Arginine methylation facilitates the nuclear export of hnRNP proteins

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Eukaryotic mRNA processing and export is mediated by various heterogeneous nuclear ribonucleoproteins (hnRNPs). Many of these hnRNPs are methylated on arginine residues. In the yeast, *Saccharomyces cerevisiae*, the predominant enzyme responsible for arginine methylation is Hmt1p. Hmt1p methylates both Npl3p and Hrp1p, which are shuttling hnRNPs involved in mRNA processing and export. Here, we employ an in vivo nuclear export assay to show that arginine methylation is important for the nuclear export of these hnRNPs. Both Npl3p and Hrp1p fail to exit the nucleus in cells lacking Hmt1p, and overexpression of Hmt1p enhances Npl3p export. The export of a novel hnRNP-like protein, Hrb1p, which does not bind poly(A)⁺ RNA, however, is not affected by the lack of methylation. Furthermore, we find a genetic relationship between Hmt1p and cap-binding protein 80 (CBP80). Together, these findings establish that one biological role for arginine methylation is in facilitating the export of certain hnRNPs out of the nucleus.

[**Key Words:** HMT1; NPL3; HRP1; CBP80; heterogeneous ribonucleoprotein (hnRNP); arginine methylation; mRNA export]

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While in the nucleus, mRNA precursors, referred to as pre-mRNAs or heterogeneous nuclear RNAs (hnRNAs), undergo a series of processing events before traveling to the cytoplasm. These maturation events include capping at the 5' end, splicing, and 3'-end cleavage followed by polyadenylation. From the time they leave the transcription complex, hnRNAs are associated with proteins, some of which have been proposed to be mediators of RNA export (for review, see Lee and Silver 1997). The set of proteins that bind hnRNAs, with the exception of small nuclear RNPs, are referred to as heterogeneous nuclear ribonucleoproteins (hnRNPs).

Among the most abundant proteins in the nucleus (Kiledjian et al. 1994), there are over 20 mammalian hnRNPs, proposed to function in nearly every step of mRNA maturation including export (Piñol-Roma 1997). One of the best-studied hnRNPs, hnRNP A1, travels back and forth between the nucleus and the cytoplasm in a process termed nucleocytoplasmic shuttling (Piñol-Roma and Dreyfuss 1992). In addition, an hnRNP A1-like protein in the insect *Chironomus tentans* can be seen by immunoelectron microscopy to be associated with pre-mRNA traveling through the nuclear pore to the cytoplasm (Visa et al. 1996a).

The discovery of a nuclear export signal (NES) in some hnRNPs has suggested further that these hnRNPs could play an active role in RNA export. A 38-amino-acid sequence within hnRNP A1 termed M9 has been found to be necessary and sufficient for export of the protein to the cytoplasm (Michael et al. 1995). Microinjection experiments have shown that saturating amounts of the M9 domain block mRNA export in *Xenopus* oocytes (Izaurre et al. 1997). A model for mRNA export has been proposed in which the export signals on the shuttling hnRNPs are directly responsible for the translocation of bound mRNAs into the cytoplasm (Fischer et al. 1996; Nigg 1997). This model is best supported by studies of the HIV Rev protein. Through its leucine-rich NES, Rev facilitates the nuclear export of partially spliced and unspliced viral RNAs containing the Rev response element (Fischer et al. 1996).

Further evidence for the involvement of hnRNPs in mRNA export comes from studies in the yeast *Saccharomyces cerevisiae*. Npl3p is a yeast hnRNP (Bossie et al. 1992; Russell and Tollervey 1992) with structural features similar to those found in some mammalian hnRNPs, such as hnRNP A1. These include two RNP-type RNA-recognition motifs (RRMs), and a glycine-rich domain in the form of 15 arginine-glycine-glycine (RGG) repeats (Birney et al. 1993). Although Npl3p appears to localize to the nucleus in a distinct non-nuclear pattern similar to human hnRNPs (Flach et al. 1994), it actually shuttles between the nucleus and the cyto-

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plasm (Flach et al. 1994; Lee et al. 1996). Strains bearing mutant alleles of *NPL3* accumulate poly(A)⁺ RNA in the nucleus (Russell and Tollervy 1992; Singleton et al. 1995; Henry et al. 1996; Lee et al. 1996). Export of these mutant Npl3p proteins from the nucleus is also defective, providing further evidence that Npl3p is a poly(A)⁺ RNA carrier (Lee et al. 1996). Moreover, Npl3p export depends on ongoing RNA polymerase II transcription, suggesting a tight association between the export of poly(A)⁺ RNA and Npl3p (Lee et al. 1996).

A second yeast hnRNP, *HRP1*, was identified in a screen for suppressors of the temperature-sensitive mutant, *npl3-1* (Henry et al. 1996). This essential gene encodes a protein with two RNA-recognition motifs most similar to those found in human hnRNPs A1, A2/B1, and D/AUF1. In addition, the carboxyl terminus of the protein is arginine and glycine rich. Hrp1p has recently been shown to be required for proper 3' end formation in *S. cerevisiae* (Kessler et al. 1997). As such, it binds mRNA at the UA-rich efficiency element, which is required for both cleavage and polyadenylation. Although Hrp1p localizes to the nucleus at steady state, it has also been found to efficiently exit the nucleus (Kessler et al. 1997). These data suggest a link between polyadenylation and mRNA export in yeast.

It has become increasingly apparent that hnRNPs play key regulatory roles at all levels of RNA processing (for review, see Dreyfuss et al. 1993). Such regulation may be mediated by post-translational modifications of the hnRNPs themselves. In fact, most hnRNPs do undergo such modifications including phosphorylation (e.g., Cobianchi et al. 1993; Piñol-Roma and Dreyfuss 1993), glycosylation (Soulard et al. 1993), and methylation (e.g., Liu and Dreyfuss 1995). Partially purified mammalian hnRNP complexes contain proteins with a high level of the unusually modified amino acid N^G,N^G-dimethylarginine (Beyer et al. 1977). In fact, hnRNPs contain about 65% of methylated arginines in the cell nucleus (Boffa et al. 1977), indicating that the methylation state of these proteins is likely to have an important effect on their functions. In addition, these methylated arginine residues are found in other non-hnRNP RNA-binding proteins, all of which appear to contain RGG repeats (Najbauer et al. 1993 and references therein). Despite speculation that methylation affects the RNA-binding activity of hnRNPs (Rajpurohit et al. 1994), or the interactions of hnRNPs with other proteins (Liu and Dreyfuss 1995), no in vivo function has been defined.

The recent identification of the enzymes responsible for arginine methylation provides an important gateway to further our understanding of its true biological role. Protein arginine methyltransferases specific for hnRNP A1 have been partially purified from several sources (Ghosh et al. 1988; Rawal et al. 1994; Liu and Dreyfuss 1995). The cDNAs encoding human and rat arginine methyltransferases have been identified (Lin et al. 1996; Nikawa et al. 1996; Katsanis et al. 1997). In *S. cerevisiae*, a homolog of the rat protein arginine methyltransferase (PRMT1) termed *HMT1/RMT1* has been identified in a synthetic lethal screen with the temperature-sensitive

mutant, *npl3-1* (Henry and Silver 1996). *HMT1/RMT1* was independently identified as a gene whose protein product represents the major arginine methyltransferase activity present in *S. cerevisiae* (Gary et al. 1996). Hmt1p has been shown to methylate Npl3p both in vitro and in vivo (Henry and Silver 1996; Siebel and Guthrie 1996), and to localize to the nucleus (Henry and Silver 1996). In a strain containing a null allele of *HMT1*, methylation of Npl3p is no longer detectable (Henry and Silver 1996). Taken together, these data suggest that Hmt1p may play a role in the regulation of Npl3p function.

In this investigation, we show that, like Npl3p, Hrp1p is methylated by Hmt1p. More importantly, we demonstrate that, in the absence of Hmt1p methylation, the ability of Npl3p and Hrp1p to exit the nucleus is impaired. This study is the first in vivo demonstration of a function for hnRNP arginine methylation and implicates this modification as part of the mechanism by which protein-RNA complexes are recognized for nuclear export.

Results

Effect of methylation on the nuclear export of mRNA-binding proteins

We have shown previously that both Npl3p and Hrp1p exit the nucleus (Flach et al. 1994; Lee et al. 1996; Kessler et al. 1997). To test whether methylation of Npl3p and Hrp1p has an effect on their export, we utilized a nuclear export assay (Lee et al. 1996). This assay exploits the properties of a temperature-sensitive mutant of the nucleoporin Nup49p (Doye et al. 1994). At the nonpermissive temperature of 36.5°C, import of proteins into the nucleus is blocked in the *nup49-313* strain, whereas nuclear export is not appreciably affected. Because of this phenotype, shuttling proteins accumulate in the cytoplasm after exit from the nucleus. To monitor this export visually, the green fluorescent protein (GFP) is fused to the protein of interest and placed under a galactose inducible (*GAL1*) promoter. After induction, cells are either shifted to the nonpermissive temperature or maintained at the permissive temperature of 25°C.

The deletion of *HMT1* alone results in no poly(A)⁺ RNA export defects or nuclear protein mislocalization (Henry and Silver 1996). To study the effect of methylation through the use of this export assay, a strain containing the *nup49-313* mutation and a deletion of the *HMT1* gene was constructed (see Materials and Methods).

To monitor the localization of Npl3p, GFP was fused to its amino terminus, and the resulting fusion protein was placed under the *GAL1* promoter (Lee et al. 1996). GFP-Npl3p accurately reflects the behavior of its endogenous counterpart (Lee et al. 1996) under all conditions examined. The nuclear export assay was performed in the *nup49-313 ΔHMT1* strain, as well as the *nup49-313* strain. At the permissive temperature, GFP-Npl3p localizes entirely in the nucleus in both strains (Fig. 1 B,F). Although GFP-Npl3p accumulates in the cytoplasm of

Figure 1. Export of Npl3p and Hrp1p is blocked in the absence of Hmt1p. Either *nup49-313* cells (A–D and I–L) or the *nup49-313* strain in which *HMT1* has been deleted (E–H and M–P) were incubated in the export assay. The reporter used was GFP–Npl3p (pPS811; A–H) or GFP–Hrp1p (pPS1358; I–P). Cells were incubated at 25°C (A,B,E,F,I,J,M,N) or at 36.5°C (C,D,G,H,K,L,O,P) for 5 hr. Cells were photographed by use of Nomarski optics (A,C,E,G,I,K,M,O) or for GFP fluorescence (B,D,F,H,J,L,N,P).

the *nup49-313* cells at 36.5°C (Fig. 1D), the signal remains completely nuclear in the *nup49-313* Δ *HMT1* cells (Fig. 1H). As in the Δ *HMT1* strain (Henry and Silver 1996), Npl3p is no longer methylated in the double mutant (data not shown). These results indicate that the export of Npl3p out of the nucleus is affected by methylation.

To determine whether this effect extended to another shuttling hnRNP, Hrp1p (Henry et al. 1996; Kessler et al. 1997), the nuclear export assay was performed using GFP–Hrp1p as the reporter protein. The localization patterns observed for GFP–Hrp1p paralleled those seen previously with myc–Hrp1p (Henry et al. 1996), and the fusion protein has been shown to be functional (Kessler et al. 1997). When the cells were incubated at 25°C, GFP–Hrp1p was seen in the nucleus in both strains (Fig. 1J,N). As shown in our previous study (Kessler et al. 1997), GFP–Hrp1p accumulated in the cytoplasm in *nup49-313* cells (Fig. 1L) following shift to 36.5°C. Conversely, the fusion protein failed to leave the nucleus in the *nup49-313* Δ *HMT1* strain (Fig. 1P). These results demonstrate that nuclear export of Hrp1p, like that of Npl3p, is affected by methylation. The failure of GFP–Hrp1p and GFP–Npl3p to leave the nucleus at the restrictive temperature may be attributable to a slower rate of export in the strain lacking Hmt1p.

Hrp1p is methylated by *Hmt1p*

Previous work has shown that Hmt1p is required for Npl3p methylation and that this methylation occurs in the RGG-containing carboxyl terminus (Henry and Sil-

ver 1996; Siebel and Guthrie 1996). Because *HRP1* was originally identified because of its genetic interactions with *NPL3* and the encoded protein (Hrp1p) contains three RGGs, we tested whether Hmt1p was able to methylate Hrp1p as well. Recombinant Hmt1p and Hrp1p, both purified from *Escherichia coli*, were incubated with [methyl³H]-S-adenosylmethionine ([methyl³H]-SAM; see Materials and Methods). The proteins were resolved by SDS-PAGE and labeling was detected by fluorography. Under these conditions, Hrp1p is methylated by Hmt1p (Fig. 2A, lane 2). In addition, the assay was repeated with Npl3p purified from *E. coli* (Fig. 2A, lane 1). Quantitation of the incorporated label indicates that Npl3p is methylated approximately sixfold more than as Hrp1p. When the assay was performed in the absence of Hmt1p, no labeling of Npl3p or Hrp1p was detected (Fig. 2A, lanes 5,6).

Methylation is not required for the export of all hnRNPs

The novel hnRNP, *HRB1* (*TOM34*, YNL004w; Philippsen et al. 1997), was identified during the course of a screen for chromosomal mutations that are lethal in combination with a deletion of the nonessential nucleoporin *NUP2*. *HRB1* was cloned as a very weak suppressor of the synthetically lethal combination of Δ *NUP2* and a currently unknown mutant (M.S. Lee and P.A. Silver, unpubl.). The protein contains three RRM, and an amino-terminal domain that contains two repeats of the tripeptide RGG. *HRB1* is not essential for vegetative growth (see Materials and Methods).

To examine the subcellular localization of Hrb1p, an epitope-tagged version of this protein was generated with a single myc epitope fused to the carboxyl terminus of the protein. Tagged and untagged versions of Hrb1p un-

Figure 2. Npl3p, Hrp1p and Hrb1p are methylated by Hmt1p. Recombinant substrates Npl3p (lanes 1,5), Hrp1p (lanes 2,6), Hrb1p (lanes 3,7) were purified from *E. coli* and BSA was used as a negative control (lanes 4,8). Substrates were incubated with [methyl³H]-SAM in the presence (lanes 1–4) or absence (lanes 5–8) of Hmt1p purified from *E. coli*. Samples were resolved by SDS-PAGE. Proteins were detected by Coomassie staining (B) and labeling was detected by fluorography (A). Protein molecular weights are indicated in kilodaltons.

der the control of the inducible *GAL1* promoter were constructed and harbored in a strain lacking the chromosomal copy of *HRB1*. Indirect immunofluorescence microscopy performed on these cells with the anti-Myc monoclonal 9E10 indicates that Hrb1p-Myc is located primarily in the nucleus (Fig. 3I). This is similar to the localization of Npl3p-Myc (Fig. 3C). Virtually no staining is observed in cells containing Hrb1p lacking the epitope tag (Fig. 3F). Nuclear Hrb1p-Myc is also seen with a centromeric plasmid containing *HRB1* under its own promoter, although the signal is weaker (data not shown).

To examine whether Hrb1p exits the nucleus, the nuclear export assay was performed with a GFP-Hrb1p fusion (Fig. 4). The localization of GFP-Hrb1p was first examined in a wild-type strain. After induction of the fusion protein and incubation at either 25°C or 36.5°C, GFP-Hrb1p is found predominantly in the nucleus with some faint cytoplasmic signal (Fig. 4B,D). The same predominantly nuclear localization is seen when the *nup49-313* strain containing this reporter is induced followed by incubation at 25°C (Fig. 4F). When *nup49-313* cells are induced followed by a shift to 36.5°C, however, GFP-Hrb1p accumulates in the cytoplasm, indicating export from the nucleus (Fig. 4H). Furthermore, GFP-Hrb1p is still capable of export from the nucleus in a *nup49-313* strain lacking *HMT1* (Fig. 4L). These results indicate that although Hrb1p is located primarily in the nucleus, its localization represents a dynamic state within the cell. In addition, in contrast to the situation found with Hrp1p and Npl3p, the absence of functional Hmt1p has no effect on the ability of Hrb1p to exit the nucleus.

Hrb1p is methylated by Hmt1p

Because Hrb1p contains RGG repeats, we tested whether this protein is also methylated by Hmt1p. Recombinant

Hrb1p was purified from *E. coli* and incubated with [methyl³H]-SAM in the presence or absence of bacterially produced Hmt1p (Fig. 2). Reaction products were separated by SDS-PAGE, and labeling was detected by fluorography. In the presence of Hmt1p, Hrb1p is methylated (Fig. 2A, lane 3). When Hmt1p is omitted from the reaction, no labeling of Hrb1p is seen (Fig. 2A, lane 7). Note that in the autoradiograph in Figure 2A, lanes 3–8 were exposed eight times longer than Figure 2A, lanes 1 and 2, although equal amounts of substrate protein were present in each reaction (Fig. 2B). Quantitation of the amount of radioactivity incorporated into the individual substrate in each reaction indicates that Npl3p contains 26 times as much label as Hrb1p. Similarly, Hrp1p contains 4.5 times as much label as Hrb1p.

Nuclear protein export and RNA synthesis

Because the nuclear export of Npl3p requires ongoing RNA synthesis, we tested whether the same is true for Hrp1p and Hrb1p. To accomplish this, we employed a temperature-sensitive allele of RNA Pol II termed *rpb1-1* (Nonet et al. 1987). In the *rpb1-1* strain, RNA Pol II transcription is inhibited at the nonpermissive temperature. The nuclear export assay was performed with a strain containing both the *nup49-313* and *rpb1-1* mutant alleles (Lee et al. 1996). Consistent with previous observations (Lee et al. 1996), GFP-Npl3p failed to exit the nucleus in this strain at the nonpermissive temperature (Fig. 5D). Furthermore, both GFP-Hrp1p and GFP-Hrb1p also failed to accumulate in the cytoplasm (Fig. 5, H and L, respectively) at the nonpermissive temperature. This result is not attributable to a lack of protein synthesis because, when the assay was performed in the presence or absence of the protein synthesis inhibitor cycloheximide, GFP-Hrp1p or GFP-Hrb1p accumulated in the cytoplasm at equal levels (data not shown). These results indicate that RNA Pol II transcription, but not new protein synthesis, is required for the export of Hrp1p and Hrb1p from the nucleus.

Both Npl3p and Hrp1p bind poly(A)⁺ RNA (Wilson et al. 1994; Kessler et al. 1997). Hrb1p was also examined for its ability to bind to poly(A)⁺ RNA by irradiation of cells with ultraviolet light to crosslink RNA to proteins. Poly(A)⁺ RNA was then isolated from these cells and analyzed by Western blotting for the presence of Myc-tagged proteins. Hrb1p was not detected in the poly(A)⁺ RNA fraction when expressed either from its own promoter or from the high expression *GAL1* promoter under conditions in which Npl3p was readily detectable (data not shown).

Suppression of export defects by HMT1

We previously generated and characterized a temperature-sensitive allele of *NPL3*, *npl3-17* (Lee et al. 1996). This mutant displays a fully recessive phenotype that includes accumulation of poly(A)⁺ RNA in the nucleus at the nonpermissive temperature. This allele contains

Figure 3. Hrb1p is a novel nuclear hnRNP. Cells harboring plasmids expressing Npl3p-Myc expressed from its endogenous promoter (A–C), or Hrb1p (D–F) and Hrb1p-Myc (G–I) under control of the inducible *GAL1* promoter were subjected to immunofluorescence microscopy with the 9E10 monoclonal antibody. The Myc-tagged proteins were visualized with FITC labeled anti-mouse antibody (C,F,I). DNA was visualized using DAPI (B,E,H). Cells were photographed by use of Nomarski optics (A,D,G).

Figure 4. Hrb1p export is unaffected by the absence of Hmt1p. Wild-type cells (A–D), *nup49-313* cells (E–H), and *nup49-313* Δ HMT1 double-mutant cells (I–L) containing the reporter plasmid pPS1341 expressing GFP-Hrb1p were subjected to the export assay. Cells were incubated at 25°C (A,B,E,F,I,J) or 36.5°C (C,D,G,H,K,L). Cells were photographed either using Nomarski optics (A,C,E,G,I,K) or for the GFP signal (B,D,F,H,J,L).

two amino-acid substitutions, one in the first RRM, phenylalanine 160 changed to leucine (F160L), and one in the second RRM, isoleucine 268 changed to threonine (I268T). Despite the presence of mutations in both RRMs, this protein can still be crosslinked to poly(A)⁺ RNA in vivo (data not shown).

Overproduction of Hmt1p either on a high-copy plasmid (Fig. 6a), or by induction with the strong *GAL1* promoter (data not shown) suppresses the temperature-sensitive growth defect of *npl3-17* cells. The associated RNA export defect in *npl3-17* is also suppressed by the overproduction of Hmt1p from a high-copy vector (data not shown) or with the *GAL1* promoter (Fig. 6b, panel H), but not by the vector alone (Fig. 6b, panel D). We have shown previously that the single mutation in the first RRM, F160L, when present in the GFP-Npl3p reporter protein (GFP-F160L) results in a mutant protein that is

defective in export from the nucleus (Lee et al. 1996). Taken together, these observations suggest the possibility that overproduction of Hmt1p rescues the temperature-sensitive growth and RNA export defects of *npl3-17* by restoring the ability of this mutant to exit the nucleus at the nonpermissive temperature.

To address this possibility, we constructed high-copy plasmids that carry *HMT1* under its own promoter in addition to the GFP-Npl3p or GFP-F160L fusion proteins under control of the *GAL1* promoter. We then compared the export of GFP-Npl3p and GFP-F160L in the nuclear export assay in the presence or absence of this additional plasmid-borne copy of *HMT1* (Fig. 6c). As we have reported previously, there is often a nuclear GFP-Npl3p signal still visible at the time that export is examined in the assay (Lee et al. 1996; Fig. 6c, panel B), indicating that not all the Npl3p was exported during the course of this experiment. Interestingly, when *HMT1* is included on the reporter plasmid, the accumulation of GFP-Npl3p in the cytoplasm is enhanced and most of the nuclear signal is gone (Fig. 6c, panel D). This result suggests that the methylation of Npl3p is limiting in this assay, possibly because of the high overexpression of the GFP-Npl3p reporter in this system. Consistent with previous observations, the GFP-F160L reporter fails to accumulate in the cytoplasm after temperature shift in the export assay (Lee et al. 1996; Fig. 6c, panel F). The presence of *HMT1* on the reporter plasmid, however, was able to rescue the defective export of GFP-F160L, resulting in accumulation of this reporter in the cytoplasm (Fig. 6c, panel H). These results indicate that overproduction of Hmt1p rescues the defective nuclear export of the *npl3-17* temperature-sensitive mutant.

Figure 5. Export is blocked in the absence of RNA Pol II transcription. Double-mutant *nup49-313 rpb1-1* cells carrying reporter plasmids expressing GFP-Npl3p (A–D), GFP-Hrp1p (E–H), or GFP-Hrb1p (I–L) were subjected to the export assay. Cells were incubated at 25°C (A,B,E,F,I,J) or 36.5°C (C,D,G,H,K,L) for 5 hr. Living cells were photographed by Nomarski optics (A,C,E,G,I,K) or for the GFP signal (B,D,F,H,J,L).

Synthetic lethality with HMT1

Given the role of Hmt1p in the export of Npl3p and Hrp1p from the nucleus, it is puzzling that *HMT1* is not an essential gene. To identify genes that function in conjunction with Hmt1p in nuclear export, a synthetic lethal screen was conducted with a strain that contains a

Figure 6. Overproduction of Hmt1p rescues the export defects of *npl3-17*. (a) A *npl3-17* strain was transformed with the following plasmids and streaked on synthetic complete plates lacking leucine and containing glucose as the carbon source: CEN vector alone (pRS315; panels A,C), CEN *HMT1* (pPS1305; panels B,D), 2μ vector alone (YE351; panels E,G), 2μ *HMT1* (pPS1308; panels F,H). Plates were incubated at either 25°C (panels A,B,E,F) or 36°C (panels C,D,G,H) for 3 days. (b) *npl3-17* cells harboring *GAL1* vector pPS311 (panels A–D) or *GAL1* *HMT1* pPS1141 (panels E–H). Transformants were induced for protein expression for 2 hr then incubated at 36°C for 4 hr, fixed with formaldehyde and processed for in situ hybridization and immunofluorescence microscopy to detect Npl3p (panels C,G) and poly(A)⁺ RNA (panels D,H). DNA was visualized by DAPI staining (panels B,F), and cells were photographed by use of Nomarski optics (panels A,E). (c) *nup49-313* cells containing GFP–Npl3p (pPS811; panels A,B), GFP–Npl3p with *HMT1* (pPS1348; panels C,D), GFP–F160L (pPS879; panels E,F), and GFP–F160L with *HMT1* (pPS1349; panels G,H) were subjected to the export assay. Cells shifted to 36.5°C for 5 hr are shown. Cells were photographed by use of Nomarski optics (panels A,C,E,G) and for the GFP signal (panels B,D,F,H).

deletion of the *HMT1* gene and a wild-type copy of *HMT1* on a *URA3* plasmid. This strain was mutagenized with ethyl methanesulfonate (EMS) to kill ~50% of the cells. Cells containing mutations that are synthetically lethal with the *HMT1* deletion were selected by the colony color assay (see Materials and Methods). Following mutagenesis, only one recessive mutant was found to require the wild-type *HMT1* gene for viability. The corresponding wild-type gene was cloned from a yeast genomic library by screening for restoration of the sectoring phenotype. Subcloning of complementing plasmids localized the minimal region required to a 4.2-kb *Clal* DNA fragment. DNA sequence analysis and BLASTn searches revealed a single complete ORF that encodes the yeast homolog of the human cap-binding protein 80 (*CBP80*) (Izaurralde et al. 1994).

To establish linkage to the synthetic lethality, wild-type *CBP80* was marked on the chromosome with the *LEU2* gene in a Δ *HMT1* strain (see Materials and Methods). The resulting haploid was crossed to the synthetic lethal mutant strain. The diploid was sporulated, and tetrads were analyzed. Each spore carried the null allele of *HMT1*. Assuming linkage, the spores that carried the *LEU2*-marked wild-type copy of *CBP80* would be able to lose the *URA3 HMT1* plasmid. Those that carried the mutant form of *CBP80* from the synthetic lethal strain, however, would require the plasmid to remain viable. To

test for the ability to lose the plasmid, complete tetrads were streaked on plates containing 5-fluoro-orotic acid (5-FOA). The presence of 5-FOA in the plates selects against cells that carry the *URA3* plasmid. Fourteen tetrads were analyzed, and all displayed the behavior predicted for linkage; two *Leu*⁺ *FOA*^R; two *leu*[–] *FOA*^S spores.

Given that a mutant form of *CBP80* is synthetically lethal with the deletion of Δ *HMT1*, we also tested whether the null allele of *CBP80* would give the same result. It had been shown previously that *CBP80* is a nonessential gene and that the deletion of *CBP80* results in a slow growth phenotype (Uemera and Jigami 1992). We observed the same growth characteristics when constructing a null allele in our strain background (see Materials and Methods). A haploid containing the null allele of *CBP80* was crossed to the Δ *HMT1* strain. The diploid was sporulated and tetrads were dissected. Of the 47 tetrads analyzed, no spore with the genotype of Δ *CBP80* Δ *HMT1* was found, indicating that the null alleles are also synthetically lethal. To verify this result, we introduced a copy of *HMT1* on a *URA3* plasmid into the Δ *HMT1* strain and crossed it to the Δ *CBP80*. The diploid was sporulated, and tetrads were subjected to analysis. The presence of the plasmid rescued the synthetic lethality. In addition, when tetrads were streaked on FOA plates, the double mutant failed to grow, confirming our

conclusion that $\Delta CBP80$ is synthetically lethal with $\Delta HMT1$ (Fig. 7).

Discussion

In this report we have investigated the effect of arginine methylation on yeast hnRNPs. Previous studies have shown that the essential shuttling hnRNP, Npl3p, is modified by the arginine methyltransferase, Hmt1p. We now demonstrate that Hmt1p is also able to methylate another essential shuttling hnRNP, Hrp1p. By use of a nuclear export assay, the ability of Npl3p and Hrp1p to exit the nucleus was shown to depend on their methylation by Hmt1p. The export of a novel shuttling protein, Hrb1p, however, is not impaired by lack of methylation. Taken together, these data indicate that methylation is critical for the export of certain proteins that bind mRNAs.

Our discovery of genetic interactions between *HMT1* and *NPL3*, in combination with our data that Npl3p is a substrate of Hmt1p, provide the first insight into at least one biological role of arginine methylation. We have been able to study the effect of methylation on Npl3p export by using a nuclear export assay that effectively uncouples export from reimport. Our results suggest that the rate of export is decreased in a strain lacking *HMT1*. Under the conditions of our assay, Npl3p does not leave the nucleus when *HMT1* is absent, thereby indicating a role for methylation in its export. Because *HMT1* is not an essential gene (Henry and Silver 1996), however, RNA export must still be occurring in vivo. One possibility is that when *NPL3* is not methylated, it does not exit the nucleus, and its role in export is provided by another protein that does not require arginine methylation by Hmt1p. In our assay, GFP-Npl3p needs to reach a threshold level in the cytoplasm before it can be visually de-

tected. In a strain lacking *HMT1*, this threshold is not attained, suggesting that Npl3p is exiting the nucleus at a slower rate. Consistent with this idea, when the nuclear export assay was performed with cells overexpressing Hmt1p, there was an increase in the cytoplasmic accumulation of GFP-Npl3p at the nonpermissive temperature (Fig. 6c, panel D). This result argues in favor of a role for methylation in the rate of export.

Is methylation affecting Npl3p export directly or through its effects on another protein? We have shown previously that overexpression of Hmt1p can suppress the temperature-sensitive growth of certain *npl3* mutant alleles (Henry and Silver 1996). In this study, we have found that overexpression of Hmt1p is able to suppress the temperature-sensitive growth and mRNA export defect associated with the *npl3-17* mutant allele. Suppression of these defects correlates with the restoration of the ability of mutant Npl3 to exit the nucleus. It is possible that the *npl3-17* protein product is undermethylated in comparison to wild-type Npl3p. Hypermethylation by overproduction of Hmt1p could be enhancing the activity of a partially defective Npl3 encoded by the mutant allele of *NPL3*. The suppression does not occur by bypassing the need for Npl3p because overexpression of Hmt1p is unable to suppress a null allele of *NPL3* (P. Chui and P.A. Silver, unpubl.). In sum, these data support the idea that methylation directly affects the export of Npl3p.

Interestingly, Npl3p is not the only protein whose export is affected by methylation. The export of another methylated mRNA-binding protein known to shuttle between the nucleus and the cytoplasm, Hrp1p, is also impaired when *HMT1* is missing. Hrp1p binds specifically to pre-mRNA and promotes its proper polyadenylation (Kessler et al. 1997). Furthermore, mutations in *HRP1* can suppress mRNA export defects associated with certain *npl3* temperature-sensitive alleles (Henry et al. 1996). It may be that, in addition to its role in polyadenylation, Hrp1p acts together with Npl3p to promote mRNA export. Methylation of Hrp1p is not required for reconstitution of the cleavage and polyadenylation reactions (Kessler et al. 1997), suggesting further that this modification functions specifically in nuclear export. Thus, the efficient export of at least two shuttling hnRNPs implicated in RNA processing and transport requires arginine methylation.

The idea that methylation by Hmt1p has a direct effect on the export of Npl3p and Hrp1p is supported by our studies of Hrb1p. This novel hnRNP-like protein maintains its ability to exit the nucleus in the presence or absence of Hmt1p. One possibility is that although Hrb1p is methylated in vitro, it may not be a bona fide substrate of Hmt1p in vivo. In addition, unlike Npl3p and Hrp1p, we do not detect Hrb1p bound to poly(A)⁺ RNA. Nevertheless, ongoing RNA Pol II transcription is required for nuclear export of Hrb1p. One explanation for these results is that Hrb1p binds a different Pol II transcript such as snRNAs. These results demonstrate that methylation does not affect the export of all shuttling proteins. Furthermore, the effect of methylation appears

Figure 7. Deletion of both *CBP80* and *HMT1* is lethal. One tetrad from a cross of $\Delta HMT1$ covered with wild-type *HMT1* and $\Delta CBP80$ is shown. Two spores are wildtype for both alleles (A,D), and two contain both deletion mutants (B,C). Spores were streaked on synthetic complete media lacking uracil (*top*) or containing 5-FOA (*bottom*). Plates were incubated at 25°C for 2 days.

to be specific for at least two proteins that bind poly(A)⁺ RNA.

There are several possibilities by which methylation could mediate an effect on a substrate. First, the modification could regulate protein–protein interactions. Methylation could lead to the formation and stabilization of an interaction or could disrupt a pre-existing interaction. Second, the modification could be involved in protein–RNA interactions (Calnan et al. 1991; Rajpurohit et al. 1994). It has been proposed that arginine methylation could regulate the specificity of RNA binding (Calnan et al. 1991). Alternatively, arginine methylation could also play a role in protein stability. It has been shown that methylated hnRNP A1 is more susceptible to trypsin digestion than its unmethylated counterpart (Rajpurohit et al. 1994). The stability of Npl3p, however, is unaffected in the absence of methylation (M.F. Henry and P.A. Silver, unpubl.). Finally, the presence of the N^G, N^G-dimethylarginine could help determine the localization of the modified protein (Pintucci et al. 1996) as demonstrated here. For instance, it could be mediating interactions of hnRNPs with the nuclear export machinery (Ullman et al. 1997).

Given the effect of methylation by Hmt1p on the exit of Npl3p and Hrp1p from the nucleus, it is surprising that *HMT1* is not an essential gene. However, we have discovered a situation where *HMT1* is essential. In strains containing a mutant allele of the gene encoding *CBP80*, *HMT1* is required for viability. Cbp80p and another protein termed Cbp20p form the cap-binding protein complex (CBC) (Izaurrealde et al. 1994; Lewis et al. 1996). Cbp80p and Cbp20p have been isolated in yeast by both genetic and biochemical approaches (Uemera and Jigami 1992; Colot et al. 1996; Gorlich et al. 1996). Homologs of both proteins have been identified in other organisms, including amphibia and insects (Izaurrealde et al. 1995; Visa et al. 1996b). Studies in *Xenopus* oocytes and HeLa cell extracts have shown that CBC mediates

efficient pre-mRNA splicing, as well as snRNA export (for review, see Lewis and Izaurrealde 1997). Studies of the association of Cbp20p with a specific hnRNP complex, the Balbiani ring particle in *C. tentans*, indicate that Cbp20p binds to nascent hnRNA very soon after transcription initiation and remains attached during translocation through the nuclear pore (Visa et al. 1996b). While the 5'-cap structure is not required for successful export, it has been shown to enhance the rate of mRNA export from the nucleus (Hamm and Mattaj 1990). Thus, the CBC is a good candidate for the role of the mediator of this effect.

How do Cbp80p, Hrp1p, and Npl3p all affect the export of mRNA from the nucleus? From our genetic data, it is possible that Cbp80p, Hrp1p, and Npl3p all act in a linear pathway of export, with each step being affected by Hmt1p (Fig. 8A). As mentioned previously, a mutant allele of *HRP1* is able to suppress a temperature-sensitive allele of *NPL3*. Suppression could occur by the ability of the mutant Hrp1p to compensate for the defective Npl3p. In this scheme, methylation would play a role at the different levels of mRNA metabolism including capping, polyadenylation, and export. First, Hmt1p has an effect on *CBP80* function, as seen by the synthetic lethal interactions. Second, Hmt1p interacts biochemically to modify Hrp1p, a protein necessary for cleavage and polyadenylation. Finally, Hmt1p also modifies Npl3p, a potential carrier of mRNA out of the nucleus. Successful completion of each step, mediated by Hmt1p, could lead to efficient export of the mRNA.

A molecular model supported by the data is that proteins such as Cbp80p, Npl3p, and Hrp1p package the RNA for export, and that methylation plays a role in the efficiency of this packaging (Fig. 8B). All three factors are known to exit the nucleus and to bind pre-mRNAs (Flach et al. 1994; Gorlich et al. 1996; Lee et al. 1996; Kessler et al. 1997), thus supporting the possibility that they could be actively involved in export. The presence

Figure 8. Models for *HMT1* function in pre-mRNA metabolism, packaging, and export. (A) Genetic data suggest that *HMT1* affects diverse functions in RNA metabolism including capping, polyadenylation, and export. (B) Hmt1p methylates at least two nuclear RNA binding proteins that play a role in the export of RNA, possibly providing a signal to facilitate association of properly packaged RNAs with the export machinery. Once in the cytoplasm, RNA binding proteins are released and re-enter the nucleus.

of proteins associated with capping and polyadenylation could lead to more efficient packaging and export of the RNA to the cytoplasm. Consistent with this concept, we have found that a null allele of *CBP80* is synthetically lethal with a temperature-sensitive allele of *NPL3* (E.C. Shen and P.A. Silver, unpubl.). In this case, the presence of one defective packaging protein would cause the cell to be sick but still viable. In the presence of more than one defective packaging protein, however, RNA export would be so impaired as to result in cell death.

In this packaging model, Hmt1p could affect the functions of these proposed carriers of mRNA in several ways. One possibility is that methylation of Hrp1p and Npl3p could be affecting the interaction of these factors with each other. In addition, the modification could mediate interactions between these proteins and Cbp80p, or other hnRNPs that are presumably binding pre-mRNAs. Formation of the proper export complex would be crucial for efficient exit of mRNA out of the nucleus. Another possibility is that methylation mediates interactions of these factors with the export machinery such as the recently identified nuclear export receptor, Xpo1p/Crm1p

(Adachi and Yanagida 1989; Fornerod et al. 1997; Neville et al. 1997; Ossareh-Nazari et al. 1997; Stade et al. 1997). Modification by Hmt1p could also be mediating the specificity of RNA binding by Hrp1p and Npl3p and hence formation of a functional transport unit. Lack of modification by Hmt1p could result in less efficient packaging of this transport unit, and lead to slower export of mRNA out of the nucleus.

Conservation of the players in the packaging model from yeast to mammals suggests that this model can be applied to higher eukaryotes. The knowledge that human arginine-methylated hnRNPs (Liu and Dreyfuss 1995) are involved in mRNA processing makes it likely that some are involved in the packaging of the RNA. Notably, some viruses exploit the cellular export machinery to facilitate the maturation of their RNAs. Finally, viral RNA-binding proteins such as the herpes simplex virus ICP27 protein are known to be arginine-methylated (Mears and Rice 1996). We should be able to extend our new understanding of the biological role of Hmt1p and arginine methylation to virally infected cells and the design of possible therapeutics.

Table 1. *Plasmids used in this study*

Plasmid	Features	Source
pETHisHrp1	HisHRP1 in pET15b	M. Kessler and C. Moore (Tufts Medical School, Boston, MA)
pET9c-NPL3	<i>NPL3</i> in pET9c	A. Krainer and A. Mayeda (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY)
pPS293	yeast <i>URA3</i> 2 μ <i>GAL1</i> promoter expression vector	Lee et al. (1996)
pPS311	yeast CEN <i>URA3</i> <i>GAL1</i> promoter expression vector	Flach et al. (1994)
pPS709	yeast 2 μ <i>URA3</i> <i>ADE3</i>	Koepp et al. (1996)
pPS811	yeast 2 μ <i>URA3</i> GFP-Np13p under <i>GAL1</i> promoter	Lee et al. (1996)
pPS877	<i>np13-17</i> allele in pRS306	Lee et al. (1996)
pPS879	yeast 2 μ <i>URA3</i> GFP-F160L under <i>GAL1</i> promoter	Lee et al. (1996)
pPS892	yeast 2 μ <i>URA3</i> <i>GAL1</i> GST fusion expression vector	Koepp et al. (1996)
pPS1141	<i>HMT1</i> ORF in pPS311	Henry and Silver (1996)
pPS1303	yeast 2 μ <i>URA3</i> <i>GAL1</i> sGFP fusion expression vector	this study
pPS1305	2.7-kb <i>XbaI</i> <i>HMT1</i> fragment in pRS315	Henry and Silver (1996)
pPS1307	2.7-kb <i>XbaI</i> fragment containing <i>HMT1</i> in pRS316	Henry and Silver (1996)
pPS1308	2.7-kb <i>XbaI</i> - <i>HindIII</i> <i>HMT1</i> fragment in YE351	Henry and Silver (1996)
pPS1319	<i>NdeI</i> - <i>Bam</i> HI His <i>HMT1</i> PCR fragment in PET15b	this study
pPS1320	1.8-kb <i>Bam</i> HI- <i>Avr</i> II <i>HRB1</i> fragment in pPS892	this study
pPS1321	1766-bp PCR product containing <i>HRB1</i> in pBSK ⁺	this study
pPS1323	2.3-kb <i>Bal</i> I- <i>Sal</i> I GST- <i>HRB1</i> fragment in pGEX-4T-1	this study
pPS1324	12.5-kb genomic insert in YCp50 (original <i>HRB1</i> clone)	this study
pPS1325	3.6-kb <i>Xho</i> I <i>HRB1</i> fragment from pPS1324 in pRS315	this study
pPS1327	2.6-kb <i>Spe</i> I- <i>Avr</i> II <i>HRB1</i> fragment in pRS315	this study
pPS1332	1.8-kb <i>Bam</i> HI- <i>Avr</i> II <i>HRB1</i> fragment in pPS293	this study
pPS1333	1.3-kb <i>HRB1</i> -myc PCR product in pPS1332	this study
pPS1340	1-kb <i>Bsa</i> BI- <i>Sal</i> I <i>HRB1</i> -myc fragment in pPS1327	this study
pPS1341	1.1-kb <i>HRB1</i> PCR product in pPS1303 (<i>HRB1</i> -sGFP)	this study
pPS1347	2.5-kb <i>Bam</i> HI <i>HMT1</i> fragment from pPS1307 in pPS709	this study
pPS1348	2.7-kb <i>Xba</i> I <i>HMT1</i> fragment from pPS1307 in pPS811	this study
pPS1349	2.7-kb <i>Xba</i> I <i>HMT1</i> fragment from pPS1307 in pPS879	this study
pPS1352	4.2-kb <i>Cl</i> aI fragment containing <i>CBP80</i> in pRS316	this study
pPS1353	<i>Pst</i> II- <i>Xho</i> I <i>CBP80</i> fragment from pPS1352 in pRS305	this study
pPS1356	3.5-kb <i>Sc</i> aI <i>NLP3</i> -myc fragment in pRS315	this study
pPS1357	<i>NPL3</i> -myc in pET9c-Np13	this study
pPS1358	<i>Hrp1</i> fused to the carboxyl terminus of GFP (pCGF-1C- <i>HRP1</i>)	Kessler et al. (1997)

Materials and methods

Plasmids and strains

Plasmids used in this study are listed in Table 1. Yeast strains used are listed in Table 2. The diploid JU4-2xJR26-19B (gift from Ed Hurt, Biochimie-Zentrum Heidelberg, Germany) was used to generate wild-type haploids PSY603 and PSY609. The *npl3-17* allele was integrated by homologous recombination. To create the *nup49-313 ΔHMT1* double mutant, *nup49-313* was mated to PSY1122, a strain created exactly as described for PSY865 (Henry and Silver 1996). A PCR strategy (Baudin et al. 1993) was employed to disrupt *HRB1* in the wild-type strain PSY615. After sporulation, all tetrads had four viable spores, indicating that *HRB1* is not essential for vegetative growth. All other strain constructions are described under headings for individual experiments.

Export assay

The nuclear export assay was performed exactly as described (Lee et al. 1996). Briefly, cells containing various reporter plasmids are induced for 2 hr in medium containing galactose to express the reporter followed by repression for 2 hr in medium containing glucose. Cells are then incubated at 25°C or 36.5°C for 5 hr and examined for GFP fluorescence.

Purification of recombinant proteins from *E. coli*

HisHmt1 was purified from *E. coli* by binding to a charged nickel column (Pharmacia). Bound proteins were eluted with

500 mM NaCl, 50 mM EDTA and dialyzed against buffer B (20 mM MOPS at pH 7.2, 50 mM NaCl, 2 mM EDTA, 5 mM DTT) containing 50 mM EDTA, then against buffer B. HisHrp1 was purified from *E. coli* like HisHmt1 except that eluted proteins were dialyzed against buffer C (20 mM potassium phosphate at pH 7.4, 50 mM KCl, 0.2 mM EDTA, 5 mM DTT) containing 50 mM EDTA and then against buffer C. HisHrp1 was then bound to a SP Sepharose column (Pharmacia) equilibrated with buffer C, and eluted with a step gradient of buffer C containing 250 mM, 500 mM, and then 750 mM KCl. Proteins were then dialyzed into buffer D (20 mM Tris at pH 8.0, 50 mM KCl, 0.2 mM EDTA, 5 mM DTT). GST-Hrb1p was purified from *E. coli* by affinity to glutathione-sepharose (Pharmacia) and released from GST by cleavage with thrombin (Sigma) in the presence of 20 mM glutathione. Free GST was removed by incubation with glutathione-agarose, and the remaining protein was dialyzed against methylation assay buffer (50 mM MOPS at pH 7.2, 2 mM EDTA, 300 mM NaCl). HisHmt1p, HisHrp1p, and Hrb1p were concentrated with a Centriprep-10 ultra centrifugation unit (Amicon). Npl3P-Myc was purified from *E. coli* by cesium chloride gradient fractionation as described (Krainer et al. 1991) by monitoring fractions with the mouse monoclonal anti-c-Myc 9E10 antibody. Fractions containing Npl3p-Myc were purified further by anion-exchange chromatography on a Mono-Q column (Pharmacia) and cation-exchange chromatography on a Mono-S column (Pharmacia).

Methylation assay

Methylation of recombinant proteins was performed as de-

Table 2. Yeast strains used in this study

Strain	Genotype	Source
FY23	<i>MATa ura3-52 leu2Δ1 trp1Δ63</i>	Fred Winston (Harvard Medical School, Boston, MA)
<i>nup49-313</i>	<i>MATα nup49::TRP1 ura3 leu2 his3 ade2 ade3</i> plus pUN100- <i>nup49^{ts}-LEU2</i>	Doye et al. (1994)
CH1305	<i>MATa ura3 leu2 his3 ade2 ade3 can1</i>	Kranz and Holm (1990)
JU4-2 × JR26-19B	<i>MATa/α ura3-52/-leu2-3/-his3/+ his4/+ trp1/+lys1-1/-ade2-1/- ade8/+ can1-100/-</i>	Ed Hurt
PSY603	<i>MATα ura3-52 leu2-3 his4 trp1 lys1-1 ade2-1 ade8 can1-100</i>	this study
PSY609	<i>MATα ura3-52 leu2-3 his3 lys1-1 ade2-1 ade8 can1-100</i>	this study
PSY615	<i>MATa/α ura3-52/- leu2-3/-his3/- trp1/+ lys1-/- ade2-1/- ade8/-</i>	Henry et al. (1996)
PSY814	<i>MATa npl3::HIS3 ura3-52 leu2-3 his3 trp1 lys1-1 ade2-1 ade8</i> plus YCpNPL3-3	Henry et al. (1996)
PSY865	<i>MATα hmt1::HIS3 ura3 leu2 his3 lys1 ade2 ade8</i>	Henry and Silver (1996)
PSY902	<i>MATa/α ura3-52/- leu2Δ1/- his3Δ200/-trp1/+ lys2/+ade2/+ ade3/+</i>	this study
PSY992	<i>MATa/α npl3::HIS3/npl3-17 ura3-52/- leu2-3/- his3/- trp1-1/+ lys1-1/- ade2-1/- ade8/-can1-100/-</i>	this study
PSY1023	<i>MATa npl3-17 ura3-52 leu2-3 his3 lys1-1 ade2-1 ade8 can1-100</i>	this study
PSY1096	<i>MATa hmt1::HIS3 nup49::TRP ura3-52 leu2 lys⁻ ade2 ade8</i> plus pUN100- <i>nup49-313-LEU2</i>	this study
PSY1115	<i>MATa hmt1::HIS3 ura3 leu2 lys⁻ ade2 ade3</i> plus pPS1347	this study
PSY1116	<i>MATa hmt1::HIS3 cbp80-1 ura3 leu2 lys⁻ ade2 ade3</i> plus pPS1347	this study
PSY1117	<i>MATa hmt1::HIS3 cbp80-1 ura3 leu2 lys⁻ ade2 ade3</i> plus pPS1351	this study
PSY1118	<i>MATα hmt1::HIS3 CBP80:LEU2 ura3 leu2 his3 lys1 ade2</i>	this study
PSY1119	<i>MATa cbp80::HIS3 ura3 leu2 his3 trp1 ade2</i>	this study
PSY1120	<i>MATα cbp80::HIS3 ura3 leu2 lys1 ade2 ade8</i>	this study
PSY1121	<i>MATa/α npl3::HIS3/NPL3 ura3-52/- leu2-3/- his3/- trp1-1/+ lys1-1/- ade2-1/- ade8/-can1-100/-</i>	this study
PSY1122	<i>MATa hmt1::HIS3 ura3 leu2 his3 lys1 ade2 ade8</i>	Henry and Silver (1996)
PSY1123	<i>MATa/α hrb1::HIS3/HRB1 ura3/- leu2/- his3/- trp1-1/+ lys1/- ade2/- ade8/-can1-100/-</i>	this study
PSY1124	<i>MATa hrb1::HIS3 ura3 leu2 his3 trp1-1 lys1 ade2 ade8 can1-100</i>	this study
PSY1129	<i>MATa npl3::HIS3 ura3-52 leu2-3 his3 trp1 lys1-1 ade2-1 ade8 can1-100</i> plus pPS1356	this study

scribed (Henry and Silver 1996). Briefly, the 40- μ l reaction contained 50 mM MOPS at pH 7.2, 2 mM EDTA, 300 mM NaCl, 30 μ M SAM, 5.5 μ Ci [methyl³H]-SAM (NEN Dupont, 80 Ci/mmol), and 3 μ g of substrate protein. Npl3p-Myc, HisHrp1p, and Hrb1p were prepared from *E. coli*, and the BSA control was purchased from Sigma. Reactions were initiated by the addition of 4 μ g of HisHmt1p or methylation assay buffer, and incubated at 30°C for 40 min. Reactions were terminated by the addition of SDS-PAGE sample buffer and boiling for 5 min at 100°C. Proteins were resolved by SDS-PAGE followed by Coomassie staining and fluorography.

Indirect immunofluorescence

Indirect immunofluorescence microscopy was performed exactly as described (Lee et al. 1996). Affinity purified rabbit anti-Npl3p (Bossie et al. 1992) was used at 1:1000, and 9E10 mouse monoclonal anti-Myc (Department of Molecular Biology, Princeton University, NJ) was used at 1:2. In situ hybridization to poly(A)⁺ RNA was performed exactly as described (Lee et al. 1996).

CBP80 synthetic lethal screen

To create the parental strain for the synthetic lethal screen, PSY865 was mated with CH1305 (Kranz and Holm 1990), diploids were selected, tetrads were dissected, and one Δ HMT1::HIS3 *ade2 ade3* spore was selected and transformed with pPS1347 to generate PSY1115. pPS1347, a 2 μ URA3 ADE3 HMT1 plasmid, was generated by subcloning of a 2.5-kb *Bam*HI fragment from pPS1307 (Henry and Silver 1996) into the *Bam*HI site of pPS709 (Koepp et al. 1996).

Cells were mutagenized with EMS to 50% killing. Approximately 53,000 colonies were screened. Three hundred and sixteen non-sectored colonies were streaked on fresh YEPD medium plates. One candidate satisfied all the criteria (Henry and Silver 1996; Koepp et al. 1996) for synthetic lethality with Δ HMT1. A 4.2-kb *Clal* fragment containing one complete ORF in pRS316 (pPS1352) was the minimal region required to restore sectoring. Linkage of the synthetic lethality to *CBP80* was checked by mating PSY1118 with PSY1116. The diploid was sporulated and tetrads analyzed for FOA sensitivity. PSY1118 contains a *LEU2* marked copy of *CBP80* generated by homologous recombination. To disrupt the *CBP80* gene, a PCR strategy was performed (Baudin et al. 1993). To check synthetic lethality between Δ HMT1 and Δ CBP80, PSY1119 was outcrossed three times to the wild-type strain PSY609. The resulting Δ CBP80 strain, PSY1120, was crossed to PSY865, and tetrads were analyzed.

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