Sequence-specific and Shape-selective RNA Recognition by the Human RNA 5-Methylcytosine Methyltransferase NSun6*

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Human NSun6 is an RNA methyltransferase that catalyzes the transfer of the methyl group from S-adenosyl-L-methionine (SAM) to C72 of tRNA^{Thr} and tRNA^{Cys}. In the current study, we used mass spectrometry to demonstrate that human NSun6 indeed introduces 5-methylcytosine (mC) into tRNA, as expected. To further reveal the tRNA recognition mechanism of human NSun6, we measured the methylation activity of human NSun6 and its kinetic parameters for different tRNA substrates and their mutants. We showed that human NSun6 requires a well folded, full-length tRNA as its substrate. In the acceptor region, the CCA terminus, the target site C72, the discriminator base U73, and the second and third base pairs (2:71 and 3:70) of the acceptor stem are all important RNA recognition elements for human NSun6. In addition, two specific base pairs (11:24 and 12:23) in the D-stem of the tRNA substrate are involved in interacting with human NSun6. Together, our findings suggest that human NSun6 relies on a delicate network for RNA recognition, which involves both the primary sequence and tertiary structure of tRNA substrates.

Post-transcriptional modifications frequently occur in all types of cellular RNAs (for RNA modification databases, see Refs. 1 and 2). Currently, more than 130 RNA modifications have been identified, and these modifications are involved in many crucial life processes (3–5). Methylation of cytosine at the 5′ position of DNA is an important epigenetic marker that affects the expression of genes (6). dm5C modifications have been extensively studied in both DNA and RNA (dm5C3 and mC), is notable because it is widespread in both coding and non-coding RNAs (9, 10). Among all cellular RNAs, tRNAs contain the greatest number of modified nucleosides (4). As an adaptor molecule between amino acids and mRNA, tRNA plays a pivotal role in protein synthesis (11). Nucleoside modifications affect every aspect of tRNA function, from biogenesis and structural stability to the efficiency and accuracy of decoding (12–15). Meanwhile, tRNA modifications have been shown to play regulatory roles in cellular stress, immune responses, diabetes, cancers, and other diseases (16–20). The mC modification is also present in tRNA but only in archaea and eukaryotes, not in bacteria (1, 2, 9). For eukaryotic tRNA, mC is most abundant in the junction region between the variable loop and the T-stem, usually at position 48 and/or 49 and sometimes at position 50 (9, 10). The mC modification also occurs in the anticodon loop of tRNA, frequently at position 34 or 38 (9, 21, 22). In mice, mC modifications in the variable region and anticodon loop promote tRNA stability and protein synthesis (23). The amino acid acceptor of the tRNA is rarely modified; only a few modifications have been identified in this region. However, an mC modification at position 72 in the acceptor stem of many tRNAs is conserved in higher eukaryotes (1, 2). The function of this mC72 modification remains elusive, and the enzyme that is thought to establish this modification has only recently been identified (24). Through cross-linking and deep sequencing methods, the putative human RNA mC methyltransferase (MTase) NSun6 (NSun family, member 6) has been found to catalyze the methylation at position 72 in some human tRNAs (24).

The NSun family includes the main RNA mC MTases in eukaryotes and contains seven members: NSun1–NSun7 (25). Each of these proteins contains an NOL1/NOP2/Sun (NSun) domain, which is an S-adenosyl-L-methionine (SAM)–dependent MTase domain. RNA substrates have been identified for six NSuns, with the exception of NSun7. NSun1, NSun4, and...
NSun5 catalyze m^5C modifications at specific sites in tRNA (26–27), and NSun2, NSun3, and NSun6 modify tRNA (24, 28, 29). The biological functions of some NSun members have been investigated, revealing roles in protein biosynthesis, cell proliferation and differentiation, and organ development (30–33). The genetic mutation and aberrant expression of several NSun family members also cause many human diseases. For example, the NSUN2 and NSUN5 genes are related to intellectual disability; NSUN2 mutations cause autosomal recessive non-syndromic mental retardation (34, 35), and NSUN5 is among the genes that are completely deleted in Williams-Beuren syndrome (36). Additionally, mutations in NSUN7 cause male infertility in mice and humans (37, 38), and increased NSun1 and NSun2 protein expression has been observed in various cancers (39, 40). However, the modification mechanism, tertiary structure, and RNA recognition mechanism of most NSun MTases remain largely unexplored.

In higher eukaryotes, RNA m^5C modifications are widely found in both coding and non-coding RNAs. All known eukaryotic RNA m^5C MTases are members of the NSun family, along with a tRNA 38: m^5C MTase from the DNA m^5C MTase (Dnmt) family, Dnmt2 (21). The RNA recognition mode and substrate specificity of these enzymes, particularly how they recognize RNA substrates and whether they methylate other types of RNAs, are highly intriguing questions in the field. NSun6 is a newly identified tRNA MTase. Haag et al. (24) have determined that human NSun6 (hNSun6) interacts with tRNA\(^{\text{Cys}}\) and tRNA\(^{\text{Thr}}\) in vitro and methylates C72 of these tRNAs in vitro. By investigating the subcellular locations of overexpressed hNSun6 in HEK293 cells, this group has shown that hNSun6 localizes to the cytoplasm, particularly to the Golgi apparatus and pericentriolar matrix (24). However, the biological function, modification mechanism, and RNA recognition specificity of NSun6 all remain elusive. In the current study, we focused on the RNA recognition mechanism of hNSun6. Our results showed that hNSun6 uses a complicated network for RNA recognition and that the tertiary structure and some characteristic elements of the tRNA are all required. Other cellular RNAs are unlikely to satisfy all of the elements required for hNSun6 recognition; thus, we conclude that hNSun6 is a tRNA-specific MTase.

**Results**

**hNSun6 Catalyzes the m^5C Modification of tRNA\(^{\text{Thr}}\) and tRNA\(^{\text{Cys}}\)—**Previous research has shown that tRNA\(^{\text{Thr}}\)(UGU), tRNA\(^{\text{Thr}}\)(AGU), and tRNA\(^{\text{Cys}}\)(GCA) are methylated at position C72 by hNSun6 (24). Thus, we prepared these tRNA substrates of hNSun6 by using in vitro transcription with T7 RNA polymerase (Fig. 1A). Recombinant hNSun6, which is 469 amino acids long and included a C-terminal His\(_6\) tag (8 amino acids), was purified to very high purity, as determined by SDS-PAGE (Fig. 1B). Recombinant hNSun6 was eluted at 16.42 ml, corresponding to a calculated molecular mass of 54.4 kDa, as compared with the theoretical molecular mass of recombinant hNSun6 of 52.8 kDa, suggesting that hNSun6 protein exists mainly as a monomer in solution (Fig. 1C). This purified hNSun6 was used for the subsequent methyl transfer assays. Consistent with results from previous research, our results showed that hNSun6 independently catalyzed methyl transfer from SAM to tRNA\(^{\text{Thr}}\)(UGU), tRNA\(^{\text{Thr}}\)(AGU), and tRNA\(^{\text{Cys}}\)(GCA) in vitro (Fig. 1D) (24). We then performed mass spectrometry to confirm the identity of the modification as m^5C. Only one peak for the modified nucleoside was detected by ultra-performance liquid chromatography (UPLC)-MS/MS in solutions of the tRNA substrates that had been reacted with hNSun6 and digested by RNases, and the peak exactly matched with the m^5C standard (Fig. 2A); however, no such peak for the modified nucleoside was detected with tRNA substrates that were not reacted with hNSun6 (Fig. 2B). Thus, our data showed that hNSun6 catalyzes the m^5C modification of tRNA\(^{\text{Thr}}\)(UGU), tRNA\(^{\text{Thr}}\)(AGU), and tRNA\(^{\text{Cys}}\)(GCA) in vitro. Subsequently, we measured the steady-state kinetic parameters of hNSun6 for these substrates. hNSun6 catalyzed the m^5C modification of all three tRNAs with high efficiency, with \(k_{cat}\) values of \(\sim 0.5\) min\(^{-1}\) (Table 1). However, the \(K_m\) values showed some differences. The \(K_m\) value for tRNA\(^{\text{Cys}}\)(GCA) was 0.89 \(\mu\)M, which was much lower than those for tRNA\(^{\text{Thr}}\)(UGU) (3.02 \(\mu\)M) and tRNA\(^{\text{Thr}}\)(AGU) (1.58 \(\mu\)M) (Table 1). We therefore chose tRNA\(^{\text{Cys}}\)(GCA), which had a lower \(K_m\) value, as a substrate for hNSun6 to further study hNSun6 tRNA recognition.

**The Acceptor Region of tRNA Is Critical for Recognition by hNSun6—**Human cytosolic tRNA\(^{\text{Phe}}\)(GAA) shares 72.4% sequence identity with tRNA\(^{\text{Cys}}\)(GCA), but hNSun6 did not detectably methylate tRNA\(^{\text{Phe}}\)(GAA) (Fig. 3, A and B). To identify the elements involved in enzyme recognition, several chimeric tRNAs were constructed from active tRNA\(^{\text{Cys}}\)(GCA) and inactive tRNA\(^{\text{Phe}}\)(GAA) as substrates for hNSun6 (Fig. 3C). Each chimeric tRNA had one stem/loop from the active tRNA\(^{\text{Cys}}\)(GCA) and other regions from the inactive tRNA\(^{\text{Phe}}\)(GAA), resulting in C-Acc-F with the accepting stem of tRNACys(GCA), C-Dsl-F with the D-stem and loop of tRNACys(GCA), C-Asl-F with the anticodon stem and loop, and C-Tsl-F with the TΨC stem and loop of tRNA\(^{\text{Cys}}\)(GCA) (Fig. 3C). Interestingly, only C-Acc-F in which the acceptor region of tRNA\(^{\text{Cys}}\)(GCA) was swapped for the equivalent region of tRNA\(^{\text{Phe}}\)(GAA) was methylated by hNSun6 at similar levels to tRNA\(^{\text{Cys}}\)(GCA) methylation; however, the other chimeric tRNAs were not methylated by hNSun6 (Fig. 3B). Furthermore, the steady-state kinetic constants for C-Acc-F were similar to those of hNSun6 for tRNA\(^{\text{Cys}}\)(GCA) (Table 2). Thus, our results showed that substrate recognition by hNSun6 requires the presence of correct identity elements within the amino acid acceptor region of the tRNA.

**Essential Nucleotide Residues within the Acceptor Region of the tRNA for hNSun6 Recognition—**We next examined the tRNA acceptor region in detail to identify crucial residues involved in RNA recognition by hNSun6. Previous experiments have shown that the CCA terminus of tRNA is essential for recognition by hNSun6 (24). Although all tRNAs contain a CCA terminus within the acceptor, only a subset of C72-containing tRNAs are methylated by hNSun6, thus indicating that the CCA terminus is not sufficient for hNSun6 to distinguish its substrates from other tRNAs. Thus, additional essential recognition residues within the acceptor region of the tRNA substrate must play a role. Aside from the CCA terminus, the dis-
FIGURE 1. hNSun6 methylates tRNA^Thr and tRNA^Cys. A, the cloverleaf structures of three tRNA transcripts that are substrates of hNSun6. B, SDS-PAGE analysis of hNSun6 from purified proteins. The hNSun6 protein was stained with Coomassie Brilliant Blue. The theoretical molecular mass of recombinant hNsun6 was 52.8 kDa. C, recombinant hNsun6 was purified and analyzed by gel filtration chromatography on a Superdex™ 200 column. hNsun6 was eluted at 16.42 ml, corresponding to a calculated molecular mass of 54.4 kDa. The locations of the marker proteins are shown above the graph. D, the capacity of tRNA^Thr(UGU), tRNA^Thr(AGU), and tRNA^Cys(GCA) to be methylated by hNSun6. * no tRNA. Error bars, S.E. of three independent experiments.

FIGURE 2. hNSun6 catalyzes the m^5C modification. A, the m^5C modification of tRNA substrates after hNSun6 incubation was detected by mass spectrometry. B, the m^5C modification of tRNA substrates without hNSun6 incubation was detected by mass spectrometry.
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criminator base at position 73 and a seven-base pair acceptor stem in the acceptor of tRNA may be involved in recognition. From sequence alignments and secondary structure analyses of these tRNA substrates, we determined that the fourth, fifth, sixth, and seventh base pairs of the acceptor stem are divergent (Fig. 1A), whereas other regions, such as base 73 and the top three base pairs of the acceptor stem, are conserved. Therefore, we focused on these regions of the hNSun6 tRNA substrate.

Position 73 is located just between the methylation site C72 and the CCA terminus. The nucleotide residue at site 73 in tRNA^Thr(UGU), tRNA^Thr(AGU), and tRNA^Cys(GCA) is consistently G2:C71 (Fig. 1A) (41). To determine whether U73 is essential for hNSun6 catalysis, three mutations were generated, resulting in tRNA^Cys(GCA)-U73C, tRNA^Cys(GCA)-U73A, and tRNA^Cys(GCA)-U73G mutants (Fig. 4A). Surprisingly, methylation assays showed that none of these mutant tRNAs were methylated by hNSun6 (Fig. 4B). These data suggest that U73 is a critical element for tRNA recognition by hNSun6.

The second base pair in the acceptor stem of tRNA^Thr(UGU), tRNA^Thr(AGU), and tRNA^Cys(GCA) is consistently G2:C71 (Fig. 1A) (41), but it is C2:G71 in the inactive tRNA^Phe(GAA) (Fig. 3A). Therefore, we performed G2A:C71, G2U:C71, G2U:C71G substitutions in tRNA^Cys(GCA) to form four mutants: tRNA^Cys(GCA)-G2A:C71U, tRNA^Cys(GCA)-G2U:C71A, tRNA^Cys(GCA)-G2U:C71G, and tRNA^Cys(GCA)-G2C:C71G (Fig. 4A). Unexpectedly, hNSun6 methylated all of these mutants. The tRNA^Cys(GCA)-G2A: C71U, -G2U:C71A, and -G2U:C71G mutants were methylated more efficiently than the WT tRNA^Cys(GCA) (Fig. 4C). Consistent with these findings, the K_m values of hNSun6 for tRNA^Cys(GCA)-G2A:C71U, -G2U:C71A, and -G2U:C71G were decreased ~3-fold compared with WT tRNA^Cys(GCA) (Table 3). However, the last mutant, tRNA^Cys(GCA)-G2C:C71G, was a poor substrate for hNSun6 (Fig. 4C), possibly because the K_m value of hNSun6 for tRNA^Cys(GCA)-G2C:C71G was >2-fold higher than that of WT tRNA^Cys(GCA) (Table 3). The k_cat values of hNSun6 for all four mutants were similar to that of WT tRNA^Cys(GCA), thus suggesting that the second base pair does not affect the catalytic rate (Table 3). However, the decrease or increase in the K_m values of hNSun6 for these mutants strongly suggested that the second base pair in the acceptor stem influences the binding of hNSun6 to tRNA (Table 3). Furthermore, the A:U, U:A, and U:G substitutions, which form two hydrogen bonds, induced lower K_m values for hNSun6 than did C:G or G:C base pairs.

TABLE 1
Kinetic parameters of hNSun6 for various tRNA substrates in the methyl transfer reaction

| tRNAs         | K_m  | k_cat | k_cat/K_m |
|---------------|------|-------|----------|
| tRNA^Thr(UGU)| 3.02 | 0.50  | 0.17     |
| tRNA^Thr(AGU)| 1.58 | 0.79  | 0.50     |
| tRNA^Cys(GCA)| 0.89 | 0.54  | 0.61     |

| tRNAs         | K_m  | k_cat | k_cat/K_m |
|---------------|------|-------|----------|
| tRNA^Thr(UGU)| 0.02 | 0.61  |          |
| tRNA^Thr(AGU)| 0.02 | 0.50  |          |
| tRNA^Cys(GCA)| 0.03 | 0.17  |          |

FIGURE 3. Recognition elements of tRNA by hNSun6. A, the schematic diagrams of hypothesized secondary and tertiary structures of tRNA^Cys(GCA) (black) and tRNA^Phe(GAA) (red). B, the methyltransferase activity of hNSun6 toward tRNA^Cys(GCA) (●), tRNA^Phe(GAA) (▲), and chimeras of tRNA^Phe(GAA) with each separate stem/loop of tRNA^Cys(GCA): C-Acc-F (●), C-Dsl-F (●), C-Asl-F (●), and C-Tsl-F (●). Error bars, S.E. of three independent experiments. C, the schematic diagrams of hypothesized tertiary structures of chimeras of tRNA^Phe(GAA) with each separate stem/loop of tRNA^Cys(GCA) (black and red): C-Acc-F, tRNA^Phe(GAA) with the acceptor region of tRNA^Cys(GCA); C-Dsl-F, tRNA^Phe(GAA) with the D-stem/loop of tRNA^Cys(GCA); C-Asl-F, tRNA^Phe(GAA) with the anticodon stem/loop of tRNA^Cys(GCA); and C-Tsl-F, tRNA^Phe(GAA) with the T-stem/loop of tRNA^Cys(GCA). The schematic diagrams of hypothesized tertiary structures of all tRNAs are derived from the crystal structure of the cysteinyl-tRNA^Cys,EFTu-GDPNP complex (Protein Data Bank entry 1B23) (60).
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pairs with three hydrogen bonds, thus suggesting that hNSun6 prefers to bind to a flexible base pair next to the C72 site. Together, these results clearly showed that the second base pair in the tRNA acceptor stem is important for RNA recognition by hNSun6.

Similarly, the third base pair in the acceptor stem was C3:G70 in tRNA^{Thr} (UGU) and tRNA^{Thr} (AGU) and G3:C70 in tRNA^{Cys} (GCA) (Fig. 1A) (41). To determine whether hNSun6 recognizes this base pair, we mutated G3:C70 from tRNACys-G3:G70 in tRNA^{Cys} (GCA) to A:U, U:A, or U:G, resulting in tRNACys-G3:A:C70, tRNACys-G3:U:C70, and tRNACys-G3:G:C70 (Fig. 4A). Methylation assays using hNSun6 and tRNA^{Cys} (GCA)-G3:C70G showed a clear decrease in methylation activity compared with that of WT tRNA^{Cys} (GCA) (G3:C70G, 0.89 min⁻¹, K_m = 0.89 μM, and k_cat/K_m = 0.61 min⁻¹ μM⁻¹) (Table 3). Furthermore, the methylation activity of hNSun6 toward tRNA^{Cys} (GCA)-G3:A:C70U and tRNA^{Cys} (GCA)-G3:U:C70A was completely undetectable (Fig. 4D). These data implied that hNSun6 prefers to methylate substrates with a rigid G:C or C:G at the third base pair in the acceptor stem as opposed to substrates with flexible base pairs (A:U, U:A, and U:G).

On the basis of the above observations, we tried to convert the inactive tRNA^{Phe} (GAA) into an hNSun6 substrate. Because the third base pair in the acceptor stem of tRNA^{Phe} (GAA) is C3:G70, we needed to mutate only position 73 and the second base pair in the acceptor stem of tRNA^{Phe} (GAA). Initially, we mutated position 73 and the second base pair separately, obtaining tRNA^{Phe} (GAA)-A73U and tRNA^{Phe} (GAA)-C2G:G71C, respectively (Fig. 5A). Neither of these mutants was efficiently methylated by hNSun6 (Fig. 5B). However, when we combined these two mutations into tRNA^{Phe} (GAA)-A73U/C2G:G71C, hNSun6 was able to efficiently methylate this variant (Fig. 5B). In the steady-state kinetic assays, the k_cat value of hNSun6 for tRNA^{Phe} (GAA)-A73U/C2G:G71C was 7.53 μM, which was higher than that of tRNA^{Cys} (GCA) (0.89 μM), whereas the k_cat value of hNSun6 for tRNA^{Phe} (GAA)-A73U/C2G:G71C was 0.52 min⁻¹, which was similar to that for tRNA^{Cys} (GCA) (0.54 min⁻¹) (Table 4). This finding further confirmed that U73 and the second base pair are important for RNA recognition by hNSun6.

In conclusion, we identified three components in the acceptor region that are important for hNSun6 recognition: a U base at position 73, a preference for a flexible base pair at the second base pair, and a preference for a rigid base pair at the third base pair.

hNSun6 Recognizes a Well Folded, Full-length tRNA Substrate—In general, tRNA MTases fall into two groups that differ in their sensitivity to structural perturbations in the tRNA...
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**Figure 5. A mutant of tRNA^{Pho}(GAA) is an hNSun6 substrate.**

*Panel A:* secondary structure of tRNA^{Pho}(GAA) highlighting the mutation in the acceptor region. *Panel B:* the capacity of tRNA^{Pho}(GAA) mutants to be methylated by hNSun6. 

| tRNAs                  | $K_m$  | $k_{cat}$ | $k_{cat}/K_m$ |
|------------------------|--------|-----------|---------------|
| tRNA^{Pho}(GAA)        | 0.89 ± 0.13 | 0.54 ± 0.02 | 0.61          |
| tRNA^{Pho}(GAA)-A73U   | ND     | ND        | ND            |
| tRNA^{Pho}(GAA)-C2G:G71C | ND    | ND        | ND            |
| tRNA^{Pho}(GAA)-A73U/C2G:G71C | 7.53 ± 0.33 | 0.52 ± 0.03 | 0.07          |

**Discussion**

NSun6 belongs to the NSun family of putative eukaryotic RNA m^5^C MTases (25). Recent research has shown that hNSun6 methylates tRNA^{Thr}(UGU), tRNA^{Thr}(AGU), and tRNAs. The nucleotide at position 11 is a pyrimidine nucleotide (C or U), and that at position 24 is a purine nucleotide (G or A), and these positions always form a typical Watson-Crick base pair (C11:G24 or U11:A24). However, the base pair between site 12 and site 23 is not conserved in all tRNAs. Thus, we constructed a mutant in the C11:G24 base pair and a series of mutants in the U12:A23 base pair, resulting in tRNA^{Cys}(GCA)-C11U:G24A, -U12A:A23U, -U12C:A23G, -U12G:A23C, -A23G, and -U12G:A23U (Fig. 7A). The tRNA^{Cys}(GCA)-C11U:G24A mutant was methylated by hNSun6 in vitro (Fig. 7B), although the $K_m$ value of hNSun6 for tRNA^{Cys}(GCA)-C11U:G24A increased 3-fold compared with WT tRNA^{Cys}(GCA) (2.72 and 0.89 μM, respectively) (Table 5). This finding indicated that substituting C11:G24 for U11:A24 affected the methylation activity of hNSun6 by impairing the binding between the tRNA and the enzyme. The methylation activity of hNSun6 was variably affected by the substitutions of the U12:A23 base pair. When U12:A23 was mutated to A12:U23, the corresponding tRNA^{Cys}(GCA)-U12A:A23U was methylated by hNSun6 as efficiently as WT tRNA^{Cys}(GCA) (Fig. 7B and Table 5). However, when U12:A23 was mutated to C12:G23 or G12:C23, the methylation of the corresponding tRNA mutants by hNSun6 was significantly reduced compared with WT tRNA^{Cys}(GCA) (Fig. 7B). Moreover, the $K_m$ values of hNSun6 for tRNA^{Cys}(GCA)-U12C:A23G (5.35 μM) and tRNA^{Cys}(GCA)-U12G:A23C (6.07 μM) were >6-fold higher than that of WT tRNA^{Cys}(GCA) (0.89 μM) (Table 5). Furthermore, when the wobble base pair G:U or U:G was introduced into site 12 and site 23, neither of these tRNA mutants (tRNA^{Cys}(GCA)-A23G and tRNA^{Cys}(GCA)-U12G:A23U) was methylated by hNSun6 (Fig. 7B). These results implied that hNSun6 methylates C72 when a Watson-Crick base pair between positions 12 and 23 is present in the tRNA and prefers A:U and U:A base pairs over G:C and C:G base pairs but cannot methylate tRNAs containing a wobble base pair. Hence, our results suggest that the C11:G24 and U12:A23 base pairs in the D-stem of tRNA^{Cys}(GCA) are also very important for hNSun6 recognition.
Here, we used mass spectrometry to confirm that hNSun6 indeed catalyzes m5C methylation. The intriguing question of how hNSun6 distinguishes its substrates from other tRNAs remains unanswered. Through domain-swapping experiments, we showed that the acceptor region is crucial for hNSun6 recognition. Previous work has shown that the common CCA terminus and the C72 target site in tRNA substrates are required for hNSun6 recognition. Furthermore, we identified three additional components in the acceptor region of the tRNA that are involved in recognition by hNSun6. First, U73 functions as a discrimination factor for hNSun6 recognition. tRNA variants with U73 mutated to A, C, or G were not methylated by hNSun6. This result may explain the previous finding that hNSun6 did not methylate an isode-
discriminator of tRNA\textsuperscript{Cys}(GCA) with A73 (24). Likewise, tRNA\textsuperscript{A\textsubscript{22}} isoacceptors that do not possess U73 were not methylated, despite weakly interacting with hNSun6 (24). Interestingly, sequence analyses show that only a few tRNAs, tRNA\textsuperscript{Thr}, tRNA\textsuperscript{Cys}, and tRNA\textsuperscript{Gln} isoacceptors, possess a U at position 73. The strict requirement for U73 in tRNAs by hNSun6 highlights U73 as a discrimination factor that can distinguish substrates from other tRNAs. Interestingly, U73 is also the discrimination base of tRNA\textsuperscript{Cys} for cysteinyl-tRNA synthetase and reportedly brings flexibility to the acceptor of tRNA, which further facilitates the aminoacylation by cysteinyl-tRNA synthetase (43, 44). Second, hNSun6 prefers to methylate tRNA substrates that contain a flexible base pair with two hydrogen bonds, such as A:U and U:A, at the second base pair in the acceptor stem. Conformational change of some base pairs near the modification site in tRNA substrates during catalysis has been observed in some modification enzymes, such as TruB (45). In our study, the second base pair in the acceptor stem is near the modification site C72. Therefore, it is tempting to hypothesize that conformational change of the second base pair in the acceptor stem of tRNA substrates is also required by hNSun6 during its methylation process. This may explain why tRNA substrates with a weak hydrogen bond base pair next to the methylation site C72 are preferred by hNSun6. From these observations, we successfully converted the inactive tRNA\textsuperscript{Phe}(GAG) into an hNSun6 substrate, tRNA\textsuperscript{Phe}(GAG)-A73U/G3A:C70U. Furthermore, in contrast to the second base pair in the acceptor stem, hNSun6 prefers to methylate tRNA substrates with a rigid base pair, such as G:C or C:G, at the third base pair in the acceptor stem. In particular, hNSun6 is completely unable to methylate mutants containing either A:U or U:A as the third base pair. These results suggested that the third base pair in the acceptor stem also functions as a discriminatory element for hNSun6 recognition. This result may explain why tRNA\textsuperscript{Gln} isoacceptors with U73 are not hNSun6 substrates, because the third base pair in their acceptor stems is U:A. Surprisingly, although it is important for hNSun6 recognition, the acceptor region alone is not sufficient for methylation by hNSun6. Indeed, hNSun6 requires a well folded, full-length tRNA as its substrate. Furthermore, we identified additional recognition elements for hNSun6, including the C11:G24 and U12:A23 base pairs in the D-stem. Specifically, the preference for A:U and U:A base pairs between positions 12 and 23 makes this base pair the third discrimination element for hNSun6 recognition. Actually, it is not uncommon for tRNA modification enzymes to require RNA tertiary structure. For example, the m\textsuperscript{1}G37 methyltransferase Trm5 and the pseudouridine synthase TruA both recognize the elbow junction of D and T loops of L-shaped tRNA (46, 47). Introduction of some small mutations into tRNAs could result in misfolding. Therefore, we checked whether some of the mutated tRNAs in our study, including those with a large domain exchange, were well folded by native polycravelilide gel assays. The chimeric and mutated tRNAs we checked all had the same mobility as their corresponding wild type tRNAs in a native polycravelilide gel assay (Fig. 8), suggesting that these tRNA mutants folded normally. Because the tertiary structures are unknown, many details regarding how hNSun6 recognizes and interacts with tRNA substrates remain elusive. Future structural and biochemical studies will be needed to further understand the process.

In summary, our current and previous results reveal that hNSun6 recognizes substrate tRNAs based on (i) a well folded, full-length tRNA, (ii) the CCA terminus and the methylation site C72 (24), (iii) the discriminator base U73, (iv) the second and third base pairs (2:71 and 3:70) of the acceptor stem, and (v) two base pairs (11:24 and 12:23) in the D-stem. Hence, we propose a model for tRNA binding to hNSun6 in which hNSun6 makes a number of contacts with the tRNA substrate (Fig. 9). Sequence analyses have suggested that NSun6 contains a pseudouridine synthase and archaeosine transglycosylase (PUA) domain, which usually contributes to RNA binding (48). In our model, hNSun6 binds both the acceptor and D-stem regions of the tRNA, and this may explain why hNSun6 requires well folded tRNA substrates. tRNA is one of the most highly modified cellular RNAs. Many studies have shown that some tRNA modifications are dependent on the presence of other prior modified nucleotides in tRNA substrates (6, 49). However, in our study, those recognition sites of hNSun6 are located in selected modified regions, and no modifications in those sites have ever been reported for eukaryotic tRNA\textsuperscript{Cys} and tRNA\textsuperscript{Thr} according to the tRNA databases (1, 2, 41). Furthermore, hNSun6 could well methylate the transcripts that are lacking modifications. Therefore, we propose that it is very likely that hNSun6 does not need to recognize other modifications in

### TABLE 5

**Kinetic parameters of hNSun6 for tRNA\textsuperscript{Cys}(GCA) with mutations in the D-stem in the methyl transfer reaction**

All parameters represent the average of three independent trials, and the S.D. values are indicated. ND, not detectable.

| tRNAs                        | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|------------------------------|-------|-----------|---------------|
| tRNA\textsuperscript{Cys}(GCA) | 0.89 ± 0.13 | 0.54 ± 0.02 | 0.61          |
| tRNA\textsuperscript{Cys}(GCA)-C11U:G24A | 2.72 ± 0.37 | 0.40 ± 0.03 | 0.15          |
| tRNA\textsuperscript{Cys}(GCA)-U12A:G23U | 1.16 ± 0.08 | 0.44 ± 0.01 | 0.38          |
| tRNA\textsuperscript{Cys}(GCA)-U12C:G23G | 5.35 ± 0.38 | 0.33 ± 0.04 | 0.06          |
| tRNA\textsuperscript{Cys}(GCA)-U12G:G23C | 6.07 ± 0.19 | 0.50 ± 0.03 | 0.08          |
| tRNA\textsuperscript{Cys}(GCA)-A23G | ND     | ND        | ND            |
| tRNA\textsuperscript{Cys}(GCA)-U12G:G23U | ND     | ND        | ND            |

**FIGURE 8. Mobility of various tRNAs by native polyacrylamide gel assay.** Shown are WT-tRNA\textsuperscript{Phe}(GAA) (lane 1), C-Acc-F (tRNA\textsuperscript{Phe}(GAA) with the acceptor region of tRNA\textsuperscript{Cys}(GCA)) (lane 2), WT-tRNA\textsuperscript{Cys}(GCA) (lane 3), and its inactive mutants tRNA\textsuperscript{Cys}(GCA)-U73A, tRNA\textsuperscript{Cys}(GCA)-G3A:C70U, and tRNA\textsuperscript{Cys}(GCA)-A23G (lanes 4–6, respectively).
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**FIGURE 9. A proposed model of hNSun6 bound to tRNA.** The structure of tRNA is derived from the crystal structure of the cysteiny1-tRNA^Met-EF-Tu-GDNP complex (Protein Data Bank entry 1B23) (60). The tRNA elements that are recognized by hNSun6 are summarized here and highlighted in sticks.

vitro, although this does not rule out the possibility that some modifications at other locations of tRNA could optimize the recognition and interaction with hNSun6 in vivo.

In this study, we showed that hNSun6 recognizes several elements in tRNA substrates, including some characteristic tRNA residues and even the tertiary structure of the tRNA. Other cellular RNAs, such as mRNA, rRNA, and microRNA, are unlikely to contain all of the elements required for hNSun6 recognition. We therefore conclude that NSun6 is a tRNA-specific m^5^C MTase, thus making NSun6 different from the other known tRNA m^5^C MTases NSun2, NSun3, and Dnmt2. In addition to tRNA substrates, NSun2 has been reported to methylate mRNA and microRNA (50, 51). Moreover, NSun2 can methylate multiple sites (C34 and C48/49/50) in multiple tRNA substrates (23). Dnmt2 is the tRNA m^5^C38 MTase in eukaryotes and is a member of the Dnmt family (21, 52). Dnmt2 shows robust methylation activity toward tRNA substrates; however, many studies have suggested that Dnmt2 also methylates DNA substrates in vitro and in vivo (53–55). NSun3 is recently reported to methylate C34 of mitochondrial tRNA^Met^, but the substrate specificity and recognition mechanism of NSun3 have not been elucidated (29). Therefore, NSun6 may be the only known tRNA m^5^C MTase that methylates only a subset of tRNAs at a single site. Further biochemical and structural studies are needed to investigate how NSun6 uses such a delicate network to recognize its tRNA substrates as well as to uncover the biological functions of this m^5^C modification at position 72 in a subset of tRNAs. Methylation affects multiple aspects of tRNA function, and its physiological significance has been exemplified by studies of relevant tRNA methyltransferases (6). Moreover, emerging evidence supports that tRNA methyltransferases have important roles in human health. For example, mutations in many tRNA methyltransferase genes, such as FT5J1 and NSUN2, are linked to intellectual disability (34, 56); and the dysfunction of tRNA methyltransferase TRMT10A is associated with abnormality in glucose homeostasis (57). Therefore, study of the physiological functions of NSun6 and the modifications it introduces is important for human health and should be pursued further in the future.

**Experimental Procedures**

**Materials—**m^3^C, DTT, 5′-GMP, Tris-base, β-mercaptoethanol, KCl, benzonase, and phosphodiesterase I were purchased from Sigma-Aldrich. Bacterial alkaline phosphatase was obtained from Invitrogen (Shanghai, China). MgCl2, NaCl, ATP, CTP, GTP, and UTP were purchased from Sangon Biotech (Shanghai, China). [methyl-^3^H]SAM (78.0 Ci/mmol) was purchased from PerkinElmer Life Sciences. SAM was purchased from New England Biolabs, Inc. PCR primers were synthesized by BioSune (Shanghai, China). Isopropyl β-D-thiogalactoside was obtained from AMRESCO. Nitrocellulose membranes (0.22 μm) were purchased from Merck-Millipore. Dnmt2 shows robust methylation activity toward tRNA substrates; however, many studies have suggested that Dnmt2 also methylates DNA substrates in vitro and in vivo (53–55). NSun3 is recently reported to methylate C34 of mitochondrial tRNA^Met^, but the substrate specificity and recognition mechanism of NSun3 have not been elucidated (29). Therefore, NSun6 may be the only known tRNA m^5^C MTase that methylates only a subset of tRNAs at a single site. Further biochemical and structural studies are needed to investigate how NSun6 uses such a delicate network to recognize its tRNA substrates as well as to uncover the biological functions of this m^5^C modification at position 72 in a subset of tRNAs. Methylation affects multiple aspects of tRNA function, and its physiological significance has been exemplified by studies of relevant tRNA methyltransferases (6). Moreover, emerging evidence supports that tRNA methyltransferases have important roles in human health. For example, mutations in many tRNA methyltransferase genes, such as FT5J1 and NSUN2, are linked to intellectual disability (34, 56); and the dysfunction of tRNA methyltransferase TRMT10A is associated with abnormality in glucose homeostasis (57). Therefore, study of the physiological functions of NSun6 and the modifications it introduces is important for human health and should be pursued further in the future.

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tRNA Methyl Transfer Assay—To measure the methyl transfer activity of hNSun6, 5 μM tRNAs were used as substrates. The reactions were performed at 37 °C under the same conditions in a 25-μl reaction mixture containing 200 μM [3H]SAM, 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM MgCl2, 100 μg/ml BSA, and 5 mM DTT. Reactions were initiated by the addition of 500 nM hNSun6. At time intervals ranging between 5 and 20 min, 5-μl aliquots were removed to glass fiber filter discs and soaked in 5% trichloroacetic acid to precipitate the labeled methylated tRNA. After washing, the amount of radioactive [3H]SAM using Lineweaver-Burk plots. When the data of a mutant could not distinguish it from background without tRNA, we considered that the kinetic parameters of this mutant were not detectable.

**Mass Spectrometry Analysis of m5C Modifications—**After the SAM-dependent methylation of tRNA by hNSun6, 10 μg of tRNA^Thr(UGU), tRNA^Thr(AGU), and tRNA^Glu(GCA) were hydrolyzed by benzophenone, phosphodiesterase I, and bacterial alkaline phosphatase, respectively, in a 60-μl reaction containing 20 mM Tris-HCl, pH 8.0, 2 mM MgCl2, and 20 mM NaCl. After complete hydrolysis of the methylated tRNA at 37 °C for 24 h, 1 μl of the solution was applied to UPLC-MS/MS. The nucleosides were separated by UPLC on a C18 column (Agilent Zorbax Eclipse Plus C18, 2.1 × 50 mm, 1.8 μm) and then detected by a triple-quadrupole mass spectrometer (Agilent 6495 QQQ) in the positive ion multiple reaction-monitoring mode. Mass transitions from m/z 258 to 126 (m°C) were monitored and recorded.

**Native Polyacrylamide Gel Assay—**tRNAs (100–200 ng each) were loaded to a 6% native polyacrylamide gel. The electrophoresis was carried out at 4 °C at a constant voltage of 60 V for 90 min, using 50 mM Tris/glycine buffer (pH 8.2). The gel was stained with ethidium bromide for detection of tRNA.

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