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Report

NSun2-Mediated Cytosine-5 Methylation of Vault Noncoding RNA Determines Its Processing into Regulatory Small RNAs

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SUMMARY

Autosomal-recessive loss of the *NSUN2* gene has been identified as a causative link to intellectual disability disorders in humans. NSun2 is an RNA methyltransferase modifying cytosine-5 in transfer RNAs (tRNAs), yet the identification of cytosine methylation in other RNA species has been hampered by the lack of sensitive and reliable molecular techniques. Here, we describe miCLIP as an additional approach for identifying RNA methylation sites in transcriptomes. miCLIP is a customized version of the individual-nucleotide-resolution crosslinking and immunoprecipitation (iCLIP) method. We confirm site-specific methylation in tRNAs and additional messenger and noncoding RNAs (ncRNAs). Among these, vault ncRNAs contained six NSun2-methylated cytosines, three of which were confirmed by RNA bisulfite sequencing. Using patient cells lacking the NSun2 protein, we further show that loss of cytosine-5 methylation in vault RNAs causes aberrant processing into Argonaute-associated small RNA fragments that can function as microRNAs. Thus, impaired processing of vault ncRNA may contribute to the etiology of NSun2-deficiency human disorders.

INTRODUCTION

Cytosine-5 methylation (m^5C) is a common epigenetic modification found in DNA with important regulatory roles in transcription (Suzuki and Bird, 2008). The cellular and molecular functions of m^5C-modified nucleobases in RNA, however, remain largely unknown. Dnmt2 and NSun2 are currently the only known m^5C RNA methyltransferases in higher eukaryotes, and transfer RNA (tRNA) is the confirmed target substrate for both enzymes (Brzezicha et al., 2006; Goll et al., 2006). The regulatory functions of m^5C modifications in tRNA are not fully understood but have been reported to regulate tRNA stability and cleavage (Schaefer et al., 2010; Tuorto et al., 2012). Deletion of Dnmt2 or NSun2 in yeast, flies, and mice impairs cellular differentiation pathways in skin, testes, and brain (Blanco et al., 2011; Hussain et al., 2013; Rai et al., 2007; Tuorto et al., 2012). In humans, mutations in the *NSUN2* gene can cause disorders that are associated with intellectual disability (Abbasi-Moheb et al., 2012; Khan et al., 2012; Martinez et al., 2012).

Although NSun2-dependent deposition of m^5C into tRNAs has been widely confirmed, global identification of m^5C in RNA has been hampered by the lack of suitable molecular techniques. Recent high-throughput RNA methylation profiling by bisulfite sequencing and the chemical modification of cytosine-5 by 5-azacytidine increased the repertoire of RNAs carrying m^5C modifications (Khoddami and Cairns, 2013; Squires et al., 2012). In this report, we combine various transcriptome-wide methodologies to identify NSun2-specific RNA methylation sites independent of any chemical modification of RNA. CLIP (crosslinking immunoprecipitation) is a stringent technique devised to identify RNA-protein interactions and uses UV crosslinking to induce a covalent bond between protein and RNA (Ule et al., 2003). Combined with next-generation sequencing, the iCLIP protocol enables genome-wide analysis of crosslink sites at nucleotide resolution (iCLIP) (König et al., 2010).

We modified the iCLIP protocol to identify additional RNA methylation targets of NSun2 and termed it miCLIP (methylation iCLIP). In addition to the established tRNA target substrates of NSun2, miCLIP identified coding RNAs and noncoding RNAs (ncRNAs). We establish vault ncRNAs as NSun2-specific methylated targets and confirm the deposition of m^5C by RNA bisulfite sequencing. Finally, we provide evidence that m^5C controls the processing of vault ncRNAs into small regulatory RNAs with microRNA functions.

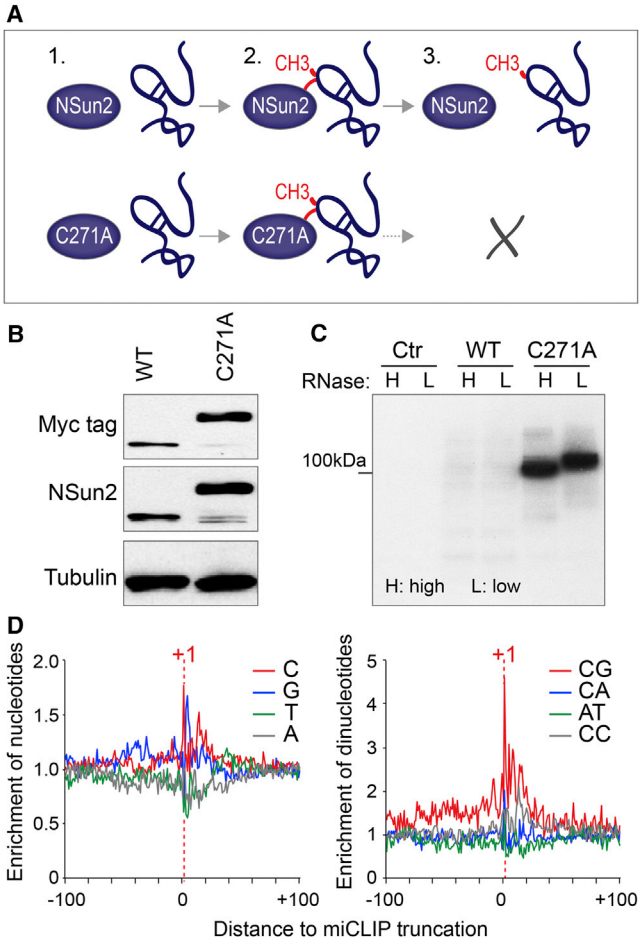

Figure 1. miCLIP Identifies Cytosine-5-Methylated Nucleosides

(A) Schematics of NSun2-mediated cytosine-5 methylation and how the C271A mutation causes irreversible covalent crosslinks between the protein and substrate.

(B) Western blot detecting wild-type (WT) and mutant (C271A) NSun2 proteins using an antibody for the Myc tag (top) or NSun2 (middle). Tubulin (bottom) serves as a loading control.

(C) Detection of radiolabeled immunoprecipitated protein-RNA complexes (^{32}P-ATP) after transfection of an empty vector control (Ctr), wild-type NSun2 (WT), or mutant NSun2 (C271A) using a Myc antibody. Lysates were incubated with high (H) or low (L) concentration of RNase.

(D) Enrichment of nucleotides (left) and dinucleotides (right) in the region up to 100 nt around all crosslink sites. Only the top 4 dinucleotides at position +1 are shown (see also Table S1).

See also Figure S1.

RESULTS

miCLIP: A Technique to Identify m^{5}C in the Transcriptome at Nucleotide Resolution

Cytosine methylation at carbon 5 (m^{5}C) is initiated by the formation of a covalent bond between cysteine 321 of NSun2 and the cytosine pyrimidine ring (Figure 1A) (Liu and Santi, 2000). The release of the methylated RNA depends on a second conserved cysteine at position 271 (C271) (Figure 1A) (King and Redman, 2002; Redman, 2006). Mutation of C271 (C271A) stabilizes the covalently linked protein-RNA catalytic intermediate, which can be detected as higher-molecular-weight complexes by western blot (Figures 1A and 1B) (Hussain et al., 2009).

Because the formation of the protein-RNA covalent bond allowed direct immunoprecipitation of the Myc-tagged C271A NSun2 without UV crosslinking, we named our method miCLIP (methylation iCLIP). The protein-RNA complex was detected by radiolabeling, and a shift in molecular weight in response to a high concentration of RNase I confirmed the presence of the NSun2-RNA complex (Figure 1C). We extracted the RNA from the purified complex and amplified the libraries for 25 or 35 PCR cycles, followed by high-throughput sequencing (Figures S1A and S1B) (König et al., 2010; Sugimoto et al., 2012). We used at least three independent replicates per cell line for all analyses. Analyses of the complementary DNA libraries showed strong cytosine enrichment at position +1 (Figure 1D, left panel), which corresponds to the first nucleotide of all sequence reads (Sugimoto et al., 2012). Thus, reverse transcription terminates precisely at the polypeptide-nucleotide (C271A-cytosine-5) crosslink site. We further detected enrichment of CG dinucleotide at position +1, indicating that deposition of m^{5}C occurs preferably at this dinucleotide (Figure 1D, right; Table S1).

miCLIP-Identified NSun2 Targets Are tRNAs, mRNAs, and ncRNAs

The vast majority of miCLIP reads (>80%) mapped to tRNAs (Figures 2A and 2B). RNA bisulfite conversion identified tRNA Asp^{GTC}, Val^{AAC}, Gly^{GCC}, and Leu^{CAA} as methylation substrates of NSun2 in mouse (Tuorto et al., 2012), and miCLIP precisely mapped the expected m^{5}C sites in these tRNAs (Figure 2A; Figure S2A). When all tRNA reads were mapped, miCLIP identified a total of 41 isoacceptors (Figure S2B). These results are in good agreement with the recently developed 5-azacytidine-mediated RNA immunoprecipitation method (Aza-IP), where the majority of tRNAs were found to be methylated by NSun2 (Khoddami and Cairns, 2013). miCLIP consistently detected NSun2-targeted sites within the variable arm at cytosines 48, 49, and 50 (Figure S2B). However, it does not detect any additional NSun2 target sites outside the variable arm (Khoddami and Cairns, 2013).

The specificity of the interaction between the C271A mutant protein and its target RNA was further confirmed by the very low number of reads mapped to ribosomal RNAs (rRNAs) (Figure 2B). The total number of reads mapping to other ncRNAs and messenger RNAs (mRNA) was consistently less than 20% (Figure 2C; Table S2). It has been recently suggested that NSun2-mediated methylation of mRNAs may increase their half-life (Zhang et al., 2012); yet gene expression assays in various tissues including testis and liver failed to uncover any major changes in mRNA abundance when NSun2 was deleted (Hussain et al., 2013; Tuorto et al., 2012). The only mRNA identified by miCLIP that was differentially expressed when NSun2 was inhibited by RNAi in HEK293 cells was NSun2 itself (Figure S3A; Table S3). We also sequenced cDNA from total RNA isolated from human skin fibroblasts carrying a heterozygous or homozygous loss-of-function mutation in the *NSUN2* gene (Figure S3B; Table S4) (Martinez et al., 2012). The vast majority of the 312 miCLIP-identified mRNAs (>90%) remained