Arginine methylation can affect both nucleocytoplasmic transport and protein-protein interactions of RNA-binding proteins. These effects are seen in cells that lack the yeast hnRNP methyltransferase (HMT1), raising the question of whether effects on specific proteins are direct or indirect. The presence of multiple arginines in individual methylated proteins also raises the question of whether overall methylation or methylation of a subset of arginines affects protein function. We have used the yeast mRNA-binding protein Npl3 to address these questions in vivo. Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry was used to identify 17 methylated arginines in Npl3 purified from yeast: whereas 10 Arg-Gly-Gly (RGG) tripeptides were exclusively dimethylated, variable levels of methylation were found for 5 RGG and 2 RG motif arginines. We constructed a set of Npl3 proteins in which subsets of the RGG arginines were mutated to lysine. Expression of these mutant proteins as the sole form of Npl3 specifically affected growth of a strain that requires Hmt1. Although decreased growth generally correlated with increased numbers of Arg-to-Lys mutations, lysine substitutions in the N terminus of the RGG domain showed more severe effects. Npl3 with all 15 RGG arginines mutated to lysine exited the nucleus independent of Hmt1, indicating a direct effect of methylation on Npl3 transport. These mutations also resulted in a decreased, methylation-independent interaction of Npl3 with transcription elongation factor Tho2 and inhibited Npl3 self-association. These results support a model in which arginine methylation facilitates Npl3 export directly by weakening contacts with nuclear proteins.

Protein-arginine methylation by type I methyltransferases, which add one or two methyl groups to one of the guanidino nitrogens of arginine, has been shown to affect a number of euarkotic processes including protein transport, transcription, and cell signaling (reviewed in Refs. 1–3). Although many substrates for arginine methyltransferases are RNA-binding proteins, to date methylation has been shown to have only relatively minor effects on the affinity of target proteins for RNA (4–7). Many studies, however, point to a role for arginine methylation in modulating protein-protein interactions (reviewed in Ref. 2). The observation of both positive and negative effects of arginine methylation on protein-protein interactions has led to models for roles of arginine methylation in cell signaling and transcription, through the modification of histones, RNA-binding proteins, signaling proteins, and proteins involved in transcription (1, 2, 8).

Over 25 years ago heterogeneous nuclear ribonucleoproteins (hnRNPs) were found to contain the majority of asymmetric dimethylarginine in HeLa cell nuclei (9). Subsequent studies of hnRNPs and related messenger RNA (mRNA)-binding proteins have revealed an intricate and evolving picture of nuclear mRNA metabolism from transcription to processing to nuclear export (10). Methylation has been implicated in the movement of hnRNPs across the nuclear envelope. In mammalian cells, arginine methylation has been linked to nucleocytoplasmic distribution of hnRNP2 (11). In yeast, deletion of the predominant arginine methyltransferase, termed Hmt1 or Rmt1, inhibits nuclear export of hnRNP-like proteins (12, 13). The molecular interactions underlying these effects of arginine methylation on protein transport, however, remain to be elucidated. The yeast hnRNP-like protein Npl3, a known Hmt1 substrate (14, 15), provides an excellent system for exploring these mechanisms, because of its extensive arginine-glycine-rich domain.

Npl3 is a major yeast mRNA-binding protein that shuttles between the nucleus and the cytoplasm (16, 17). Although it is predominantly nuclear at steady state, Npl3 has been implicated in processes in both compartments, from linking transcription and mRNA export (18, 19) to acting as a translational repressor (20). Npl3 contains two central RNA-recognition motifs (RRMs) and a C-terminal domain rich in arginine (R) and glycine (G), including 15 RGG tripeptides that are likely targets for arginine methylation (21). Whereas a number of mutations within the RRMs of Npl3 block mRNA export (22), the C terminus plays a role in the nucleocytoplasmic transport of Npl3 (17, 23). The initial discovery of a genetic interaction between the temperature-sensitive npl3-1 allele and a null

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† The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; CBC, cap binding complex; Cbp80, cap-binding protein, 80 kDa; DHB, 2,5-dihydroxybenzoic acid; DMA, dimethylamine; POA, 5-fluoroorotic acid; FTMS, Fourier transform mass spectrometry; HPA, 3-hydroxypropionilic acid; ICAS, internal calibration on adjacent samples; MALDI, matrix-assisted laser desorption/ionization; PAD, protein arginine deiminase; PrA, protein A; RRM, RNA-recognition motif; TREX, transcription-export.

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Arginine Methylation of Yeast mRNA-binding Protein Npl3 Directly Affects Its Function, Nuclear Export, and Intranuclear Protein Interactions*§
allele of HMT1 (14), led to experiments showing that Hmt1 facilitates nuclear export of Npl3 (12). In addition, the interaction of Npl3 with two nuclear proteins, Npl3 itself and transcription elongation factor Tho2, is decreased by the presence of the arginine methyltransferase (19). These interactions, combined with the recruitment of both Npl3 and Hmt1 to actively transcribed genes (18, 19), suggest that Npl3 and Hmt1 may be combined with the recruitment of both Npl3 and Hmt1 to actively facilitate nuclear export of Npl3 (12). In addition, the interactions of arginines important for protein function? We have used mass spectrometry, mutagenesis and assays for Npl3 activity, transcription and amino-acid substitutions in a conserved protein complex named TREX (transcription-remodeling complex). The TREX complex, which associates with RNA-binding proteins Yra1 and Sub2, forms a plethora of factors influencing mRNA export, a key question in vivo.

Here we present mass spectrometric evidence for methylation of all arginines within RGG tripeptides and of two arginines in RG dipeptides, with variable levels of methylation detected for at least five locations. We present evidence that overall methylation is important for protein function through the introduction of systematic arginine-to-lysine mutations in RGG tripeptides followed by tests of Npl3 function in a yeast strain that requires Hmt1. We also describe experiments that suggest that arginine methylation has a direct effect on Npl3 export, its self-association and its interaction with Tho2, through the use of a mutant Npl3 protein with all RGG arginines changed to lysines (Npl3-RK1-15). These data support a model in which methylation directly affects Npl3 export by weakening contacts with nuclear proteins.

**Experimental Procedures**

**Yeast Strains and Plasmids—**Yeast strains used in this study are listed in Table I. All strains were grown and genetic manipulations performed as previously described (30). YAM505 (pSil3-cbp80Δ) was obtained by mating YAM159 (cbp80Δ) and PSY814 (pSil3). The strains used for mass spectrometric analysis, which express the protein A (PrA)-Npl3 fusion protein as the sole form of Npl3, were constructed by transforming the PrA-Npl3 plasmid pPS2389 into pPS2389-His3 (18) and npl3Δ::mttΔ (PSY1943), respectively, and selecting for loss of the URA3 maintenance plasmid, resulting in strains YAM569 and YAM570. Plasmids used in this study are listed in Table II. Oligonucleotides used for plasmid construction and sequencing were synthesized at Integrated DNA Technologies, Inc. and are shown in Table III. Mutations that result in arginine-to-lysine substitutions within RGG tripeptides (RK mutations) were introduced by QuikChange mutagenesis (Stratagene). The seven oligonucleotide primer pairs that targeted RGG arginines 1–14 introduced two RK substitution mutations while the final pair (RK15, RK16) mutated the fifteenth RGG arginine. All primers also introduced silent base pair changes that resulted in restriction sites for identification of positive clones. The order of primer pairs used was RK9/10, RK7/8, RK5/6, RK 1/2, RK3/4a, RK11/12, RK13/14, RK15/16. All mutations were verified after each round of mutagenesis by sequencing the 3′-end of NPL3 (including the entire RGG coding region and 3′-untranslated region); the entire NPL3 open reading frame was sequenced in the final npl3-RK1-15 plasmid, pAM409. The only additional mutation detected in this region was the insertion of an extra T in a stretch of 10 T nucleotides in the 3′-untranslated region in plasmids pAM407-pAM409. Given the identical growth phenotypes seen in such studies and the numerous targets for arginine methylation raise two questions. First, are the effects of removing the methyltransferase direct, through eliminating Npl3 methylation, or indirect, through eliminating methylation of other substrates? Second, given the presence of an extensive arginine-glycine-rich domain in Npl3, is overall methylation of the domain or methylation of a subset of arginines important for protein function? We have used mass spectrometry, mutagenesis and assays for Npl3 activity, transport, and protein-protein interactions to address these questions in vivo.

| Strain                  | Genotype                        | Source                  |
|------------------------|---------------------------------|-------------------------|
| nup49–313              | MATα nup49::TRP1 ade2 ade3 ura3 leu2 his2 + pUN100-nup49ts-LEU2 | (62)                    |
| PSY865                 | MATα hmt1Δ::HIS3 ade2 ade8 ura3 leu2 his3 lys1 | (14)                    |
| PSY867                 | MATα ade2 ade8 ura3 leu2 his3 lys1 | (14)                    |
| PSY814                 | MATα npl3Δ::HIS3 ade2 ade8 can1 ura3 leu2 his3 lys1 trp1 + YCP50-NPL3-3 | (63)                    |
| PSY196                 | MATα hmt1Δ::HIS3 nup49::TRP1 ade2 ade8 ura3 leu2 lys2 + pUN100-nup49ts-LEU2 | (12)                    |
| PSY1943                | MATα npl3Δ::HIS3 hmt1Δ::HIS3 ade2 ade8 ura3 leu2 his3 lys1 + YCp50-NPL3-3 | (12)                    |
| PSY3210                | MATα THO2-9xmyc::TRP1 ura3 leu2 his3 lys1 trp1 | (19)                    |
| PSY3211                | MATα hmt1Δ::HIS3 THO2-9xmyc::TRP1 ura3 lys1 | (19)                    |
| YAM519                 | MATα cbp80Δ::URA3 ade2 ade2 leu2 his3 | This study              |
| YAM505                 | MATα npl3Δ::HIS3 cbp80Δ::URA3 ade2 ade8 can1 ura3 leu2 his3 lys1 | This study              |
| YAM533                 | MATα hmt1Δ::HIS3 NPL3-RK1–15::URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| YAM534                 | MATα hmt1Δ::HIS3 npl3-RK1–15::URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| YAM535                 | MATα NPL3-myc::URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| YAM536                 | MATα npl3-RK1–15-myc::URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| YAM537                 | MATα THO2-9xmyc::TRP1 NPL3::URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| YAM538                 | MATα THO2-9xmyc::TRP1 npl3-RK1–15·URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| YAM539                 | MATα hmt1Δ::HIS3 THO2-9xmyc::TRP1 NPL3::URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| YAM540                 | MATα hmt1Δ::HIS3 THO2-9xmyc::TRP1 npl3-RK1–15·URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| YAM569                 | MATα npl3Δ::HIS3 ade2 ade8 can1 ura3 leu2 his3 lys1 trp1 + pPS2389 | This study              |
| YAM570                 | MATα npl3Δ::HIS3 hmt1Δ::HIS3 ade2 ade8 ura3 leu2 his3 lys1 + pPS2389 | This study              |

| Strain                  | Genotype                        | Source                  |
|------------------------|---------------------------------|-------------------------|
| PSY865                 | MATα hmt1Δ::HIS3 cbp80Δ::URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| PSY1943                | MATα hmt1Δ::HIS3 npl3-RK1–15::URA3 ade2 ade8 ura3 leu2 his3 lys1 | This study              |
| PSY3210                | MATα THO2-9xmyc::TRP1 ura3 leu2 his3 lys1 trp1 | (19)                    |
Arginine Methylation and Npl3 Function in Vivo

### Table II

| Plasmid | Features | Source |
|---------|----------|--------|
| pAM399  | CEN LEU pNop-PrA-npl3-RK7–10 AmpR | This study |
| pAM400  | CEN LEU pNop-PrA-npl3-RK5–10 AmpR | This study |
| pAM402  | CEN LEU pNop-PrA-npl3-RK1–2.5–10 AmpR | This study |
| pAM404  | CEN LEU pNop-PrA-npl3-RK1–10 AmpR | This study |
| pAM406  | CEN LEU pNop-PrA-npl3-RK5–14 AmpR | This study |
| pAM407  | CEN LEU pNop-PrA-npl3-RK1–12 AmpR | This study |
| pAM408  | CEN LEU pNop-PrA-npl3-RK1–14 AmpR | This study |
| pAM409  | CEN LEU pNop-PrA-npl3-RK1–15 AmpR | This study |
| pAM410  | 2μ URA3 pGal GFP-Npl3 AmpR | This study |
| pAM421  | URA3 NPL3 integrating AmpR | This study |
| pAM422  | URA3 Npl3-RK1–15 integrating AmpR | This study |
| pAM423  | URA3 NPL3-myc integrating AmpR | This study |
| pAM424  | URA3 Npl3-RK1–15-myc integrating AmpR | This study |
| pAM431  | CEN LEU pNop-PrA-npl3-RK1–6 AmpR | This study |
| pNCPPPATA | CEN LEU pNop-PrA AmpR vector | (36) |
| pPS430  | CEN LEU pGal NPL3-myc | (17) |
| pPS811  | URA3 Npl3-RK1–15 Integrating AmpR | (22) |
| pPS239  | CEN LEU pNop-PrA-NPL3 AmpR | (19) |
| pRS306  | URA3 AmpR vector | (32) |

### Table III

Oligonucleotides used in this study

| Oligo | Sequence (5’–3’) | Site/mutation |
|-------|------------------|---------------|
| AM215 | ATAGGCCGAAATTGGATCC | |
| AM216 | CCTGCGCTTATGTTGAAAG | |
| RK1   | CCAATCGAGAATTTATATATAAAGTGGCTCTTACAGGTTAGG | BglII (+) R3284K, R3290K |
| RK5   | CAGATGGATTTAACCCCTTACAGGGTTCG | DraI (+) R3294K, R3298K |
| RK3a  | GCTAAAGGCCGTTTCAAGGCGCCTTTATTAGTGG | DraI (+) R3294K, R3298K |
| RK4a  | CCTTAAACCTCCATTAAAGCGGCTTTTACC | DraI (+) R3294K, R3298K |
| RK5   | CAGATGGATTTAACCCCTTACAGGGTTCG | DraI (+) R3294K, R3290K |
| RK6   | CCAAGCCGCTTTGAGAGAAGCGCTTTTACGTC | DraI (+) R3294K, R3290K |
| RK7   | CGGATGCGCTTTAGGGAGAGGAGCTTTTACGTC | DraI (+) R3294K, R3290K |
| RK9   | CCGCTTTAGGCATAGTCTAGTGTTGCTCAGTCTTACAGGATTAGT | BatBI (+) R3293K, R337K |
| RK10  | CCGCTTTAGGCATAGTCTAGTGTTGCTCAGTCTTACAGGATTAGT | BatBI (+) R3293K, R337K |
| RK11  | CGGATGCGCTTTAGGGAGAGGAGCTTTTACGTC | BatBI (+) R3294K, R335K |
| RK12  | CCGCTTTAGGCATAGTCTAGTGTTGCTCAGTCTTACAGGATTAGT | BatBI (+) R3294K, R335K |
| RK13  | CGGATGCGCTTTAGGGAGAGGAGCTTTTACGTC | BatBI (+) R3294K, R335K |
| RK14  | CCGCTTTAGGCATAGTCTAGTGTTGCTCAGTCTTACAGGATTAGT | BatBI (+) R3294K, R335K |
| RK15  | CCGCTTTAGGCATAGTCTAGTGTTGCTCAGTCTTACAGGATTAGT | BatBI (+) R3294K, R335K |
| RK16  | CCGCTTTAGGCATAGTCTAGTGTTGCTCAGTCTTACAGGATTAGT | BatBI (+) R3294K, R335K |

containing Myc-tagged Tho2, plasmid pAM422 was constructed by ligating the 795-bp KpnI-BamHI fragment from pAM420 into KpnI-BamHI-digested pRS306. This plasmid, containing the last 175 codons of the NPL3 open reading frame, was linearized by HindIII digestion and transformed into PSY3210 and PSY3211. Ura+ colonies (YAM538 and YAM540) were tested for proper integration by PCR and sequencing. DNA sequencing was performed either at the University of Arizona DNA sequencing facility or GeneGateway. Similarly, the KpnI-BamHI fragment from pPS2389 was subcloned into pRS306 (resulting in pAM421), linearized with HindIII, and integrated into Myctagged THO2 strains to produce wild-type NPL3 control strains YAM537 and YAM539. Genomic Myc-tagged NPL3 and npl3-RK1-15 strains (YAM533-536) were produced by the same strategy with integration plasmids pAM423 and pAM424. These plasmids were constructed by ligating the 481-bp KpnI-NsiI fragment from pPS2369 or pAM402 and the ~300-bp NsiI-BamHI fragment from pPS430 (which contains a Myc tag inserted into the PmlI site at the 3′-end) into KpnI-BamHI-digested pRS306.

Npl3 Purification for Mass Spectrometry—PrA-Npl3 was purified from HMT1 and hmt1Δ cells according to published protocols (31). In brief, spheroplasts were prepared from either wild-type (YAM569) or hmt1Δ (YAM570) cells expressing PrA-Npl3, stored at −80°C, and lysed by Dounce homogenization in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml each of pepstatin, leupeptin, antipain, and chymostatin). To affinity purify PrA-Npl3, IgG-Sepharose beads (Amersham Biosciences) were incubated with clarified lysates at 4 °C for 1.5–2 h and, after extensive washing, Npl3 was eluted using the Tobacco Etch Virus (TEV) protease or PrA-Npl3 was eluted with acetic acid. Npl3 protein was concentrated by trichloroacetic acid precipitation (15%, v/v), resolved by SDS-PAGE and observed by Coomassie Brilliant Blue or zinc staining (Bio-Rad zinc stain kit).

**Proteolytic Digests and MALDI-FTMS Analysis**—PrA-Npl3 or Npl3 was excised from gels, desalted with 50% methanol, 5% acetic acid (Coomassie Blue stain) or Bio-Rad zinc destain solution (according to the manufacturer’s instructions) prior to proteolytic digestion. In-gel trypsin digests were performed according to the UCSF in-gel digest procedure using 12.5 μg/trypsin (Sigma) in 25 mM ammonium carbonate. Chymotryptic digests of purified protein (in-gel) or tryptic fragments were performed in 25 mM ammonium carbonate (pH 7.8) or in 100 mM Tris-HCl/10 mM CaCl2 with a 1:20 to 1:200 chymotrypsin (Sigma) to substrate ratio. Reactions were stopped by boiling digests or adding formic acid to a final total volume of 5%. Digests were incubated at 30 °C or 37 °C overnight. After incubation, each digest solution was removed, and the remaining peptides extracted with 50% acetonitrile, 5% formic acid. Digest solutions and extracts were pooled, lyophilized, and stored at −20 °C. Some digests were further purified with C18 Zip Tips® (Millipore).

For mass spectrometric analysis, dried peptides were diluted in 0.1% trifluoroacetic acid, 50% acetonitrile and loaded onto a MALDI probe tip (HPA) matrix (0.5M in 50% acetonitrile, 0.1% trifluoroacetic acid). Some DHB matrix solutions also contained 0.5M fructose or fucose. Samples were then air-dried and analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS).
Arginine Methylation and Npl3 Function in Vivo

The peptide peaks attributed to the sequence where modifications were detected are shown; complete peak list is available as Supplementary Data. DHB was used as the MALDI matrix unless otherwise noted. Monoisotopic masses, [M+H]+ or fragment, are shown. Dimethylated arginines are shown in bold and underlined; monomethylarginines are underlined; modifications that cannot be localized to one residue are in italics.

TABLE IV

Summary of peptide masses detected in the in-gel tryptic digest of Npl3 using MALDI-FTMS

| Observed mass | Predicted mass | Error | Residue numbers | Peptide sequence | No. C18 | Notes |
|---------------|----------------|-------|-----------------|-----------------|---------|-------|
| 905.4843      | 905.4840       | 0.4   | 258-264         | (R) LNNIFPR     | 0       |       |
| 860.4863      | 860.4836       | 3.1   | 265-272         | (R) GIVTVGER    | 0       |       |
| 863.4057      | 863.4052       | 1.8   | 275-280         | (D) NPPFPR      | 2       | 5     |
| 821.4419      | 821.4377       | 5.1   | 282-288         | (R) SNOGFR      | 2       | 2, 1.5|
| 776.3902      | 776.3798       | 0.7   | 282-388         | (R) SDNIFR      | 2       |       |
| 1351.7370     | 1351.7342      | 2.1   | 282-293         | (R) SFRGFRQGFRQGF | 6 |       |
| 1306.6805     | 1306.6763      | 3.2   | 282-305         | (R) SFRGFRQGFRQGF | 6 | 1     |
| 2687.4612     | 2687.4654      | -1.6  | 282-305         | (R) SFRGFRQGFRQGF | 12 | 2    |
| 2642.4066     | 2642.4076      | 3.4   | 282-305         | (R) SFRGFRQGFRQGF | 12 | 1    |
| 2041.1445     | 2041.1467      | 0.9   | 282-305         | (R) GOFPRQGFRQGF | 10 | 2    |
| 1996.0838     | 1996.0889      | -2.5  | 285-302         | (R) DFQGFRQGFRQGF | 10 | 1    |
| 1354.7502     | 1354.7491      | 0.8   | 291-302         | (R) GFFGFRQGF   | 6       |       |
| 1309.6925     | 1309.6912      | 1.0   | 291-302         | (R) GFFGFRQGF   | 6       |       |
| 2157.0778     | 2157.0729      | 1.9   | 308-329         | (R) GGFQFGFRQGF | 6       | 1     |
| 2112.0145     | 2112.0158      | -0.6  | 308-329         | (R) GGFQFGFRQGF | 6       | 1     |
| 844.3974      | 844.3948       | 3.1   | 322-329-330-337 | (R) GYGYSKR     | 2       |       |
| 359.2031      | 359.2037       | -1.8  | 349-351-356-358 | (D) APR         | 0       |       |
| 589.5986      | 589.5992       | 0.6   | 373-377-384-389 | (Y) GSPFR       | 0       | 3     |
| 1085.4672     | 1085.4647      | -0.9  | 378-388         | (R) GYGYSGYD    | 0       | 4     |
| 329.1929      | 329.1932       | -0.9  | 388-391         | (D) GPR         | 0       | 3     |

Notes: 1. peptide with loss of NH(CH2)2(OA); 2. HPA, not DHB, used as matrix; 3. cleavage C-terminal to aspartate; 4. cleavage C-terminal to aspartate with loss of H2O, 5. one dimethylated residue at either Arg264 or Arg266.

RESULTS

Identification of Methylated Arginines in Npl3—To investigate the molecular effects of arginine methylation on Npl3 function in vivo, it was necessary to identify those residues that are modified. Methylated and unmethylated Npl3 proteins were purified from HMT1 (YAM569) and hmt1Δ (YAM570) lysates, respectively, and analyzed following electrophoretic separation and in-gel protease digestion by MALDI-FTMS. FTMS is a high-resolution mass spectrometric technique that provides excellent mass accuracy (38).

Enzymatic digestions were performed with trypsin and chymotrypsin to increase sequence coverage. The most relevant mass spectral data for peptides from the C-terminal region of methylated Npl3 are reported in Tables IV and V. Complete peak analysis and representative mass spectra for Npl3 isolated from HMT1 and hmt1Δ cells can be found as supplemental data (JBC online). The data include peaks resulting from the loss of dimethylamine (DMA) from asymmetrically dimethylated arginine residues (39–41) and cleavages at aspartate residues (Asp-Xxx cleavage) (42, 43), which result from gas phase metastable decay on vacuum MALDI-FTMS instruments (33). Sequence coverage for the full methylated protein (74%) is summarized in Fig. 1, and details regarding the C-terminal RGG-rich region are described below.

The data shown in Tables IV and V and summarized in Fig. 1 provide direct evidence for dimethylation of 12 of 15 arginines in RGG tripeptides and indirect evidence for dimethylation of the other RGG motif arginines. In addition, these data provide evidence for variable levels of methylation for arginine.
Arginine Methylation and Npl3 Function in Vivo

The peptide peaks attributed to the sequence where modifications were detected are shown; complete peak list available as Supplementary Data. DHB was used as the MALDI matrix unless otherwise noted. Monoisotopic masses, [M+H]⁺ or fragment, are shown. Dimethylated arginines are shown in bold and underlined; monomethylarginines are underlined; modifications that cannot be localized to one residue are in italics; s indicates the phosphorylated serine. When more than one level of methylation was detected, the masses and number of methyl groups for the most intense peak are shown and the number of methyl groups for other peaks are shown in parentheses (see Supplementary Mass Spectral Data).

| Observed mass (Da) | Predicted mass (Da) | Error (ppm) | Residue numbers | Peptide sequence | No. CH₃ | Notes |
|-------------------|-------------------|------------|----------------|----------------|--------|-------|
| 842.4724          | 842.4730          | -0.8       | 264–271        | (F) RGSVITVE   | 0      | 4     |
| 705.4163          | 705.4155          | -1.1       | 288–293        | (F) RGSQGF    | 4      | 1     |
| 464.2628          | 464.2616          | 2.6        | 294–297        | (F) RSGF      | 2      |       |
|                   |                   | 298–301    | 302–305        |                |        |       |
| 551.2926          | 551.2936          | -1.8       | 306–310        | (F) SGGGF     | 2      |       |
| 1207.6325         | 1207.6331         | 0.5        | 306–317        | (F) SGGQGFPGF | 4      | 1     |
| 675.3573          | 675.3573          | 0.03       | 311–312        | (F) PGGF      | 2      |       |
| 1347.6949         | 1347.6916         | -2.4       | 311–324        | (F) PGGQFGGFQ | 4      | 1     |
| 691.3516          | 691.3522          | -0.01      | 318–324        | (F) PGGF      | 2      |       |
| 968.4571          | 968.4585          | 1.4        | 318–327        | (F) PGGQGYGQY | 2      |       |
| 1121.5021         | 1121.5011         | 0.9        | 325–335        | (Y) GYSGGYGQY | 2      |       |
| 844.3950          | 844.3948          | -0.2       | 325–332        | (Y) GYSGGYQY  | 2      |       |
|                   |                   | 328–335    | 328–332        | (Y) GYSGGYQY  | 2      |       |
| 567.2871          | 567.2885          | -2.5       | 328–332        | (Y) GYSGGYQY  | 2      |       |
|                   |                   | 336–340    | 336–340        | (Y) GYSGGYQY  | 2      |       |
| 681.3291          | 681.3315          | 3.5        | 341–347        | (Y) GYSGGYQY  | 2      | 0(0)  |
|                   |                   | 381–387    |                |                |        |       |
| 1385.6252         | 1385.6193         | -4.3       | 341–354        | (Y) GGSGQGDSPFGG | 0 (1,2) | 2, 5  |
| 1483.6533         | 1483.6561         | 1.9        | 348–361        | (Y) DSPGQDGFQGGQG | 0 (1,2) | 2     |
| 778.3490          | 778.3478          | -1.4       | 341–347        | (Y) GYSGGYQY  | 2      | 0(1)  |
|                   |                   | 381–388    |                |                |        | 4; isobaric with unmethylated 367–373 |
| 733.3265          | 733.3264          | -0.2       | 345–354        | (Y) DSPQFGQG  | 0      | 0(2)  |
|                   |                   | 355–361    |                |                |        |       |
| 664.3412          | 664.3411          | 0.2        | 349–354        | (D) SQFGQG    | 2      | 0(1)  |
|                   |                   | 356–361    |                |                |        |       |
| 1326.6189         | 1326.6185         | -1.2       | 382–373        | (Y) SGQGQGPRNDY | 2      | 2     |
| 597.2740          | 597.2440          | -0.1       | 367–372        | (Y) GPRNDYQPGQ | 0      | 4     |
| 1492.6943         | 1492.6928         | -1.1       | 367–380        | (Y) GPRNDYQPGQ | 0      | Isobaric with 333–347 with 3 CH₃ |
| 1506.7137         | 1506.7084         | -3.5       | 367–380        | (Y) GPRNDYQPGQ | 1      | Isobaric with 333–347 with 4 CH₃ |
| 1520.7291         | 1520.7241         | -3.3       | 367–380        | (Y) GPRNDYQPGQ | 2      |       |
| 924.4563          | 924.4574          | 1.2        | 373–380        | (D) YPPQFGQG  | 0      | 0(1)  |
| 2198.0075         | 2198.0001         | -2.9       | 381–401        | (Y) GGQDGQGDPQG | 2 (0,1)| 3     |
| 1535.6877         | 1535.6873         | -0.2       | 388–401        | (Y) DSPRGQGPDQDAY | 0     | 2     |
| 1677.8293         | 1677.8293         | -0.02      | 402–414        | (Y) TRDPAPEPRPTR | 0     | 2     |
| 1579.8520         | 1579.8524         | -0.2       | 402–414        | (Y) TRDPAPEPRPTR | 0     | 2, 7  |

Notes: 1, peptide with loss of NH(CH₃)₂ (DMA); 2, HPA, not DHB, used as matrix; 3, cleavage C-terminal to aspartate; 4, cleavage C-terminal to aspartate with loss of H₂O; 5, modification cannot be localized to one residue; 6, peptide with loss of H₂O; 7, peptide with loss of H₃PO₄.

Fig. 1. Location of methylarginine residues in Npl3. The amino acid sequence of Npl3 is shown. Residues detected in tryptic or chymotryptic peptides of methylated Npl3 are underlined once, and those detected in both digests are underlined twice. Dimethylarginine residues are marked with a filled circle; residues that are seen to be dimethylated with additional evidence for possible monomethylation or lack of methylation are marked with open circles.

Arginines in both RGG and RG contexts. There was no evidence for methylation of other arginine residues within or outside the RGG domain of Npl3 isolated from wild-type cells. The lack of evidence for methylarginine residues in Npl3 isolated from hmt1Δ cells (see Supplemental Data) also suggests that neither of the other two known Saccharomyces cerevisiae protein arginine methyltransferases, Hsl7 (44) and Rmt2 (45), methylates Npl3 in vivo.

Asymmetrical dimethylation of arginines Arg³⁴ through Arg³⁴ is strongly supported by the detection of 14 peptides, with associated losses of DMA, in the tryptic and chymotryptic digests (Tables IV and V). Two of the detected peptides show
masses indicating cleavage C-terminal to phenylalanine (Table IV). Although trypsin has stringent specificity for arginine and lysine residues (46), the low mass measurement errors indicate that these peaks are correctly assigned. We also detected a peak at m/z 821.44 in the trypptic digest, with a DMA loss at m/z 776.39. These peaks likely specify a peptide sequence with only one arginine residue, Arg$_{284}$ or Arg$_{288}$, which is dimethylated. In contrast with evidence for this single peptide with one unmethylated arginine, four Arg$_{284}$/Arg$_{288}$ peptides in the tryptic and chymotryptic digests showed evidence for dimethylation of both residues. In summary, we have strong support for eight consecutive RGG motif arginines (Arg$_{359}$-Arg$_{359}$) that are exclusively dimethylated (no evidence for mono- or nonmethylated). Additionally, residues Arg$_{344}$ and Arg$_{288}$ are dimethylated, with the detection of one peptide providing evidence for nonmethylation of either Arg$_{284}$ or Arg$_{288}$.

Peaks from the chymotryptic, not trypptic, digest provided the most informative peptide peaks for arginines beginning with Arg$_{344}$ and extending through to the C terminus of the sequence (Table V). Our data provide strong evidence for the dimethylation state of arginines beginning at Arg$_{363}$ and proceeding through to the C terminus. Dimethylation of arginine Arg$_{363}$ is supported by the detection of a peptide at m/z 1326.62. The site of dimethylation is localized to Arg$_{363}$ by the detection m/z 597.24, which is found as a nonmethylated peak. The data support non-, mono-, and dimethylation of arginine Arg$_{377}$, which is located in an RG motif. The most intense peaks, which correspond to the nonmethylated product, appear at m/z 1492.69 and 896.43. Mono- and dimethylation are indicated by lower intensity peaks at m/z 1506.71 and 1520.73. While the m/z 1506.71 peak is not unique (isobaric with the chymotryptic peptide for residues 333–347), the m/z 1520.73 peak and m/z 910.44 and 924.46 fragments uniquely support mono- and dimethylation at this residue. Additionally, associated with the m/z 1506.71 and 1520.73 peaks are losses of H$_2$O and NH$_3$ that parallel those observed for m/z 1492.69 when it was detected in the nonmethylated Npl3 digest (see Supplemental Data), reflecting losses from aspartate and asparagine residues. Collectively, these data support our conclusion that Arg$_{377}$ is present in unmethylated, monomethylated, and dimethylated forms. The intensities of the unmethylated peptides peaks (m/z 1492.69 and 896.43) suggest that the RG motif Arg$_{377}$ is found largely in an unmethylated state.

The most intense peak attributed to the RGG motif arginine Arg$_{384}$, m/z 2198.00, reflects dimethylation of this residue; however, we also detect lower abundance peaks showing that this residue also appears in a mono- and nonmethylated state (Tables IV and V, and Supplemental Data). The data also provide strong support for our conclusion that the seven remaining arginine residues at the C terminus of the protein are unmethylated (Tables IV and V, and Supplemental Data), including an additional RG and two RXR arginines. Whereas methylated RXR motifs were found in poly(A)-binding protein II (47), none of the arginines in the three RXR motifs is methylated in Npl3. We also detect phosphorylation of the C terminus serine residue, in agreement with previous findings (48, 49).

Our results for arginine residues Arg$_{337}$, Arg$_{344}$, Arg$_{351}$, and Arg$_{358}$ are less definitive. Dimethylation of Arg$_{337}$ is supported by the exclusive detection of m/z 844.40, not m/z 816.36, in both the tryptic and chymotryptic digests; however, for both digests this mass reflects a redundant region of the sequence and does not uniquely specify the methylation state of Arg$_{337}$. Although indirect, we find no evidence to disprove the conclusion that this residue is dimethylated. For arginines Arg$_{344}$, Arg$_{351}$, and Arg$_{358}$ we find a significant number of peaks that suggest lower and variable levels of methylation associated with these three arginines. Two longer peptides, m/z 1385.62 (for Arg$_{344}$/Arg$_{351}$) and m/z 1483.65 (for Arg$_{344}$/Arg$_{351}$) (Table V) are detected as unmodified. In addition they are found as mono- or dimethylated peptides that appear at m/z 1399.63 and 1413.65 (for Arg$_{344}$/Arg$_{351}$) and m/z 1497.67 and 1511.69 (for Arg$_{344}$/Arg$_{351}$) (Supplemental Data). A number of lower mass peaks, attributed to shorter peptides containing one arginine residue, also show variable levels of methylation. These include the dimethylated versions at m/z 681.33 and 778.35 (for Arg$_{344}$/Arg$_{351}$) and 664.34 (for Arg$_{351}$/Arg$_{358}$) (Table V), as well as peaks including m/z 733.33 (Arg$_{351}$/Arg$_{358}$) that show zero or one methyl group addition (Table V and Supplemental Data). The peak at m/z 359.20 (Arg$_{351}$/Arg$_{356}$) from the trypptic digest (Table IV) was found exclusively as the unmethylated version. Because of the repetitive nature of sequences in this region and isobaric masses, we cannot distinguish contributions from each residue (Arg$_{344}$/Arg$_{351}$/Arg$_{358}$). We conclude that Arg$_{344}$, Arg$_{351}$, and Arg$_{358}$ are present in variable methylation states with evidence to suggest that di-, mono-, and unmethylated versions of these residues may be present, and use indirect evidence to support dimethylation of Arg$_{337}$.

In summary, the mass spectral data reveal direct evidence for dimethylation of 12–15 arginines in RGG tripeptides, and indirect support for dimethylation of an additional residue (Arg$_{337}$), as summarized in Fig. 1. Evidence was found suggesting variable levels of methylation for two RG dipeptides. In one case (Arg$_{377}$), lower abundance mono- and dimethylated peaks were localized to this residue. In the second case, dimethylation was present for all but one detected peptide, and for that instance Arg$_{288}$ (RG) could not be distinguished from Arg$_{288}$ as the unmethylated residue. While nine consecutive N-terminal RGG motif arginines were found to be exclusively dimethylated, we found evidence for variable levels of methylation associated with four C-terminal RGG motif arginines (Arg$_{344}$, Arg$_{351}$, Arg$_{358}$, and Arg$_{364}$). For Arg$_{344}$, Arg$_{351}$, and Arg$_{358}$ we were unable to localize the level of methylation to a specific residue because of redundant sequence masses. Additionally, there was no evidence of methylation of arginines outside the RGG domain.

Effect of Arginine-to-Lysine Mutations on Npl3 Function in Vivo—The presence of 15 RGG tripeptides within Npl3 raises the question of whether specific arginines or their methylation are important for Npl3 function or whether overall methylation of Npl3 plays a key role. With the identification of numerous methylated arginines in the C terminus of Npl3, we decided to mutate all the arginines within RGG contexts to lysines to test the effect of methylation on Npl3 function. The conservative Arg-to-Lys (RK) mutation retains the positive charge of the residue (Fig. 2A) while removing its ability to be targeted by the arginine methyltransferase (50). Arginines within RGG peptides have been numbered 1-15 from the N terminus to simplify mutant nomenclature (Fig. 2B). We created a set of 20 Npl3 mutants by adding two RK mutations at each round of mutagenesis (Fig. 2B). Mutations were introduced into a plasmid that expresses a functional protein A (PrA)-Npl3 fusion protein (pPS2389) to facilitate subsequent protein-protein interaction analysis (19).

RK mutants could impact protein function either due to the need for arginine at a certain position or to the importance of methylation of that residue. To distinguish between these two effects, we tested Npl3-RK mutants for function in two different strain backgrounds. Although most strains lacking HMT1 are viable, a strain in which the 80 kDa cap-binding protein gene (CBP80) has been deleted requires the arginine methyltransferase for growth (12). In addition, a cold-sensitive hmt1 allele identified in the cbp80Δ background showed a specific
defect in Npl3 methylation (29). These genetic and biochemical findings suggest a complementary role for Npl3 methylation and Cbp80 in these strains. Therefore, if Npl3 methylation is important in strains lacking Cbp80, Npl3-RK mutants, which should not be methylated, would be predicted to support growth of an npl3/H9004 strain but show poorer rescue of an npl3/H9004 cbp80/H9004 strain.

Fig. 3 shows the growth of the npl3Δ mutant (PSY814) expressing each of the Npl3-RK mutants. The Npl3 protein with all 15 arginines within RGG peptides mutated to lysines (RK1-15) is able to support growth of npl3Δ at 30 °C to the same extent as wild-type Npl3, with only a slight growth defect at 20 °C and 37 °C. Thus the RK mutants are still functional. The deletion of HMT1 does not affect the growth of strains expressing either wild-type PrA-Npl3 or PrA-Npl3-RK1-15 (data not shown), as expected. In contrast, the expression of PrA-Npl3-RK mutant proteins in the npl3Δ cbp80Δ strain (YAM505) has a significant effect on growth (Fig. 4). This effect is significantly more pronounced at low and high temperature (Fig. 4). Therefore in a background in which HMT1 is essential, the substitution of multiple lysines for arginines at methylation sites results in a significant loss of Npl3 function.

Although mutating two neighboring or a single RGG arginine (RK1–2, RK3–4, RK5–6, RK7–8, RK9–10, RK11–12, RK13–14, and RK15) showed no effect on Npl3 protein function as assessed by growth of npl3Δ cbp80Δ cells (data not shown), increasing the number of RK mutations increased the severity of the phenotype (Fig. 4). This result suggests that cumulative methylation of Npl3 is important for protein function. When mutations in the first and second RGG arginines were added to Npl3-RK5–10, however, there was a more severe effect on growth than when either mutations in arginines 11–12 or 11–14 were added (Fig. 4, 20 °C, 37 °C). This result suggests the relative increased importance of the N-terminal RGG arginines, particularly at low temperature where the effect is most pronounced. Indeed, while PrA-Npl3-RK1–6 supports equivalent growth to PrA-Npl3-RK5–14 at 37 °C, PrA-Npl3-RK1–6 grows significantly more poorly at 20 °C (data not shown). Therefore, although the total number of arginines that can be methylated is important for Npl3 function, it appears that the N-terminal RGG arginines may play a specific role.

Arginine-to-Lysine Mutations Result in Methylation-independent Export of Npl3—Because the arginine methyltransferase facilitates the export of Npl3 (12), we wished to use the Npl3-RK1–15 mutant to test whether the Hmt1 effect on export was direct, because of methylation of Npl3, or indirect, through the methylation of another protein. To examine export we used an established assay in which the import of GFP-Npl3 is blocked by shifting a strain with a mutation in a nuclear pore protein
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Arginine-to-lysine mutations in NPL3 are deleterious in the absence of the 80 kDa cap-binding protein. A strain lacking the 80-kDa cap-binding protein gene, YAM505 (npl3Δ cbp80Δ + NPL3 URA3), was transformed with PrA plasmids containing mutant forms of npl3 and positive (WT) and negative (PrA vector) controls. The function of mutant Npl3 proteins was tested by a plasmid shuffle assay as described in the legend for Fig. 3.

The nuclear export of GFP-Npl3-RK1-15 in the nup49-313 mutant with or without HMT1 is compared with that of wild-type GFP-Npl3 in Fig. 5. Like GFP-Npl3, GFP-Npl3-RK1-15 is predominantly nuclear in wild-type and hmt1/H9004 cells at 25 °C. The cytoplasmic GFP-Npl3-RK1-15 signal at high temperature in nup49-313 cells indicates that methylarginine residues in Npl3 are not required for its export (Fig. 5A). However, some cytoplasmic GFP-Npl3-RK1-15 is also seen at the non-permissive temperature in nup49-313 lacking the methyltransferase (nup49-313 hmt1Δ; PSY1096) as tested in A.

Methylation-independent Interaction of Npl3-RK1-15 with Tho2—Our results suggest that Npl3 should interact with nuclear molecules and that these interactions should be decreased by methylation. We have previously identified two molecular partners of Npl3 with these characteristics: the transcription elongation factor Tho2 and Npl3 itself (19). To test whether the Tho2 interaction is affected by the Npl3-RK1-15 mutations, this mutant was integrated into HMT1 and hmt1/H9004 strains expressing Myc-tagged Tho2. Following immunoprecipitation of Tho2-Myc from these strains and strains expressing wild-type Npl3, copurifying Npl3 was detected with a polyclonal Npl3 antiserum (Fig. 6). While wild-type Npl3 copurifies with Tho2-Myc from hmt1/H9004 strains (lane 1), the Npl3-RK1-15 mutant protein shows a reduced interaction with Tho2-Myc (lane 2). This reduced interaction is not caused by differential recognition by the Npl3 antiserum, which was raised against a truncated Npl3 lacking the RGG domain (37), nor is the mutant protein expressed at lower levels than the wild-type protein (Fig. 6, lysate). In addition, the Tho2-Myc interaction with wild-type Npl3 is not detected in HMT1 cells whereas the reduced interaction with Npl3-RK1-15 is still observed (lanes 3 and 4). These results support the possibility that lysine, like dimethylarginine, loosens a nuclear Npl3 complex that contains Tho2-Myc, which could facilitate export even in the presence of the arginine methyltransferase.

RK1-15 Mutations Block Npl3-Npl3 Interactions—To test the effect of the Npl3-RK1-15 mutation on Npl3 self-association, Myc-tagged forms of the wild-type and mutant NPL3 genes were integrated into hmt1Δ and HMT1 genes were integrated into hmt1Δ and HMT1 strains and the resulting strains transformed with the PrA-Npl3 plasmids. IgG-Sepharose was then used to isolate PrA-Npl3 and associated proteins and PrA-Npl3 and Npl3-Myc were detected with a polyclonal Myc antiserum. When the RK1-15 mutations are present in both forms of Npl3, no Npl3-Npl3 interaction is observed.
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**DISCUSSION**

Npl3 is a particularly interesting model to explore the effects of arginine methylation on protein function because of its extensive arginine-glycine-rich domain, which has 15 RGG, 3 RG, and 2 RAR peptides (Fig. 1). This long C-terminal domain allowed us to address the question whether overall methylation or methylation of a subset of arginines influenced protein function. Using mass spectrometry, we identified 16 arginine residues that are dimethylated in vivo, and one additional arginine that is likely to be dimethylated. Whereas evidence pointed to variable levels of methylation for two RG dipeptides and at least three RGG tripeptides, the vast majority of dimethylarginine in Npl3 is found in the RGG context (Fig. 1). Mutating these residues to lysine affected general Npl3 function only in a strain background that requires the methyltransferase. Whereas increasing the total number of lysine substitutions generally increased the severity of the growth phenotype, mutations in the four RGG tripeptides at the N terminus of the domain had a greater influence on Npl3 function than mutating RGGs more C-terminal (Fig. 4), suggesting a specific role for these residues.

The seven N-terminal RGG arginines are followed by phenylalanine (F) residues, including tandem RGGF repeats from Arg$_{280}$–Arg$_{302}$ (RGG2–5), with an average of two residues between RGG tripeptides (Fig. 1). The next seven RGGs are all followed by tyrosine residues, with an average spacing of four residues between RGG tripeptides. Although the three-dimensional structure of Npl3 has not been determined, these results suggest that the influence of arginine methylation or lysine substitution on Npl3 structure and activity may be greater at the N terminus of this domain where the arginines are more closely spaced in the primary structure.

Lysine substitutions are commonly used both to determine sites of arginine methylation and to probe the functional significance of methylarginine residues within a protein (27, 51–54). This choice is based on 1) both lysine and arginine being long chain amino acids with a positive charge at physiological pH and 2) lysine not being a target for protein-arginine methyltransferases (50). Although methylation should not alter the positive charge of arginine, asymmetric dimethylation increases the hydrophobicity of the residue, is likely to have steric effects on inter- or intramolecular contacts and may also influence hydrogen bonding (2, 6, 55). Whereas lysine maintains a positive charge and should not incur the same steric effects that occur upon arginine methylation, this substitution could still decrease arginine-specific contacts of the protein. The challenge of using RK mutations to distinguish between direct and indirect effects of arginine methylation on protein function is to try to separate effects of loss of arginine function from loss of methylation of the arginine in question.

The ability of lysine to substitute for arginine in the C-terminal domain of Npl3 is supported by the robust growth of npl3Δ strains expressing the Npl3-RK1-15 mutant protein and the steady-state nuclear localization of this protein (Figs. 3 and 5). Xu and Henry have also used an elegant cloning strategy to construct an Npl3 mutant with all RGG arginines mutated to lysine (Npl3(KGG)) by introducing similar missense mutations and four fewer silent mutations (27). They detected steady-state nuclear localization of Npl3(KGG) and reported that this protein was functional and was not methylated in vivo (27). In their experiments, Npl3(KGG) expressed from a plasmid rescued the synthetic lethality of an npl3-1 hmt1Δ strain, and we have also found that Npl3-RK1-15 supports growth of an npl3Δhmt1Δ strain (data not shown). These results were expected given the non-essential nature of HMT1 and the viability of the npl3Δ strain expressing Npl3-RK1-15. Because the arginine methyltransferase gene is not essential, these results do not address whether arginine methylation of Npl3 in particular has functional consequences for cell growth.

When a set of Npl3-RK mutant proteins are expressed in a strain lacking the 80-kDa cap-binding protein, however, mutant proteins with increased numbers of lysine substitutions show a more severe growth defect (Fig. 4). Because the cbp80Δ background requires HMT1 for viability, these results suggest that methylation of Npl3 in particular is important in this background. Whereas the cbp80Δ strain that expresses Npl3-RK1-15 grows slowly at 30 °C (Fig. 4), the deletion of HMT1 in the cbp80Δ background is lethal (12). This result could either reflect the importance of methylation of Arg$_{280}$ and Arg$_{377}$, which are variably methylated in vivo and were not mutated in Npl3-RK1-15, or methylation of another protein in the cbp80Δ strain. Alternatively, the greater severity of the hmt1Δ deletion may be explained by lysine exerting an intermediate effect, failing functionally between unmethylated and methylated arginine.

The importance of the Hmt1 arginine methyltransferase for the export of its mRNA-binding substrates Npl3, Hrp1, and Nab2 (12, 13) suggests a role for this post-translational modification in the process of directing export or remodeling nuclear complexes to facilitate export. Deletion analysis of Nab2 (56) and the identification of Npl3 protein-protein interactions that are decreased by methylation (19) pointed to the second model. Whereas Npl3 phosphorylation at a C-terminal serine plays a role in nuclear import by promoting dissociation from mRNA and binding to the Mtr10 import receptor (48, 49), in vitro data also suggest that Hmt1 has an indirect effect on Npl3 binding to Mtr10 (48). The identification of specific sites of methylation in Npl3 has now allowed us to test whether methylation effects on Npl3 export are direct or indirect.

Although the RGG domain of Npl3 is necessary for nuclear import (17, 23), the nuclear localization of GFP-Npl3-RK1-15 in nup49-313 strains at 25 °C (Fig. 5) indicates that these muta-
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Fig. 7. Arginine-to-lysine mutations block Npl3 self-association. Hmt1Δ and hmt1Δ strains expressing Myc-tagged wild-type (WT) or RK1-15 (RK) Npl3 proteins from the endogenous locus were transformed with PrA-Npl3 or PrA-Npl3-RK1-15 plasmids. YAM7 (hmt1Δ) with an untagged genomic NPL3 gene was used to control for PrA-Npl3 degradation (lanes 9 and 10). Cells were lysed, and lysates were normalized for total protein prior to isolation of PrA-Npl3-associated proteins with IgG-Sepharose beads. Bound proteins were eluted with 3 M MgCl₂, precipitated with trichloroacetic acid, resuspended in Laemmli buffer, and analyzed by anti-Myc immunoblotting. Npl3 protein levels in lysates are also shown.

Signals do not have a severe effect on Npl3 import into the nucleus. The Npl3-RK1-15 mutant protein also can exit the nucleus even in the absence of the methyltransferase, as also seen for Npl3(KGG) by Xu and Henry (27). This result suggests that, whereas methylation may block specific nuclear contacts made by arginine in Npl3, the lysine substitutions may promote export in this assay through their inability to mediate nuclear protein-protein interactions. Whereas RK mutations in Npl3 directly affect Npl3 export, similar mutations in the three RGG tripeptides in Hrp1 do not influence its export (27). Intriguingly, the expression of the Npl3(KGG) mutant protein facilitated the export of Hrp1 in the absence of Hmt1, suggesting that the methylation of Npl3 is important for the export of an interacting protein (27).

Npl3 copurifies with Hrp1 and the nuclear cap-binding complex, but these interactions are not influenced by the presence of Hmt1 (19, 26, 27). In contrast, interactions of Npl3 with Tho2 and Npl3 are affected either by the presence of the methyltransferase (19) or by the presence of RK mutations within Npl3 (Figs. 6 and 7), arguing for a direct effect of Npl3 methylation on nuclear protein complex formation. The Npl3 self-association is more severely affected by RK mutations than the Npl3-Tho2 interaction. This difference may be because of the C-terminal Myc tag used in self-association studies (see “Results”). Alternatively, different contacts may be involved in the two interactions, a likely possibility given the RNase sensitivity of the Npl3-Tho2 interaction and RNase resistance of self-association (19). Xu and Henry found that whereas HMT1 deletion did not affect UV-cross-linking of Npl3 to bulk poly(A) RNA in vivo, more Npl3(KGG) than wild-type Npl3 copurified with poly(A) RNA after cross-linking (27). Therefore, even though the RK mutations may decrease protein-protein interactions that are important for the copurification of Tho2 with Npl3, the Tho2-Npl3-RK1-15 interaction may still be detected because of increased binding of the mutant Npl3 to pre-mRNA. In combination these data suggest a model in which arginine methylation of Npl3 specifically modulates mRNP formation by loosening contacts with nuclear proteins, thereby facilitating export. The synthetic lethality of npl3Δ and cbp80Δ with hmt1Δ may reflect the combination of decreased binding of export-facilitating proteins to mRNA with stronger interactions of Npl3 with a specifically nuclear complex.

Chromatin immunoprecipitation experiments have also revealed interesting binding patterns for proteins involved in mRNA metabolism. Whereas Hmt1 and Npl3 are present at the promoter and toward the 5′-ends of highly transcribed genes (18, 19), polyadenylation factors bind preferentially to the 3′-end (57). TREX components are cross-linked to coding regions but binding is much lower at the promoter or downstream of polyadenylation signals (18, 25, 57). In addition, recent work has shown that mutations in Npl3 enhance transcription termination, pointing to a role for Npl3 in antagonizing mRNA 3′-end formation (58). Taken together, these results support a model in which a series of proteins associates with DNA and likely nascent mRNA at the site of transcription, with Npl3 associating near the beginning of transcription, followed by TREX components and then polyadenylation factors. By decreasing association with Tho2 and potentially other TREX proteins, methylation of Npl3 near the 5′-end of actively transcribed genes may help remodel mRNPs and thus facilitate export.

The discovery of protein-arginine deiminases (PADs) that can convert methylarginines to citrulline within histones (59, 60), raises the tantalizing possibility that arginine methylation may be reversible. Non-histone substrates of both type I and type II methyltransferases can also be targets for deimination (54, 61), but in vitro data suggest that arginine dimethylation inhibits deimination (60, 61). We did not detect citrulline residues within Npl3 by mass spectrometry and S. cerevisiae has no obvious PAD homolog. Therefore, whereas we cannot rule out the possibility that Npl3 deimination may allow increased nuclear interactions, Npl3 methylation is likely to have a more long term effect on the balance between transcriptional and export complexes.

Numerous type I arginine methyltransferase substrates are involved in mRNA transcription and export, from histones to transcription factors to hnRNPs. Therefore the various effects seen in hmt1Δ cells, from synthetic lethal interactions to altered mRNA binding profiles to protein export defects, likely reflect altered methylation of proteins in addition to Npl3. Nab2, Yra1, and Tho2, for example, are all targets for in vivo methylation (13, 19). The identification and mutagenesis of target arginines within these and other proteins will greatly enhance our understanding of mRNP complex formation as well as the coupling of transcription, processing, and export.

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Arginine Methylation of Yeast mRNA-binding Protein Npl3 Directly Affects Its Function, Nuclear Export, and Intranuclear Protein Interactions
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