1   **PCIF1 catalyzes m6Am mRNA methylation to regulate gene expression**

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24   **Highlights**

25   • PCIF1 is an evolutionarily conserved mRNA m6Am methyltransferase

26   • Loss of PCIF1 leads to a complete loss of m6Am, whereas m6A level and

27   distribution are not affected

28   • PCIF1 mediated m6Am does not affect RNA Pol II transcription or mRNA stability

29   • m6Am-Exo-Seq is a robust methodology that enables global m6Am mapping

30   • m6Am suppresses cap dependent translation

31

32   **Summary**

33   mRNA modifications play an important role in regulating gene expression. One of the

34   most abundant mRNA modifications is N6,2-O-dimethyladenosine (m6Am). Here, we

35   demonstrate that m6Am is an evolutionarily conserved mRNA modification mediated by

36   the Phosphorylated CTD Interacting Factor 1 (PCIF1), which catalyzes m6A methylation

37   on 2-O-methylated adenine located at the 5' ends of mRNAs. Furthermore, PCIF1

38   catalyzes only 5' m6Am methylation of capped mRNAs, but not internal m6A

39   methylation *in vitro* and *in vivo*. Our global mRNA methylation analysis revealed that

40   there is no crosstalk between m6Am and m6A mRNA methylation events, suggesting

41   that m6Am is functionally distinct from m6A. Importantly, our data indicate that m6Am

42   negatively impacts translation of methylated mRNAs by antagonizing cap binding

43   protein eIF4E. Together, we identify the first and only human mRNA m6Am

44   methyltransferase and demonstrate a novel mechanism of gene expression regulation

45   through PCIF1-mediated m6Am mRNA methylation in eukaryotes.

46

47   **Keywords:**

48   mRNA modifications, m6Am, PCIF1, translational regulation, transcriptional regulation,
49   epitranscriptomics, cap-dependent translation, eIF4E, gene expression, m6Am-Exo-Seq

Introduction

An important step in the regulation of gene expression is accomplished through chemical modifications of mRNAs. One of the best studied internal mRNA modifications is N6-methyladenosine (m6A), which is present on a diverse set of mRNAs, typically clustered around stop codons (Dominissini et al., 2012; Meyer et al., 2012). m6A is catalyzed by METTL3/METTL14 complex, and this modification regulates gene expression by influencing localization, stability, splicing or translation of mRNAs (Yang et al., 2018). Another abundant mRNA modification is N6,2-O-dimethyladenosine (m6Am), which occurs near the mRNA cap (Wei et al., 1975). 5' ends of eukaryotic mRNAs typically carry a 7-methylguanosine (m7G) cap linked to the rest of the mRNA by a triphosphate linkage. The first nucleotide after the m7G cap can be methylated on the ribose sugar. If this first nucleotide is 2-O-methyladeonisine (Am), it can be further methylated at its N6 position to generate m6Am. While the methylase mediating Am methylation is known, the methyltransferase responsible for generating m6Am has not been identified (Keith et al., 1978; Mauer et al., 2017). As a consequence, the impact of m6Am on gene regulation is still largely unexplored.

Using multiple approaches, we provide both *in vitro* and *in vivo* evidence supporting Phosphorylated CTD Interacting Factor 1 (PCIF1) as the one and only mammalian m6Am methyltransferase. In order to investigate the distribution of m6Am, we developed a novel transcription-wide mapping technique we name m6Am-Exo-Seq. PRO-Seq, RNA-seq and m6Am-Exo-Seq analyses carried out in wildtype and PCIF1

72   null cells show that mRNA stability of m6Am-enriched genes is largely unaffected by the

73   loss of PCIF1. In contrast, reporter assays carried out in PCIF1 null cells using mRNA

74   transcripts with and without m6Am suggest that m6Am may function to suppress protein

75   translation. Taken together, our findings identify a novel enzyme that mediates m6Am

76   methylation in the nucleus, which impacts translation in the cytoplasm.

77

Results

m6Am is an evolutionarily conserved RNA modification

To study the biological significance of m6Am, we developed a highly sensitive mass spectrometry approach (LC-MS/MS) to detect and quantify m6Am (Figure S1). The triphosphate bond between the m7G cap and the first nucleotide of the mRNA is not cleaved by the enzymes typically used to generate single nucleosides for LC-MS/MS analysis. Therefore, without the removal of the m7G cap, only the internal m6A and Am modifications are detectable in human mRNA (Figure 1B). On the other hand, m6Am is detectable only after treatment with a de-capping enzyme, indicating that m6Am is restricted to the first nucleotide adjacent to the m7G cap of the mRNA (Figure 1B) (Linder et al., 2015). Using this protocol, we analyzed mRNA isolated from various model organisms by LC-MS/MS. While m6Am was not detected in fission yeast, nematode or Drosophila samples, mRNA isolated from zebrafish, mouse and human cells yielded m6Am, indicating that m6Am is an evolutionary conserved mRNA methylation (Figure 1C).

PCIF1 is required for mRNA m6Am methylation *in vivo*

A putative methyltransferase enzyme that is conserved in zebrafish, mouse and human, but absent in yeast and nematodes, is PCIF1 (Phosphorylated CTD Interacting Factor 1) (Figure S2) (Iyer et al., 2016). PCIF1 contains a N-terminal WW domain, which is believed to mediate interactions with RNA Polymerase II (Pol II) phospho-CTD, and also a putative C-terminal methyltransferase domain (Ebmeier et al., 2017; Fan et al., 2003).

5

100   To determine whether PCIF1 is a m6Am methyltransferase, we generated several

101   independent PCIF1 knockout (KO) clonal cell lines in human melanoma MEL624 cells

102   and determined its mRNA m6Am level by LC-MS/MS (Fig. 2A and 2B). mRNA isolated

103   from a control MEL264 cell line yielded around 0.04% m6Am/A ratio. Strikingly,

104   knocking out PCIF1 resulted in a complete loss of this modification in mRNA samples

105   (Figure 2B). We further verified the absence of m6Am in PCIF1 KO cells by performing

106   thin layer chromatography (TLC) (Figures 2C and S3A). Although PCIF1 KO resulted in

107   a complete loss of m6Am, global m6A levels did not change, suggesting that PCIF1 is

108   not required for m6A modification and that m6A is not dependent on m6Am levels

109   (Figure S3B).

110

111   To determine whether it is the intrinsic enzymatic activity of PCIF1 that is responsible for

112   m6Am methylation, we carried out rescue experiments by introducing either wild-type

113   (WT) or catalytically inactive PCIF1 (APPA) back into the KO cells. While the expression

114   of wild-type PCIF1 restored the global m6Am level in the KO cells, expression of the

115   catalytic mutant with the conserved methyltransferase NPPF motif altered to APPA did

116   not result in m6Am detectable by mass spectrometry or TLC (Figures 2C and 2D).

117   PCIF1 contains a nuclear localization signal and has been suggested to be a nuclear

118   protein (Ebmeier et al., 2017; Fan et al., 2003; Hirose et al., 2008). Consistently, we

119   confirmed that PCIF1 is predominantly nuclear localized in both rescue cell lines by

120   immunofluorescence (Figure S3C). These results indicate that PCIF1 is required for

121   mRNA m6Am modification, which likely takes place in the nucleus *in vivo*.

6

Recombinant PCIF1 methylates capped mRNA *in vitro*

To determine whether this putative methyltransferase can directly catalyze m6Am, we purified full length recombinant PCIF1 from bacteria (Figure S4A) and performed *in vitro* methylation assays employing firefly luciferase mRNA with m7G-Am 5' cap as a substrate. Wild-type recombinant enzyme generated m6Am efficiently, whereas the APPA catalytic mutant did not show any activity (Figures 3A and S4B). Furthermore, PCIF1 did not generate m6A or Am modifications. On the other hand, generation of m6Am was accompanied by a decrease in the Am level, indicating that PCIF1 catalyzed conversion of Am to m6Am (Figure 3A). Consistent with previous findings (Kruse et al., 2011; Mauer et al., 2017), we failed to detect cap-adjacent m6A by TLC and PCIF1 KO cells show a greater accumulation of cap-adjacent Am (Figure S3A). These *in vitro* and *in vivo* data collectively suggest that PCIF1 specifically methylates Am-marked mRNAs.

We next investigated whether the m7G cap structure is required for the catalytic activity of PCIF1, using de-capped luciferase mRNA with 5' Am in *in vitro* methylation assays. The Am residue next to the intact m7G cap was readily converted to m6Am by recombinant PCIF1. In contrast, the Am residue on de-capped mRNA was not converted to m6Am, indicating that m7G cap is required for PCIF1 catalytic activity (Figure 3B). To determine whether there is a size requirement for capped mRNA to serve as a substrate, we used a capped RNA dinucleotide (m7G-AmG) in *in vitro* methylation assays. PCIF1 efficiently generated m6Am with the dinucleotide as a substrate with a K_m of 208 nM and K_{cat} at 0.34 min^{-1} (Figure 3C). This indicates that

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144 capped transcripts as short as 2 nucleotides can be efficiently methylated by PCIF1

145 (Figure 3D). Given that recombinant PCIF1 efficiently methylates capped mRNAs *in*

146 *vitro* and that deletion of PCIF1 results in a complete loss of mRNA m6Am methylation

147 *in vivo*, we conclude that PCIF1 is the only mRNA m6Am methyltransferase in human

148 cells.

149

150 **Loss of PCIF1 does not affect global mRNA m6A distribution *in vivo***

151 In order to identify the mRNAs that carry m6A and m6Am, we performed MeRIP-Seq

152 with an antibody against m6A in control and PCIF1 KO cell lines. Given that m6A and

153 m6Am are structurally similar, antibodies against m6A are expected to enrich for m6Am

154 at the 5' end of the mRNAs, as has been previously suggested (Linder et al., 2015).

155 However, although we were able to identify internal m6A loci enriched around stop

156 codons, as reported previously for other cell lines (Dominissini et al., 2012; Meyer et al.,

157 2012), we observed a very modest enrichment at the 5' end of mRNAs that was mostly

158 maintained in the PCIF1 KO cells (Figures 4 and 5A). Therefore, it appears that the

159 regular m6A MeRIP-Seq protocol does not efficiently capture m6Am-enriched

160 transcripts. In agreement with our *in vitro* and *in vivo* results (Figures 3A and S3B),

161 global m6A distribution did not change in two independent PCIF1 KO lines, compared to

162 control cells (Figure 4). Deletion of PCIF1, and hence complete loss of m6Am, altered

163 neither global m6A level (Figure S3B) nor genome-wide m6A distribution (Figure 4A),

164 suggesting that m6A and m6Am are independent mRNA modifications.

165 **Development of m6Am-Exo-Seq as a robust methodology that enables**

166 **global m6Am mapping**

167 To be able to map m6Am modification transcriptome-wide, we developed a novel

168 exonuclease assisted high-throughput sequencing methodology that we call m6Am-

169 Exo-Seq (Figure S5). We performed m6Am-Exo-Seq on mRNA from control and PCIF1

170 KO cells using 8 different m6A antibodies. Our new technique allowed us to observe a

171 clear enrichment of 5' ends immediate to transcription start sites only in mRNAs from

172 control cells, but not in mRNAs from PCIF1 KO cells, suggesting that m6Am-Exo-Seq

173 specifically pull-down m6Am-enriched transcripts (Figure 5A). Seven out of the eight

174 m6A antibodies tested with m6Am-Exo-Seq resulted in a significant enrichment of

175 m6Am only in control mRNA, demonstrating the robustness of this novel methodology

176 (Figure S5).

177

178 To globally map m6Am, we applied m6Am-Exo-Seq with a monoclonal m6A antibody in

179 MEL624 cell line using three biological replicates of control and PCIF1 KO cells (Figure

180 5). We identified 640 genes that are enriched for this modification (Table S1). These

181 transcripts are among the highly expressed genes in MEL624 cell line (Figure 5B), and

182 are in diverse pathways including splicing, vesicular transport and mitochondrial

183 metabolism (Figures 5B and 5C). m6Am was also mapped in HEK293T cells previously

184 (Linder et al., 2015). While most of the m6Am transcripts are methylated in both

185 HEK293T and MEL624 cells, around 40% of m6Am transcripts are unique to MEL624

186   cells (Figure S6A), suggesting that m6Am is a dynamic epitranscriptomic mark with cell-

187   type specific patterns.

188

189   **PCIF1-mediated deposition of m6Am does not alter transcription or**

190   **stability of target genes.**

191   PCIF1 interacts with the phosphorylated C-terminal domain (CTD) of Pol II, and has

192   been implicated in negative regulation of gene expression (Ebmeier et al., 2017; Fan et

193   al., 2003; Hirose et al., 2008). However, recent studies suggest that deposition of m6Am

194   stabilizes mRNA or stimulates translation (Mauer et al., 2017), both of which would

195   increase the expression of target genes. On the other hand, a separate study failed to

196   find a correlation between m6Am methylation and mRNA stability (Wei et al., 2018). We

197   thus wished to investigate the mechanism of m6Am activity in our system, where we

198   can precisely abolish m6Am by PCIF1 KO, without perturbing m6A levels. We first

199   performed RNA-Seq in PCIF1 KO MEL624 cells and a control cell line. We defined 236

200   mRNA species that were significantly more abundant in both PCIF1 KO cell lines, and

201   304 mRNAs with reduced abundance (Figure 6A, Tables S2 and S3). To distinguish

202   whether these changes in mRNA levels were due to altered transcription mediated by

203   m6Am, we next performed PRO-Seq in control and PCIF1 KO cells. PRO-Seq

204   specifically measures engaged Pol II across the genome, providing definitive

205   information on levels of active transcription (Kwak et al., 2013). These assays were

206   spike normalized to enable accurate quantification and comparisons between samples.

207   PRO-Seq signals across the 236 up-regulated genes from RNA-seq revealed that these

208 genes were more highly transcribed in PCIF1 KO cells (Figure 6B). Likewise, down-

209 regulated genes were less actively transcribed (Figure 6B). Importantly, there was a

210 strong correlation between the changes observed in RNA-Seq and PRO-Seq, indicating

211 that changes in RNA abundance could be explained fully by altered transcription

212 (r=0.67, Figure 6D).

213

214 Furthermore, we noted no significant overlap between m6Am-enriched genes and

215 genes with significantly altered signals in RNA-Seq or PRO-Seq assays (Figure 6A,

216 Tables S1-S5). PCIF1 KO did not change RNA-seq or PRO-Seq signal within m6Am-

217 enriched genes as compared to controls (Figure 6C). Moreover, the loss of PCIF1 did

218 not alter Pol II levels or distribution at noncoding snRNA genes that are marked by

219 m6Am (Figure S6B) (Mauer et al., 2018; Wei et al., 2018). Taken together, our data

220 suggest that although PCIF1 has been reported to bind RNA Pol II, it does not play a

221 significant role in transcription at m6Am target genes. Moreover, steady-state transcript

222 levels of m6Am-marked genes, as determined by RNA-Seq in PCIF1 KO cells, are not

223 affected by the loss of this modification, suggesting that stability of m6Am-marked

224 mRNAs is not significantly affected by PCIF1-mediated methylation.

225

226 **m6Am suppresses cap-dependent translation**

227 Regulation of cap binding protein eIF4E during translation initiation is a key event in

228 protein translation control. eIF4E binds m7G cap of mRNAs and recruits other

229 translation initiation factors and ribosomes for translation initiation (Sonenberg and

230   Gingras, 1998). The C terminal loop of cap bound eIF4E is in close proximity to the first

231   nucleotide of the mRNA (Tomoo et al., 2002, 2005). The N6 residue of the cap-adjacent

232   adenine of mRNA forms a critical hydrogen bond with threonine 205 residue of human

233   eIF4E (Figure S7) (Tomoo et al., 2002, 2005). We thus hypothesized that modification

234   of this adenine by PCIF1 to form m6Am may affect this interaction and hence impact

235   translation at the initiation step. To test this, we used well established reporter assays to

236   analyze the effect of m6Am on translation efficiency. We first transfected PCIF1 KO

237   cells with EGFP mRNA with either m7G-Am or m7G-m6Am 5' ends and determined the

238   GFP signal by microscopy and flow cytometry (Figure 7A and S8). Although both

239   mRNAs are transfected with similar efficiencies, m6Am-EGFP transfected cells showed

240   diminished GFP signal (Figures 7A-D and S8), suggesting that m6Am-containing

241   transcripts are translated less efficiently that the unmethylated counterparts *in vivo*. To

242   further validate the effect of m6Am on translation and to investigate whether this effect

243   is independent of the mRNA sequence, we performed *in vitro* translation assays using

244   varying amounts of firefly luciferase mRNAs with cap adjacent Am or m6Am

245   modifications, followed by luminometric quantification. Similar to our *in vivo* reporter

246   assays, m6Am suppressed translation of luciferase mRNA *in vitro* (Figure 7E). Next, we

247   investigated whether m6Am-mediated suppression is specific to cap-dependent

248   translation by employing Am or m6Am methylated bi-cistronic mRNA transcripts in *in*

249   *vitro* translation assays. m6Am suppressed the translation of cap-dependent firefly

250   luciferase whereas IRES mediated translation of renilla luciferase was unaffected,

251   indicating that m6Am specifically suppresses cap-dependent translation (Figure 7F).

252

253   Given that m6Am is a cap proximal RNA modification that suppresses cap-dependent

254   translation we next determined whether m6Am affects the interaction between mRNA

255   and cap binding protein eIF4E. Specifically, we analyzed binding dynamics between

256   recombinant human eIF4E protein and capped Am and m6Am RNA oligos, as well as

257   de-capped RNA oligos, by microscale thermophoresis (MST). As expected, the affinity

258   of eIF4E to the RNA decreased significantly when the mRNA cap was removed.

259   Importantly, we found that m6Am also decreased the affinity of eIF4E to the capped

260   RNA oligo, in comparison to capped Am RNA (Figure S9). Taken together, these data

261   suggest that m6Am modification suppresses cap-dependent mRNA translation, possibly

262   by decreasing eIF4E binding.

263

264 **Discussion**

265 In this study, we uncovered a new regulatory mechanism mediated by PCIF1 m6Am

266 deposition on mRNAs. Our *in vivo* and *in vitro* data demonstrate that PCIF1 is the only

267 methyltransferase necessary and sufficient for the conversion of cap-Am mRNAs to

268 cap-m6Am mRNAs in human cells. Moreover, we provide evidence that m6Am

269 negatively regulates cap dependent translation by decreasing the binding of eIF4E to

270 m6Am-containing transcripts. Our findings highlight a previously unknown translational

271 regulatory pathway that begins with a modification in the nucleus, opening new avenues

272 of investigation in the epitranscriptome and translation fields.

273

274 Given that m6Am marks diverse transcripts including noncoding snRNAs and histone

275 mRNAs that are translated independently of eIF4E, we predict that this modification will

276 play diverse functions, which include but are not limited to protein translation. m6Am

277 has been suggested to play a role in regulating the stability of modified mRNAs in

278 HEK293T cells (Mauer et al., 2017) . However, our analyses show that neither

279 transcription nor abundance of m6Am-enriched transcripts are affected in PCIF1 KO

280 MEL624 cells (Figure 6). However, we cannot rule out a potential role of m6Am in

281 regulating mRNA stability under conditions that were not tested in this study.

282

283 Rather than altering mRNA stability, we found that m6Am suppresses cap dependent

284 translation, possibly by reducing binding of eIF4E to the capped mRNAs. Our findings

285 strongly suggest a role for PCIF1 in protein translation control, which will be validated by

14

286   investigation of translation of endogenous mRNAs by PCIF1. Collectively, the

287   identification of PCIF1 as the sole, evolutionarily conserved, mRNA m6Am

288   methyltransferase paves the way for investigating the impact of PCIF1 on gene

289   expression of m6Am-marked transcripts in diverse biological contexts.

290

Acknowledgments

Author contributions:

E.S., D.V-G. and Y.S. conceived and designed the study. E.S., D.V-G., A.D., H.C., T.H. and W.S. performed the research. T.H. and K.A. performed and analyzed PRO-Seq. E.S., D.V-G. and Y.S. wrote the manuscript and all authors contributed to the final version of the manuscript.

16

313 **Declaration of interests**

314 Y.S. is a cofounder of Constellation Pharmaceuticals and Athelas Therapeutics, and a

315 consultant for Active Motif, Inc.

316

317 **Data availability**

318 Once the paper is published, all the genome-wide data will be accessible through GEO

319 with accession number GSE122803.

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References

Dominissini, D., Moshitch-Moshkovitz, S., Schwartz, S., Salmon-Divon, M., Ungar, L., Osenberg, S., Cesarkas, K., Jacob-Hirsch, J., Amariglio, N., Kupiec, M., et al. (2012). Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. Nature *485*, 201–206.

Ebmeier, C.C., Erickson, B., Allen, B.L., Allen, M.A., Kim, H., Fong, N., Jacobsen, J.R., Liang, K., Shilatifard, A., Dowell, R.D., et al. (2017). Human TFIIH Kinase CDK7 Regulates Transcription-Associated Chromatin Modifications. Cell Rep. *20*, 1173–1186.

Fan, H., Sakuraba, K., Komuro, A., Kato, S., Harada, F., and Hirose, Y. (2003). PCIF1, a novel human WW domain-containing protein, interacts with the phosphorylated RNA polymerase II. Biochem. Biophys. Res. Commun. *301*, 378–385.

Hirose, Y., Iwamoto, Y., Sakuraba, K., Yunokuchi, I., Harada, F., and Ohkuma, Y. (2008). Human phosphorylated CTD-interacting protein, PCIF1, negatively modulates gene expression by RNA polymerase II. Biochem. Biophys. Res. Commun. *369*, 449–455.

Iyer, L.M., Zhang, D., and Aravind, L. (2016). Adenine methylation in eukaryotes: Apprehending the complex evolutionary history and functional potential of an epigenetic modification. Bioessays *38*, 27–40.

Keith, J.M., Ensinger, M.J., and Moss, B. (1978). HeLa Cell RNA(2'-0-methyladenosine-W-)-methyltransferase Specific for the Capped &End of Messenger RNA*.

Kruse, S., Zhong, S., Bodi, Z., Button, J., Alcocer, M.J.C., Hayes, C.J., and Fray, R. (2011). A novel synthesis and detection method for cap-associated adenosine

343   modifications in mouse mRNA. Sci. Rep. *1*, 126.

344   Kwak, H., Fuda, N.J., Core, L.J., and Lis, J.T. (2013). Precise maps of RNA polymerase

345   reveal how promoters direct initiation and pausing. Science *339*, 950–953.

346   Linder, B., Grozhik, A. V, Olarerin-George, A.O., Meydan, C., Mason, C.E., and Jaffrey,

347   S.R. (2015). Single-nucleotide-resolution mapping of m6A and m6Am throughout the

348   transcriptome. Nat. Methods *12*, 767–772.

349   Mauer, J., Luo, X., Blanjoie, A., Jiao, X., Grozhik, A. V., Patil, D.P., Linder, B., Pickering,

350   B.F., Vasseur, J.-J., Chen, Q., et al. (2017). Reversible methylation of m6Am in the 5′

351   cap controls mRNA stability. Nature *541*, 371–375.

352   Mauer, J., Sindelar, M., Guez, T., Vasseur, J.-J., Rentmeister, A., Gross, S., Pellizzoni,

353   L., Debart, F., Goodarzi, H., and Jaffrey, S.R. (2018). The RNA demethylase FTO

354   targets m 6 Am in snRNA to establish distinct methyl isoforms that influence splicing.

355   Meyer, K.D., Saletore, Y., Zumbo, P., Elemento, O., Mason, C.E., and Jaffrey, S.R.

356   (2012). Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs

357   and near stop codons. Cell *149*, 1635–1646.

358   Sonenberg, N., and Gingras, A.C. (1998). The mRNA 5' cap-binding protein eIF4E and

359   control of cell growth. Curr. Opin. Cell Biol. *10*, 268–275.

360   Tomoo, K., Shen, X., Okabe, K., Nozoe, Y., Fukuhara, S., Morino, S., Ishida, T.,

361   Taniguchi, T., Hasegawa, H., Terashima, A., et al. (2002). Crystal structures of 7-

362   methylguanosine 5'-triphosphate (m(7)GTP)- and P(1)-7-methylguanosine-P(3)-

363   adenosine-5',5'-triphosphate (m(7)GpppA)-bound human full-length eukaryotic initiation

364   factor 4E: biological importance of the C-terminal flexible region. Biochem. J. *362*, 539–

365   544.

366   Tomoo, K., Matsushita, Y., Fujisaki, H., Abiko, F., Shen, X., Taniguchi, T., Miyagawa,

367   H., Kitamura, K., Miura, K.-I., and Ishida, T. (2005). Structural basis for mRNA Cap-

368   Binding regulation of eukaryotic initiation factor 4E by 4E-binding protein, studied by

369   spectroscopic, X-ray crystal structural, and molecular dynamics simulation methods.

370   Biochim. Biophys. Acta *1753*, 191–208.

371   Wei, C.M., Gershowitz, A., and Moss, B. (1975). N6, O2'-dimethyladenosine a novel

372   methylated ribonucleoside next to the 5' terminal of animal cell and virus mRNAs.

373   Nature *257*, 251–253.

374   Wei, J., Liu, F., Lu, Z., Fei, Q., Ai, Y., He, P.C., Shi, H., Cui, X., Su, R., Klungland, A., et

375   al. (2018). Differential m6A, m6Am, and m1A Demethylation Mediated by FTO in the

376   Cell Nucleus and Cytoplasm. Mol. Cell *71*, 973–985.e5.

377   Yang, Y., Hsu, P.J., Chen, Y.-S., and Yang, Y.-G. (2018). Dynamic transcriptomic m6A

378   decoration: writers, erasers, readers and functions in RNA metabolism. Cell Res. *28*,
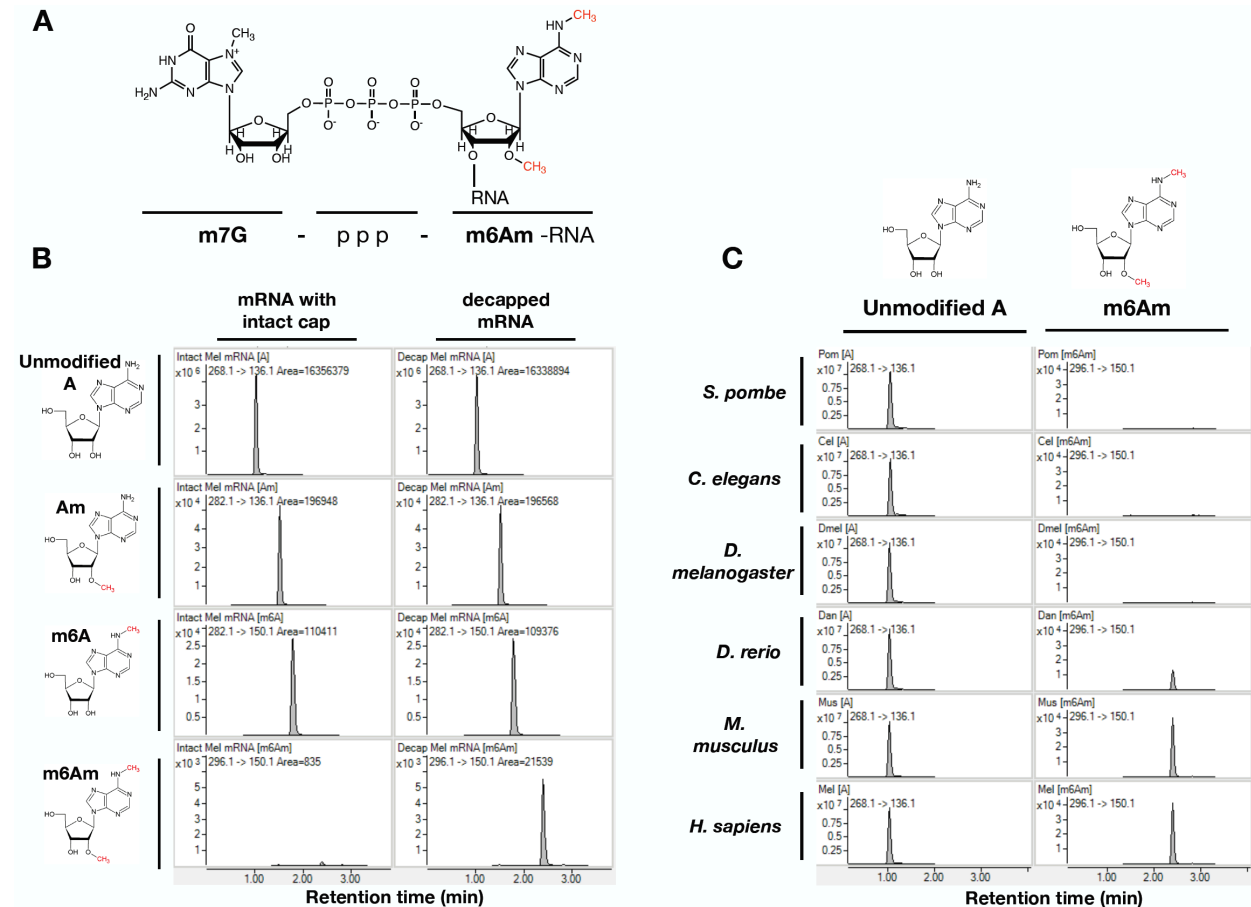
379   616–624.

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Figures

Figure 1. m6Am is an evolutionarily conserved RNA modification that is restricted to 5' end of mRNAs

1A. Chemical structure of 5' end of eukaryotic mRNA with m6Am methylation.

1B. MS spectra of mRNA from MEL624 human melanoma cell line with or without treatment by de-capping enzyme to remove m7G cap prior to digestion for MS analysis. m6Am is detectable only when mRNA is de-capped.

1C. MS spectra of de-capped mRNAs isolated from indicated organisms.
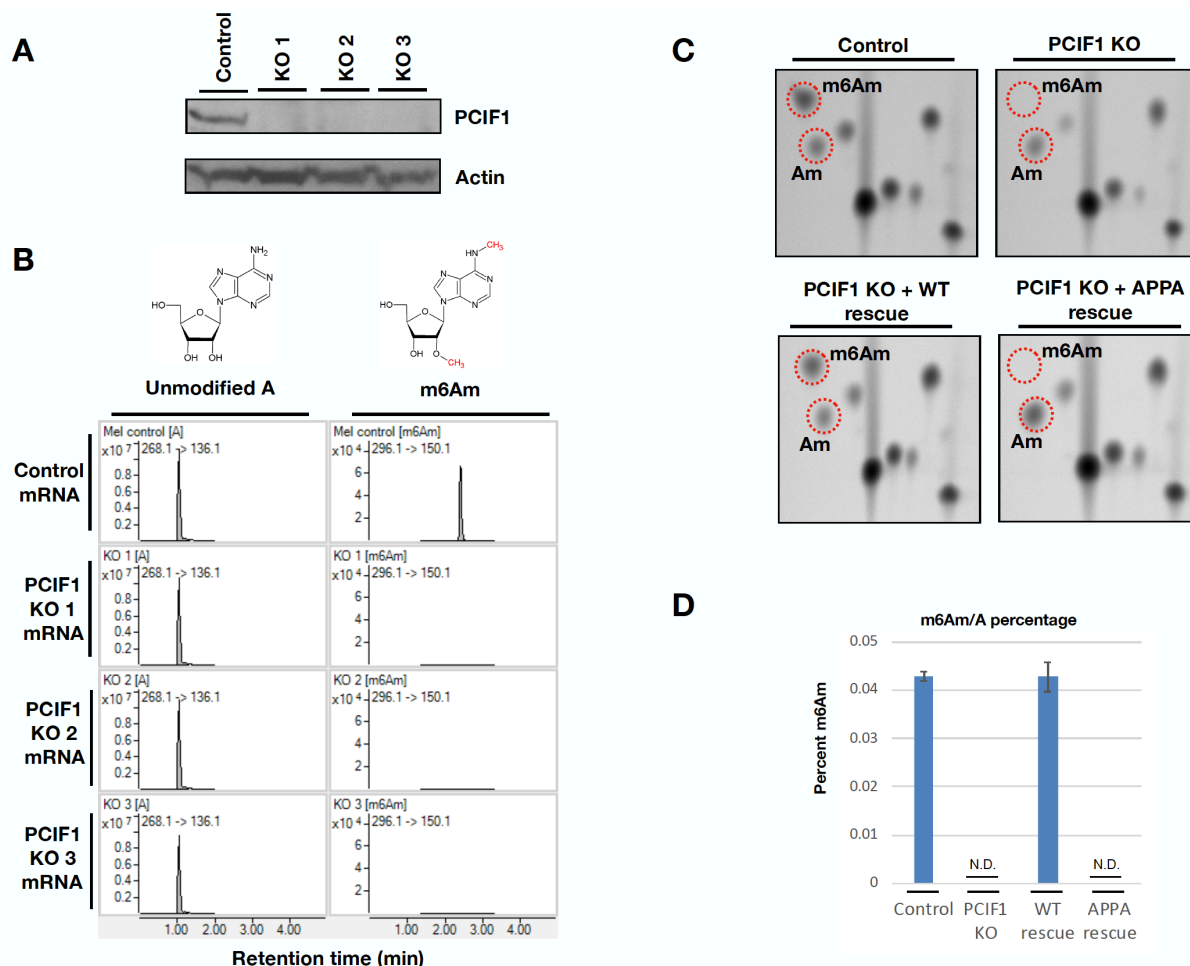
Figure 2. PCIF1 is required for mRNA m6Am methylation *in vivo*

2A. Western blot analysis of control and three independent PCIF1 KO MEL624 cell lines with antibodies against endogenous PCIF1. Beta-actin is shown as a loading control.

2B. MS spectra of de-capped mRNA from control and three independent PCIF1 KO MEL624 cell lines.

2C. Thin-layer chromatography analysis of cap-adjacent nucleotides from mRNA isolated from control, PCIF1 KO and KO cells rescued with wild type or catalytic mutant PCIF1 (APPA).

2D. HPLC-MS/MS m6Am/A percentages of mRNAs from control, PCIF1 KO and KO cells rescued with wild type or catalytic mutant PCIF1 (APPA). (N.D., Not Detected). (n=3). Error bars depict standard error.
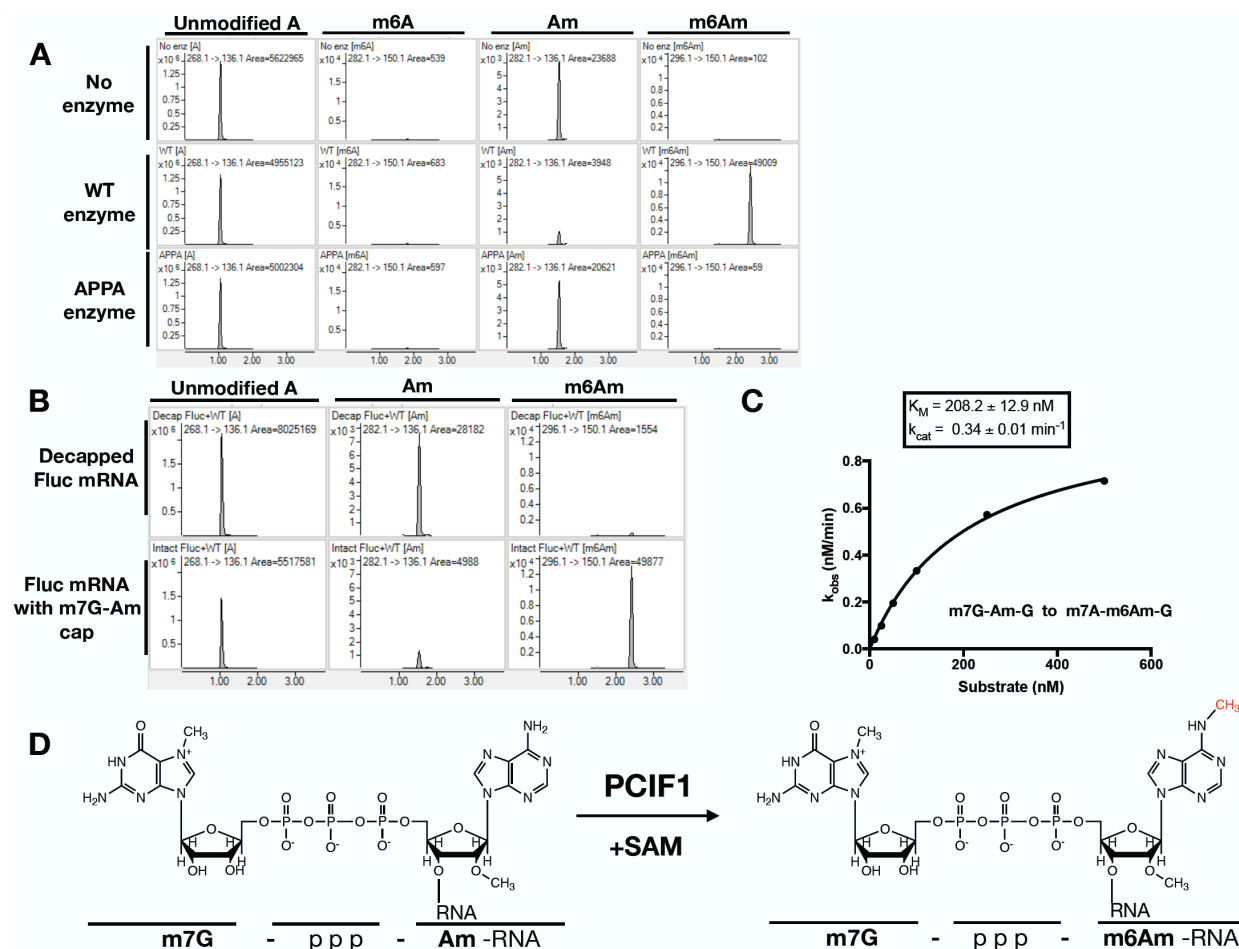
Figure 3. Recombinant PCIF1 methylates capped mRNA *in vitro*.

3A. Firefly luciferase mRNA with m7G-Am cap structure is methylated with wild-type or catalytic mutant recombinant PCIF1 *in vitro*. The methylation pattern of mRNA was analyzed by LC-MS/MS.

3B. In vitro methylation assays were performed with recombinant PCIF1 enzyme using de-capped firefly luciferase mRNA or mRNA with intact m7G-Am cap.

3C. Michaelis–Menten kinetics of recombinant PCIF1 was determined using m7G-Am capped dinucleotide RNA oligo by LC-MS/MS analysis.

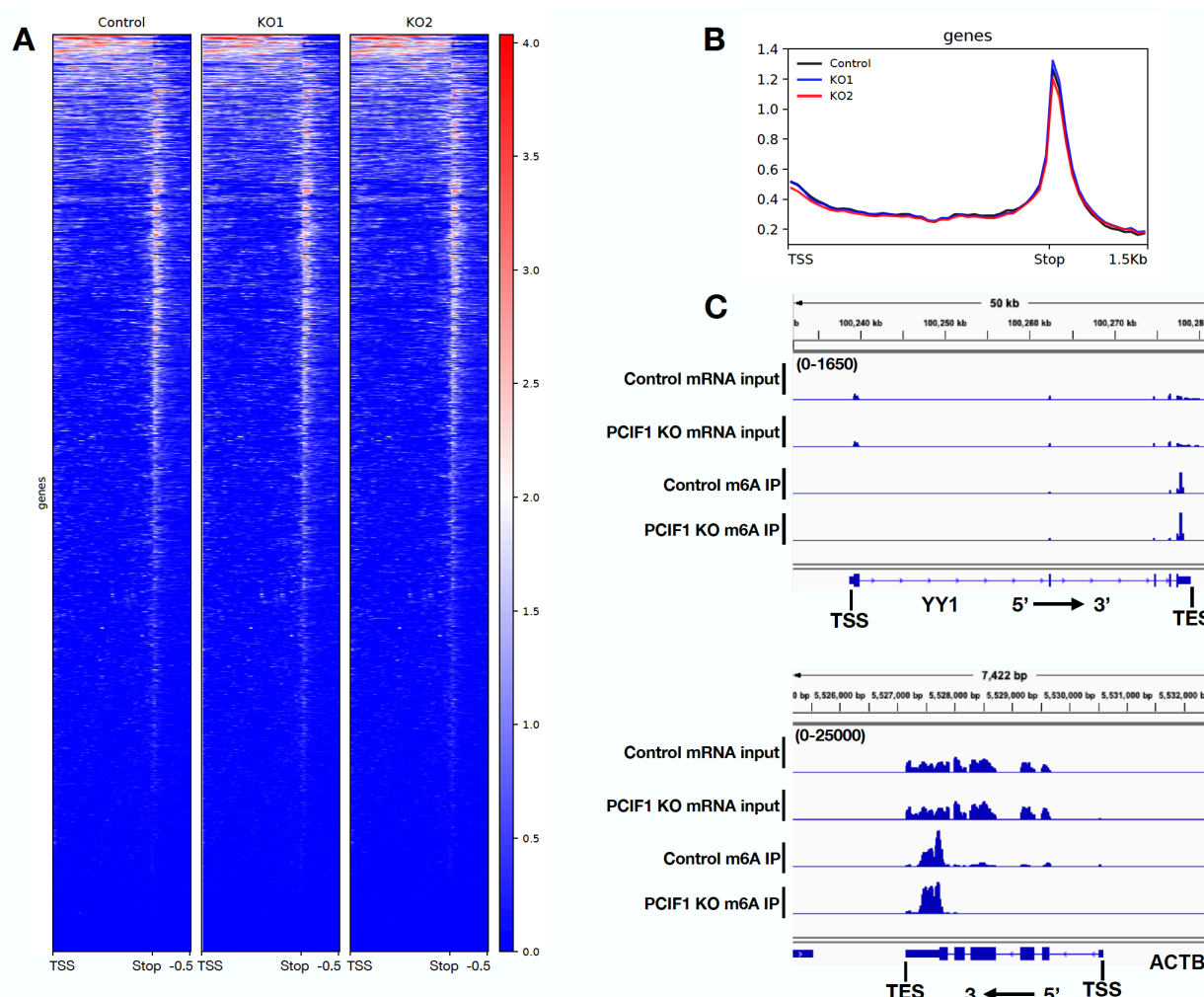3D. Chemical structures of PCIF1 substrate and product.

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Figure 4. Loss of PCIF1 does not affect global mRNA m6A distribution *in vivo*.

4A. Heat map of m6A enrichment over Input of genes overlapping with m6A peaks (n=4,460) in MEL624 cell line determined by m6A MeRIP-Seq in control and two independent PCIF1 KO cell lines.

4B. Metagene plots of m6A enrichment over Input of genes overlapping with m6A peaks (n=4,460) in MEL624 cell line determined by m6A MeRIP-Seq in control and two independent PCIF1 KO cell lines.

4C. Genome browser views of two examples genes with m6A enrichment near stop codon.
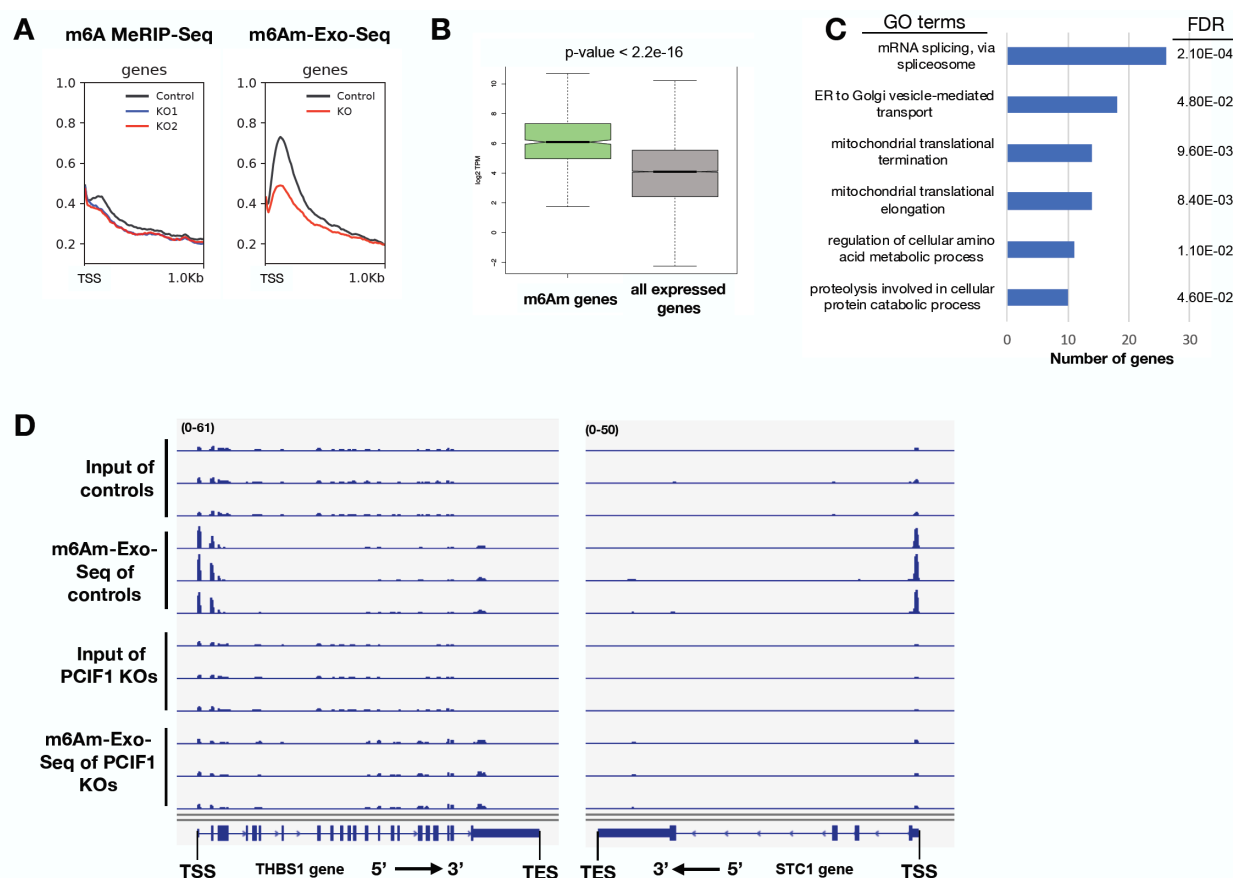
24

433
434
Figure 5. m6Am-Exo-Seq as a robust methodology that enables global m6Am mapping.

436
5A. Metagene plots of m6A(m) enrichment over Input near TSS of all expressed genes
(n=15,258) in MEL624 cells employing either m6A MeRIP-Seq or m6Am-Exo-Seq methodology
in control and PCIF1 KO cells.
5B. Boxplots of expression level (log2 TPM) of m6Am mRNAs (n=640) vs. all mRNAs
expressed in MEL624 cell line (n=15,258). The observed difference is significant (p-value <
2.2e-16) using a Mann-Whitney test. Box plots show the 25th–75th percentiles and error bars
depict the 10th–90th percentiles.
5C. Biological process Gene Ontology (GO) terms that have FDR<0.05 identified by DAVID 6.8
5D. Genome browser views of 2 example genes with m6Am enrichment using m6Am-Exo-Seq
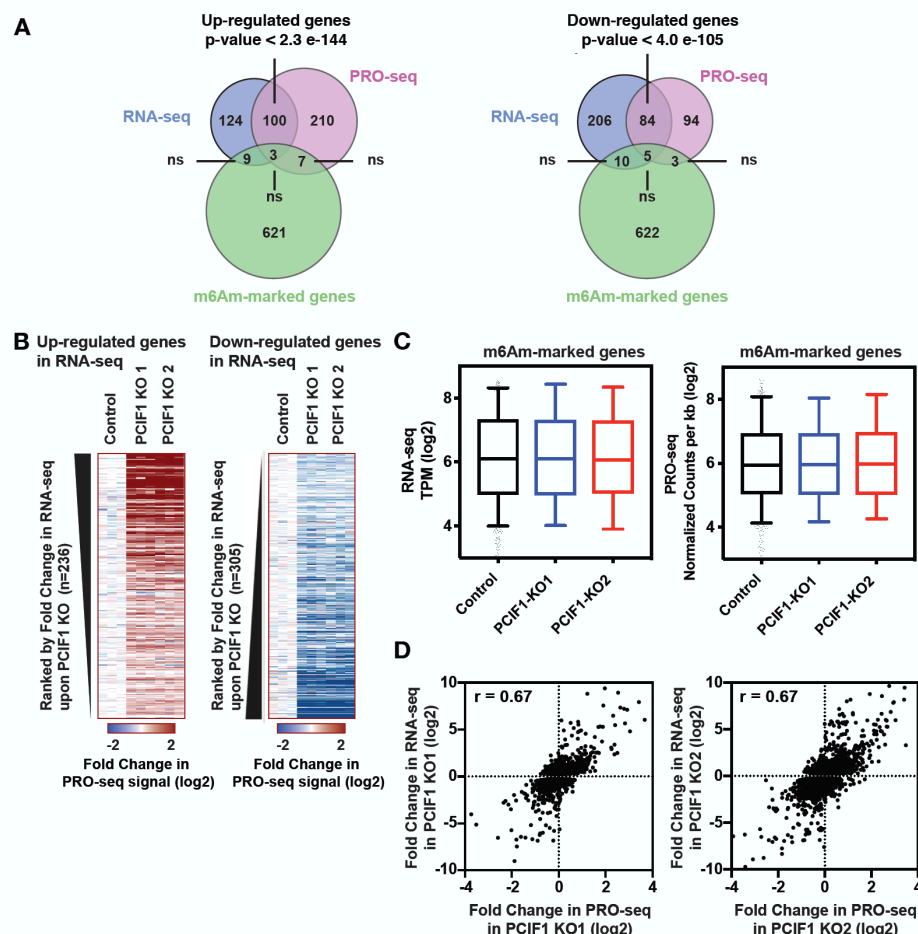446

447
448
Figure 6. PCIF1-mediated deposition of m6Am does not alter transcription or stability of target genes.

451
6A. Venn diagrams displaying overlap between m6Am-enriched genes in MEL624 cells and genes identified as significantly upregulated (left) or downregulated (right) in both PCIF1 KO MEL624 cell lines, by RNA-Seq and PRO-Seq. P-values are calculated with a hypergeometric test.
6B. Heatmaps of PRO-Seq signal at genes found to be up- or down-regulated using RNA-Seq in PCIF1 KO cells. Shown are the fold changes in PRO-Seq signal across the bodies of genes up-regulated in RNA-Seq (left) or down-regulated in RNA-Seq (right). Genes are ranked from most upregulated to least (at left) and least down-regulated to most (at right), demonstrating that PRO-Seq signal scales with results from RNA-seq.
6C. RNA-Seq signal (left) and normalized PRO-Seq signal (right) within 640 m6Am-enriched genes in Control MELF624 cells, as compared to PCIF1 KO cells. Box plots show the 25th–75th percentiles and error bars depict the 10th–90th percentiles. Differences are not significant (P > 0.01). P-values were calculated using the Mann-Whitney test.
6D. Scatter plots displaying the correlation of RNA-Seq vs PRO-Seq KO/control fold change signal. Only genes that have a fold change > 1.2 according to RNA-Seq in KO1 or KO2 were plotted. The total number of genes used in KO1 plot is 5,774 and KO2 is 5,580. Both KOs have the same Pearson correlation value of 0.67.
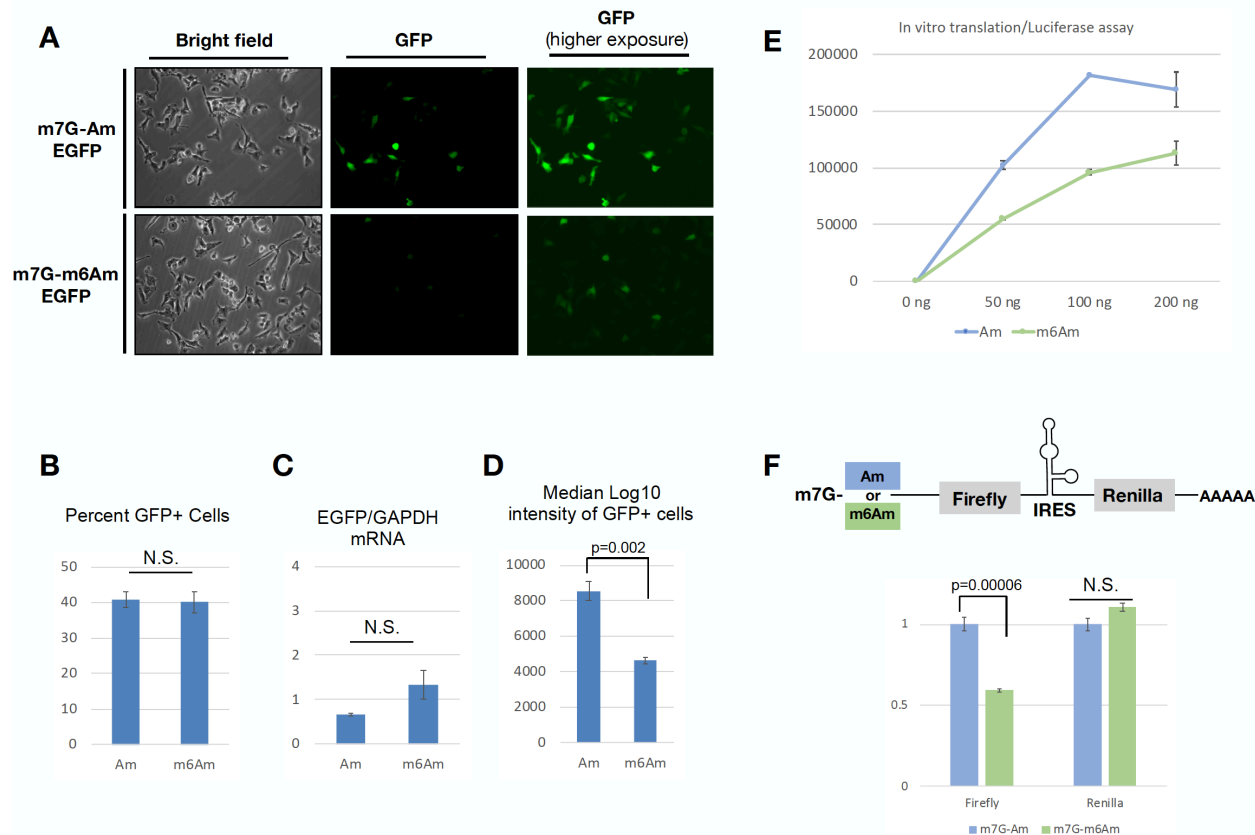469

26

Figure 7. m6Am suppresses cap-dependent translation.

7A. EGFP mRNA that starts with either m7G-Am or m7G-m6Am was transfected into PCIF1 KO MEL624 cells. GFP signal was determined by fluorescence microscopy 24 hours post-transfection.

7B. The percentage of GFP positive cells from the experiment in 7A determined by flow cytometry (n=3 replicates).

7C. EGFP mRNA level from the cells in experiment 7A as determined by RT-qPCR. (n=3 replicates).

7D. Median fluorescence intensity of the cells from the experiment in 7A determined by flow cytometry. P-values in 7B-D are calculated using unpaired t-tests. (n=3 replicates).

7E. *In vitro* translation assays were carried out with indicated amounts of luciferase mRNA starting with m7G-Am or m7G-m6Am using rabbit reticulocyte system. Resulting luciferase signal was quantified on a plate reader. (n=4 replicates)

7F. In vitro translation assays were carried out with bicistronic construct that starts with m7G-Am or m7G-m6Am using rabbit reticulocyte system. Resulting firefly and renilla luciferase signals were quantified on a plate reader. (n=4 replicates). The p-values were calculated with an unpaired t-test. All error bars in this figure depict standard error.

27

491   **Methods**

492

493   **Cell culture**

494   MEL624 (RRID: CVCL_8054) and HEK293T (RRID:CVCL_0063) cells are grown in

495   DMEM (Gibco) supplemented with 10% FBS and 1x pen/strep solution (Gibco) at 37°C

496   in a CO_2 incubator. Cells are split once they reach ~90% confluence with a 1:8 ratio.

497   Cells are routinely checked for mycoplasma infection.

498

499   **mRNA purification**

500   Total RNA is isolated from MEL624 cells with TRIzol reagent following manufacturer's

501   instructions. 200 µg of total RNA are subjected to two rounds of Poly(A) mRNA

502   purification using the magnetic mRNA isolation kit from (NEB). 100 µL of oligo d(T)

503   magnetic beads are used per purification. mRNA is eluted in a final volume of 50 µL, in

504   elution buffer.

505

506   **Processing of RNA Samples for Mass Spectrometry**

507   250 or 500 ng of mRNA is de-capped with a decapping enzyme in 20 µL reaction

508   volume for an hour at 37°C. Then the RNA is digested with 0.5U of nuclease P1 (Sigma)

509   in P1 buffer in 50 uL reaction at 37°°C for 2 hours. To dephosphorylate the single

510   nucleotides, 1U of rSAP (NEB) is added to a final reaction volume of 100 µL with

511   CutSmart buffer (NEB) and incubated for an hour at 37°°C. The 100 µL samples are

512   filtered with Millex-GV 0.22u filters.

28

HPLC-MS/MS Analysis

10 uL from each sample is injected into Agilent 6470 Triple Quad LC/MS instrument.

The samples are run in mobile phase buffer A (water with 0.1% Formic Acid) and 2 to

20% gradient of buffer B (Methanol with 0.1% Formic Acid). MRM transitions are

measured for adenosine (268.1 to 136.1, retention time 1.03 min), guanosine (284.1 to

152.1, retention time 1.15 min), 2'-O-methyladenosine (Am) (282.1 to 136.1, retention

time 1.52 min), N6-methyladenosine (m6A) (282.1 to 150.1, retention time 1.79 min),

N6,2-O-dimethyladenosine (m6Am) (296.1 to 150.1, retention time 2.40 min). The

concentrations of each compound in the samples are calculated using calibration curves

constructed with standard compounds of adenosine (Abcam), N6-methyladenosine

(Abcam), N6,2'-O-dimethyladenosine (Toronto Research Chemicals). For LC/MS-MS

data collection and analysis, Agilent Mass Hunter LC/MS Data Acquisition Version

B.08.00 and Quantitative Analysis Version B.07.01 softwares are used.

mRNA purification from different species

Total RNA was extracted with TRIzol from the following sources:

Human: MEL624 melanoma cell line (RRID: CVCL_8054)

Mouse: C2C12 mice myoblast cell line (ATCC Cat# CRL-1772, RRID:CVCL_0188)

Zebrafish: WT adult whole fish (ZFIN Cat# ZDB-GENO-990623-3, RRID:ZFIN_ZDB-

GENO-990623-3)

Worm: Adult N2 (WT) *C. elegans* (WB Cat# N2, RRID:WB-STRAIN:N2)

Fly: KC drosophila cell line (RRID:CVCL_Z833)

29

535   Yeast: *S. pombe,* ED666 h+ ade6-M210 ura4-D18 leu1-32 from Bioneer Inc.

536   mRNA was extracted from 50-100 μg of total RNA as described above.

537

538   **Generation of PCIF1 KO cell lines**

539   Two complementary strategies are followed to generate clonal PCIF1 MEL624 cell lines.

540   3 sgRNAs were cloned into the lentiCRISPR V2 vector (RRID:Addgene_52961):

541   PCIF1 sgRNA-1: TAGCGGTAAAGGAGCCACTG

542   PCIF1 sgRNA-2: CGGTTGAAAGACTCCCGTGG

543   PCIF1 sgRNA-3: ATTCACCAACCAGTCCCTGT

544   Additionally, a random non-targeting sgRNA was cloned into lentiCRISPR V2 as a

545   control:

546   Random sgRNA-1: ATCGTTTCCGCTTAACGGCG

547   For the first strategy, 1 μg of lentiCRISPR V2 plus 0.5 μg of psPAX2

548   (RRID:Addgene_12260) and 0.2 μg of pMD2.G (RRID:Addgene_12259) packaging

549   plasmids were mixed with 3 μg of polyethylenimine (PEI) in 100 ul of OptiMEM (Gibco)

550   and added into 6-well plates of 30% confluent HEK293T cells (RRID:CVCL_0063). 4

551   hours post-transfection, the transfection media was replaced with 1.5 mL of regular

552   media (DMEM, 10% FBS, 1X pen/strep). 48 hours post-transfection the media

553   containing viral particles was passed through a 0.45 um filter and the viral particles from

554   the 3 PCIF1 sgRNAs were combined. 200 μL of the pooled viral particles were used to

555   infect a 6-well plate of ~30% confluent MEL624. At the same time, another 6-well plate

556   was infected with 200 μl from the non-targeting sgRNA. Under these conditions we

30

557   reached an infection rate of ~50%. 12 hours post-infection, cells were washed with PBS

558   and media with 1 μg/mL of puromicyn was added to select infected cells. 48 hours post-

559   selection, cells were grown with regular media for additional 48 hours and single-cell

560   sorted in a 96-well plate in a FACS Aria cell sorter. Individual clones were validated as

561   true KOs by western blot with a PCIF1 specific antibody (Bethyl A304-711A). This

562   strategy generated PCIF1 KO1 and PCIF1 KO2 cell lines, as well as the control cell line

563   from the non-targeting sgRNA clones.

564

565   In the second strategy, and to avoid integration of cas9 into the genome, 2 μg of

566   lentiCRISPRv2 from the PCIF1 sgRNA-2 were transfected into a ~30% confluent 6-well

567   plate of MEL624 cells using 4 μl of lipofectamine 2000 according to manufacturer's

568   instructions. 24 hours post-transfection, cells were selected with 1 μg/mL of puromycin

569   for 48 hours and growth for additional 48 hours without selection. Cells were single-cell

570   sorted and evaluated as described above. This strategy generated the PCIF1 KO used

571   for the rescues and m6Am mapping experiments.

572

573   **PCIF1 KO cell line rescue**

574   WT and catalytic-deficient versions of PCIF1 coding sequence were cloned into the

575   PHAGE-puro lentiviral plasmid with a C-terminal HA-FLAG tag. Lentiviruses were

576   prepared as described above. 500 μL of lentiviral particles were used to infect ~30%

577   confluent MEL624 KO cells. 12 hours post-infection, viruses were washed out with PBS

578   and cells were selected with media containing 1 μg/mL of puromycin for 48 hours. After

31

579   selection, rescue cell lines were continuously growth in media containing 0.5 μg/mL of

580   puromycin to avoid the silencing of PCIF1 transgene.

581

582   **PCIF1 immunofluorescence**

583   PCIF1 KO and rescue cell lines were seeded in a 96-well plate to a ~70% confluence.

584   Cells are fixed for 10 mins in 3.7% formaldehyde in PBS at room temperature. Cells are

585   washed twice with PBS and permeabilized for 10 mins with 0.1% saponin in PBS. Cells

586   are blocked at room temperature for 1 hour with IF blocking buffer (10% BSA, 0.3%

587   triton in PBS) and then are incubated overnight at 4°C with a monoclonal anti-FLAG

588   antibody (Sigma-Aldrich Cat #F1804, RRID:AB_262044) diluted 1:500 in IF buffer (10%

589   FBS, 1% BSA, 0.3% triton in PBS). Primary antibody is washed three times with 0.05%

590   PBS-Tween. Cells are incubated with Alexa 488 goat anti-Mouse (Thermo Fisher

591   Scientific Cat #A-11029, RRID:AB_2534088) 1:1000 in IF buffer for 1 h at room

592   temperature in the dark. DAPI is added to a final concentration of 0.2 μg/mL and

593   incubated for additional 10 mins. Cells are washed three times with 0.05% PBS-Tween,

594   once with PBS and then imaged in a Nikon Eclipse Ti fluorescent microscope. Pictures

595   of representative fields are taken with a S Plan Fluor 20X/0.45 objective with the NIS

596   Elements v4.20 software (DAPI channel exposure: 500 msec. GFP channel exposure:

597   600 msec.).

598

599

600

Thin Layer Chromatography

1 μg of mRNA is cleaned, labeled and digested as previously described (Kruse et al., 2011; Mauer et al., 2017). 10% of the starting material is loaded into a TLC cellulose glass plate and first and second dimensions are run for 14 and 19 hours, respectively. Plates are exposed to a phosphor imager screen for 24 hours and read in a Typhoon scanner. Pictures are analyzed and quantified using Fiji v2.0.0 (RRID:SCR_002285).

Recombinant Protein Purification

Full-length human PCIF1 gene is cloned into pGEX-4T1 vector. It is expressed overnight in 500 mL Rosetta bacteria (Novagen) culture induced with 0.1 mM IPTG at 18°C. The cells are lysed in 15 mL cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.05% NP-40, 1mM PMSF) with 0.25 mg/mL chicken lysozyme on ice for 30 min. The cell lysate is sonicated on ice and cleared with centrifugation for 20 min at 10000 rpm at 4°C. The GST tagged recombinant protein is bound to Glutathione Sepharose 4B beads (GE Healthcare) at 4°C with rotation for 3 hours. The beads are washed with lysis buffer containing 500 mM NaCl. The untagged recombinant protein is eluted off the beads with overnight rotation at room temperature with 5U of thrombin in thrombin cleavage buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5mM CaCl$_2$). The protein preparation is supplemented with 10% glycerol and stored at -80°C.

Full-length human EIF4E gene with N terminal 6xHis tag is cloned into pGEX-4T1 vector. The same purification protocol for recombinant PCIF1 is followed with the

33

623   following exceptions. The initial lysis buffer is supplemented with 10U RNaseA/T1 mix.

624   The cleared lysate is first bound to Ni-NTA beads at 4°C for 3 hours to purify using the

625   6xHis tag, washed in lysis buffer with 750 mM NaCl and eluted in His elution buffer (50

626   mM Tris pH 7.4, 150 mM NaCl, 150 mM imidazole). The eluted protein is bound to

627   Glutathione Sepharose 4B beads (GE Healthcare) at 4°C with rotation for 1 hour and

628   eluted off the beads with overnight rotation at room temperature with 5U of thrombin in

629   thrombin cleavage buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5mM $CaCl_2$). The protein

630   preparation is supplemented with 10% glycerol and stored at -80°C.

631

632   **Enzyme Kinetics**

633   The enzymatic parameters for m7G-Am methylation were determined by incubating full-

634   length untagged recombinant PCIF1 enzyme (3 nM) with increasing concentrations of

635   RNA dinucleotide (10 – 500 nM) at 37 C in 50 mM Tris-HCl (pH 7.5 @ 25 °C), 5 mM β-

636   ME, 10 mM EDTA and 80 µM S-adenosylmethionine (SAM) buffer. Aliquots were

637   withdrawn at t = 0, 2, 5, 10, 30 and 60 min and boiled for 3 min to stop all enzymatic

638   activity. The aliquots were further processed as described in Processing of RNA

639   samples for mass spectrometry methods section. The levels of the final product (m7G-

640   m6AmG) formed at each time point was determined using mass spectrometry by

641   monitoring the amount of m6Am in each aliquot. The observed rate of product formation

642   (kobs) was determined by plotting the concentration of m6Am against time for each

643   concentration of the substrate (10, 25, 50, 100, 250, 500 nM). The kobs vs substrate

644   concentration curve was fit to the Michaelis-Menten equation using the Graphpad Prism

645   software to obtain the final enzymatic parameters. The same protocol was followed for

646   kinetics using EGFP mRNA with indicated concentrations.

647

648   ***In vivo* translation assays**

649   200 ng of either Am-, or m6Am-EGPF mRNA are transfected into a 6-well plate with

650   MEL624 at ~30% confluence. Transfection is done with 1 μl of lipofectamin 2000

651   according to manufacturer's instructions. Untransfected wells are used as negative

652   controls. 24 h post-transfections, cells are imaged in a Nikon Eclipse Ti fluorescent

653   microscope and pictures of representative fields are taken with a S Plan Fluor 20X/0.45

654   objective with the NIS Elements v4.20 software (GFP channel low exposure: 500 msec.

655   GFP channel high exposure: 3 sec. bright field exposure: 20-40 msec.). At the same

656   time, a second set of transfected wells is trypsinized, washed once with PBS and

657   resuspended in 500 μl of PBS for flow cytometry. Cells are analyzed in a FACS Canto II

658   and percentages of GFP positive cells as well as GFP intensity values are calculated

659   with FloJo 8.7. Finally, a third set of transfected wells is lysed with 200 μL of TRIzol and

660   total RNA is purified following manufacturer's instructions. Quantitative PCR is

661   performed with primers specific for GFP and GAPDH as internal control in order to

662   determine the level of mRNA that was transfected into the cells.

663

664   ***In vitro* Translation**

665   In vitro translation reactions are carried out using Retic Lysate IVT kit (Invitrogen

666   Ambion) in 25 μL or 10 μL reactions with varying indicated mRNA concentrations (50,

35

667   100 or 200 ng). The translation is carried out at 30°C for 1 hour. For the firefly luciferase

668   mRNA translation reactions, luciferase reagent supplied by Firefly Luc One-Step Glow

669   Assay Kit (Pierce Thermo Fisher Scientific) is used. For the in vitro transcribed

670   bicistronic firefly-IRES-renilla luciferase mRNA translation reactions, luciferase reagents

671   supplied by Dual-Luciferase Reporter Assay System (Promega) are used and

672   luminescence is measured on a plate reader.

673

674   **m6A MeRIP-Seq**

675   m6A MeRIP experiments were performed following the recommendations from the

676   EpiMark N6-Methyladenosine Enrichment Kit (NEB #E1610). Briefly: 10 μg of mRNA

677   were incubated at 98°C for 3 min in fragmenting buffer (10 mM Tris pH 7.4, 10 mM

678   ZnCl$_2$) to generate fragments of ~100-150 bp. Fragmented RNA is incubated with

679   protein G magnetic beads conjugated to 1 μL (250 μg) of anti-m6A rabbit monoclonal

680   antibody from the EpiMark kit for 1 h at 4°C. Beads were washed twice with 200 μL of

681   reaction buffer, twice with 200 μL of low salt buffer and twice with 200 μL of high salt

682   buffer. Immunoprecipitated RNA was eluted with TRIzol and purified. Illumina

683   sequencing libraries are prepared with the NEBNext Ultra II Directional Library Prep Kit

684   for Illumina from NEB (#E7760), following manufacture's instructions. RNA-Seq libraries

685   are generated from three biological replicates for Input (RNA-Seq) and m6A-

686   immunoprecipitated mRNA. Libraries are sequenced in a HiSeq 2000 with an average

687   coverage of 20 million reads per sample.

688

689   Reads are aligned against hg38 genome assembly with hisat 2.0.4 using the following

690   parameters: --no-unal --rna-strandness R. SAM files are sorted and converted to BAM

691   with samtools v1.7. Reads with QS < 10 are excluded, Normalized bigWig files are

692   generated from BAM files with bamCoverage from the deepTools suite v3.0.2 with the

693   following parameters: -bs 20 --normalizeUsing BPM --skipNAs. Reads that overlap with

694   the blacklisted regions from ENCODE project are excluded. Average bigWig files for the

695   biological replicates are generated using the bigwigCompare program from the

696   deepTools suite v3.0.2. m6A-enriched genes are obtained by calling significant peaks

697   form the BAM files with macs2 (v2.1.1) with a q-value < 1e-10 for each individual

698   biological replicate. A gene is considered to be enriched for m6A only if a peak was

699   called in all the replicates. Metagene and heatmap plots were generated with the

700   deepTools suite v3.0.2 and in-house scripts.

701

702   **PRO-Seq sequencing and analysis**

703   PRO-Seq libraries from 3 independent biological replicates were generated for Control

704   MEL624 cells, PCIF1-KO clone 1 and PCIF1-KO clone 2, using 1 million cells per

705   sample. Samples were spiked with 40,000 Drosophila S2 cells as a normalization spike-

706   in control. Samples were sequenced on the NextSeq using a High Output 75-cycle kit,

707   generating paired end 42 nt reads. Libraries were sequenced to an average read depth

708   of 50 million mappable reads per sample.

709

710　Paired-end reads were trimmed to 40 nt. To remove adapter sequence and low quality

711　3' ends we used cutadapt 1.14, discarding reads shorter than 20 nt (-m 20 -q 10), and

712　removing a single nucleotide from the 3' end of all trimmed reads to allow successful

713　alignment with Bowtie 1.2.2. Remaining read pairs were paired-end aligned to the

714　*Drosophila* dm3 genome index to determine spike-normalization ratios based on

715　uniquely mapped reads. Counts of pairs mapping uniquely to spike-in RNAs (drosophila

716　genome) were determined for each sample. In this case, the samples displayed highly

717　comparable recovery of spike-in reads, thus only depth normalization was used for each

718　bedGraph.

719

720　Reads mapped to dm3 were excluded from further analysis, and unmapped pairs were

721　aligned to the hg38 genome assembly. Identical parameters were utilized in each

722　alignment described above: up to 2 mismatches, maximum fragment length of 1000 nt,

723　and uniquely mappable, and unmappable pairs routed to separate output files (-m1, -v2,

724　-X1000, --un). Pairs mapping uniquely to hg38, representing biotin-labeled RNAs were

725　separated, and strand-specific counts of the 3'-end mapping positions determined at

726　single nucleotide resolution, genome-wide, and expressed in bedGraph format with

727　"plus" and "minus" strand labels, as appropriate. Combined bedGraphs were generated

728　after deduplication by summing counts per nucleotide of all 3 replicates for each

729　condition.

730

731 Read counts were calculated per gene (from transcription start site to transcription end

732 site), in a strand-specific manner, based on default annotations (ensembl hg38), using

733 featureCounts. Differentially expressed genes were identified using DESeq2 v1.18.1

734 under R 3.3.1. PRO-Seq size factors were determined based on DESeq2 (for Control:

735 0.9690449, 0.8752877, 0.9008983; PCIF1-KO1: 1.1669854, 0.9802904, 1.3105898;

736 PCIF1-KO2: 0.9866223, 1.0047620, 0.9516695). At an adjusted p-value threshold of

737 <0.01, 731 affected genes in KO clone 1 and 1406 in KO clone 2 were identified as

738 differentially expressed upon PCIF1-KO in MEL624 cells. After overlapping both lists

739 there are 506 genes that pass the padj < 0.01 threshold, from which 320 are

740 upregulated in both KO clone 1 and clone 2 (Table S4), 182 are downregulated in both

741 clones (Table S5) and 4 are downregulated in KO1 and upregulated in KO2. UCSC

742 Genome Browser tracks were generated from the combined replicates per condition,

743 normalized as in the differential expression analysis.

744

745 **RNA-Seq analysis**

746 Read counts for the RNAseq inputs from the m6A mapping experiment are calculated

747 from BAM files with featureCounts from the Rsubread package v1.22.3 in an R 3.3.3

748 environment. The read count table is analyzed with edgeR v3.12.1 and log2 TPM

749 measurements are generated. After calculating normalization factors and estimating

750 dispersion, DEG genes between KOs and Control cells were calculated using glm

751 modeling with a FDR < 1e-5. A final list of DEG shared between PCIF1 KO1 and PCIF1

752 KO2 is generated using in-house scripts, which gives a total of 236 upregulated genes

753   (Table S2) and 305 downregulated genes (Table S3). Boxplots of average expression

754   are generated with R 3.3.3 and in-house scripts from the log2 TPM values calculated in

755   edgeR. Mann-Whitney tests to compare average expression levels between different

756   samples and hyper geometric test to calculate p-values for overlaps between datasets

757   in Venn diagrams are performed in R 3.3.3.

758

759   **Microscale Thermophoresis (MST)**

760   RNA oligos used in MST analysis correspond to the first 219 nt 5' end of bicistronic

761   mRNAs used in Figure 7F. The 219 nucleotide m7G-Am capped RNA oligos are

762   transcribed using a PCR generated DNA template using the bicistronic construct

763   plasmid (pFR_CrPV_xb). Am methylated RNA oligo is further in vitro methylated to

764   m6Am using recombinant PCIF1. To generate decapped RNA control, m7G-Am

765   methylated RNA is decapped using a decapping enzyme. The RNA constructs are

766   column purified using Zymo RNA Clean & Concentrator kit after each transcription, in

767   vitro methylation step and decapping reaction.

768

769   The full-length human recombinant N terminal 6xHis tagged EIF4E protein is

770   fluorescently labelled in PBST with 0.1% PEG8000 using His-Tag Labelling Kit RED-

771   Tris-NTA (2nd Generation) (Nanotemper MO-L018). The RNA oligos are denatured at

772    $65^{o}C$ for 3 minutes followed by 1 minute immediate ice incubation. Then 2-fold serial

773   dilutions of RNA are carried out in PBST with 0.1% PEG800 at room temperature using

774   LoBind microfuge tubes. Labelled protein is added at 25 nM dye and 100 nM protein

775   final concentration to each RNA dilution. After 5 minute room temperature incubation,

776   premium capillaries are loaded and MST is carried out with 100% LED power and 40%

777   laser (20 seconds ON and 5 seconds OFF) using NanoTemper Control 1.1.9 software.

778   The binding constants are calculated using MO.Affinity Analysis V2.3 software using 10

779   dilution points carried out in triplicate independent binding assays for each RNA oligo.
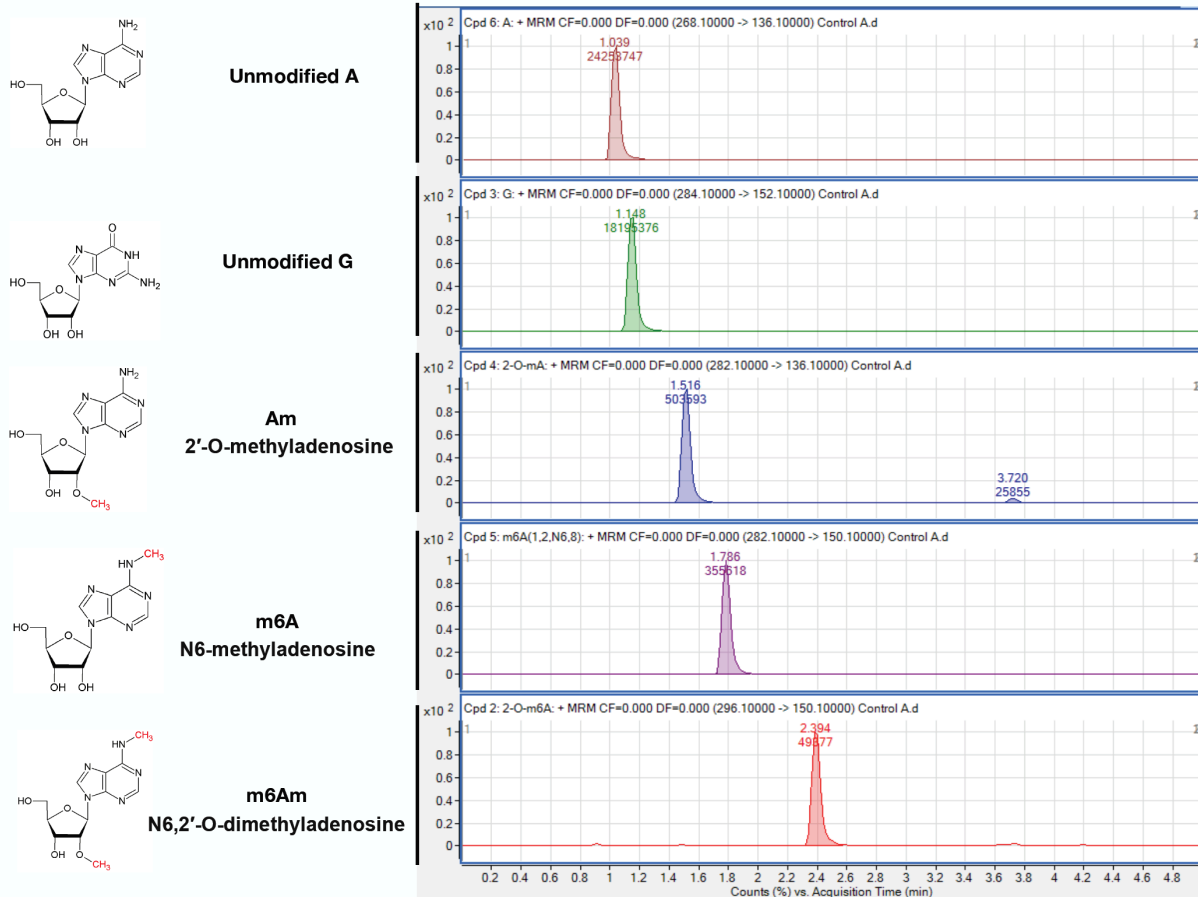
Supplemental Information

Figure S1. Related to Figure 1. Representative MS spectra of de-capped mRNA from Mel624 human melanoma cell line.

787
788
789 **Figure S2. Related to Figure 2.** Alignment of protein sequences of zebrafish,
790 mouse and human PCIF1 using ClustalW. The conserved methyltransferase motif is
791 highlighted.
792
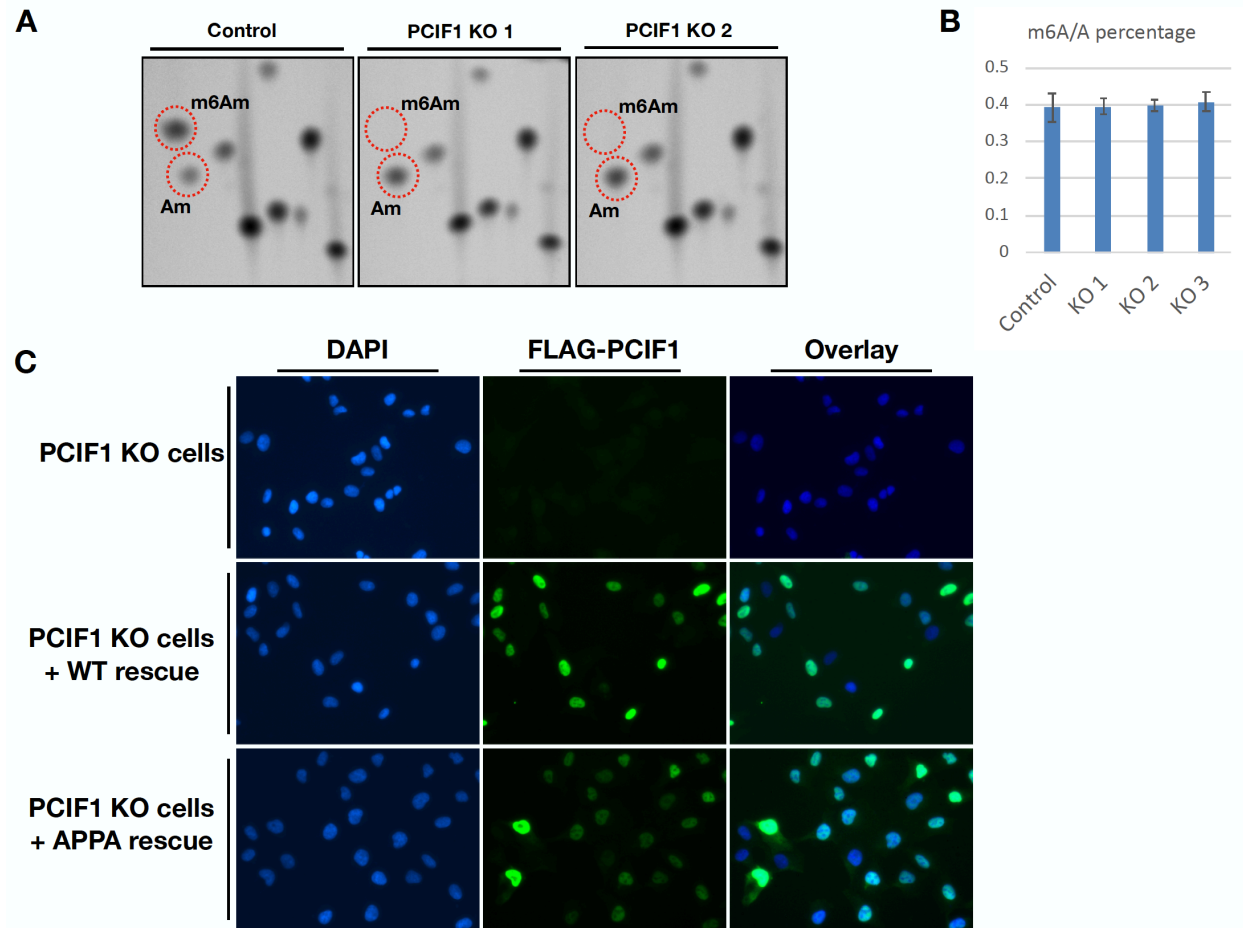
Figure S3. Related to Figure 2. PCIF1 is required for mRNA m6Am methylation in vivo.

3A. Thin-layer chromatography analysis of cap-adjacent nucleotides from mRNA isolated from control and two independent PCIF1 KO cell lines.

3B. LC-MS/MS m6A/A percentages of mRNAs from control and three independent PCIF1 KO cell lines

3C. Immunofluorescence images of PCIF1 KO and FLAG-PCIF1 rescue cell lines with anti-FLAG antibody.

44
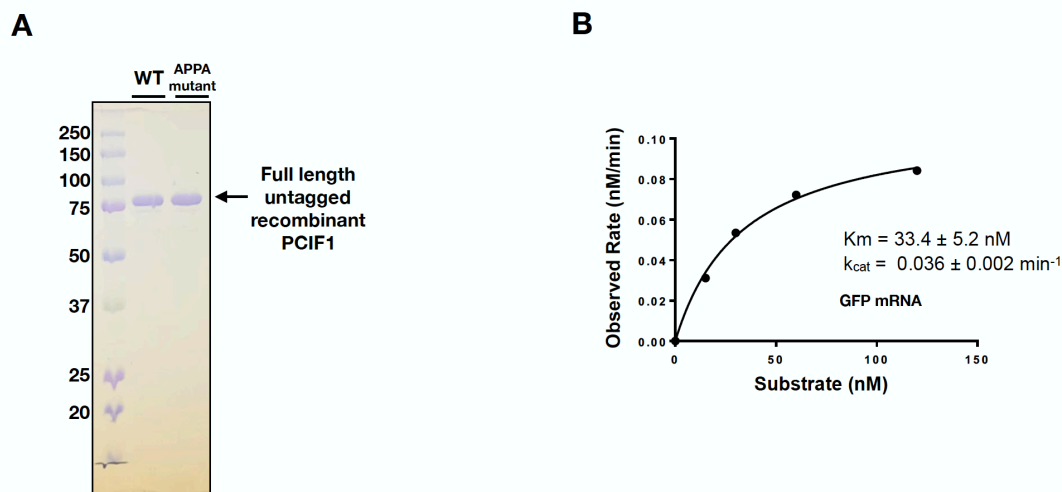
805
806
807 **Figure S4. Related to Figure 3. *In vitro* enzyme kinetics with recombinant**
808 **PCIF1**
809
810 4A. GST tagged PCIF1 was expressed in bacteria and purified using Glutathine
811 Sepharose beads and eluted using overnight thrombin cleavage. Around 3 μg of purified
812 recombinant PCIF1 was run on PAGE gel and stained with Coomassie Blue.
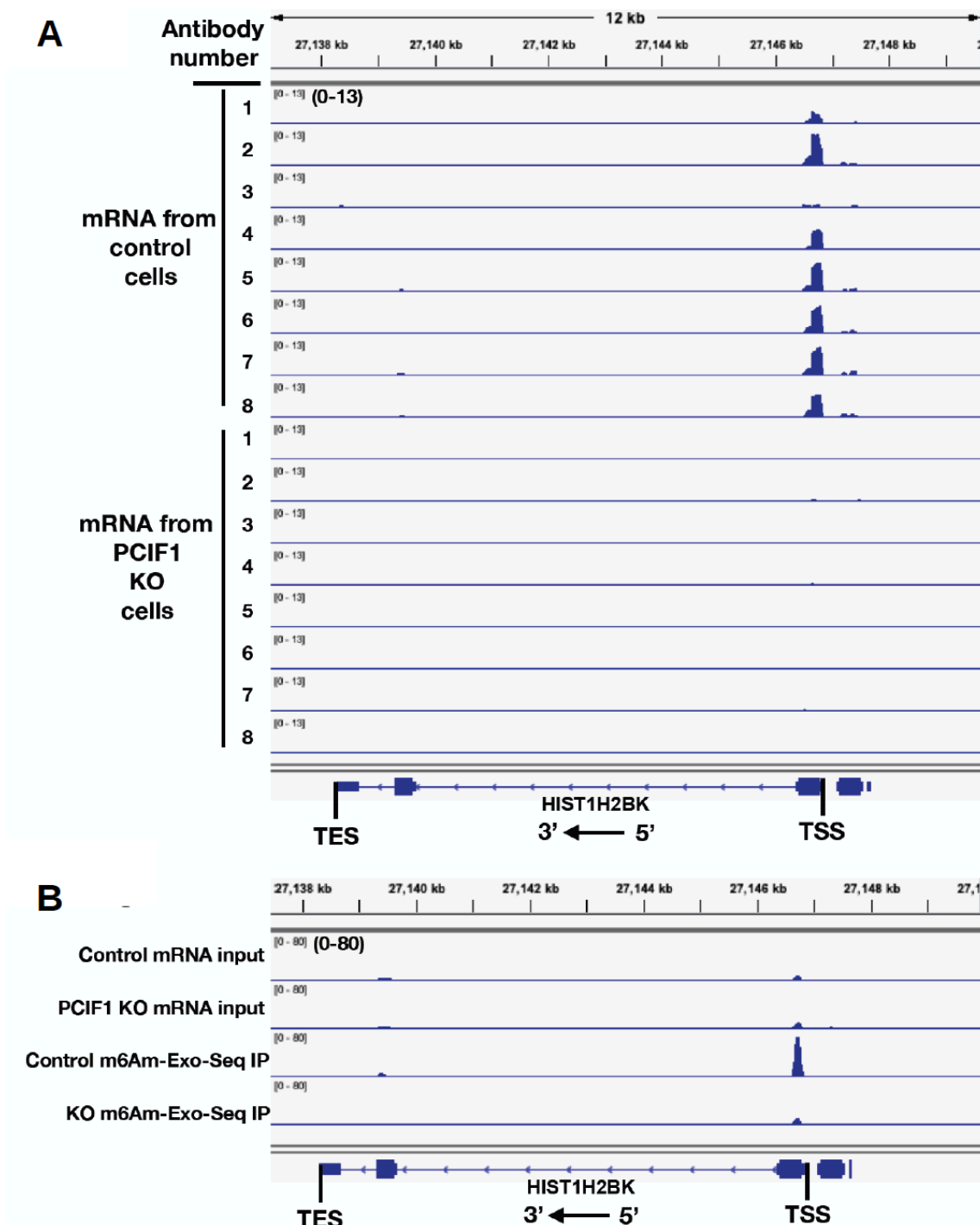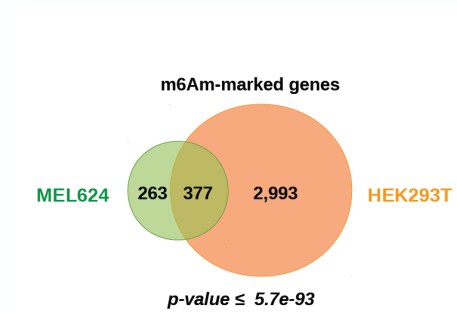813 4B. Michaelis–Menten kinetics of recombinant PCIF1 is determined using m7G-Am
814 capped EGFP mRNA.
815

Figure S5. Related to Figure 5. Global mapping of m6Am through m6Am-Exo-Seq

5A. Genome browser view of m6Am-Exo-Seq fold change over input employing 8 different antibodies against m6A in control and PCIF1 KO cells.

5B. Genome browser view of reads per million in inputs and corresponding m6Am-Exo-Seq IPs from control and PCIF1 KO cells.
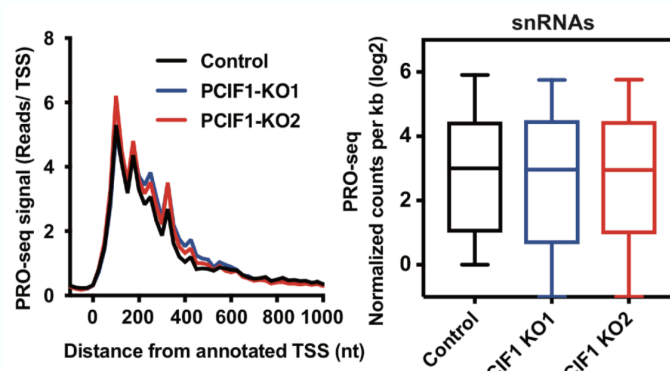
46

A

m6Am-marked genes

MEL624   263 | 377   2,993   HEK293T

p-value ≤ 5.7e-93

B

snRNAs

Control
PCIF1-KO1
PCIF1-KO2

825
826
827 **Figure S6. Related to Figure 6. m6Am is cell type specific and does not**
828 **affect snRNA transcription**
829
830 6A. Venn diagram depicting overlap between 640 m6Am enriched genes in MEL624
831 and 3370 identified in HEK293T cells. P-values are calculated with a hypergeometric
832 test.
833 6B. PRO-Seq signal is shown at snRNA genes (N=667 snRNA genes expressed in
834 MEL624) in control and PCIF1 KO cells. Shown are a composite metagene distribution
835 (left) or boxplot of PRO-Seq signal within snRNA genes. Boxplots are shown as in 7D.
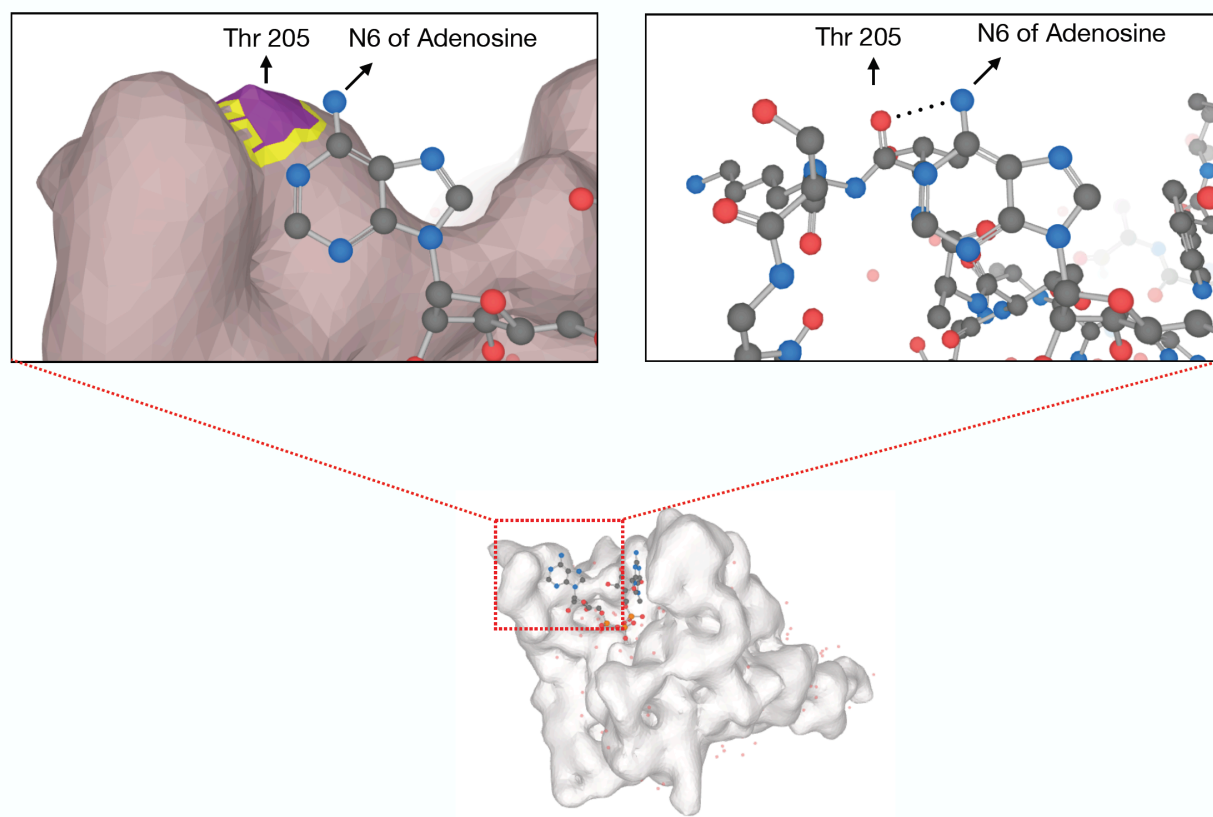836 No significant differences were detected.
837

838
839
840 **Figure S7. Related to Figure 7.** N6 residue of cap adjacent adenosine of mRNA,
841 which gets m6A methylated by PCIF1, forms a hydrogen bond with threonine 205
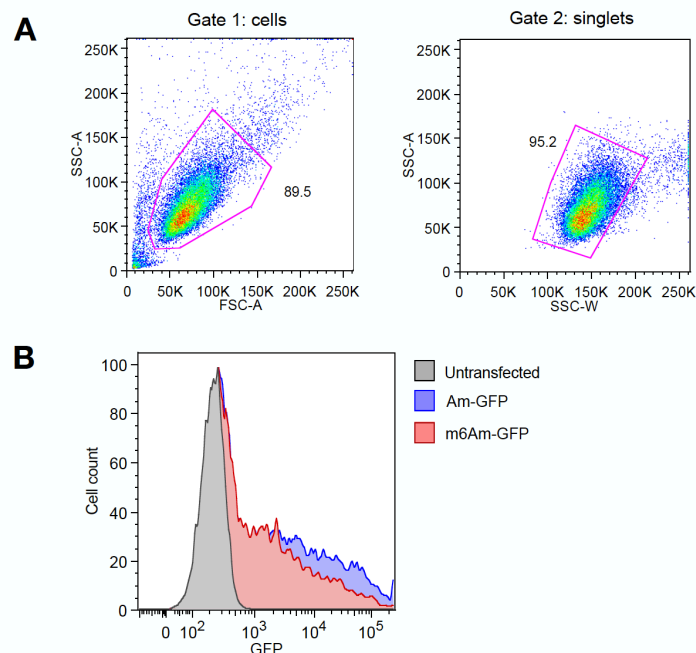842 residue of human eIF4E.
843

Figure S8. Related to Figure 7. FACS analysis of Am or m6Am EGFP transfected MEL624 cell lines.

8A. Gating strategy for selecting singlet cells. Cells are gated based on FCS-A and SSC-A (gate 1). Singlets are selected from gate 1 by gating SSC-W vs SSC-A. Cells from gate 2 are used for all quantifications shown in Figure 7.

8B. Representative fluorescent profile of MEL624 cells transfected with Am- or m6Am-EGFP mRNA. Untransfected cells are used as negative control.
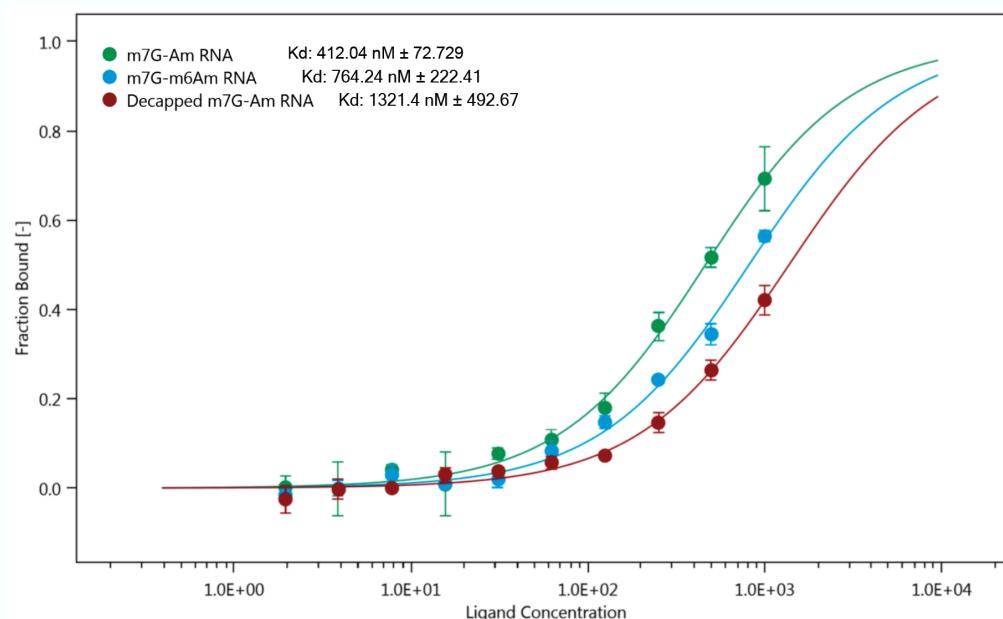
49

Figure S9. Related to Figure 7. Kd constants of fluorescently labelled N-terminal His tagged recombinant human eIF4E with 217 nt RNA oligos with either m7G-Am or m7G-m6Am 5' ends measured by microscale thermophoresis. De-capped m7G-Am RNA was used as a control. The 217 nucleotide RNA oligo sequence corresponds to the first 217 nt of bicistronic construct that was used in in vitro translation assays in Fig.7F. Three independent binding assays were performed with freshly labelled protein for each RNA oligo.

865   **Table S1. Related to Figure 5.** List of m6Am-marked genes.

866

867   **Table S2. Related to Figure 6.** List of Upregulated genes according to RNA-Seq

868

869   **Table S3. Related to Figure 6.** List of Downregulated genes according to RNA-Seq

870

871   **Table S4. Related to Figure 6.** List of Upregulated genes according to PRO-Seq

872

873   **Table S5. Related to Figure 6.** List of Downregulated genes according to PRO-

874   Seq

875