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Identification of N⁶-methyladenosine reader proteins

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Abstract

The reversible N⁶-methyladenosine (m⁶A) modification of eukaryotic messenger RNAs (mRNAs) is a widespread regulatory mechanism that impacts every step in the mRNA life cycle. The effect of m⁶A on mRNA fate depends on the binding of “m⁶A reader” proteins – RNA binding proteins that specifically bind to RNAs containing m⁶A. Here, we describe an RNA pull-down method that can be used to identify novel m⁶A reader proteins starting from a known m⁶A-modified site in cellular or viral RNA. We further describe how a combination of immunoprecipitation-based sequencing methods can be used to identify m⁶A-modified sites bound by an m⁶A reader protein on a transcriptome-wide level. The discovery of new m⁶A reader proteins and their m⁶A-modified targets would provide further insight into the mechanisms and functions of m⁶A in the cell.

Keywords

N⁶-methyladenosine (m⁶A); RNA binding proteins; m⁶A reader proteins; RNA pull-down; photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP); m⁶A-specific methylated RNA immunoprecipitation (MeRIP)

1. Introduction

N⁶-methyladenosine (m⁶A) is a highly abundant modification in eukaryotic messenger RNA (mRNA) and long noncoding RNA (lncRNA). It is dynamically regulated through the opposing actions of the m⁶A methyltransferase complex and m⁶A demethylases [1–4]. Reversible m⁶A modification of RNA transcripts influences their splicing, translation, and decay [5–8], with important consequences for processes including cell differentiation, spermatogenesis, and circadian rhythms [3,9–12]. The impact of m⁶A on RNA fate depends

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on the recruitment of proteins that selectively bind m⁶A-modified RNAs, termed “m⁶A reader” proteins.

RNA binding proteins can selectively bind m⁶A-modified RNAs using two primary mechanisms: direct binding to the m⁶A base, or binding to motifs that become accessible upon m⁶A modification. Proteins containing YT521-B homology (YTH) domains selectively bind methylated RNAs by directly recognizing the N⁶-methyl group [13–15]. By sorting m⁶A-modified RNAs for rapid translation and decay, the YTH domain family of proteins seems to function to generate short pulses in gene expression [16]. So far, the YTH domain is the only domain that has been shown to directly and selectively bind the m⁶A base. Other RNA binding proteins preferentially bind to m⁶A-modified RNAs in which m⁶A modification increases the accessibility of their binding motifs. For instance, m⁶A modification can destabilize an RNA duplex and thereby increase accessibility of a single-stranded U-tract motif recognized by heterogeneous nuclear ribonucleoprotein C (hnRNP C) [17,18]. In turn, hnRNP C binding influences the expression and splicing of thousands of m⁶A-modified transcripts [17]. Other m⁶A-dependent RNA–protein interactions have been shown to regulate mRNA translation, pri-microRNA processing, and the nuclear export of viral RNA [8,19–21].

Several different approaches have led to the discovery of the currently known m⁶A reader proteins. The YTH domain family, hnRNP C, and hnRNP G were first identified as m⁶A reader proteins because they were differentially pulled down by the m⁶A-modified and unmodified forms of synthetic RNA stem-loop structures [17,22,23]. Other m⁶A reader proteins were examined as potential m⁶A reader proteins due to their role in processes that are influenced by m⁶A, including cap-independent translation and viral RNA export [8,21]. The overlap of protein binding sites with m⁶A-modified sites led to the identification of hnRNP A2B1 as an m⁶A reader protein [20]. High-throughput sequencing of m⁶A-modified sites and protein binding sites is also used to further study m⁶A reader proteins previously identified using other methods. These studies focus on the intersection between protein binding sites and m⁶A-modified sites, the enrichment of m⁶A among protein-bound RNAs, and the influence of altering m⁶A methyltransferase or m⁶A demethylase expression on protein binding sites [6,8,17].

Here, we first describe an RNA pull-down method in which an m⁶A site of interest is used as a starting point for the identification of candidate m⁶A reader proteins. Next, we examine how high-throughput sequencing can be used to identify transcriptome-wide m⁶A-modified sites bound by a candidate m⁶A reader protein (Figure 1). Given the crucial role of m⁶A reader proteins in mediating the effects of m⁶A, the discovery of m⁶A reader proteins and their targets will help elucidate the mechanisms behind the influence of m⁶A on diverse cellular processes.

2. Methods

2.1. RNA pull-down

The RNA pull-down method enables the discovery of RNA binding proteins that specifically bind to an RNA of interest. Published protocols have described the use of this method to

identify proteins that bind to specific cellular RNAs or RNA structures [24,25]. With an m⁶A-modified RNA as the bait, the RNA pull-down method can be used to discover novel m⁶A reader proteins [17,22,23].

The pull-down experiment requires two components: an m⁶A-modified RNA of interest, and a cellular extract containing potential m⁶A reader proteins. The m⁶A-modified bait RNA is designed based on an m⁶A site that has been evaluated for its *in vivo* modification status and structural context. To ensure that the bait is fully m⁶A-modified, the RNA is chemically synthesized. The unmodified form of the same RNA is used as the control bait, and both RNAs are biotinylated. The m⁶A-modified and unmodified bait RNAs are then incubated with cell extract. In this protocol, we incubate with nuclear extract from the human embryonic kidney cell line HEK293T, but extracts from different cellular compartments and cell types can be used. Following pull-down with streptavidin beads, proteins that preferentially bind to the m⁶A-modified bait are identified by mass spectrometry.

2.1.1. Select and synthesize bait RNA—In order to identify physiologically relevant m⁶A-modified RNA–protein interactions, the bait RNA should be designed based on an m⁶A site that is modified *in vivo*, as determined by both sequencing and biochemical validation methods. Candidate m⁶A sites can be identified based on sequencing methods such as m⁶A-seq, MeRIP, or miCLIP [22,26,27]. While miCLIP has single-nucleotide resolution, m⁶A-seq and MeRIP have 100–200 nucleotide resolution, so candidate m⁶A sites are identified by searching for the m⁶A consensus motif RRACH (R = A/G, H = A/C/U) within m⁶A enrichment peaks. It is highly recommended to validate the m⁶A site at single-nucleotide resolution using an orthogonal method. For example, m⁶A site can be directly confirmed by site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) [28]. This unbiased biochemical method can both verify that a candidate site is m⁶A-modified and evaluate its m⁶A modification fraction in the cell type of interest. Sites with a high m⁶A modification fraction would be preferred for subsequent studies described below, since these sites could presumably have stronger effects on RNA–protein interactions in the cell. We recommend using an m⁶A site that is modified in the cell type of interest because this site would be a candidate endogenous target for any m⁶A reader proteins identified in the pull-down experiment. However, it is not always necessary to use an endogenous m⁶A site. Notably, the YTH domain family proteins were identified as m⁶A readers by using m⁶A-modified and unmodified RNA from the Rous sarcoma virus genome as bait to pull down proteins from HepG2 cell lysate [22].

Once an m⁶A site is selected, a sequence including the methylated site should be used as the bait. To minimize background due to binding away from the m⁶A, a short RNA fragment of ~30 nucleotides is typically used [17,22,23]. To ensure that the bait RNA mimics its *in vivo* structure, the structure of the RNA fragment can be evaluated by computational prediction or *in vitro* structure probing, while the structure of its source RNA sequence can be evaluated by *in vivo* structure probing or co-variation analysis. Structured RNA baits are expected to yield lower background from the abundant single-stranded RNA binding proteins in the cell. Since m⁶A modification can influence the stability of RNA duplexes [29,30], an RNA bait with an m⁶A site within a stem structure might be biased toward identifying m⁶A reader

proteins that recognize an m⁶A-induced change in RNA structure rather than directly binding the m⁶A base. These biases should be taken into account when designing the bait RNA.

Chemical synthesis of the 5'- or 3'-biotin-labeled m⁶A-modified and unmodified bait RNAs can be performed using m⁶A phosphoramidite that is either purchased from Glen Research or prepared as described [31]. Alternatively, custom synthesis of RNA oligonucleotides containing m⁶A at specific positions is available at Dharmacon and Integrated DNA Technologies. Although a variety of different biotin labels can be used, we prefer biotin modifications with long linkers such as triethylene glycol to minimize steric hindrance. In order to maximize sensitivity for the detection of m⁶A reader proteins, the bait RNAs for the pull-down experiment are synthesized as completely m⁶A-modified or completely unmodified. However, these conditions differ from the cellular environment, in which m⁶A sites are dynamically and incompletely modified. This difference between the *in vitro* and *in vivo* m⁶A modification level should be taken into account when considering potential *in vivo* roles for candidate m⁶A readers identified in the pull-down experiment.

2.1.2. Obtain pre-cleared cell extracts—The cell extract used for the pull-down experiment should come from the same cell type as was used to identify the m⁶A site. Depending on the goal of the experiment, whole cell lysate or cell extract from a particular compartment can be used. In our case, the m⁶A-modified RNA of interest was a hairpin from a nuclear long noncoding RNA [17], so we aimed to pull down m⁶A reader proteins from nuclear extracts from HEK293T cells (CRL-11268, ATCC).

The nuclear extracts can be isolated using published protocols [25] or with the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (78833, Thermo Scientific). We recommend using 1.5 packed cell volumes of nuclear extraction reagent supplemented with 1% v/v protease inhibitor (25955-11, Nacalai USA) in order to obtain nuclear extracts of sufficiently high concentration (~3.2 mg/mL). The relative concentration of protein to RNA can greatly affect the signal-to-noise ratio and needs to be optimized for each cell lysate and bait. The optimal protein-to-RNA ratio likely depends on the abundance of the m⁶A reader protein and its affinity for the methylated and unmethylated bait RNAs. Prior to the RNA pull-down, pre-clear the cell extract under the following conditions: 0.4 U/ μ L RNase inhibitor (N2615, Promega), 0.25 mg yeast tRNA (10109517001, Sigma) per 1 mg protein extract, and 1 mg streptavidin beads (11206D, Life Technologies) per 1 mg protein extract, rotating for 1 hour at 4 °C (415110, Barnstead Thermolyne).

2.1.3. Pull down proteins with biotinylated bait RNA and streptavidin beads—Next, the m⁶A-modified and unmodified bait RNAs are used to pull down proteins from the pre-cleared cell extract using a procedure adapted from [22]. First, refold 1.5 μ g (~150 pmol) of each biotinylated RNA bait in 20 mM Tris-Cl (pH 7.5) in a total volume of 8 μ L by denaturing at 90 °C for 1 minute, then incubating at ambient temperature for 5 minutes. Add 140 μ L (~450 μ g) of pre-cleared nuclear extract to each 8 μ L of refolded RNA. Rotate for 30 minutes at ambient temperature, then for 2 hours at 4 °C to allow RNA binding proteins to bind to the biotinylated RNA. A control experiment without RNA bait should be included.

In the meantime, prepare 450 µg of streptavidin beads (11206D, Life Technologies) for each pull-down sample, by washing the beads with sodium hydroxide as described in the manual. The beads should be blocked with non-specific protein and RNA under the following conditions: 0.2 mg/mL BSA (UltraPure BSA, AM2616, Thermo Fisher), 1% v/v protease inhibitor, 50 µg/mL yeast tRNA, and 0.2 U/µL RNase inhibitor in binding buffer [10 mM Tris-Cl (pH 7.5), 150 mM KCl, 1.5 mM MgCl₂, 0.05% v/v IGEPAL CA-630 (I8896, Sigma)] in a total volume of 90 µL, rotating for 2 hours at 4 °C. Following the blocking step, resuspend the beads in 9 µL of binding buffer with 1% v/v protease inhibitor and 0.4 U/µL RNase inhibitor.

Add 9 µL of blocked beads to 148 µL of nuclear extract with biotinylated bait RNA and rotate for 30 minutes at ambient temperature. Remove the unbound solution. Wash the beads 5 times with 900 µL wash buffer [binding buffer + 100 mM NaCl: 10 mM Tris-Cl (pH 7.5), 100 mM NaCl, 150 mM KCl, 1.5 mM MgCl₂, 0.05% v/v IGEPAL CA-630]. For each wash, resuspend the beads in wash buffer, and collect the supernatant on the magnetic stand, making sure to remove as much of the supernatant as possible on the last wash. Elute the bound proteins with 13.5 µL of high-salt elution buffer [binding buffer + 500 mM NaCl: 10 mM Tris-Cl (pH 7.5), 500 mM NaCl, 150 mM KCl, 1.5 mM MgCl₂, 0.05% v/v IGEPAL CA-630]: vortex for 30 seconds, incubate for 5 minutes at 37 °C and 1000 rpm, and then collect the eluate on the magnetic stand.

2.1.4. Analyze by SDS-PAGE and mass spectrometry—Add 5.25 µL of 4× NuPAGE LDS sample buffer (NP0008, Thermo Fisher) to the eluate and incubate for 10 minutes at 70 °C. Use an unstained protein size marker, since pre-stained markers can result in negative staining. We use 0.3 µL of BenchMark Unstained Protein Ladder (10747-012, Thermo Fisher) with 13.2 µL of elution buffer and 5.25 µL of 4× NuPAGE LDS sample buffer, heated for 10 minutes at 70 °C, in order to mimic the buffer conditions of the eluate. A small amount of protein ladder is used so that the intensity of the marker bands are in the same range as the intensity of the protein bands in the eluate.

Separate the unstained marker and eluted proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), stain with SYPRO Ruby Protein Gel Stain (S-12000, Life Technologies) using either the rapid or basic protocol described in the manual, and scan the gel (Bio-Rad FX Pro Plus Imager). Protein bands that are enriched in the pull-down with the m⁶A-modified bait RNA relative to the pull-down with the unmodified bait are candidate m⁶A reader proteins. The enrichment of these bands can be estimated by densitometry (ImageQuant), with normalization to a protein band that is equally pulled down by both bait RNAs. To identify the m⁶A reader protein, cut out the enriched bands from the gel, digest with trypsin, and identify the proteins using liquid chromatography tandem mass spectrometry (LC-MS/MS). Alternatively, the entire eluted sample can be digested and analyzed by LC-MS/MS. Reasonable thresholds for the identification of proteins by LC-MS/MS are 99% protein probability, with a minimum of 2 peptides at 80% probability, and false discovery rate <1%, as determined by Scaffold software. Although primarily geared towards protein–protein interaction experiments, the CRAPome repository can also be used to filter out background contaminants, such as proteins that interact with the solid-phase support [32]. The pull-down with the unmethylated bait serves as an additional negative

control, and the ratio of spectral counts in the elutions from the methylated versus unmodified baits can be used as a semi-quantitative measure of enrichment. The identity of a candidate m⁶A reader protein can also be confirmed and its enrichment quantified by Western blotting.

The RNA pull-down method described here allows for the discovery of m⁶A readers without previously identifying candidate proteins. Instead, this method requires the identification of a specific m⁶A site of interest and can be geared toward identifying m⁶A reader proteins in a particular cellular compartment or cell type. This approach has been used to identify m⁶A reader proteins that either directly bind the m⁶A base or bind to motifs exposed upon m⁶A-induced changes in RNA structure [17,22,23]. Further *in vitro* experiments are necessary to confirm that the enriched protein is an m⁶A reader and to determine the mechanism by which the candidate m⁶A reader protein specifically recognizes m⁶A-modified RNAs. Some methods that can be used to measure binding of the candidate m⁶A reader to the methylated and unmethylated bait RNAs include electrophoretic mobility shift assay, fluorescence anisotropy, and isothermal titration calorimetry [33–36]. Chemical and enzymatic RNA structure probing experiments can be used to determine whether m⁶A induces changes in RNA structure, while mutagenesis of the RNA can be used to assess the dependence of protein binding on RNA structure and sequence [37,38].

2.2. PAR-CLIP–MeRIP

Once a candidate m⁶A reader protein has been identified by RNA pull-down and confirmed as an m⁶A reader protein through *in vitro* binding assays, a reasonable next step is to identify the m⁶A-modified sites bound by the m⁶A reader on a transcriptome-wide level. These sites cannot be identified by simply taking the overlap between protein binding sites and m⁶A sites, since both protein binding and m⁶A modification are incomplete at any given site, and therefore an overlap does not imply that the site is simultaneously methylated and bound. Moreover, the binding sites of an m⁶A reader protein might not all be m⁶A-modified, since m⁶A might increase the binding affinity of reader proteins without being absolutely required for protein binding, and since m⁶A reader proteins could have multiple RNA binding domains with only a subset of domains binding in an m⁶A-dependent manner. Here, we describe how to specifically identify the m⁶A-modified binding sites of an m⁶A reader protein through the sequential application of two methods: photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) and m⁶A-specific methylated RNA immunoprecipitation (MeRIP).

2.2.1. PAR-CLIP—The PAR-CLIP step in PAR-CLIP–MeRIP is used to isolate RNAs bound by the candidate m⁶A reader protein. The RNAs isolated by PAR-CLIP experiments are usually short RNA fragments (30–50 nucleotides long) that are cross-linked to RNA binding proteins and thus survive the RNase digestion step. Hence, the PAR-CLIPed RNA samples represent the RNA binding sites. We choose to use PAR-CLIP rather than other immunoprecipitation methods because cross-linking-based methods specifically identify direct interactions *in vivo* and also reveal RNA binding sites at higher resolution; PAR-CLIP also has higher efficiency than cross-linking methods that do not use photoreactive

nucleosides [39,40]. The high cross-linking efficiency of PAR-CLIP is particularly important given that the PAR-CLIP step must yield enough RNA to be used as the input for MeRIP.

The m⁶A abundance in PAR-CLIPed RNAs can be evaluated by traditional biochemical methods, such as two-dimensional thin-layer chromatography (TLC) using the specific solvent conditions for m⁶A [17,41]. Since the RNA binding sites of a true m⁶A reader protein are expected to be enriched in m⁶A, one would only proceed to the MeRIP step if the PAR-CLIPed RNAs have a higher level of m⁶A than the cellular poly(A) RNA control.

PAR-CLIP procedures have been described in detail elsewhere [40,42]. To ensure sufficient yield, we start with sixteen 15-cm plates of HEK293T cells: 8 plates for PAR-CLIP and 8 plates for PAR-CLIP–MeRIP [17]. However, the efficiency of PAR-CLIP depends on several factors, including protein abundance, RNA binding affinity, RNA binding site occupancy, and antibody quality, so the amount of starting material might need to be adjusted depending on the candidate m⁶A reader protein under investigation. The cells are first grown to 80% confluence in normal media, then grown for 15 hours in media supplemented with 100 μM 4-thio-uridine (T4509, Sigma), and finally cross-linked at 365 nm for 2–5 minutes. Following cell lysis, an antibody specific for the candidate m⁶A reader protein is used for immunoprecipitation. The specificity of the antibody should be confirmed by Western blotting and by knockout or knockdown of the target protein. Next, we partially digest the cross-linked, immunoprecipitated RNA with 20–30 U (10–15 U/mL) RNase I (AM2295, Thermo Fisher) and 4–8 U (2–4 U/mL) Turbo DNase (AM2238, Thermo Fisher) per 15-cm plate of HEK293T cells for 3–4 minutes at 37 °C and 1100 rpm. Alternatively, other nucleases, such as RNase T1, RNase A, or micrococcal nuclease, can be used [39].

The RNAs cross-linked to the candidate m⁶A reader protein are further isolated by SDS-PAGE, followed by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The RNA pellet is resuspended in 10 μL of H₂O and split into two halves. The first half is used for library preparation using the TruSeq Small RNA Sample Preparation Kit (RS-200-0012, Illumina) and submitted for single-end sequencing with a read length of 50 base pairs (Illumina HiSeq2000). The cross-linked RNAs are identified based on characteristic transition mutations at the cross-linking sites using PARalyzer [43]. The other half of the resuspended RNA is used as the input for MeRIP.

2.2.2. MeRIP—Once the RNAs bound by the candidate m⁶A reader protein have been isolated by PAR-CLIP, the m⁶A-modified protein-bound RNAs can be isolated by conducting MeRIP on the PAR-CLIP sample. MeRIP protocols have previously been described elsewhere [22,26,44]. In short, the resuspended PAR-CLIP RNA is incubated with m⁶A antibody, and then with pre-blocked protein A beads, and the RNA is eluted by competition with m⁶A. The eluted RNA fragments are used for library preparation and sequencing in parallel with the PAR-CLIP sample prepared in the previous section.

PAR-CLIP–MeRIP allows for the identification of m⁶A-modified sites bound by the candidate m⁶A reader protein. The enrichment of m⁶A in RNAs cross-linked to the candidate m⁶A reader can be determined by comparing the PAR-CLIP–MeRIP reads with

the PAR-CLIP reads: sites with $\log_2(\text{Counts}_{\text{PAR-CLIP-MeRIP}} / \text{Counts}_{\text{PAR-CLIP}}) > 0.5$ are considered to be m⁶A-modified binding sites of the m⁶A reader protein.

3. Results and Conclusion

We previously identified nucleotide 2515 in the long noncoding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) as an m⁶A-modified site that is 41% methylated in HEK293T cells [28]. We selected a 34-nucleotide region of MALAT1 that includes the 2515 m⁶A site and that folds into a hairpin structure, based on computational prediction by Mfold [45] and based on co-variation analysis [28]. Since MALAT1 is localized in the nucleus, we searched for RNA binding proteins that selectively bind to the m⁶A-modified MALAT1 hairpin by pulling down proteins from HEK293T nuclear extract. The RNA pull-down experiment identified hnRNP G as a protein that was enriched in the pulldown with the m⁶A-modified hairpin by SDS-PAGE and Western blotting (Figure 2), and by mass spectrometry [23]. Additional structural probing and mutagenesis experiments revealed that hnRNP G does not directly recognize the m⁶A base, but instead recognizes a purine-rich motif that is exposed upon m⁶A modification of the MALAT1 hairpin [23].

For Western blotting, the entire eluate from the RNA pull-down with no RNA, unmodified MALAT1 hairpin, or m⁶A-modified MALAT1 hairpin bait was separated by SDS-PAGE, with 2 μ L of MagicMark XP Western Protein Standard (LC5602, Thermo Fisher) in an adjacent lane as a size marker. The proteins were transferred to a polyvinylidene difluoride membrane (IPVH00010, Millipore), blocked in 5% w/v milk (170-6404, Bio-Rad), 1 $\times$ TBST [20 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1% v/v Triton X-100], and agitated in 1/1000 v/v rabbit monoclonal anti-hnRNP G antibody (ab190352, Abcam), 5% w/v milk, 1 $\times$ TBST at 4 °C overnight. The membrane was subsequently agitated in 1/30000 goat anti-rabbit antibody conjugated to horseradish peroxidase (ab97051, Abcam), 5% w/v milk, 1 $\times$ TBST and visualized by enhanced chemiluminescence (RPN2109, GE Healthcare).

Post-transcriptional m⁶A modifications influence every step in the life cycle of a cellular RNA transcript [5]. The influence of m⁶A on RNA fate depends on the specific binding of m⁶A reader proteins. RNA pull-downs using chemically synthesized m⁶A-modified bait RNAs can identify novel m⁶A reader proteins, while PAR-CLIP–MeRIP can identify m⁶A-modified sites bound by candidate m⁶A readers on a transcriptome-wide level. The discovery of m⁶A reader proteins and their m⁶A-dependent binding sites will enhance our understanding of the mechanisms and functions of m⁶A in the cell.

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Highlights

- We describe methods to discover m⁶A readers and their m⁶A-modified targets.
- RNA pull-down with an m⁶A-modified RNA bait can identify novel m⁶A readers.
- PAR-CLIP followed by MeRIP identifies m⁶A-modified protein binding sites.

Figure 1.

Flow diagram of the procedure for the identification of m⁶A reader proteins and their targets by RNA pull-down followed by PAR-CLIP-MeRIP. The cartoon shapes representing proteins in the cell extract include examples of an m⁶A reader protein (blue), a non-m⁶A-dependent bait-RNA-binding protein (brown), a non-binding protein (violet), and a non-specific streptavidin-bead-binding protein (dark red).

Figure 2.

Identification of hnRNPG as m⁶A reader protein. (A) MALAT1 hairpin used for RNA pull-down. The m⁶A-modified base corresponding to position 2515 in MALAT1 lncRNA is shown in red. (B) SDS-PAGE showing that one specific protein band is enriched in the pull-down with the m⁶A-modified bait (2515-m⁶A) compared to the pull-down with the unmodified bait (2515-A). The protein was identified as hnRNPG using mass spectrometry. In contrast, Histone H1.2 is equally pulled down by both bait RNAs. (C) Western blot showing hnRNPG pull-down with no RNA (control), unmodified MALAT1 hairpin (2515-A), or m⁶A-modified MALAT1 hairpin (2515-m⁶A) as bait.