Protein Arginine Methylation of Npl3 Promotes Splicing of the *SUS1* Intron harboring Non-consensus 5' Splice Site and Branch Site

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Abstract

Protein arginine methylation occurs on spliceosomal components and spliceosome-associated proteins, but how this modification contributes to their function in pre-mRNA splicing remains sparse. Here we provide evidence that protein arginine methylation of the yeast SR-/hnRNP-like protein Npl3 plays a role in facilitating efficient splicing of the *SUS1* intron that harbors a non-consensus 5' splice site and branch site. In yeast cells lacking the major protein arginine methyltransferase *HMT1*, we observed a change in the co-transcriptional recruitment of the U1 snRNP subunit Snp1 and Npl3 to pre-mRNAs harboring both consensus (*ECM33* and *ASC1*) and non-consensus (*SUS1*) 5' splice site and branch site. Using an Npl3 mutant that phenocopies wild-type Npl3 when expressed in *Δhmt1* cells, we showed that the arginine methylation of Npl3 is responsible for this. Examination of pre-mRNA splicing efficiency in these mutants reveals the requirement of Npl3 methylation for the efficient splicing of *SUS1* intron 1, but not of *ECM33* or *ASC1*. Changing the 5' splice site and branch site in *SUS1* intron 1 to the consensus form restored splicing efficiency in an Hmt1-independent manner. Results from biochemical studies show that methylation of Npl3 promotes its optimal association with the U1 snRNP through its association with the U1 snRNP subunit Mud1. Based on these data, we propose a model in which Hmt1, via arginine methylation of Npl3, facilitates U1 snRNP engagement with the pre-mRNA to promote usage of non-consensus splice sites by the splicing machinery.

1. Introduction

Eukaryotic gene expression requires functional integration of splicing and transcription of precursor mRNAs (pre-mRNAs) [reviewed in 1, 2]. During pre-mRNA splicing, it is critical that introns are precisely removed from the precursor transcript via the spliceosome, a macromolecular RNA-protein complex whose recruitment and activities are controlled by *cis* sequence elements and by *trans*-acting factors [reviewed in 3, 4]. In *Saccharomyces cerevisiae*, five small nuclear ribonucleoproteins (snRNPs) comprise the core of the spliceosome. In the consensus model of spliceosome assembly, the U1 snRNP first recognizes the 5' splice site in the pre-mRNA through base-pairing between the 5'-end of the U1 snRNA and the 5' splice site sequence, forming the earliest commitment complex (CC1) [5-7]. The branch site is then recognized by the branchpoint binding protein (BBP/Msl5) and its binding partner Mud2 to form commitment complex 2 (CC2) [8-10]. The BBP-Mud2 heterodimer then interacts with the U1 snRNP, forming a bridge between the 5' splice site and the branch site [11]. Prp5 and Sub2, both of which are DEAD/DECD box proteins, then promote displacement of BBP and Mud2 by the U2 snRNP at the branch site in an ATP-dependent manner, resulting in the pre-spliceosome or A complex [12-14]. The preformed U4/U6.U5 tri-snRNP then joins to form the assembled spliceosome [15-18]. Following the binding of the tri-snRNP, major structural rearrangements occur within the spliceosome, resulting in the release of U1 and U4 snRNPs and formation of new base-pairings between U6 and the 5' splice site and between U2 and U6 snRNA [19, 20]. A protein complex called the nineteen complex (NTC) then stabilizes the association of U5 and U6 with the spliceosome, thereby allowing the formation of an activated spliceosome to catalyze the actual splicing

reaction [21]. The snRNPs and non-snRNP-associated factors are co-transcriptionally recruited through an ordered process [22-24]. Although splicing can occur post-transcriptionally, the coupling of this process to transcription likely maximizes its fidelity and efficiency [25].

While the basic components of the splicing machinery are conserved between *S. cerevisiae* and mammals, splicing regulators of the serine/arginine-rich (SR) protein family are present only in the latter [26]. Previous studies in mammalian cells showed that SR proteins stabilize interactions between the U1 snRNP and pre-mRNAs [reviewed in 27, 28]. It is thought that SR proteins bind to the exonic sequences adjacent to suboptimal splice sites, consequently promoting the recruitment of U1 and U2 snRNPs and thereby playing a significant role in constitutive splicing of pre-mRNAs as well as regulating alternative splicing. In yeast, most splice sites obey a strict consensus, and since only a handful of intron-containing genes possess more than a single intron it was thought that the lack of SR proteins in the budding yeast reflects lack of a need for alternative splicing [29]. Nevertheless, a number of yeast proteins such as Npl3, Hrb1, and Gbp2 possess characteristics of mammalian SR proteins [30]. Interestingly, Npl3 additionally shares characteristics with heterogeneous nuclear ribonucleoproteins (hnRNPs) – a large number of RGG boxes and RNA-recognition motifs (RRMs) [reviewed in 31, 32].

Several post-translational modifications have been implicated in the control of pre-mRNA splicing [reviewed in 33]. For example, ubiquitination of the DExD/H box family protein Brr2 suppresses its ability to unwind U4/U6 RNA duplex [34]. Recently, protein arginine methylation has been linked to the control of pre-mRNA splicing in

several model systems: budding yeast [35], cultured mammalian cells [36, 37], plants [38], and mice [39]. Protein arginine methylation alters the biochemical properties of the side chain of the amino acid arginine [reviewed in 40]. This modification is catalyzed by members of the protein arginine methyltransferase (PRMT) family of enzymes, which are divided into four subtypes based on the specific type of methylarginine that is formed. The type I PRMTs transfer either one or two methyl groups from S-adenosyl-*L*-methionine (AdoMet) to a single guanidino nitrogen on a protein-incorporated arginine residue, thus forming monomethylarginine (MMA) or asymmetric dimethylarginine (aDMA), respectively. Type II PRMTs likewise catalyze monomethylation, but then add the second methyl group from AdoMet to the opposing guanidino nitrogen within the arginine residue, thereby forming symmetric dimethylarginine (sDMA). Sm proteins, which are critical to the assembly of functional U snRNPs in the cell, contain sDMAs that are modified by PRMT5, a type II PRMT [41, 42]. In *S. cerevisiae*, Hmt1 (also termed Rmt1) is the only known type I PRMT and it is the yeast homolog of mammalian PRMT1 [43, 44]. Hmt1 has been shown to methylate the SR-/hnRNP-like Npl3 [44, 45] and the vast majority of aDMAs are present within the RGG context [46].

Previously, we showed that Hmt1 methylates the U1 snRNP subunit Snp1 and that Hmt1 is required for proper co-transcriptional recruitment of specific snRNP subunits and snRNP-associated factors to their genomic targets [35]. In the current study, we demonstrate that Hmt1 plays a role in facilitating the spliceosome usage of non-consensus splice sites, and that it does so via methylation of the SR-/hnRNP-like protein Npl3. Our data show that $\Delta hmt1$ cells display altered recruitment of U1 snRNP subunit Snp1 and SR-like protein Npl3 to intron-containing genes *SUS1*, *ECM33*, and *ASC1* in an

RNA-dependent manner. However, only *SUS1* intron 1, which harbors a non-consensus 5' splice site and branch site, exhibits a decreased splicing efficiency in these cells. Using an Npl3 mutant that phenocopies wild-type Npl3 behavior when expressed in $\Delta hmt1$ cells, we show that the arginine methylation of Npl3 is responsible for the recruitment and splicing efficiency changes observed in $\Delta hmt1$ cells. Biochemical characterizations using methylated and unmethylated form of Npl3 indicate a methylation-dependent association between Npl3 and the U1 snRNP via Mud1, a subunit of U1 snRNP. Overall, our data support a model in which Hmt1, via methylating Npl3, promotes optimal U1 snRNP engagement with non-consensus 5' splice site and branch site in *SUS1* in order to achieve its efficient splicing.

2. Materials and Methods

2.1. Yeast strains used in this study

All yeast strains used are listed in Table S1. All plasmids used are listed in Table S2. All primers used are listed in Table S3. Cells were grown at 30°C on YPD medium (1% yeast extract, 2% bactopectone, 2% glucose, w/v) unless otherwise stated. Genomic deletions and integration of epitope tag cassettes was performed as previously described [47, 48]

2.2. Chromatin immunoprecipitation (ChIP)

ChIPs were performed as described previously [49, 50], except for the sonication condition (Branson Digital Sonifier 450, 3mm tapered microtip, 20% amplitude, 20 sec pulse followed by 55 sec pause for 15 cycles). For each IP, anti-myc (Thermo-Fisher cat# MS127P), α -Rpb3 (Neoclone cat#W0012) or anti-IgG (Jackson ImmunoResearch cat# 209-005-082) antibodies were pre-coupled to protein A-sepharose beads. qPCR was

performed on a Bio-Rad MyiQ5 real-time thermal cycler in 20 μ l reactions with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 2 μ l of DNA sample (input or IP), and primers at a final concentration of 0.25 μ M for each primer. Percent of input was calculated using the ΔC_T method and statistical testing was performed by Student's t-test. All values reported are the mean of three biological replicates (n=3), with error bars indicating the standard error of the mean. For RNase treatment, sonicated chromatin samples were treated with 400 μ g of RNase A and 800 units of RNase A/T1 Cocktail (Ambion #AM2286) prior to IP. IPs and qPCR were performed on RNase treated samples as described above. All the primers used in the real-time PCR experiments are listed in Table S3.

2.3. Reverse transcription and quantitative real-time PCR analysis (RT-qPCR)

Overnight yeast cultures were subcultured 1:100 into YEPD and grown to $OD_{600} \approx 0.35$ -0.45, whereupon they were harvested and cell pellet flash-frozen in liquid nitrogen. Total RNA was extracted with TRIZOL reagent, followed by treatment with 10U of RNase-free DNase for 30 minutes at 37°C. The resulting mixture was then further extracted with phenol:chloroform:isoamyl alcohol and resuspended in nuclease-free water. Reverse transcription was performed on 250 ng of RNA with Superscript III (Thermo-Fisher), primed with 0.25 μ g oligo-dT(12-18) and 0.05 μ g random hexamer, for 1 hour at 50°C. The resulting cDNA was treated with 5U of RNaseH for 30 minutes at 37°C and then heat-inactivated for 20 minutes at 70°C. qPCR was done as described in section 2.1, using 2 μ l of cDNA (which equals to approximately 2.5 ng of reverse-transcribed RNA). No-RT controls were performed in parallel. The cDNA and the corresponding No-RT control had at least an 8 C_T difference for all samples. Intron retention percentage was

calculated by the ΔC_T method, with intron minus total mRNA, after correcting for primer efficiency. The retention percentage was subtracted from 100% to obtain the reported values for percentage of intron spliced. Statistical testing was performed using Tukey's HSD test, with the R statistical analysis software. All values reported are the mean of three biological replicates (n=3), with error bars indicating the standard error of the mean.

2.4. Measurement of *SUS1* Mutant Splicing Efficiency

A *SUS1* genomic fragment was amplified from BY4741 genomic DNA, including 226 bp upstream of the ORF and 228 bp downstream, and cloned into pRS415 by PIPE [51].

Site-directed mutagenesis was then performed on this fragment to generate pRS415-*SUS1*[cons], which contains consensus 5' splice site and branch site sequences. pRS415-*SUS1* and pRS415-*SUS1*[cons] were transformed into *Δsus1* and *Δsus1/Δhmt1* strains.

These strains were then grown overnight in SD/Leu⁻ media, subcultured 1:100 into SD/Leu⁻, and grown to an OD₆₀₀ ≈ 0.35-0.45, whereupon they were harvested and flash-frozen in liquid nitrogen. RNA extraction, reverse transcription, and qPCR were performed as described above.

2.5. Npl3 Protein Pulldown

1.5 mg of yeast whole-cell extract in 100 μl of storage buffer D (20 mM HEPES-KOH pH 7.8, 50 mM KCl, 20% Glycerol, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF) was incubated with 5 μl of Npl3-sepharose beads (which contains 2.5 μg Npl3 protein) for 20 minutes at room temperature. RNase A-treated samples were supplemented with 1 mM MgCl₂, 20 U of DNase I, and 1 μg/mL of RNase A. After incubation, Npl3-sepharose beads were washed five times with IPP300 (25 mM Tris pH 8.0, 300 mM NaCl, 0.1% NP-40), eluted by boiling in 25 μl of 2X Gel Loading Buffer, and resolved by SDS-

PAGE using a Novex 4-12% Bis-tris pre-cast gel (Life Technologies). The gel was then fixed, silver stained, and imaged. Additional experimental details are included in the Supplemental Information.

2.6 Co-immunoprecipitation (coIP)

Affinity resin for TAP coIPs was generated by covalently linking 500 μ g of human serum IgG (Sigma cat#I4506) in PBS + 0.01% Triton X-100 to 30 mg tosylactivated Dynabeads M-280 (Life Technologies cat#14203) overnight at 37°C. Beads were then washed and blocked with 1 mg/ml BSA in 100 mM Tris pH 8.0 for 4 hrs at room temperature, then washed again and stored in 1 ml of PBS + 0.01% Triton X-100, 20% ethanol, and 0.1% NaN_3 . Overnight grown yeast cultures were subcultured 1:100 into 50 ml YPD and grown to $\text{OD}_{600} \approx 1.0$, then harvested and washed in PBS with the final cell pellet snap-frozen in liquid nitrogen. Lysates were made by FastPrep (MP Biomedical) in PBSMT (1X PBS, 3 mM KCl, 2.5 mM MgCl_2 , 0.5% Triton X-100) completed with 10 μ g/mL RNase A and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 1.3mM benzamidine, 2.5 μ g/ml each of leupeptin, chymostatin, antipain, pepstatin A, and aprotinin). Lysates were normalized to 5 mg/ml protein concentration by Bradford assay and 5 mg of the lysate was bound to 10 μ l of pre-washed IgG-Dynabead slurry (300 μ g beads) for 2 hrs at 4°C. Beads were then washed twice with 1 ml IPP300, twice with 1 ml IPP150 (25 mM Tris pH 8.0, 150 mM NaCl, 0.1% NP-40), eluted in 25 μ l of 1X Gel Loading Buffer, and resolved on a 4-12% Novex Bis-Tris gel. Following SDS-PAGE, the proteins were transferred to nitrocellulose membrane. The blot was probed with α -Npl3 antibody and then with α -Protein A (Sigma cat#P3775) antibody.

2.8. Npl3 Pulldown with U-snRNPs

1.5 mg of yeast whole-cell extract prepared as previously described for protein pulldown was incubated with 5 μ l of Npl3-bound sepharose beads (containing approximately 2.5 μ g Npl3 protein) for 20 minutes at room temperature. Beads were then washed five times with ice cold IPP300 and eluted with 200 μ l of RNA Extraction Buffer (50 mM NaOAc pH 5.5, 50 mM NaCl, 1% SDS) and 200 μ l acid P:C:I (125:24:1 phenol:chloroform:isoamyl alcohol pH 4.5) (Thermo-Fisher cat#AM9720) for 30 minutes at 37°C. 100 ng of control RNA (generated by *in vitro* transcription from a fragment of the GFP gene) was also added to the elution. Input RNA was extracted by adding 1.5mg whole cell extract to 200 μ l RNA Extraction Buffer and 200 μ l acid P:C:I, with 100 ng spike-in control RNA. The RNA was extracted, precipitated, and then DNase-treated using the DNase Turbo Kit (Thermo-Fisher cat#AM1907). Reverse transcription was carried out as before with 2.5ng of RNA as template, and pulldown was quantified as % of input by $\Delta\Delta C_T$ using the spike-in RNA control to normalize pulldown.

2.9. Npl3 pulldown with U1 snRNP subunits

GST-tagged protein constructs were generated as follows: pGEX-5X-SNP1 was generated by amplifying *SNP1* from BY4741 genomic DNA and cloning it between BamHI and NotI sites in pGEX-5X-3. pGEX-4T-MUD2, -LUC7, and -NAM8 were generated by amplification of the respective gene from BY4741 genomic DNA and cloning it between BamHI and XhoI sites in pGEX-4T-1. pGEX-4T-YHC1 and -SNU56 were generated by amplification of the respective gene from BY4741 genomic DNA and cloning it into pGEX-4T-1 by PIPE. pGEX-4T-MUD1 were generated by amplification of the respective gene from BY4741 cDNA and cloning it into pGEX-4T-1 by PIPE. All constructs were confirmed by sequencing prior to transforming into the Rosetta 2 (DE3)

expression strain of *E. coli*. For each GST-construct, 1 mg of induced and lysed *E. coli* cell lysate was bound to 50 μ l of GSH-magnetic beads (Pierce cat#88821) for 1 hr at 4°C. Beads were then washed three times with 1 ml PBS with 1M NaCl, 0.1% Triton X-100, and 1 mM DTT, followed by two washes with 1 ml PBS with 0.1% Triton X-100 and 1 mM DTT. 2.5 μ g Npl3 was then incubated with the GST-coupled GSH-magnetic beads in 1 mL of PBS with 0.1% Triton X-100, 1 mM DTT for 30 minutes at room temperature. Beads were then washed four times with 1 ml PBS containing 0.1% Triton X-100, 1 mM DTT, and eluted in 25 μ L 1x Gel Loading Buffer. Samples were resolved by the SDS-PAGE, transferred to a nitrocellulose membrane, Ponceau stained to check equal loading, and then blotted with α -Npl3. Additional experimental details are included in the Supplemental Information.

3. Results

3.1. Loss of Hmt1 results in reduced *in vivo* occupancy of intron-containing genes by the U1 snRNP subunit Snp1

To determine how Hmt1 affects the co-transcriptional recruitment of snRNP subunits and snRNP-associated proteins involved in the early steps of the spliceosome assembly, we measured the *in vivo* occupancy of intron-containing genes *ECM33*, *SUS1*, and *ASC1* by Npl3, Snp1, Mud2, and the U2 snRNP subunit Lea1 using chromatin immunoprecipitation (ChIP) (Figure 1A and Figure S1A). Both Npl3 [44, 45] and Snp1 [35] are established substrates of Hmt1. *ECM33* and *ASC1* are examples of typical budding yeast intron-containing genes: they have a single intron with consensus 5' splice site, branch site, and 3'SS (Figure 1B). However, *ECM33* has a much shorter first exon

in comparison with *ASCI* (58 bp vs. 537 bp). *SUSI* is one of only 10 intron-containing genes with more than one intron in the budding yeast and *SUSI* contains a non-consensus 5' splice site and branch site at its first intron but retains the consensus sequences for its 5' splice site, branch site, and the 3' splice site at its second intron (Figure 1B).

Interestingly, only about 50% of two-intron genes in the budding yeast contain non-consensus branch region sequences versus that of just 7.5% for single intron genes [52].

Yeast strains of the *HMT1* and $\Delta hmt1$ backgrounds were engineered to express C-terminal myc-tagged version of the protein. In $\Delta hmt1$ cells, occupancy by Npl3 was significantly higher across all three intron-containing genes (Figure 2A and Figure S1B; compare Hmt1 “+” bars to Hmt1 “-“ bars above myc IP). Previously, we had observed an increase in the biochemical association between Npl3 and Snp1 in $\Delta hmt1$ cells [35]. As such, we anticipated seeing a higher level of gene occupancy by Snp1 in cells that lack Hmt1. However, the converse was true; Snp1 occupancy was significantly lower across all genes tested in $\Delta hmt1$ cells (Figure 2B and Figure S1C; compare Hmt1 “+” bars to Hmt1 “-“ bars above myc IP), approaching near background levels.

We next examined Mud2 (Figure 2C and Figure S1D) and Lea1 (Figure 2D and Figure S1E). No change in Mud2 occupancy was observed (Figure 2C and Figure S1D; compare Hmt1 “+” bars to Hmt1 “-“ bars above myc IP) and a small increase in Lea1 occupancy occurred near the 5'-end of the second *ECM33* exon (Figure 2D; compare Hmt1 “+” bars to Hmt1 “-“ bars in primer set A3). In the case of *ASCI*, the *in vivo* occupancy by the tested splicing factors (Figure S1B-E) was similar to that for *ECM33*, except for the observation of an increase in Snp1 occupancy in the downstream regions of *ASCI*. This observation is consistent with a previously published result showing

increased occupancy for three other U1 snRNP proteins at the same region [22]. As a control, we also examined the recruitment pattern of Snp1 and Npl3 along an intronless gene *PMA1* and did not find their occupancies to be affected in *Δhmt1* cells (Figure S2B and S2C). Their recruitment patterns along *PMA1* were similar to those seen in previously published reports for U1 snRNP [22] and Npl3 [53]. To test for potential differences in transcriptional output that may account for the differences in their recruitment pattern, we measured occupancy of RNA Polymerase II subunit Rpb3 across these genes and found no difference between *HMT1* and *Δhmt1* cells (Figure 2E and Figure S1F). To determine whether the difference seen in the recruitment of Npl3 and Snp1 is due to the presence of RNA, we incorporated an RNase treatment step in our ChIP protocol and repeated the ChIP experiment. For both Npl3 and Snp1, the differences observed between *HMT1* and *Δhmt1* cells for *ECM33* and *SUS1* are no longer present (Figure 2F-G). Thus, Hmt1 plays a role in promoting optimal level of co-transcriptional recruitment for Snp1 and Npl3 across intron-containing genes of either consensus or non-consensus splice sites in an RNA-dependent manner.

3.2. Arginine methylation of Npl3 affects recruitment of Snp1 to intron-containing genes

Since Hmt1 is an enzyme that methylates a number of RNA-binding proteins known to play a role in splicing, we wanted to see whether one such substrate, Npl3, is responsible for the differences in the occupancy of splicing factors observed in a *Δhmt1* background (Figure 2). To this end, we replaced the genomic copy of *NPL3* with the *npl3^{RK}* mutant in our strains with myc-tagged splicing factors. Npl3^{RK} is a mutant form of Npl3 in which 15 of the 17 identified Npl3 arginines that undergo arginine methylation

had been mutated to lysines [46]. Substitution of the arginine with lysine retains the positive charge of the arginine residue [54, 55]. Based on characterizations done by other labs, it was revealed that Npl3^{RK} phenocopies wild-type Npl3 behavior when expressed in *Δhmt1* cells [46, 55, 56]. Thus, the use of Npl3^{RK} mutant makes it possible for us to probe the specific impact of wild-type Npl3 in isolation.

We tested Npl3^{RK} occupancy across the same intron-containing genes in both *HMT1* and *Δhmt1* cells (Figure 3 and Figure S3) and, as expected, the expression of Npl3^{RK} in *Δhmt1* cells restored occupancy of both Npl3 (Figure 3A; compare Hmt1 “+” bars above Npl3 WT to Hmt1 “-“ bars above Npl3 RK) and Snp1 (Figure 3B; compare Hmt1 “+” bars above Npl3 WT to Hmt1 “-“ bars above Npl3 RK) to the levels seen in the *HMT1* strain for *ECM33*, *SUS1*, and *ASC1* (Figure S3A and B; compare Hmt1 “+” bars above Npl3 WT to Hmt1 “-“ bars above Npl3 RK). Similar as Npl3 and Snp1, recruitment of Lea1 across the same three intron-containing genes in *Δhmt1* cells expressing Npl3^{RK} was restored to the level seen in *HMT1* cells (Figure 3C and Figure S3D, compare Hmt1 “+” bars above Npl3 WT to Hmt1 “-“ bars above Npl3 RK). Thus, we attribute the changes in the recruitment pattern for Snp1, Npl3, and Lea1 to the lack of Npl3 arginine methylation in *Δhmt1* cells.

3.3. Loss of Hmt1 results in decreased splicing of pre-mRNAs harboring non-consensus splice sites

We inferred that the observed changes in *in vivo* occupancy of intron-containing genes by splicing factors affect the efficiency of splicing of transcripts produced from the tested intron-containing genes above. To test this hypothesis, we assessed the splicing efficiency of these transcripts at 30°C and 22°C by RT-qPCR, based on the percentage of

intron spliced, as previously described [57]. For *ECM33* (Figure 4A) and *ASCI* (Figure S4A) pre-mRNAs, we observed no difference in their efficiency of splicing between *Hmt1* and Δ *hmt1* cells (Figure 4A; compare Hmt1 “+” to Hmt1 “-“ bars above Npl3 WT; and Figure S4A; compare Hmt1 “+” to Hmt1 “-“ bars). In contrast, loss of Hmt1 affected the efficiency of splicing of the first intron of *SUS1* pre-mRNA at both 30°C and 22°C (Figure 4B; *SUS1* (Intron 1) panel, compare Hmt1 “+” to Hmt1 “-“ bars above Npl3 WT). Notably, the second intron of the *SUS1* pre-mRNA, which harbors consensus 5’ splice site and branch site, did not display an Hmt1-dependent decrease in splicing efficiency at these temperatures (Figure 4B; *SUS1* (Intron 2) panel, compare Hmt1 “+” to Hmt1 “-“ bars above Npl3 WT). Thus, our data implicate a role for Hmt1 in promoting efficient splicing of introns with a non-consensus 5’ splice site and a branch site, but not those with consensus sequences.

3.4. Requirement of Npl3 arginine methylation for efficient splicing of the *SUS1* pre-mRNAs

In mammalian cells, the SR proteins stabilize the association between the U1 snRNP and the 5’ splice site [58], and they are thought to function by binding to exonic sequences adjacent to suboptimal SS and promoting recruitment of the U1 and U2 snRNPs. While the budding yeast lacks homologs of these SR proteins, Npl3, a substrate of Hmt1 [44, 45], is considered an SR-like protein [26, 59]. Previously, Npl3 has been shown to physically association with the U1 snRNP on the chromatin and facilitates splicing of a specific set of intron-containing transcripts [57]. Thus, we hypothesized that the arginine methylation of Npl3 may be critical for efficient splicing of introns with non-consensus splice sites. To probe the impact of Npl3 arginine methylation on the splicing

efficiency of the same intron-containing transcripts, we expressed Npl3^{RK} in the *HMT1* and *Δhmt1* backgrounds, as Npl3^{RK} phenocopies wild-type Npl3 behavior when expressed in the *Δhmt1* background.

Employing the same RT-qPCR approach, we found that the efficiency of splicing of the *ECM33* pre-mRNA did not differ between the *HMT1* and *Δhmt1* cells expressing Npl3^{RK} (Figure 4A; compare the bars in Hmt1 “-“ above Npl3 RK to Hmt1 “+” above Npl3 WT). In the case of *SUS1* intron 1, the expression of Npl3^{RK} in *Δhmt1* cells restored the efficiency of pre-mRNA splicing to a similar level as those in the *HMT1* background with wild-type Npl3 expression (Figure 4B; compare the bars in Hmt1 “-“ above Npl3 RK to Hmt1 “+” above Npl3 WT in *SUS1* (Intron 1) panel). Similar to *ECM33*, expressing Npl3^{RK} in *Δhmt1* cells does not affect the efficiency of splicing of *SUS1* intron 2 (Figure 4B, compare the bars in Hmt1 “-“ above Npl3 RK to Hmt1 “+” above Npl3 WT in *SUS1* (Intron 2) panel). The same is true for *ASC1* (Figure S4B). Thus, the use of an Npl3^{RK} mutant that phenocopies wild-type Npl3 behavior when expressed in *Δhmt1* cells allows us to conclude that arginine methylation of Npl3 positively influences the splicing of *SUS1* intron 1.

3.5. Changing a non-consensus splice site in *SUS1* to its consensus counterpart rescues splicing efficiency in the *Δhmt1* background

Whereas the introns of both *ECM33* and *ASC1* have consensus 5' splice site (GUAUGU) and branch site sequences (UACUAAC), the first intron of *SUS1* has a non-consensus 5' splice site (GUAUGA) and branch site sequence (UACUGAC) (Figure 1A). To determine if Hmt1 plays a role in the splicing of pre-mRNAs containing non-consensus splice sites, we mutated the non-consensus 5' splice site and the branch site

sequence in the *SUS1* intron 1 to the consensus sequence and ectopically expressed this clone (Figure 5A) in a Δ *sus1* strain. We then determined the efficiency of splicing of *SUS1* intron 1 in this strain by RT-qPCR as described above (Figure 5B, CONS lanes). The *SUS1* intron 1 with a consensus 5' splice site and branch site sequence was more efficiently spliced than its wild-type, non-consensus counterpart in both *HMT1* and Δ *hmt1* backgrounds at both temperatures tested (Figure 5B; compare WT to CONS lanes). Thus, the splicing efficiency of this modified *SUS1* construct is refractory to Hmt1 status, similar to what was observed with *ECM33* and *ASC1*. These data support a role for Hmt1 in promoting the utilization of a non-consensus 5' splice site and branch site by the spliceosome.

3.6. Methylation of Npl3 does not directly affect its binding to *SUS1* RNA

Arginine methylation has previously been implicated as a means of regulating how a substrate interacts with nucleic acids and proteins [60, 61]. Since Npl3 is an RNA-binding protein that harbors RRM motifs [62], we hypothesize that a mechanism by which Hmt1-mediated methylation of Npl3 affects pre-mRNA splicing of *SUS1* may be through its ability to bind *SUS1* pre-mRNA. To this end, we tested the RNA-binding capacity of recombinant Npl3 containing either methylated or unmethylated arginines that were generated by an *E. coli* co-expression system similar to the one described previously [63]. Based on the pattern of migration shift of recombinant Npl3 in the immunoblot, we conclude that these recombinant Npl3 proteins are likely all fully methylated. We then performed an electrophoretic gel mobility shift assay (EMSA) with the recombinant methylated or unmethylated Npl3 and *in vitro* transcribed *SUS1* (Figure S5A). Binding of both forms of Npl3 to the tested RNA was robust and specific, as

determined by competition experiments with either specific or non-specific competitors (Figure S5B). We did not detect any significant differences in the binding of methylated versus unmethylated Npl3 to *SUS1* under any of the several experimental conditions. Thus, methylation of Npl3 does not affect its direct binding to *SUS1* RNA and likely affects *SUS1* pre-mRNA splicing via perturbing its interaction with a protein partner.

3.7. Methylation of Npl3 is critical for specific interaction with Mud1, a subunit of the U1 snRNP

To determine if the methylation status of Npl3 affects the interaction between Npl3 and the U1 snRNP, we performed a pulldown experiment with immobilized methylated or unmethylated Npl3, measuring the amount of snRNA as a readout for snRNPs associated with Npl3 (Figure 6A; bars above U1). We detected more U1 snRNA in the pulldown with unmethylated Npl3 versus methylated Npl3, suggesting that methylation promotes the dissociation between U1 snRNPs and Npl3 (Figure 6A; compare the bar from Npl3 methylation “+” to Npl3 methylation “-” above U1). This effect is specific for the U1; no notable difference in pulldown of either U2 or U4 snRNAs was observed when using methylated versus unmethylated Npl3 (Figure 6A; compare the bar from Npl3 methylation “+” to Npl3 methylation “-” above U2 and U4).

Having demonstrated that the methylation of Npl3 interferes with its interaction with the U1 snRNP, we sought to determine which subunit(s) mediates this interaction. We first expressed and purified individual U1 snRNP subunits as GST fusion constructs in *E. coli*, and then tested their individual interactions with methylated or unmethylated Npl3 (Figure 6B). For Luc7 or Nam8, we did not detect any interaction (Figure 6B; GST-Luc7 and GST-Nam8 panels, compare the band intensities from Npl3 “+” lanes to

Npl3 “-“ lanes under GST-Protein Pulldown). The interaction between Npl3 and the U1 snRNP subunit Snu56 was not affected by methylation (Figure 6B; GST-Snu56 panel, compare the band intensities from Npl3 “+ lanes to Npl3 “-“ lanes under GST-Protein Pulldown) whereas the interaction with Snp1 and Yhc1 were slightly increased (Figure 6B; GST-Snp1 and GST-Yhc1 panels; compare the band intensities from Npl3 “+ lanes to Npl3 “-“ lanes under GST-Protein Pulldown). The greatest change in the level of interaction was detected for Mud1 and unmethylated Npl3 relative to Mud1 and methylated Npl3 (Figure 6B; GST-Mud1 panel; compare the band intensities from Npl3 “+ lanes to Npl3 “-“ lanes under GST-Protein Pulldown). Thus, arginine methylation of Npl3 is critical for its interaction with the U1 snRNP, and this is likely accomplished via its association with Mud1.

4. Discussion

The splicing architecture in the budding yeast is simple relative to that of metazoans, and yeast introns conform to tight consensus sequences at their splice sites. One outstanding question with respect to the yeast splicing machinery is whether it is capable of regulating splice-site selection, with an implication that such regulation could impact gene expression. The presence of non-consensus splice-site sequences at certain intron-containing genes supports the hypothesis that the yeast splicing machinery is capable of regulating splice-site selection. One potential means by which yeast splicing machinery can regulate splice site selection is by post-translational modifications on core components of the spliceosome or proteins that interact with the spliceosome. Our present work demonstrates that Hmt1 promotes the spliceosome usage of non-consensus splice sites in *SUS1* intron 1. This is in contrast to *ECM33*, *ASC1*, and *SUS1* intron 2, all

of which harbors a consensus 5' splice site and branch site (Figure 1A). When the non-consensus 5' splice site and branch site were changed back to consensus in *SUS1* intron 1, its splicing efficiency in *Δhmt1* cells was restored to the level seen in *HMT1* cells and refractory to Hmt1-status, further supporting a role for Hmt1 in promoting utilization of a non-consensus 5' splice site and branch site by the spliceosome.

In addition to having its majority of splice sites conform to a strict consensus, most of the intron-containing genes in the budding yeast contains only a single intron and do not undergo alternative splicing. This may explain a lack of SR proteins and hnRNPs that normally control transcript-specific splicing in higher eukaryotes. In mammalian cells, SR proteins play key regulatory roles in constitutive and alternative splicing [27, 28, 64-66]. These proteins have a modular domain structure, including one or more RNA recognition motifs (RRMs), as well as a C-terminal RS domain that is enriched for arginine and serine residues, which are often sites of post-translational modifications. SR proteins stabilize the association between the U1 snRNP and the 5' splice site [58], and they are thought to function by binding to exonic sequences adjacent to suboptimal splice sites and promoting recruitment of the U1 and U2 snRNPs. Npl3 is an SR-like protein [26, 59] that shares structural features with members of the hnRNP family [67-69]. It has two RRM domains and a C-terminal domain that contains multiple RGG tripeptides and RG dipeptides. The vast majority of aDMAs of Npl3 are present within the RGG context [46]. As Npl3 is required for the splicing of a subset of pre-mRNAs [57], we posited that Hmt1-mediated methylation of Npl3 could be a means of regulating the splicing efficiency of pre-mRNAs with non-consensus splice sites. Employing a previously characterized Npl3 mutant (Npl3^{RK}) that phenocopies wild-type Npl3 when expressed in

a *Δhmt1* background [46, 55, 56], we demonstrated that Hmt1, via its methylation of Npl3, promotes efficient splicing of *SUS1* intron 1. When expressed in a *Δhmt1* background, Npl3^{RK} mutant mimics wild-type Npl3 behavior in its subcellular localization and protein-protein interactions [46, 56]. In our present study, we further show that the co-transcriptional profile for Npl3^{RK}, when expressed in *Δhmt1* background, mimics wild-type Npl3. A similar approach was previously employed to study the mammalian SR-protein SF2/ASF, which is a substrate of human PRMT1 [70]. Characterization of a SF2/ASF triple point mutant in which the three identified methylarginines were replaced by lysines revealed this mutant to be functionally indistinguishable from wild-type SF2/ASF in its ability to carry out general and alternative splicing *in vitro* [70]. Thus, the use of Npl3^{RK} mutant makes it possible for us to probe the specific impact of wild-type Npl3 in isolation and define what contributes to the co-transcriptional recruitment and splicing efficiency phenotypes observed in *Δhmt1* cells.

Protein arginine methylation has been known to play a key role in the protein-nucleic acids and protein-protein interactions [71-73], as methylation of the terminal amino group in the arginine side chain does not alter its positive charge but increases the hydrophobicity, resulting in a bulkier side chain and thereby blocking any potential formation of hydrogen bonds. Our RNA gel shift assays indicate that arginine methylation of Npl3 does not impact its ability to associate with *SUS1* introns (Figure S5). Turning our attention to its potential impact on protein-protein interactions, our *in vitro* data shows an increased biochemical association between unmethylated Npl3 and the U1 snRNP, relative to methylated Npl3 and the U1 snRNP, and this observation is

made only with the U1 snRNP, not U2 nor U4 snRNP (Figure 6A). Subsequent pairwise testing with individual U1 snRNP subunits revealed that Mud1, the yeast homolog of human U1-A, is the subunit that demonstrates an increased association with unmethylated Npl3, relative to its association with methylated Npl3 (Figure 6B). Mud1 is a subunit of the U1 snRNP and is important for 5' splice site selection and the splicing efficiency of intron-containing transcripts in yeast [74, 75]. *In vivo* splicing efficiency assays using intron-containing reporters have shown that yeast cells with defective Mud1 adversely affect splicing of an inefficient intron-containing reporter versus that of an efficient spliced intron-containing reporters [76]. Therefore, it is possible that the methylation of Npl3 plays an important role in promoting how U1 snRNP engages with the pre-mRNA via its interaction with Mud1. Further experiments will need to be carried out in order to better understand the mechanistic details of how arginine methylation of Npl3 impacts Mud1 function within the U1 snRNP.

Many post-translational modifications of spliceosomal components have been reported, but both the mechanism whereby these modifications of the splicing factors are regulated and their functional consequences for pre-mRNA splicing remain obscure. The work we report here provides insight into how one such modification controls pre-mRNA splicing. We predict that the establishment of this yeast mechanism will facilitate the analysis of similar mechanisms in higher eukaryotes, given the strong conservation of PRMT-mediated arginine methylation in mammalian cells.

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Figure and Table Legends

Figure 1: Schematic representation of the intron-containing genes used in chromatin immunoprecipitation (ChIP) experiments.

(A) For the ChIP experiments described in this study, the primer sets used for qPCR detection of specific regions within the intron-containing genes *ECM33* and *SUS1* are denoted by corresponding letters and numbers.

(B) The consensus sequences of the yeast splice sites, compared to the ones present in the intron-containing genes *ECM33* and *SUS1*. The red colored nucleotide highlights the difference of such nucleotide from the consensus sequence.

Figure 2: Co-transcriptional recruitment of snRNP subunit and snRNP-associated proteins to intron-containing genes is altered in *Δhmt1* cells

qPCR results for products of chromatin immunoprecipitation, performed on either wild-type or *Δhmt1* cells using an α -myc antibody (9E11) or α -human IgG (as a negative control IP). Immunoprecipitated DNA was normalized to input chromatin DNA. (A-D) snRNP subunit and snRNP-associated proteins examined in each panel is indicated. (E) Rpb3 was tested, using an α -Rpb3 antibody, as a control for RNA polymerase II occupancy. (F-G) An RNase treatment step was added to the ChIP protocol for Npl3 and Snp1. Letters and numbers (e.g. A1) under the graphs correspond to the specific primer sets indicated in Figure 1A. Percentage of input is calculated by ΔC_T , with error bars representing standard error of the mean of three biological replicates (n=3). *P*-value: *<0.05; and **< 0.01.

Figure 3: Arginine methylation of Npl3 promotes proper co-transcriptional recruitment of U1 snRNP subunit Snp1 and SR-like protein Npl3.

qPCR results for products of chromatin immunoprecipitation performed on *NPL3* strains of either the *HMT1* or $\Delta hmt1$ background, and *npl3RK* strains of either the *HMT1* or $\Delta hmt1$ background. (A) Npl3, (B) Snp1, and (C) Lea1. Letters and numbers (e.g. A1) under the graphs correspond to the specific primer sets indicated in Figure 1A.

Percentage of input is calculated by ΔC_T , with error bars representing SEM of three biological samples (n=3). *P*-value: * <0.05 ; ** <0.01 , and *** <0.001 .

Figure 4: Arginine methylation of Npl3 promotes efficient splicing of the *SUS1* intron harboring a non-consensus 5' splice site and branch site.

Pre-mRNA splicing efficiency of *ECM33* (A) and both *SUS1* introns (B) is determined by qPCR on reverse-transcribed total RNA from yeast cells of either *HMT1* or $\Delta hmt1$ background expressing either Npl3 (WT) or Npl3^{KK} (RK). Percentage of introns spliced was determined by ΔC_T after efficiency correction between an exonic primer pair and an intronic primer pair, and shown as a bar graph. Cultures were grown at 30°C and 22°C. Error bars represent the SEM of three biological replicates (n=3). *P*-value: * <0.05 and ** <0.01 .

Figure 5: Replacement *SUS1* intron 1 non-consensus 5' splice site and branch site with consensus sequences restores its efficient splicing independent of Hmt1

(A) Comparison of yeast consensus 5' splice site and branch site to that of *SUS1* intron 1. Underlined *SUS1* bases have been mutated to the consensus sequence shown above.

(B) Splicing efficiency of *SUS1* (WT) and *SUS1* (CONS; with the *SUS1* bases in the 5' splice site and branch site in intron 1 mutated to the consensus sequence) as quantified by qPCR on reverse-transcribed total cellular RNA as in Figure 4. Error bars represent the SEM of three biological replicates (n=3). *P*-value: * <0.05 .

Figure 6: Arginine methylation of Npl3 plays a role in its biochemical association with the U1 snRNP.

(A) qPCR results of U-snRNA levels from either methylated or unmethylated Npl3 pulldown of yeast whole-cell extract are shown. The results were normalized to a spiked-in control RNA, and then to input RNA. The negative controls are beads only.

(B) Individual GST-tagged U1 snRNP subunit was immobilized on GSH-magnetic beads and used as bait for pulldown with methylated or unmethylated Npl3. The methylated and unmethylated Npl3 pulldown signals are quantitated and the $\log_2$ ratio of the two signals is shown. A negative ratio indicate that unmethylated Npl3 interacts more strongly with the bait protein than methylated Npl3; a positive ratio indicates that methylated Npl3 interacts more strongly than unmethylated Npl3, and a ratio near 0 indicates that the level of interaction is the same.

A)

B)

A)

B)

C)

A)

B)

SUS1 (intron 1) **SUS1 (intron 2)**

B)