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Analysis of the Yeast Arginine Methyltransferase Hmt1p/Rmt1p and Its in Vivo Function

COFACTOR BINDING AND SUBSTRATE INTERACTIONS*

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Many eukaryotic RNA-binding proteins are modified by methylation of arginine residues. The yeast *Saccharomyces cerevisiae* contains one major arginine methyltransferase, Hmt1p/Rmt1p, which is not essential for normal cell growth. However, cells missing *HMT1* and also bearing mutations in the mRNA-binding proteins Npl3p or Cbp80p can no longer survive, providing genetic backgrounds in which to study Hmt1p function. We now demonstrate that the catalytically active form of Hmt1p is required for its activity in vivo. Amino acid changes in the putative Hmt1p S-adenosyl-L-methionine-binding site were generated and shown to be unable to catalyze methylation of Npl3p in vitro and in vivo or to restore growth to strains that require *HMT1*. In addition these mutations affect nucleocytoplasmic transport of Npl3p. A cold-sensitive mutant of Hmt1p was generated and showed reduced methylation of Npl3p, but not of other substrates, at 14 °C. These results define new aspects of Hmt1 and reveal the importance of its activity in vivo.

Post-translational modifications are frequently used by cells to expand the repertoire of proteins to control their activity temporally or spatially or to target their degradation. The methylation of the guanidino group of arginine residues was first recognized 30 years ago (1), but recently more work has focused on this modification. Protein arginine methyltransferases have been identified and cloned from many eukaryotes (2–6). In addition, the number of potential substrates for these enzymes has grown as genome sequencing projects have revealed numerous proteins containing the RGG motif common to substrates for arginine methylation, many of which are RNA-binding proteins (7, 8). Although it is not yet clear what effect methylation may have on the activity of such proteins, the yeast arginine methyltransferase Hmt1p has been shown to facilitate the export of at least two of its substrates from the nucleus, pointing to the importance of methylation in nucleocytoplasmic transport (9).

Arginine methyltransferases were initially identified biochemically in mammalian cell lysates by their ability to transfer a radiolabeled methyl group from *S*-adenosyl-L-methionine (SAM)1 to histones, myelin basic protein, or the heterogeneous nuclear ribonucleoprotein A1 (10–14). In addition to monomethylated forms of these proteins, heterogeneous nuclear ribonucleoprotein A1 undergoes asymmetric dimethylation, resulting in N4,N4-dimethylarginine residues, whereas myelin basic protein is symmetrically methylated (Nε,Nε)-methylarginine (3, 11, 15). Sequence comparison of methyltransferases has revealed motifs common to enzymes that methylate different molecules (17); at least one of these motifs has been implicated in binding to their common co-factor, SAM (18). Novel proteins that contain these motifs have since been identified in general data base searches and in genetic screens (2–6, 19).

The first protein arginine methyltransferase gene in the yeast *Saccharomyces cerevisiae*, *HMT1* (heterogeneous nuclear ribonucleoprotein methyltransferase), was identified in a screen for genes that interacted with *NPL3*, which encodes an RNA-binding protein (4). The same methyltransferase gene, alternatively called *RMT1*, was also found in a systematic search of the yeast genome for proteins containing methyltransferase motifs (3). *HMT1* is not an essential gene in yeast, but it is required in at least two separate genetic backgrounds: in strains with the temperature-sensitive *npl3–1* allele or in strains lacking the 80-kDa cap-binding protein gene *CBP80* (3cbp80) (4, 9). A search of ESTs for human homologs of *HMT1* revealed two human methyltransferase cDNAs, HRMT1L1 and HRMT1L2, and HRMT1L2 was shown to substitute for *HMT1* in *npl3–1* strains (19). The ability of a human arginine methyltransferase to function in yeast indicates that cellular mechanisms involving protein methylation are conserved throughout eukaryotes.

The *S. cerevisiae* Hmt1 protein has been shown to have methyltransferase activity in vitro, methylating a range of RGG-containing proteins including yeast proteins Npl3p, Hrp1p and Hrb1p, and human heterogeneous nuclear ribonucleoprotein A1 (4, 9, 20). A major poly(A) RNA-binding protein in yeast, Npl3p, contains 15 RGG motifs and has been implicated in many cellular processes including nuclear transport, ribosome biogenesis, and silencing (21–25). Hrp1p has three RGG motifs and is a component of cleavage factor 1, which is required for mRNA cleavage and polyadenylation (26). In vitro methylation of recombinant Hrp1p had no effect on its specific binding to the polynucleotidy efficiency element; however, binding of Hrp1p to RNA inhibits its methylation by Hmt1p (27). Npl3p has been shown to be methylated in vivo (4, 20), and there are at least three other proteins that are substrates for Hmt1p (3, 28).

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§ The abbreviations used are: SAM, *S*-adenosyl-L-methionine; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBST, phosphate-buffered saline with 0.25% Tween; MOPS, 4-morpholinepropanesulfonic acid; 5-FOA, 5-fluoro-orotic acid.
Arginine Methyltransferase Hmt1 Function

Many known and potential substrates of arginine methyltransferases, including Npl3p, Hrp1p, and Hrb1p, are proteins that shuttle between the nucleus and the cytoplasm (9, 21, 26). The first evidence for the importance of Hmt1p in cellular processes was the finding that it is required for efficient export of Npl3p and Hrp1p from the nucleus (9). The role of Hmt1p in protein export is also specific; Hrb1p export is not inhibited by the deletion of HMT1 (9).

Similar to Npl3p and Hrp1p, eukaryotic Cbp80p is involved in RNA processing and nuclear export of RNA-protein complexes (29–32). The lack of RGG tripeptides in Cbp80p, however, suggests that this protein is not likely to be a substrate for methylation. The lethality of Δhmt1Δcbp80 strains may reflect the importance of methylation of Npl3p or other proteins involved in these processes in the absence of Cbp80p. Alternatively, Hmt1p may have cellular functions in addition to its role as a methyltransferase that are required in strains lacking HMT1.
We have now tested directly whether the methyltransferase activity is crucial for its biological function. In addition we have discovered a new mutation that affects the ability of Hmt1p to methylate certain substrates and have defined its effect in vivo.

**MATERIALS AND METHODS**

**Yeast Strains and Media**—The yeast strains used in this study are listed in Table I. All strains were grown and genetic manipulations performed as described previously (33). The plasmids used here are listed in Table II. Oligonucleotides used in plasmid construction and sequencing were synthesized at Integrated DNA Technologies, Inc. and verified by automatic sequencing at the Dana-Farber Cancer Institute Molecular Biology Core Facility.

**Construction of G68 Mutant hmt1 Alleles—**

HMT1 sequences were amplified by PCR from pPS1307 with oligonucleotides 5′ HMT1a and 3′ HMT3. Amplified sequences were verified by automated sequencing at the Dana-Farber Cancer Institute Molecular Biology Core Facility.

**In Vitro Methyltransferase Activity Assays—**

Methyltransferase activity assays were performed in a 15-μl reaction containing 20 mM MOPS, pH 7.2, 400 mM KCl, 2 mM EDTA, 30 μM SAM (Sigma), 3.5 μCi of [methyl- 3H]SAM (NEN Life Science Products, 80 Ci/mmol) in addition to enzyme and substrate proteins. The protein concentration of the supernatant was determined by Bio-Rad protein assay. Material was pelleted by centrifugation at 4 °C for 10 min, and the protein concentration of the supernatant was determined by Bio-Rad protein assay. The final concentration for the peptide was 100 μM each leupeptin, chymostatin, antipain, peptatin A, and aprotenin using a Fast-Prep instrument (Bio 101) set at 6.5 for 30 s. Insoluble material was pelleted by centrifugation at 4 °C for 10 min, and the protein concentration of the supernatant was determined by Bio-Rad assay. Indicated amounts of total protein were resolved by SDS, 10% PAGE (34) and immunoblotted essentially as described previously (35).

**Immunoblot Analysis—**

Strains were grown overnight in the appropriate medium and then diluted and grown at the appropriate temperature for the peptide. Western blots were incubated overnight in phosphate-buffered saline with 0.25% Tween (PBST), 2.5% milk powder and 0.05% Tween 20. After washing in PBST, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody solution (1:5000 in PBST with milk; Jackson Immunoresearch Laboratories) and washed, and proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). All reactions were terminated by the addition of SDS-PAGE sample buffer and boiling. Samples were resolved by SDS-10% PAGE (15% for peptide) followed by Coomassie staining and fluorography (Enten- sify; NEN Life Science Products). Autoradiographs were exposed for 4–16 h at −80 °C.

**Immunofluorescence Microscopy—**

Localization of Npl3p by immunofluorescence microscopy was performed essentially as described previously (37). Wild-type and npl3Δ strains were grown to mid-log phase at 25 °C and then shifted to 37 °C for 30 min. Cells were fixed in formaldehyde for 1 h at 37 °C before preparation for microscopy. A 1:1000 dilution of anti-Npl3p and a 1:100 dilution of Texas red-conjugated anti-rabbit antibody were used to detect Npl3p.

**Generation of Conditional hmt1 Alleles—**

HMT1 sequences were amplified from pPS1307 by PCR using a variety of conditions to optimize the degree of mutagenesis. All 100-μl reactions contained 1 μl of Taq enzyme and 2 μl of each primer. Conditions used were 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C for 40 cycles. Sequencing primers were verified by automatic sequencing at the Dana-Farber Cancer Institute Molecular Biology Core Facility.
Arginine Methyltransferase Hmt1 Function

Mutations in the SAM Binding Motif Disrupt Activity of Hmt1p—To test the importance of methyltransferase activity of Hmt1p in vivo, we wished to design point mutations in Hmt1p that would disrupt its catalytic activity (Fig. 1). Sequence alignments of methyltransferases have revealed sequence motifs shared by a diverse set of proteins (17), and the location of these motifs in Hmt1p is shown schematically (Fig. 1A). Although residues involved in substrate binding vary among methyltransferases that target nucleic acids, proteins, and small molecules, motifs I and post-I (Fig. 1B) are involved in binding to the cofactor SAM (3, 18, 39, 40).

A mutation in the EcoP15I DNA methyltransferase, chang-
ing the highly conserved penultimate glycine in motif I (bold, Fig. 1) to a serine or an arginine, reduced the in vitro methyltransferase activity more than 400-fold (41). We therefore chose to mutate the equivalent residue in Hmt1p, G68. This residue was mutated to either an arginine or an alanine, as a more conservative change, and the mutant proteins were tested for methyltransferase activity and biological function.

To determine expression levels of wild-type and mutant Hmt1 proteins, an antiserum was raised in rabbits immunized with recombinant, histidine-tagged Hmt1p. Fig. 2A demonstrates that the antiserum recognizes both the original antigen (lanes 1–4), which migrates as a 45-kDa protein, and endogenous yeast Hmt1p (lanes 5 and 6), which migrates as a 40-kDa protein. The absence of a band in lysate from cells lacking HMT1 (Dhmt1) and the lack of other molecular weight bands indicates the high specificity of the antiserum. Comparison of the His-Hmt1p and wild-type lysate dilution series allowed an approximate calculation of the amount of Hmt1p in the cell, which was $4 \times 10^5$ molecules/cell.

To test the in vivo expression and activity of mutant Hmt1 proteins, mutated alleles were integrated into the genome of Δhmt1 cells such that only mutant forms were expressed. Both mutant alleles expressed Hmt1p to the same level as the wild-type strain (top panel, Fig. 2B). In addition, the anti-Npl3p immunoblot shows that equivalent amounts of Npl3p were found in the lysates (middle panel, Fig. 2B).

Swanson and co-workers (36) raised a monoclonal antibody, 1E4, against purified yeast Npl3p. The ability of this antibody to recognize Npl3p in yeast lysates but not recombinant bacterially expressed Npl3p led to a series of experiments showing that this antibody specifically recognizes methylated Npl3p, although its exact epitope has not been elucidated (20). We used 1E4 to determine the activity of the mutant Hmt1 proteins in vivo. The 1E4 immunoblot (bottom panel, Fig. 2B) reveals that the methylation of Npl3p in strains expressing mutant Hmt1 proteins is significantly reduced compared with that in the wild-type strain at 25 °C. As expected, Hmt1p-G68A, with its less disruptive amino acid change, retains some activity (down >5- but <25-fold), whereas Hmt1p with arginine at position 68 has no detectable activity (>25-fold less than wild type).

The loss of in vitro methyltransferase activity of recombinant G68 mutant Hmt1 proteins correlates with their reduced in vivo activities (Fig. 3). Histidine-tagged wild-type and mutant Hmt1 proteins were expressed in bacteria, purified, and tested for methyltransferase activity. Equal amounts of His-Hmt1p and substrate were incubated with [methyl-³H]SAM to test if mutant proteins were able to methylate either His-Hrp1p or Npl3-myc. Hmt1p-G68A (lanes 2 and 5) was less active than wild-type Hmt1p (lanes 1 and 4) when either His-Hrp1p or Npl3-myc was

Fig. 3. Methyltransferase activity of Hmt1p affects localization of Npl3p. Yeast strains PSY667 (wild type (WT)), 1031 (npl3-27), 1826 (npl3-27, G68A), and 1825 (npl3-27, G68R) were grown to mid-log phase at 25 °C and then shifted to 37 °C for 30 min. After a 1-h fixation in formaldehyde at 37 °C, cells were prepared for immunofluorescence microscopy. Npl3p was detected with the anti-Npl3p polyclonal antibody and a Texas red-conjugated anti-rabbit antibody. Nuclei were visualized by staining with 4,6-diamidino-2-phenylindole (DAPI).

Fig. 4. Mutations in G68 are synthetically lethal with npl3–1 and Δcbp80. Synthetic lethal strains Δhmt1 npl3–1 (PSY966; A) and Δhmt1 Δcbp80 (PSY1191; B) bearing a URA3 HMT1 CEN plasmid that express either no Hmt1p (vector), wild-type (WT) Hmt1p, or mutant Hmt1 proteins (G68A or G68R). Cells were transferred to 5-FOA plates and tested for their ability to grow at 25 °C (3–4 days).
used as a substrate (Fig. 3). Hmt1-G68R, however, failed to methylate either substrate (lanes 3 and 6).

**SAM-binding Site Mutations Disrupt Hmt1p Function in Vivo**—To study the in vivo effect of these mutations that inhibit methyltransferase activity, we took advantage of strain backgrounds that require \( HMT1 \). Plasmids that express the mutant proteins were transformed into \( \text{Dhmt1 npl3–1} \) and \( \text{Dhmt1 cbp80} \) strains bearing an \( HMT1 \text{ URA3} \) plasmid to test for their ability to suppress the synthetic lethality. The growth of strains that had lost the \( \text{URA3} \) plasmid bearing the wild-type \( HMT1 \) was monitored on FOA plates at 25 °C (Fig. 4). Neither mutant \( hmt1 \) was able to suppress the \( \text{Dhmt1 npl3–1} \) synthetic lethality after 3 days of growth (Fig. 4A), and \( hmt1\text{-G68A} \) only partially suppressed the \( \text{Dhmt1 cbp80} \) synthetic lethality after 4 days of growth (Fig. 4B). Thus, methyltransferase activity correlates with growth in the \( \text{Delta cbp80} \) background, whereas complete wild-type methyltransferase activity appears to be necessary in the \( \text{npl3–1} \) background. Therefore, the methyltransferase activity of Hmt1p is important for its function in vivo.

To test whether methyltransferase activity of Hmt1p is important for Npl3p export from the nucleus, we used the \( \text{npl3–27} \) allele, which encodes a mutant Npl3 protein that is imported slowly into the nucleus (37). In the presence of \( HMT1 \), wild-type Npl3p is predominantly nuclear at 37 °C, whereas npl3–27p is found throughout the cell (Fig. 5 (21)). In the presence of G68 mutant \( hmt1 \) alleles, however, the steady state localization of npl3–27p becomes more nuclear. The severity of this phenotype correlates with loss of methyltransferase activity. Nuclear npl3–27p is found in <50% of hmt1-G68A cells, whereas npl3–27p is concentrated in the nucleus of 60–80% of hmt1-G68R cells. Thus methyltransferase activity of Hmt1p
Arginine Methyltransferase Hmt1 Function

**FIG. 7. In vivo methylation of Npl3p by cold-sensitive Hmt1p.** Cold-sensitive *hmt1* alleles were integrated into the genome as in Fig. 2C. Cells were grown and lysed, and total protein was used for immunoblot analysis of the expression and activity of the mutant proteins. Lysate from Δhmt1 strains and a 5-fold dilution series of wild-type HMT1 lysate (same as Fig. 2) are shown for comparison. Lanes 1, 2, 5–9, 12–14 contain 5 μg of total protein, lanes 3 and 10 contain 1 μg, and lanes 4 and 11 contain 0.2 μg of total protein for anti-Hmt1p and anti-methyl-Npl3p. Half as much lysate was used for anti-Npl3p blots.

**A. Npl3p**

| Enzyme : Substrate | 1:10 | 1:1 | 10:1 |
|--------------------|------|-----|------|
| 14°C               | WT E18 | WT E18 | WT E18 |
| 30°C               | 1 | 2 | 3 |

**B. Hrp1p**

| Enzyme : Substrate | 1:10 | 1:1 | 10:1 |
|--------------------|------|-----|------|
| 14°C               | WT E18 | WT E18 | WT E18 |
| 30°C               | 1 | 2 | 3 |

**C. FGGRGGGF**

| Enzyme : Substrate | 1:1000 | 1:100 | 1:10 |
|--------------------|--------|-------|------|
| 14°C               | WT E18 | WT E18 | WT E18 |
| 30°C               | 1 | 2 | 3 |

**FIG. 8. In vitro activity of wild-type and E18V Hmt1p.** A, recombinant Npl3-myc (0.7 μM) was incubated in the presence of [methyl-3H]SAM and increasing amounts of wild-type (WT; lanes 1, 3, 5) or E18V (lanes 2, 4, 6) mutant His-Hmt1p (lanes 1 and 2, 0.07 μM; lanes 3 and 4, 0.7 μM; lanes 5 and 6, 7 μM enzyme) at 14 °C or 30 °C for 30 min. Proteins were resolved by SDS-PAGE and visualized by fluorography. B, recombinant His-Hrp1p was tested for methylation by wild-type and mutant His-Hmt1 proteins exactly as described in A. C, a synthetic peptide with a single site for methylation (100 μM) was incubated in the presence of increasing amounts of wild-type (lanes 1, 3, 5) or E18V (lanes 2, 4, 6) His-Hmt1p (lanes 1 and 2, 0.1 μM; lanes 3 and 4, 1 μM; lanes 5 and 6, 10 μM enzyme). Methylation of the peptide was detected as in A.

Generation of Cold-sensitive Alleles of Hmt1p—To investigate further the in vivo function of HMT1, we again took advantage of its being essential in Δcbp80 strains to generate conditional alleles of *hmt1*. Error-prone PCR was used to amplify and to introduce random mutations into HMT1 sequences. The entire HMT1-coding region and some 5′- and 3′-noncoding sequences were excised from a LEU2 HMT1 CEN plasmid. The gapped plasmid and PCR product were co-transformed into a Δhmt1 Δcbp80 strain that contained a URA3 HMT1 CEN plasmid (PSY1191). After in vivo gap repair (42), the URA3 plasmid was eliminated by transferring Leu+ colonies to FOA. LEU2 plasmids bearing temperature-sensitive or cold-sensitive mutant *hmt1* alleles were then identified by plating on rich medium at different temperatures.

Two plasmids, pPS1762 and 1763, still showed conditional suppression of synthetic lethality when retransformed into the Δhmt1 Δcbp80 strain. The *hmt1* alleles in these plasmids were named *hmt1*-20 and *hmt1*-46, Δhmt1 Δcbp80 strains bearing these alleles can grow on FOA plates at 25 °C but not at 14 °C, demonstrating the cold sensitivity of these alleles (compare *hmt1*, upper left section and wild-type HMT1, top section, Fig. 6A).

Given that the conditions used for mutagenesis were predicted to result in multiple mutations, the N- and C-terminal mutations were subcloned separately into the wild-type HMT1 plasmid pPS1305. The cold sensitivity of each allele mapped to the N terminus (Fig. 6A, compare lower left and bottom sections). Sequencing of the 5′-noncoding region, the open reading frame and a portion of the 3′-noncoding region revealed that each allele did contain multiple mutations (Fig. 6B). Remarkably, the two alleles shared one mutation in common, A to T at nucleotide 53 of the open reading frame. This mutation resulted in the substitution of a valine for glutamate 18 of Hmt1p (E18V). Introduction of this point mutation into the HMT1 and expression of the resultant *hmt1*-E18V allele in PSY1191 demonstrated that cold sensitivity is mapped to this residue (Fig. 6A, lower right section).

The E18V Mutation in Hmt1p Reduces Methylation of Npl3p—Although E18 does not lie within predicted methyltransferase motifs of Hmt1p, mutating this residue might affect methyltransferase activity of the protein. To test this possibility in vivo, the original mutant alleles and *hmt1*-E18V were integrated into the genome of a Δhmt1 strain (PSY865), and lysates were immunoblotted for expression and activity of the mutant proteins (Fig. 7). The activity of all three mutant alleles, as detected by the relative abundance of methyl-Npl3p, is reduced at 14 °C compared with 25 °C, from slightly less than wild type to more than 20-fold less than wild type (Fig. 7, bottom panel). Thus cold-sensitive growth correlates with cold-
sensitive methyltransferase activity.

Histidine-tagged E18V Hmt1p was expressed in bacteria, purified, and tested for methylation of recombinant Npl3p-myc and His-Hrp1p. Although methylation of Npl3-myc by recombinant E18V Hmt1p was equivalent to that by wild-type Hmt1p at 30 °C, [methyl-3H]Npl3-myc was significantly reduced for E18V reactions incubated at 14 °C (Fig. 8A). To test whether the cold sensitivity of methyltransferase activity was substrate-specific, methylation of His-Hrp1p, a synthetic peptide substrate, and GST-Hrb1p were tested at two temperatures (Fig. 8B, C, and data not shown). A higher concentration of the peptide substrate was used to allow detection of the tritiated peptide within a responsive range of enzyme-to-substrate ratios. Notably, hmt1-E18V appeared to methylate these substrates as well as, if not better than, wild-type Hmt1p at both temperatures, suggesting that the hmt1-E18V defect is specific for Npl3p (Fig. 8F, B and C, and data not shown).

To test whether the hmt1-E18V allele was synthetically lethal with npl3–1, a hmt1-E18V strain (PSY1686) was crossed to a npl3–1 strain (PSY773; Ref. 43). After sporulation of the diploid, 20 tetrads were dissected: 6 parental ditypes, 3 nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 20 tetrads were dissected: 6 parental ditypes, 3 nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes, and 11 tetratypes. 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FIG. 9. Model: Hmt1p and nuclear transport. Proteins involved in nuclear transport that interact with Hmt1p are shown schematically. Hrp1p and Npl3p are Hmt1p substrates, and the cap-binding protein gene CBP80 interacts genetically with HMT1. These proteins are thought to play a role in packaging mRNA for export into the cytoplasm for subsequent translation (the ribosome is indicated by 60 S and 40 S subunits).
Arginine Methyltransferase Hmt1 Function

These genes implicated in RNA binding and nuclear transport. Future studies should help to define the interactions among these genes implicated in RNA binding and nuclear transport.

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Analysis of the Yeast Arginine Methyltransferase Hmt1p/Rmt1p and Its in Vivo Function: COFACTOR BINDING AND SUBSTRATE INTERACTIONS
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