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Supplemental information

SINEUP non-coding RNA activity depends on specific N6-methyladenosine nucleotides

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SUPPLEMENTAL MATERIAL

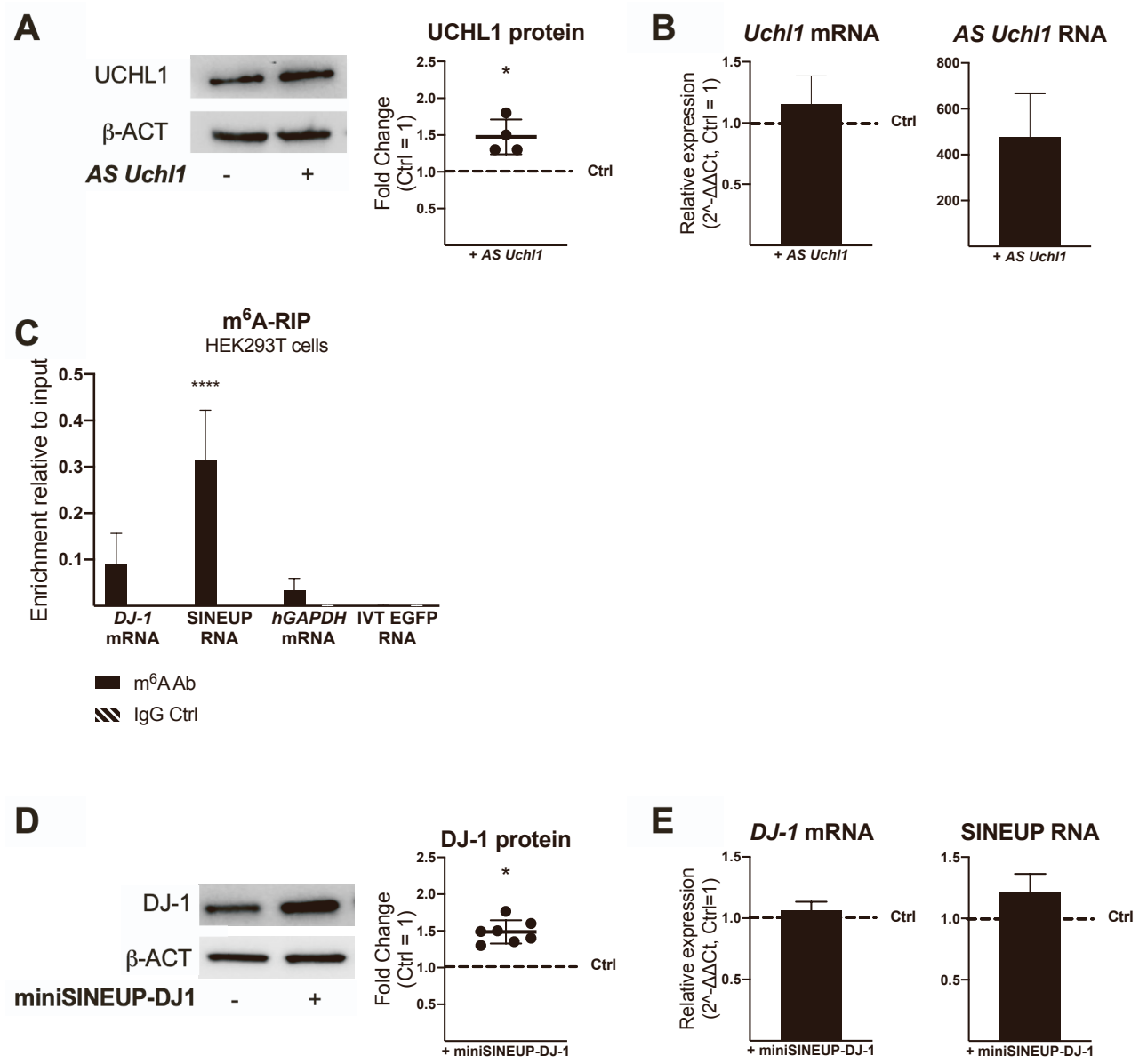


Figure S1. A-B) *AS Uchl1* upregulates UCHL1 protein expression. MN9D cells were transfected with empty control or *AS Uchl1* overexpressing plasmids. Whole-cell lysates were analysed by western blotting with anti-UCHL1 and anti-β-actin antibodies. One representative experiment is shown (left). First, UCHL1 band intensity was normalized to the relative β-actin band. Then, fold change values were calculated normalizing to control cells. Data (right) indicate single replicate values and mean ± SEM and are relative to $n = 4$ independent experiments. p values are calculated by one-sample t and Wilcoxon test, *p<0.05 **B**) qRT-PCR Real-time analysis of *Uchl1* and *AS Uchl1* RNA levels. Columns represent mean ± SEM and are relative to $n = 4$ independent experiments. Transcripts were analysed using *mGAPDH* expression as the internal control. **C) m^{6}A-RIP-qPCR on miniSINEUP-DJ-1 transfected HEK293T cells.** Eluates from IgG immunoprecipitation were used as negative controls. IVT *EGFP* mRNA was spiked in total RNA extract to assess the specificity of the immunoprecipitation reaction. Data indicate mean ± SEM and are relative to $n = 3$ independent experiments. p values are calculated by two-way ANOVA and Dunnett's multiple comparisons test. ***$p < 0.001$, ****$p < 0.0001$ **D-E) miniSINEUP-DJ-1 upregulates DJ-1 protein expression.** A549 cells were transfected with miniSINEUP-DJ-1 or ΔBD (i.e. miniSINEUP-DJ-1 deprived of BD, negative control) overexpressing plasmids. **D**) Whole-cell lysates were analysed by western blotting with anti-DJ-1 and anti-β-actin antibodies. One representative experiment is shown (left). First, DJ-1 band intensity was normalized to the relative β-actin band. Then, fold change values were calculated normalizing to control cells. Data (right) indicate single replicate values and mean ± SEM and are relative to $n = 7$ independent experiments. p values are calculated by one-sample t and Wilcoxon test, *p<0.05 **E**) qRT-PCR Real-time analysis of *DJ-1* and miniSINEUP RNAs level, respectively. Columns represent mean ± SEM and are relative to $n = 4$ independent experiments. Transcripts were analysed using *GAPDH* expression as the internal control. p values are calculated by one-sample t and Wilcoxon test, none of them being statistically significant.

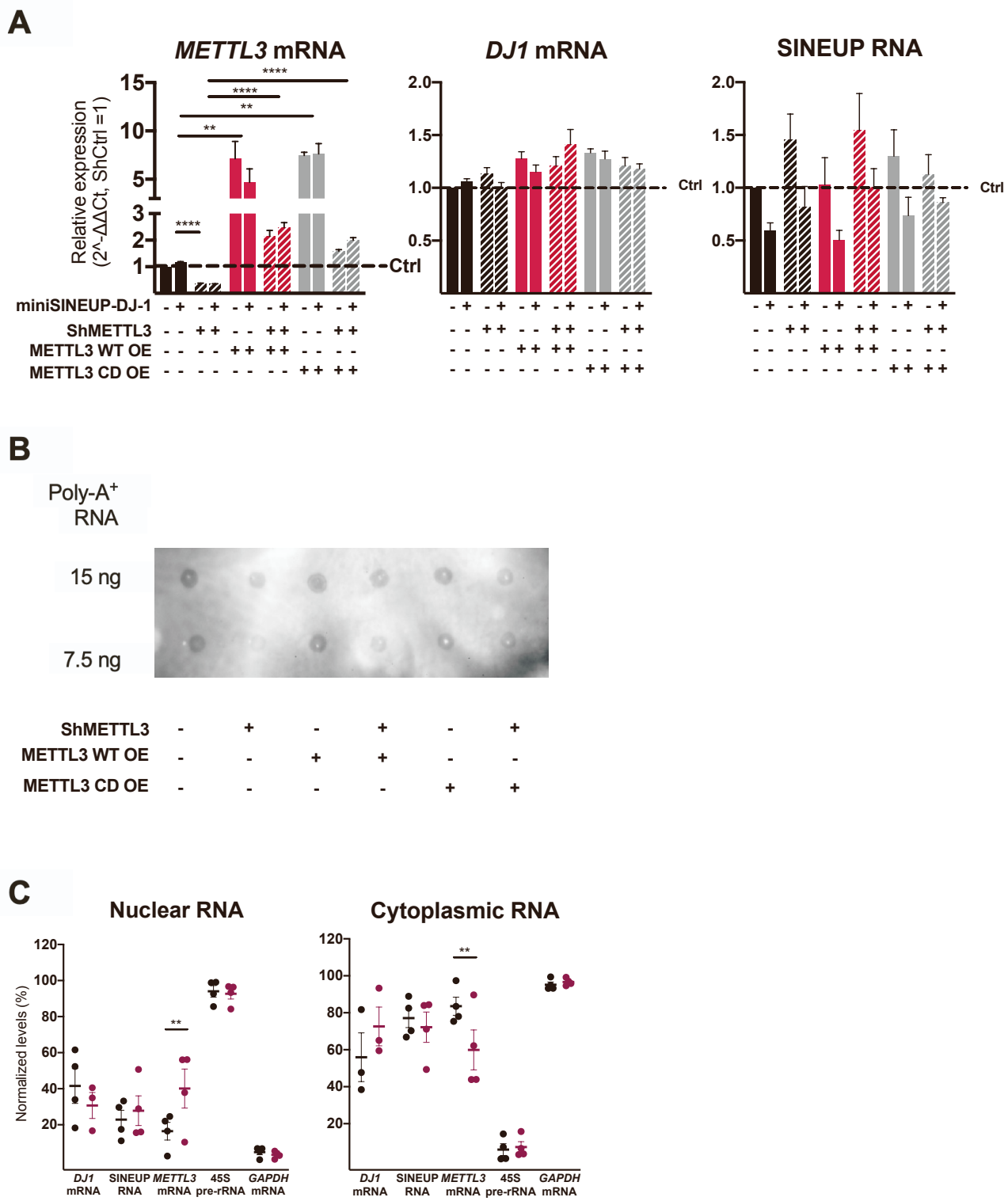


Figure S2. A549 ShCtrl and ShMETTL3 cells were transfected with control, METTL3 WT or METTL3 Catalytically Dead (CD) on day 7 of induction. 24 h after the first transfection, miniSINEUP-DJ-1 or miniSINEUP-□BD (i.e. miniSINEUP-DJ-1 deprived of BD, negative control) plasmids were transfected. **A) qRT-PCR Real-time analysis of total RNA.** *METTL3* mRNA, (left), *DJ-1* mRNA (left) and miniSINEUPs RNA (right) levels were respectively analysed in total RNA extract from experiments reported in Figure 2**.** Columns represent mean ± SEM and are relative to $n = 3$ independent experiments. Transcripts were analysed using *GAPDH* expression as the internal control. *p* values are calculated by two-way ANOVA two-way ANOVA and Sidak's multiple comparisons test. ** p<0.01, **** p>0.0001 **B) m^{6}A RNA Dot Blot.** Poly-A+ RNA was purified to evaluate METTL3-dependent modification levels and stained with m^{6}A antibody. **C) qRT-PCR Real-time analysis of subcellular RNA distribution.** Nuclear (left) and cytoplasmic (right) cellular fractions were separated, and RNA expression levels were analysed by real-time quantitative PCR. Purity of cellular fractions was checked by monitoring

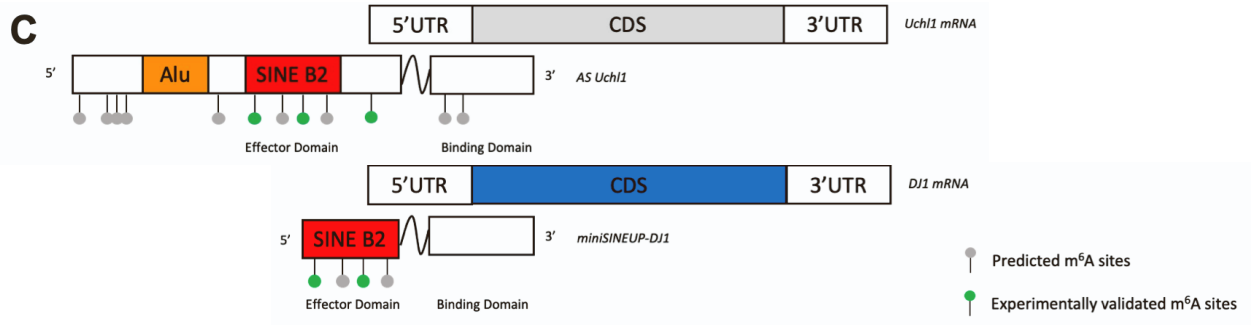
levels of *GAPDH* and 45S pre-RNA. Data are expressed as percentages of total RNA and indicate single replicate values and mean ± SEM and are relative to $n = 4$ independent experiments. p values are calculated by two-way ANOVA and Dunnett's multiple comparisons test. $^{**}p < 0.01$.

A *AS Uchl1* putative m^{6}A sites

Position	Sequence	Confidence	SRAMP
165	AAACA	Low	✓
197	GAACU	High	✓
275	GGACU	Very High	✓
390	GAACU	Moderate	✓
407	UAACC	-	-
425	GAACU	-	-
455	AAACU	-	-
544	GAACA	Low	✓
759	AGACU	Low	✓
801	GGACA	Low	✓
845	AGACU	Moderate	✓
1231	AGACU	Moderate	✓

B invSINEB2 putative m^{6}A sites

Position	Sequence	Confidence	SRAMP
46	GAACU	Low	✓
61	UAACC	-	-
81	GAACU	-	-
111	AAACU	-	-



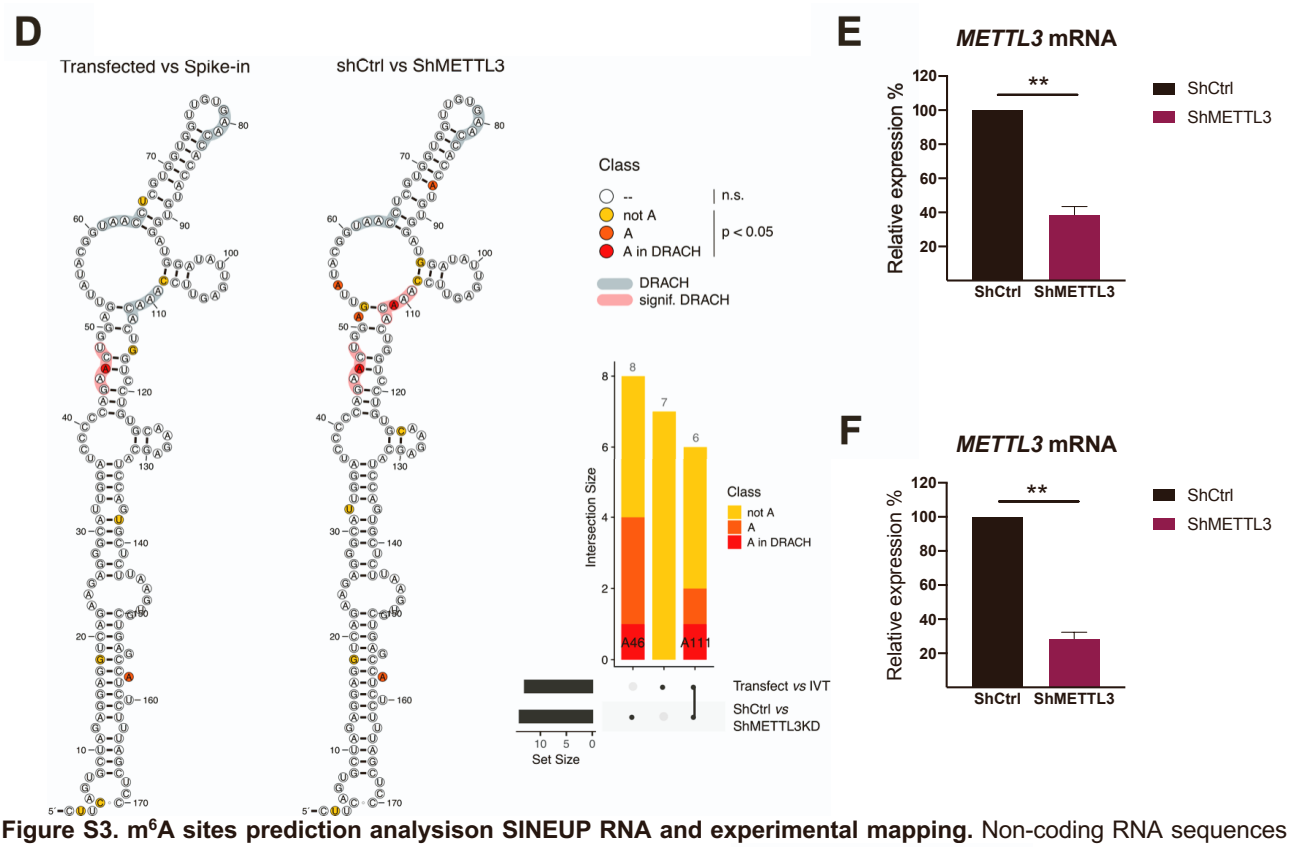


Figure S3. m^{6}A sites prediction analysison SINEUP RNA and experimental mapping. Non-coding RNA sequences were submitted in FASTA format in the SRAMP prediction server (http://www.cuilab.cn/sramp/) and additional putative

sites were manually annotated along invSINEB2 sequence. **A**) Predicted m^{6}A sites on *AS Uchl1* sequence are listed. **B**) Predicted m^{6}A sites on miniSINEUP-DJ-1 sequence are listed. **C**) A schematic representation of *AS Uchl1* and miniSINEUP-DJ-1 with respective target mRNA (*Uchl1* and *DJ-1*) is reported. The binding domain provides SINEUP specificity and is in antisense orientation relative to the sense protein-coding target mRNA (*Uchl1* and *DJ-1*). The inverted SINEB2 element (SINEB2) is the effector domain (red) and confers enhancement of protein synthesis. 5' to 3' orientation of sense and antisense RNA molecules are indicated. Structural elements of target mRNAs are shown: 5' untranslated region (5'UTR, white), coding sequences (CDS, grey and blue) and 3' untranslated region (3'UTR, white). The scheme is not drawn in scale. **D**) **Nanopore sequencing m^{6}A mapping.** Statistically significant modified residues are highlighted along the invSINEB2 sequence structure. DRACH consensus sites are indicated within the structure in grey (= not significantly modified) and red (= significantly modified) (legend upper right). Resulting hits derived from each comparison are reported (bottom right): spiked-in *versus* transfected (left), and ShCtrl *versus* ShMETTL3 (right). Results from final intersection are reported in Figure 3B: two modification sites were identified within 5 nucleotide distance from DRACH consensus: A46, exactly falling within a DRACH consensus sequence (significative from both comparisons), and C108, likely accounting for A111 site that resides in the next DRACH consensus (significative in ShCtrl *versus* ShMETTL3). **E**) **qRT-PCR Real-time analysis of total RNA.** *METTL3* mRNA levels were analysed in total RNA extract from experiments reported in Figure 3A-C and Supplementary Figure 3D**.** Columns represent mean ± SEM and are relative to $n = 3$ independent experiments. Transcripts were analysed using *GAPDH* expression as the internal control. *p* values are calculated by one sample t and Wilcoxon test. ** $p<0.01$ **F**) **qRT-PCR Real-time analysis of total RNA.** *METTL3* mRNA levels were analysed in total RNA extract from experiments reported in Figure 3D**.** Columns represent mean ± SEM and are relative to $n = 3$ independent experiments. Transcripts were analysed using *GAPDH* expression as the internal control. *p* values are calculated by one sample t and Wilcoxon test. ** $p<0.01$.

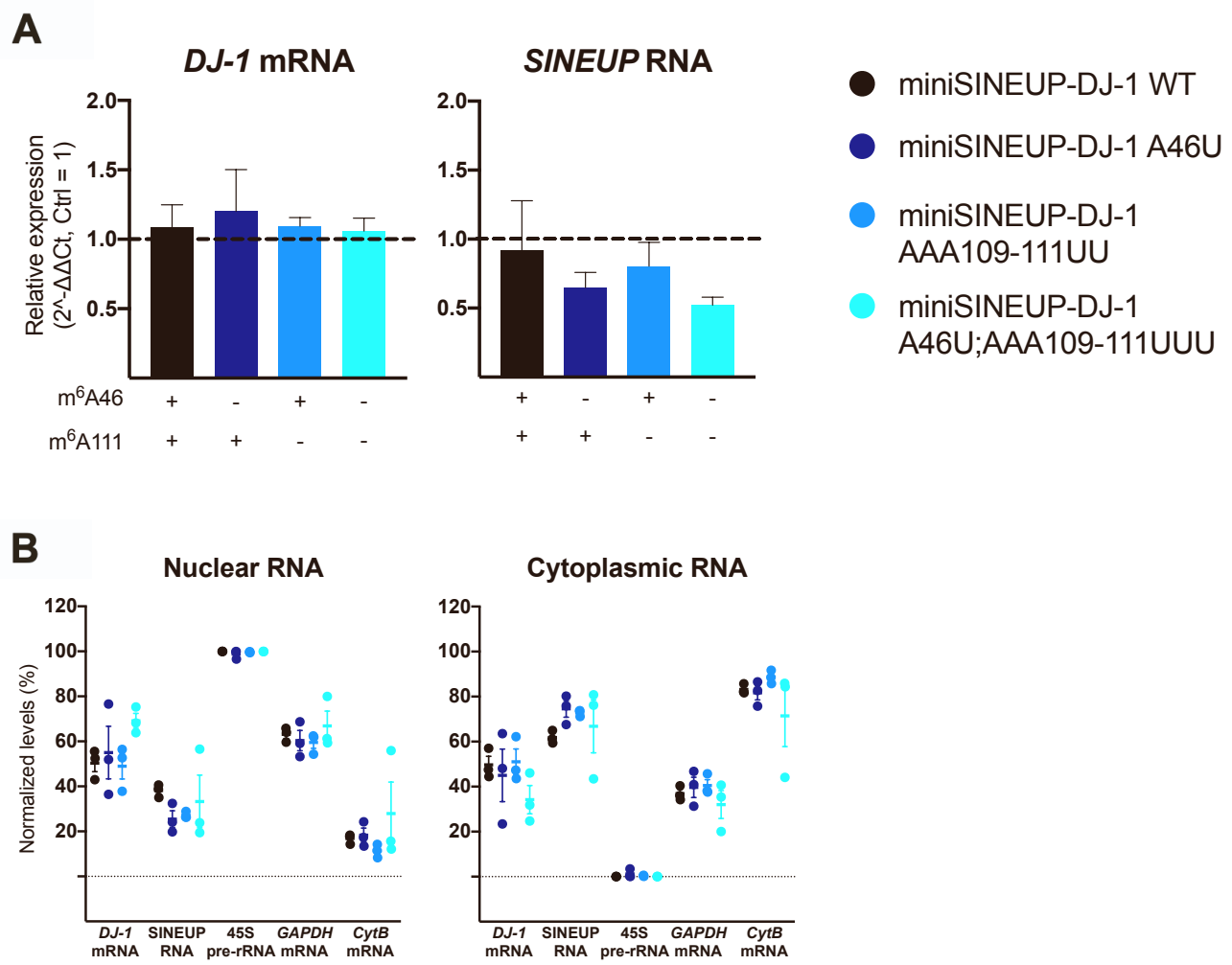
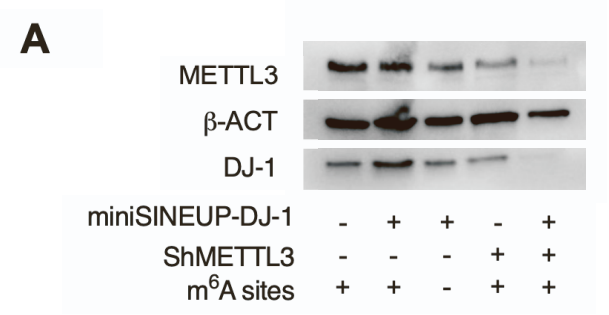
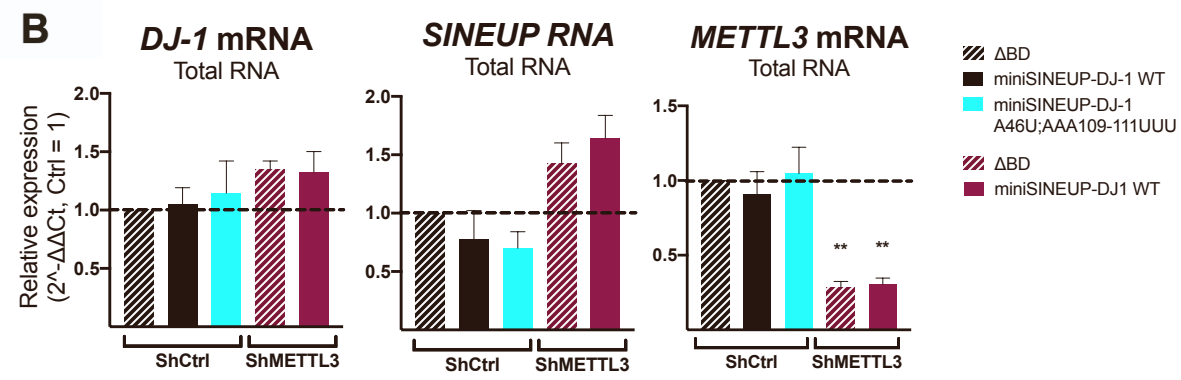
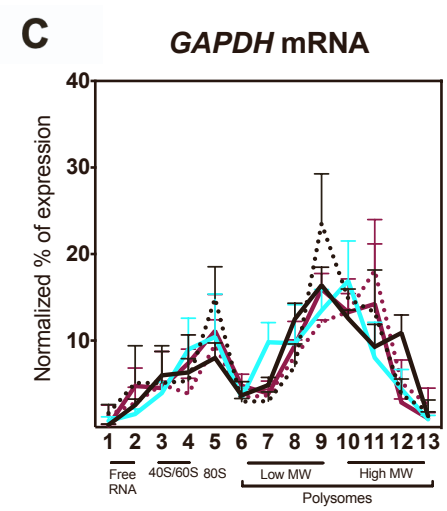
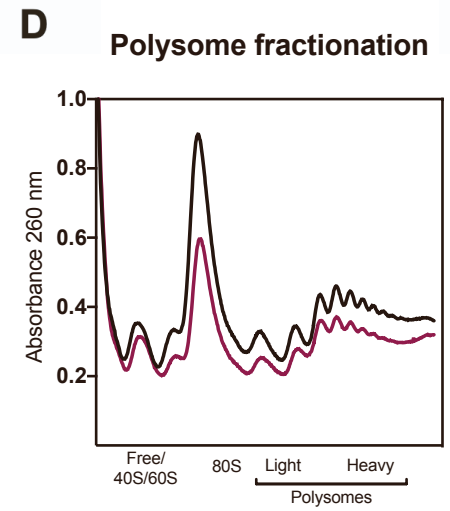


Figure S4. A) qRT-PCR Real-time analysis of total RNA. *DJ-1* mRNA (left) and miniSINEUPs (right) levels were respectively analysed in total RNA extracts from experiments reported in Figure 4**.** Columns represent mean ± SEM and are relative to *n* = 4 independent experiments. Transcripts were analysed using *GAPDH* expression as the internal control. *p* values are calculated by one-sample t and Wilcoxon test, none of them being statistically significant. **C-B) qRT-PCR Real-time analysis of subcellular RNA distribution.** Nuclear (left) and cytoplasmic (right) cellular fractions were separated, and RNA expression levels were analysed by real-time quantitative PCR. Purity of cellular fractions was checked by monitoring levels of *GAPDH*, *CytB* and 45S pre-RNA. Data are expressed as percentages of total RNA and indicate single replicate values and mean ± SEM and are relative to *n* = 4 independent experiments. *p* values are calculated by two-way ANOVA and Dunnett's multiple comparisons test, none of them being statistically significant.


A
METTL3
β-ACT
DJ-1
miniSINEUP-DJ-1 - + + - +
ShMETTL3 - - - + +
m6A sites + + - + +


B
DJ-1 mRNA
Total RNA
SINEUP RNA
Total RNA
METTL3 mRNA
Total RNA
Relative expression (2^-ΔΔCt, Ctrl = 1)
ShCtrl
ShMETTL3
ΔBD
miniSINEUP-DJ-1 WT
miniSINEUP-DJ-1 A46U;AAA109-111UUU
ΔBD
miniSINEUP-DJ1 WT


C
GAPDH mRNA
Normalized % of expression
Free RNA
40S/60S
80S
Low MW
High MW
Polysomes


D
Polysome fractionation
Absorbance 260 nm
Free/ 40S/60S
80S
Light
Heavy
Polysomes

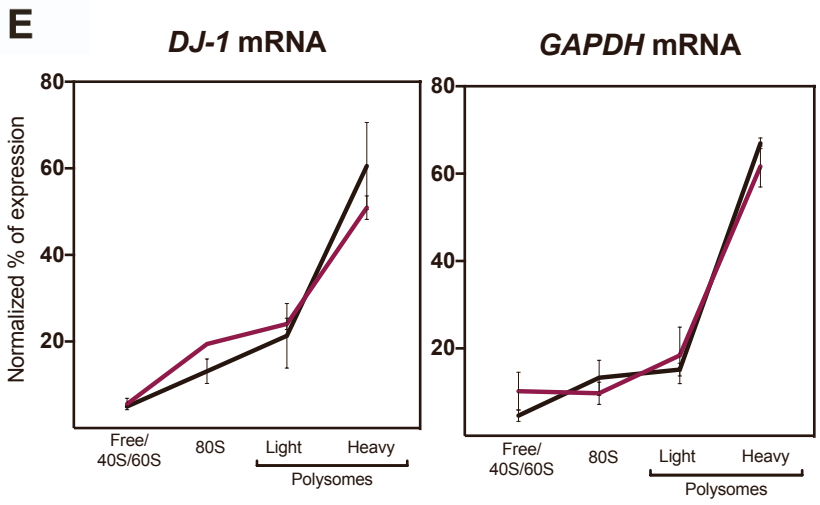

E
DJ-1 mRNA
GAPDH mRNA
Normalized % of expression
Free/ 40S/60S
80S
Light
Heavy
Polysomes

Figure S5. Polysome fractionation analysis. A) Protein expression was analysed by western blotting cell extracts subsequently used for polysome fractionation analysis. One representative image is shown. **B**) **qRT-PCR Real-time analysis of total RNA.** Total RNA extracts from ShCtrl cells transfected with ΔBD, miniSINEUP-DJ-1 WT or A46U;AAA109-111UUU and ShMETTL3 cells transfected with ΔBD and miniSINEUP-DJ-1 WT subsequently used for polysome fractionation were analysed. *DJ-1* mRNA (left), miniSINEUPs RNA (centre) and *METTL3* mRNA (right) levels were respectively analysed in total RNA extract from experiments reported in Figure 5**.** Columns represent mean ± SEM and are relative to $n = 3$ independent experiments. Transcripts were analysed using *GAPDH* expression as the internal control. *p* values are calculated by one-sample t and Wilcoxon test, ** $p<0.01$. **C) Polysome fractionation analysis**. *GAPDH* mRNA distribution in ShCtrl and ShMETTL3 cells transfected with miniSINEUP-DJ-1 WT (black = ShCtrl, purple = ShMETTL3), miniSINEUP-DJ-1 A46U;AAA109-111UUU (light blue line) or negative control (i.e. ΔBD, dotted black line = ShCtrl, dotted purple line = ShMETTL3) is reported for each fraction. Data indicate mean ± SEM and are relative to $n = 3$ independent experiments. *p* values are calculated by two-way ANOVA and Dunnett's multiple comparisons test, none of them being statistically significant. **D-E) Polysome fractionation analysis of untransfected ShCtrl and ShMETTL3 A549 cells.** As a control, polysome fractionation was performed on untransfected ShCtrl (black line) and ShMETTL3 (purple) A549 cells. 3 subsequent fractions were pulled for Free RNA/40S/60, 80S, light and heavy polysomes analysis. **D**) One representative polysome profile is reported. **E**) *DJ-1* mRNA (left) distribution and *GAPDH* (right) mRNA distribution are reported. No significant difference was observed between samples. Data indicate mean ± SEM and are relative to $n = 2$ independent experiments. *p* values are calculated by two-way ANOVA and Dunnett's multiple comparisons test, none of them being statistically significant.

Table S1 List of primers. Complete list of oligonucleotides used in this study.

SybrGreen qRT-PCR Oligo Name:	*Forward (5'→3')*	*Reverse (5'→3')*
hGAPDH	TCTCTGCTCCTCCTGTTC	GCCCAATACGACCAAATCC
mACT	CACACCCGCCACCAGTTC	CCCATTCCCACCATCACACC
mGAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
CytB	CAATGGCGCCTCAATATTCT	AATGTATGGGTGGCGGATA
45S rRNA	GAACGGTGGTGTGTCGTT	GCGTCTCGTCTCGTCTCACT
hMETTL3	CTGAGGCAGGAGAATTGCTT	GGCAGCCATACACGTTAAGA
hDJ1	GAGACGGTCATCCCTGTAG	CATCTTCAAGGCTGGCATC
hSON	TGACAGATTTGGATAAGGCTCA	GCTCCTCCTGACTTTTTAGCAA
AS Uchl1	CTGGTGTGTATCTCTTATGC	CTCCCGAGTCTCTGTAGC
mUchl1	CCCGCCGATAGAGCCAAG	ATGGTTCACTGGAAAGGG
pTS invB2	CAGTGCTAGAGGAGGTCAGAAGA	GGAGCTAAAGAGATGGCTCAGCACT
Overlap	CTCGGGGTTAATCTCCATCGGC	TCTGCTCCCGTCTCCC
m^{6}A amp	ATATGTTTACAAGCCCCACACCA	TCTGACCTCCTCTAGCACTGA
ivt EGFP	AGGAGCGCACCATCTTC	GATGCCCTTCAGCTCGAT
BstI RT Assay Oligo Name:	*Reverse (5'→3')*	
invB2 m^{6}A -	GAGCTAAAGAGATGGCT	
A46+	GGTTACCGTATAACTCC	
A63+	TTCACAACCACCACGA	
A81+	CTCAATATCCATCCACATG	
A111+	TCTTGCACAGGACCAG	
A165+	CCTCCTCTGCTTGT	
A197+	GTGCATGGGGGAG	
A275+	GCTACCATGCCCAG	
A390+	GGTTACCGTATAACTCCAG	
A801+	CTCCCTCTCTGCTTG	
A845+	CAGTTTGCTAAGGAACATAG	
A1231+	CATCGGTTCAATGGAAG	
A759+	AAAGGGCCTTATTACAAAG	
A544+	AGCTCCCTTGCTG	
ASUchl1 m^{6}A -	GGAGCTAAAGAGATGGC	
Nanopore Targeting Adapters Oligo Name:	*5' → 3'* *(**bold**: complementary sequence)*	
BC1_A	5Phos/CCTCCCCTAAAAACGAGCCGCATTTGCGTAGTAGGTTC	
BC2_A	5Phos/CCTCGTCGGTTCTAGGCATCGCGTATGCTAGTAGGTTC	
BC3_A	5Phos/CCTCCCACTTTCACACGCACTAACCAGGTAGTAGGTTC	
BC1_B_mSUP	GAGGCGAGCGGTCAATTTTCGCAAATGCGGCTCGTTTTTAGGGGAGG**AAGCTTGGAGCTAAAGAGATGG**	
BC2_B_mSUP	GAGGCGAGCGGTCAATTTTGCATACGCGATGCCTAGAACCGACGAGG**AAGCTTGGAGCTAAAGAGATGG**	
BC3_B_mSUP	GAGGCGAGCGGTCAATTTTCCTGGTTAGTGCGTGTGAAAGTGGGAGG**AAGCTTGGAGCTAAAGAGATGG**	