

1 **Title: N6-methyladenosine regulates the stability of RNA:DNA hybrids in**
2 **human cells.**

3 **Authors:** Abdulkadir Abakir¹, Tom C. Giles^{2,3}, Agnese Cristini⁴, Jeremy M. Foster⁵, Nan
4 Dai⁵, Marta Starczak⁶, Alejandro Rubio-Roldan⁷, Miaomiao Li^{8,9}, Maria Eleftheriou¹, James
5 Crutchley¹, Luke Flatt¹, Lorraine Young¹, Daniel J. Gaffney¹⁰, Chris Denning¹, Bjørn
6 Dalhus^{8,11}, Richard D. Emes^{2,12}, Daniel Gackowski⁶, Ivan R. Corrêa Jr.⁵, Jose L. Garcia-
7 Perez^{7,13}, Arne Klungland^{8,9*}, Natalia Gromak^{4*} and Alexey Ruzov^{1*}.

8 **Affiliations:**

9 ¹Department of Stem Cell Biology, University of Nottingham, Nottingham, UK.

10 ²Advanced Data Analysis Centre, University of Nottingham, Sutton Bonington, UK.

11 ³Digital Research Service, University of Nottingham, Sutton Bonington, UK.

12 ⁴Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

13 ⁵New England Biolabs, Inc., Ipswich, Massachusetts, USA.

14 ⁶Department of Clinical Biochemistry, Nicolaus Copernicus University in Toruń, Bydgoszcz,
15 Poland

16 ⁷GENYO, Centre for Genomics and Oncological Research: Pfizer/University of
17 Granada/Andalusian Regional Government, Granada, Spain.

18 ⁸Department of Microbiology, Oslo University Hospital, Oslo, Norway.

19 ⁹Department of Molecular Medicine, University of Oslo, Oslo, Norway

20 ¹⁰Wellcome Sanger Institute, Hinxton, Cambridge, UK.

21 ¹¹Department of Medical Biochemistry, University of Oslo, Oslo, Norway.

22 ¹²School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington,
23 UK.

24 ¹³MRC Human Genetics Unit, University of Edinburgh, Edinburgh, UK.

25 *Correspondence to: Alexey Ruzov, email: alexey.ruzov@nottingham.ac.uk, Natalia
26 Gromak, email: natalia.gromak@path.ox.ac.uk and Arne Klungland, email:
27 arne.klungland@medisin.uio.no

28 **Running title:** m⁶A regulates stability of R-loops.

29 **Introductory paragraph:** R-loops are nucleic acid structures formed by an RNA:DNA
30 hybrid and unpaired single stranded DNA that represent a source of genomic instability in
31 mammalian cells¹⁻⁴. Here we show that N6-methyladenosine (m⁶A) modification,
32 contributing to different aspects of mRNA metabolism^{5, 6}, is detectable on the majority of
33 RNA:DNA hybrids in human pluripotent stem cells (hPSCs). We demonstrate that m⁶A-
34 containing R-loops accumulate during G₂/M and are depleted at G₀/G₁ phases of the cell
35 cycle and that the m⁶A reader promoting mRNA degradation, YTHDF2⁷, interacts with R-
36 loops-enriched loci in dividing cells. Consequently, YTHDF2 knockout leads to increased R-
37 loop levels, cell growth retardation and accumulation of γ H2AX, a marker for DNA double-
38 strand breaks, in mammalian cells. Our results suggest that m⁶A regulates accumulation of R-
39 loops, implying a role for this modification in safeguarding genomic stability.

40 **Main Text:** Dynamic methylation of adenosine in RNA (N6-methyladenosine, m⁶A) has
41 been implicated in regulation of different aspects of mRNA metabolism in mammals by
42 numerous studies^{5,6}. Although m⁶A is abundant in eukaryotic transcriptomes, its DNA
43 counterpart, N6-methyldeoxyadenosine (6mA) was previously thought to be restricted to
44 unicellular organisms and only recently has been shown to exist in non-negligible quantities
45 in metazoan DNA⁸⁻¹⁰. Despite the fact 6mA is reportedly widespread in fungal genomes¹¹, its
46 prevalence in mammalian systems is currently poorly understood. This modification
47 accumulates in preimplantation pig embryos¹²; however, evidence for its presence in mouse
48 tissues is contradictory^{13,14}. In this study, we initially aimed to examine if this mark is
49 detectable in human cell lines using a sensitive immunostaining method that we have
50 previously employed to detect modified forms of cytosine in vertebrate models¹⁵.

51 To confirm that we can differentiate between m⁶A-modified mRNAs and 6mA
52 present on genomic DNA, we performed immunostaining of hPSCs using previously
53 validated anti-m⁶A/6mA antibody¹¹ without the DNA denaturation step which is required for
54 the immunochemical detection of modified bases in genomic DNA^{11,15,16}. In these
55 experiments, we observed prominent m⁶A staining that disappeared upon pre-treatment of the
56 samples with RNase A (Supplementary Note). Next, we immunostained several human cell
57 lines with the same antibody but after treatment of the samples with 4 M HCl, which allows
58 denaturing double stranded nucleic acids and is routinely used for detection of cytosine
59 modifications and 6mA in genomic DNA^{11,15,16}. In these conditions, we also detected strong
60 m⁶A signal in both nuclei and the cytoplasm of hPSCs and cancer cell lines. Notably, high
61 levels of m⁶A staining were still evident in the mitotic chromatin in all our samples processed
62 after RNase A treatment (Fig. 1a; Supplementary Note). To examine if the mitotic staining
63 we observed indicates the presence of 6mA in the human genome, we performed LC-MS/MS
64 quantification of 6mA and modified forms of cytosine in the DNA of two hPSCs lines either
65 cultured under standard conditions or after enrichment for mitotic cells using colcemid
66 treatment¹⁷. Unlike the species of modified cytosine, 6mA was not detectable by LC-MS/MS
67 in hPSCs under both experimental conditions even at low parts per million (ppm) levels,
68 suggesting that this modification, if present in the hPSCs genomes, only occurs at levels
69 substantially lower than that of 5-formylcytosine¹⁸ (Fig. 1b). These results confirmed
70 previously published LC-MS data indicating the absence of 6mA in the genome of mouse
71 embryonic stem cells and tissues¹⁴.

72 Attempting to explain the discrepancy between our LC-MS/MS data and
73 immunostaining results, we hypothesized that the mitotic anti-m⁶A/6mA antibody-specific
74 signal was caused by the presence of this modification on the RNA component of R-loops¹.
75 R-loops are specific nucleic acid structures formed by an RNA:DNA hybrid and an unpaired
76 single stranded DNA that contribute to a number of important biological processes ranging
77 from transcriptional regulation to DNA repair, and represent a source of genomic instability
78 in mammalian cells¹⁻⁴. To test this hypothesis, we immunostained hPSCs using m⁶A antibody
79 after treatment of the samples with *E. coli* RNase H, an enzyme that specifically degrades
80 RNA molecules present in RNA:DNA hybrids. Notably, mitotic m⁶A staining significantly
81 decreased or disappeared in the hPSCs pre-treated with RNase H, corroborating the presence
82 of this modification on the RNA strand of RNA:DNA hybrids (Fig. 1c-d; Supplementary
83 Note). Confirming our immunostaining results, we also detected a release of m⁶A (but not of
84 ribo-5-methylcytidine, ribo-m⁵C) to filtrate by stable-isotope dilution ultra-performance
85 liquid chromatography with tandem mass spectrometry (SID-UPLC-MS/MS) upon treatment
86 of hPSCs-derived nucleic acids with RNase H (Fig. 1e, f; Supplementary Note). Overall,
87 these results strongly suggested that m⁶A modification is associated with the RNA
88 components of RNA:DNA hybrids in hPSCs.

89 To examine the genomic distribution of m⁶A-marked RNA:DNA hybrids, we
90 modified a previously published DNA:RNA immunoprecipitation technique (DRIP, referred
91 here as S9.6 DRIP)^{2, 19} by replacing anti-RNA:DNA hybrid S9.6 antibody²⁰ with anti-m⁶A
92 antibody (designated here as m⁶A DNA immunoprecipitation, m⁶A DIP). After validation of
93 this technique using synthetic spike-in RNA:DNA hybrids and single-stranded
94 oligonucleotides (Extended Data Fig. 1a-d), we performed m⁶A DIP in parallel with S9.6
95 DRIP coupled with high-throughput sequencing on hiPSCs (Fig. 2a; Extended Data Fig. 2).
96 Although both types of IP resulted in generation of large peak datasets, the majority of m⁶A
97 DIP and S9.6 DRIP peaks were not detectable in the control samples pre-treated with RNase
98 H, confirming that the presence of methylated adenosine is correlated to the RNA component
99 of R-loops in hPSCs (Fig. 2b, Supplementary Note). Both m⁶A- and S9.6 peaks exhibited
100 virtually identical distribution across various genomic features and repetitive elements and
101 were enriched in transcribed regions of the human genome (Fig. 2c, 3a; Supplementary
102 Note). Despite the number of m⁶A DIP peaks being approximately fourfold greater relative to
103 S9.6 DRIP, both sets of peaks displayed an essentially complete overlap at the sequence level
104 (Fig. 2b, d). Since the presence of both m⁶A- and S9.6 peaks was RNase H-dependent, and
105 the density of S9.6 DRIP reads was noticeably increased across the m⁶A peaks that do not
106 overlap with S9.6 peaks (Fig. 2e, Extended Data Fig. 3a), we concluded that difference in the
107 peak numbers we observed was likely due to different sensitivity of the corresponding
108 antibodies and, therefore, our results imply that m⁶A marks most of the RNA:DNA hybrids in
109 hPSCs. In line with this explanation, m⁶A DIP demonstrated approximately 3.6-fold more
110 efficient enrichment for the synthetic m⁶A-containing RNA:DNA hybrid compared with S9.6
111 DRIP in our spike-in experiments (Extended Data Fig. 1b). We also observed similar
112 distribution of common m⁶A/S9.6- and m⁶A-only peaks amongst different genomic features
113 (Extended Data Fig. 3b, c).

114 Since the RNase H-sensitive m⁶A immunostaining signal was particularly high in
115 mitotic chromatin (Fig. 1a, c), we hypothesized that this modification may accumulate on
116 RNA:DNA hybrids in a cell cycle-specific manner. To examine the dynamics of m⁶A-
117 containing R-loops during cell cycle, we performed m⁶A DIP and S9.6 DRIP on G₀/G₁, S and
118 G₂/M flow cytometry-sorted hPSCs populations (Extended Data Fig. 4a), followed by
119 quantitative PCR (qPCR) of LINE-1 repeats and individual intronic sequences enriched in
120 both m⁶A- and S9.6 peaks (Fig. 3a; Extended Data Fig. 4b). These experiments demonstrated
121 that RNA:DNA hybrids accumulate on LINE-1 retrotransposons during S phase, max out at
122 G₂/M and drastically decrease at G₀/G₁ phases of the cell cycle in hPSCs (Fig. 3b).
123 Consistently, a recent study demonstrated that retrotransposition active LINE-1-derived
124 mRNAs are enriched in cells exiting mitosis²¹. The intronic R-loops were found in high
125 levels at both S and G₂/M phases, but were also significantly depleted at G₀/G₁ phase (Fig.
126 3c, d). Importantly, these cell cycle-specific changes were essentially equivalent in both m⁶A
127 DIP and S9.6 DRIP, suggesting that m⁶A is present on RNA:DNA hybrids throughout all
128 stages of the cell cycle (Fig. 3b-d). Notably, m⁶A DIP qPCR enrichment substantially
129 increased on the repetitive and intronic loci upon small interfering RNA (siRNA)-mediated
130 knock down of RNase H1 in hPSCs (Extended Data Fig. 5a-d). Moreover, the intronic and
131 repetitive m⁶A DIP-containing sequences were also enriched in the two round IP (S9.6 DRIP
132 followed by m⁶A DIP or m⁶A RNA IP) procedures, further confirming the presence of m⁶A
133 on the RNA components of R-loops (Extended Data Fig. 6a-e). In sum, these results
134 suggested that the turn-over rates of m⁶A-marked R-loops vary for cell cycle phases.

135 Given that deposition of m⁶A is known to affect stability of mRNAs⁵⁻⁷, we
136 hypothesized that this mark may also modulate the stability of R-loops. Since siRNA-
137 mediated knockdown of m⁶A methyltransferase METTL3 led to accumulation of RNA:DNA

138 hybrids in hPSCs (Extended Data Fig. 7a-b, 8a-f; Supplementary Note), we next enquired if
139 any of the previously characterized m⁶A reader proteins may interact with mitotic chromatin
140 enriched in m⁶A-containing R-loops. First, we examined for the presence of the m⁶A readers
141 in proteins interacting with RNA:DNA hybrids immuno-precipitated from HeLa cells using
142 S9.6 antibody²². The analysis showed an enrichment of YTHDF1 – a protein promoting
143 translation of m⁶A-containing mRNAs²³, HNRNPA2B1 – a nuclear m⁶A reader previously
144 implicated in mRNA processing²⁴, and YTHDF2 – an m⁶A-interacting protein that regulates
145 degradation of cytoplasmic mRNAs⁷ as well as METTL3 in the R-loop IP, suggesting that
146 these proteins interact with RNA:DNA hybrids (Fig. 4a). Our subsequent immunostaining
147 experiments showed that, while YTHDF1 exhibited predominantly cytoplasmic localization
148 in both interphase and mitotic hPSCs (Fig. 4b, c) and HNRNPA2B1 was specifically
149 excluded from the chromatin during mitosis (Fig. 4d, e), YTHDF2 migrated to mitotic
150 chromatin in dividing hiPSCs (Fig. 4f, g). Moreover, the nuclear fraction of YTHDF2
151 exhibited a high degree of co-localization with RNA:DNA hybrids in interphase cells
152 (Extended Data Fig. 9a-e). In line with this, we also observed preferential interaction of
153 YTHDF2 with m⁶A-containing synthetic RNA:DNA substrates in electrophoretic mobility-
154 shift assays (Extended Data Fig. 9f, g; Supplementary Note) and in MicroScale
155 Thermophoresis (MST) analysis that demonstrated that YTHDF2 shows comparable
156 dissociation constant values for its interaction with m⁶A-marked single-stranded RNA and
157 m⁶A-RNA:DNA duplexes in this assay (Fig. 4h). Furthermore, YTHDF2 ChIP showed that
158 this m⁶A reader interacts with both LINE-1s and intronic genomic regions enriched in
159 RNA:DNA hybrids in these cells (Extended Data Fig. 10a). In contrast, we did not observe
160 any interaction of HNRNPA2B1 with LINE-1 repeats but were able to detect binding of this
161 protein to R-loops-containing intronic regions in ChIP experiments (Extended Data Fig. 10b).
162 Interestingly, although the recruitment of both these proteins to R-loop-containing loci was
163 reduced upon METTL3 knock-down, confirming their interaction with m⁶A in chromatin-
164 bound RNAs (Extended Data Fig. 10c, d), the accumulation of YTHDF2 (but not of
165 HNRNPA2B1) at LINE-1s and intronic loci was dramatically increased in siRNaseH1
166 hPSCs, strongly suggesting the association of this m⁶A reader with R-loops *in vivo* (Extended
167 Data Fig. 10e, f). To assess the functional significance of YTHDF2 migration to mitotic
168 chromatin, we performed its siRNA-mediated depletion (siYTHDF2) in hPSCs. S9.6 DRIP-
169 and m⁶A DIP qPCR showed a significant enrichment in both repetitive and individual
170 intronic R-loops sequences in siYTHDF2 hPSCs relative to siCTL cells (Extended Data Fig.
171 10g-i). To further confirm these results, we next assessed the levels of R-loops in *YTHDF2*
172 knockout (KO) HAP1²⁵ cells expressing a truncated version of this protein that does not co-
173 localize with mitotic chromatin (Fig. 5a, Supplementary Note). These experiments showed
174 both the elevated levels of S9.6 immunostaining and dramatic 5-50 fold increase in R-loops
175 at Alu-S, Alu-Y, LINE-1s and intronic sequences in *YTHDF2* KO compared with isogenic
176 wild type (WT) parental HAP1 cells (Fig. 5b, c). Moreover, YTHDF2 depletion in HAP1
177 cells also resulted in increased accretion of m⁶A on RNA:DNA hybrids (Fig. 5d) and cell
178 growth retardation (Fig. 5e). Subsequent analysis of recently published *Ythdf2* constitutive
179 knockout²⁶ mice-derived neural stem cells (mNSCs) confirmed these results demonstrating
180 increased levels of S9.6 immunostaining and accumulation of RNA:DNA hybrids in LINE-
181 1open reading frames upon depletion of *Ythdf2* in this system (Fig. 5f, g). In line with these
182 results, *YTHDF2* KO HAP1 cells displayed an increased accumulation of a marker for DNA
183 double-strand breaks, phosphorylated (ser139) histone variant H2AX (γ H2AX)²⁷ both at the
184 nucleus-wide level and at R-loop-enriched loci (Fig. 6a, b). Correspondingly, we also
185 observed elevated levels of γ H2AX staining in the cortex of *Ythdf2* KO embryos and *Ythdf2*
186 KO mNSCs (Fig. 6c) as well as, to a lesser extent, in hPSCs upon siRNA-mediated
187 depletions of METTL3 and HNRNPA2B1 (Fig. 6d, Supplementary Note). Moreover, the

188 γ H2AX intensity significantly decreased upon overexpression of RNase H1 in *YTHDF2* KO
 189 HAP1 cells (Fig. 6e). Overall, these results suggest that *YTHDF2* prevents accumulation of
 190 m⁶A-containing RNA:DNA hybrids contributing to inhibition of R-loop-dependent DNA
 191 damage in mammalian cells. Correspondingly, *YTHDF2* has been previously identified as
 192 one of the factors promoting genomic stability in a genome-wide siRNA screen²⁸.

193 The nature of the techniques we used for m⁶A mapping is limited by the specificity
 194 and sensitivity of the available antibody. Even so, our results show that m⁶A modification is
 195 present on the RNA within R-loops, potentially contributing to various aspects of their
 196 biology (Supplementary Note). In this context, the *YTHDF2*-mediated regulation of
 197 RNA:DNA hybrids may represent a specific mechanism of preventing accumulation of co-
 198 transcriptional R-loops during mitosis. Together with previously described factors
 199 suppressing formation of these structures²⁹⁻³¹, *YTHDF2* plays a role in safeguarding genomic
 200 stability.

201 **Acknowledgments:** We thank V. Wright, S. Malla, M. Loose, S. Rajani, D. Mosqueira, L.
 202 Lewis, R. Marcus, D. Onion, D. Bates and B. Coyle for technical help, D. Huangfu
 203 (Memorial Sloan Kettering Cancer Center) for hPSCs promoters dataset and S. Peña Perez
 204 for animal care. This work was supported by Medical Research Council IMPACT DTP PhD
 205 Studentship [grant number MR/N013913/1] to A.A. and by Biotechnology and Biological
 206 Sciences Research Council [grant number BB/N005759/1] to A.R. N.G.'s lab is supported by
 207 a Royal Society University Research Fellowship and a John Fell award [grant number
 208 BVD07340] to N.G. J.L.G.P.'s lab is supported by MINECO-FEDER [grant number
 209 SAF2017-89745-R] to J.L.G.P., the European Research Council [grant number ERC-
 210 Consolidator ERC-STG-2012-233764] to J.L.G.P., and a private donation from Ms Francisca
 211 Serrano (Trading y Bolsa para Torpes, Granada, Spain). C.D. is supported by British Heart
 212 Foundation [SP/15/9/31605, PG/14/59/31000], Medical Research Council [MR/M017354/1],
 213 NC3Rs [CRACK-IT:35911-259146], and Heart Research UK [TRP01/12]. L.Y. was
 214 supported by Medical Research Council [grant number MR/M017354/1]. A.K. is supported
 215 by the Norwegian Research Council [grant number 275286] and the Health Authority South-
 216 East [grant number 2018086]. B.D. is supported by the Norwegian Health Authority South-
 217 East (Regional Core Facility for Structural Biology) [grant number 2015095] to B.D. D.J.G.
 218 is funded by Wellcome Trust [grant number WT206194]. This work was partially supported
 219 by Health Data Research UK, an initiative funded by UK Research and Innovation,
 220 Department of Health and Social Care (England) and the devolved administrations, and
 221 leading medical research charities.

222 **Author contributions:** A.A. performed immunostaining, microscopy, DRIP, DIP, RNAseq,
 223 ChIP, qPCR, FACS sorting, cell culture experiments and contributed to bioinformatics
 224 analysis and data interpretation. T.C.G., A.R.R., J.L.G.P. and R.D.E. performed
 225 bioinformatics analysis. A.C. and N.G. performed S9.6 IP and western blots. J.M.F., N.D.
 226 and I.R.C. performed LC-MS/MS. M.S. and D.G. performed SID-UPLC-MS/MS. A.R.,
 227 A.A., M.L. and A.K. contributed to EMSA and mouse KO experiments. M.L. B.D. and A.K.
 228 generated His-fused *YTHDF2* and performed MST. M.E. provided cell lines samples. J.C.,
 229 L.F., L.Y., C.D. and D.J.G. provided wild type REBL-PAT transcriptome dataset. A.R.
 230 conceived, designed and coordinated the study and drafted the manuscript together with A.A.,
 231 N.G., J.L.G.P. and I.R.C. All authors read and approved the final manuscript.

232 **Competing interests:** Authors declare no competing interests.

233 **References:**

- 234 1. Chédin, F. Nascent Connections: R-Loops and Chromatin Patterning. *Trends Genet.* **32**,
235 828-838 (2016).
- 236 2. Sanz, L. A. et al. Prevalent, Dynamic, and Conserved R-Loop Structures Associate with
237 Specific Epigenomic Signatures in Mammals. *Mol Cell.* **63**, 167-178 (2016).
- 238 3. Lu, W. T. et al. Drosha drives the formation of DNA:RNA hybrids around DNA break
239 sites to facilitate DNA repair. *Nat Commun.* **9**, 532, (2018).
- 240 4. Santos-Pereira, J. M., Aguilera, A. R loops: new modulators of genome dynamics and
241 function. *Nat Rev Genet.* **16**, 583-597 (2015).
- 242 5. Yue, Y., Liu, J., He, C. RNA N6-methyladenosine methylation in post-transcriptional
243 gene expression regulation. *Genes Dev.* **29**, 343-355 (2015).
- 244 6. Roundtree, I. A., Evans, M. E., Pan, T., He, C. Dynamic RNA Modifications in Gene
245 Expression Regulation. *Cell.* **169**, 1187-1200 (2017).
- 246 7. Wang, X. et al. N6-methyladenosine-dependent regulation of messenger RNA stability.
247 *Nature.* **7481**, 117-120 (2014).
- 248 8. Zhang, G. et al. N6-methyladenine DNA modification in Drosophila. *Cell.* **161**, 893-906
249 (2015).
- 250 9. Greer, E. L. et al. DNA Methylation on N6-Adenine in *C. elegans*. *Cell.* **161**, 868-78
251 (2015).
- 252 10. Fu, Y. et al. N6-methyldeoxyadenosine marks active transcription start sites in
253 *Chlamydomonas*. *Cell.* **161**, 879-892 (2015).
- 254 11. Mondo, S. J. et al. Widespread adenine N6-methylation of active genes in fungi. *Nat*
255 *Genet.* **49**, 964-968 (2017).
- 256 12. Liu, J. et al. Abundant DNA 6mA methylation during early embryogenesis of zebrafish
257 and pig. *Nat Commun.* **7**, 13052, (2016).
- 258 13. Yao, B. et al. DNA N6-methyladenine is dynamically regulated in the mouse brain
259 following environmental stress. *Nat Commun.* **8**, 1122, (2017).
- 260 14. Schiffers, S. et al. Quantitative LC-MS Provides No Evidence for m(6) dA or m(4) dC in
261 the Genome of Mouse Embryonic Stem Cells and Tissues. *Angew Chem Int Ed Engl.* **56**,
262 11268-11271 (2017).
- 263 15. Wheldon, L. M. et al. Transient accumulation of 5-carboxylcytosine indicates
264 involvement of active demethylation in lineage specification of neural stem cells. *Cell*
265 *Rep.* **7**, 1353-1361 (2014).
- 266 16. Santos, F., Dean, W. Using immunofluorescence to observe methylation changes in
267 mammalian preimplantation embryos. *Methods Mol Biol.* **325**, 129-137 (2006).
- 268 17. Rieder, C. L., Palazzo, R. E. Colcemid and the mitotic cycle. *J Cell Sci.* **102**, 387-92
269 (1992).
- 270 18. Pfaffeneder, T. et al. The discovery of 5-formylcytosine in embryonic stem cell DNA.
271 *Angew Chem Int Ed Engl.* **50**, 7008-7012 (2011).
- 272 19. Chan, Y. A. et al. Genome-wide profiling of yeast DNA:RNA hybrid prone sites with
273 DRIP-chip. *PLoS Genet.* **10**, e1004288 (2014).
- 274 20. Boguslawski, S. J. et al. Characterization of monoclonal antibody to DNA:RNA and its
275 application to immunodetection of hybrids. *J Immunol Methods* **89**, 123-130 (1986).

- 276 21. Mita, P. et al. LINE-1 protein localization and functional dynamics during the cell cycle.
277 *Elife*. **7**, pii: e30058 (2018).
- 278 22. Cristini, A., Groh, M., Kristiansen, M.S., Gromak, N. RNA/DNA Hybrid Interactome
279 Identifies DXH9 as a Molecular Player in Transcriptional Termination and R-Loop-
280 Associated DNA Damage. *Cell Rep*. **23**, 1891-1905 (2018).
- 281 23. Wang, X. et al. N(6)-methyladenosine Modulates Messenger RNA Translation
282 Efficiency. *Cell*. **161**, 1388-1399 (2015).
- 283 24. Alarcón, C. R. et al. HNRNPA2B1 Is a Mediator of m(6)A-Dependent Nuclear RNA
284 Processing Events. *Cell*. **162**, 1299-12308 (2015).
- 285 25. Carette, J.E. et al. Haploid genetic screens in human cells identify host factors used by
286 pathogens. *Science*. **5957**, 1231-1235 (2009).
- 287 26. Li, M., Zhao, X., Wang, W., Shi, H., Pan, Q., Lu, Z., Perez, S.P., Suganthan, R., He, C.,
288 Bjørås, M., Klungland, A. Ythdf2-mediated m(6)A mRNA clearance modulates neural
289 development in mice. *Genome Biol*. **19**, 69 (2018).
- 290 27. Bonner, W.M. et al. GammaH2AX and cancer. *Nat Rev Cancer*. **8**, 957-967 (2008).
- 291 28. Paulsen, R. D. et al. A genome-wide siRNA screen reveals diverse cellular processes and
292 pathways that mediate genome stability. *Mol Cell*. **35**, 228-239 (2009).
- 293 29. Salas-Armenteros, I., Pérez-Calero, C., Bayona-Feliu, A., Tumini, E., Luna, R., Aguilera,
294 A. Human THO-Sin3A interaction reveals new mechanisms to prevent R-loops that cause
295 genome instability. *EMBO J*. **36**, 3532-3547 (2017).
- 296 30. Bayona-Feliu, A., Casas-Lamesa, A., Reina, O., Bernués, J., Azorín, F. Linker histone H1
297 prevents R-loop accumulation and genome instability in heterochromatin. *Nat Commun*.
298 **8**, 283 (2017).
- 299 31. Benitez-Guijarro, M. et al. RNase H2, mutated in Aicardi-Goutières syndrome, promotes
300 LINE-1 retrotransposition. *EMBO J*. **37** pii: e98506. doi: 10.15252/embj.201798506
301 (2018).

302 Figure Legends:

303 **Fig. 1.** m⁶A marks the RNA components of RNA:DNA hybrids in hPSCs. (a) m⁶A and 5-
304 methyldeoxycytosine (5mC) co-immunostaining of KaryoMAX-treated hiPSCs without
305 RNases and after RNase A treatment. Merged images are shown. Mitotic cells are arrowed.
306 (b) The ratios of the indicated deoxynucleotides obtained from the quantification of LC-
307 MS/MS peaks in KaryoMAX-treated and untreated hiPSCs/hESCs DNA. Data are means ±
308 SD, n=2 MS experiments. (c) Immunostaining of hiPSCs using anti-m⁶A and anti-phospho-
309 Histone H3 antibodies without RNases and after RNase A or combined RNases A/H
310 treatments. Merged views are presented. (d) Box plots showing quantification of m⁶A signal
311 intensity in the interphase and mitotic hiPSCs at indicated immunostaining conditions. The
312 elements of the box plots are: center line, median; box limits, upper and lower quartiles;
313 whiskers, minimum and maximum of all the data; n=20 nuclei for each condition.
314 Significance was determined by unpaired two-tailed Student's t-test. No adjustments were
315 made for multiple comparisons. (e) Schematic illustrating design of the experiment on SID-
316 UPLC-MS/MS analysis of hPSCs-derived nucleic acids released and retained upon RNase H
317 treatment. (f) SID-UPLC-MS/MS quantification of m⁶A and ribo-m⁵C in the fractions of
318 hESCs- and hiPSCs-derived nucleic acids released upon RNase H treatment. Data are shown
319 as means ± SD, n=4/n=3 MS experiments for m⁶A/ribo-m⁵C quantification. Scale bars are 10

320 μm in (a) and 5 μm in (c). KaryoMAX treatment was used to enrich hPSCs for mitotic cells
 321 in (a, b). The experiments shown in (a, c) were repeated independently 6 times with similar
 322 results.

323 **Fig. 2.** m^6A is present on the majority of the RNA:DNA hybrids in hPSCs. (a) Schematic
 324 illustrating the m^6A DIP technique. See also Extended Data Fig. 2. (b) The coverage plots of
 325 m^6A DIP and S9.6 DRIP densities (CPK) in the intron of *CAMTA1-201* gene. m^6A DIP and
 326 S9.6 DRIP peaks are marked with red and blue rectangles. (c) Distribution of m^6A and S9.6
 327 peaks at the indicated genomic features in hiPSCs. (d) Venn diagram illustrating an overlap
 328 between m^6A DIP and S9.6 DRIP consensus peaks in REBL-PAT hiPSCs. (e) Heatmaps
 329 showing the distribution of density of m^6A DIP and S9.6 DRIP reads across genomic regions
 330 containing the peaks (3 kb around peak center) of the three categories: m^6A peaks
 331 overlapping with S9.6 peaks ($\text{m}^6\text{A}/\text{S9.6}$), m^6A peaks that do not overlap with S9.6 DRIP
 332 peaks (m^6A only) and S9.6 peaks that do not correspond to m^6A peaks (S9.6 only). The color
 333 of each line represents the density of reads for a given peak. The width of the heatmaps is
 334 normalized by peak length. Median numbers of reads per normalized region within each of
 335 the peak subsets are plotted over the top of the heatmaps. As the exact mode of genomic
 336 distribution of m^6A -containing RNA:DNA hybrids was initially unknown, we performed
 337 detection of both narrow and broad peaks in the datasets. The results shown were obtained
 338 from analyses of the narrow m^6A and S9.6 peaks.

339 **Fig. 3.** RNA:DNA hybrids exhibit cell cycle-specific dynamics in hPSCs. (a) The m^6A DIP
 340 and S9.6 DRIP consensus narrow peak counts of the indicated repetitive elements in hiPSCs.
 341 (b) The results of m^6A DIP and S9.6 DRIP qPCR of the indicated repeats performed on
 342 hiPSCs sorted at different cell cycle phases. Generic primers amplifying evolutionarily young
 343 L1Hs were used. (c, d) The results of S9.6 DRIP (c) and m^6A DIP qPCR (d) of the
 344 RNA:DNA peaks localized in the introns of the indicated genes (See Extended Data Fig. 9b)
 345 performed on hiPSCs sorted at different phases of the cell cycle. Data are means \pm SD, $n=3$
 346 independent experiments in (b-d).

347 **Fig. 4.** m^6A reader proteins interact with RNA:DNA hybrids. (a) Western blot of RNA:DNA
 348 hybrids protein co-IP probed with indicated antibodies. Top1 and Lamin B1 serve as positive
 349 and negative controls for R-loop IP, respectively. The experiments were repeated
 350 independently 2 times for METTL3 and 3 times for other proteins with similar results. The
 351 blots were cropped. The full scans of the blots are shown in Source Data 1. (b, c)
 352 Immunostaining of hiPSCs using anti-YTHDF1 and anti-phospho-Histone H3 antibodies
 353 imaged at two different magnifications. (d, e) Immunostaining of hiPSCs for HNRNPA2B1
 354 and m^6A imaged at two different magnifications. (f, g) Immunostaining of hiPSCs for
 355 YTHDF2 and phospho-Histone H3 imaged at two different magnifications. Merged views
 356 and YTHDF1/HNRNPA2B1/YTHDF2 channels (b, d, f) or merged views and individual
 357 channels (c, e, g) are shown. The locations of the views shown in (c, e, g) are marked with
 358 dotted rectangles in (b, d, f). Scale bars are 10 μm . The experiments shown in (b-g) were
 359 repeated independently 4 times with similar results. (h) Microscale thermophoresis binding
 360 curves for YTHDF2 interaction with m^6A -containing/non-modified RNA:DNA hybrid and
 361 m^6A -marked/non-modified ssRNA synthetic substrates. The binding is shown as fraction
 362 protein bound as a function of substrate concentration. Binding curves are fitted to the data
 363 points from experiments for m^6A -containing (filled circles/triangles) and unmodified (open
 364 circles/triangles) substrates. Dissociation constant values are shown for each of the
 365 interactions. Error bars show SD, the centre values are means, $n=6$ independent series of
 366 experiments.

367 **Fig. 5.** YTHDF2 depletion leads to accumulation of R-loops, increased accretion of m⁶A on
 368 RNA:DNA hybrids and cell growth retardation. (a) Immunostaining of WT and *YTHDF2* KO
 369 HAP1 for YTHDF2 and phospho-Histone H3. The experiments were repeated independently
 370 3 times with similar results. (b) Immunostaining of WT and *YTHDF2* KO HAP1 for R-loops
 371 alongside the quantification of S9.6 nuclear signal. (c) DRIP qPCR of the indicated
 372 sequences performed on WT and *YTHDF2* KO HAP1. RANBP17 and HECW1 downstream
 373 regions lacking DRIP peaks were used as controls. (d) SID-UPLC-MS/MS quantification of
 374 m⁶A and ribo-m⁵C in S9.6-IPs performed on WT and *YTHDF2* KO HAP1 and normalized for
 375 dA or rA. RNase H-pre-treated samples were used as controls. Data are means \pm SD, n=5
 376 (left) and n=7 (right panel) measurements of 4 independent samples. (e) The growth curves of
 377 WT and *YTHDF2* KO HAP1. (f) Immunostaining of WT/*Ythdf2* KO mNSCs for R-loops
 378 alongside the quantification of S9.6 nuclear signal. (g) DRIP and m⁶A DIP qPCR of mouse
 379 LINE-1 ORF1 performed on WT and *Ythdf2* KO mNSCs. Individual channels (a) or S9.6
 380 channel (b, f) with merged views are shown. Scale bars are 10 μ m. Data are means \pm SD, n=3
 381 independent experiments in (c, e, g). The elements of the box plots (b, f) are: centre line,
 382 median; box limits, upper and lower quartiles; whiskers, minimum and maximum of all the
 383 data; n, sample size, ***p < 0.0001. Significance was determined by unpaired two-tailed
 384 Student's (b, f) or unpaired two-tailed Welch's (e) t-test.

385 **Fig. 6.** YTHDF2 depletion leads to elevated levels of H2AX phosphorylation in human and
 386 mouse cells. (a) Representative images of WT and *YTHDF2* KO HAP1 cells immunostained
 387 for γ H2AX, and quantification of γ H2AX signal intensity in these cells. (b) The results of
 388 γ H2AX ChIP qPCR of the indicated sequences performed on WT and *YTHDF2* KO HAP1.
 389 Generic primers amplifying Alu elements from the indicated families and evolutionarily
 390 young L1Hs were used. Data are means \pm SD, n=3 independent experiments. (c)
 391 Representative images of WT/*Ythdf2* KO embryonic brain cortex and mNSCs immunostained
 392 for γ H2AX alongside quantification of γ H2AX signal intensity in these tissues/cells. (d)
 393 Immunostaining of siCTL, siMETTL and siHNRNPA2B1 hPSCs for γ H2AX and
 394 quantification of γ H2AX signal intensity in these cells. (e) Representative images of
 395 *YTHDF2* KO HAP1 cells transfected with GFP-RNase H1 and GFP-only expression
 396 constructs immunostained for γ H2AX, alongside the quantification of γ H2AX signal
 397 intensity in the GFP-positive cells. P value is indicated. Examples of the nuclei used for
 398 signal quantification are marked with dotted shapes. Merged images and S9.6 channel views
 399 are shown in (a, c-e). Scale bars are 10 μ m. The elements of the box plots shown in (a, c-e)
 400 are: centre line, median; box limits, upper and lower quartiles; whiskers, minimum and
 401 maximum of all the data, n, sample size, ***p < 0.0001. Significance was determined by
 402 unpaired two-tailed Student's t-test, no adjustments were made for multiple comparisons.

403

404 Methods

405 Cell culture, flow cytometry and RNA-interference-mediated knockdowns.

406 REBL-PAT hiPSCs and HUES7 hESCs were maintained in Essential 8TM (E8) medium with
 407 supplement (#A1517001) on MatrigelTM-coated tissue culture flasks at 37 °C with 5 % CO₂.
 408 Use of the HUES7 hESC line was approved by the UK Medical Research Council Steering
 409 Committee, in association with the UK Stem Cell Bank. Cells were passaged every 3–4 d
 410 using TrypLETM Select Enzyme (#12563029). hiPSCs were treated with 1:100 dilution of
 411 KaryoMAX[®] ColcemidTM Solution (Thermo Fisher Scientific, catalogue number 15212012)
 412 for 3 h. HeLa, LN-18 and U87MG cells were maintained on DMEM (GIBCO) supplemented
 413 with 10 % bovine serum. G₀/G₁, S and G₂/M phases flow cytometry sorting was performed

414 according to the previously described method³². Briefly, enzymatically dissociated hPSCs
 415 were washed in PBS and fixed in 70 % ethanol for 2 h, washed with PBS again and stained
 416 with 10 µg/ml propidium iodide (PI) (Sigma-Aldrich, catalogue number P3566) in PBS
 417 supplemented with 0.1 % Triton X-100 and 100 µg/ml RNase A (Qiagen, catalogue number
 418 19101). PI treated hPSCs were sorted based on the DNA content into G₀/G₁, S and G₂/M cells
 419 using Beckman Coulter Astrios EQ and Beckman Coulter Kaluza 2.1 software. For *METTL3*
 420 and *YTHDF2* depletion, hiPSCs were transfected with 50 pmol of siRNA duplexes against
 421 human *METTL3* (Dharmacon™, catalogue number 56339), human *HNRNPA2B1*
 422 (Thermofisher, Catalogue number 4390824 siRNA, ID: s6714), human *YTHDF2* (Qiagen,
 423 catalogue number GS51441), human *RNase H1* (Dharmacon, Catalogue number M-012595-
 424 00-0010) and nontargeting siRNA #2 (Thermo Fisher Scientific, catalogue number D-
 425 001210-02) using DharmaFECT™ (GE Lifesciences) in antibiotic-free medium. Cells were
 426 collected for analysis 72 h after transfection. Expression of *METTL3*, *HNRNPA2B1*, *RNase*
 427 *H1* and *YTHDF2* was analysed by qPCR, according to standard procedures. Gene expression
 428 was normalized by comparison to levels of *GAPDH* gene expression. The primers used for
 429 qPCR are listed in Supplementary Table 1.

430 *YTHDF2* KO (CRISPR/Cas9-mediated deletion of 140 bp in the exon 3 leading to frameshift
 431 and generating premature stop codon) HAP1 cells (Horizon Discovery, catalogue number
 432 HZGHC006678c001) and their isogenic wild type parental HAP1 cells (Horizon Discovery)
 433 were cultured on DMEM/F12 (Gibco Life Technologies, Catalog number 11320033)
 434 supplemented with 20 % heat-inactivated foetal bovine serum containing 1 % pen/strep at 37
 435 °C in a humidified incubator with 5 % CO₂. Culture medium was changed daily and the cells
 436 were passaged using trypsin every 48 h. For determining the growth curve cells were counted
 437 using haemocytometer. Statistical significance was determined using 2-tailed t-test following
 438 assessment of the variance with F-test. The deletion in the 3rd exon of *YTHDF2* gene was
 439 validated by PCR (See Supplementary Table 1 for primer sequences) and by sequencing.
 440 For overexpression of RNase H1 in mammalian cells we C-terminally eGFP-tagged human
 441 RNASEH1 (nuclear isoform) pEGFP-RNASEH1 plasmid³³. This construct was a gift from
 442 Andrew Jackson & Martin Reijns (Addgene plasmid # 108699 ;
 443 <http://n2t.net/addgene:108699> ; RRID:Addgene_108699). pmaxGFP™ (Lonza) was used as a
 444 control GFP-only plasmid.

445 **Animals and *Ythdf2* KO mouse model.** Generation of the *Ythdf2* conditional
 446 knockout mice, followed by cre-mediated deletion and derivation of mNSCs from E14.5
 447 embryonic forebrains were described previously²⁶. All mouse experiments were approved by
 448 the Norwegian Animal Research Authority by Norwegian Food Safety Authority and done in
 449 accordance with institutional guidelines at the Centre for Comparative Medicine at Oslo
 450 University Hospital. Animal work was conducted in accordance with the rules and
 451 regulations of the Federation of European Laboratory Animal Science Association's
 452 (FELASA).

453 **Immunocytochemistry, immunohistochemistry, confocal microscopy and image**
 454 **quantification.** Immunocytochemistry and immunohistochemistry were performed as
 455 described^{15, 34}. Sections of paraffin-embedded E14.5 wild type and *Ythdf2* KO mouse
 456 embryonic brain were used for γH2AX immunohistochemistry. The sections were dewaxed
 457 according to standard procedures. Cells were fixed in 4 % formaldehyde for 15 min. Cells
 458 and tissue sections were permeabilised with PBS containing 0.5 % Triton X-100 for 15 min.
 459 After permeabilisation, cells were treated with 25 mg/ml RNase A (Qiagen, catalogue

460 number 19101) in PBS or with a mixture of 25 mg/ml RNase A and 10 U of RNase H in 1X
461 RNase H buffer (NEB, catalogue number M0297S) overnight at 37 °C. DNase I (Qiagen,
462 catalogue number 79254) treatment (20 U per sample) was carried out for 4 h at room
463 temperature. The samples were incubated in 4N HCl for 1 h at 37 °C. Competition
464 experiments were performed as described previously¹⁵ using N⁶-Methyl-2'-deoxyadenosine-
465 5'-triphosphate (Trilink, catalogue number NU-949S) or unmodified dATP and dTTP from
466 dNTP set (NEB, catalogue number N0446S). Immunostaining for RNA:DNA hybrids was
467 performed according to previously published protocol³⁵ using S9.6 antibody (Merck
468 Millipore, catalogue number MABE1095). The antibodies used for immunochemistry and
469 their dilutions are provided in Supplementary Note. Control staining without primary
470 antibodies produced no detectable signal. Images (500 nm optical sections) were acquired
471 with a Zeiss LSM 700 AxioObserver confocal microscope using a Plan-Apochromat 63x/1.40
472 Oil DIC M27 objective and processed using Image J and Adobe Photoshop. 2.5XD signal
473 intensity plots and intensity profiles were generated using ZEN Zeiss LSM 700 imaging
474 software as described previously^{15, 36}. Confocal raw data are available upon request. Co-
475 localization coefficients were determined using the inbuilt analysis function of ZEN as
476 described^{15, 34}. Quantification of the m⁶A, γ H2AX and S9.6 signal intensities was performed
477 according to the previously described method³⁴. Mean values of the average of 18-60 nuclei
478 signal intensities were calculated for each experimental point.

479 **Liquid chromatography-tandem mass spectrometry (LC-MS/MS).** DNA and total
480 RNA were isolated according to standard procedures. Up to 15 μ g of purified DNA was
481 digested to nucleosides for subsequent LC-MS analysis. Genomic DNAs and RNAs were
482 digested to nucleosides by treatment with the Nucleoside Digestion Mix (NEB, M0649S)
483 overnight at 37 °C. LC-MS/MS analysis was performed in duplicate by injecting digested
484 DNAs and RNAs on an Agilent 1290 UHPLC equipped with a G4212A diode array detector
485 and a 6490A Triple Quadrupole Mass Detector operating in the positive electrospray
486 ionization mode (+ESI). UHPLC was carried out using a Waters XSelect HSS T3 XP column
487 (2.1 \times 100 mm, 2.5 μ m) with the gradient mobile phase consisting of methanol and 10 mM
488 aqueous ammonium formate (pH 4.4). MS data acquisition was performed in the dynamic
489 multiple reaction monitoring (DMRM) mode. Each nucleoside was identified in the extracted
490 chromatogram associated with its specific MS/MS transition: dC at m/z 228 \rightarrow 112, d5mC at
491 m/z 242 \rightarrow 126, d5hmC at m/z 258 \rightarrow 142, d5fC at m/z 256 \rightarrow 140, dA at 252 \rightarrow 136, d6mA at
492 266 \rightarrow 150, rC at m/z 244 \rightarrow 112, m⁵C at 258 \rightarrow 126, Cm at 258 \rightarrow 112, rA at 268 \rightarrow 136, m¹A at
493 282 \rightarrow 150, Am at 282 \rightarrow 136, m⁶A at 282 \rightarrow 150, and m⁶₂A at 296 \rightarrow 164. External calibration
494 curves with known amounts of the corresponding nucleosides were used to calculate the
495 ratios within the samples analysed.

496 **Stable-isotope dilution ultra-performance liquid chromatography with tandem**
497 **mass spectrometry (SID-UPLC-MS/MS).** The cells were resuspended in ice-cold buffer
498 containing 10 mM Tris-HCl, 5 mM Na₂EDTA, 0.15 mM deferoxamine mesylate (pH 8.0)
499 and 0.5 % SDS. The samples were incubated at 37 °C for 30 min followed by addition of 2.5
500 mg/ml Proteinase K and further incubation at 37 °C for 1.5 h. The nucleic acids were isolated
501 using phenol/chloroform extraction and precipitated using ethanol. The precipitate was
502 removed to another tube with plastic spatula, washed with 70 % ethanol and dissolved in
503 MilliQ-grade deionized water. 5-10 μ g of nucleic acids were treated with 5 U of RNase H
504 (NEB) overnight in RNase H Reaction Buffer (NEB) at 37 °C. After incubation samples were
505 ultrafiltered using Amicon Ultra-0.5 MWCO 3 kDa Centrifugal Filter (Merck) at 14000 g for

506 10 min. Subsequently, the samples were rinsed twice with MilliQ-grade deionized water
507 (14000 g for 15 min). To recover the nucleic acids the filter was placed upside down in a
508 clean microcentrifuge tube and centrifuged at 1000 g for 3 min. The ultrafiltrates containing
509 released (oligo)nucleotides with molecular weight less than 3 kDa and the remaining nucleic
510 acids were treated with 1U of nuclease P1 for 1 h in a buffer containing 200 mM ammonium
511 acetate, 0.2 mM ZnCl₂ (pH 4.6) and 10 µg/sample tetrahydrouridine at 37°C, followed by
512 addition of 10 % NH₄OH and 1.3 U of alkaline phosphatase and subsequent additional 1 h
513 incubation at 37 °C. Chromatographic analysis was performed using previously described
514 method³⁷ adapted for determination of m⁶A, m⁷C and adenosine (See details in
515 Supplementary Note).

516 **RNA:DNA hybrids and protein co-immunoprecipitation with S9.6 antibody** was
517 performed from non-cross-linked HeLa cells as described previously²². The genomic DNA
518 was isolated from HeLa cells and sonicated as described³⁸. The western blots of RNA:DNA
519 hybrid IP samples were probed with the following antibodies: Top1 (Abcam, catalogue
520 number ab109374, dilution 1:2000), YTHDF1 (ProteinTech, catalogue number 17479-1-AP,
521 dilution 1:1000), YTHDF2 (ProteinTech, catalogue number 24744-1-AP, dilution 1:500),
522 METTL3 (Bethyl Laboratories, catalogue number A301-567A, dilution 1:2000),
523 HNRNPA2B1 (Novus, catalogue number NB120-6102SS, dilution 1:500) and Lamin B1
524 (Abcam, catalogue number ab16048, dilution 1:2000). Images were acquired by
525 chemiluminescence using autoradiography.

526 **m⁶A DIP and S9.6 DRIP.** Genomic DNA was isolated from REBL-PAT hiPSCs by
527 SDS/Proteinase K treatment at 37 °C followed by incubation with 100 µg/ml RNase A
528 (Qiagen, catalogue number 19101) for 30 min in lysis buffer, phenol-chloroform extraction
529 and ethanol precipitation. The DNA was fragmented to 300-600 bp using Covaris S2
530 ultrasonicator (Covaris Inc). Genomic DNA of the control samples was treated with 10 U of
531 RNase H (NEB, catalogue number M0297S) in 1x RNase H buffer overnight at 37 °C before
532 the immunoprecipitation. 10 µg of genomic DNA was used for immunoprecipitation. S9.6
533 DRIP was carried out essentially as described in the previously published protocol³⁹ using
534 S9.6 antibody (Merck Millipore, catalogue number MABE1095) and anti-mouse magnetic
535 Dynabeads (Invitrogen). m⁶A DIP was performed using anti-m⁶A rabbit polyclonal antibody
536 (Synaptic systems, catalogue number 202003) and magnetic anti-rabbit Dynabeads
537 (Invitrogen, M-280; polyclonal sheep anti-rabbit IgG; catalogue number 10716653) with
538 denaturation step before the IP (10 min at 95° C) analogously to meDIP technique⁴⁰
539 (Supplementary Fig. 6). The corresponding primary IgG-only and secondary IgG only
540 (Dynabeads only) DRIP reactions were used in control immunoprecipitations. For the two
541 round (S9.6 DRIP followed by m⁶A DIP) DRIP/DIP, approximately 500 ng of the nucleic
542 acids recovered from multiple DRIP reactions performed in parallel were used for m⁶A DIP
543 followed by qPCR analysis. For S9.6 DRIP followed by m⁶A RIP experiment, nucleic acids
544 recovered from DRIP were denatured for 30 min at 95 °C followed by digestion of the DNA
545 components of RNA:DNA hybrids using Turbo DNase (Invitrogen, catalogue number:
546 AM1907) for 30 min at 37 °C. After inactivation of DNase, approximately 100 ng of the
547 recovered RNA was used for the m⁶A RIP performed using EpiMark® N6-Methyladenosine
548 Enrichment Kit (NEB, catalogue number E1610S). The eluted RNA was reverse transcribed
549 using SuperScript III Reverse Transcriptase (Invitrogen, catalogue number 2072529A) and
550 random hexamers (Invitrogen, catalogue number 1831815) and analysed by qPCR. IgG-only
551 reactions and reactions carried out without reverse transcription were used as controls.

552 For validation of m⁶A-DIP using synthetic oligonucleotides, 0.1-1 pmol of m⁶A-containing-
 553 and non-modified RNA:DNA hybrids and individual single stranded RNA or DNA
 554 oligonucleotides were spiked-in with 5 µg of mouse genomic DNA. The RNA and DNA
 555 oligonucleotides used for spike-in experiments and primers used to amplify spike-in controls
 556 are listed in Supplementary Table 1. The RNA oligonucleotides were synthesised by
 557 Dharmacon. To generate RNA:DNA hybrids, the RNA and DNA oligonucleotides were
 558 mixed in equimolar concentrations, incubated for 20 min at 98° C, slowly cooled down in a
 559 heating block and placed on ice. Quantitative PCR analysis of m⁶A DIP and S9.6 DRIP
 560 samples was carried out with SYBR Green PCR Master Mix (Sigma) according to standard
 561 procedures. Fold enrichment was calculated as 2^{ddCt} , where $\text{dCt}=\text{Ct}(\text{enriched})-\text{Ct}(\text{input})$ and
 562 $\text{ddCt}=\text{dCt} - \text{Ct}(\text{IGG})$. The primers used for DRIP/DIP-qPCR and qPCR analysis of the
 563 corresponding transcripts are listed in Supplementary Table 1. The primers for α -satellites
 564 were obtained from Novus (catalogue number NBP1-71654SS). Generic primers amplifying
 565 Alu elements from the indicated families and evolutionarily young L1Hs were used. Human
 566 LINE-1 primers were designed to detect L1PA1 and L1PA2 classes of these retroelements.
 567 The primers for mouse LINE-1 ORF1 were previously published⁴¹. The primers used for
 568 DRIP-qPCR of Alu-Y, Alu-S and LINE-1s were also employed for qPCR. Gene expression
 569 was normalized by comparison to levels of *GAPDH* gene expression.

570 **Chromatin immunoprecipitation (ChIP)** was performed using EZ-Magna ChIP™
 571 A/G Chromatin Immunoprecipitation Kit (Merck, catalogue number 17-10086) according to
 572 manufacturer's instructions using anti-YTHDF2 rabbit polyclonal (ProteinTech, catalogue
 573 number 24744-1-AP), anti-HNRNPA2B1 mouse monoclonal (Novus, catalogue number
 574 NB120-6102SS) and anti- γ H2AX mouse monoclonal (Merck, catalogue number 05-636,
 575 clone JBW301) primary antibodies. ChIP was analysed by quantitative PCR carried out with
 576 SYBR Green PCR Master Mix (Sigma) according to standard procedures. Fold enrichment
 577 was calculated as 2^{ddCt} , where $\text{dCt}=\text{Ct}(\text{enriched})-\text{Ct}(\text{input})$ and $\text{ddCt}=\text{dCt} - \text{Ct}(\text{IGG})$. The
 578 primers used for DRIP-qPCR were also used for ChIP-qPCR analysis.

579 **Purification of recombinant YTHDF2.** Full-lengths YTHDF2 was cloned into pET-
 580 28b with N-terminal His-tag (Genescript). The plasmid was expressed in BL21(DE3) cells
 581 and incubated with LB-medium (Puls medical, 244610) with sorbitol at 37° C until OD600
 582 0.7. Expression was induced with 300 µM IPTG overnight at 18° C. Cells were pelleted by
 583 centrifugation at 3000 g for 10 min at 4° C, resuspended in lysis buffer (50 mM Tris pH 8.0,
 584 300 mM NaCl, 10 mM ME, 10 mM imidazole) and sonicated. After sonication, the extract
 585 was centrifuged at 19000 g for 20 min at 4° C. The supernatant was loaded to Protino® Ni-
 586 NTA agarose prepared as described by the producer (Macherey-nagel, 745400.100) in a 50
 587 ml tube and incubated at 4° C with rotation for 30 min. After centrifugation at 3000 g for 2
 588 min, the Ni-agarose-bound YTHDF2 was washed with the buffer containing 50 mM Tris pH
 589 8.0, 300 mM NaCl, 10 mM ME and 50 mM imidazole. Recombinant YTHDF2 was eluted
 590 with 5 washes (1.5 ml each) of elution buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM
 591 ME and 300mM imidazole).

592 **Microscale thermophoresis (MST)** was employed to study the interaction of
 593 YTHDF2 with modified or unmodified- RNA and RNA:DNA hybrid synthetic substrates
 594 used in EMSA experiments. The purified full-lengths YTHDF2 with N-terminal His-tag was
 595 labelled with the NT-647 RED-tris-NTA dye for His-tagged proteins following the
 596 recommended labelling protocol (Nanotemper Technologies). 100 µl of 200 nM protein in
 597 PBS buffer was mixed with 100 µl of 100 nM dye in the supplemented PBS-T buffer and
 598 incubated at room temperature for 30 minutes. The labelled protein was then added to a 1:1
 599 dilution series of the respective substrate in UltraPure DNase/RNase-Free Distilled Water
 600 (FisherScientific) to a final concentration of 50 nM. The different protein-substrate samples

601 were loaded into standard NT.115 MST capillaries (Nanotemper). Fluorescence profiles were
602 measured at 25° C in a Monolith NT.115 instrument using the red channel. Data was
603 collected at 20 % (single-strand substrates) or 40 % (hybrid substrates) MST power and 60 %
604 excitation power. The changes in fluorescence (ΔF_{norm}) due to thermophoresis were measured
605 as the signal difference between time points 0 and 10 seconds. Data were normalized and
606 plotted as a function of the ligand concentration, and a binding curve was fitted to the average
607 of six independent dilution series of each substrate.

608 **Library preparation and high throughput sequencing.** Sequencing libraries were
609 prepared according to the NEB Next DNA Ultra Library Preparation Kit for Illumina (NEB,
610 E7370). DNA was sonicated to 400-600 bp (Covaris S2) and adapters were ligated (NEB,
611 E73355S) according to the protocol. Adapter ligated DNA was digested with USER enzyme
612 as stated in the protocol. Following immunoprecipitation, the enriched adapter ligated DNA
613 was amplified for 15 cycles and libraries were quantified using the Kapa Library
614 Quantification Kit (Kapa Biosystems, KK4823). Sequencing was performed using the
615 Illumina NextSeq500 platform to generate 2 x 150 bp reads. Primary IgG-only DRIP
616 reactions resulted in the DNA amounts insufficient for successful library production even
617 with maximum number (15) of amplification cycles recommended by NEB.

618 **Whole transcriptome sequencing.** Total RNA was isolated from REBL-PAT hiPSCs
619 according to standard procedures. RNA-seq libraries were constructed using the Illumina
620 TruSeq Stranded Total RNA sample preparation kits (Illumina, Inc., San Diego, CA),
621 according to the manufacturers guidelines, and then sequenced on Illumina HiSeq 4000
622 generating 20–50 million 75 bp paired-end reads per sample.

623 **Bioinformatics analysis.** The 150 bp Illumina paired end reads were trimmed using
624 Skewer to remove low quality sequences⁴². Reads that passed filtering were aligned to the
625 human Ensembl genome (build hg38.89) using BWA with default parameters⁴³. As each
626 biological sample was split across multiple lanes of sequencing, the corresponding
627 alignments were merged with Samtools⁴⁴ and de-duplicated to remove PCR artefacts with
628 picard-tools MarkDuplicates⁴⁵. The impact of each pulldown was assessed using
629 Phantompeakqualtools⁴⁶ and the highly modified regions (HMRs, peaks) were identified
630 using MACS2.1.1^{46, 47}. As the exact mode of genomic distribution of m⁶A-containing
631 RNA:DNA hybrids was initially unknown, we performed detection of both narrow and broad
632 peaks using -q 0.01 settings for narrow peaks and --broad-cutoff 0.1 (q 0.01) for broad peaks.
633 High confidence peaks and consensus peaks were identified using the bioconductor package
634 DiffBind⁴⁸. We performed peak calling against input DNA and against secondary IgG-only
635 control samples. More than 96 % of the m⁶A DRIP peaks called against input were also
636 identified using IgG-only controls. Peaks called against input were used for further analysis.
637 Consensus peaks were defined using the dba.peakset() function to select for peaks
638 overlapping in both replicates. In each instance the replicate sample BAM/bed files along
639 with the corresponding input samples were used as input. Additional details of bioinformatics
640 analysis are provided in Supplementary Note. Details on software and data deposition are
641 listed in the Life Sciences Reporting Summary.

642 **Statistics and reproducibility.** At least 2 and typically 3 independent experiments were
643 carried out for most of the assays. DRIP and DIP were performed in two and RNaseq in three
644 biologically independent experiments. All experiments were replicated independently. We
645 observed generally good correlation between the replicates. Statistical tests used for
646 individual experiments are described in corresponding figure legends. For quantification of
647 the m⁶A, γ H2AX and S9.6 signal intensities, statistical significance was determined using
648 unpaired two-tailed Student's t-test or unpaired two-tailed Welch's t-test. Signal intensity and
649 qPCR data were plotted and analyzed in GraphPad Prism 7.04.

650 **Ethics statement:** Use of the HUES7 hESC line was approved by the UK Medical Research
 651 Council Steering Committee, in association with the UK Stem Cell Bank. All mouse
 652 experiments were approved by the Norwegian Animal Research Authority by Norwegian
 653 Food Safety Authority and done in accordance with institutional guidelines at the Centre for
 654 Comparative Medicine at Oslo University Hospital. Animal work was conducted in
 655 accordance with the rules and regulations of the Federation of European Laboratory Animal
 656 Science Association's (FELASA).

657 **Data and materials availability:** The confocal raw data that support the findings of this
 658 study are available from the corresponding author upon request due to size considerations.
 659 The deep sequencing data have been deposited in the NCBI Sequence Read Archive (SRA)
 660 with the Bioproject ID: PRJNA474076
 661 (<https://submit.ncbi.nlm.nih.gov/subs/sra/SUB4074125>). The annotated bed files have been
 662 deposited to the following online repository ([https://bitbucket.org/ADAC_UoN/adac1075-
 663 bed-files/src](https://bitbucket.org/ADAC_UoN/adac1075-bed-files/src)). The in-house scripts used for the analysis can be found in the following online
 664 repository (https://bitbucket.org/ADAC_UoN/adac0175-code/src).

665 **Code availability:** The in-house scripts used for the analysis can be found in the following
 666 online repository (https://bitbucket.org/ADAC_UoN/adac0175-code/src).

667 **Methods-only References:**

- 668 32. Pozarowski, P., Darzynkiewicz, Z. Analysis of cell cycle by flow cytometry. *Methods*
 669 *Mol Biol.* **281**, 301-311 (2004).
- 670 33. Bubeck, D., Reijns, M.A., Graham, S.C., Astell, K.R., Jones, E.Y., Jackson, A.P. PCNA
 671 directs type 2 RNase H activity on DNA replication and repair substrates. *Nucleic Acids*
 672 *Res.* **39**, 3652-3266 (2011).
- 673 34. Abakir, A., Wheldon, L., Johnson, A. D., Laurent, P., Ruzov, A. Detection of Modified
 674 Forms of Cytosine Using Sensitive Immunohistochemistry. *J Vis Exp.* **114** (2016).
- 675 35. Hamperl, S., Bocek, M.J., Saldivar, J.C., Swigut, T., Cimprich, K.A. Transcription-
 676 Replication Conflict Orientation Modulates R-Loop Levels and Activates Distinct DNA
 677 Damage Responses. *Cell.* **170**, 774-786 (2017).
- 678 36. Ramsawhook, A. H. et al. Immunostaining for DNA Modifications: Computational
 679 Analysis of Confocal Images. *J Vis Exp.* **127** (2017).
- 680 37. Gackowski, D. et al. Accurate, Direct, and High-Throughput Analyses of a Broad
 681 Spectrum of Endogenously Generated DNA Base Modifications with Isotope-Dilution
 682 Two-Dimensional Ultraperformance Liquid Chromatography with Tandem Mass
 683 Spectrometry: Possible Clinical Implication. *Anal Chem.* **88**, 12128-12136 (2016).
- 684 38. Groh, M., Lufino, M. M., Wade-Martins, R., Gromak, N. R-loops associated with triplet
 685 repeat expansions promote gene silencing in Friedreich ataxia and fragile X syndrome.
 686 *PLoS Genet.* **10**, e1004318 (2014).
- 687 39. Ginno, P. A., Lott, P. L., Christensen, H. C., Korf, I., Chédin, F. R-loop formation is a
 688 distinctive characteristic of unmethylated human CpG island promoters. *Mol Cell.* **45**,
 689 814-825 (2012).
- 690 40. Weber, M. et al. Chromosome-wide and promoter-specific analyses identify sites of
 691 differential DNA methylation in normal and transformed human cells. *Nat Genet.* **37**,
 692 853-862 (2005).

- 693 41. Jachowicz, J.W., Bing, X., Pontabry, J., Bošković, A., Rando, O.J., Torres-Padilla, M.E.
694 LINE-1 activation after fertilization regulates global chromatin accessibility in the early
695 mouse embryo. *Nat Genet.* **49**, 1502-1510 (2017).
- 696 42. Jiang, H., Lei, R., Ding, S. W., Zhu, S. Skewer: a fast and accurate adapter trimmer for
697 next-generation sequencing paired-end reads. *BMC Bioinformatics.* **15**, 182, (2014).
- 698 43. Li, H., Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler
699 transform. *Bioinformatics.* **25**, 1754-1760 (2009).
- 700 44. Li, H. et al. 1000 Genome Project Data Processing Subgroup. The Sequence
701 Alignment/Map format and SAMtools. *Bioinformatics.* **25**, 2078-2079 (2009).
- 702 45. Chang, V.Y., Federman, N., Martinez-Agosto, J., Tatishchev, S.F., Nelson, S.F. Whole
703 exome sequencing of pediatric gastric adenocarcinoma reveals an atypical presentation of
704 Li-Fraumeni syndrome. *Pediatr Blood Cancer.* **60**, 570-574 (2013).
- 705 46. Marinov, G. K., Kundaje, A., Park, P. J., Wold, B. J. Large-scale quality analysis of
706 published ChIP-seq data. *G3 (Bethesda)* **4**, 209-223 (2014).
- 707 47. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* **9**, R137
708 (2008).
- 709 48. Ross-Innes, C.S., et al. Differential oestrogen receptor binding is associated with clinical
710 outcome in breast cancer. *Nature.* **7381**, 389-393 (2012).
- 711