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Genetic, biochemical and genomic analyses of RNP biogenesis in Saccharomyces cerevisiae



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# Genetic, Biochemical and Genomic Analyses of RNP Biogenesis in *S. cerevisiae*

by

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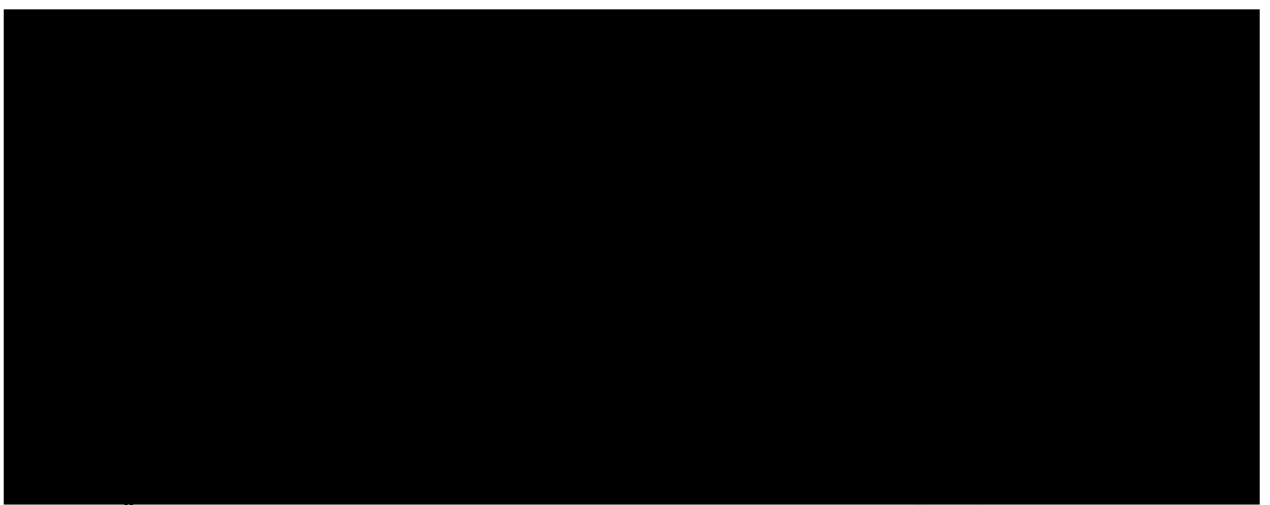

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**Genetic, biochemical and genomic analyses of RNP biogenesis in *S. cerevisiae***

**Maki Inada**

## ABSTRACT

The assembly of noncoding RNAs into ribonucleoprotein particles (RNPs) is a complex, multi-step process required for many steps in gene expression. The spliceosomal small nuclear RNPs (snRNPs) must bind common core proteins and undergo 5' cap and 3' processing steps in the cytoplasm of mammalian cells in order to re-enter the nucleus for function in splicing. To examine whether biogenesis of the spliceosomal snRNPs similarly occurs in the cytoplasm in yeast, we developed an *in situ* hybridization assay for examining snRNA localization and transport. However, we did not observe any evidence for a cytoplasmic phase, suggesting that snRNP biogenesis is restricted to the nucleus in *S. cerevisiae.*

To investigate the interphase between snRNP biogenesis and splicing, we conducted genetic and biochemical analyses of the snRNP biogenesis factor Brr1p for a role in splicing; *brr1* mutant cells display defects both in levels of newly synthesized snRNAs and also in splicing. We observed *in vitro* splicing defects in *brr1* mutant extracts, but since snRNP levels were also decreased, we could not assign a direct role for Brr1p in splicing. However, we identified genetic interactions between *BRR1* and factors involved in triple snRNP addition during spliceosome assembly (Sad1p, Snu66p, Snu114p, Sub2p), suggesting either Brr1p is playing a direct role in splicing or these splicing factors play a role in biogenesis. Thus, steps involved in assembling snRNPs and assembling the spliceosome may be common and require similar factors.

Lastly, to broaden our understanding of the function of RNP biogenesis factors, we used a genomics approach to examine the complete RNA binding profile of Lhp1p, a factor involved in stabilizing precursors of multiple noncoding RNAs. We confirmed Lhp1p association with

multiple noncoding RNAs and identified nearly class-wide association with multiple tRNAs and snoRNAs. Intriguingly, we identify Lhp1p association with a subset of mRNAs, such as ribosomal protein genes and HAC1 mRNA. Our analysis suggests that Lhp1p may play a novel role in the translation of one or more cellular mRNAs.

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Thank you.

## PROLOGUE

The goal of this thesis work was to gain deeper understanding into how the cell insures proper assembly of ribonucleoprotein particle (RNP) machinery, the workhorses of the cell. The central dogma describes gene expression as a linear path from DNA to mRNA to protein. However, in order to achieve this, many noncoding or functional RNAs must be transcribed, processed, assembled with their associated proteins and targeted to their correct cellular compartment for function at nearly every step in gene expression. Known RNPs include ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) as integral components of the translational apparatus; small nucleolar RNAs (snoRNAs), RNaseP and RNase MRP guide modifications of the rRNAs and tRNAs; SRP RNA promotes protein translocation; telomerase RNA is involved in complete DNA replication and small nuclear RNAs (snRNAs) are required for splicing pre-mRNAs to mature mRNAs.

The spliceosome is a highly dynamic machine that undergoes multiple RNP assembly events, requiring the energy of ATP for rearrangements at multiple steps (Staley and Guthrie 1998). Not only do the five snRNAs (U1, U2, U4, U5 and U6), with their associated proteins, together known as small nuclear ribonucleoprotein particles (snRNPs), come together to form an active spliceosome, but they must also be released and reassembled on each new mRNA substrate. At the time I joined the lab, progress towards understanding the roles of the snRNAs was being made (Madhani and Guthrie 1994), but much less was known about how the individual snRNPs are initially formed, a complex process known as snRNP biogenesis.

The first studies of snRNP biogenesis, shown to consist of multiple steps and bidirectional nucleocytoplasmic transport, were carried out in vertebrate cells where microinjection manipulations and cell fractionation were possible (Mattaj et al. 1993). These experiments demonstrated that snRNAs are transcribed in the nucleus and exported to the cytoplasm where core Sm proteins bind. This is then the signal for trimethylation of the cap, which together with

the Sm core constitute a bipartite nuclear localization signal. The snRNAs are trimmed at their 3' end, assembled with their associated proteins and delivered to their site of function, the nucleus (Will and Luhrmann 2001). At the time I began my thesis research, while these snRNP biogenesis steps had been identified, none of the mediating factors were known. Moreover, the field of nucleocytoplasmic transport was in its infancy. A few factors involved in protein transport had been identified, setting up a new paradigm. Carrier proteins bound to adaptors were shown to mediate transport of signal-containing cargo through nuclear pores with the assistance of the Ran GTPase (Gorlich and Mattaj 1996). Very little was known about RNA transport and while no RNA transport factors had been identified, it was hypothesized to occur via a similar, carrier-mediated mechanism (Jarmolowski et al. 1994).

The genetically tractable budding yeast (*S. cerevisiae)* was our amenable system of choice for identifying novel snRNP biogenesis and transport factors. Its cytological properties compared to mammalian cells are somewhat limited due to its small size, but progress with *in situ* hybridization techniques for visualizing bulk polyA+ mRNA using an oligo dT50 probe (Amberg et al. 1992; Kadowaki et al. 1994) suggested that visualizing specific RNAs such as snRNAs were potentially within reach.

Additionally, factors involved in snRNP biogenesis in yeast were beginning to be identified. Homologs of two mammalian Sm proteins were identified in yeast and were shown to affect snRNA stability and cap hypermethylation, indicating a role in snRNP biogenesis (Rymond 1993; Roy et al. 1995). Also, Brr1p, the first non-Sm snRNP biogenesis factor, was characterized in our laboratory (Noble and Guthrie 1996). Brr1p was shown to specifically affect the stability of newly synthesized snRNAs (Noble and Guthrie 1996). While its exact function was still unknown, Brr1p was also shown to affect 3' end trimming of U2 snRNA (Noble and Guthrie 1996). Although it was not yet known if snRNP biogenesis in yeast similarly consisted of a cytoplasmic phase, since the major landmarks of Sm core binding, cap hypermethylation and 3' end trimming were conserved, this was a likely scenario.

Chapter I of this thesis describes the development of *in situ* hybridization assays for localizing snRNAs designed to ask whether snRNP biogenesis occurs in the cytoplasm in yeast and to identify mediating factors. While we successfully localized yeast snRNAs to the nucleus, we did not observe any evidence for snRNA transport. We examined a battery of mutants and assayed for snRNA transfer in a binucleate system, but were unable to detect a cytoplasmic phase, suggesting that snRNP biogenesis is restricted to the nucleus in yeast. Indeed, subsequent work from others identifying metazoan snRNP transport factors has found that there are no known yeast homologs, suggesting a divergent pathway. One consequence of such a pathway is that snRNP synthesis, function and regeneration all take place within the nucleoplasm, suggesting that mechanisms for insuring proper RNP assembly before splicing may exist.

Chapter II describes an investigation at the interface between snRNP biogenesis and splicing via biochemical and genetic analyses of the role of Brr1p in splicing. Brr1p was shown to be involved in the stability of newly synthesized snRNAs (Noble and Guthrie 1996), but was also originally isolated as a factor defective in pre-mRNA splicing (Noble and Guthrie 1996). We examined splicing in *brr1*Δ extracts to ask whether Brr1p played a direct role in splicing. Since snRNP levels were also compromised, it remained difficult to determine whether the defects we observed were due to Brr1p function or an indirect effect of decreased snRNP levels. However, *brr1* alleles displayed genetic interactions with spliceosome assembly factors integral to the splicing pathway. There are several possible explanations for these observations: 1) factors such as Sub2p, Snu66p, and Snu114p may play roles in snRNP biogenesis, 2) Brr1p is playing a role in splicing, and/or 3) snRNP biogenesis and spliceosomal reassembly are closely related processes and may utilize similar factors.

Chapter III describes our investigation of RNP biogenesis on a global scale. To broaden our understanding of the function of RNP biogenesis factors, we used a genomics approach to identify the complete RNA binding profile of Brr1p and Lhp1p, another known RNP biogenesis factor involved in the processing of multiple noncoding RNAs (Wolin and Cedervall 2002).

While Brr1p was too low in abundance for analysis, we confirmed Lhp1p interaction with many noncoding RNAs and identified new Lhp1p interactions with multiple tRNAs and snoRNAs, suggestive of class-wide recognition. Strikingly, we also identified Lhp1p interaction with a subset of mRNAs. Specifically, we observe Lhp1p association with HAC1 mRNA, which encodes a transcription factor required for the unfolded protein response (Cox and Walter 1996). Contrary to our original hypothesis that Lhp1p may be involved in the processing of this unusual tRNA-like RNA, which is spliced by tRNA machinery (Sidrauski et al. 1996), we describe evidence for a novel role for Lhp1p in translation. Lastly, we observed Lhp1p interaction with many ribosomal protein genes. While this had been observed in mammalian systems, it was unexpected in yeast since yeast ribosomal protein genes lack the 5' terminal oligo pyrimidine binding sequence of metazoans (Powers and Walter 1999; Cardinali et al. 2003). Our data suggest that the function of RNP biogenesis factors is not restricted to noncoding RNAs, but is also important for mRNAs. Moreover, our data suggest an intriguing connection among Lhp1p-interacting RNAs. Coordinated regulation of gene expression by RNP assembly may be carried out by factor(s) involved in the biogenesis of noncoding RNAs and by modulation of translation of their associated proteins.

## Chapter I – snRNA biogenesis may be restricted to the nucleus in *S. cerevisiae*


### Abstract

Small nuclear ribonucleoprotein particles (snRNPs) must be properly assembled and processed in order to function in pre-mRNA splicing. In metazoan cells, snRNP biogenesis is a complex, multi-step process, which requires a cytoplasmic assembly phase and subsequent import to the nucleus. At the outset of this work, it was not known whether snRNP biogenesis in yeast also involved a cytoplasmic phase. In addition, none of the metazoan factors involved in mediating nucleocytoplasmic transport of snRNAs were known. To ask whether snRNP biogenesis occurred similarly in yeast and to identify factors involved in snRNA transport, we developed a fluorescence *in situ* hybridization assay for snRNAs in *S. cerevisiae*. We observed nuclear localization for snRNAs, but we did not observe any evidence for transport. We examined a battery of mutant strains defective in nucleocytoplasmic transport of both protein and RNA cargo, such as nucleoporins, protein transport factors, RNA export factors and all known importin β homologs. However, we were unable to detect mislocalization of snRNAs, either cytoplasmic accumulation for snRNA import mutants or an increase in nuclear signal for snRNA export mutants. We also developed an assay to examine U2 snRNA localization in a binucleate cell, but we were unable to detect U2 snRNA transfer from one nucleus to the other, again suggesting that snRNAs are not transported in yeast. Taken together with the finding that there are no known yeast homologs of the snRNA transport factors that have been identified by others in mammalian systems, these data suggest that snRNA biogenesis in *S. cerevisiae* may be restricted to the nucleus.


### Introduction

The biogenesis and assembly of small nuclear ribonucleoprotein particles (snRNPs) is necessary to produce the proper machinery for pre-mRNA splicing and hence for gene expression. In

metazoan cells, snRNP biogenesis is a multi-step process shown to require nucleocytoplasmic transport for cytoplasmic assembly and for return of the snRNPs to the nucleus where splicing occurs (Mattaj et al. 1993; Will and Luhrmann 2001). Briefly, snRNAs are first transcribed in the nucleus and exported to the cytoplasm where Sm core proteins bind in order for trimethylation of the cap to occur (Mattaj 1986; Hamm et al. 1987). The Sm core and trimthylated cap then act as a bipartite signal for reimport into the nucleus (Fischer and Luhrmann 1990; Hamm et al. 1990). Finally, although it is not known whether the final steps occur before, during or after reimport, 3' end trimming and snRNP specific protein binding occurs for final assembly of the mature snRNP (Neuman de Vegvar and Dahlberg 1990). While the steps of snRNP biogenesis had been thus characterized, none of the factors involved in mediating snRNA transport were known.

In fact, very little was known about RNA transport. But a paradigm for protein transport was emerging; a receptor, importin β/importin α, that binds basic nuclear localization signal sequences (NLSs) on protein cargo and directs their transport had been discovered (Gorlich and Mattaj 1996). Subsequently, a family of homologous receptors such as importin/transportin that bind to other signal sequences (M9) on other protein cargo (hnRNPA1) had also been identified (Pollard et al. 1996). These receptors continuously shuttle between the nucleus and the cytoplasm, modulating their affinity for cargo based on the nucleotide-bound state of Ran GTPase (Kuersten et al. 2001). All nucleocytoplasmic transport occurs through large channel structures called nuclear pore complexes (NPC), whose many components were also being identified (Rout and Wente 1994). As for RNA transport, the first competitive inhibition studies by microinjection of RNAs into oocyte nuclei, suggested that different classes of RNAs have separable carriers involved in mediating their export (Jarmolowski et al. 1994), and that perhaps RNA transport was analogous to protein transport in that it utilized cargo carriers and import/export receptors.

To identify factors involved in snRNA transport we developed an *in situ* hybridization assay to localize snRNAs in *S. cerevisiae*. Although it had never been demonstrated that snRNAs undergo

a similar bidirectional transport during biogenesis in yeast cells, the major landmark steps of Sm core assembly, trimethylation of the cap and 3' end trimming are conserved between yeast and mammals, suggesting that other aspects are also conserved. With the assay in hand we were poised to characterize the yeast snRNP biogenesis pathway.

## Materials and Methods

### Yeast strains

See Table 1 for yeast strains used in this study.

### ssDNA probe preparation

To make ssDNA probes, PCR reactions were performed using sense strand primers phosphorylated at their 5' end and incorporating digoxygenin-dUTP (4:1 dTTP:digoxygenin-dUTP) (Roche). Sense strands were subsequently specifically digested with lambda exonuclease (Pharmacia Biotech).

### *In situ* hybridization assay

Yeast cells were grown, fixed, washed and placed on polylysine treated glass slides as previously described (Takizawa et al. 1997). Cells were incubated in hybridization mix (5μg/ml each probe, 50% formamide, 20% dextran sulfate, 2xSSC, 0.4% BSA, 1mg/ml tRNA) overnight at 37°C and then washed in 2xSSC/50% formamide, 2xSSC, 1xSSC and equilibrated in PBST. Cells were then incubated with anti-digoxygenin antibody conjugated with FITC (Roche) diluted 1:100 in PBST for 1hr at 37°C. Cells were washed in PBS and mounted for visualization with a Zeiss Axioscope microscope using a 100x/1.3 NA lens. Images were captured using a Sensys CCD camera (Photometrics).

### Heterokaryon assay

Heterokaryons were made as previously described (Flach et al. 1994), mating a strain containing wildtype U2 snRNA (yES18) or a strain deleted for the U2 fungal domain (yES77) (Shuster and

Guthrie 1988) with *kar1-Δ13* (Vallen et al. 1992). Fixation and *in situ* hybridization procedures were performed as described above.

## Results

### *In situ* hybridization of specific RNA LacZ

Our first goal was to visualize the subcellular localization of snRNAs. While bulk polyA+ mRNAs had been successfully localized using an oligo dT50 probe against their polyA tail (Amberg et al. 1992; Kadowaki et al. 1994), less was known about the requirements for localizing specific RNAs. We attempted to visualize abundant endogenous mRNAs such as PGK1, TUB1 and ACT1. However, signal was low and without a negative control strain lacking the target RNA for these essential genes, we could not tell if the observed signal was significantly above background. We also attempted to visualize the localization of CUP1 and SSA4 mRNAs that are respectively nonessential and inducible, but again signal was weak and difficult to discern. To develop this assay we examined a highly expressed exogenous mRNA, LacZ. A strain lacking the LacZ expressing plasmid served as a negative control (Legrain and Rosbash 1989). Since signal strength was a limiting factor for specific RNAs, we started with enzymatic signal amplification of a diffusible substrate (alkaline phosphatase and BCIP/NBT). We observed specific whole cell staining of LacZ that was absent with the negative control strain (data not shown). However, in an mRNA export mutant such as *brr3-1* where polyA+ mRNAs were accumulated in the nucleus (data not shown, Awabdy and Guthrie personal communication), we also observed whole cell staining, most likely due to diffusion of the substrate (data not shown). As these results were promising, we decided to turn directly to snRNA targets for assay optimization.

**ssDNA probes were developed for *in situ* hybridization of snRNAs**

At this time, others had begun to develop both short DNA probes and long RNA probes generated by T7 transcription for localizing specific RNAs. Singer and colleagues had used a highly light sensitive microscope and short DNA probes to visualize ASH1 mRNA to the bud tip (Long et al. 1997). Conversely, Vale and colleagues had used a standard microscope and long RNA probes to visualize the same ASH1 mRNA (Takizawa et al. 1997). While RNA probes form stronger hybrids with their targets than DNA probes, they are often unstable. Since amplification of the entire target sequence for maximum signal coverage was possible by PCR and DNA probes were more reliably stable than RNA probes, we developed single stranded DNA (ssDNA) probes by eliminating the competing sense-strand to maximize signal. We initially used biotin-labeled sense-strand oligos in the PCR reactions and attempted to purify the probe strands by streptavidin pull-downs, but the separation and purification steps were inefficient (data not shown). Instead we utilized the ability of lambda exonuclease to specifically cleave DNA strands terminated with 5' phosphates. By including sense-strand 5' phosphate-containing oligos in our PCR reactions, we could efficiently synthesize the desired ssDNA probes (data not shown). In addition, rather than using diffusible substrates for visualization, we incorporated digoxygenin labeled nucleotides during the PCR reactions and visualized the probes by fluorescently labeled antibodies against digoxygenin.

**A strain deleted for the fungal domain of U2 snRNA serves as a negative control for *in situ* hybridization assays**

Since *in situ* hybridization signal for specific RNAs is often low, it was critical to have a strain lacking the target RNA in order to assess background levels. However, the snRNA genes are essential in *S. cerevisiae*. Interestingly, while core sequences are conserved between yeast and human snRNAs, some yeast snRNAs have up to 1kb long intervening sequences that were found to be nonessential for growth and were dubbed 'fungal domains' (Shuster and Guthrie 1988; Roiha et al. 1989; Liao et al. 1990; Siliciano et al. 1991). We designed *in situ* hybridization

probes to the 945 nucleotide nonessential fungal domain of U2 snRNA and used a strain deleted for this region as our negative control (Shuster and Guthrie 1988). We used five tandem ~190 nucleotide ssDNA probes covering the entire length of the fungal domain. We initially found that a tertiary antibody signal amplification setup using mouse anti-digoxygenin, rabbit anti-mouse, and Rhodamine-conjugated goat anti-rabbit was required for visualization (Figure 2B). After further optimization of hybridization and fixation procedures we were able to use a single anti-digoxygenin Fluorescein-conjugated antibody for snRNA localization (Figure 3).

**snRNAs are localized to the nucleus in *S. cerevisiae***

Using ssDNA probes for the U2 snRNA fungal domain (Figure 2A), we were able to localize U2 snRNA to the nucleus in yeast cells (Figure 2B). A similar strategy for U1 snRNA using the fungal domain, which is considerably shorter (315nt) than the U2 fungal domain (Liao et al. 1990; Siliciano et al. 1991), also localized U1 snRNA to the nucleus (Figure 3A). However, detection of U1 snRNA required tailing of the PCR products with DIG labeled nucleotides for further signal amplification. For the snRNAs without sizeable fungal domains, we were able to visualize dim nuclear signal for U4, U5 and U6 snRNAs using full length probes (160, 214, and 112 nucleotides respectively) (Figure 4B and data not shown). Using probes that covered the full length of U3 snoRNA (334 nucleotides), we were also able to localize U3 snoRNA to a crescent shaped structure overlapping, but not fully concomitant with DAPI signal, which we presume to be the nucleolus (Figure 4B).

**Transport mutants do not display any defects in snRNA localization**

To identify factors involved in snRNA transport, we examined a battery of mutant strains previously shown to be defective in transport processes, looking for either increased nuclear signal (snRNA export mutants) or increased cytoplasmic signal (snRNA import mutants). We first examined mutant strains known to block general nucleocytoplasmic transport – nucleoporin components of the nuclear pore complex, Ran GTPase effectors and importin $\alpha/\beta$ protein import factors (see Table 1). Mutant strains we examined were *nup116*$\Delta$ (blocked pores by EM), *nup49-*

*313* (protein import), *rna1-1* (Ran GAP), *prp20-1* (Ran guanine nucleotide exchange factor), *srp1-49* (importin α), *kap95-1* (importin β) (Figure 3 and data not shown). In each of these mutant strains, U1 and U2 snRNA remained nuclear even after shift to nonpermissive temperatures (Figure 3B). Nuclear accumulation of polyA+ mRNA was confirmed by *in situ* hybridization with the dT50 assay for those strains with mRNA export defects (data not shown). With some nucleoporin mutant strains, *nup116Δ*, *nup49-313*, *nup188Δ*, and *cse1-1*, an importin β homolog (see below) we believe we observed a slight increase in nuclear signal (Figure 3B and data not shown). However, it was unclear if this observation was significant in the absence of quantitative methodologies, which were unavailable at the time.

Brr1p was shown to play a role in snRNP biogenesis since *brr1* mutant strains are defective in producing mature snRNPs from newly synthesized snRNAs (Noble and Guthrie 1996). Because Brr1p could potentially play a role in snRNP transport during biogenesis, we also examined U1 and U2 snRNA localization in both *brr1-1* and *brr1Δ* strains, but we did not observe U1 or U2 snRNA mislocalization (data not shown).

Although data from mammalian systems suggested that different classes of RNAs are exported by separable pathways (Jarmolowski et al. 1994), factors involved in mRNA export could also be involved in snRNA transport. Therefore, we examined mRNA export mutants shown by accumulation of polyA signal in the nucleus via the dT50 assay (de Bruyn Kops and Guthrie personal communication). We examined *brr3-1*, *brr4-1*, *brr6-1*, *brr6-2*, *brr7-1* and *brr8-1* under conditions with nuclear accumulation of bulk mRNAs (data not shown), but we only observed nuclear localization for U1 and U2 snRNAs (data not shown).

**Importin β homologs are not involved in snRNA transport in yeast**

In 1997, the entire yeast genome was sequenced and we and others were able to perform genome-wide searches for homologous proteins (Cherry et al. 1997). Using BLAST, 14 yeast homologs of importin β were identified (Altschul et al. 1990). To examine whether the cargoes of any of

these putative carrier proteins were in fact carriers for snRNAs, we assayed snRNA localization with 11 of the 14 importin β homologs for which mutant strains were available, namely *kap95-1*, *kap104Δ*, *pse1-1*, *kap123Δ*, *pse1-1/kap123Δ*, *cse1-1*, *sxm1Δ*, *los1-1*, *mtr10Δ*, *crm1-1*, *nmd5Δ* and *msn5Δ* (see Table 1). However we did not observe any changes in U1 or U2 snRNA localization (Figure 3 and data not shown). We constructed deletion strains of the three remaining importin β homologs (*pdr6Δ*, *ygl241Δ*, and *lph2Δ)*, which were nonessential and assayed for mislocalization of U1 and U2 snRNAs. We did not observe any changes in snRNA localization for the eight candidate deletion strains we tested for each homolog (data not shown). This data suggested that importin β homologs are not required for the transport of snRNAs in yeast.

**Crm1p is not involved in snRNA export in yeast**

During the course of this work, the mammalian importin β homolog CRM1 was shown to be involved in export of protein cargo containing leucine-rich nuclear export signals (Fornerod et al. 1997). Moreover, it had been shown in mammalian cells that Leptomycin B, a cytotoxin that binds specifically to CRM1, inhibited U snRNA export, suggesting that snRNA export may be mediated by an NES-containing factor bound to CRM1 (Fornerod et al. 1997). To test whether this was also true in yeast, we examined U1 and U2 snRNA localization in a *crm1-1* mutant strain under conditions previously shown to block mRNA export (Stade et al. 1997). However, we did not observe any increase in nuclear snRNA signal or any change in snRNA localization (Figure 3B and data not shown), suggesting that Crm1p is not required for snRNA export in yeast. During snRNP biogenesis in metazoan cells, some steps of 3'end trimming have been shown to occur in the cytoplasm (Neuman de Vegvar and Dahlberg 1990). Therefore, as an alternative to *in situ* hybridization assays, we attempted to use 3' end processing as a biochemical marker for export of snRNAs. To assay 3' end processing of newly synthesized U2 snRNA, we induced expression of a U2 snRNA with altered mobility due to a short deletion, under the control of the GAL4 promoter in galactose-containing media (Noble and Guthrie 1996). To assay U2 3' end

formation in a strain predicted to block snRNA export, we examined newly synthesized U2 snRNA 3' end formation in a *crm1-1* strain upon shift to nonpermissive temperature (37°). However, we did not observe accumulation of a pre-U2 snRNA species, (data not shown). We observed that the total levels of U2 snRNA were decreased in the *crm1-1* strain, but the ratios of pre-U2 to mature U2 snRNA and the kinetics of U2 3' end trimming were similar to that of wildtype. This suggested that either 3' end trimming is not a true marker for snRNA export to the cytoplasm (see Discussion) or that Crm1p is not involved in snRNA export in yeast as predicted from mammalian studies.

**Cell wall morphology may confound the *in situ* hybridization assay**

Most mutant strains we examined with our snRNA *in situ* hybridization assay displayed no change in U1 or U2 snRNA localization (Figure 3, data not shown). However, one mutant, *ssr1-1*, displayed striking whole cell signal of snRNAs when shifted to the nonpermissive temperature (Figure 4A), suggesting a role for Ssr1p in snRNP import. The *ssr1-1* mutant was found to suppress the temperature sensitivity of a U1 snRNA deletion mutation, but only in combination with another mutant *tom1-1* (Umen and Guthrie 1995), suggesting that these factors could indeed be playing a role in snRNA metabolism. Interestingly, mutants in *tom1*, encoding a ubiquitin ligase, had been previously shown to be defective in mRNA export (Duncan et al. 2000). The *tom1-1* mutant displayed nuclear U1 and U2 snRNA localization, while *ssr1-1* displayed whole cell signal, and the double mutant displayed an intermediate phenotype (Figure 4A). We also examined the localization of U3 snoRNA and U6 snRNA, which unlike U1 and U2 snRNAs are restricted to the nucleus and are not predicted to accumulate in the cytoplasm (Vankan et al. 1990; Speckmann et al. 1999). We observed both U3 snoRNA and U6 snRNA to display similar whole cell signal (Figure 4B), suggesting that either U3 snoRNA and U6 snRNA undergo a previously undescribed cytoplasmic phase or that the whole cell signal we observed under these conditions was an artifact.

To determine if the whole cell signal was U2 snRNA-dependent, we constructed an *ssr1-1* strain also deleted for the fungal domain of U2 and conducted U2 snRNA *in situ* hybridization experiments. We observed partial whole cell signal even in the absence of U2 snRNA target (data not shown), suggesting that the signal we observed was an artifact and not a true indication of snRNA localization. At this time Ssr1p was shown to be a factor involved in cell wall biosynthesis, Kre6p, a 1,4 β glucan synthase (Duncan and Guthrie personal communication). Identifying Ssr1p as a factor involved in cell wall metabolism was consistent with a model whereby changes in cell wall composition could cause nonspecific interactions between the probe and the outside of the cell, mimicking whole cell signal. We observed that *ssr1-1* cells also displayed abnormally bright whole cell signal with a dT50 probe when shifted to the nonpermissive temperature (37°) (data not shown), suggesting that the *in situ* hybridization assay is not robust under these conditions.

Another mutant strain *prp40-10* also displayed whole cell signal of U1 and U2 snRNA upon shift to the nonpermissive temperature (37°) (Figure 5). Prp40p is a U1 snRNP protein, which contains two leucine-rich NES sequences (Murphy et al. 2004). Intriguingly, mutants of *prp40* with deletions of either of the NES sequences are synthetically lethal with *crm1* mutations (Murphy et al. 2004). Siliciano and colleagues have proposed that Prp40p may be exported in a Crm1p-dependent manner and that U1 snRNA may be exported via its interaction with Prp40p (Murphy et al. 2004). However, we observed that increasing digestion of the cell wall by zymolyase reduced U1 and U2 snRNA whole cell signal and increased nuclear signal (Figure 5, data not shown), suggesting that the state of the cell wall can have profound effects on apparent *in situ* hybridization signal.

**Overexpressing U2 snRNA or depleting SmD1p did not increase U2 snRNA signal in the cytoplasm**

In addition to examining a battery of mutant strains for snRNA mislocalization, we attempted to find evidence for snRNA transport by two other means. First, we attempted to visualize

cytoplasmic snRNA signal by overexpressing U2 snRNA and increasing overall levels of U2 snRNA through the biogenesis pathway. We induced U2 snRNA from a plasmid containing full-length U2 snRNA under control of the GAL4 promoter in both a wildtype and a strain lacking the fungal domain grown in galactose media. With the wildtype strain, we observed no change in nuclear signal (data not shown). With the strain lacking the fungal domain, we observed appearance of full-length U2 snRNA in the nucleus upon induction. However, we did not observe any increase in cytoplasmic signal despite an increase in total U2 snRNA levels as assayed by Northern analysis (data not shown).

Second, we attempted to visualize cytoplasmic snRNA signal by depleting Sm protein SmD1p. In mammalian cells, Sm proteins bind to snRNAs in the cytoplasm and are required for reimport (Hamm et al. 1990). To test if depleting Sm proteins could lead to accumulation of precursor snRNAs in the cytoplasm, we examined U1 and U2 snRNA localization in a strain containing SmD1 under the control of a GAL4 promotor grown in the presence of glucose. Although we did not independently confirm depletion of Smd1p, we did not observe an increase in cytoplasmic signal (data not shown).

***In situ* hybridization of U2 snRNA in binucleate heterokaryon cells suggest that snRNAs are not transported in yeast**

To assay for snRNA transport, we developed an alternative *in situ* hybridization assay using binucleate cells. It had been previously demonstrated that strains defective in nuclear fusion, karyogamy mutants, can still mate to produce a yeast cell with shared cytoplasm and two nuclei called a heterokaryon (Conde and Fink 1976). These mutants had been used to demonstrate nucleocytoplasmic shuttling of certain proteins (Flach et al. 1994). To see if U2 snRNA could be transferred by export from one nucleus and import into a second nucleus, we mated a *kar1-Δ13* strain containing wild type U2 snRNA with a strain containing U2 snRNA lacking the fungal domain and asked whether U2 snRNA signal could be detected in both nuclei of the resulting heterokaryons (Figure 6A). While we observed rapid NPL3-GFP protein shuttling within 1 hour

of heterokaryon formation (data not shown), when we fixed the heterokaryons and performed *in situ* hybridization, we consistently observed U2 snRNA localization in only one of two nuclei (Figure 6B). In the event that accumulation of snRNAs into the second nucleus required further incubation, we examined heterokayrons 5 hours after mating, but we nonetheless only observed U2 snRNA localization in one of two nuclei (data not shown). Even after 24 hrs, when the heterokaryon nuclei were replicating and budding multiple nuclei, we were still unable to observe heterokaryons without at least one unlabeled nucleus (data not shown). Although it is conceivable that snRNA transport may be coupled to the originating nucleus and may not be released into the cytoplasm allowing for import into a second nucleus, this data suggests that snRNAs are not transported in *S. cerevisiae*.

**Discussion**

While snRNAs undergo a cytoplasmic assembly and biogenesis phase in metazoan cells (Figure 1), we and others have accumulated increasing evidence for the conclusion that yeast snRNAs are not transported during biogenesis. First, we detect no evidence for a cytoplasmic phase for yeast snRNAs using a fluorescence *in situ* hybridization assay. We looked for mislocalization of U1 and U2 snRNAs in a battery of transport mutants including nucleoporins, Ran GTPase, effectors, protein import factors, mRNA export factors and all 14 known importin β homologs, but we only observed nuclear localization (Figure 3, data not shown). It is possible that our *in situ* hybridization assay is not sensitive enough to detect subtle increases in already nuclear signal for export mutants or low levels of cytoplasmic pre-snRNA signal for import mutants. Development of quantitative measurements combined with microscopic localization may elucidate subtle changes in signal (Brendt personal communication).

We also did not detect U2 snRNA transfer in heterokaryon yeast cells containing two nuclei fused with a common cytoplasm (Figure 6), suggesting a solely nuclear biogenesis pathway. In a recent

analogous study, Siliciano and colleagues have observed transfer of U1 snRNA in heterokaryon cells (Olson and Siliciano 2003). Their finding that Prp40p, a U1-associated protein, contains two NES sequences that genetically interact with CRM1, suggests that Prp40p may be exported in a Crm1p-dependent manner (Murphy et al. 2004). They propose that U1 snRNA may be uniquely exported in association with Prp40p as no other snRNP proteins with NES sequences have been characterized (Olson and Siliciano 2003; Murphy et al. 2004). Contrary to our data, they observed that U2 snRNA is also transferred (Olson and Siliciano 2003). However, they observed that U6 snRNA and several snoRNAs not known to be transported to the cytoplasm (Vankan et al. 1990; Speckmann et al. 1999) were also transferred (Olson and Siliciano 2003), suggesting leakage of RNAs. Further work is required to understand the differences in our results, but their longer fixation procedure is a possible explanation.

Second, as further evidence for a nuclear biogenesis pathway in yeast, some of the recently identified biogenesis factors involved in 3' end trimming of snRNA precursors and trimethylation of the cap have been localized to the nucleolus and/or the nucleus. Rnt1p, the yeast homolog of RNase III, which has been shown to cleave 3' end stem loop structures in snRNA and snoRNA precursors (Chanfreau et al. 1997; Abou Elela and Ares 1998), has been localized to the nucleus and nucleolus (Huh et al. 2003), suggesting that this cleavage step occurs within the nucleoplasm. The Parker and Tollervey groups have shown that nuclear exosome factors (Rrp6p and Mtr4p) are involved in 3' end trimming of U4 and U5 snRNAs (Allmang et al. 1999; van Hoof et al. 2000; van Hoof et al. 2000). In addition, the snRNA and snoRNA trimethylating enzyme, Tgs1p, has been recently identified and localized to the nucleolus (Mouaikel et al. 2002). Intriguingly, deletion of *tgs1* causes U1 snRNA to accumulate in the nucleolus, suggesting that trimethylation of the snRNA cap occurs in the nucleolus and the precursors become blocked or trapped in the absence of trimethylation (Mouaikel et al. 2002). These data suggest that major snRNA biogenesis steps may occur specifically in the nucleolus in yeast.

Lastly, since the time of our work, progress toward identifying the metazoan factors involved in snRNA transport has been significant (Figure 1). Both the snRNP import and export receptors have been identified as snurportin and PHAX respectively (Huber et al. 1998; Ohno et al. 2000). Snurportin binds the trimethyl G cap structure of snRNPs cooperatively with importin β to mediate snRNP import and PHAX contains an NES sequence bound by CRM1/XPO1, which together bind the cap binding complex of newly transcribed snRNAs and mediates snRNA export (Huber et al. 1998; Ohno et al. 2000). Strikingly, there are no yeast homologs for either snurportin or PHAX despite conservation of both the recognition elements on the snRNAs, the trimethylated cap and the cap binding complex, and the transport receptors, Kap95p and Xpo1p. This indicates that the yeast biogenesis pathway may either undergo transport via different factors from mammalian cells or alternatively, snRNAs may remain restricted to the nucleus. Moreover, a large complex called the survival of motor neurons complex (SMN) involved in cytoplasmic snRNP assembly in mammlian cells, is not present in *S. cerevisiae* (Fischer et al. 1997), suggesting that budding yeast lack machinery required for cytoplasmic snRNP assembly. SMN is a critical cellular component as it has been identified as the disease gene for Spinal Muscular Atrophy (SMA), a common neurodegenerative disease (Meister et al. 2002). Specifically, the SMN complex has been shown to bind to newly exported snRNAs associated with PHAX in the cytoplasm (Massenet et al. 2002). SMN binds directly to dimethylarginine-modified domains of certain Sm proteins, which do not exist in yeast Sm proteins, and are believed to modulate cytoplasmic Sm core assembly (Friesen et al. 2001). Interestingly, SMN has also been shown to function in the nucleus in recycling of snRNPs during pre-mRNA splicing (Liu et al. 1997). While no SMN homologs have been identified in *S. cerevisiae,* SMN interacting protein, SIP1 was believed to have distant homology to *S. cerevisiae* snRNP biogenesis factor, Brr1p (Liu et al. 1997). Subsequent work in *S. pombe* has identified homologs for SMN and SIP1 in fission yeast as YAB8 and YIP1 respectively (Hannus et al. 2000). However, YIP1 bears no resemblance to BRR1 (Hannus et al. 2000). While Brr1p and SIP1 may

not be closely related by sequence homology, they may nonetheless be functional homologs. Brr1p has been shown to be involved in the biogenesis of newly synthesized snRNAs and also in snRNP recycling during splicing in *S. cerevisiae* (Noble and Guthrie 1996) (see Chapter II). One difference between yeast and metazoan cells that may offer an explanation for the divergence in nuclear and cytoplasmic snRNP assembly is that budding yeast undergo closed mitosis whereas mammalian cells undergo open mitosis in which the nuclear envelope is dissolved and all nuclear components must be subsequently reimported. Mammalian cells require mechanisms for reimporting snRNPs during mitosis that they may have adapted to compartmentalize snRNP biogenesis. The fission yeast *S. pombe* also undergo closed mitosis, but unlike *S. cerevisiae. S. pombe* have SMN complex homologs (Hannus et al. 2000). It has yet to be determined if snRNAs are transported to the cytoplasm during biogenesis in *S. pombe*, but since pombe snRNP biogenesis is more closely related to metazoan biogenesis mediated by SMN (Hannus et al. 2000), it is therefore likely to be cytoplasmic. This suggests that snRNP assembly in the cytoplasm is not necessarily dictated by open mitosis. If so, then what does determine the localization of the snRNP assembly pathway?

Another striking and provocative difference between budding yeast U snRNAs and both the mammalian and *S. pombe* snRNAs is the presence of elongated 'fungal domains' in *S. cerevisiae* (Roiha et al. 1989). The fungal domains may act as chaperones or as binding sites for chaperones in assisting RNA folding for proper snRNP assembly and biogenesis and bypass the need for an SMN-mediated cytoplasmic assembly phase. Further investigation is required to dissect the function of the fungal domains.

Whatever the reason(s) for a nuclear assembly phase may be, if biogenesis occurs in the same subcellular compartment as splicing, mechanisms to insure proper biogenesis has occurred before entry into splicing may exist. The localization of the yeast trimethylating enzyme to the nucleolus suggests that at least some biogenesis steps are shunted to the nucleolus (Mouaikel et al. 2002) and may serve to compartmentalize snRNP assembly from snRNP activity.

Interestingly, it has been observed that 3' elongated pre-snRNAs can be assembled into spliceosomal complexes that are functional (Abou Elela and Ares 1998; Xue et al. 2000), indicating that some snRNA precursors can enter the splicing pathway. However, other snRNAs are targeted for degradation as in the case of newly synthesized snRNAs in a *brr1-1* mutant strain (Noble and Guthrie 1996), suggesting that there may be factors involved in quality control. Intriguingly, *brr1* mutant strains also display defects in splicing (Noble and Guthrie 1996; Noble and Guthrie 1996). In *S. cerevisiae,* the steps involved in snRNP biogenesis and the steps involved in regenerating or recycling snRNPs during pre-mRNA splicing presumably occur in the nucleus and may share common steps. Investigation of the mechanistic links between snRNP recycling and snRNP biogenesis may elucidate steps required for insuring completion of snRNP assembly before entry into splicing.

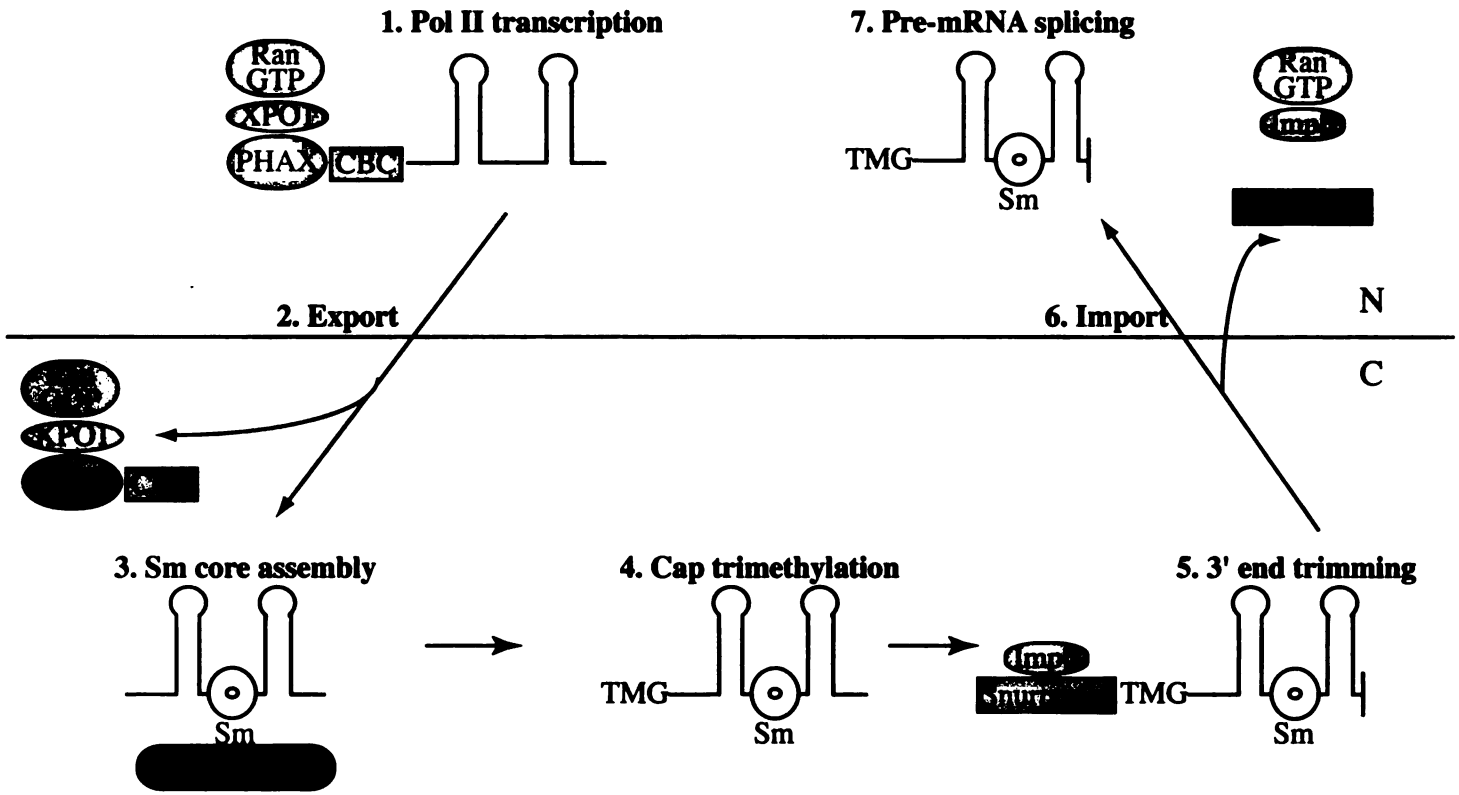


**Figure 1** snRNA tranport and biogenesis in mammalian cells. 1) snRNAs are transcibed in the nucleus, 2) exported to the cytoplasm, 3) bound by Sm proteins, 4) trimethylated at the cap, 5) 3' end trimmed and 6) reimported into the nucleus for 7) splicing. The mediating factors were identified after our studies (blue and red). Factors in red have no yeast homolog.

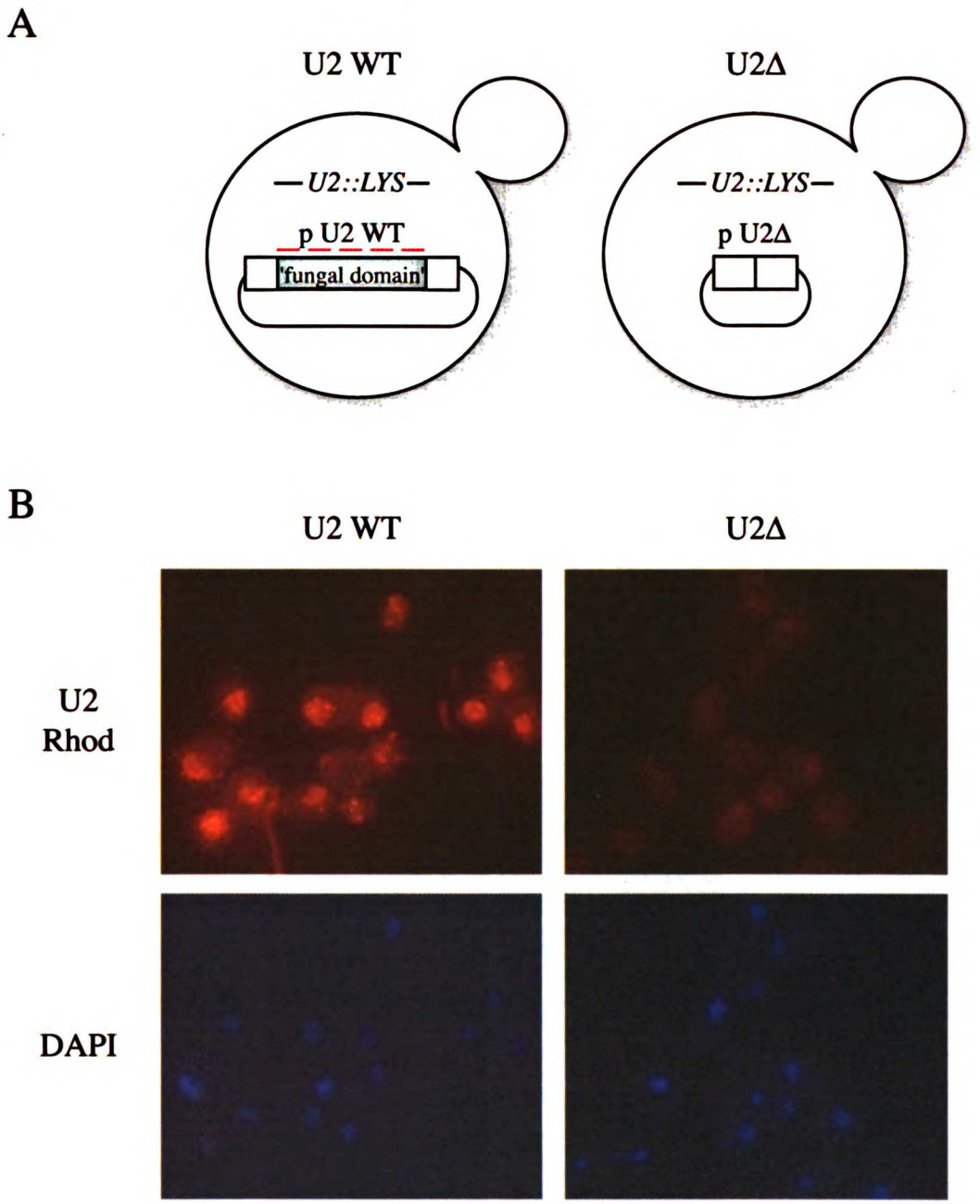


**Figure 2** U2 snRNA is localized to the nucleus in *S. cerevisiae*. A) U2 snRNA probes designed to target the nonessential fungal domain (red) B) specifically localize U2 snRNA to the nucleus (stained with DAPI) in cells containing full length U2 (U2 WT), but not in cells with U2 deleted for the fungal domain (U2Δ).

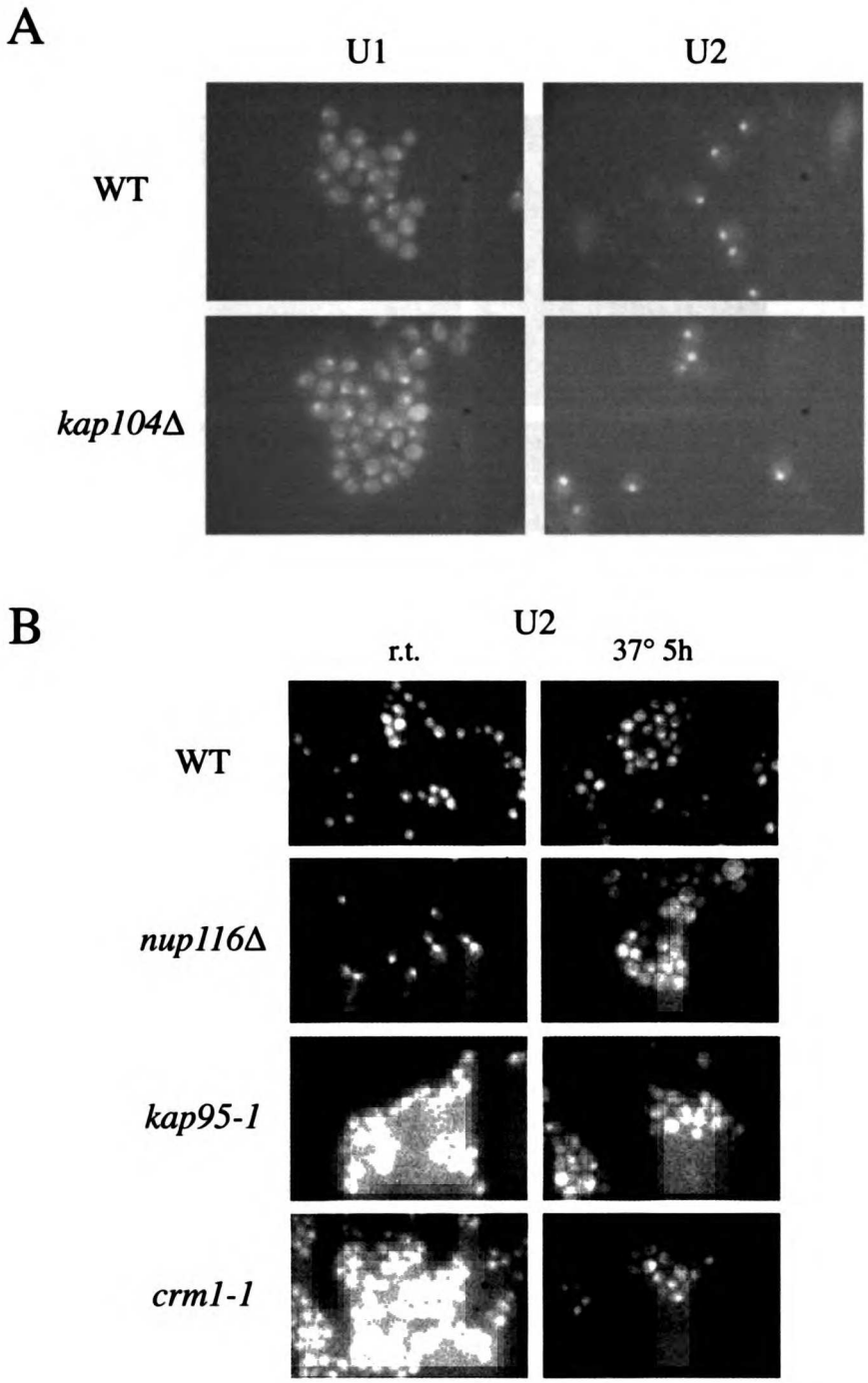


**Figure 3** U1 and U2 snRNAs are localized to the nucleus in transport mutant strains. A) U1 and U2 snRNA are nuclear localized in *kap104Δ* strain shifted to nonpermissive temperature (37°) for 1hr. B) U2 snRNA is localized to the nucleus in nucleoporin *nup116Δ*, importin beta homolog *kap95-1*, and nuclear export factor *crm1-1* strains shifted to nonpermissive temperature (37°) for 5hrs.

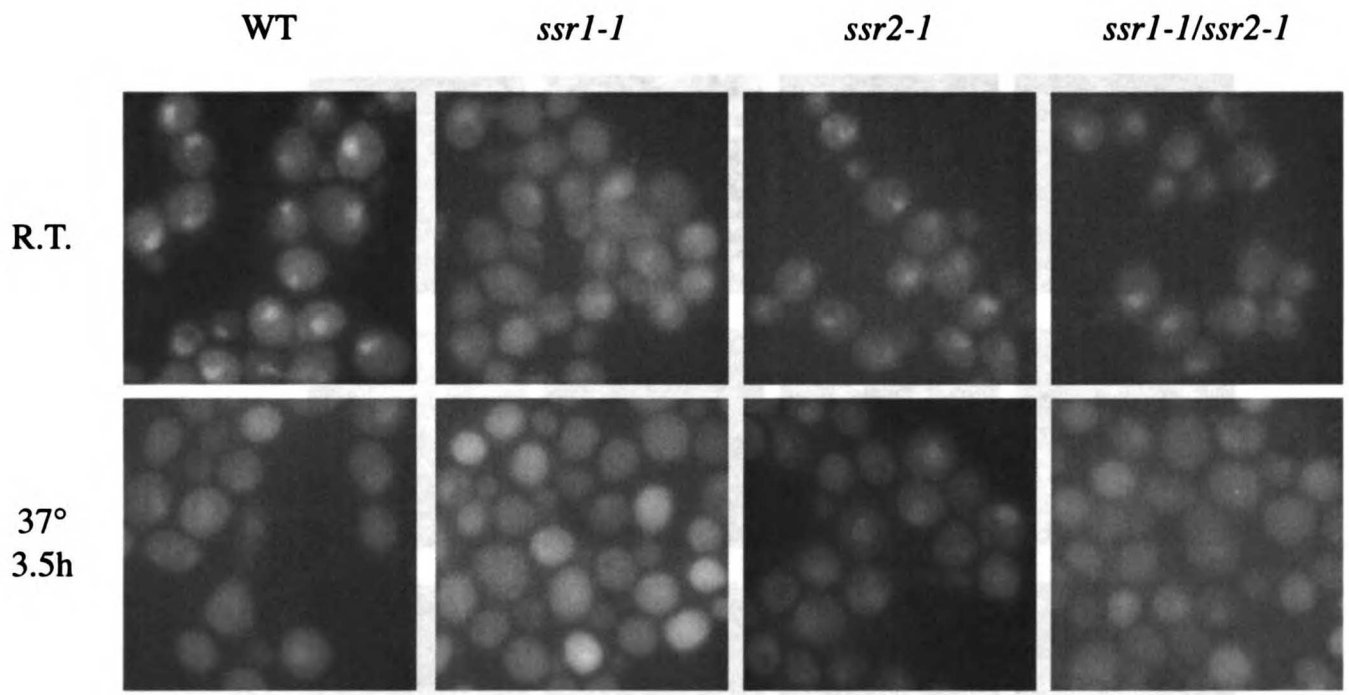


**Figure 4A** *ssr1-1* cells display bright whole cell U2 snRNA signal upon shift to the nonpermissive temperature. *ssr2-1* cells display nuclear U2 snRNA localization, while *ssr1-1/ssr2-1* double mutant cells display an intermediate phenotype.

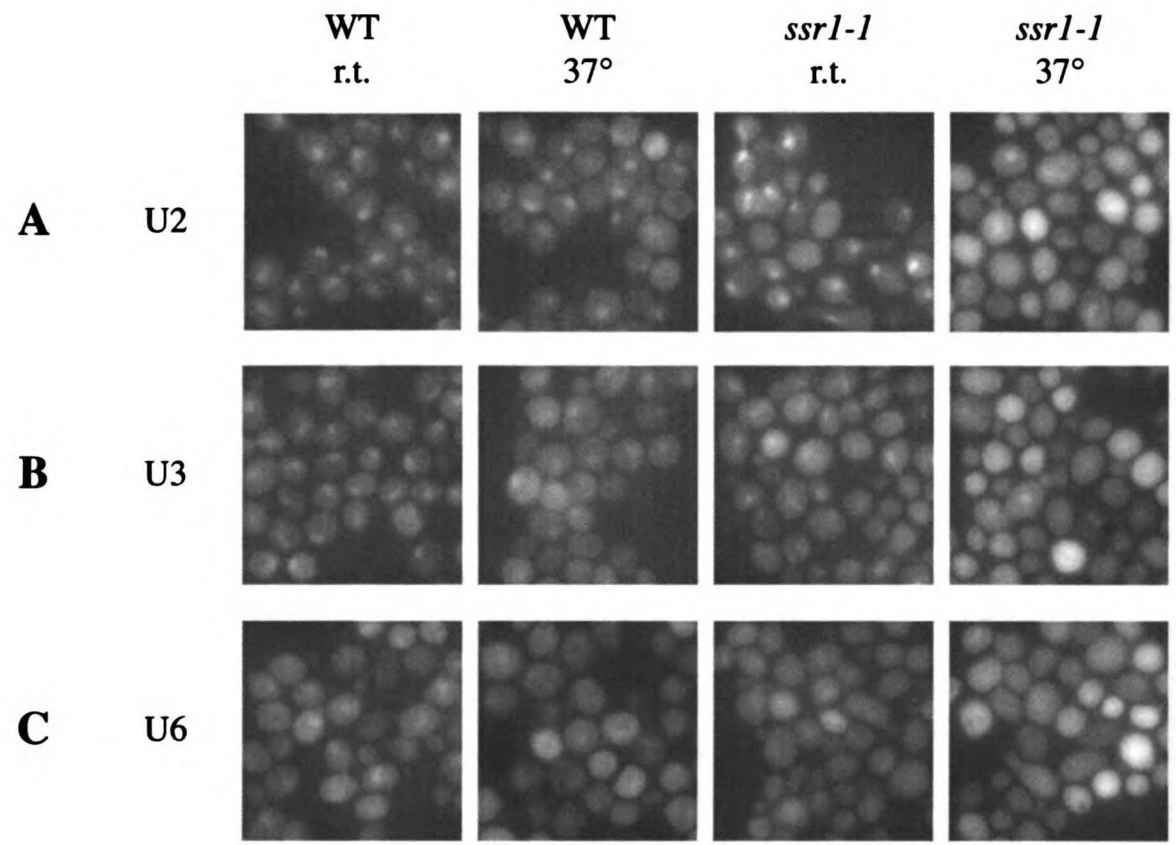


**Figure 4B** A) *ssr1-1* cells shifted to the nonpermissive temperature (37°) for 3.5h display bright whole cell U2 snRNA signal, as well as whole cell B) U3 snoRNA and C) U6 snRNA signal.

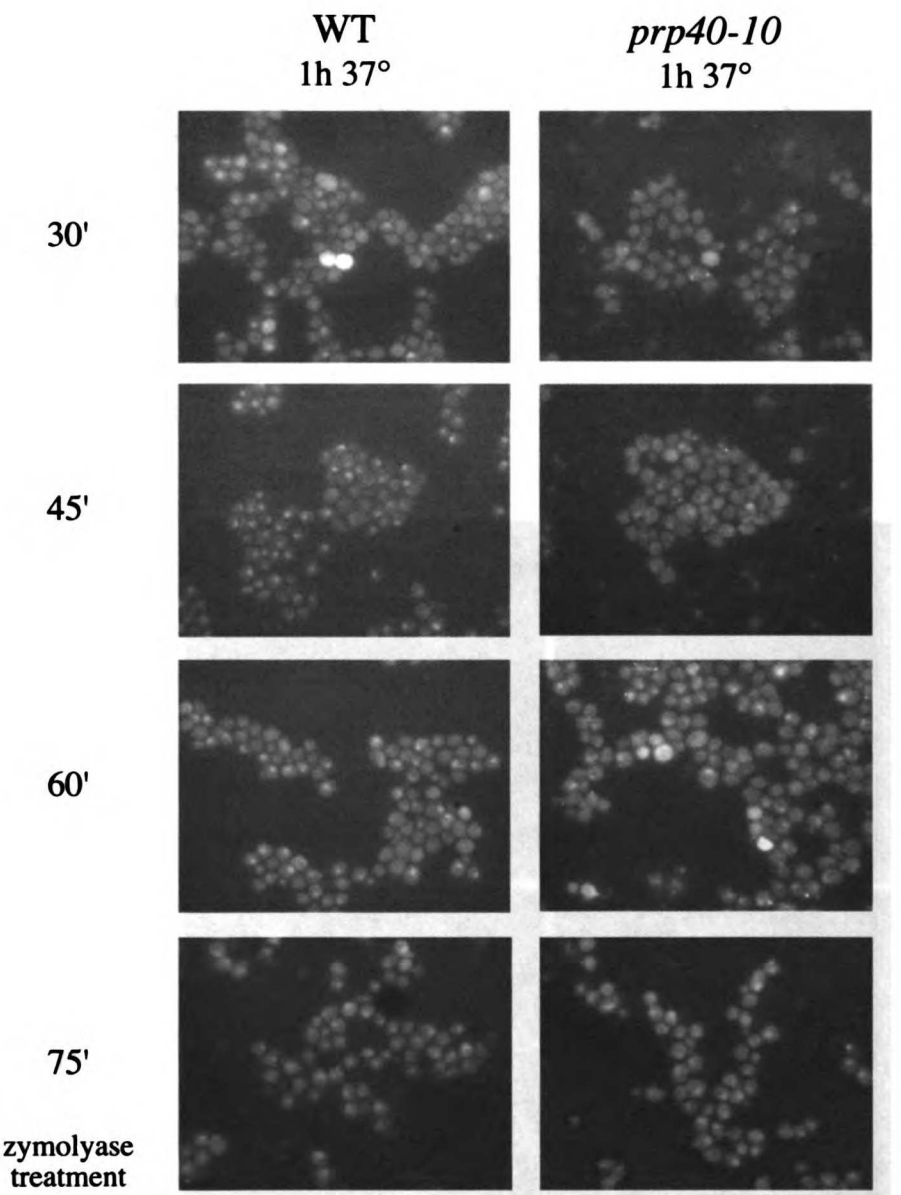


**Figure 5** Nuclear localization of U1 snRNA becomes apparent in *prp40-10* cells with 60 minutes of zymolyase treatment. *prp40-10* cells were shifted to 37° for 1hr, fixed, digested with zymolyase for the indicated times and hybridized with probes targeted for U1 snRNA.

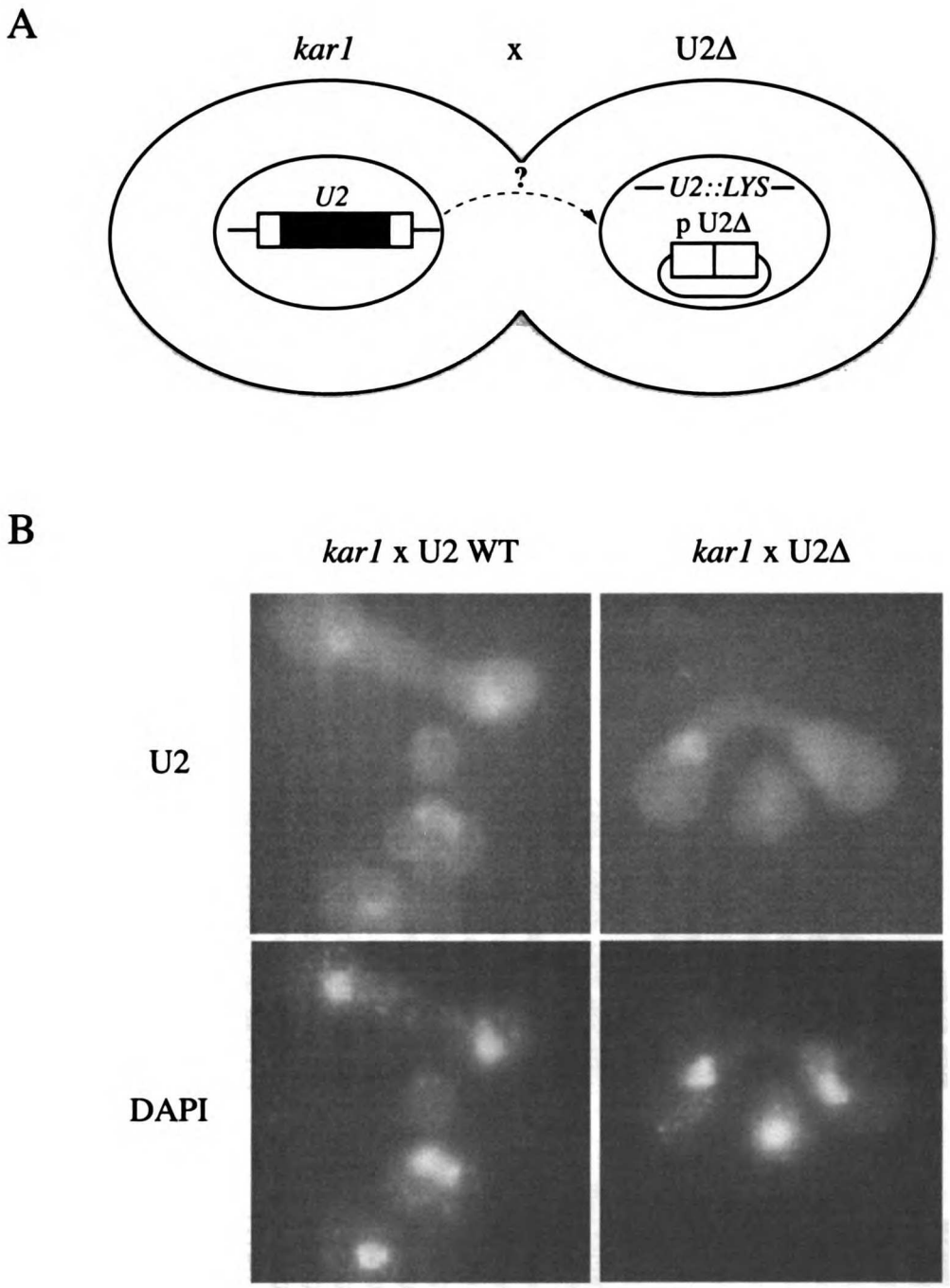


**Figure 6** U2 snRNA is localized to only one nucleus in heterokaryon experiments. A) A karyogamy mutant strain (*kar1*) is crossed with another strain containing either full length U2 snRNA (U2 WT) or U2 deleted for its fungal domain (U2Δ) to form heterokayrons and B) are subsequently hybridized with probes for U2 snRNA.

**Table 1 - Strains tested for snRNA localization**

| function | mutant | source |
|---|---|---|
| nucleoporin | *nup116Δ* | Wente and Blobel 1993 |
| | *nup49-313* | Doye and Hurt 1994 |
| | *nup188Δ/brr7-1* | Zabel & Hurt 1996, Nehrbass & Wozniak 1996, de Bruyn Kops & Guthrie |
| Ran effector | *prp20-1* | Amberg and Aebi 1993 |
| | *rna1-1* | Hopper and Dunst 1990 |
| snRNA biogenesis | *brr1-1/brr1Δ* | Noble and Guthrie 1996 a,b |
| mRNA export | *brr3-1* | de Bruyn Kops and Guthrie personal communication, Awabdy & Guthrie |
| | *brr4-1* | de Bruyn Kops and Guthrie personal communication |
| | *brr6-1* | de Bruyn Kops and Guthrie personal communication |
| | *brr6-2* | de Bruyn Kops and Guthrie personal communication |
| | *brr8-1* | de Bruyn Kops and Guthrie personal communication |
| protein import | *srp1-49* | Yano and Nomura 1994 |
| | *kap95-1* | Iovine and Wente 1997 |
| importin beta | *kap104Δ* | Aitchison, Blobel and Rout 1996 |
| | *pse1-1* | Seedorf and Silver 1997 |
| | *kap123Δ* | Seedorf and Silver 1997 |
| | *pse1-1/kap123Δ* | Seedorf and Silver 1997 |
| | *cse1-1* | Xiao and Fitzgerald-Hayes 1993 |
| | *sxm1Δ* | Rosenblum and Blobel 1997 |
| | *los1-1* | Hopper and Shapiro 1980 |
| | *mtr10Δ* | Kadowaki and Tartakoff 1994 |
| | *crm1-1* | Stade and Guthrie 1997 |
| | *nmd5Δ* | Ferrigno and Silver 1998 |
| | *msn5Δ* | Kaffman and O'Shea 1998 |
| | *pdr6Δ* | this study |
| | *ygl241Δ* | this study |
| | *lph2Δ* | this study |

## Chapter II – The snRNP biogenesis factor Brr1p may play a role in pre-mRNA splicing


### Abstract

The *brr1-1* mutant was originally isolated as a strain defective in pre-mRNA splicing. However, Brr1p was subsequently characterized as a factor involved in the production of mature spliceosomal snRNP levels. This raised the question of whether Brr1p was playing a direct or indirect role in splicing. We found that extracts made from *brr1* mutant cells display *in vitro* splicing and spliceosome assembly defects. However, *brr1* mutant extracts also contained decreased snRNP levels even at permissive temperatures. Intriguingly, our genetic analyses with snRNP biogenesis factors (LHP1, LSM5, LSM6, LSM7, SAD1) and splicing factors (PRP24, SAD1, SNU66, SNU114, SUB2) suggest a role for Brr1p in splicing, specifically U4/U6 snRNP assembly and/or triple snRNP addition. In addition, interactions with SUB2 suggest other possible roles for Brr1p in recombination, transcription and/or export. These data suggest that some splicing factors play a role in snRNP biogenesis or Brr1p plays a role in splicing and thus, step(s) in biogenesis are likely to be in common with step(s) required for the regeneration of snRNPs.


### Introduction

The process of pre-mRNA splicing requires five small nuclear RNAs (snRNAs) and approximately 100 associated proteins (Jurica and Moore 2003), together comprising a macromolecular machine called the spliceosome. The spliceosome is highly dynamic; it must be assembled, activated and disassembled upon each new mRNA substrate (Staley and Guthrie 1998). The spliceosome carries out two consecutive transesterification reactions to remove an intervening intronic sequence and to ligate the 5' and 3' ends of RNAs to supply the cell with mature mRNAs. The spliceosomal components - the snRNAs and their associated proteins together called small nuclear ribonucleoproteins (snRNPs) - have been demonstrated to assemble

in a highly ordered and stepwise manner (Staley and Guthrie 1998). First U1 and then U2 snRNAs base pair to and identify the 5' splice site and the branch site, respectively. U4 and U6 snRNAs come together by an extensive base pairing interaction to form the U4/U6 disnRNP. Together with U5 snRNP, they form the triple snRNP, which adds to the pre-mRNA. U1 and U4 snRNAs are unwound, catalysis occurs, and the snRNPs must be released, rearranged and reassociated for the next mRNA. While studies investigating the activities of the snRNPs are imperative for our understanding of the mechanics of this essential biological process, examining the steps requisite for *de novo* production of proper snRNPs as well as those required for regeneration is also an essential part of understanding this highly iterative operation. As testament to the importance of assembling proper snRNPs, survival of motor neurons (SMN), the disease gene for spinal muscular atrophy (SMA), is required for spliceosomal snRNP assembly in higher eukaryotes (Mattaj 1998; Terns and Terns 2001; Meister et al. 2002; Paushkin et al. 2002). The biogenesis of snRNPs in metazoans is a complex process involving many steps including transit to and from the cytoplasmic compartment (Mattaj et al. 1993; Will and Luhrmann 2001). In short, snRNAs are transcribed in the nucleus and exported to the cytoplasm where a common set of proteins called Sm proteins bind in order for trimethylation of the cap to occur, which together constitute a bipartite nuclear localization signal (Mattaj 1986; Hamm et al. 1987; Fischer and Luhrmann 1990; Hamm et al. 1990). The snRNPs are then trimmed at their 3' end and reimported into the nucleus, during which time snRNP specific proteins associate in final preparation for splicing (Neuman de Vegvar and Dahlberg 1990). While snRNP biogenesis may be restricted to the nucleus in *S. cerevisiae* (See Chapter I), the major landmarks of Sm core assembly, trimethylation of the cap and 3' end trimming are conserved, suggesting that other steps may also be conserved.

At the time of onset of this work, Brr1p was the only known snRNP biogenesis factor in *S. cerevisiae* (Noble and Guthrie 1996). It had been demonstrated that in *brr1-1* cells the levels of snRNAs were compromised (Noble and Guthrie 1996). Pulse-chase analysis further

demonstrated that only newly synthesized snRNAs were affected, suggesting a function in biogenesis rather than maintenance of mature snRNP stability (Noble and Guthrie 1996). Further dissection of specific biogenesis steps showed that 3' end trimming of U2 snRNA was slowed in *brr1-1* cells (Noble and Guthrie 1996). However, Brr1p's exact role in biogenesis remained unclear.

Since Brr1p was first identified in a screen for factors involved in splicing by searching for mutants with *in vivo* splicing defects (Noble and Guthrie 1996), it remained an open question if the decreased snRNA levels in *brr1-1* cells subsequently led to a defect in splicing or if Brr1p had an independent role in splicing. Notably, snRNA levels in *S. cerevisiae* are believed to be in great excess over levels necessary for splicing, because depletion of snRNPs is not detrimental until levels are reduced to 10% (Patterson and Guthrie 1987; Liao et al. 1990; Seraphin et al. 1991), suggesting the possibility for an alternative or additional role for Brr1p. To determine whether Brr1p could be playing a direct role in splicing we conducted biochemical and genetic analyses and provide evidence for Brr1p function in steps integral to the splicing pathway.

## Materials and Methods

### *In vitro* splicing assays

Splicing extracts from wildtype (YPH399), *brr1-1* (ySN186) and *brr1Δ::LEU* (ySN226) yeast strains (Noble and Guthrie 1996; Noble and Guthrie 1996) grown at permissive temperature (30°C) were prepared (Umen and Guthrie 1995). *In vitro* spliceosome assembly assayed by native gel analysis was performed (Cheng and Abelson 1987). *In vitro* snRNP status was assayed in the absence of spliceosome assembly by an alternative native gel system (Raghunathan and Guthrie 1998). Velocity sedimentation analysis of spliceosomal complexes was performed (Madhani et al. 1990). snRNA levels assayed by primer extension and Northern analysis were

performed (Noble and Guthrie 1996; Staley and Guthrie 1999). *In vitro* snRNP recycling assays were also performed (Raghunathan and Guthrie 1998).

***In vitro* snRNP assembly assays**

snRNP assembly was assayed by incubating T7-transcribed radioactively labeled snRNAs of mature length with 4nM cold actin pre-mRNA and 2mM ATP in splicing extracts at 16°C or on ice for 30 minutes and separated by native gel (Cheng and Abelson 1987). To prepare U4 snRNA-depleted extracts, extracts were incubated with a mixture of U4 specific oligos for 1hr at 16°C to digest endogenous U4 snRNA by endogenous RNase H activity (Kim et al. 1997). Depletion of U4 snRNA levels was assayed by Northern analysis and loss of splicing activity was assayed by *in vitro* splicing assays (data not shown, Rader and Guthrie personal communication). snRNP assembly with Sm RNA oligos was performed with yeast splicing extracts (Raker et al. 1999).

**Genetic interactions**

To examine genetic interactions, *brr1* mutant strains containing wildtype BRR1 on a CEN plasmid (pSN185) (Noble and Guthrie 1995) were mated with various mutant strains, sporulated, dissected and analyzed by standard techniques (Guthrie and Fink 1991).

## Results

### I. Biochemical Analyses of Brr1p's Role in Splicing

***brr1-1* and *brr1*Δ extracts display *in vitro* splicing defects**

The *brr1-1* strain was first identified in a bank of cold sensitive strains screened for defects in pre-mRNA splicing (Noble and Guthrie 1996). When the *brr1-1* strain was grown and shifted to nonpermissive temperature (16°C), U3 snoRNA precursor accumulated as determined by primer extension and Northern analysis (Noble and Guthrie 1996). Subsequent analysis determined that *BRR1* was a nonessential gene and that the *brr1*Δ strain also displayed cold sensitivity (Noble and

Guthrie 1995). To assess whether the *in vivo* splicing phenotype could be recapitulated *in vitro*, we examined splicing efficiency by assaying formation of spliced products of radioactively labeled ACT1 pre-mRNA incubated in wildtype, *brr1-1* and *brr1*Δ extracts. We observed that splicing efficiency in *brr1-1* and *brr1*Δ extracts was modestly, but reproducibly decreased compared to that in wildtype extracts (Figure 1A). Although *brr1-1* and *brr1*Δ strains are cold sensitive for growth, the splicing phenotype was not significantly exacerbated at the nonpermissive temperature of 16°C (Figure 1B). Since *brr1-1* and *brr1*Δ were both cold sensitive and displayed similar *in vitro* splicing phenotypes, this suggested that the *brr1-1* mutant was a null mutation. Although this has not been formally demonstrated, since the *brr1-1* mutation has not been mapped, subsequent analysis was done primarily with *brr1*Δ extracts.

**_brr1_Δ extracts display decreased levels of spliceosome assembly, but kinetics were unchanged**

To further dissect a potential cause for the decrease in splicing efficiency, we examined whether the spliceosome was being properly assembled onto radioactively labeled ACT1 pre-mRNA, by assaying spliceosomal complex formation using native gel analysis (Cheng and Abelson 1987). In *brr1*Δ extracts, the levels of spliceosomal complexes were decreased compared to those in wildtype extracts at either the permissive (Figure 2A) or nonpermissive (Figure 2B) temperatures. Addition of increasing amounts of heparin to the formed complexes before native gel electrophoresis caused a small increase in mobility of the A1 complex (U2, U5, U6) in the *brr1*Δ extract (Figure 2A and B) and at 16°C there appeared to be proportionally less A2 complex (U2, U4, U5, U6) in *brr1*Δ extracts (Figure 2B), suggesting that some *brr1*Δ complexes may be less stable than those formed in wildtype extracts. However, since mobility differences are difficult to resolve and assessing levels of complexes is not quantitative by this assay, further analysis is required to assess the Brr1p-dependent stability of the spliceosomal complexes. Alternatively, the observed decrease in A2 complex could be due to a block in formation. To examine whether the decrease in spliceosome assembly levels resulted from a defect in the kinetics of complex

formation, we observed the kinetics of spliceosome assembly during a 1 hour time course at 16°C (Figure 3). The similar rates of assembly we observed for both wildtype and *brr1*Δ extracts suggested that there were no blocks to any particular assembly steps and that the existing snRNPs were functional.

**snRNP levels are decreased in *brr1*Δ extracts**

The decreased levels of spliceosome assembly despite normal kinetics of formation (Figure 3) suggested that the snRNP levels may be decreased in *brr1*Δ extracts. To examine snRNP levels, we conducted additional spliceosome assembly experiments using unlabelled ACT1 pre-mRNA and assaying the mobility and levels of the snRNP complexes by snRNA Northern analysis. Indeed, we observed that although the mobilities of snRNPs in *brr1*Δ extracts were similar to wildtype, there were dramatic decreases in the levels of all snRNP complexes (Figure 4). We quantitated the levels of U2-containing complexes to be decreased approximately 10-fold in *brr1*Δ extracts compared to wildtype extracts. Notably, the snRNP levels did not decrease during a 90 minute time course, suggesting that this was not a defect in snRNP stability, but a defect in snRNP levels originally present in extract (Figure 4). We next examined snRNP levels by native gel and Northern analysis in the absence of spliceosome assembly on exogenous ACT1 pre-mRNA via a different native gel system (Raghunathan and Guthrie 1998). We again observed decreases in individual snRNP levels, but normal snRNP mobilities for *brr1*Δ extracts (Figure 5). Lastly, in case the snRNP complexes were unstable to migration through the matrix of native gels, we examined snRNP levels by velocity sedimentation analysis in a glycerol gradient (Madhani et al. 1990), and again observed similar mobilities, but decreased overall snRNP levels in *brr1*Δ extracts (Figure 6). These results indicated that the snRNPs were of normal mobility, but confirmed our suspicion that they were decreased in overall levels.

**snRNA levels in extract are not indicative of stable snRNP levels**

We observed dramatic decreases of snRNPs in *brr1*Δ extracts by native gel analyses, on the order of 10-fold for U2 snRNP complexes (Figure 4). To more precisely quantify snRNA levels, we

obtained total RNA from extracts with or without incubation in the presence or absence of ATP as we had for splicing and assembly assays, and separated them by denaturing gel electrophoresis. We analyzed snRNA levels by Northern or primer extension analyses and found that all snRNA levels in *brr1*Δ extracts were indeed decreased compared to wildtype extracts (see Table 1). However, the defects were more dramatic for *in vitro* snRNPs by native gel analyses (~10-fold) than the decreased levels of snRNAs in extracts (Figure 4, Figure 5, Table 1). The snRNA levels did not significantly decrease during incubation, suggesting that they are stable in extracts during the course of the experiments. However, our data suggested that the levels of snRNPs are compromised in *brr1*Δ extracts. Discrepancies between snRNA levels *in vivo* and snRNP levels in extracts have been previously observed by Wolin and colleagues with two other snRNP biogenesis factors, *smd1-1* and *lsm8-1* (Pannone et al. 1998; Xue et al. 2000). Since they observed that the levels of snRNAs in extracts is decreased from those extracted directly from cells, they conclude that the reason for their observed decrease of snRNP levels in extracts was that a subset of the snRNAs are unstable during their extract preparation (Xue et al. 2000). Since we observed a difference in levels between snRNAs in extracts and snRNPs on native gels, this suggested that a subset of the snRNAs may not form snRNPs stable to native gel electrophoresis. Previously, it had been shown that the snRNA levels were decreased *in vivo,* 10-fold for U4 snRNA and approximately 2-fold for the other snRNAs (U1, U2, U5 and U6), in a *brr1-1* mutant (Noble and Guthrie 1996). Since U4 snRNA was the most decreased and is believed to be limiting because there is very little free U4 snRNP (Li and Brow 1993), we asked whether GAL induction of U4 snRNA could suppress the cold sensitive growth phenotype of *brr1-1* or *brr1*Δ. Interestingly, while we did not independently assess the levels of U4 snRNA, we observed that conditions under which we induced expression of U4 snRNA did not suppress the cold sensitive growth phenotype of *brr1-1* or *brr1*Δ (data not shown). One possibility is that other snRNAs also need to be overexpressed for restoration of wildtype growth. A recent study demonstrated that overexpression of U1 and U4 snRNAs were sufficient to suppress *smd1-1* cold sensitive growth

phenotype (Xue et al. 2000). Alternatively, our *brr1-1* and *brr1*Δ extracts did not display a 10-fold decrease in U4 snRNA and we observed stronger decreases with the levels of U1, U2 and U6 snRNAs (Table 1), suggesting that U4 snRNA levels may not be limiting and that there may be other essential role(s) for Brr1p.

Our studies indicate that snRNA and snRNP levels in *brr1*Δ extracts are compromised and at least a subset of snRNPs may be unstable either during extract preparation or during native gel electrophoresis. Although we observed a splicing defect *in vitro* in *brr1-1* and *brr1*Δ mutant extracts, we could not attribute this solely to a defect in Brr1p function since the levels of snRNAs, and more dramatically the levels of snRNPs were also decreased.

**snRNP assembly assays revealed no differences between wildtype and *brr1*Δ extracts**

Since snRNP levels were compromised in *brr1*Δ extracts, we hypothesized that perhaps not all of the required snRNP proteins were being properly bound during assembly and that this was affecting snRNP stability. To assay snRNP formation we developed three *in vitro* snRNP assembly assays using gel shift mobility to test if Brr1p was required for the association of factors with snRNAs. First, we incubated radioactively labeled T7 transcribed U snRNA transcripts in extracts at 16°C for 30 minutes and assayed snRNP complexes by native gel mobility shift analyses. While we did observe complex formation as deduced by the appearance of shifts of U snRNA transcripts to higher mobility complexes, we did not observe any differences in *brr1*Δ extracts compared to wildtype (Figure 7). Second, to control for the possibility that free snRNP factors may be limiting for new assembly, we examined gel mobility shifts of exogenously added T7-transcribed snRNAs in extracts depleted of endogenous snRNAs. Indeed, although the mobility of U4 snRNA decreased in extracts depleted of endogenous U4 snRNA (Figure 8, Complex B) versus untreated extracts (Figure 8, Complex A), there were no differences in mobility between wildtype and *brr1*Δ extracts incubated either on ice (Figure 8) or at 16°C (data not shown). Titration experiments to determine U4 anti-sense oligo concentrations required for endogenous U4 snRNA depletion by RNaseH and for U4 snRNA addition for reconsititution

were performed (data not shown). Excess cold U4 snRNA was added to demonstrate U4 complex specificity by the ability to compete for any assembled complexes (Figure 8). Third, at this time Luhrmann and colleagues demonstrated that an RNA nonamer with an Sm site sequence was sufficient for Sm core assembly when incubated with partially purified human snRNP proteins (Raker et al. 1999). We attempted to take advantage of this minimal system and examine Sm core assembly by incubating Sm site sequence RNA oligos in yeast extract and similarly assay for gel shift mobility. Unfortunately, the wildtype Sm RNA oligo (AAUUUUUGG) was degraded both in wildtype and *brr1*Δ extracts despite addition of RNase inhibitors (data not shown), perhaps due to the lack of additional purification of snRNP proteins in yeast whole cell extracts.

Since we did not characterize the composition of the observed snRNP complexes, we do not know if they were *bona fide* snRNP complexes. However, these results suggest that there were no gross Brr1p-dependent changes in *in vitro* association of factors on various snRNA transcripts.

**_In vitro_ splicing analysis suggests that *brr1*Δ extracts are more sensitive to multi-round conditions**

Since we could not detect a snRNP assembly defect by the available assays, we decided to examine Brr1p's role in splicing in a more demanding, multi-round assay. Conceivably, steps at the beginning of a snRNP lifecycle during biogenesis are related or may even be common to steps required for snRNP recycling for each new round of splicing. Since Brr1p is known to be involved in snRNP biogenesis, we examined its role in snRNP recycling by an assay developed to examine later rounds of splicing by challenging the extracts with excess cold precursor during a preincubation (Raghunathan and Guthrie 1998). We observed that *brr1*Δ extracts were modestly, but reproducibly more sensitive to the addition of excess cold precusor than wildtype extracts (Figure 9), suggesting a defect in the recycling of snRNPs. This phenotype was the same whether splicing was assayed at permissive (25°C) or nonpermissive (16°C) temperatures (Figure 9, data not shown). However, since snRNP levels are decreased in *brr1*Δ extracts, the decrease in

splicing could be due to titration of lower levels of spliceosomal components. Therefore, although these results suggest a role for Brr1p in recycling, we again cannot rule out an indirect effect of the decreased levels of snRNPs.

## II. Genetic Analysis of BRR1 with Biogenesis and Splicing Factors

To further investigate Brr1p function we tested for genetic interactions with different subclasses of factors to implicate a role for Brr1p in specific processes. During the course of this work, additional factors involved in steps of snRNP biogenesis in yeast were discovered and characterized by others. Since Brr1p was shown to be involved in snRNP biogenesis (Noble and Guthrie 1996), but a specific function was not yet identified, we examined snRNP biogenesis mutants for genetic interactions with *BRR1*.

### *brr1*Δ is not synthetically lethal with *lhp1*Δ, a factor involved in stabilizing snRNA precursors

While our work was in progress, Lhp1p, the yeast La homologous protein, was characterized to bind and stabilize newly synthesized 3' extended snRNA precursors with 3' UUU-OH sequences and was proposed to act as a chaperone for facilitating proper biogenesis (Pannone et al. 1998; Kufel et al. 2000; Xue et al. 2000; Wolin and Cedervall 2002). Previously, *LHP1* was identified as an overexpression suppressor of *brr1-1*'s cold sensitive growth phenotype (Noble and Guthrie 1995). To investigate the mechanism of suppression, we examined the effect of *LHP1* overexpression on snRNA levels in the *brr1-1* strain by Northern analysis. We found that *LHP1* overexpression not only rescued the decreased levels of snRNAs in the *brr1-1* strain but also accumulated high levels of mature and 3' extended precursors of snRNAs (Kistler and Guthrie 2001). Overexpression of *LHP1* appeared to bypass the requirement of Brr1p by maintaining snRNA levels in a *brr1-1* strain.

Previously, the *brr1-1* strain had also been shown to slow the kinetics for U2 snRNA 3' end processing (Noble and Guthrie 1996), suggesting that Brr1p may play a role in 3' end processing

of snRNAs. To further investigate a role for Brr1p in 3' end processing of snRNAs, we examined genetic interaction between *LHP1* and *BRR1*. However, we observed that *brr1Δ* is not synthetically lethal in combination with *lhp1Δ* (Table 2). This is in contrast to two other mutant strains found to be defective in snRNP stability, *smd1-1* and *lsm8-1*, which have been shown to be synthetically lethal with *lhp1Δ* (Pannone et al. 1998; Xue et al. 2000). While the role of Brr1p in 3' end processing is still unclear, our data suggests that Brr1p is not directly involved in stabilization of 3' extended precursors of snRNAs with Lhp1p.

Rnt1p, the yeast homolog for RNase III, has been shown to cleave a stem-loop structure in the 3' extended precursors of snRNAs en route to a proper mature end (Chanfreau et al. 1997; Abou Elela and Ares 1998; Seipelt et al. 1999; Kufel et al. 2000). Since *brr1-1* was shown to slow kinetics for U2 3' end processing (Noble and Guthrie 1996), we attempted to test a genetic interaction between *rnt1Δ* and *brr1Δ*. However, the *rnt1Δ* strain was very sick, thwarting any analysis. We did not observe any additional evidence for an interaction between Brr1p and the 3' end processing machinery.

***BRR1* has genetic interactions with Sm core protein *SMD1***

The Sm proteins are a set of seven proteins that bind a common Sm sequence in U1, U2, U4 and U5 snRNAs as one of the first steps in snRNP biogenesis that acts to stabilize snRNAs from degradation (Rymond 1993; Roy et al. 1995; Will and Luhrmann 2001). Previously, the gene encoding one Sm protein, *SMD1*, was identified as both a low and high copy suppressor of *brr1-1's* cold sensitive growth phenotype (Noble and Guthrie 1995). Since Sm proteins have been shown to stabilize snRNAs (Rymond 1993; Roy et al. 1995) and snRNA levels have been shown to be compromised in the *brr1-1* mutant strain (Noble and Guthrie 1996), a likely mechanism of suppression was stabilization of snRNAs via increased Sm core assembly. To examine the effect of *SMD1* overexpression on the levels of snRNAs in a *brr1-1* strain we conducted Northern analysis. However, we observed that *SMD1* overexpression did not rescue the decreased levels of snRNAs in the *brr1-1* strain (Kistler and Guthrie 2001). Our data suggest that Brr1p may be

involved in an Sm core function other than in stabilizing snRNAs. It has been shown previously that depletion of SmD1p and SmD3p led to decreases specifically in the Sm site containing snRNAs (U1, U2, U4, U5) but not U6 snRNA (Rymond 1993; Roy et al. 1995). However, the levels of all snRNAs are decreased in *brr1* mutant strains (Noble and Guthrie 1996) (Figure 4, 5 and 6, Table 1). Moreover, it has been proposed that Sm proteins may play roles directly in splicing (Zhang et al. 2001). These data suggest that Brr1p may be involved in functions other than maintenance of snRNA stability.

**BRR1 has weak genetic interactions with U6 snRNA associated LSM proteins**

In 1999, during the course of our analyses, a set of like Sm proteins (LSM) homologous to the Sm proteins were identified and were found to bind specifically to U6 snRNA (Mayes et al. 1999; Salgado-Garrido et al. 1999). Lsm proteins were shown to be involved in U6 snRNA stability and biogenesis (Pannone et al. 1998; Pannone et al. 2001), and also in annealing of U4/U6 snRNAs (Achsel et al. 1999; Mayes et al. 1999). We looked for genetic interactions between *brr1*Δ and the nonessential *lsm* deletion strains, *lsm5*Δ, *lsm6*Δ, and *lsm7*Δ. Work from others demonstrated that deletions of each of *lsm5, lsm6,* and *lsm7* were synthetically lethal with *lhp1*Δ, presumably due to their common function in U6 snRNA stability and biogenesis (Pannone et al. 1998). In contrast, we observed only weak interactions with each combination of mutant strains with *brr1*Δ. Brr1p had previously been shown to be physically associated with Sm-site containing snRNAs (U1, U2, U4, U5) and to be only associated with U6 snRNA via its interaction with U4 snRNA (Noble and Guthrie 1995). These results suggest that while Brr1p is unlikely to be involved directly in U6 snRNA biogenesis, Brr1p may play a role in U4/U6 assembly via its interaction with U4 snRNA.

In addition, we examined genetic interactions with *lsm1*Δ, the one Lsm protein that has been shown to be uniquely part of a cytoplasmic mRNA decapping and degradation complex (Bouveret et al. 2000; Tharun et al. 2000), in contrast to Lsm5p, Lsm6p, and Lsm7p, which are part of both the degradation complex and U6 snRNP (Mayes et al. 1999; Salgado-Garrido et al.

1999). However, we find that *lsm1Δ* and *brr1Δ* are not synthetically lethal, suggesting that the genetic interactions between *lsm5Δ*, *lsm6Δ*, *lsm7Δ* and *brr1Δ* are likely to be specific for the U6-associated Lsm complex and not related to function of the Lsm complex in mRNA degradation.

**BRR1 has genetic interactions with U4/U6 annealing factor PRP24**

While Prp24p is often categorized as a splicing factor, *in vitro* it is only required under conditions where recycling of snRNPs is necessary (Raghunathan and Guthrie 1998). Prp24p is involved in annealing U4/U6 snRNAs (Raghunathan and Guthrie 1998), which occurs after every round of splicing for regeneration, but is also likely to occur during biogenesis. To see if Brr1p might play a role in recycling of snRNPs, we examined genetic interactions between *brr1* and *prp24* alleles. We observed that *brr1Δ* is synthetically lethal with *prp24* mutations in RNA recognition motifs (RRM) 1 and 3 (See Table 2, Rader and Guthrie personal communication), believed to be involved in U4/U6 annealing activity (Shannon and Guthrie 1991; Vidaver et al. 1999; Rader and Guthrie 2002). This genetic interaction suggested that Brr1p may play a role in U4/U6 annealing. In addition, we have observed that *PRP24* overexpression suppresses the cold sensitive growth defect of *brr1Δ* (Rader and Guthrie personal communication). The deletion of a conserved C-terminal motif in PRP24 (*prp24Δ10*) (Rader and Guthrie 2002) believed to play a role in U4/U6 association abolishes the suppression, suggesting that U4/U6 annealing activity is important for this interaction (Rader and Guthrie personal communication). Increased annealing of U4/U6 snRNAs may compensate for the lack of proper U4 levels (Noble and Guthrie 1996). Alternatively, Brr1p may function in steps involved in recycling of snRNPs and perhaps in U4/U6 reannealing.

***brr1Δ* is not synthetically lethal with *brr2-1*, a factor involved in U4/U6 release**

Since we identified genetic interactions between *BRR1* and factors involved in U4/U6 assembly, namely Lsm proteins and Prp24p, we were interested to examine whether Brr1p was also involved in disassembly. To investigate a role for Brr1p in this process we looked for genetic interactions with Brr2p, a factor involved in U4/U6 release during activation of the spliceosome

(Raghunathan and Guthrie 1998). However, we observed that *brr1*Δ was not synthetically lethal with *brr2-1* (See Table 2), suggesting that Brr1p may be specifically involved in the annealing of U4/U6 snRNP, but not the release.

***BRR1* has genetic interactions with snRNP biogenesis factors that are also integral to splicing – *SAD1* and *SNU66***

Sad1p was identified as a snRNP biogenesis factor in *S. cerevisiae* (Lygerou et al. 1999). The *sad1-1* strain was identified in a screen devised to identify mutants blocked for assembly of newly synthesized U4 snRNA into U4/U6 snRNP by pulse-chase analysis of a tagged U4 snRNA under inducible control (Lygerou et al. 1999). While *prp3-1*, *prp4-1* and *prp24-1* showed defects in converting both nascent and recycled U4 snRNA into U4/U6 snRNP, *sad1-1* specifically affected assembly of newly synthesized U4 snRNA (Lygerou et al. 1999). We examined genetic interactions between *SAD1* and *BRR1* and found that the combination of *sad1-1* and *brr1*Δ was synthetically lethal. Intriguingly, although *sad1-1* specifically affects newly synthesized U4 assembly, *sad1-1* also displays a strong block to splicing both *in vivo* and *in vitro*, suggesting a role integral to splicing (Lygerou et al. 1999). Subsequent mammalian studies with hSad1 revealed that depletion of hSad1p blocked splicing at the first step and that triple snRNP addition was impaired (Makarova et al. 2001). This was the first example of a snRNP biogenesis factor playing a role in splicing. Sad1p was identified as part of the penta-snRNP, a complex of all five snRNPs believed to associate with pre-mRNA as a whole (Stevens et al. 2002), suggesting that Sad1p is in active spliceosomes, consistent with the model that Sad1p is required during splicing. In the same study investigating hSad1, the human homolog of Snu66p, hSnu66, was also demonstrated to be required for splicing and specifically block triple snRNP addition in mammalian extracts (Makarova et al. 2001). In yeast, Snu66p has been shown to be required for splicing *in vivo* and *in vitro* by immunoinhibition and complementation studies (Gottschalk et al. 1999; Stevens et al. 2001). Also, Snu66p has been identified as part of triple snRNP complexes (Gottschalk et al. 1999; Stevens and Abelson 1999; Stevens et al. 2001) and to be involved in U5

snRNP particle stability (Stevens unpublished data). We examined genetic interactions between *SNU66* and *BRR1* and found that the combination of *snu66Δ* and *brr1Δ* was also synthetically lethal. Although snRNP biogenesis assays have not been performed with Snu66p to test if Snu66p like Sad1p functions in both splicing and snRNP biogenesis, these data suggest that Brr1p may function in steps required for splicing and perhaps more specifically in triple-snRNP addition.

## Discussion

While traditional screens for splicing mutants and subsequent biochemical analysis have been fruitful methods for characterizing many splicing factors (Hartwell et al. 1970; Vijayraghavan et al. 1989; Noble and Guthrie 1996), dissecting a role for snRNP biogenesis factors in splicing remains a challenge. Mutations in snRNP biogenesis factors such as Brr1p cause defects in snRNP levels making it difficult to determine whether any downstream defects in splicing are just an indirect consequence of decreased levels or if they indicate a separate role. However, our genetic analyses indicate that Brr1p is playing a direct role in splicing and that other factors may also perform dual functions in splicing and snRNP assembly.

### Brr1p role in splicing: genetic evidence

We identified a synthetic lethal interaction between *brr1Δ* and *sad1-1* (see Table 2). Since Sad1p was identified as a snRNP biogenesis factor specifically involved in assembly of newly synthesized U4 snRNA into U4/U6 snRNP (Lygerou et al. 1999), this interaction was not unexpected. However, subsequent analysis of both Sad1p and Snu66p, another factor whose deletion we found to be synthetically lethal with *brr1Δ* (see Table 2), identified them as splicing factors. Sad1p and Snu66p were found to be required for splicing in yeast and in mammalian extracts, specifically in triple snRNP addition in mammalian extracts (Gottschalk et al. 1999;

Lygerou et al. 1999; Makarova et al. 2001). This suggested that Brr1p may also be involved in triple snRNP addition.

Other genetic interactions we found with *brr1Δ* suggested a role for Brr1p in recycling of snRNPs, specifically U4/U6 annealing. We found *brr1Δ* to be synthetically lethal with several RRM mutations in *prp24*, known to be involved in RNA annealing for U4/U6 snRNP formation (Raghunathan and Guthrie 1998). Interestingly, *brr1Δ* did not show genetic interactions with *brr2-1*, involved in U4 release from the spliceosome (Raghunathan and Guthrie 1998), suggesting a specific role for Brr1p in the annealing or assembly aspects of the splicing cycle. We also identified weak synthetic interactions with deletions of *lsm5, 6*, and *7* (see Table 2), which are known to be involved in U4/U6 annealing in addition to U6 snRNP biogenesis and stability (Pannone et al. 1998; Achsel et al. 1999; Mayes et al. 1999; Pannone et al. 2001). Intriguingly, a recent study has shown that the Lsm proteins may also be destabilized from the end of U6 snRNA during spliceosomal activation, suggesting a role for Lsm proteins in spliceosome activation (Chan et al. 2003). Since our genetic interaction suggest that Brr1p is involved in Lsm protein function, Brr1p may be involved in U4/U6 annealing during recycling and/or steps integral to splicing.

Since the completion of our work, a number of genetic interactions with other splicing factors have been identified which further implicate Brr1p in splicing (see Table 2). Brenner and Guthrie find genetic interactions between *brr1Δ* and a specific allele of *snu114-60*, a U5 snRNP GTPase required for splicing (Bartels et al. 2003). Interestingly, *snu114-60* has allele-specific interactions both with factors implicated in snRNP biogenesis and a number of DEAD box helicases, namely *sad1, snu66, prp24, brr2, prp28* and *prp8* (Brenner and Guthrie personal communication). Moreover, *brr1Δ* is not synthetically lethal with *snu114* alleles with mutations in the GTPase domain, which display defects in triple snRNP levels, suggesting that the genetic interaction observed is not simply due to decreased snRNA levels (Brenner and Guthrie personal communication). In addition, *brr1Δ* has been shown to be synthetic in combination with *prp8-*

*brr* (Collins and Guthrie 2000), another U5 snRNP component believed to function in the catalytic core (Collins and Guthrie 1999). Taken together, these data either suggest that factors believed to play roles primarily in splicing may actually participate in snRNP biogenesis or that Brr1p may play a role in splicing. Alternatively, but not mutually exclusively, snRNP biogenesis and snRNP recycling may utilize equivalent pathways or factors, which ultimately are required for splicing. Notably, a block to triple snRNP addition is a common phenotype for many of the splicing factors genetically linked with *BRR1* – namely *sad1, snu66, snu114* and *sub2*. A block to triple snRNP addition may be a broad indicator of many spliceosomal, recycling and/or biogenesis defects.

**Brr1p has been further implicated in splicing: genomic and proteomic evidence**

Comprehensive studies identifying protein-protein interactions by 2-hybrid analyses and immunoaffinity purification followed by mass spec identification have applied 'guilt by association' to identify genome-wide relationships (Fromont-Racine et al. 2000; Gavin et al. 2002). In addition, genomic expression studies under a battery of environmental conditions have used 'guilt by expression' to similarly identify functional relationships (Ball et al. 2001). While 2-hybrid and genomic expression studies have not yet yielded any Brr1p interactions or related BRR1 expression profiles (Fromont-Racine et al. 2000; Ball et al. 2001), comprehensive TAP tagged affinity pull-downs have identified at least two complexes containing Brr1p with a number of spliceosomal factors that correspond to U1 snRNP and penta-snRNP-like complexes (Gavin et al. 2002; Jurica and Moore 2003). Intriguingly, the complexes contain many proteins that have been implicated by genetic interactions with Brr1p such as Smd1p, Snu66p, Snu114p, and Prp19p (Gavin et al. 2002). Curiously, however, other studies purifying spliceosomal complexes have not identified Brr1p as a stable component (Stevens and Abelson 1999; Stevens et al. 2001; Ohi et al. 2002; Stevens et al. 2002), suggesting weak or transient interactions between Brr1p and the spliceosome. However, the presence of integral splicing factors suggests that Brr1p may be

associated with active spliceosomes, consistent with our hypothesis that Brr1p may play an integral role in splicing.

With the advancement of microarray analysis, one now has the ability to examine the splicing efficiency of the approximately 200 known spliced mRNAs in the yeast genome in a single experiment (Clark et al. 2002) (Pleiss and Guthrie personal communication). In such a study with 18 nonessential splicing factors, those implicated in related steps of splicing such as Prp17 and Prp18 have been shown to have similar splicing profiles, while unrelated factors show more distant profiles (Clark et al. 2002). Intriguingly, *brr1*Δ, *snu66*Δ, and *snt309*Δ, a Prp19 complex protein, were shown to display similar splicing defect profiles, suggesting a cooperative function for Brr1p, Snu66p, and the Prp19 complex in splicing (Clark et al. 2002). The Prp19 complex is known to associate with the spliceosome during activation (Makarov et al. 2002) and more recently has been shown to be involved in Lsm protein dissociation from U6 snRNA during spliceosome activation (Chan et al. 2003), while Snu66p is known to be required for triple snRNP addition (Gottschalk et al. 1999; Stevens et al. 2001). Further study is required to dissect a potential common role for these factors, but these results strongly suggest that Brr1p plays a direct role in splicing.

**SMN suggests a mechanistic link between splicing and snRNP assembly**

Recent studies of the human disease gene SMN have elucidated a large complex of proteins involved in the assembly of snRNPs that are also involved in splicing (Mattaj 1998; Matera 1999), adding to accumulating evidence for the existence of factors with dual roles in snRNP assembly and splicing . In human cells, a dominant negative form of SMN (SMNΔN) was shown to cause a dramatic mislocalization of snRNPs in both the nucleus and the cytoplasm, consistent with SMN's role in Sm core assembly in the cytoplasm, but implicating SMN function in the nucleus (Pellizzoni et al. 1998). Strikingly, when SMNΔN was added to splicing extracts, a detrimental effect was observed only when the mutant protein was preincubated in the extract prior to assaying splicing, suggesting that SMN may be involved in snRNP recycling (Pellizzoni

et al. 1998). Lastly, *in vitro* spliceosome assembly assays revealed that SMNΔN also caused a block to spliceosome assembly (Pellizzoni et al. 1998). Intriguingly, there is no clear homolog of SMN in *S. cerevisiae*. SMN has been shown to mediate Sm core assembly via dimethylarginine modifications (Friesen et al. 2001), which do not exist in *S cerevisiae* Sm proteins. It was originally thought that an SMN interacting protein (SIP1) had distant homology to Brr1p (Liu et al. 1997). However, this is likely to have been a red herring in that *S. pombe* does have SMN and SIP1 homologs, but *pombe* SIP1 is not related to BRR1 (Hannus et al. 2000). Although the exact machineries may be different between yeast and mammals, there may be functionally similar factors involved in snRNP assembly and recycling that directly impact steps during splicing.

**Brr1p role in splicing: biochemical evidence and implications for Brr1p's role in biogenesis**

We characterized the *in vitro* splicing phenotypes of *brr1-1* and *brr1*Δ extracts and observed decreased levels of splicing, spliceosome assembly and recycling (Figure 1, 2, 9). However, further analysis revealed that all snRNP levels were also dramatically decreased in *brr1*Δ extracts (Figure 4, 5, 6). Mutations in *brr1* cause defects in snRNP levels making it difficult to determine whether Brr1p function is 'upstream' of splicing simply in decreasing the levels of splicing components, or if Brr1p may play a role during splicing.

Notably, unlike other splicing factors where complete blocks to specific splicing or assembly steps have been observed in mutant extracts, for *brr1* the kinetics of both splicing and spliceosome assembly were primarily unaffected (Figure 1 and 3), suggesting that the existing snRNPs were of normal composition and function. What is then the role for Brr1p in biogenesis? One possibility is that Brr1p is involved in proper association of snRNP components. We did not observe any major snRNP assembly defects by various gel mobility shift assays (Figure 7, 8, 9), which may simply reflect insufficient sensitivity of our assays. Indeed, the stability of some spliceosomal complexes was perhaps compromised in *brr1*Δ mutant extracts (Figure 2) and may be the reason why we observe fewer snRNPs by native gel (Figure 4, 5, 6). Another possibility is that Brr1p is acting as a chaperone by stabilizing snRNAs during snRNP biogenesis. Indeed, the

levels of newly synthesized snRNAs are specifically affected (Noble and Guthrie 1996). However, while Lhp1p interacts with 3' extended pre-snRNAs (Pannone et al. 1998; Xue et al. 2000), Brr1p has been found to associate with 3' end mature species (Noble and Guthrie 1995), suggesting that Brr1p is not exclusively associated with snRNA precursor species.

To ask whether Brr1p may be associated with RNAs other than snRNAs, we attempted to identify the complete RNA binding profile of Brr1p by whole genome IP microarray analysis (see Chapter III). However, we found that Brr1p-associated RNAs were too low in abundance to identify even after PCR amplification. Further assay optimization was impeded by the inability to readily detect Brr1p levels due to low levels of protein. Since previous analysis found that the levels of SCR1, U3 snoRNA, SNR5 and U14 were unaffected in the *brr1-1* strain (Noble and Guthrie 1996), it is likely that Brr1p function is primarily limited to spliceosomal snRNAs.

Our genetic analyses with BRR1 and snRNP biogenesis factors did not reveal specific roles in biogenesis, but additional factors have been identified that remain to be examined. Although previous data suggested a role for Brr1p in 3' end trimming (Noble and Guthrie 1996), we found that *brr1*Δ is not synthetically lethal with *lhp1*Δ,, and Rrp6p, an exosome component involved in 3' end trimming of snRNAs to their mature end (Allmang et al. 1999), has yet to be examined. Previously, *brr1-1* was shown to display normal U4 snRNA trimethylation kinetics (Noble and Guthrie 1996), suggesting that Brr1p is not likely to play an essential role in snRNA trimethylation. However, the yeast snRNA (and snoRNA) trimethylating enzyme has been identified as Tgs1p (Mouaikel et al. 2002). Interestingly, deletion of *tgs1* confers a cold sensitive growth phenotype and a defect in *in vivo* splicing, but displays normal snRNP mobilities (Mouaikel et al. 2002), reminiscent of *brr1* phenotypes. But unlike *brr1*, *tgs1* cells display normal snRNP profiles by velocity sedimentation analysis (Mouaikel et al. 2002). A genetic interaction with *tgs1* has yet to be examined. Further work to determine the role that Brr1p plays in snRNP biogenesis and whether/how it may impact splicing is required.

**Summary of genetic interactions between BRR1 and SUB2: a Brr1p perspective**

***Genetic interaction between BRR1 and SUB2 suggest a role for Sub2p in snRNP biogenesis***

Sub2p, the yeast homolog of UAP56, is a highly abundant DEAD box helicase factor. Sub2p has been shown to be required for stable binding of U2 snRNP to the pre-mRNA (Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001). Intriguingly, *SUB2* was originally isolated as a high copy suppressor of *brr1-1* cold sensitive growth phenotype (Noble and Guthrie 1995), suggesting a role for Sub2p in biogenesis or a role for Brr1p in splicing. Further analysis demonstrated that *SUB2* overexpression can also bypass the requirement for *BRR1* at low temperatures (data not shown) (Kistler and Guthrie 2001). Since Sub2p plays a role in splicing, the effect of overexpressing *SUB2* on splicing in the *brr1-1* strain was examined. *SUB2* overexpression only partially suppressed the *brr1-1* splicing defect at nonpermissive temperatures and exacerbated the *brr1-1* splicing defect at permissive temperatures (Kistler and Guthrie 2001). This suggested that the mechanism of suppression was by another means than splicing. Further analysis demonstrated that SUB2 overexpression in the *brr1-1* strain partially suppressed the decreased U5 snRNA levels, but not U4 snRNA levels, suggesting that Sub2p may play a role in maintaining snRNA levels (data not shown) (Kistler and Guthrie 2001). Intriguingly, *sub2-1* extracts, but not *sub2-5* extracts, phenocopy decreases in snRNP levels seen with *brr1*Δ extracts (Kistler and Guthrie 2001). This suggested that Sub2p may play a role in snRNP biogenesis. Additionally, the *sub2-1* temperature sensitive phenotype is suppressed by *brr1*Δ (Kistler and Guthrie 2001), suggesting antagonistic functions between Sub2p and Brr1p. However, the *sub2-1* and *sub2-5* cold sensitive phenotype is exacerbated by *brr1*Δ (Kistler and Guthrie 2001), suggesting a complex relationship between *brr1* and *sub2* alleles. As further evidence for an antagonistic relationship between Sub2p and Brr1p, *BRR1* overexpression exacerbated the *sub2-1* temperature sensitive growth phenotype and *SUB2* overexpression growth phenotype was partially suppressed by *brr1*Δ at 37°C (data not shown) (Kistler and Guthrie 2001). The nature of the antagonistic relationship between Brr1p and Sub2p is still unknown.

***Various phenotypes for Sub2 suggest other roles for Brr1***

In addition to Sub2's role in splicing, Sub2p is a ubiquitous DEAD box ATPase family member that has been implicated in a wide range of biological processes throughout gene expression, starting with DNA recombination followed by transcription, splicing, and export of mRNAs. Sub2p is implicated in recombination as *SUB2* was identified as a high copy suppressor of both the growth and genome instability defects conferred by *hpr1*Δ, as assayed by maintenance of directed repeats and plasmid loss (Fan et al. 2001; Merker and Klein 2002). Sub2p is also implicated in transcription as *sub2* alleles have been shown to have defects in transcribing GC rich or long mRNAs (Jimeno et al. 2002; Rondon et al. 2003). Sub2p has also been implicated in mRNA export since *sub2* mutants accumulate mRNAs in the nucleus as detected by a dT50 probe (Jensen et al. 2001; Strasser and Hurt 2001). In addition, Sub2p/UAP56 is a component of the spliced mRNP and is believed to be involved in recruiting the exon junction complex for facilitating export of mRNAs (Gatfield et al. 2001; Keys and Green 2001; Luo et al. 2001; Gatfield and Izaurralde 2002). Lastly, Sub2p is part of the THO/TREX complex (Strasser et al. 2002), that functions at the interface of transcription and mRNA metabolism (Aguilera 2002). Since *brr1*Δ suppresses the growth phenotype conferred by *SUB2* overexpression (Kistler and Guthrie 2001), which itself displays defects in mRNA export and affects genome stability and transcription elongation (Fan et al. 2001; Strasser and Hurt 2001; Jimeno et al. 2002; Merker and Klein 2002), deletion of *brr1* may relieve defects in export, recombination and/or transcription. We have observed that *brr1-1* and *brr1*Δ strains do not display mRNA export phenotypes by the dT50 assay on their own (data not shown), but *brr1*Δ may be relieving the mRNA export defects conferred by overexpression of *SUB2* and warrants examination. While a role of Brr1p in recombination and transcription has not been directly investigated, there are a number of interesting links. Another high copy suppressor of *brr1-1*, identified in the same screen as SUB2, is the TFIIH component, TBF4 (Noble and Guthrie 1995), involved in basal level transcription and recombination repair (Zurita and Merino 2003). Since snRNA levels were decreased in a

*brr1-1* mutant, it was hypothesized early on that Brr1p could be a transcription factor for snRNAs (Noble and Guthrie 1996). Interestingly, it has recently been shown in mammals that U1 snRNA associates with TFIIH and modulates transcription initiation (Kwek et al. 2002). However, pulse-chase analysis indicated that transcription rates of U2 snRNA were unaffected in the *brr1-1* mutant (Noble and Guthrie 1996), suggesting that Brr1p did not directly affect transcription of snRNAs. Intriguingly, mutations in another TFIIH component, *rad3*, involved in DNA repair, have genetic interactions with *sub2* alleles (Jensen et al. 2001). Lastly, SMN which has been shown to be involved in snRNP assembly in mammalian cells has been shown to interact with RNaseA and the RNA pol II transcription machinery (Pellizzoni et al. 2001). The links between Brr1p and transcription are particularly intriguing since transcription is upstream, but perhaps coupled to a role in pre-mRNA splicing. It will be interesting to investigate the connections between Brr1p, transcription and splicing.

**Future Directions**

**Splicing Biochemistry**

The role that Brr1p plays in splicing in addition to its role in biogenesis still remains unclear. There are a number of biochemical assays that have yet to be tested. For example, examining conditions where snRNA levels start at wildtype levels would greatly improve these analyses. Immnodepletion of Brr1p from BRR1-HA-tagged extracts to examine splicing defects in 'wildtype' extracts would be a good starting point. Although Brr1p has been shown to be associated with snRNAs, the associated levels are a small fraction (<1%) and the association has been shown to be salt sensitive (Noble and Guthrie 1995). To deplete Brr1p without depleting snRNAs, it may be possible to immunodeplete Brr1p in high salt conditions and then dialyze the extracts to physiological salt conditions for subsequent splicing analysis. Also, one could genetically deplete *BRR1 in vivo* by glucose depletion, since pGAL-BRR1 constructs were isolated in the screen for high copy suppressors of *brr1-1*. If the kinetics of Brr1p depletion are faster than the destabilization of snRNAs, then this could be a fruitful means for examining the effects of Brr1p on splicing before depletion of snRNAs. It has been previously observed that the half-life of snRNPs is on the order of hours (Patterson and Guthrie 1987; Liao et al. 1990; Seraphin et al. 1991).

Another useful tool for assigning a function for Brr1p would be recombinant Brr1 protein. With the ability to add back Brr1p to either mutant or immunodepleted extracts to test for restoration of splicing or snRNA level activity one could begin to assign functions for Brr1p. It would be informative to see if adding Brr1p to even the current mutant extracts is sufficient for restoration of wildtype levels of splicing or spliceosome assembly, or if other factors or snRNAs are required. In the absence of such a tool, one could start with mixing *brr1*Δ extracts with micrococcal nuclease snRNA depleted extracts to see if Brr1p alone and not the presence of additional snRNAs can complement splicing activity of *brr1*Δ extracts in order to assign a splicing role for Brr1p.

**snRNP Biogenesis Biochemsitry**

The role that Brr1p plays in biogenesis is also still unknown. Subsequent to our studies, Wolin and colleagues have utilized two different methods to examine snRNP biogenesis and integrity *in vitro*, which may prove to be useful for Brr1p snRNP biogenesis analyses. First, they *in vitro* transcribed U6 snRNA in extracts in the presence of radiolabelled nucleotide and examined U6 snRNA stability (Pannone et al. 1998). They found that the stability of newly transcribed U6 snRNA in *lsm8-1/lhp1Δ* extracts was enhanced by the presence of additional Lhp1p (Pannone et al. 1998). A similar analysis could be tried with snRNAs such as U4 in *brr1Δ* extracts examining effects of addition of Brr1p. Second, they added *in vitro* transcribed 3' extended precursors of U4 and U5 snRNAs to extracts and assayed their biogenesis status by examining their immunoprecipitability with anti-SmD1, anti-trimethyl G cap, and anti-Lhp1p antibodies (Xue et al. 2000). If Brr1p is affecting Sm core assembly, trimethylation or Lhp1p association to pre-snRNAs, it may be possible to dissect specific roles in this pathway by such immunological methods in *brr1Δ* extracts. While the effects of adding Lhp1p to mutant extracts on the immunoprecipitability of pre-U snRNAs were modest, this assay is likely to be more sensitive than gel mobility shift assays. Such biochemical methods may prove to be useful for identifying a role for Brr1p in biogenesis.

**Brr1 Genetic Analysis**

Genetic analyses proved to be very fruitful for identifying interactions with Brr1p. Extending these analyses would continue to pinpoint the steps in which Brr1p function is involved. Directed genetic interactions with snRNP biogenesis factors not yet tested are those with *smd1-1, rnt1-1, rrp6Δ,* and *tgs1Δ* for investigating Brr1p function in Sm core assembly, 3' end trimming and trimethylation (See Table 2). Given the genetic interaction web that is forming with Brr1p and a number of splicing factors involved in triple snRNP addition and penta-snRNP factors, additional genetic analysis with the *snu114-60* interacting factors such as *prp28*, as well as other splicing factors would elucidate the strength and specificity of our identified interactions. Alternatively,

an open-ended synthetic lethal screen, which has proven to be fruitful for Lhp1p (Pannone et al. 1998; Xue et al. 2000), may be an efficient method for identifying factors connected with Brr1p to elucidate a role in splicing or other processes such as transcription. Current methods for high-throughput genome-wide screening of factors, while only investigating nonessential deletions that may miss allele specific interactions, are nonetheless rapid and broad reaching (Tong et al. 2001). In fact, a synthetic lethal screen starting with a number of known mitotic and structural factors with nonessential deletion strains identified an interaction between *brr1Δ* and *cdc45-1*, a DNA replication initiation factor (Tong et al. 2004)..

**Splicing Microarrays**

Another recent technological development has allowed for a large advancement in the simultaneous analysis of all of the ~230 yeast introns in a single experiment by microarray analysis (Clark et al. 2002). This global analysis is in stark contrast to previous splicing analyses that usually examined only a single message from a handful of working choices (ACT1, RP51, U3) by Northern or primer extension analyses. Intriguingly, by splicing microarray analysis Ares and colleagues detected defects in the splicing of many introns in a *brr1Δ* strain compared to a wildtype strain. Moreover, they also observed that the splicing profile clustered with *snu66Δ*, for which we have observed a genetic interaction with *brr1Δ*, and also with *snt309Δ* a component of the PRP19 complex, amongst the ~20 nonessential splicing factors they examined (Clark et al. 2002). As we now have the technology to conduct such experiments in our lab (Pleiss and Guthrie personal communication), repeating the examination of *brr1Δ* splicing profiles with other nonessential and conditional splicing mutants as well as a battery of other factors involved in multiple aspects of RNA metabolism could identify relationships between Brr1p and factors in splicing and/or other metabolic processes.

**Investigation of SUB2 related phenotypes**

Splicing and biogenesis are processes in which Brr1p and Sub2p are known to interact. Further biochemical analysis of the snRNA and snRNP profiles in various mutant combinations may

elucidate the nature of the antagonistic relationship previously observed. However, while SUB2 was originally isolated as a high copy suppressor of *brr1-1*'s and subsequently *brr1*Δ's cold sensitive growth phenotype (Noble and Guthrie 1995; Kistler and Guthrie 2001), upon further analysis, it has been found that *SUB2* overexpression is toxic and displays phenotypes in mRNA export, recombination and transcription (Strasser and Hurt 2001; Jimeno et al. 2002; Merker and Klein 2002; Rondon et al. 2003). These data suggest that a hypomorphic allele of *brr1* could be relieving the toxicity of *SUB2* overexpression. It would be elucidating to examine the mechanism of *SUB2* overexpression suppression in *brr1-1* and *brr1*Δ strains with regards to mRNA export (by dT50 assay), recombination (by 6-azauracil sensitivity), and transcription (by LacZ/GC rich reporter assays). While both Brr1p and Sub2p were identified to be involved in splicing, their primary defect may be in one of these other processes. Given the range of processes that Sub2 is involved, further dissection of their relationship may be required to identify the primary role of Brr1p.

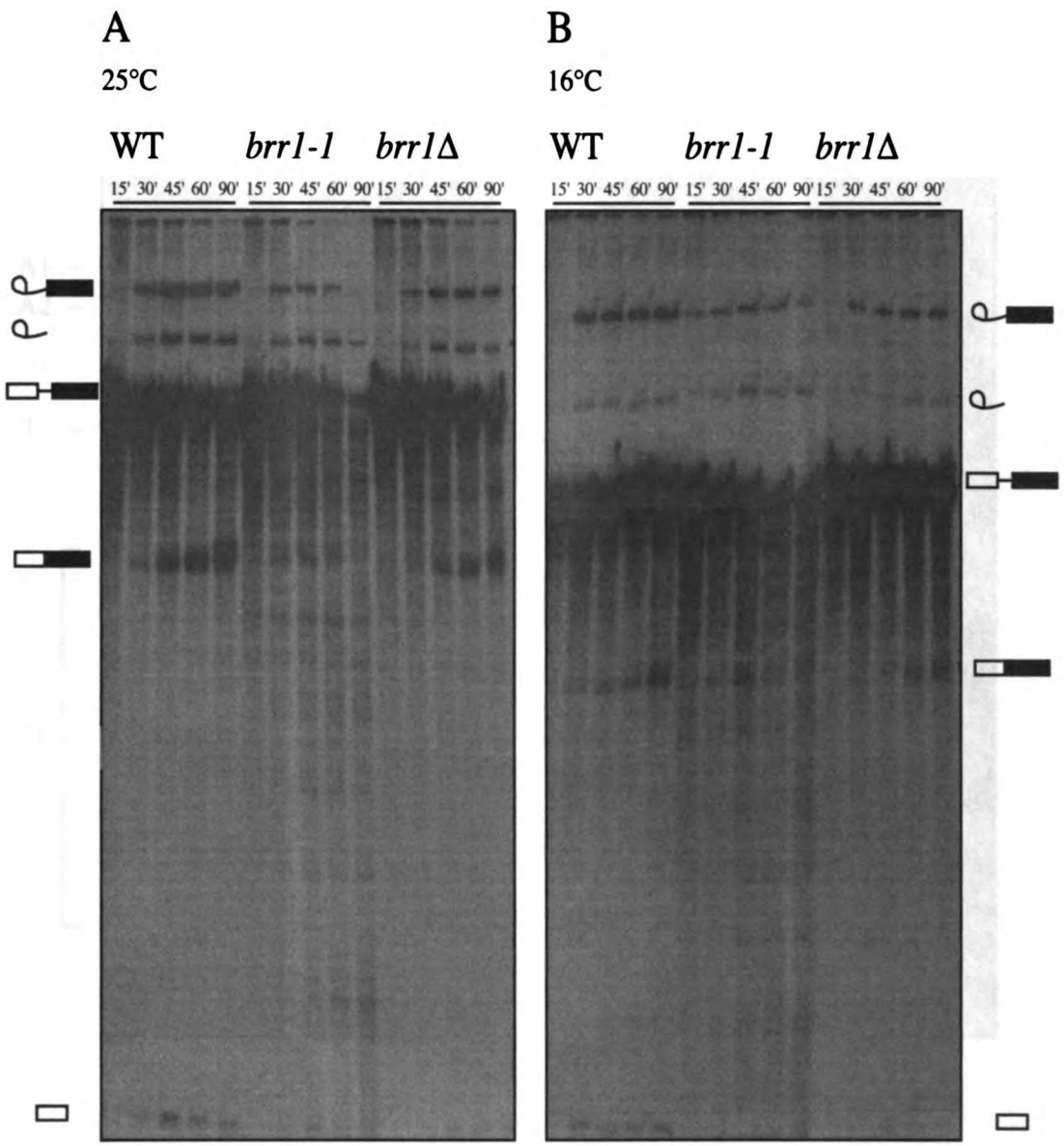


**Figure 1** - *In vitro* splicing of ACT1 pre-mRNA incubated in *brr1-1* and *brr1Δ* mutant extracts from 0 to 90 minutes is decreased compared to wildtype (WT) extracts at both A) permissive (25°C) and B) nonpermissive (16°C) temperatures.

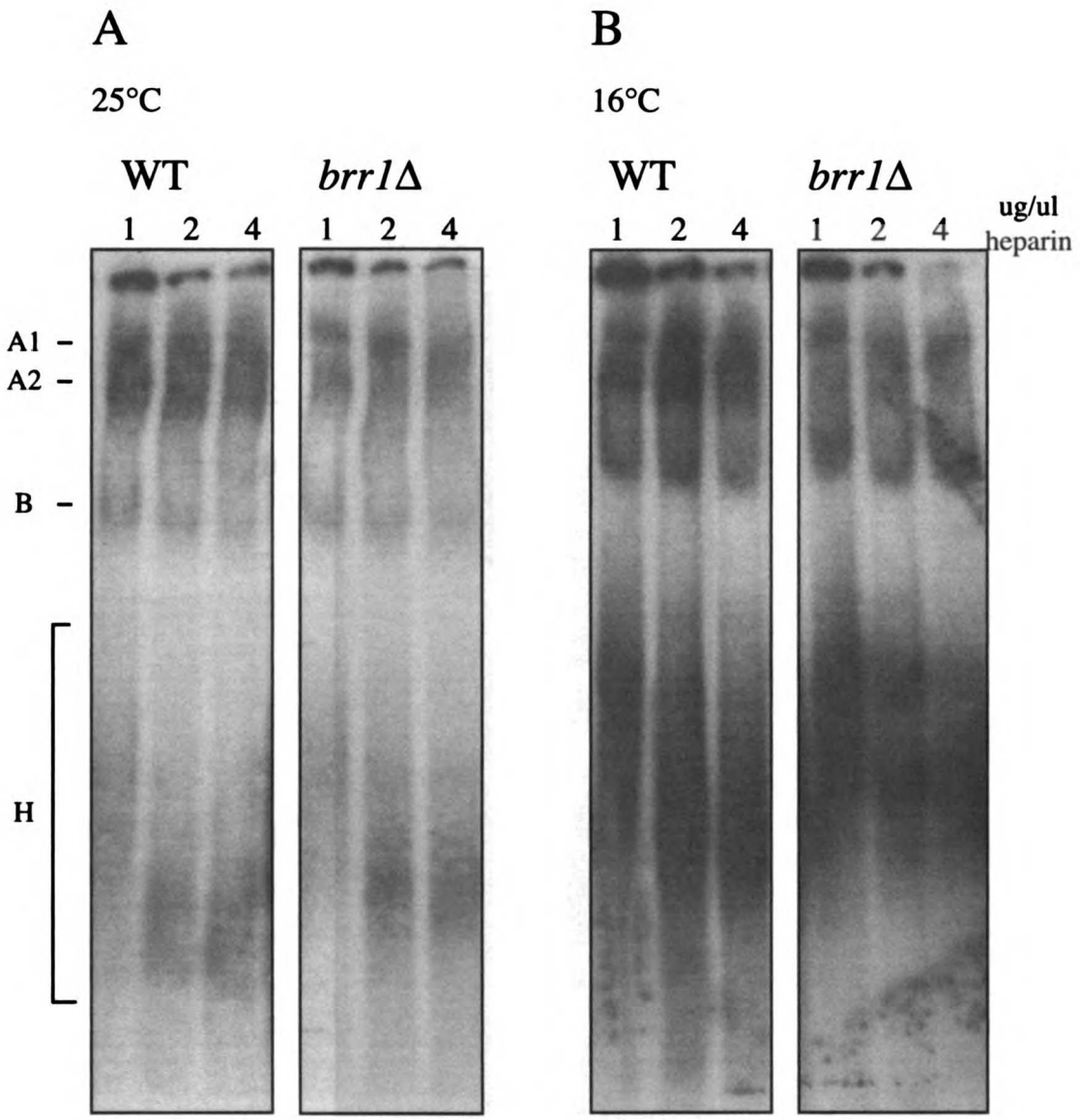


**Figure 2** - Spliceosome assembly levels on ACT1 pre-mRNA are decreased in *brr1*Δ extracts compared to wildtype (WT) extracts at both A) permissive (25°C) and B) nonpermissive (16°C) termperatures. After A) 15 minutes at 25°C or B) 60 minutes at 16°C, 1 to 4 ug heparin/ul extract were added.

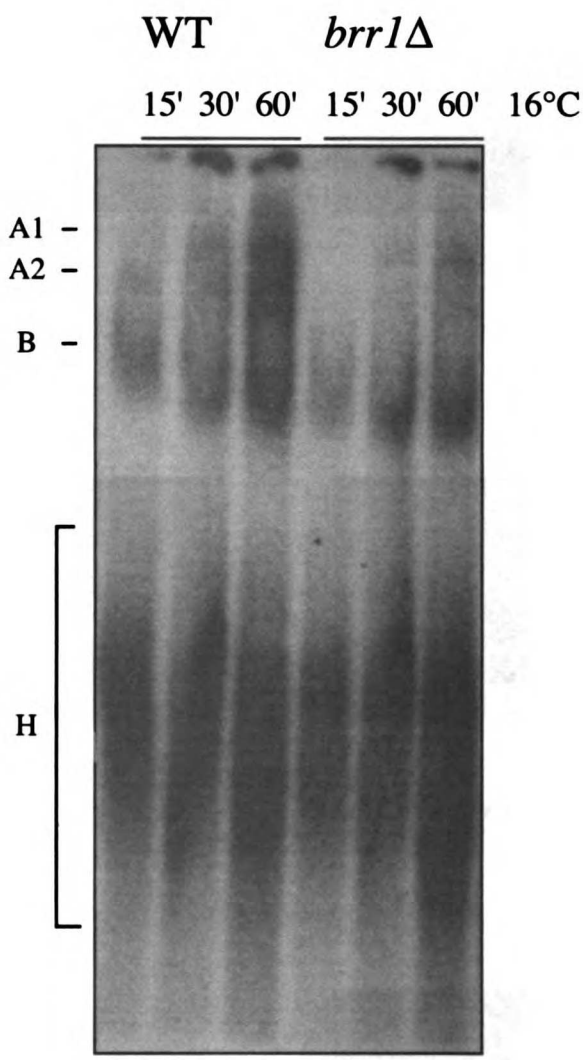


**Figure 3** - Kinetics of spliceosomal complex formation in wildtype (WT) and *brr1Δ* extracts are similar, although levels are decreased in the *brr1Δ* extract. Complex formation was carried out at 16°C with radioactively labelled ACT1 pre-mRNA and 1ug heparin /ul extract was added before gel electrophoresis.

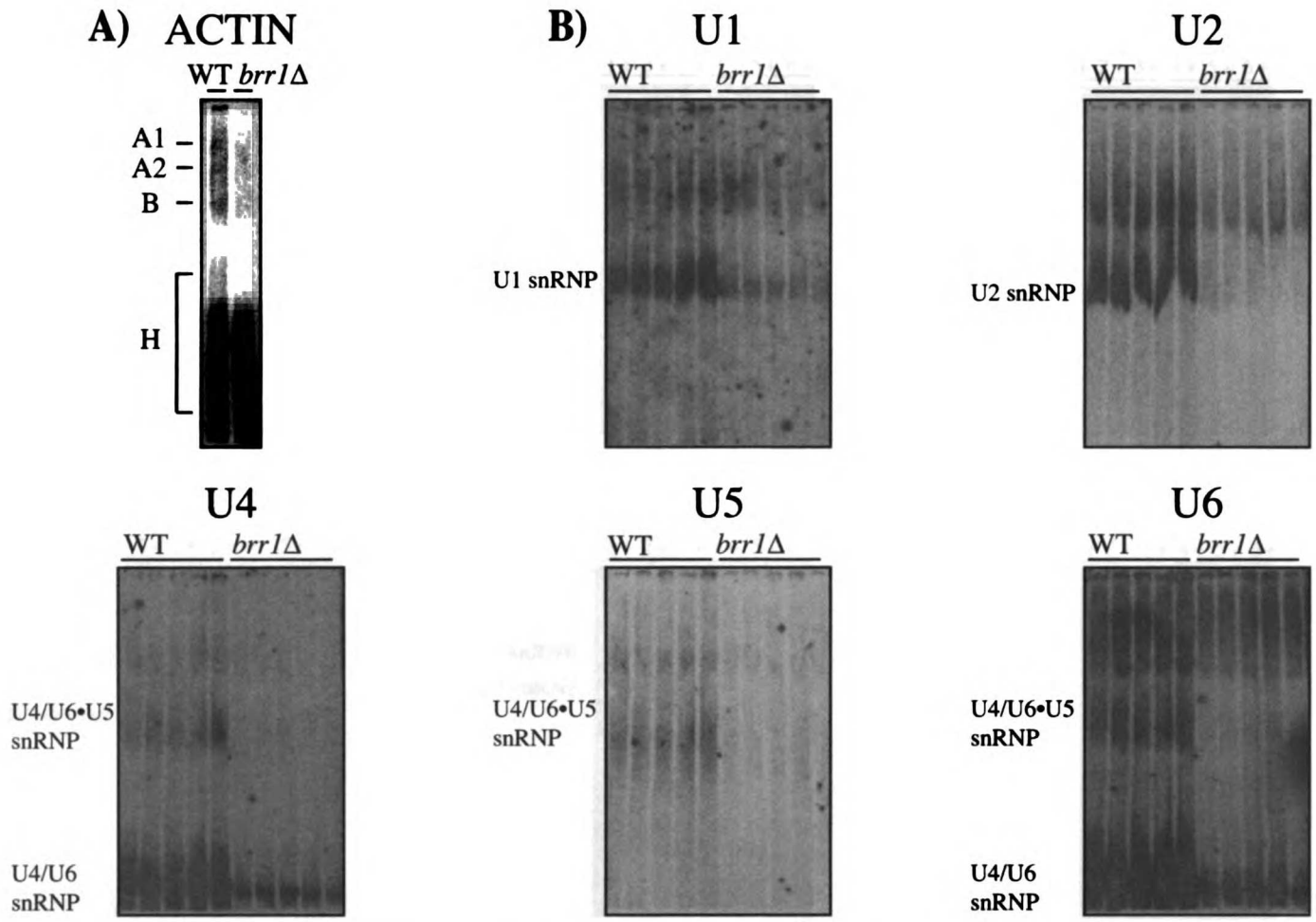


**Figure 4** - snRNP levels are severly decreased in *brr1*Δ mutant extracts by spliceosome assembly native gel analysis. A) Radioactively labelled ACT1 pre-mRNA (90 minute incubation) or B) unlabelled ACT1 pre-mRNA was incubated in extracts for 15, 30, 45, 60 and 90 minutes at 16°C followed by snRNA Northern analysis.

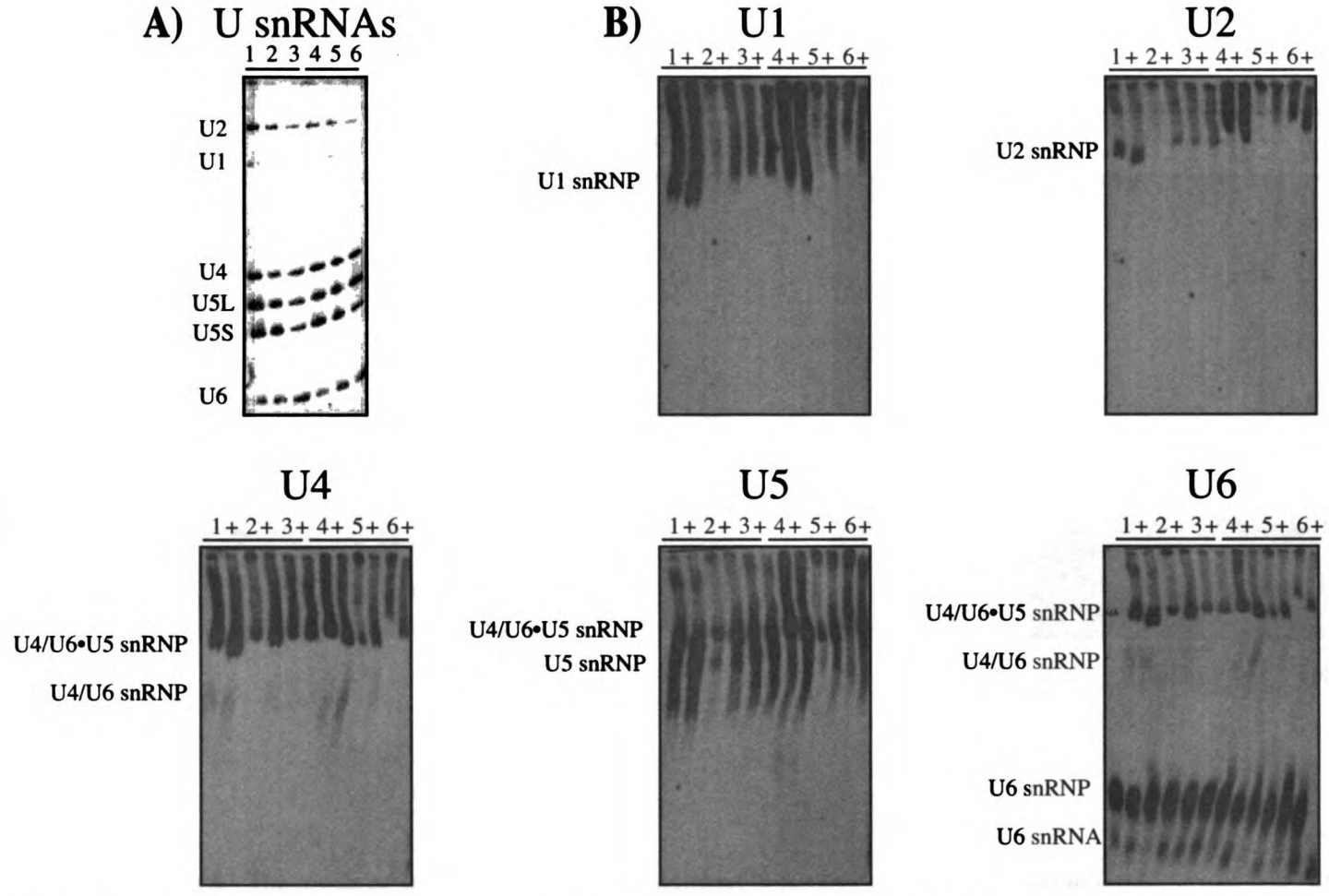


**Figure 5** - A) snRNA and B) snRNP levels are decreased in *brr1-1* (lanes 2 and 5) and *brr1*Δ (lanes 3 and 6) mutant extracts compared to wildtype extracts (WT) (lanes 1 and 4) in duplicate samples. A) snRNA levels were determined by denaturing gel and B) snRNP levels were assayed by native gel both followed by Northern analysis. (+) lanes are + ATP.

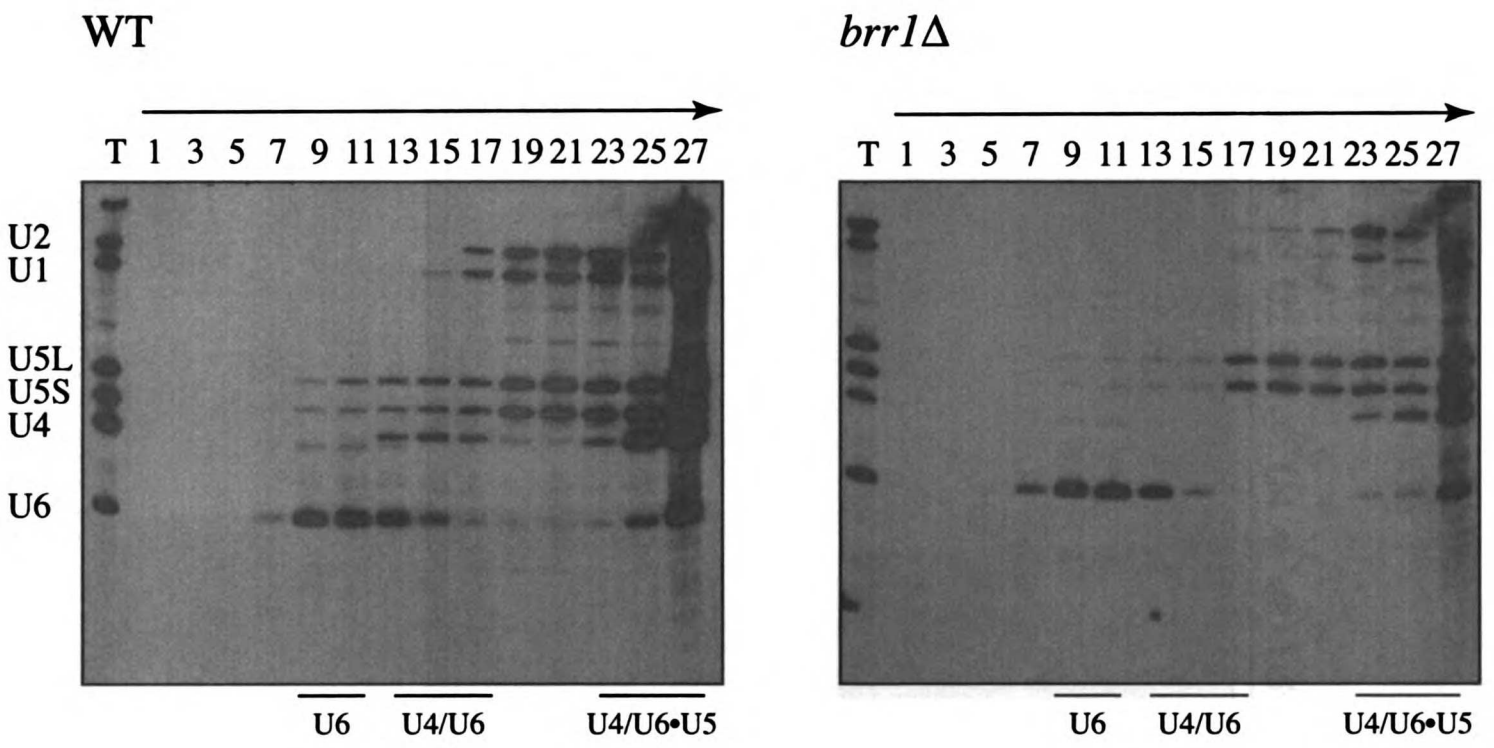


**Figure 6** - The levels of snRNP complexes are reduced in *brr1Δ* extracts compared to wildtype extracts (WT) by velocity sedimentation analysis. RNA from odd numbered fractions of 10-30% glycerol gradients were analyzed by Northern analysis. Input RNA (1/20th total extract (T)), snRNAs, and the approximate mobility of snRNP complexes are indicated.

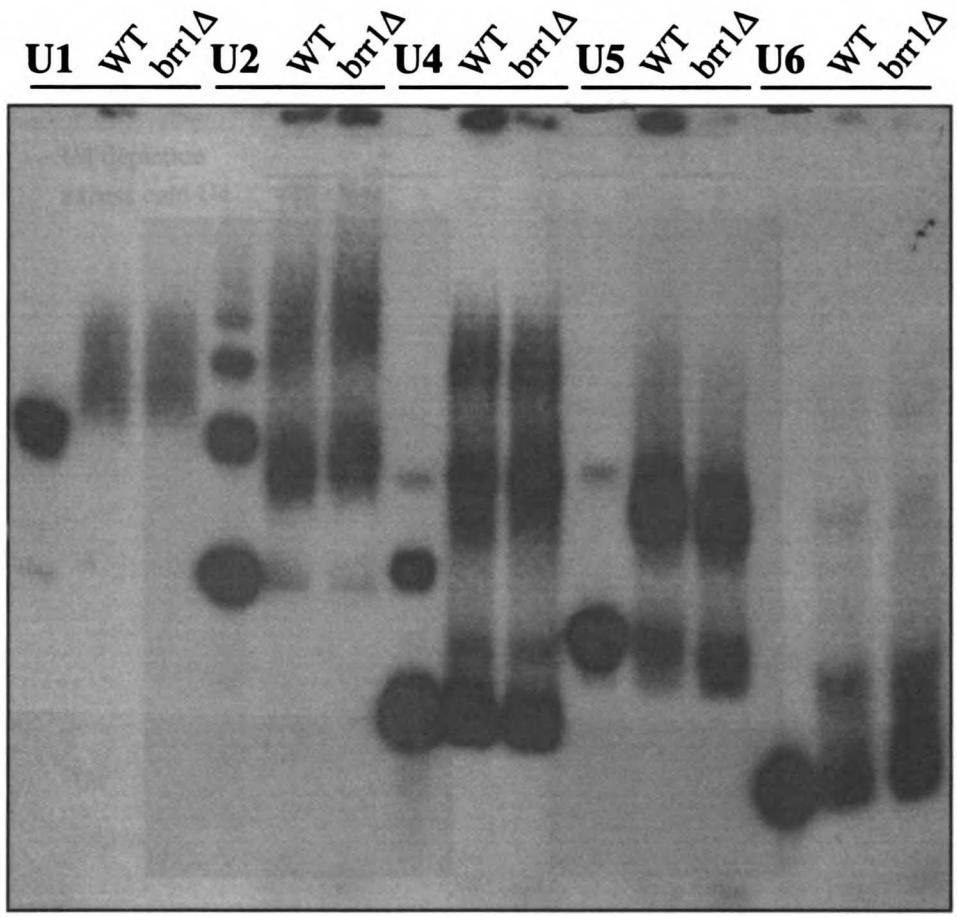


**Figure 7** - In vitro snRNP assembly with T7 transcribed snRNAs.
The mobilities of radioactively labelled U snRNA transcripts (~1-10nM) were similar whether incubated in wildtype (WT) or brr1Δ extracts at 16°C for 30 minutes.

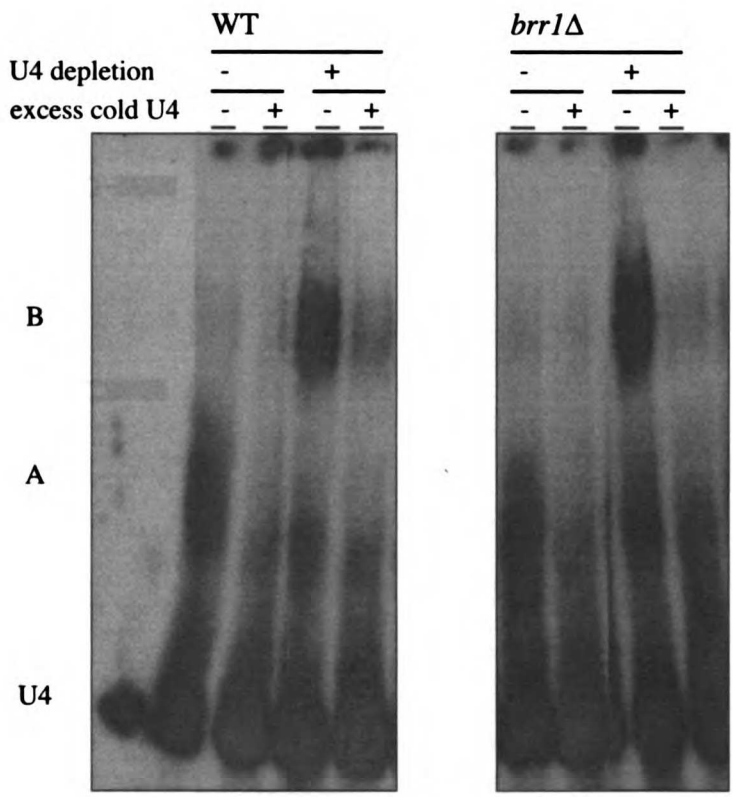


**Figure 8** - In vitro snRNP assembly with U4 depleted extracts.
The mobility of radioactively labelled U4 snRNA decreased in extracts depleted of endogenous U4 snRNA (complex B) compared to untreated extracts (complex A). However, the mobilities of U4 snRNA were simlar with both wildtype (WT) and *brr1*Δ extracts that were incubated on ice.

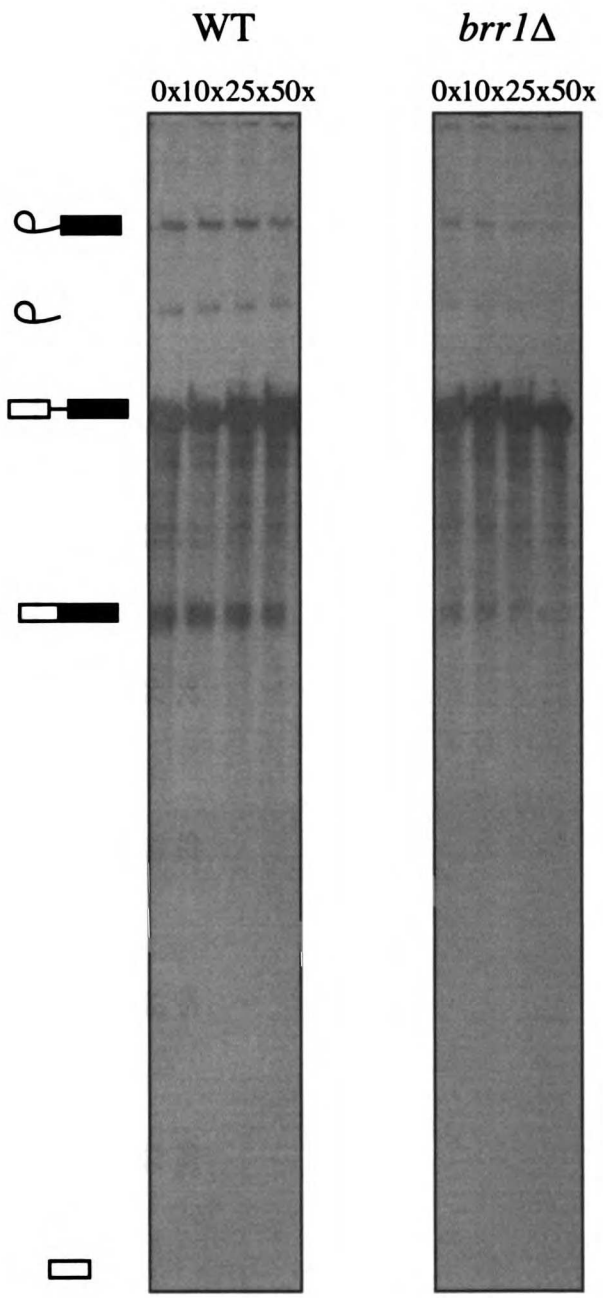


**Figure 9** - *In vitro* splicing in *brr1*Δ extracts is more susceptible to the challenge of multi-round splicing than wildtype extract (WT). Unlabelled ACT1 pre-mRNA was added to the indicated fold excess amounts during a 30 minute preincubation at 16°C and then splicing of radioacitvely labelled ACT1 was assayed at 25° for 30 minutes.

**Table 1 - snRNA and snRNP levels in brr1-1 and brr1Δ extracts as a percentage of wildtype levels**

| gel | denaturing | denaturing | denaturing | denaturing | denaturing | denaturing | native |
|---|---|---|---|---|---|---|---|
| strain | *brr1-1* | *brr1-1* | *brr1-1* | *brr1Δ* | *brr1Δ* | *brr1Δ* | *brr1Δ* |
| incubation | - | + | + | - | + | + | + |
| ATP | - | - | + | - | - | + | + |
| U1 | 37 | 29 | 30 | 29 | 22 | 23 | |
| U2 | 65 | 49 | 64 | 41 | 30 | 37 | 10 |
| U4 | 49 | 66 | 61 | 50 | 79 | 58 | |
| U5 | 75 | 73 | 81 | 81 | 79 | 90 | |
| U6 | 57 | 56 | 54 | 25 | 28 | 28 | |

**Table 2 - BRR1 Genetic Interactions**

| Factor | Function | brr1-1 c.s. interaction | brr1Δ c.s. interaction | brr1-1 snRNAs | BRR1 o/x |
|---|---|---|---|---|---|
| *lhp1Δ* | 3' end stabilization | n/a | none | | |
| *rnt1Δ t.s. c.s.* | 3' cleavage | n/a | n/a | | |
| *lsm1Δ t.s. c.s.* | mRNA degradation | n/a | none | | |
| *lsm5Δ* | U6 biogenesis and mRNA degradation | n/a | sick | | |
| *lsm6Δ t.s.* | U6 biogenesis and mRNA degradation | n/a | sick | | |
| *lsm7Δ t.s.* | U6 biogenesis and mRNA degradation | n/a | sick | | |
| [1] *prp24 rrm1-sub* | U4/U6 annealing | none | none | | |
| [1] *prp24 rrm1-B4 c.s.* | U4/U6 annealing | lethal | lethal | | |
| [1] *prp24 rrm1-B5 c.s.* | U4/U6 annealing | sick | sick | | |
| [1] *prp24 rrm3-sub t.s.* | U4/U6 annealing | lethal | lethal | | |
| [1] *prp24 Δ10 c.s.* | U4/U6 annealing | none | none | | |
| *brr2-1 c.s.* | U4/U6 release | n/a | none | | |
| *sad1-1 c.s.* | snRNP biogenesis and triple snRNP | none (?) | lethal | | |
| *snu66Δ c.s.* | triple snRNP | n/a | lethal | | |
| [2] *snu114-60* | U5 snRNP, recycling | n/a | sick | | |
| [3] *sub2-1 t.s. c.s.* | splicing, export, recombination, transcription | sick | suppr/sick | | sick |
| [3] *sub2-5 c.s.* | splicing, export, recombination, transcription | sick | sick | | |
| [4] *prp8-brr c.s.* | U5 snRNP, splicing | n/a | sick | | |
| [5] *cdc45-1* | DNA replication | n/a | sick | | |
| SMD1 o/x | Sm core | suppression | none | none | |
| LHP1 o/x | 3' end stabilization | suppression | none | suppression | |
| SUB2 o/x | splicing, export, recombination, transcription | suppression | suppression | partial (U5) | |
| TFB4 o/x | TFIIH trxn factor | suppression | suppression | n/a | |
| [1]PRP24 o/x | U4/U6 annealing | n/a | suppression | n/a | |
| [1]prp24 Δ10 o/x | U4/U6 annealing | n/a | none | n/a | |

1 Rader and Guthrie personal communication

2 Brenner and Guthrie personal communication

3 Kistler and Guthrie Ph. D. Thesis

4 Collins and Guthrie Ph. D. Thesis

5 Tong et al.

## Chapter III - Identification of Lhp1p-associated RNAs by microarray analysis in *S. cerevisiae* reveals association with coding and noncoding RNAs


### ABSTRACT

La is a conserved eukaryotic RNA-binding protein best known for its role in the biogenesis of noncoding RNAs transcribed by RNA polymerase III. To broaden our understanding of the function of the La homologous protein (Lhp1) in *S. cerevisiae* we have taken a genomics approach. Lhp1 RNP complexes were immunoprecipitated and bound RNAs examined by hybridization to whole genome microarrays that include over 6000 ORFs, documented noncoding RNAs and the intervening intergenic regions. Demonstrating the validity of this approach, associations with previously known Lhp1p-associated RNAs were detected and associations with additional noncoding RNAs, including multiple tRNAs and snoRNAs, were revealed. Indicating that this approach provides a robust method for discovering new RNAs, the data also identify associations between Lhp1p and several intergenic regions, three of which encode the recently annotated putative snoRNAs - *RUF1, RUF2* and *RUF3*. Unexpectedly, we find that Lhp1p is also associated with a subset of coding mRNAs. These include many ribosomal protein transcripts as well as the mRNA encoding Hac1p, a transcription factor required during the unfolded protein stress response (UPR). In cells lacking Lhp1, Hac1p levels are decreased two- to three-fold, while no changes are detected in the levels of spliced or unspliced HAC1 mRNA or in the stability of Hac1p. Finally, although *LHP1* is dispensable for growth under standard conditions, we find that it is required when the UPR is induced at elevated temperatures. These results suggest that Lhp1p may play a novel role in the translation of one or more cellular mRNAs.

## INTRODUCTION

La is an abundant eukaryotic RNA binding protein implicated in multiple steps of RNA metabolism, including transcription and 3' end processing of RNA polymerase III (RNA pol III) transcripts, as well as translation of certain viral and endogenous mRNAs containing internal ribosome entry sites (IRES) sequences (Wolin and Cedervall 2002). The best characterized role of La is in the biogenesis and processing of a variety of noncoding RNAs (Wolin and Cedervall 2002). La binds the 3' terminal $UUU_{OH}$ sequences of newly synthesized RNA pol III transcripts such as pre-tRNAs and pre-5S rRNA and protects them from degradation (Rinke and Steitz 1982; Yoo and Wolin 1994). In yeast, La homologous protein (*LHP1*) is nonessential, which is surprising given that its metazoan counterparts are involved in processing such a wide range of essential, noncoding RNAs. Nevertheless, genetic and biochemical analyses in *S. cerevisiae* have confirmed that Lhp1p is involved in the processing of newly synthesized RNA pol III transcripts (Yoo and Wolin 1997; Pannone et al. 1998), and revealed a similar role with noncoding RNAs generated by RNA pol II, spliceosomal snRNAs (Xue et al. 2000) and U3 snoRNA (Kufel et al. 2000). Lhp1p is known to bind these noncoding RNA precursors and is thought to facilitate their maturation by stabilizing them from digestion. Since Lhp1p is not required for maintaining normal levels of mature RNPs, it has been suggested that Lhp1p acts as an RNA chaperone, enhancing the efficiency of biogenesis (Pannone et al. 1998; Xue et al. 2000).

Additional metazoan studies (Wolin and Cedervall 2002) have indicated that La may function at the 5' end of some RNAs to play a role in translation. By *in vitro* studies, La has been shown to stimulate translation and influence IRES selection of some viral RNAs (Meerovitch et al. 1993; Ali et al. 2000) and of some cellular RNAs containing IRES elements (Holcik and Korneluk 2000; Kim et al. 2001). However, it remains unclear if La plays these roles *in vivo*. More recently La has been proposed to influence the translation of mRNAs containing 5' terminal oligopyrimidine (TOP) sequences, which include mRNAs encoding ribosomal proteins that are translationally regulated in a growth-dependent manner (Meyuhas 2000). However, one study

examining La's effect on TOP mRNA translation by *in vivo* transfection experiments observed modest positive effects (Crosio et al. 2000), while another group has observed inhibitory effects of La on TOP mRNA translation (Zhu et al. 2001). Although La has recently been demonstrated to be associated with TOP mRNAs *in vivo* and in active polysomes (Keene and Tenenbaum 2002; Cardinali et al. 2003; Intine et al. 2003), La's specific role with TOP mRNAs remains unclear. To gain a comprehensive view of Lhp1p function, we set out to characterize the complete binding profile of Lhp1p using a genomics approach. We have immunoprecipitated Lhp1p and identified associated RNAs by microarray analysis. We find that Lhp1p is associated not only with a large number of noncoding RNAs but with coding RNAs as well. Thus, Lhp1p may have a broader function than previously thought.

## MATERIALS AND METHODS

### Oligos

See Table 1.

### Strains

The following yeast strains were used: *LHP1-myc$_3$* and its isogenic wildtype (Noble and Guthrie 1995), *lhp1Δ::LEU* and its isogenic wildtype (Yoo and Wolin 1994), *lhp1Δ::KAN* and its isogenic wildtype (Invitrogen).

### Immunoprecipitation and Microarray Analysis of Lhp1p-Associated RNAs

Isogenic wildtype and *LHP1-myc$_3$* genomically epitope-tagged cells were grown to saturation in rich media at 30°C overnight, diluted to $OD_{600}$=0.1 in 1 liter, grown to exponential phase ($OD_{600}$=0.75-1.0), and harvested by centrifugation for extract preparation and immunoprecipitations as previously described (Takizawa and Vale 2000) with minor modifications. Immunoprecipitated RNAs were reverse transcribed into cDNA as previously described (Takizawa et al. 2000), but without PCR amplification, in the presence of aminoallyl-

dUTP (Sigma-Aldrich) using random sequence nonamers as primers (0.25mg/ml). The cDNA samples were labeled with either Cy3 or Cy5 fluorescent dyes (Amersham Biosciences) and purified. Fluorescently labeled cDNAs were hybridized to whole genome microarrays as previously described (DeRisi et al. 1997). See also http://microarrays.org/ for microarray printing and preparation protocols. Whole genome primer sets and sequences are available upon request from Invitrogen. The microarrays were scanned with a GenePix 4000a scanner and analyzed with GenePixPro 3.0 software (Axon Instruments). Cy3 and Cy5 fluorescent signals were normalized so that the total signal ratio for all good quality features was equal to one. Therefore, the fluorescence signal intensity from the relatively low abundance untagged control is artificially inflated and the data do not necessarily reflect true enrichment values. For each feature we calculated an average $\log_2$ transformed ratio from two independent experiments by which we determined an overall rank order. See Table 2, which is published as Supporting Information on the PNAS website for primary Lhp1p IP microarray data.

**QPCR**

Cell equivalent fractions of isolated RNA from *LHP1*-myc$_3$ tagged and untagged samples were reverse transcribed with a sequence specific cocktail of reverse primers (250nM each) (see Table 1). We found that quantitative PCR (QPCR) results were more linear with input template when reverse transcription was done with sequence specific primers, rather than with random primers (data not shown). The cDNA was ethanol precipitated twice and used as template for QPCR. The QPCR reactions contained 250nM primer concentration and 0.75x SYBR Green (Sigma-Aldrich) for fluorescent detection of product formation. Standard curves were generated by making a dilution series of template cDNA, which was reverse transcribed from total RNA. Fold enrichments between tagged and untagged samples were calculated based on standard curves when the data were within the linear range. The presence of single PCR products was confirmed both by analyzing melting curve transitions and by electrophoresing completed QPCR on 2.2%

96-well agarose gels (Amersham Biosciences). All QPCR reactions were carried out and analyzed with Opticon Detection Systems (MJ Research).

**UPR Growth Assays**

Wildtype and *lhp1*Δ strains were grown in rich or synthetic media. Five-fold serial dilutions were made in 96-well plates and spotted on SD complete plates lacking inositol and top spread with 0 to 1.5μg/ml tunicamycin, equilibrated for 5 hours. The plates were incubated at room temperature, 30°C and 37°C.

**HAC1 Northern and Western Analysis**

Wildtype and *lhp1*Δ strains were grown in synthetic media at room temperature and then shifted to 37°C with addition of 1.0μg/ml tunicamycin or in a *sec14-3*$^{ts}$ strain background to induce the UPR. Aliquots were harvested at time points from 0 to 3 hours. Total RNA was prepared and Northern analysis was carried out as described (Ruegsegger et al. 2001). For Western analysis, protein was prepared as described (Ruegsegger et al. 2001). Samples were separated by SDS-PAGE and transferred to nitrocellulose and immunodetected using anti-Hac1p$^{i}$ (a gift from P. Walter, UCSF), anti-Npl3p mAb 1E4 (a gift from M. Swanson, U. of Florida), ECL and ECF detection systems (Amersham Biosciences).

**RESULTS**

To identify Lhp1p-associated RNAs, we performed immunoprecipitation (IP) assays followed by microarray analysis (see Materials and Methods). Lhp1p-associated RNAs were immunoprecipitated by anti-myc antibodies from *LHP1-myc*$_3$ genomically epitope-tagged cell extracts under native conditions. They were then extracted and converted to cDNA by reverse transcription without PCR amplification, which had been required in other studies (Takizawa et al. 2000; Hieronymus and Silver 2003). In our hands this step decreased data reproducibility (data

not shown) and was eliminated because we found it to be unnecessary. The cDNA from Lhp1p myc-tagged and untagged control IPs were fluorescently labeled with Cy5 and Cy3 dyes respectively and analyzed by simultaneous hybridization to *S. cerevisiae* whole genome microarrays (Fig. 1). These microarrays were designed to cover the entire genome according to the Saccharomyces Genome Database (SGD) annotation in May 1999 with approximately 13,000 unique features, corresponding to over 6000 ORFs, known noncoding RNAs and their intervening intergenic regions (Iyer et al. 2001). Although SGD annotation has been constantly updated since the time of design, newly annotated genes are nonetheless represented on the microarray, because they are encoded within the PCR fragments of intergenic region features.

For each microarray feature we obtained a Cy5 to Cy3 fluorescence signal ratio, representing the relative abundance of each RNA in the Lhp1p versus control IP. Since the data are normalized so that the total signal ratio is equal to one, the intensity in the relatively low abundance control IP is artificially inflated. Therefore the normalized data do not necessarily reflect true enrichment values. Data from two independent IP experiments were linearized by $\log_2$ transformation, averaged, and found to be highly correlated (Pearson Correlation R=0.85) (data not shown). The dynamic range of the average $\log_2$ ratios spanned from +4.4 to –4.6 and defined a rank order of Lhp1p IP enriched features (see Table 2 Supplementary Information). The data displayed a roughly Gaussian distribution centered around –1.2 (mean), with an extended tail comprising approximately 100 of the most highly enriched features or the top 1% of the dataset (Fig. 2A). Moreover, since most known Lhp1p-associated RNAs appeared reproducibly in the top 100 (see Table 3), we interpreted this subset as highly likely to be associated with Lhp1p. While distinguishing between true associations and false positives becomes more difficult further down the list, we have arbitrarily classified the top 10% or 1000 features as significantly enriched. For ease of discussion, we will present our results for three classes of Lhp1p-associated RNAs: noncoding, intergenic and coding mRNAs (Fig. 2A).

**Class I – Noncoding RNAs**

In accordance with previous results that all mammalian RNA pol III transcripts are believed to interact with La (Wolin and Cedervall 2002), we find several yeast RNA pol III transcripts are also highly enriched in Lhp1p IPs. Three known Lhp1p-associated RNA pol III transcripts are among the 10 most highly enriched RNAs. These are U6 snRNA, SCR1, the RNA component of the signal recognition particle, and RPR1, the RNA component of RNase P (see Table 3). Lhp1p has been shown to bind and stabilize newly synthesized U6 snRNA (Pannone et al. 1998). Lhp1p has also been shown to associate with SCR1 (Yoo and Wolin 1994). The feature corresponding to SCR1 itself was not included on the microarray, but the intergenic region immediately downstream was identified with high enrichment (Fig. 3A). We infer that this reflects association of Lhp1p with a pre-SCR1 species elongated at its 3' end. Previous work has indicated that Lhp1p binds and may function to stabilize pre-RPR1 (Calvo et al. 1999; Kufel et al. 2002). We observe an interaction with the adjacent intergenic region directly downstream of RPR1 and believe this to be an association with 3' extended pre-RPR1.

There are 42 unique tRNA features on the microarray, representing at least one tRNA for 38 of 41 total anticodon isoacceptor types. In addition, because of tRNA gene duplication events and the high level of homology between tRNA isoacceptors, the 42 tRNA features on the microarray represent the vast majority of the 274 total tRNA genes present in the *S. cerevisiae* genome. We find that 22 of the 42 tRNA features appear in the 100 most highly enriched RNAs, with 33 in the top 1000 (see Table 4). Significantly, $tRNA^{Ser}$(CGA), previously shown to be associated with Lhp1p (Yoo and Wolin 1997), is in the top 100. Of the11 other tRNAs previously suggested to be associated with Lhp1p, based on the observation that aberrant pre-tRNA processing was observed in strains deleted for *LHP1* (Yoo and Wolin 1997; Calvo et al. 1999), 10 are in the top 100 and one was undetected. Interestingly, $tRNA^{Leu}$(CAA), which was not shown to require *LHP1* for processing (Yoo and Wolin 1997), is Lhp1p-associated. In addition to the tRNA features, Lhp1p is associated with 8 intergenic regions directly 5' or 3' of tRNAs. While unannotated RNAs

encoded within these intergenic regions may be responsible for the observed enrichment (see Class II: Intergenic Regions), it is equally plausible that although yeast tRNA leader and trailer sequences are generally short, these associations reflect interaction with the 5' and/or 3' extended pre-tRNA species. Also, since the microarray consists of double-stranded PCR products, we believe we detect three tRNAs by way of ORF features residing on the opposite strand or overlapping with the tRNAs. For example, the microarray feature for *YNL017c* is highly enriched, most likely because *tI(AAU)N2* is located on the opposite strand (Fig. 3B). All three of these ORFs were recently annotated as Dubious ORFs by comparative genomic analysis (Brachat et al. 2003; Cliften et al. 2003; Kellis et al. 2003), strongly suggesting that the associations detected are with the tRNAs. Finally, our failure to see significant enrichment of Lhp1p with the remaining nine tRNAs on the microarray may indicate low binding, low signal from low abundance, or low reverse transcription efficiency of a highly structured RNA.

The feature corresponding to 5S rRNA shows only a relatively modest enrichment compared to other RNA pol III transcripts, even though an association of Lhp1p with pre-5S rRNA has been previously observed (Yoo and Wolin 1994; Kufel et al. 2002). We find 5S rRNA and two other intergenic regions encoding highly homologous 5S rRNA variants (Johnston et al. 1997) within the 700 most highly enriched RNAs (Fig. 3C and Table 3).

In addition to RNA pol III–transcribed noncoding RNAs, we also detect association with RNA pol II-transcribed noncoding RNAs, including U2, U4 and U5 snRNAs (see Table 3). Lhp1p has been shown to bind and stabilize 3' extended precursors of RNA pol II-transcribed spliceosomal snRNAs (U1, U2, U4 and U5) (Xue et al. 2000), as well as U3 snoRNA (Kufel et al. 2000). While the genes encoding U1 snRNA and U3 snoRNA were not included as unique features on our microarray due to incomplete noncoding RNA annotation, we detect enrichment with the intergenic regions encoding these RNAs. We have not excluded the possibility that other unannotated RNAs within these regions may contribute to the high enrichment ratios, but the most likely explanation is Lhp1p association with the snRNAs.

Whereas U3 was the only snoRNA previously shown to be associated with and its processing facilitated by Lhp1p (Kufel et al. 2000), we find a large number of snoRNAs associated with Lhp1p. There are currently 66 total known snoRNA genes in the *S. cerevisiae* genome. Approximately one-third (20) are found in the 100 most enriched RNAs with more than half (38) in the top 1000 (see Table 5). On the microarray, the snoRNAs are represented as unique features, encoded within intergenic regions, or for 7 intronically-encoded snoRNAs are included within host ORF PCR products. For example, *SNR18* is encoded in the intron of *YAL003w*, which is highly enriched (Fig. 3D). For the 5 out of 7 intronically-encoded snoRNAs found in the top 1000 via their host ORF feature, we have not determined if the enrichment is due solely to the snoRNA, the host ORF (see Class III: Coding mRNAs) or both.

**Class II – Intergenic Regions**

An advantage of the whole genome microarray is the potential to discover new, uncharacterized RNAs within intergenic regions. Indeed, 11 intergenic regions in the top 100 do not contain any known RNAs. Notably, the immediately surrounding features are not significantly enriched, suggesting that the signal in the intergenic region is not due to hybridization from the 5' or 3' UTR of a neighboring feature. Remarkably, the three most highly enriched intergenic regions encode genes annotated only after our analysis was completed. They are *RUF1*, *RUF2* and *RUF3*, which correspond to three putative H/ACA snoRNAs identified computationally by phylogenetic analysis and confirmed by Northern analysis (McCutcheon and Eddy 2003). The convergence of discovering these new genes within Lhp1p-enriched intergenic regions strongly suggests that the other intergenic regions may also contain novel RNAs (see Table 6).

**Class III – Coding mRNAs**

Unexpectedly, 26 coding mRNAs appear in the 100 most Lhp1p-enriched RNAs with up to 300 in the top 500 (see Table 7). Previous descriptions of La interactions with mRNAs have been restricted to virally encoded RNAs or endogenous mRNAs with IRES sequences or mRNAs with

TOP sequences, which have not been documented in *S. cerevisiae* (Lascaris et al. 1999; Zhou et al. 2001).

We used QPCR as an independent method for assessing RNA association, by reverse transcribing cell-equivalent fractions of immunoprecipitated RNA from tagged and untagged cells (see Materials and Methods). We then determined fold enrichments for 18 RNAs from the microarray data list (see Table 8). The values determined by QPCR generally correlated well with the ratios generated from the microarray results (Pearson Correlation R=0.8) (Fig. 4). As expected, as a result of data normalization (see Materials and Methods), the enrichment ratios obtained from the microarray data generally underestimated fold enrichments determined by QPCR. Nevertheless, although not as highly enriched as many of the noncoding RNAs, the QPCR data confirmed significant association between Lhp1p and coding mRNAs.

***Ribosomal Protein Genes***

The predominant highly enriched class of coding mRNAs in the microarray data set is the ribosomal protein genes (RPGs) (Fig. 2B). Of the 130 RPGs, 14 are in the 100 most highly enriched RNAs and 100 are in the top 1000. Although RPGs encode some of the most abundant transcripts, we did not observe a general bias for Lhp1p association with highly expressed genes (data not shown) (Velculescu et al. 1997; Holstege et al. 1998). Moreover, there does not seem to be any bias for Lhp1p association with the most abundant RPGs, the small versus large subunit or intron-containing versus nonintron-containing RPGs (data not shown).

***HAC1 mRNA***

One of the most intriguing mRNAs we found to be associated with Lhp1p was HAC1 RNA, which encodes a transcription factor required for induction of the unfolded protein stress response (UPR). We sought to demonstrate a functional link between Lhp1p and HAC1 mRNA by examining the cellular response to unfolded proteins in the absence of Lhp1p. We plated serial dilutions of wildtype and *lhp1*Δ yeast cells on yeast media supplemented with tunicamycin (0 to 1.5μg/ml), which impairs protein folding by inhibiting glycosylation (Cox et al. 1993), to induce

the UPR. Whereas *lhp1*Δ cells grew like wildtype at 30°C, *LHP1* is essential for growth upon induction of the UPR at 37°C (Fig. 5). Although we cannot rule out indirect effects, these results suggest that Lhp1p is required for proper induction of the UPR at elevated temperatures. Interestingly, HAC1 mRNA is spliced only upon induction of the UPR by a novel mechanism that does not use the spliceosome (Sidrauski et al. 1996). Rather, the HAC1 transcript is cleaved by an endonuclease and ligated by tRNA ligase (Sidrauski and Walter 1997). Since the mechanism of HAC1 mRNA splicing partially resembles that of pre-tRNA splicing (Gonzalez et al. 1999), this raised the possibility that HAC1 RNA processing could likewise be facilitated by Lhp1p. To determine if Lhp1p is involved in the splicing of HAC1 mRNA, we examined HAC1 RNA levels by Northern analysis in an *lhp1*Δ strain at time points from 0 to 3 hours after induction of the UPR. Two methods of induction were used: addition of 1μg/ml tunicamycin (data not shown), and shifting to 37°C in a *sec14-3*$^{ts}$ mutant background, which arrests the secretory pathway (Ruegsegger et al. 2001; Chang et al. 2002). We observe no *LHP1*-dependent change in either the HAC1 total mRNA levels or the ratio of spliced to unspliced HAC1 RNA (Fig. 6A). Thus, Lhp1p has no detectable effect on the processing of HAC1 RNA.

To determine if Lhp1p is involved in post-processing steps of HAC1 gene expression, we examined Hac1p levels by Western analysis. In an *lhp1*Δ strain, upon induction of the UPR by 1μg/ml tunicamycin (data not shown) or shifting to 37°C in a *sec14-3*$^{ts}$ mutant background we observe a reproducible two- to three-fold reduction in Hac1p levels (Fig. 6B and data not shown). This observation is consistent with a role for Lhp1p either in Hac1p translation or post-translational stability of Hac1p. By Western analysis, we examined the decay of Hac1p levels (Kawahara et al. 1997) in an *lhp1*Δ strain after addition of cycloheximide to inhibit new protein synthesis and found no *LHP1*-dependent change in Hac1p stability (data not shown). Taken together, these data suggest a role for Lhp1p in translation of HAC1 mRNA.

## DISCUSSION

We have employed a genomics approach to identify Lhp1p-associated RNAs. By exploiting the whole genome microarray, which includes intergenic regions and noncoding RNAs in addition to ORFs (Iyer et al. 2001), we were able to survey Lhp1p association with the entire transcribed yeast genome in a single experiment. The wealth of available genetic and biochemical evidence for Lhp1p association with many noncoding RNAs provided robust positive controls for our study. Indeed, we detected association with many known Lhp1p substrates, including $tRNA^{Ser}$(CGA), U6 snRNA, SCR1, RPR1 and 5S rRNA as well as the RNA pol II-transcribed spliceosomal snRNAs and U3 snoRNA.

It is important to note that some RNAs that are Lhp1p-associated may not be identified by our assay as a consequence of insufficient signal due to low abundance, inadequate hybridization from a short RNA, or poor reverse transcription efficiency of a structured RNA. Moreover, only associations stable during the course of the IP assay will be detectable. Therefore, the absence of significant enrichment by our assay does not necessarily indicate lack of an association. For example, at least one RNA predicted to be associated with Lhp1p, NME1 (Calvo et al. 1999), the RNA component of ribonuclease MRP, was not significantly enriched in our IPs. Furthermore, some genes, such as TLC1, which encodes the RNA component of telomerase, were omitted either as unique features or within intergenic regions on the microarray. With these caveats we were able to survey nearly the whole genome for Lhp1p-association to gain a broader view of its binding profile.

### Discovery of new noncoding RNA binding partners

While previous work had demonstrated that 3' end processing of multiple tRNAs is facilitated by Lhp1p (Yoo and Wolin 1997; Calvo et al. 1999), it remained an open question whether the interaction with U3 snoRNA (Kufel et al. 2000) was unique or indicative of class-wide recognition for snoRNAs. Our detection of Lhp1p interaction with the majority of tRNAs and snoRNAs by the IP microarray assay suggests a class-wide role for Lhp1p in the processing of

these stable, noncoding RNAs. Interestingly, although no changes in the pre-tRNA patterns were previously detected for $tRNA^{Leu}$(CAA) in *lhp1*Δ strains (Yoo and Wolin 1997), we nonetheless detected association with this tRNA by the IP microarray assay. While 3' processing defects may simply not be rate-limiting under the conditions assayed, another possibility is that Lhp1p plays a role in step(s) further downstream. One such possible role is the facilitation of proper pre-tRNA folding for aminoacylation (Chakshusmathi et al. 2003). While our work has expanded the number of RNAs associated with Lhp1p, it remains to be determined if Lhp1p plays similar or different roles for all tRNAs and snoRNAs.

Our approach provides the unique opportunity to explore uncharacterized regions in the *S. cerevisiae* genome in search of novel RNAs on a genomic scale. The discovery of novel RNAs by probing unusually large intergenic regions (Olivas et al. 1997), underscored the fact that our current annotations are incomplete. Subsequently, others have employed computational approaches to look for new members of specific classes of RNAs by consensus sequences (Lowe and Eddy 1999) or to find novel noncoding RNAs within structurally conserved intergenic sequences among related species (McCutcheon and Eddy 2003). In contrast, the whole genome microarray approach is unbiased for sequence information. Recent studies have productively utilized a combination of computational and biochemical methods using similar microarrays in prokaryotes (Wassarman et al. 2001; Li et al. 2003). To our knowledge, however, ours is the first search for new RNAs using a complete eukaryotic genome array. Because the SGD noncoding RNA annotation was deficient at the time of design of the whole genome microarray, a number of known snRNAs and snoRNAs were omitted as specific features. Nonetheless, intergenic regions encoding these RNAs were detected as Lhp1p-associated by the microarray assay. As further validation of the utility of this approach for gene discovery, we identified three intergenic regions, which were independently found to encode novel snoRNA genes (McCutcheon and Eddy 2003). We are currently mapping the other Lhp1p-associated intergenic regions to look for novel RNAs.

Interestingly, we find some of the intergenic regions are highly conserved among fungal species (data not shown).

**Lhp1p is associated with coding mRNAs**

Our data indicate that Lhp1p is associated with coding mRNAs in addition to noncoding RNAs. In particular, we detect an interaction with HAC1 mRNA, which encodes an activating transcription factor for the UPR. Importantly, we demonstrate that Lhp1p is required for growth under UPR conditions at high temperature (Fig. 6). Most simply, Lhp1p could be involved in facilitating processing of HAC1 mRNA as it is with many noncoding RNAs. However, we did not observe an effect on HAC1 RNA levels or HAC1 mRNA splicing due to deletion of *LHP1* (Fig. 5A). Moreover, HAC1 mRNA is polyadenylated (Cox and Walter 1996) and although HAC1 mRNA splicing is carried out in part by tRNA ligase (Sidrauski et al. 1996), HAC1 RNA is not believed to be acted upon by other 3' end tRNA processing enzymes. As the steady state levels of many RNAs whose processing are known to be facilitated by Lhp1p remain unaffected in an *lhp1*Δ strain (Pannone et al. 1998; Xue et al. 2000), it is still possible that Lhp1p plays a role in HAC1 RNA processing, but its effects are undetected by our assays. However, we consistently observe a two- to three-fold decrease in Hac1p levels in the absence of *LHP1* (Fig. 5B and data not shown), suggesting a role for Lhp1p in Hac1p production. Since La has been implicated in enhancing the translation rates of certain mRNAs in mammals (Crosio et al. 2000; Kim et al. 2001), it is intriguing to speculate that Lhp1p may be involved in formation of a structure required for efficient Hac1p translation. Notably, translation of HAC1 is attenuated by way of a base-paired interaction between the 5'UTR and the intron (Ruegsegger et al. 2001). Thus Lhp1p could influence translation by destabilization of an inhibitory interaction such as between the 5'UTR and intron or by stabilization of a competing alternative structure. The latter mechanism would be consistent with Lhp1p's proposed role in stabilizing the anticodon stem of $tRNA^{Ser}$(CGA) (Yoo and Wolin 1997). Similarly, loss of Lhp1p may destabilize such a structure in HAC1 RNA and compromise Hac1p translation.

As the vast majority of mRNAs in *S. cerevisiae* are not believed to include IRES elements (Zhou et al. 2001), and yeast RPGs lack 5' TOP sequences (Lascaris et al. 1999), we were surprised to identify Lhp1p association with multiple mRNAs, particularly RPGs. Although yeast RPGs lack 5' TOP sequences, they are nonetheless regulated in a growth dependent manner (Powers and Walter 1999). However, unlike mammalian RPGs that are primarily translationally regulated via polysome association (Meyuhas 2000), yeast RPGs are primarily regulated at the level of transcription (Planta 1997). While future experiments are required to determine how association with yeast RPGs may affect their regulation, our data suggest an intriguing connection among Lhp1p-interacting RNAs. These RNAs are not only components of the translational apparatus (5S rRNA and tRNAs) and involved in processing the RNA components of the translational apparatus (snoRNAs and RNase P RNA), but include transcripts encoding the protein components of translational machinery (RPGs). Thus, Lhp1p may contribute to the coordination of both RNA and protein components of translation for general cell metabolism. Intriguingly, LHP1 mRNA is expressed during logarithmic growth but repressed during diauxic shift and stationary phase (DeRisi et al. 1997; Gasch et al. 2000), suggesting that Lhp1p function may only be utilized during times of rapid growth, which is particularly suggestive in the case of RPGs. Moreover, Lhp1p function may only become critical during challenging growth situations as we have uncovered in the case of the unfolded protein stress response. Further experiments are required to assess the functional relationship between Lhp1p and coding mRNAs.

## ACKNOWLEDGMENTS

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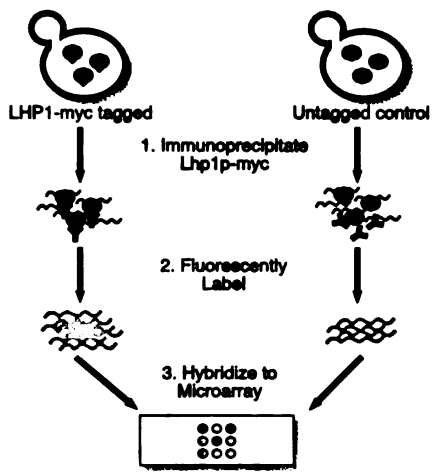


Figure 1 Schematic of Lhp1p IP microarray assay. Features enriched in the Lhp1p IP versus the untagged control appear as red, while others appear yellow or green.

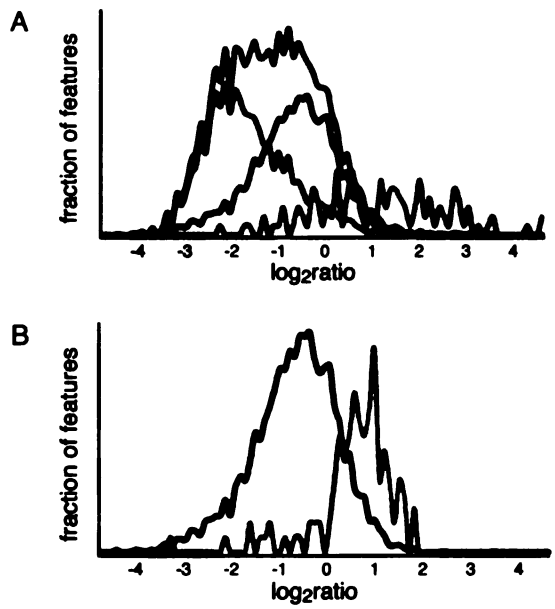


Figure 2 Histograms of RNA classes. Different RNA classes are depicted on scaled y-axes. A) The distribution of all features is shown in blue, the noncoding RNAs in orange, the intergenic regions in green and the coding mRNAs in black. B) The ribosomal protein gene transcripts (red) are more enriched in the Lhp1p IP compared to the distribution of all ORFs (black).

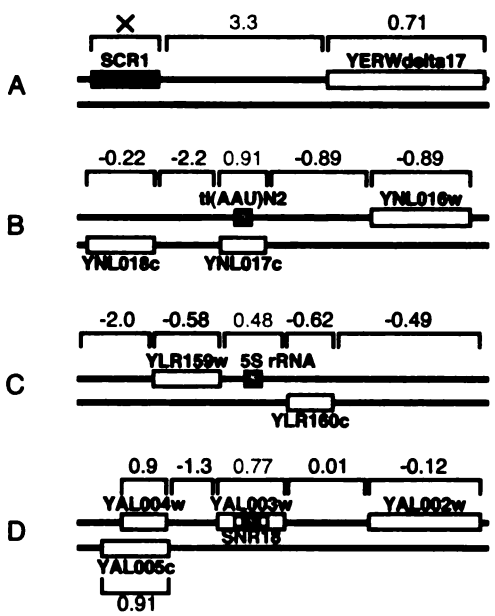


Figure 3 Chromosomal maps of Lhp1p IP microarray enrichment. Feature boundaries and corresponding average log2 ratios are indicated. A) While the feature corresponding to SCR1 is absent from the microarray (X), the intergenic feature iSCR1 immediately 3' of SCR1 shows high enrichment. B) The feature representing YNL017c shows high enrichment, most likely because tI(AAU)N2 is located on the opposite strand.
C) The intergenic region encoding a highly homologous 5S rRNA variant, iYLR159w, shows modest enrichment. D) The snoRNA SNR18 lies within the intron of YAL003w, which shows high enrichment.

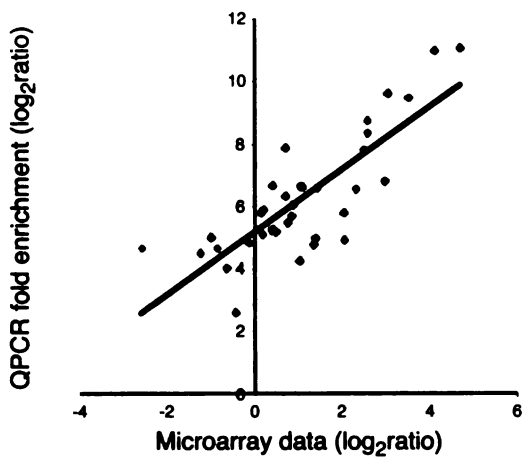


Figure 4 Lhp1p IP microarray data and QPCR data are highly correlated (Pearson Correlation R=0.8). A subset of eighteen genes was chosen (see Table 8).

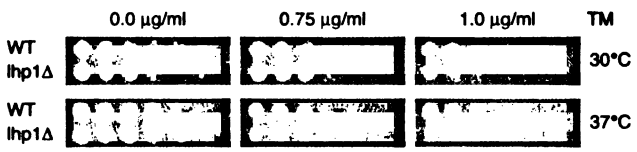


Figure 5 LHP1 is required for growth during the unfolded protein response at 37°C. Serial dilutions of wildtype (WT) and lhp1Δ::KAN strains (or lhp1Δ::LEU, data not shown) were grown at 30°C and 37°C on yeast media plates containing 0.0, 0.75, and 1.0 µg/ml tunicamycin (TM) to induce the UPR.

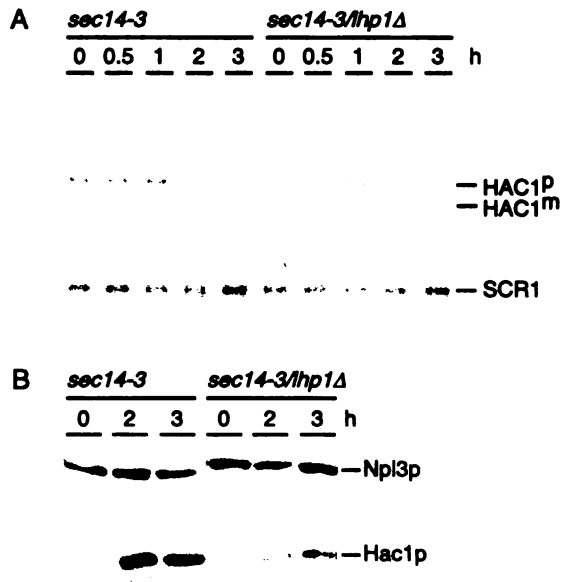


Figure 6 A) HAC1 RNA levels and rate of splicing are unaffected in an lhp1Δ::LEU strain upon induction of the UPR by shifting to 37°C in a sec14-3ts mutant strain background. HAC1 precursor (HAC1p) and HAC1 mature (HAC1m) were detected, and SCR1 served as a loading control. B) Hac1p levels are consistently reduced by at least two-fold. Npl3p served as a loading control. Similar results were seen with an lhp1Δ::KAN strain and with 1μg/ml tunicamycin induction of the UPR (data not shown).

**Table 1 - Oligos used in this study for QPCR**

| Oligos | | |
|---|---|---|
| **Gene** | **Forward** | **Reverse** |
| CUP1 | GTTCAGCGAATTAATTAACTTCC | CATTTCCCAGAGCAGCATGAC |
| HAC1 | CGTCAACCTTGAAAAACTGGCTGACC | GGCTCCATTGTACAGTTCAGAGGTGAA |
| NOG2 | CAGCTTGGGTCAAACATTTGTC | AAGAGTTGGTAATAGATGCGTG |
| PGK1 | CAAAGGCTAAGACCATTGTC | CACCAGTAGAGACATGGGAG |
| RPC11 | TGATGTTCTTGGTGGTGGTTGGG | TCGGCTCATCAGCTGACCTAATTTGTA |
| RPL11A | CTATGTCGTCATGAACAGACC | CTTTTGCTTGAACCAAGAGACG |
| RPL31 | CCGATGTTTTTGGCGTGATGCT | GCATCTTCTTCAACGACAACGGTTTGT |
| RPS22A | GAAAAATGGACTGCCAACTTGTTG | CTTTCTTCTGGCTTCTTCATGG |
| RPS4B | GAAGCATCTAAAGAGATTAGCAGC | GCAATTTGTGTGGACCAGCAG |
| SCR1 | GAGGCGGGAGAGTCCGTTCTGAAGTG | AAACTCCCCTAACAGCGGTGAAGG |
| SEC14 | CAACAAGAAAAGGAATTTTTAGAATCC | TGCCTTCTCTTGAGCGCTGTC |
| SNR189 | AGGGGATTTGTGACTTTCAAGGCAA | TGTCAGGCCCGTGTGAAGTACTTGA |
| U2 | CTCGGTTTGAGGGGGTGTAG | TATTCTCACAAACACATCTCCTC |
| U3 | TAGGATCATTTCTATAGGAATCG | ATAGATGGCCGAACCGCTAAG |
| U4 | ATCCTTATGCACGGGAAATACG | AAAGGTATTCCAAAAATTCCCTAC |
| U5 | CAAGCAGCTTTACAGATCAATGG | AGTTCCAAAAAATATGGCAAGCC |
| U6 | GTTCGCGAAGTAACCCTTCG | AAAACGAAATAAATCTCTTTGTAAAAC |
| UBC5 | GTTCAGCAGGACCTGTAGG | GTGAATAGACAAAAAGAAAACGCC |

**Table 2 - Lhp1p IP microarray data**

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**Table 3 - Previously known Lhp1p-associated noncoding RNAs**

| Rank | Noncoding | Feature | Exp.1 E | Exp. 2 E | average | Feat | Feature S | Feature End |
|---|---|---|---|---|---|---|---|---|
| 1 | U6 | SNR6 | 4.136 | 4.724 | 4.430 | 12 | 366236 | 366347 |
| 5 | SCR1 | iSCR1 | 3.070 | 3.542 | 3.306 | 5 | 442412 | 442731 |
| 8 | RPR1 | irpr1 | 2.915 | 3.239 | 3.077 | 5 | 118035 | 118230 |
| 43 | tS(CGA)C | tS(CGA)C | 1.891 | 1.731 | 1.811 | 3 | 226666 | 226766 |
| 615 | 5S rRNA | RDN5-1 | 0.000 | 0.816 | 0.408 | 12 | 459677 | 459797 |
| 527 | 5S rRNA | iYLR159W | -0.152 | 1.104 | 0.476 | 12 | 485690 | 486202 |
| 407 | 5S rRNA | iYLRCdelta8 | 0.214 | 0.956 | 0.585 | 12 | 481898 | 482550 |
| 56 | U1 | iYNL222W | 1.269 | 1.856 | 1.563 | 14 | 229713 | 231067 |
| 48 | U2 | LSR1 | 1.373 | 2.057 | 1.715 | 2 | 680643 | 681817 |
| 17 | U4 | snR14 | 2.519 | 2.602 | 2.560 | 5 | 167426 | 167585 |
| 39 | U4 | iYER007C-A | 1.840 | | 1.840 | 5 | 166884 | 167426 |
| 34 | U5 | SNR7-L | 1.433 | 2.602 | 2.017 | 7 | 939451 | 939665 |
| 13 | U3A | iYOR235W | 2.339 | 3.000 | 2.670 | 15 | 780182 | 780904 |
| 28 | U3B | iYPL144W | 2.546 | 1.860 | 2.203 | 16 | 280925 | 282121 |

**Table 4 - Lhp1p IP microarray tRNAs**

| Rank | Feature | Exp. 1 Er | Exp. 2 Er | average | Fea | Feature S | Feature End |
|---|---|---|---|---|---|---|---|
| 2 | tS(GCU)L | 4.633 | 4.181 | 4.407 | 12 | 784353 | 784452 |
| 10 | iYBR044C | 3.318 | 2.339 | 2.828 | 2 | 326016 | 326749 |
| 12 | tW(CCA)P | 3.060 | 2.365 | 2.712 | 16 | 56169 | 56274 |
| 15 | tP(AGG)C | 2.438 | 2.824 | 2.631 | 3 | 123178 | 123249 |
| 18 | tV(AAC)M3 | 3.216 | 1.880 | 2.548 | 13 | 586635 | 586708 |
| 20 | tK(UUU)P | 2.430 | 2.646 | 2.538 | 16 | 769204 | 769299 |
| 21 | tS(UGA)P | 2.154 | 2.856 | 2.505 | 16 | 689560 | 689641 |
| 22 | tP(UGG)M | 2.807 | 2.114 | 2.461 | 13 | 196068 | 196170 |
| 23 | iYOR072W-1 | 2.348 | | 2.348 | 15 | 463133 | 464450 |
| 25 | tT(UGU)P | 2.664 | 1.872 | 2.268 | 16 | 744281 | 744352 |
| 29 | tR(ACG)O | 2.208 | 2.087 | 2.148 | 15 | 340299 | 340371 |
| 31 | tQ(UUG)L | 1.580 | 2.483 | 2.031 | 12 | 448651 | 448722 |
| 35 | tL(GAG)G | 2.406 | 1.556 | 1.981 | 7 | 700669 | 700750 |
| 36 | tR(UCU)M2 | 1.795 | 2.108 | 1.951 | 13 | 131825 | 131896 |
| 37 | tY(GUA)M2 | 2.114 | 1.664 | 1.889 | 13 | 837928 | 838016 |
| 40 | tI(UAU)L | 1.824 | 1.816 | 1.820 | 12 | 605300 | 605432 |
| 43 | tS(CGA)C | 1.891 | 1.731 | 1.811 | 3 | 226666 | 226766 |
| 45 | iYMRWdelta1 | 3.362 | 0.251 | 1.806 | 13 | 379197 | 379302 |
| 46 | itH(GUG)G2 | 2.420 | 1.176 | 1.798 | 7 | 319850 | 320353 |
| 54 | tF(GAA)P2 | 2.202 | 1.014 | 1.608 | 16 | 622535 | 622626 |
| 58 | tV(UAC)B | 2.101 | 1.014 | 1.558 | 2 | 326749 | 326822 |
| 59 | iYLRCdelta19 | 1.541 | | 1.541 | 12 | 734671 | 734802 |
| 64 | itE(UUC)E1 | 1.956 | 0.971 | 1.463 | 5 | 177169 | 177834 |
| 67 | tL(CAA)C | 1.257 | 1.604 | 1.431 | 3 | 90474 | 90587 |
| 79 | tL(CAA)N | 0.986 | 1.736 | 1.361 | 14 | 443005 | 443118 |
| 81 | tL(UAG)L2 | 1.239 | 1.465 | 1.352 | 12 | 732090 | 732190 |
| 88 | tN(GUU)C | 0.816 | 1.799 | 1.307 | 3 | 127317 | 127390 |
| 95 | tR(CCG)L | 0.678 | 1.888 | 1.283 | 12 | 818608 | 818679 |
| 103 | tM(CAU)O2 | 1.251 | 1.227 | 1.239 | 15 | 976412 | 976484 |
| 108 | tE(UUC)C | | 1.214 | 1.214 | 3 | 82424 | 82495 |
| 141 | tM(CAU)P | 0.782 | 1.339 | 1.061 | 16 | 338847 | 338918 |
| 152 | tV(CAC)D | 1.705 | 0.322 | 1.013 | 4 | 1075508 | 1075580 |
| 159 | tH(GUG)M | 1.220 | 0.766 | 0.993 | 13 | 363063 | 363134 |
| 190 | YNL017C | | 0.911 | 0.911 | 14 | 602134 | 602472 |
| 218 | tE(UUC)P | 0.275 | 1.444 | 0.859 | 16 | 210191 | 210262 |
| 245 | YDR278C | 0.367 | 1.257 | 0.812 | 4 | 1016992 | 1017309 |
| 262 | tL(UAA)N | -0.304 | 1.844 | 0.770 | 14 | 726132 | 726215 |
| 319 | tC(GCA)P2 | 0.740 | 0.623 | 0.681 | 16 | 775762 | 775833 |
| 329 | YGR164W | 0.227 | 1.104 | 0.665 | 7 | 828619 | 828954 |
| 340 | iYDR419W | 1.070 | 0.239 | 0.655 | 4 | 1305106 | 1305664 |
| 426 | tI(AAU)P2 | 0.057 | 1.091 | 0.574 | 16 | 880291 | 880364 |
| 429 | itM(CAU)C | 0.263 | 0.880 | 0.571 | 3 | 149697 | 149926 |
| 639 | tN(GUU)P | 0.433 | 0.356 | 0.395 | 16 | 810671 | 810744 |
| 972 | tK(CUU)P | -0.515 | 0.895 | 0.190 | 16 | 582057 | 582129 |
| 1563 | tT(CGU)K | -0.644 | 0.575 | -0.034 | 11 | 46736 | 46807 |
| 1689 | tG(GCC)P2 | -0.916 | 0.766 | -0.075 | 16 | 860374 | 860444 |
| 2428 | tT(AGU)O2 | | -0.304 | -0.304 | 15 | 354040 | 354112 |
| 2459 | tG(CCC)D | 0.322 | -0.943 | -0.311 | 4 | 1257042 | 1257113 |
| 2492 | tD(GUC)O | -1.358 | 0.722 | -0.318 | 15 | 571956 | 572027 |
| 2707 | tA(UGC)O | -1.120 | 0.356 | -0.382 | 15 | 854182 | 854254 |
| 2932 | tG(UCC)O | -0.415 | -0.474 | -0.444 | 15 | 110961 | 111032 |
| 3366 | tS(AGA)M | -1.184 | 0.070 | -0.557 | 13 | 259158 | 259239 |
| 5219 | tA(AGC)P | -1.089 | -0.916 | -1.003 | 16 | 856897 | 856969 |

**Table 5 - Lhp1p IP microarray snoRNAs**

| Rank | snoRNA | Feature | Exp. 1 | Exp. 2 | averag | Feat | Feature | Feature End |
|---|---|---|---|---|---|---|---|---|
| 3 | SNR3 | snR3 | 4.681 | 3.432 | 4.057 | 10 | 663437 | 663630 |
| 4 | SNR32 | iYHR140W | 3.487 | 3.237 | 3.362 | 8 | 381291 | 381990 |
| 7 | SNR43 | YCL006C | 2.570 | 3.866 | 3.218 | 3 | 107245 | 107574 |
| 9 | SNR37 | iYJL104W | 2.755 | 3.014 | 2.885 | 10 | 227473 | 228122 |
| 11 | SNR11 | iYMR194W | 2.444 | 3.162 | 2.803 | 13 | 651909 | 652911 |
| 13 | SNR17a | iYOR235W | 2.339 | 3.000 | 2.670 | 15 | 780182 | 780904 |
| 14 | SNR46 | SNR46 | 2.409 | 2.880 | 2.644 | 7 | 545363 | 545559 |
| 24 | SNR5 | iYOR277C | 2.325 | 2.331 | 2.328 | 15 | 841818 | 842812 |
| 24 | SNR31 | iYOR277C | 2.325 | 2.331 | 2.328 | 15 | 841818 | 842812 |
| 26 | SNR34 | SNR34 | 2.004 | 2.480 | 2.242 | 12 | 898986 | 899535 |
| 28 | SNR17b | iYPL144W | 2.546 | 1.860 | 2.203 | 16 | 280925 | 282121 |
| 30 | SNR190 | SNR190 | 1.316 | 2.842 | 2.079 | 10 | 139459 | 139648 |
| 33 | SNR49 | iYNR050C | 2.449 | 1.595 | 2.022 | 14 | 715387 | 716779 |
| 38 | SNR70 | SNR70 | 1.098 | 2.621 | 1.859 | 16 | 718884 | 719048 |
| 47 | SNR13 | SNR13 | 1.316 | 2.227 | 1.771 | 4 | 1402953 | 1403076 |
| 61 | SNR42 | SNR42 | 1.322 | 1.646 | 1.484 | 11 | 558655 | 559005 |
| 68 | SNR4 | SNR4 | 1.163 | 1.664 | 1.414 | 5 | 424695 | 424880 |
| 77 | SNR10 | YGL088W | 0.731 | 2.018 | 1.375 | 7 | 345834 | 346199 |
| 96 | SNR128 | snR128 | 1.138 | 1.428 | 1.283 | 10 | 139263 | 139390 |
| 98 | SNR9 | iYOR040W | 0.888 | 1.655 | 1.271 | 15 | 407920 | 408357 |
| 128 | SNR8 | SNR8 | 0.832 | 1.345 | 1.088 | 15 | 832327 | 832516 |
| 137 | SNR30 | iYLR027C | 0.642 | 1.496 | 1.069 | 12 | 198128 | 199541 |
| 153 | SNR69 | SNR69 | 0.496 | 1.531 | 1.013 | 11 | 364418 | 364518 |
| 202 | SNR54 | YML056C | 1.566 | 0.214 | 0.890 | 13 | 162194 | 164176 |
| 244 | SNR189 | SNR189 | 0.895 | 0.731 | 0.813 | 3 | 177338 | 177526 |
| 263 | SNR18 | YAL003W | 1.007 | 0.526 | 0.767 | 1 | 142172 | 143158 |
| 270 | SNR24 | YMR116C | 0.748 | 0.757 | 0.753 | 13 | 499455 | 500687 |
| 363 | SNR59 | YPL198W | 1.064 | 0.189 | 0.626 | 16 | 173151 | 174701 |
| 400 | SNR33 | SNR33 | 0.880 | 0.310 | 0.595 | 3 | 141963 | 142145 |
| 605 | SNR44 | SNR44 | 0.299 | 0.526 | 0.412 | 12 | 856708 | 856918 |
| 608 | SNR61 | SNR61 | | 0.411 | 0.411 | 12 | 794485 | 794574 |
| 636 | SNR41 | iSNR70 | 0.411 | 0.379 | 0.395 | 16 | 719048 | 719555 |
| 699 | SNR35 | iYOR222W | 0.556 | 0.163 | 0.360 | 15 | 759251 | 759780 |
| 786 | SNR39b | iYGL076C | 0.070 | 0.546 | 0.308 | 7 | 365994 | 366923 |
| 792 | SNR45 | SNR45 | 0.422 | 0.189 | 0.306 | 16 | 821727 | 821898 |
| 912 | SNR52 | SNR52 | 0.070 | 0.390 | 0.230 | 5 | 431126 | 431217 |
| 984 | SNR57 | SNR57 | -0.152 | 0.526 | 0.187 | 12 | 794936 | 795023 |
| 991 | SNR55 | SNR55 | -0.415 | 0.782 | 0.184 | 12 | 794696 | 794793 |
| 1504 | SNR38 | YKL081W | 0.014 | -0.044 | -0.015 | 11 | 282533 | 284097 |
| 1665 | SNR63 | SNR63 | -0.322 | 0.189 | -0.066 | 4 | 323216 | 323470 |
| 2150 | SNR53 | SNR53 | | -0.218 | -0.218 | 5 | 61699 | 61789 |
| 2151 | SNR64 | SNR64 | | -0.218 | -0.218 | 11 | 38812 | 38912 |
| 2432 | SNR62 | SNR62 | | -0.304 | -0.304 | 15 | 409764 | 409863 |
| 2519 | SNR39 | YGL076C | -0.252 | -0.396 | -0.324 | 7 | 364333 | 365994 |
| 2530 | SNR76 | iYMR013C-( | -0.474 | -0.184 | -0.329 | 13 | 296737 | 297802 |
| 2530 | SNR77 | iYMR013C-( | -0.474 | -0.184 | -0.329 | 13 | 296737 | 297802 |
| 2530 | SNR78 | iYMR013C-( | -0.474 | -0.184 | -0.329 | 13 | 296737 | 297802 |
| 2701 | SNR72 | iYMR013C-' | -0.286 | -0.474 | -0.380 | 13 | 297802 | 298867 |
| 2701 | SNR73 | iYMR013C-' | -0.286 | -0.474 | -0.380 | 13 | 297802 | 298867 |
| 2701 | SNR74 | iYMR013C-' | -0.286 | -0.474 | -0.380 | 13 | 297802 | 298867 |
| 2701 | SNR75 | iYMR013C-' | -0.286 | -0.474 | -0.380 | 13 | 297802 | 298867 |
| 3334 | SNR66 | SNR66 | -0.971 | -0.120 | -0.546 | 14 | 586088 | 586173 |
| 3647 | SNR48 | SNR48 | -0.358 | -0.889 | -0.624 | 7 | 609578 | 609690 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 4506 | SNR79 | iYLR105C | | -0.837 | -0.837 | 12 | 348181 | 349007 |
| 5037 | SNR47 | SNR47 | -1.252 | -0.667 | -0.959 | 4 | 541641 | 541701 |
| 5088 | SNR56 | SNR56 | -0.971 | | -0.971 | 2 | 88181 | 88268 |
| 5089 | SNR67 | SNR67 | -0.971 | | -0.971 | 5 | 61352 | 61433 |
| 5953 | SNR40 | iYNL289w-0 | | -1.184 | -1.184 | 14 | 88734 | 89517 |
| 5999 | SNR60 | SNR60 | -1.286 | -1.089 | -1.188 | 10 | 348826 | 348929 |
| 6305 | SNR36 | iYOR184W | -1.515 | -1.029 | -1.272 | 15 | 680542 | 681442 |
| 6504 | SNR51 | SNR51 | -1.322 | | -1.322 | 16 | 718697 | 718803 |
| 7840 | SNR50 | SNR50 | -1.358 | -1.943 | -1.651 | 15 | 259489 | 259578 |
| 8275 | SNR71 | SNR71 | -1.556 | -2.000 | -1.778 | 8 | 411228 | 411317 |
| 10161 | SNR65 | SNR65 | -2.396 | -2.120 | -2.258 | 3 | 175907 | 176006 |
| N/A | SNR58 | SNR58 | N/A | N/A | N/A | N/A | N/A | N/A |
| N/A | SNR68 | SNR68 | N/A | N/A | N/A | N/A | N/A | N/A |

**Table 6 - Lhp1p-enriched intergenic regions (approximately 50 in top 400)**

| Rank | Feature | Exp. 1 | Exp. 2 | Average | Feat | Feature | Feature End |
|---|---|---|---|---|---|---|---|
| 32 | iYNL006W | 1.731 | 2.325 | 2.028 | 14 | 620977 | 621312 |
| 41 | iYMR246W | 1.824 | 1.816 | 1.820 | 13 | 761890 | 763350 |
| 42 | iYEL018W | 1.811 | | 1.811 | 5 | 122310 | 122798 |
| 44 | iYEL055C | 2.094 | 1.521 | 1.808 | 5 | 51539 | 52721 |
| 62 | iYDR342C-0 | 1.491 | 1.470 | 1.480 | 4 | 1155963 | 1157189 |
| 70 | iYER030W | | 1.411 | 1.411 | 5 | 213897 | 214075 |
| 73 | iYEL035C | 1.401 | | 1.401 | 5 | 85545 | 85676 |
| 78 | iYGL009C | 0.993 | 1.753 | 1.373 | 7 | 478648 | 479906 |
| 80 | iYDR524C | 1.536 | 1.183 | 1.359 | 4 | 1489038 | 1489953 |
| 89 | iYJR114W | 1.305 | | 1.305 | 10 | 638740 | 639631 |
| 99 | iYERWdelta22 | 1.176 | 1.362 | 1.269 | 5 | 488161 | 488853 |
| 102 | iYOR181W | 1.239 | | 1.239 | 15 | 677838 | 678189 |
| 112 | iYNL190W | 1.362 | 1.007 | 1.184 | 14 | 283005 | 284256 |
| 114 | iYDR439W-0 | 0.356 | 1.993 | 1.174 | 4 | 1340753 | 1341640 |
| 116 | iYERComega1- | 0.705 | 1.637 | 1.171 | 5 | 564020 | 565123 |
| 118 | iYER015W | 1.516 | 0.799 | 1.158 | 5 | 186774 | 188276 |
| 143 | iYGL224C | 1.816 | 0.287 | 1.051 | 7 | 78855 | 79110 |
| 144 | iYDR382W | | 1.050 | 1.050 | 4 | 1239858 | 1239994 |
| 156 | iYEL074W | 0.310 | 1.683 | 0.996 | 5 | 6464 | 7230 |
| 161 | iYBR161W | 0.934 | 1.043 | 0.988 | 2 | 562718 | 563157 |
| 172 | iYPL146C | 1.941 | -0.015 | 0.963 | 16 | 277528 | 278394 |
| 178 | iYEL072W-1 | 2.087 | -0.218 | 0.935 | 5 | 15385 | 16355 |
| 183 | iYER019C-A | 0.926 | | 0.926 | 5 | 194538 | 195167 |
| 184 | iYLR166C | 0.444 | 1.406 | 0.925 | 12 | 498046 | 498949 |
| 187 | iYFL031W | 0.941 | 0.895 | 0.918 | 6 | 75780 | 76829 |
| 189 | iYEL060C | 0.333 | 1.496 | 0.915 | 5 | 41953 | 42400 |
| 200 | iYJR075W | | 0.895 | 0.895 | 10 | 574859 | 575042 |
| 208 | iYMR122C-0 | 0.748 | 1.007 | 0.878 | 13 | 511074 | 512333 |
| 214 | iYJRWdelta17 | 0.465 | 1.269 | 0.867 | 10 | 538122 | 538243 |
| 220 | iYER012W | | 0.856 | 0.856 | 5 | 178430 | 178840 |
| 225 | iYAR033W | 0.903 | 0.791 | 0.847 | 1 | 188806 | 189421 |
| 229 | iYGL007W-1 | 0.422 | 1.251 | 0.837 | 7 | 484774 | 485916 |
| 236 | iYJR094W-A | 1.036 | 0.614 | 0.825 | 10 | 608549 | 609464 |
| 252 | iYDR231C | | 0.799 | 0.799 | 4 | 926946 | 927488 |
| 257 | iYEL009C | 0.782 | | 0.782 | 5 | 139763 | 140512 |
| 267 | iYEL073C-2 | 0.757 | | 0.757 | 5 | 10019 | 11252 |
| 266 | iYPL063W | 0.757 | | 0.757 | 16 | 431364 | 431890 |
| 277 | iYLR035C-A-0 | 1.131 | 0.356 | 0.744 | 12 | 218908 | 219788 |
| 302 | iYGR287C | 0.705 | 0.696 | 0.700 | 7 | 1068987 | 1070289 |
| 305 | iYPL087W | 0.604 | 0.791 | 0.697 | 16 | 384403 | 384768 |
| 312 | iYDR345C-0 | 1.021 | 0.356 | 0.689 | 4 | 1164695 | 1165972 |
| 316 | iYLL048C | | 0.687 | 0.687 | 12 | 46264 | 46671 |
| 322 | iYDR033W | 1.091 | 0.263 | 0.677 | 4 | 509148 | 509776 |
| 328 | iYJR105W | 0.299 | 1.036 | 0.667 | 10 | 624290 | 624525 |
| 357 | iYLR035C-A-1 | 0.714 | 0.556 | 0.635 | 12 | 219788 | 220669 |
| 371 | iYJR109C | 1.422 | -0.184 | 0.619 | 10 | 632630 | 633304 |
| 372 | iYER161C | 0.660 | 0.575 | 0.618 | 5 | 500344 | 500626 |
| 379 | iYGR124W | | 0.614 | 0.614 | 7 | 741656 | 742319 |
| 378 | iYPR079W | 0.614 | | 0.614 | 16 | 700010 | 700590 |
| 382 | iYIL174W-0 | 0.189 | 1.029 | 0.609 | 9 | 9696 | 10594 |
| 392 | iYCR024C | 0.740 | 0.465 | 0.602 | 3 | 161930 | 162653 |
| 393 | iYPL183W-A | 0.506 | 0.696 | 0.601 | 16 | 199375 | 199494 |
| 396 | iYOR207C | 0.239 | 0.956 | 0.597 | 15 | 733455 | 733923 |

**Table 7 - Lhp1p-enriched coding mRNAs (approximately 300 in the top 500)**

| Rank | Feature | Gene | SGD De: | Exp. | Exp. | avera | Fea | Feature | Feature End |
|---|---|---|---|---|---|---|---|---|---|
| 27 | YDR538W | PAD1 | | | 2.21 | 2.211 | 4 | 1510903 | 1511631 |
| 49 | YNL297C | MON2 | unknown | 1.7 | | 1.696 | 14 | 71671 | 76581 |
| 50 | YOR352W | unknown | | | 1.68 | 1.683 | 15 | 997204 | 998235 |
| 52 | YOL039W | RPP2A | ribosome | 1.3 | 1.99 | 1.642 | 15 | 254295 | 254615 |
| 53 | YLR167W | RPS31 | ribosome | 2 | 1.23 | 1.618 | 12 | 498949 | 499407 |
| 55 | YBR189W | RPS9B | ribosome | 2.18 | 1.04 | 1.606 | 2 | 604467 | 605467 |
| 57 | YNR053C | NOG2 | unknown | 2.06 | 1.06 | 1.56 | 14 | 721117 | 723108 |
| 60 | YLR110C | CCW12 | unknown | 1.2 | 1.8 | 1.502 | 12 | 369698 | 370099 |
| 63 | YDR406W | PDR15 | | | 1.46 | 1.465 | 4 | 1279199 | 1283788 |
| 66 | YIL018W | RPL2B | ribosome | 1.6 | 1.26 | 1.431 | 9 | 316766 | 317930 |
| 69 | YOR293W | RPS10A | ribosome | 1.87 | 0.96 | 1.412 | 15 | 867093 | 867847 |
| 72 | YER131W | RPS26B | ribosome | 1.34 | 1.46 | 1.402 | 5 | 423949 | 424308 |
| 74 | YOR063W | RPL3 | ribosome | 1.62 | 1.17 | 1.396 | 15 | 444687 | 445850 |
| 76 | YPL048W | CAM1 | translatic | 1.38 | | 1.384 | 16 | 464396 | 465643 |
| 77 | YGL088W | unknow | unknown | 0.73 | 2.02 | 1.375 | 7 | 345834 | 346199 |
| 82 | YMR303C | ADH2 | alcohol d | 1.3 | 1.37 | 1.336 | 13 | 873291 | 874337 |
| 83 | YBR300C | unknow | unknown | 1.83 | 0.83 | 1.332 | 2 | 808556 | 809053 |
| 84 | YLR170C | APS1 | AP-1 con | 0.64 | 2.01 | 1.324 | 12 | 500581 | 501051 |
| 86 | YGR118W | RPS23A | ribosome | 1.7 | 0.93 | 1.317 | 7 | 726967 | 727724 |
| 90 | YML063W | RPS1B | ribosome | 1.58 | 1.03 | 1.304 | 13 | 146482 | 147249 |
| 91 | YEL054C | RPL12A | ribosome | 1.33 | 1.28 | 1.304 | 5 | 52721 | 53218 |
| 92 | YBR191W | RPL21A | ribosome | 1.81 | 0.8 | 1.303 | 2 | 606229 | 607099 |
| 93 | YDR134C | unknow | unknown | 1.17 | 1.42 | 1.296 | 4 | 721064 | 721474 |
| 94 | YJL052W | TDH1 | glycerald | 1.23 | 1.33 | 1.283 | 10 | 337967 | 338965 |
| 97 | YML123C | PHO84 | inorganic | 1.57 | 0.99 | 1.279 | 13 | 24038 | 25801 |
| 100 | YDR025W | RPS11A | ribosome | 1.36 | 1.17 | 1.266 | 4 | 491510 | 492319 |
| 101 | YOL040C | RPS15 | ribosome | 1.16 | 1.35 | 1.254 | 15 | 253147 | 253575 |
| 104 | YFL031W | HAC1 | transcrip | 1.07 | 1.41 | 1.238 | 6 | 75178 | 75780 |
| 105 | YDR216W | ADR1 | ADH2 an | 1.39 | 1.08 | 1.237 | 4 | 895026 | 898997 |
| 106 | YMR075W | RCO1 | (YMR075 | 1.23 | | 1.227 | 13 | 413981 | 416035 |
| 107 | YCL043C | PDI1 | protein d | 1.1 | 1.34 | 1.221 | 3 | 48630 | 50198 |
| 111 | YKR075C | unknown | | 0 | 2.38 | 1.188 | 11 | 579463 | 580386 |
| 113 | YOR133W | EFT1 | translatic | 1.17 | 1.18 | 1.176 | 15 | 575096 | 577624 |
| 115 | YHR216W | PUR5 | IMP deh | 1.74 | 0.6 | 1.172 | 8 | 554391 | 555962 |
| 117 | YMR121C | RPL15B | ribosome | 1.33 | 0.99 | 1.16 | 13 | 509733 | 510347 |
| 119 | YOR153W | PDR5 | transport | 1.72 | 0.58 | 1.147 | 15 | 619838 | 624373 |
| 120 | YDR382W | RPP2B | ribosome | 1.11 | 1.15 | 1.131 | 4 | 1239481 | 1239813 |
| 122 | YBL072C | RPS8A | ribosome | 1.23 | 1.01 | 1.12 | 2 | 88518 | 89120 |
| 123 | YLR448W | RPL6B | ribosome | 0.94 | 1.3 | 1.12 | 12 | 1028847 | 1029761 |
| 124 | YPL090C | RPS6A | ribosome | 1.54 | 0.7 | 1.119 | 16 | 377286 | 378390 |
| 125 | YBR078W | ECM33 | unknown | 1.08 | 1.11 | 1.098 | 2 | 393083 | 394819 |
| 126 | YBR054W | YRO2 | putative | 1.09 | | 1.091 | 2 | 343061 | 344095 |
| 127 | YAR002C | ERP1 | unknown | 1.44 | 0.74 | 1.089 | 1 | 154062 | 154721 |
| 129 | YCR021C | HSP30 | plasma n | 1.08 | 1.08 | 1.084 | 3 | 155817 | 156815 |
| 130 | YPL248C | GAL4 | transcrip | 1.08 | | 1.084 | 16 | 79711 | 82356 |
| 131 | YPL131W | RPL5 | ribosome | 0.93 | 1.24 | 1.082 | 16 | 303120 | 304013 |
| 133 | YDR385W | EFT2 | translatic | 0.72 | 1.43 | 1.075 | 4 | 1243219 | 1245747 |
| 134 | YCL040W | GLK1 | glucokina | 1.24 | 0.91 | 1.075 | 3 | 50812 | 52314 |
| 135 | YIR033W | MGA2 | chromati | 1.44 | 0.7 | 1.074 | 9 | 416121 | 419462 |
| 136 | YDL130W | RPP1B | ribosome | 1.45 | 0.7 | 1.072 | 4 | 229906 | 230527 |
| 138 | YLR249W | YEF3 | translatic | 1.26 | 0.87 | 1.067 | 12 | 636780 | 639914 |
| 140 | YGL031C | RPL24A | ribosome | 1.17 | 0.96 | 1.063 | 7 | 437463 | 437930 |
| 142 | YPL106C | SSE1 | HSP70 fa | 1.58 | 0.53 | 1.053 | 16 | 350189 | 352270 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 146 | YIL052C | RPL34B | ribosoma | 1.5 | 0.59 | 1.048 | 9 | 256224 | 257061 |
| 147 | YDR154C | unknow | unknown | 1.03 | 1.05 | 1.039 | 4 | 768396 | 768746 |
| 148 | YLR333C | RPS25B | ribosoma | 0.46 | 1.61 | 1.039 | 12 | 795572 | 795898 |
| 149 | YIL148W | RPL40A | ribosoma | 1.41 | 0.66 | 1.036 | 9 | 68708 | 69528 |
| 150 | YNL209W | SSB2 | cytosolic | 0.93 | 1.12 | 1.025 | 14 | 252058 | 253899 |
| 151 | YNL190W | unknow | unknown | 1.14 | 0.91 | 1.024 | 14 | 282391 | 283005 |
| 154 | YHR085W | IPI1 | unknown | 0.89 | 1.14 | 1.013 | 8 | 276765 | 277769 |
| 155 | YLR325C | RPL38 | ribosoma | 1.2 | 0.81 | 1.004 | 12 | 781142 | 781378 |
| 157 | YGR282C | BGL2 | endo-bet | 1.06 | 0.93 | 0.995 | 7 | 1057779 | 1058720 |
| 158 | YJL138C | TIF2 | translatic | 1.37 | 0.61 | 0.993 | 10 | 153202 | 154389 |
| 160 | YPL155C | KIP2 | kinesin-r | 0.96 | 1.03 | 0.992 | 16 | 257215 | 259335 |
| 162 | YBR118W | TEF2 | translatic | 0.76 | 1.21 | 0.986 | 2 | 477630 | 479006 |
| 163 | YPR028W | YIP2 | unknown | 0.99 | | 0.986 | 16 | 623522 | 624197 |
| 164 | YPR102C | RPL11A | ribosoma | 1.11 | 0.86 | 0.984 | 16 | 731221 | 731745 |
| 165 | YGR192C | TDH3 | glycerald | 1.12 | 0.85 | 0.983 | 7 | 882806 | 883804 |
| 166 | YIL151C | unknonw | | | 0.98 | 0.978 | 9 | 57338 | 60694 |
| 168 | YCL020W | transpo | unknown | 1.58 | 0.37 | 0.976 | 3 | 84714 | 86030 |
| 169 | YHR080C | unknow | unknown | 0.92 | 1.02 | 0.97 | 8 | 262802 | 266839 |
| 170 | YKL056C | unknow | unknown | 1.07 | 0.86 | 0.967 | 11 | 334054 | 334557 |
| 171 | YLR134W | PDC5 | pyruvate | 1.03 | 0.9 | 0.966 | 12 | 410724 | 412415 |
| 173 | YFR046C | unknow | unknown | 0.58 | 1.33 | 0.954 | 6 | 243061 | 244146 |
| 174 | YIL053W | RHR2 | DL-glyce | 0.89 | 1.01 | 0.951 | 9 | 255050 | 255865 |
| 175 | YER165W | PAB1 | cleavage | 1.32 | 0.58 | 0.949 | 5 | 510369 | 512102 |
| 176 | YPR156C | TPO3 | major fa | 1.07 | 0.82 | 0.943 | 16 | 837904 | 839772 |
| 177 | YPL061W | ALD6 | acetaldel | 1.23 | 0.65 | 0.942 | 16 | 432583 | 434085 |
| 179 | YGR293C | unknow | unknown | 1.38 | 0.49 | 0.935 | 7 | 1079881 | 1080342 |
| 180 | YKL097W | CWP2 | | 0.96 | 0.9 | 0.933 | | | |
| 181 | YGL008C | PMA1 | plasma n | 0.52 | 1.35 | 0.933 | 7 | 479906 | 482662 |
| 182 | YNL301C | RPL18B | ribosoma | 1.29 | 0.58 | 0.931 | 14 | 63569 | 64561 |
| 185 | YHR010W | RPL27A | ribosoma | 1.1 | 0.74 | 0.922 | 8 | 126513 | 127484 |
| 186 | YOR232W | MGE1 | could cha | 1.29 | 0.56 | 0.921 | 15 | 774571 | 775257 |
| 188 | YDL119C | unknow | unknown | 0.96 | 0.87 | 0.918 | 4 | 246688 | 247611 |
| 191 | YJR009C | TDH2 | glycerald | 0.97 | 0.85 | 0.909 | 10 | 453370 | 454368 |
| 192 | YAL005C | SSA1 | cytosolic | 0.99 | 0.82 | 0.908 | 1 | 139501 | 141429 |
| 193 | YGR085C | RPL11B | ribosoma | 1.01 | 0.81 | 0.907 | 7 | 648377 | 648901 |
| 194 | YJL172W | CPS1 | vacuolar | 0.48 | 1.34 | 0.907 | 10 | 97730 | 99460 |
| 195 | YER187W | unknow | KHS1 overlap | | 0.9 | 0.903 | 5 | 566226 | 566651 |
| 196 | YMR143W | RPS16A | ribosoma | 1.02 | 0.78 | 0.902 | 13 | 552282 | 552902 |
| 197 | YNL172W | APC1 | anaphase | 0.82 | 0.98 | 0.901 | 14 | 310633 | 315879 |
| 198 | YAL004W | SSA1 | unknown | 0.92 | 0.88 | 0.899 | 1 | 140758 | 141405 |
| 199 | YOL098C | unknow | unknown | 0.79 | 1 | 0.895 | 15 | 132724 | 135837 |
| 201 | YGL055W | OLE1 | delta-9-f | 0.82 | 0.96 | 0.894 | 7 | 398625 | 400157 |
| 202 | YML056C | SNR54 | unknown | 1.57 | 0.21 | 0.89 | 13 | 162194 | 164176 |
| 203 | YJL012C | VTC4 | unknown | 0.63 | 1.14 | 0.888 | 10 | 411141 | 413087 |
| 204 | YMR120C | ADE17 | 5-aminoi | 0.9 | 0.88 | 0.888 | 13 | 507501 | 509279 |
| 205 | YGR027C | RPS25A | ribosoma | 0.87 | 0.9 | 0.887 | 7 | 534126 | 534452 |
| 206 | YGR148C | RPL24B | ribosoma | 1.49 | 0.28 | 0.883 | 7 | 787306 | 787773 |
| 207 | YPR097W | unknow | unknown | 1.82 | -0.1 | 0.878 | 16 | 725391 | 728612 |
| 209 | YFL039C | ACT1 | actin : cy | 0.81 | 0.94 | 0.874 | 6 | 53261 | 54696 |
| 210 | YDR368W | YPR1 | unknown | 0.87 | | 0.872 | 4 | 1213893 | 1214831 |
| 211 | YGL189C | RPS26A | ribosoma | 1.03 | 0.71 | 0.871 | 7 | 148233 | 148592 |
| 212 | YGL123W | RPS2 | ribosoma | 1.09 | 0.65 | 0.871 | 7 | 277618 | 278382 |
| 213 | YPL250C | ICY2 | unknown | 1.45 | 0.29 | 0.868 | 16 | 74309 | 74719 |
| 215 | YGL135W | RPL1B | ribosoma | 0.9 | 0.82 | 0.863 | 7 | 254644 | 255297 |
| 216 | YLR262C | unknow | <a targe | 1.33 | 0.39 | 0.861 | 12 | 669468 | 669662 |
| 217 | YBL030C | PET9 | mitochor | 1.47 | 0.25 | 0.86 | 2 | 163004 | 163960 |

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|---|---|---|---|---|---|---|---|---|---|---|
| 221 | YMR173V | DDR48 | induced l | 0.85 | 0.86 | 0.856 | 13 | 608688 | 609980 |
| 222 | YLR161W | unknown | | 1.47 | 0.24 | 0.854 | 12 | 488998 | 489342 |
| 223 | YLR150W | STM1 | unknown | 0.79 | 0.91 | 0.851 | 12 | 440468 | 441289 |
| 224 | YLL048C | YBT1 | bile acid | 1.26 | 0.44 | 0.85 | 12 | 41279 | 46264 |
| 226 | YJL177W | RPL17B | ribosoma | 1.04 | 0.64 | 0.842 | 10 | 90783 | 91654 |
| 227 | YBR181C | RPS6B | ribosoma | 1.37 | 0.31 | 0.839 | 2 | 591671 | 592733 |
| 228 | YOL086C | ADH1 | alcohol d | 0.41 | 1.26 | 0.837 | 15 | 159547 | 160593 |
| 230 | YMR173V | DDR48 | unknown | 0.61 | 1.06 | 0.835 | 13 | 608896 | 610080 |
| 231 | YMR085V | unknow | unknown | 0.42 | 1.24 | 0.834 | 13 | 437490 | 438788 |
| 232 | YNL300W | unknow | unknown | 0.82 | 0.84 | 0.832 | 14 | 65743 | 66051 |
| 233 | YPR132W | RPS23B | ribosoma | 1.18 | 0.48 | 0.829 | 16 | 794960 | 795762 |
| 234 | YOR369C | RPS12 | ribosoma | 0.45 | 1.2 | 0.828 | 15 | 1028186 | 1028617 |
| 235 | YLR193C | unknow | unknown | 1.28 | 0.38 | 0.827 | 12 | 540011 | 540538 |
| 237 | YBR145W | ADH5 | alcohol d | 1.01 | 0.64 | 0.824 | 2 | 533721 | 534776 |
| 238 | YPL220W | RPL1A | ribosoma | 1.28 | 0.37 | 0.824 | 16 | 135789 | 136442 |
| 239 | YHL015W | RPS20 | ribosoma | 0.63 | 1.01 | 0.823 | 8 | 75408 | 75773 |
| 240 | YNR050C | LYS9 | saccharo | 1.26 | 0.39 | 0.823 | 14 | 714045 | 715385 |
| 241 | YKR082W | NUP133 | nuclear p | 0.48 | 1.16 | 0.819 | 11 | 592461 | 595934 |
| 242 | YOR221C | MCT1 | malonyl- | 0.99 | 0.64 | 0.817 | 15 | 756474 | 757829 |
| 243 | YHL033C | RPL8A | ribosoma | 0.93 | 0.7 | 0.815 | 8 | 35253 | 36023 |
| 246 | YDR224C | HTB1 | histone H | 0.95 | 0.67 | 0.809 | 4 | 914308 | 914703 |
| 247 | YDL185W | TFP1 | vacuolar | 1.02 | 0.59 | 0.808 | 4 | 126788 | 130003 |
| 248 | YLR301W | unknow | unknown | 0.81 | | 0.807 | 12 | 730825 | 731559 |
| 249 | YJL089W | SIP4 | RNA pol II acti | 0.81 | 0.807 | 10 | 265622 | 268111 |
| 250 | YLR029C | RPL15A | ribosoma | 1.21 | 0.4 | 0.807 | 12 | 201977 | 202591 |
| 251 | YKR094C | RPL40B | ribosoma | 0.87 | 0.74 | 0.806 | 11 | 617630 | 618384 |
| 253 | YGL067W | NPY1 | unknown | 1.04 | 0.56 | 0.796 | 7 | 376099 | 377253 |
| 254 | YPR074C | TKL1 | transketo | 0.44 | 1.14 | 0.794 | 16 | 692791 | 694833 |
| 255 | YOL072W | THP1 | unknown | 0.85 | 0.72 | 0.785 | 15 | 194969 | 196336 |
| 256 | YMR305C | SCW10 | glucanas | 1.04 | 0.53 | 0.784 | 13 | 879063 | 880232 |
| 258 | YGR214W | RPS0A | ribosoma | 1.32 | 0.24 | 0.78 | 7 | 920569 | 921782 |
| 259 | YOR065W | CYT1 | cytochro | 0.96 | 0.6 | 0.78 | 15 | 447440 | 448369 |
| 261 | YGR240C | PFK1 | phosphof | 1.04 | 0.51 | 0.774 | 7 | 970766 | 973729 |
| 263 | YAL003W | EFB1 | translatic | 1.01 | 0.53 | 0.767 | 1 | 142172 | 143158 |
| 264 | YIL069C | RPS24B | ribosoma | 1.33 | 0.19 | 0.758 | 9 | 231550 | 232366 |
| 268 | YBR084C | RPL19A | ribosoma | 1.5 | 0.01 | 0.755 | 2 | 414145 | 415220 |
| 269 | YLR286C | CTS1 | endochiti | 0.99 | 0.52 | 0.754 | 12 | 708448 | 710136 |
| 270 | YMR116C | ASC1 | G-beta li | 0.75 | 0.76 | 0.753 | 13 | 499455 | 500687 |
| 271 | YCL039W | MOH2 | unknown | 0.9 | 0.6 | 0.75 | 3 | 52619 | 54856 |
| 272 | YML073C | RPL6A | ribosoma | 1.19 | 0.31 | 0.75 | 13 | 123227 | 124172 |
| 273 | YOR155C | unknow | unknown | 0.75 | | 0.748 | 15 | 626626 | 627978 |
| 274 | YLR075W | RPL10 | ribosoma | 0.58 | 0.91 | 0.748 | 12 | 282928 | 283593 |
| 275 | YGL147C | RPL9A | ribosoma | 1.15 | 0.34 | 0.748 | 7 | 227757 | 228332 |
| 276 | YDR417C | RPL12b | unknown | 0.61 | 0.88 | 0.747 | 4 | 1301544 | 1301915 |
| 279 | YJR051W | OSM1 | fumarate | 0.33 | 1.15 | 0.742 | 10 | 529549 | 531054 |
| 280 | YHR109W | CTM1 | unknown | 0.88 | 0.6 | 0.742 | 8 | 330312 | 332069 |
| 281 | YOR257W | CDC31 | | | 0.74 | 0.74 | 15 | 811004 | 811489 |
| 282 | YPR080W | TEF1 | translatic | 0.64 | 0.83 | 0.737 | 16 | 700590 | 701966 |
| 283 | YLR202C | unknow | unknown | 0.97 | 0.5 | 0.733 | 12 | 550196 | 550638 |
| 284 | YLR048W | RPS0B | ribosoma | 0.59 | 0.87 | 0.733 | 12 | 242233 | 243350 |
| 285 | YBR279W | PAF1 | RNA poly | 0.65 | 0.82 | 0.733 | 2 | 761215 | 762552 |
| 286 | YNL289W | PCL1 | G1/S cyc | 0.73 | | 0.731 | 14 | 87895 | 88734 |
| 287 | YPL221W | BOP1 | unknown | 1.26 | 0.2 | 0.729 | 16 | 133042 | 135423 |
| 288 | YIL011W | TIR3 | unknown | 1.18 | 0.28 | 0.729 | 9 | 333724 | 334533 |
| 289 | YBR246W | unknow | unknown | 1.11 | 0.32 | 0.716 | 2 | 711550 | 712713 |
| 291 | YGR241C | YAP180 | clathrin a | 0.61 | 0.82 | 0.715 | 7 | 974870 | 976576 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 292 | YOR102W | unknow | unknown | 0.29 | 1.13 | 0.709 | 15 | 516421 | 516771 |
| 293 | YDR077W | SED1 | putative | 0.75 | 0.67 | 0.709 | 4 | 600787 | 601803 |
| 296 | YLR340W | RPP0 | ribosoma | 0.7 | | 0.705 | 12 | 805886 | 806824 |
| 297 | YML026C | RPS18B | ribosoma | 1.26 | 0.15 | 0.704 | 13 | 222987 | 223828 |
| 298 | YKL153W | unknow | unknown | 0.91 | 0.5 | 0.703 | 11 | 163602 | 164111 |
| 300 | YNL006W | LST8 | unknown | 1.07 | 0.33 | 0.702 | 14 | 620064 | 620975 |
| 303 | YNL119W | unknow | unknown | 1.06 | 0.33 | 0.698 | 14 | 401037 | 402518 |
| 304 | YAR073W | IMD1 | unknown | 1.14 | 0.25 | 0.698 | 1 | 227728 | 228939 |
| 306 | YDR450W | RPS18A | ribosoma | 1.05 | 0.34 | 0.697 | 4 | 1359912 | 1360787 |
| 307 | YML107C | unknown | | | 0.7 | 0.696 | 13 | 55265 | 56425 |
| 308 | YBR221C | PDB1 | pyruvate | 1.21 | 0.18 | 0.692 | 2 | 665112 | 666212 |
| 309 | YML007W | YAP1 | transcrip | 0.73 | 0.65 | 0.691 | 13 | 253848 | 255800 |
| 310 | YOR095C | RKI1 | ribose-5- | 1.12 | 0.26 | 0.69 | 15 | 503550 | 504326 |
| 311 | YKL060C | FBA1 | aldolase | 0.55 | 0.83 | 0.689 | 11 | 326050 | 327129 |
| 313 | YNL067W | RPL9B | ribosoma | 1.23 | 0.15 | 0.689 | 14 | 499678 | 500253 |
| 314 | YCL041C | unknow | unknown | 0.25 | 1.12 | 0.688 | 3 | 50110 | 50604 |
| 315 | YPR125W | unknow | suppress | 0.69 | | 0.687 | 16 | 787956 | 789320 |
| 317 | YIL123W | SIM1 | unknown | 0.42 | 0.95 | 0.685 | 9 | 128151 | 129578 |
| 318 | YMR142C | RPL13B | ribosoma | 0.87 | 0.5 | 0.684 | 13 | 550205 | 551206 |
| 320 | YHR023W | MYO1 | myosin h | 0.39 | 0.97 | 0.68 | 8 | 151657 | 157443 |
| 321 | YNL241C | ZWF1 | glucose-( | 0.77 | 0.58 | 0.679 | 14 | 196425 | 197942 |
| 323 | YIL039W | unknow | unknown | 0.67 | 0.68 | 0.674 | 9 | 278425 | 279846 |
| 324 | YMR297W | PRC1 | carboxyp | 1.21 | 0.14 | 0.673 | 13 | 861922 | 863520 |
| 325 | YNL160W | YGP1 | unknown | 0.91 | 0.43 | 0.672 | 14 | 336542 | 337606 |
| 326 | YDL051W | YLA1 | RNA binc | 1.03 | 0.31 | 0.669 | 4 | 363951 | 364778 |
| 327 | YOR127W | RGA1 | putative | 1.31 | 0.03 | 0.669 | 15 | 561168 | 564191 |
| 330 | YLL024C | SSA2 | cytosolic | 0.78 | 0.55 | 0.664 | 12 | 95565 | 97484 |
| 331 | YOR122C | PFY1 | profilin : | 1.25 | 0.07 | 0.661 | 15 | 552296 | 552885 |
| 332 | YDR248C | unknow | unknown | 0.66 | | 0.66 | 4 | 957749 | 958330 |
| 333 | YER110C | KAP123 | beta-kar | 0.66 | | 0.66 | 5 | 378758 | 382099 |
| 334 | YDL014W | NOP1 | fibrillarin | 0.68 | 0.64 | 0.66 | 4 | 427360 | 428343 |
| 335 | YOR261C | RPN8 | 26S prot | 1.24 | 0.07 | 0.658 | 15 | 815911 | 816927 |
| 336 | YKL080W | VMA5 | vacuolar | 0.81 | 0.51 | 0.657 | 11 | 284672 | 285850 |
| 337 | YPR029C | APL4 | AP comp | 1.43 | -0.1 | 0.656 | 16 | 624464 | 626962 |
| 338 | YGL215W | CLG1 | cyclin-lik | 1.05 | 0.26 | 0.656 | 7 | 87979 | 89337 |
| 339 | YGR234W | YHB1 | flavoherr | 0.82 | 0.5 | 0.656 | 7 | 959897 | 961096 |
| 341 | YIL078W | THS1 | tRNA syr | 0.83 | 0.48 | 0.653 | 9 | 212496 | 214700 |
| 342 | YKL181W | PRS1 | phospho | 0.41 | 0.9 | 0.653 | 11 | 107318 | 108601 |
| 343 | YAL023C | PMT2 | dolichyl p | 0.84 | 0.46 | 0.652 | 1 | 106274 | 108550 |
| 344 | YOR317W | FAA1 | long chai | 1.03 | 0.28 | 0.652 | 15 | 909334 | 911436 |
| 345 | YIL105C | unknow | unknown | 1.18 | 0.12 | 0.65 | 9 | 167578 | 169638 |
| 346 | YDR050C | TPI1 | triosephc | 0.44 | 0.86 | 0.65 | 4 | 555721 | 556467 |
| 347 | YIR035C | unknow | unknown | 0.99 | 0.3 | 0.646 | 9 | 421023 | 421787 |
| 348 | YMR182C | RGM1 | putative | 0.14 | 1.15 | 0.644 | 13 | 624531 | 625166 |
| 349 | YNL327W | EGT2 | unknown | 1.08 | 0.2 | 0.643 | 14 | 24047 | 27172 |
| 350 | YIL086C | unknow | unknown | 0.64 | | 0.642 | 9 | 200150 | 200458 |
| 351 | YIL143C | SSL2 | TFIIH he | 0.6 | 0.68 | 0.641 | 9 | 80510 | 83041 |
| 352 | YNL071W | LAT1 | dihydroli | 0.95 | 0.33 | 0.641 | 14 | 491520 | 492968 |
| 353 | YOR312C | RPL20B | ribosoma | 1.24 | 0.04 | 0.641 | 15 | 900241 | 901172 |
| 354 | YIL104C | SHQ1 | unknown | 0.91 | 0.37 | 0.639 | 9 | 169979 | 171502 |
| 355 | YPR181C | SEC23 | vesicle c | 1.1 | 0.18 | 0.637 | 16 | 897356 | 899662 |
| 356 | YBL045C | COR1 | ubiquinol | 0.85 | 0.42 | 0.635 | 2 | 134106 | 135479 |
| 358 | YMR181C | unknow | unknown | 0.86 | 0.41 | 0.634 | 13 | 623615 | 624079 |
| 359 | YBR286W | APE3 | vacuolar | 0.63 | | 0.632 | 2 | 774580 | 776271 |
| 360 | YPR145W | ASN1 | asparagi | 1.01 | 0.25 | 0.629 | 16 | 822615 | 824333 |
| 361 | YLR451W | LEU3 | transcrip | 0.83 | 0.42 | 0.627 | 12 | 1036087 | 1038747 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 362 | YLR441C | RPS1A | ribosome | 0.8 | 0.45 | 0.627 | 12 | 1018135 | 1018902 |
| 363 | YPL198W | RPL7B | ribosome | 1.06 | 0.19 | 0.626 | 16 | 173151 | 174701 |
| 364 | YOR326W | MYO2 | myosin, | 0.87 | 0.38 | 0.625 | 15 | 925712 | 930436 |
| 365 | YCL037C | SRO9 | actin fila | 0.87 | 0.38 | 0.625 | 3 | 57347 | 58747 |
| 366 | YBR150C | TBS1 | unknown | 0.71 | 0.54 | 0.625 | 2 | 541168 | 544452 |
| 367 | YNL104C | LEU4 | 2-isopro | 0.85 | 0.4 | 0.624 | 14 | 424892 | 426751 |
| 368 | YDL172C | unknow | unknown | 1.01 | 0.24 | 0.623 | 4 | 148608 | 149087 |
| 369 | YGR071C | unknown | | | 0.62 | 0.623 | 7 | 631475 | 634057 |
| 370 | YPL082C | MOT1 | putative | 1.14 | 0.1 | 0.621 | 16 | 398475 | 404078 |
| 373 | YOR187W | TUF1 | translatic | 1.08 | 0.15 | 0.617 | 15 | 684028 | 685341 |
| 375 | YMR008C | PLB1 | phosphol | 0.86 | 0.37 | 0.616 | 13 | 280590 | 282584 |
| 376 | YMR061W | RNA14 | cleavage | 0.61 | | 0.614 | 13 | 392754 | 394787 |
| 377 | YJR123W | RPS5 | | | 0.61 | 0.614 | 10 | 651590 | 652267 |
| 380 | YNL228W | unknow | unknown | 0.61 | | 0.614 | 14 | 220644 | 221420 |
| 381 | YMR295C | unknow | unknown | 1.11 | 0.11 | 0.611 | 13 | 858297 | 858890 |
| 383 | YDL140C | RPO21 | RNA poly | 0.57 | 0.65 | 0.608 | 4 | 205361 | 210562 |
| 384 | YOR167C | RPS28A | ribosome | 1.1 | 0.11 | 0.608 | 15 | 648802 | 649005 |
| 386 | YMR011W | HXT2 | hexose p | 1.14 | 0.07 | 0.607 | 13 | 288078 | 289703 |
| 387 | YCL030C | HIS4 | histidinol | 1.11 | 0.1 | 0.604 | 3 | 65906 | 68305 |
| 388 | YPL112C | PEX25 | unknown | 0.93 | 0.28 | 0.604 | 16 | 337435 | 338619 |
| 389 | YPR035W | GLN1 | glutamine | synt | 0.6 | 0.604 | 16 | 642203 | 643315 |
| 390 | YJL076W | NET1 | unknown | 1.15 | 0.06 | 0.604 | 10 | 294941 | 298510 |
| 391 | YOL139C | CDC33 | translatic | 0.48 | 0.73 | 0.603 | 15 | 60382 | 61023 |
| 394 | YGR124W | ASN2 | asparagi | 1.06 | 0.14 | 0.601 | 7 | 739938 | 741656 |
| 395 | YBL002W | HTB2 | histone H | 1.18 | 0.01 | 0.599 | 2 | 236455 | 236850 |
| 397 | YPR043W | RPL43A | ribosome | 1.24 | -0 | 0.597 | 16 | 654161 | 654842 |
| 398 | YIL074C | SER33 | 3-phosph | 0.87 | 0.32 | 0.597 | 9 | 221078 | 222487 |
| 399 | YBR121C | GRS1 | tRNA syr | 0.94 | 0.25 | 0.596 | 2 | 481323 | 483326 |
| 402 | YOL036W | | unknown | 0.42 | 0.77 | 0.594 | 15 | 256743 | 259028 |
| 404 | YCL025C | AGP1 | amino ac | 1.34 | -0.2 | 0.588 | 3 | 75985 | 77886 |
| 405 | YDL191W | RPL35A | ribosome | 1.19 | -0 | 0.587 | 4 | 117665 | 118518 |
| 406 | YOL100W | PKH2 | protein k | 0.82 | 0.36 | 0.586 | 15 | 129236 | 132481 |
| 410 | YOR096W | RPS7A | ribosome | 0.58 | | 0.585 | 15 | 505792 | 506765 |
| 411 | YKR057W | RPS21A | ribosome | 0.53 | 0.64 | 0.584 | 11 | 551293 | 551878 |
| 413 | YGL036W | MTC2 | unknown | 0.52 | 0.65 | 0.583 | 7 | 428597 | 431326 |
| 414 | YPL014W | | unknown | 1.24 | -0.1 | 0.582 | 16 | 527542 | 528687 |
| 415 | YCR024C | PMP1 | regulates | 0.48 | 0.69 | 0.581 | 3 | 162653 | 162775 |
| 416 | YKR042W | UTH1 | unknown | 0.91 | 0.25 | 0.581 | 11 | 518909 | 520261 |
| 417 | YGR155W | CYS4 | cystathio | 0.82 | 0.34 | 0.58 | 7 | 798537 | 800060 |
| 421 | YDL195W | SEC31 | vesicle c | 1.29 | -0.1 | 0.575 | 4 | 107209 | 111030 |
| 424 | YJL121C | RPE1 | Ribulose- | 0.99 | 0.16 | 0.574 | 10 | 190074 | 190790 |
| 427 | YMR309C | NIP1 | unknown | 1.05 | 0.1 | 0.574 | 13 | 892988 | 895426 |
| 428 | YCL009C | ILV6 | acetolact | 0.99 | 0.15 | 0.572 | 3 | 104230 | 105159 |
| 430 | YIL047C | SYG1 | pheromo | 0.81 | 0.33 | 0.57 | 9 | 265114 | 267822 |
| 431 | YCR048W | ARE1 | acyl-CoA | 0.55 | 0.59 | 0.57 | 3 | 210660 | 212492 |
| 432 | YMR145C | | unknown | 0.63 | 0.51 | 0.569 | 13 | 554792 | 556474 |
| 433 | YOR198C | BFR1 | unknown | 1.3 | -0.2 | 0.568 | 15 | 718651 | 720063 |
| 434 | YKR059W | TIF1 | translatic | 0.4 | 0.73 | 0.566 | 11 | 554623 | 555810 |
| 435 | YDL055C | PSA1 | mannose | 0.58 | 0.56 | 0.566 | 4 | 355673 | 356758 |
| 439 | YNL178W | RPS3 | ribosome | 0.29 | 0.84 | 0.563 | 14 | 302677 | 303399 |
| 440 | YNL045W | unknow | unknown | 1.33 | -0.2 | 0.563 | 14 | 542959 | 544974 |
| 442 | YLR264W | RPS28B | ribosome | 0.48 | 0.65 | 0.563 | 12 | 673131 | 673334 |
| 444 | YPL117C | IDI1 | isopente | 0.56 | | 0.556 | 16 | 327863 | 328729 |
| 445 | YJR096W | | | | 0.56 | 0.556 | 10 | 610886 | 611734 |
| 447 | YBR156C | SLI15 | unknown | 0.86 | 0.25 | 0.553 | 2 | 551063 | 553159 |
| 448 | YJL054W | TIM54 | inner me | 0.75 | 0.36 | 0.552 | 10 | 333961 | 335397 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 449 | YLR293C | GSP1 | GTP-binc | 0.49 | 0.61 | 0.549 | 12 | 720771 | 721430 |
| 452 | YGR243W | | unknown | 0.55 | | 0.546 | 7 | 977331 | 977771 |
| 453 | YGR138C | | major fa | 0.99 | 0.1 | 0.545 | 7 | 763756 | 765600 |
| 454 | YMR046C | | unknown | 0.72 | 0.37 | 0.545 | 13 | 361304 | 362626 |
| 455 | YML040W | | unknown | 0.82 | 0.26 | 0.543 | 13 | 196628 | 197950 |
| 457 | YBL076C | ILS1 | tRNA syr | 0.8 | 0.29 | 0.543 | 2 | 81038 | 84256 |
| 458 | YCL008C | STP22 | unknown | 0.03 | 1.06 | 0.543 | 3 | 105307 | 106464 |
| 459 | YGL245W | | tRNA syr | 0.83 | 0.25 | 0.541 | 7 | 38975 | 41149 |
| 460 | YBL075C | SSA3 | cytosolic | 0.82 | 0.26 | 0.539 | 2 | 84494 | 86443 |
| 461 | YGR224W | | major fa | 0.89 | 0.19 | 0.538 | 7 | 942798 | 944639 |
| 462 | YHL019C | APM2 | AP-2 con | 0.64 | 0.43 | 0.537 | 8 | 67727 | 69544 |
| 465 | YJR013W | | unknown | 0.54 | | 0.536 | 10 | 460364 | 461281 |
| 466 | YML045W | | unknown | 0.58 | 0.5 | 0.536 | 13 | 184461 | 189729 |
| 467 | YFL045C | SEC53 | phosphoi | 0.81 | 0.26 | 0.535 | 6 | 43628 | 44392 |
| 468 | YDR408C | ADE8 | phosphoi | 0.71 | 0.36 | 0.535 | 4 | 1288204 | 1288848 |
| 470 | YER120W | SCS2 | regulator | 0.82 | 0.25 | 0.533 | 5 | 401131 | 401865 |
| 471 | YHR018C | ARG4 | arginino: | 0.86 | 0.2 | 0.533 | 8 | 140002 | 141393 |
| 472 | YPR185W | APG13 | unknown | 1.01 | 0.06 | 0.532 | 16 | 907213 | 909429 |
| 473 | YOR099W | KTR1 | mannosy | 1.06 | 0 | 0.532 | 15 | 511823 | 513004 |
| 474 | YLR044C | PDC1 | pyruvate | 0.55 | 0.52 | 0.531 | 12 | 232391 | 234082 |
| 475 | YBR086C | IST2 | unknown | 0.91 | 0.15 | 0.531 | 2 | 420160 | 423000 |
| 476 | YBL101W-A | | unknown | 1.01 | 0.04 | 0.528 | 2 | 29927 | 31243 |
| 477 | YOR230W | WTM1 | transcrip | 0.92 | 0.14 | 0.528 | 15 | 770798 | 772111 |
| 478 | YOL075C | | putative | 0.14 | 0.92 | 0.528 | 15 | 189657 | 193541 |
| 479 | YPL281C | ERR2 | unknown | 0.76 | 0.3 | 0.528 | 16 | 9557 | 10870 |
| 481 | YFL022C | FRS2 | tRNA syr | 0.86 | 0.19 | 0.523 | 6 | 93497 | 95008 |
| 482 | YDR074W | TPS2 | trehalose | 0.88 | 0.16 | 0.522 | 4 | 593887 | 596577 |
| 483 | YHL001W | RPL14B | ribosoma | 0.82 | 0.23 | 0.521 | 8 | 104270 | 105084 |
| 484 | YEL034W | HYP2 | translatic | 0.77 | 0.28 | 0.52 | 5 | 85676 | 86149 |
| 485 | YOR129C | | unknown | 1.38 | -0.3 | 0.519 | 15 | 566875 | 569556 |
| 487 | YLR109W | AHP1 | alkyl hyd | 0.44 | 0.59 | 0.519 | 12 | 368782 | 369312 |
| 488 | YJR024C | | unknown | 1.4 | -0.4 | 0.518 | 10 | 469918 | 470652 |
| 489 | YBR283C | SSH1 | ER prote | 1.14 | -0.1 | 0.516 | 2 | 768901 | 770373 |
| 490 | YJR059W | | | | 0.52 | 0.516 | 10 | 545475 | 547931 |
| 491 | YDL015C | | unknown | 0.54 | 0.5 | 0.516 | 4 | 425998 | 426930 |
| 492 | YHR019C | DED81 | tRNA syr | 0.85 | 0.18 | 0.512 | 8 | 141885 | 143549 |
| 493 | YDR086C | SSS1 | ER prote | 0.59 | 0.42 | 0.508 | 4 | 616921 | 617163 |
| 494 | YLR413W | | unknown | 0.8 | 0.21 | 0.507 | 12 | 951150 | 953177 |
| 495 | YAR075W | | unknown | 1.21 | -0.2 | 0.507 | 1 | 228830 | 229303 |
| 496 | YHL020C | OPI1 | negative | 0.51 | | 0.506 | 8 | 66238 | 67452 |
| 497 | YPR021C | | unknown | 0.51 | | 0.506 | 16 | 600644 | 603352 |
| 498 | YER055C | HIS1 | ATP phos | 0.51 | | 0.506 | 5 | 264891 | 265784 |
| 499 | YDR471W | RPL27B | ribosoma | 0.78 | 0.23 | 0.504 | 4 | 1401759 | 1402553 |
| 500 | YFR031C | RPL2A | ribosoma | 0.24 | 0.77 | 0.502 | 6 | 220494 | 221405 |

**Table 8 - Microarray and QPCR data for 18 chosen RNAs**

| Gene | Microarray 1 | Microarray 2 | QPCR 1 | QPCR 2 |
|---|---|---|---|---|
| CUP1 | 0.73 | -0.42 | 6.32 | 2.58 |
| HAC1 | 1.07 | 1.41 | 6.63 | 4.95 |
| NOG2 | 2.06 | 1.06 | 4.91 | 4.25 |
| PGK1 | 0.16 | 0.51 | 5.78 | 5.17 |
| RPC11 | -0.97 | -2.56 | 5.00 | 4.64 |
| RPL11A | 1.11 | 0.86 | 6.60 | 5.67 |
| RPL31 | 0.42 | -0.10 | 6.64 | 4.81 |
| RPS22A | 0.77 | 0.23 | 5.47 | 5.88 |
| RPS4B | 0.41 | 0.42 | 5.21 | 5.25 |
| SCR1 | 3.07 | 3.55 | 9.59 | 9.45 |
| SEC14 | 0.20 | -0.84 | 5.09 | 4.64 |
| SNR189 | 0.90 | 0.73 | 6.02 | 7.85 |
| U2 | 1.36 | 2.06 | 4.75 | 5.78 |
| U3 | 2.34 | 3.00 | 6.54 | 6.78 |
| U4 | 2.52 | 2.60 | 7.78 | 8.71 |
| U5 | 1.43 | 2.60 | 6.55 | 8.32 |
| U6 | 4.14 | 4.72 | 10.97 | 11.04 |
| UBC5 | -0.62 | -1.22 | 4.01 | 4.49 |

## EPILOGUE

This thesis work represents several different approaches for investigating the mechanisms of how cells assemble RNAs into RNPs that have broadened our understanding of RNP biogenesis with several unexpected outcomes.

### Divergent snRNP biogenesis pathways

Restriction of snRNP biogenesis to the nucleus in yeast was unexpected given that the major assembly steps of Sm core binding, 5' cap hypermethylation and 3' end trimming that occur in the cytoplasm of metazoan cells all occur in yeast. Factors identified to be involved in 3' end processing in yeast, the cleavage factor Rnt1p and the 3' end precursor stabilizing factor Lhp1p, both have mammalian homologs – RNase III and La (Yoo and Wolin 1994; Elela et al. 1996). The 5' monomethyl cap, the cap binding complex and the trimethyl cap recognition elements for PHAX and snurportin are present in yeast (Huber et al. 1998; Ohno et al. 2000), suggesting that processing occurs, but diverges only at nucleocytoplasmic transport. However, for Sm core assembly, the symmetric dimethylarginines in the C terminal tails of SmB/B', SmD1, SmD3 and Lsm4 are absent in yeast Sm and Lsm proteins (Brahms et al. 2000). Likewise, SMN that binds the dimethylarginines of Sm proteins to mediate Sm assembly is absent in yeast (Friesen et al. 2001). Intriguingly, the C terminal tails of SmB and SmD1 in yeast display basic nuclear localization properties (Bordonne 2000), suggesting that yeast Sm proteins can and may enter the nucleus in the absence of RNA. While metazoan Sm core assembly on U snRNAs using purified proteins occurs spontaneously *in vitro* (Raker et al. 1996), core assembly *in vivo* is mediated by SMN and may be regulated by methylation (Brahms et al. 2001). In the same way that eukaryotes have compartmentalized DNA from the translational machinery for regulation opportunities and insurance that RNA synthesis is completed before translation can occur, cytoplasmic snRNP biogenesis may be a quality control mechanism to prevent aberrant snRNPs from entering the splicing pathway. Notably, *Xenopus* oocytes are known to store large quantities of cytoplasmic snRNPs for developmentally regulated bursts of splicing activity.

How then does yeast insure such a mechanism? One possibility is that the nucleolus is the site for snRNP assembly and biogenesis in *S. cerevisiae*. The trimethylating enzyme Tgs1p is localized to the nucleolus, and U1 snRNA precursors accumulate in the nucleolus in a *tgs1Δ* strain (Mouaikel et al. 2002), suggesting that unprocessed precursors may be blocked there. The 3' end processing factors Rnt1p and Lhp1p have also been localized to the nucleus and do not appear to be excluded from the nucleolus (Huh et al. 2003), suggesting that other snRNP biogenesis steps may also be shunted to the nucleolus in yeast.

Elucidation of such a pathway may broaden our understanding snRNP quality control. Current *in situ* hybridization techniques do not readily allow for visualization of nucleolar localization in the presence of intense nuclear signal. However, live visualization of RNA localization by introducing a protein binding site in the RNA and a GFP-conjugated RNA binding protein in combination with Fluorescence Resonance Energy Transfer (FRET) techniques may allow for snRNA nucleolar visualization (Brodsky and Silver 2000). By designing nucleolar snRNP processing factors such as Tgs1p as the acceptor molecule and snRNAs bound by a GFP labelled protein as the donor molecule, this may allow specific visualization of nucleolar snRNAs. In combination with snRNP biogenesis mutants, it may then be possible to identify the location and order of Sm core binding, trimethylation and 3' end trimming steps. Intriguingly, U3 snoRNA which is similarly trimethylated at its cap, but is not Sm-associated, has been shown to be targeted to the nucleolus by specific C'/D cis sequences (Narayanan et al. 1999). Finding the determinants for snRNA nucleolar localization and the requirements for return to the nucleus for splicing may reveal the steps necessary for proper quality control of snRNP assembly in *S. cerevisiae*.

**The interface between snRNP biogenesis and snRNP recycling during splicing in *S. cerevisiae***

From our work and others, snRNA synthesis, assembly, function and regeneration may occur within the nucleoplasm in *S. cerevisiae*. One view asserts that steps required to insure proper

biogenesis of snRNPs are common with steps for regenerating snRNPs for subsequent rounds of splicing and that these processes may utilize common factors. Previously, function in snRNP biogenesis was assigned to factors affecting stability of snRNAs, such as Sm proteins (Rymond 1993; Roy et al. 1995), and recycling function was assigned to 'late' acting factors such as Prp24p involved in annealing of mature U4 and U6 snRNPs (Raghunathan and Guthrie 1998). However a new snRNP assembly and snRNP recycling prototype factor has been found. Sad1p, which was identified as a snRNP biogenesis factor involved in formation of newly synthesized U4/U6 snRNP, also displays defects in triple snRNP addition (Lygerou et al. 1999). This suggests that there are step(s) during snRNP assembly which when defective do not lead to degradation, but rather lead to dire consequences during assembly of the spliceosome. In contrast, in the absence of Brr1p, newly synthesized snRNPs that are improperly assembled are seemingly rapidly targeted for degradation masking any later function in splicing.

To identify and characterize the steps during snRNP assembly required for proper spliceosome assembly, development of biochemical assays is imperative. The current methods for examining snRNP status and spliceosome assembly by native gel are crude and cannot detect subtle changes in composition or structure. Adaptation of recent advances in affinity purification and mass spec analysis may begin to identify changes in composition of snRNPs (Gottschalk et al. 1999; Stevens and Abelson 1999; Stevens et al. 2001). However, there is evidence that the spliceosome associates with pre-mRNA as a whole complex known as the penta-snRNP (Stevens et al. 2002), suggesting that the composition of such a complex may not change significantly during the course of the splicing pathway. Multiple rearrangements in snRNA:snRNA or snRNA:protein interactions occur during spliceosome assembly (Staley and Guthrie 1998) and are likely to occur during biogenesis. The fact that a GTPase (Snu114p) and an ATPase (Sub2p) genetically interact with BRR1 suggest that conformational rearrangements may be required for biogenesis. Indeed, the allele specific mutant that is synthetic with *brr1Δ, snu114-60,* displays an aberrant U5 snRNP whose composition has yet to be determined (Brenner and Guthrie personal communication).

Other methodologies for examining spliceosome assembly include affinity purification of pre-mRNA associated spliceosomes and probing of snRNA composition by QPCR (Staley and Guthrie 1999) (Brenner and Guthrie personal communication). However, if critical assembly steps occur when the spliceosome is not yet associated with pre-mRNA, development of new methodologies will be required to further our understanding of snRNP assembly, biogenesis and recycling steps and their impact on splicing.

**Novel RNAs**

The sequencing of genomes and development of computational algorithms has caused a boom in the identification of new RNAs. In the past several years, these methods have been used to identify multitudes of novel, noncoding RNAs in various organisms, many with unknown function (Eddy 2002). In contrast, we have used a biochemical approach to identify a number of intergenic regions that are likely to contain novel noncoding RNAs. Mapping the start and end sequences of these RNAs if the first step towards identification. Although we attempted to map RNAs in a few intergenic regions by making a Northern probe to the entire region and looking for stable RNAs, we observed multiple bands (data not shown). A reverse transcription primer 'walk' across the region with subsequent PCR analysis may identify the sizes of the RNAs. Identification of these novel noncoding RNAs may be particularly challenging since they may be low in abundance and/or short in length. This may explain why they have previously escaped detection by other methods in contrast to ours, which includes an enrichment step. The next step will be to knockout the identified novel RNAs and attempt to assign function.

**Novel function for Lhp1p with noncoding RNAs**

We observe nearly class-wide association of Lhp1p with snoRNAs and tRNAs. Directed interactions with the remaining snoRNA and tRNAs could be assayed to determine if Lhp1p association is indeed class-wide. Repeating the Lhp1p IP microarrays with lower O.D. growth conditions may reveal such interactions since Lhp1p is downregulated at diauxic shift (DeRisi et al. 1997) and Lhp1p association with $tRNA^{Ser}$(CGA) is only observed at O.D.s below 0.5 (Wolin

personal communication). It also remains to be seen if Lhp1p plays a universal role in stabilization of precursors or if Lhp1p harbors different roles for different snoRNAs and tRNAs. Lhp1p has been recently shown to affect the aminoacylation of some tRNAs (Chakshusmathi et al. 2003). We find association with a $tRNA^{Leu}$(CAA) that doesn't display processing defects, suggesting a different role for Lhp1p with this tRNA.

**Function for Lhp1p in translation during the unfolded protein response**

We have accumulated evidence suggesting that Lhp1p may be involved in modulating translation of Hac1p. To test this, one can examine translation rates of Hac1p in wildtype and *lhp1Δ* extracts. Both spliced and unspliced HAC1 mRNA have been shown to be associated with polysomes (Cox and Walter 1996). To see if Lhp1p impairs polysome association of HAC1 mRNA, one can examine the location of HAC1 mRNA in wildtype and *lhp1Δ* polysome profiles. Since it has been shown that the intron:5'UTR interaction attenuates translation (Ruegsegger et al. 2001) and the absence of Lhp1p causes a decrease in translation of Hac1p, this suggests that Lhp1p may be involved in release of translational inhibition upon splicing. Walter and colleagues have observed that hyperstabilization of the intron:5'UTR interaction delays the kinetics of Hac1p translation (Aragon and Walter personal communication). To see if Lhp1p is involved in unwinding of this helix, one can examine induction of the UPR in the absence of Lhp1p with the hyperstabilized HAC1 mRNA. Lastly, Lhp1p may be playing a role upstream of translation, but downstream of RNA processing/splicing, for example in HAC1 mRNA export. Localization of HAC1 mRNA in *lhp1Δ* can be examined by *in situ* hybridization (Chapman and Walter 1997).

**Function for Lhp1p with the ribosomal protein genes and other mRNAs**

Our finding that Lhp1p associates with ribosomal protein genes in yeast was unexpected, because while mammalian La is associated with 5' TOP mRNAs (Cardinali et al. 2003), which include ribosomal protein genes, TOP sequences are not believed to exist in yeast (Powers and Walter 1999). This suggests that either Lhp1p is binding ribosomal protein genes via a different element

in yeast or that Lhp1p is perhaps binding a structural element conserved between yeast and mammalian RPGs. Mapping the binding location of Lhp1p in these mRNAs would begin to address this question. One could roughly map the interaction domain by immunoprecipitating Lhp1p associated RNAs and using site directed RNase H cleavage at different locations of the mRNAs during the immunoprecipitation to crudely map whether Lhp1p is associated with the 5' UTR, the body of the message, the intron or the 3' UTR. Various HAC1 and ACT1 chimeras exist in the Walter lab for examining the Lhp1 binding site of HAC1 mRNA (Ruegsegger et al. 2001). Preliminary results examining Lhp1p association with HAC1 RNA during induction of the UPR, suggested that Lhp1p is not associated exclusively with the intron because association with both the pre-mRNA and mature mRNA is maintained (data not shown). Once a region is narrowed down, computational analysis may be used to reveal a sequence or structural motif.
In addition, while La is believed to modulate translation of TOP mRNAs in mammalian systems (Cardinali et al. 2003), yeast ribosomal protein genes are known to be transcriptionally regulated (Powers and Walter 1999). Therefore, whether Lhp1p association with ribosomal protein genes reflects a novel transcriptionally related function or a translationally related function has yet to be determined.

**Global regulation of gene expression by RNP assembly**

Lastly, our experiments bring to light an intriguing connection between Lhp1p associated RNAs. These RNAs are not only part of the translational apparatus (5S rRNA and tRNAs) and involved in processing RNAs in the translational apparatus (snoRNAs and RNase P), but include transcripts encoding the protein components of the translational machinery (RPGs). Lhp1p may contribute to the coordination of both RNA and protein components of translation for general cell metabolism. Understanding the coordinated relationship between regulating cell growth and assembly of RNP machinery will require continued study.

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