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FTO-mediated cytoplasmic m⁶A_m demethylation adjusts stem-like properties in colorectal cancer cell

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ABSTRACT

Cancer stem cells (CSCs) are a small but critical cell population for cancer biology since they display inherent resistance to standard therapies and give rise to metastases. Despite accruing evidence establishing a link between deregulation of epitranscriptome-related players and tumorigenic process, the role of messenger RNA (mRNA) modifications dynamic in the regulation of CSC properties remains poorly understood. Here, we show that the cytoplasmic pool of fat mass and obesity-associated protein (FTO) impedes CSC abilities in colorectal cancer through its m⁶A_m (N⁶,2'-O-dimethyladenosine) demethylase activity. While m⁶A_m is strategically located next to the m⁷G-mRNA cap, its biological function is not well understood and has not been addressed in cancer. Low FTO expression in patient-derived cell lines elevates m⁶A_m level in mRNA which results in enhanced *in vivo* tumorigenicity and chemoresistance. Inhibition of the nuclear m⁶A_m methyltransferase,

31 **PCIF1/CAPAM, partially reverses this phenotype. FTO-mediated regulation of m⁶A_m marking**
32 **constitutes a novel, reversible pathway controlling CSC abilities that does not involve transcriptome**
33 **remodeling, but could fine-tune translation efficiency of selected m⁶A_m marked transcripts.**
34 **Altogether, our findings bring to light the first biological function of the m⁶A_m modification and its**
35 **potential adverse consequences for colorectal cancer management.**

36

37 Despite significant advances in diagnosis and therapy, colorectal cancer (CRC) remains a major
38 cause of mortality and morbidity worldwide. CRC survival is highly dependent upon early diagnosis.
39 Patients with localized cancer exhibit 70 to 90% 5-year survival. Survival from metastatic cancer
40 plummets to 10%. Metastasis is a multistep process encompassing local infiltration of tumor cells into
41 adjacent tissues, transendothelial migration into vessels, survival in the circulatory system,
42 extravasation, and colonization of secondary organs [1]. This process entails constant reprogramming
43 of gene expression to enable tumor adaptation in different environments, a peculiar trait of cancer
44 stem cells (CSCs). CSC constitute a minor subpopulation of tumor cells endowed with self-renewal and
45 multi-lineage differentiation capacity [2]. The most clinically relevant trait of CSCs is their ability to
46 metastasize and escape from standard chemotherapy [3]. Understanding the molecular mechanisms
47 that participate to the CSC phenotype is critical to designing improved cancer therapeutics.

48 Among more than 100 post-transcriptional modifications reported to occur on RNA [4], N⁶-
49 methyladenosine (m⁶A) is the most frequent epigenetic modification of mammalian messenger RNAs
50 (mRNAs) [5]. m⁶A is involved in all post-transcriptional steps of gene expression (mRNA splicing,
51 transport, stability and translation) and plays a role in pleiotropic biological processes including
52 development, immunology, and stem cell biology [5]. Therefore, it comes at no surprise that m⁶A
53 dysregulation is intricately involved in the progression of several solid and non-solid tumors while the
54 underlying functional mechanism differs from one malignancy to another [6-11].

55 Discovered several decades ago [12-16], the function of m⁶A remained obscure until the identification
56 of the first m⁶A demethylase, the fat mass and obesity-associated protein (FTO) [17]. The marriage of
57 immunochemical approaches with next-generation sequencing (NGS) technologies revealed the
58 unique topology of m⁶A distribution along mRNA. m⁶A is a dynamic reversible chemical modification
59 catalyzed by a protein complex consisting of the methyltransferase-like 3 and 14 (METTL3 and
60 METTL14), and several auxiliary proteins such as the Wilms' tumor 1-associating protein (WTAP) [18-
61 21]. Effects of m⁶A involve recruitment of reader proteins, e.g. YTHDF1 [22] or YTHDF2 [23], that lodge
62 the modified adenosine in the hydrophobic pocket of their YTH domain. Finally, m⁶A is removed by the
63 AlkB homolog 5 (ALKBH5) [24] and FTO [17] (**Figure 1a**). m⁶A modification generally occurs in a subset
64 of RRA*CH consensus sites (R, purine; A*, methylable A; C, cytosine; H, non-guanine base), at the
65 beginning of the 3'-UTR near the translation termination codon [25, 26] with one exception. Indeed,
66 besides internal adenosine, 2'-O-methyladenosine (A_m) residue adjacent to the N7-methylguanosine
67 (m⁷G) cap, can be further methylated at the N6 position and become the N6,2'-O-dimethyladenosine
68 (m⁶A_m) [27]. m⁶A_m can be deposited by the recently identified PCIF1/CAPAM [28] and removed by FTO
69 [29]. Unlike m⁶A, the biological function of m⁶A_m mRNA modification is poorly understood and its
70 potential involvement in cancer onset or evolution has never been addressed.

71 Here, we uncover the importance of cytoplasmic FTO-mediated m⁶A_m level adjustment to colorectal
72 CSC. Using patient-derived cell lines, we establish that FTO activity hampers CSC abilities through a
73 novel, unconventional process that does not involve transcriptional reprogramming but, most likely,
74 fine-tuning translation of m⁶A_m marked transcripts.

75 **RESULTS**

76 **FTO inhibition promotes stem-like traits in colorectal cancer cell lines**

77 We initially evaluated the involvement of m⁶A modification in generating the CSC phenotype. Due to
78 their inherent plasticity, CSCs are best identified via their functional abilities, such as tumorigenic
79 potential and chemoresistance, rather than surface biomarkers. We examined the ability of short

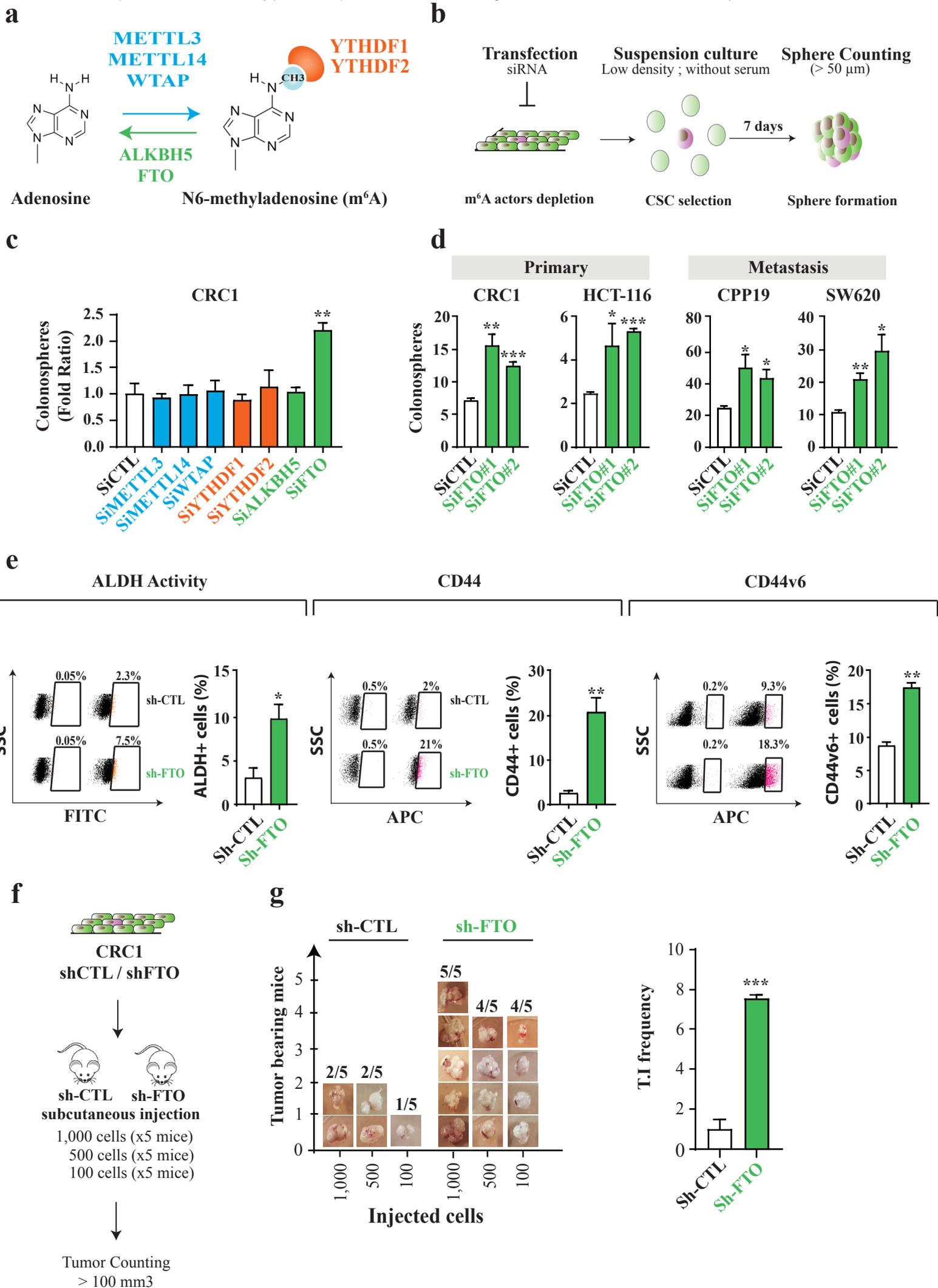


Figure 1. FTO inhibition promotes stem-like traits in colorectal cancer cell lines. (a) Actors of m⁶A modification targeted by the siRNA screening. The writer complex METTL3 – METTL14 – WTAP deposits m⁶A while ALKBH5 and FTO erases m⁶A. Both YTHDF1 and YTHDF2 are readers of m⁶A modification. **(b) Sphere forming assay.** siRNA-transfected cells were seeded at low density (1 cell/ μ L) in non-adherent conditions and serum-deprived medium. This type of suspension culture allows the survival and growth of stem-like/progenitor cells. Following 7 days of culture, the number of spheres correlates with the initial number of CSC. **(c) FTO silencing increases the sphere forming potential of CRC1 cell line.** Colonosphere formation was quantified following knockdown of the main m⁶A actors. Results are expressed in fold change compared to si-CTL. n = 3 biological replicates. Mean +/- S.E.M, ** p-value < 0.01. Two-sided Unpaired T-test. **(d) FTO silencing increases the sphere forming potential in four CRC cell lines.** Colonospheres quantification after silencing of FTO by two distinct siRNA, in four cell lines derived from primary tumors or metastasis. Results are expressed in fold change compared to si-CTL (n = 3 biological replicates). Mean +/- S.E.M, * p-value < 0.05 ; ** p-value < 0.01. Two-sided Unpaired T-test. **(e-g) Expression of CSC biomarkers.** Level of ALDH activity **(e)** as well as cell-surface expression of CD44 **(f)** and CD44v6 **(g)** were evaluated by flow cytometry in CRC1- sh-FTO cell line vs sh-CTL. Graphs show one representative biological replicate (n = 3). Bar plots show quantification of the number of ALDH **(e)**, CD44 and CD44v6 positive cells from three biological replicates. Mean +/- S.E.M, *p-value < 0.05, **p-value < 0.01. Two-sided Unpaired T-test. **(f) In vivo tumor initiation assay.** Either 1,000, 500 or 100 CRC1 sh-FTO / sh-CTL cells were subcutaneously injected into nude mice. After 7 weeks, the number of tumor-bearing mice (tumor > 100 mm³) was evaluated. **(g) FTO silencing increases tumor initiation in vivo.** Picture represents the number of tumor bearing mice for each group (5 mice per group) after injection of sh-FTO or sh-CTL CRC1 cell line. Bar plot represents the quantification of Tumor Initiation (T.I) frequency obtained by ELDA software. *** p-value < 0.001, chi2 test.

80 interfering RNAs (siRNA) targeting known m⁶A mediators –writers, readers and erasers- to alter
81 sphere-forming potential (SFP) (**Figure 1b**). SFP is the ability of cancer cells -from either conventional
82 cell lines or patient exeresis- to form microtumor-like spheroids (colonospheres) from a single cancer
83 progenitor cell [30]. This model is often used as a surrogate to evaluate the tumorigenic potential of
84 solid tumors (5). We used CRC1 cells, a colorectal cancer cell line established in the lab from colorectal
85 cancer exeresis [31]. The various siRNAs significantly silenced expression of individual target genes:
86 METTL3, METTL14, WTAP, YTHDF1, YTHDF2, ALKBH5 and FTO (**Figure S1a**). Only FTO knockdown
87 affected SFP, nearly doubling colonospheres numbers (**Figure 1c**). We confirmed the importance of
88 FTO using a different targeting siRNA (**Figure 1d, S1b**) and three other cell lines, derived from primary
89 (HCT-116) and metastatic (CPP19, SW620) tumors (**Figure 1d**).

90 Next, we generated stable CRC1 cell lines expressing GFP with either a short hairpin RNA targeting FTO
91 (sh-FTO) or an irrelevant scrambled short hairpin control (sh-CTL), and selected transfected clones by
92 cell sorting for GFP expression (**Figure S1c**). Sh-FTO expressing cell lines established from individual
93 cells exhibited a three to ten-fold decrease of FTO expression as shown by immunoblot analysis (**Figure**
94 **S1d**) and displayed increased SFP, confirming the siRNA findings (**Figure S1e**). Importantly, FTO
95 knockdown does not influence cell growth (**Figure S1f**), which differs from the AML (acute myeloid
96 leukemia) model [8]. Then, we evaluated the level of several stemness-related markers associated with
97 CSC features. FTO knockdown cells displayed enhanced aldehyde dehydrogenase (ALDH) activity, a
98 hallmark of CSC [32] (**Figure 1e**), and increased expression of CD44 and CD44v6 (**Figure 1e**), membrane
99 receptors associated with tumor progression [33] and indispensable for CSC tumor initiation,
100 chemoresistance, and epithelial to mesenchymal transition. CRC1 sh-FTO cells demonstrated a ten-
101 fold increase in the number of CD44+ cells (from 2.1% to 21%) (**Figure 1e**) and CD44v6 isoform
102 expression was doubled (**Figure 1e**). FTO knockdown in SW620 cells phenocopied the CRC1 cells
103 (**Figure S1c-h**).

104 To connect FTO levels with *in vivo* tumor initiation potential we inoculated immunodeficient mice
105 (athymic nude) with increasing numbers of sh-FTO or sh-CTL cells (**Figure 1f**). Seven weeks later, tumor
106 xenografts (diameter > 100mm³) were counted and harvested. Remarkably, as few as hundred sh-FTO
107 cells were capable of initiating tumor formation in 4/5 mice vs. 1/5 for sh-CTL cells (**Figure 1g**). Extreme
108 limiting dilution software analysis (ELDA) shows that the frequency of tumor-initiating cells is seven-
109 fold higher in sh-FTO cells, with a highly significant p-value (p=0.00035) (**Figure 1g**).

110 Based on these findings, we conclude that diminished FTO expression promotes the CSC phenotype.

111 **FTO silencing confers resistance to chemotherapy in colorectal cancer cell lines**

112 We extended these findings to chemoresistance, another hallmark of CSC. We employed FIRI
113 treatment, a combination of 5-fluorouracil and irinotecan, used to treat metastatic colorectal cancer
114 [34]. We followed a standard protocol for treating colorectal cancer cell lines based on 3 days
115 treatment with FIRI (50µM 5-fluorouracil (5-FU) + 500nM SN38, active metabolite of irinotecan) (**Figure**
116 **2a**). siRNA mediated FTO targeting conferred significant chemoprotective effects on CRC1 cells (2 to 3-
117 fold) in comparison with control cells (si-CTL)(**Figure 2a**). As above, we obtained a similar effect in
118 SW620 (**Figure 2a**), as well as in stable sh-FTO models (**Figure 2b**).

119 Importantly, chemoprotection bestowed by FTO silencing extended to FOX treatment (50µM 5-
120 fluorouracil (5-FU) + 1 µM oxaliplatin), another advanced stage colorectal cancer therapy [35, 36]
121 (**Figure S2a**). To evaluate the effect of FTO knockdown *in vivo* we injected sh-CTL or sh-FTO cells (50,000
122 cells) into nude mice (6 mice per group). When the tumors reached about 100 mm³ (day 21), we
123 treated mice with FIRI (50mg/kg 5-FU + 30mg/kg irinotecan) and measured tumors twice a week for
124 four and a half weeks (**Figure 2c**). While FIRI treatment stabilized tumor size in sh-CTL mice, sh-FTO
125 tumors displayed substantial chemoresistance maintaining consistent growth (**Figure 2c**).

126 Do chemoresistant cells display low FTO levels? To produce CSC-enriched chemoresistant cells, we
127 treated CRC1 and SW620 cells for three days with sub-lethal doses of FIRI (0.2 X FIRI = 10 µM 5-FU +

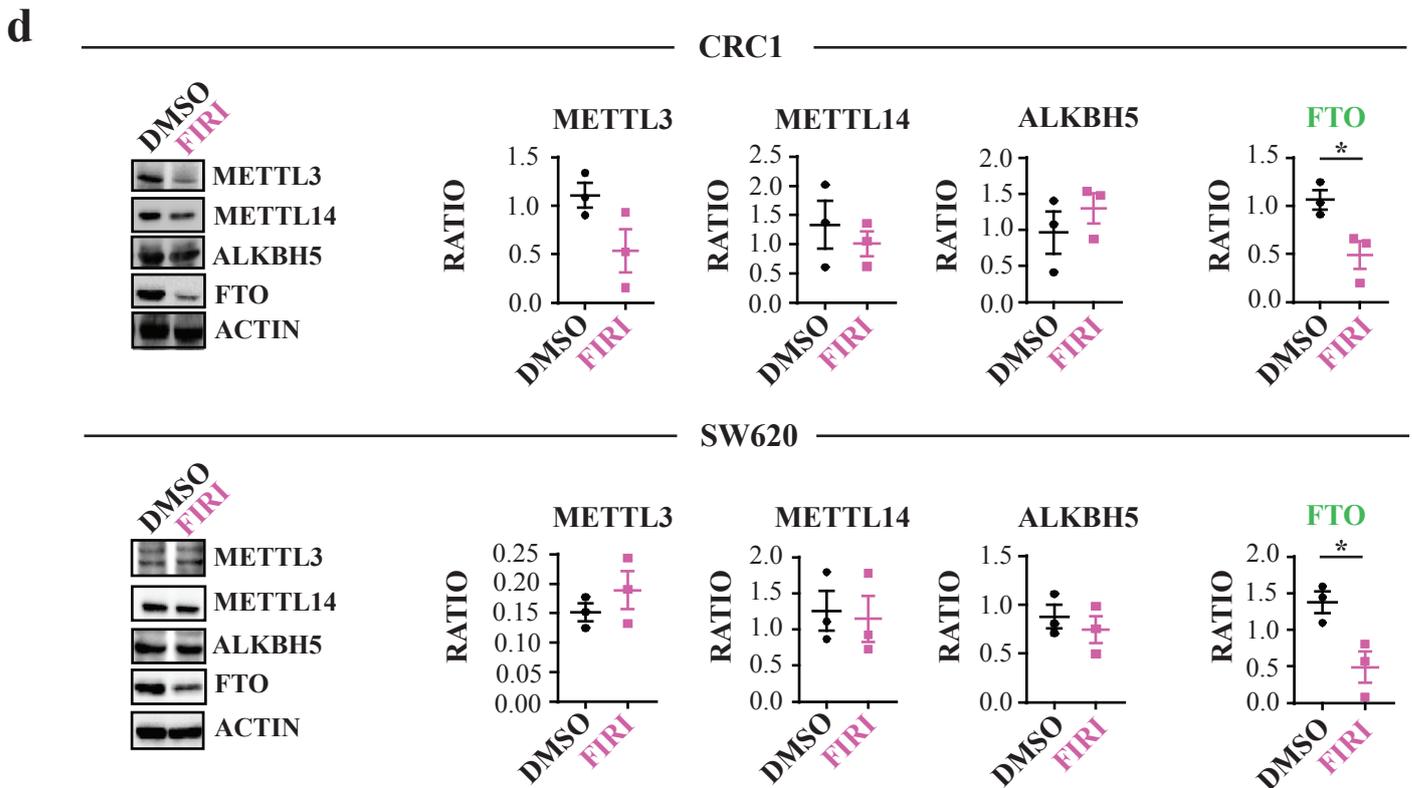
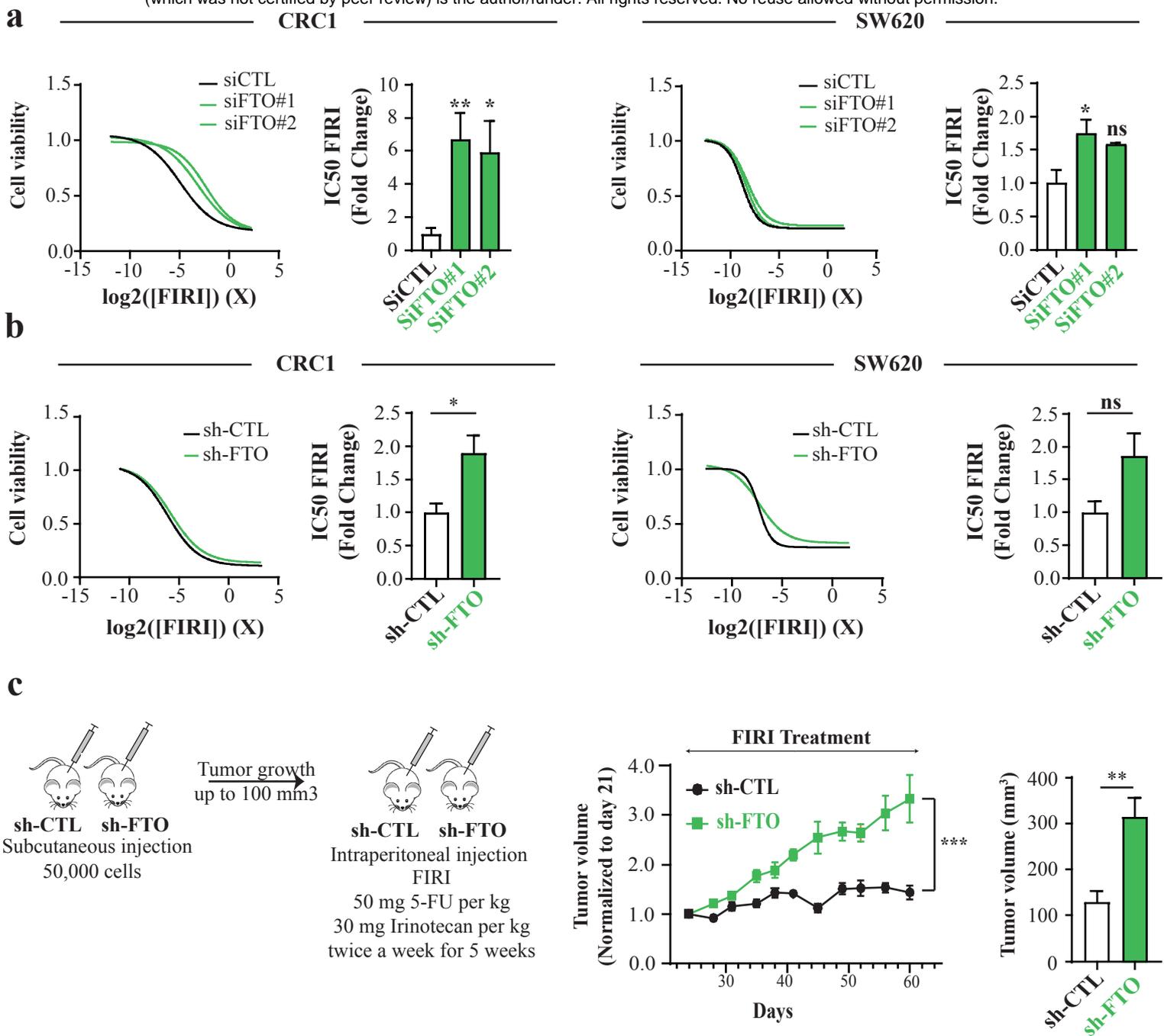


Figure 2. FTO silencing confers resistance to chemotherapy. (a) Transient FTO silencing increases chemoresistance to FIRI. Graphs illustration of FIRI toxicity on either si-FTO or si-CTL-transfected cells (CRC1 (left) and SW620 (right) cell lines). Toxicity was measured using Sulforhodamine B assay. Bar plot represents quantification of IC_{50} of three biological replicates. FIRI 1 X = 50 μ M 5-FU, 0.5 μ M SN38. Mean +/- S.E.M, **p-value < 0.01, *p-value < 0.05, Two-sided Unpaired T-test. **(b) Stable FTO silencing increases chemoresistance to FIRI.** Same as (a) with stable cell lines (sh-FTO or sh-CTL) from two distinct backgrounds (CRC1 (left) and SW620 (right)). Mean +/- S.E.M, *p-value < 0.05, Two-sided Unpaired T-test. **(c) FTO silencing increases *in vivo* chemoresistance.** 50,000 of either sh-FTO or sh-CTL cells were subcutaneously injected into the flank of nude mice. After 21 days (tumor size about 100mm³), mice were treated with FIRI (50 mg FIRI per kg, 30 mg irinotecan per kg) and tumor growth was measured twice a week for 5 weeks. *** p-value < 0.001, two-way ANOVA test. Bar plot represent mean +/- S.E.M of tumor volume measured at the last time point. **p-value < 0.01. **(d) FIRI-resistant cells display decreased FTO expression.** Immunoblot analysis of METTL3 – METTL14, ALKBH5 and FTO levels after 72 h of 0.2 X FIRI treatment. Pictures are representative of 3 experiments in CRC1 cell line. Protein level quantification is mean +/- S.E.M of FTO normalized to ACTIN of three biological replicates. * p-value < 0.05, ns = not significant, Two-sided Unpaired T-test.

128 0.1 μ M SN38). As expected, 0.2 X FIRI treatment triggered cell cycle arrest at G2/M phase and increased
129 subG1 cells (**Figure S2b**). Surviving cells demonstrated increased ALDH activity (**Figure S2c**) and
130 enhanced resistance to chemotherapy (**Figure S2d**). While variations of METTL3 and ALKBH5 levels
131 could be noticed following chemoresistance acquisition, they were not consistent between cell lines
132 (**Figure 2d**). By contrast, FTO level was decreased by half in both cell lines (**Figure 2d**). Noteworthy, the
133 correlation between FTO mRNA expression levels and protein abundance varies from one cell line to
134 another (**Figure S2e**).

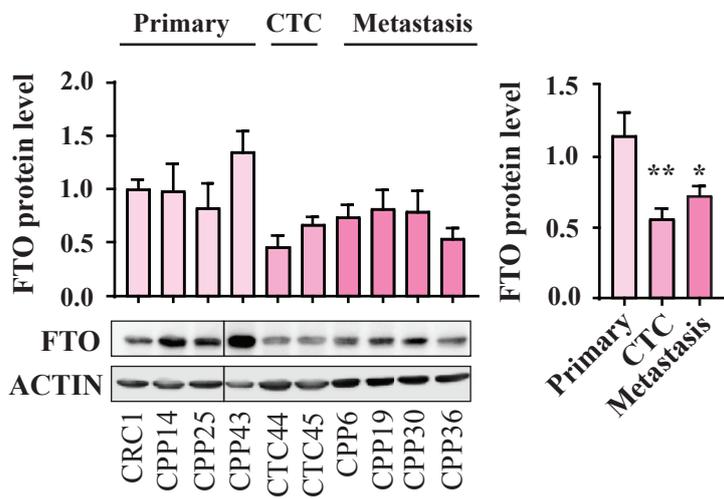
135 Together, these findings demonstrate that FTO expression is tightly linked to the CSC chemoresistant
136 phenotype.

137 **FTO expression regulates CSC phenotype in circulating tumor cells**

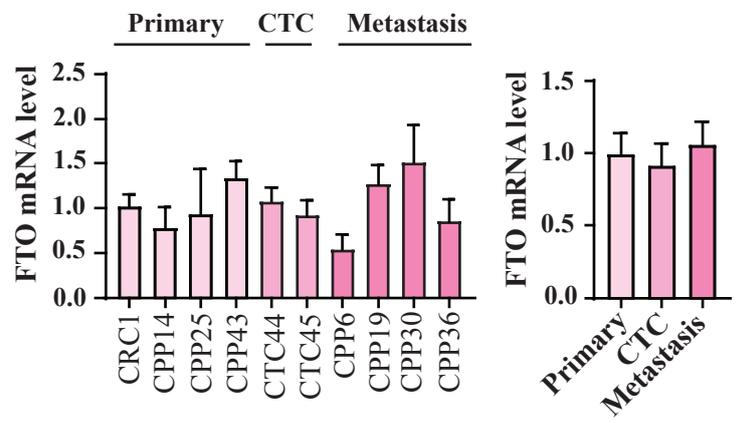
138 Circulating tumor cells (CTCs) are often detected in the bloodstream of colorectal cancer patients,
139 sometimes even at early stages of the disease [37]. CTC are responsible for metastasis and high
140 mortality. We previously established CTC lines from chemotherapy-naïve stage IV (metastatic) CRC
141 patients that display a strong CSC phenotype [31]. Immunoblotting revealed that FTO protein levels
142 are reduced by ~50% in CTC lines in comparison with primary and metastatic patient derived lines
143 (**Figure 3a**). Decreased expression is achieved post transcriptionally, since quantitative PCR (qPCR)
144 analysis of FTO mRNA levels showed no significant differences between CTC lines and
145 primary/metastatic patient derived lines (**Figure 3b**). A similar disconnect in FTO mRNA/protein was
146 reported in gastric tumors [38].

147 Despite this, we could increase FTO protein by transfecting cells with a plasmid containing a FTO cDNA
148 under a strong promoter. Increasing FTO in CTC lines (CTC44 and CTC45) did not affect cell proliferation
149 in monolayer culture but decreased SFP (**Figure 3c**), chemoresistance to FIRI treatment (**Figure 3d**) and
150 ALDH activity (**Figure 3e**) in both cell lines.

a

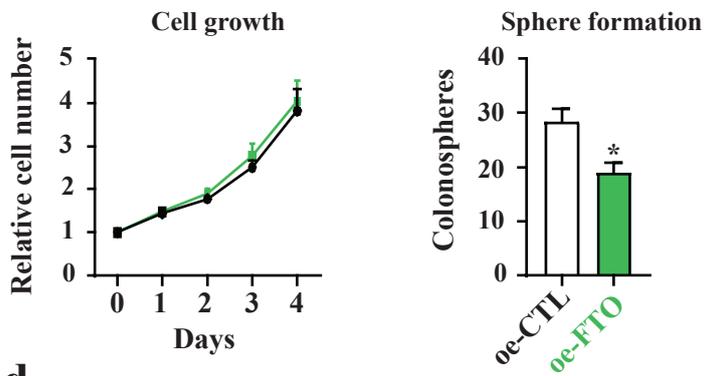


b

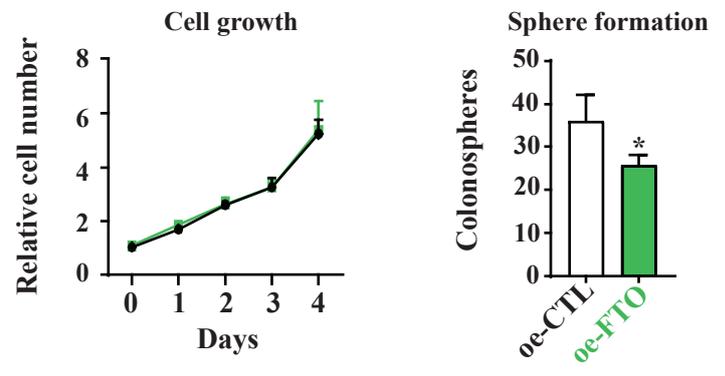


c

CTC44

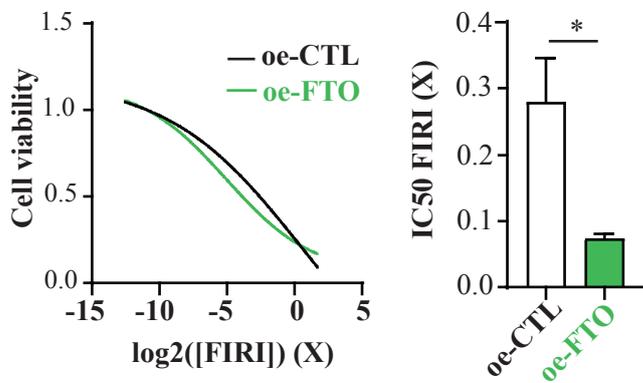


CTC45

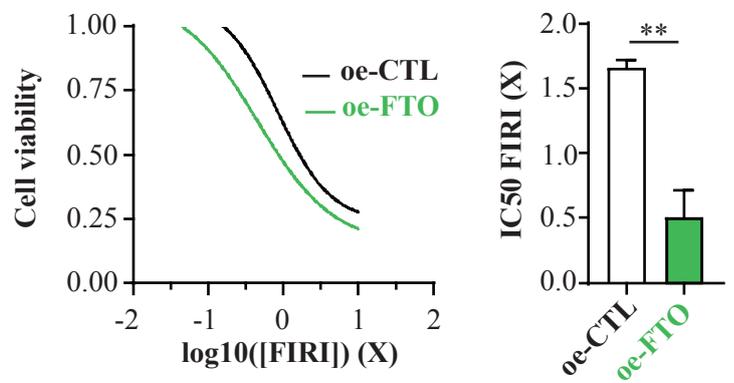


d

Resistance to FIRI

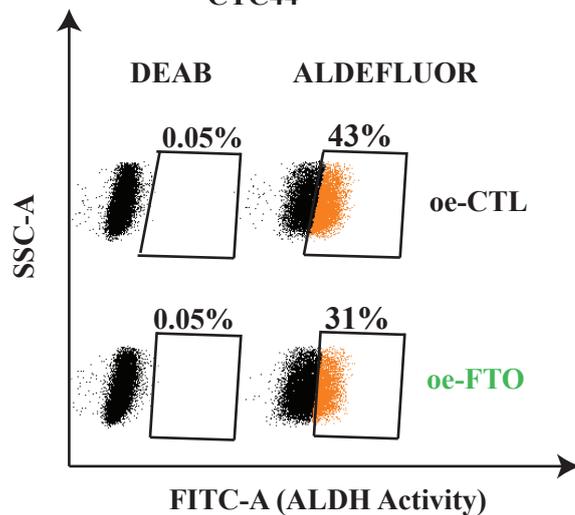


Resistance to FIRI



e

CTC44



CTC45

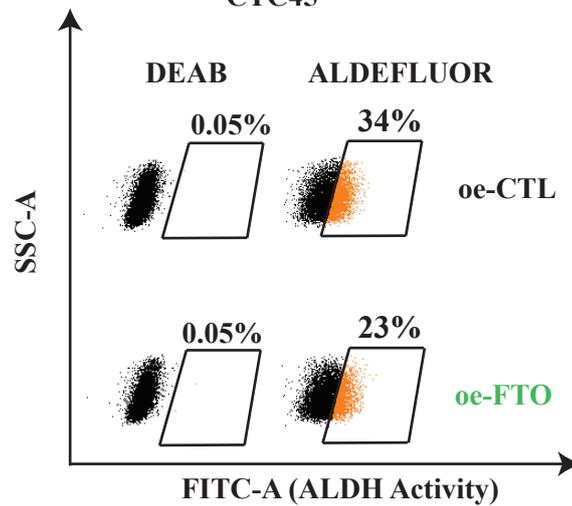


Figure 3. FTO expression is lower in “circulating tumor cells” (CTC) than in “solid” tumor cells. (a) FTO protein level is the lowest in Circulating Tumor Cell lines. FTO protein level quantification by western blot in several patient derived cell lines from distinct origin: primary tumor, blood, and metastasis. The first graph represents the level of FTO protein in each individual cell lines. The following bar plot represents the quantification by groups. Mean +/- S.E.M of three biological replicates, ** p-value < 0.01, * p-value < 0.05, One-way Anova followed by multiple comparison. **(b) FTO mRNA level barely fluctuates throughout colon cancer progression.** Level of FTO mRNA was evaluated by RT-PCR in the same cell lines as in (a). Left graph represents of FTO level in each individual cell line. Right bar plot represents the quantification by groups. Mean +/- S.E.M of 3 biological replicates, ns = not significant, One way Anova. **(c) Increase of FTO level decreases the sphere forming potential of CTC cell lines.** Cell growth and sphere forming potential evaluation after overexpression of FTO in CTC44 and CTC45 lines. **(d) Increase of FTO level sensitizes CTC cell lines to chemotherapy.** FIRI toxicity assay on CTC44 or CTC45 after overexpression of FTO. Mean +/- S.E.M, ** p-value < 0.01, *p-value < 0.05, Two-sided Unpaired T-test. **(e) FTO overexpression decreases ALDH activity in CTC cell lines.** Flow cytometry quantification of ALDH positive cells after FTO overexpression. Graphs show the number of ALDH positive cells in one representative biological replicate out of three for CTC44 and CTC45.

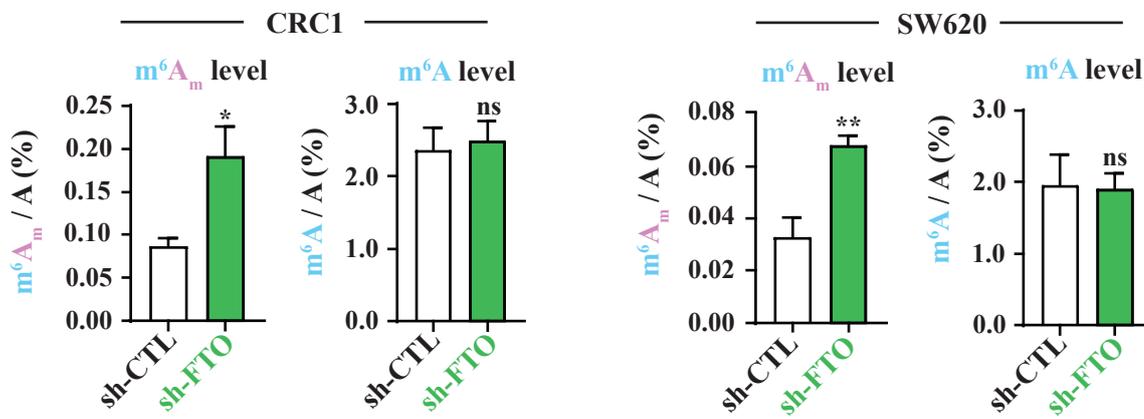
151 Thus, multiple lines of evidence point to the conclusion that FTO is a key factor in maintaining the CSC
152 phenotype.

153 **FTO functions via its m⁶A_m demethylase activity**

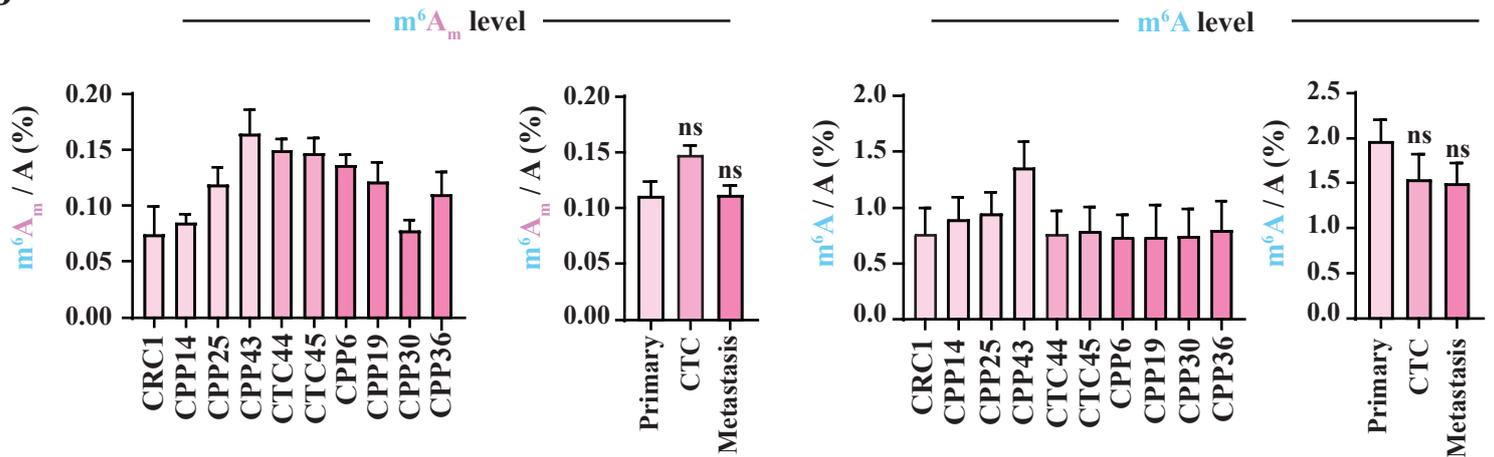
154 How does FTO modulate CSC functions? First, we evaluated whether its catalytic activity was essential
155 to achieve this phenotypic outcome. As FTO can demethylate m⁶A and m⁶A_m [39-41], we measured
156 their levels in RNase digested polyadenylated mRNA from both sh-CTL and sh-FTO cells using high-
157 performance liquid chromatography-coupled to tandem mass spectrometry (LC-MS/MS) analysis. At
158 first, we isolated mRNA from cell pellets and verified the purity of our sample preparation by LC-
159 MS/MS (**Figure S3a**) and qPCR (**Figure S3b**). To detect m⁶A_m proximal to m⁷G-Cap, we used a previously
160 published method based on including a decapping step (RNA 5' Pyrophosphohydrolase, RppH
161 treatment) prior to Nuclease P1 treatment [41]. We calibrated the assay using standard curves created
162 with synthetic nucleoside standards (**Figure S3c**).

163 In both CRC1 and SW620 lines, FTO knockdown did not impact internal m⁶A / A level (**Figure 4a**).
164 However, we clearly observed a significant increase of m⁶A_m / A ratio (**Figure 4a**). We performed
165 controls to strengthen our observation: first, FTO silencing affected neither m⁶A / A nor m⁶A_m / A ratio
166 in small RNA species, another potential target of FTO (**Figure S3d**); second, to ensure our ability to
167 detect m⁶A variation, we silenced METTL14, an m⁶A writer. As anticipated, METTL14 targeting
168 triggered a significant decrease of m⁶A level (but not m⁶A_m) by LC-MS/MS (**Figure S4a**). Next, we
169 performed a similar analysis with mRNA extracted from our panel of patients derived cell lines (**Figure**
170 **3b**). We observed an increased level of m⁶A_m / A ratio in CTC lines (**Figure 4b**) which was concomitant
171 with low FTO levels (**Figure 3a**). By contrast, m⁶A / A ratio was rather decreased (**Figure 4b**).
172 Importantly, FTO overexpression in two CTC lines triggered the opposite effect and decreased
173 tremendously m⁶A_m / A ratio while m⁶A / A ratio remained unchanged (**Figure 4c**). Altogether, these
174 observations established a first connecting thread between FTO-mediated m⁶A_m dynamic and the
175 acquisition of cancer stem ability in colorectal cancer.

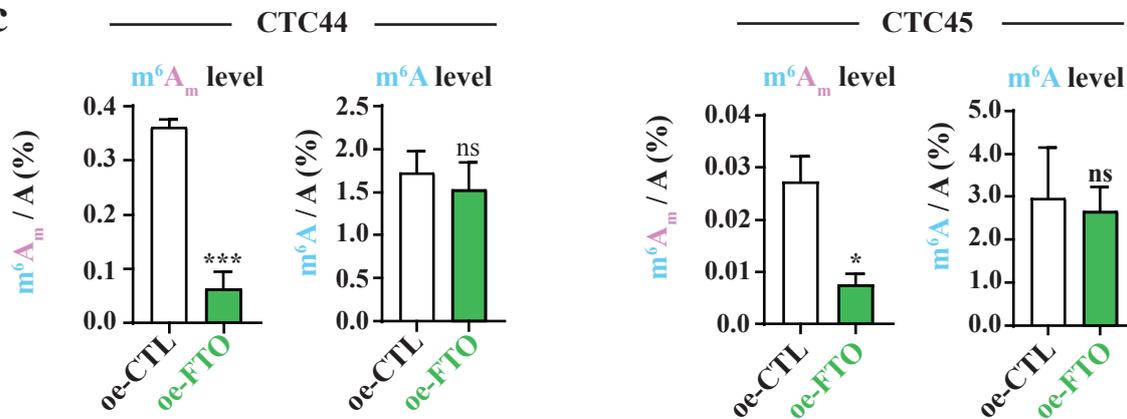
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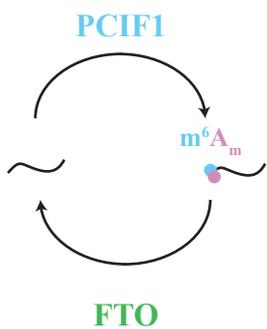
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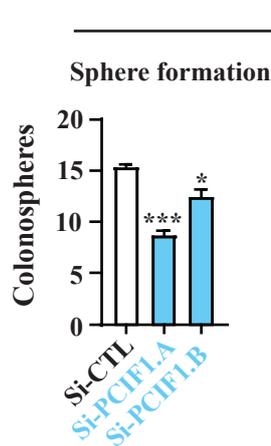
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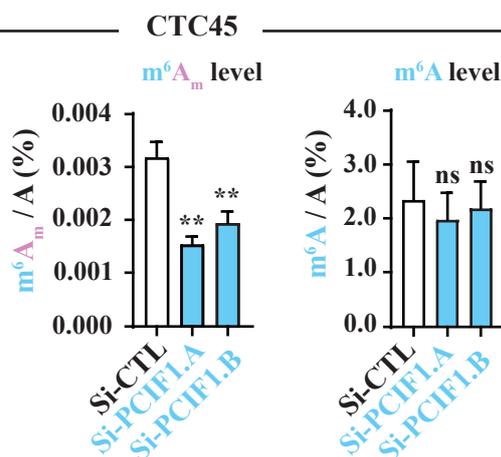
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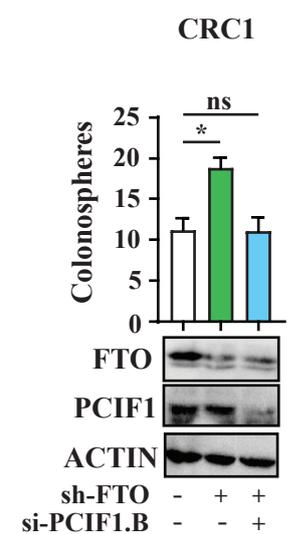


Figure 4. FTO affects stem-like properties through m⁶A_m demethylase activity. (a) FTO silencing increases m⁶A_m level rather than m⁶A. LC-MS/MS mRNA quantification of m⁶A_m/A and m⁶A/A ratios in either CRC1 sh-FTO or sh-CTL and SW620 sh-FTO or sh-CTL cell line. Bar plot represents Mean +/- S.E.M of at least three biological replicates. ** p-value < 0.01, * p-value < 0.05, ns = not significant. Two-Sided Unpaired T-test. **(b) m⁶A_m tends to be increased in CTC lines but not m⁶A.** LC-MS/MS analysis of mRNA from patient derived cell lines (same as in **Figure 3**). m⁶A_m/A and m⁶A/A were evaluated. Graphs represent mean +/- S.E.M of m⁶A_m/A or m⁶A/A level for each cell line or group of cell lines. Three biological replicates. **(c) FTO overexpression decreases m⁶A_m rather than m⁶A in CTC lines.** mRNA quantification of m⁶A_m/A and m⁶A/A after FTO overexpression in CTC44 line. Graphs represent mean +/- S.E.M of at least 3 biological replicates. *** p-value < 0.001, * p-value < 0.05, ns = not significant. Two-sided Unpaired T-test. **(d) Effectors of m⁶A_m modification. (e) PCIF1 silencing decreases colonosphere formation.** Sphere forming ability of CTC44 line after silencing of the m⁶A_m writer PCIF1. Bar plot represents mean +/- S.E.M of three experiments. *** p-value < 0.001, * p-value < 0.05. Two-sided Unpaired T-test. **(f) PCIF1 silencing decreases only m⁶A_m level.** LC-MS/MS mRNA quantification of m⁶A_m/A and m⁶A/A after PCIF1 silencing in CTC44 line. Graphs represent mean +/- S.E.M of 3 biological replicates. *p-value < 0.05, ns = not significant. Two-Sided Unpaired T-test. **(g) PCIF1 silencing “rescues” sphere forming ability in CRC1 sh-FTO cell line.** PCIF1 was depleted by siRNA treatment in sh-FTO cell line and sphere formation assay was performed. Results are mean +/- S.E.M of three independent experiments. *p-value < 0.05, ns = not significant. One-way Anova followed by multiple comparisons.

176 Recent reports identified PCIF1/CAPAM as the m⁶A_m methyltransferase (“writer”) [28, 42] (**Figure 4d**)
177 and proposed that PCIF1/CAPAM activity is involved in cellular resistance to oxidative stress response
178 induction [28]. It is well known that elevated reactive oxygen species (ROS) production impairs self-
179 renewal and promote cell differentiation in stem cells and their malignant counterpart [43]. To
180 examine the contribution of PCIF1/CAPAM to CSC phenotype, we silenced PCIF1 in two CTC lines
181 (**Figure S4b**) and tested both SFP and resistance to chemotherapy. As expected, PCIF1/CAPAM
182 knockdown reduced sphere number by 30% (**Figure 4e, S4c**) as well as m⁶A_m / A ratio while m⁶A level
183 remained unaffected (**Figure 4f, S4d**). Along the same vein, PCIF1 depletion in sh-FTO cells rescued the
184 phenotype (**Figure 4g, S4e**). PCIF1 is also expressed at lower level in metastatic cell lines (**Figure S4f &**
185 **g**), concomitantly with reduced FTO expression (**Figure 3a**). Balanced decrease of m⁶A_m writer and
186 eraser (**Figure S4h**) would explain the lack of change of m⁶A_m level with respect to cell lines originating
187 from primary tumor (**Figure 4b**).

188 Yet, PCIF1/CAPAM level did not affect CTC resistance to FIRI treatment (**Figure S5a**). Likewise, sub-
189 lethal doses of FIRI, which promote CSC phenotype (**Figure 2d**), did not modify PCIF1 expression (**Figure**
190 **S5b**). Nevertheless, these cells displayed reduced FTO expression (**Figure S5b**) as well as increased
191 m⁶A_m level (**Figure S5c**). This suggests that FTO and PCIF1 do not exhibit mere antagonistic activities
192 but rather share a partially overlapping enzyme substrate specificity. Distinct cellular distribution of
193 these two effectors may explain such functional difference.

194 **FTO mediated m⁶A_m demethylation takes place in the cytoplasm**

195 While FTO sequence carries a Nuclear Localization Signal (NLS), its cellular localization varies among
196 several mammalian cell lines [39, 40]. This spatial regulation may result in distinct substrate
197 preference: a recent report shows that FTO catalyzes both m⁶A and m⁶A_m demethylation of mRNA in
198 cytoplasm but targets preferentially m⁶A in cell nucleus [41]. In our cell lines (CRC1 and SW620), FTO
199 is present in nuclear speckles [17] as well as in cell cytoplasm (**Figure 5a**). We employed detergent-
200 based cell fractionation procedure to separate nuclei from cytoplasm from sh-CTL and sh-FTO cells.

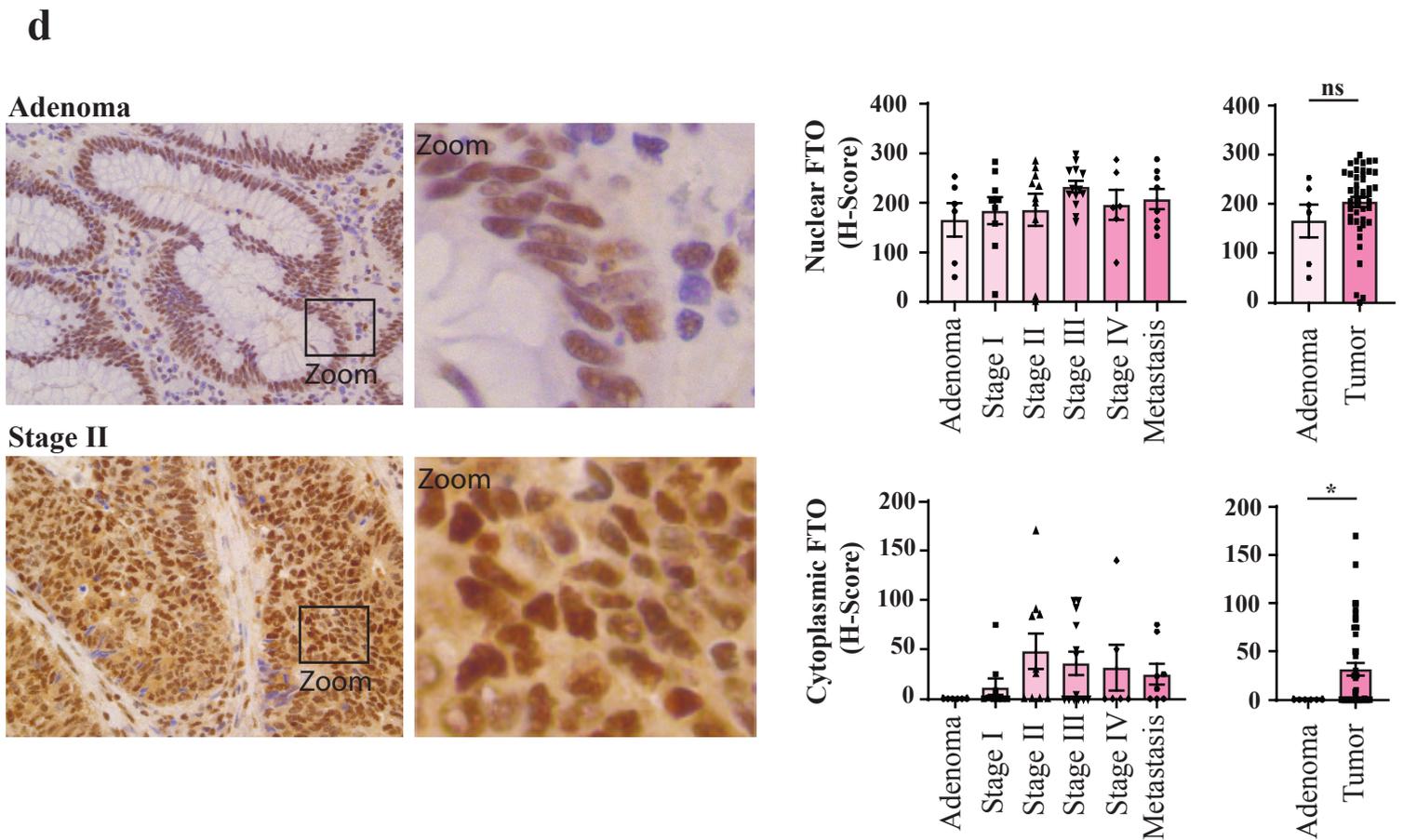
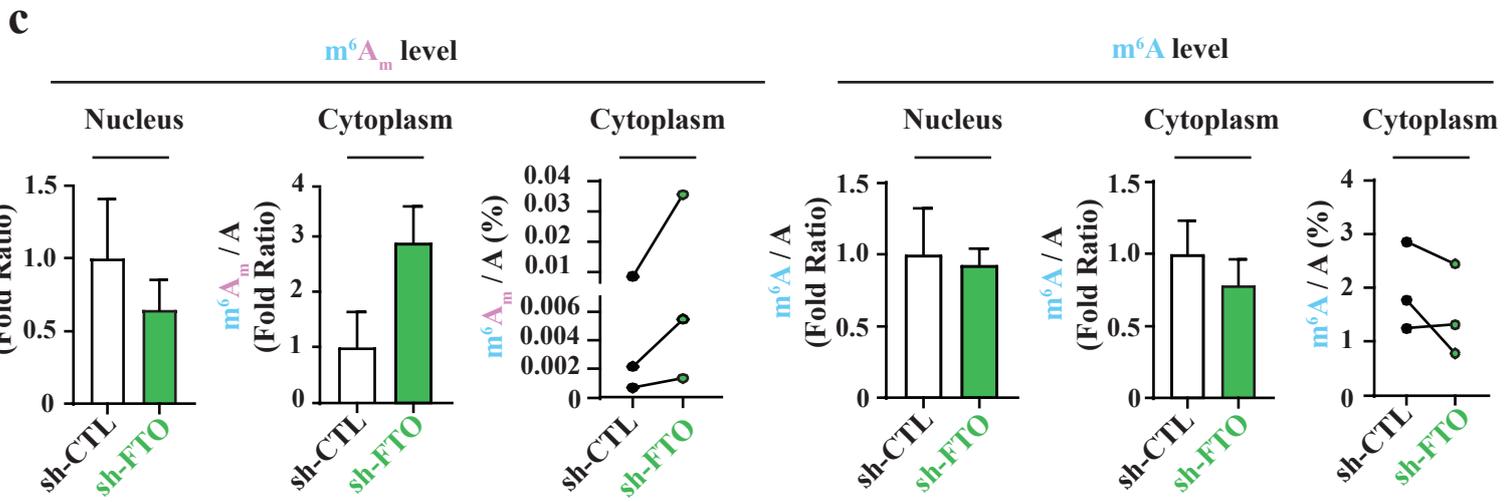
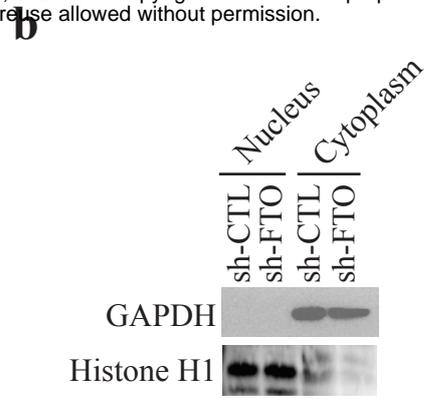
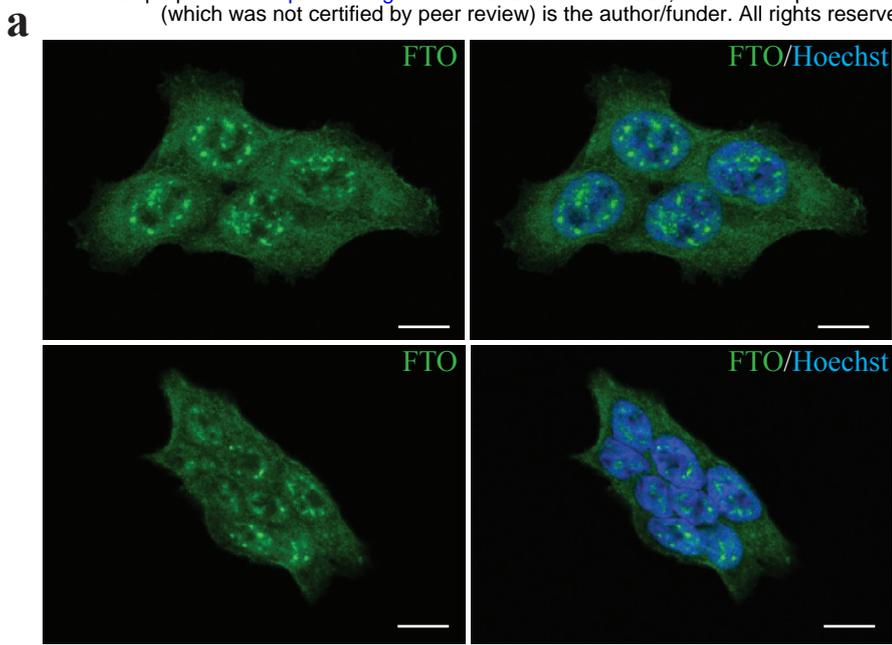


Figure 5. FTO mediated m^6A_m demethylation takes place in the cytoplasm. (a) FTO localizes both in nucleus and cytoplasm. Immunofluorescence staining of FTO (green) in CRC1 and SW620 cell lines show presence of FTO in cytoplasm and in nuclear speckles. Nucleus of cells were stained with Hoechst (blue). Scale bar 10 μ m. **(b) Verification of the effective cell fractionation procedure by immunoblot.** Effective separation of cytoplasmic and nuclear fractions was evaluated by immunoblot using cytoplasmic marker (GAPDH) and nuclear marker (Histone H1). **(c) FTO silencing increases cytoplasmic m^6A_m level.** mRNA quantification of m^6A_m / A and m^6A / A level for nuclear fraction and cytoplasmic fraction. Bar plots represents mean \pm S.E.M of three biological replicates. Before – After plots represents the same data as bar plot with raw values. **(d) FTO relocates to the cytoplasm during tumorigenesis.** FTO expression and localization was evaluated by IHC on TMA from CRC patient. Then, nuclear and cytoplasmic level of FTO were quantified. Pictures are representative of stage 0 and stage 2. Bar plots represents mean \pm S.E.M of H-score based on nuclear and cytoplasmic intensity of FTO staining. Each dot corresponds to individual value. *p-value < 0.05, ns = not significant, Mann-Whitney test.

201 The efficacy of this protocol was evaluated by immunoblot using cytoplasm- and nucleus-specific
202 markers, respectively GAPDH and Histone H1 (**Figure 5b**). Then, we extracted mRNA from both
203 compartment and quantified m^6A_m and m^6A by LC-MS/MS (**Figure 5c**). We observed a significant
204 increase (almost 3 fold) of m^6A_m / A ratio in sh-FTO cytoplasm (in comparison with sh-CTL) while this
205 ratio remained steady in the nucleus. By opposition, neither of these two cell compartments displays
206 alteration of m^6A / A ratio (**Figure 5c**). This suggests that FTO-mediated m^6A_m demethylation takes
207 place in the cytoplasm in colorectal cancer cell lines.

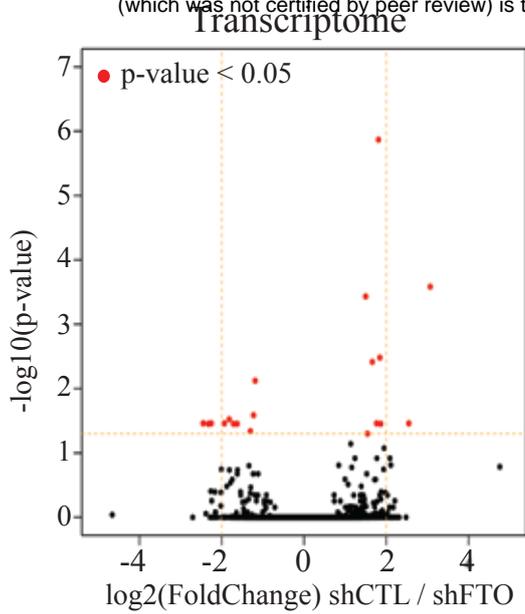
208 Next, we evaluated FTO expression and localization in tumor microarrays (TMA) from different
209 colorectal stages: adenoma, 1, 2, 3, 4 and metastases (n=52). Global FTO expression did not show any
210 significant change over the course of tumor evolution (**Figure 5d**). Yet, subcellular distribution analysis
211 unveiled interesting features. FTO expression is strictly nuclear in healthy adjacent tissue as well as in
212 precursor lesions of CRC (adenoma, **Figure 5d**). Then, following submucosal invasion (stage 1), FTO was
213 also found in the cytoplasm (**Figure 5d**). These observations suggest that the tumorigenic process alters
214 sub-cellular FTO distribution.

215 **FTO activity does not affect transcription process but rather translational control**

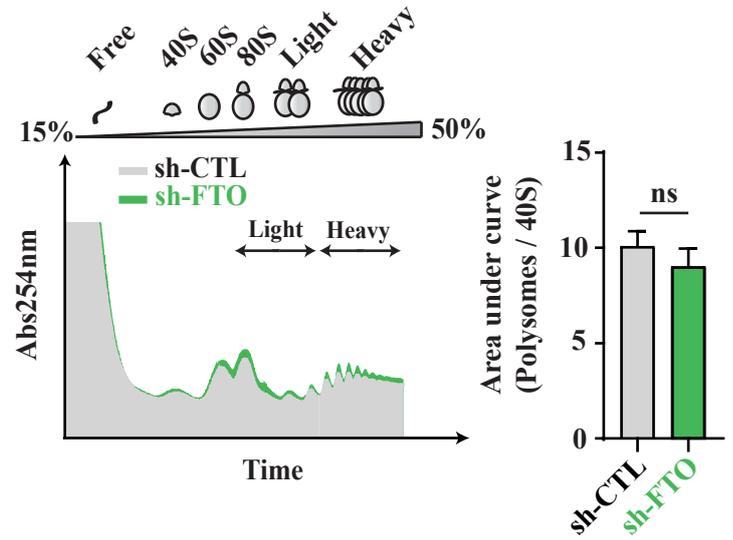
216 FTO activity is associated with regulating post-transcriptional processes including mRNA splicing [44],
217 stability [29] and translation [41]. To investigate the molecular mechanism of FTO regulation of
218 colorectal CSCs we performed whole transcriptome sequencing (RNA-seq, 125 bp, paired-end, n=3) of
219 sh-FTO and sh-CTL cells. The volcano plot of the transcriptomic variation between sh-CTL and sh-FTO
220 cells (**Figure 6a**) reveals that only few mRNAs exhibit a fourfold change, and among those only 5 reach
221 the standard $\log_{10}(p\text{-value})$ threshold of 1.5 (with n=3 replicates) for statistical significance: PUF60,
222 GFOD1, PAK6, COL13A1 and BMP2K (**Table S4**).

223 Such minor differences in mRNA levels suggest that FTO may act in CSCs by modulating translation
224 efficiency of individual mRNAs. This scenario is consistent with the proximity of m^6A_m adjacent to the
225 m^7G -Cap, as well as a recent study on PCIF1 suggesting a role of m^6A_m modification in translational

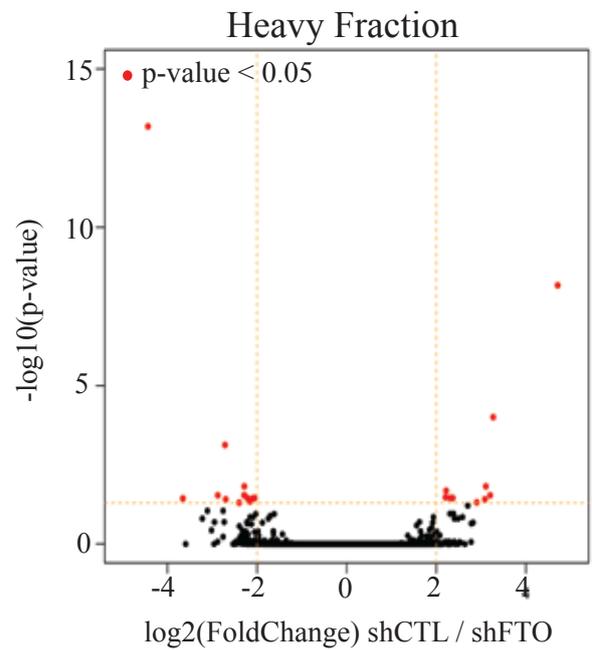
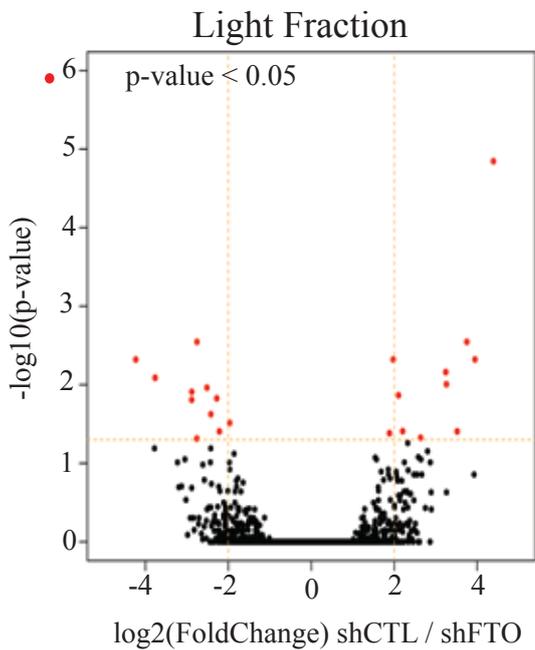
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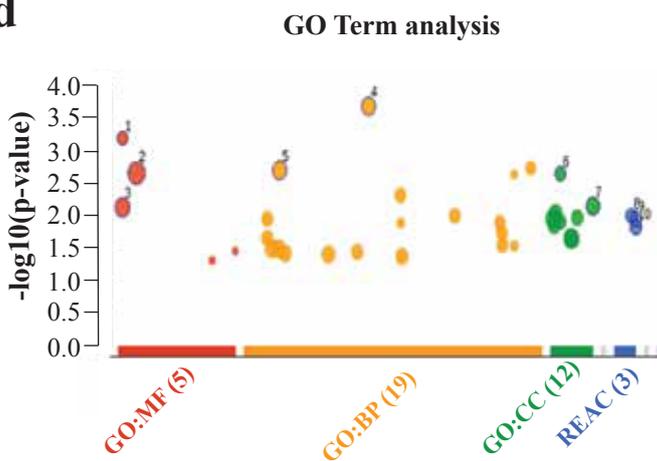
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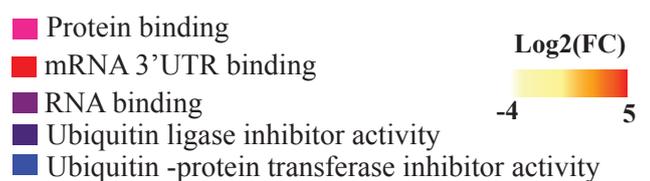
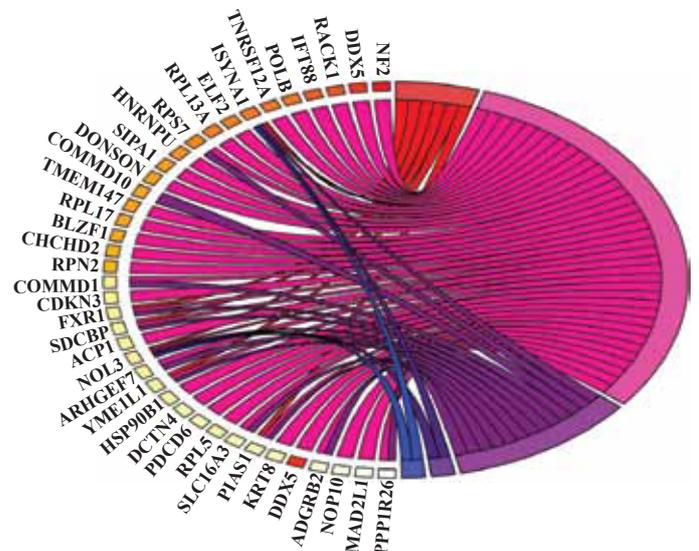
c



d



GO Term analysis : Molecular Function



source	term names	p-value
GO:MF	mRNA 3'UTR binding	6.8×10^{-4}
GO:MF	Protein binding	2.3×10^{-3}
GO:MF	RNA binding	7.7×10^{-3}
GO:BP	Cellular macromolecule catabolic process	2.2×10^{-4}
GO:BP	Macromolecule catabolic process	2.1×10^{-4}
GO:CC	Cytosolic ribosome	2.4×10^{-3}
GO:CC	Ribonucleoprotein complex	7.3×10^{-3}
REAC	Co-translational protein targeting to membrane	1.0×10^{-2}
REAC	rRNA processing in the nucleus and cytosol	1.2×10^{-2}
REAC	rRNA processing	1.6×10^{-2}

Figure 6. FTO silencing triggers subtle changes at the translational level. (a) FTO silencing does not affect global transcript level. MA plot analyses of transcripts changes. Red dot represent transcripts with an adjusted p-value < 0.05. Pie Chart shows the percentage of transcripts with fold change > 2 or < 0.5; CRC1 line n = 3 individual experiments. **(b) FTO does not affect global translation activity.** Polysome profile of sh-CTL and sh-FTO CRC1 cell lines. Light fraction (1 to 3 ribosomes/mRNA) and heavy fractions are indicated. Bar plot represents polysome level quantification of three experiments. ns = p-value > 0.05. **(c) FTO silencing impairs translation of specific mRNA.** MA plot analyses of transcripts changes in Light and Heavy fractions. Same representation as in **(a)**; CRC1 line, n = 3. **(d) Gene ontology analysis.** GO Term analysis was performed with gProfileR. Graph on the left shows significant GO terms of the three main categories (Molecular Function, MF; cellular compartment CC; Biological Process, BP). The table indicates the three most significant GO Terms for each category. MF GO Terms are detailed in the pie chart (right). Each color represents one GO Term that associates with the corresponding transcripts. Level of transcript changes is also indicated (below; right).

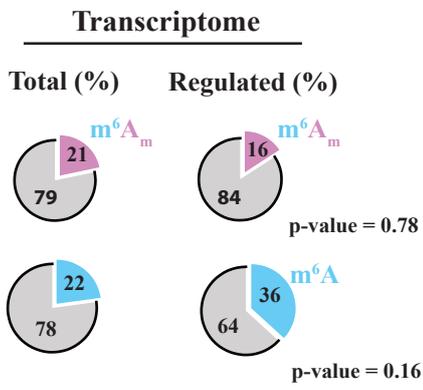
226 control of certain mRNAs [28]. Consistent with the lack of effect of FTO modulation on proliferation
227 **(Figure S1e)**, polysome fractionation profile did not reveal significant changes in global translation
228 activity, which would be expected to modify the ratio between unassembled ribosome subunits,
229 monosome and polysome size and numbers **(Figure 6b)**.

230 We therefore performed RNA-seq on mRNA co-sedimenting with heavy polysomes (4+ ribosomes
231 /mRNA) or light polysomes/monosomes (1-3 ribosomes per mRNA). We mapped reads to the human
232 reference transcriptome to determine mRNAs that were differentially translated between sh-CTL and
233 sh-FTO cell lines. The volcano plots of the light and heavy analyses **(Figure 6c)** show stronger changes
234 in translation than in transcription **(Figure 6a)**. Globally, 23 mRNAs were differentially translated in the
235 heavy fraction, and 26 in the light fraction ($|\log_2(\text{FC})| > 2$ and $(-\log_{10}(\text{p-value})) > 1.5$), including ribosome
236 related proteins (RPL13A, RPS7, FXR1, UTP4, or NOP10) **(Table S5 and S6)**. We confirmed this selective
237 translation engagement for several genes by qRT-PCR **(Figure S6)** Gene set enrichment analyses of
238 differentially translated genes indicate their involvement in mRNA/macromolecule catabolic process
239 (and regulation), in apoptotic process, and molecular functions such as mRNA 3'UTR binding **(Figure**
240 **6d)**. Analysis with gProfiler indicated three altered pathways, all related to translation: rRNA processing
241 in the nucleus and cytosol and SRP-dependent co-translational protein targeting to membrane
242 (Reactome ids: R-HSA-72312, R-HSA-8868773, R-HSA-1799339). Taken together, these results point to
243 an impact of FTO activity on the fine-tuning of translation process in colorectal cancer cells.

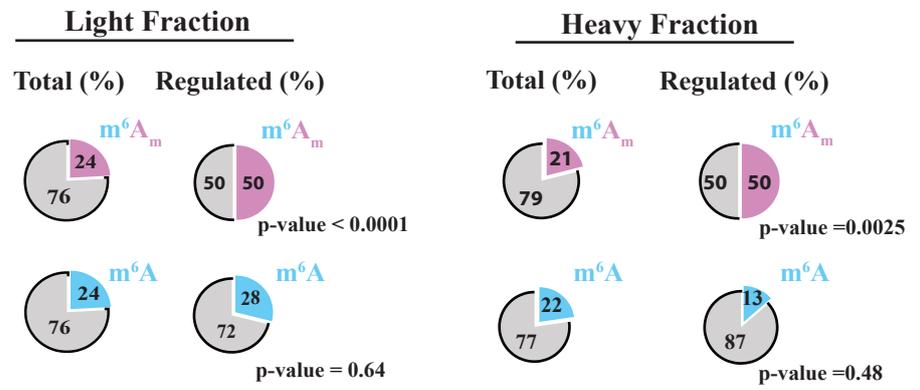
244 **m^6A_m -modified transcripts are enriched in translationally up-regulated genes**

245 Next, we examined whether the FTO effect on translational control could be mediated by m^6A_m
246 modification. We used data from Wei *et al.* [41] to identify m^6A_m modified transcripts among either
247 transcriptionally or translationally regulated genes. We could not see any enrichment of m^6A_m genes
248 in the shortlist of transcriptionally regulated mRNAs **(Figure 7a)**. However, m^6A_m marked genes were
249 significantly enriched in the regulated mRNA populations of mRNA from light and heavy fractions
250 **(Figure 7b)**. The cumulative distribution of transcripts correlated m^6A_m , but not m^6A marking, with

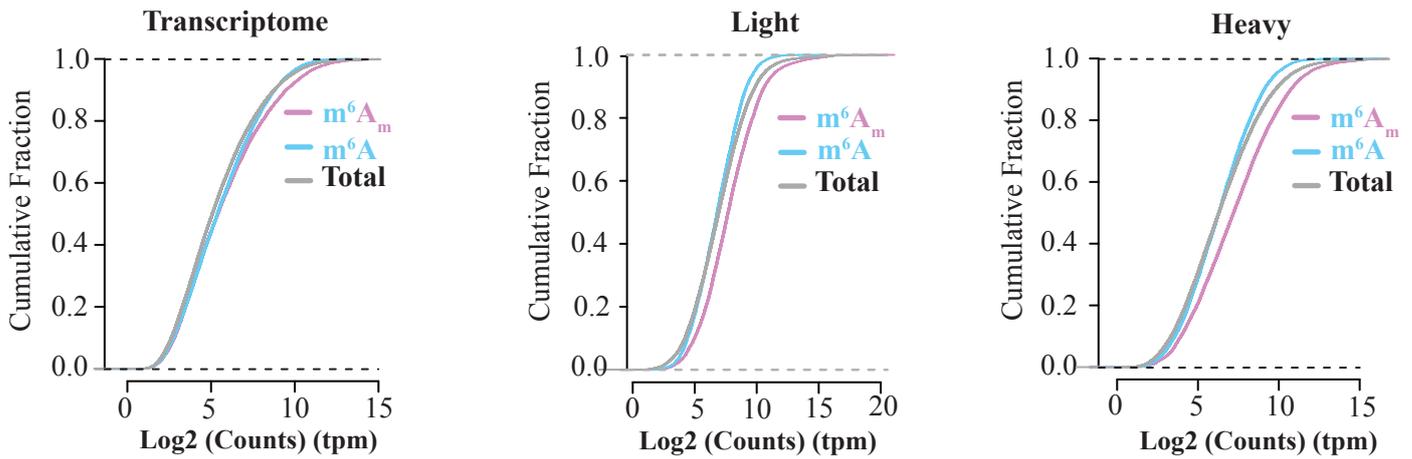
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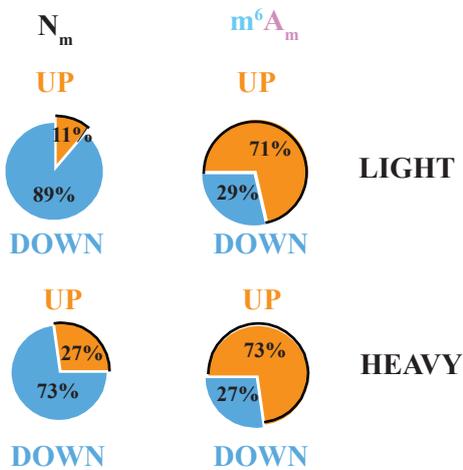


Figure 7. m⁶A_m marking correlates with enhanced translation. (a) m⁶A_m marking does not correlate with transcription regulation. Fraction of potential m⁶A_m marked transcripts (identified by Wei J *et al.* [41]) in total mRNA and significantly regulated transcripts (sh-FTO CRC1 cell line, FDR < 0.05). P-values were obtained using Fisher exact test. **(b) Translationally regulated transcripts are enriched in m⁶A_m transcripts.** Same as in (a) with translatoome data from light and heavy fractions (sh-FTO CRC1 cell line, FDR < 0.05). P-values were obtained using Fisher exact test. **(c) m⁶A_m transcripts are enriched in polysome fractions from CRC1 cell line.** Cumulative distribution plot that represents the fold change of mRNA that starts with either m⁶A_m or m⁶A from sh-FTO vs sh-CTL cells. Both m⁶A_m (pink) and m⁶A (blue) transcripts were identified based on data from Wei J *et al.* The level of m⁶A_m transcripts is more important in polysome fractions (light and heavy) in comparison with total transcripts. Graph represents the number of counts for m⁶A_m, m⁶A and total transcripts in transcriptomic data, light and heavy fractions. n = 3, CRC1 cell line. **(d) m⁶A_m marking tends to favor mRNA translation.** Translationally deregulated transcripts were divided into two categories: upregulated or downregulated genes. Most of m⁶A_m modified transcripts belong to the upregulated category. Other transcripts (Nm) show the opposite effect.

251 enhanced translation (**Figure 7c**) as previously suggested by Akichika *et al.* [28]. Accordingly, FTO
252 silencing triggers enrichment of m⁶A_m-modified transcripts in upregulated genes but not in
253 downregulated ones (**Figure 7d**).

254 **DISCUSSION**

255 Our study is the first to address the specific function of FTO in recently established colorectal cancer
256 cell lines with a focus on CSC phenotype. We show that decreased FTO activity plays a critical role in
257 colorectal cancer by enhancing CSC properties including sphere forming, *in vivo* tumorigenesis and
258 chemoresistance. The underlying mechanism takes place in the cytoplasm and appears to involve
259 demethylating m⁶A_m residues adjacent to the m⁷G-cap in highly selected transcripts, most likely to
260 adjust their translation efficiency.

261 As the initially characterized m⁶A demethylase, FTO has been studied in various types of cancers, often
262 reported as a pro-oncogenic factor [45]. Inhibiting FTO in glioblastoma impairs self-renewal and cancer
263 progression [46]. FTO is highly expressed in some AML types where it suppresses all-trans retinoic acid-
264 induced cell differentiation and promotes oncogene-mediated cell transformation and
265 leukemogenesis [45]. High levels of FTO are characteristic in cervical squamous cell carcinomas where
266 it promotes resistance to chemo/radiotherapy and increases DNA damage responses [47]. More
267 recently, high FTO expression was associated with lower survival rates in patients with breast cancer
268 [48]. Finally, FTO promotes breast cancer cell proliferation, colony formation and metastasis *in vitro*
269 and *in vivo* [48]. By contrast, our data support an anti-oncogenic role of FTO in colorectal cancer,
270 emphasizing the importance of tissular context. This observation is consistent with previous studies
271 showing cancer-specific effects of m⁶A regulators. METLL3 activity promotes tumorigenesis in AML by
272 enhancing BCL2 and PTEN translation [49], whereas in glioblastoma stem cells, it suppresses growth
273 and self-renewal by reducing expression of ADAM19 [50].

274 Interestingly, Kaplan-Meier survival analysis from cancer database shows that overall survival of
275 colorectal cancer is higher with lower FTO. This is contradictory with our data that connects reduced

276 FTO expression with enhanced chemoresistance and tumor initiation, as emphasized by our results
277 from cell lines derived from CTCs, the source of lethal metastases. At least two parameters can account
278 this inconsistency. First, we show that FTO expression in colon cancer cells is finely regulated at the
279 post-transcriptional level, though the precise underlying mechanism remains to be determined. This
280 observation is in agreement with a recent report in gastric tissue [38] and stresses the importance of
281 quantifying gene expression at the protein level for diagnostic purpose. Second, as will be discussed
282 below, FTO activity and substrate specificity may vary with its subcellular distribution. This parameter
283 is even more critical in the context of colorectal cancer where a fraction of FTO relocate from the
284 nucleus to the cytoplasm at early stage of the disease. Whether the cause may be due to neoplastic
285 transformation process remains to be determined.

286 Despite being ubiquitously expressed, FTO activity and function vary widely among tissues, as
287 illustrated by transcriptome-wide mapping of m⁶A modifications [25, 26]. To better comprehend the
288 biological function of FTO, extensive efforts have been made to identify relevant RNA substrate(s). No
289 clear consensus emerges from recently published studies [29, 41, 51, 52]. Following the identification
290 of FTO as the first m⁶A mRNA demethylase [17], several reports connected demethylation of internal
291 m⁶A nucleotides with a wide range of biological processes such as viral infection, stress- and DNA UV
292 damage-responses [53-55]. More recently, Mauer *et al.* threw a stone into the pond by suggesting that
293 FTO preferentially demethylates m⁶A_m residues closely adjacent to the cap [29]. The higher *in vitro*
294 demethylation efficiency reported was confirmed by Wei *et al.*, although the conclusion was
295 moderated in the cellular context, since the level of internal m⁶A marking is ten-fold higher than m⁶A_m
296 marking [41]. Discrepancies between studies on FTO may arise from several known biological
297 parameters. A recent review addresses this ambiguity and emphasize the context-dependent functions
298 of RNA methylation effectors [56]. First, FTO displays a wide substrate spectrum: besides mRNA (and
299 tRNA for m¹A [41]), FTO can demethylate m⁶A and m⁶A_m in snRNAs [51]. Second, its spatial distribution
300 dictates substrate preferences: cytoplasmic FTO catalyzes demethylation of both m⁶A and cap-m⁶A_m
301 whereas nuclear FTO demethylates preferentially m⁶A [41], most likely owing to accessibility

302 constraints to the cap-moiety. In colorectal cancer cell lines, FTO activity displays a remarkable
303 selectivity for cap-m⁶A_m which concurs with its presence in the cytoplasm. LC-MS/MS analysis of small
304 RNA species did not reveal any change of m⁶A and m⁶A_m levels following FTO silencing. Also, our
305 bioinformatic analysis of sh-FTO vs sh-CTL RNA-seq data failed to identify significant alterations of
306 mRNA splicing that might result from altered snRNA methylation. While we cannot exclude subtle
307 alteration of internal m⁶A marks, our data clearly establishes cap-m⁶A_m residues of mRNA as the main
308 substrate of cytoplasmic FTO in colorectal cancer cells. Extending this observation, targeting either the
309 m⁶A writer complex or ALKBH5 does not significantly affect CSC phenotype. By contrast, inhibiting
310 PCIF1/CAPAM partially recapitulated the inhibiting effect of FTO overexpression on the CSC
311 phenotypes. PCIF1/CAPAM catalyzes cap-A_m methylation in the nucleus while FTO demethylates m⁶A_m
312 in the cytoplasm. Compartment-specific enzymatic activity implies partially overlapping mRNA
313 substrates and explains why m⁶A_m writer and eraser do not display mere antagonistic activities.

314 In this study, we clearly identify m⁶A_m as a critical epitranscriptomic mark for controlling the stem cell
315 phenotype of human colon cancer cells. While m⁶A_m was first identified in mRNA from mammalian
316 cells and viruses in the 70s [27], the enzymes catalyzing m⁶A_m modifications, PCIF1/CAPAM and FTO,
317 were only recently identified, and the study of m⁶A_m role in mRNA metabolism and cellular function is
318 still in its infancy. Due to its abundance and its strategical location in the cap structure [57], this
319 chemical modification holds an inherent potential in gene expression control. Mauer *et al.* reported
320 that FTO-catalyzed m⁶A_m demethylation reduced mRNA stability by rendering it more vulnerable to
321 DCP2-mediated decapping process [29]. While our study supports the function of FTO as m⁶A_m
322 demethylase, we noticed minor changes at the transcriptome level following FTO knockdown. Yet, FTO
323 depletion enhanced translation of known m⁶A_m marked transcripts, which concurs with the study from
324 Akichika *et al.* that connected loss of m⁶A_m modification in PCIF1/CAPAM knockout cells with decreased
325 translation of mRNA [28]. Several deregulated genes relate to translation machinery (e.g. RPL13A,
326 RPS7, FXR1, UTP4, or NOP10) which underscores the role of FTO/m⁶A_m axis in fine-tuning the
327 translation process. Whether this involves subtle change in the composition of the translation

328 machinery, that would alter its ability to filter mRNA [58], remains to be assessed. Nevertheless, none
329 of the key regulatory genes generally associated with CSC traits, such as the octamer-binding
330 transcription factor 4 (OCT4), SOX2 and NANOG [59, 60], were altered in sh-FTO cells. This suggests
331 that, in colorectal cancer cell, FTO-mediated m⁶A_m demethylation restrains CSC abilities through
332 reversible, unconventional mechanism that may involve reprogramming of gene expression.
333 Nevertheless, while we see modest but significant differences at the translation level, we cannot
334 exclude the involvement of other mechanism(s).

335 In summary, we have shown that cytoplasmic FTO activity regulates m⁶A_m modification of selected
336 mRNAs and subtly but surely is necessary for maintaining the CSC phenotype *in vitro* and *in vivo* for
337 human colon cancers. The extent to which this applies to other cancers remains to be determined:
338 given the complexity of cancer, there will likely be a great variability between cancer types. Even if
339 limited to colon cancer, however, our findings point the way to deeper understanding of the CSC
340 phenotype and have exciting implications for developing therapies that decrease m⁶A_m modification
341 to cripple CSC-based metastases and resistance.

342 **ONLINE CONTENT**

343 The online version of the paper contains additional methods, along with supplementary figures and
344 tables that support the findings. References unique to these sections appear only in the online paper.

345 **SUPPLEMENTARY INFORMATION** is available in the online version of the paper.

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351 *whose servers hosted our bioinformatic analyses, and which belongs to the “France Génomique”*
352 *network and to Institut Français de Bioinformatique.*

353 *The authors declare no conflict of interest.*

354 **AUTHOR CONTRIBUTIONS**

355 *A.D., A.B., E.R., S.R. designed experiments and analysed the results. S.R., H.G., A.Am., F.B., A.At., J.V.,*
356 *F.D., A.C., F.M. performed experiments. A.D., A.B., E.R., S.R., C.H., E.C., J.J.V., E.S., J.P. performed data*
357 *analyses. J.R. and S.R. designed bioinformatics pipelines and performed bioinformatics analysis. A.D.,*
358 *A.B., E.R., S.R. wrote the manuscript. All the authors reviewed the final version of the manuscript.*

359 **METHODS**

360 **Cell lines.** Patient-derived colon cancer cell lines (CRC1, CPP-14/25/43/6/19/30/36) were derived from
361 CRC surgeries provided by CHU-Carêmeau (Nîmes, France, ClinicalTrial.gov Identifier#NCT01577511)
362 within an approved protocol. CRC1, CPP-14/25/43 cell lines were derived from primary tumors and
363 CPP-6/19/30/36 from metastatic tumors. CTC44 and CTC45 are circulating tumor cell lines derived
364 from blood of metastatic chemotherapy-naïve stage IV CRC patients [31]. HCT-116 (ATCC® CCL-247™)
365 and SW620 (ATCC® CCL-227™) are commercially available colon cancer cell lines derived respectively
366 from primary and metastatic human tumors.

367 **Cell culture and generation of stable cell lines.** Cells were maintained at 37 °C under humidified 5%
368 CO₂ in DMEM medium supplemented with 10% FCS (Invitrogen) and 2 mM glutamine. Stable
369 knockdown of FTO was achieved by lentiviral delivery (5 D.O.I) of anti-FTO sh-RNA (Origene,
370 #TL308064, sh-FTO#B). Isolation of infected cells was performed by GFP positive cells sorting on
371 FACSaria.

372 **Plasmid constructions.** FTO coding sequence was amplified from pDONOR plasmid (Montpellier
373 Genomic Collection MGC Facility) and inserted into pCDNA3-Flag Cter plasmid into *HindIII* and *XbaI*
374 restriction sites.

375 **Transfections.** Transfection of 100 nM of si-RNA duplex was performed using Lipofectamine RNAimax
376 (Invitrogen) according to the manufacturer's instructions. The sequences of siRNA are provided in
377 **Table S3.** 300,000 cells in 6-well plate were transfected for 48 h with 2 µg of plasmid DNA using
378 Lipofectamine 2 000 (Invitrogen) according to the manufacturer's instructions.

379 **FIRI treatment.** Cells were treated for 72 h at 37 °C under humidified 5% CO₂ with 10 µM of 5-
380 Fluorouracile coupled with 0.1 µM of SN38.

381 **RNA extraction and RT-qPCR.** Total RNA was extracted using TRIzol reagent (Invitrogen) according to
382 the manufacturer instructions. For RT-qPCR analyses, 1 µg of RNA was reverse-transcribed into cDNA
383 using random hexamer (Invitrogen) and 1 U of MML-V reverse transcriptase (Invitrogen). Quantitative
384 gene expression was performed using SYBR Green master mix (Roche) on LightCycler 480 Instrument
385 (Roche). Results were normalized to actin expression and analyzed using the $\Delta\Delta C_t$ method. Primer
386 sequences are provided in **Table S1.**

387 **Protein extraction and western blot.** Cells were washed twice with ice cold phosphate-buffered saline
388 (PBS) and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate,
389 2 mM sodium orthovanadate, 50 mM Sodium fluoride, 50 mM β -glycerophosphate, 2 mM EGTA; pH
390 7.5). Samples were separated on a 12% SDS–polyacrylamide gel electrophoresis, transferred to
391 nitrocellulose membrane, blocked for 1 h in 5% (w/v) non-fat dry milk in PBS and probed overnight at
392 4 °C with primary antibodies (**Table S2**). Membranes were incubated with secondary antibody for 1 h
393 and proteins were revealed by ECL Prime (Amersham) using ChemiDoc Touch imager (Biorad).

394 **Proliferation assay.** 1,000 cells were seeded in triplicate in 96-well plate for 24, 48, 72 and 96 h. Cells
395 were fixed for at least 2 h at 4 °C in 10% TCA. After three washes in miliQ water, cells were incubated
396 in 0.4% Sulforhodamine B solution for 30 min at room temperature followed by three washes with
397 miliQ water. 562nm absorbance was measured after resuspension in 10 mM Tris-Base.

398 **Sphere formation assay.** This test was performed as previously described [61]. Number of sphere
399 forming cells were determined after plating of 100 cells / 100 μ L of M11 medium (DMEM/F12 (1:1)
400 Glutamax medium, N2 Supplement, Glucose 0.3 %, insulin 20 μ g/ml, hBasic-FGF 10 ng/ml, hEGF 20
401 ng/ml) in ultra-low attachment 96 well-plates. Spheres > 50 μ m were counted after 5 – 7 days.

402 **Cytotoxicity assay.** 2,000 cells were seeded in a 96-well plate. After 24 h, cells were treated with
403 decreasing doses of 5-Fluorouracile (5-Fu) coupled with SN38 (FIRI) for 72 h (1/3 dilution from 3.3 X to
404 0 X ; 1 X = 50 μ M 5-FU + 0.5 μ M SN38). FOX toxicity was assessed with decreasing doses of 5-
405 Fluorouracile coupled with oxaliplatin for 72 h (1/3 dilution from 3.3 X to 0 X ; 1 X = 50 μ M 5-FU + 1 μ M
406 oxaliplatin). Cell viability was measured using Sulforhodamine B assay as previously described and IC₅₀
407 was determined graphically.

408 **Flow cytometry.** The ALDH activity of adherent cells was measured using the ALDEFLUOR kit (Stem Cell
409 Technologies), according to the manufacturer's instructions. CD44 and CD44v6 were stained using anti-
410 CD44 antibody and anti-CD44v6 antibody for 15 min at 4 °C (**Table S2**). As a reference control, anti-
411 IgG2a and REA-S control Isotype was used under identical conditions. The brightly fluorescent ALDH,
412 CD44 or CD44v6 positive cells were detected using a MACSQuant Analyzer (Miltenyi Biotec). To exclude
413 nonviable cells, Sytox blue was added at a concentration of 0.1 μ g/ml.

414 **Immunofluorescence.** Cells were fixed in PBS containing 4% paraformaldehyde at room temperature
415 for 15 min, wash twice in PBS, permeabilized with 0.1% NP-40 in PBS for 10 min, wash twice in PBS and
416 blocked with 5% FCS for 30 min. Coverslips were incubated 1h with primary antibody
417 (Phosphosolution, 597-FTO) at RT. After washing three times with PBS, coverslips were incubated for
418 1 h with Alexa Fluor®-conjugated secondary antibody (Alexa Fluor® 488 Goat Anti-Mouse (IgG),
419 Invitrogen) at RT. For nuclei staining, coverslips were washed twice and incubated with 1 μ g/ml
420 Hoechst 3358 for 5 min at RT. After two washes with distilled water, coverslips were mounted on slides
421 with Fluoromount-G (Invitrogen). Fluorescent pictures were acquired at room temperature on an
422 AxioImager Z1 microscope (Carl Zeiss, Inc.) equipped with a camera (AxioCam MRm; Carl Zeiss, Inc.)

423 and Plan Apochromat (63×, NA 1.4) objective, the Apotome Slider system equipped with an H1
424 transmission grid (Carl Zeiss, Inc.), and Zen 2 imaging software (Carl Zeiss, Inc.).

425 **mRNA purification.** mRNA was purified from total RNA with 2 rounds of GeneElute mRNA purification
426 kit (Sigma). rRNA was removed using Ribominus kit (Invitrogen) according to the manufacturer's
427 instructions.

428 **Nucleoside mass-spectrometry analysis.** This part was performed as previously described [41]. Briefly,
429 400 ng of RNA was digested by 5 U of RppH (New England Biolabs) for 2 h at 37 °C. Decapped mRNA
430 were then digested by 1 U of Nuclease P1 (Sigma) for 2 h at 42 °C in NH₄OAc buffer (10 mM, pH 5.3).
431 Nucleotides were dephosphorylated for 2 h at 37 °C by 1 U of Alkaline phosphatase in 100 mM of
432 NH₄OAc. The sample was then filtered (0.22 μm pore size, 4 mm diameter, Millipore), and 10 μL of the
433 solution was injected into LC-MS/MS. The nucleosides were separated by reverse phase ultra-
434 performance liquid chromatography on a C18 column with online mass spectrometry detection using
435 Agilent 6490 triple-quadrupole LC mass spectrometer in multiple reactions monitoring (MRM) positive
436 electrospray ionization (ESI) mode. The nucleosides were quantified by using the nucleoside-to-base
437 ion mass transitions of 282.1 to 150.1 (m⁶A), 268 to 136 (A), 296 to 150 (m⁶A_m) and 282 to 136 (A_m).

438 **Tumor initiation assay.** Decreasing amount of cells (1,000 , 500 , 100) were subcutaneously injected
439 into nude mice (Hsd:Athymic Nude-Foxn1nu nu/nu, 6 weeks, females, 5 mice per group) in Matrigel -
440 DMEM (v : v). Tumor sizes were measured twice a week for 50 days. After 50 days, the mice were
441 sacrificed and tumors were taken out. The number of mice bearing growing tumor (size > 100 mm³)
442 was counted. Tumor apparition frequency was determined using online ELDA (extreme limiting
443 dilution analysis) software (<https://bioinf.wehi.edu.au/elda/software>).

444 **Chemoresistance *in vivo*.** 50,000 cells were subcutaneously injected into nude mice in Matrigel –
445 DMEM (v : v). 50mg/kg 5-FU + 30mg/kg Irinotecan treatment (two i.p injection a week), was initiated
446 once tumor reached 100 mm³ [62]. These studies were approved by the ethics committee of the

447 Languedoc Roussillon Region and carried out in compliance with the CNRS and INSERM ethical
448 guidelines of animal experimentation (CEEA-LR-12051).

449 **Purification of nucleus and cytoplasm.** Cells were washed twice in ice-cold PBS. Cells were
450 resuspended in fractionation buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 7.4) and left on ice
451 for 3 min. Cytoplasm was then extracted and nuclei were washed twice in wash buffer (150 mM
452 NaCl, 50 mM Tris-HCl pH 7.4). Nuclei were resuspended in fractionation buffer and left on ice for 2 h.
453 Cell debris were removed by centrifugation at 13 200 rpm for 10 min at 4 °C.

454 **Tissue Microarray (TMA).** Tissue microarray was constructed with FFPE tumor samples collected in the
455 frame of the Clinical and Biological Database BCBCOLON (registered at ClinicalTrials.gov as
456 NCT03976960). Adenomas, primary adenocarcinomas and metastatic lesions were sampled as two
457 cores of 1mm diameter. All samples were chemo-naive. Tumor samples were collected following
458 French laws under the supervision of an investigator and declared to the French Ministry of Higher
459 Education and Research (declaration number DC-2008–695). The study was approved by the local
460 translational research committee (ICM-CORT-2018-28).

461 **FTO detection by immunohistochemistry.** Three- μ m thin sections of formalin-fixed paraffin-
462 embedded tissues were mounted on Flex microscope slides (Dako) and allowed to dry overnight at
463 room temperature before immunohistochemistry processing, as previously described [63]. Briefly, PT-
464 Link® system (Dako) was used for pre-treatment. Then, heat-induced antigen retrieval was executed
465 for 15 minutes in High pH Buffer (Dako) at 95°C. Immunohistochemistry procedure was performed
466 using the Dako Autostainer Link48 platform. Endogenous peroxidase was quenched using Flex
467 Peroxidase Block (Dako) for 5 min at room temperature. Slides were then incubated with the anti-FTO
468 rabbit monoclonal antibody (AbCam, Clone EPR6895; 1/1000) for 20 min at room temperature. After
469 two rinses in buffer, the slides were incubated with a horseradish peroxidase-labeled polymer coupled
470 to secondary anti-mouse and anti-rabbit antibodies for 20 min, followed by application of 3,3'-
471 Diaminobenzidine for 10 min as substrate. Counterstaining was performed using Flex Hematoxylin

472 (Dako) followed by washing the slides under tap water for 5 min. Finally, slides were mounted with a
473 coverslip after dehydration.

474 Two independent observers analyzed the TMA slide in a blinded manner. The semi quantitative H-
475 score method [64] was used to convert the expression of FTO to continuous values, based on both the
476 staining intensity and the percentage of cells at that intensity. Nuclear and cytoplasmic signals were
477 taken into account separately. Staining intensity was scored as no staining (0), weak staining (1),
478 moderate staining (2), or intense staining (3). The percentage of cells stained at certain intensity was
479 determined and multiplied by the intensity score to generate an intensity percentage score. The final
480 staining score of each tissue sample was the sum of the four intensity percentage scores, and these
481 scores ranged from 0 (no staining) to 300 (100% of cells with intense staining). In all cases with
482 discrepant results, a consensus was reached between both investigators. Averaged FTO H-score was
483 given when both cores from a single sample were assessable.

484 **Transcriptome Analysis.** Total RNA was extracted using TRIzol method. RNA samples were send to
485 Eurofins Genomics /GATC to perform next generation sequencing on Illumina platform.

486 **Polysome fractionation.** This procedure was performed as previously described [65]. Briefly, 6 plates
487 (150 cm²) were seeded with 2 x 10⁶ cells. After 72 h, cells were treated with 20 µg / ml emetine for 5
488 min at 37°C, washed twice with ice-cold PBS, and scraped in ice cold PBS. Cells were centrifuged,
489 resuspended into 1mL of polysome lysis buffer, homogenized by hard shaking with 1,4mm ceramix
490 spheres (Lysing matrix D MPBio) in FastPrep machine (MPBio), centrifuges 10 min at full speed at 4°C.
491 Lysates were loaded on 15-50% sucrose gradient and centrifuged at 35,000 rpm for 2.5 h at 4°C in a
492 SW41 rotor (Beckman Coulter). Polysomes were separated through a live optical density (OD) 254 nm
493 UV spectrometer and collected with an ISCO (Lincoln, NE) density gradient fractionation system. The
494 absorbance at 254 nm was measured continuously as a function of gradient depth.

495 **Translatome Analysis.** mRNA associated with 1 to 3 ribosomes “Light polysomes” and mRNA
496 associated with more than 3 ribosomes “Heavy polysomes” were extracted using TRIzol LS (Invitrogen)

497 according to manufacturer's instructions. Library preparation was performed using TruSeq Stranded
498 mRNA Sample Preparation kit (Illumina) according to the manufacturer's protocol. cDNA libraries were
499 sequenced using the sequencer HiSeq 2500 (Illumina).

500 **Bioinformatic pipeline.** Transcriptome and translome libraries read quality were assessed using
501 FastQC v0.11.5 (Babraham Institute, Cambridge, UK). Ribosomal RNA were discarded using SortMeRNA
502 v2.1b [66]. High quality reads were then aligned on the *Homo sapiens* reference transcriptome, version
503 GRCh38.cdna, and quantified using pseudocounts with Kallisto v0.45.0 [67]. Kallisto quantification
504 parameters were fixed at 25 for k-mer size for the index, 20 for standard deviation and 100 for
505 bootstraps. Statistical differential analyses were performed on each dataset using Wald test from
506 DESeq2 R package [68]. Each count dataset was filtered at 1 count per millions per biological sample
507 after size factors estimation, then dispersion was estimated. Primary risk of probabilities to false
508 discovery fold change was corrected by Benjamini and Hochberg multiple test adjustment. Corrected
509 p-values < at 0.05 % were kept. Volcano plots were realized with ggplot2 R package [69]. Gene
510 identifications were performed with biomaRt R package [70]. Functional annotations were performed
511 with online gProfileR [71] using a g:SCS threshold < at 0.05.

512 **DATA AVAILABILITY**

513 Data collection will be freely available by DOI hosted in DRYAD (<https://datadryad.org/stash>) and/or
514 on NCBI BioProject. Three links will be provided soon, one for transcriptome data, one for light
515 translome fraction and one for heavy translome fraction.

516 **CODE AVAILABILITY**

517 Data analysis was performed using free software detailed in the material and methods (i.e. FastQC
518 v0.11.5, SortMeRNA v2.1b, Kallisto v0.45.0 and R v3.5.1). Statistics and graphics were performed
519 using R packages (DESeq2, ggplot2, biomaRt) and an online program gProfileR. All the scripts used
520 are hosted on a private gitlab depository and could be available from the corresponding author on
521 reasonable request (contact: rivals@lirmm.fr).

522 **BIOLOGICAL MATERIAL AVAILABILITY**

523 Any material used in this study is available from commercial source (indicated in the Materiel and
524 Method section) or from the authors upon request.

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