Interactions Affected by Arginine Methylation in the Yeast Protein–Protein Interaction Network*§

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Protein–protein interactions can be modulated by the methylation of arginine residues. As a means of testing this, we recently described a conditional two-hybrid system, based on the bacterial adenylate cyclase (BACTH) system. Here, we have used this conditional two-hybrid system to explore the effect of arginine methylation in modulating protein–protein interactions in a subset of the *Saccharomyces cerevisiae* arginine methylproteome network. Interactions between the yeast hub protein Npl3 and yeast proteins Air2, Ded1, Gbp2, Snp1, and Yra1 were first validated in the absence of methylation. The major yeast arginine methyltransferase Hmt1 was subsequently included in the conditional two-hybrid assay, initially to determine the degree of methylation that occurs. Proteins Snp1 and Yra1 were confirmed as Hmt1 substrates, with five and two novel arginine methylation sites mapped by ETD LC-MS/MS on these proteins, respectively. Proteins Ded1 and Gbp2, previously predicted but not confirmed as substrates of Hmt1, were also found to be methylated with five and seven sites mapped respectively. Air2 was found to be a novel substrate of Hmt1 with two sites mapped. Finally, we investigated the interactions of Npl3 with the five interaction partners in the presence of active Hmt1 and in the presence of Hmt1 with a G68R inactivation mutation. We found that the interaction between Npl3 and Air2, and Npl3 and Ded1, were significantly increased in the presence of active Hmt1; the interaction of Npl3 and Snp1 showed a similar degree of increase in interaction but this was not statistically significant. The interactions of Npl3 and Gbp2, along with Npl3 and Yra1, were not significantly increased or decreased by methylation. We conclude that methylarginine may be a widespread means by which the interactions of proteins are modulated. *Molecular & Cellular Proteomics* 12: 10.1074/mcp.M113.031500, 3184–3198, 2013.

Proteins participate in a wide variety of protein–protein interactions, stable and transient. Some have multiple proteins with which they can interact, depending on cellular requirements, the environment, and the spatial and temporal availability of partner proteins. Thus far, the data obtained from two-hybrid and tandem affinity purification experiments predominately represent protein interactions of gene products expressed under one condition; the resulting interaction networks are therefore static. In recent years, attempts have been made to explore the dynamics of the interactome. For example, the correlation of cell cycle gene expression data with protein interaction networks (1, 2) showed that not all subunits of a protein complex are expressed at the same time, revealing patterns of “just in time” assembly of large complexes. Analysis of hub proteins in the cell cycle revealed the presence of static hubs with dynamic interaction partners and dynamic hubs with static interaction partners (1, 3). Post-translational modifications (PTMs)† also play a widespread role in the dynamics of the interactome, with many protein–protein interactions being “conditional” on the presence or absence of a PTM (4). The presence of modifications such as phosphorylation, methylation, ubiquitylation and acetylation can alter the structure of proteins and, either by allosteric change or participation in a binding domain or motif, affects their capacity to interact (5). In some cases, a single modification to one amino acid can act as an on/off switch that controls a domain-motif interaction. Protein phosphorylation, for example, modulates interactions of proteins containing SH2, 14-3-3, and other phosphoamino acid-specific domains (6). In other cases, multiple modifications may be required on a protein before allosteric change and interaction occurs. The histone code is the best-characterized example, where combinations of phosphorylation, methylation, ubiquitylation and acetylation on histones impact on their interaction with other

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*The abbreviations used are: C2H, conditional two-hybrid; ETD, electron-transfer dissociation; DMA, dimethylamine; DMG, dimethylguanidine; GAR, glycine-arginine-rich; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; PGM, proline-, glycine- and methionine; PTM, post-translational modification; MMA, monomethylaniline; MMG, monomethylguanine; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.*
proteins, which in turn affects gene expression (7). Although, the role of PTMs such as phosphorylation and acetylation are quite well-understood, both in terms of their predominance in the proteome and their role, protein methylation is a more recent focus (8, 9).

Arginine methylation is present in eukaryotes but not found in prokaryotes (10). It can modulate protein–protein, protein–RNA and protein–DNA interactions. There are four different forms of methylarginines that have been identified; $\text{N}^\omega\text{G}$-monomethylarginine, $\text{N}^\delta\text{-}\text{monomethylarginine, } \omega\text{-}\text{N}^\delta\text{, N}^\omega\text{G}-\text{asymmetric dimethylarginine, and } \omega\text{-}\text{N}^\delta, \text{N}^\gamma\text{-symmetric dimethylarginine.}$

Four arginine methyltransferases (Hmt1, Rmt2, Hsl7, and Sfm1) have been identified in Saccharomyces cerevisiae (recently reviewed in (8)). Of these, Hmt1, is responsible for the methylation of 16 of the 20 known methylarginine-containing proteins in yeast (8). Methylation of arginine residues on S. cerevisiae proteins is associated with several biological functions including nucleocytoplasmic shuttling, RNA processing (mRNA and rRNA) and transcription (through histone and non-histone proteins) (reviewed in (8)). Its involvement in other biological functions such as splicing and translation are just beginning to be elucidated.

Recently, we constructed the Saccharomyces cerevisiae methylproteome network—this combined the links between known methyltransferases and their substrates with protein–protein interactions in the yeast interactome (9) (Fig. 1). Through the study of the arginine methylproteome network, it emerged that a number of Hmt1 substrates interact with many proteins, raising the question as to whether methylation modulates these interactions. This is possible as a number of domains have been reported to bind to methylated residues. The interactions of GAR and PGM motifs with Tudor domains are facilitated by arginine methylation. Symmetric dimethylation of SmB is a requirement for its interaction with the Tudor domains of SMN, SPF30 and TDRD3 (11). Conversely, arginine methylation has also been shown to block some protein–protein interactions. Asymmetric dimethylation of Sam68 block its interactions with the SH3 domain but does not affect interaction with the WW domain (12). The dimethylation of arginine residues increases residue bulkiness and hydrophobicity and can affect protein–protein interactions through loss of hydrogen bonding (13). In the yeast arginine methylproteome network, Npl3, an RNA-binding protein has characteristics of a “hub” as it has been documented to have numerous interactions (Fig. 1). It is methylated by Hmt1, which is known to affect its interactions with other proteins and self-association (14–16).

To determine whether Hmt1-mediated arginine methylation modulates protein–protein interactions, we recently developed a conditional two-hybrid (C2H) system (Fig. 2). This system assays protein–protein interactions in the presence or absence of PTMs, including methylarginine (14). Based on the Escherichia coli bacterial adenylyl cyclase (BACTH) system ((17), reviewed in (18)), a modifying enzyme can be co-expressed along with the two hybrid proteins. For this study, if an interaction is methylarginine-mediated, it should occur in the presence of the methyltransferase enzyme. If an interaction is not methylarginine-associated, interactions will occur in the presence or absence of the methyltransferase. If an interaction is blocked by arginine methylation, reporter gene expression will

![Fig. 1. A subset of the methylproteome network in S. cerevisiae.](image)
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Construction of C2H Vectors Carrying Interacting Proteins and Modifying Enzymes—Plasmids used in this study are listed in supplemental Table S1. To create the expression plasmids for the N-terminal T25 fusion of Air2, Ded1, Gbp1, Snp1, and Yra1, AIR2, DED1, GBP2, SNP1, and YRA1 were amplified from either S. cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0) genomic DNA or cDNA using primers with the appropriate flanking restriction enzyme sites (supplemental Table S2). The PCR fragments were digested with the appropriate restriction enzymes and inserted into pRsfT25MCS1, which had been linearized with the same enzymes to create RsfT25Air2, RsfT25Ded1, RsfT25GGBP2, RsfT25SnP1, and RsfT25Yra1. As a positive control, the Gcn4 leucine zipper was cloned into pDuE2T18MCS1 and pRsfT25MCS1 to create N-terminal T18/T25 fusions to the Gcn4 leucine zipper. As a negative control for the methyltransferase, we used a plasmid expressing inactivated Hmt1 (14). All inserts in all vectors were verified via colony PCR and capillary sequencing.

Production of Recombinant Protein for Western Blotting and Mass Spectrometry—The relevant plasmids were transformed into the expression strain, E. coli Rosetta (DE3). Recombinant protein production was achieved by induction with 1 mM IPTG at 30 °C for 4 h. Cells were collected and resuspended in Binding Buffer (BB) [50 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 40 mM imidazole, 20% v/v glycerol, 0.25% v/v Triton X-100 and 10 mM β-mercaptoethanol, 1 tablet of Roche Complete EDTA-free per 50 ml buffer], lysis by sonication (Branson digital sonifier) and the resulting lysate clarified by centrifugation at 22,000 × g for 30 min on 4 °C. The supernatants were electrophoresed using 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) in 1× MOPS running buffer according to the manufacturer’s instructions.

Verification of Methylation—The methylation states of the proteins were verified via Western blotting (14), and methylated arginine residues were confirmed using electron-transfer dissociation (ETD) liquid chromatography (LC)-tandem mass spectrometry (MS/MS) (20). Where appropriate, proteins were purified via his-tag purification before ETD LC-MS/MS.

Polyacrylamide gel samples for ETD LC-MS/MS were destained, reduced and alkylated following the procedure described by Shevchenko et al. (21). For protein digestion, 4 ng of trypsin (Promega, Madison, WI) in 40 μl of 0.1 M NH₄HCO₃ was used and incubation was for 16 h at 37 °C. The digest solutions were removed to new microfuge tubes and the gel slices treated with each of the following solutions sequentially for 30 min each: 50 μl 0.1% (v/v) trifluoroacetic acid (TFA); 50 μl 0.1% (v/v) TFA/60% (v/v) acetonitrile (ACN); and 50 μl 100% ACN. The pooled digest and peptide extraction solutions were then dried (Savant SPD1010, Thermofisher Scientific) before resuspending in 20 μl of 1% (v/v) formic acid and 0.05% (v/v) heptafluorobutyric acid (HFBA).

Proteolytic peptide samples were separated by nano-LC using an Ultimate 3000 HPLC and autosampler system (Dionex, Amsterdam, Netherlands), and ionized using positive ion mode electrospray following experimental procedures described previously (22). MS and ETD MS/MS were performed using an LTQ Orbitrap Velos (Thermo Electron, Bremen, Germany) hybrid linear ion trap and Orbitrap mass spectrometer using previously described experimental conditions (14, 20). The precursor ions were measured in the Orbitrap and the ETD fragments were measured in the linear trap.

Peak lists derived from LC-MS/MS were generated using Mascot Daemon/ExtractMSn.exe (Matrix Science, Thermo Electron) and submitted to the database search program Mascot (version 2.3, Matrix Science) (23). The following search parameters were employed: instrument type was set as ETD-TRAP; peptide and peptide fragment mass tolerances were ±4 ppm and ±0.4 Da respectively; acrylamide (C), carbamidomethyl (C), oxidation (M), methylation (R) and dimethylation (R) were specified as variable modifications; enzyme specificity was set to tryptic.
was trypsin with up to 4 missed cleavages; and all taxonomies in the Swiss-Prot database (July 2012 release, 536789 sequence entries) were searched.

For arginine methylated peptides identified at Mascot expect-values (E-values) <0.05, c- and z- ion series were manually inspected to confirm unambiguous localizations of modification sites, and the observations of methylarginine-associated neutral losses from charge-reduced precursor ions were used to further support modified peptide characterizations (24).

Transformation Procedure and Screening for Interactions Through Plate Assays—Relevant plasmids were co-transformed into the cy deficient reporter strain E. coli DHM1 (DE3) pRARE, harboring the bacteriophage λDE3 (14), following standard molecular biology techniques (25, 26). Transformants were plated onto LB agar containing ampicillin (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹) and chloramphenicol (17 μg ml⁻¹). To test for interacting proteins, the resulting cotransformants were then struck out onto LB10 agar with the appropriate antibiotics, IPTG and X-gal as previously described (14). Positive controls were N-terminal T18/T25 Gcn4 leucine zipper motifs fusions cloned into pETDuet-1 and pRSFDuet-1 (blue colonies). Negative controls were the empty “bait” and “prey” vectors DuetMCS2T18 and RSFT25MCS1 (white colonies). Assays for β-galactosidase Activity—To quantify the degree of protein–protein interaction, quadruplicate β-galactosidase assays were carried out as described in (14). Statistical significance of the results of the assays was determined using ANOVA and Student’s t-tests.

Calmodulin Affinity Purification—Plasmid expression vectors were constructed by the insertion of expression plasmids into the bacterial C2H system before the effect of arginine methylation was examined. We constructed plasmids expressing N-terminal fusions of the T25 domain of adenylate methylation was examined. We constructed plasmids expressing N-terminal fusions of the T25 domain of adenylate

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through 1-D SDS-PAGE, we verified that Npl3-T18 and its partner proteins were successfully co-expressed in E. coli (results not shown). To check for interactions, we then cotransformed DuetNpl3T18 with either RsfT25Air2, RsfT25Ded1, RsfT25Gbp2, RsfT25Snp1, or RsfT25Yra1 into the C2H reporter strain, E. coli DHM1 (DE3) pRARE. As predicted, we observed positive interactions of Npl3 with Ded1, Gbp2, Snp1, and Yra1 as evidenced by blue colonies of varying intensity on LB-X-gal IPTG indicator media (Fig. 3). Air2, however, did not show evidence of interaction; it remained white, similar to the vector-only T18+T25 control. The positive control, whereby N-terminal T18 and N-terminal T25 fusions were created to the leucine zipper of Gcn4 (17), showed strong interaction as evidenced by blue colonies.

Hmt1 Can Modify Yra1 and Snp1 in E. coli—Before we could interrogate the role of Hmt1-mediated arginine methylation on the interactions of Npl3, we needed to first verify that Hmt1 was capable of recognizing and adding methyl groups to substrates in the C2H system. Snp1 and Yra1 have previously been described as Hmt1 substrates, however methylarginine sites have not been localized (8, 16, 30). Hmt1 was co-expressed with N-terminal T25 fusion proteins of Snp1 and Yra1. ETD LC-MS/MS analysis was used to localize sites of arginine methylation on Snp1 and Yra1. We found five monomethyl arginine sites and a dimethylarginine site on Snp1, which correlated with those known for U1-70K, the human ortholog of this protein (16) (e.g. Fig. 4, Table I). We also localized two dimethylarginine and one monomethylarginine sites on Yra1 (Fig. 5, Table I). These findings, and our previous observation that the C2H system could accurately methylate Npl3 (14), showed that the C2H system is capable of producing appropriately methylated Hmt1-methylated proteins in E. coli.

Air2, Ded1, and Gbp2 are New Substrates of Hmt1—Although Air2, Ded1, and Gbp2 are known to interact with Npl3, they have not been previously identified to be substrates of Hmt1 (8, 27). Air2 is functionally redundant with Air1, which has been found to interact with Hmt1 to inhibit its activity (27). Ded1 is an ATP-dependent DEAD-box RNA helicase (31) whereas Gbp2 is a Poly(A⁺)-binding protein; we note that both proteins have glycine and arginine-rich domains, similar to those methylated by Hmt1 in Npl3 (32). To determine whether these proteins are arginine-methylated, Hmt1 was co-expressed with N-terminal T25 fusion proteins of Air2, Ded1 and Gbp2. Through Western blot and antibodies against monomethylarginine, we found that Gbp2 was methylated by Hmt1 in vivo in E. coli (supplemental Fig. S1). We were unable to detect Hmt1-mediated monomethylation of Air2 or Ded1 via this method, however the antibody used is not known to effectively detect asymmetric dimethylation or methylation in non-RGG domains. Through ETD LC-MS/MS analysis, we were able to localize sites of methylation for Air2, Ded1, and Gbp2 (Table I). We identified a single monomethyl arginine site and a dimethyl arginine site for Air2 (Fig. 6), two monom-
ethyl arginine and three dimethylarginine sites for Ded1 (Fig. 7), and seven dimethyl arginine sites for Gbp2 (Fig. 8). These observations, along with the fact that there are no known arginine methyltransferases in *E. coli* (33) and thus no methylation in the absence of Hmt1, confirmed these proteins as new substrates of Hmt1.

**Methylation Affects the Interactions of Npl3 With Other Proteins**—Having determined that Hmt1 could effectively methylate substrate proteins in the C2H system, we determined whether the interactions of Npl3 with Air2, Ded1, Gbp2, Snp1, and Yra1 in the presence and/or absence of active Hmt1, alongside positive (Gcn4 leucine zipper) and negative (vector only) controls. The previously reported Npl3 self-association is also shown here (14). Blue colonies indicate interactions, as measured by the expression of the reporter gene, β-galactosidase. Vector-only transformants (T18 + T25) appear as white colonies, indicating no interaction, and the positive controls of Gcn4 leucine zippers fused to T18 and T25 (Gcn4) are blue, indicating positive interaction. Npl3 shows interaction with Ded1, Gbp2, Snp1 and Yra1 and is not visibly changed by arginine methylation as colonies appeared blue in the absence as well as in the presence of Hmt1. In contrast, some evidence of the interaction of Npl3 with Air2 occurred in the presence of active Hmt1 but not in the absence of Hmt1 or the presence of inactive Hmt1.

**Fig. 3.** Conditional two-hybrid (C2H) plate assays can detect methylation-dependent protein–protein interactions. The effect of methylation on Npl3 association with its partner proteins can be assayed by C2H. Interaction of Npl3 with Air2, Ded1, Gbp2, Snp1, and Yra1 in the presence and/or absence of active Hmt1, alongside positive (Gcn4 leucine zipper) and negative (vector only) controls. The previously reported Npl3 self-association is also shown here (14). Blue colonies indicate interactions, as measured by the expression of the reporter gene, β-galactosidase. Vector-only transformants (T18 + T25) appear as white colonies, indicating no interaction, and the positive controls of Gcn4 leucine zippers fused to T18 and T25 (Gcn4) are blue, indicating positive interaction. Npl3 shows interaction with Ded1, Gbp2, Snp1 and Yra1 and is not visibly changed by arginine methylation as colonies appeared blue in the absence as well as in the presence of Hmt1. In contrast, some evidence of the interaction of Npl3 with Air2 occurred in the presence of active Hmt1 but not in the absence of Hmt1 or the presence of inactive Hmt1.

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Notes:
1 Diagnostic neutral losses observed: mono-methyllarginine (MMA)
2 Loss of MMA is denoted by an asterisk (*)
3 Charged reduced species denoted by +

Notes:
1 Diagnostic neutral losses observed: mono-methylamine (MMA)
2 Loss of MMA is denoted by an asterisk (*)
3 Charged reduced species denoted by +

Notes:
1 Diagnostic neutral losses observed: dimethylarginine (DMA), dimethylguanidine (DMG)
2 Loss of DMA is denoted by an asterisk (*)
3 Charged reduced species denoted by +

Notes:
1 Diagnostic neutral losses observed: mono-methylarginine (MMA)
2 Loss of MMA is denoted by an asterisk (*)
3 Charged reduced species denoted by +

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Gbp2, Gcn4, and Snp1, Yra1 and the interactions were measured using the C2H system and plate-based assays. The association between Npl3 and Air2 was lost in the absence of Hmt1 catalytic activity as evidenced by the appearance of white colonies. This suggests that the association between Npl3 and Air2 is not because of bridging by Hmt1 but is conditional on arginine methylation. For Npl3 with Ded1, Gbp2, Snp1 and Yra1, positive interactions still occurred in the absence of Hmt1 catalytic activity (Fig. 3), as was seen with the interactions undertaken in the complete absence of enzyme.

**Methylation Significantly Increases the Interactions of Npl3 with Air2 and Ded2**—Plate-based assays give a qualitative indication of whether or not an interaction occurs between two proteins. To quantitatively measure the extent of interaction, the C2H system also allows the activity of β-galactosidase to be directly measured (14). Using a microtitre plate-based β-galactosidase assay (35) with modifications as described in Erce et al. (14), we analyzed liquid cultures of relevant transformants, which had been IPTG-induced. The Miller units of β-galactosidase activity were calculated from four biological replicates. For each Npl3 protein pair, we analyzed β-galactosidase levels without Hmt1, with active Hmt1 and with the G68R inactivated Hmt1. In all cases, the presence of active Hmt1 resulted in higher levels of β-galactosidase activity than the presence of inactive Hmt1 or the complete absence of Hmt1 (Fig. 9). The increase was substantial for Npl3 - Air2, Npl3 - Ded1, Npl3 - Snp1, and the Npl3 - Npl3 control (1.7- to 2.1-fold increase) but small for Npl3 - Gbp2 and Npl3 - Yra1 (1.1-fold increase). We statistically compared the β-galactosidase levels of each Npl3 - partner pair, in the presence of active Hmt1 and the inactivated G68R Hmt1. This revealed a significant increase in interaction, as assayed by β-galactosidase levels, for the pairs of Npl3 - Air2 (p = 0.009), Npl3 - Ded1 (p = 0.003), and the Npl3 - Npl3 control (p = 0.003). No significant increase was observed for the interactions of Npl3 - Gbp2 or Npl3 - Yra1. The interaction of Npl3 and Snp1 was also not statistically significant in the presence of methylation (p = 0.092) despite an average of 1.7-fold increase when methylated. As all interaction pairs showed levels of β-galactosidase that were near-identical in the absence of Hmt1 or with inactive Hmt1, this is strong evidence that the increase in interactions seen above were because of the presence of arginine methylation. For these assays, an interaction pair was considered to be positive as long as the β-galactosidase levels were twofold greater than the vector-only control.

The Associations Between Npl3 and Its Partners Do Not Involve RNA—The Npl3 partner proteins we have investigated are all RNA binding proteins. As such, it was possible that the in vivo associations we observed using the C2H system were because of bridging of the proteins via RNA. To this end, we sought to confirm that the associations between Npl3 and the partner proteins are not RNA-mediated and involve physical associations between the proteins. For this, we took advantage of the calmodulin-binding property of T18 (36) and pulled-down Npl3-T18 from each C2H pair via affinity purification, using calmodulin affinity resin. We then checked for the presence of his-tagged partner proteins in the eluates via immunoblot with a Penta- His-HRP conjugated antibody (supplemental Fig. S3). We confirmed the physical interactions of the partner proteins with Npl3 in all cases and we observed no difference in association of Npl3 with its partner proteins with or without benzonase nuclease treatment. This suggests that these interactions are not RNA mediated and indicate a direct physical association between Npl3 and its partner proteins.

**DISCUSSION**

The construction of protein interaction networks in *S. cerevisiae* has been made possible using data from proteome-scale two-hybrid assays and affinity purification of protein complexes coupled to MS (AP-MS) (37–42). However,
### Table I

Methylated tryptic peptides identified via ETD LC-MS/MS. Modified amino acid residues are underlined. Missed cleavages marked with an asterisk occur after methylarginine residues, which are known to affect the cleavage site specificity of trypsin (66). Methylarginine-associated neutral losses observed from charge-reduced precursor ions (24) are abbreviated as follows: monomethylamine (MMA), monomethylguanidine (MMG), dimethylamine (DMA), and dimethylguanidine (DMG). Disorder (D) was predicted using PONDR VL-XT Predictor (67–69)

| Protein | Peptide sequence | Methylated residue (amino acid #) | Missed cleavages | Charge state | Theoretical m/z | Mascot ion score | Mascot Expect value | Methylation-specific neutral losses | Disorder (D)/Order (O) |
|---------|-----------------|-----------------------------------|------------------|--------------|----------------|------------------|---------------------|-------------------------------------|----------------------|
| Air2    | SNVIQPTIRGETLSNNISK + methyl | 274 | 1* | 3+ | 771.4249 | 41 | 0.0034 | MMA, MMG | D |
|         | NYNSYQPYBSSTLGBK + dimethyl     | 336  | 1* | 2+ | 888.4402 | 25 | 0.025 | DMA, DMG | D |
| Ded1    | NNESSYNNNNIHYYNHCHGGGSSFNRR + dimethyl | 51 | 1* | 3+ | 956.4137 | 65 | 1.7 × 10⁻⁶ | DMA | O |
|         | RGGYNGGGFFGNGGSR + dimethyl     | 62 | 1* | 2+ | 879.9064 | 61 | 6.3 × 10⁻⁶ | DMA, DMG | O |
|         | SGGSRWIDGK + methyl             | 87 | 1* | 2+ | 495.2616 | 30 | 4.9 × 10⁻² | MIA | D |
|         | DNSFRGGGSWGDSK + dimethyl       | 578 | 1* | 3+ | 528.9059 | 66 | 1.6 × 10⁻⁵ | O | O |
|         | DNSFRGGSSWGDSK + methyl         | 578 | 1* | 2+ | 785.8480 | 45 | 1.4 × 10⁻⁴ | none | O |
| Gbp2    | GSFNDRYDSQYSYGGSR + dimethyl    | 54 | 2* | 3+ | 631.6260 | 55 | 2.4 × 10⁻⁵ | none | D |
|         | FNDRYDSQYSYGGSR + dimethyl      | 58 | 1* | 3+ | 526.9025 | 54 | 1.2 × 10⁻⁶ | DMA | D |
|         | RGGGGGSSR + 3 dimethyl          | 78, 81, 83 | 3* | 3+ | 367.2234 | 48 | 0.051 | DMA, DMG | D |
|         | SFRGGGGRGRG + 2 dimethyl        | 90, 93 | 3* | 2+ | 531.7994 | 31 | 0.0082 | DMA, DMG | D |
| SnIP1   | KGVDERR + methyl                | 187 | 0 | 2+ | 516.2811 | 29 | 0.053 | MMA | D |
|         | RLGGLGGR + methyl               | 198 | 1* | 2+ | 428.7590 | 60 | 0.0018 | MMA | D |
|         | LGGGLGGR + methyl               | 206 | 0 | 2+ | 350.7086 | 56 | 0.00043 | MMA, MMG | D |
|         | LGGGLGGRGYSNKR + methyl         | 206 | 1* | 3+ | 426.5619 | 80 | 1.3 × 10⁻⁶ | MIA, MMG | D |
|         | LGGGLGGRGYSNKR + dimethyl       | 206 | 1* | 3+ | 431.2336 | 69 | 3.6 × 10⁻⁵ | DMA, DMG | D |
|         | LPQFASATSNPAER + methyl          | 218 | 1* | 2+ | 837.9306 | 60 | 4.8 × 10⁻⁵ | MMA, MMG | O |
|         | FASATSNPAER + methyl             | 230 | 0 | 2+ | 626.3019 | 54 | 5.9 × 10⁻⁵ | MIA | D |
| Yra1    | VGSFSGNPR + dimethyl + methyl    | 29, 35 | 1* | 2+ | 584.8365 | 41 | 0.017 | MMA, MMG, DMG | D |
|         | APPNAVARVAK + dimethyl           | 67 | 1* | 3+ | 374.5641 | 28 | 0.025 | DMA | D |

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protein–protein interactions, like all biological processes, are not static and focus is shifting from the cataloguing of protein–protein interactions and studies of network topology toward studying the subtleties of these interactions. In this study, we have investigated how post-translational arginine methylation affects protein–protein interactions in a subset of the yeast interactome.

The methylation of arginine residues is known to either facilitate or block interactions between proteins. For example, the dimethylation of SmB by PRMT5 is required for its interaction with Smd1.

**Fig. 5.** Electron-transfer dissociation (ETD) tandem MS spectra of the Yra1 derived tryptic peptides, confirming the methylation of Yra1. A. Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Yra1 peptide VGTR(dimethyl)GNPRPR(methyl) observed at 584.6533 m/z. In this peptide, a single dimethylarginine site and a single monomethylarginine site were identified and diagnostic neutral losses for methyllarginines were observed. B. Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Yra1 peptide APPNA-VAR(dimethyl)VAK observed at 374.3408 m/z. In this peptide, a single dimethylarginine site was identified and diagnostic neutral losses for methyllarginines were observed. Included in the above spectra are the summarized ion-fragment coverage where c- and z-ions and their derivatives are shown. Precursor and charge-reduced precursor ions, c- and z-ions, prominent ions resulting from -NH3 and methylarginine-associated losses and their measured masses are labeled in the spectra. The theoretical masses for peptide backbone fragments are listed in the tables to the right of each spectra for comparison and those that correspond to masses measured are bolded. Methylarginine-associated neutral losses are abbreviated as follows: monomethylamine (MMA), monomethylguanidine (MMG), dimethylamine (DMA), and dimethylguanidine (DMG). Losses from NH3 are shown as ‘*’.

**Fig. 6.** Electron-transfer dissociation (ETD) tandem MS spectra of the Air2 derived tryptic peptides, confirming the methylation of Air2. A. Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Air2 peptide SNIVQPTIR(monomethyl)GETLSLNNNISK observed at 771.4895 m/z. In this peptide, a single dimethylarginine site was identified and diagnostic neutral losses for methyllarginines were observed. B. Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Air2 peptide NYNSYQPYR(dimethyl)SGTLGK observed at 888.4326 m/z. In this peptide, a single dimethylarginine site was identified and diagnostic neutral losses for methyllarginines were observed. Included in the above spectra are the summarized ion-fragment coverage where c- and z-ions and their derivatives are shown. Precursor and charge-reduced precursor ions, c- and z-ions, prominent ions resulting from -NH3 and methylarginine-associated losses and their measured masses are labeled in the spectra. The theoretical masses for peptide backbone fragments are listed in the tables to the right of each spectra for comparison and those that correspond to masses measured are bolded. Methylarginine-associated neutral losses are abbreviated as follows: monomethylamine (MMA), monomethylguanidine (MMG), dimethylamine (DMA), and dimethylguanidine (DMG). Losses from NH3 and H2O are shown as ‘*’ and ‘0’ respectively.

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protein–protein interactions, like all biological processes, are not static and focus is shifting from the cataloguing of protein–protein interactions and studies of network topology toward studying the subtleties of these interactions. In this study, we have investigated how post-translational arginine methylation affects protein–protein interactions in a subset of the yeast interactome.

The methylation of arginine residues is known to either facilitate or block interactions between proteins. For example, the dimethylation of SmB by PRMT5 is required for its inter-
Electron-transfer dissociation (ETD) tandem MS spectra of the Ded1 derived tryptic peptides, confirming the methylation of Ded1.

A, Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Ded1 peptide NNSSNYNNNNHHHYNHHR(dimethyl)GGGSFFSNNR observed at 956.7968 m/z. In this peptide, one dimethylarginine site was identified and a diagnostic neutral loss for methylarginines was observed.

B, Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Ded1 peptide R(dimethyl)GGYGNGGFFG- GNNGGSR observed at 880.4504 m/z. In this peptide, one dimethylarginine site was identified and diagnostic neutral losses for methylarginine were observed.

C, Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Ded1 peptide SGGR(monomethyl)WIDGK observed at 495.3793 m/z. In this peptide, one monomethylarginine site was identified and a diagnostic neutral loss for methylarginine was observed.

D, Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Ded1 peptide DNSFR(dimethyl)GGSGWGSDK observed at 529.2772 m/z. In this peptide, one dimethylarginine site was identified and diagnostic neutral losses for methylarginine were observed.

E, Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Ded1 peptide DNSFR(monomethyl)GGSGWGSDK observed at 786.3884 m/z. In this peptide, one monomethylarginine site was identified but no diagnostic neutral losses for methylarginine were observed. Included in the above spectra are the summarized ion-fragment coverage where c- and z-ions and their derivatives are shown. Precursor and charge-reduced precursor ions, c- and

Fig. 7. Electron-transfer dissociation (ETD) tandem MS spectra of the Ded1 derived tryptic peptides, confirming the methylation of Ded1. A, Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Ded1 peptide NNSSNYNNNNHHHYNHHR(dimethyl)GGGSFFSNNR observed at 956.7968 m/z. In this peptide, one dimethylarginine site was identified and a diagnostic neutral loss for methylarginines was observed. B, Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Ded1 peptide R(dimethyl)GGYGNGGFFG-GNNGGSR observed at 880.4504 m/z. In this peptide, one dimethylarginine site was identified and diagnostic neutral losses for methylarginine were observed. C, Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Ded1 peptide SGGR(monomethyl)WIDGK observed at 495.3793 m/z. In this peptide, one monomethylarginine site was identified and a diagnostic neutral loss for methylarginine was observed. D, Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Ded1 peptide DNSFR(dimethyl)GGSGWGSDK observed at 529.2772 m/z. In this peptide, one dimethylarginine site was identified and diagnostic neutral losses for methylarginine were observed. E, Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Ded1 peptide DNSFR(monomethyl)GGSGWGSDK observed at 786.3884 m/z. In this peptide, one monomethylarginine site was identified but no diagnostic neutral losses for methylarginine were observed. Included in the above spectra are the summarized ion-fragment coverage where c- and z-ions and their derivatives are shown. Precursor and charge-reduced precursor ions, c- and
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Fig. 8. Electron-transfer dissociation (ETD) tandem MS spectra of the Gbp2 derived tryptic peptides, confirming the methylation of Gbp2. A, Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Gbp2 peptide GSR(dimethyl)FNDRYDQSYGGSR observed at 631.5281 m/z. In this peptide, one dimethylarginine site was identified but no diagnostic neutral losses for methylarginines were observed. B, Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Gbp2 peptide FDNDYDQSYGGSR observed at 526.2153 m/z. In this peptide, one dimethylarginine site was identified and a diagnostic neutral loss for methylarginine was observed. C, Annotated ETD-MS/MS spectrum obtained for the triply charged tryptic Gbp2 peptide R(dimethyl)GGR(dimethyl)GR(dimethyl)GGSR observed at 367.4809 m/z. In this peptide, three dimethylarginine sites were identified and diagnostic neutral losses for methylarginines were observed. D, Annotated ETD-MS/MS spectrum obtained for the doubly charged tryptic Gbp2 peptide SFR(dimethyl)GGR(dimethyl)GGGRGR observed at 531.6411 m/z. In this peptide, two dimethylarginine sites were identified and diagnostic neutral losses for methylarginines were observed. Included in the above spectra are the summarized ion-fragment coverage where c- and z-ions and their derivatives are shown. Precursor and charge-reduced precursor ions, c- and z-ions, prominent ions resulting from -NH3 and methylarginine-associated losses and their measured masses are labeled in the spectra. The theoretical masses for peptide backbone fragments are listed in the tables to the right of each spectra for comparison and those that correspond to masses measured are bolded. Methylarginine-associated neutral losses are abbreviated as follows: monomethylamine (MMA), monomethylguanidine (MMG), dimethylamine (DMA), dimethylguanidine (DMG) and interfering species from co-eluting ion (I). Losses from NH3 are shown as ‘*’.

methylated with the Tudor domains of SMN, SPF30 and TRD3 (11) and Spt5 interaction with RNAPII is regulated by methylation (43). In contrast, arginine methylation of the nuclear poly(A) binding protein weakens its interaction with transportin (44) and methylation of heterogeneous nuclear ribonucleoprotein K (hnRNK K) interferes with the binding of tyrosine kinase c-Src (45). In a more complicated scenario, asymmetric di-

methylated of Sam68 blocks its interactions with the SH3 domain but does not affect its interaction with the WW domain (12). Together, these examples show that the methylation of arginine is a subtle and multifaceted means by which protein–protein interactions can be modulated.

Npl3 is an RNA-binding protein that has numerous interaction partners in the yeast interactome (Fig. 1). Included in the above spectra are the summarized ion-fragment coverage where c- and z-ions and their derivatives are shown. Precursor and charge-reduced precursor ions, c- and z-ions, prominent ions resulting from -NH3 and methylarginine-associated losses and their measured masses are labeled in the spectra. The theoretical masses for peptide backbone fragments are listed in the tables to the right of each spectra for comparison and those that correspond to masses measured are bolded. Methylarginine-associated neutral losses are abbreviated as follows: monomethylamine (MMA), monomethylguanidine (MMG), dimethylamine (DMA), dimethylguanidine (DMG) and interfering species from co-eluting ion (I). Losses from NH3 are shown as ‘*’.
a number of gene expression processes including transcription elongation and termination, 3’ end formation, splicing and mRNA export and translation (46–51). Defective methylation of Npl3 affects its nuclear export and affects its interaction with Tho2 (15). Evidence from pull-down analyses also suggests that Npl3’s interactions with other proteins can be modulated by Hmt1-mediated methylation (15, 30, 52). Along with our recent observation that many of the substrates of Hmt1 interact in the methylproteome network, this motivated us to determine which of Npl3’s interactions in the network are affected by methylation.

Air1 is a RING-finger containing protein that has been suggested to interact with the RGG domain of Npl3 via bridging by Hmt1 (27). Via AP-MS, the Air1 homolog, Air2 was found to interact with Npl3 in two separate studies (28, 38). Using Y2H, both Air1 and Air2 were found to only associate with Npl3 in yeast in the presence of Hmt1 suggesting that Hmt1 was required for the interaction (27). Using plate and β-galactosidase assays, we observed that Npl3-Air2 in the presence of active Hmt1 resulted in a twofold increase in β-galactosidase activity compared the absence of Hmt1 or its catalytic activity. Through our analysis, we can be certain that it is indeed Hmt1 activity that is directly affecting the association between Npl3 and Air2. It has been suggested that the association of Air1 with Hmt1 served to inhibit Npl3 methylation (27). In our study, we did not observe this with Air2, as the co-expression of Npl3, Hmt1 and Air2 resulted in the methylation of Npl3 (supplemental Fig. S2). In fact, we found that Air2 is a novel substrate of Hmt1 with the identification of a dimethyl arginine site and one monomethyl arginine site.

Ded1 is an ATP-dependent helicase belonging to the DEAD-box family (53). The association between Npl3 and Ded1 was first identified via tandem affinity purification of Npl3 (29) and later via AP-MS along with cross-linking (28). We found this interaction was affected by methylation \( P = 0.003\) with a 1.8 fold increase in β-galactosidase activity in the presence of Hmt1catalytic activity. Ded1, which has been previously predicted but not confirmed to be a substrate of Hmt1 (54), was confirmed be Hmt1-methylated with the localization of three dimethylarginine and two monomethyl arginine sites occurring mainly within its RGG regions.

Npl3 has been found to associate with Snp1, a component of the U1 snRNP in a salt sensitive manner (55). Tandem affinity purification of Snp1 in wildtype, null Hmt1 and inactive Hmt1 yeast resulted in a decreased Npl3-Snp1 association when methylated (30). However, in our hands, we were unable to detect this - instead, we found that Hmt1 catalytic activity increased Npl3-Snp1 association 1.7-fold, although this was not statistically significant \( P = 0.092\). Neither the plate assays nor β-galactosidase assays showed a decrease in Npl3-Snp1 association in the presence of Hmt1 catalytic activity. The calmodulin pulldown we performed showed an association between Npl3 and Snp1 in the absence of RNA indicating that there is a physical association between the two proteins. Methylarginine sites have been localized on the U1-70K, the human homolog of Snp1 but no sites have been reported for Snp1. We have identified one dimethylarginine and five monomethyl arginine sites for Snp1. Two of these monomethylarginine sites are on sites conserved with those reported for the human homolog U1-70K (16).
Gbp2, a poly(A+) RNA-binding protein was previously reported to associate with TAP-Npl3 and with the RGG domain of Npl3 (residues 281 to 414) independently of Hmt1 and Hmt1-mediated methylation (27, 29). This was confirmed using the C2H system where no significant difference was observed with the Gbp2-Npl3 association in the presence of methylation ($p = 0.811$). Although Gbp2 was previously predicted to be a substrate of Hmt1 (54), no sites have been reported. We identified seven dimethyl arginine sites in Gbp2.

The association of the RNA binding protein, Yra1 with Npl3 was reported in a number of AP-MS studies (28, 56). Up until this study, it was unclear whether this association was affected by methylation. In the aforementioned study by Yu et al. (30), TAP-tagged Yra1 was purified from yeast with either wild type or null Hmt1 and in both instances, Npl3 was found to associate with Yra1. Using the C2H system, we determined that the Npl3-Yra1 association showed a subtle but not statistically significant change in the presence of methylation ($p = 0.393$). Previously shown to be a substrate of Hmt1 in vivo but with no sites reported (30), in our study we identified two dimethylarginine sites on Yra1.

Taken together, the above data suggest that the C2H system is an accurate means of monitoring methylation-associated protein–protein interactions. It should be noted that in Y2H experiments, the presence of native enzymes, the possibility of endogenous proteins acting as bait-prey bridges and the action of other post-translational modifications may affect results. Small scale immunoprecipitations and large scale AP-MS experiments are usually performed only in wild-type yeast and do not take into account “conditional interaction effects” such as those conferred by PTMs. The design of our study allows the effects of a single modification type, in this case Hmt1-mediated methylation on the interaction of Npl3 with its partners to be investigated.

Using the C2H system, we were able to identify many new sites of arginine methylation on Hmt1 substrates. At this moment, it is unclear as to whether the number of methylated arginines or the type of methylated arginine residues affects the manner in which Npl3 interacts with its partner proteins. Structure data is only available for the RRM1 and RRM2 domains of Npl3 (57, 58) and residues 57–198 of Air2 (59, 60). The analysis of the location of the methylated residues on the proteins revealed that they mostly reside in regions of disorder (Table I). This is consistent with the fact that PRMT1, the human homolog of Hmt1, has an active site that is smaller than the smallest globular protein fold (61). In support of the notion that Npl3 is a “hub,” so termed as it has numerous interaction partners, is the fact that it has a long region of disorder in its RGG domain (62). The RGG motif-containing proteins are defined by single or multiple-RGG/RGX motifs, which are positively charged and known to interact electrostatically with other proteins and RNA (63) and recently reviewed in (64, 65)). A number of proteins are known to interact with the RGG domain of Npl3 (27, 52). Often, as is the case in this study, the partner proteins themselves contain RGG motifs.

In conclusion, we have shown that arginine methylation can affect interactions by use of a generalizable two-hybrid method. This study is another step toward the integration of this type of information with the interactome, which will help to further understand how conditional interaction effects alter protein–protein interaction networks.

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[This article contains supplemental Figs. S1 to S3 and Tables S1 and S2.]

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