Principles of mRNA targeting via the Arabidopsis m^{6}A-binding protein ECT2

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Abstract

Specific recognition of *N6*-methyladenosine (m^6A) in mRNA by RNA-binding proteins containing a YT521-B homology (YTH) domain is important in eukaryotic gene regulation. The Arabidopsis YTH-domain protein ECT2 is thought to bind to mRNA at URU(m^6A)Y sites, yet RR(m^6A)CH is the canonical m^6A consensus site in all eukaryotes and ECT2 functions require m^6A binding activity. Here, we apply iCLIP (individual-nucleotide resolution cross-linking and immunoprecipitation) and HyperTRIBE (targets of RNA-binding proteins identified by editing) to define high-quality target sets of ECT2, and analyze the patterns of enriched sequence motifs around ECT2 crosslink sites. Our analyses show that ECT2 does in fact bind to RR(m^6A)CH. Pyrimidine-rich motifs are enriched around, but not at m^6A-sites, reflecting a preference for *N6*-adenosine methylation of RRACH/GGAU islands in pyrimidine-rich regions. Such motifs, particularly oligo-U and UNUNU upstream of m^6A sites, are also implicated in ECT2 binding via its intrinsically disordered region (IDR). Finally, URUAY-type motifs are enriched at ECT2 crosslink sites, but their distinct properties suggest function as sites of competition between binding of ECT2 and as yet unidentified RNA-binding proteins. Our study provides coherence between genetic and molecular studies of m^6A-YTH function in plants, and reveals new insight into the mode of RNA recognition by YTH-domain-containing proteins.

Introduction

N6-methyladenosine (m^6A) is the most abundant modified nucleotide in eukaryotic mRNA bodies. It is required for embryonic development and stem cell differentiation in several animals and plants (Zhong et al. 2008; Batista et al. 2014; Ping et al. 2014; Geula et al. 2015; Zhang et al. 2017) and for the control of the meiotic program in yeast (Shah and Clancy 1992; Clancy et al. 2002; Agarwala et al. 2012). Most *N6*-adenosine methylation of mRNA is catalyzed in the nucleus (Salditt-Georgieff et al. 1976; Ke et al. 2017; Huang et al. 2019) by a highly conserved, multimeric methylase (the m^6A "writer") (Balacco and Soller 2019) whose catalytic core consists of the heterodimer METTL3/METTL14 (MTA/MTB in plants) (Bokar et al. 1997; Zhong et al. 2008; Liu et al. 2014). In addition, a number of highly conserved proteins is required for *N6*-methylation *in vivo* (Balacco and Soller 2019). The strong conservation of these core factors suggests that the biochemical basis of *N6*-adenosine methylation is common in eukaryotes and indeed, m^6A occurs in the consensus site RR($m^6\underline{A}$)CH (R=G/A, H=A/C/U), primarily in 3'-UTRs in animals (insects, mammals and fish), plants (maize and Arabidopsis) and fungi (yeast) that possess the canonical METTL3/METTL14 methyltransferase (Dominissini et al. 2012; Meyer et al. 2012; Schwartz et al. 2013; Luo et al. 2014; Lence et al. 2016; Zhao et al. 2017; Li et al. 2019; Miao et al. 2019; Parker et al. 2020). Conversely, the characteristic motif and gene-body location is not detected in organisms that lack METTL3/METTL14 homologs, such as the nematode *Caenorhabditis elegans* (Sendinc et al. 2020) and bacteria (Deng et al. 2015).

m^6A may impact mRNA function by different mechanisms, including the creation of binding sites for reader proteins that specifically recognize m^6A in mRNA (Dominissini et al. 2012; Fu et al. 2014; Meyer and Jaffrey 2014). The best understood class of readers contains a so-called YT521-B homology (YTH) domain (Stoilov et al. 2002) of which two phylogenetic groups, YTHDF and YTHDC, have been defined (Patil et al. 2018; Balacco and Soller 2019). The YTH domain harbors a hydrophobic methyl-binding pocket that increases the affinity of m^6A-containing RNA by more than 10-fold compared to unmethylated RNA (Li et al. 2014b; Luo and Tong 2014; Theler et al. 2014; Xu et al. 2014; Zhu et al. 2014). Apart from interactions with the methylated adenosine and the purine at the -1 position, YTH-RNA domain interactions mostly involve the sugar-phosphate backbone of the RNA (Luo and Tong 2014; Theler et al. 2014; Xu et al. 2014). That is consistent with only mild reductions in the binding affinity of the YTH domain of human YTHDC1 upon substitution of nucleotides -2, +1 and +3 that abrogate the canonical RR($m^6\underline{A}$)CH motif (Xu et al. 2014), and poor sequence specificity of RNA binding by isolated YTH domains of human YTHDF1, YTHDF2 and YTHDC1 (Arguello et al. 2019). Thus, the methyltransferase complex gives the

72 sequence specificity, while YTH domain proteins may bind to m^{6}A-containing RNA regardless of
73 the identity of the immediately adjacent nucleotides.

74 YTHDF proteins are typically cytoplasmic and consist of a long N-terminal intrinsically
75 disordered region (IDR) followed by the globular YTH domain (Patil et al. 2018). Because the
76 affinity of isolated YTH domains for m^{6}A-containing RNA is modest, typically with dissociation
77 constants on the order of 0.1-1 μM (Li et al. 2014b; Luo and Tong 2014; Theler et al. 2014; Xu et al.
78 2014; Zhu et al. 2014), it has been suggested that the IDR may participate in RNA binding (Patil et
79 al. 2018). Nonetheless, the clearest evidence for functions of the IDRs in YTHDF proteins reported
80 thus far includes direct interactions with effectors such as the CCR4-NOT complex in mammalian
81 cells (Du et al. 2016), and the ability to cause liquid-liquid phase transition when sufficiently high
82 local concentrations are reached (Arribas-Hernández et al. 2018; Gao et al. 2019; Ries et al. 2019;
83 Fu and Zhuang 2020; Wang et al. 2020).

84 The YTHDF family comprises 11 proteins in *Arabidopsis* that are referred to as
85 EVOLUTIONARILY CONSERVED C-TERMINAL REGION1-11 (ECT1-11) (Li et al. 2014a;
86 Scutenaire et al. 2018). ECT2, ECT3 and ECT4 are expressed in rapidly dividing cells of root, leaf
87 and flower primordia and genetic analyses have revealed their general importance in development.
88 Simultaneous inactivation of *ECT2* and *ECT3* causes slow organogenesis and abnormal
89 morphology of leaves, roots, stems, flowers, and fruits, and these defects are generally enhanced
90 by additional mutation of *ECT4* (Arribas-Hernández et al. 2018; Arribas-Hernández et al. 2020).
91 Importantly, the biological functions of ECT2/3/4 described thus far are shared with those of m^{6}A
92 writer components and, where tested, have been shown to depend on intact m^{6}A-binding pockets,
93 strongly suggesting that the basis for the observed phenotypes in *ect2/3/4* mutants is defective
94 regulation of m^{6}A-modified mRNA targets (Bodi et al. 2012; Shen et al. 2016; Růžička et al. 2017;
95 Arribas-Hernández et al. 2018; Scutenaire et al. 2018; Wei et al. 2018; Arribas-Hernández et al.
96 2020). Despite this progress in identifying biological functions of plant m^{6}A-YTHDF axes, a number
97 of fundamental questions regarding their molecular basis remains unanswered. For example, it is
98 unclear whether sequence determinants in addition to m^{6}A are important for mRNA target
99 association of ECT proteins *in vivo*, the mRNA targets of ECT2/3/4 responsible for the
100 developmental delay of *ect2*/*ect3*/(*ect4*) mutants have not been identified, and it is not clear what
101 the effects of ECT2/ECT3/ECT4 binding to them may be (Arribas-Hernández and Brodersen 2020).
102 Clearly, robust identification of the mRNA targets directly bound by ECT proteins is key to obtain
103 satisfactory answers to all of these questions. Towards that goal, formaldehyde crosslinking and
104 immunoprecipitation (FA-CLIP) was used to identify mRNA targets of ECT2 (Wei et al. 2018).

105 Nonetheless, because formaldehyde, in contrast to UV illumination, generates both protein-protein
106 and protein-RNA crosslinks, it is not an ideal choice for identification of mRNAs bound directly by a
107 protein of interest (see Arribas-Hernández and Brodersen (2020) for a discussion). In particular,
108 this problem concerns the unexpected conclusion that ECT2 binds to the 'plant-specific consensus
109 motif' URU(m^6A)Y (Y=U/C), not RR(m^6A)CH (Wei et al. 2018). Thus, the field of gene regulation via
110 m^6A-YTHDF modules in plants is in a state of confusion: On the one hand, m^6A mapping (Luo et al.
111 2014; Wan et al. 2015; Shen et al. 2016; Duan et al. 2017; Anderson et al. 2018; Miao et al. 2019;
112 Parker et al. 2020) and phenotypes of mutants defective in m^6A writing (Bodi et al. 2012; Shen et
113 al. 2016; Růžička et al. 2017) or m^6A-binding of ECT2/ECT3/ECT4 (Arribas-Hernández et al. 2018;
114 Arribas-Hernández et al. 2020) suggest that these YTHDF proteins should act via recognition of
115 m^6A in the RRACH context. On the other hand, the only attempt at a mechanistic understanding of
116 ECT2 function via mRNA target identification concluded that ECT2 binds to a sequence element
117 different from RRACH (Wei et al. 2018). To complicate matters further, a number of motifs including
118 not only URUAY, but also UGUAMM (M=A/C), UGWAMH (W=A/U), UGUAWA and GGAU have
119 been reported to be enriched around m^6A sites in *Arabidopsis* and other plant species (Li et al.
120 2014c; Anderson et al. 2018; Miao et al. 2019; Zhang et al. 2019; Zhou et al. 2019), but it remains
121 unclear whether the adenosines in such motifs are methylated *in vivo*. Alternatively, these
122 sequence contexts may play a role in guiding m^6A deposition or ECT recognition nearby, either
123 directly by ECT interaction or indirectly via additional RNA binding proteins assisting or competing
124 with ECT binding.

125 To clarify principles underlying mRNA recognition by ECT2, we undertook rigorous analysis
126 of its mRNA binding sites using two orthogonal methods, the proximity-labeling method
127 HyperTRIBE (targets of RNA binding proteins identified by editing) (McMahon et al. 2016; Xu et al.
128 2018), and iCLIP (individual nucleotide resolution crosslinking and immunoprecipitation) (König et
129 al. 2010). This resulted in identification of high-quality target sets as judged by mutual overlaps and
130 by overlaps with previously reported m^6A maps from plants at a similar developmental stage (Shen
131 et al. 2016; Parker et al. 2020). Relying on this high-quality target set, we used the position
132 information inherent to iCLIP and a single-nucleotide resolution m^6A dataset (Parker et al. 2020) to
133 establish six properties of m^6A-containing mRNA and mRNA targeting by ECT2. (1) RRACH and its
134 variant DRACH (D=R/U) are unequivocally the most highly enriched motifs at m^6A sites in
135 Arabidopsis. (2) ECT2 binds to m^6A sites in the canonical RRACH context, as ECT2 crosslinking
136 sites are preferentially found immediately 5' to m^6A sites, and RRACH is enriched immediately 3' to
137 ECT2 crosslinking sites. (3) GGAU is a minor m^6A consensus site in plants. (4) U- and U/C-rich

138 motifs are enriched around, but not at, m^6A sites, and, together with RRACH and GGAU, constitute
139 core elements that distinguish m^6A-containing 3'-UTRs from non-m^6A-containing 3'-UTRs in plants.
140 (5) The IDR of ECT2 participates in RNA binding as it crosslinks to target mRNAs at U-rich
141 elements highly abundant upstream of m^6A-sites. (6) Although URUAY, URURU and similar motifs
142 may crosslink to ECT2, their presence in m^6A-containing mRNA disfavors ECT2 binding, consistent
143 with those motifs acting predominantly as sites of interaction for RNA-binding proteins that may
144 compete with ECT2.
145

146 **Results**

147 ***ADARcd fusions to ECT2 are functional in vivo***

148 HyperTRIBE uses fusion of RNA binding proteins to the hyperactive E488Q mutant of the catalytic
149 domain of the *Drosophila melanogaster* adenosine deaminase acting on RNA (*Dm*ADAR^{E488Q}cd)
150 (Kuttan and Bass 2012) to achieve proximity labeling *in vivo* (McMahon et al. 2016; Xu et al. 2018).
151 Targets are identified as those mRNAs that contain adenosine-inosine sites significantly more
152 highly edited than background controls, measured as A-G changes upon reverse transcription and
153 sequencing. To develop material suitable for ECT2 HyperTRIBE, we expressed
154 *AtECT2pro:AtECT2-FLAG-DmADAR^{E488Q}cd-AtECT2ter* (henceforth "*ECT2-FLAG-ADAR*") in the
155 single *ect2-1* and triple *ect2-1/ect3-1/ect4-2* (*te234*) knockout backgrounds (Arribas-Hernández et
156 al. 2018; Arribas-Hernández et al. 2020). We identified lines exhibiting nearly complete rescue of
157 *te234* mutant seedling phenotypes, indicating that the fusion protein was functional (*Figure 1A*). We
158 then used the expression level in complementing lines as a criterion to select lines in the *ect2-1*
159 single mutant background, for which no easily scorable phenotype has been described (*Figure 1—*
160 *figure supplement 1A*). Lines expressing free *Dm*ADAR^{E488Q}cd under the control of the endogenous
161 *ECT2* promoter (*AtECT2pro:FLAG-DmADAR^{E488Q}cd-AtECT2ter;* henceforth *FLAG-ADAR*) at levels
162 similar to or higher than those of the fusion lines (*Figure 1—figure supplement 1A,B*) were used to
163 control for background editing after verification that *FLAG-ADAR* expression did not result in
164 phenotypic abnormalities in Col-0 WT plants (*Figure 1A*).

165

166 ***The ECT2-ADARcd fusion imparts adenosine-to-inosine editing of target mRNAs in planta***

167 To identify ECT2 HyperTRIBE targets (HT-targets), we sequenced mRNA from dissected root tips
168 and shoot apices of 10-day-old seedlings of *ect2-1*/*ECT2-FLAG-ADAR* and *FLAG-ADAR*
169 transgenic lines, using five independent lines of each type as biological replicates to prevent line-
170 specific artifacts. Next, we generated nucleotide base counts for all positions with at least one
171 mismatch across the full set of samples of mapped reads (*Figure 1B*), resulting in a raw list of
172 potential editing positions. This revealed that the amount of editing was clearly higher in the lines
173 expressing the ECT2-FLAG-ADAR fusion protein than in the negative control lines (*Figure 1C,*
174 *Figure 1—figure supplement 1C*). To identify positions with significantly higher editing rates in
175 ECT2-FLAG-ADAR lines compared to controls, we developed a new approach to detect differential
176 editing (*Figure 1B*) that will be described in detail in a subsequent report. Briefly, the hyperTRIBE**R**
177 (https://github.com/sarah-ku/hyperTRIBER) method of detecting differential editing exploits the
178 powerful statistical capabilities of a method originally designed to detect differential exon usage

179 (Anders et al. 2012). It efficiently takes replicates and possible differences in expression into
180 account, resulting in high power to detect sites despite the generally low editing proportions that we
181 found in our data (*Figure 1D*). As expected, the tendency towards higher editing proportions in
182 fusion lines compared to controls was even more pronounced after filtering non-significantly edited
183 sites (*Figure 1C, Figure 1—figure supplement 1C*). Three additional properties of the resulting
184 editing sites indicate that they are the result of ADARcd activity guided by its fusion to ECT2. First,
185 the vast majority of significant hits corresponded to A-to-G transitions (*Figure 1—figure supplement*
186 *1D*). Second, the consensus motif at the edited sites matched the sequence preference of
187 *Dm*ADAR^{E488Q}cd (5' and 3' nearest-neighbor preference of U>A>C>G and G>C>A~U, respectively
188 (Xu et al. 2018)) (*Figure 1E, Figure 1—figure supplement 1E*), with highly edited sites more closely
189 matching the optimal sequence context than lowly edited ones (*Figure 1—figure supplement 1F*).
190 Third, principal component analysis of editing proportions at significant sites over the different lines
191 clearly separated the ECT2-FLAG-ADAR fusion lines from the control lines (*Figure 1F, Figure 1—*
192 *figure supplement 1G*). Application of subsequent filtering steps, including removal of non-(A-to-G)
193 mismatches and of potential line-specific single-nucleotide variants (see *Methods*), resulted in a
194 final list of 16,176 edited sites for aerial tissues and 19,242 for roots, corresponding to 4,864 and
195 5,052 genes (ECT2 HT-targets), respectively (*Figure 1B, Supplementary file 1*). In both cases, this
196 represents 27% of all expressed genes. We note that the editing proportions were generally low
197 (*Figure 1D*) compared to previous work in Drosophila (Xu et al. 2018), perhaps in part due to the
198 limited number of cells that express ECT2 (Arribas-Hernández et al. 2018; Arribas-Hernández et al.
199 2020). Indeed, the *ADAR* expression level (TPMs) correlated strongly with editing proportions
200 among *ECT2-FLAG-ADAR* lines (*Figure 1G, Figure 1—figure supplement 1H*), and editing
201 proportions were higher for target mRNAs that are co-expressed with *ECT2* in a large percentage
202 of cells according to single-cell RNA-seq (Denyer et al. 2019) (*Figure 1H*), lending further support to
203 the conclusion that the observed editing is ADAR-specific and driven to target mRNAs by ECT2.
204 Hence, HyperTRIBE can be used to identify targets of RNA binding proteins *in planta*.
205
206 ***HyperTRIBE is highly sensitive and identifies primarily m^{6}A-containing transcripts as ECT2***
207 ***targets***
208 To evaluate the properties of ECT2 HT-targets, we first noted that most of them were common
209 between root and aerial tissues (*Figure 2A*), as expected given the recurrent function of ECT2 in
210 stimulating cell division in all organ primordia (Arribas-Hernández et al. 2020). In agreement with
211 this result, most of the targets specific to root or aerial tissue were simply preferentially expressed

212 in either tissue (*Figure 2B*). Moreover, the significant editing sites in roots and aerial tissues had a
213 considerable overlap (*Figure 2A*), and their editing proportions were similar in the two tissues
214 (*Figure 2C*). Of most importance, we observed a large overlap between the ECT2 HT-targets and
215 m^6A-containing transcripts mapped by different methods in seedlings (Shen et al. 2016; Parker et
216 al. 2020), as more than 76% of ECT2 HT-targets had m^6A support by either study (*Figure 2D*).
217 These results validate our HyperTRIBE experimental setup and data analysis, and confirm that
218 ECT2 binds predominantly to m^6A-containing transcripts *in vivo*.
219
220 ***ECT2-mCherry can be specifically UV-crosslinked to target RNA in vivo***
221 We next moved on to independent target and binding site identification via iCLIP (*Figure 3A*). We
222 used transgenic lines expressing functional ECT2-mCherry under the control of the endogenous
223 *ECT2* promoter in the *ect2-1* knockout background (Arribas-Hernández et al. 2018; Arribas-
224 Hernández et al. 2020) to co-purify mRNAs crosslinked to ECT2 for iCLIP. Lines expressing the
225 *ECT2^{W464A}-mCherry* variant were used as negative controls, because this Trp-to-Ala mutation in the
226 hydrophobic methyl-binding pocket of the YTH domain abrogates the increased affinity for m^6A-
227 RNA (Li et al. 2014b; Xu et al. 2014; Zhu et al. 2014). Accordingly, the point mutant behaves like a
228 null allele in plants, despite its wild type-like expression pattern and level (Arribas-Hernández et al.
229 2018; Arribas-Hernández et al. 2020).
230 To test the feasibility of iCLIP, we first assessed the specificity of RNA co-purified with
231 ECT2-mCherry after UV-illumination of whole seedlings by 5'-radiolabeling of the
232 immunoprecipitated RNP complexes followed by SDS-PAGE. These tests showed that
233 substantially more RNA co-purifies with wild type ECT2 than with ECT2^{W464A} upon UV-crosslinking,
234 and that no RNA is detected without UV irradiation, or from irradiated plants of non-transgenic
235 backgrounds (*Figure 3B, Figure 3—figure supplement 1A*). RNAse and DNAse treatments also
236 established that the co-purified nucleic acid is RNA (*Figure 3—figure supplement 1B*). Thus, UV
237 crosslinking of intact *Arabidopsis* seedlings followed by immunopurification successfully captures
238 ECT2-RNA complexes that exist *in vivo*. Curiously, although the pattern of ECT2-RNA complexes
239 with bands migrating at ~110 and 55 kDa is highly reproducible, it does not correspond to the
240 majority of the purified ECT2-mCherry protein which runs at ~125 kDa in SDS-PAGE (*Figure 3B,C*).
241 A variety of control experiments (*Figure 3—figure supplement 1C-E*), most importantly the
242 disappearance of additional bands with use of an N-terminal rather than a C-terminal tag (*Figure*
243 *3C,D*), indicates that the band pattern arises as a consequence of proteolytic cleavage of the N-
244 terminal IDR in the lysis buffer, such that fragments purified using the C-terminal mCherry tag

245 include the YTH domain with portions of the IDR of variable lengths (*Figure 3—figure supplement*
246 *2*). Comparative analysis of RNA in 55 kDa and 110-125 kDa complexes may, therefore, provide
247 insight into the possible role of the N-terminal IDR of ECT2 in mRNA binding (*Figure 3E*), an idea
248 consistent with the comparatively low polynucleotide kinase labeling efficiency of full-length ECT2-
249 mCherry-mRNA complexes (~125 kDa) (*Figure 3B, Figure 3—figure supplement 2*). Thus, we
250 prepared separate iCLIP libraries from RNA crosslinked to ECT2-mCherry/ECT2^{W464A}-mCherry that
251 migrates at ~110-280 kDa ('110-kDa band'), and at ~55-75 kDa ('55-kDa band') (*Figure 3—figure*
252 *supplement 3*) to investigate the possible existence of IDR-dependent crosslink sites, and thereby
253 gain deeper insights into the mode of YTHDF-binding to mRNA *in vivo*.
254
255 ***ECT2-mCherry iCLIP peaks are enriched in the 3'-UTR of mRNAs***
256 We identified a total of 15,960 iCLIP 'peaks' or crosslink sites (i.e. single nucleotide positions called
257 by PureCLIP from mapped iCLIP reads (Krakau et al. 2017)) in 2,281 genes from the 110-kDa
258 band of wild type ECT2-mCherry (henceforth referred to as ECT2 iCLIP peaks and targets,
259 respectively). In the corresponding 55-kDa band, 4,549 crosslink sites in 1,127 genes were called,
260 93% of them contained in the 110-kDa target set (*Figure 3F,G, Figure 3—figure supplement 4,*
261 *Supplementary file 2*). We note that these numbers perfectly agree with the idea of the 55-kDa
262 band containing only YTH domain crosslink sites, while the full length may also include IDR
263 crosslink sites. Importantly, for both libraries, the majority of crosslink sites mapped to the 3'-UTRs
264 of mRNAs (*Figure 3H*, see *Figure 4A, Figure 4—figure supplement 1* for examples), coincident with
265 the main location of m^{6}A (*Figure 4B*) (Parker et al. 2020). Accordingly, the 3'-UTR specificity was
266 largely lost in RNA isolated from 55-kDa ECT2^{W464A} (*Figure 3H*), for which neither YTH-domain nor
267 IDR binding to RNA can be expected. Finally, iCLIP targets in full length (110-kDa band) ECT2 WT
268 and ECT2^{W464A} overlapped only marginally (*Figure 3G*), providing molecular proof of the
269 dependence of m^{6}A-binding activity for ECT2 function. Nonetheless, the bias towards occurrence in
270 the 3'-UTR was only reduced, not abolished, for crosslinks to the full-length ECT2^{W464A} protein,
271 providing another indication that the IDR itself is able to associate with RNA-elements in 3'-UTRs
272 (*Figure 3H*). We elaborate further on this important point by analysis of IDR-specific crosslinks to
273 wild type ECT2 after in-depth validation of sets of ECT2 target mRNAs, and determination of the
274 sequence motifs enriched around m^{6}A and ECT2 crosslink sites.
275
276
277

278 ***iCLIP sites tend to be in the vicinity of HyperTRIBE editing sites***

279 To evaluate the congruence of the results obtained by iCLIP and HyperTRIBE, we investigated the
280 cumulative number of iCLIP sites as a function of distance to the nearest editing site determined by
281 HyperTRIBE. This analysis showed a clear tendency for iCLIP peaks called with ECT2^{WT}-mCherry,
282 but not for ECT2^{W464A}-mCherry, to be in the vicinity of editing sites (*Figure 4C*), indicating that the
283 majority of called iCLIP peaks identify genuine ECT2 binding sites on mRNAs. Similar tendencies
284 of proximity between iCLIP peaks and HyperTRIBE editing sites were previously observed for a
285 *Drosophila* hnRNP protein (Xu et al. 2018). Although manual inspection of individual target genes
286 confirmed these tendencies, it also revealed that ADAR-edited sites are too dispersed around iCLIP
287 peaks to give precise information on the actual ECT2-binding sites (*Figure 4A, Figure 4—figure*
288 *supplement 1*). Therefore, we used both HyperTRIBE and iCLIP for gene target identification, but
289 relied on iCLIP peaks for motif analyses.

290

291 ***ECT2 targets identified by iCLIP and HyperTRIBE overlap m^{6}A–containing transcripts***

292 To examine the quality of our target identification in further detail, we analyzed the overlap between
293 ECT2 targets identified by iCLIP and HyperTRIBE. This analysis also included m^{6}A mapping data
294 obtained with either m^{6}A-seq (Shen et al. 2016) or the single-nucleotide resolution methods miCLIP
295 and Nanopore sequencing (Parker et al. 2020), as young seedlings were used in all cases. ECT2
296 targets identified by iCLIP and HyperTRIBE showed clear overlaps, both with each other and with
297 m^{6}A-containing transcripts, further supporting the robustness of ECT2 target identification via
298 combined iCLIP and HyperTRIBE approaches (*Figure 4D upper panel, Figure 4—figure*
299 *supplement 2*). Importantly, although some m^{6}A-targets are expected not to be bound by ECT2
300 because of the presence of MTA in cells that do not express ECT2 (Arribas-Hernández et al. 2020),
301 only 18% of the high-confident set of m^{6}A-containing genes (with support from miCLIP and
302 Nanopore) did not overlap with either ECT2 iCLIP or HT target sets (*Figure 4—figure supplement 2,*
303 *arrow*). We also observed that HyperTRIBE identifies ~3 times more ECT2 targets than iCLIP,
304 possibly because of the bias towards high abundance inherent to purification-based methods like
305 iCLIP (Wheeler et al. 2018). To test this idea, we compared the distribution of target mRNAs
306 identified by the different techniques across 9 expression bins. As expected, a bias towards highly
307 abundant transcripts was evident for iCLIP-identified targets compared to HyperTRIBE (*Figure 4E*).
308 We also observed a similar bias for m^{6}A-containing transcripts detected by miCLIP, another
309 purification-based method, and in the Nanopore dataset (*Figure 4E*), probably explained by its
310 relatively low sequencing depth (Parker et al. 2020). These observations also suggest that the

311 higher sensitivity of HyperTRIBE (analyzed in detail in *Figure 4—figure supplement 3*) explains the
312 lack of m^{6}A support (by Nanopore or miCLIP) for 28% of ECT2 HT-targets (1,689) compared to
313 only 4% (83) of ECT2 iCLIP targets (*Figure 4D, Figure 4—figure supplement 2, upper row*), since
314 HT-targets may simply include genes that escape detection by m^{6}A mapping methods due to low
315 expression. Indeed, ECT2-HT targets without any m^{6}A support were distributed in lower-expression
316 bins compared to those with m^{6}A support (*Figure 4F*). Intriguingly, ECT2 FA-CLIP targets (Wei et
317 al. 2018) did not show a bias towards highly expressed genes, as their distribution over expression
318 bins largely reflected that of the total number of genes (*Figure 4E*), and as many as 37% of FA-
319 CLIP targets did not have m^{6}A support (*Figure 4D, Figure 4—figure supplement 2, upper row*). In
320 summary, these analyses show that ECT2 iCLIP and HT target sets are in excellent agreement
321 with each other and with independently generated m^{6}A maps, and that HyperTRIBE identifies
322 targets below the detection limit of other techniques.
323
324 ***ECT2 crosslink sites coincide with m^{6}A miCLIP-sites and are immediately upstream of***
325 ***Nanopore m^{6}A-sites***
326 To characterize the sequence composition and exact positions of ECT2 binding sites relative to
327 m^{6}A, we first used the high resolution of iCLIP data to examine the position of ECT2 crosslink sites
328 relative to m^{6}A sites, determined at single-nucleotide resolution (Parker et al. 2020). This analysis
329 showed that ECT2 crosslinks in the immediate vicinity, but preferentially upstream (~11 nt) of
330 Nanopore-determined m^{6}A sites, with a mild depletion at the exact m^{6}A site (*Figure 5A*, upper
331 panel). Furthermore, while m^{6}A-miCLIP sites corresponded to m^{6}A Nanopore sites overall, a subset
332 of m^{6}A-miCLIP sites were located upstream of m^{6}A-Nanopore sites and coincided well with ECT2-
333 iCLIP peaks (*Figure 5A*). This pattern is probably explained by the fact that the UV illumination
334 used in both iCLIP and miCLIP preferentially generates RNA-protein crosslinks involving uridine
335 (Hafner et al. 2021), also detectable in the datasets analyzed here (*Figure 5B,C*). Thus, the
336 depletion of ECT2-iCLIP sites at Nanopore-, but enrichment at miCLIP-determined m^{6}A sites
337 (*Figure 5A*) might be explained by the absence of uridine within the RR<u>A</u>C core of the m^{6}A
338 consensus motif, and perhaps also to some extent by reduced photoreactivity of the m^{6}A base
339 stacking with indole side chains of the YTH domain. Furthermore, the fact that nucleotides at -2, +1,
340 and +2 positions are only expected to contribute sugar-phosphate backbone interactions with the
341 YTH domain (Luo and Tong 2014; Theler et al. 2014; Xu et al. 2014), may also contribute to the
342 absence of direct crosslinks at the m^{6}A site relative to the adjacent bases.
343

344 ***DRACH, GGAU and U/Y-rich motifs are the most enriched around m^{6}A/ECT2-sites***

345 The 5' shift observed for iCLIP and miCLIP sites relative to Nanopore sites might be explained by a
346 higher occurrence of uridines upstream of m^{6}A sites, a particularly interesting possibility given the
347 numerous reports of U-rich motifs enriched around m^{6}A sites in plants (Li et al. 2014c; Anderson et
348 al. 2018; Miao et al. 2019; Zhang et al. 2019; Zhou et al. 2019; Luo et al. 2020) and animals (Patil
349 et al. 2016). To investigate the sequence composition around m^{6}A and ECT2 sites, we first
350 performed exhaustive unbiased *de novo* motif searches using Homer (Heinz et al. 2010) (*Figure*
351 *5—figure supplement 1*) and extracted all candidate motifs, including the m^{6}A consensus motif
352 RRACH, as well as GGAU (Anderson et al. 2018), URUAY (Wei et al. 2018) and several other U-
353 rich sequences. Combined with manually derived candidate motifs (*Figure 5—figure supplement*
354 *1B*), we then calculated position weight matrices (PWM) for a final set of 48 motifs and scanned for
355 their occurrences genome-wide using FIMO (Grant et al. 2011) (*Figure 5—figure supplements 1,2*).
356 This allowed us to determine three key properties. First, the global enrichment of the motifs at
357 locations across the gene body. Second, the total count of occurrences of each motif at m^{6}A sites
358 and ECT2 iCLIP crosslink sites compared to a set of sites in non-target mRNAs matching the
359 location within gene bodies of m^{6}A/ECT2 iCLIP sites (expected background). Third, the distribution
360 of the motifs relative to m^{6}A and ECT2 iCLIP sites. The results of this systematic analysis
361 (*Supplementary file 3*) were used to select those with a more prominent enrichment at or around
362 m^{6}A and ECT2 sites (*Figure 5D*). This approach defined two major categories of motifs of
363 outstanding interest, RRACH-like and GGAU on the one side, and a variety of U/Y-rich motifs on
364 the other. *Figure 5D* shows a minimal selection of such motifs, while a more comprehensive
365 compilation is displayed in *Figure 5—figure supplements 3,4*. Not surprisingly, RRACH-like motifs
366 were the most highly enriched at m^{6}A sites and showed a clear enrichment immediately
367 downstream of ECT2 crosslink sites in our analyses, with the degenerate variant DRACH being the
368 most frequently observed (*Figure 5D, Figure 5—figure supplement 3*). Motifs containing GGAU
369 behaved similarly to DRACH, with a sharp enrichment exactly at m^{6}A sites and mild enrichment
370 downstream of ECT2 peaks (*Figure 5D*), supporting a previous suggestion of GGAU as an
371 alternative methylation site (Anderson et al. 2018). The possible roles of the U/Y-rich motifs in m^{6}A
372 deposition and ECT2 binding are analyzed in the following sections.

373

374 ***Neighboring U/Ys results in enriched RRACH- and GGAU-derived motifs***

375 We first noticed that several motifs retrieved around ECT2 crosslink sites by Homer constituted
376 extended versions of **DRACH/GGAU** with *U*s upstream (e.g. *U***GAAC**/*U***GGAU**), or remnants of

377 DR**ACH** with *U/C*s (*Y*s) downstream (e.g. **ACU***CU*). To test whether these motifs are indeed
378 located adjacent to m^6A, we examined their distribution and enrichment around ECT2 and m^6A
379 sites. The distributions showed a clear enrichment at m^6A positions with a shift in the direction of
380 the U/Y-extension (see *Figure 5D* for ACUCU and *Figure 5—figure supplement 4* for others). An
381 enrichment over location-matched background sites close to ECT2-iCLIP sites was also apparent
382 (see *Figure 5D* for ACUCU and *Figure 5—figure supplement 4* for others), further supporting that
383 ECT2 preferentially crosslinks to uridines located in the immediate vicinity of DRACH (/GGAU).
384 Thus, several enriched motifs around ECT2 crosslink sites are DRACH/GGAU-derived, and their
385 detection in unbiased searches simply reflects a tendency of methylated DRACH/GGAU sites to be
386 flanked by U/Y.
387
388 ***Nature of U/Y-rich motifs more distant from m^6A sites***
389 U/R rich motifs without traces of adjacent DRACH (e.g. YUGUM, URUAY, URURU) showed a
390 characteristic enrichment around, but depletion at, m^6A sites. For some motifs, the enrichment was
391 more pronounced 5' than 3' to m^6A sites (see *Figure 5D* for URUAY and *Figure 5—figure*
392 *supplement 4* for others). The distance between the site of maximal motif occurrence and the m^6A
393 site roughly coincided with the shift observed in ECT2 crosslink sites relative to m^6A (*Figure 5A,*
394 *upper panel*). Accordingly, these motifs were enriched exactly at ECT2 crosslink sites (see *Figure*
395 *5D* for URUAY and *Figure 5—figure supplement 4* for others), suggesting that they may constitute
396 additional m^6A-independent sites of interaction with ECT2. We also observed that the 3' enrichment
397 of YYYYY was closer to m^6A than that of UUUUU/URURU/URUAY (*Figure 5—figure supplement 4,*
398 *2nd row from the top*), indicating a preference for hetero-oligopyrimidine tracts immediately
399 downstream the m^6A site, as suggested by the 3'-enrichment of DRACUCU-type motifs as
400 described above.
401 Taken together, these results suggest that *N6*-adenosine methylation preferentially occurs
402 in DRACH/GGAU sequences surrounded by stretches of pyrimidines, with a preference for YYYYY
403 (e.g. CUCU) immediately downstream, URURU (including URUAY) immediately upstream, and
404 UUUUU/UNUNU slightly further away in both directions. The enrichment of ECT2 crosslink sites at
405 these motifs, and the fact that the m^6A-binding deficient mutant of ECT2 (W464A) crosslinks
406 preferentially to 3'UTRs through its N-terminal IDR, indicate IDR-mediated binding to U/R- and Y-
407 rich motifs around m^6A.
408
409

410 ***DRACH/GGAU motifs are determinants of m^{6}A deposition at the site, while flanking U(/Y)-***
411 ***rich motifs are indicative of m^{6}A presence and ECT2 binding***
412 Since our analysis thus far uncovered several motifs of potential importance for m^{6}A deposition and
413 ECT2 binding, we employed machine learning to distinguish m^{6}A and ECT2 iCLIP sites from
414 random location-matched background sites using motif-based features. Importantly, the underlying
415 classification model includes all motif features within the same model, allowing an evaluation of the
416 importance of the motifs relative to each other. We used as features the number of matches to
417 each of the 48 motifs (*Figure 5—figure supplement 2*) in three distinct regions relative to the
418 methylated site according to Nanopore sequencing (Parker et al. 2020), defined as position 0: ”at” [-
419 10 nt; +10 nt], “down” [-50 nt; -10 nt], or “up” [+10 nt; +50 nt] (*Figure 6A*). The model involving all
420 motifs could successfully distinguish the methylated sites from the background as indicated by an
421 area under the receiver operating characteristic curve (true positive rate versus false positive rate,
422 AUC) of 0.93, and even a reduced model incorporating only the top 10 features from the full model
423 classified sites largely correctly (AUC = 0.86; *Figure 6—figure supplement 1*). The top 16 features
424 ordered by importance from the full model confirmed that RRAC/DRACH or GGAU at the site was
425 indicative of the presence of m^{6}A (*Figure 6B*). Interestingly, U/Y-rich sequences (UNUNU and
426 YYYYY in particular) flanking the site were also strongly indicative (*Figure 6B*). Some motifs
427 showed a skew in their feature importance score, with UNUNU and YUGUM showing a preference
428 to be upstream, and YYYYY downstream (*Figure 6B*), thus corroborating our previous observations
429 (*Figure 6C*).
430 We used a similar modeling approach to identify non-m^{6}A determinants of ECT2 binding, in
431 this case comparing m^{6}A sites within 10nt-distance of ECT2-iCLIP sites to m^{6}A sites without ECT2-
432 iCLIP sites nearby (AUC=0.94, and AUC=0.84 using only the top 10 features, *Figure 6—figure*
433 *supplement 1*). In agreement with previous observations, this model showed flanking U/Y-rich
434 sequences as the main determinants for ECT2 crosslinking (*Figure 6D*).
435
436 ***The U(-R) paradox: URURU-like sequences around m^{6}A sites repel ECT2 binding, while U-***
437 ***rich sequences upstream enhance its crosslinking***
438 To investigate the idea of URURU-like motifs as additional sites of ECT2 binding upstream of the
439 m^{6}A-YTH interaction site, we split Nanopore-m^{6}A sites according to two criteria: 1) whether they
440 occur in ECT2-target transcripts (both permissive and stringent sets analyzed separately), and 2)
441 for ECT2 targets, whether there is an ECT2 crosslink site within 25 nt of the m^{6}A site (‘near’) or not
442 (‘far’). Although there was no obvious difference between these categories for most of the motifs

443 (*Supplementary file 3, page 2*), some U-rich sequences displayed distinctive features (*Figure 7A,*
444 *Figure 7—figure supplement 1*) that can be summarized as follows. If a transcript has m^{6}A and
445 ECT2 sites in close proximity, it is: i) more likely to have UNUNU/UUUUU/YYYYY sequences
446 upstream of the m^{6}A site than targets with distantly located ECT2 binding sites or than non-ECT2
447 targets; ii) less likely to have UUUUU/URURU sequences downstream of the m^{6}A site, possibly
448 because ECT2 prefers CUCU-like sequences downstream; iii) less likely to have URURU/URUAY-
449 like motifs upstream of the m^{6}A site. The latter observation is striking, because for the specific
450 subset of ECT2-bound m^{6}A-sites with URURU/URUAY upstream of m^{6}A, these sequences tend to
451 crosslink to ECT2, as seen by the enrichment spike at ECT2 crosslink sites (*Figure 5D, Figure 7—*
452 *figure supplement 1, bottom panels*). Although these two results seem contradictory at first glance,
453 they may be reconciled by a model in which a URURU/URUAY-binding protein would compete with
454 ECT2 for binding adjacent to m^{6}A. If that protein is absent, ECT2 may bind to the site, potentially
455 via its IDR, to stabilize the low-affinity YTH-m^{6}A interaction and crosslink efficiently due to the U-
456 content. Conversely, if occupied by the alternative interacting protein, the site might repel ECT2
457 (see discussion and *Figure 7B*).
458
459 ***The N-terminal IDR of ECT2 is involved in preferential crosslinking at U-rich sequences and***
460 ***URURU-repulsion immediately upstream m^{6}A sites***
461 We reasoned that insights into contacts between ECT2 and mRNA may be gained by analysis of
462 the iCLIP libraries prepared with the 'YTH-mCherry' truncation devoid of the N-terminal IDR ('55
463 kDa band') compared to the full-length ECT2-mCherry ('110 kDa') (*Figure 3, Figure 3—figure*
464 *supplements 2-4*). Initial inspection of the distribution of ECT2 peaks relative to Nanopore-m^{6}A sites
465 showed that the 5'-3' asymmetry observed with full-length ECT2 was largely reduced with the
466 truncated protein (*Figure 7C*), as was the bias towards uridines (*Figure 7—figure supplement 2A*).
467 These observations suggest that the IDR indeed is implicated in binding to U-rich regions upstream
468 of m^{6}A. We next split the full-length ECT2 iCLIP peaks according to whether they are present in
469 libraries from both full length and truncated forms ('IDR-independent'), or exclusively in the full
470 length ('IDR-dependent') (distance > 10 nt), and plotted the enrichment of the studied motifs
471 relative to the crosslink site (*Figure 7D, Figure 7—figure supplement 2B; Supplementary file 3-page*
472 *2*). UUUUUU/UNUNU-like motifs were more enriched at and immediately upstream of IDR-
473 dependent crosslink sites relative to the IDR-independent ones, supporting preferential crosslinking
474 of the IDR to Us in this region. Remarkably, the exact opposite was true for URURU/URUAY motifs
475 that showed modest depletion 5' to IDR-dependent crosslink sites relative to their IDR-independent

476 counterparts (*Figure 7D*). These observations are consistent with a model of an RNA binding
477 protein competing with the ECT2 IDR for interaction with upstream URURU/URUAY motifs (*Figure*
478 *7B*).
479

480 **Discussion**

481 *Methodology for mapping protein-RNA interactions in plants*

482 Our work establishes experimental and computational approaches to implement HyperTRIBE for
483 unbiased and sensitive mapping of direct targets of RNA binding proteins in plants. Two points are
484 particularly relevant in this regard. First, the examples studied here show that stable transgenic
485 expression of *Dm*ADARcd does not lead to detrimental phenotypes, perhaps because of the
486 generally low editing proportions obtained *in vivo*. Second, the rigorous statistical approach
487 developed to call editing sites makes HyperTRIBE powerful, despite the low editing proportions
488 observed. We also note that ECT2 is well suited to verify that HyperTRIBE mostly recovers directly
489 bound target RNAs, because of the possibility to cross-reference the data with independently
490 obtained m^6A maps (Parker et al. 2020). The combination of iCLIP and HyperTRIBE for unbiased
491 mapping of targets proved particularly attractive for at least two reasons. First, the convergence on
492 overlapping target sets by orthogonal methods strengthens the confidence that the identified
493 targets are biologically meaningful. Second, HyperTRIBE, especially with the novel computational
494 approach for calling of editing sites developed here, offers higher sensitivity than iCLIP, while iCLIP
495 is unmatched in providing information on binding sites within target RNAs. It is possible that better
496 positional information on binding sites may be obtained from HyperTRIBE data using maximal
497 editing proportions rather than statistical significance as the parameter to call editing sites. Indeed,
498 recent work on the use of HyperTRIBE to identify targets of the RNA-binding protein MUSASHI-2
499 (MSI-2) in leukemic stem cells recovered the known MSI-2 binding site as enriched around editing
500 sites in targets (Nguyen et al. 2020). Nonetheless, our data shows that highly edited sites match
501 the ADAR substrate consensus site better than lowly edited sites, suggesting that site proximity to
502 ADAR is not the only determinant of editing proportions. Finally, our work also clearly indicates that
503 FA-CLIP, now used in at least two studies involving YTH domain proteins (Wei et al. 2018; Song et
504 al. 2021), is not a recommendable technique, as it recovers many false positives and fails to
505 include many genuine targets. Thus, with the possible exception of cases in which evidence for
506 indirect association is specifically in demand, such as the recent study in human cells of mixed
507 tailing of viral RNA by the cellular terminal nucleotidyltransferase TENT4 (Kim et al. 2020), FA-CLIP
508 should not be used for identification of RNAs associating with a particular RNA-binding protein of
509 interest.

510

511

512

513 *Core elements in m^{6}A writing: DRACH, GGAU and U/Y-rich motifs*

514 Our analyses of motif enrichments around m^{6}A and ECT2 crosslink sites clarify roles of previously
515 reported motifs and uncovers new motifs of importance in m^{6}A writing and ECT2 binding. Since
516 m^{6}A is a prerequisite for ECT2 binding, any analysis of determinants of ECT2 binding must
517 consider determinants of *N6*-adenosine methylation separately. Three conclusions stand out from
518 our analysis in this regard. First, the major *N6*-adenosine methylation site is DRACH, consistent
519 with conclusions from multiple other studies. Second, GGAU is a minor *N6*-adenosine methylation
520 site, as seen by its enrichment directly at m^{6}A-sites. Third, m^{6}A occurs in DRACH/GGAU islands
521 embedded in U-rich regions. Such U-rich regions around m^{6}A sites emerged from sorting of
522 methylated from non-methylated transcripts by machine learning as being of similar importance for
523 recognition of m^{6}A-containing transcripts from sequence features as DRACH and GGAU at m^{6}A
524 sites, suggesting their implication in MTA/MTB-catalyzed adenosine methylation (*Figure 6C*). This,
525 in turn, may also explain the pronounced 3'-UTR bias of m^{6}A occurrence, as extensive poly-U and
526 poly-pyrimidine tracts are rare in coding regions (*Figure 5D, 2nd row; Supplementary file 3-page 1)*.
527 As a special case in this context, our analyses suggest a simple explanation for the tendency of
528 m^{6}A to occur at stop codons. UAA and UGA correspond to DRA, increasing the frequency of
529 occurrence of DRACH directly at stop codons (*Figure 5D, 2nd row)*, many of which have adjacent U-
530 rich elements in the 3'-UTRs. We note that the observed pattern is in agreement with a role of the
531 poly(U)-interacting proteins RBM15A/B associated with the methyltransferase complex in
532 mammalian cells in guiding methylation (Patil et al. 2016). Whether a similar mechanism operates
533 in plants, potentially via the distant RBM15A/B homologue FPA (Arribas-Hernández and Brodersen
534 2020), remains to be investigated.

535

536 *Reading of DR(m^{6}A)CH in 3'-UTRs of target mRNAs by ECT2*

537 It is a major conclusion of the present work that ECT2 binds to m^{6}A predominantly in the
538 DR(m^{6}A)CH sequence context *in vivo*, consistent with reading of m^{6}A written by the conserved
539 nuclear MTA/MTB methyltransferase. This key conclusion refutes the claim by Wei et al. (2018)
540 that ECT2 binds to the supposedly plant-specific m^{6}A-containing sequence motif URU(m^{6}A)Y, and
541 it thereby reconciles knowledge on m^{6}A-YTHDF axes in plants specifically and in eukaryotes more
542 broadly. The phenotypic similarity of plants defective in MTA/MTB writer and ECT2/3/4 reader
543 function is now coherent with the locations of MTA/MTB-written m^{6}A and ECT2 binding sites
544 transcriptome-wide, and it is now clear that plants do not constitute an exception to the general

545 biochemical framework for eukaryotic m^{6}A-YTHDF function in which YTHDF proteins read the m^{6}A
546 signal written by the MTA/MTB methyltransferase.
547
548 *The role of U-rich motifs 5' to m^{6}A sites in ECT2 binding: direct interaction of the IDR of ECT2 with*
549 *mRNA*
550 The pronounced protease-sensitivity of IDRs, leading to limited proteolysis of ECT2 upon cell lysis
551 after *in vivo* crosslinking allowed us to extract information on the mode of ECT2-RNA binding from
552 different observations, all converging on the conclusion that the IDR of ECT2 participates in RNA
553 binding. First, RNA-complexes with YTH-mCherry were 5'-labeled by polynucleotide kinase much
554 more efficiently than RNA-complexes with full-length ECT2-mCherry, indicating that the IDR limits
555 accessibility to the 5' of bound mRNAs. Second, in contrast to the m^{6}A-binding deficient YTH^{W464A}-
556 mCherry truncation, the full-length ECT2^{W464A}-mCherry mutant retained an enrichment of crosslink
557 sites in 3'-UTRs. Third, crosslinks specific to the IDR (i.e. observed only with full-length ECT2-
558 mCherry-RNA complexes, but not with YTH-Cherry-RNA complexes) could be assigned, and have
559 two notable properties. They are mainly 5' to m^{6}A sites, and thereby cause a conspicuously
560 asymmetric distribution of ECT2 crosslink sites around m^{6}A sites, not seen with crosslinks to the
561 YTH-mCherry fragment. In addition, the IDR-specific crosslinks are specifically enriched in U-rich
562 elements of the type UUUUUU and UNUNU immediately upstream. Taken together, these
563 observations suggest that the IDR of ECT2 participates in locating ECT2 to 3'-UTRs by association
564 with U-rich elements. Thus, ECT2, and perhaps YTHDF proteins more generally given their highly
565 similar YTH domains, appears to bind RNA through multivalent interactions among which the YTH
566 domain is responsible for m^{6}A-binding, and the IDR is responsible for interaction with adjacent
567 elements. We note that the notion of RNA-interaction by IDRs has precedent (Corley et al. 2020), is
568 consistent with the modest affinity of isolated YTHDF domains for m^{6}A-containing oligonucleotides
569 (Patil et al. 2018), and is reminiscent of the recent demonstration that transcription factors use their
570 globular DNA-binding domains to recognize core sequence elements of promoters, and their IDRs
571 to provide additional DNA contacts, contributing to specificity (Brodsky et al. 2020). We stress that
572 although our data point to an important role of the IDR in RNA binding, it does not in any way
573 suggest that this is the only function of the IDR, and protein-protein interactions involving the IDR
574 are likely to be key to understanding YTHDF function molecularly.
575
576
577

578 *URUAY as sites of competitive interaction between ECT2 and other RNA-binding proteins*
579 Despite the conclusions that URUAY does not contain m^6A in Arabidopsis, and that ECT2 binds to
580 DR(m^6A)CH, our detailed analysis of sequence motifs enriched around m^6A and ECT2 iCLIP
581 crosslink sites shows that additional motifs, including URUAY, are likely to be implicated in m^6A
582 reading by ECT2, even if not directly. In contrast to other m^6A-proximal, pyrimidine-rich sequences
583 (e.g. UNUNU, YYYYY) that may be of importance for both m^6A writing and ECT2 binding, URUAY
584 appears to have ties more specifically to ECT2 binding thanks to three properties. (1) When present
585 5' to m^6A sites, it crosslinks to ECT2, suggesting that some part of the protein can be in contact
586 with URUAY. (2) URUAY is more enriched close to m^6A-sites for which there is no evidence of
587 ECT2-binding, suggesting that it weakens ECT2 binding. This latter point is also consistent with the
588 distinction of ECT2-bound from non-ECT2 bound m^6A sites by machine learning that did not find
589 URUAY to be of importance for ECT2-bound sites. (3) The URUAY enrichment 5' to ECT2
590 crosslink sites is observed only when crosslinks to both full-length protein and the YTH-mCherry
591 fragment are considered (IDR-independent), but disappears when crosslinks specific to the full-
592 length protein (IDR-dependent) are analyzed. Although these observations may be explained by
593 multiple scenarios, we find a simple, yet at present speculative, model attractive: URUAY may be a
594 site of competition between the IDR of ECT2 and another, as yet unknown, RNA binding protein.
595 We also note that the idea of a URUAY-binding protein influencing ECT2-binding and/or regulation
596 is consistent with the recovery of formaldehyde crosslinks between ECT2 and URUAY (Wei et al.
597 2018), in this case indirectly. Finally, it is intriguing that URUAY resembles part of a Pumilio binding
598 site (Hafner et al. 2010; Huh et al. 2013), raising the tantalizing possibility of functional interaction
599 between YTHDF and Pumilio proteins. In any event, the functional dissection of the URUAY
600 element in m^6A-reading now constitutes a subject of major importance, emphasized by the broad
601 conservation of its enrichment around m^6A sites across multiple plant species, including rice (Li et
602 al. 2014c; Zhang et al. 2019), maize (Luo et al. 2019; Miao et al. 2019), tomato (Zhou et al. 2019),
603 and Arabidopsis (Miao et al. 2019).
604
605

606 **Methods**
607
608 All data analyses were carried out using TAIR 10 as the reference genome and Araport11 as the
609 reference transcriptome. Unless otherwise stated, data analyses were performed in R
610 (https://www.R-project.org/) and plots generated using either base R or ggplot2.
611 (https://ggplot2.tidyverse.org).
612
613 *Plant material*
614 All lines used in this study are in the *Arabidopsis thaliana* Col-0 ecotype. The mutant alleles or their
615 combinations: *ect2-1* (SALK_002225) (Arribas-Hernández et al. 2018; Scutenaire et al. 2018; Wei
616 et al. 2018), *ect3-1* (SALKseq_63401), *ect4-2* (GK_241H02), and *ect2-1/ect3-1/ect4-2* (*te234*)
617 (Arribas-Hernández et al. 2018) have been previously described. The transgenic lines
618 *ECT2pro:ECT2-mCherry-ECT2ter*, *ECT2pro:ECT2^{W464A}-mCherry-ECT2ter, ECT2pro:3xHA-ECT2-*
619 *ECT2ter*, or *ECT2pro:3xHA-ECT2^{W464A}-ECT2ter* have also been described or generated by floral
620 dip in additional mutant backgrounds using the same plasmids and methodology (Arribas-
621 Hernández et al. 2018; Arribas-Hernández et al. 2020).
622
623 *Growth conditions*
624 Seeds were surface-sterilized, germinated and grown on vertically disposed plates with Murashige
625 and Skoog (MS)-agar medium (4.4 g/L MS, 10 g/L sucrose, 10 g/L agar) pH 5.7 at 20°C, receiving
626 ~70 µmol m^{-2} s^{-1} of light in a 16 hr light/8 hr dark cycle as default. To assess phenotypes of adult
627 plants, ~8-day-old seedlings were transferred to soil and maintained in Percival incubators also
628 under long day conditions. Additional details and variations of growth conditions for specific
629 experiments can be found in Supplemental Methods.
630
631 *Generation of transgenic lines for HyperTRIBE*
632 We employed USER cloning (Bitinaite and Nichols 2009) to generate *ECT2pro:ECT2-FLAG-ADAR-*
633 *ECT2ter* and *ECT2pro:FLAG-ADAR-ECT2ter,* constructs in pCAMBIA3300U (pCAMBIA3300 with a
634 double PacI USER cassette inserted between the *Pst*I-*Xma*I sites at the multiple cloning site (Nour-
635 Eldin et al. 2006)). Details on the cloning procedure can be found in Supplemental Methods.
636 *Arabidopsis* stable transgenic lines were generated by floral dip transformation (Clough and Bent
637 1998) of Col-0 WT, *ect2-1*, or *te234*, and selection of primary transformants (T1) was done on MS-
638 agar plates supplemented with glufosinate ammonium (Fluka) (10 mg/L). We selected 5
639 independent lines of each type based on segregation studies (to isolate single T-DNA insertions),

640 phenotypic complementation (in the *te234* background) and transgene expression levels assessed
641 by FLAG western blot (see Supplemental Methods).
642
643 *HyperTRIBE library preparation*
644 We extracted total RNA (see Supplemental Methods) from manually dissected root tips and apices
645 (removing cotyledons) of 5 independent lines (10-day-old T2 seedlings) of each of the lines used
646 for ECT2-HT, to use as biological replicates. Illumina mRNA-Seq libraries were then prepared by
647 Novogene (see Supplemental Methods) after mRNA enrichment with oligo(dT) beads (18-mers).
648
649 *HyperTRIBE editing site calling*
650 Significant differentially edited sites between *ECT2-FLAG-ADAR* (fusion) and *FLAG-ADAR* (control)
651 samples were called according to our hyperTRIBE**R** pipeline (https://github.com/sarah-
652 ku/hyperTRIBER), testing all nt positions with some evidence of differential editing across multiple
653 samples. Significant (adjusted-*p*-value < 0.01 and $\log_2$FC > 1) A-to-G hits were further filtered and
654 annotated according to Araport11 by integrating quantification information generated using Salmon
655 (Patro et al. 2017), based on the Araport11 transcriptome with addition of *FLAG-ADAR* sequence.
656 See Supplemental Methods for additional details.
657
658 *Analysis of HyperTRIBE sites*
659 For all significant editing sites (sig. E.S.), editing proportions (E.P.) where calculated as G/(A+G)
660 where A, G are the number of reads covering the E.S. with A or G at the site, respectively. Sample-
661 specific E.P.s for all sig. E.S. were used for principal component analyses and correlations between
662 *FLAG-ADAR* TPM and E.P., and replicate-averaged E.P. was used for density plots, condition- or
663 cell-type-based comparisons and comparisons over expression bins. For expression bins, the
664 $\log_2$(TPM+1) values for all expressed genes in either aerial tissues, roots or combined were split
665 into 9 bins of increasing expression. The Support of ECT2 target or m^6A gene sets was calculated
666 by the proportion of genes in a given bin out of the total number of genes in that bin. See
667 Supplemental Methods for additional details and methods.
668
669 *CLIP experiments and iCLIP library preparation*
670 *In vivo* UV-crosslinking of 12-day-old seedlings and construction of iCLIP libraries were optimized
671 for ECT2-mCherry from the method developed by Prof. Staiger's group for Arabidopsis GRP7-GFP
672 (Meyer et al. 2017; Köster and Staiger 2020). Details can be found in Supplemental Methods.

673

674 *iCLIP data analysis and peak calling*

675 Sequenced reads were mapped to TAIR10 after being processed by trimming, demultiplexing and
676 discarding short reads. Peak calling of uniquely mapped reads was done using PureCLIP 1.0.4
677 (Krakau et al., 2017) after removal of PCR duplicates. Gene annotation of peaks was carried out
678 using the hyperTRIBE**R** pipeline. Details can be found in Supplemental Methods.

679

680 *Analysis of publicly available data*

681 Single cell expression data and marker genes associated with 15 clusters annotated to cell types in
682 roots was downloaded from Denyer et al. (2019). Single nucleotide resolution locations of m^6A sites
683 (defined according to Nanopore or miCLIP) were downloaded from Parker et al., 2020. Intervals
684 defining m^6A locations based on m^6A-seq were downloaded from Shen et al. 2016, and intervals
685 defining locations of ECT2-bound sites as determined by FA-CLIP were downloaded from Wei et
686 al., 2018. For consistency with HyperTRIBE and ECT2-iCLIP, all sets of m^6A or ECT2-bound sites
687 were gene annotated using the hyperTRIBE**R** pipeline.

688

689 *Motif analysis*

690 A list of 48 motifs was compiled from multiple sources and for each motif a position weight matrix
691 (PWM) was generated based on local sequence frequencies around ECT2-iCLIP peaks and used
692 as input to FIMO (Grant et al. 2011) to detect genome-wide occurrences. In order to account for
693 location-specific sequence contexts (typically 3'UTR), each site from iCLIP or m^6A (Parker et al.
694 2020) sets was assigned a random 'matched background' site, in a non-target gene, at the same
695 relative location along the annotated genomic feature (5'UTR, CDS or 3'UTR) of the site.
696 Distributions of motifs per 1000 sites over distance, centering on ECT2-iCLIP or m^6A sites and the
697 respective matched backgrounds were generated using a custom R-script
698 (https://github.com/sarah-ku/targets_arabidopsis). Sets were further split according to IDR or target
699 status (see Supplemental Methods for further details).

700 For machine learning, features were generated from motifs according to their relative locations in
701 windows from m^6A or ECT-iCLIP sites. Importance scores were generated using gradient boosting
702 *gbm* (https://github.com/gbm-developers/gbm), with performance statistics based on the AUC
703 calculated from held-out data. See Supplemental Methods for additional details.

704
705

706

Data Access

Accession numbers

The raw and processed data for HyperTRIBE (ECT2-HT) and ECT2-iCLIP have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under the accession number PRJEB44359.

Code availability

The code for running the hyperTRIBE**R** pipeline and for bioinformatics analyses is available at https://github.com/sarah-ku/targets_arabidopsis.

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Author Contributions

P.B. and L.A.-H. designed and coordinated the study. L.A.-H. built the biological material for HyperTRIBE and S.R. developed the hyperTRIBE**R** pipeline, called edited sites to define HyperTRIBE target sets and assessed their veracity. L.A.-H. and T.K. performed iCLIP experiments and produced iCLIP libraries, M.L. analyzed iCLIP data, C.P. and S.R. executed *de novo* motif discovery. and S.R. studied the overlap between m^6A and ECT2 target sets and performed motif enrichment analyses. R.A. supervised work related to hyperTRIBE**R** development and sequence motif analysis and D.S. supervised work related to iCLIP data acquisition and analysis. P.B., L.A.-H. and S.R. wrote the manuscript with input from all authors.

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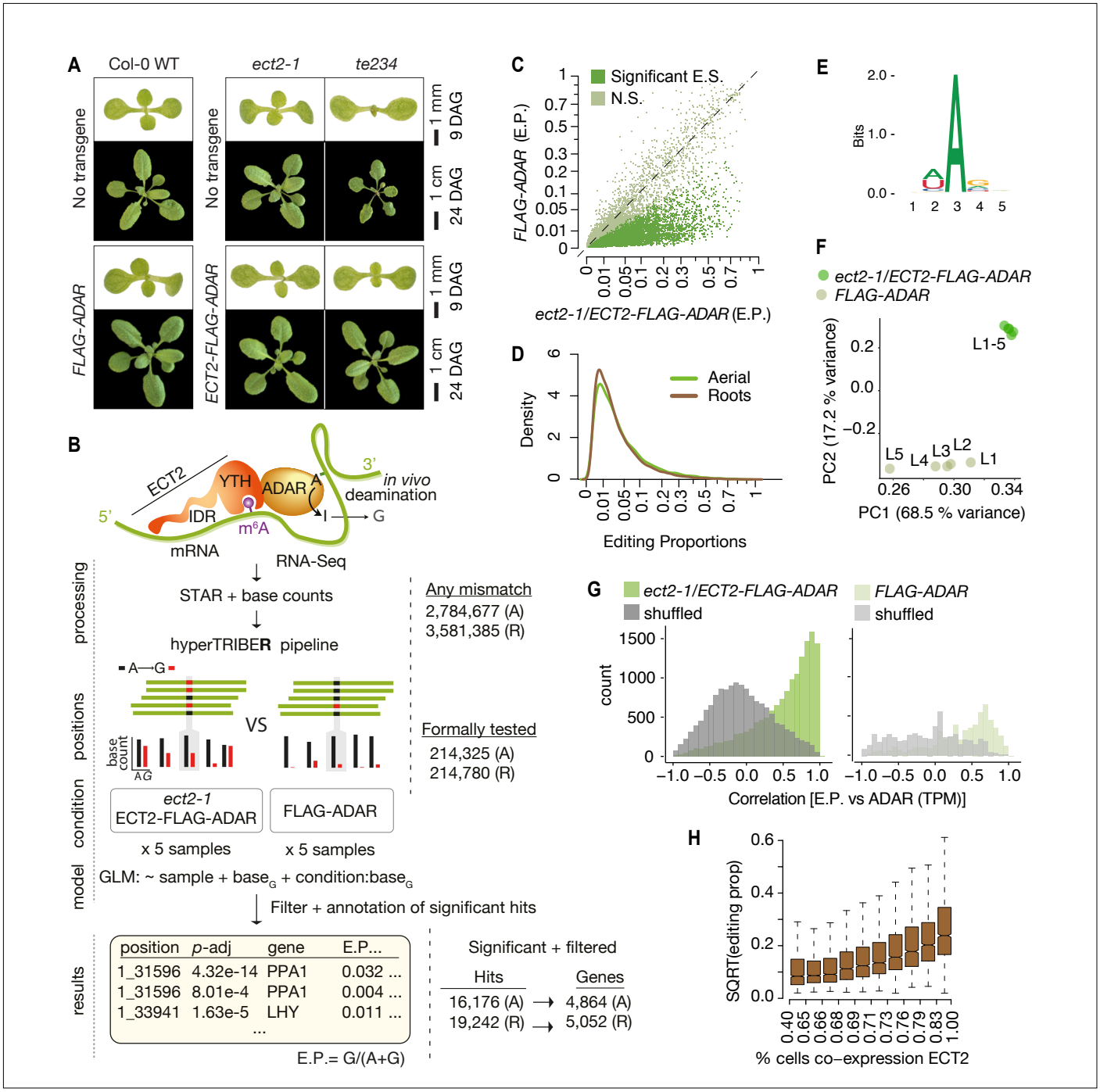

Figure 1. ***Drosophila* ADARcd fused to ECT2 can edit target mRNAs *in vivo* in plants. (A)** Phenotypes of wild type, *ect2-1* and *te234* mutants with (lower panels) or without (upper panels) *ECT2-FLAG-ADAR* or *FLAG-ADAR* transgenes, at 9 or 24 days after germination (DAG). **(B)** Experimental design for ECT2-HyperTRIBE (ECT2-HT) target identification and hyperTRIBE**R** pipeline. Nucleotide base counts quantified from mapped RNA-seq libraries were passed into the HyperTRIBE**R** pipeline to call significant editing sites, which were further filtered and annotated. The number of sites in either aerial (A, dissected apices) or root (R, root tips) tissues considered at each stage of the analysis is indicated. GLM, generalized linear model; E.P., editing proportion. **(C)** Scatterplot of the editing proportions of potential and significant editing sites (E.S.) in aerial tissues of *ect2-1/ECT2-FLAG-ADAR* lines compared to the *FLAG-ADAR* controls. Significant sites are highlighted in vivid green. N.S., not significant. **(D)** Consensus motif identified at significant editing sites in aerial tissues of *ect2-1/ECT2-FLAG-ADAR* lines. **(E)** Principal component analysis of editing proportions at significant editing sites in samples with aerial tissue. **(F)** Density of editing proportions for significant editing sites in aerial tissues and roots of *ect2-1/ECT2-FLAG-ADAR* lines. **(G)** Distribution of the correlations between editing proportions and ADAR expression (TPM) for significant editing sites in aerial tissues of either *ect2-1/ECT2-FLAG-ADAR* or *FLAG-ADAR* lines. Background correlations are based on randomly shuffling ADAR expression for each site. **(H)** Boxplots showing the mean editing proportions as a function of the proportion of cells co-expressing *ECT2*, calculated based on single cell RNA-seq in roots (Denyer et al., 2019). For panels C, E, and G, comparable analyses in both aerial and root tissues are shown in the figure supplement 1.

Figure supplement 1. *Drosophila* ADARcd fused to ECT2 can edit target mRNAs *in vivo* in plants (extended data, aerial and root tissues).

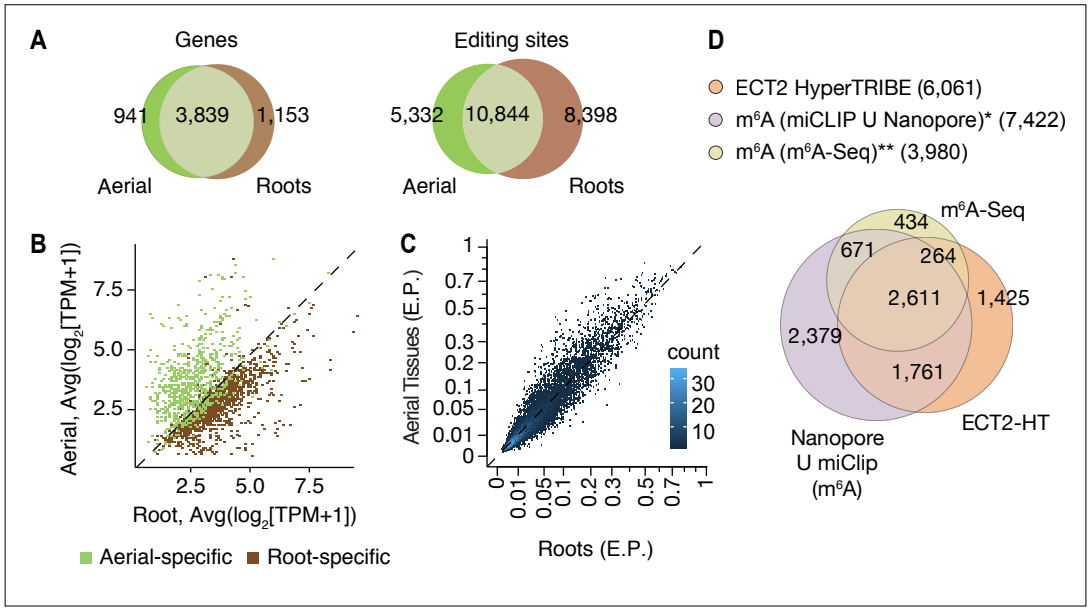

Figure 2. **HyperTRIBE identifies m^{6}A-reader targets in plants. (A)** Overlap between ECT2-HT targets (genes and editing sites) in roots and aerial tissues, based on genes commonly expressed in both tissues. **(B)** Scatterplot showing the expression levels in roots and aerial tissues (mean $\log_2$(TPM+1) over the five ECT2-HT control samples) of the genes identified as aerial or root-specific targets. **(C)** Scatterplot of the editing proportions (E.P.) of significant editing sites in ECT2-HT for aerial vs root tissues. **(D)** Overlap between ECT2-HT targets and m^{6}A-containing genes. *Parker et al. (2020); ** Shen et al. (2016).

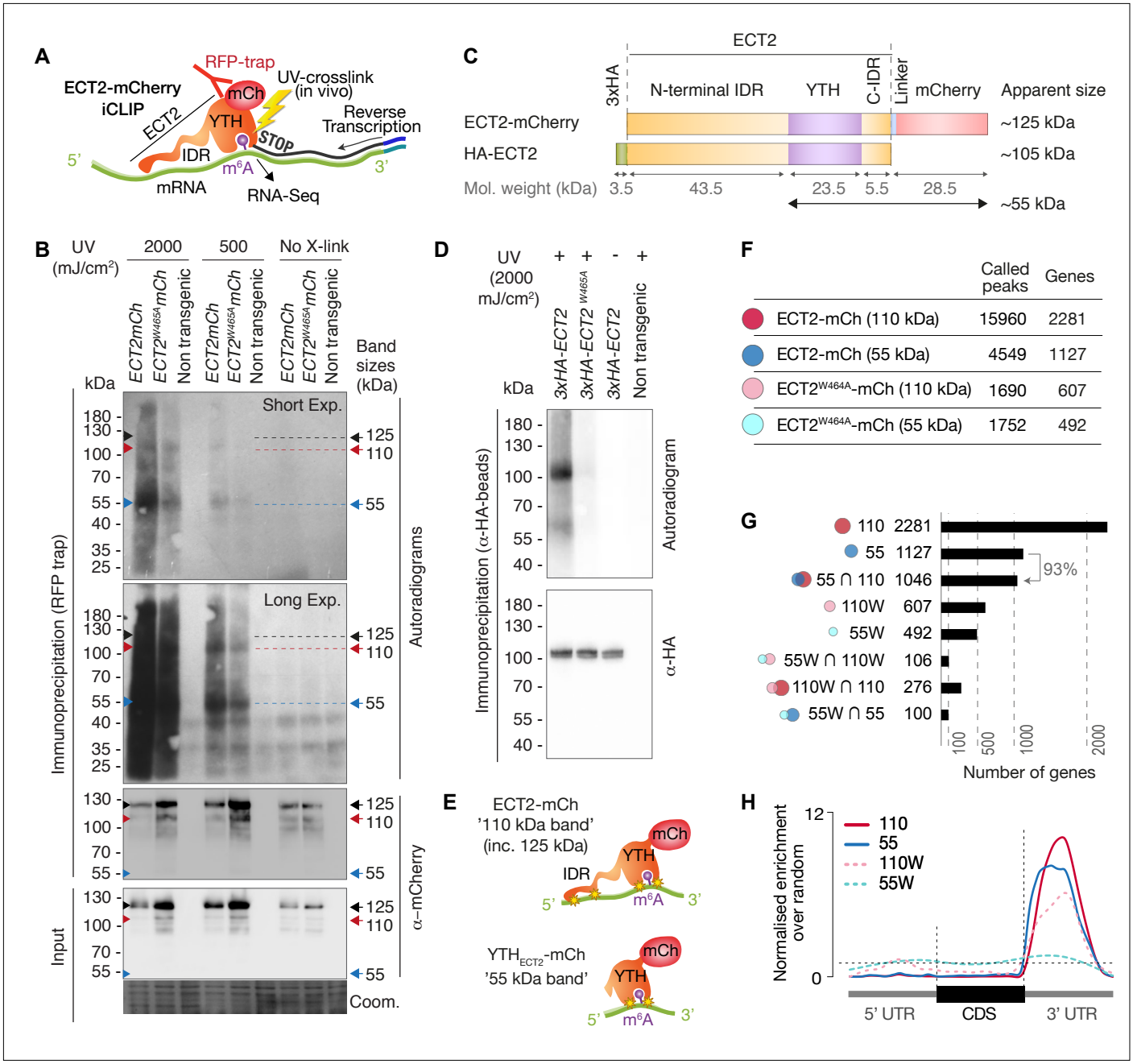

Figure 3. RNA-binding properties of ECT2 revealed by CLIP. (A) iCLIP experimental design. **(B)** Upper panels: autoradiogram (top) and α-mCherry protein blot (below) of RFP-trap immuno-purifications. Samples are cell extracts from 12-day-old seedlings expressing *ECT2-mCherry* or *ECT2^{W464A}-mCherry* in the *ect2-1* mutant background after in vivo UV-crosslinking as indicated, and subjected to DNase digestion, partial RNase digestion, and 5'-^{32}P labeling of RNA. Non-transgenic, Col-0 wild type. Lower panels: α-mCherry protein blot of the same extracts before immunoprecipitation (input) and Coomassie staining of the membrane. Sizes corresponding to full length ECT2-mCherry (~125 kDa) and the most apparent RNA bands are indicated with arrows. A repeat of the experiment with independently grown and crosslinked tissue is shown in the figure supplement 1A. **(C)** Schematic representation of ECT2-mCherry and HA-ECT2 fusion proteins with their apparent size (electrophoretic mobility). The molecular weight of each region is indicated. Notice that IDRs tend to show higher apparent sizes (low electrophoretic mobility) than globular domains. **(D)** Equivalent to B with lines expressing *3xHA-ECT2* variants in the *ect2-1* background, α-HA immuno-purifications and α-HA detection by western blot. **(E)** Cartoon illustrating the bands of labelled RNA co-purifying with ECT2-mCherry. Yellow stars indicate possible crosslinking sites. **(F)** Number of called peaks and genes detected from the 4 iCLIP libraries sequenced for this study (figure supplement 3). **(G)** Upset plot showing single and pairwise combinations of genes for the 4 sequenced iCLIP libraries. Additional intersections can be found in the figure supplement 4. **(H)** Metagene profiles depicting the enrichment along the gene body (5'UTR, CDS or 3'UTR) of the called iCLIP peaks detailed in F.

Figure supplement 1. UV-crosslinked RNA co-purifies with ECT2-mCherry in a pattern that depends on the proteolytic cleavage of the ECT2 IDR.

Figure supplement 2. Illustration of RNA-binding properties of ECT2 revealed by CLIP.

Figure supplement 3. ECT2 iCLIP libraries.

Figure supplement 4. Analysis of ECT2 iCLIP Libraries.

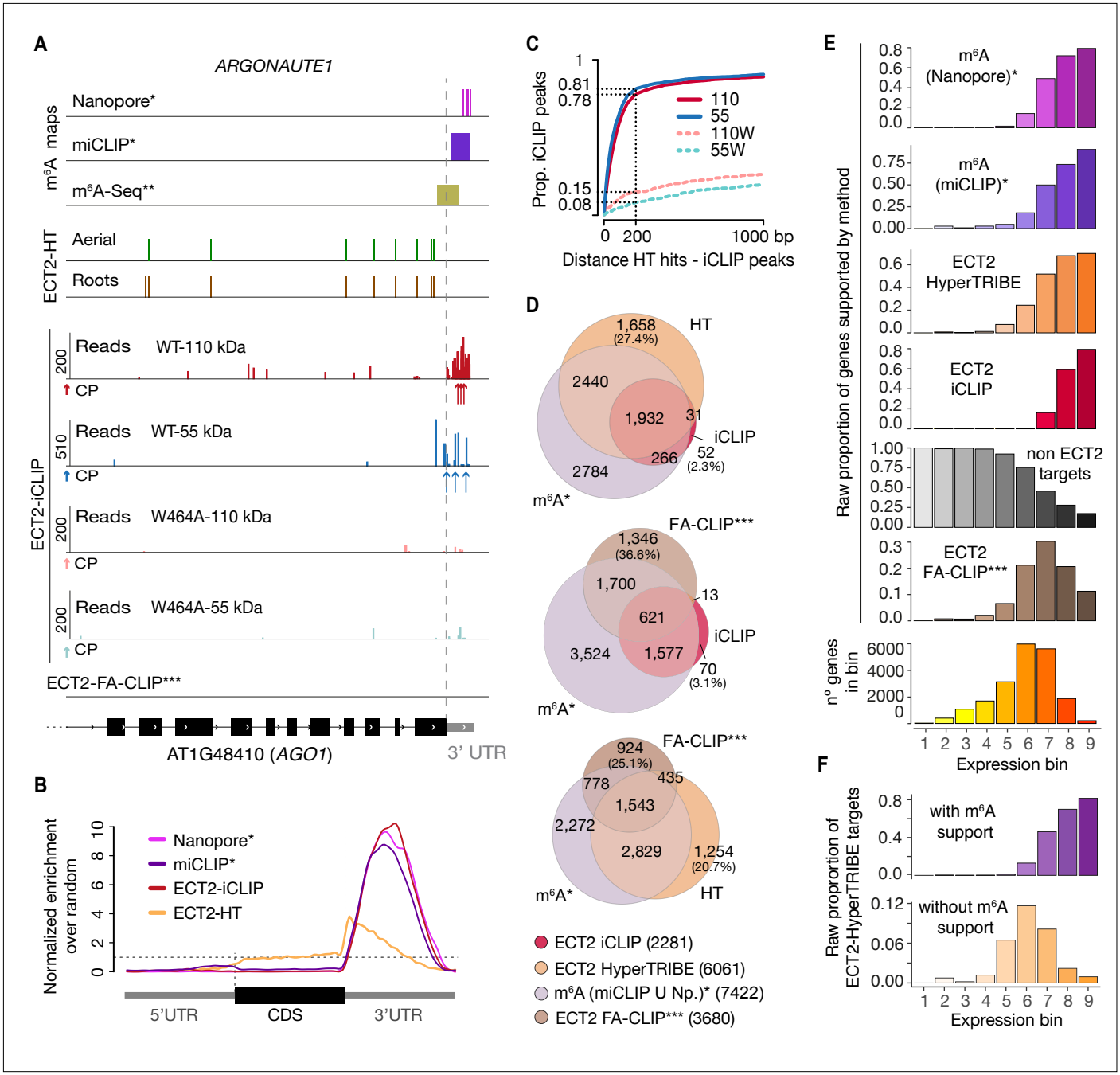

Figure 4. iCLIP identifies bona-fide ECT2 targets. (A) Example of an ECT2 target (*AGO1*) showing the distribution of m^{6}A sites*, **, ECT2-iCLIP reads and peaks, ECT2-HT edited sites, and FA-CLIP peaks*** along the transcript. CP, called peaks. See more examples in the figure supplement 1. **(B)** Metagene profiles comparing the distributions along the gene body of ECT2-mCherry iCLIP peaks (wild type, 110-kDa band), ECT2-HT editing sites (in roots and aerial tissues) and m^{6}A sites*. **(C)** Proportion of ECT2 iCLIP peaks within a given distance from the nearest ECT2-HT edited site. Numbers indicated on the y-axis show the proportion of ECT2 iCLIP peaks less than or equal to 200 nt from the nearest ECT2-HT edited site. **(D)** Overlap between genes supported as containing m^{6}A or ECT2 targets by the different techniques indicated. The ECT2-HT target set includes the sum of targets identified in root and aerial tissues. Additional overlaps are shown in the figure supplement 2. **(E)** Proportions of genes in each expression bin either containing m^{6}A or supported as ECT2 targets by the indicated techniques. **(F)** Proportion of ECT2-HT targets with or without support from m^{6}A data (Nanopore*, miCLIP* or m^{6}A-Seq**) in each expression bin. * Parker et al. (2020); ** Shen et al. (2016); *** Wei et al. (2018).

Figure supplement 1. Distribution of m^{6}A and ECT2 sites on ECT2 targets.

Figure supplement 2. Overlaps between m^{6}A-containing genes and ECT2-targets datasets.

Figure supplement 3. Characteristics of ECT2-HyperTRIBE editing sites relative to target expression levels.

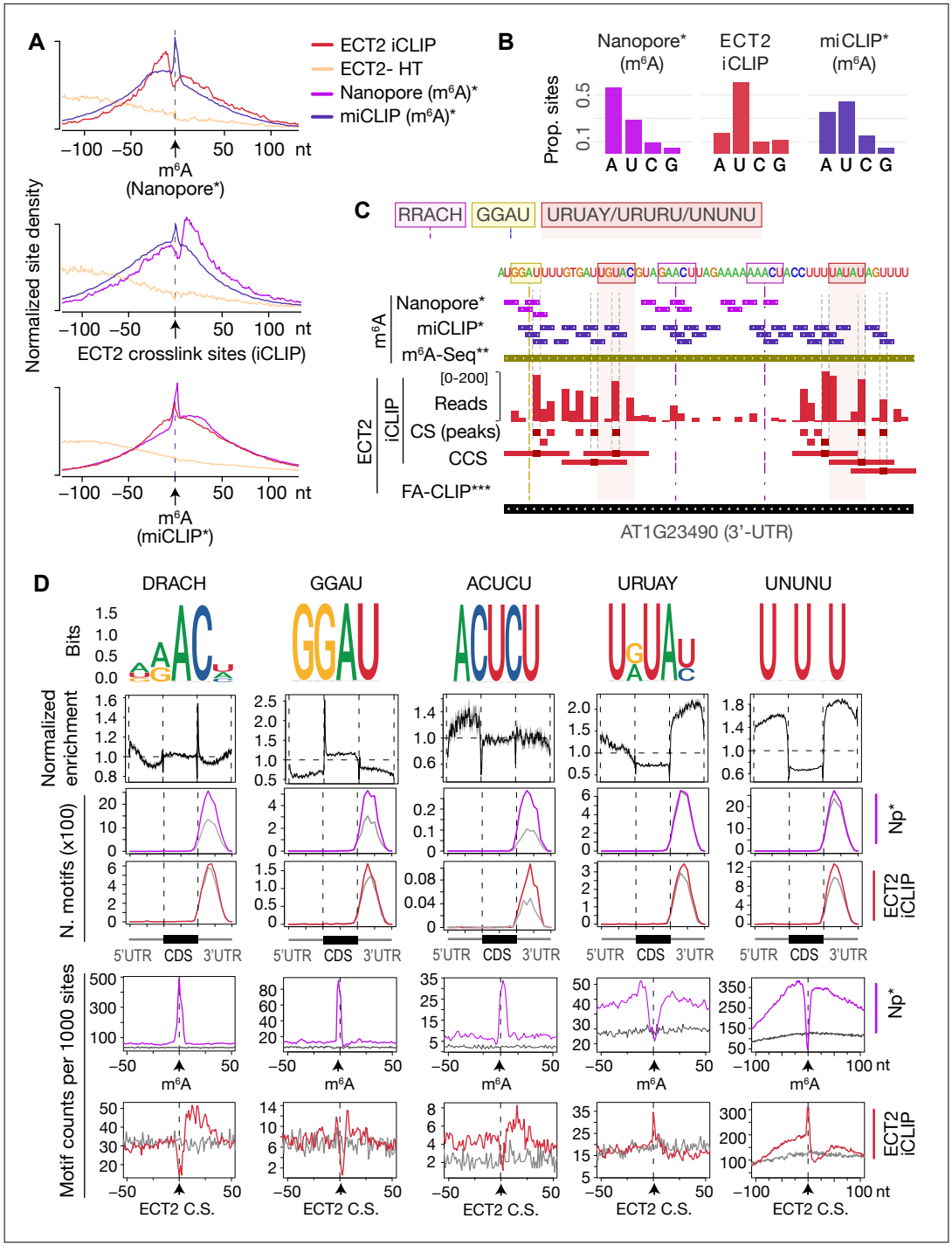

Figure 5. ECT2 UV-crosslinks to uridines in the immediate vicinity of DR(m^{6}A)CH or GG(m^{6}A)U sites. **(A)** Normalized density of sites at and up to +/-100 nt of either m^{6}A Nanopore*, m^{6}A miCLIP* or ECT2 iCLIP sites. **(B)** Proportion of m^{6}A and ECT2 iCLIP sites at each nucleotide by the different methods. **(C)** View from IGV browser illustrating the presence of RRACH, GGAU and U-rich motifs in the vicinity of m^{6}A and ECT2 sites in the 3'-UTR of AT1G23490 (*ARF1*). CS, crosslink sites; CSS, collapsed crosslink sites. **(D)** Key motifs analyzed in this study. From top to bottom: (1) motif logos for derived position weight matrices (PWMs); (2) normalized enrichment of motif locations across gene body; (3-4) total number of the relevant motif found at m^{6}A-Nanopore* (3) or ECT2-iCLIP (4) sites according to gene body location. Grey lines indicate numbers found in a gene-body location-matched background set of sites of equivalent number; (5-6) distribution of the relevant motif relative to m^{6}A-Nanopore* (5) or ECT2–iCLIP (6) sites. Grey lines represent the distribution for the same gene-body location-matched set as derived in the panels above. * Parker et al. (2020); ** Shen et al. (2016); *** Wei et al. (2018).

Figure supplement 1. Sources of motifs and generation of position weight matrices.

Figure supplement 2. Motif logos generated from position weight matrices

Figure supplement 3. Enrichment of DRACH variants around m^{6}A and ECT2 sites.

Figure supplement 4. Uridines flanking DRACH result in additional motifs enriched at ECT2 iCLIP sites.

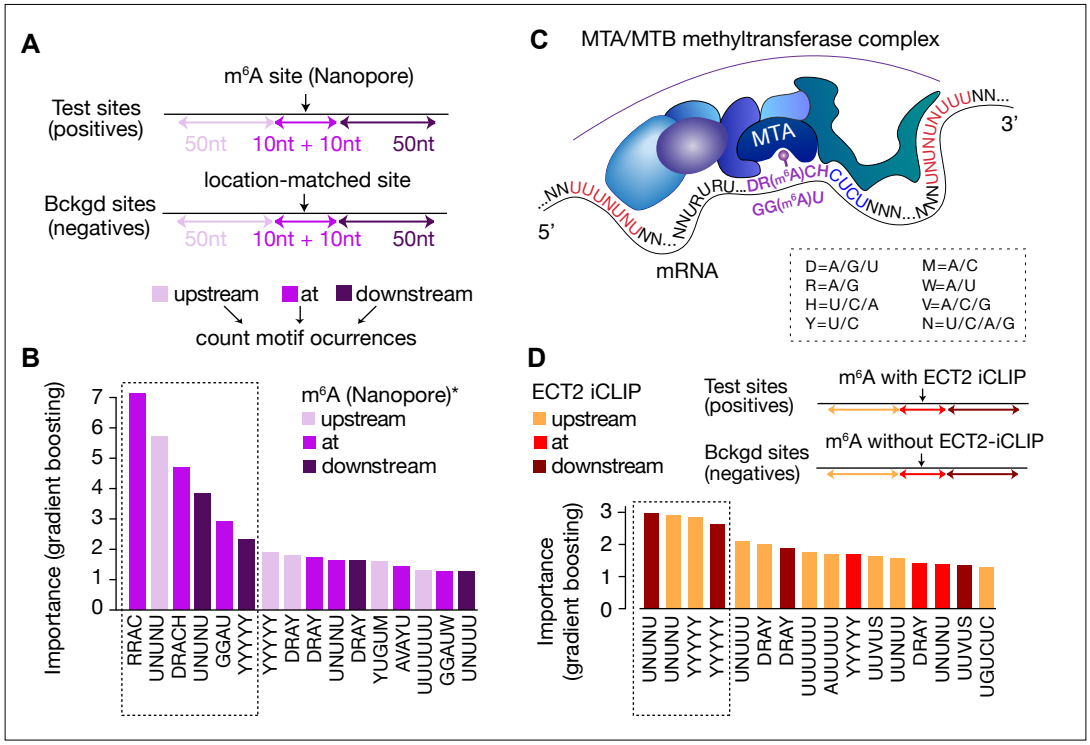

Figure 6. Distal U-rich motifs and at-the-site DRACH/GGAU are determinants for m^{6}A deposition. (A) Diagram representing strategy for machine learning model trained to distinguish m^{6}A Nanopore* sites from their respective gene-body location matched background sets. **(B)** Bar plots showing top 16 motif feature importance scores from the m^{6}A model, ordered from left to right by importance. Dotted rectangle highlights motifs with outstanding importance compared to the rest. **(C)** Cartoon representing the most important motifs found at and around m^{6}A sites. UPAC-IUB codes to define multiple nucleotide possibilites in one position are indicated. **(D)** Machine learning model trained to distinguish between m^{6}A sites with and without ECT2 crosslink sites, and the resulting bar plot showing top 16 motif feature importance scores. Nucleotide distances for intervals, order and dotted box are as in A.

* Parker et al. (2020).

Figure supplement 1. Model performance ROC curves for distinguishing sequence preferences of m^{6}A or ECT2-bound sites.

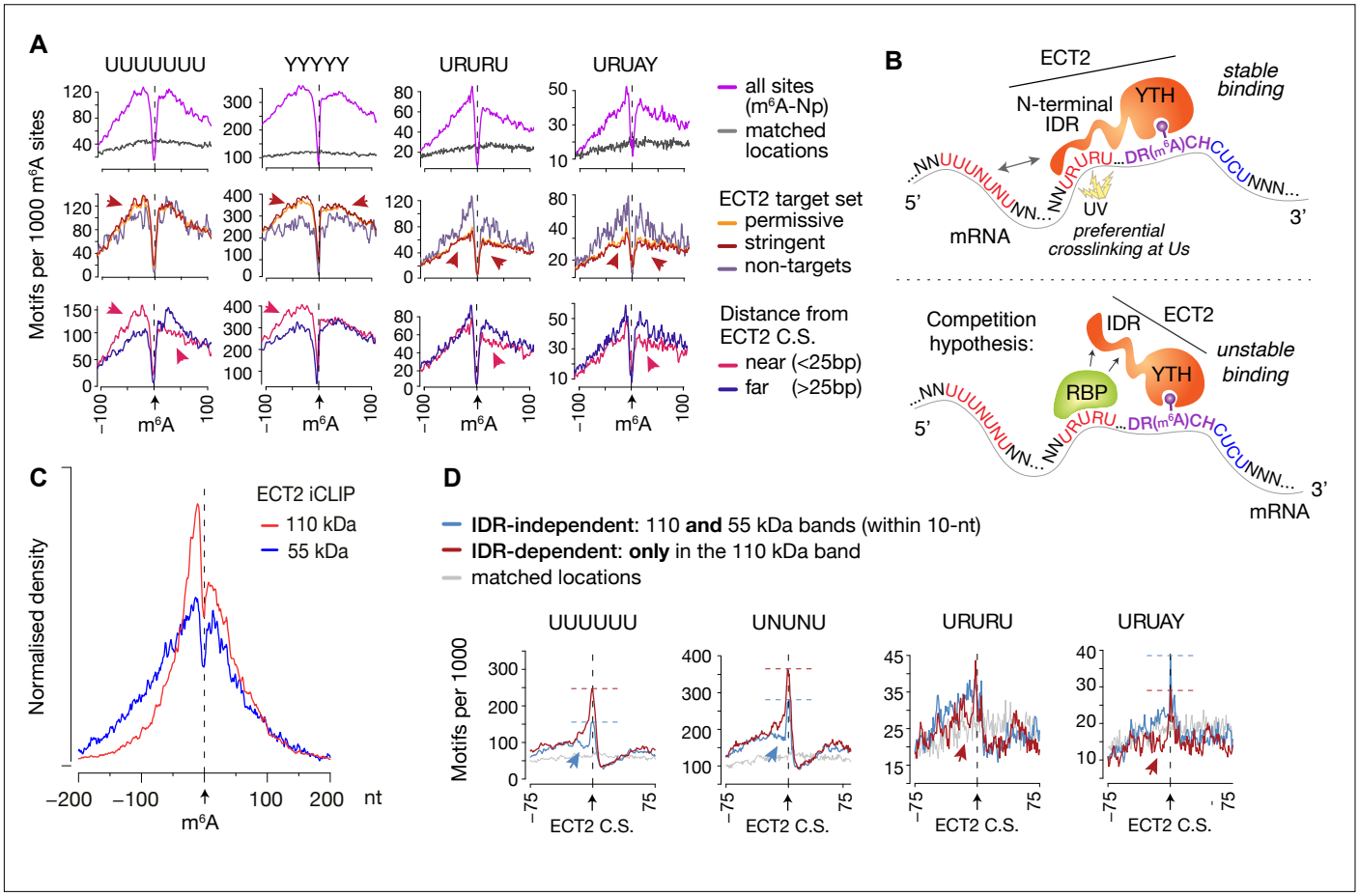

Figure 7. IDR-dependent binding of ECT2 to U-rich motifs 5' of m^{6}A. (**A**) Top panels: Distance-based enrichment of motifs at and around m^{6}A-Nanopore (Np, Parker et al., 2020) sites, plotted as motif counts per 1000 m^{6}A sites (purple lines). Grey lines indicate the enrichment in a location-matched background set as in Figure 5D. Middle and bottom panels: sites are split according to whether they sit on ECT2 targets (middle), or to distance from the nearest ECT2 crosslink site (for ECT2-iCLIP targets only) (bottom). Additional motifs are shown in the figure supplement 1. (**B**) Cartoon illustrating the ECT2 IDR RNA-binding and competition hypotheses. (**C**) Normalized density of ECT2 iCLIP crosslink sites identified in the libraries corresponding to the 110 and 55 kDa bands (Figure 3B) at and up to +/-200 nt of m^{6}A Nanopore* sites. (**D**) Motifs per 1000 ECT2 iCLIP crosslink sites (CS) split according to whether they are found in libraries from both 110 kDa and 55 kDa bands (IDR-independent'), or exclusively (distance > 10 nt) in the 110 kDa band ('IDR-dependent'). Grey lines indicate the enrichment in a location-matched background set as in Figure 5D. Additional motifs are shown in the figure supplement 2 and supplementary file 3.

Figure supplement 1. Motif preferences around m^{6}A sites according to ECT2 binding.

Figure supplement 2. Dependency of the ECT2 IDR for motif enrichment.