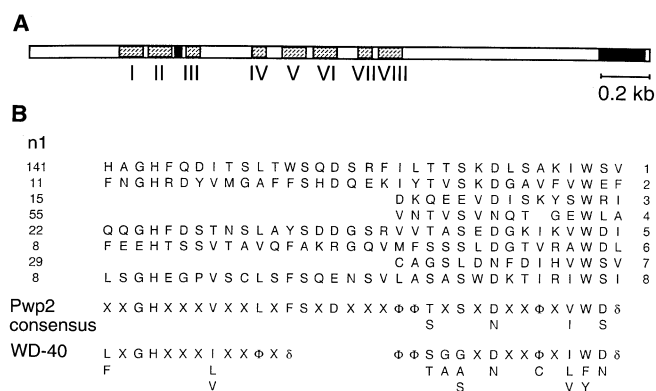
To generate an HA-tagged Pwp2 polypeptide, oligonucleotides encoding the HA epitope of influenza hemagglutinin were fused in frame to the 5' end of the *PWP2* ORF and subcloned in YCplac111-*GAL* (pRS35) to allow expression under control of the inducible *GAL1* promoter. A strain carrying pRS35 was grown on galactose medium and subjected to *in vivo* <sup>35</sup>S labeling and immunoprecipitation. As shown in Fig. 1D, the anti-HA monoclonal antibody (12CA5) immunoprecipitated a single polypeptide with an apparent molecular weight of 104 kDa. No such band was detected in extracts from a control strain that expressed the untagged version of *PWP2*. This result demonstrates that the *PWP2* gene encodes a polypeptide of 104 kDa, consistent with the size of the product of the 3.2-kb mRNA detected by *in vitro* translation.

### Structural features of *PWP2*

The predicted amino acid sequence of *PWP2* was used to search the database of nonredundant sequences using the BLAST and FASTA algorithms. This revealed a weak similarity at the C-terminus to neurofilament proteins and to Asp/Glu-rich proteins related to nucleolin. Significant homology was found with G protein  $\beta$ -subunits, including human GBB2 (Accession No. P11016, BLAST score  $p = 10^{-24}$ ) which shared 32% identity and 77% similarity with the sequence of

Pwp2p between residues 140 to 500. This central region of Pwp2p was strongly related (BLAST scores  $p < 10^{-15}$ ) to other subtypes of mammalian  $\beta$ -subunits; GBB1 (P04901), GBB3 (P16520) and GBB4 (P29387), as well as the homologous  $\beta$ -subunits from *Drosophila* (P26308), *Caenorhabditis elegans* (P17343), *Loligo forbesi* (P23232), *Dictyostelium discoideum* (P36408) and the *S. cerevisiae* Ste4 protein (A30102). Also related but with lower identity scores (BLAST scores  $p < 10^{-7}$ ) were *TUP1* (P16649), *Espl* (P16371), *LIS1* (P43034), *AAC3* (P14197), *CDC4* (P07834), *COP $\beta$ '* (P35605), *MET30* (P39014), and *CDC20* (P26309). Graphical self-comparison analysis (Maizel and Lenk 1981) revealed that Pwp2p contained internal repeats in the central region and at the C-terminus. The central repeats (Figs. 3, 4A) correspond to eight copies of the WD motif that was first identified as a repeating unit in the  $\beta$ -subunit of transducin (Fong et al. 1986). Five of these are full-length WD-repeats and three are "incomplete" half-repeats. As shown in Fig. 4B, the eight sequences are conserved with respect to each other as well as to the consensus WD motif (Voorn and Ploegh 1992).

A statistical search for additional features of the predicted sequence using the SAPS algorithm (Brendel et al. 1992) revealed a high proportion of Asp residues (8.1% overall), particularly between amino acids 225-235 and near the C-terminus (Fig. 4A). Such acidic clusters occur in less than 4% of proteins from yeast and humans (Sapolsky et al. 1993) and occur in several other WD-repeat proteins (Durino et al. 1992).



**Fig. 4A, B** Structure of the Pwp2 polypeptide. **A** The shaded blocks indicate the WD-repeats and the black bars indicate the clusters of negatively charged residues. **B** Alignment of WD-repeats in Pwp2p. The WD-repeats revealed by matrix analysis of the deduced amino acid sequence of *PWP2* were aligned manually for comparison with the consensus WD-repeat. n1 indicates the number of residues between the repeats. The Pwp2 consensus sequence represents the consensus for the 8 internal repeats. The WD-40 consensus sequence, shown at the bottom, represents the consensus for fourteen proteins (Voorn and Ploegh 1992); hydrophobic amino acids are represented by  $\phi$ ;  $\delta$ , indicates a noncharged side chain and x, any amino acid

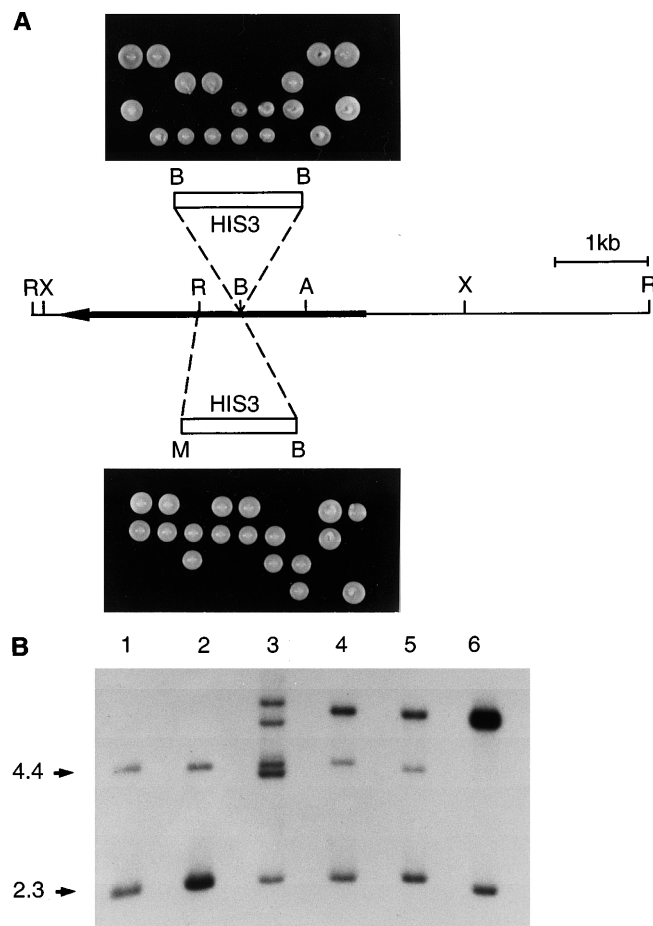
Although an N-terminal signal sequence was not detected, a possible subcellular localization to the mitochondrial matrix was predicted by the PSORT program (Goffeau et al. 1993; Nakai and Kanehisa 1992). Since the C-terminal region of Pwp2p had weakly repetitive character and a number of proteins related to neurofilaments have coiled-coil domains, the Pwp2p sequence was analysed with an algorithm to identify regions with heptad repeats (Lupas et al. 1991). This analysis predicted two regions near the C-terminus with 70–80% probability of forming coiled-coil structures.

#### *PWP2* is essential for growth

To investigate the effect of loss of *PWP2* function, the chromosomal copy of the gene was inactivated using the one-step gene disruption method (Rothstein 1991). Two disrupted alleles of *PWP2* were constructed (see Fig. 5A, and Materials and methods). A deletion-disruption mutation was created (*pwp2Δ1::HIS3*) by replacing the 467-bp *Bam*HI-*Eco*RI fragment by the *HIS3* gene. The other mutation (*pwp2-1::HIS3*), was created by insertion of the *HIS3* gene at the *Bam*HI site in the coding sequence. A restriction fragment containing each of these constructions was purified and introduced into a diploid strain (JRY182) by transformation, followed by selection for histidine prototrophy. Transformants ( $\text{His}^+$ ) in which one copy of the *PWP2* locus had been disrupted were identified by Southern analysis (Fig. 5B, lanes 3, 4 and 5). Strains of heterozygous *pwp2Δ1::HIS3/PWP2* diploids (RSY15) and *pwp2-1::HIS3/PWP2* diploids (RSY12) were sporulated, dissected on non-selective (YPD) medium and incubated at 15, 25 or 37° C. At each temperature and in all tetrads examined (45) only two out of four spores produced colonies (Fig. 5A). Viable cells were auxotrophic for histidine and contained the wild-type allele of *PWP2* as shown by Southern analysis (Fig. 5B, lane 2). This indicated that spore progeny carrying the disrupted *pwp2* gene were inviable; *PWP2*, therefore, is an essential gene. Similar results were obtained following disruption and sporulation of a diploid with a different genetic background (RAY3A).

Microscopic examination of the non-growing progeny revealed either swollen spores (20%), enlarged spores which had germinated with one large bud (60%), and spores with two or more large buds which could not be separated by micromanipulation (20%). Most spores lacking *PWP2* underwent one or two mitotic duplications before arresting growth with one or two large buds.

Plasmids containing fragments of the *PWP2* gene were introduced into the diploid strain RSY15 (heterozygous for the *PWP2* locus) and tested for complementation (Fig. 2B). Tetrads carrying either pRS9 (YCR57c), pRS12 (YCR57c, YCR55c) or pRS25 (C-



**Fig. 5A, B** *PWP2* disruption. **A** Diploid yeast strains were transformed with restriction fragments containing the indicated *pwp2Δ1::HIS3* deletion or *pwp2-1::HIS3* insertion allele and  $\text{His}^+$  transformants were selected. Sporulation and dissection of strains heterozygous for the *PWP2* locus, yielded a 2:2 segregation of viability to non-viability. Shown are 18 tetrads from the diploid strain JRY182. The four spores of each tetrad were positioned vertically. All viable spores were  $\text{His}^-$ , indicating that *PWP2* is an essential gene. **B** For Southern blotting analysis of the disrupted *PWP2* gene, total cellular DNA was prepared from the wild-type diploid strain JRY182 (lane 1), the heterozygous insertion diploid strain RSY12 (*PWP2/pwp2-1::HIS3*) (lane 3), the heterozygous deletion diploid strain RSY15 (*PWP2/pwp2Δ1::HIS3*) (lanes 4 and 5), one of its haploid progeny (lane 2) and the haploid disruption strain RSY18 (*pwp2Δ1::HIS3* carrying pRS10(YCplac22[*PWP2*])) (lanes 6). DNA was digested with *Eco*RI, transferred to the nylon membrane and hybridized with the radiolabelled 1.4-kb fragment of YCR57c. The resulting autoradiogram shows patterns of hybridization consistent with the DNA sequence: bands of 2.3-kb and 4.4-kb are derived from the intact *PWP2* gene (lanes 1 and 2); insertion of the *HIS3* gene in *PWP2* produced an additional band of 8 kb (lanes 4, 5 and 6) and eliminated the 4.4-kb band (lane 6)

terminal deletion derivative of *PWP2*) produced only two viable spores that were wild type for *PWP2*. In contrast, tetrads derived from pRS10 (*PWP2*), and pRS18 (*GALI-PWP2*) produced four viable progeny, two of which were disrupted in the chromosomal copy

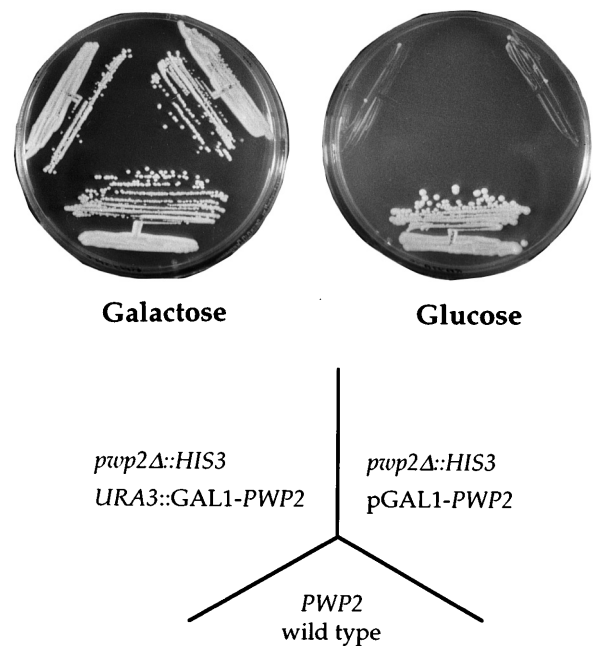
of *PWP2* (Fig. 5B, lane 6). As judged by retention of the auxotrophic markers, pRS10 or pRS18 were not lost from these haploid cells even after prolonged growth in rich medium. These results confirm that *PWP2* is an essential gene and demonstrate that the entire ORF of *PWP2* is required to rescue the *pwp2Δ1::HIS3* mutation.

#### Reduced expression of *PWP2* results in formation of cell chains and multibudded clusters

To further characterize the effects of Pwp2 protein depletion, the *PWP2* gene was placed under control of the *GAL1* promoter in plasmid pRS18. On solid media containing glucose, RSY24 cells (*pwp2Δ1::HIS3*/pRS18) were markedly inhibited, with no colony formation visible, whereas colonies on galactose medium grew to normal size (Fig. 6). In YPGal liquid broth, RSY24 grew normally. After an exponentially growing culture was shifted from galactose to glucose (YPD), the optical density increased over a 24 h period but cell division, as measured by the number of individual cells, was dramatically reduced. Microscopic examination of the same cells grown for 8 h after transfer to YPD revealed chains of 3 to 8 connected cells (Fig. 7) that were absent in control cultures (SP1 or RSY18). The proportion of connected cells and the number of cells in each chain increased dramatically over a period of 36 h. While only 8% of the cells had one or more buds attached before the shift, 18 h after shift to YPD, more than 60% of the cells remained connected in chains of 3 or more. Connected cells formed chains or branched chains which could not be separated by sonication. The average number of cells in a cluster was 4 (300 clusters counted). In most clusters, some buds appeared elongated rather than round (35% of cells in 75 clusters). These results suggested that depletion of Pwp2p resulted in defects in bud morphology and mother-daughter cell separation.

#### Pwp2p depleted cells exhibit defects in cytokinesis

To test whether the cells connected in chains and clusters had completed cytokinesis, they were fixed with formaldehyde and treated with zymolyase to remove the cell wall (Healy et al. 1991). This treatment reduced the number of cells in the chain and produced individual spheroplasts or pairs of spheroplasts (20% pairs in 150 cells treated) joined by internal connections. Inspection of spheroplasts after treatment with DAPI (to visualize the nucleus) revealed that most pairs had individual nuclei (64%), some had only one nucleus (32%) or two nuclei in one cytoplasm (4%) (data not shown). This indicated that cytokinesis was defective in some cells, whereas nuclear division was relatively normal in most cells and continued even in the absence of



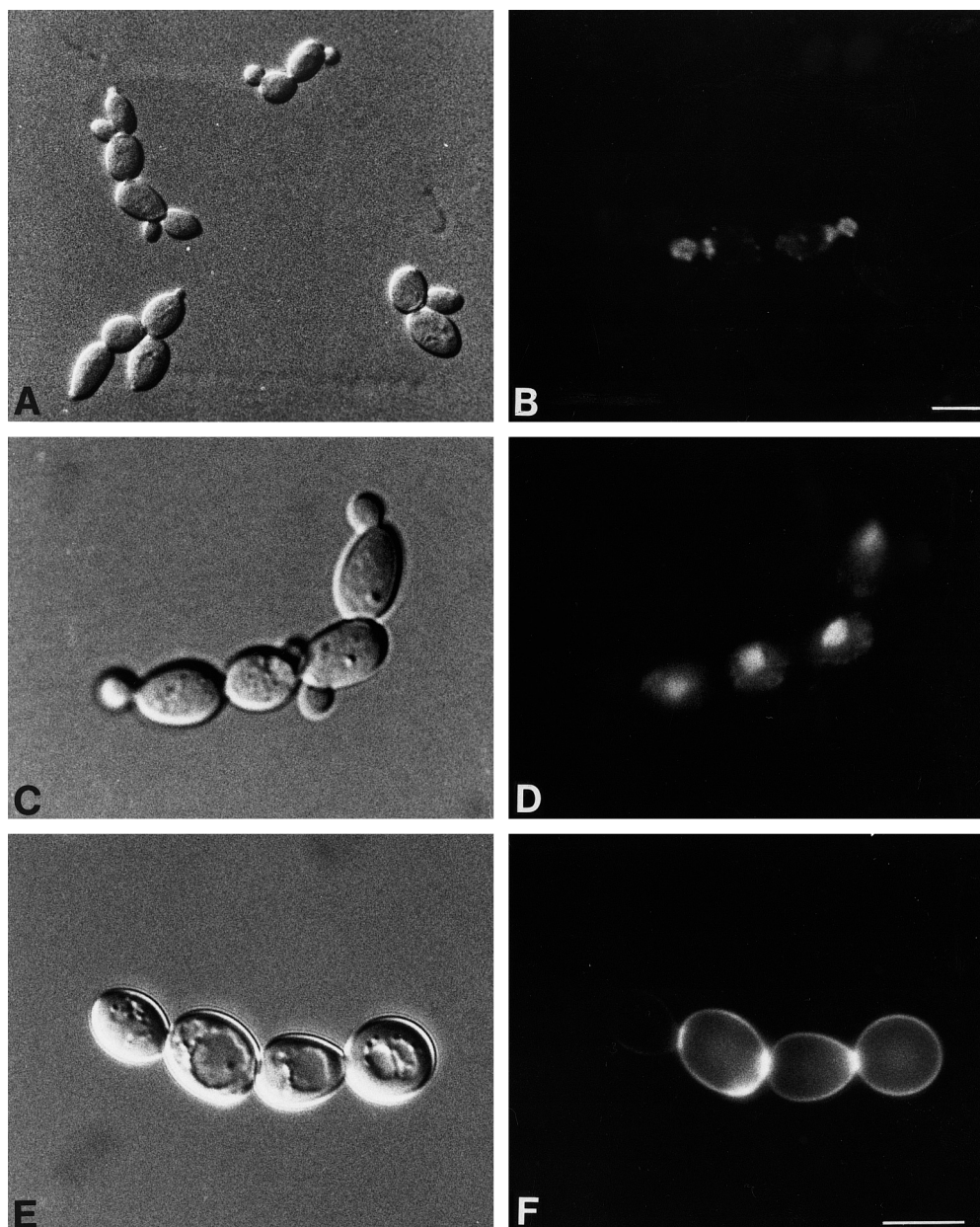
**Fig. 6** Complementation *pwp2Δ1::HIS3* by *GAL1-PWP2*. Strains RAY3A (wild type), RSY41 (*pwp2Δ1::HIS3*[*pGAL1-PWP2*]) and RSY59 (*pwp2Δ1::HIS3* carrying *GAL1-PWP2* integrated at the *URA3* locus) are shown after incubation for 3 days at 30°C on YPGal and YPD plates. The growth of RSY41 and RSY59 was inhibited on glucose plates, with no individual colonies visible

cytokinesis. Although most clusters could not be separated by micromanipulation, a few detached cells placed on solid medium containing galactose and incubated at 30°C gave rise to colonies (in 9 out of 12 cases), which suggested that at least some of the cells in the clusters contained a viable nucleus. The distribution of nuclei was examined by DAPI staining 18 h after shifting RSY24 cells to glucose medium. Most cells in clusters contained a single nucleus (50%); some cells contained two nuclei (33%) and less frequently large buds were anucleate (17%) (Fig. 7D). Together these results confirmed that nuclear division and bud emergence continued in spite of defects in cytokinesis and cell separation.

Rings of cell wall chitin that form at the nascent site of bud emergence and the septal junction can be visualized by Calcofluor staining. Chains of glucose-cultured RSY24 cells stained with Calcofluor exhibited normal staining at the sites of budding. Mother cells, identified by the presence of multiple bud scars, stained relatively intensely, suggesting that chitin deposition may be somewhat delocalized whereas chitin deposition at the septal junction between cells appeared normal (Fig. 7F). Treatment of chains of glucose-cultured RSY24 cells with chitinase resulted in a dramatic reduction in the proportion of chains and clusters of cells (Fig. 8). Twenty-four percent ( $n = 3$ ) of cells incubated with buffer (control) contained clusters of 3–15



**Fig. 7A–F** Micrographs of Pwp2p-depleted cells. Cells of RSY24 (*pwp2Δ1::HIS3[pGAL-PWP2]*) 8 h after transfer to YPD are shown, viewed by differential interference contrast microscopy (**A**, **C**, and **E**) or fluorescence microscopy after treatment with rhodamine-phalloidin which stains actin (**B**), DAPI which stains DNA (**D**), or Calcofluor which stains chitin (**F**). Bar, 5  $\mu$ m



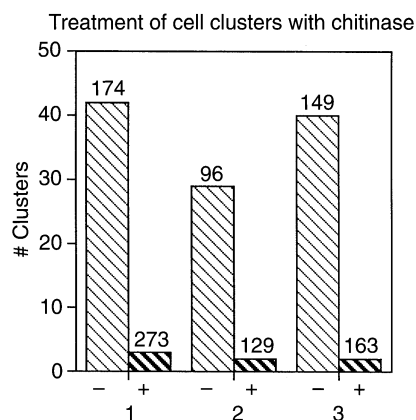
unseparated cells, whereas only 1.3% ( $n = 3$ ) clusters were present in equivalent cultures incubated with chitinase. Chitinase treatment increased the number of cells per ml; most of the resulting cells were either unbudded or contained a single bud. A similar result was obtained following chitinase treatment of clusters produced by depletion of Pwp2 protein in cells (RSY41) of a different genetic background (RAY3A). These data indicate that the formation of cells in chains and clusters results from incomplete hydrolysis of the chitinous septum between mother and daughter cells.

In wild-type cells the actin cytoskeleton functions in directing polarized cell-surface growth. To analyze the pattern of actin distribution RSY24 cells were stained

with rhodamine phalloidin. Compared to galactose-grown RSY24 cells, glucose cultured cells exhibited a normal staining pattern with intensely staining cortical patches in buds and long fibers that extended from mother cells into buds (Fig. 7B).

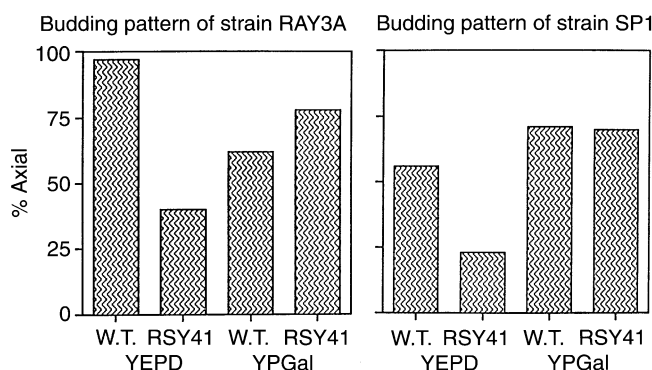
#### *PWP2* depletion alters bud site selection

Since mutations in genes involved in cytokinesis have been reported to affect bud-site selection (Flescher et al. 1993), we examined the budding pattern of Pwp2p-depleted cells. The abnormal cells that resulted from culturing strain RSY24 in glucose medium were



**Fig. 8** Separation of multibudded clusters by chitinase. RSY24 cells were cultured in YPD for 10–12 h prior to incubation with chitinase (+) or buffer (–) as described in Materials and methods. The results from three independent experiments are shown. Numbers indicate the total number of single cells, chains, and clusters in each experiment. A group of 3 or more cells was counted as a cluster. The average number of cells per cluster was 8 in these experiments

attached either in linear chains or (less commonly) in branched chains. This arrangement suggested that many cells grew buds at their poles, a pattern suggestive of the bipolar bud site selection exhibited by diploid cells. As judged by its ability to mate and to respond to  $\alpha$ -mating factor, however, RSY24 is a *MATa* haploid strain. To analyze the pattern of bud site selection in RSY24 cells, the location of buds in mother cell containing one bud and one bud scar (4–12% of the population) was determined after staining with Calcofluor to identify the bud scar. Such cells were classified as exhibiting either an axial, central, or polar budding pattern. In galactose-grown RSY24 cells, most mother cells (70%) had budded adjacent to the scar (axial), 19% budded near the scar (central) and 11% budded at the opposite pole (polar). Control cells (SP1) wild-type for *PWP2*, as well as SP1 cells carrying pRS18 (RSY39), which overexpress *PWP2* mRNA when grown in galactose medium, exhibited a similar, predominantly axial budding pattern. In contrast, 18 h after shifting RSY24 cells to glucose medium, the majority of such mothers had formed buds at the opposite pole (51%) and relatively fewer had budded at sites either axial (23%) or central (26%) to the bud scar (Fig. 9). In agreement with these observations, in glucose-cultured RSY24 cells with two large buds, 14% of the buds were adjacent, 12% were central, and 74% had buds at opposite poles. Thus, mother cells wild-type for *PWP2*, exhibited a predominantly axial pattern of bud site selection, whereas the majority of mother cells with reduced expression of *PWP2* had budded at the opposite pole. Since SP1 control cells cultured in glucose medium showed a surprisingly high frequency of polar budding (23%), the analysis of bud site selection was repeated in isogenic strains from a different genetic background (RAY3A). In glucose-



**Fig. 9** Abnormal bud position in *Pwp2p*-depleted haploid cells. The budding pattern of mother and daughter cells stained with Calcofluor was determined as described in Materials and methods and designated as “axial”, “polar” or “central”. Bud position was counted as the number of mothers with one bud scar and one bud that exhibited each pattern. The percentages of buds in the axial position in the combined results of three experiments are shown

grown RAY3A haploid cells, nearly all mothers with one bud and a single bud scar had budded adjacent to the scar (97%), whereas 12 h after shifting RSY41 cells to glucose medium such mothers exhibited a random pattern and formed buds at either polar (31%), axial (40%), or central (29%) sites (Fig. 9). These results confirmed the observation that the pattern of bud site selection was abnormal (random) in *Pwp2p*-depleted haploid cells. The homozygous *pwp2Δ1/pwp2Δ1* diploid strain (RSY50) carrying pRS18 also produced unseparated cells after shifting to glucose medium, but these cells maintained the normal bipolar budding pattern observed for diploid wild type cells, RAY3A-D and JRY182.

#### Subcellular location of *Pwp2p*

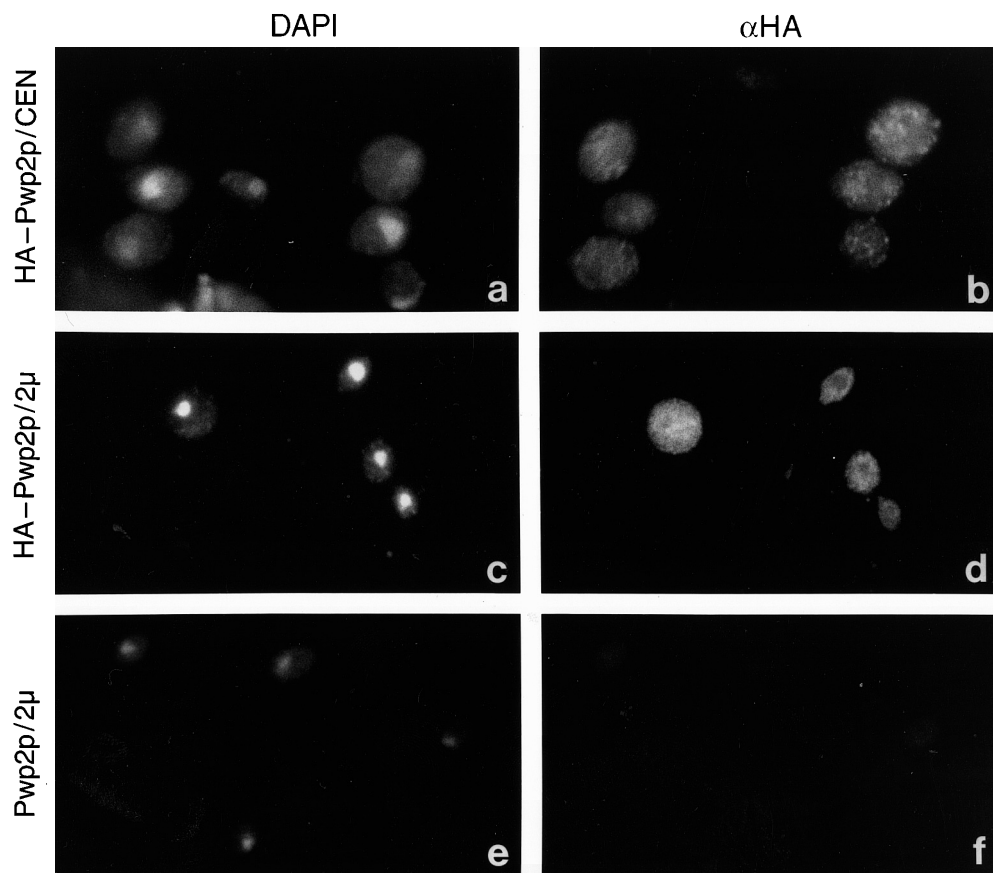
The *HA-PWP2* gene with the intact promoter region of *PWP2* was subcloned in high- and low-copy-number vectors containing the *LEU2* marker to generate, respectively, pRS41 and pRS42 (Materials and methods). To test if *HA-PWP2* was biologically active, strain RSY41 (*pwp2Δ1::HIS3*) carrying the *GAL1-PWP2* expression plasmid with the *URA3* marker (pRS18) was transformed with pRS41 or pRS42. Transformants that grew on galactose medium lacking both uracil and leucine were subsequently plated on medium containing 5-fluorouracil (5-FOA) to identify cells that had lost the *URA3* plasmid. This generated strain RSY54 carrying pRS42, and strain RSY55 carrying pRS41. Both RSY54 and RSY55 cells were morphologically indistinguishable from wild-type control cells. Therefore, expression of *HA-PWP2* can complement the *pwp2Δ1::HIS3* mutation.

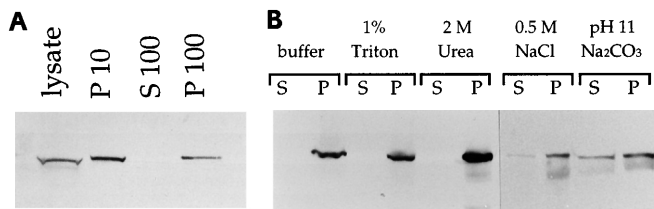
The intracellular location of Pwp2p was examined by indirect immunofluorescence microscopy. Cells of RSY54 and RSY55, showed staining at multiple points dispersed throughout the cytoplasm (Fig. 10b, d). Cells carrying *HA-PWP2* on the high-copy-number plasmid (RSY55) exhibited more intense staining and a greater number of the cytoplasmic dots. Wild-type cells or RSY41 cells expressing an untagged version of *PWP2* treated with the 12CA5 anti-HA antibody gave very weak staining. Cells were also co-stained with DAPI to visualize DNA (Fig. 10a, c, e). As judged by the lack of correlation between the pattern of DAPI staining and the pattern of the antibody staining, HA-Pwp2p did not colocalize with the nucleus or mitochondria. Cells with large buds exhibited the same staining pattern as the unbudded cells, suggesting that the intracellular location of Pwp2p did not change during the cell cycle.

The subcellular distribution of HA-Pwp2p was further examined by fractionation of RSY54 cells (low-copy-number *HA-PWP2*) using differential centrifugation (Epenshade et al. 1995; Singer and Riezman 1990). Logarithmically growing cells were lysed by shaking with glass beads and centrifuged at 500 g to remove unbroken cells. The cleared supernatants were then spun at  $10\,000 \times g$  yielding the S10 supernatant and the P10 pellet enriched in nuclei, mitochondria,

vacuoles and large structures of both the endoplasmic reticulum and cytoskeleton. Finally, the S10 was centrifuged at  $100\,000 \times g$  to produce the S100 fraction and the P100 pellet containing Golgi particles, small vesicles and small cytoskeletal elements. Each subcellular fraction was resolved by SDS-PAGE and immunoblotted with 12CA5 antibody. As illustrated in Fig. 11A, HA-Pwp2p was enriched in the insoluble fractions P10 and P100. In contrast, HA-Pwp2p was barely detectable in the S100 cytosolic fraction, even when over-produced in strain RSY55. As a control for cell lysis and fractionation, glucose-6-phosphate dehydrogenase was found enriched in the S100 fraction in these experiments. To determine the nature of the association of HA-Pwp2p with the particulate cell fraction, cell lysates were pretreated with various reagents for 20 min prior to centrifugation at  $100\,000 \times g$  and subsequent Western blot analysis. The results of this experiment (Fig. 11B) indicate that Pwp2p was partially solubilized by pretreatment with 0.1 M  $\text{Na}_2\text{CO}_3$  (pH 11) or 0.5 M NaCl at room temperature, but was unaffected by treatment with lysis buffer, 1% Triton X-100 or 2 M urea. These results indicate that the strong associations between Pwp2p and insoluble components involve electrostatic and pH-sensitive forces rather than simple hydrophobic interactions. Together with the

**Fig. 10a-f** Intracellular localization of *HA-PWP2*. Haploid *pwp2Δ1::HIS3* cells carrying low-copy (CEN), high-copy ( $2\mu$ ) *HA-PWP2* or untagged *PWP2* as indicated, were photographed after staining with anti-HA antibodies (b, d, f) and DAPI (a, c, e)





**Fig. 11A, B** Subcellular fractionation of *HA-PWP2*. **A** Extracts from RSY54 cells were prepared and fractionated by differential centrifugation as described in Materials and methods. Equivalent volumes of total lysate, P10, P100 and the corresponding supernatant (S100) prepared from approximately  $10^8$  cells were resolved by SDS-PAGE and probed with 12CA5 antibody. The 104-kDa HA-Pwp2p band from each fraction is shown. **B** Cell lysates were separated into pellet (P) and supernatant (S) fractions by centrifugation at  $100\,000\times g$  after treatment with lysis buffer, 1% Triton X-100, 2 M urea, 0.5 M NaCl or 0.1 M sodium carbonate (pH 11). The distribution of HA-Pwp2p after extraction is shown

immunolocalization results, these data suggest that Pwp2p is associated with a large proteinaceous complex, possibly involving the yeast cytoskeleton, rather than membrane structures such as the plasma membrane or organelles.

## Discussion

In this report, we have described a new gene, *PWP2*, that was cloned after identifying and correcting errors in the coding sequence of YCR57c and in the flanking sequences predicted to encode YCR55c and YC58c (Oliver et al. 1992). This protein (Pwp2p) is predicted to have an N-terminal extension preceding a central region with eight WD-repeats that share homology with the G protein  $\beta$ -transducin, followed by possible coiled-coil structures at the C-terminal region. The present work shows that Pwp2p is part of an insoluble complex located at multiple points in the cytoplasm, where it is involved in regulation of processes that are essential for growth, cytokinesis, and cell separation.

Previously known WD proteins have been found in a variety of cellular locations, where they are involved in diverse processes including cell cycle regulation, transcription regulation, signal transduction and RNA splicing. Most of them seem to have a regulatory function and many are known to exist in large protein complexes (Neer et al. 1993). Another structure predicted to be present in Pwp2p, the coiled-coil domain, is also found in proteins that exist in complexes. Coiled-coil domains have been found in filament-forming proteins, G protein  $\beta$ -subunits, and in the dimerization domains of several transcriptional regulatory proteins (Lupas et al. 1991). These observations raise the possibility that Pwp2p may exist as part of a multiprotein complex, possibly interacting with other proteins through the coiled-coil and WD-repeat regions.

This idea is supported by the results of subcellular localization studies. Indirect immunofluorescence methods using biologically active HA-tagged Pwp2p, revealed a number of small, brightly staining structures throughout the cytoplasm, which did not coincide with the nucleus, mitochondria or plasma membrane. The multidot pattern did not change significantly during the cell cycle or upon overproduction of *PWP2*. Despite its hydrophilic, charged character, Pwp2p was found to be strongly associated with the insoluble fraction of the cell. Even after repeated differential centrifugation steps, the particles were found equally in the P10 and the P100 fractions, indicating that they do not have a uniform size or density. Based on several observations, we conclude that these particles are unlikely to be associated with large organelles like mitochondria, nuclei, vacuoles or large membrane structures of the plasma membrane and endoplasmic reticulum. The fact that reagents known to perturb protein-protein interactions, such as alkaline buffer and high salt were able partially to solubilize Pwp2p suggests that Pwp2p is part of a protein complex. The finding that the association between Pwp2p and the insoluble fraction is completely resistant to treatment with Triton X-100 is suggestive of cytoskeletal associations, which, in yeast as in other eukaryotic cells, remain intact following extraction with nonionic detergents (Branton et al. 1981; Herman and Emr 1990). Our localization and fractionation evidence indicates, therefore, that Pwp2p is not associated with membrane vesicles but rather forms part of a proteinaceous complex, potentially including cytoskeletal elements of the cell. Further biochemical experiments are necessary to determine the precise nature of the association of Pwp2p with this insoluble complex. In an effort to identify the specific components that interact with Pwp2p *in vivo*, we have initiated a search for genes that suppress *pwp2* ts mutants in multiple copies and we have identified peptides that interact with Pwp2p in the two-hybrid system. The combined results of these genetic and biochemical studies should lead to a better understanding of the role of *PWP2* in control of growth and morphology.

What is the function of *PWP2*? The *PWP2* gene is expressed in all cell types and is essential for growth. Spores carrying a *PWP2* gene disruption can germinate, but after one or two rounds of replication they arrest growth with one or more large buds. A similar phenotype was observed in detail following down-regulation of *PWP2* gene expression with the *GALI* promoter. Cell separation is severely defective and cannot easily be accomplished by micromanipulation or sonication. Bud site selection is also abnormal and follows a random pattern, in contrast to the axial pattern typical of haploid wild-type cells. Although some of the buds are elongated, which suggest that bud growth is overpolarized, the formation of actin cytoskeletal elements and the chitin ring at the base of the bud appear to be unaffected. Thus, continued DNA synthesis,

nuclear division, and bud emergence in combination with defective cell separation and abnormal bud site selection results in the formation of chains and clusters of cells connected at the bud neck.

The cell separation defect in cells depleted of Pwp2p protein could reflect either the formation of an abnormal septum or the lack of hydrolysis of the junction between the mother and bud. An endochitinase is involved in hydrolysis of the primary septum and cells that lack the chitinase gene, *CTS1*, are unable to separate (Kuranda and Robbins 1991). *CTS1* transcription is regulated by *ACE2* and cells that carry an *ace2* mutation also display a clumpy phenotype (Dohrmann et al. 1992). The ability of exogenous chitinase to release Pwp2p-depleted cells from clusters is consistent with the idea that hydrolysis of the septum is delayed in the absence of *PWP2*. In contrast to *PWP2*, however, strains that lack the *CTS1* gene do not have an abnormal budding pattern (Kuranda and Robbins 1991) and neither *CTS1* nor *ACE1* are essential genes. Thus, although *PWP2* may not participate directly in chitin hydrolysis, it may possibly mediate the localization or the activity of factors involved in cleavage of the septum.

The morphology of Pwp2p-depleted cells also resembles phenotypes that have been observed in cells with defects in the cell division cycle genes *CDC3*, *CDC10*, *CDC11*, and *CDC12* (Hartwell 1971). These genes encode proteins of the ring of 10 nm filaments that appears as a cortical ring at the bud site before bud emergence; cells carrying temperature-sensitive mutations in any of these four genes display essentially identical pleiotrophic phenotypes (Flescher et al. 1993). With respect to organization of the cell wall at the base of the bud, they seem to have more severe defects in cytokinesis, bud elongation and formation of the chitin ring. However, in the formation of unseparated cells and the abnormal pattern of bud site selection, they resemble cells depleted of Pwp2p. Flescher et al. (1993) have proposed that *CDC10* and other neck filament proteins required for cytokinesis are involved in determining the next site of bud emergence. The finding that Pwp2p-depleted cells also show defects in both cytokinesis and bud site selection is consistent with the idea that these processes are related.

Another instance in which cells fail to separate occurs in strains that have undergone a dimorphic transition and form pseudohyphae that penetrate the growth medium (Gimeno et al. 1992). Phenotypically related defects in cell separation and abnormally elongated buds were reported for haploid cells carrying mutant alleles of *CDC55* (Healy et al. 1991), *YCK1*, *YCK2* (Robinson et al. 1993), and *ELM1*, *ELM2*, and *ELM3* (Blacketer et al. 1993). However, RSY24 cells that formed microcolonies on YPD plates did not show the distinctive pattern of growth below the surface of the agar medium, referred to as foraging, that is a property of the pseudohyphal form, nor do they follow the

normal budding pattern which is required for the dimorphic transition.

The finding of numerous sequences related to  $\beta$ -transducin indicates that the family of WD-repeat proteins is both ancient and diverse. In *S. cerevisiae*, where many WD-repeat sequences have been identified by systematic sequencing, the relative ease of genetics provides an attractive system for functional analysis. In addition, when homologous genes have been identified in other organisms, cross-species complementation can offer a paradigm for further experimentation. Thus, after completion of this manuscript we were interested to discover human ESTs (T16114, T75342, R20872, F13143) in the XREF database (Tugendreich et al. 1994) with significant identity to segments of *PWP2*. It will be important to obtain the full sequences of the human cDNAs to verify that as seems likely, homologs of *PWP2* exist in more complex eucaryotes.

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#### Note added in proof

Recent reports have identified PWP2-related genes in the filamentous fungus *Ashbya gossypii* (Altmann-Johl R, Philippsen P (1996) *Mol Gen Genet* 250:69–80), and in humans (Lalioti M, Chen H, Rossier C, Shafaatian R, Reid JD, Antonarakis SE (1996) *Genomics*, in press).