Supplementary Materials for

Regulation of telomere homeostasis and genomic stability in cancer by N\textsuperscript{6}-adenosine methylation (m\textsuperscript{6}A)

Ji Hoon Lee, Juyeong Hong, Zhao Zhang, Bábara de la Peña Avalos, Cecilia J. Proietti, Agustina Roldán Deamicis, Pablo Guzmán G., Hung-Ming Lam, Jose Garcia, Martine P. Roudier, Anthony E. Sisk, Richard De La Rosa, Kevin Vu, Mei Yang, Yiji Liao, Jessica Scheirer, Douglas Pechacek, Pooja Yadav, Manjeet K. Rao, Siyuan Zheng, Teresa L. Johnson-Pais, Robin J. Leach, Patricia V. Elizalde, Eloïse Dray, Kexin Xu*

*Corresponding author. Email: xuk3@uthscsa.edu

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Fig. S1. Proteins involved in m^6^A regulation are misregulated in a wide range of cancers.

(A-D) Expression of METTL14 (A), WTAP (B), FTO (C) and ALKBH5 (D) by RT-qPCR in the indicated normal and cancer cell lines. (E) METTL3 expression in different kinds of human tumors.
and matched normal tissues in The Cancer Genome Atlas (TCGA). Cancer types in which expression of METTL3 is significantly elevated are highlighted in red. (F-H) Comparison of METTL3 expression between tumor and normal tissues. Data were retrieved from two independent cohorts of patients with prostate (F) (15, 16), liver (G) (17, 18), or lung (H) (19, 20) cancer. N, case numbers in each specified group of clinical samples.
Fig. S2. METTL3 is required for the growth of several types of cancer cells both in vitro and in vivo.

(A) Immunoblotting with indicated antibodies in LNCaP, Huh-7 and A549 cells that were infected with control shRNA (shCtrl) or two independent shRNAs targeting METTL3 (shM3#1 and #2). Molecular weights of the blotted proteins were marked. (B) Representative images (left panel) and quantitative analysis (right panel) of the colony formation assays in LNCaP, Huh-7 and A549 cells infected with shRNAs as specified in (A). (C-D) Tumor sizes (C) and animal weights (D) in the 22Rv1 xenograft model. 22Rv1 cells were infected with control shRNA (shCtrl) or METTL3-specific shRNA (shM3#1), and meanwhile overexpressed control vector (Vec.), wild-type METTL3 (M3-WTR) or catalytic-dead mutant (M3-CDR), which are resistant to shM3#1. Data in (D) are presented as the mean body weight in gram (g) ± standard error of the mean (SEM). Photo credit in (C): Ji Hoon Lee, University of Texas Health Science Center at San Antonio (UTHSCSA).
Fig. S3. Analysis of McRIP/m\(^6\)A-seq data reveals transcriptome-wide m\(^6\)A patterns in RWPE-1 and LNCaP cells.

(A-C) Top motifs enriched at the m\(^6\)A modification sites (A), metagene profiles of m\(^6\)A peaks across the transcriptome (B) and distribution of m\(^6\)A peaks in different transcript regions (C) in RWPE-1 and LNCaP cells. 5’ (3’)-UTR, 5’ (3’) untranslated region; CDS, coding sequence. (D) Gene Ontology analysis of mRNAs with similar (“Common” cluster) or differential (“LNCaP” and “RWPE-1” clusters) m\(^6\)A intensities in LNCaP and RWPE-1 cells. (E) The Integrative Genomics Viewer (IGV) browser tracks showing m\(^6\)A peaks at the genomic location of *HMBOX1* (highlighted in red) in Huh-7 (GSE130891) (24), HepG2 (GSE37005) (2) and A549 (GSE76367) (25) cells. Replicates of inputs and m\(^6\)A pull-downs are shown. *P* values in (A) were calculated by Fisher’s exact test. The adjusted *P* values in (D) were determined by hypergeometric test.
Fig. S4. The m^6^A modification of HMBOX1 transcripts regulates the mRNA stability in cancer cells.

(A-B) Levels of indicated RNA species by RT-qPCR in LNCaP cells transfected with control siRNA (siCtrl) or siRNA targeting METTL14 (A, siM14) or ALKBH5 (B, siA5) for 72 hours. (C) Immunoblotting with specified antibodies in LNCaP cells transfected with control siRNA (siCtrl) or siRNA targeting METTL3 (siM3), METTL14 (siM14) or ALKBH5 (siA5) for 72 hours. Molecular weights of each blotted protein were indicated aside. (D) Half-life of HMBOX1 pre-mRNA by RT-qPCR in LNCaP cells that were transfected with control siRNA (siCtrl) or METTL3-specific siRNA (siM3) for 72 hours and meanwhile treated with 5 μg/mL Actinomycin D (ActD) for the times indicated.
Fig. S5. Removal of the m6A mark on HMBOX1 abrogates YTHDF2-mediated degradation of HMBOX1 mRNA.

(A) Protein (left panel) and mRNA (right panel) levels of indicated molecules in LNCaP cells that were transfected with control siRNA (siCtrl) or siRNA targeting YTHDC2 (siDC2) for 72 hours. Molecular weights of the blotted proteins were marked. (B) Expression of YTHDF2 by RT-qPCR in LNCaP cells stably expressing control backbone (vector), wild-type METTL3 (M3-WT) or catalytic-dead mutant (M3-CD) in the presence of control siRNA (siCtrl) or siRNA targeting YTHDF2 (siDF2). (C) Immunoblotting using indicated antibodies in LNCaP cells transfected with empty backbone (vector) or dCas9 proteins fused to wide-type ALKBH5 (dCas9-A5-WT) or enzymatically dead mutant (dCas9-A5-HA) for 72 hours. Molecular weights of the blotted proteins were marked. (D) Schematic illustration of a second set of HMBOX1-targeting sgRNA (sgHMBOX1) that is effective (B) or nonfunctional (B-10nt). The targeted adenosine is highlighted in green and the flanking sequences are also specified. Positions of the primers designed for detecting methylated HMBOX1 are indicated by the arrows. (E-F) MeRIP-qPCR analysis of m6A signals on HMBOX1 (E) or expression of HMBOX1 (F) in LNCaP cells expressing dCas9 fusion proteins as explained in (C) together with control sgRNA (sgCtrl), sgHMBOX1-B or sgHMBOX1-B-10nt. GAPDH was detected in (E) as a negative control. (G) Immunoblotting with indicated antibodies in dCas9-based, m6A-editing system that is elucidated in (C) in the presence of control sgRNA (sgCtrl), two efficacious sgHMBOX1 (A and B) or two ineffective sgHMBOX1 (A-10nt and B-10nt). Molecular weights of each blotted protein were marked.
Fig. S6. Downregulation of HMBOX1 by METTL3 has no effect on the intrinsic activity of telomerase in cancer cells.

(A) Immunoblotting with indicated antibodies in LNCaP (left panel) and A549 (right panel) cells stably expressing empty backbone (−, −), HA-tagged METTL3 alone or with myc-tagged HMBOX1. Molecular weights of each blotted protein were indicated aside. (B) Growth curves of the stable clones of LNCaP (left panel) and A549 (right panel) cells as described in (A) by recording the population doublings (PD) over time. Arrows delineate the selections of early passage cells (EPC) or late passage cells (LPC). (C) Images from the agarose gel electrophoresis
showing integrity of genomic DNA extracted from LNCaP (left panel) and A549 (right panel) stable clones as elucidated in (A) at low (EPC) or high (LPC) passage numbers. The size markers of 1 kb DNA ladder were indicated aside. (D) Measurement of the telomerase activity in LNCaP cells that are described in (A) by examining the addition of the 6-nt telomeric repeats (TTAGGG) based on the telomeric repeat amplification protocol (TRAP). NC, negative control with no cell lysates. ITAS, internal telomerase assay standard serving as an internal control. (E-F) Protein (left panels) and mRNA (right panels) levels of indicated molecules in LNCaP (E) and A549 (F) cells that were infected with control shRNA (shCtrl) or two independent shRNAs targeting HMBOX1 (shHM1#1 and #2), or transiently transfected with control siRNA (siCtrl) or siRNA targeting TERT (siTERT) for 72 hours. Molecular weights of each blotted protein were marked. (G) Representative images and quantification of TERC RNA FISH with TRF2 immunostaining in A549 cells as described in (A) and in BEAS-2B cells. Telomeres were indicated by the immunofluorescence staining with anti-TRF2 antibody (green) and locations of telomerase complex by FISH with TERC-specific probes (Quasar 570 labeled) (red). DNA was stained with DAPI (blue). At least 70 nuclei were counted in each biological replicate for quantification purpose. (H-I) Representative images (H) and quantification (I) of the telomere dysfunction-induced foci (TIF) assay in early (EPC) and late (LPC) passage LNCaP cells expressing empty backbone (Vector), METTL3 alone or with HMBOX1 (METTL3+HMBOX1). TIFs were detected by immunofluorescence-FISH with anti-γH2AX antibody (red) and a peptide nucleic acid (PNA) probe (TelG-FITC) (green). DNA was stained with DAPI (blue). Cells with five or more colocalization of γH2AX foci and telomeres were scored as TIF positive and at least 60 nuclei were counted in each biological replicate.
Fig. S7. HMBOX1 transcriptionally represses MDM2 expression by binding at the promoter regions of MDM2 in cancer cells.

(A) The Integrative Genomics Viewer (IGV) snapshot showing the chromatin-binding signals of HMBOX1 at the genomic loci of MDM2. HMBOX1 ChIP-seq data are retrieved from (GSE96356) (41). Input and duplicates of ChIP samples (1 and 2) are shown. Black bars (denoted as P1, P2 and P3) indicate the chromatin regions detected in ChIP-qPCR. (B) Expression of indicated genes by RT-qPCR in LNCaP (top panels) and A549 (bottom panels) cells that were infected with control shRNA (shCtrl) or two independent shRNAs targeting HMBOX1 (shHM1#1 and #2). (C) Immunoblotting with indicated antibodies in LNCaP (left panel) and A549 (right panel) cells that are described in (B). Molecular weights of each blotted protein were indicated aside. (D) HMBOX1 binding at the promoter of MDM2 (P1, P2 and P3) by ChIP-qPCR in LNCaP (left panel) and A549 (right panel) cells that are described in (B). Promoter of GAPDH was included as a negative control. (E) The Integrative Genomics Viewer (IGV) browser tracks showing m^6A peaks at the genomic location of MDM2 (highlighted in red) in Huh-7 (GSE130891) (24), HepG2 (GSE37005) (2) and A549 (GSE76367) (25) cells. Replicates of inputs and m^6A pull-downs are shown. (F) MeRIP-qPCR analysis of m^6A signals at two identified sites (A and B) on MDM2 mRNAs in LNCaP, Huh-7 and A549 cells, which were infected with control shRNA (shCtrl) or shRNA targeting METTL3 (shM3#1), followed by immunoprecipitation of total mRNAs with either control IgG antibody (IgG) or m^6A-specific antibody (m^6A). (G) MeRIP-qPCR analysis of m^6A intensities on MDM2 as described in (F) in LNCaP cells expressing dCas13b proteins with a nuclear localization signal (nls) fused to either the wild-type METTL3 (dCas13b-M3^WT^nls) or the catalytically dead mutant (dCas13b-M3^CD^nls), together with control sgRNA (sgCtrl) or sgHMBOX1-A. (H) Representative images (left panel) and quantification (right panel) of the anaphase bridges (arrowhead) in late passage LNCaP cells expressing empty backbone (Vector), METTL3 alone or with HMBOX1 (METTL3+HMBOX1). DNA was stained with DAPI (blue). Scale bars in (E), 10 µm. At three random microscopic fields were viewed in each biological replicate for quantification purpose.
Fig. S8. Both METTL3 and HMBOX1 play important roles in the growth of human cancer cells.

(A-B) Dot plots demonstrating the dependency scores (CERES) of METTL3 and HMBOX1 in a wide variety of cancer cell lines based on Avana (A) and GeCKO (B) CRISPR-Cas9 knockout screening data (DepMap: https://depmap.org/portal/). (C) Expression of HMBOX1 by RT-qPCR in the indicated normal and cancer cell lines. (D-F) Comparison of HMBOX1 expression between tumors and corresponding normal tissue counterparts in patient cohorts of prostate (D) (16), liver
(E) (17) and lung (F) (20) cancer. N, case numbers in each specified group of clinical samples.
| Names                                      | 5’-3’ Sequences                                      |
|--------------------------------------------|------------------------------------------------------|
| METTL3-Forward                             | CCTCTAGAATGTACCCATACGACGTCGCCAGA                    |
|                                            | CTACGCTTCGGACACGTTGGAGCTCTATCTATC                   |
| METTL3-Reverse                             | CGCGTCGACCTAAAAATTCTTAGGTTTAGAG                     |
|                                            | ATGA                                                 |
| METTL3-CD-Forward                          | CCCGCAGATATTGCATGGAACTGCC                           |
| METTL3-CD-Reverse                          | TGGTGCAGCCATCAACTGCAAAC                             |
| METTL3-shRNA-resistant-Forward              | GCTGCACTTCCGAGGATTATCAAATGACAC                    |
|                                            | ACTGATGAG                                             |
| METTL3-shRNA-resistant-Reverse              | TTGCGACAGGGGTCGATCA                                  |
| HMBOX1-Forward                             | CCGGATCCATGGGAACGAGAAACTGATTAGCGA                   |
|                                            | AGAAGATCTGCTTTAG                                    |
|                                            | TCCTTTCCAGTGGTTTG                                   |
| HMBOX1-Reverse                             | CCCTCGAGTGTCATCATCCAGGGCTC                          |
| dCas13b-METTL3CDnls-Forward                 | CCGGCGGATATCCATGGAGCTGC                             |
| dCas13b-METTL3CDnls-Reverse                 | CGGTGCCGCCCATCACAACAGCAAA                           |
Table S2. sgRNA oligos and PAMers used in the CRISPR-based, m6A-editing tools.

| Names                        | 5’-3’ Sequences                                      |
|------------------------------|------------------------------------------------------|
| dCas9-sgControl-Sense        | GGACGGGAATAGCTCAGAGGCGAGGT                          |
| dCas9-sgControl-Anti-Sense   | TTTCCACTCGGCTCTGAGCTATCC                            |
| dCas9-sgHMBOX1-A-Sense       | GGACGCACCTCTGAGAGGCTACTAGAT                         |
| dCas9-sgHMBOX1-A-Anti-Sense  | TTTCACTCTAGTGACCTCTCACAGATGC                        |
| dCas9-sgHMBOX1-A-10nt-Sense  | GGACGGGAAGCCCCCTACTCTGAGAT                         |
| dCas9-sgHMBOX1-A-10nt-Anti-Sense | TTTCACTCTAGAGGAGGGGGCTTCC                        |
| dCas9-sgHMBOX1-B-Sense       | GGACGGGCTGTATGAGAACACGATGT                        |
| dCas9-sgHMBOX1-B-Anti-Sense  | TTTTACATCTGTCTCTACAGCCCC                        |
| dCas9-sgHMBOX1-B-10nt-Sense  | GGACGCTACATTGTGCTGTATGAT                         |
| dCas9-sgHMBOX1-B-10nt-Anti-Sense | TTTTACATACAGGGACACGATGT                        |
| HMBOX1-A PAMer               | mUdCmAdGmAdGmUdGmGdGmGdGmGdC                       |
| HMBOX1-A-10nt PAMer          | mGdGmGdGmCdTmUdGmGdGmUdGmAdTmAdA                   |
| HMBOX1-B PAMer               | mAdAmGmUdGmAdGmGdGmGdAmAdA                        |
| HMBOX1-B-10nt PAMer          | mUdTmAdAmGdTmUdGmGdGmGdAmTmAdA                     |
| dCas13b-sgControl-Sense     | CACCCGAGGTTTTCTTCCAGTCAGCAGTGT                     |
| dCas13b-sgControl-Anti-Sense | CAACTTTTTACACGTCGACTGGGAAAA                       |
| dCas13b-sgHMBOX1-A-Sense     | CACCCGACCTCTAGAGGAGGGGGGCTTCC                      |
| dCas13b-sgHMBOX1-A-Anti-Sense | CAACCTAAACGGAGCCCCCTACTCTGAGGTC                    |

HMBOX1-A PAMer: mUdCmAdGmAdGmUdGmGdGmGdGmGdC
HMBOX1-A-10nt PAMer: mGdGmGdGmCdTmUdGmGdGmUdGmAdTmAdA
HMBOX1-B PAMer: mAdAmGmUdGmAdGmGdGmGdAmAdA
HMBOX1-B-10nt PAMer: mUdTmAdAmGdTmUdGmGdGmGdAmTmAdA
Table S3. Primers for RT-qPCR and MeRIP-qPCR.

| Primer Names | 5’-3’ Sequences |
|--------------|-----------------|
| GAPDH-F      | TGCAACCACCAACTXGCTTAGC |
| GAPDH-R      | GGCATGGACTGTTGGTCTAGAG |
| METTL3-F     | TCAGCATCGGAAACCAGCAAG |
| METTL3-R     | TCCTGACTGACCTTCTTGCTC |
| METTL14-F    | GTTGGGAAACTGATAGGAGCCGC |
| METTL14-R    | CAATGCTGTCGGCAGCCTTCA |
| WTAP-F       | CTTCCCAAGAGGATTTCGATAGA |
| WTAP-R       | TCAGACTCTCTTTAGGCCAGTTAC |
| ALKBH5-F     | CCTGGACAGCTCGTGTTAC |
| ALKBH5-R     | CCAGGATCGTGGCGCTGTA |
| FTO-F        | GACTGCGAGGAAAGCACAGAGC |
| FTO-R        | GGGTGCAGATAAGGAGGAGCAAG |
| YTHDF2-F     | CCTCCATTGGCTTCTCTATT |
| YTHDF2-R     | GTTGCTCAGCTGTCATAAGA |
| YTHDC2-F     | GTGGCAGGATGTATCCTAAT |
| YTHDC2-R     | CTGAGAAGCTAGGAGGAGGAA |
| HMBOX1-mRNA-F| CCTCAGAAAGGAGCCTAGATGT |
| HMBOX1-mRNA-R| TCCTCCAGATGGCAAGAAG |
| HMBOX1-pre-mRNA-F | TAGCATGGGACATACTTCATT |
| HMBOX1-pre-mRNA-R | GAAGTCGTGTAAGCAGATCTAT |
| TP53-F       | GGAAATTTGCGTTGGAGTATTT |
| TP53-R       | GGTGTAGTGGATGGTTACAG |
| CDKN1A-F     | GTCACTGCTCTTGATACCTTT |
| CDKN1A-R     | GGCCTTTGGAGTGGTAGAAA |
| MDM2-F       | GGTGAGGAGCCAGCATAT |
| MDM2-R       | CTGGAATCTGTGAGGAGTGT |
| MDM2-pre-mRNA-F | ATGGTGAGGAGCAGTACT |
| MDM2-pre-mRNA-R | GGGTGCGAAGCAGCATAG |
| TERT-F       | CCCATTTCATCAGGTAGTATT |
| TERT-R       | CTTGGCCTTCCAGGATGAGTAG |
| TERC-F       | TTTGCTCAACCTAACCTGAGAAG |
| TERC-R       | CTCTAGAATGAAACGCTGGAAG |
| qTelomere-hTEL-F | CGGTCTTGGGTGCCCTTGGGTT |
| qTelomere-hTEL-R | GGGCTGCTTACCCCTTACCTTACCTTACCT |
| qTelomere-hBG-F | GCTTCTGACACAACCTCGTGTCATAGC |
| qTelomere-hBG-R | CACCAACCTTCACGCTTACCC |
| Gene   | Forward Primers                      | Reverse Primers                      |
|--------|--------------------------------------|--------------------------------------|
| GAPDH  | A-GATTCAAGGCTGAGAACGGGAAG            | GGACTCCACGACGTACTCAG                 |
| HMBOX1 | A-ACCTTGCAGTGTTTGTATCCATT            | GGACGTCTCTCCTACGACATT               |
| HMBOX1 | A-CAGTGCTTCTGTCTGATAAG              | GGGACGTCTCTCCACTACATT               |
| HMBOX1 | A-GGGACGTTTCCTACTACATT              | TTTTCACCTCTCCAAAGGAC                |
| MDM2   | A-GACTATTCTCAGCCATCAAACCTCT         | GACTAGTGCTTGACACCTGAC              |
| MDM2   | A-TCCACACTCTCTTTTTTGTCTTG          | TCCACACTCTCTTCTTTGTTGCTTG         |
| MDM2   | A-AGTTTTTTGACCCCATCTAC             | AGTTTTTTGACCCCATCTAC               |
| MDM2   | A-TGCTTTAGTCCACCTAACCCTTT       | TGCTTTAGTCCACCTAACCCTTT           |
| Gene Names                  | 5’-3’ Sequences                          |
|-----------------------------|------------------------------------------|
| MDM2_P1_ChIP-F             | CCAGCCAAACCCAAACACATTTC                  |
| MDM2_P1_ChIP-R             | TAGCACTTAAGGAGCGTGTTAC                   |
| MDM2_P2_ChIP-F             | GTTAAGTCTGACTTGGTCTCCA                   |
| MDM2_P2_ChIP-R             | CTTACCTGGATCAGCAGAGAAAA                  |
| MDM2_P3_ChIP-F             | GAGGCACGATGATAGTAGGAAG                  |
| MDM2_P3_ChIP-R             | CCCAAGCTGACAAGGTAAT                     |
| GAPDH promoter_ChIP-F      | GCTTGGGCTCTGGAATCATA                    |
| GAPDH promoter_ChIP-R      | GCTCACCACAGGTCAAGATTAT                  |
Table S5. Sequences of Oligonucleotide probes for *TERC* RNA FISH.

| Probe Names | 5'-3’ Sequences                                      |
|-------------|------------------------------------------------------|
| Probe-1     | CAAAAAATGGCCACCACCC                                  |
| Probe-2     | CCTTCTCAGTTAGGTTAG                                   |
| Probe-3     | CAAAAGCACGGCGCCTACG                                  |
| Probe-4     | AGAAAAACAGCGCGCGGG                                   |
| Probe-5     | AATGAACGCTGGAAGGCAG                                  |
| Probe-6     | CCAGCAGCTGACATTTTTT                                 |
| Probe-7     | TGACAGAGCCCAACTCTTTC                                |
| Probe-8     | CTGAAAGGCCTGAACCTCG                                  |
| Probe-9     | CACAGCTCAGGGAATCGCG                                  |
| Probe-10    | ATGTGTGACCGCGAGTCTG                                  |