Cell, Vol. 109, 835–848, June 28, 2002, Copyright ©2002 by Cell Press

Yph1p, an ORC-Interacting Protein: Potential Links between Cell Proliferation Control, DNA Replication, and Ribosome Biogenesis

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Summary

Immunoprecipitation of the origin recognition complex (ORC) from yeast extracts identified Yph1p, an essential protein containing a BRCT domain. Two Yph1p complexes were characterized. Besides ORC, MCM proteins, cell-cycle regulatory proteins, checkpoint proteins, 60S ribosomal proteins, and preribosome particle proteins were found to be associated with Yph1p. Yph1p is predominantly nucleolar and is required for 60S ribosomal subunit biogenesis and possibly for translation on polysomes. Proliferating cells depleted of Yph1p arrest in G_1 or G_2, with no cells in S phase, or significantly delay S phase progression after release from a hydroxyurea arrest. Yph1p levels decline as cells commit to exit the cell cycle, and levels vary depending on energy source. Yph1p may link cell proliferation control to DNA replication, ribosome biogenesis, and translation on polysomes.

Introduction

DNA replication is a critical event during the cell cycle of proliferating cells. Faithful inheritance of genetic information requires DNA to be accurately copied once during the S phase of each cell cycle. The mechanism and cell cycle control of the initiation of eukaryotic DNA replication have been extensively studied in the budding yeast *Saccharomyces cerevisiae* (Kelly and Brown, 2000). The origin recognition complex (ORC) was first identified from *S. cerevisiae* as the initiator protein that directly binds to autonomously replicating sequences (ARS) that function as origins in chromosomes. ORC contains six subunits (Orc1p–Orc6p) that bind to the ARS elements in the genome throughout the entire cell cycle. ORC serves as a landing pad for the assembly of a series of different protein complexes on origins of DNA replication at different times during the cell division cycle, including the MCM (mini-chromosome maintenance) proteins.

In addition to its role in DNA replication, ORC is involved in transcriptional silencing at the silent mating-type loci in *S. cerevisiae* (Haber, 1998), heterochromatin binding in *Drosophila* (Pak et al., 1997), and possibly for G_2 to M phase transition (Dillin and Rine, 1998) and checkpoint regulation (Weinberger et al., 1999). Therefore, ORC-interacting proteins may well have many functions.

Proliferating cells must increase their mass prior to cell division. Cell proliferation is primarily regulated during G_1 before DNA replication (Norbury and Nurse, 1992). Such a control mechanism ensures that a critical cell size has been achieved to allow for the completion of cell division. The time in G_1 at which cells decide to undergo cell division is called START in yeast and the restriction point in animal cells. This is a critical regulatory mechanism in which a cell responds to its environment to control alternative cell fates such as cell division, quiescence, or differentiation. In an environment that allows rapid proliferation, cells must achieve a critical size in order to commit to cell division, probably to ensure that there are sufficient cellular components to ensure survival of the daughter cells. Likewise, cells reentering the cell division cycle from a period of quiescence must coordinate DNA replication with duplication of other cellular machineries, such as the protein synthesis machinery, but how this is achieved is not known.

Because ORC is the platform for the assembly of different protein complexes at origins of DNA replication, searching for proteins that interact with ORC has long been hypothesized to be an effective approach to identify other protein components involved in DNA replication and associated regulatory pathways. In this study, we performed ORC immunoprecipitation (IP) to identify its interacting proteins in *S. cerevisiae*. One of these proteins, Yph1p (YGR103W), is a conserved protein containing a BRCT domain. Recent evidence showed that the mammalian ortholog of Yph1p is upregulated in metastatic cancer tissues (Charpentier et al., 2000; Kinoshita et al., 2001). We demonstrate Yph1p is present in two multiprotein complexes. Mass spectrometric analysis of the Yph1p complexes reveals that Yph1p associates with proteins involved in ribosome biogenesis, DNA replication, regulation of chromatin structure, and cell cycle regulation, as well as checkpoint control. Genetic analyses show that Yph1p is critical for cell proliferation, DNA replication, and 60S ribosomal subunit biogenesis, and possibly translation on polysomes. The ORC-Yph1p interaction is increased in *sir2*Δ cells. The results suggest insights into the coordination of cell proliferation control with many cellular processes such as DNA replication and ribosome biogenesis.

Results

Identification of ORC-Interacting Proteins

To identify ORC-interacting proteins, IP experiments were performed from yeast whole-cell extracts using a monoclonal antibody against the largest ORC subunit, Orc1p (Figure 1). Wild-type and *orc2-1* mutant cells were grown at the permissive temperature of 25°C overnight and then shifted to a semipermissive temperature of 30°C for 3 hr. The cells were harvested and whole-cell extracts were prepared by bead beating using a lysis buffer containing ethidium bromide, which helps to extract proteins from DNA, as ORC is a chromatin-binding protein. IP was performed using an anti-Orc1p antibody.

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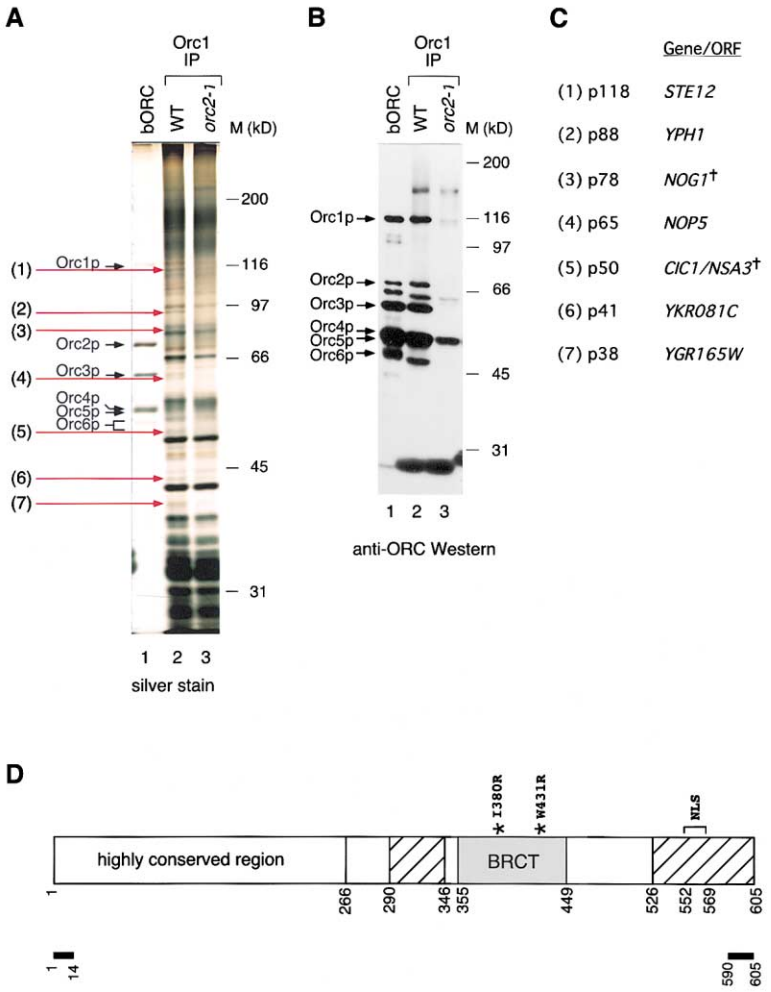

Figure 1. An Anti-Orc1 Antibody Precipitates Yph1p from Wild-Type, but Not from *orc2-1* Cell Extracts

(A and B) An anti-Orc1p antibody (SB35) was used to perform IP from W303-1A or *orc2-1* cell extracts. The immunoprecipitates were boiled in Laemmli buffer and resolved on 10% SDS-PAGE. The gels were subjected to silver staining (A) or Western blotting using a mixture of anti-Orc1p to -Orc6p antibodies as probes (B). bORC, baculovirus-expressed and purified ORC; M, molecular weight markers. The band between Orc2p and Orc3p is the 65 kDa degradation product of Orc1p.

(C) List of the putative ORC-interacting protein bands in (A) identified by peptide microsequencing analysis. Superscript dagger indicates Yph1p-interacting proteins in Figure 4C.

(D) Diagram of Yph1p. Two coiled-coil domains are marked with slanted lines. The regions corresponding to the synthetic peptides used to raise polyclonal antibodies are marked on the bottom.

After the IP, the antibody bound complexes were boiled off from the beads, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by silver staining (Figure 1A). Many bands besides the ORC subunits were present in the IP. ORC is unstable even in extracts prepared at the permissive temperature from *orc2-1* cells (Bell et al., 1993), so little or no ORC subunits were detected in the IP from these cells (Figures 1A and 1B). Therefore, we expected that ORC-interacting proteins would also be absent or much less prominent in the ORC IP from the *orc2-1* extracts. Indeed, seven bands were specifically precipitated with ORC from the wild-type extracts, but not from the mutant extracts (Figure 1A, lane 2). The identities of these seven putative ORC-interacting proteins were obtained by peptide microsequencing analysis (Figure 1C). We initially focused our effort on one novel protein with an apparent molecular weight of 88 kDa because of its potentially interesting properties.

Features of Yph1p

The 88 kDa protein shared 40% amino acid identity to an essential zebrafish protein, pescadillo (Allende et al., 1996), and is identical to the yeast pescadillo homolog Yph1p (Kinoshita et al., 2001). Yph1p is highly conserved among species with highly related orthologs found in *S. pombe*, *C. elegans*, *Drosophila*, zebrafish, mouse, and human. Motif analysis revealed that Yph1p contains two coiled-coil domains, one BRCT domain, and a putative bipartite nuclear localization signal (Figure 1D). The BRCT domain was first described at the C terminus of the breast cancer protein BRCA1 and has been found in many proteins involved in DNA damage repair and cell cycle checkpoint control. Because of the BRCT domain and the observation that the zebrafish pescadillo was essential for cell proliferation, we studied Yph1p further.

ORC and Yph1p Interact with Each Other In Vivo and In Vitro

To further demonstrate that Yph1p interacts with ORC, reciprocal IP experiments were carried out (Figures 2A–2D). Polyclonal antibodies against Yph1p were raised using synthetic peptides from the N and C terminus of the protein (indicated in Figure 1D). All generated antibodies recognized Yph1p in Western blot analysis. The antibodies, CS965 and CS967, against the C terminus of Yph1p were able to precipitate Yph1p and ORC (Figures 2A and 2B). About 3% of ORC from yeast whole-cell extracts was coprecipitated with Yph1p. The anti-

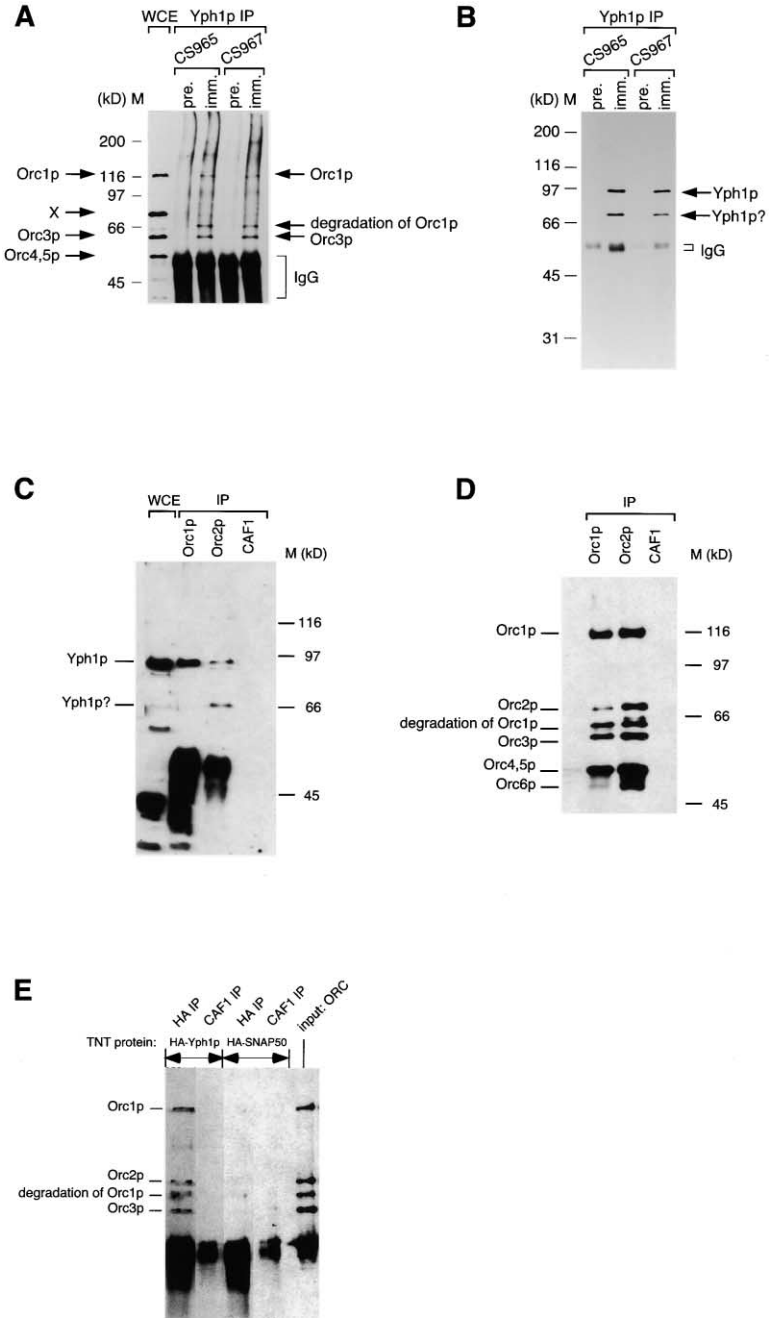

Figure 2. Yph1p Interacts with ORC In Vivo and In Vitro

(A and B) Two anti-Yph1p antibodies (CS965 and CS967) and their preimmune sera were used to perform IP from W303-1A cell extracts. The precipitates were subjected to Western blotting using a mixture of anti-ORC antibodies (A) or an anti-N terminus-Yph1p antibody (CS959) (B). WCE, whole-cell extracts; X, a crossreacting protein detected by the anti-Orc3p antibody.

(C and D) Anti-Orc1p, anti-Orc2p, and control anti-CAF1 IP from W303-1A extracts was analyzed by Western blotting using an anti-N terminus-Yph1p antibody (CS959) (C) or a mixture of anti-ORC antibodies (D).

(E) HA-Yph1p and HA-SNAP50 were in vitro transcribed/translated and mixed with purified ORC. An anti-HA IP and a control anti-CAF1 IP were performed. The immunoprecipitates were subjected to Western blotting using a mixture of anti-ORC antibodies.

body CS959 against the N terminus of Yph1p sometimes recognized an additional band corresponding to a molecular weight of ~68 kDa in both yeast whole-cell extracts and the anti-Yph1p IP. Although it has been shown that some human pescadillo proteins have a covalent attachment of the ubiquitin-like protein, SUMO-1, which causes an increase of ~20 kDa (Kinoshita et al., 2001), we did not detect SUMOlation of Yph1p using an anti-Smt3p (yeast SUMO-1) antibody in Western blot analysis against whole-cell extracts (data not shown). In addition, no modification of ubiquitin, or another ubiquitin-like protein, Rub1p, on Yph1p was detected in Western blot analysis (data not shown). More importantly, in vitro expressed Yph1p ran at 88 kDa on SDS gels, even though its calculated molecular weight is closer to 68 kDa (data not shown). Thus, the 68 kDa form of yeast Yph1p may be the cleavage or modified product (see below).

The anti-Orc1p and anti-Orc2p antibodies precipitated Yph1p and ORC, but the control anti-CAF1 antibody did not (Figures 2C and 2D). The anti-Orc2p antibody, which precipitated slightly more ORC than did the anti-Orc1p antibody, precipitated less Yph1p. It is possible that the anti-Orc2p antibody affects the interaction between ORC and Yph1p.

We then tested whether ORC and Yph1p interacted with each other without the assistance of any other yeast proteins. *YPH1* was cloned into an in vitro transcription/

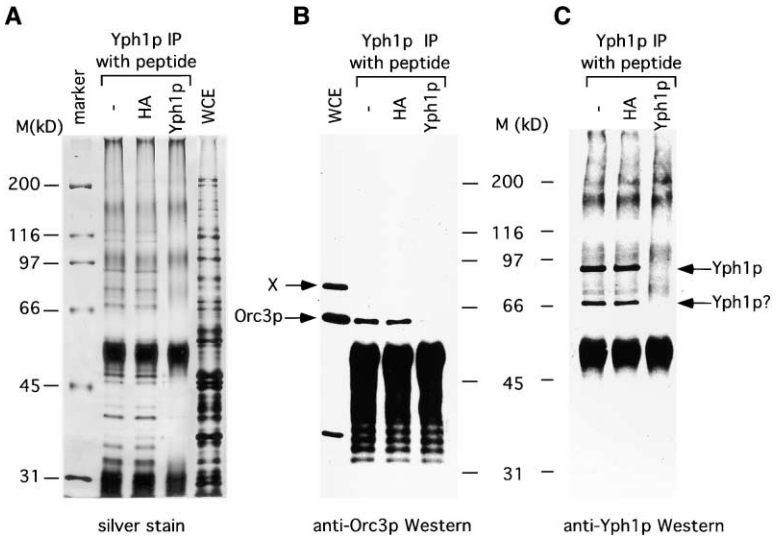

Figure 3. Yph1p Specifically Associates with a Group of Proteins

The anti-Yph1p IP was performed from W303-1A cell extracts in the absence of any additional peptide or in the presence of the HA peptide or the Yph1p-specific peptide. The immunoprecipitates were subjected to silver staining (A) or Western blotting using an anti-Orc3p antibody (B) or anti-Yph1p antibody (CS959) (C). WCE, whole-cell extracts; X, a crossreacting protein detected by the anti-Orc3p antibody.

translation vector with one copy of the hemagglutinin (HA) epitope at the N terminus. HA-Yph1p was expressed in a rabbit reticulocyte lysate system and mixed with yeast ORC purified from recombinant baculovirus-infected Sf9 cells. An in vitro transcribed/translated HA-SNAP50 protein was used as a negative control. An anti-HA IP and a control anti-CAF1 IP were performed (Figure 2E). ORC was precipitated only in the presence of HA-Yph1p by the anti-HA antibody, but not with HA-SNAP50. Furthermore, ORC was absent in the anti-CAF1 IP. Therefore, ORC specifically interacted with Yph1p in vitro, although it remains possible that the interaction involved rabbit reticulocyte proteins. Altogether, both in vivo and in vitro IP results suggest that ORC and Yph1p interact with each other.

Proliferating Cells Contain Two Yph1p Complexes

The anti-Yph1p antibody directed against the Yph1p C-terminal peptide (Figure 1D) immunoprecipitated a group of proteins from yeast whole-cell extracts (Figure 3A). To test if all these proteins precipitated specifically with Yph1p, a peptide competition experiment was performed. Nonspecific HA peptide or the Yph1p C-terminal peptide antigen was mixed with the anti-Yph1p antibody before the addition of yeast whole-cell extracts. The same group of proteins was brought down in the presence or absence of the HA peptide, and these proteins in the IP were not abundant yeast proteins compared to those in the whole-cell extracts (Figure 3A). In contrast, the IP was blocked by preincubation with the Yph1p-specific peptide antigen. Only the background pattern of the boiled-off antibodies was detected. Furthermore, Figures 3B and 3C show that (1) a crossreacting 70 kDa protein detected by the anti-Orc3p antibody was not coprecipitated with Yph1p, and (2) the precipitation of Orc3p and Yph1p was blocked by the specific peptide, but not by the HA peptide. Therefore, this group of proteins specifically associates with Yph1p.

To distinguish further whether the proteins associated with Yph1p constitute one or multiple Yph1p-containing complexes, Yph1p immunoprecipitates were eluted from the antibody beads with an excess of the Yph1p-specific peptide antigen and fractionated by sedimentation through a glycerol gradient. Western blot analysis with anti-Yph1p antibodies of the fractions from the glycerol gradient revealed the existence of two major Yph1p complexes (Figure 4A). One complex had a sedimentation rate slightly slower than that of catalase (200 kDa), while the other containing Yph1p, Orc3p, and MCM6 (Figure 4A) was larger than 2 MDa. Proteins present in the small and large complexes are shown in Figures 4B and 4C, respectively.

To understand the possible functions of Yph1p through its interacting partners, major bands in the two Yph1p complexes were excised from the gels and were analyzed by mass spectrometry. The small Yph1p complex contained three proteins, Erb1p, Yph1p, and Ytm1p (Figure 4B), whereas the large complex contained many more proteins, as expected (Figure 4C). *ERB1* is an essential gene required for 60S ribosome biosynthesis (Pestov et al., 2001a). Ytm1p, cited as a microtubule-associated protein essential for the G_1/S transition (Hodges et al., 1999), is also involved in 60S ribosomal subunit biogenesis (Harnpicharnchai et al., 2001). Both of these proteins were also found in the large Yph1p complex. In addition, proteins in the large complex include nucleolar proteins, proteins involved in 60S ribosome biosynthesis, and the 60S ribosomal proteins (Supplemental Table S1 available at http://www.cell.com/cgi/content/full/109/7/835/DC1; Bassler et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001). Therefore, Yph1p may have a role in ribosome biogenesis. Altogether, these results suggest that two Yph1p complexes exist in yeast, and the smaller complex may be the core of the larger one, since they share the three proteins.

In addition to the major proteins potentially stoichiometric with Yph1p, many other proteins were associated with Yph1p. Direct mass spectrometric analysis of Yph1p complexes was performed to identify these sub-stoichiometric proteins (Supplemental Table available at above website, group 2). Orc6p, MCM6, cell-cycle regulatory proteins, checkpoint proteins, and proteins

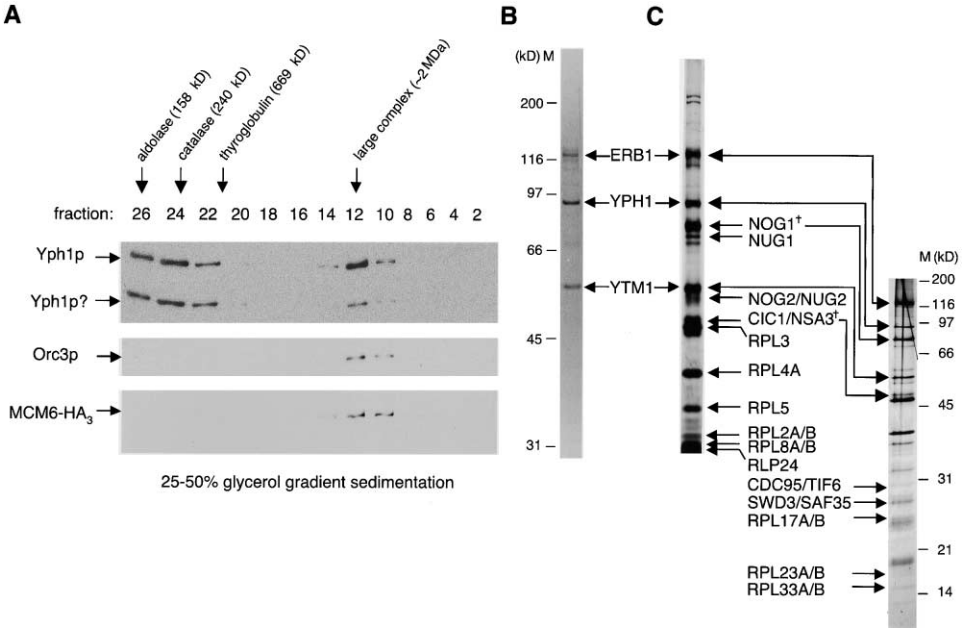

Figure 4. The Existence of Two Yph1p Complexes

(A) Yph1p immunoprecipitates were eluted from the antibody beads using the Yph1p-specific peptide and were fractionated in a 25%–50% glycerol gradient. Yph1p, Orc3p, and MCM6-HA_3 across the gradient were analyzed by Western blotting.

(B) Coomassie blue-stained gel of the small Yph1p complex (fraction 22 in a 15%–35% glycerol gradient).

(C) Silver-stained gels of the large Yph1p complex. Right, 10% gel; left, 17% gel. Labeled bands were identified by mass spectrometry. Superscript dagger indicates putative ORC-interacting proteins in Figures 1A and 1C.

involved in chromatin remodeling were also present in the Yph1p immunoprecipitates. The presence of Erb1p, Rpl3p, MCM6, Cdc28p, Mec1p, Dun1p, and Rad23p in Yph1p complexes was confirmed by IP with the anti-Yph1p antibody and Western blot analysis (Figure 4A and data not shown). In addition to MCM6, we also detected MCM4, 5, and 7 in the Yph1p IP by Western blot analysis (data not shown). The association of Yph1p with replication and checkpoint proteins suggests a potential role of Yph1p in DNA replication and checkpoint control.

yph1-td Is Defective for 60S Ribosomal Subunit Biogenesis

The presence of many ribosomal proteins in the large Yph1p complex prompted us to examine the ribosome profile in *yph1-td*, a temperature-inducible degron mutant strain. The *yph1-td* mutant cell had a relatively smaller size and grew more slowly than its isogenic wild-type cell at the permissive temperature of 25°C. Extracts prepared from the *yph1-td* and isogenic wild-type cells grown to log phase at 25°C and shifted to the nonpermissive temperature of 37°C for 3 hr before harvest were fractionated by sucrose velocity gradient centrifugation. At the nonpermissive temperature, Yph1p protein was degraded in mutant cells (data not shown). Compared to the profile of wild-type cells (Figure 5A), in the Yph1p-depleted cells free 60S ribosomal subunits were not detected, polysomes were reduced substantially, and 80S ribosome levels were increased (Figure 5B). This result suggests that Yph1p is required for 60S ribosomal subunit synthesis or stability, and the absence of polysomes in the mutant suggests a reduction in the rate of protein synthesis. The position of Yph1p in a sucrose gradient of the wild-type cell extracts (shown in Figure 5A) was examined and interestingly, the 88 kDa form of Yph1p cosedimented with 80S ribosomes, but the 68 kDa form cosedimented with polysomes (Figure 5C). It is possible that Yph1p is a component of preribosomal particles, then remains associated with 80S ribosomes, but is modified into the 68 kDa form in polysomes. In this experiment, cosedimentation of Yph1p with 60S ribosomal subunits was not observed, but it is probably because of the relatively low amount of material in the 60S.

Yph1p Is an Essential Protein Located Predominantly in the Nucleolus

To investigate the phenotype of a *YPH1* null mutant, the entire coding sequence of one *YPH1* gene in a diploid strain was replaced with a selectable kanMX6. Tetrad analysis of the heterozygous knockout demonstrated that *YPH1* is essential, consistent with previous findings (Kinoshita et al., 2001). Microscopic examination of the meiotic segregants revealed that none of the nonviable spores divided, and 8 of the 54 spores had a dumbbell-shaped terminal morphology while the rest remained as single unbudded cells. A complementation analysis revealed that the lethal phenotype could be rescued by an additional copy of *YPH1* on a plasmid (data not shown). In a separate genetic analysis, when one of two separate, plasmid-borne mutations (W431R and I380R) within the BRCT domain of Yph1p was used to replace the wild-type gene in a haploid strain, both strains,

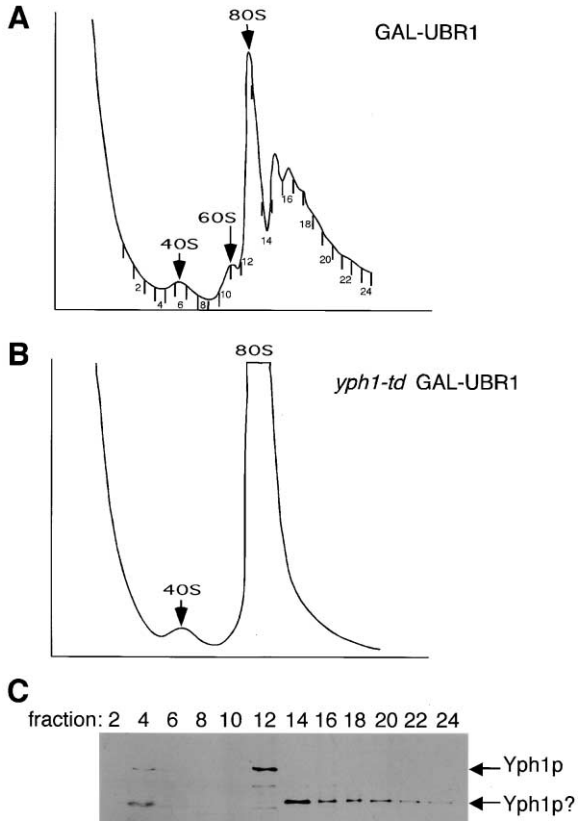

Figure 5. Yph1p Is Required for Biogenesis of 60S Ribosomal Subunit

The isogenic wild-type (A) and *yph1-td* (B) strains growing at 25°C in YPRaf were shifted to 37°C in YPGal for 3 hr. Extracts were resolved in a 7%–47% sucrose gradient. The peaks of free 40S and 60S ribosomal subunits and 80S ribosomes are indicated.

(C) Yph1p across the gradient from (A) was analyzed by Western blotting using anti-N terminus antibody (CS959).

yph1-1 and *yph1-2*, were temperature sensitive at 37°C (data not shown). W431 is the most conserved residue of the BRCT domain, and W431R corresponds to a cancer-predisposing mutation (W1837R) in BRCA1. These results demonstrate that *YPH1* is an essential gene and the BRCT domain is crucial.

Since Yph1p is an ORC-interacting protein, we were interested in studying its role in DNA replication. The temperature-sensitive mutant, *yph1-td*, and isogenic wild-type cells were inoculated into media, producing separate log phase cultures at 25°C, and then shifted to 37°C for 3 hr. The DNA content was analyzed by flow cytometry. The *yph1-td* mutant yeast arrested with either a 1C or 2C DNA content at 37°C, with no cells in S phase (Figure 6A), similar to other temperature-sensitive alleles (Kinoshita et al., 2001). Furthermore, inactivation of *yph1-td* caused very slow S phase progression after cells were released from a hydroxyurea (HU) block, whereas the isogenic wild-type cells completed the S phase normally (Figure 6B). Because Yph1p was required for 60S ribosomal subunit biogenesis, it was possible that the S phase delay of *yph1-td* mutant yeast was merely an indirect effect due to a defect in protein synthesis or ribosome biogenesis, rather than a direct effect of Yph1p on DNA replication. To test this possibility, the protein synthesis inhibitor cycloheximide and a *nop1-7* mutant strain with a defect in 60S ribosomal subunit assembly (Tollervey et al., 1993) were used. Cells that were released from HU block progresses through S phase normally in the presence of cycloheximide or *nop1-7* at the nonpermissive temperature (Figure 6B). Therefore, Yph1p was required for normal S phase progression and the S phase delay in the *yph1-td* mutant was not an indirect effect of impaired protein synthesis or ribosome biogenesis.

The BRCT domain has been found in many nuclear proteins involved in DNA damage checkpoint and cell cycle control. The presence of a BRCT domain and a bipartite nuclear localization signal within Yph1p raised the possibility that Yph1p was a nuclear protein. To address the subcellular localization of Yph1p, a C-terminal GFP- tagged endogenous *YPH1* diploid strain was generated. Figure 6C shows that Yph1p is located predominantly in or near the crescent-shaped nucleolus and, to a lesser extent, in the nucleoplasm.

Yph1p Levels Are Downregulated before the Diauxic Shift and Respond to Energy Source

We noticed that the protein levels of Yph1p varied in cells grown under different conditions and it was traced to the carbon source used for growth. Cells grown in glucose and that do not respire had higher levels of Yph1p than cells grown in glycerol or acetate where respiration is required for growth (data not shown). Eventually, cells grown in glucose accumulate ethanol and undergo a diauxic shift. We followed Yph1p levels during the diauxic shift by growing a wild-type yeast culture at 30°C for 26 hr (Figure 7A). After an initial 12 hr proliferation, cells were harvested for eight successive 2 hr intervals, and whole-cell extracts were prepared. The expression levels of Yph1p, Nop1p, Nop5p, Rpl3p, Orc3p, and α-tubulin were analyzed by Western blotting (Figure 7B). During the exponential growth phase, Yph1p levels were roughly constant; however, Yph1p levels dropped as cells committed to exit the proliferation phase as indicated by the DNA content profiles and the increased doubling time (Figure 7C). Interestingly, Yph1p disappeared at exactly at the same time as cells with a 1C DNA content and reduced S phase began accumulating (compare 20 hr with 22 hr, Figure 7C). Nop1p, required for 35S rRNA processing, and Nop5p, required for pre-18S rRNA processing, disappeared in the stationary culture after Yph1p. In contrast, Rpl3p, a 60S ribosomal protein, Orc3p, and α-tubulin levels slightly decreased in the stationary culture. Note that although the *RPL3* mRNA level was significantly repressed during the diauxic shift (DeRisi et al., 1997), its protein levels did not decline much. Like cells exiting cell division, cells grew more slowly when Yph1p was overexpressed (data not shown). The protein data combined with microarray data showing Yph1p mRNA levels declining during a diauxic shift (DeRisi et al., 1997) demonstrate that Yph1p levels correlate with the rate of proliferation in response to energy source.

Yph1p Is Required to Initiate Proliferation after Exit from G_0

The demonstration that Yph1p disappears upon commitment to reduced proliferation raised the issue of

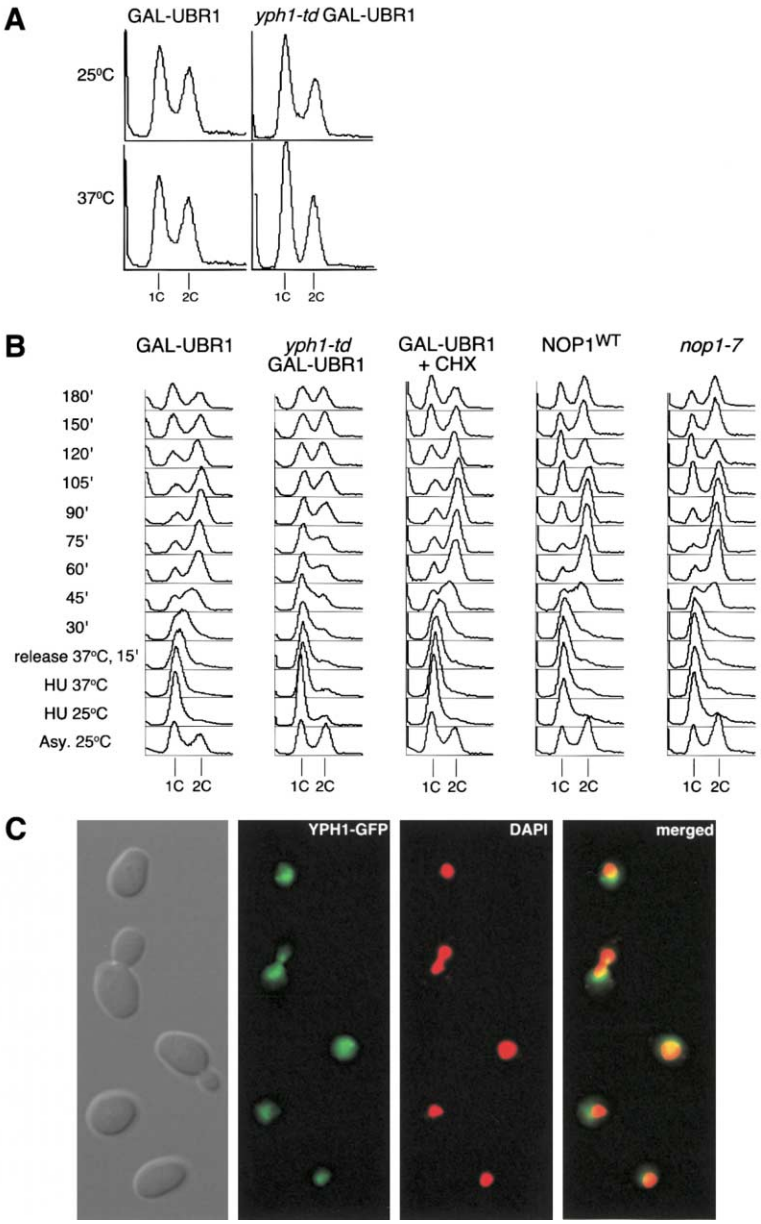

Figure 6. Yph1p Is an Essential Nucleolar Protein Required for Normal S Phase Progression

(A) *yph1-td* mutant arrests at G_1 or G_2 with no S phase. The *yph1-td* and isogenic wild-type strains growing at 25°C in YPRaf were shifted to 37°C in YPGal for 3 hr. Aliquots of cultures were analyzed by FACS.

(B) The indicated strains were arrested in early S phase with HU at 25°C in YPRaf for 2 hr and shifted to 37°C in YPGal for 1 hr. Then, they were released into fresh YPGal at 37°C. For the cycloheximide treatment, 10 μg/ml of cycloheximide was added 1 hr prior to release and was present in the fresh YPGal medium. Aliquots of the culture were analyzed by FACS.

(C) Yph1p is located predominantly in or near the nucleolus. GFP signal from exponentially growing YPH1-GFP diploid cells was visualized by fluorescence microscopy. The DNA staining by DAPI was represented by the red color to identify the overlapping regions with the green signal from YPH1-GFP.

whether Yph1p was required for entry into the cell cycle from G_0. To this end, an exponentially proliferating *yph1-td* mutant and its isogenic wild-type cells were shifted from YP-raffinose to nitrogen-free medium (YN′) to induce cells to enter stationary phase. The cultures were split in two and then released into rich medium (YP) in the presence (raffinose, 25°C) or absence (galactose, 37°C) of newly synthesized Yph1p. Without newly synthesized Yph1p, the mutant cells remained unbudded with a 1C DNA content and were unable to enter the cell division cycle even after 12 hr in rich medium at 37°C (Figure 7D). These cells were viable when plated onto YPRaf plates at 25°C (data not shown). Therefore, the proliferation inhibition was reversible.

An important consideration was whether the requirement of Yph1p to initiate cell proliferation from G_0 was only an indirect effect due to a defect in ribosome biogenesis. Note that a population of ribosomes still exists in the stationary cells (Rpl3p in Figure 7B). To address this question, *nop1-7*, the mutant strain with a defect in 60S ribosomal subunit assembly, was used. A stationary phase culture of *nop1-7* mutant or isogenic wild-type cells was released into rich medium at permissive (25°C) or nonpermissive (37°C) temperatures. Because Nop1p protein has already disappeared in the stationary culture (Figure 7B), any newly synthesized Nop1p will be the mutant form in *nop1-7* cells. However, *nop1-7* cells were still able to exit from G_0 to enter the cell division cycle at 37°C. These results indicate that Yph1p, but not new ribosome biogenesis, is crucial for cells to exit from the G_0 state and initiate cell proliferation.

Increased Interaction of Yph1p and ORC in *sir2*Δ Strain

Each rDNA repeat in the *S. cerevisiae* genome contains an origin of DNA replication and hence an ORC binding

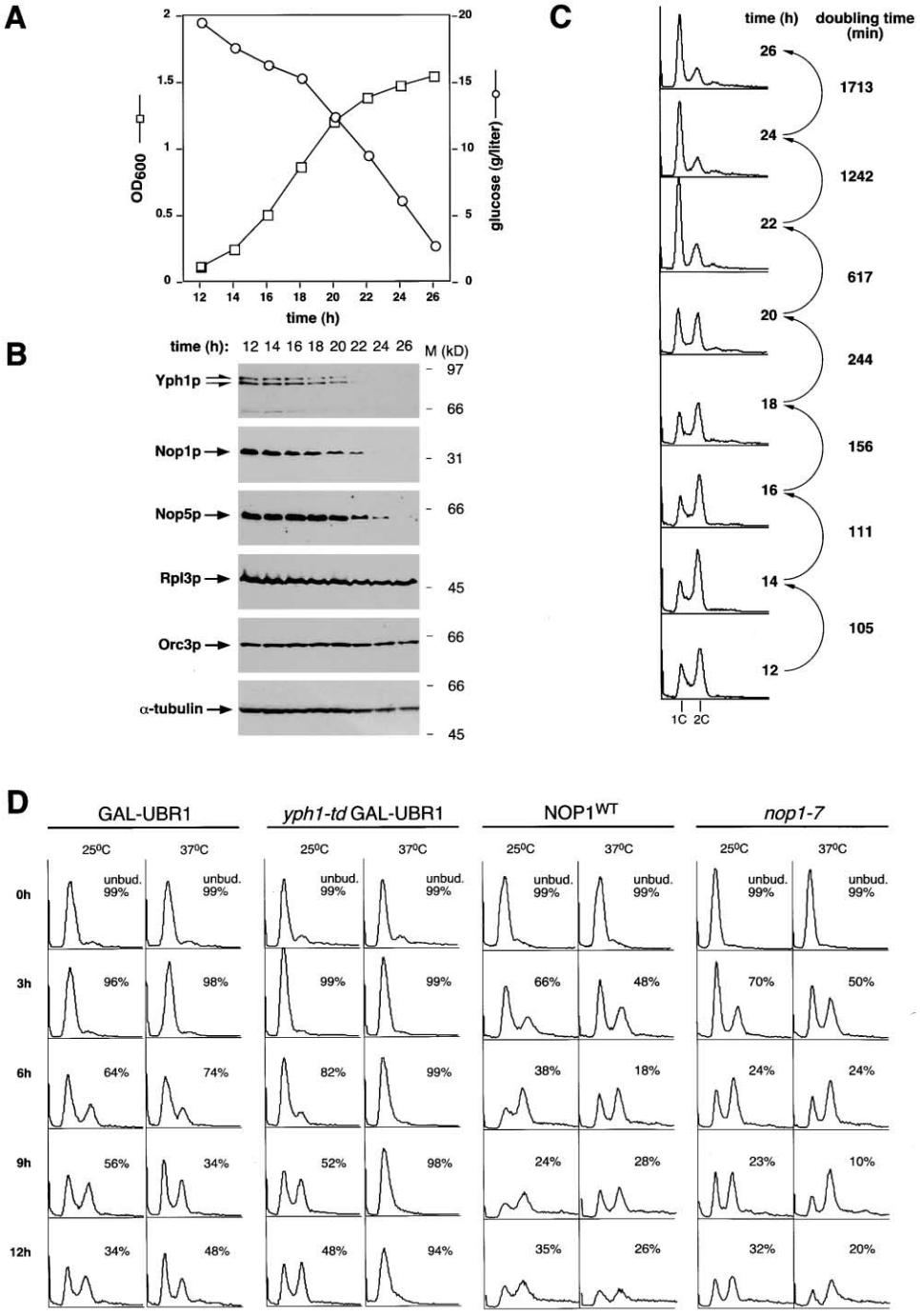

Figure 7. Yph1p Is Linked to Cell Proliferation Stages

(A–C) Yph1p is downregulated during the diauxic shift.

(A) W303-1A cells were grown at 30°C in YPD and were harvested at eight successive 2 hr intervals. Cell density was measured by OD at 600 nm wavelength. The glucose concentration of the media was measured with a UV test kit (Boehringer Mannheim, #716251).

(B) Western blot analysis of Yph1p, Nop1p, Nop5p, Rp13p, Orc3p, and α-tubulin during the course of the experiment.

(C) Aliquots of the culture were analyzed by FACS. The doubling time of the culture between each 2 hr interval is indicated on the side.

(D) Yph1p is required to initiate growth after G_0. Indicated strains in YPRaf were washed three times by nitrogen-free medium (YN′) and were resuspended in YN′ followed by incubation at 25°C for 39 hr. Resulting G_0 cells were released from YN′ to YPRaf at 25°C or to YPGal at 37°C. Samples were taken every 3 hr after the release. Aliquots of cultures were analyzed by FACS, and the percentage of unbudded cells in these cultures was determined by light microscopy.

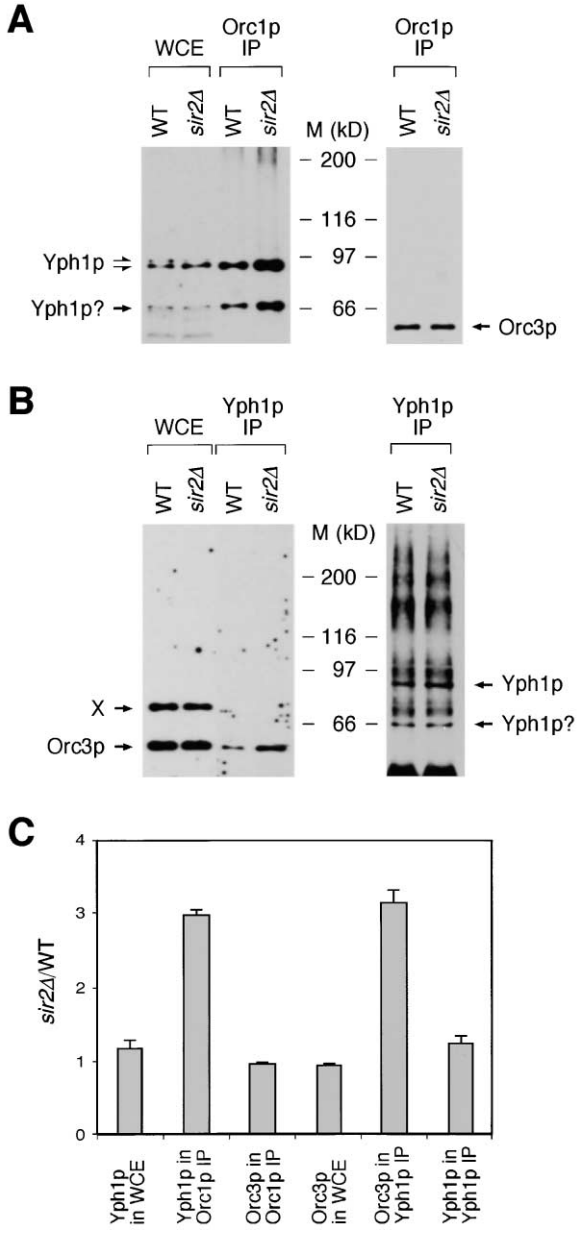

Figure 8. The Interaction of Yph1p and ORC Is Increased in sir2Δ Strain

An anti-Orc1p antibody (A) or an anti-Yph1p antibody (B) was used to perform IP from W303-1B or *sir2*Δ cell extracts. The immunoprecipitates were boiled in Laemmli buffer and resolved on 10% SDS-PAGE. The gels were subjected to Western blotting using anti-Yph1p or anti-Orc3p antibody as probes. WCE, whole-cell extracts; X, a crossreacting protein detected by the anti-Orc3p antibody.

(C) The ratio of the indicated proteins from *sir2*Δ versus wild-type was quantified. The figure shows the mean of three determinations ± SD.

site, a feature conserved in all rDNA repeats in every eukaryote examined. Since the NAD-dependent histone deacetylase Sir2p regulates the rDNA locus, we tested whether Sir2p influenced the interaction between Yph1p and ORC. Whole-cell extracts of *sir2*Δ and wild-type strains were prepared and an anti-Orc1p IP or an anti-Yph1p IP were performed (Figure 8). The anti-Orc1p antibody precipitated the same amount of Orc3p in both extracts, but 3-fold more Yph1p was precipitated from *sir2*Δ extracts than from wild-type extracts. Similarly, the anti-Yph1p antibody precipitated 3-fold more Orc3p from *sir2*Δ extracts than from wild-type cell extracts. Thus, the interaction between Yph1p and ORC is increased when the rDNA locus is derepressed in the absence of Sir2p.

Discussion

Possible Links between Ribosome Biogenesis, DNA Replication, and Cell Proliferation Control

The search for ORC-interacting proteins led to the surprising discovery that ORC links to a large protein complex through an interaction with Yph1p. Characterization of Yph1p also suggested links between cell proliferation control in response to energy sources and DNA replication, ribosome biosynthesis, and efficient translation on polysomes. Two Yph1p interdependent complexes exist and share the common proteins Yph1p, Erb1p, and Ytm1p, suggesting that the smaller complex forms a core for assembly of the larger one. The observation that Yph1p mutants arrest at G_1 and G_2 phases without any cells in S phase (Figure 6 and Kinoshita et al., 2001) and the observation that Yph1p mutants cells remain viable but do not enter the cell division cycle from a G_0 state suggests that Yph1p may be involved in sensing pathways that coordinate cell proliferation control with many important cellular processes.

We define cell proliferation control as mechanisms that determine whether cells are quiescent or are progressing through the cell cycle. In yeast, proliferation is primarily regulated by available energy sources and it makes sense to link high energy-requiring processes such as DNA replication, ribosome biosynthesis, and rapid protein synthesis to a regulatory mechanism that senses energy levels. Yph1p levels were sensitive to carbon source, being high in glucose when respiration is suppressed and low or absent when slow proliferation is triggered by poor energy sources. The levels of Yph1p in a cell affect cell proliferation since we found that overexpression of Yph1p causes slow proliferation. As cells undergo the diauxic shift, converting from fermentation to gluconeogenesis and respiration, Yph1p levels dramatically fall and concomitantly the rate of proliferation slows. Previous studies of global gene expression during the diauxic shift using DNA microarrays revealed that the *YPH1* mRNA level was repressed before the diauxic shift and continued to be repressed upon entry into stationary phase (DeRisi et al., 1997). Other genes encoding proteins in the small and large Yph1p complexes we have identified are transcriptionally coregulated (Wade et al., 2001). Genes that have a similar temporal pattern as *YPH1* are mostly of unknown function, but genes encoding ribosomal proteins are repressed at a later time (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/109/7/835/DC1).

During the diauxic shift, cells respond to the stress of energy reduction. Yph1p may be responsive to energy source stress, since Yph1p levels are reduced as proliferating cells begin to run out of glucose and are also low in cells grown on poor energy sources such as

acetate and glycerol. One of the proteins we found linked to the Yph1p complex was Sok1p. Mutations in *SOK1* suppress depletion of cyclic-AMP protein kinase (Ward and Garrett, 1994), possibly linking cAMP signaling to the function of the Yph1p complex. In addition, Yvh1p, which interacts with the BRCT domain of Yph1p (Sakumoto et al., 2001), may also participate in the cAMP-PKA pathway regulation of Yph1p functions (Beeser and Cooper, 2000).

Yph1p and Ribosome Biogenesis

A flurry of recent reports describe overlapping, but different large protein complexes containing Yph1p (also called Nop7p) and other proteins found in the Yph1p complexes described herein, including Erb1p, Ytm1p, Nog1p, Nog2p/Nug2p, Nug1p, Cic1p/Nsa3p, Rlp24p, and Cdc95p/Tif6p (Bassler et al., 2001; Basu et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001; Senger et al., 2001; Warner, 2001; Wood et al., 1999). A tagged version of Yph1p/Nop7p was shown to be a nucleolar protein that coprecipitated with many proteins, including all 40S and 60S ribosomal proteins, some of the proteins we identified, but also many that were not identified herein (Adams et al., 2002; Harnpicharnchai et al., 2001). Yph1p was present in 60S-66S preribosome particles that were involved in the processing of rRNA from its precursor forms in the nucleolus and transport of the assembled preribosomes to the cytoplasm. However, many proteins that were found in the 60S or 66S preribosomal particles were not present in our large Yph1p complex, for example, the 40S ribosomal proteins found in the 66S particle and many nonribosomal proteins (e.g., Loc1p, Mrt4p, etc.). Note that a glycerol gradient step and different buffer compositions were employed in our purification of Yph1p complexes; thus, it is not surprising that the 66S particle, identified by purifying a tagged version of Yph1p/Nop7p (Harnpicharnchai et al., 2001), yielded a different complex to that described herein. Since we found many nuclear proteins associated with Yph1p, it is possible that under our conditions, only a subset of the potential Yph1p complexes was identified. Furthermore, Yph1p has been found in a fraction enriched for nuclear pore complexes, probably trapped during transport to the cytoplasm with the rDNA (Rout et al., 2000). A role for Yph1p in 60S ribosome particle biogenesis is consistent with these observations. What emerges from all these results, however, is that Yph1p may exist in a series of dynamic, partially related complexes.

Erb1p, a component of the small and large Yph1p complexes identified herein, is the yeast ortholog of mouse Bop1 (Pestov et al., 2001a). Expression of an N-terminally truncated form of Bop1 causes G_1 arrest of mouse cells and a deficit in 60S ribosomal subunit biogenesis (Pestov et al., 2001b; Strezoska et al., 2000). Interestingly, the G_1 arrest only occurs in cells with intact p53 tumor suppressor protein. Components of the p53 pathway, p53, Mdm2, and $p19^{Arf}$ have been shown to be located in the nucleolus (Tao and Levine, 1999). Previous studies have shown that p53 associates with some components of ribosomes. For example, the p53-Mdm2 complex interacts with ribosomal protein L5 and 5S rRNA (Marechal et al., 1994), and p53 was shown to bind to DNA sequences near replication origins in the ribosome gene cluster (RGC; Kern et al., 1991). Interestingly, p53 inhibits DNA replication of plasmids containing the RGC DNA sequences (Miller et al., 1995), suggesting that p53 may regulate DNA replication via specific sequences. Furthermore, p53 is negatively regulated by Sir2α, the mammalian homolog of Sir2p (Luo et al., 2001). Since deletion of *SIR2* in yeast causes an increase in the interaction between ORC and Yph1p (Figure 8), we suggest that mammalian Yph1p and ORC might be linked to p53 and Sir2α control of DNA replication or ribosome biosynthesis.

There are other intriguing links to unregulated proliferation among the group of proteins in the large Yph1p complex. Nog2p/Nug2p is the yeast ortholog of a human breast tumor-associated autoantigen (Racevskis et al., 1996). The human Yph1/pescadillo protein, also localized in the nucleolus, is upregulated in malignant mouse astrocytes following the loss of the tumor suppressor protein p53 (Kinoshita et al., 2001). We suggest that pathways involving Yph1p and its associated proteins are controlled by cell proliferation signals that are disrupted in tumor cells. Yph1p itself may be a key regulator or sensor of this pathway since cells lacking this protein, but not those lacking functional Nop1p, fail to enter the cell division cycle when released from a G_0 arrest.

DNA Replication

The role of Yph1p in DNA replication was suggested because Yph1p directly associates with ORC and exists in a complex containing at least four of the MCM proteins, as well as proteins having roles in DNA metabolism, cell cycle control, and checkpoint pathways (Supplemental Table S1 available at http://www.cell.com/cgi/content/full/109/7/835/DC1). Whether the putative nonstoichiometric group of proteins exists in a single complex or in many different dynamic complexes remains to be determined. In proliferating cells, depletion of Yph1p arrests cells specifically either in G_1 or G_2. Similar results were obtained when two different temperature-sensitive alleles of *YPH1* were shifted to the nonpermissive temperature (Kinoshita et al., 2001). In the absence of Yph1p, cells failed to progress normally though S phase following release from a HU block, even though ribosome biosynthesis and protein synthesis were not required for S phase progression. Therefore, we suggest that Yph1p plays a role in ensuring efficient DNA replication in proliferating cells.

Nog1p and Cic1p/Nsa3p were not only present in the large Yph1p complex, but they were also identified as ORC-interacting proteins in the original anti-Orc1p IP (Figure 1). The association of Nog1p and Cic1p/Nsa3p with both complexes provides further evidence for the existence of a common complex containing ORC, Yph1p, Nog1p, and Cic1p/Nsa3p in vivo. Two facts have recently been shown (1) Cic1p/Nsa3p associates with the 26S proteasome and links Cdc4p and Grr1p, two F box proteins, to ubiquitin-mediated protein degradation (Jager et al., 2001). Cdc4p and Grr1p are important for cell proliferation since Cdc4p is involved in controlling the levels of two key DNA replication proteins Cdc6p and Sic1p, and Grr1p is required for glucose signaling (Dutta and Bell, 1997; Hodges et al., 1999; Kelly and

Brown, 2000). (2) Nog1p interacts with the Nog2p-TAP complex and also interacts with MCM6 in a two-hybrid assay (Schwikowski et al., 2000). In fact, we detected substoichiometric amounts of MCM6 in the Yph1p complex. MCM6 also has a two-hybrid interaction with Krr1p (Uetz et al., 2000), whose ortholog Mis3, the *S. pombe* mini-chromosome instability protein, is essential for ribosome biogenesis and S phase checkpoint regulation (Kondoh et al., 2000). How all these proteins actually coordinate together remains to be determined, but they potentially link many cellular processes.

The rDNA repeats in all eukaryotic cells that have been characterized contain an origin of DNA replication within each repeat, and the direction of transcription and DNA replication is coincident. In *S. cerevisiae*, Fob1p functions as a DNA replication fork arrest protein that ensures that DNA replication does not conflict with rDNA transcription (Kobayashi and Horiuchi, 1996). Interestingly, Nog1p, a putative GTP binding protein that is present in the large Yph1p complex and the anti-Orc1p IP, has a two-hybrid interaction with Fob1p (Schwikowski et al., 2000). We suggest that in rapidly proliferating cells, the ORC-Yph1p interaction may link rDNA transcription to replication of the locus. In fact, coincident replication and transcription of rDNA genes in yeast has been observed by electron microscopy (Saffer and Miller, 1986). We suggest that one reason why DNA replication and transcription of rDNA may be linked is that proliferating cells require a large amount of new ribosome synthesis so that the daughter cells inherit sufficient ribosomes. In contrast, in nonproliferating cells where only maintenance of ribosome levels is necessary, rDNA transcription would be significantly downregulated, similar to the transcription of ribosomal protein genes (DeRisi et al., 1997).

In many species, such as *Xenopus* and *Tetrahymena*, rDNA is amplified when more ribosomes are needed (Engberg, 1985; Stark and Wahl, 1984). Interestingly, the direction of transcription and replication are coordinated, even though the position of the origins of rDNA replication is different in the various amplicons. It is therefore possible that Yph1p orthologs coordinate rDNA amplification with ribosome biosynthesis. In mammalian cells, where amplification does not normally occur, DNA replication initiates in distinct foci that surround the nucleolus in primary mammalian cells, but interestingly, not in tumor or immortal cells (Kennedy et al., 2000). Each focus may represent a large number of clustered origins that contain sites of rDNA transcription since the nucleolar UBF transcription factor colocalized with the replication foci. We suggest that mammalian tumor and immortal cells, which continually proliferate, may have lost a fundamental regulatory system that links DNA replication and rDNA transcription. In normal cells that can cease proliferation, the processes may be linked to cell signaling pathways that are disrupted in tumor cells. As stated above, the p53 tumor suppressor pathway may be a critical component of this regulatory system.

Translation on Polysomes

Although not studied extensively here, we did observe a dramatic reduction in the number of polysomes in proliferating cells when Yph1p was depleted. We also found that Yph1p was modified to a lower molecular mass form on polysomes in wild-type cells compared to the Yph1p present on 80S ribosomes or preribosomal particles. It is possible that Yph1p modification (cleavage) may occur only when efficient translation of mRNA occurs on polysomes, perhaps linking efficient translation to other cells processes and cell proliferation. But clearly more needs to be done to determine if this is the case.

Perspective

The primary function of the nucleolus is a factory for ribosome assembly, but recent studies have attributed additional functions to the nucleolus, including regulation of the cell cycle, telomerase activity, p53 activity, signal recognition particle biogenesis, small RNA processing, and mRNA transport (Olson et al., 2000). Although we have favored the view that ORC and Yph1p may serve a critical role in coordinating the processes of ribosome biosynthesis and DNA replication with cell proliferation control, it is possible that Yph1p could be involved in these processes independently. Further studies will be required to distinguish these possibilities.

The Yph1p family is highly conserved in eukaryotes. The high degree of sequence identity among species suggests that the functions of the homologs may be also evolutionarily conserved. The zebrafish homolog, pescadillo, has been shown to be required for cell proliferation during embryonic development (Allende et al., 1996). Some regions of the embryo express pescadillo strongly, and in the mutant, these particular regions fail to proliferate and the embryo dies on the sixth day of development. Mammalian homologs of Yph1p were found to be upregulated in metastatic cancer tissues. For instance, mouse pescadillo is upregulated in malignant $p53^{-/-}$ astrocytes, and human pescadillo is overexpressed in various cancer cell lines (Kinoshita et al., 2001). In fact, out of 12,550 transcripts examined in breast cancer cells, human pescadillo was one of only nine transcripts induced more than 10-fold at 3 hr postestrogen treatment (Charpentier et al., 2000). None of these nine transcripts is related to protein synthesis or ribosome biogenesis except for human pescadillo. We suggest that this unusual regulation reflects the above mentioned coupling of Yph1p with ribosome biogenesis, DNA replication, and unchecked cell proliferation.

Experimental Procedures

Strains and Media

The strains used here were W303-1A (*MAT*a *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*), W303-1 (*MAT*a/α), or their derivatives. To generate a heterozygous *YPH1* knockout diploid strain (YND001), a deletion cassette was generated by fusion PCR as described previously (Amberg et al., 1995). The deletion cassette consists of 200 bp of YPH1 upstream region followed by the kanMX module and then 200 bp of YPH1 3′-UTR. To generate mutations within the BRCT domain, a QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used, and the resulting strains, *yph1-1* (YND006) and *yph1-2* (YND007), are *yph1*Δ in W303-1A containing pRS415-YPH1^{W431R} and ^{I380R}, respectively. To generate YPH1-GFP haploid strains, a PCR-based gene modification method was performed as described previously (Longtine et al., 1998), except that the gene-specific sequences included in the primers were 70 nucle-

otides in length. A YPH1-GFP diploid strain (YND010) was generated by crossing YPH1-GFP haploid cells. The *yph1-td* mutant and isogenic wild-type strains (YND005 and YND003) were constructed by a heat-inducible degron method (Labib et al., 2000). YP medium contained 1% yeast extract, 2% bacto peptone, and was supplemented with glucose (YPD), raffinose (YPRaf), or galactose (YPGal) to a final concentration of 2%. Nitrogen-free medium (YN′) was a modification of Y-N medium (Rivin and Fangman, 1980), and contained per liter, 1.45 g Difco yeast nitrogen base (Difco Laboratories) without amino acids and without ammonium sulfate, 10 g succinic acid, 6 g NaOH, 20 mg adenine, 20 mg uracil, 40 mg tryptophan, 100 mg leucine, and 20 g glucose.

Protein Identification

The putative ORC-interacting proteins were excised from a Coomassie blue-stained gel and in-gel digested by lysylendopeptidase. The resulting peptides were separated by reverse-phase chromatography and sequenced by automated Edman degradation. The Coomassie blue- or silver-stained protein bands of Yph1p IP were digested with modified trypsin (Roche) and analyzed by the following two methods. (1) Liquid chromatography-mass spectrometry (LCQ MS/MS; Hewlett-Packard HP1100 connected to a Thermo-Finnigan LCQdeca electrospray ionization ion trap mass spectrometer), wherein the acquired MS data were analyzed by SEQUEST software (Thermo-Finnigan) against the National Center for Biotechnology Information (NCBI) database. (2) Matrix-assisted laser desorption ionization—time of flight (MALDI-TOF). The program PROWL (http://prowl.rockefeller.edu) was used to identify the proteins.

Generation of Anti-Yph1p Antibodies

Two synthetic peptides with extra cysteine on the terminus (MRIKK KNTRGNARNC and CQIAKQKAKLNKLDSKK) derived from Yph1p were conjugated to KLH (Pierce) and used for inoculation into rabbits for polyclonal antibodies production (Covance).

Immunoprecipitation, Glycerol Gradient Sedimentation, and Western Blot Analysis

The following procedures were carried out on ice or at 4°C except for elution. Yeast whole-cell extracts were prepared in L100 buffer (20 mM Tris-HCl [pH 7.8], 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 0.1% Triton X-100, 100 mM $(NH_4)_2SO_4$, 5 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM $NaVO_3$, and protease inhibitors, including 0.5 mM PMSF, 2 μg/ml pepstatin A, 20 μg/ml leupeptin, 1 mM benzamidine HCl, 0.1 mg/ml bacitracin, 10 μg/ml aprotinin, and 0.1 mM prefabloc) by bead beating and clarified by centrifugation. Ethidium bromide was added to a final concentration of 300 μg/ml in L100 buffer before bead beating. The extracts were precleared with protein A Sepharose beads for 1 hr and then were mixed with antibodies crosslinked to protein A Sepharose beads for 9 hr. After extensive washes, the precipitates were boiled off from the beads in Laemmli buffer. For glycerol gradient sedimentation of the Yph1p complexes, proteins were eluted from antibody-beads with A500 buffer (500 mM NaCl, 50 mM Tris-HCl [pH 7.8], 10% glycerol, 2 mM EDTA, 0.2 mM EGTA, 0.2% NP-40) plus 3 mg/ml Yph1p peptide at 37°C for 5 min. The eluates were applied to a 5 ml, 25%–50% glycerol gradient. The glycerol gradient was centrifuged at 35,000 rpm for 9 hr in an SW55Ti rotor. For a better separation of the small Yph1p complex from dissociated proteins, a 15%–35% glycerol gradient was used and was centrifuged at 45,000 rpm for 9 hr. Fractions (200 μl) were collected from the bottom of the gradient and proteins were precipitated by acetone.

For Western blotting, samples were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with the appropriate antibodies, including monoclonal anti-ORC antibodies (Liang and Stillman, 1997), 12CA5 (dilution 1:5000 for HA-ERB1, MCM6-HA_3, and MEC1-HA_9), anti-Rpl3p antibody (1:20,000), anti-Nop1p antibody (1:5000), and anti-Nop5p antibody (1:5000), YOL1/43 α-tubulin (1:100), polyclonal anti-Yph1p antibody CS959 (1:5000), anti-Smt3p antibody (1:1500), anti-cdc2 antibody (1:5000 for Cdc28p, Upstate Biotechnology), anti-Dun1p antibody (1:10,000), and anti-Rad23p antibody (1:200,000).

In Vitro Expression of HA-Yph1p and ORC Purification

YPH1 ORF was PCR amplified and cloned into pNcite vector, which contained a single HA epitope at the N-terminal fusion sequence. The resulting plasmid (PND001) was used to express HA-Yph1p in TNT Quick Coupled systems (Promega). ORC complexes were expressed and purified using recombinant baculoviruses as described previously (Bell et al., 1995) except that MgOAc was omitted from all chromatography buffers, the double-stranded DNA-cellulose column was omitted, and a Superdex 200 16/60 column (Pharmacia) replaced glycerol gradient separation.

Sucrose Gradient Fractionation of Ribosomes

The *yph1-td* mutant and isogenic wild-type stains growing in YPRaf at 25°C were shifted to YPGal at 37°C for 3 hr before harvest. Ribosomes were prepared and fractionated as described previously (Baim et al., 1985) with minor modifications: they were centrifuged on a 16 ml, 7%–47% sucrose gradient at 4°C for 5.2 hr at 27,000 rpm in an SW28 rotor. Gradients were fractionated from the top in an ISCO gradient collector while scanning at 254 nm.

Other Techniques

For fluorescence microscopy, exponentially growing YPH1-GFP cells in YPD were collected, sonicated, and washed in phosphate-buffered saline (PBS). The cells were resuspended in PBS containing 1% Triton X-100 and incubated for 10 min. Then, cells were resuspended in PBS and mixed with equal volume of mounting medium containing 0.045 mg/ml 4′,6-diamidine-2-phenylinodole-dihydrochloride (DAPI), 1 mg/ml p-phenylenediamine, and 90% glycerol. YPH1-GFP and DNA staining were examined with fluorescence microscopy. FACS was performed as described previously (Liang and Stillman, 1997).

Acknowledgments

We are grateful to members of the Stillman laboratory for discussions. We thank R. Kobayashi and S. Lopez for advice and technical expertise in peptide sequencing; J.F. Diffley, M.S. Longtine, N. Hernandez, B. Ma, E. Hurt, L.F. Lau, J. Callis, M.P. Longhese, P. Meluh, J.R. Warner, S. Elledge, L. Prakash, and J. Aris for reagents; J. Duffy and P. Renna for art work and photography; and J. Kuo, T.C. Sung, W. Chen, O. Cohen-Fix, A. Stenlund, and T. Wei for suggestions. This work was supported by National Institutes of Health grant GM 45436.

Received: November 1, 2001
Revised: May 9, 2002

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