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The YTHDF1-3 proteins are bidirectionally influenced by the codon content of their mRNA targets

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33 **ABSTRACT**

34 *N*⁶-methyladenosine (m⁶A) is the most abundant modification in eukaryotic mRNAs and plays critical roles in a
35 broad variety of biological processes. Recognition of m⁶A by the YTHDF1-3 proteins results in the alteration of
36 the translation efficiency and stability of methylated mRNAs, although their mode of action is still matter of
37 debates. To decode the molecular basis of YTHDF1-3 action in human cells, we performed an unbiased proteomic
38 screen of their full spectrum of interacting proteins using BioID (proximity-dependent biotin identification). Our
39 systematic BioID mapping revealed that each YTHDF protein is a dynamic hub that associates with both mRNA
40 silencing machineries and the translation apparatus. Based on this, we identified a direct interaction between
41 YTHDF2 and the ribosomal protein RACK1, and found that the silencing activity of YTHDF2 is bidirectionally
42 modulated by the codon content of its targeted mRNAs. Using a tethering reporter system that recapitulates this
43 phenomenon, we confirmed that the three YTHDF proteins selectively repress mRNAs enriched in optimal
44 codons, while they activate those enriched in non-optimal codons. Altogether, these results have important
45 implications for understanding the underlying multiplicity of YTHDF1-3 and could reconcile seemingly
46 contradictory data.

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60 INTRODUCTION

61 Post-transcriptional silencing mechanisms modulate global and transcript-specific mRNA stability and translation,
62 contributing to the rapid and flexible control of gene expression. mRNA silencing relies on a variety of non-coding
63 RNAs, such as microRNAs (miRNAs), mRNA modifications and RNA-binding proteins (RBPs) which target the
64 3'-untranslated regions (3'UTRs) of specific mRNAs to recruit silencing effector machineries. Among these
65 modifications, *N*⁶-methyladenosine (m⁶A) was identified as the most abundant modification in eukaryotic
66 mRNAs^{1,2}. Deposition of m⁶A occurs cotranscriptionally and is determined by the coordinated action of
67 methyltransferases (m⁶A writers) and demethylases (m⁶A erasers)³⁻⁵. Their detailed mapping showed an
68 enrichment of m⁶A near the stop codon and in the 3' untranslated region of the target mRNAs^{2,6}.

69 The impact of m⁶A on mRNAs depends on their recognition by different classes of proteins called “readers”.
70 Among the best characterized readers, the YTHDF1, 2 and 3 paralogs directly recognize m⁶A to change the
71 translation efficiency and stability of m⁶A-containing RNAs^{7,8}. Human YTHDF1, 2 and 3 are made of a C-terminal
72 YTH-domain that binds to m⁶A separated from a N-terminal proline and glutamine-rich low-complexity domain
73 with ~ 70% amino acid similarity⁹⁻¹¹. Despite their high level of similarity, the multiplicity of their mode of action
74 is intensively disputed. Some studies claim their dissimilar roles, where YTHDF1 promotes mRNA translation
75 through interactions with translational factors, YTHDF2 induces mRNA degradation, and YTHDF3 assists
76 YTHDF1 and YTHDF2 to regulate mRNA fate^{10,12,13}. By contrast, recent papers point out their redundancy with
77 the prevailing view that YTHDF1-3 share highly similar mRNA targets and promote their degradation in a largely
78 redundant manner¹⁴⁻¹⁶. To clarify their degree of similarity, the interactome of the YTHDF1-3 proteins has been
79 mapped using BioID (proximity-dependent biotin identification) through their fusion with the promiscuous biotin
80 protein ligase BirA. To date, these BioID mapping have led to the publication of contradictory observations.
81 Zaccara et al. reported a high level of resemblance between the YTHDF interaction networks by comparing
82 proteins enriched with C-terminal BirA fusion of YTHDF1 to those with the N-terminal BirA fusion of YTHDF2
83 and YTHDF3¹⁴. This mapping identified high-scoring interactions with mRNA silencing factors such as the
84 CCR4-NOT deadenylase complex, the exoribonuclease XRN1, and the translational repressor DDX6¹⁴. By
85 contrast, Zou et al. argued that YTHDF1, 2 and 3 have distinct partners using C-terminal BirA fusions, assuming

86 that YTHDF1 is found with translation machineries such as initiation factors (eIFs), while YTHDF2 associates
87 better with mRNA silencing factors, and YTHDF3 is in the margin between YTHDF1 and 2¹⁷.

88 Mechanistically, YTHDF2 remains the best-characterized m⁶A reader of the YTHDF family. Its knock-down
89 results in the accumulation of its targets in translatable or actively translating polysome pools, pointing to a crucial
90 role in the translational repression of its targets. Accordingly, this defect in translation is also accompanied by an
91 increase in the global abundance of m⁶A-modified mRNAs, confirming the link existing between the number of
92 m⁶A sites and the degradation rates of the targeted mRNA¹⁰. This role of YTHDF2 in the degradation of m⁶A-
93 containing mRNAs is further supported by its capacity to recruit various mRNA silencing machineries through an
94 effector region, namely a low-complexity segment of 385 amino acids that comprises the remainder of the protein
95 outside of the YTH domain¹⁰. These silencing machineries include the CCR4-NOT complex, the RNase P/MRP
96 endoribonuclease, and the decapping-promoting proteins UPF1/PNRC2/DCP1a¹⁸⁻²⁰. Evidence also suggests that
97 YTHDF2 transports its targets from the translatable pool to mRNA decay sites, such as processing bodies (P-
98 bodies), although a recent study argues that it may only have a negligible impact in mRNA partitioning into
99 granules²¹⁻²³.

100 Here, in an attempt to decrypt the mechanism behind the multiplicity of YTHDF1-3, we present a comprehensive
101 clarification of their interactome through an systematic BioID strategy. Notably, we report the proteomic mapping
102 of the “effector” N-terminus of YTHDF2, which appears as a nexus for contacting both mRNA silencing factors
103 and the translational apparatus. The spatial organization of this interactome was assessed through mapping both
104 the N- and C-terminus of YTHDF2, and concomitantly compared with those of YTHDF1 and YTHDF3. We then
105 describe a direct interaction between YTHDF2 and the ribosomal protein RACK1, and find that the silencing
106 activity of YTHDF2 is selectively modulated by the codon content of its targeted mRNAs. Overall, these results
107 have important implications for understanding the underlying multiplicity of YTHDF1-3 and could reunify
108 apparently contradictory models.

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112 RESULTS

113 Defining the interactome of YTHDF2

114 A BioID proximity mapping was first used to obtain an integrative view of the interaction network of YTHDF2.
115 Full-length YTHDF2 (FL) was fused at its N-terminus with the abortive biotin ligase BirA and expressed as bait
116 to capture interactors in close proximity of its N-terminus. The contribution of the effector region of YTHDF2
117 (AA: 1-385) was also evaluated through the generation of a truncated version of BirA-tagged YTHDF2, termed
118 YTHDF2^{ΔNT}, in which the 1-385 region was deleted. Both BirA alone and BirA-fused eGFP were selected as
119 negative controls to monitor the non-specific background of our BioID mapping (Supplementary Figure S1A).
120 When expressed in HEK293T cells in presence of biotin, both negative controls and BirA-fused YTHDF2 (FL and
121 ΔNT) led to an efficient activation of biotinylation (Figure 1A). Biotinylated proteins of five independent
122 replicates, alongside negative controls, were isolated using streptavidin-affinity purification under denaturing
123 conditions and analyzed by quantitative mass spectrometry (MS). Our differential and statistical analysis revealed
124 that the high-confident interactome of YTHDF2 is composed of ~400 proteins including a high amount of factors
125 involved in mRNA deadenylation (CCR4-NOT complex), decapping (DCP1a, EDC3), 5' to 3' decay (XRN1) and
126 translational repression (GIGYF1/2, 4EHP, 4E-T, DDX6) (Figure 1B and Supplementary table 1). Numerous
127 components of the translational apparatus, including ribosomal proteins and translation factors (eIF4G2, eIF4A,
128 eIF3 subunits), were also detected as high-confident hits. Gene ontology (GO) analysis confirmed that both
129 “negative regulation of translation” and “mRNA catabolic process” were among the biological processes with the
130 most significant representation among YTHDF2 interactions (Supplementary Figure S1B)

131 BioID mapping of YTHDF2^{ΔNT} was performed concomitantly with its full-length version. By plotting the MS data
132 of YTHDF2^{ΔNT} over the negative controls, we found an interactome of 106 proteins, with only 34 in common with
133 the one of FL YTHDF2 (Figure 1C, Supplementary Figure S1C-D and Supplementary Table 2). This YTHDF2^{ΔNT}
134 dataset showed a substantial enrichment in RNA splicing and processing factors, while neither mRNA silencing
135 factors nor ribosomal proteins were detected. To get a complementary perspective on this interaction network,
136 BirA was also fused to the C-terminus of YTHDF2 (YTHDF2-HA-BirA). When computed over its respective
137 negative controls (HA-BirA alone and eGFP-HA-BirA; Supplementary Figure S1E), the C-terminal BirA fusion
138 of YTHDF2 was found to biotinylate a list of 78 proteins including 50 that were also detected with the N-terminal

139 BirA fusion of YTHDF2 (Supplementary Figure S1F-H and Supplementary Table 3). This dataset was globally
140 enriched in mRNA silencing factors and RNA-binding proteins such as Roquin, Pumilio and the microRNA-
141 associated factors TNRC6A/B/C, although neither ribosomal proteins nor translation factors were detected,
142 suggesting that the translation apparatus might be spatially too far from the BirA labelling radius at the C-terminus
143 of YTHDF2. Overall, these data indicate that the N-terminal BirA fusion of YTHDF2 provides a broader
144 interaction map than that of its C-terminal equivalent.

145 Since the deletion of the N-terminus of YTHDF2 prevents its interaction with cytoplasmic mRNA-associated
146 machineries to favor its proximity with nuclear factors, we sought to compare the subcellular distribution of the
147 FL and Δ NT versions of YTHDF2. In agreement with our BioID, we found that an eGFP-tagged version of
148 YTHDF2 $^{\Delta$ NT predominantly accumulates in the nucleus of HEK293T cells, while its FL counterpart is only
149 detected in the cytoplasm both as a diffuse signal and in punctuated foci alongside the P-body resident protein
150 DDX6 (Supplementary Figure S1I). We then examined whether YTHDF2 utilizes its N-terminus to nucleate
151 interactions with both mRNA silencing factors and ribosomal proteins. The stable and physical association of a
152 Flag-tagged version of YTHDF2 with its high confident proximal interactors was examined using co-
153 immunoprecipitation experiments (co-IP). As expected, an interaction was detected between Flag-YTHDF2 and
154 the endogenous CNOT1 and CNOT3 subunits of the CCR4-NOT complex, the translational repressors 4EHP,
155 GIGYF1/2 and DDX6, and the ribosomal proteins RACK1 and RPL10 (Figure 1D). Notably, these co-IPs were
156 observed in RNase A-treated lysates, indicating that these interactions occur in an RNA-independent manner. By
157 contrast, none of these proteins were detected following Flag-YTHDF2 $^{\Delta$ NT IP, confirming the importance of the
158 N-terminus of YTHDF2 in mediating the interaction with both silencing factors and the ribosome.

159 **The ribosome is a high-confident proximal interactor of YTHDF1-3**

160 We then sought to compare the interactome of the three YTHDF1-3 paralogs through a BioID mapping of their
161 N-terminus (Supplementary Figure S2A). When compared to the negative controls (Myc-BirA alone and Myc-
162 BirA-eGFP), the N-terminal BirA fusions of YTHDF1, 2 and 3 biotinylated a common set of 313 proteins showing
163 a comparable over-representation of proteins from both mRNA silencing machineries and ribosomes
164 (Supplementary Figure S2B-C and Supplementary Tables 4-6). In particular, global comparison of the enrichment

165 of proteins from the ribosome among the three datasets revealed the presence of 33 ribosomal proteins with
166 YTHDF1, 40 with YTHDF2, and 15 with YTHDF3, including 14 in common (Figure 1E). Among them, RACK1,
167 a highly conserved factor positioned at the back side of the 40S head in the vicinity of the mRNA exit channel^{24,25},
168 was found as the most abundant ribosomal protein shared between the YTHDF1-3 BioID (Supplementary Figure
169 S2D).

170 Our BioID mapping pointed to the ribosome as a high-confident proximal interactor of YTHDF1-3. To evaluate
171 the association of endogenous YTHDF proteins with ribosomes, HEK293T extracts were fractionated using Ribo
172 Mega-SEC, which allows the separation of polysomes and ribosomal subunits using Size Exclusion
173 Chromatography (SEC)²⁶. The collected fractions, ranging from polysomes to smaller protein complexes, were
174 analyzed by Western blot to visualize the fractionation profile of endogenous YTHDF1-3 along with ribosomes.
175 Although the majority of the YTHDF1-3 pool resides in smaller complexes which did not fractionate with
176 ribosomal components (fractions 25-31; Figure 1F and Supplementary Figure S2E), significant amounts of
177 YTHDF2, and in a smaller extent YTHDF1, were detected in the fractions containing the ribosomal proteins
178 (fractions 7-12). By contrast, we failed to detect YTHDF3 amongst the latter. In agreement with the BioID
179 mapping, our co-fractionation profiles therefore indicated that the endogenous YTHDF proteins, YTHDF1 and
180 YTHDF2 at least, can co-exist along with ribosomes.

181 **The ribosomal protein RACK1 directly interacts with YTHDF2**

182 Our BioID mapping pointed out the ribosomal protein RACK1 as a high-confident interactor of the YTHDF
183 proteins. To test whether this interaction depends on the ribosome, we generated vectors encoding Flag-tagged
184 versions of RACK1, Wild-Type (WT) or carrying the R36D/K38E substitutions which prevent its ribosomal
185 incorporation²⁷. These vectors were expressed in HEK293T cells and RNase A-treated extracts were used for Flag
186 IP. Following Western blot, the co-immunoprecipitation of endogenous YTHDF1 and YTHDF2 was observed
187 with both Flag-RACK1 and its mutated version, indicating that their interaction can occur independently of the
188 incorporation of RACK1 in the ribosome (Figure 2A). In agreement with the Ribo-Mega-SEC data (Figure 1F),
189 we could not detect any interaction of endogenous YTHDF3 with Flag-RACK1. By contrast, CNOT1 and CNOT3,
190 subunits of CCR4-NOT and high-confident interactors of the YTHDF proteins, were only detected with ribosomal

191 Flag-RACK1, along with the ribosomal proteins RPS3 and RPL10, used as controls. We next sought to evaluate
192 the contribution of RACK1 in mediating the interaction between the YTHDF proteins and the rest of the ribosome.
193 For this purpose, RACK1-knock-out (KO) HEK293T cells (RACK1^{KO}) were generated using a CRISPR/Cas9-
194 based strategy (Supplementary Figure S3A). Vectors expressing Flag-YTHDF2, or Flag as a control, were
195 transiently transfected in either RACK1^{KO} cells or their WT counterpart. Following Flag IP, the interaction of
196 Flag-YTHDF2 with the ribosomal proteins RPS3 and RPL10 was more than 70% reduced in RACK1^{KO} cells in
197 comparison with WT (Supplementary Figure S3A). A minimal co-IP remained detectable following RACK1 KO,
198 suggesting that RACK1-independent contacts may exist between YTHDF2 and the ribosomes.

199 We then investigated which part of YTHDF2 binds RACK1 using Flag-tagged YTHDF2 truncations and co-IP
200 experiments. Our incremental N-terminal deletions showed that the region 200-275 of YTHDF2 is required for
201 the maximal co-IP of endogenous RACK1 (Figure 2B). In an attempt to narrow down this minimal region, we
202 used the AlphaFold2 structure prediction tool^{28,29}. A single polypeptide comprising the RACK1 sequence fused
203 C-terminally to the region 200-275 of YTHDF2 via a polyglycine linker was provided to AlphaFold2, and five
204 models were generated (Supplementary Figure S3B). Remarkably, in each of the five models, YTHDF2²⁰⁰⁻²⁷⁵ was
205 predicted as an unstructured region with the exception of a short segment (W²³⁹ADIAS) folding into an α -helix
206 and positioned on the outer circumference of RACK1 (Figure 2C, Supplementary Figure S3B). RACK1 is known
207 to adopt a seven-bladed β -propeller structure, each propeller blade consisting of a four-stranded antiparallel β -
208 sheet³⁰. In this context, the W²³⁹ADIAS peptide of YTHDF2 is predicted to occupy an inter-blade spacing between
209 blades 5 and 6 with multiple contacts involving tyrosine 246 of RACK1 (Y246) stacked between tryptophan 239
210 (W239) and isoleucine 242 (I242) of YTHDF2 WADIAS motif. Alignment of this predicted RACK1-binding
211 region showed that W239 and I242 are both conserved across human YTHDF1 and YTHDF3, as well as in the
212 mouse, zebrafish and fly orthologs (Supplementary Figure S3C).

213 Superposition of the AlphaFold2-based model with the structure of a ribosome also showed that the YTHDF2
214 binding surface is accessible in ribosomal RACK1 (Supplementary Figure S3D). To validate this predicted
215 interface, pull-down assays were performed to evaluate the interaction between recombinant Glutathione S-
216 transferase (GST)-fused RACK1 and hexahistidine (His₆)-YTHDF2. GST-RACK1 was incubated with His₆-
217 YTHDF2, WT or mutants harboring either a deletion of the 239-245 segment (Δ 239-245) or the substitutions

218 W239A/I242A. In agreement with the prediction, we observed a specific binding of WT His₆-YTHDF2 to GST-
219 RACK1, thus confirming their direct interaction (Figure 2D). By contrast, neither the W239A/I242A nor the Δ 239-
220 245 mutants of His₆-YTHDF2 were bound to GST-RACK1, confirming the importance of the 239-245 region of
221 YTHDF2 in contacting RACK1. Consistently, we also performed an Ni-NTA pull-down assay which confirmed
222 a specific retention of WT GST-RACK1 by His₆-YTHDF2 (Figure 2E). By contrast, the substitution of Y246 of
223 GST-RACK1 with an alanine (Y246A) led to pronounced inhibition of this retention. Overall, these data indicate
224 a direct interaction between the 239-245 region of YTHDF2 and an inter-blade spacing in RACK1.

225 **Synonymous codon usage influences YTHDF2-mediated silencing**

226 Prior studies have reported that the degree to which mRNAs are impacted by m⁶A is dependent on their respective
227 coding sequences in vertebrates³¹. The proximity of YTHDF2 with the ribosome prompted us to explore the
228 possible existence of a functional connection between the mRNA coding sequence and the repressive activity of
229 YTHDF2. The ribosome is a central determinant of mRNA stability as its translation speed directly influences the
230 half-life of the translated mRNA (for review, see³²). Ribosome velocity depends on the identity of the codons it
231 translates and in particular on their optimality. To test whether YTHDF2 is involved in this process, we first
232 interrogated how the sensitivity of mRNAs to YTHDF2 could be influenced by their codon composition using
233 published mRNA lifetime profiling. We focused on endogenous mRNA stability profiles that have been previously
234 published after blocking transcription in HeLa cells invalidated for YTHDF2¹⁰. As a metric of codon usage, the
235 Codon Adaptation Index (CAI) of each transcript was computed and plotted based on the alteration of its mRNA
236 lifetime following YTHDF2 knockdown. Based on this, we found that YTHDF2 preferentially induces the
237 degradation of mRNAs with high CAI scores, while low CAI scores are enriched within the repertoire of mRNAs
238 destabilized by the loss of YTHDF2 (Figure 3A-B), suggesting a bidirectional modulation of YTHDF2 activity
239 based on codon usage. Early studies have shown that synonymous codon usage is correlated to GC content at third
240 position of codons, termed GC3³³. Accordingly, a comparable relationship was also observed when plotting GC3
241 levels, as well as total GC levels in the coding sequence, versus the differential mRNA lifetime following YTHDF2
242 knockdown (Figure 3C-D and Supplementary Figure S4A). YTHDF2-regulated mRNAs were also displayed on a
243 scatter plot displaying the CAI of all the mRNAs detected in HeLa cells versus their GC3 score. Based on this, we
244 confirmed the opposite distribution between the mRNAs whose lifetime was increased (high CAI, high GC3) and

245 those having a decreased lifetime following YTHDF2 knockdown (low CAI, low GC3; Figure 3E-F). Since
246 YTHDF2 knockdown is also known to alter translation efficiency of its targets, a similar analysis was performed
247 using data obtained with ribosome profiling¹⁰. For this, we categorized the transcripts whose translation efficiency
248 was either increased or reduced following YTHDF2 knockdown, and computed their GC3/CAI scores. In the same
249 way as for mRNA lifetime, we found that YTHDF2 selectively favors the translation efficiency of mRNAs with
250 low GC3/CAI scores, while it reduced that of mRNAs with high GC3/CAI scores (Supplementary Figure S4B-D).
251 Overall, these data suggest the existence of a bidirectional activity of YTHDF2 which is correlated with the codon
252 composition in its targeted mRNAs.

253 **Assessing the bidirectional codon-driven activity of YTHDF with the λ N/BoxB system**

254 To experimentally assess whether codon optimality influences the activity of the YTHDF proteins, we used the
255 λ N/BoxB system^{34,35}, which is based on the artificial tethering of YTHDF onto a reporter mRNA encoding Renilla
256 luciferase (RLuc). For this assay, YTHDF1, 2 and 3 were individually fused to the λ N peptide, which has a high
257 affinity for BoxB sequences inserted into the 3' UTR region of the mRNA encoding luciferase (RLuc-BoxB). Co-
258 expression of λ N-YTHDFs with RLuc-BoxB mRNA induces their recruitment at the 3' UTR, mimicking their
259 interaction with m⁶A. To include codon usage as a parameter, we used the iCodon tool to introduce two degrees
260 of codon optimality in the RLuc-BoxB mRNA³⁶. Synonymous codon substitutions were introduced into its coding
261 sequence to generate an optimized reporter with a high CAI score (RLuc^{High}; CAI: 0.886), and a deoptimized low-
262 score variant (RLuc^{Low}; CAI: 0.733), both encoding for the same RLuc protein (Figure 4A). These reporters were
263 individually expressed along with the λ N-YTHDF proteins in HEK293T cells, together with a firefly luciferase
264 construct (FLuc) as a transfection control (Figure 4B). As expected, we observed that the recruitment of λ N-
265 YTHDF1, 2 and 3 to the 3' UTR of the optimized RLuc^{High} reporter markedly reduced RLuc activity compared
266 with cells expressing only λ N (~2-fold repression, Figure 4C). Interestingly, tethering the λ N-YTHDF proteins on
267 the deoptimized RLuc^{Low} mRNA relieved their silencing activity and resulted in a ~50% increase of RLuc
268 production. Measurements of mRNA level by RT-qPCR showed that tethering λ N-YTHDF reduced the
269 accumulation of RLuc^{High} mRNA by ~50%, and to a lesser extent, by only 10% that of RLuc^{Low} (Figure 4D),
270 confirming that codon optimality selectively dictates the capacity of YTHDF1-3 to induce mRNA degradation.
271 Besides, the YTHDF-induced upregulation in RLuc^{Low} production was thus not accompanied by a proportional

272 increase of its mRNA level, suggesting that the translation efficiency of the non-optimal reporter mRNA was
273 augmented by YTHDF1-3. We therefore hypothesized that this effect could be mediated by their connection with
274 the ribosome. To test this, λ N-YTHDF2 tethering was employed with the RACK1^{KO} cells in which we found that
275 the interaction between YTHDF2 and ribosomal proteins is reduced (Supplementary figure S3A). We found that
276 the λ N-YTHDF-induced repression of the RLuc^{High} reporter remains unchanged between WT and RACK1^{KO} cells
277 (Supplementary Figure S5A). Conversely, the upregulation of RLuc^{Low} by λ N-YTHDF1, 2 and 3 was reduced by
278 ~50% in absence of RACK1 (Figure 4E). RT-qPCR analysis of RLuc^{Low} following λ N-YTHDF2 tethering showed
279 that this effect is not due to a significant reduction of its mRNA level in RACK1^{KO} cells compared with WT
280 (Supplementary Figure S5B). Altogether, our tethering assays on the RLuc^{High} and RLuc^{Low} reporters indicate the
281 existence of a selectivity in the repressive capacity of YTHDF1-3 that is directly influenced by the codon content
282 of the targeted mRNA.

283 DISCUSSION

284 The action of the YTHDF1-3 proteins is a subject of continuous debate. Here, we present evidence to support a
285 functional duality in their capacity to influence mRNA translation and stability, with an unexpected contribution
286 of the codon content of the targeted mRNA. Based on this, we propose to derive an extended model in which the
287 YTHDF1-3 proteins selectively induce the degradation of mRNAs enriched in optimal codons, while they promote
288 the translation of mRNAs enriched in non-optimal codons. In this regard, our data point toward a relative
289 redundancy of the YTHDF proteins, as we found a high degree of similarity in their interactomes and activities
290 when tethered onto the RLuc^{High} and RLuc^{Low} reporters.

291 Mapping the interactomes of YTHDF1-3 revealed their unexpected proximity with the ribosomes. More that
292 proximal, this interaction turned out to be direct through RACK1, and not brought by interactions with mRNAs,
293 as our co-IP experiments were made with RNase A-treated extracts. Of note, the mode of interaction between
294 YTHDF2 and the ribosome may involve multiple interfaces, and could be more sophisticated *in cellulo* than in
295 our *in vitro* binding assays, as an interaction between YTHDF2 and the ribosomal proteins RPS3 and RPL10 was
296 still detectable in absence of RACK1 (Supplementary Figure S3A). Beyond the complexity of this interaction
297 surface, our findings are consistent with previous proteomics data describing the mammalian “ribo-interactome”,

298 namely the identification of ribosomes associated factors through affinity purification of endogenous ribosomal
299 proteins³⁷. In this report, YTHDF1/3 were listed as direct ribosome interactors, and it was proposed that they could
300 act as anchor points on the ribosome to recruit mRNA decay factors. While it is generally assumed that mRNAs
301 undergoing translation are protected from decay, recent work has revealed that the ribosome is a master arbiter of
302 mRNA decay, wherein ribosome velocity serves as a major determinant of transcript half-lives³⁸. Central to this
303 emerging concept is the fact that codon usage controls ribosome traffic on mRNAs and that rare codons result in
304 ribosome pausing³⁹⁻⁴¹. Previous reports showed that ribosomes experiencing stalls during translation require
305 RACK1 for proper resolution, and that RACK1 is located at the interface between collided ribosomes^{42,43}. In this
306 context, anchoring YTHDF2 onto ribosomal RACK1 could serve as a proxy to sense ribosome traffic in order to
307 coordinate the fate of the targeted mRNA. Since slowing ribosome movement is also sensed by the CCR4-NOT
308 complex to elicit decay of the mRNA⁴⁴, it is tempting to speculate that the recruitment of CCR4-NOT on
309 ribosomes, as well as its deadenylase activity, could be altered by the YTHDF2/RACK1 interaction on mRNAs
310 enriched in non-optimal codons. Further investigation will be needed to fully resolve the interplay between the
311 YTHDF proteins, the silencing machineries and ribosomes, and thus elucidate their impact on mRNA fate.

312 In agreement with previous studies⁴⁵, our reanalysis of published datasets indicated that YTHDF2 preferentially
313 induces the degradation of GC-rich mRNAs at transcriptome-wide level. Conversely, a striking feature of the
314 mRNAs that tend to be less stable, as well as less translated, following YTHDF2 knock-down is that they
315 correspond to an AU-rich subset of the transcriptome. Based on our tethering assay, this codon-driven selectivity
316 of YTHDF2 can be extrapolated to YTHDF1 and YTHDF3, and turned out to be RACK1-dependent in the case
317 of the AU-rich RLuc^{Low} reporter. Beyond its impact in mRNA translation and stability, AU content is known to
318 target mRNA for P-body localization. Notably, P-bodies accumulate mostly AU-rich mRNAs with a specific
319 codon usage associated with a low protein yield^{45,46}. The YTHDF proteins can also promote m⁶A-modified
320 mRNAs entering P-bodies. Central to this phenomenon is the propensity of the YTHDF proteins to undergo liquid-
321 liquid phase separation (LLPS) through a disordered proline-glutamine-rich region in their effector domain (AA:
322 230–383 in YTHDF2)^{21,22,47}. Since the ribosome is not detected in P-bodies⁴⁶, it is plausible that the RACK1-
323 dependent activation of AU-rich mRNAs by YTHDF2 occurs outside P-bodies. The RACK1-binding region of
324 YTHDF2 (AA: 239-245) is located within its LLPS-inducing segment, and it is therefore tempting to speculate

325 that the YTHDF2/RACK1 interaction could reduce condensate assembly around the methylated mRNAs in order
326 to isolate it from silencing granules and to maintain it in the translatable pool of transcripts. Additional experiments
327 will be mandatory to test if RACK1 affects the partitioning of AU-rich targets of YTHDF2 out of P-bodies.
328 Alternatively, further investigation will be needed to verify whether YTHDF2 and RACK1 could co-exist in phase
329 separated condensates. Recent studies found that granules can positively impact co-translation events, and active
330 translation can occur in granules⁴⁸⁻⁵¹. These findings may open the door to understanding how a physical intimacy
331 between YTHDF action and translation apparatus shapes the fate of mRNAs.

333 **AUTHORS CONTRIBUTIONS**

334 C.M., L.Z. N.P. and Cl.C. performed the experiments. L.Z., M.G., N.P. and Ch.C. and Cl.C. designed research and
335 analyzed the experiments, G.B. helped with Ribo Mega-SEC experiments, and C.M., L.Z., and Cl.C. wrote the
336 paper.

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351 **Materials availability**

352 All newly created cell populations generated in this study are available upon request.

353 **Data availability**

354 All data reported in this paper and any additional information required to reanalyze the data will be shared by the
355 lead contact upon request.

356

357 **METHODS**

358 **Cell culture**

359 HEK293T cells (Sigma-Aldrich) were routinely maintained in DMEM supplemented with 10% FBS and 2%
360 penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

361 **Plasmid cloning**

362 For fusion to the C-terminus of BirA(R118G), the human YTHDF1, 2, 3 ORFs provided by Mission TRC3 Human
363 LentiORF Clones TRCN0000477232, TRCN0000468616, TRCN0000473510, respectively, were cloned by PCR
364 as EcoRI–BamHI fragments into the vector pcDNA3.1 mycBioID⁵² (Addgene plasmid #35700) in frame with
365 BirA. Similarly, to generate the BirA-fused eGFP used as a negative control for BioID, a fragment containing the
366 eGFP coding sequence was obtained by PCR from the pEGFPc1 and inserted at the EcoRI–BamHI sites of the
367 pcDNA3.1 mycBioID vector. pCI-eGFP-YTHDF2 was generated by inserting the eGFP sequence at the NheI–
368 XhoI site of the pCI-Neo vector (Promega) and then by adding a fragment encoding YTHDF2 at the XhoI–NotI
369 site. To create 3xFlag expression plasmids, an annealed oligonucleotide encoding the triple Flag peptide was
370 inserted as a NheI–XhoI fragment into the pCI-Neo plasmid. YTHDF1, 2 and 3 sequences were then inserted in
371 frame with the triple Flag sequence at the XhoI–NotI sites. PCR amplifications were also used to create truncated
372 fragments of YTHDF2 which were inserted in the pCI-3xFlag vector. For tethering assays, fragments containing
373 the YTHDF1, 2 or 3 sequence were obtained by PCR and inserted into the pCI-λNV5 at the XhoI–NotI sites. As
374 previously described³⁵, a pCI-RLuc-BoxB vector containing RLuc^{High} (CAI: 0.886) was used. The deoptimized
375 low-score RLuc variant (RLuc^{Low}; CAI: 0.733) was synthesized as a gBlock gene fragment (Integrated DNA

376 Technologies) and inserted at the NheI-XbaI of pCI-RLuc-BoxB instead of the RLuc^{High} fragment. For bacterial
377 expression of His-tagged YTHDF2, the pET-28c(+)-YTHDF2 vector (Addgene plasmid #102275) was used⁵³. For
378 LLPS assays, a PCR fragment encoding a monomeric eGFP (A207K) was inserted at the NdeI site of pET-28c(+)-
379 YTHDF2. The full-length cDNA encompassing the coding region of human RACK1 was obtained by RT-PCR
380 using total RNA from HEK293T cells and inserted at the XhoI-NotI sites of the pCI-Neo-3xFlag vector, and at
381 the Sall-NotI of the pGEX-6P-1 vector (Amersham) in frame with the GST coding sequence. For the bacterial
382 expression of GST-fused mCherry2-RACK1, a gBlock gene fragment (Integrated DNA Technologies) encoding
383 the mCherry2-RACK1 fusion protein was also inserted at the Sall-NotI of the pGEX-6P-1. Amino acid
384 substitutions and deletions were introduced by site-directed mutagenesis using the QuikChange Kit (Agilent).
385 Sequences of the primers used are listed in the Supplementary Table S7.

386 **BioID and affinity purification**

387 BioID experiments were performed as previously described with minor changes⁵⁴. Briefly, HEK293T cells were
388 grown in 15 cm dishes and transfected with 15 µg of plasmids expressing the BirA-fused baits. The day after,
389 biotinylation was induced by adding biotin (50 µM) in the medium. After 24 h of treatment, cells were rinsed once
390 on the plate with 20 mL of PBS, then scraped into 1 mL of PBS. Cell pellets were collected by centrifugation (500
391 × g for 5 min) and resuspended in 1 mL of ice-cold lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1%
392 IGEPAL® CA-630, 0.4% SDS, 1.5 mM MgCl₂, 1 mM EGTA, benzonase, and cOmplete EDTA-free Protease
393 inhibitor). Cells were dispersed with a P1000 pipette tip (~10–15 aspirations), lysates were rotated at 4 °C for 30
394 min and then centrifuged at 16,000 × g for 20 min at 4 °C. Supernatant was collected into new tubes for affinity
395 purification. Samples were incubated with 20 µL (packed beads) of streptavidin-Sepharose (GE) (equilibrated in
396 lysis buffer) with rotation overnight at 4 °C. Beads were collected (500 × g for 2 min), the supernatant was
397 discarded, and the beads were transferred to new tubes in 500 µL of lysis buffer. Beads were washed once with
398 SDS wash buffer (50 mM Tris·HCl, pH 7.5, and 2% SDS), twice with lysis buffer, and three times with 50 mM
399 ammonium bicarbonate pH 8.0 (all wash volumes: 500 µL with centrifugations at 500 × g for 30 s).

400

401

402 **Proteomic sample preparation**

403 Affinity purified proteins retained on beads were resuspended in 100 μ L of NH_4HCO_3 at 25 mM containing 2 μ g
404 of Trypsin/Lys-C mix (Mass Spec Grade, Promega, Madison, WI, USA). Digestion was performed under agitation
405 at 37°C during 4h. Then, the same enzyme amount was added to the solution and the second digestion step was
406 conducted overnight at 37°C under agitation. Supernatant was collected after a centrifugation at 500 x g for 2 min,
407 and beads were washed with water. Both solutions were pooled. The enzymatic digestion was stopped by adding
408 6 μ L of pure formic acid (2% final concentration). Samples were then centrifuged at 16,000 x g for 5 min and
409 supernatants were collected (90% of the solution). Finally, the samples were vacuum dried, and then resolubilized
410 in 400 μ L of H_2O /acetonitrile/formic acid (98/2/0.1 v/v/v).

411 **NanoLC-MS/MS Analysis**

412 NanoLC-MS/MS analyses were performed on a nanoAcquity UPLC device (Waters Corporation, Milford, USA)
413 coupled to a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were
414 loaded on a symmetry C18 pre-column (20 mm \times 180 μ m with 5 μ m diameter particles, Waters) before being
415 separated on an ACQUITY UPLC BEH130 C18 column (250 mm \times 75 μ m with 1.7 μ m diameter particles). The
416 solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent
417 B). The samples were loaded into the enrichment column over 3 min at 5 μ L/min with 99% of solvent A. Peptides
418 were then eluted at 450 nL/min with the following gradient of solvent B: from 1 to 8% over 2 min, 8 to 35% over
419 77 min, and 35 to 90% over 1 min. The MS capillary voltage was set to 2 kV at 250 °C. The system was operated
420 in a data-dependent acquisition (DDA) mode with automatic switching between MS (mass range 375-1500 m/z
421 with R = 120,000 at 200 m/z, automatic gain control fixed at 3×10^6 ions, and a maximum injection time set at 60
422 ms) and MS/MS (mass range 200-2000 m/z with R = 15,000 at 200 m/z, automatic gain control fixed at 1×10^5 ,
423 and the maximal injection time set to 60 ms) modes. The twenty most abundant peptides were selected on each
424 MS spectrum for further isolation and higher energy collision dissociation fragmentation (normalized collision
425 energy set to 27), excluding unassigned, singly charged and over seven times charged ions. The dynamic exclusion
426 time was set to 40 sec, and peptide match selection was turned on preferred.

427

428

429 **Quantitative proteomic data analysis**

430 Raw data collected during nanoLC-MS/MS were processed using MaxQuant software (version 1.6.6.0)⁵⁵. Peaks
431 were assigned with the Andromeda search engine with trypsin specificity. The database used for the searches was
432 extracted from UniProtKB-SwissProt and included all *Homo sapiens (Human)* entries (22 July 2019; Taxonomy
433 ID=9606; 20,409 entries). The minimum peptide length required was seven amino acids and a maximum of one
434 missed cleavage was allowed. The precursor mass tolerance was set to 20 ppm for the first search and 4.5 ppm for
435 the main search. The fragment ion mass tolerance was set to 20 ppm. Methionine oxidation and acetyl (Protein N-
436 term) were set as variable modifications. The maximum false discovery rate was 1% for peptides and proteins with
437 the use of a decoy strategy. The “match between runs” option was deactivated. Unique peptides were used but
438 modified peptides as well as their unmodified counterparts, were excluded from protein quantification. We used
439 the “proteingroups.txt” file with intensities (non-normalized intensities). The dataset was deposited to the
440 ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD046957.

441 **Statistical data analysis of proteomic data**

442 Statistical analyses were performed using ProStaR software⁵⁶. Proteins identified in the reverse and contaminant
443 databases and those only identified by site were removed. Moreover, only proteins for which 5 intensity values
444 were available in a single condition were kept. After log₂ transformation, intensities were normalized within
445 condition using vsn method and imputation of missing values were performed. For each sample, the slsa algorithm
446 was used for the POV (Partially Observed Values) imputation, and missing values were replaced by the 2.5
447 percentile value for the MEC (Missing on the Entire Condition); statistical testing was performed using Limma.
448 Benjamini-Hochberg method was used to adjust p-values for multiple testing and differentially expressed proteins
449 were sorted out using a p-value threshold that guarantees a FDR below 1%.

450 **Western blot and antibodies**

451 Proteins were separated by SDS-PAGE and transferred onto low-fluorescence PVDF membranes. The membranes
452 were blocked in PBS containing 5 % non-fat milk and 0.1% Tween 20 for 30 min at room temperature. Blots were
453 probed with the following antibodies: rabbit anti-eIF4E2 (4EHP) (Proteintech, catalog no. 12227-1-AP), rabbit
454 anti-YTHDF1 (Proteintech, catalog no. 17479-1-AP), rabbit anti-YTHDF2 (Proteintech, catalog no. 24744-1-AP),
455 rabbit anti-YTHDF3 (Proteintech, catalog no. 25537-1-AP), rabbit anti-eIF4ENIF1 (4E-T; Abcam, catalog no.

456 ab55881), rabbit anti-DDX6 (Proteintech, catalog no. 14632-1-AP), rabbit anti-CNOT1 (Proteintech, catalog no.
457 14276-1-AP), mouse anti-Flag (Sigma, catalog no. F1804), rabbit anti-CNOT3 (Proteintech, catalog no. 11135-1-
458 AP), rabbit anti-GIGYF1 (Bethyl, catalog no. A304-133A), rabbit anti-GIGYF2 (Proteintech, catalog no. 24790-
459 1-AP), mouse anti-GAPDH (Proteintech, catalog no. 60004-1-Ig), rabbit anti-XRN1 (Proteintech, catalog no.
460 23108-1-AP), rabbit anti-RACK1 (Cell Signaling Technology, catalog no.5432), rabbit anti-RPL10 (Cell
461 Signaling Technology, catalog no. 72912), rabbit anti-RPS3 (Proteintech, catalog no. 11990-1-AP), anti-V5 tag
462 (Invitrogen, catalog no.R960-25).

463 **Extract Preparation and Immunoprecipitation**

464 Cells were resuspended in a lysis buffer containing 20 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 2.5 mM MgCl₂,
465 0.5% IGEPAL® CA-630, 0.25% sodium deoxycholate, supplemented with cOmplete EDTA-free Protease
466 inhibitor and phosphatase inhibitor Cocktail (Roche), and incubated for 20 min on ice. The lysate was clarified by
467 centrifugation at 15,000 × g for 10 min at 4 °C. One milligram of extract was used for immunoprecipitation with
468 the indicated antibodies. Thirty microliters of pre-equilibrated Anti-FLAG® M2 Magnetic Beads (Millipore,
469 catalog no. M8823) and RNase A (Thermo Scientific) were added, and the mixtures were rotated overnight at 4
470 °C. Beads were washed five times with lysis buffer and directly resuspended in protein sample buffer for Western
471 blot analysis.

472 **CRISPR/cas9-mediated genome editing**

473 CRISPR-Cas9-mediated genome editing of HEK293 cells was performed as previously described⁵⁷. The following
474 oligonucleotides encoding a small guide RNA cognate to the coding region of *Rack1* gene were used: 5'-
475 CACCGTGTCAACCGCACGTCTATGC and 5'-AAACGCATAGACGTGCGGTTGACAC. These oligos which
476 contain BbsI restriction sites were annealed creating overhangs for cloning of the guide sequence oligos into
477 pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene plasmid #62988) by BbsI digestion. To generate KO HEK293T
478 cells, we transfected 700,000 cells with the pSpCas9(BB)-2A-Puro plasmid. 24 hr after transfection, puromycin
479 was added in the cell medium to 1.5 µg/mL final concentration. After 72 h, puromycin-resistant cells were isolated
480 into 96-well plates to obtain monoclonal colonies. Clonal cell populations were analyzed by WB for protein
481 depletion.

482 **Polysome fractionation by Ribo Mega-SEC**

483 Ribo Mega-SEC was performed as previously described with minor changes²⁶. Cells in two 15 cm dishes (70%
484 confluency) were treated with 100 µg/ml cycloheximide for 5 min, washed with ice-cold PBS containing 50 µg/ml
485 cycloheximide, scraped on ice, collected by centrifugation, lysed in 600 µl of polysome extraction buffer (20 mM
486 Hepes-NaOH (pH 7.4), 130 mM NaCl, 10 mM MgCl₂, 1% CHAPS, 2.5 mM DTT, 50 µg/ml cycloheximide, 20 U
487 RNaseIn RNase inhibitor, cOmplete EDTA-free Protease inhibitor), incubated for 15 min on ice, and centrifuged
488 at 17,000 g for 10 min (all centrifugations at 4°C). Supernatants were filtered through 0.45 µm Ultrafree-MC HV
489 centrifugal filter units by 12,000 g for 2 min before injecting to SEC. For SEC, an Agilent Bio SEC-5 2,000 Å
490 column (7.8 × 300 mm with 5 µm particles) was equilibrated with two column volumes of filtered SEC buffer (20
491 mM Hepes-NaOH (pH 7.4), 60 mM NaCl, 10 mM MgCl₂, 0.3% CHAPS, 2.5 mM DTT) and 100 µl of 10 mg/ml
492 of filtered bovine serum albumin (BSA) solution diluted by PBS was injected once to block the sites for non-
493 specific interactions. 500 µl of cell lysates were injected onto the column. The flow rate was 0.2 ml/min and 48 ×
494 100 µl fractions were collected in a low-protein binding 96-deep-well plate (Eppendorf) at 4°C. The chromatogram
495 was monitored by measuring UV absorbance at 215, 260 and 280 nm. 20 µl of each fraction were used for western
496 blotting.

497 **Structure prediction using AlphaFold2**

498 The predicted structure of the YTHDF2/RACK1 interaction was performed using AlphaFold2 as implemented in
499 ColabFold^{28,29}. A chimeric sequence in which full-length RACK1 was concatenated at the C-terminal end to
500 YTHDF2 (AA: 200-275) with a 30-glycine linker, and used as input in default settings and in absence of any
501 imposed constraints to generate five models.

502 **Codon usage analysis**

503 BioMART was used to retrieve the coding sequences of 19,068 human transcripts (GRCh38.p14) from *Ensembl*.
504 The codon adaptation index and GC level were calculated using the CAIcal tool (genomes.urv.es/CAIcal/).
505 Statistical analysis and plots were obtained with GraphPad Prism software.

506

507

508 **Confocal microscopy scanning**

509 HEK293T cells were grown in U-Slide ibiTreat coverslip multi-well chambers and transfected with 20 ng of pCI-
510 eGFP-YTHDF2 (FL or Δ NT) vector. After 24 h, cells were fixed with 4% paraformaldehyde and permeabilized
511 in PBS containing 0.1% Triton X100, blocked in blocking buffer (1% BSA, 22.5 mg/mL glycine in PBS + 0.1%
512 Tween 20) for 30 min. Anti-DDX6 antibody was added onto the cells in blocking buffer for 1 h at 37 °C. After
513 three washes in PBS, cells were incubated with secondary antibody coupled with Alexa Fluor 568 (Thermo Fisher
514 Scientific, catalog no. A-11011) for 45 min at 37°C, and mounted on glass slides in mounting solution containing
515 DAPI (Fluoroshield, Sigma-Aldrich). Fluorescence was visualized under a LEICA-SP8ST-WS confocal
516 microscope.

517 **Expression and purification of His₆-YTHDF2**

518 His₆-YTHDF2, FL or its mutants (W239A/I242A and Δ 239-245) were expressed in *E. coli* BL21 (DE3) Codon+
519 (Agilent). Large-scale expression was done in 1 L of auto-inducible terrific broth media (ForMedium
520 AIMTB0260) supplemented with kanamycin (50 μ g/mL) and chloramphenicol (25 μ g/mL), first at 37°C for 3 h
521 and then at 18°C overnight. Cells were collected, pelleted and then resuspended in 30 mL of lysis buffer (25 mM
522 HEPES pH 7.5, 300 mM NaCl and 20 mM Imidazole) supplemented with one protease inhibitor tablet (Roche),
523 0.5 mM PMSF and 30 μ L benzonase nuclease (Millipore Sigma). The cells were lysed by sonication on ice and
524 the lysate clearance was performed by centrifugation at 20,000 g for 30 min. The supernatant was applied on Talon
525 Metal Affinity Resin (Clontech) pre-equilibrated with the lysis buffer and incubated at 4°C on a rotating wheel for
526 1 h, followed by a washing step with 30 mL of washing buffer (25 mM HEPES pH 7.5 and 1 M NaCl). His₆-
527 YTHDF2 was eluted by the addition of 15 mL of elution buffer (25mM HEPES pH 7.5, 300 mM NaCl and 400
528 mM Imidazole), followed by concentrating up to 10 mL by a 30 kDa cutoff concentrator. The sample was then
529 diluted to 50 mL using Hitrap S buffer A (50 mM MES pH 6.0 and 50mM NaCl), followed by loading on a 5 mL
530 an HiTrap S FF column (Cytiva) and eluted using a NaCl linear gradient from 50 mM (100% Hitrap S buffer A)
531 to 1 M (100% Hitrap S buffer B: 50 mM MES pH 6.0, and 1 M NaCl). The fractions containing His₆-YTHDF2
532 protein were collected, concentrated up to 5 mL, and injected on a Superdex 200 increase 10/300 size-exclusion
533 column (Cytiva) with Gel filtration buffer (25 mM HEPES pH 7.5, 300 mM NaCl and 5 mM β -mercaptoethanol).
534 The fractions containing His₆-YTHDF2 were collected and concentrated.

535 **Expression and purification of GST-RACK1**

536 Expression of GST-RACK1 and its mutant Y246A was carried out in *E. coli* BL21 (DE3) Codon+ (Agilent) in 1
537 L of auto-inducible terrific broth media (ForMedium AIMTB0260) supplemented with ampicillin at 100 µg/mL
538 and chloramphenicol at 25 µg/mL. Cultures were incubated at 18°C for 20 h. Bacteria were harvested by
539 centrifugation and resuspended in lysis buffer (25 mM HEPES pH 7.5, 50 mM NaCl, 5 mM β-mercaptoethanol).
540 Cell lysis was performed by sonication on ice. After centrifugation for 30 min at 20,000 g at 4°C, clarified samples
541 were transferred to batch-bind with Glutathione Sepharose™ 4B (Cytiva) resin for 1 h at 4°C followed by a
542 washing step with 30 mL of washing buffer (25 mM HEPES pH 7.5, 50mM NaCl and 5 mM β-mercaptoethanol)
543 supplemented with 10 mM ATP. GST-RACK1 was eluted with lysis buffer supplemented with 20 mM GSH (pH
544 7.0) and further purified on an HiTrap Q FF column (Cytiva) using a linear gradient of 100% Hitrap buffer A (20
545 mM Tris/HCl pH 8.0, 50 mM NaCl and 5 mM β-mercaptoethanol) to 100% Hitrap buffer B (20 mM Tris/HCl pH
546 8.0, 1 M NaCl and 5 mM β-mercaptoethanol). The peak fractions corresponding to GST-RACK1 were pooled,
547 concentrated up to 5 mL, and injected on a Superdex 75 increase 10/300 size-exclusion column (Cytiva) with Gel
548 filtration buffer (20 mM Tris/HCl pH 8.0, 200 mM NaCl and 5 mM β-mercaptoethanol). The fractions containing
549 GST-RACK1 were concentrated and used for pull-down assays.

550 **Pull-down assays**

551 Both Ni-NTA and GST pull-down experiments were performed by incubating 1 nmol of His₆-YTHDF2, FL or
552 mutants, with the same molar amount of GST-RACK1. GST was used as negative control. All proteins were free
553 of nucleic acids according to the OD 280 nm /OD 260 nm ratio. For Ni-NTA pull-down, binding buffer (25 mM
554 HEPES pH 7.5, 150 mM NaCl and 50 mM imidazole) was added to a final volume of 60 µL. The reaction mixtures
555 were incubated on ice for 1 h. After that, 10 µL was withdrawn and used as an input fraction for SDS-PAGE
556 analysis. The remaining 50 µL were incubated at 4°C for 2 h with 40 µg of pre-equilibrated HisPur Ni-NTA
557 magnetic beads (Thermo Scientific) in a final volume of 200 µL. Beads were then washed three times with 500
558 µL of binding buffer. Bound proteins were eluted with 50 µL of elution buffer (25 mM HEPES pH 7.5, 150 mM
559 NaCl, 250 mM imidazole and 10% glycerol). Samples were resolved on SDS-PAGE and visualized by Coomassie
560 blue staining. For GST pull-down, the supernatant was applied on pre-equilibrated Glutathione Sepharose™ 4B
561 resin (Cytiva) and incubated at 4°C on a rotating wheel for 1 h, followed by a washing step with 1 mL of lysis

562 buffer (50 mM HEPES pH 7.5, 150mM NaCl and 10% Glycerol). Retained proteins were eluted by 50 μ L of
563 elution buffer (50 mM HEPES pH 7.5, 150mM NaCl, 10% Glycerol and 20mM GSH), followed by SDS-PAGE
564 and Coomassie blue staining.

565 Tethering and luciferase assays

566 HEK293T cells were transfected with 20 ng of RLuc-5BoxB, 5 ng of FLuc, and 100 ng of λ N-fusion constructs
567 per well in a 24-well plate by using Lipofectamine 2000 (Thermo Scientific, 11668019) according to the
568 manufacturer's instructions. Cells were lysed 24 h after transfection and luciferase activities were measured with
569 the Dual-Luciferase Reporter Assay System (Promega) in a GloMax 20/20 luminometer (Promega). RLuc/FLuc
570 activity values represent the mean ratio of RLuc luminescence normalized against FLuc expressed as percentage
571 of the ratio of the cells transfected with the λ N-V5 empty vector.

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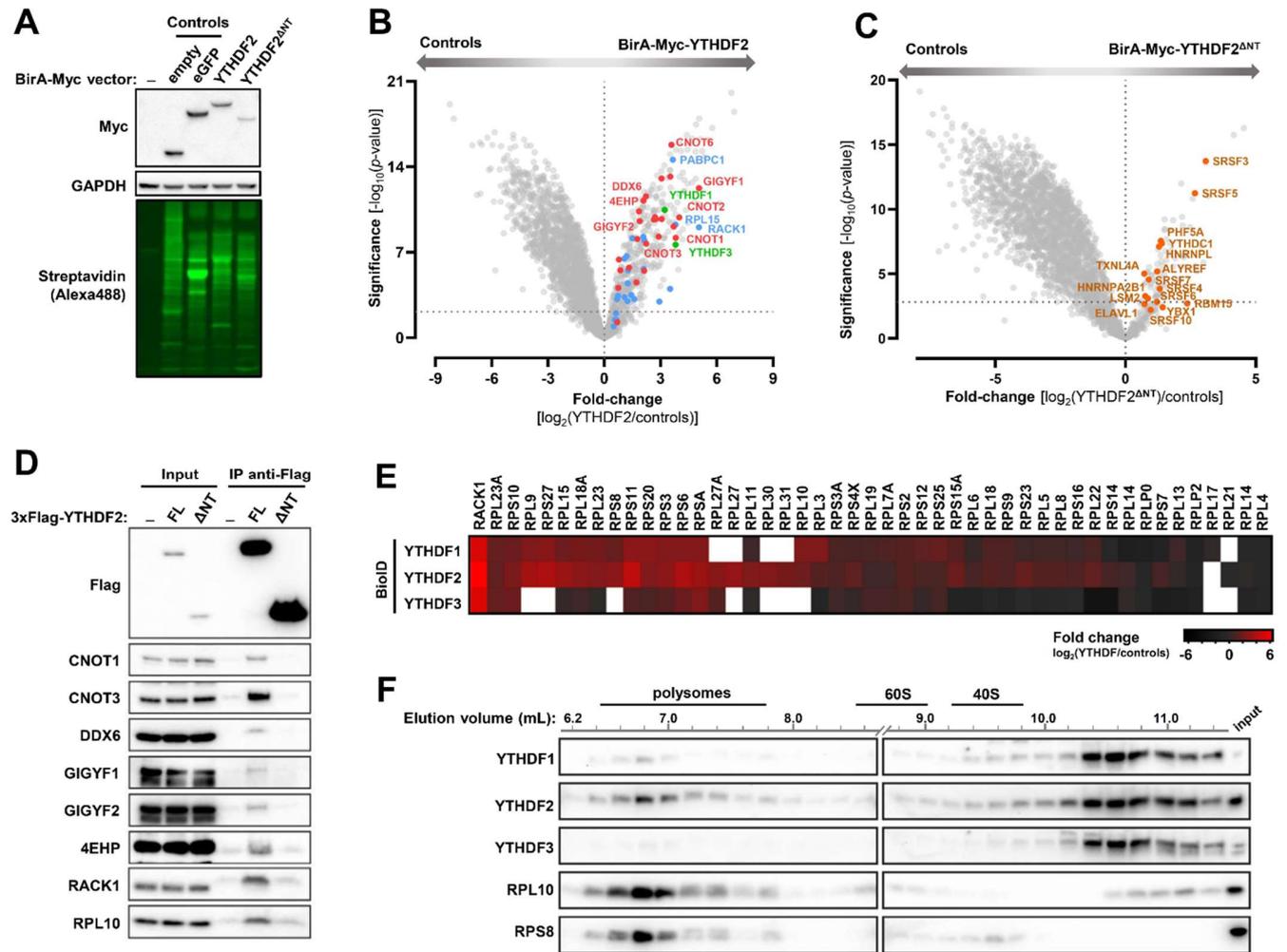
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Figure 1



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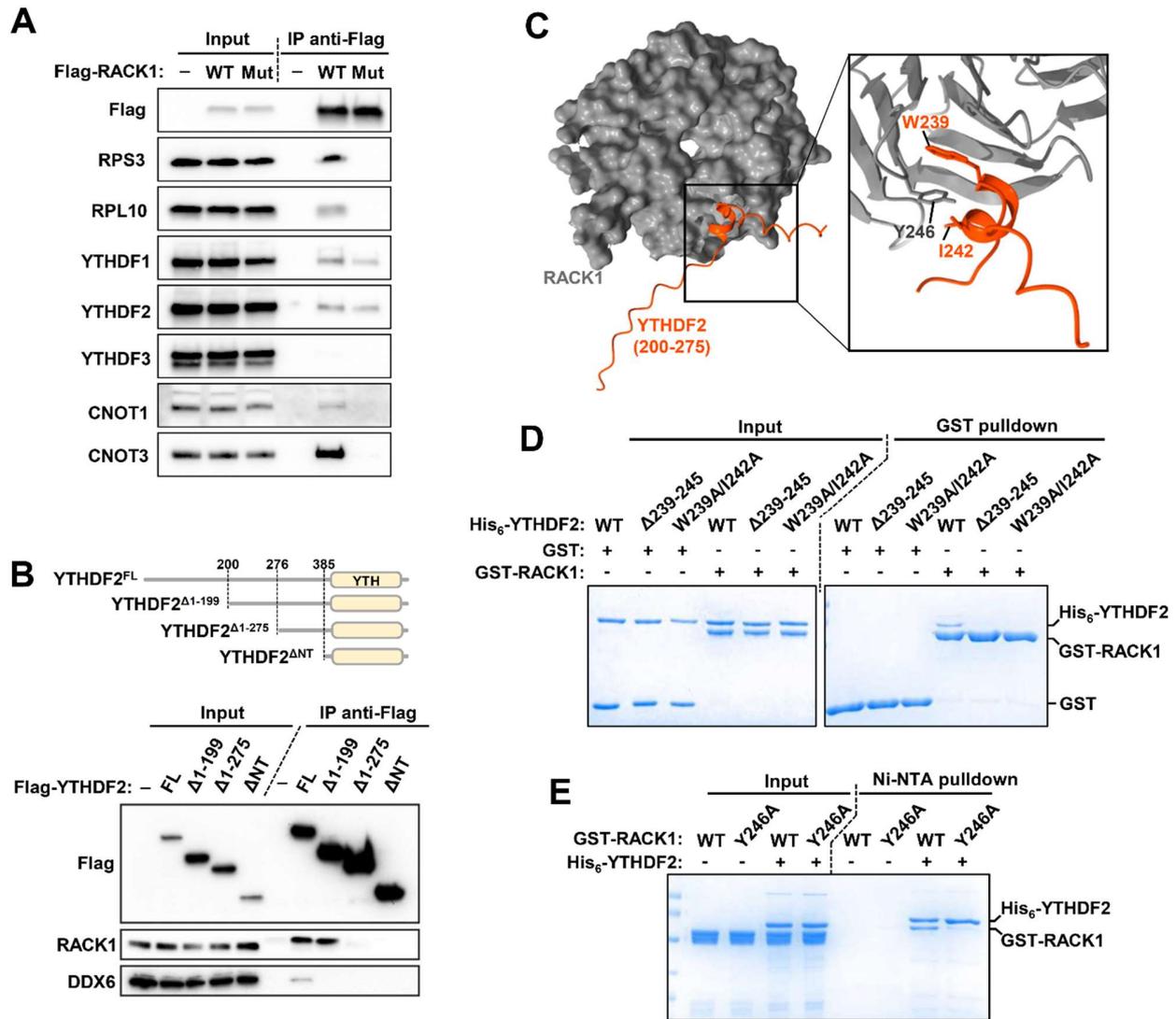
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Figure 2



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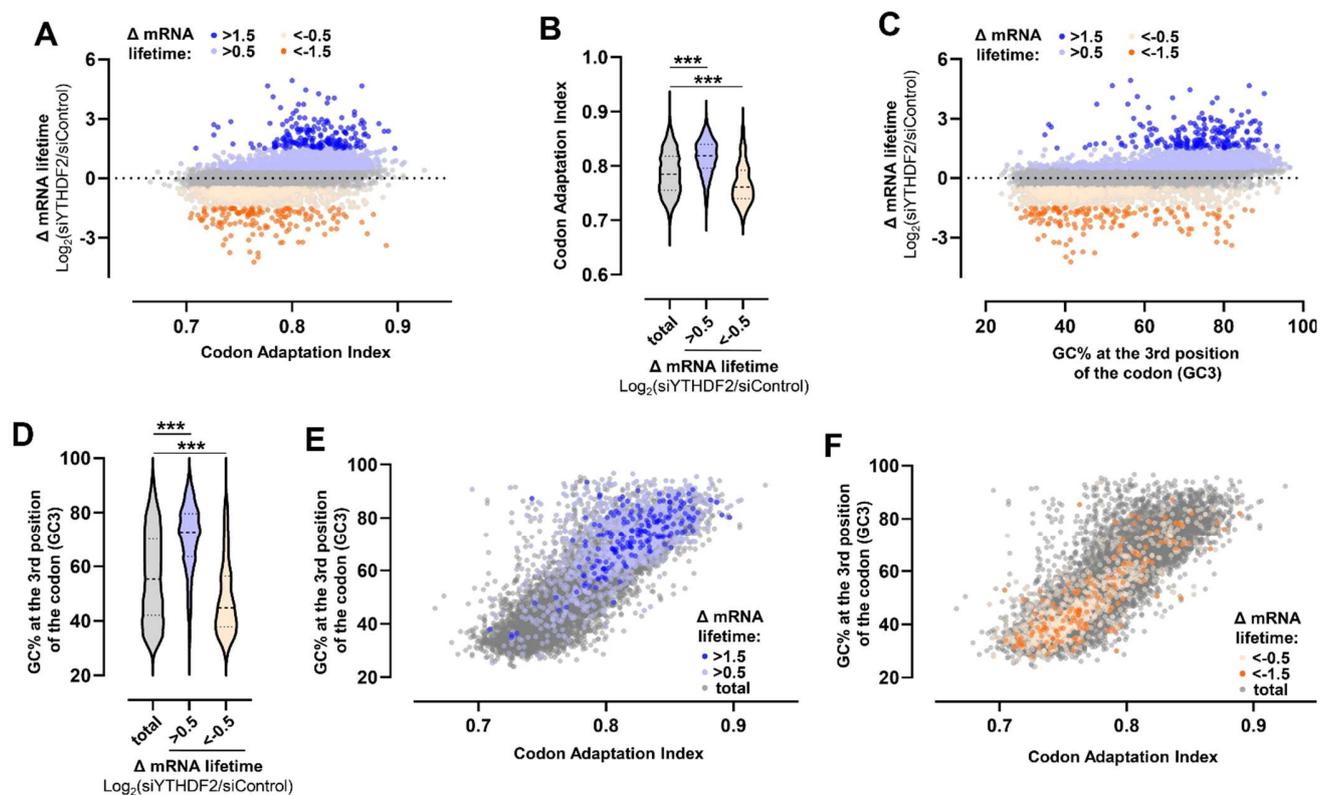
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Figure 3



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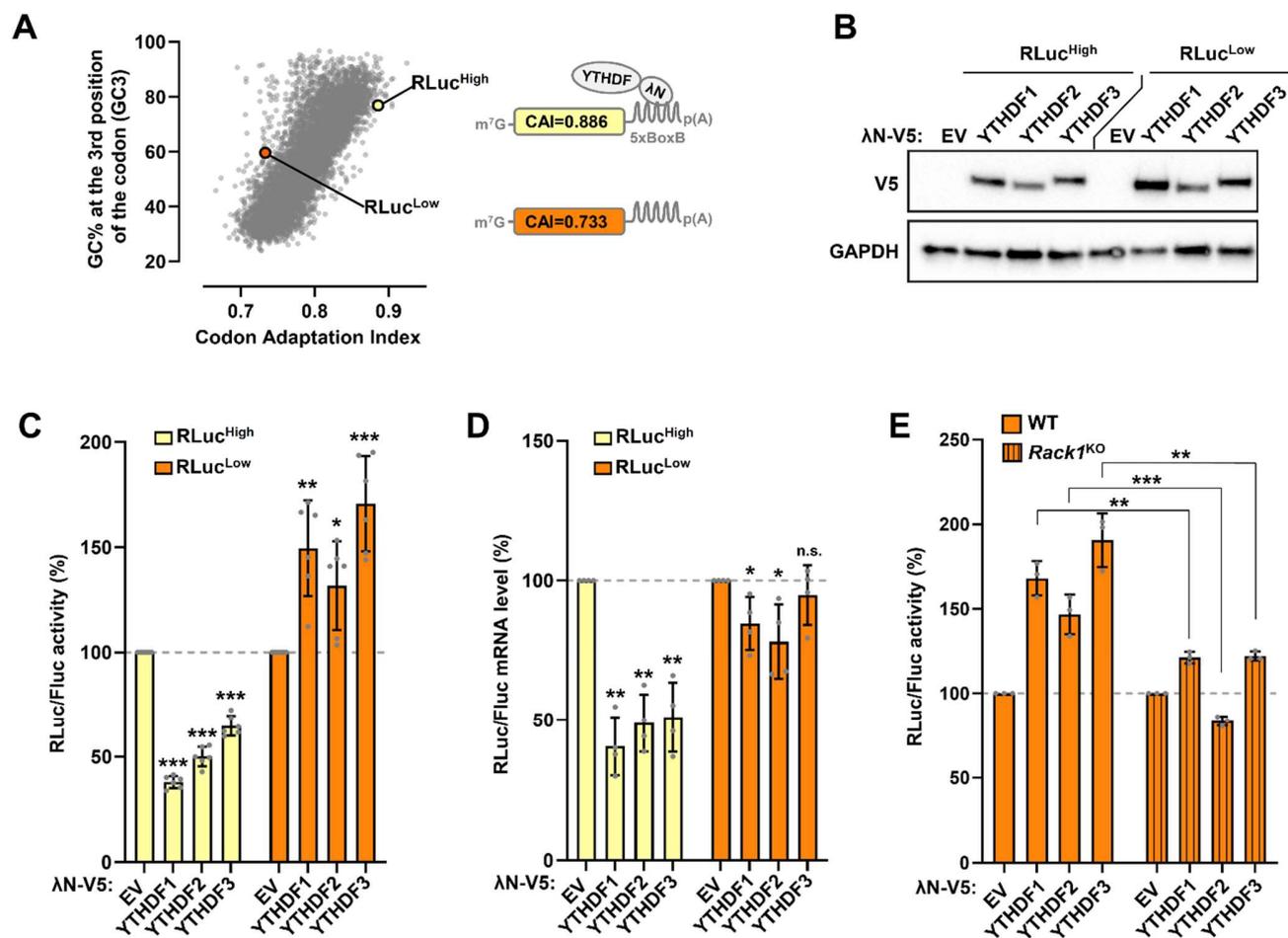
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Figure 4



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741 **FIGURE LEGENDS**

742 **Figure 1 – BioID mapping of the YTHDF1-3 proteins reveals their close proximity with the translation**
743 **apparatus**

744 (A) Western blot of lysates of non-transfected HEK293T cells (-) or transiently transfected with constructs to
745 express either BirA-Myc alone (empty), BirA-Myc-fused eGFP, or BirA-Myc-fused YTHDF2 or a version without
746 its N-terminus (Δ NT). Biotinylation was analysed using Alexa488-labelled streptavidin, and expression levels of
747 the fusion proteins were analyzed with antibodies against the Myc tag. GAPDH was used as a loading control.

748 (B) Volcano plot showing proteins enriched in the YTHDF2 BioID over the control BioID samples (BirA-Myc
749 alone + BirA-Myc-fused eGFP). The logarithmic fold-changes were plotted against negative logarithmic P values
750 of a two-sided two samples t-test. A selection of silencing factors (red) and translation factors, including ribosomal
751 proteins (blue) is indicated. The YTHDF paralogs are shown in green.

752 (C) Volcano plot showing of the interactome of a version of YTHDF2 deleted of its N-terminus (region 1-389).
753 The plot represents the differential enrichment of proteins detected in the BirA-Myc-YTHDF2 ^{Δ NT} dataset (positive
754 values) calculated against data obtained with the negative controls, BirA-Myc alone and BirA-Myc-eGFP
755 (negative values). Proteins involved in RNA splicing are shown in orange.

756 (D) Interaction of YTHDF2 with its high confidence preys. Vectors expressing 3xFlag-tagged YTHDF2, either
757 Full-Length (FL) or Δ NT, or 3xFlag as control (-), were transfected into HEK293T cells. Extracts were
758 immunoprecipitated with anti-Flag antibody. Total lysates (input) and IP extracts were analyzed by Western blot
759 (WB) with the indicated antibodies.

760 (E) Heatmap showing the logarithmic fold-changes of the ribosomal proteins detected in the BioID datasets of
761 BirA-Myc-fused YTHDF1, 2 or 3 over the control BioID samples (BirA-Myc alone and BirA-Myc-fused eGFP).
762 Note that the white color indicate that the protein was not present in the MS dataset.

763 (F) Ribo Mega-SEC fractionation of a HEK293T cell lysate. Extract was fractionated using a flow rate of 0.2
764 ml/min on a 2,000 Å SEC column. The chromatogram is shown in Supplementary Figure S2E. Western blot was
765 performed with the indicated antibodies.

766 **Figure 2 – The N-terminus of YTHDF2 directly interacts with the ribosomal protein RACK1**

767 (A) Western blot showing the ribosome-independent interaction between 3xFlag-RACK1 and YTHDF2. RNase
768 A-treated extracts from cells expressing 3xFlag-RACK1, WT or carrying the R36D/K38E substitution (Mut), were
769 used for Flag IP. Inputs and bound fractions were analyzed by Western blotting using the indicated
770 antibodies. Empty vectors served as negative controls (-).

771 (B) Top: schematic cartoon of FLAG-tagged YTHDF2 truncations immunoprecipitated in experiments shown in
772 the bottom panel. Bottom: co-immunoprecipitation of 3xFlag-tagged versions of YTHDF2. Total lysates (input)
773 and IP extracts were analyzed by Western blot (WB) with the indicated antibodies.

774 (C) AlphaFold2-predicted structure of the 200-275 segment of YTHDF2 (orange) bound to RACK1 (gray). The
775 insert shows an enlarged view of the putative interface involving tyrosine 246 of RACK1 (Y246) stacked between
776 tryptophan 239 (W239) and isoleucine 242 (I242). Ribbon representations were developed using ChimeraX.

777 (D) GST pull-down assay showing the interaction between recombinant GST-fused RACK1 and His₆-YTHDF2.
778 GST-fused RACK1 was incubated with His₆-YTHDF2, WT or its versions harboring the double substitution
779 W239A/I242A or deletion of the 239-245 segment (Δ 239-245). GST served as negative control. The starting
780 material (Input) and bound fractions (GST pulldown) were analyzed by SDS-PAGE followed by Coomassie blue
781 staining.

782 (E) Ni-NTA pull-down assay showing the interaction between recombinant His₆-YTHDF2 and GST-RACK1.
783 His₆-YTHDF2 was incubated with GST-RACK1, WT or with the substitution Y246A. The starting material
784 (Input) and bound fractions (Ni-NTA pull-down) were analyzed by SDS-PAGE followed by Coomassie blue
785 staining.

786 **Figure 3 – YTHDF2 sensitivity among the transcriptome is correlated with by the codon usage**

787 (A) Scatter plot representing mRNA lifetime change following YTHDF2 knockdown in HeLa cells (publicly
788 available data¹⁰) versus the Codon Adaptation Index of each transcript. A total of 7699 mRNAs are represented
789 and colored according to the Δ mRNA lifetime ($\text{Log}_2(\text{siYTHDF2}/\text{siControl})$), including 1602 mRNAs with a Δ
790 greater than 0.5, (197 above 1.5), and 938 with a Δ lower than -0.5 (149 below -1.5).

791 (B) Violin Plot showing the CAI score for each category of mRNAs shown in (A). P value was determined by a
792 Kolmogorov-Smirnov test: (***) $P < 0.0001$.

793 (C) Scatter plot representing Δ mRNA lifetime following YTHDF2 knockdown (publicly available data¹⁰) versus
794 the percentage of GC at the third position of the codons (GC3) of each transcript. mRNAs are colored according
795 to the Δ mRNA lifetime ($\text{Log}_2(\text{siYTHDF2}/\text{siControl})$), same as in (A).

796 (D) Violin Plot showing the GC3 score for each category of mRNAs shown in (C). P value was determined by a
797 Kolmogorov-Smirnov test: (***) $P < 0.0001$.

798 (E-F) The subsets of YTHDF2-targeted mRNAs described in (A-D) were indicated on a scatter plot showing the
799 CAI of each mRNA detected in HeLa cells versus their GC3 score (represented as grey dots). The subsets of
800 mRNAs whose lifetime was increased (E) or decreased (F) following YTHDF2 knockdown are colored in orange
801 and blue, respectively.

802 **Figure 4 – Codon usage drives YTHDF-mediated silencing of a reporter mRNA**

803 (A) Schematic of the λ N/BoxB tethering assay using a RLuc-5boxB reporter construct. Two degrees of codon
804 optimality were designed for the coding region of the Renilla Luciferase (RLuc) mRNA: RLuc^{High} and RLuc^{Low}
805 with a CAI of 0.886 and 0.733, respectively. GC3 and CAI scores of each reporter are indicated in the scatter plot
806 representing the percentage of GC at the third position of the codon (GC3) and the CAI of the coding region for
807 each transcript described in the UCSC database (represented as grey dots). For the tethering assay, recruitment of
808 the YTHDF proteins to the Renilla Luciferase (RLuc) mRNA was mediated by the fused λ N peptide which has a
809 high affinity for the BoxB sequence.

810 (B) Western blot showing the expression of λ N-V5-fused YTHDF proteins used in the λ N/BoxB tethering assay.
811 Extracts of HEK293T cells transfected with an empty vector (EV) or plasmids encoding the λ N-V5-YTHDF
812 proteins were analyzed by Western blot with the indicated antibodies.

813 (C) λ N/BoxB tethering assay of the YTHDF protein on the RLuc^{High} and RLuc^{Low} reporters. HEK293T cells were
814 co-transfected with plasmids encoding λ N-V5-YTHDF1-3, RLuc^{High} or RLuc^{Low}, and Firefly luciferase (FLuc).
815 RLuc/FLuc activity values represent the mean ratio of RLuc luminescence normalized against FLuc expressed as

816 percentage of the ratio of the cells transfected with the λ N-V5 empty vector (EV). The mean values (\pm SD) from
817 six independent experiments are shown and the P value was determined by two-tailed Student's t-test: (***) P <
818 0.001; (**) P < 0.01; (*) P < 0.05 (compared to EV for each reporter).

819 **(D)** Measurements by RT-qPCR to estimate the RLuc and FLuc mRNA levels from the λ N/BoxB tethering assay
820 of the YTHDF protein on the RLuc^{High} and RLuc^{Low} reporters. The mean values (\pm SD) from four independent
821 experiments are shown and the P value was determined by two-tailed Student's t-test: (**) P < 0.01; (*) P < 0.05
822 and n.s. means not significant (compared to EV for each reporter).

823 **(E)** λ N/BoxB tethering assay of the YTHDF protein on the RLuc^{Low} reporter in RACK1^{KO} cells. WT and *Rack1*^{KO}
824 cells were co-transfected with indicated vectors, and RLuc/FLuc activities were analyzed as in (C).

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