METTL1 Promotes *let-7* MicroRNA Processing via m7G Methylation

**Highlights**
- Internal m7G is identified in miRNAs by two independent sequencing techniques
- Methyltransferase METTL1 mediates m7G modification of specific miRNAs
- METTL1 promotes miRNA maturation and suppresses lung cancer cell migration
- m7G promotes processing by antagonizing G-quadruplex structures in miRNA precursors

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**In Brief**
Pandolfini, Barbieri, et al. show that a subgroup of tumor suppressor microRNAs, including *let-7e*, contain 7-methylguanosine (m7G). Methyltransferase METTL1 is required for m7G modification of miRNAs, their efficient processing, and the inhibition of lung cancer cell migration. Structurally, m7G in miRNA precursors antagonizes RNA secondary structures that would otherwise inhibit their maturation.

**Data Resource**
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METTL1 Promotes let-7 MicroRNA Processing via m7G Methylation

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SUMMARY

7-methylguanosine (m7G) is present at mRNA caps and at defined internal positions within tRNAs and rRNAs. However, its detection within low-abundance mRNAs and microRNAs (miRNAs) has been hampered by a lack of sensitive detection strategies. Here, we adapt a chemical reactivity assay to detect internal m7G in miRNAs. Using this technique (Borohydride Reduction sequencing [BoRed-seq]) alongside RNA immunoprecipitation, we identify m7G within a subset of miRNAs that inhibit cell migration. We show that the METTL1 methyltransferase mediates m7G methylation within miRNAs and that this enzyme regulates cell migration via its catalytic activity. Using refined mass spectrometry methods, we map m7G to a single guanosine within the let-7e-5p miRNA. We show that METTL1-mediated methylation augments let-7 miRNA processing by disrupting an inhibitory secondary structure within the primary miRNA transcript (pri-miRNA). These results identify METTL1-dependent N7-methylation of guanosine as a new RNA modification pathway that regulates miRNA structure, biogenesis, and cell migration.

INTRODUCTION

Post-synthesis covalent modification of biological molecules is a key aspect of intracellular signaling, and it is critically important in many biological processes. RNA molecules, similar to proteins, are subject to a vast array of post-synthesis covalent modifications, which together constitute the epitranscriptome. To date, >100 RNA modifications have been identified, which are spread throughout every class of RNA and are evolutionarily conserved throughout all kingdoms of life (Carell et al., 2012; Machnicka et al., 2013).

RNA modifications have the potential to affect all RNA processes, including splicing, stability, and localization (Roundtree et al., 2017). Many RNA modifications have been identified by mass spectrometry (MS), and complex epitranscriptomes of tRNA and rRNA have been thoroughly studied. However, this represents a mere snapshot of a much bigger picture, with the clear majority of modifications remaining uncharacterized. This is predominantly due to a lack of sensitive methodologies with which to detect the modifications at a high resolution. Even now, MS methodologies are largely unable to generate transcriptome-wide modification profiles. However, a few very recent analyses have used anti-modification antibodies (e.g., against N1-methyladenosine [Dominissini et al., 2016], N6-methyladenosine [Dominissini et al., 2012], and 5-hydroxymethylcytosine [Delatte et al., 2016]) or chemical reactivity of the modification (for pseudouridine [Carlile et al., 2014; Schwartz et al., 2014], m2C [Schaefer, 2015], and 2’-O-methylation [Dai et al., 2017]). Their results clearly suggest that many of the modifications identified on rRNA and tRNA are also present on other RNA classes. Therefore, the development of epitranscriptomic methodologies (e.g., new antibody and chemical methods coupled to next-generation sequencing [NGS]) represents a bottleneck in deciphering the function of new RNA modifications.

Certain nucleotides, such as 7-methylguanosine (m7G), display specific modification-dependent chemistries that can be exploited to study their prevalence and transcript location. m7G is present in eukaryotic mRNA 5’ caps and at defined internal positions within tRNAs and rRNAs across all domains of life. The best-characterized enzyme mediating internal m7G methylation is the TRMT8 yeast enzyme homolog METTL1 (methyltransferase-like 1), which, together with its co-factor WDR4...
Figure 1. Detection of m7G in Specific miRNAs in A549 Cells

(A) Schematic of a novel chemical method to detect internal m7G RNA modification.

(B) Schematic representation of the procedure used to identify the m7G modified miRNAs in A549 cells.

(C) Immuno-dot blot of total decapped INPUT RNA (10%) or RNA immunoprecipitated with anti-m7G antibody or control immunoglobulin G (IgG).

(D) Immunoprecipitation with anti-m7G antibody enriches for m7G-containing RNAs as determined by mass spectrometry (MS; see also Figure S1). The average of two biological replicates ± SDs is shown.

(E) Scatterplot showing a high degree of consistency between the BoRed-seq approach and RIP-seq in detecting miRNAs harboring m7G (upper right quadrant). Goodness of fit is calculated as R² Pearson correlation coefficient.

(F) RNA immunoprecipitation with the anti-m7G antibody coupled to qRT-PCR was used to validate five m7G-containing miRNAs and four negative miRNAs, which are identified in (E). The average of two biological replicates ± SDs is shown. The distributions of mean enrichments in m7G+ and m7G/C0 miRNAs are significantly different, as evaluated by the two-tailed Wilcoxon test (*p < 0.05).

(G) Venn diagram showing the overlap between miRNAs significantly enriched in m7G-RIP of A549 and Caco-2 cells, respectively (see also Figure S2). The p value is obtained by Fisher’s exact test.

(H) Western blot showing METTL1 protein levels in A549 cells infected with METTL1-specific (sh1, sh2) or control (Scramble) tetracycline (TET)-inducible shRNAs 5 days after doxycycline treatment. A representative experiment of four independent biological replicates is shown.

(legend continued on next page)
(WD repeat domain 4), catalyzes m7G at G46 of specific tRNAs, such as tRNA\textsuperscript{Thi} (Alexandrov et al., 2002).

In contrast to deoxy-m7G, m7G in RNA is highly stable in neutral aqueous solution (Kriek and Emmelot, 1964). The methylation significantly alters the charge density of RNA, potentially serving as a molecular handle, but it does not impair Watson-Crick G:C base complementarity. It does, however, interfere with non-canonical base pairing (i.e., Hoogsteen pairs), possibly affecting the secondary structure of RNA. Although relatively abundant, m7G has proved very difficult to study so far. Being neutral to Watson-Crick base pairing, it does not interfere with reverse transcription, rendering it invisible to detection by standard sequencing-based technologies.

miRNAs (miRNAs) are short single-stranded RNA molecules (18–24 nucleotides [nt]) that target the RNA interference silencing complex (RISC) to specific mRNAs. Their specificity depends on miRNA guide strands (18–24 nucleotides [nt]) that target the RNA interference pathway. The interaction generally occurs with non-canonical base pairing (i.e., Hoogsteen pairs), possibly affecting the secondary structure of RNA. Although relatively abundant, m7G has proved very difficult to study so far. Being neutral to Watson-Crick base pairing, it does not interfere with reverse transcription, rendering it invisible to detection by standard sequencing-based technologies.

To detect N7-methylguanosine within low-abundant RNAs, we adapted an existing strategy (Zueva et al., 1985) to allow the profiling of m7G in eukaryotic RNAs. In this reaction, m7G residues are prone to nucleoside hydrolysis when reduced by treatment with NaBH\textsubscript{4}. The resulting abasic sites can be revealed by aniline-induced cleavage of the RNA chain by β-elimination. This reaction is the basis of direct RNA sequencing (RNA-seq) by the Maxam and Gilbert method (Peattie, 1979) and has been used for mapping m7G residues in highly abundant rRNAs and tRNAs at single nucleotide resolution (Zueva et al., 1985). We optimized the reaction conditions for the reduction of mammalian total RNA in the absence of methylated carrier RNA, which would interfere with the NGS analysis. As proof of principle, reduced 18S rRNA was cleaved by aniline treatment into two fragments, in agreement with the known position of m7G (Piekná-Przybylska et al., 2008; Figure S1A).

The above strategy is not suitable for very short RNAs such as miRNAs, because the resulting cleavage fragments would be too small to be unequivocally mapped to the human transcriptome. Therefore, we developed a new protocol, based on the above, to detect m7G within miRNAs, which we refer to as Borohydride Reduction (BoRed-seq) (Figure 1A). Total RNA from a human lung cancer cell line (A549 cells) was decapped, treated with NaBH\textsubscript{4}, and exposed to low pH to generate abasic sites at positions harboring m7G. These sites were exposed to a biotin-coupled aldehyde reactive probe [N-(aminooxyacetyl)-N'-(p-biotinyl) hydrazine; ARP] that covalently binds to abasic RNA sites (Tanaka et al., 2011). Modified RNAs were then pulled down using streptavidin beads, small RNA libraries were prepared, and RNAs were identified by high-throughput sequencing. Using this approach (Figure 1B), a number of mature miRNAs likely to contain m7G were identified (Table S1).

To confirm the validity of this technique and to provide an independent verification of m7G-methylated miRNAs, we performed an RNA immunoprecipitation sequencing (RIP-seq) experiment using an antibody that recognizes m7G in RNA (Figure 1C). This antibody immunoprecipitates m7G-containing RNAs, but not other methylated G-containing RNAs (as judged by MS; Figures 1D and S1B–S1D), and it specifically enriches m7G-containing 18S rRNA and tRNAs (Figures S2A and S2B). RIP-seq with this antibody identified a second cohort of mature miRNAs containing m7G (Table S2).

We then compared the results from the BoRed-seq and RIP-seq approaches and found there was a significant overlap of m7G-modified miRNAs detected by each technique (Figures 1E, upper right quadrant, S2C, and S2D). We regard these miRNAs as high-confidence m7G-modified miRNAs (Table S3), five of which were validated by RIP-qPCR analysis (Figure 1F). m7G is found on miRNAs of any abundance, bearing no correlation with any particular expression level (Figure S2E).

We extended these analyses to an unrelated colorectal cancer cell line (Caco-2 cells), which expresses METTL1 at levels comparable to those observed in A549 cells. This identified significantly overlapping m7G-modified miRNAs (Figures 1G and

### RESULTS

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suggest that METTL1 specifically influences cell migration via m7G methylation of miRNAs.

To further explore this possibility, we performed a global gene expression analysis to identify transcripts affected by the depletion of METTL1 (Figure 2D; Table S6). This revealed 254 upregulated and 60 downregulated transcripts. Gene Ontology analysis indicated the upregulation of pathways involved in cellular migration (Figures 2E, and S4B–S4E; Table S7), in agreement with our phenotypic characterization of METTL1 knockdown cells (Figures 2A and 2B).

We then asked whether METTL1-regulated transcripts are also targets of the m7G-modified miRNAs. In silico-predicted mRNA targets for these miRNAs are differentially expressed upon METTL1 knockdown, whereas mRNA targets of control miRNAs are not (Figure 2F). We confirmed these findings using an unbiased approach that identified let-7(5p) seed sequence as the most significantly enriched in upregulated miRNAs (Figure S3F), a number of which are individually shown in Figure S3G. The presence of the let-7 target sequence within the 3’ UTR of mRNAs represents the strongest predictive factor for their upregulation upon METTL1 knockdown (Figure S3H).

The above results indicate that many of the genes involved in cell migration and upregulated upon METTL1 depletion are targets of METTL1-dependent miRNAs. This suggests that METTL1 regulates gene expression via the control of miRNA function. To investigate this possibility, we focused on HMGA2, one of the most upregulated transcripts following METTL1 depletion, and whose 3’ UTR is significantly enriched for evolutionarily conserved target sites of several m7G-containing miRNAs, including let-7(5p), mir-125(5p), and mir-92(3p) (Figure 3A; odds ratio [OR] = 5.46, p = 0.001). First, we confirmed that METTL1 knockdown increases HMGA2 mRNA expression and protein levels in both A549 cells (Figures 3B and 3C) and Caco-2 cells (Figures S4A and S4B). Second, we confirmed that HMGA2 mRNA is not m7G modified itself by performing BoRed-qPCR and RIP-qPCR (Figure S4C).

To demonstrate that the effect of METTL1 was mediated, at least in part, by a miRNA pathway, we generated a stable A549 cell line containing a reporter construct consisting of the 3’ UTR of Hmga2 linked to the coding sequence of luciferase. Knock down of METTL1 in this reporter line increases luciferase activity, confirming that the 3’ UTR of HMGA2 confers responsiveness to METTL1 (Figures 3D and 3E). To confirm that METTL1 regulation of the 3’ UTR of HMGA2 is mediated through the action of miRNAs, we concentrated on let-7, which has seven binding sites within this 3’ UTR. Deletion of the let-7 seed sequences from the 3’ UTR leads to increased luciferase activity and uncouples it from METTL1 regulation (Figures 3E and S4D).

Figure 3F shows that the introduction of mature let-7e-5p miRNA into A549 cells reduced HMGA2 protein expression as expected (Mayr et al., 2007). Transfection of mature let-7e miRNA reverts the upregulation of HMGA2 protein caused by METTL1 depletion (Figure 3F). Expression of a short hairpin RNA (shRNA)-resistant version of WT METTL1 also reverts HMGA2 upregulation due to METTL1 depletion, and this requires the catalytic activity of the methyltransferase (Figure 3G). These data confirm that METTL1 methyltransferase activity regulates the expression of HMGA2 in a let-7-dependent manner.
We next explored the functional role of let-7 methylation by METTL1. We used UV cross-linking and immunoprecipitation (CLIP) to confirm in our system previous findings (Bao et al., 2018) that METTL1 binds directly to miRNA precursors, including pri-let-7e and pri-miR-125a hairpins (Figures 4A and S4E). To date, there are two known methylations of miRNA: m6A catalyzed by METTL3 (Alarco´n et al., 2015) and 5′-phosphate methylation catalyzed by BCDIN3D (Xhemalce et al., 2012). In both cases, methylation regulates miRNA processing. To test whether METTL1 may also be involved in such a pathway, we asked whether it regulates the processing of m7G-containing miRNAs. Depletion of METTL1 significantly reduced the levels of the premature and mature forms of let-7e-5p and miR-125a-5p, whereas their primary transcripts were unaffected (Figures 4B, 4C, and S4F). This is unlikely to be due to a general defect in processing since METTL1 depletion does not affect the levels of various miRNA processing factors (Figure S4G).

The above findings indicate that METTL1 activity is required for pri- to pre-processing of miRNAs, implying that pri-miRNAs are directly m7G modified by METTL1 and that the modification is subsequently retained through to the mature miRNA (Figure S4H). m7G RIP experiments show that pri-miRNAs are enriched in m7G, which decreases upon METTL1 knockdown (Figures 4D, S4I, and S4J).

To further demonstrate the METTL1-dependent effect on miRNA processing, we used an in vitro assay (Lee et al., 2002) to test the efficiency of cellular extracts to process a radioactive pri-let-7e transcript into precursor and mature miRNAs. Cellular extracts devoid of METTL1 process precursor transcripts less efficiently than control extracts (Figure S5A). Thus, METTL1 is required for the efficient processing of target miRNAs such as let-7.

To confirm that pri-let-7e is directly methylated by METTL1, we performed an in vitro methyltransferase assay using a pre-assembled recombinant METTL1/WDR4 complex (Figure S5B) and pri-let-7e hairpin oligonucleotides, tRNA Phe, and an unrelated negative control miRNA (cel-miR-67 hairpin) as substrates. Using MS, we detected m7G in let-7e RNA and tRNA Phe, but not in the control miRNA (Figures 4E and S5C).

To directly assess whether m7G affects miRNA processing, we prepared radiolabeled m7G containing pri-let-7e RNA, as shown in Figure 4F. Briefly, radioactive pri-let-7e hairpin
RNA was methylated in vitro by incubation with the METTL1/ WDR4 complex, and m7G containing RNA was enriched via RIP. The resulting methylated and non-methylated (control) pri-miRNAs were then subjected to a DROSHA processing assay. The results indicate that m7G methylated pri-let-7e RNA was more efficiently processed by DROSHA in vitro (Figure 4G).

To probe the mechanism by which m7G methylation of let-7e affects its processing in vivo, we sought to identify the position of the methylation site within the let-7e miRNA. We set up spectral sequencing of RNA methylation and applied it to mature miRNAs purified from A549 cells. This targeted MS method allows the mapping of modification sites within a specific sequence at single base resolution. This approach highlighted one methylated guanosine at position 11 (G11) of let-7e-5p (Figures 5A, 5B, and 5C), which is also required for efficient DROSHA cleavage (Figure 4G).

G11 is part of a short 16-nt-long G-rich sequence of the form G2+N4G2+N4G2+N4G2+, where N is any base. This type of motif is known to fold into the alternative Hoogsteen base-paired G-quadruplex structure (Kwok et al., 2016b). It is noteworthy that the formation of a G-quadruplex structure has been documented in three miRNA precursors, namely miR-92b (Mirihana Arachchilage et al., 2015), miR-149 (Kwok et al., 2016b), and let-7e (Pandey et al., 2015). According to our previous results, all of these miRNAs are both m7G modified and METTL1 dependent (Table 1), suggesting that G-quadruplex formation may be involved in the regulation of m7G-modified miRNAs.

To explore the connection between G-quadruplexes and m7G, we analyzed the base composition of m7G harboring miRNAs, assessing the potential enrichment of G-quadruplex motifs. We found that m7G-modified miRNAs are characterized by a bias in nucleotide content toward increased G-richness (Figure S6A) and G-skewness (Figure 5C). These observations suggest that m7G harboring miRNAs display sequences with the propensity to form G-quadruplexes. Moreover, using a G-quadruplex-predicting algorithm, we found that miRNAs containing at least one predicted G-quadruplex are significantly enriched in m7G (Figures 5D and S6B). The G-quadruplexes are predicted to fold at a very similar relative position within different m7G-containing miRNA hairpins (Figure 5E), which overlaps the 5′ site of pri-miRNA (DROSHA cleavage). Overall, these analyses suggest...
Figure 4. METTL1 Directly Modifies let-7e pri-miRNA and Regulates Its Processing

(A) CLIP-qPCR using a METTL1-specific antibody or a non-specific IgG. The levels of immunoprecipitated pri-let-7e and pri-mir-125a hairpins are shown. The average of two independent immunoprecipitation reactions ± SEMs is shown (*p < 0.05, two-tailed t test).

(B) qRT-PCR showing the levels of either LET7E/125A primary transcript (blue) or let-7e and mir-125a precursors (gray) upon METTL1 knockdown in A549 cells. The average of five to six independent biological replicates ± SDs is shown (*p < 0.05, **p < 0.01, two-tailed t test).

(C) qRT-PCR quantification of let-7e-5p and miR-125a-5p upon METTL1 knockdown. The average of five independent biological replicates ± SDs is shown (**p < 0.01, ***p < 0.001, two-tailed t test).

(D) m7G RNA immunoprecipitation and qRT-PCR of LET7A3, LET7B, and LET7E/125A primary transcripts in A549 cells upon METTL1 knockdown. The average of three independent biological replicates ± SEMs is shown (*p < 0.05, **p < 0.01, ***p < 0.001, two-tailed t test).

(F) In vitro methylation reaction using recombinant METTL1/WDR4 pre-assembled complex on let-7e or cel-miR-67 primary hairpin (negative control). MS analysis shows specific m7G methylation of the let-7e hairpin. The average of three independent experiments ± SDs is shown.

(G) In vitro processing assay of pri-let-7e: control (Ctrl) or in vitro methylated pri-miRNAs were incubated in the presence of immunoprecipitated DROSHA. Autoradiography reveals that IVm7G-pri undergoes more efficient processing, yielding the expected cleavage pattern shown in the illustration. The histogram shows the relative quantification of the resulting pre-let-7e from four samples obtained in two independent experiments (*p < 0.05, two-tailed t test). Autoradiography images are composites of different molecular weight regions and exposure times. Full, unprocessed images are deposited on Mendeley Data.

See also Figure S5.
that G-quadruplexes contribute to the METTL1-mediated regulation of miRNA activity.

To support a role for G-quadruplex formation in the processing of pri-let-7e, we carried out a biophysical analysis of the short 16-nt-long G-rich sequence, referred to as rG4-let-7e. Using circular dichroism (CD), we found that the CD spectrum of rG4-let-7e is cation dependent and is characterized by a maximum ellipticity at 263 nm and a minimum ellipticity at 240 nm (Figure S6C). This observation is consistent with G-quadruplex formation (Kypr et al., 2009). Denaturation experiments revealed a potassium-dependent transition at 48.1°C (Figure S6C). We then assessed the ability of this sequence to fold into a G-quadruplex in the context of pri-let-7e. The CD spectrum of pri-let-7e also displayed a maximum at 263 nm and a local minimum at 210 nm indicative of a more complex structure (Figure S6D). Denaturation experiments of pri-let-7e, followed by CD spectroscopy at 263 or 210 nm in the presence of KCl, revealed two transitions indicating the presence of two structures in equilibrium (Figure 5F). While the second transition is centered...
around ~75°C and correspond to the expected hairpin structure, a first transition was observed at ~48°C, similar to the melting transition of rG4-let-7e, and may correspond to the formation of the rG4-let-7e-quadruplex structure. To support this point, we introduced G-to-A mutations in pri-let-7e at positions that are expected to destabilize the rG4-let-7e-quadruplex (Figure S6 E). We found that G-to-A mutants display single-phase melting curves with transitions >70°C (Figure S6 F). This result demonstrates that G-to-A mutations in pri-let-7e impede G-quadruplex formation.

We hypothesized that m7G may affect the stability of G-quadruplexes in vivo by disrupting the N7 H-bonds and Hoogsteen base pairing while preserving Watson-Crick base pairing (Figure 6A). To support this hypothesis, we used 7-deaza-deoxyguanosine (DAG) as a mimic of m7G, since synthesis of m7G-containing oligonucleotides is currently unavailable and because, like m7G, DAG weakens secondary structures supported by Hoogsteen base pairing (Figure 6A; Römmler et al., 2013). We found that the rG4-let-7e quadruplex structure containing a single G-to-DAG substitution at the G11 position was significantly less stable than the WT sequence (Figure S6C). We then generated G-to-DAG mutant versions of pri-let-7e (Figure 6B). While the D1 and D2 oligonucleotides displayed two G-to-DAG mutations at G4-G5 and G11-G12, respectively, one oligonucleotide bears a single G-to-DAG mutation at position G11. Denaturation experiments followed by CD spectroscopy were performed to assess the quadruplex:stem-loop equilibrium within pri-let-7e and the contribution of each guanosine to pri-let-7e folding (Figures 6C and 6D). We observed that mutation of G11 (in both D2 and G11 mutants) affects the folding of pri-let-7e by shifting the structural equilibrium toward the canonical stem-loop structure. In contrast, mutating G4 and G5 (D1 mutant) did not significantly affect the structural equilibrium. These results suggest that the methylation of G11 favors the stem-loop structure of let-7e.

We next used the DAG-containing pri-let-7e hairpin oligonucleotides to establish whether the induced change in structure affects the processing of the precursor RNAs in vivo. We transfected either WT or the DAG-containing oligonucleotides into A549 cells and we measured the levels of mature let-7e-5p by

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**Figure 6. m7G Position Is Essential for let-7e Quadruplex:Stem-Loop Equilibrium and Promotes miRNA Processing**

(A) Schematic representation of a guanine tetrad, highlighting Hoogsteen base pairing involving the N7 of guanosine that stabilizes the G–quadruplex structure, together with a stabilizing monovalent cation (M⁺, usually potassium). Both 7-methylguanosine and 7-deaza-guanosine are able to destabilize the hydrogen bond involving N7.

(B) Illustration depicting the pri-miRNA hairpins used in the following experiments.

(C) Thermal denaturation studies of RNA oligonucleotides as described in (B). While GG-to-DAG-DAG mutation at the D1 position does not significantly affect the contribution of G4 in the G4:stem-loop equilibrium, GG-to-DAG-DAG mutation at the D1 position and a single G11-to-DAG mutation affect the contribution of rG4 in the structural equilibrium by shifting it toward the hairpin form.

(D) First derivative plot of the denaturation experiment in (C) helps visualize the decrease in rG4 contribution to the equilibrium (red arrow).

(E) qRT-PCR showing the levels of let-7e-5p 72 h after transfection with either WT, D1, D2, or G11 oligonucleotides. The average of six independent transfections ± SDs is shown (**p < 0.01, ***p < 0.001, two-tailed t test).

(F) Western blot showing the rescue of HMGA2 upregulation upon transfection of D2, but not WT let-7e primary hairpin in A549 METTL1 knockdown cells. Two representative biological replicates of a total of three independent experiments are shown.
either WT containing G11 DAG, were more efficiently processed than
and measured the levels of HMGA2 by immunoblotting. In agree-
consistent with a G-quadruplex structure and a canonical
the formation of a G-quadruplex structure ( Pandey et al.,
the G-quadruplex motif in let-
Figure 6 F). Finally, the
miRNAs, m6A and 5
alternative structures involving
m7G in miRNA Biogenesis
Figure 7. Role of m7G in miRNA Biogenesis
Proposed model of the role of METTL1-mediated m7G in promoting miRNA processing and suppressing migration phenotype.
qRT-PCR 72 h after transfection. Furthermore, we transfected
methyl phosphate represses the processing of
estimated, whereas the WT did not (Figure 6F).
Our data suggest that the methylation of pri-let-7e at G11 promotes its processing via disruption of local G-quadruplex structures.

**DISCUSSION**

In the present study, we use two independent unbiased tech-
qRT-PCR 72 h after transfection. Furthermore, we transfected
methyl phosphate represses the processing of
miR-145 by inhibiting the binding of DICER to the pre-miRNA. Here, we show that m7G promotes miRNA processing in a
unique manner, by directly affecting the secondary structure of
a specific set of pri-miRNAs that share a common functional signature: suppression of cell migration.

The model proposed here could represent a widespread molecular mechanism to safeguard the levels and activity of important tumor-suppressive G-rich miRNAs. In this scenario, the formation of G-quadruplexes is a “side effect” of the miRNA sequence, and the m7G pathway is required to maintain pri-miRNAs in a functional state. Alternatively, but not mutually exclusively, the presence of G-quadruplexes itself may represent a novel additional layer of regulation to control functionally related miRNAs, which, for example, inhibit cell migration.

Overall, our data suggest that the methylation of
miRNA biogenesis.

**Figure 7. Role of m7G in miRNA Biogenesis**
Proposed model of the role of METTL1-mediated m7G in promoting miRNA processing and suppressing migration phenotype.
Many mechanisms control cell migration and tumor invasiveness, and one of the most important drivers is the AKT oncogenic signaling pathway (Irie et al., 2005). Notably, AKT has been implicated in neurodegenerative diseases, such as Alzheimer’s disease, in which it is significantly upregulated (Lehmann et al., 2012). Furthermore, low levels of let-7 are implicated in neurodegenerative diseases, such as Alzheimer’s disease, in which it is significantly upregulated (Lehmann et al., 2012). Additionally, low levels of let-7 have been shown to improve tissue repair through reprogramming cellular metabolism (McDaniel et al., 2016; Shyh-Chang et al., 2013). Therefore, direct targeting of METTL1 could represent a valid and unexplored therapeutic strategy in these pathological contexts.

This report identifies the m7G pathway as a novel regulator of miRNA function. Considering the interest in miRNAs as targets for therapeutic intervention (Chakraborty et al., 2017), our findings could be exploited in many miRNA-related disease settings to open up new therapeutic avenues.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
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  - Generation of conditional knockdown cells
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  - In vitro RNA methylation assays
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- Isolation of DROSHA by IP
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  - Bioinformatic analysis of smallRNA sequencing
  - Global gene expression analysis
  - Bioinformatic prediction of G-quadruplexes
  - Statistical analysis
- DATA AND SOFTWARE AVAILABILITY
  - Data Resource
  - Supplemental Information

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.molcel.2019.03.040.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS


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REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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**Deposited Data**

| METTL1 Knockdown Expression Microarray data                      | This study        | GEO: GSE112180   |
| BoRed-seq and m7G-RIP-seq in A549                                | This study        | GEO: GSE112181   |
| m7G-RIP-Seq in Caco-2                                            | This study        | GEO: GSE120454   |
| m7G-RIP-Seq in A549 METTL1 Knockdown                            | This study        | GEO: GSE120455   |
| Unprocessed imaging data                                         | This study        | https://data.mendeley.com/datasets/yscng45zgj/1 |

**Experimental Models: Cell Lines**

| HEK-293T (Human embryonic kidney)                                | ATCC              | RRID: CVCL_0063  |
| A549 (Human lung adenocarcinoma)                                | ATCC              | RRID: CVCL_0023  |
| Caco-2 (Human colorectal adenocarcinoma)                        | ATCC              | RRID: CVCL_0025  |

**Oligonucleotides**

| DNA and RNA oligonucleotides are listed in Table S8              | This study        | N/A              |
| GFP MISSION esiRNAs                                              | Sigma-Aldrich     | Cat# EHUEGFP-50UG|
| METTL1 MISSION esiRNAs                                           | Sigma-Aldrich     | Cat# EHU076851-50UG|
| miRIDIAN Control miRNA mimic                                    | Dharmacon         | Cat# CN-001000-01-05|
| miRIDIAN hsa-let-7e-5p miRNA mimic                              | Dharmacon         | Cat# C-300479-05-0002|

**Recombinant DNA**

| Hmga2-Luc-m7                                                    | Addgene           | #14788           |
| Hmga2-Luc-wt                                                    | Addgene           | #14785           |
| PAX2                                                            | Addgene           | #12260           |
| pcDNA3-pri-let-7e                                               | Addgene           | #51380           |
| pcDNA4/TO/CMycDrosa                                             | Addgene           | #10828           |
| pHIV-ZsGreen                                                    | Addgene           | #18121           |
| pLKO-TETon-Puro                                                 | Addgene           | #21915           |
| pMD2.G                                                          | Addgene           | #12259           |
| pMirGlo                                                         | Promega           | Cat# E1330       |

**Software and Algorithms**

| Bioconductor                                                     | https://www.bioconductor.org/ | N/A              |
| DESeq2                                                          | Love et al., 2014          | N/A              |
| FastQQC                                                         | https://github.com/s-andrews/FastQQC | N/A              |
| featureCounts                                                    | Liao et al., 2014          | N/A              |
| G4Hunter                                                        | Bedret et al., 2016        | N/A              |
| Gage                                                            | Luo et al., 2009           | N/A              |
| gbm                                                             | Freund and Schapire, 1997  | N/A              |
| limma                                                           | Smyth et al., 2005         | N/A              |
| miRWalk 2.0                                                     | Dweep and Gretz, 2015      | N/A              |
| Pathwiew                                                        | Luo et al. 2017            | N/A              |

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EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
HEK293T (RRID:CVCL_0063) and A549 cells (RRID:CVCL_0023) were cultured in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/glutamine (PSQ). Caco-2 cells (RRID:CVCL_0025) were cultured in Eagle’s Minimum Essential Medium, supplemented with 20% FBS and 1% PSQ. Cell lines were obtained from the ATCC and tested negative for mycoplasma contamination. Human cell lines used are not listed in the cross-contaminated or misidentified cell lines database curated by the International Cell Line Authentication Committee (ICLAC).

Lentiviral vector preparation and cell transduction
For virus production, HEK293T cells were transfected with the lentiviral vector pLKO-TETOn-Puro for METTL1 knockdown, or Zs-Green-HIV for METTL1 rescue experiments, together with the packaging plasmids PAX2 (Addgene Plasmid #12260) and pMD2.G (Addgene Plasmid #12259) at a 1:1.5:0.5 ratio using Lipofectamine 2000 reagent (Invitrogen) according to the...

CONTACT FOR REAGENT AND RESOURCE SHARING
Queries and reagent requests may be directed and will be fulfilled by the lead contact, Tony Kouzarides (tony.kouzarides@gurdon.cam.ac.uk).
manufacturer’s instructions. Supernatants were harvested 48 h and 72 h after transfection. Cells ($5 \times 10^5$) were mixed in 2 ml viral supernatant supplemented with 8 μg/ml polybrene (Millipore), followed by spinfection (60 min, 900 g, 25°C) and further incubated overnight at 37°C. For METTL1 knockdown experiments, cells were replated in fresh medium containing 1 μg/ml puromycin and kept in selection medium for 7 days. For METTL1 rescue experiments, GFP+ cells were isolated using a SONY SH800 cell sorter 48 h after infection.

**Generation of conditional knockdown cells**
A549 or Caco-2 cells were infected with pLKO-TET-on-Puro lentiviral vectors (Addgene Plasmid #21915) expressing shRNAs against the coding sequence of human METTL1 or a scrambled control as described above. The shRNA sequences are listed in Table S8. shRNA was induced by treatment with 200 ng/ml doxycycline for the indicated times.

**METHOD DETAILS**

**BoRed-Seq and m7G-RIP-Seq**
The detailed protocol of all the procedures required to perform BoRed-Seq and m7G RNA immunoprecipitation experiments on small RNAs is described in Methods S1. Single-end 50-bp stranded smallRNA libraries were prepared using the NEBNext SmallRNA kit (NEB) according to the manufacturer’s recommendations and sequenced on a HiSeq 4000 (Illumina).

**RNA immunoblots and dot blots**
RNA was resolved by denaturing polyacrylamide gel electrophoresis using Novex TBE-urea 15% precast gels (Thermo Fisher). Equal loading was checked by staining with ethidium bromide (Sigma-Aldrich), then RNA was transferred to a nylon membrane (Amersham Hybond-N+, GE Healthcare) by wet electro-blotting in TBE (45 min at 400 mA).

For dot-blot analysis, input RNA or RNA immunoprecipitated with either anti-m7G or isotypic non-specific antibodies was spotted onto a nitrocellulose membrane and UV cross-linked at 254 nm (120 mJ/cm²). The membranes were blocked in Denhart’s solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin; Thermo Fisher) for 1 h at room temperature and incubated with m7G antibody for 1 h at room temperature. Signal was detected using HRP conjugated secondary antibodies and ECL (GE Healthcare) and developed on a Chemidoc MP machine (BioRad).

**Global gene expression profiling**
Cells were lysed in Qiazol (QIAGEN) and total RNA was extracted with miRNEasy mini kit (QIAGEN). RNA quality was assessed using an Agilent Tapestation RNA; 50 ng of RNA were labeled with Low Input QuickAmp Labeling Kit, One-Color (Agilent Technologies), purified and hybridized overnight on an Agilent SurePrint G3 Human Gene Expression Array v3 (8x60K) before detection according to the manufacturer’s instructions. An Agilent DNA microarray scanner (model G2505C) was used for slide acquisition.

**RT-qPCR**
Cells were lysed in Qiazol (QIAGEN) and total RNA was purified using the miRNEasy mini kit (QIAGEN) according to the manufacturer’s instructions. For mRNA detection, 1 μg of purified total RNA was reverse transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems). To quantify gene expression, we used probes from Universal ProbeLibrary (UPL; Roche) with TaqMan Fast Advanced Master Mix (Thermo Fisher).

For specific pri- and pre-miRNA quantification, we size-fractionated large (> 200nt, containing the pri-miRNAs) and small RNAs (< 200nt, containing pre-miRNAs) using RNA Clean & Concentrator 5 column kits (Zymo), as per the manufacturer’s instructions. Pri-miRNAs were reverse transcribed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems), which employs random nonamer priming and favors long molecules. Pre-miRNAs were reverse transcribed with miScript II RT kit (QIAGEN), which is more efficient on short RNAs. Primers were designed to anneal either within the stem loop (pre-miRNAs) or to overlap the DROSHA cleavage sites (pri-miRNAs). Both pri- and pre-miRNAs were quantified using Fast SybrGreen PCR mastermix (Applied Biosystems) according to the manufacturer’s instructions.

For mature miRNA detection, total RNA was reverse transcribed and amplified using the Taqman Advanced miRNA cDNA Synthesis Kit from Thermo Fisher Scientific. The levels of specific miRNAs were measured with Taqman advanced miRNA Assays from Thermo Fisher Scientific. All the RT-qPCR experiments were run on an ABI 7900 real-time PCR machine (Applied Biosystems). GAPDH and RNY1 were used as housekeeping genes for RT-qPCR normalization of long and small RNAs, respectively. Primer sequences, UPL probe numbers and assay IDs are listed in Table S8.

**Western blotting**
For total cell protein extraction and western blot analysis, cells were lysed in a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, Complete protease inhibitor cocktail (Roche) and cleared by centrifugation. The protein concentration was determined by Bradford assay (Bio-Rad). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Hybond-C Extra, GE Healthcare). Membranes were blocked with 5% milk proteins in TBST (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween-20), and probed with
primary antibodies overnight. Membranes were then washed three times with TBST (15 min each) and probed with a HRP-conjugated secondary anti-rabbit antibody for 1 h. After three more washes, signal was detected using HRP conjugated secondary antibodies and ECL (GE Healthcare) and developed on a BioRad Chemidoc MP machine.

Migration assays
For transwell migration assays, the lower surface of the transwell inserts (8 μm; Corning) was coated with human recombinant fibronectin (1 μg/ml, 1 h at RT; Sigma-Aldrich). A549 cells were serum-starved overnight and seeded (3 × 10^4) in serum-free medium on transwell inserts. After 7 h incubation in wells containing DMEM+20% FBS, inserts were stained with crystal violet and cells on the lower surface were counted (blindly).

Proliferation assays
4 days after shRNA induction, 10^5 A549 METTL1 knockdown or Ctrl cells were plated in each well of 6-well cell culture plates in normal culture medium. Cells were counted daily for the following three days using a Countess II cell counter (Thermo Fisher).

Mature miRNA/hairpin transfection
20 pmol of has-let-7e-5p miRNA mimic or control miRNAs (miRIDIAN, Dharmacon) were transfected into 2.5 × 10^5 A549 cells using 2 μL of Lipofectamine 2000 (Thermo Fisher). 10 pmol of pri-let-7e hairpins (Integrated DNA Technologies) were transfected into 1.5 × 10^5 A549 cells using the same amount of Lipofectamine as above.

Luciferase assay
A dual luciferase reporter harboring the 3′-UTR of mouse Hmga2 was generated extracting the XbaI-NotI digestion fragments from either Hmga2-Luc-wt or Hmga2-Luc-m7 (Mayr et al., 2007; Addgene plasmids #14785 and #14788) and subcloning them into pMirGlo (Promega). In order to generate stable reporter lines, A549 cells were transfected with the pMirGlo-hmga2(3′-UTR) constructs. 24 h after transfection, cells were selected using 300 μg/ml G418 for 7 days. Subsequently, cells were transfected with 20 pmol of MISSION esiRNAs (Sigma-Aldrich) against human METTL1 or GFP, 24 h after transfection, Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) on a CLARIOstar microplate reader (BMG Labtech).

METTL1 rescue experiments
cDNA was obtained by reverse transcription of A549 RNA with Superscript III (Thermo Fisher), then the METTL1 full-length coding sequence was amplified by PCR and cloned into pHIV-ZsGreen plasmid (Addgene plasmids #18121) using restriction sites XbaI and NotI. In order to generate an shRNA-resistant METTL1 sequence, synonymous substitutions were introduced in the codons corresponding to shRNA binding sites by long DNA fragment synthesis (GeneArt Strings; Thermo Fisher) of the N-terminal portion of METTL1 (up to NheI site). The in vitro synthesized fragment was swapped into pHIV-ZsGreen-METTL1 using restriction sites NotI and NheI. The same approach was combined to codon mutagenesis to generate the catalytically inactive METTL1 variant E107A (amino acids 107-109, that form the SAM-binding pocket of the enzyme; see Figure S2I). Primer and long oligonucleotide sequences are listed in Table S8.

METTL1 UV-CLIP
Adherent cells in a 15 cm dish were rinsed twice in ice-cold PBS, cross-linked at 254 nm (120 mJ/cm^2), scraped and lysed on ice for 10 min in 1 mL of fresh lysis buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM MgCl2, 0.5% NP-40, 5 mM DTT) supplemented with protease inhibitors (Complete tablets, Roche) and RNase inhibitors (RNaseOUT; Thermo Fisher). Lysates were vortexed, then centrifuged at max speed at 4°C for 5 min. The supernatants were used in immunoprecipitation (IP) assay with 5 μg of anti-METTL1-antibody (sheep polyclonal obtained by MRC Protein Phosphorylation Unit, #588192; Cartlidge et al., 2005) or control isotypic IgG at 4°C for 90 min, with rotation. 80 μL of Protein G Dynabeads (Invitrogen) per IP reaction were rinsed 2 times with lysis buffer and blocked with 1 mg/ml BSA (NEB) for 2 h at 4°C. Beads were resuspended in 100 μL lysis buffer, then added to the IP tube. The reaction was incubated at 4°C for 2 h with rotation. Beads were then washed twice with high-salt buffer (25 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM MgCl2, 1% NP-40, 5 mM DTT) and three times with lysis buffer. Immunoprecipitated samples were treated with DNase I (QIAGEN) followed by digestion with Proteinase K (NEB). RNA was purified using an RNeasy MinElute column kit (QIAGEN).

Expression and purification of recombinant METTL1/WDR4 (Evotec)
The constructs EV4866 (his-METTL1) and EV4868 (flag-WDR4) were cloned into plasmid pTriJ-HV (Evotec). Recombinant virus was produced by co-transfecting transfer plasmid DNA and bacmid DNA in insect cells. 100 ng bacmid DNA and 500 ng transfer plasmid DNA were mixed with 2 μl Cellfectin II transfection reagent (Invitrogen) in 200 μl TC100 media (Sigma) and incubated at room temperature for 2-3 h. SF21 insect cells, grown to 80%-90% confluency in 24 well plates were washed with TC100 before adding 0.2 mL TC100 and 0.2 mL co-transfection mix. After overnight incubation, 0.6 mL SF900 II SFM media containing 5 μg/ml gentamicin was added and the cells were incubated at 27°C for six days with humidity. The cells were observed under an inverted microscope.
and compared to the mock-transfected control. BluoGal (2%) was added to the LacZ positive control well and blue coloration was observed within 1 h. Following confirmation of successful transfection, the medium containing the recombinant virus (P0) was harvested into a sterile deep well block and stored in the dark at 4 °C. P1 BIICs were centrifuged for 72-120 h at 27 °C with shaking at 360 rpm. P1-BIICs were harvested by centrifugation of the cell block and virus supernatant was removed to a fresh block and stored at 4 °C. The cells were then re-suspended in freezing mix (S900 II + 10% heat inactivated FBS + 10% DMSO) and frozen gradually to −80 °C in the block. Working P2-BIICs were amplified by infecting Sf21 cells grown in shake flasks at MOI-0.1 using P1-BIICs and incubated for 72 h. P2-BIICs were harvested by centrifugation and infected cells were resuspended in freezing media and stored at −80 °C. Sf21 cells grown in S900 II SFM media plus 5 µg/ml gentamicin were infected with both EV4866 and EV4868 P2 BIICs at an MOI of 2 (1+1). The infected culture was incubated for 72 h at 27 °C with shaking at 110 rpm, before harvesting by centrifugation and storing at −80 °C. Thawed cells were lysed in 25 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM TCEP, 5% glycerol, 0.25% CHAPS supplemented with Complete EDTA-free protease inhibitor tablets (Roche). Samples were homogenized for 20-30 s with an IKA Ultra-Turrax and sonicated in a Branson probe sonicator (cycles of 30 s on, 30 s off for 5 min at 40% amplitude). Samples were centrifuged at 45000 rpm for 50 min to remove insoluble material. Purification was carried out by sequential Ni-affinity and size-exclusion chromatography on an ÄKTA Xpress system (GE). Samples were bound to 1 mL HisTrap FF column, washed with 25 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM TCEP, 5% glycerol, 20 mM imidazole and eluted with a step elution over 20 CV of 25 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM TCEP, 5% glycerol and 500 mM imidazole at a flow rate of 0.8 ml/min. This was followed by size-exclusion on a 16/60 S200 column (25 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM TCEP, 5% glycerol) at a flow rate of 1 ml/min. Purified protein was analyzed by SDS-PAGE, western blotting and measurement of A260/A280 to estimate levels of contaminating nucleosides. Aliquotted protein was snap-frozen in liquid nitrogen and stored at −80 °C.

**In vitro RNA methylation assays**

Recombinant METTL1/WDR4 (300 nM; Evotec) was incubated for 2 h with S-adenosylmethionine (15 µM) and oligonucleotide (1 µM) in a Tris-HCl pH 8.0 buffer (20 mM) supplemented with 1 mM DTT and 0.01% Triton X-100.

**Mass spectrometry analysis of RNA nucleoside m7G modification**

Nucleosides were prepared from enzyme-processed RNA by enzymatic digestion, using a cocktail of Benzonase (Merck), Phosphodiesterase 1 (Merck), and Antarctic Phosphatase (New England Biolabs) as described previously (van Delft et al., 2017). The reactions were filtered using an Amicon 30kDa MWCO spin-column (Merck) to remove protein and the filtrate was mixed with a 2x loading buffer containing 0.1% formic acid and an internal standard (13C-labeled uridine generated from 645672-1MG Merck KGaA, previously treated with Antarctic Phosphatase). The samples were loaded onto an ACQUITY UPLC HSS T3 Column, 100 Å, 1 mm X 100 mm (Waters Corp., Milford, MA, USA) and resolved using a gradient of 2.5% to 20% B at 200 nl/min on a QExactive HF (Thermo Fisher, Waltham, MA, USA) mass spectrometer. Standard dilutions of all analytical nucleosides were prepared and analyzed in parallel. There were three technical replicates of each sample and the analytical processing was performed using XCalibur Software (Thermo Fisher).

**Mature miRNA isolation**

Mature miRNA fraction was isolated from total RNA by gel extraction: RNA was denatured by incubation at 73 °C for 3 min in 2X Urea Sample Buffer (Thermo Fisher) and run in a 10% TBE–urea precast polyacrylamide gel (Thermo Fisher) at 250V for 12 min. Gel was visualized by ethidium bromide staining and the region corresponding to the expected size of mature miRNAs was excised using a guanidinium isothiocyanate (Guanidine thiocyanate, 5% sodium dodecylsulfate) by freeze-thawing once on dry ice and incubating at 4 °C for 2 min in 2X Urea Sample Buffer (Thermo Fisher). Precipitated RNA was washed by ethanol precipitation.

**Context-specific mass spectrometry analysis of miRNA m7G modification**

Oligonucleotides were prepared from miRNA fraction using RNase A (Thermo Fisher) and chromatographically separated by ion pair reverse phase chromatography (200 mM Hexafluoroisopropanol [HFIP], 8.5 mM triethylamine [TEA] in water as eluent A, and 100 mM HFIP, 4.25 mM TEA in methanol as eluent B). The oligonucleotides were resolved by a gradient of 2.5% to 20% B at 200 nl/min on Acclaim PepMap C18 solid phase (Thermo Fisher) and characterized by negative ion tandem LC-MS in a hybrid quadrupole – orbitrap (QExactive HF, Thermo Fisher). Data were collected in data-dependent acquisition mode in pathfinding experiments before subsequent hybrid acquisition investigation. Full scan MS1 data were acquired between 700 and 3500 m/z and extracted ion chromatograms from these data were used for label-free quantification of oligonucleotides derived from let-7. MS2 data were collected in subsequent scan events for the same let-7 oligonucleotides by targeted, multiplexed, data-independent acquisition on filtered precursor ion masses multiplexed from the double and triple charge states of unmodified and monomethylated AGGAGGU (m/z of 1180.158, 786.436, 1187.166 and 791.108, with a window of 3 m/z). Technical replicates of n = 3 were acquired, with MS2 ions matched with to within 5 ppm.
Preparation of naive and in vitro methylated [α-32P]-pri-let-7e
Plasmid pcDNA3-pri-let-7e (Addgene #51380; Heo et al., 2008) linearized by digestion with Xbal (NEB) was used as a template for RNA in vitro transcription in the presence of [α-32P]-GTP (Perkin Elmer) using TranscriptAid T7 High Yield Kit (Thermo Fisher). The resulting RNA (369bp) was denatured by incubation at 73°C for 3 min in 2X Urea Sample Buffer (Thermo Fisher) and run in a 6% TBE–urea precast polyacrylamide gel (Thermo Fisher). The band corresponding to the expected size of pri-let-7e transcript was excised using autoradiography and, after breaking the gel, RNA was eluted as described above for mature miRNA isolation. 2.5 μg of [32P]-labeled pri-let-7e (1 μM) were incubated for 3 h at 37°C in the presence of recombinant METTL1/WDR4 (300 nM) and S-adenosylmethionine (1 mM) in a methylation buffer (85 mM Tris–HCl pH 8.0, 1.4 mM DTT, 0.07 mM EDTA, 1 mM spermidine). Methylated pri-let-7e was isolated by immunoprecipitation using m7G-specific antibody, purified on RNA Clean & Concentrator - 5 columns (Zymo) and quantified by scintillation counting (Hitex 300 SL).

Isolation of DROSHA by IP
Immunopurification of DROSHA and in vitro processing assays were performed according to a published protocol (Lee et al., 2002). HEK293T cells transfected with pcDNA4/TO/cmycDrosha plasmid (Landthaler et al., 2004; Addgene plasmid #10828) were lysed after 48 h in buffer D (20 mM HEPES–KOH pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 5% glycerol) by sonication (Bioruptor: 5 min; 30 s ON / 30 s OFF, 200W) followed by centrifugation. 2 mg of crude extract were incubated with 12 μg of anti-myc-tag antibody (Abcam) for 2 h at 4°C, then the recombinant enzyme was pulled-down with 30 μL Protein G Sepharose 4 Fast Flow beads (GE Healthcare) and washed 4 times in buffer D. Whole-cell extracts were prepared from control and METTL1 knockdown A549 cells using the same lysis protocol as above.

In vitro miRNA Processing Assays
For evaluating the processing efficiency of DROSHA, 30,000 cpm (50-100 ng) of either naive or in vitro methylated pri-let-7e were incubated with 15 μL of DROSHA-IP beads, 6.4 mM MgCl₂ and 1U/μL RNaseOUT RNase inhibitor (ThermoFisher) at 30 min at 37°C.

For evaluating the processing activity of METTL1 depleted cells, 10 μL of processing reaction containing 5 μL of whole-cell extract, 1 μL of solution A (32 mM MgCl₂, 5 mM ATP, 200 mM creatine phosphate), 1 μL 32 mM S-adenosyl methionine, 1 U/μL RNase inhibitor (Thermo Fisher) and the labeled transcripts (10²–10⁵ c.p.m.) were incubated at 37°C for 120 min.

All processing reactions were purified with RNA Clean & Concentrator - 5 column kit (Zymo), measured and equalized by scintillation counting, resolved on a 6% TBE–urea precast polyacrylamide gel (Thermo Fisher) and developed by autoradiography exposing the dried gel on Amersham Hyperfilm high-sensitivity film (GE Healthcare).

Circular dichroism spectroscopy
CD experiments were conducted on a Chirascan Plus spectropolarimeter. Oligonucleotide solutions were prepared at a final concentration of 10 μM (G44-let-7e oligonucleotides) or 2.5 μM (let-7e hairpins) in 10 mM lithium cacodylate (pH 7.2) containing 1mM EDTA and supplemented with 100 mM LiCl, NaCl or KCl. Oligonucleotides were annealed by heating at 95°C for 3 min and cooling the solutions at 4°C for 4 h. Scans were performed over the range of 200–320 nm at 5°C. Each trace was the result of the average of three scans taken with a step size of 1 nm, a time per point of 1 s and a bandwidth of 1 nm. A blank sample containing only buffer was treated in the same manner and subtracted from the collected data. The data were finally baseline corrected at 320 nm. Denaturation experiments were performed by heating the samples to 95°C using the stepped temperature ramping mode, a setting time of 10 s and with data collection every 1°C monitoring the CD signal at 263 and 210 nm. Differential melting curves (quantification of folded fractions) were obtained by subtracting the upper baseline to the signal and dividing by the difference between the upper and lower baseline. Melting temperatures ($T_m$) values were extracted as the local minima of the first derivatives of a Boltzman or bi-phasic dose-response fittings of the differential curves.

Polysome fractionation
Control or METTL1 knockdown cells (n = 2) were treated 5 days after doxycycline induction with 0.1 mg/ml cycloheximide for 5 min at 37°C, then they were lysed and polysomes were fractionated on a sucrose gradient while measuring absorbance at 254 nm (Panda et al., 2017).

QUANTIFICATION AND STATISTICAL ANALYSIS
Bioinformatic analysis of smallRNA sequencing
Multiplexed reads were split on the basis of their barcodes using Illumina Basespace. Read quality was assessed using FastQC program (https://github.com/s-andrews/FastQC). Library adaptors were trimmed with Trimomatic (Bolger et al., 2014), and reads were mapped to the human genome (NCBI GRCh38/hg38) with STAR (Dobin et al., 2013), using the parameters of ENCODE guidelines: --runThreadN 15 --sjdbGTFfile /path/to/GENCODE_miRNA_subset.gtf --alignEndsType EndToEnd --outFilterMismatchNmax 1 --outFilterMultimapScoreRange 0 --outSAMtype BAM SortedByCoordinate --outFilterMultimapNmax 10 --outSAMunmapped Within --outFilterScoreMinOverLread 0 --outFilterMatchNminOverLread 0 --outFilterMatchNmin
and quantile normalized among arrays using the Bioconductor package limma (Smyth and Speed, 2003; Smyth et al., 2005). The Microarray spot analysis was performed with Feature Extraction software (Agilent Technologies). Data were background-corrected

Global gene expression analysis

Microarray spot analysis was performed with Feature Extraction software (Agilent Technologies). Data were background-corrected and quantile normalized among arrays using the Bioconductor package limma (Smyth and Speed, 2003; Smyth et al., 2005). The statistical significance of differential gene expression was calculated with the empirical Bayes method implemented in limma. KEGG pathway ontologies over-represented in the subset of genes upregulated or downregulated upon METTL1 knockdown were evaluated using the R package Gage (Luo et al., 2009, 2017).

In order to identify the mRNAs that are predicted to be targets of selected miRNAs, we extracted the positive hits of at least 3 out of 6 in silico prediction algorithms (namely miRWalk, miRanda, miRDB, PicTar, RNA22 and Targetscat) using miRWalk 2.0 web server (Dweep and Gretz, 2015).

For unbiased analysis of miRNA seed sequences enriched in the top upregulated genes upon METTL1 knockdown, we took advantage of the Sylamer online software (van Dongen et al., 2008).

To produce a classification model predicting mRNA upregulation upon METTL1 knockdown, we extracted the subset of genes expressed above the background (log2 Average expression > 6). Genes displaying a log2FoldChange > 1 and a FDR < 0.05 were considered upregulated, and then a model employing the presence of putative miRNA target sites as predictors was generated by gradient boosting using R package gbm (Freund and Schapire, 1997).

Bioinformatic prediction of G-quadruplexes

Stem-loop and mature miRNA sequences were recovered from miRBase (Kozomara and Griffiths-Jones, 2014) release 22. These sequences were used to calculate the quantitative parameters used to describe the different miRNA features discussed in this manuscript. Base composition was assessed using a custom Python script. G-score, a quantitative estimation of G-richness and G-skewness, is based on the G4Hunter algorithm (Bedrat et al., 2016). Briefly, each position in a sequence is given a score between −4 and 4. To account for G-richness, a single G is given a score of 1, in a GG sequence each G is given a score of 2; in a GGG sequence each G is given a score of 3; and in a sequence of 4 or more Gs each G is given a score of 4. To account for G-skewness, Cs are scored similarly but their values are negative. The Gscore is the maximum value obtained while scanning miRNA sequences using a 20nt window and averaging the score of each nucleotide over the considered window.

G-quadruplex forming motifs, G2N7, G2N3, G3N7 and G3N12 are sequences of the form G2,N1−7G2,N1−7G2,N1−7G2+, G2,N1−3G2,N1−3G2,N1−3G2+, G2,N1−3G2,N1−7G3,N1−7G3,N1−7G3+, and G2,N1−12G3,N1−12G3,N1−12G3, respectively, where N is any base. G3N7 represents the strict definition of G4 forming sequences according to the Quadparser algorithm (Huppert and Balasubramanian, 2003). Other motifs represent the loose definition of G4 forming sequences (Kwok et al., 2016a). The presence of each motif within miRNA sequences was assessed using the re.finder function in custom python scripts.

RNA secondary structures were predicted using the RNAfold 2.2.10 algorithm of the ViennaRNA package (Lorenz et al., 2011). RNAfold computes the minimum free energy (MFE) of optimal secondary structures via estimation of base pairing probabilities. MFEs of dsRNA secondary structures (∆G_{dsRNA}) were computed at 37°C. MFEs of G4 secondary structures (∆G_{G4}) were computed by subtracting MFEs obtained when considering G4 formation into the structure prediction algorithm to the previous values (∆G_{G4} = ∆G_{dsRNA} - ∆G_{dsRNA + G4}). RNAfold was used to assess the stability of predicted RNA structures within miRNA sequences using a 20nt sliding window. m7G-containing miRNAs are the subset of miRNAs that have been enriched from the total population of miRNAs using both the BoRed-Seq and m7G-RIP-Seq protocols. Background is the rest of annotated miRNAs. For assessing the local enrichment of structures within m7G-containing precursor miRNAs, pre-miRNA sequences were piled up and centered, MFE values were then averaged at each position. Data were compiled and plotted using R.

Statistical analysis

All general statistical analyses were performed using either a two-tailed Student’s t test or a Wilcoxon test (when distributions were assessed not to be normal and homoscedastic) at a confidence interval of 95%, unless otherwise specified. No statistical methods were used to predetermined sample size.

DATA AND SOFTWARE AVAILABILITY

Data Resource

Raw genomic data have been deposited in the Gene Expression Omnibus database with accession number GSE112182 (Expression Microarray data: GSE112180; BoRed-seq and m7G-RIP-seq in A549: GSE112181; m7G-RIP-Seq in Caco-2: GSE120454; m7G-RIP-Seq in A549 METTL1 knockdown: GSE120455). Unprocessed imaging data are deposited on Mendeley Data: (https://data.mendeley.com/datasets/ysng45zgi/1). All other data and analysis scripts are available from the corresponding author upon reasonable request.
Supplemental Information

METTL1 Promotes *let*-7 MicroRNA Processing via m7G Methylation

Figure S1 | High-throughput profiling of m7G modification in miRNAs. Related to Figure 1.

A, Schematic representation (top) of the expected cleavage of 18S rRNA after NaBH₄ + aniline treatment and electropherogram analysis (bottom) of untreated and treated total RNA from A549 cells. B, Immunoprecipitation with anti-m7G antibody does not enrich RNAs containing five different guanine modifications (including four methylations) as determined by mass spectroscopy. Error bars represent S.E.M. C, HPLC trace of the four canonical RNA nucleosides along with 7-methylguanosine, showing retention times that are an exact match with pure nucleosides run as quantitation standards. D, Extracted ion chromatogram of 7-methylguanosine, with the accompanying mass spectra showing the intact protonated precursor ion of the nucleoside [M+H] (middle panel) and the 7-methylguanine ion generated by in-source fragmentation as an internal verification of its identity.
Figure S2 | Validation of BoRed-Seq and m7G-RIP-Seq. Related to Figure 1.

A, Electropherogram analyses of total RNA before and after immunoprecipitation with an m7G-specific antibody showing specific enrichment for 18S rRNA in the immunoprecipitate. B, Read counts for lncRNAs from the BoRed-Seq and m7G-RIP-Seq experiments are shown. Error bars represent S.E.M. C, Venn diagram showing the high overlap between lncRNAs significantly enriched in either m7G-RIP-Seq or BoRed-Seq of A549 cells, respectively. Confidence interval of the odds ratio (OR) and P-value, as obtained by Fisher’s exact test, are indicated in the table below. D, Read counts for the indicated lncRNA from the BoRed-Seq and RIP-Seq experiments are shown. Error bars represent S.E.M. E, Scatter plot showing the absence of correlation between m7G and miRNA expression levels. Labels show the m7G containing miRNAs amongst the top 20 most expressed ones and their absolute ranking. F, Scatter plot of m7G-RIP-Seq miRNA enrichment in Caco-2 versus A549 cells, highlighting a high degree of correlation, as indicated by R² Pearson correlation coefficient. G, Immunoblot using an m7G-specific antibody shows a reduction of m7G levels in tRNAs upon METTL1 knockdown. Right graph shows the average ± S.E.M. digital quantification of the m7G signal (*P<0.05, two tailed t-test). H, RT-qPCR quantification of let-7e-5p, miR-125a-5p and miR-148a-3p upon METTL1 KD in Caco-2 cells. The average of four independent biological replicates ± S.D. is shown (***P<0.01, two tailed t-test). I, Crystal structure of METTL1 in complex with S-adenosylmethionine (PDB file: 3CKK). The EIR (107-109) amino acids mutated to alanine to generate the catalytically inactive mutant are shown in red. J, RNA immunoblot with an m7G-specific antibody on total RNA from METTL1 knockdown or control cells over-expressing either WT METTL1 or a catalytically inactive METTL1 (c.i.). K, Polysome fractionation analysis. Cell extracts from control or METTL1 knockdown cells were prepared and resolved in a 5–50% sucrose gradient. The absorbance at 254 nm was continuously measured. The peaks corresponding to free 40S and 60S subunits, 80S and polysomes are indicated. Shaded area in grey indicates the S.E.M. of two independent samples for each condition.
**Figure S3**

*Effects of targeting METTL1 in A549 cells.* Related to Figure 2.

A, migration assay of A549 cells. Increased migration due to METTL1 KD is rescued by the expression of WT METTL1 (+), but not by a catalytically inactive version of the enzyme (c.i.).

B,C,D,E, Graphical representation of KEGG pathway regulation, showing up-regulation of ECM-receptor interaction (B), adherens junction (C), axon guidance (D) and focal adhesion (E). Data were obtained by comparing RNA expression data from METTL1 KD and control A549 cells (up-regulated genes, red; down-regulated genes, green).

F, Sylamer miRNA target seed motif analysis on up-regulated mRNAs upon METTL1 KD, showing a specific enrichment for the let-7 (5p) seed motif.

G, representative cell migration genes whose expression is upregulated upon METTL1 KD, which are targets of let-7e-5p, miR-125a-5p or both (for all of them FDR<0.05, as evaluated with limma).

H, A gradient boosting classifier was used to model the mRNA expression fold changes observed by microarray analysis as a function of the presence of seed sites for different miRNAs in 3’-UTRs. Receiver operating characteristic (ROC) curve displays the goodness of the model, with an area under ROC (AUC) of 0.68. The presence of let-7 (5p) seed sequence within an mRNA represents the strongest predictive factor for its up-regulation upon METTL1 KD, as shown by the top 20 predictor list. The marginal effect plot shows that there is a positive correlation between the number of seeds in the 3’-UTR and mRNA up-regulation.
Figure S4 | miRNA processing is dependent on m7G. Related to Figure 3 and 4.
A, HMGA2 expression measured by RT-qPCR in Caco-2 cells infected with METTL1-specific or control (Scr) TET-inducible shRNAs five days after doxycycline treatment. The average of five biological replicates ± S.D. is shown (***p<0.001, two tailed t-test).
B, Western blot showing METTL1, HMGA2 and β-tubulin protein levels in Caco-2 cells infected with METTL1-specific or control (Scramble) TET-inducible shRNAs five days after doxycycline treatment. Two representative biological replicates of a total of four are shown.
C, BoRed and m7G-RIP coupled to RT-qPCR of HMGA2 in A549 cells. 18S rRNA is used as a positive control; GAPDH is a negative control. let-7e levels are replotted alongside as a positive reference. The average of three biological and technical replicates ± S.E.M. are shown.
D, Western blot showing METTL1 down-regulation upon transfection with METTL1 specific siRNAs in A549 cells stably expressing a luciferase cDNA with a Hmga23′-UTR with mutated let-7 seed sites. Two independent transfections of a total of four replicates are shown.
E, CLIP-qPCR using a METTL1-specific antibody or a non-specific IgG. The levels of immunoprecipitated mir-148a primary hairpin are shown. The average of two independent immunoprecipitation reactions ± S.E.M. is presented (*P<0.05, two tailed t-test).
F, RT-qPCR showing the levels of mir-148a-3p upon METTL1 KD in A549 cells. The average of five independent biological replicates ± S.D. is shown.
G, Western blot of DICER, DGC8R and ARGONAUTE (pan-AGO) upon METTL1 KD in A549 cells.
H, Box plots visualizing the normalized smallRNA-Seq counts of INPUT and m7G-RIP miRNAs from either control (Scr) or METTL1 KD A549 cells.
I, m7G RNA immunoprecipitation and RT-qPCR of LET7A3, LET7B, LET7E/125A and MIR23B primary transcripts in A549 cells. MIR148A is shown as a negative control.
J, m7G-RIP and RT-qPCR of MIR23B and MIR148A primary transcripts upon METTL1 KD in A549 cells. The average of three independent biological replicates ± S.E.M. is shown (*P<0.05, **P<0.01, ***P<0.001, two tailed t-test).
Figure S5 | METTL1-mediated miRNA methylation. Related to Figure 4 and 5.
A, Autoradiography and digital quantification of the results from in vitro processing of a radioactively labelled pri-let-7e incubated with cell extracts from control (Ctrl) or METTL1 KD A549 cells. The average of three independent experiments ± S.D. is shown (*P<0.05, **P<0.01, two tailed t-test). Autoradiography images are composite of different molecular weight regions and exposure times. Full, unprocessed images are deposited on Mendeley Data. B, Coomassie staining and Western blot of co-expressed recombinant 6xHis-tagged METTL1 and FLAG-tagged WDR4 from baculovirus-infected insect cells. C, In vitro methylation reaction using recombinant METTL1/WDR4 pre-assembled complex on pre-tRNA\textsuperscript{Phe} or a hairpin shRNA targeting GFP (negative control). Mass spectrometry analysis shows specific m7G methylation of pre-tRNA\textsuperscript{Phe}. D,E, Representative extracted ion chromatograms of collated charge states of unmodified (left chromatogram) and methylated AGGAGGU (right chromatogram) oligonucleotides. Precursor ion areas were quantified from technical triplicates (quantified area shown in grey). Chromatograms shown are from a single replicate. E, in vitro methylation reaction using recombinant METTL1/WDR4 pre-assembled complex on WT and G11-DAG let-7 oligonucleotides.
**Figure S6**

**A, B, Box plot showing the base content in primary hairpins of either unmodified or m7G containing miRNAs (P-values are calculated with Wilcoxon test).**

**B, Proportion of G-quadruplex forming motifs detected within primary hairpins of either unmodified or m7G containing miRNAs (see STAR Methods for details; *P<0.05, **P<0.01, ***P<0.001; Fisher’s exact test).**

**C, Circular dichroism (CD) of the minimal rG4-let-7e sequence reveals that its structure is cation-dependent and its spectra display a minimum and maximum at 240 and 263 nm, respectively. These are feature of G-quadruplex formation. The stability of rG4-let-7e WT structure is cation-dependent, with a thermal stability ($T_m$) of 48.1 °C in the presence of 100 mM KCl. While G11 to DAG substitution in rG4-let-7e DAG does not completely abolishes rG4 formation, it greatly destabilizes it. The thermal stability decreases from 48.1 °C to 35.5 °C in the presence of 100 mM KCl and the oligonucleotide is no longer folded in the presence of LiCl or NaCl.**

**D, CD spectra of pri-let-7e hairpin. The minimum at 210 nm indicates the expected stem-loop structure.**

**E, Cartoon depicting the pri-miRNA hairpins employed in the following panel.**

**F, Thermal denaturation studies of GG to AA mutants at the D1 or D2 position, supporting the formation of a G-quadruplex within pri-let-7e. Melting profiles of both mutants display a single transition at high temperature characteristic of the canonical hairpin structure. The transition attributed to the rG4 motif is no longer observed. Both mutations destabilize the hairpin structure as expected (decreased GC content).**

**Figure S6 | m7G modified miRNAs are prone to G-quadruplex formation.** Related to Figure 5.

A, Box plot showing the base content in primary hairpins of either unmodified or m7G containing miRNAs (P-values are calculated with Wilcoxon test). B, Proportion of G-quadruplex forming motifs detected within primary hairpins of either unmodified or m7G containing miRNAs (see STAR Methods for details; *P<0.05, **P<0.01, ***P<0.001; Fisher’s exact test). C, Circular dichroism (CD) of the minimal rG4-let-7e sequence reveals that its structure is cation-dependent and its spectra display a minimum and maximum at 240 and 263 nm, respectively. These are feature of G-quadruplex formation. The stability of rG4-let-7e WT structure is cation-dependent, with a thermal stability ($T_m$) of 48.1 °C in the presence of 100 mM KCl. While G11 to DAG substitution in rG4-let-7e DAG does not completely abolishes rG4 formation, it greatly destabilizes it. The thermal stability decreases from 48.1 °C to 35.5 °C in the presence of 100 mM KCl and the oligonucleotide is no longer folded in the presence of LiCl or NaCl. D, CD spectra of pri-let-7e hairpin. The minimum at 210 nm indicates the expected stem-loop structure. E, Cartoon depicting the pri-miRNA hairpins employed in the following panel. F, Thermal denaturation studies of GG to AA mutants at the D1 or D2 position, supporting the formation of a G-quadruplex within pri-let-7e. Melting profiles of both mutants display a single transition at high temperature characteristic of the canonical hairpin structure. The transition attributed to the rG4 motif is no longer observed. Both mutations destabilize the hairpin structure as expected (decreased GC content).
| Table S8 | DNA and RNA oligonucleotides employed in this study. Related to STAR Methods |
Methods S1 – m7G Profiling Protocol

Related to STAR Methods

1. Total RNA extraction

2. RNA decapping

3. NaBH₄ reduction or Mock reaction

4. Abasic site production

5. ARP coupling and pulldown

6. m7G-RIP

7. SmallRNA library preparation

8. High-throughput sequencing
**BoRed-Seq Protocol**

**1 RNA extraction from A549 cells**

*Total RNA, including miRNAs, is extracted from mammalian cells. To ensure optimal results, it is advisable to always start with freshly extracted RNA.*

1. Culture up to $1.5 \times 10^7$ A549 cells to a confluency of $\sim 80\%$.
2. Remove the medium and rinse the cells twice in PBS.
3. Extract RNA using the miRNeasy Mini Kit (Qiagen). Elute RNA in $30\ \mu l$ RNAse-free water.

**2 RNA decapping**

*Cap-Clip Acid Pyrophosphatase is used to remove the m7GpppG cap of eukaryotic mRNA in order to avoid cross-reactivity to non-internal m7G.*

1. Prepare the RNA decapping mix according to Table 1.
2. Incubate for 1 h at 37°C.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>20\ µg</td>
<td>RNA</td>
</tr>
<tr>
<td>2\ µl</td>
<td>10× Reaction buffer</td>
</tr>
<tr>
<td>1\ µl</td>
<td>CapCLIP (5 U; CellScript)</td>
</tr>
<tr>
<td>1\ µl</td>
<td>RNAsin Plus (Thermo Fisher)</td>
</tr>
<tr>
<td>20\ µl</td>
<td>Final vol. with ddH$_2$O</td>
</tr>
</tbody>
</table>

**3 NaBH$_4$ Reduction**

*m7G residues are specifically reduced by treatment with NaBH$_4$ in the presence of high concentrations of free m7GTP. Control samples are treated with acid-quenched NaBH$_4$. The NaBH$_4$ treatment also reduces aldehydes in RNA (e.g. 5-formylcytosine or 5-formyluracil), thereby preventing unwanted covalent binding of ARP with them in the downstream stages.*

1. Throughout the entire protocol, always use nuclease-free not DEPC-treated water (Ambion) as even small traces of DEPC lead to RNA degradation after NaBH$_4$ reduction.
2. Prepare 3 M Tris-HCl (pH 8.2) and 1 M sodium acetate (NaOAc; pH 4.5). Filter through 0.22\ µm filter and autoclave. Store aliquots at -20°C.
3. Prepare the RNA mix according to Table 2. For each sample prepare n × 2 reaction mixes: one reduction reaction (RED) and one control reaction (CTRL). At least three parallel reactions are recommended to obtain sufficient reduced RNA for subsequent steps.
4. Prepare a fresh 1 M sodium borohydride solution (NaBH$_4$; Sigma-Aldrich) immediately before use. The NaBH$_4$ powder should be stored at 4°C in a desiccator.
Table 2: Reaction mix for 4 µg of RNA

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>up to 4 µg RNA</td>
<td></td>
</tr>
<tr>
<td>3 µl</td>
<td>37 mM m7GTP (Sigma-Aldrich)</td>
</tr>
<tr>
<td>5 µl</td>
<td>3 M Tris-HCl (pH 8.2)</td>
</tr>
<tr>
<td>15 µl</td>
<td>Final vol. with ddH₂O</td>
</tr>
</tbody>
</table>

5. Add 15 µl 1 M NaBH₄ solution to the reduction reaction. Vortex and spin briefly. Do not add NaBH₄ to the CTRL reaction and keep it on ice until quenching.

6. Incubate on ice for 40 min. Open the lid of the tube, debubble and spin down every 10 min.

7. Meanwhile prepare the quenched NaBH₄ solution (QB1) for the control reaction (non-reduced RNA) according to Table 3.

8. Incubate QB1 at 90 °C for 30 min.

9. Place QB1 on ice and add 1 µl of glycogen (20 µg/µl; Roche).

Table 3: Preparation of QB1 solution

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>180 µl</td>
<td>1 M NaOAc (pH 4.5)</td>
</tr>
<tr>
<td>120 µl</td>
<td>ddH₂O</td>
</tr>
<tr>
<td>15 µl</td>
<td>1 M NaBH₄</td>
</tr>
</tbody>
</table>

10. Prepare the quenching solution for the reduction reaction (QB2) according to Table 4. Keep it on ice until used.

11. After the incubation of the reduction reaction (RED) on ice for 40 min, add 300 µl of QB2 quenching solution drop-wise, to avoid a violent reaction.

12. Add 990 µl of ice-cold absolute ethanol.

13. Add 315 µl of QB1 solution and 990 µl of ice-cold absolute ethanol to the control reaction (CTRL).

14. Incubate both reactions (RED and CTRL) for 1 h at -20 °C to precipitate RNA.

15. Centrifuge at ≥15,000 g for 30 min at 4 °C. Carefully decant the supernatant.

16. Wash the RNA with 1 ml of 70% ethanol. Vortex to detach the pellet for 5 min at room temperature.

17. Centrifuge at ≥15,000 g for 5 min at 4 °C.

18. Carefully remove the supernatant and air-dry the pellet.

19. Dissolve the pellet in 10 µl ddH₂O and quantify by Qubit RNA HS Assay (Thermo Fisher).

4 RNA abasic site production

The reduced m7G residues are subjected to nucleoside hydrolysis at low pH.

1. Prepare a sodium acetate solution (pH 3.9) by mixing 3.5 ml ddH₂O, 1.5 ml acetic acid and
300 µl 10 N sodium hydroxide (NaOH).

2. Add 1 µl 7-methyl-GTP (37 mM; Sigma-Aldrich) and 11 µl of acetate solution to the RNA dissolved in 10 µl of ddH₂O (CTRL or RED).

3. Incubate the mix for 15 min at 37 °C.

4. Precipitate by adding 200 µl ddH₂O, 20 µl NaOAc (pH 4.5), 660 µl of ice-cold absolute ethanol and 1 µl glycogen.

5. Incubate for 1 h at -20 °C.

6. Centrifuge at ≥15,000 g for 30 min at 4 °C. Carefully decant the supernatant.

7. Wash the RNA with 1 ml of 70% ethanol. Vortex to detach the pellet.

8. Centrifuge at ≥15,000 g for 5 min at 4 °C.

9. Carefully remove the supernatant and air-dry the pellet.

10. Dissolve the pellet in 10 µl ddH₂O and quantify by Qubit RNA HS Assay.

5 ARP coupling and pulldown

A biotin-coupled aldehyde reactive probe covalently binds to the abasic RNA sites resulting from m7G reduction and hydrolysis. This allows pull-down with streptavidin-conjugated beads.

1. Prepare a 22.5 mM stock solution (4.5×) of Aldehyde Reactive Probe (ARP) by adding 1 ml ddH₂O to 10 mg of ARP powder (N-(aminooxyacetyl)-N'- (D-Biotinoyl) hydrazine, Trifluoroacetic Acid Salt; Thermo Fisher).

2. Prepare the 10× Reaction buffer containing 50 mM Tris-HCl (pH 6.8) and 10 mM EDTA.

3. Denature the RNA at 80 °C for 2 min.

4. Incubate RNA on ice for 5 min.

5. Prepare the ARP reaction according to Table 5.

Table 5: Preparation of ARP reaction mix

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 µg</td>
<td>RNA (CTRL or RED)</td>
</tr>
<tr>
<td>8 µl</td>
<td>22.5 mM ARP (3 mM final)</td>
</tr>
<tr>
<td>6 µl</td>
<td>10× Reaction buffer</td>
</tr>
<tr>
<td>60 µl</td>
<td>Final vol. with ddH₂O</td>
</tr>
</tbody>
</table>

6. Incubate for 1 h at 30 °C.

7. Meanwhile prepare 1.33 M formaldehyde solution in water (dilution of 1:10 from 37% formaldehyde stock; Sigma-Aldrich).

8. To stop the reaction, add 2.25 µl 1.33 M formaldehyde solution, then add 40 µl ddH₂O, 10 µl 3 M sodium acetate and 300 µl absolute ethanol to the ARP samples.

9. Precipitate RNA at -20 °C for 1 h.

10. Pellet RNA by centrifugation at ≥15,000 g for 30 min at 4 °C.

11. Decant supernatant carefully and wash the pellet twice with 70% ethanol (step 7 of section 4).

12. Dissolve RNA in 50 µl ddH₂O.
13. Filter through an Illustra MicroSpin G-25 spin column (GE Healthcare) to remove the remaining unreacted ARP.

14. Prepare the 5× Pulldown (PD) buffer containing 50 mM Tris-HCl (pH 7.4), 750 mM sodium chloride (NaCl) and 0.5% NP-40 (v/v).

15. Wash 50 µl of Dynabeads MyOne C1 Streptavidin beads (Thermo Fisher) three times with 1× PD buffer.

16. Resuspend the beads in 1 ml 1× PD buffer + 0.5 mg/ml BSA (NEB).

17. Incubate for 1 h at 4°C in rotation.

18. Prepare the PD reaction according to Table 6.

19. Add the blocked beads to the PD reaction.

20. Incubate at room temperature for 25 min in rotation.

21. Wash four times with ice-cold 1× PD buffer.

22. Add 700 µl QIAzol (Qiagen) and 140 µl chloroform to the beads.

23. Purify using the RNA Clean and Concentrator - 25 Kit (Zymo Research) according to the manufacturer’s instructions.


6 7-Methylguanosine RNA Immunoprecipitation

Heat-denatured RNA is immunoprecipitated with a specific antibody against m7G. Both Input and IP samples are then purified using the same strategy in order to avoid any bias.

1. Denature decapped RNA for 2 min at 80°C and place immediately on ice.

2. Save a 1-2.5 µg aliquot of RNA as INPUT control.

3. Prepare the 5× RIP buffer according to Table 7.

4. Prepare the RIP reaction according to Table 8.

5. Incubate the RIP reaction for 2 h at 4°C in rotation.

6. Wash n×80 µl Dynabeads G (Thermo Fisher) twice with 1 ml 1× RIP buffer.

7. To block the Dynabeads, resuspend them in 1 ml 1× RIP buffer and add 50 µl 20 µg/µl BSA (NEB), then incubate for 2 h at 4°C in rotation.

8. Wash the beads twice with 1× RIP buffer and resuspend them in n×100 µl 1× RIP buffer.

9. Add 100 µl beads to each RIP reaction.

10. Incubate for 2 h at 4°C in rotation.

Table 7: Preparation of 5× RIP reaction buffer

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml</td>
<td>1 M Tris-HCl (pH 7.4)</td>
</tr>
<tr>
<td>1.5 ml</td>
<td>5 M NaCl</td>
</tr>
<tr>
<td>0.5 ml</td>
<td>10% NP-40 (v/v)</td>
</tr>
<tr>
<td>7.5 ml</td>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

Table 8: Preparation of RIP reaction

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µg</td>
<td>total RNA</td>
</tr>
<tr>
<td>10 µg</td>
<td>anti-m7G antibody (RNO17M)</td>
</tr>
<tr>
<td>10 µl</td>
<td>RNaseOUT (Thermo Fisher)</td>
</tr>
<tr>
<td>200 µl</td>
<td>5× RIP buffer</td>
</tr>
<tr>
<td>1 ml</td>
<td>Final vol. with ddH₂O</td>
</tr>
</tbody>
</table>
11. Wash three times with 1 ml ice-cold 1× RIP buffer, changing the 1.5 ml tube in the first wash.
12. Resuspend the beads in 100 μl 1× RIP buffer + 2 μl RNaseOUT (Thermo Fisher) + 6.7 mM 7-methyl-GTP (Sigma-Aldrich).
13. Incubate for 30 min at 37°C to specifically elute m7G-harbouring RNAs, then collect the supernatant.
14. Bring the volume of the INPUT RNA control to 100 μl in 1× RIP buffer + 2 μl RNaseOUT + 6.7 mM 7-methyl-GTP.
15. Purify both INPUT and RIP samples with the RNA Clean and Concentrator-25 Kit.

7 SmallRNA Sequencing Library Preparation

Sequencing libraries are prepared from resulting BoRed or m7G-RIP RNA with a commercial kit optimized to generate size-selected Small RNA libraries.

Prepare high-throughput libraries using NEBNext Small RNA Library Preparation kit for Illumina (NEB) starting from 1 μg of RNA for each sample and employing gel extraction as the final purification step, according to the manufacturer’s protocol.

8 High Throughput Sequencing

Multiplexed libraries are sequenced by high-throughput sequencing-by-synthesis (Illumina).

Sequence the pooled libraries on Illumina platform running in 50-bp single-read mode. Plan the multiplexing strategy in order to get at least 15-20M raw reads per sample for BoRed-Seq and 5-10M reads for m7G-RIP.