The Yeast rRNA Biosynthesis Factor Ebp2p is also Required for Efficient Nuclear Division

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Ionescu, Costin N.; Origanti, Sofia; and McAlear, Michael A., "The Yeast rRNA Biosynthesis Factor Ebp2p is also Required for Efficient Nuclear Division" (2004). *Biological Sciences Faculty Research and Publications*. 523.  
https://epublications.marquette.edu/bio_fac/523
The Yeast rRNA Biosynthesis Factor Ebp2p Is Also Required for Efficient Nuclear Division

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Abstract
Molecular genetic analysis of the yeast Ebp2 protein has revealed that it is an essential, nucleolar protein that functions in the rRNA biosynthesis pathway. Temperature-sensitive ebp2-1 mutants are defective in the processing of the 27S precursor rRNA, and the point substitutions that disrupt this activity cluster towards the central, more highly conserved region of the Ebp2 protein. We report here that other ebp2 mutants exhibit deficiencies associated with defects in chromosome segregation. Yeast cells bearing a 50 amino acid C-terminal truncation allele (ebp2ΔC50) display a slow-growth phenotype and exhibit an increased percentage of cells with the nucleus positioned at the bud neck. The ebp2-1 and ebp2ΔC50 alleles genetically complement each other,
and ebp2ΔC50 mutants exhibit nuclear division defects that are distinct from the rRNA biosynthesis-related phenotypes of ebp2-1 mutants. Cytological and FACS analysis of the ebp2ΔC50 deletion mutants indicate that the chromosome segregation related activities of the Ebp2 protein are monitored by Mad2p, a mitotic checkpoint protein. The finding that yeast Ebp2p functions in nuclear division is consistent with the growing body of evidence that supports the role that human EBP2 plays in chromosome segregation. Copyright © 2004 John Wiley & Sons, Ltd.

Introduction
Homologues of the EBP2 gene are widely conserved among eukaryotes (Henning and Valdez, 2001), and they can be found in organisms ranging from budding and fission yeasts, through worms, flies and humans (Baim et al., 1985). The first report on a protein product encoded by a member of this family of genes was the description of a nucleolar antigen that was associated with proliferating human cells. Subsequently, studies on the Ebp2 protein from S. cerevisiae have revealed that it is an essential, nucleolar protein that functions in the rRNA biosynthesis pathway. Much of this insight has come from the phenotypic analysis of temperature-sensitive ebp2-1 mutants (Huber et al., 2000). Upon shifting to the restrictive temperature, ebp2-1 mutants suffer defects in 60S ribosome subunit biogenesis and become depleted of ribosomes. Detailed analysis of the rRNA processing pathway revealed that Ebp2p is required for the efficient processing of the 27S pre-rRNA intermediates. Additionally, other genetic and biochemical studies have clearly placed yeast Ebp2p within the ribosome and rRNA biosynthesis pathways (Mizuta and Warner, 1994; Wade et al., 2001).

Interestingly, the human EBP2 protein was independently isolated from a two-hybrid screen that was designed to identify proteins that interact with the Epstein–Barr virus (EBV)-encoded EBV nuclear antigen 1 (EBNA1) protein (Shire et al., 1999). Worldwide, over 90% of the population is infected with EBV (Faulkner et al., 2000) and, after initial infection, the virus remains in a latent stage in a small number of B lymphocytes. EBV genomes are maintained in these cells as double-stranded, circular DNA episomes, which replicate only once per cell cycle and segregate efficiently to the daughter cells (Adams, 1987; Yates and Guan, 1991). Given that the replication and segregation of the EBV episomes requires only one viral protein, EBNA1, and the viral origin of DNA replication, oriP (Yates et al., 1985), interacting host factors are critical for viral maintenance. Human EBP2 is one such host factor that is vital for the proper segregation of EBV episomes. EBNA1 mutants that are defective in interacting with EBP2 are unable to properly segregate oriP plasmids, but are not defective in DNA replication (Shire et al., 1999). Since human EBP2 is a nucleolar protein in interphase, but co-localizes with EBNA1 on the chromosomes during mitosis (Wu et al., 2000), one model is that the EBNA1–EBP2 interaction facilitates viral episome segregation by ‘piggy backing’ on the host cell mitotic chromosomes (Kapoor and Frappier, 2003; Shire et al., 1999; Wu et al., 2000). This model is supported by the observations that the partitioning of oriP containing plasmids can be reconstituted in yeast, and that this segregation system is dependent on EBNA1 and human EBP2 (Kapoor and Frappier, 2003; Kapoor et al., 2001).

Until now, there has been little to suggest that the yeast Ebp2 protein plays a role in chromosome segregation. Nuclear division is a complex and highly regulated process in yeast, and there are a number of pathways devoted to ensuring that the replicated sister chromatids are properly attached to the spindle apparatus, aligned and segregated during mitosis (Petronczki et al., 2003; McInish et al., 2003). Central to these processes is the spindle assembly checkpoint (SAC) and the so-called MAD and BUB gene products, which were identified as being crucial for preventing mitosis in response to treatment with microtubule poisons (Li and Murray, 1991; Hoyt et al., 1991). The transition from metaphase to anaphase is regulated by a group of gene products (including Mad2p) that ensure that the cohesive proteins that hold sister chromatids together are not degraded by the anaphase-promoting complex before all of the chromatids are properly attached to the mitotic spindle (Musacchio and Hardwick, 2002; Gardner and Burke, 2000). The BUB2 branch of the SAC inhibits the exit from
mitosis in response to failures of nuclear migration through the bud neck. Bub2p, Tem1p and components of the mitotic exit network (MEN) work to ensure that the exit from mitosis does not proceed until after the spindle pole body enters the daughter cell (Morgan, 1999; Stegmeier et al., 2002).

In this study we show, through the analysis of a new set of ebp2 mutants, that the Ebp2 protein does indeed play a role in nuclear division in yeast and that this function is separable from the role it plays in rRNA biosynthesis. Specific ebp2 mutants exhibit defects in chromosome segregation and the MAD2 checkpoint pathway monitors these defects. These results strengthen the functional links between the yeast and human EBP2 proteins, and add to a growing list of nucleolar, rRNA biosynthesis-related proteins that also function in mitosis.

Experimental

Strains, plasmids and media

The genotypes of the strains used in this study are listed in Table 1, and the plasmids used in this study are indicated in Table 2. Standard media preparation and yeast genetic techniques were used throughout (Sherman et al., 1986). Restriction enzymes were purchased from New England BioLabs, and Taq polymerase was purchased from Promega.

<table>
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<th>Strain</th>
<th>Alias</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
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<tr>
<td>yMM49</td>
<td>MATα ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM113 EBP2, URA3)</td>
<td>Huber et al. (2000)</td>
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<td>yMM113</td>
<td>MATα ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM112 EBP2 LEU2)</td>
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<td>Huber et al. (2000)</td>
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<td>Huber et al. (2000)</td>
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<td>Huber et al. (2000)</td>
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<td>yMM361</td>
<td>MATα ebp2-1.Kan’ leu2Δ1 trp1Δ63 ura3-52 (pMM 131 ebp2ΔC50 LEU2)</td>
<td>This study</td>
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</tr>
<tr>
<td>yMM362</td>
<td>MATα ebp2-1.Kan’ leu2Δtrp1Δ63 ura3-52 (pMM 112 EBP2 LEU2)</td>
<td>This study</td>
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<td>yMM383</td>
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<td>yMM392</td>
<td>MATα ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM113 EBP2 URA3) (pMM169 GFP-EBP2 LEU2)</td>
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<td>yMM393</td>
<td>MATα ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM176 GFP-ebp2ΔN62ΔC50 LEU2)</td>
<td>This study</td>
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<td>yMM394</td>
<td>MATα ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM169 GFP-ebp2ΔN62 LEU2)</td>
<td>This study</td>
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yMM395  MATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM176 GFP-ebp2ΔN62ΔC50 LEU2)  This study

yMM396  MATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM113 EBP2 URA3) (pMM199 GFP-ebp2-1ΔN62 LEU2)  This study

yMM408  MATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM199 GFP-ebp2-1ΔN62 LEU2)  This study

yMM445  MATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM354 ebp2ΔN62ΔC50 LEU2)  This study

yMM447  MATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM353 ebp2ΔN127ΔC50 LEU2)  This study

yMM472  MAY2055 MATa ura3 his3 leu2 ade2 GAL+ bub2::URA3  Farr and Hoyt (1998)

yMM473  MAY4428 MATa ura3 his3 leu2 ade2 GAL + mad2::URA3  Farr and Hoyt (1998)

yMM474  MATaGAL + bub2::URA3 ebp2::HIS3 ade2 (pMM169 GFP-ebp2ΔN62 LEU2 CEN ARS, ori, ampR)  This study

yMM476  MATaGAL + mad2::URA3 ebp2::HIS3 trp1Δ63 ade2 (pMM169 GFP-ebp2ΔN62 LEU2 CEN ARS, ori, ampR)  This study

yMM478  MATaGAL+ bub2::URA3 ebp2::HIS3 lys2-1285 (pMM176 GFP-ebp2ΔN62ΔC50, CEN LEU ARS, ori, ampR)  This study

yMM480  MATa GAL + mad2::URA3 ebp2::HIS3 lys2-1285 ade2 trp1Δ63 (pMM176 GFP-ebp2ΔN62ΔC50, CEN LEU ARS, ori, ampR)  This study

yMM484  2268 MATa ade2 can1 his3 leu2 trp1 ura3 GAL + ctf19::KAN  Gardner et al. (2001)

yMM485  Mif2-3B MATa mif2-3 his3 ade2 trp1 leu2 ura3 can1 sap3  Brown et al. (1993)

yMM486  MATa ade2 can1 his3 leu2 trp1 ura3 GAL + ctf19::KANMATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM169 GFP-ebp2ΔN62 LEU2 CEN ARS, ori, ampR)  This study

yMM487  MATa ade2 can1 his3 leu2 trp1 ura3 GAL + ctf19::KANMATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM176 GFP-ebp2ΔN62ΔC50, CEN LEU ARS, ori, ampR)  This study

yMM490  1cAS281 MATa cep3-1 ade2 his3 leu2 lys2 trp1 ura3  Strunnikov et al. (1995)

yMM492  MATa cep3-1 ade2 his3 leu2 lys2 trp1 ura3 MATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM169 GFP-ebp2ΔN62 LEU2 CEN ARS, ori, ampR)  This study

yMM493  MATa cep3-1 ade2 his3 leu2 lys2 trp1 ura3 MATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM176 GFP-ebp2ΔN62ΔC50, CEN LEU ARS, ori, ampR)  This study

yMM496  MATa mif2-3 his3 ade2 trp1 leu2 ura3 can1 sap3 MATa ebp2::HIS3 his3 ura3-52 leu2Δtrp1Δ63 lys2-1285 (pMM169 GFP-ebp2ΔN62 LEU2 CEN ARS, ori, ampR)  This study
Creation of EBP2 alleles and strains

The ebp2-1 strain (yMM179) was transformed with plasmid pMM112 (EBP2 LEU2) to generate the EBP2/ebp2-1 strain yMM362, or with plasmid pMM131 (ebp2ΔC50 LEU2) to produce the ebp2-1/ebp2ΔC50 strain yMM361. The ebp2ΔN62ΔC50 (pMM354) and ebp2ΔN127ΔC50 (pMM353) alleles were created by partially digesting plasmids pMM147 (ebp2ΔN62 LEU2) or pMM156 (ebp2ΔN127) with NdeI, and inserting the resulting fragments into plasmid pMM264 (ebp2ΔC50), which was also treated with NdeI.

The green fluorescent protein (GFP)–Ebp2p fusion constructs were created previously (Huber et al., 2000) and all contain an N-terminal 62 amino acid deletion of the Ebp2 protein. To create new GFP–EBP2 fusion strains, we used the plasmid shuffle technique. Strain yMM113 (with plasmid pMM112 EBP2 LEU2) was transformed with plasmid pMM113 (EBP2 URA3). After growing the cells on synthetic minimal media containing leucine, the resultant Leu" strain (yMM383) was transformed with LEU2 plasmids containing GFP–EBP2 (pMM169), GFP–ebp2ΔC50 (pMM176) or GFP–ebp2-1 (pMM199) alleles, yielding strains yMM392, yMM393 and yMM396. The final strains bearing only the GFP–EBP2 (yMM394), GFP–ebp2ΔC50 (yMM395) or GFP–ebp2-1 (yMM408) alleles were created by selecting against cells bearing the EBP2–URA3 plasmids (pMM113) on 5-FOA plates.

Immunofluorescence microscopy

Cells were grown in synthetic complete media lacking leucine (yMM361) or YPD media (yMM202, yMM179, yMM160) at 28 °C to the early log phase, and then 1 ml of culture was collected, sonicated and fixed in 3.7% formaldehyde. The cells were washed once in phosphate-buffered saline (PBS:150 mM NaCl, 10 mM sodium phosphate, pH 7.4) containing 1% Triton X-100 and resuspended in 10 µl PBS. Mounting medium (9 µl) containing 1 mg/ml 4',6'-diamidino-2-phenylindole (DAPI) and 50% glycerol was then added to 1 µl cells. The cells were analysed using a Zeiss Axioplan microscope and categorized according to their cellular and nuclear morphologies (Huber et al., 2000). To visualize the spindles, the cells were treated with anti-α-tubulin antibodies (Sigma), as described previously (Adams and Pringle, 1984). The cells were fixed in formaldehyde and potassium phosphate buffer for 30 min. Following the fixation, the cells were treated with zymolase (50 µg/ml) for 30 min.
Sensitivity to the drugs nocodazole, benomyl, paromomycin and hygromycin
Nocodazole and benomyl (Sigma) were added to the YPD media just before the pouring of the plates. Serial dilutions of the strains were inoculated onto the indicated plates and incubated at 32 °C for 3 days. For the sensitivity to paromomycin and hygromycin, 1 × 10^7 cells from the indicated strains were spread across YPD plates. GF/C glass filter discs of 0.6 cm diameter were placed on the agar, and 10 µl of the indicated concentrations for either drug paromomycin (Sigma) or hygromycin (Sigma) were added to the centre of the disks. The plates were incubated at 32 °C for 4 days.

RNA preparations and Northern blotting
Cultures of EBP2 (yMM202), ebp2-1 (yMM203) and ebp2ΔC50 (yMM160) strains were grown to early log phase at 28 °C and then half of the cultures were shifted to 36 °C for 2 h. Total RNA preparation and the Northern blotting analysis was done as described previously (Huber et al., 2000).

Flow cytometry analysis
Flow cytometry analysis was performed on cells grown to mid-log phase. One ml of culture was collected and fixed at room temperature on a roller drum in 1 ml 95% ethanol for at least 2 h. The cells were pelleted again and resuspended in 1 ml 50 mM sodium citrate, pH 7.1. After sonication, the cells were pelleted and resuspended in 1 ml of 50 mM sodium citrate containing 0.25 mg/ml RNase A and then incubated at 50 °C for 1 h. Proteinase K was added to 1 mg/ml, and the cells were incubated for a further 1 h at 50 °C. After two washes in sodium citrate, propidium iodide was added to a final concentration of 16 µg/ml and the tubes containing the cells were wrapped in aluminum foil and incubated overnight at 4 °C. Flow cytometric analysis was performed using a Becton-Dickinson FACS Vantage flow cytometer [Becton-Dickinson Immunocytometry Systems (BDIS), San Jose, CA]. The cells were excited at 488 nm, and the propidium iodide fluorescence collected through a 630/30 nm band-pass filter. 50 000 cells were observed for each samples. Data collection and analysis were performed using CellQuestPro software (BDIS).

Results
Different ebp2 alleles give rise to different phenotypes
In order to determine whether the Ebp2 protein may have more than one activity in yeast cells, we analysed a selection of ebp2 mutants beyond the originally characterized ebp2-1 strain. Previous deletion analysis revealed that whereas the N-terminal 178 amino acids of the Ebp2 protein are dispensable, the C-terminal end of the protein is critical for some essential function. A strain bearing a 50 amino acid C terminal ebp2 deletion allele grew slowly, and the 105 and 151 amino acid deletion constructs were non-functional (Huber et al., 2000). We also previously created and tested fusion constructs containing the GFP coding sequence from A. victoria joined to EBP2, ebp2-1 and ebp2ΔC50 alleles (Huber et al., 2000) (Figure 1A). Each of these alleles was able to complement an ebp2::HIS3 disruption allele, and we observed that, like the ebp2ΔC50 mutant, the GFP–ebp2ΔC50 strain was similarly slow-growing.
Different ebp2 alleles give rise to different phenotypes. (A) Schematic representations of various Ebp2p constructs are shown with respect to the basic (hatched), and acidic (stippled) regions of the Ebp2 protein. GFP fusion residues are indicated in grey, and the putative nuclear localization signals are indicated with an N. Stars represent the positions of the ebp2-1 point substitutions. (B) Wild-type (yMM202) and ebp2ΔC50 (yMM160) cells were collected, stained with DAPI to visualize the DNA, and observed under a fluorescence microscope using Nomarski optics.

To further characterize the slow-growth phenotype of the 50 amino acid C-terminal ebp2 deletion mutants, we analysed the cellular and nuclear morphologies of cells taken from log-phase cultures. EBP2, ebp2-1 and ebp2ΔC50 strains were grown in YPD media at 30 °C and the cells were collected, stained with DAPI, and visualized by fluorescent microscopy. We could observe the expected proportions of unbudded and budded cells in the dividing EBP2 and ebp2-1 cultures, including a small percentage of cells (5% or less) with the characteristic nucleus-through-the-neck phenotype indicative of cells going through mitosis. In contrast, 17% of the cells from the ebp2ΔC50 strain appeared to be large-budded, with the nucleus directly at, or across, the neck (Figure 1B). These same cells were also larger than the wild-type or ebp2-1 cells. Since nuclear division is normally a relatively quick process in dividing yeast cells, the increased proportion of cells with this morphology indicated that ebp2ΔC50 mutants might be defective in proceeding through mitosis.

To assess the relationship between the apparent nuclear division defects of ebp2ΔC50 mutants and the previously characterized temperature sensitivity of ebp2-1 mutants, we carried out intragenic complementation studies. We transformed an ebp2-1 strain with a plasmid copy of ebp2ΔC50 to generate an ebp2-1/ebp2ΔC50 strain (yMM361), and separately with an EBP2 plasmid to generate an ebp2-1/EBP2 strain (yMM362). Both strains were able to grow at 36 °C, indicating that the ebp2ΔC50 deletion allele was able to complement the temperature sensitivity of ebp2-1 mutants (Figure 2A). Also, by examining the nuclear morphology of the ebp2-1/ebp2ΔC50 strain, we observed that the ebp2-1 allele is able to complement for the ebp2ΔC50 deletion. Since the ebp2-1 ebp2ΔC50 double mutant strain exhibited wild-type levels of cells having their nucleus through the bud neck, the nuclear division and the temperature-sensitive phenotypes are genetically separable (Figure 2B).
Intragenic complementation between the *ebp2-1* and *ebp2ΔC50* alleles. (A) Strains yMM160, yMM202, yMM203, yMM361, yMM362 were inoculated onto YPD plates and incubated at either 28 °C or 36 °C for 2 days. (B) Samples from log cultures of the indicated strains were collected, stained with DAPI and visualized by fluorescence microscopy. The nuclear and cellular morphologies from three independent experiments were used to determine the percentage of cells with the various morphologies consistent with different stages of the cell cycle.

The nuclear division defect is separable from the rRNA processing defect

To determine the extent to which the C-terminal end of Ebp2p is required for ribosome biogenesis or rRNA processing, we monitored ribosome function by assessing the sensitivity of the *EBP2*, *ebp2-1* and *ebp2ΔC50* strains to aminoglycoside antibiotics (Carter et al., 2000; Brodersen et al., 2000). Log cultures of the strains were spread across plates containing filter disks spotted with increasing concentrations of the antibiotics paromomycin and hygromycin. As apparent by the zone of growth inhibition surrounding the discs (Figure 3A, b), the *ebp2-1* strain was the most sensitive to the drugs. The *ebp2ΔC50* strain was less sensitive, yet slightly more sensitive than the wild-type strain. These results are consistent with the known ribosome biogenesis defect of *ebp2-1* mutants, and also indicate that the *ebp2ΔC50* strain is less impaired for this function.
Figure 3 ebp2-1 mutants are the most sensitive to aminoglycoside antibiotics. Strains yMM160, yMM202, yMM203 were spread onto YPD plates and then filter disks containing increasing concentrations of either paromomycin (A) or hygromycin B (B) were placed on top of the lawn of cells. Zones of growth inhibition were observed after incubation for 3 days at 32 °C.

To more specifically determine whether the ebp2ΔC50 mutation affects rRNA biosynthesis, we monitored the rRNA processing pathway by Northern blotting. rRNA biosynthesis in yeast involves a complex series of endo- and exonucleolytic cleavages whereby the 35S primary rRNA transcript is processed into the mature 25S, 18S and 5.8S rRNAs (reviewed in (Kressler et al., 1999)). Intermediates in the rRNA-processing pathway can be detected by probing Northern blots with specifically designed oligonucleotides (Beltrame and Tollervey, 1992; Dunbar et al., 1997). Total RNA was extracted from GFP–EBP2, GFP–ebp2-1, and GFP–ebp2ΔC50 strains growing at either 28 °C or 36 °C, and Northern blots were probed with oligonucleotides designed to hybridize to the mature 25S and 18S rRNAs, as well as the 27S intermediate (oligo e) (Figure 4). We could detect mature 18S and 25S rRNAs in all three strains with 25 and 18 specific probes, and with oligonucleotide e, which hybridizes to the 3’ end of the 5.8S rRNA, we could detect the presence of 27SA, and 27SB rRNA intermediates. We had previously observed that upon shifting to the restrictive temperature, ebp2-1 mutants exhibit an accumulation of the 35S pre-rRNA, as well as a significant block in the processing of the 27S A to the 27S B rRNA intermediate (Huber et al., 2000). While we could observe a slight accumulation of the 35S pre-rRNA in the ebp2ΔC50 strain, the levels of the 27S A and 27S B precursor rRNAs were similar to those in the wild-type strain. Thus, while the ebp2ΔC50 strains do exhibit minor rRNA- and ribosome-related defects, they are much less perturbed in this regard than are ebp2-1 mutants.
Figure 4 ebp2ΔC50 mutants are proficient at processing rRNA precursors. A simplified overview of the rRNA processing pathway, containing the relevant precursors species is depicted (A). Total RNA was prepared from samples of cells taken from strains yMM160, yMM202, and yMM203 grown at 28 °C and 36 °C. Northern blots were prepared and hybridized to probes recognizing the 35S primary transcript, the mature 25S and 18S rRNAs (B) and the 27 S rRNA intermediates (oligo ‘e’) (C).

A common characteristic of yeast mutants that are defective in nuclear division is their sensitivity to antimicrotubule drugs, such as nocodazole or benomyl. These drugs interfere with the polymerization of the microtubules, thereby compromising spindle function. We assessed the sensitivity of our strains to these drugs on a plate assay (Figure 5), and we monitored the drug sensitivity of both the GFP tagged (Figure 5A) as well as non-tagged ebp2 alleles, including N-terminal and C-terminal deletions (Figure 5B). Whereas the ebp2-1 strains exhibited drug sensitivities similar to the wild-type EBP2 strain, all of the ebp2ΔC50 alleles were markedly sensitive to both nocodazole and benomyl. Thus, the nuclear division defect, as well as the microtubule poison sensitivity phenotype, is associated only with the ebp2ΔC50 strains.

Figure 5 ebp2ΔC50 mutants are hypersensitive to microtubule depolymerizing drugs. (A) Serial dilutions of cells from strains yMM160, yMM202, yMM203, yMM445 and yMM447 were inoculated onto plates containing increasing concentrations of nocodazole (A), or benomyl (B) and incubated at 32 °C for 3 days.

To investigate whether the nuclear division defects were related to abnormal spindle integrity, we examined wild-type and mutant cells by immunofluorescence microscopy. Anti-tubulin antibodies were used to visualize the spindle, DAPI was used to stain the DNA, and the GFP signal was used to localize the Ebp2 protein. We could readily visualize the GFP-tagged Ebp2 protein in the wild-type and ebp2ΔC50 strains (Figure 6A), and no discernable difference was noted in the distribution of the protein between the two strains. Likewise, no abnormal spindle or nuclear morphologies were observed in the ebp2ΔC50 mutants, suggesting that the spindle structure was not greatly disturbed in these cells (Figure 6B). Co-staining the cells with anti-Nop1p antibodies revealed that, although for the most part the GFP–Ebp2p signal overlapped that of the nucleolar protein Nop1p, we could detect extranucleolar GFP–Ebp2p signal spanning the bud neck in dividing cells (Figure 6C).
Figure 6 Cytological examination of ebp2ΔC50 mutants. Cells from strains yMM160 and yMM202 were prepared for immunofluorescence microscopy and visualized for GFP–Ebp2p signal (A), anti-tubulin staining (B), or GFP–Ebp2p and anti-Nop1p staining (C).

The nuclear division delay seen in ebp2ΔC50 mutants is mediated by Mad2p, but not Bub2p.

Another way to assess cell cycle progression is to monitor the DNA content profile of a culture by FACS analysis (Figure 7). The proportion of cells with G1, S and G2/M DNA contents can be used to gauge the length of time the cells spend in each phase of the cell cycle. Consistent with our cytological observations of a nuclear division delay, ebp2ΔC50 cultures had an increased proportion of cells with a G2/M DNA content than did wild-type or ebp2-1 cells (data not shown). Similarly, the observation that the ebp2ΔC50 FACS profile was slightly skewed to the right is consistent with the larger size of the ebp2ΔC50 mutants. In order to determine whether the delay in the cell cycle seen in our ebp2ΔC50 mutant was caused by a mitotic checkpoint, we constructed double mutant haploid yeast strains bearing the ebp2ΔC50 and mad2 or bub2 deletion alleles (Farr and Hoyt, 1998). Mad2p and Bub2p are key components of checkpoint pathways that supervise the progression through mitosis (Li and Murray, 1991; Hoyt et al., 1991). We observed that the mitotic delay phenotype was abrogated when the ebp2ΔC50 allele was combined with the mad2 mutation, but not when it was combined with the bub2 mutation (Figure 7). On their own, the mad2 and bub2 single mutants exhibited wild-type-like FACS profiles (data not shown).
The *ebp2ΔC50* associated mitotic delay is abrogated by the *mad2* mutation. Cells from strains yMM160, yMM202, yMM474, yMM476, yMM478 and yMM480 were grown to early log phase, prepared for FACS analysis, and the DNA content histograms were determined for at least 50 000 cells.

To further test the relationship between the observed mitotic delay and the MAD checkpoint, we examined the cellular and nuclear morphologies of the *ebp2ΔC50 mad2* double mutants. If the MAD checkpoint plays a role in delaying mitosis in response to the defects associated with the truncated Ebp2 protein, one might expect that the *ebp2ΔC50 mad2* double mutants would not have an elevated number of cells with the nucleus spanning the bud neck. Indeed, we observed many fewer large-budded cells with the nucleus either at or through the bud neck in the *ebp2ΔC50 mad2* strain than with any of the other strains (Figure 8A). Close examination revealed that the *ebp2ΔC50 mad2* culture contained an increased number of anucleate cells, and cells with either multiple or fragmented nuclei. To investigate this further, we monitored the viability of the single and double mutant *ebp2* and *mad2* strains. Log cultures were sonicated, diluted and plated onto YPD plates (Figure 8B). Whereas the wild-type and single mutant strains exhibited cell viability of over 60%, the *ebp2ΔC50 mad2* double mutant culture contained many fewer viable cells. On their own, the *mad2* and *bub2* strains did not show this marked inviability (data not shown and Krishnan et al., 2000; Hardwick et al., 1999). These results are consistent with the hypothesis that the MAD checkpoint prevents *ebp2ΔC50* mutants from undergoing aberrant nuclear divisions.
Discussion

Previously, yeast Ebp2p was characterized as an essential, nucleolar protein that functions in ribosome biogenesis and the processing of the 27S pre-rRNA intermediates (Huber et al., 2000). The finding that yeast Ebp2p is also required for efficient nuclear division helps to establish a functional link between the yeast and human EBP2 proteins. Starting from its characterization as an EBNA1-binding protein, there has been clear evidence supporting a role for human EBP2 in the segregation of EBV episomes. Human EBP2 localizes to condensed mitotic chromosomes, and the EBNA1–EBP2 interaction facilitates the partitioning of EBV episomes by ‘piggy-backing’ on segregating host chromosomes (Kapoor and Frappier, 2003; Shire et al., 1999). Our observations that specific yeast ebp2 mutants are sensitive to microtubule poisons and that they exhibit nuclear division defects that are monitored by the MAD2 mitotic checkpoint pathway are also consistent with a function for the yeast Ebp2 protein in chromosome segregation. The specific role that yeast Ebp2p plays in this regard remains to be determined, as is the role that human EBP2 plays in host cell chromosome segregation. Similarly, although human EBP2 does localize to the nucleolus in interphase cells, it has yet to be determined whether, as in yeast, the human EBP2 protein also functions in the rRNA biosynthesis pathway.

Our genetic dissection of the yeast EBP2 gene reveals that the ribosome biogenesis and nuclear division functions of the Ebp2 protein are, for the most part, separable. The C-terminal residues of Ebp2p appear to be critical for the nuclear division activities, whereas the rRNA-processing defects associated with the ebp2-1 allele are the result of point substitutions in the central, more highly conserved portion of the protein. Although the C-terminal deletion mutants do show an elevated sensitivity to aminoglycoside antibiotics, they do not exhibit the same ribosome biogenesis or rRNA processing defects of ebp2-1 mutants. Even though the C-terminal 50 amino acid deletion removes one of the putative Ebp2p nuclear localization signals, we did not detect any differences between the cellular distributions of the wild-type and mutant GFP-tagged Ebp2 proteins. Since two-hybrid analysis indicates that Ebp2p interacts with itself (Tsujii et al., 2000), it is possible that the observed intragenic complementation is related to Ebp2p acting as a dimer.

Figure 8 The MAD checkpoint monitors the ebp2ΔC50 mediated defect. (A) Strains yMM151, yMM160, yMM478 and yMM480 were grown to early log phase and for each strain, 500 cells were scored for their nuclear and cellular morphologies by fluorescence microscopy. (B) Cultures were grown to mid-log phase, sonicated, counted, diluted and plated onto YPD plates. Average viabilities were determined for three independent experiments after incubation for 3 days at 30 °C.
Our findings that the \textit{MAD2} gene monitors the defects associated with the \textit{ebp2ΔC50} allele offer a clue as to the role that Ebp2p plays in the cell. There are several proteins that have been implicated to control the metaphase–anaphase transition in yeast, including Mad1p, Mad2p, Mad3p, Bub1p, Bub3p and Mps1p (Li and Murray, 1991; Hoyt \textit{et al.}, 1991; Weiss and Winey, 1996). Mad2p binds to unattached kinetochores (Rieder \textit{et al.}, 1995; Howell \textit{et al.}, 2000) and regulates progression through the cell cycle by inhibiting the anaphase-promoting complex (APC) (Fang \textit{et al.}, 1998). One possibility, therefore, is that even though the spindle morphology appears intact in \textit{ebp2ΔC50} mutants, there may be improper attachments between the chromosomes and the spindle apparatus. In contrast, \textit{BUB2} is part of the mitotic exit network (MEN) that controls the degradation of cyclins and exit from mitosis (reviewed in Gardner and Burke, 2000). Because \textit{ebp2ΔC50} mutants display phenotypes that have been associated with mutations in genes encoding proteins that function at the kinetochore, we tested for synthetic lethal interactions between \textit{ebp2ΔC50} and known kinetochore-related mutants. We investigated proteins that are implicated in binding CDE II (Mif2p) (Brown \textit{et al.}, 1993), CDE III (Cep3p) (Strunnikov \textit{et al.}, 1995) and a component of the central kinetochore, Ctf19p (Gardner \textit{et al.}, 2001) (reviewed in Cheeseman \textit{et al.}, 2002; Cleveland \textit{et al.}, 2003). Crosses were made between the wild-type and \textit{ebp2ΔC50} and \textit{cep3-1} (yMM492, yMM493) \textit{ctf19::KAN} (yMM486, yMM487) and \textit{mif2-3} (yMM496, yMM497) mutants and, in each case, we were able to isolate double mutants carrying both mutations. From this limited analysis, it appears that Ebp2p may not function directly at the kinetochore.

There are precedents for other ribosome biogenesis-related nucleolar proteins that, after interphase, also play a role in mitosis. Cbf5p (first described as a centromere binding factor) interacts with the kinetochore proteins that bind to conserved centromeric DNA sequences (Jiang \textit{et al.}, 1993). Interestingly, \textit{cbf5} mutants lacking the C-terminal 50 amino acids of the Cbf5 protein grow slower, exhibit a G2/M cell cycle delay, and have normal spindles. Like Ebp2p, Cbf5p is also required for efficient rRNA processing (Cadwell \textit{et al.}, 1997). Similarly, Nme1p, a component of the mitotic exit network, is involved in rRNA processing (Schmitt and Clayton, 1993; Cai \textit{et al.}, 2002). The link between Ebp2p and components of the mitotic machinery was also supported by the observation that the microtubule-associated protein Ytm1p can be co-immunoprecipitated with Ebp2p (K. Shire and L. Frappier, unpublished results). Ytm1p was isolated in a screen for genes that affect chromosome stability when overexpressed in yeast and it is also implicated to function in the rRNA processing pathway (Ouspenski \textit{et al.}, 1999). In another study, both Ebp2p and Ytm1p were found to associate with Nop7p in the 66S pre-ribosomal intermediate (Harnpicharnchai \textit{et al.}, 2001). At this point, however, it is not clear what larger connection there may be between the seemingly disparate processes of ribosome biogenesis and chromosome segregation.

\section*{Acknowledgements}

We would like to thank Phil Hieter, Dan Burke, Megan Brown, Doug Koshland and Andy Hoyt for yeast strains, and Lori Frappier for sharing unpublished results. This work was supported in part by Grant MCB-9875283 (to M.M.) from the National Science Foundation.

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