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► **To cite this version:**

Sébastien Relier, Eric Rivals, Alexandre David. The multifaceted functions of the Fat mass and Obesity-associated protein (FTO) in normal and cancer cells. *RNA Biology*, 2022, 19 (1), pp.132-142. 10.1080/15476286.2021.2016203 . hal-03545725

**HAL Id: hal-03545725**

**<https://hal.science/hal-03545725>**

Submitted on 3 Feb 2022

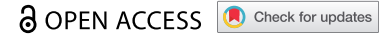
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REVIEW



# The multifaceted functions of the Fat mass and Obesity-associated protein (FTO) in normal and cancer cells

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## ABSTRACT

The last decade has seen mRNA modification emerge as a new layer of gene expression regulation. The Fat mass and obesity-associated protein (FTO) was the first identified eraser of N6-methyladenosine (m6A) adducts, the most widespread modification in eukaryotic messenger RNA. This discovery, of a reversible and dynamic RNA modification, aided by recent technological advances in RNA mass spectrometry and sequencing has led to the birth of the field of epitranscriptomics. FTO crystallized much of the attention of epitranscriptomics researchers and resulted in the publication of numerous, yet contradictory, studies describing the regulatory role of FTO in gene expression and central biological processes. These incongruities may be explained by a wide spectrum of FTO substrates and RNA sequence preferences: FTO binds multiple RNA species (mRNA, snRNA and tRNA) and can demethylate internal m6A in mRNA and snRNA, N6,2'-O-dimethyladenosine (m6Am) adjacent to the mRNA cap, and N1-methyladenosine (m1A) in tRNA. Here, we review current knowledge related to FTO function in healthy and cancer cells. In particular, we emphasize the divergent role(s) attributed to FTO in different tissues and subcellular and molecular contexts.

## ARTICLE HISTORY

Received 4 August 2021  
Revised 21 October 2021  
Accepted 2 December 2021

## KEYWORDS

FTO; N6-methyladenosine; RNA modification; cancer; epitranscriptomics; transcription

## INTRODUCTION

The discovery of the Fat mass and obesity-associated protein (FTO) originated from a fused-toe (Ft) mouse mutation that lacks several hundred kb off chromosome 8 [1]. The Fatso (FTO) gene was cloned and studied in mouse. Based on its expression throughout embryonic development and the Ft mouse phenotype, FTO was assumed to be linked to programmed cell death and craniofacial development. Almost a decade later, genome-wide association studies established a strong correlation between single-nucleotide polymorphisms of the FTO gene and human obesity [2–5]. It was then renamed the ‘Fat mass and Obesity-associated’ (FTO) gene. This connection was strengthened by a study on FTO KO mice in which the loss of Fto triggers postnatal growth retardation and decreased body mass [6]. Contrastingly, overexpression of Fto promotes food intake and results in increased body and fat mass [7]. However, the real function of Fto in obesity remains controversial to date. While the Fto single-nucleotide polymorphism does not affect Fto itself, it promotes the expression of the neighbouring IRX3 gene. An IRX3-dependent mechanism is supported by IRX3 knockout mice, which tend to be leaner, protected from diet-induced obesity and whose metabolism is disrupted (Smemo et al., Nature 2014). Genetic variants in the FTO gene are also associated with several pathologies such as metabolic disorders [8–11], neurological diseases [12] and cancers [13,14]. Altogether, these phenotypic observations have attracted interest from the research community and led to substantial

investment in identifying the physiological substrate(s) of Fto as well as the downstream molecular mechanisms.

Fto was initially shown to catalyse oxidative demethylation of 3-methyl thymine on synthetic single-stranded DNA (ssDNA) and 3-methyl uracil of synthetic single-stranded RNA (ssRNA) *in vitro* [15]. Shortly afterwards, the N6-methyladenosine (m<sup>6</sup>A) mark, decorating messenger RNA (mRNA), was identified as the main physiological substrate of FTO [16]. Identified a few decades earlier, m<sup>6</sup>A was shown to regulate every step of mRNA processing: splicing, stability and translation [17]. The discovery of reversible modification in mRNA was a major breakthrough in the field of RNA modification as it gave birth to ‘epitranscriptomics’. Nevertheless, since its discovery, the purported functions of FTO in living cells have been repeatedly challenged.

So far, FTO has been shown to target five distinct methylation types, in various RNA species as well as in DNA. However, the individual FTO targets were identified in distinct experimental settings and/or cell models, lending towards the possibility of a context-dependent specificity of FTO towards its substrates. The aim of this review is to provide an overview of our current knowledge regarding FTO function and activity across cell lines and tissues. After a global presentation of known FTO targets, we discuss the molecular mechanisms that could be involved in fine-tuning substrate selection. Finally, we present evidence supporting the importance of tissue context for FTO function, especially in cancer.

## 1. Targets of FTO

FTO is a homolog of the AlkB Family of Fe(II)/ $\alpha$ -Ketoglutarate ( $\alpha$ -KG)-dependent Dioxygenases. This class of ubiquitous DNA repair enzymes removes alkyl adducts from nucleobases through oxidative dealkylation [18]. FTO has an amino-terminal AlkB-like domain and a carboxy-terminal domain that interact with each other, as well as an extra loop that distinguishes it from other AlkB members [19]. FTO was originally reported to catalyse the demethylation of various RNA and DNA substrates [15] (Table 1). Nevertheless, recent studies point towards m<sup>6</sup>A modification in mRNA as its most relevant biological substrate [16]. These discoveries stem from recent progress in detection techniques such as high-throughput sequencing and mass spectrometry [20].

### 1.1. FTO targets 3-methyl-thymine and 3-methyl-Uracil *in vitro*

The first two targets of FTO were identified more than a decade ago as the 3-meThymine (3-meT) and the 3-meUracil (3meU) [15]. The authors synthesized *in vitro* methylated single-stranded DNAs and RNAs and incubated them in the presence of purified mouse or human FTO proteins. High-performance liquid chromatography (HPLC) chromatograms of the modifications revealed reduced ssDNA 3-meT and ssRNA 3meU but stable ssDNA 3-meC nor 5mdc [21]. While FTO can demethylate 3-meT in ssDNA *in vitro*, it has negligible activity towards double-stranded DNA [15]. So far, FTO-mediated 3 meT demethylation in living cells has not been reported.

### 1.2. FTO targets N<sup>6</sup>-methyladenosine in various RNA species

More recently, m<sup>6</sup>A modification, which functions to shape RNA splicing, stability and translation, has been identified as the major substrate of FTO. Jia G. et al. used HPLC to evaluate the effect of FTO on m<sup>6</sup>A-modified synthetic oligonucleotides: ssRNA and ssDNA [16]. FTO treatment decreased m<sup>6</sup>A level in ssRNA but not in ssDNA. These observations were substantiated by *in cellulo* experiments performed in HeLa and 293 T cells, where silencing of FTO increased m<sup>6</sup>A level in mRNA while FTO overexpression resulted in the opposite effect [16]. In addition to mass spectrometry, methylated RNA immunoprecipitation and sequencing (m<sup>6</sup>A-seq) was developed to identify FTO targets at genome scale. In various system, FTO activity has been shown to regulate m<sup>6</sup>A level in hundreds of mRNAs [22–25]. In a cell type-dependent manner, FTO binds to some sequences containing a consensus motif RRACH or DRACH (R = G or A; D = A or G or T; H = A, C, or U), which was thus dubbed the m<sup>6</sup>A consensus motif [26]. However, m<sup>6</sup>A-seq require the usage of an antibody whose specificity can be questioned. m<sup>6</sup>A antibody cross-reacts with m<sup>6</sup>Am, a subtype of m<sup>6</sup>A modification that locates at the 5' end of mRNA. Hence, m<sup>6</sup>Am modification at alternative transcription start sites could be mistaken for internal m<sup>6</sup>A modification

(Boulias et al., Mol Cell 2019, 10.1016/j.molcel.2019.06.006). Despite the bias, other approaches have been used to confirm the effect of Fto on m<sup>6</sup>A methylation.

Transcriptome-wide analysis of FTO binding sites uncovered its remarkable ability to bind a wide range of m<sup>6</sup>A-containing RNAs, such as pre-mRNA, mature mRNA, transfer RNA (tRNA), as well as various other non-coding RNA (ncRNA), suggesting potent activity towards those species [16]. This initial observation was further refined by experiments combining FTO silencing with purification of RNA species followed by m<sup>6</sup>A quantification by mass spectrometry. This approach confirmed that FTO efficiently targets m<sup>6</sup>A in 16–28 nucleotides long RNAs [27] and U6 snRNAs [28]. Yet, quantification of global m<sup>6</sup>A in 18S and 28S ribosomal RNAs (rRNAs) was not impaired by FTO silencing, suggesting that rRNA is not a substrate of FTO [28].

There is no clear evidence that could establish a direct connection between FTO and m<sup>6</sup>A demethylation in the intronic regions. However, 75% of FTO binds to introns, whereas only 7% of m<sup>6</sup>A is found in the intronic regions of pre-mRNA at steady state [29]. This discrepancy suggests that FTO binding is not biased by the global high proportion of introns over exons. Furthermore, m<sup>6</sup>A is elevated in introns of nascent pre-mRNA compared to steady-state pre-mRNA [30]. Taken together, these observations suggest that the low proportion of m<sup>6</sup>A in introns could result from a co-transcriptional demethylation activity mediated by FTO.

To sum up, FTO displays an m<sup>6</sup>A demethylase activity towards mRNAs, U6 small RNAs, and probably microRNAs (miRNA) but not rRNA. Moreover, while FTO seems to preferentially target intronic regions, this activity remains to be confirmed.

### 1.3. FTO targets m<sup>6</sup>A<sub>m</sub> in mRNA and small RNAs

m<sup>6</sup>A<sub>m</sub> modification is a specific m<sup>6</sup>A methylation that occurs on the 2'-O methylAdenosine (A<sub>m</sub>) residue adjacent to the 7-methylguanosine (m<sup>7</sup>G) mRNA cap (only in transcripts whose +1 nucleoside is an A). Due to its particular location, m<sup>6</sup>Am has an expected role in regulating mRNA stability and/or translation. A first study by Mauer et al. has shown that m<sup>6</sup>A<sub>m</sub>, and not m<sup>6</sup>A, is the preferred cellular substrate for FTO both *in vitro* and *in vivo* [31]. Interestingly, FTO-mediated m<sup>6</sup>A<sub>m</sub> demethylation occurs preferentially on m<sup>7</sup>G-capped RNAs rather than uncapped RNAs, implying that m<sup>7</sup>G is required for FTO targeting [31]. *In cellulo* studies have quantified global m<sup>6</sup>A<sub>m</sub> level by 2D-TLC (Thin Layer Chromatography) or mass spectrometry and confirmed *in vitro* experiments: m<sup>6</sup>A<sub>m</sub> was increased in FTO KO cell lines and decreased following FTO overexpression [28,31–33]. m<sup>6</sup>A<sub>m</sub> was also mapped transcriptome-wide using miCLIP technology. miCLIP is based on m<sup>6</sup>A immunoprecipitation and further sequencing of methylated RNA fragments, to map m<sup>6</sup>A at nucleotide resolution [34]. This method allows m<sup>6</sup>A<sub>m</sub> residues at the mRNA cap, to be distinguished from internal m<sup>6</sup>A sites, which are mostly in the 3'UTR region. As expected by the authors, FTO KO significantly increased the number of m<sup>7</sup>G-m<sup>6</sup>A<sub>m</sub> sites [31]. Consistently, CLIP-seq experiment revealed an enrichment

of FTO binding at the 5' end of mRNA [29], therefore establishing a direct connection between  $m^6A_m$  level and FTO activity.

Like mRNAs, small RNAs can have an  $m^7G-m^6A_m$  cap structure. By applying miCLIP from FTO KO cell lines. Mauer J. et al. revealed enrichment of  $m^6A_m$  at the 5' end of several small RNAs including U1, U2, U4 and U5 small RNAs [35]. FTO-mediated demethylation of  $m^6A_m$  on U1 and U2 small RNAs was consolidated by 2D TLC and mass spectrometry. Interestingly, U2 snRNAs possess internal  $m^6A_m$  in addition to cap- $m^6A_m$ . Jiangbo Wei et al. applied mass spectrometry analysis of nuclease-treated RNA modifications w/o RNA decapping in order to discriminate  $m^7G-m^6A_m$  from internal  $m^6A_m$  [28]. Using this approach, they discovered that FTO preferentially demethylates cap- $m^6A_m$  in U1 snRNA, whereas it targets internal  $m^6A_m$  in U2 snRNAs.

#### 1.4. FTO targets $m^1A$ in tRNA

In a recent study, the CLIP-seq analysis revealed that a small fraction of FTO binds to tRNA [28]. *In vitro* experiments were performed in order to identify the FTO substrate in this highly modified RNA species. FTO incubation with tRNA does not affect  $m^1G$ ,  $m^7G$ ,  $m^5C$ ,  $m^2G$  or  $m^{22}G$  methylation. However, it decreases  $m^1A$  methylation, suggesting that FTO could catalyse  $m^1A$  demethylation in tRNAs. The biological relevance of this observation was confirmed in both living cells and mouse brain: FTO overexpression reduces  $m^1A$  level in tRNA from HEK293T cells [28], whereas FTO KO increases  $m^1A$  level in tRNAs extracted from mouse brain [28].

*In vitro* assays underscore the importance of structural context: FTO fails to target  $m^1A$  in linear transcripts, whereas it efficiently demethylates  $m^1A$  when present in a loop structure [28]. Eukaryotic tRNA has three potential  $m^1A$  modifications, at position A9, A22 and A58 [28]. However, only  $m^1A58$  is localized in the large stem loop of tRNA, suggesting that FTO may preferentially target  $m^1A58$  rather than  $m^1A9$  or  $m^1A22$ . Nevertheless, primer extension experiments will be required to confirm the activity of FTO towards  $m^1A58$  in living cells. Remarkably, while  $m^1A58$  is ubiquitous in tRNAs, CLIP analysis revealed that FTO only associates with 10 tRNA species, in particular, to tRNA<sup>Glu</sup>(CUC) (45%) and tRNA<sup>His</sup>(RUG) (32%), suggesting some sort of specificity [28].

#### 1.5. Compartment-specific activity of FTO

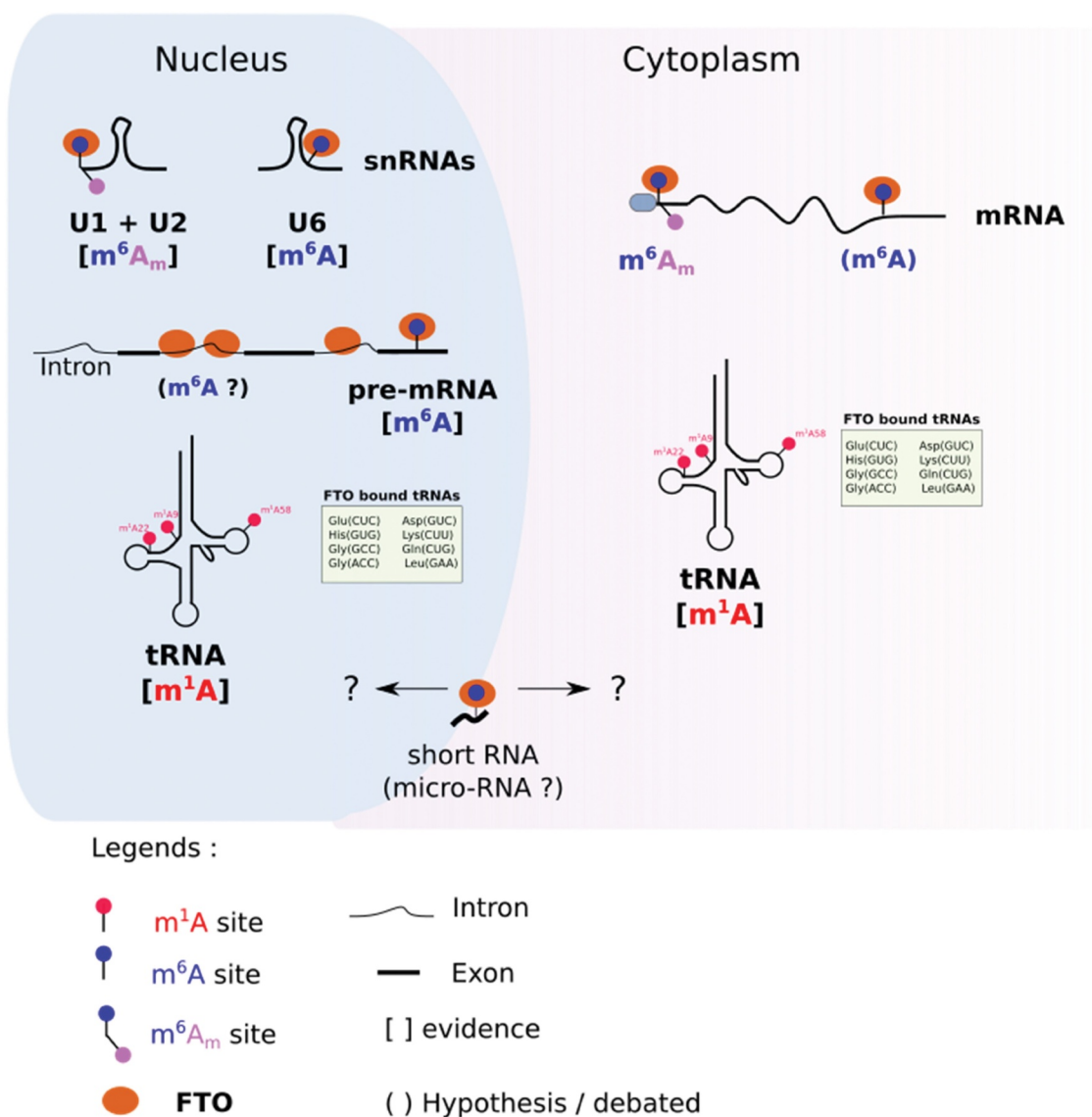
Methylases and demethylases can target different RNA species and/or modifications according to their subcellular localization [36,37]. For example, the  $m^1A$  methylase TRM61 targets tRNA in the nucleus [38,39], but it targets mRNA in the cytoplasm [40]. The eraser ALKBH1 also provides a striking example of compartment-specific activity; ALKBH1 removes  $m^1A$  of tRNA in the cytoplasm [41] and targets  $m^5C$  of tRNA in mitochondria [42]. Like many other RNA modifying enzymes, FTO has different substrate preferences, depending on the subcellular context (Fig. 1).

Various approaches have been employed to study how the activity of FTO depends on its localization. Mauer J. and colleagues fused FTO with a Nuclear Export Signal to promote its cytoplasmic translocation in HEK293T cells [31]. Then, they quantified both  $m^6A$  and  $m^6A_m$  in mRNA by the means of two-dimensional TLC. Increased expression of cytoplasmic FTO significantly reduced  $m^6A_m/m^6A$ , demonstrating that cytoplasmic FTO preferentially targets  $m^6A_m$  in mRNA. In another study, Jiangbo Wei et al. employed a biochemical procedure to isolate cell compartments, nucleus and cytoplasm, after silencing FTO in HEK293T cells [28]. From each compartment, they purified tRNA, small RNA and mRNA and analysed RNA modifications by mass spectrometry. They confirmed that FTO silencing increases cytoplasmic – but not nuclear –  $m^6A_m$  in mRNA. By contrast, only the nuclear fraction of mRNA had increased  $m^6A$  after FTO depletion. Furthermore,  $m^6A$ -seq experiments from nascent RNA, nuclear and cytoplasmic steady-state mRNA support the hypothesis of nuclear specific  $m^6A$  demethylation [43]. While  $m^6A$  sites tend to be more numerous in nascent RNA than in steady-state nuclear RNA, the differences in  $m^6A$  mapping become negligible when comparing nuclear versus cytoplasmic [43] mRNA. This observation strongly suggests that  $m^6A$  demethylation occurs co-transcriptionally in the nucleus rather than in the cytoplasm.

#### Towards tissue-specific function?

Whether FTO targets preferentially  $m^6A$  or  $m^6A_m$  has been debated for several years. *In vitro* demethylation assays demonstrated that FTO is a hundred times more efficient at demethylating  $m^6A_m$  than  $m^6A$  [31]. However, a mere buffer solution cannot recapitulate the tremendous compositional complexity of the cellular environment. Furthermore, changes of molecular environment from one subcellular compartment to another could easily alter the enzyme's behaviour [44]. *In cellulo* studies on FTO offer a striking illustration of this. First, FTO appears to have more impact on  $m^6A$  than  $m^6A_m$  in living cells, especially in leukaemic cells [45,46]. Second, FTO substrate preference may depend on subcellular context [28,36]; nuclear FTO would preferentially demethylate  $m^6A$ , while cytoplasmic FTO would rather target  $m^6A_m$ . Therefore, conflicting results about FTO *in vivo* targets may result from the use of different cell models. For instance, several studies describing FTO activity were performed in HEK293T cells where FTO is strictly nuclear and does not affect  $m^6A_m$  [47]. By contrast, in colon cancer cells, where FTO is both cytoplasmic and nuclear, FTO silencing significantly increases the level of  $m^6A_m$  [32]. Hence, regulation of FTO localization may be the key to regulating substrate preferences.

While more accurate than *in vitro* experiments, *in cellulo* studies do not necessarily reflect the complex reality of living tissue. In fact, FTO targets identified in a tissue context can differ from the ones identified from mere cell lines. For example, mapping of  $m^6A$  at nucleotide resolution in FTO KO mouse brain revealed a strong enrichment of  $m^6A_m$  over internal  $m^6A$  [48]. Moreover, the relative impact on each target might be different from the one established based on cell line



**Figure 1. Compartment specific targets of FTO.** In the nucleus, FTO demethylates m<sup>6</sup>A<sub>m</sub> in U1 and U2 snRNAs, m<sup>6</sup>A in U6 snRNAs and pre-mRNAs, and m<sup>1</sup>A in selected tRNAs. In the cytoplasm, FTO targets m<sup>6</sup>A<sub>m</sub> (and potentially m<sup>6</sup>A) on mRNA. It targets m<sup>1</sup>A in a subset of tRNAs. Compartment-specific m<sup>6</sup>A demethylation of short RNAs (20 nucleotides long) remains undocumented.

data. In cultivated cells, the demethylase activity of FTO silencing on m<sup>1</sup>A level is marginal [28]. By contrast, FTO KO in mouse brain tissue has more impact on m<sup>1</sup>A than m<sup>6</sup>A or m<sup>6</sup>A<sub>m</sub>. While a compensatory mechanism regulating the m<sup>6</sup>A/m<sup>6</sup>A<sub>m</sub> dynamic cannot be ruled out, this *prima facie* evidence suggests that FTO preferentially targets m<sup>1</sup>A in mouse brain.

Discrepancies between cell lines across research reports raise the question of FTO tissue specificity.

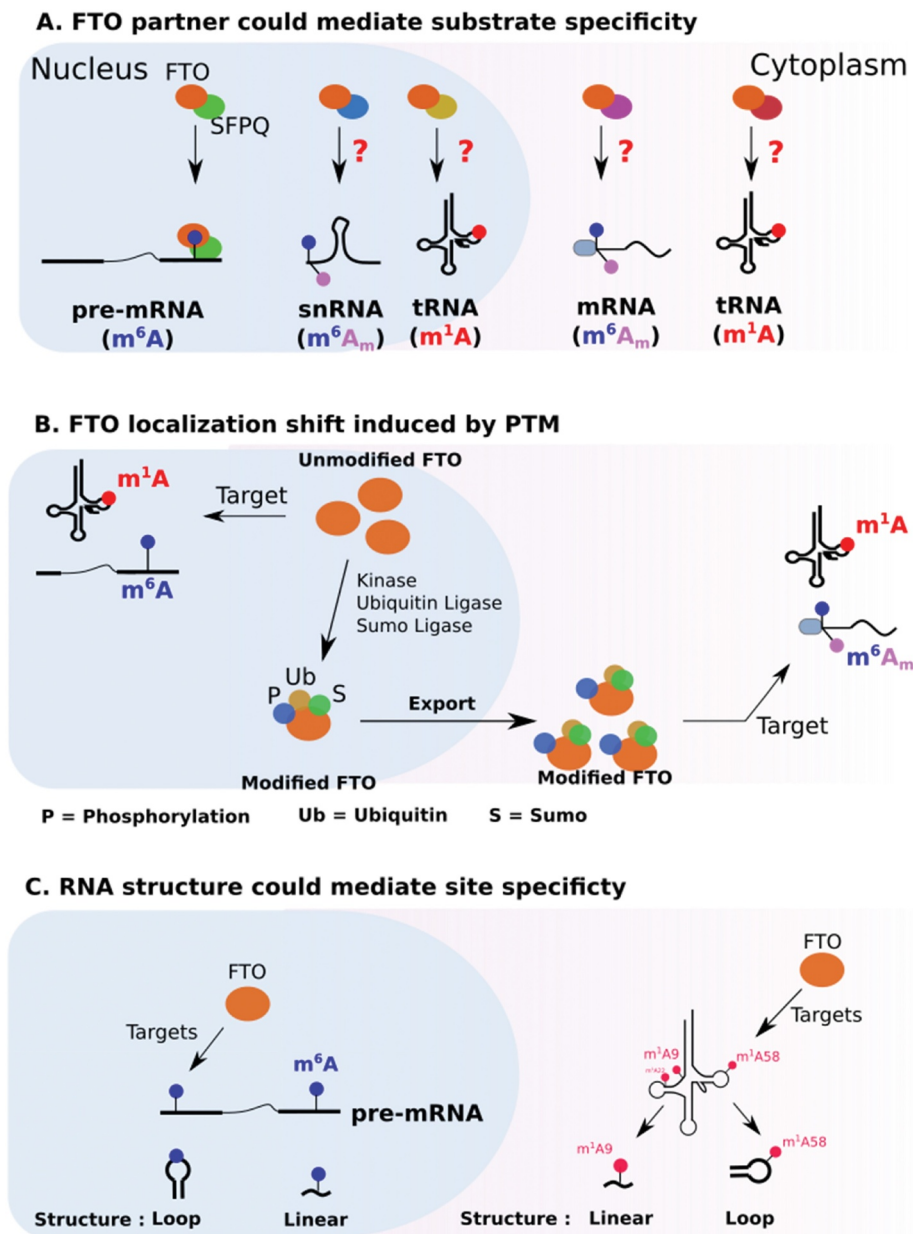
Jun Liu and colleagues compared the expression of m<sup>6</sup>A/m<sup>6</sup>A<sub>m</sub> writers and erasers with the level of m<sup>6</sup>A and m<sup>6</sup>A<sub>m</sub> across 54 human tissues and 16 mouse tissues [49]. In both human and mouse, the m<sup>6</sup>A writers METTL3-METTL14 and the eraser ALKBH5 were correlated with m<sup>6</sup>A. As expected, the m<sup>6</sup>A<sub>m</sub> writer PCIF1 was correlated with m<sup>6</sup>A<sub>m</sub>. However, FTO correlated with neither m<sup>6</sup>A nor m<sup>6</sup>A<sub>m</sub> level. This observation supports the possibility that FTO has tissue-specific target preferences.

## 2. Determinants of multi-substrate specificity of FTO

### 2.1. Structural basis for FTO catalytic activity

Solving the structure of FTO was a crucial step towards a better understanding of its interaction with methylated RNA substrates. Several groups have tackled the challenging task of crystallizing FTO in a complex with nucleic acids [19,50]. FTO is structurally composed of two main domains: the N-terminal domain from residues 32 to 326, and the C-terminal one from residues 327 to 498. Of note, the N-terminal 32 residues encode FTO's nuclear localization signal that localizes FTO to the nucleus. However, many other signal sequences throughout the FTO reading frame also affect its localization, as will be touched on later.

Inside the N-terminal domain, several residues are important for substrate recognition. Two 'pincers' composed of



**Figure 2.** Potential mechanisms regulating FTO substrate specificity.

residues K216, K88 and K316 allow FTO to interact with RNA50. Residue S229 could be involved in FTO specificity since mutation of this residue decreases FTO demethylation activity towards  $m^6A$  but not towards  $m^6A_m$  [50]. The C-terminal domain of FTO does not contain essential residues for RNA interaction or FTO activity. Noteworthy, comparative studies have shown that FTO's C-terminal domain is unique compared to the other ALKBH family members [44, 51]. The versatile activity of FTO is likely due to this domain, through its ability to associate with molecular partners.

## 2.2. Regulation by protein partners

Through its C-terminal part, FTO interacts with protein partners that regulate its subcellular localization as well as its functions (Fig. 2). In living cells, the use of RNA probes containing four repeats of the  $m^6A$  consensus sequence

GGACU failed to recruit FTO, suggesting the involvement of a third party52. Recently, a protein partner of FTO that could promote  $m^6A$  demethylation has been identified53]. SFPQ is a splicing factor that co-localizes with FTO in nuclear speckles [53]. Co-immunoprecipitation assays have demonstrated that SFPQ interacts with the C-terminal domain of FTO53. Transcriptome-wide analysis of FTO and SFPQ binding sites revealed that FTO and SFPQ bind RNA in a proximity 53. SFPQ overexpression decreases  $m^6A$  in FTO mRNA targets in various cellular models. In particular, CLIP and  $m^6A$ -IP-qPCR experiments show that increased expression of SFPQ favours the binding of FTO to myc mRNA and its subsequent  $m^6A$  demethylation.

Interestingly, only 20% of FTO binds in proximity to SFP, suggesting that the remaining pool of FTO associates with other molecular partners 53. Protein partners that would promote FTO interaction with either  $m^6A_m$  or  $m^1A$  sites

**Table 1.** Targets of FTO by RNA species and with associated evidence (experimental context).

Modification	DNA/RNA species	Evidence
3meU	ssRNA	In vitro
3 meT	ssDNA	In vitro
m <sup>6</sup> A	pre-mRNA, snRNAs	In vitro; living cells
m <sup>1</sup> A	tRNA	In vitro; living cells
m <sup>6</sup> A <sub>m</sub>	mRNA; snRNAs	In vitro, living cells
m <sup>6</sup> A	rRNA	No evidence
Intronic m <sup>6</sup> A	Intron	Many Fto binding but no evidence yet

remain to be identified. While m<sup>6</sup>A<sub>m</sub> is a structural component of the mRNA cap, FTO recruitment on this modification does not seem to involve the translation initiation machinery. Along these lines, FTO does not co-localize with stress granules. These are cytoplasmic aggregates mostly composed of stalled translation initiation complexes<sup>54</sup>.

TRMT10A, an S-adenosylmethionine-dependent methyltransferase that methylates guanosine in tRNAs at the ninth position, has also been suggested to interact with FTO and to steer its specificity towards specific m<sup>6</sup>A sites<sup>55</sup>. In this study, the authors suggested that TRMT10A coordinates the methylation status of tRNA and mRNA, to facilitate the translation of transcripts regulated by FTO.

### 2.3. Regulation of FTO by PTM

According to phosphosite plus (<https://www.phosphosite.org>), the FTO sequence harbours several potential post-translational modifications (PTMs): phosphorylation, sumoylation, ubiquitination and acetylation (Table 2). PTMs can affect protein activity, protein–protein interactions and subcellular localization<sup>56</sup>. In particular, the FTO activity is greatly influenced by its distribution, be it nuclear or cytoplasmic. Mutation of FTO lysine 216 has been shown to disrupt its nuclear localization and cause its accumulation in the cytoplasm. Three potential PTMs could be deposited at this position: ubiquitylation, sumoylation and acetylation. Interestingly, inhibiting ubiquitination with PYR-416 does not disrupt nuclear localization, but instead leads to the accumulation of a sub-pool of FTO in the perinuclear region<sup>57</sup>. Sumoylation of K216 also affects FTO localization and regulates its nuclear transport as well as its turnover<sup>58</sup>. This modification involves a direct interaction with RanBP2, a component of the SUMO E3 ligase complex (RanBP2/RanGAP1\*SUMO1/Ubc9), which localizes at the cytoplasmic side of nuclear pores and is a docking site in nucleocytoplasmic transport<sup>59</sup>. Collectively, these studies indicate that ubiquitination regulates the nucleocytoplasmic shuttling of FTO.

PTMs have also been identified inside FTO's predicted NLS. For instance, threonine 4 and threonine 6 can be phosphorylated. However, mutation of any of these threonines does not impair nuclear localization in an overexpression system, suggesting that they do not have an impact on FTO localization<sup>60</sup>. By contrast, phosphorylation of T150, located inside the catalytic domain of FTO, regulates its subcellular distribution; the FTO T150A mutant has enhanced nuclear localization, whereas the T150E mutation inhibits nuclear translocation.

Beyond localization, it is still unclear whether any FTO modification regulates its enzymatic activity. For example, the K216R mutation not only promotes the shuttling of FTO towards the cytoplasm but also decreases m<sup>6</sup>A mRNA level<sup>58</sup>. By contrast, m<sup>6</sup>A demethylase activity of FTO was previously reported to occur in the nucleus [28,31]. Both sides could be reconciled by considering that K216 directly interacts with the RNA base [33]. As such, one cannot exclude that any PTM or mutation at this position would alter the FTO affinity towards its substrate.

Other residues that play key roles in the FTO function can be post-translationally modified. Take, for example, the case of Y106, which is part of the catalytic pocket and whose mutation impairs FTO binding to ssDNA. Y106 can be phosphorylated in leukaemic cell lines (Table 2), but its function remains elusive. To take a further example, Serine 229 is involved in FTO specificity towards RNA substrates. As such, the FTO S229A mutation slightly decreases the demethylation of m<sup>6</sup>A without impairing m<sup>6</sup>A<sub>m</sub> demethylation [33]. Phosphorylation of this residue has been reported in various cancer cell lines, but the function of the phosphor adduct is not yet known. To sum up, various PTMs have been reported to occur on several residues along the FTO protein sequence. However, their impact at the molecular level and their exact function in a physio-pathological context remain to be uncovered.

### 2.4. RNA context

As well as localization and protein partners, the activity of FTO is also dependent on RNA context. A striking example comes from m<sup>1</sup>A targeting, as previously discussed in the context of tRNA (see 1.4). *In vitro*, FTO demethylates m<sup>1</sup>A on hairpin loop RNA probes but not on linear RNA probes [28]. Besides secondary structure, sequence context is also important for m<sup>6</sup>A demethylation. An *in vitro* demethylation assay on 5 nucleotide-long m<sup>6</sup>A probes revealed that the FTO activity can vary up to two-fold depending on the sequence context [33]. Furthermore, as for m<sup>1</sup>A, FTO is more efficient at targeting m<sup>6</sup>A in the context of a large loop. To demonstrate this, the authors employed probes containing a methylated GGACU motif, embedded in either a large or a closed loop, and incubated them with FTO *in vitro*. FTO's m<sup>6</sup>A demethylase activity was higher when m<sup>6</sup>A was localized in a large loop than when in a close loop or in linear RNA [33]. This predilection for targeting methylated sites in structured regions has not been demonstrated *in vivo* yet, and counterintuitively, m<sup>6</sup>A is preferentially localized in poorly structured regions [61]. How can we reconcile these two apparently contradictory observations? First, it has been proposed that m<sup>6</sup>A unfolds RNA structure through the recruitment of RNA helicase-containing m<sup>6</sup>A reader YTHDC2[61]. Second, m<sup>6</sup>A deposition can also disrupt A-U interactions to loosen RNA structure<sup>62,63</sup>. Third, the connection between m<sup>6</sup>A and RNA structure might be a result of FTO action; preferential targeting of m<sup>6</sup>A in loop structures could be what is enriching m<sup>6</sup>A in unfolded regions.

### 3. 'Context-dependent' function of FTO in cancer

#### 3.1. Pro-oncogenic role in cancer and therapeutic perspectives

Based on the current literature, the activity of FTO can have either pro-oncogenic or anti-oncogenic consequences depending on cancer type (Fig. 3). The pro-oncogenic role of FTO has been well described in acute myeloid leukaemia (AML) [25,46,47] and melanoma<sup>64</sup>. FTO expression is higher in AML with rearrangement of the mixed-lineage leukaemia (MLL) gene [25]. In these cancer cells, the ectopic expression of FTO promotes proliferation and colonogenic capacities, while it reduces apoptosis [25]. Silencing of FTO has the opposite effect, which concurs with a pro-oncogenic function of FTO in AML. Based on this conclusion, a therapeutic strategy based on FTO targeting has been elaborated to kill leukaemic cells<sup>65</sup>. The authors employed R-2-hydroxyglutarate (R-2HG), a metabolite produced by isocitrate dehydrogenase 1/2 (IDH1/2) enzymes. R-2HG is capable of inhibiting various alpha-ketoglutarate-dependent enzymes. As expected by the authors, the 2-HG treatment reduced proliferation and leukaemogenesis in an FTO-dependent manner<sup>65</sup>. As R-2HG is a broad inhibitor of alpha-ketoglutarate enzymes, subsequent studies explored the possibility of developing specific FTO inhibitors. Huang et al. identified meclofenamic acid as a highly specific inhibitor of FTO <sup>66</sup>. Meclofenamic acid is a non-steroidal, anti-inflammatory drug that competes with FTO for binding m<sup>6</sup>A sites. More recently, the same group has developed and functionally screened several derivatives of this drug and identified one of them, named FB23, as 140-fold more potent than the parent compound at inhibiting FTO-mediated demethylation [47]. A derivative of FB23, FB23-2, abrogates *in vivo* leukaemia progression and prolongs survival of leukaemic mice. On the same trend, Rui Su et al developed two FTO inhibitors for targeting leukaemic stem cells [46]. These compounds slow down AML and improve survival in a patient-derived xenograft mouse model. Importantly, FTO inhibitors are able to kill leukaemic cells without significantly affecting normal cells. Nevertheless, while FTO is a promising target in mouse models, FTO expression in men does not necessarily correlate with patient survival<sup>67</sup> and another study found no effect of Fto on AML human cell lines [68]. Hence, further studies will be required to evaluate the relevance of targeting FTO for treating AML as well as other cancers.

The oncogenic effect of FTO has also been described in melanoma, breast cancer, lung cancer, oral squamous cell carcinoma (OSCC), and glioblastoma. In melanoma, FTO silencing reduces cell proliferation *in vitro* and *in vivo* [64]. *By contrast, ectopic expression of FTO favours melanoma cell proliferation and reduces the efficiency of immunotherapy*<sup>64</sup>. *In glioblastoma cell lines, pharmacological inhibition of FTO reduces tumour growth and cripple stem-like properties* <sup>69</sup>. *In breast cancer, FTO transcript level is higher in tumours than in healthy tissue* [24]. *FTO silencing blocks cell proliferation and clonogenic abilities and induces apoptosis of breast cancer cells. In lung cancer, FTO overexpression promotes cell growth, migration and metastasis, in an E2F1-dependent manner. Concomitantly, the level of m<sup>6</sup>A level in E2F1 mRNA is reduced*

*upon FTO overexpression, suggesting that FTO's function in lung cancer depends on its m<sup>6</sup>A demethylase activity*<sup>70</sup>. *Moreover, FTO mediated m<sup>6</sup>A demethylation is oncogenic in OSCC. In this cancer type, FTO regulates autophagy and tumorigenesis by targeting m<sup>6</sup>A on the transcript encoding the eukaryotic translation initiation factor gamma 1 (eIF4G1). FTO silencing promotes the downregulation of eIF4G1 (through YTHDF2 binding) along with enhanced autophagic flux and inhibition of tumorigenesis*<sup>71</sup>.

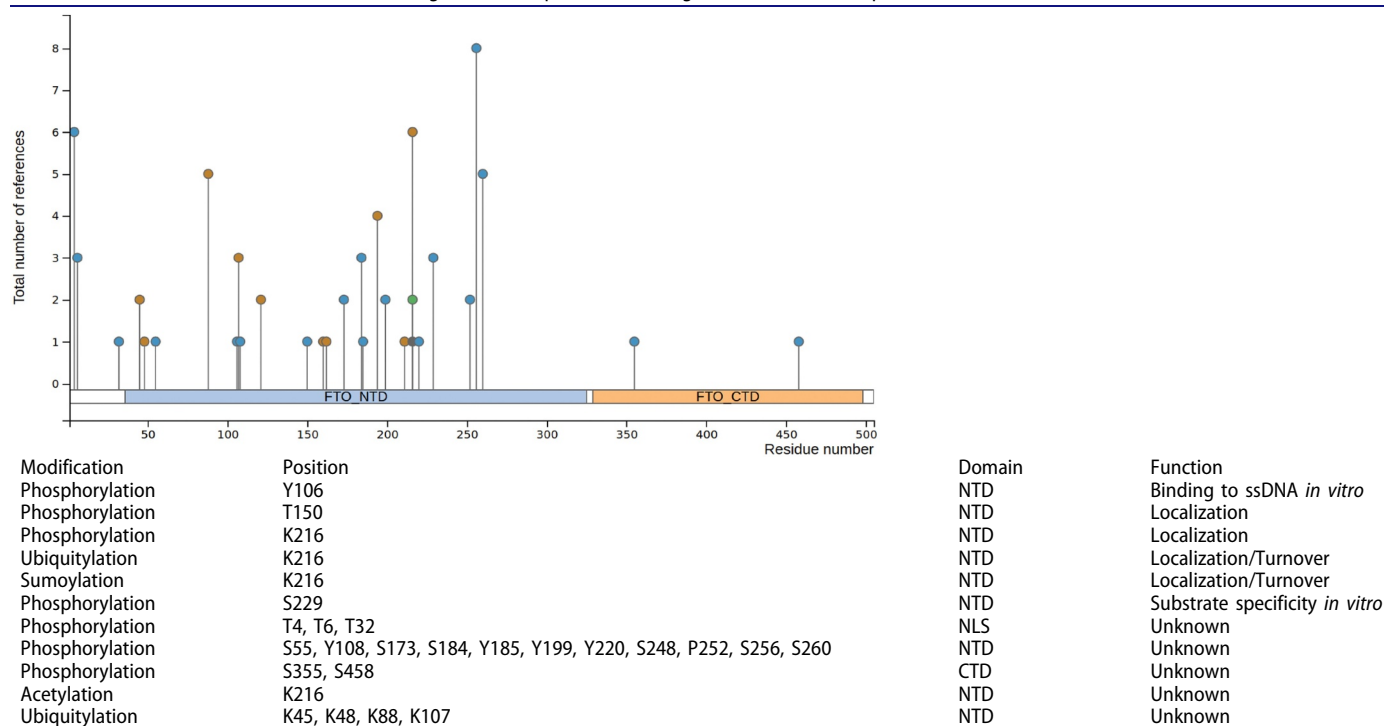
#### 3.2. Anti-oncogenic function of FTO

In some tissues, FTO exhibits anti-oncogenic functions. In colon cancer cells, for instance, FTO silencing promotes the acquisition of stem-like features: sphere formation in suspension culture, tumour initiation and chemoresistance in xenografted nude mice [32]. Remarkably, the analysis of tumour microarrays from colorectal cancer patients shows that FTO expression remains relatively constant throughout tumour onset and progression [32]. However, its cellular distribution varies significantly. While FTO is strictly nuclear in healthy tissues, a large fraction relocates to the cytoplasm during the early stages of tumour progression. Considering the compartment-specific activity of FTO, any alteration of its subcellular distribution may trigger significant functional changes and impact tumour evolution. From that point of view, FTO expression and localization may be considered of equal importance for understanding its role in cancer.

Another striking example comes from hepatocellular carcinoma, where SIRT1 acetylase promotes tumour growth by downregulating the FTO expression<sup>59</sup>. At the molecular level, SIRT1 acetylates RANBP2 that, in turn, sumoylates FTO and triggers its degradation<sup>59</sup>. Low FTO expression is also associated with poor patient outcome in renal clear cell carcinoma<sup>72</sup>. In this study, the authors demonstrate that the m<sup>6</sup>A demethylase activity of FTO suppresses tumour growth by promoting the expression of PGC-1 $\alpha$ , a transcriptional coactivator and central inducer of mitochondrial metabolism<sup>72</sup>. Last, downregulation of FTO is associated with poor prognosis in intrahepatic cholangiocarcinoma<sup>73</sup>. In this cancer type, FTO overexpression suppresses *in vitro* anchorage-independent growth as well as *in vivo* tumour growth <sup>73</sup>. In gastric cancer, a reduction of FTO protein level is associated with poor prognosis suggesting an anti-oncogenic role of FTO in this cancer<sup>74</sup>. However, this discovery remains controversial, as another study found FTO to be upregulated in gastric cancer<sup>75</sup>. Investigation of the prognosis value of FTO revealed its anti-oncogenic function in lung cancer. Indeed, high FTO expression is associated with longer survival<sup>76,77</sup>. FTO silencing promotes cell growth, cell migration and tumorigenesis *in vivo*, which indicates an anti-oncogenic function of FTO in lung cancer. Mechanistically, FTO silencing enhances the m<sup>6</sup>A levels in several critical genes, including Myc. Accrued m<sup>6</sup>A on Myc mRNA leads to YTHDF1 binding, which promotes Myc mRNA translation, increased glycolysis and cell proliferation <sup>77</sup>.

Finally, a recent report associates FTO downregulation in several epithelial cancers with enhanced cancer progression features, such as growth, invasion and metastasis, as well as



**Table 2.** Post-translational modification sites along the FTO sequence (according to UniProt and PhosphoSite).

with worse clinical outcome<sup>78</sup>. The authors show that the FTO loss promotes the implementation of an epithelial to mesenchymal programme through the activation of the Wnt signalling pathway. Indeed, FTO-dependent m<sup>6</sup>A demethylation regulates the stability of several transcripts involved in Wnt pathway regulation.

In short, FTO function in cancer varies greatly according to tissue context and further studies are required to clarify the molecular basis of this ambiguity.

### 3.3. Regulation of FTO expression in cancer

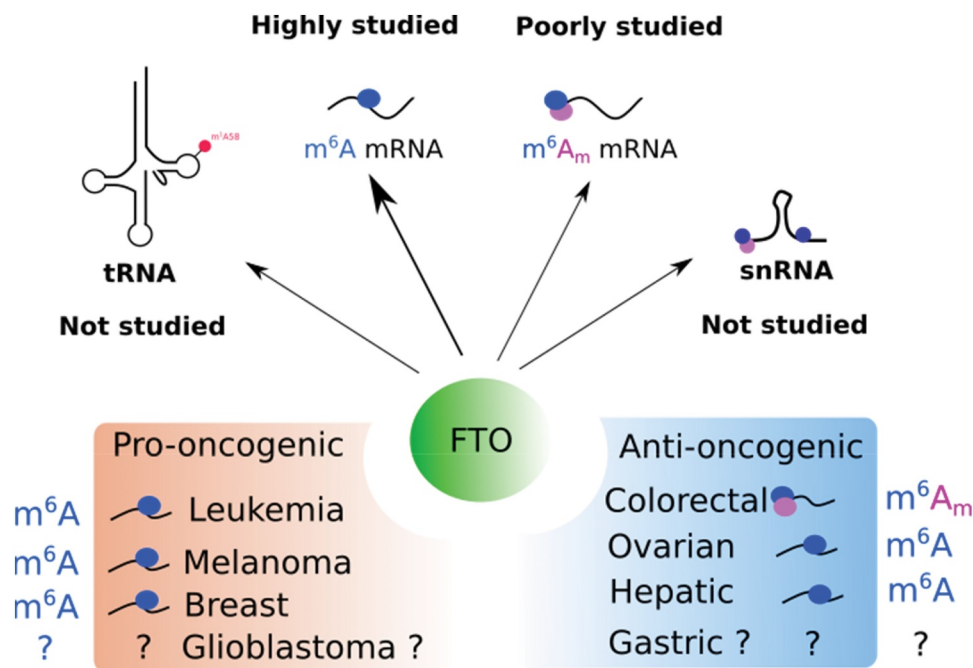
The central role of FTO in tumorigenesis is reflected by the frequent alteration of its expression and/or activity in cancer<sup>79</sup>. However, few studies have investigated the molecular mechanisms governing FTO expression in cancer. In breast cancer, STAT3 binds to the FTO promoter to induce its transcription<sup>80</sup>. FTO is also regulated at the transcriptional level in lung cancer<sup>77</sup>. Indeed, the FTO promoter has a LEF/TCF-binding element that allows the recruitment of the Bcat/TCF/EZH2 complex. The docking of this complex on the FTO promoter enhances H3K27me3 levels and inhibits the FTO expression. Transcriptional regulation of FTO also occurs in OSCC. Indeed, rapamycin treatment of OSCC lines decreases both FTO mRNA and FTO protein levels, suggesting that FTO is mainly regulated through transcription or mRNA stability in this model<sup>71</sup>. In contrast, in colorectal cancer, there is no correlation between the level of FTO mRNA and protein across patient-derived cell lines from primary tumours, in metastatic tumours or in circulating tumour cells [32], implying a regulation of FTO at the post-

transcriptional level. A similar observation was made in other cancers, such as gastric cancer<sup>74</sup>, where FTO protein expression is disconnected from its transcript level. Yet, the ‘cancerous’ signalling pathways involved in the post-transcriptional regulation of FTO must be identified in future studies.

## DISCUSSION

The molecular basis of how FTO recognizes and selects its substrates remains a matter of debate and controversy, despite significant efforts in this research area. For example, in contrast to most cancer studies showing FTO targets m<sup>6</sup>A sites, FTO preferentially targets m<sup>6</sup>A<sub>m</sub> in colorectal cancer. One possible explanation could be that most studies focus on global m<sup>6</sup>A methylation, making no distinction between m<sup>6</sup>A and m<sup>6</sup>A<sub>m</sub>, and ignoring any potential effect on m<sup>1</sup>A. Another explanation may reside in the diversity of experimental protocols for RNA extraction and processing. Taking these parameters into consideration will be essential in future studies that will require standardized protocols and the routine use of mass spectrometry for simultaneous quantification of modified nucleosides from both total and purified RNA species. Indeed, we propose to avoid creating more uncertainties and better comprehend individual FTO target’s contribution to any FTO-related phenotype.

As for many cellular enzymes, FTO can be affected by PTMs and molecular partners at the level of distribution, activity and subsequent cell phenotype. Remarkably, besides predictable discrepancies between transcript and protein level, changes in FTO localization may arise following neoplastic



**Figure 3. Function of FTO in cancers.** The top panel illustrates the targets of FTO that have been studied or not in cancer. The bottom panel shows the pro- and anti-oncogenic function of FTO with associated targets.

transformation [32], thus skewing survival statistics. This exemplifies why localization must also be evaluated in cancer studies in addition to FTO protein level. We are optimistic that pan-cancer analyses of FTO expression and localization paralleled by the identification of FTO targets will clarify the debate regarding the impact of FTO's subcellular localization on its target specificity and function.

## Acknowledgments

Our teams are generously supported by the Occitanie Region/FEDER (PPRi, SMART project), French National Cancer Institute (INCa) (DA N°2020-116), Ligue contre le Cancer, SIRIC Montpellier Cancer (INCa-DGOS-Inserm 6045), Cancéropole GSO and LabEx NUMEV (GEM flagship project, ANR 2011-LABX-076).

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

This work was supported by the LabEx NUMEV [GEM flagship project, ANR 2011-LABX-076]; SIRIC Montpellier cancer [INCa-DGOS-Inserm 6045]; Occitanie Region/FEDER [PPRi SMART]. French National Cancer Institute (INCa) [DA N°2020-116];

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