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FTO controls reversible m⁶Am RNA methylation during snRNA biogenesis
Reversible m⁶Am RNA methylation defines a novel step of snRNA biogenesis

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Summary

Small nuclear RNAs (snRNAs) are core spliceosome components and mediate pre-mRNA splicing. During their biogenesis, snRNAs acquire several constitutive nucleotide modifications. Here we show that snRNAs also contain a regulated and reversible nucleotide modification causing them to exist as two different methyl isoforms, m_1 and m_2 , reflecting the methylation state of the adenosine adjacent to the snRNA cap. We find that snRNA biogenesis involves the formation of an initial m_1 -isoform with a single-methylated adenosine (2'-*O*-methyladenosine, Am), which is then converted to a dimethylated m_2 -isoform ($N^6,2'$ -*O*-dimethyladenosine, m^6 Am). The relative m_1 - and m_2 -isoform levels are determined by the RNA demethylase FTO, which selectively demethylates the m_2 -isoform. We show FTO is inhibited by endogenous metabolites, resulting in increased m_2 -snRNA levels. Furthermore, cells that exhibit high m_2 -snRNA levels show altered patterns of alternative splicing. Together, these data reveal that FTO controls a previously unknown central step of snRNA processing involving reversible methylation, and suggest that epitranscriptomic information in snRNA may influence mRNA splicing.

Main Text

Small nuclear RNAs (snRNAs) are among the most abundant and extensively studied RNAs in eukaryotes. These uridine (U)-rich non-coding RNAs – which include U1, U2, U4, U5, and U6 – were discovered nearly fifty years ago¹, and studies of their function lead to the elucidation of the fundamental molecular mechanisms that mediate pre-mRNA splicing²⁻⁵.

snRNAs undergo a series of processing events that are required for incorporation into spliceosomes⁶⁻⁸. Except for U6 and U6atac snRNA⁹, snRNAs are synthesized by RNA polymerase II as precursors that initially contain a 3'-end extension and acquire an *N*⁷-methylguanosine (m⁷G) cap¹⁰. snRNAs are exported into the cytosol where they are incorporated into small nuclear ribonucleoproteins (snRNPs) by binding to Sm proteins¹¹. Their m⁷G cap is then further methylated to form the *N*^{2,2,7}-trimethylguanosine (TMG) cap and the snRNA 3'-end is trimmed¹²⁻¹⁴. snRNAs become highly stable upon incorporation into snRNPs and are transported back to the nucleus where snRNPs assemble into spliceosomes that mediate pre-mRNA splicing⁶⁻⁸. snRNAs that are not properly processed are not incorporated into snRNPs and are therefore unstable and degraded^{15,16}.

Importantly, snRNAs contain a set of constitutive nucleotide modifications that are highly conserved across species and essential for snRNA integrity and function^{17,18}. Thus, all mature snRNAs are thought to exist as a single molecular species, and deviations outside of this species are not thought to be utilized by spliceosomes.

Here we show that all Sm-class spliceosomal snRNAs can exist as two distinct isoforms, differing by a single methyl modification on the adenosine residue that is located at the transcription-start nucleotide of most snRNAs. This adenosine has previously been annotated to contain a constitutive 2'-*O*-methyl modification on the ribose sugar^{17,19}. We find that these adenosines are subjected to a second, reversible methylation selectively at the *N*⁶-position on the adenine base. This methylation converts the canonical single-methylated m₁-snRNA to a dimethylated m₂-snRNA. These m₂-snRNAs are major targets of the RNA demethylase FTO, which functions early during snRNA biogenesis to convert the m₂-snRNA back to m₁-snRNA. FTO-mediated demethylation of m₂-snRNA is blocked by the oncometabolite D-2-hydroxyglutarate, linking intracellular metabolism to snRNA methylation state. Upon FTO

inhibition, m₂-snRNAs accumulate and are assembled into snRNPs, which is correlated with enhanced splicing of exons that are normally poorly included. Overall these data redefine our understanding of snRNA biogenesis, by revealing the presence of an FTO-regulated methylation switch that results in the formation of previously unrecognized methyl isoforms of snRNA. Our findings point to the potential for FTO and cellular metabolites to influence transcriptome diversity by controlling the reversible methylation of snRNA.

The RNA demethylase FTO selectively demethylates snRNAs

FTO was shown to exhibit weak demethylase activity towards *N*³-methyluridine (m³U) and *N*⁶-methyladenosine (m⁶A)^{20,21}. We recently showed that FTO highly efficiently demethylates *N*⁶,2'-*O*-dimethyladenosine (m⁶Am), with nearly 100-fold higher catalytic activity towards m⁶Am compared to m⁶A (ref. 22). FTO selectively demethylates the *N*⁶-methyl, resulting in 2'-*O*-methyladenosine (Am) (ref. 22). m⁶Am is exclusively found within the “extended cap” of RNA polymerase II-transcribed RNAs, at the transcription-start nucleotide immediately adjacent to the m⁷G (ref. 23,24).

The physiological importance of FTO is indicated by analysis of FTO-deficient mice, which exhibit altered synaptic transmission, metabolic abnormalities, and growth retardation^{25,26}. Humans with loss-of-function FTO mutations show severe developmental defects and microcephaly²⁷. FTO has been proposed to regulate alternative splicing of mRNA, based on transcriptome-wide splicing alterations detected in FTO-deficient cells^{28,29}.

It remains unclear which methylated residues in the transcriptome are preferentially targeted by FTO. mRNAs seem to bypass the demethylation activity of FTO since m⁶Am is present at high stoichiometry in mRNA²². Indeed, nearly all the m⁶Am peaks in mRNA showed increases of less than 10% in *Fto* knockout brain compared to wild-type²². Although FTO clearly demethylates m⁶Am in mRNA, the moderate effect of FTO-mediated mRNA demethylation likely reflects the fact that mRNAs are predominantly cytosolic²² while FTO is nuclear in most cells and tissues^{20,26}. Thus, FTO may mediate its effects by targeting nuclear RNA.

To identify FTO targets, we used the miCLIP approach, which involves *N*⁶-methyladenine (6mAm6Am⁶A) antibodies (which bind both m⁶A and m⁶Am) that are UV-crosslinked to

purified RNA. After stringent washing, antibody-bound RNA is subjected to next generation sequencing to identify 6mA-m6Am⁶A-modified nucleotides³⁰. We used miCLIP to map 6mA-m6Am⁶A-m⁶A in wild-type and *Fto*-knockout mouse liver RNA (**Extended Data Fig. 1a**). Unlike in previous studies^{25,26}, no poly(A) purification was used, thus allowing all cellular RNAs to be analyzed. To detect m⁶Am, we searched for RNA species that show increased methylation at the transcription-start nucleotide in the *Fto* knockout transcriptome compared to wild type.

Although many RNAs showed potential methylation differences at the transcription-start nucleotide (**Fig. 1a**, grey circles), the vast majority did not pass the applied filters (see **Methods** section) and their methylation change did not reach statistical significance after multiple comparison testing. However, a small subset of methylation events was significantly increased in the *Fto* knockout transcriptome (**Fig. 1a**, red dots, adjusted *P*-value < 0.05). These RNAs were exclusively small nuclear RNAs (snRNAs), including U1, U4atac, and U5 (**Fig. 1a**). In each case, the miCLIP signal was associated with read terminations at the transcription-start nucleotide, suggesting that the first nucleotide contains an *N*⁶-methyl modification.

To test whether increased transcription-start nucleotide methylation in the *Fto* knockout transcriptome is observed in all spliceosomal snRNAs, we next looked specifically at the methylation fold change of Sm- and Lsm-class snRNAs. We observed increased transcription-start nucleotide methylation of all Sm-class snRNAs of the major and minor spliceosome (**Fig. 1b**, **Extended Data Fig. 1b**). In each case, the 6m⁶A reads were detected around the transcription-start nucleotide and were between 10-20-fold higher in the *Fto* knockout compared to wildtype (**Figs. 1c-f**, **Extended Data Figs. 2a-c**).

In addition to snRNAs, several snRNA-like transcripts showed markedly increased 6m⁶A reads in the *Fto* knockout transcriptome. These include U7, which is involved in 3'-end processing of histone mRNAs³¹ and the small nucleolar RNAs (snoRNAs) U3 and U8, which function in rRNA processing (**Extended Data Figs. 3a-c**). Notably, U3 and U8 snoRNAs are unusual in that they are not derived from intronic sequences like most snoRNAs, but instead transcribed from their own gene by RNA polymerase II using a promoter that contains sequence elements typical for snRNA genes³². No other snoRNAs exhibited significantly elevated 6m⁶A reads in the *Fto* knockout.

The increase in 6m⁶A reads was seen in all Sm-class snRNAs, but not the Lsm-class snRNAs U6 and U6atac – the only two spliceosomal snRNAs that are transcribed by RNA polymerase III rather than RNA polymerase II (ref. 9) (**Fig. 1g** and **Extended Data Figs. 1b, 2d**). U6 and U6atac do not acquire an m⁷G cap; however, U6 snRNA contains an internal m⁶A (ref. 17,33). Nevertheless, and in contrast to a recent report³⁴, FTO depletion did not lead to a statistically significant alteration in 6m⁶A reads at this internal position (**Fig. 1g** and **Extended Data Fig. 4a**).

Similarly, U2, which contains an internal m⁶Am (ref. 17, see also modomics.genesilico.pl), did not show a robust increase of 6m⁶A reads at this position (**Fig. 1d**). Notably, U1 snRNA, which is thought to not contain m⁶A or m⁶Am at internal sites based on 6m⁶A immunoprecipitation experiments and biochemical modification mapping^{33,35,36}, was recently suggested to contain m⁶Am at a previously unknown internal site³⁴. However, no significant enrichment of miCLIP reads were detected at this site in *Fto* knockout liver (**Extended Data Fig. 4b**). Overall, the miCLIP results indicate that the FTO-regulated sites are specifically localized to the transcription-start nucleotide region of Sm-class snRNAs.

miCLIP does distinguish between mature snRNA and the unprocessed longer snRNA precursors or truncated forms. Therefore, we used 6m⁶A immunoblotting to detect the length of 6mA-m⁶A-immunoreactive snRNAs. In these experiments, we immunoblotted small RNA (<200 nt) from wild-type and *FTO* knockout HEK293 cells. We observed bands corresponding to the mature snRNA forms, with significantly increased 6mA-m⁶A-immunoreactivity in *FTO* knockout cells (**Fig. 1h**).

Taken together, these data indicate that all Sm-class snRNAs are substrates for FTO-mediated demethylation during their life cycle.

The first nucleotide of snRNAs is reversibly methylated

The finding that Sm-class snRNA modification is regulated by FTO was surprising, since these snRNAs are not known to contain m⁶Am or m⁶A at the transcription-start nucleotide. Instead, Sm-class snRNAs are thought to contain 2'-*O*-methyladenosine (Am) at this position¹³. Since

the miCLIP results suggest that the first nucleotide in snRNAs in *FTO* knockout cells are either m⁶A or m⁶Am, we determined its identity using a thin-layer chromatography (TLC)-based assay²⁴. In this assay, the 5'-cap is removed from RNA, followed by radiolabeling of the exposed 5' nucleotide with [γ -³²P]-ATP. Next, the snRNAs are treated with ribonuclease and the nucleotide hydrolysate is resolved by 2D-TLC. This approach readily identifies the first nucleotide based on its migration pattern²⁴.

To determine the first transcribed nucleotide of snRNAs using the 2D-TLC assay, we first purified the small RNA fraction (<200 nt), which is highly enriched in snRNAs. In wild-type cells, the first nucleotide was predominantly Am, with low, but measurable levels of m⁶Am (**Fig. 2a**). However, when the small RNA fraction was prepared from *FTO* knockout HEK293 cells, the level of m⁶Am at the first nucleotide was substantially increased (**Fig. 2a**).

To ensure that *FTO* depletion is affecting snRNA and not some other type of small RNA, we directly measured the first nucleotide in gel-purified U1 and U2, which migrate to well-defined positions by PAGE (**Figs. 1f, 2b**). Consistent with previous publications¹³, the first nucleotide in U1 and U2 was predominantly Am in wild-type HEK293 cells (**Fig. 2b**). However, in *FTO*-knockout HEK293 cells, m⁶Am was markedly increased, with levels similar to Am (**Fig. 2b, Extended Data Fig. 4c**). These data indicate that snRNAs can contain m⁶Am at the first encoded nucleotide.

Notably, U2 is difficult to analyze by miCLIP since it contains a constitutive internal m⁶Am which causes this snRNA to be immunoprecipitated irrespective of the methylation status of its transcription-start nucleotide (**Fig. 1d**). The TLC analysis directly demonstrates that U2 also contains an m⁶Am nucleotide that is regulated by *FTO*.

To provide an independent measurement to assess whether snRNAs can contain m⁶Am at the first nucleotide, we developed a mass spectrometry-based assay. To examine m⁶Am or Am specifically in the context of the cap structure, small RNA was treated with P1 nuclease, resulting in all internal nucleotides being digested to mononucleotides. However, under these conditions, the first nucleotide remains connected to the cap in the form of a “cap-dinucleotide” structure (cap-ppp-Am or cap-ppp-m⁶Am) (**Extended Data Fig. 5a**).

Cap-dinucleotides were readily quantified by high-resolution mass spectrometry using positive ion-mode detection, since negative ion mode did not allow sensitive detection of cap-dinucleotides (**Extended Data Fig. 5b**). A multiple reaction monitoring protocol was developed based on the fragment ion transitions from distinct dinucleotide precursor species (**Extended Data Fig. 5c**). This approach exhibited high sensitivity and linear detection of cap dinucleotides, thus revealing the first nucleotide within the cap context (**Extended Data Fig. 5d**).

Using this approach, we verified that Am was associated with the cap after digestion of small RNA from wild-type HEK293 cells (**Fig. 2c, d**). However, m⁶Am was the predominant form in *FTO*-knockout HEK293 cells, with a 6.3-fold enrichment of m⁶Am over Am (**Fig. 2c**). Thus, both TLC and mass spectrometry indicate that snRNAs contain m⁶Am at the first nucleotide in *FTO*-deficient cells.

Together, these results suggest that during snRNA biogenesis, snRNAs undergo methylation to form a readily detectable dimethylated form (m₂-snRNA) with m⁶Am as the first nucleotide. The m₂-snRNA can then be demethylated by *FTO* to a single-methylated snRNA (m₁-snRNA) with Am as the first nucleotide.

m₂-snRNAs are detected in different cells and tissues

We next wanted to determine the prevalence of m₂-snRNAs in cells that are not genetically depleted of *FTO*. To test this, we isolated small RNAs and assayed the transcription-start nucleotide by mass spectrometry from various cells and tissues. In brain, only m₁-snRNAs were detected (**Fig. 2d**). However, in liver, HEK293 cells, mouse embryonic stem cells (mESCs), HT1080 fibrosarcoma and TF-1 erythroleukemia cells, m₂-snRNAs were readily detected at 1-15% of the level of m₁-snRNAs, with the highest levels in HEK293 cells (**Fig. 2d** and **Extended Data Figs. 6a-e**). UOK262 renal cell carcinoma cells showed ~3-fold higher m₂-snRNA to m₁-snRNA ratio than HEK293 cells (**Extended Data Fig. 6e**).

We next asked if the levels of m₁-snRNAs and m₂-snRNAs are regulated during embryonic stem cell development. To test this, we cultured mESCs for five days in the presence or absence of

differentiation inhibitors. Mass spectrometry revealed a two-fold increase in m₂-snRNAs upon removal of differentiation inhibitors (**Extended Data Figs. 6b, c**).

Together, these data indicate that m₂-snRNAs are nearly undetectable in most tested cells and tissues, but m₂-snRNAs can be readily detected in some cell types and can be induced in response to certain stimuli.

m₂-snRNAs are increased in response to the oncometabolite 2-hydroxyglutarate

To further understand the regulation of m₂-snRNA levels, we considered endogenous inhibitors of FTO. FTO is inhibited by natural metabolites and oncometabolites, including citrate, succinate, fumarate, and 2-hydroxyglutarate (2-HG)^{37,38}. These metabolites compete with α -ketoglutarate, an FTO co-substrate, for demethylation³⁷.

Cancer-associated mutants of isocitrate dehydrogenase (IDH) generate high levels (>10 mM) of 2-HG³⁹. To determine if 2-HG affects snRNA methylation, we first measured m₁-snRNAs and m₂-snRNAs levels in small RNA isolated from TF-1 cells expressing IDH1^{R132H} and IDH2^{R140Q} mutants^{40,41}. Expression of mutant IDH2^{R140Q} elicited a marked increase in 2-HG levels, with a stronger effect seen with mutant IDH1^{R132H} (**Extended Data Fig. 7a**). In control cells, m₁-snRNAs predominated as the major first nucleotide in small RNA. However, m₂-snRNAs were markedly elevated following expression of both IDH1^{R132H} and IDH2^{R140Q}, with a greater effect seen with IDH1^{R132H} (**Fig. 2e** and **Extended Data Fig. 7b**). Similar effects were seen using TLC analysis of U1 snRNA (**Extended Data Fig. 7c**).

To confirm that the effect of mutant IDH1 and IDH2 reflect increased 2-HG levels, we used mutant isoform-selective inhibitors. These inhibitors resulted in marked reduction of m₂-snRNAs (**Fig. 2e** and **Extended Data Figs. 7b, c**).

We also tested the effects of dimethylfumarate, a clinically used metabolite pro-drug that is converted to fumarate in cells. Treatment of HEK293 cells for 7 days with 70 μ M dimethylfumarate, a concentration used to study the cellular effects of this compound⁴², did not significantly affect m₂-snRNA levels (**Extended Data Fig. 7d**). These data point to the relative

importance of 2HG as a metabolite regulator of FTO and indicate that DMF is unlikely to mediate its clinical effects by inducing the formation of m₂-snRNAs.

Taken together, these data indicate that m₂-snRNA levels can be altered by 2-HG, and suggest the possibility that 2-HG-dependent pathogenic conditions are associated with increased levels of m₂-snRNAs.

m⁶Am is demethylated prior to cap hypermethylation during snRNA biogenesis

snRNAs biogenesis involves an initial nuclear phase⁶⁻⁸ with transcription and m⁷G capping of precursor snRNAs. After export into the cytoplasm, snRNAs acquire a TMG cap and are then subjected to 3' end trimming. Upon subsequent incorporation into small nuclear ribonucleoproteins (snRNPs), the complexes are re-imported into the nucleus^{6-8,14} and the snRNPs can function in splicing during their second nuclear phase. Since FTO is predominantly nuclear²⁰, the conversion of m₂-snRNAs to m₁-snRNAs by FTO likely takes place during one of these two nuclear phases. The absence or presence of a TMG cap is indicative of the specific nuclear phase of snRNAs.

We therefore asked if FTO demethylates m₂-snRNAs before or after the formation of the TMG cap. To determine in which phase of snRNA biogenesis FTO acts, we chemically synthesized RNA substrates mimicking the pre-export and post-import cap structure of snRNA. These 20-nucleotide long RNAs started with either an m⁷G or a TMG cap, followed by a triphosphate linker, and m⁶Am. As described previously²², FTO efficiently demethylated m⁶Am in m⁷G-capped RNA, as measured by formation of Am (**Fig. 3a**). However, FTO did not detectably demethylate m⁶Am when using the post-import RNA substrate comprising m⁶Am with a TMG cap (**Fig. 3a**). These data suggest that m₂-snRNAs are not a substrate for FTO after TMG cap formation.

Based on the m⁷G cap-specific demethylation and considering the nuclear localization of FTO, we infer the decision to demethylate m₂-snRNAs and form m₁-snRNAs is determined during a specific, early phase of snRNA biogenesis. This phase occurs in the nucleus after snRNA transcription but prior to the cytoplasmic formation of the TMG cap and assembly of snRNAs into snRNPs.

m₂-snRNAs acquire a TMG cap and assemble into snRNPs

snRNA function requires their assembly with Sm proteins into snRNPs. To test if m₂-snRNAs assemble into snRNPs, we immunoprecipitated snRNPs from cellular extracts using a small ribonucleoprotein particle protein B (SmB)-specific antibody. This approach has previously been shown to immunoprecipitate assembled snRNPs^{43,44}. We then determined the first nucleotide of snRNAs in cellular snRNPs to determine if m₂-snRNAs are present. As expected, in control HEK293 cells, m₁-snRNAs were predominant (**Fig. 3b**). However, in *FTO* knockout HEK293 cells, m₂-snRNAs were readily detected (**Fig. 3b**). Thus, m₂-snRNAs form a distinct m⁶Am-containing class of snRNPs, i.e. m₂-snRNPs.

After incorporation of snRNAs into snRNPs, their m⁷G cap is modified to form the TMG cap¹⁴. We asked if m₂-snRNAs are similarly methylated to form the TMG cap using mass spectrometry. The small RNA fraction was purified from *FTO* knockout HEK293 cells and *Fto*-knockout mouse liver and brain. The RNA was digested with nuclease P1 to liberate the cap and m⁶Am as the dinucleotide cap structure (cap-ppp-m⁶Am). The presence of m⁷G or TMG was then assessed in the liberated cap structure. In each case, the primary cap structure was the TMG cap attached to m⁶Am (TMG-ppp-m⁶Am) (**Fig. 3c, Extended Data Fig. 6a**). This indicates that m₂-snRNAs undergo normal cap hypermethylation during their biogenesis.

Taken together, these data show that m₂-snRNAs undergo normal biogenesis, including incorporation into snRNPs and acquisition of the TMG cap structure.

Altered splicing in cells expressing high levels of m₂-snRNAs

We next sought to examine the previously described splicing abnormalities seen upon *FTO* depletion. Similar to a previous study²⁹, we found that the most pronounced effect of *FTO* deficiency is increased exon inclusion (**Fig. 4a**). To further refine this analysis, we characterized the included exons for binding sites of known splicing factors in a previously published RNA-Seq analysis using control and *FTO* knockout HEK293 cells²⁸. Here, we observed a significant and specific enrichment for SRSF1, HNRNPH2 and HNRNPK-dependent exons, whereas enrichment of other exons was not detected (**Fig. 4b and Extended Data Fig. 8a**). Similar effects were also seen in our HEK293T *FTO* knockdown RNA-Seq dataset (**Extended Data Fig.**

8b). Thus, FTO depletion is associated with the enhanced inclusion of exons that would normally be inefficiently spliced into mature mRNA.

Since FTO depletion is associated with an increase in the ratio of m_2 -snRNAs to m_1 -snRNAs, as well as splicing abnormalities, this correlative evidence starts to suggest m_2 -snRNAs can affect splicing. We therefore wanted to determine how m^6 Am affects snRNAs, and if any of these changes are consistent with altered splicing phenotypes. In the case of mRNAs, DCP2-mediated decapping and mRNA degradation is impaired when m^6 Am is present at the first nucleotide²². However, northern blotting of cellular RNA harvested from *DCP2* knockout HEK293 cells, which primarily contain m_1 -snRNAs, showed no increase in snRNAs relative to wild type cells (**Extended Data Fig. 9a**). Thus, m_1 -snRNAs show negligible regulation by DCP2, making it unlikely that methylation of snRNAs confers resistance to DCP2.

When we examined our own and pre-existing²⁸ RNA-Seq datasets from FTO-depleted cells, we observed modestly increased levels of some (e.g. U1, U4, and U5) Sm-class snRNA transcripts, but not all transcripts encoded by the different genes that encode these Sm-class snRNAs (**Figs. 4c, d and Extended Data Figs. 9b, c**). These data suggesting that m^6 Am can affect snRNA levels to some extent. However, this does not seem to be a general phenomenon, since expression of U3, U7 and U8, which are functionally distinct but also acquire m^6 Am in *FTO* knockout cells (**Extended Data Figs. 3a-c**), was largely unchanged (**Figs. 4c, d and Extended Data Fig. 9d**).

Surprisingly, Lsm-class snRNAs (i.e. U6 and U6atac) showed a large increase in expression (**Figs. 4c, d and Extended Data Fig. 9c**). These snRNAs (i.e. U6 and U6atac) lack m^6 Am since they do not have adenosine at the first transcribed nucleotide, and show no alteration in $6mAm^6A$ levels in their transcript body in *FTO* knockout cells based on miCLIP (**Fig. 1g and Extended Data Fig. 4a**). Although U6 does not contain m^6 Am, U6 snRNPs assemble into multiple distinct complexes with FTO-regulated Sm-class snRNPs. The increase in U6 snRNA levels in FTO-deficient cells may therefore be the consequence of altered interactions of U6 with m_2 -snRNPs compared to m_1 -snRNPs. Overall, these data demonstrate that FTO deficiency increases levels of U6, which could therefore impact by splicing by promoting U6-dependent splicing events.

Discussion

A major class of cellular noncoding RNA are the snRNAs, which are assembled into ribonucleoproteins and have central roles in RNA processing reactions such as splicing. Although snRNAs are often regulated by trans-acting splicing factors that modulate the association of snRNPs to specific transcripts, the modified nucleotides in snRNAs have not previously been thought to be the target of regulation. Here we show that snRNAs contain a regulated nucleotide methylation switch that allows them to exist as either of two distinct methylation isoforms. This methyl modification is located at the adenosine of the first encoded nucleotide in mRNA, is reversible, and is correlated with enhanced snRNA abundance and altered splicing profiles in cells. Together, our studies provide evidence that reversible epitranscriptomic information is encoded at the first nucleotide of snRNAs and that the reversible and regulated methylation of this nucleotide is a key aspect of snRNA biogenesis.

Our study focused on snRNA biogenesis and identified the formation of m_2 -snRNAs and its subsequent demethylation to m_1 -snRNA as a previously unknown step in snRNA maturation (**Fig. 5**). snRNA biogenesis was extensively studied and involves Sm core assembly, 3' processing, nucleotide modifications at internal sites and formation of the hypermethylated guanosine cap^{6-8,11,14,17}. Although Sm-class snRNAs were known to contain a 2'-*O*-methyl modification at the evolutionarily conserved starting-adenosine, our data reveal that snRNA biogenesis also involves a previously unrecognized N^6 -methylation of this nucleotide. We find that the N^6 -methyl modification is subsequently removed by FTO, thus converting m_2 -snRNAs to m_1 -snRNAs. Our studies reveal that snRNAs exhibit a more complex process of biogenesis than previously known, involving reversible methylation of the extended snRNA cap.

Although the enzyme that forms m^6 Am in snRNAs is unknown, previous studies revealed a nuclear enzymatic activity that generates m^6 Am. Partially purified nuclear lysates are capable of performing N^6 -methylation of 2'-*O*-methyladenosine adjacent to m^7 G in RNA substrates⁴⁵. Notably this enzyme was shown to be distinct from METTL3/METTL14 that performs N^6 -methylation of internal adenosines in a purine-A-C context⁴⁶. Thus, this enzyme may also have a role in snRNA biogenesis.

FTO was previously shown to influence alternative splicing. These splicing changes were initially attributed to FTO-regulated m⁶A residues around splice-sites, since at the time, FTO was thought to target m⁶A in mRNA based on its low, but measurable, demethylation activity towards m⁶A^{28,29}. However, a role for m⁶A in alternative splicing has recently been challenged⁴⁷. Our findings potentially provide a resolution by showing that FTO regulates m⁶Am in spliceosomal snRNAs. Thus, our discovery of FTO-regulated m₂-snRNAs provides the first direct link between FTO activity and the mRNA splicing machinery.

The decision to demethylate the m₂-snRNA appears to be determined early in snRNA biogenesis. We find that FTO-mediated demethylation of m₂-snRNAs is inhibited when 2-hydroxyglutarate levels are increased due to expression of a cancer-associated mutant IDH. FTO was previously shown to be inhibited by metabolites that compete with α -ketoglutarate, including citrate, succinate, fumarate and 2-hydroxyglutarate^{37,38}. Altered levels of these metabolites are seen in diverse cancers⁴⁸⁻⁵¹, as well as during cellular differentiation^{52,53}. Our studies raise the possibility that 2-HG and potentially other metabolite abnormalities may mediate their effects, in part, by inhibiting FTO and increasing the levels of m₂-snRNAs that could in turn influence specific splicing events.

The presence of m₂-snRNAs is not restricted to cells that lack FTO or express mutant IDH. We also detected m₂-snRNAs in mouse embryonic stem cells, TF-1 cells and mouse liver tissue by miCLIP and mass spectrometry. It will be important to determine the full spectrum of cell type-specific regulation of m₂-snRNA expression *in vivo* as well as whether different levels of m₂-snRNAs accumulate in cells because of FTO inhibition or due to other mechanisms.

How does the N⁶-methyl modification affect the fate of snRNA? The altered splicing in cells expressing high levels of m₂-snRNAs are unlikely to reflect a direct effect of the N⁶-methyl modification on the snRNA:pre-mRNA hybridization steps. These hybrids mostly occur at internal sites in the snRNA. For example, U1 snRNA hybridizes to the 5' splice site using nucleotides 3-7 (ref. ⁵⁴). Therefore, snRNA binding and recognition of target sites in pre-mRNA are unlikely to be promoted by the N⁶-methylation status of the first nucleotide.

The splicing process involves dynamic assembly and disassembly of U6 and U6atac snRNPs with Sm-class snRNPs⁵⁵. It is intriguing to speculate that snRNP complex assembly dynamics are different between m₁- and m₂-snRNAs, ultimately impacting U6 expression levels. This may be mediated by dedicated reader proteins that alter the overall assembly, disassembly or turnover of multi- m₂-snRNP complexes. These alterations may in turn account for the increase in U6 snRNA levels. Therefore, it will be important to monitor the dynamics of multi-m₁- and m₂-snRNP complexes and to determine the transcriptome-wide binding properties of m₂-snRNPs.

Notably, recent studies showed that the levels of individual types of snRNAs can have large effects on splicing efficiency⁵⁶, and reduced snRNA expression is associated with severe splicing abnormalities^{44,57}. The increased expression of U6 and some Sm-class snRNAs associated with FTO-deficiency may influence splicing patterns by specifically promoting the inclusion of exons that are otherwise inefficiently spliced due to rate-limiting availability of individual snRNPs or their higher order snRNP complexes.

Although snRNAs were previously thought to be comprised of a single molecular species, the demonstration that snRNAs exist as distinct methyl isoforms indicates a potential regulatory role for methylation switches in splicing. Importantly, some snRNAs have non-splicing functions^{31,58,59} and their m₂-isoforms could therefore influence other RNA processing pathways. More research will be needed to understand how the levels of m₂-snRNPs are regulated and the diversity of RNA processing alterations associated with m₂-snRNAs.

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snRNP stability, binding and assembly. H.G. carried out the computational splicing analysis. S.R.J. and J.M. wrote the manuscript with input from all coauthors.

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Figure 1 | FTO selectively demethylates small nuclear RNAs.

a, N^6 -methyladenine ($6mAm^6A$) mapping in total RNA using miCLIP reveals FTO-dependent demethylation of small nuclear RNAs (snRNAs). To measure FTO-regulated m^6Am sites in RNAs, $6mAm^6A$ miCLIP reads were counted in a 20-nucleotide window surrounding the transcription-start nucleotide (TSN) and normalized to input RNA-Seq reads in the same region. The relative expression changes were calculated. The MA plot shows the \log_2 fold change in methylation in *Fto* knockout mouse liver (*Fto*^{-/-}) compared to wild-type liver (WT). Transcripts that showed statistically significant upregulation of transcription-start nucleotide methylation are indicated in red. These transcripts are derived exclusively from small nuclear RNA genes. Data represents the average from datasets of three independent biological replicates per genotype.

b, FTO deficiency leads to increased transcription-start nucleotide methylation of major spliceosomal snRNAs. miCLIP reads were counted in a 20-nucleotide window surrounding the transcription-start nucleotide, normalized to input RNA-Seq reads in the same region and relative expression changes were calculated. In this analysis, no *P*-value cut-off was applied. Instead, the mean \log_2 -fold change in transcription-start nucleotide methylation of specific snRNA gene classes (U1, U2, U4, U5 and U6) in *Fto* knockout mouse liver (*Fto*^{-/-}) compared to wild-type liver (WT) is shown. Notably, only snRNA genes transcribed by RNA polymerase II increased methylation, whereas U6 snRNA genes (grey), which are transcribed by RNA polymerase III and thus do not acquire a m^7G cap, show no change in transcription-start nucleotide methylation upon FTO deficiency (Data represents the average from datasets of three independent biological replicates per genotype; one-way ANOVA with Tukey's post hoc test *** $P \leq 0.001$).

c, U1 snRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U1 snRNA is shown. $6mAm^6A$ miCLIP reads are shown in the upper panel. As can be seen, miCLIP reads are readily detected at the transcription-start

nucleotide (TSN) in the U1 snRNA in *Fto*^{-/-} RNA, but is nearly undetectable in U1 snRNA from wild-type RNA. Input reads are shown in the lower panel. RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype.

d, U2 snRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U2 snRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. Although miCLIP reads are higher at the transcription-start nucleotide (TSN) and downstream region in the U2 snRNA in *Fto*^{-/-} RNA compared to wild-type RNA, these changes are not as robust as for e.g. U1 snRNA (see **Fig. 1c**). Input reads are shown in the lower panel. The transcription-start nucleotide is indicated in red and the constitutive internal N⁶,2'-*O*-dimethyladenosine (m⁶Am) residue at position 26 is indicated by the black dashed line. (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

e, U4 snRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U4 snRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. As can be seen, miCLIP reads are substantially higher at the transcription-start nucleotide (TSN) in the U4 snRNA in *Fto*^{-/-} RNA, but is reduced in the U4 snRNA from wild-type RNA. Input reads are shown in the lower panel. The transcription-start nucleotide is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

f, U5 snRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U5 snRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. As can be seen, miCLIP reads are substantially higher at the transcription-start nucleotide (TSN) in the U5 snRNA in *Fto*^{-/-} RNA, but is reduced in the U5 snRNA from wild-type RNA. Input reads are shown in the lower panel. The transcription-start nucleotide is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

g, U6 snRNA transcription-start nucleotide methylation is not affected in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U6 snRNA is shown. 6mAm⁶A miCLIP reads are shown in the upper panel. As can be seen, there are essentially no detectable miCLIP reads at the TSN in either the *Fto*^{-/-} or wildtype miCLIP samples in the U6 RNA. Therefore, U6 snRNA lacks 6mAm⁶A at the TSN. Input reads are shown in the lower panel. The constitutive internal N⁶-methyladenosine (m⁶A) residue at position 43 is indicated by the black dashed line. RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype.

h, FTO deficiency leads to increased methylation of mature snRNAs. 6mAm⁶A-immunoblot of wild-type (WT) and FTO knockout (*FTO*^{-/-}) HEK293 cells. Methylation of the most abundant snRNAs (U1, U2, U6) was detected with an antibody directed against 6mAm⁶A. The molecular weights of the 6mAm⁶A-reactive bands are consistent with the mature processed forms of the snRNA, demonstrating that the mature snRNA, rather than an snRNA precursor, contains elevated 6mAm⁶A in *FTO* knockout cells. The left panel shows a representative 6mAm⁶A-immunoblot with the positions of nucleotide size markers and the individual snRNAs indicated on the left and right, respectively. The right panel shows the quantification of 6mAm⁶A signal in U1 and U2 snRNA relative to the 6mAm⁶A signal in U6 snRNA. U6 snRNA was used for normalization since it has an internal m⁶A residue that is constitutive and lacks m⁶Am, and therefore is not regulated by FTO. Notably, we observed strong 6mAm⁶A immunoreactivity towards U2 snRNA in WT cells and increased methylation in *FTO*^{-/-} cells did not reach statistical significance. However, U2 snRNA contains a constitutive internal m⁶Am residue (see also **Fig. 1d**) that is not demethylated by FTO, which likely masks FTO deficiency-dependent effects ($n = 3$ independent biological replicates; mean \pm s.e.m.; unpaired student's *t*-test *** $P \leq 0.001$).

Figure 2 | Reversible N⁶,2'-O-dimethyladenosine (m⁶Am) in small nuclear RNAs.

a, FTO deficiency reveals the presence of m⁶Am in small RNAs. The relative abundance of modified adenosines in small RNA caps derived from wild-type (WT) and FTO-deficient (*FTO*^{-/-}) HEK293 cells was determined by thin layer chromatography. The left panel shows a TBE-Urea gel image stained with SYBR Gold, where the red dashed line indicates the small RNA fraction (all RNAs < 200 nt) that was used for the analysis. The middle panel shows a representative image of the migration pattern of radiolabeled nucleotides, where the position of

m⁶Am and 2'-*O*-methyladenosine (Am) is indicated by the dashed black circles. The level of m⁶Am is markedly increased in small RNA in *FTO* knockout cells. The right panel shows the quantification of the m⁶Am/Am ratio in small RNA ($n = 3$ independent biological replicates; mean \pm s.d; unpaired student's *t*-test $**P \leq 0.01$).

b, *FTO* deficiency reveals the presence of m⁶Am in U1 snRNA. In contrast to (a), here we isolated the U1 snRNA by excising the RNA from a TBE-Urea gel image stained with SYBR Gold (left). The red dashed line indicates the U1-enriched fraction that was gel-extracted and used for the analysis. The relative abundance of m⁶Am and Am in U1 snRNA caps derived from wild-type (WT) and *FTO*-deficient (*FTO*^{-/-}) HEK293 cells was determined by thin layer chromatography. The level of the m⁶Am cap in U1 snRNA is markedly increased in *FTO* knockout cells. The migration position of m⁶Am and 2'-*O*-methyladenosine (Am) is indicated by the dashed black circles. The right panel shows the quantification of the m⁶Am/Am ratio in the U1-enriched fraction ($n = 3$ independent biological replicates; mean \pm s.d; unpaired student's *t*-test $**P \leq 0.01$).

c, *FTO* deficiency increases the relative abundance of m₂-snRNA caps in HEK293 cells. Small RNA from WT and *FTO*^{-/-} was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the ratio of m₂-snRNA caps (m⁶Am) to m₁-snRNA caps (Am) represented by the integrated peak area ratio of the corresponding MRM transitions (recorded in positive ion mode) ($n=3$ independent biological replicates, mean \pm s.d.; unpaired Student's *t*-test, $*** p < 0.001$).

d, Differential expression of m₂-snRNA (cap-ppp-m⁶Am) caps across cells and tissues. Small RNA from wild-type HEK293 cells, naïve mouse embryonic stem cells (mESCs), TF-1 erythroleukemia cells, as well as mouse liver and brain tissue was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the ratio of m₂-snRNA caps (m⁶Am) to m₁-snRNA caps (Am) represented by the integrated peak area ratio of the corresponding MRM transitions (recorded in positive ion mode). These data suggest that the abundance of m₂-snRNAs varies in a cell-specific manner (n.d.=cap-ppp-m⁶Am not detected; $n=3$ independent biological replicates, mean \pm s.d.; unpaired Student's *t*-test, $** p < 0.01$).

e, Increased abundance of m₂-snRNA caps in oncometabolite-dependent cancer. Small RNA from wild-type (Ctrl) and mutant IDH-expressing cells was digested with nuclease P1 to

specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the ratio of m_2 -snRNA caps (m^6Am) to m_1 -snRNA caps (Am) represented by the integrated peak area ratio of the MRM transition (recorded in positive ion mode). IDH1^{R132H}- and IDH2^{R140Q}-expressing TF-1 cells have high levels of 2-hydroxyglutarate (2-HG). 2-HG is a natural inhibitor of FTO activity³⁸ and leads to increased abundance of m_2 -snRNAs. Specific inhibition of the mutant IDH1 (AGI-5027) and IDH2 (AGI-6780) isoforms shows that these effects are reversible by blocking production of 2HG ($n=3$ independent biological replicates, mean \pm s.d.; one-way ANOVA with Tukey's post hoc test $*P \leq 0.05$, $**P \leq 0.01$).

Figure 3 | m_2 -snRNAs are incorporated into snRNPs.

a, FTO does not exhibit measurable demethylation of m^6Am in RNA containing a $N^{2,2,7}$ -trimethylguanosine (TMG) cap. snRNAs contain a m^7G cap shortly after their synthesis, but acquire a TMG cap during snRNA maturation. To determine whether FTO demethylates mature snRNA, we incubated full-length human FTO with synthetic 20-mer RNA oligonucleotides starting either with a 5'- m^7G -ppp- m^6Am or 5'-TMG-ppp- m^6Am . In the context of an m^7G cap, FTO readily converted m^6Am to Am (left panels). However, the presence of an TMG cap completely blocked FTO demethylation activity towards m^6Am (right panels), indicating that snRNAs starting with a TMG-ppp- m^6Am are not a physiological target of FTO. This finding suggests that FTO demethylates m^6Am in snRNA before the formation of an TMG cap (representative HPLC track shown; $n = 3$ independent experiments).

b, m_2 -snRNAs are incorporated into small nuclear ribonucleoproteins (snRNPs). snRNA function is linked to its incorporation into spliceosomal snRNPs. To test whether m_2 -snRNAs are incorporated into snRNPs, immunoprecipitation of the SmB spliceosomal protein was performed. The relative abundance of modified adenosines in SmB-bound small RNA caps derived from wild-type (WT) and FTO-deficient ($FTO^{-/-}$) HEK293 cells was determined by thin layer chromatography. In WT cells, SmB-bound carried predominately Am caps. However, in $FTO^{-/-}$ cells, m^6Am was readily detected in the SmB-bound fraction, indicating that m_2 -snRNAs are incorporated into snRNPs and thus become part of the cellular splicing machinery. The left panel shows a representative image of the migration pattern of radiolabeled nucleotides, where the position of m^6Am and Am is indicated by the dashed black circles. The right panel shows the

quantification of the m⁶Am/Am ratio in small RNA ($n = 3$ independent biological replicates; mean \pm s.d.; unpaired student's t -test *** $P \leq 0.001$).

c, m₂-snRNAs have TMG caps. We asked if the presence of m⁶Am in snRNA blocks the maturation of the snRNA cap from m⁷G to TMG. Small RNA from FTO-deficient HEK293 cells and tissues was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Small RNA from FTO-deficient cells exhibit high levels of m⁶Am, thereby allowing an assessment of whether m⁶Am affects the formation of TMG caps. Shown is the ratio of m₂-snRNA TMG caps to m₁-snRNA TMG caps represented by the integrated peak area ratio of the multiple reaction monitoring (MRM) transition 843.2 \rightarrow 194.1 for TMG-ppp-m⁶Am and 829.1 \rightarrow 194.1 for TMG-ppp-Am (recorded in positive ion mode). These data demonstrate that m₂-snRNA contain TMG caps, thus indicating that m⁶Am does not function to regulate cap maturation ($n=3$ independent biological replicates, mean \pm s.d.).

Figure 4 | FTO depletion promotes exon inclusion and increases expression of Sm- and Lsm-class snRNAs.

a, FTO deficiency leads to exon inclusion. In line with previous reports^{28,29}, transcriptome-wide alternative splicing analysis shows increased abundance of alternatively spliced exons in FTO deficient (*FTO*^{-/-}) HEK293T cells. These data indicate that m₂-snRNAs, which are the predominant snRNA isoform in FTO deficient cells, promote the inclusion of exons that are normally excluded in wild-type cells. (PSI = percent spliced in; Bayes factor cut-off > 10).

b, m₂-snRNA-regulated exons show enrichment of specific splicing regulator binding motifs. FIRE⁶⁰ analysis revealed that the exons preferentially included in FTO-deficient HEK293T cells are enriched in binding sites for SRSF1, HNRNPH2 and HNRNPK. These data suggest that the presence of m₂-snRNAs changes binding activity of specific splicing regulators. For each motif, we indicate the mutual information (MI) value, Z-score associated with the MI value, and the robustness score ranging from 0/10 to 10/10. The enrichment score indicates over-representation of a motif; significant over-representation is indicated by red frames, whereas blue frames indicate under-representation.

c, FTO deficiency increases the abundance of Sm- and Lsm-class snRNAs. The MA plot of RNA-Seq analysis of total cellular RNA from previously published datasets²⁸ shows the transcriptome-wide log₂ fold change of gene expression in *FTO* knockout HEK293T cells (*FTO*^{-/-}).

^{-/-}) compared to wild-type HEK293T cells (WT). Significantly regulated Sm-class snRNAs (Sm-snRNAs) are indicated in red, significantly regulated Lsm-class snRNAs (Lsm-snRNAs) are indicated in orange. Notably, all significantly changed snRNAs show upregulation in *FTO*^{-/-} cells. Since snRNAs largely exist in the m₂-form in *FTO*^{-/-} cells, these data suggest that m₂-snRNAs are intrinsically more stable than m₁-snRNAs that are predominately found in WT cells. Notably, Lsm-snRNAs (U6 and U6atac) were also upregulated in *FTO*^{-/-} cells. Since Lsm-snRNAs are not directly regulated by FTO, these effects are likely to be indirect e.g. by changes in general snRNP turnover (Data represents the average of three independent biological replicates per genotype).

d, Sm- and Lsm-class snRNAs show higher increase in expression levels than m⁶Am-initiated mRNAs in *FTO*-deficient cells. mRNAs were classified based on their annotated starting nucleotide (m⁶Am, or Nm). Shown are log₂ fold changes of expression for snRNAs and mRNAs. As previously described²², *FTO* deficiency increases the abundance of m⁶Am-mRNAs compared to mRNAs starting with Am, Cm, Gm or Um (here cumulatively called Nm). However, snRNAs were upregulated even more in *FTO*-deficient cells (*FTO*^{-/-}) indicating that snRNAs are the major RNA class regulated by *FTO*. (Data represents the average from datasets of three independent biological replicates per genotype; one-way ANOVA with Tukey's post hoc test **P* ≤ 0.05, ****P* ≤ 0.0001)

Figure 5 | Proposed model of m₂-snRNA biogenesis.

Before their export to the cytosol, Sm-class snRNAs (m₁-snRNAs) can be subjected to N⁶-methylation of the m⁷G cap-adjacent 2'-*O*-methyladenosine (Am). This leads to the formation of m₂-snRNAs containing an m⁷G cap-adjacent N⁶,2'-*O*-dimethyladenosine (m⁶Am).

Under basal conditions, m₂-snRNAs can be converted back to the m₁ isoform by the nuclear RNA demethylase *FTO*. Similar to m₁-snRNAs, m₂-snRNAs are subjected to the normal biogenesis pathway, which involves Sm core assembly, hypermethylation of the m⁷G cap to N^{2,2,7}-trimethylguanosine (TMG), reimport into the nucleus, and formation of functional small nuclear ribonucleoprotein complexes (snRNPs) that carry out pre-mRNA splicing.

Notably, in conditions where specific metabolites, such as 2-hydroxyglutarate (2-HG), *FTO* activity is inhibited^{37,38}. This results in high levels of m₂-snRNAs, which leads to inclusion of alternative exons and ultimately determines transcriptome diversity.

Methods

miCLIP-based mapping of 6mA⁶A and RNA-Seq analysis

For miCLIP experiments, total RNA was extracted from *Fto* knockout mouse livers (*Fto*^{-/-}) and wild-type mouse livers using RNazol RT (Molecular Research Center, Inc.). miCLIP was carried out as described previously³⁰. In miCLIP, 6mA⁶A is mapped by immunoprecipitation and crosslinking with a 6mA⁶A-binding antibody (see below)³⁰. This mapping method detects both m⁶A and m⁶Am, the two 6mA⁶A-containing nucleotides in RNA. RNA-Seq libraries of input and 6mA⁶A immunoprecipitated and crosslinked samples were prepared using a cloning strategy according to the miCLIP protocol^{30,61} and submitted to the Weill Cornell Medicine Epigenomics Core for sequencing on the Illumina HiSeq 2500 instrument, in paired-end mode, with 50 bases per read. Three independent biological replicates were sequenced for each condition. Raw input and miCLIP reads were trimmed of their 3' adapter and demultiplexed as previously described³⁰.

To analyze 6mA⁶A coverage at the transcription start nucleotide (TSN) and internal sites, the following steps were carried out. Input and miCLIP reads were aligned to mm10 with STAR to generate RPM-normalized bedgraphs and sorted bam files⁶² (version 2.5.2a;). Bedgraphs were visualized using the Integrative Genomics Viewer⁶³ (IGV, version 2.4.4). Sorted bam files were converted to bed format and read coverage was determined in a 20-nucleotide window flanking annotated TSN (UCSC) or a 4-nucleotide window flanking the internal site of interest using bedtools (version 2.25.0). miCLIP TSN coverage reads were then counted and normalized to input coverage reads using the Deseq2 pipeline adjusted for CLIP-Seq⁶⁴ (version 1.18.1; commands: design= ~ assay + condition + assay:condition; test="LRT", reduced= ~ assay + condition). Analysis and visualization was carried out with custom in-house generated R-scripts using RStudio (Version 1.0.136). Only transcripts with normalized read counts > 10 were included in the analyses.

For all other RNA-Seq analyses, total RNA was diluted to a concentration of 50 ng/μl and submitted to the Weill Cornell Medicine Epigenomics Core for isolation of mRNA and library preparation using the Illumina TruSeq Stranded mRNA Library Prep Kit (RS-122-2101, Illumina). The libraries were sequenced on the Illumina HiSeq 2500 instrument, in paired-end

mode, with 100 bases per read. At least two independent biological replicates were sequenced for each condition.

Previously published RNA-Seq datasets utilized in the current study^{22,28} were extracted from Gene Expression Omnibus (GEO, NCBI) and, if no processed data was available, the fastq-files were reanalyzed with the pipelines described above.

Cell culture and animals

Flp-In T-REx HEK293 cells (R78007, ThermoFisher Scientific) and *DCP2* knockout (*DCP2*^{-/-}) HEK293 cells²² were maintained in DMEM (ThermoFisher Scientific) with 10% FBS and antibiotics (100 units/ml penicillin and 100 µg/ml of streptomycin) under standard tissue culture conditions. Cells were split using TrypLE™ Express (Life Technologies) according to the manufacturer's instructions.

Control, IDH1^{R132H} and IDH2^{R140Q} TF-1 erythroleukemia cells were generated and maintained as previously described⁴⁰. Intracellular 2-hydroxyglutarate concentrations were determined as previously described⁴⁰. All experiments involving TF-1 cells were carried out between passage 5-10. To inhibit production of neomorphic 2-hydroxyglutarate, IDH1^{R132H} cells or IDH2^{R140Q} cells were treated for 7 days with 1 µM AGI-5027 (SML1298, Sigma-Aldrich) or AGI-6780 (SML0895, Sigma-Aldrich), respectively.

Naive mouse embryonic stem cells (mESCs) were maintained in Knockout DMEM (ThermoFisher Scientific) with 15% FBS, antibiotics (100 units/ml penicillin and 100 µg/ml of streptomycin), 2mM L-glutamine, 50 µM β-mercaptoethanol (ThermoFisher Scientific), non-essential amino acids (ThermoFisher Scientific), recombinant mouse LIF (EMD Millipore), GSK3 inhibitor (04000402, Stemgent) and MEK1 inhibitor (04000602, Stemgent) under standard tissue culture conditions. To induce differentiation, naive mESCs were deprived of LIF, GSK3 inhibitor and MEK1 inhibitor for 7 days. Mycoplasma contamination in all cell lines used in this study was routinely tested by PCR.

Fto knockout mice were bred as previously described²⁵. Only male mice at the age of 12-16 weeks were used for the experiments. All experiments involving mice were approved by the Institutional Animal Care and Use Committee at Weill Cornell Medicine.

Antibodies

Antibodies used were as follows. For western blot analysis mouse α -FTO (ab92821, Abcam) and mouse α -ACTB (A2228, Sigma) were used. For $6mAm^6A$ -immunoprecipitation/miCLIP, rabbit α - $6mAm^6A$ (202-003, Synaptic Systems) was used. For SmB-IP, a previously described mouse α -SmB antibody (clone 18F6)⁶⁵ was used. For $6mAm^6A$ immunoblotting, a rabbit α - $6mAm^6A$ antibody (ab190886, Abcam) was used.

Generation of *FTO* knockout cells

FTO knockout cells were generated by transfecting Double Nickase plasmids (sc-403708-NIC, Santa Cruz Biotechnology) containing two guide RNAs (Strand A: 5'-CGGTCCCCTGGCCAGTGAAA-3'; Strand B: 5'-CCTGGTGTTTCAGGTACTTGT-3') into Flp-In T-REx HEK293 cells (Thermo Fisher Scientific) using LipoD293 (SigmaGen Laboratories). 24 hours after transfection, GFP-positive cells were isolated by flow cytometry and reseeded. 48 hours after transfection, cells were subjected to puromycin selection (5 μ g/ml) for three days. Cells were then reseeded at a density of 0.5 cells/well in 96-well plates for clonal selection. Loss of FTO protein expression was confirmed by Western blot. All experiments were carried out between passage 5-15.

$6mAm^6A$ immunoblot

Denatured small RNA samples (5 μ g) were separated by 10% polyacrylamide TBE-Urea denaturing gel electrophoresis at 200 V in 1x TBE. For blotting, separated RNA was transferred onto BrightStar-Plus positive charged nylon membranes (ThermoFisher Scientific) by semidry electroblotting in 0.5x TBE buffer at 300 mA for 30 min.

After UV crosslinking at 120,000 μ J/cm² in a Stratalinker UV crosslinker (Stratagene), the membrane was blocked with 1% dry milk powder in PBS-T for 1 h at room temperature and then incubated with the α - $6mAm^6A$ (1:500) overnight at 4°C. After washing with PBS-T, membranes were incubated with α -rabbit HRP-conjugated secondary antibody diluted 1:2500 with 1% dry milk powder in PBS-T for 1 h at room temperature. The membrane was visualized by Pierce

ECL Western Blotting Substrate (ThermoFisher Scientific) and a Bio-Rad Gel Doc XR system. The quantification of the band intensity was analyzed using Bio-Rad Image Lab software.

Determination of relative m⁶Am and Am levels in small RNAs by thin layer chromatography

The m⁶Am/Am ratio in small RNAs was determined as previously described²², with some modifications. The small RNA fraction (< 200 nucleotides) was isolated from total RNA using RNazol RT (Molecular Research Center, Inc.).

For the analysis of individual U-RNAs, the small RNA fraction was separated on 6% TBE-Urea gels. Small RNAs were stained with SYBR Gold (ThermoFisher Scientific) and the respective bands (~160 nucleotide for U1 snRNA, and ~180 nucleotides for U2 snRNA) were excised and extracted using ZR small-RNA PAGE Recovery kit (Zymo Research). Notably, U1 snRNA co-migrates with 5.8S rRNA. However, 5.8S rRNA is not a possible source of m⁶Am contamination, since it does not start with adenosine.

200 ng of the small RNA fraction or 40 ng of individual U-RNAs were decapped with 25 units of RppH (NEB) for 3h at 37 °C. The 5' phosphates of the exposed cap-adjacent nucleotide were removed by the addition of 5 units rSAP (NEB) and further incubated for 1 hour at 37°C. Up to this point, all enzymatic reactions were performed in the presence of SUPERase In RNase Inhibitor (ThermoFisher Scientific). After phenol-chloroform extraction and ethanol precipitation, RNA samples were resuspended in 10 µl of DEPC-H₂O and 5' ends were labeled using 30 units T4 PNK and 0.8 mBq [γ -³²P] ATP at 37°C for 30 min. This step labels the 5' ends of RNAs that are exposed as a result of RppH treatment, as well as any 5' ends that are present due to sheared RNA. PNK was heat inactivated at 65°C for 20 min and the reaction was passed through a P-30 spin column (Bio-Rad) to remove unincorporated isotope. The labeled RNA was then digested with 4 units of P1 nuclease (Sigma) for 3h at 37°C.

The nucleotide mixture was subsequently dried using an Eppendorf Vacufuge and reconstituted with 3 µL ddH₂O. 1 µl of the released 5' monophosphates from this digest were then analyzed by 2D-TLC on glass-backed PEI-cellulose plates (MerckMillipore) as described previously²². Developed TLC plates were imaged as described previously²².

Although this TLC assay can readily detect whether m⁶Am or Am is at the first nucleotide position after the cap, there are background spots in this assay since RNA is prone to low-level fragmentation and shearing which can expose 5' ends for phosphorylation. As described in the main text, a mass spectrometry approach was also used to identify the methylation state of the first nucleotide of snRNA. The results were consistent using both these different analytical approaches.

Protein expression and purification

Full-length, recombinant human FTO was expressed and purified as previously described²². Notably, to avoid any unspecific activity, it is advisable to express full-length FTO since the positively charged N-terminus of FTO may interact with the negatively charged m⁷G-ppp-cap of RNA.

Recombinant human TGS1 (hTGS1) was expressed and purified as previously described^{66,67}. Briefly, *E. coli* Tuner cells transformed with pRSET-A-hTGS1₆₁₈₋₈₅₃ were grown at 37 °C in 2YT medium until OD₆₀₀ of 0.6 and induced with 0.2 mM IPTG. After overnight expression at 18°C, cells were lysed and the protein was purified via Ni-NTA (binding buffer: 50 mM Tris, pH 8, 1 M NaCl, 10 % glycerol; elution buffer: additional 500 mM imidazole) followed by gel filtration (Superdex 75, running buffer: 50 mM Tris, pH 7.5, 200 mM NaCl, 10 % glycerol) to obtain RNase-free protein that was concentrated to ~1.5 mg/mL (MW: 27 kDa), flash frozen and stored at -20°C.

Synthesis and characterization of synthetic oligonucleotides

Synthetic RNA oligonucleotides were chemically assembled as previously described²². To generate m^{2,2,7}Gppp-capped oligonucleotides, m⁷Gppp-capped synthetic oligonucleotides (ONs) were treated with recombinant human TGS1. To do this, reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 50 mM NaCl, 1 mM AdoMet, and 150 μM pure m⁷G-ppp-ON (~50 nmoles) and 2.5 μM hTgs1 were incubated at 37°C. Aliquots were withdrawn at different times and then analyzed by RP-HPLC to monitor the reaction (20 min linear gradient of 0-24% CH₃CN in 50 mM TEAAc buffer pH 7). After 7 h incubation, the N²-Guanine-dimethylation was complete. Proteic material was then removed through a Sep PakTM C₁₈ cartridge. After

dilution with 4 mL 100 mM TEAAc, pH 7 the crude mixture was loaded onto the cartridge and a wash was performed with 10 mL of 100 mM TEAAc then ON elution was made with 10 mL 50% CH₃CN in 12.5 mM TEAAc. The solution was dried by lyophilization. Remaining AdoMet and the *S*-adenosylhomocysteine (AdoHcy) byproduct were removed as follows: the dry residue was dissolved with 1 mL H₂O and the solution was loaded onto a Sephadex G-25 gel filtration cartridge (NAPTM-10 cartridge). Elution was performed with 1 mL H₂O. When necessary, m^{2,2,7}Gppp-capped ONs were purified by IEX-HPLC and they were characterized by mass spectrometry MALDI-TOF as previously described²².

Measurement of FTO activity

In vitro demethylation measurements were performed as described previously²². Briefly, demethylation activity assay was performed in 20 µl of reaction mixture containing 5 µM synthetic RNA oligonucleotide (either 5'-m⁷G-ppp-m⁶Am-CACUUGC UUUUGACACAACU-3' or 5'-TMG-ppp-m⁶Am-CACUUGC UUUUGACACAACU-3'), 20 nM recombinant, full-length human FTO, 75 mM of (NH₄)₂Fe(SO₄)₂, 300 mM α-ketoglutarate, 2 mM sodium L-ascorbate, 150 mM KCl and 50 mM HEPES buffer, pH 7.0. The reaction was incubated at 37°C for 10 min and quenched by the addition of 1 mM of EDTA followed by inactivation of the enzyme for 5 min at 95°C.

Sample preparation for HPLC analysis

After treatment with recombinant, full-length human FTO, oligonucleotides were decapped with 25 units of RppH (NEB) in ThermoPol buffer for 3 h at 37°C. RNA was subsequently digested to single nucleotides with 200 units S1 nuclease (Takara) for 2h at 37°C. 5' phosphates were removed with 5 units rSAP (NEB) for 1h at 37°C. Before loading the samples onto the HPLC column, proteins were removed by size exclusion chromatography with a 10-kDa cut-off filter (VWR).

HPLC analysis of demethylation activity

The HPLC analysis of nucleosides was performed on an Agilent 1100 system (Agilent Technologies). Separation was performed on a Poroshell 120 EC-C18 column (4 μ m, 150 \times 4.6 mm, Agilent Technologies) equipped with an EC-C18 Guard cartridge (Agilent Technologies) at 22°C. The mobile phase consisted of buffer A (25 mM NaH₂PO₄) and buffer B (100% acetonitrile). Pump control and peak integration was achieved using the ChemStation software (Rev. A.10.02, build 1757, Agilent Technologies). Samples were analyzed at 2 ml/min flow rate with the following buffer A/B gradient: 7.5 min 95%/5%, 0.5 min 90%/10%, 2 min 10%/90%, 1 min 95%/5%. Retention times of the individual nucleosides were determined with synthetic standards (6.2 min for 2'-*O*-methyladenosine (Am), and 7.2 min for *N*⁶,2'-*O*-dimethyladenosine (m⁶Am).

Sample preparation for mass spectrometry

In addition to their modified starting nucleotides (m⁶Am or Am), most snRNAs contain additional internal 2'-*O*-methylated adenosine (Am) residues¹³. Due to possible shearing of the RNA during the extraction process, these internal Am residues could be exposed and lead to unspecific background signal in thin layer chromatography. To eliminate this possible source of error, we sought to develop an independent method to measure the precise ratio of m⁶Am and Am caps in snRNA. We therefore developed a protocol to liberate intact cap-dinucleotides from the snRNA backbone and detect these cap-dinucleotides by LC-MS/MS.

To do this, cellular small RNA (0.1-1 μ g) was digested with P1 nuclease (0.5-5 units) for 3 hours at 37°C. Notably, P1 nuclease does not cleave the triphosphate linker of the cap and thus specifically releases the cap-ppp-N dinucleotide, while digesting the RNA backbone down to single nucleotides. Proteins were removed by size exclusion chromatography with a 10-kDa cut-off filter (VWR).

The RNA was dried using an Eppendorf Vacufuge and reconstituted with 5 μ L ddH₂O. An aliquot was diluted 1:10-1:20 with 80% methanol in ddH₂O. 2 μ l of the resulting solution were subjected to LC-MS/MS analysis (see below).

Identification of TMG-ppp-m⁶Am by mass spectrometry

An aliquot of the $m^{2,2,7}G$ -ppp- m^6Am (TMG-ppp- m^6Am) standard solution (generated by subjecting our synthetic 20-mer oligonucleotides to P1 nuclease digest) was subjected to LC/MS-MS analysis by an Agilent 1200 LC-system coupled to an Agilent 6538 a quadrupole time-of-flight mass spectrometer equipped with a dual electrospray ionization (ESI) source. A second isocratic pump delivered an internal reference mass solution (ions for negative mode 119.0360 and 966.0007; ions for positive mode 121.0509 and 922.0098) over the second nebulizer into the dual ESI source for continuous calibration during sample analysis. The sample injection volume was 4 μ L. Chromatographic separation of the FTO-reaction products was performed using an aqueous normal phase (ANP) column (CogentTM Diamond Hydride, 4 μ m particle size, 150 mm x 2.1 mm; Microsolv Technology Corporation, NJ). To extend the column lifetime, a precolumn filter (0.5 μ m, Microsolv) was placed in front of the ANP column. The mobile phase consisted of (A) 50% isopropanol with 0.025% acetic acid; and (B) 90% acetonitrile containing 5 mM ammonium acetate. To eliminate the interference of metal ions on the chromatographic peak integrity and ESI ionization, EDTA was added to the mobile phase in a final concentration of 6 μ M. The final gradient applied was: 0-1.0 min 99% B, 1.0-7.0 min to 80% B, 7.0-18.0 min to 50% B, 18.0-19.0 min to 0% B and 19.1 to 33.0 min 99% B to regenerate the column. The flow rate was 0.4 mL/min. To reduce the amount of introduced salt and other interfering sample components, the first 0.5 min of the column effluent were directed to waste. Both positive and negative mass spectra were acquired in the 2 GHz extended dynamic range mode with 1.41 spectra/sec, sampled over a mass/charge range of 50-1000⁶⁸. The operating ESI-source parameters for MS-analysis were: gas temperature 300 °C; drying gas flow 10 L/min; nebulizer pressure 35 psi; TOF capillary voltage 3500 V; fragmentor voltage 140V; skimmer voltage 65 V. Data was saved in centroid mode. Data was processed using Agilent MassHunter Qualitative Analysis Software (B.7. 00 Build 7.0.7024.0, Agilent Technologies).

Analysis of TMG-ppp- m^6Am and TMG-ppp-Am by mass spectrometry

Mass spectrometry was used to quantify relative changes in the levels of TMG-ppp- m^6Am and TMG-ppp-Am between conditions indicated in each figure. Samples were injected into an LC/MS-system comprised of an Agilent 1260 HPLC and an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a JetStream electrospray ionization source, using positive ion-monitoring in multiple reaction monitoring. The RNA-caps

were resolved on an aqueous normal phase column (Cogent™ Diamond Hydride, 4 µm particle size, 150 mm x 2.1 mm; Microsolv Technology Corporation, NJ), at a column compartment temperature of 40°C. The samples were maintained at 4°C and the injection volume was 2 µL. The gradient-chromatography previously described by Chen *et al.*⁶⁸ was optimized to achieve chromatographic separation of TMG-ppp-m⁶Am and TMG-ppp-Am. The aqueous mobile phase (A) was 50% isopropanol with 0.025% acetic acid, the organic mobile phase (B) was 90% acetonitrile containing 5 mM ammonium acetate. To eliminate the interference of metal ions on the chromatographic peak integrity and ESI ionization, EDTA was added to the mobile phase in a final concentration of 6 µM. The final gradient applied was: 0-1.0 min 99% B, 1.0-7.0 min to 80% B, 7.0-18.0 min to 50% B, 18.0-19.0 min to 0% B and 19.1 to 29.0 min 99% B to regenerate the column. The flow rate was 0.4 mL/min during data acquisition and 0.6 mL/min during re-equilibration from 21.0 to 28.5 min. Data was saved in centroid mode using Agilent Masshunter workstation acquisition software (B.06.00 Build 6.0.6025.4 SP4). Acquired raw data files were processed with Agilent MassHunter Qualitative Analysis Software (B.07.00 Build 7.0.7024.0, Agilent Technologies). The operating source parameters for MS-analysis were: gas temperature 300°C; gas flow 10 L/min; nebulizer pressure 35 psi; sheath gas temperature 400°C; sheath gas flow 11 L/min; capillary voltage 3500 V; nozzle voltage 0 V; fragmentor voltage 100V; cell accelerator voltage 7 V. Multiple reaction monitoring (MRM) data was acquired for the time segment 10-15 min in which the LC-flow was directed to the MS.

Product ion scans were performed at collision energies of 10, 20, 30, 40, 50 and 60 V, selecting [M+H]⁺ 843.2 as precursor ion for TMG-ppp-m⁶Am and [M+H]⁺ 829.1 for TMG-ppp-Am. Product ions were scanned in an *m/z* range of 50 – 850.

Optimized MRM transitions resulted at a collision energy of 50 eV and represented the deglycosylated base ions: for TMG-ppp-m⁶Am the transition 843.2→194.1* represented the formation of TMG and 843.2→150.1** the formation of m⁶Am, accordingly for TMG-ppp-Am 829.1→194.1* represented the formation of TMG and 829.1→136.1** the formation of Am. The transitions 843.2→438.1 and 829.1→424.1 were acquired as additional quality indicator. * indicates quantifier transitions, ** indicates the qualifier transitions.

Immunoprecipitation of spliceosomal complexes

SmB immunoprecipitations were performed as described previously^{43,44}. Briefly, extracts were prepared in ice cold RSB-100 buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.4, 2.5 mM MgCl₂) containing 0.1% NP40, protease, phosphatase and RNase inhibitors, by passing five times through a 26-gauge needle followed by 3 x 5 seconds bursts of sonication on ice and centrifugation at 20,000 x *g* for 15 minutes at 4°C. Extract supernatant was quantified by BCA Assays (ThermoFisher Scientific).

For immunoprecipitation, a mouse monoclonal anti-SmB (18F6) antibody was bound to protein A/G-Sepharose (ThermoFisher Scientific) in RSB-100 buffer containing 0.1% NP40, protease, phosphatase and RNase inhibitors for 2 hours at 4°C. Following five washes with the same buffer, antibody-bound beads were incubated with 200 µg of cell extract rotating for 2 hours at 4°C. Following five washes with the same buffer, samples were processed for RNA extraction.

Bound RNAs were extracted by treatment with proteinase K (200 µg) for 20 minutes at 37°C followed by TRIzol LS (ThermoFisher Scientific) extraction. Immunoprecipitated RNA was then analyzed by thin layer chromatography.

Alternative splicing analysis

For alternative splicing analysis, paired reads were aligned to the human transcriptome (hg19) or mouse transcriptome (mm10) using STAR⁶² (v020201). Biological replicates were then merged and indexed using samtools (v1.5). The module exon_utils from the MISO package⁶⁹ was first used to extract the constitutive exons from the transcriptome annotation (iGenomes, UCSC build hg19). The resulting annotations were then used in conjunction with the pe_utils module (MISO) to estimate fragment length distribution. The MISO package was then used to quantify percent spliced-in values (PSI) for each splicing event annotated for hg19 (pre-built MISO annotations). For this analysis, we used skipped exons (SE) as well as alternatively spliced first and last exons (A5SS and A3SS). We then used MISO to compare changes in splicing events, which reports the change in PSI values as well as the associated Bayes factor. For motif analysis, we extracted the included exons along with 250nt flanking sequences. We then used FIRE⁶⁰, in non-discovery mode, to ask whether the presence or absence of known splicing regulator binding sites⁷⁰ were significantly associated with changes in PSI values. In addition to enrichment profiles among the

down- or up-regulated exons, FIRE also provides the mutual information (MI) values and their associated z-scores and robustness scores.

Northern blot

500 ng total RNA extracted from wild type and *DCP^{-/-}* HEK293T cells was run on the 10% TBE-Urea gel (Thermo Fisher) for 80 min at 200V. RNA was subsequently transferred onto the BrightStar-Plus nylon membrane (Thermo Fisher) in 0.5x TBE buffer for 60 min at 350 mA using the XCell SureLock Mini-gel electrophoresis apparatus (Thermo Fisher). The transferred RNA was UV crosslinked twice with 0.12 J/cm². The membrane was prehybridized in 20 mL ULTRAHyb-Oligo buffer (Thermo Fisher) at 42°C for an hour. 10 nM (final) 3'-biotinylated DNA probe for snRNAs and 5.8 S rRNA (loading control) were incubated at 42°C over-night. The membrane was washed twice (20 min each) in a buffer containing 2x SSC and 0.5% SDS. Detection was performed using Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher) according to the manufacturer's instructions.

U2 snRNA probe: 5'-TAC TGC AAT ACC AGG TCG ATG CGT-Biotin-3'

U5 snRNA probe: 5'-GAC TCA GAG TTG TTC CTC TCC ACG-Biotin-3'

5.8S rRNA probe: 5'-AGA CAG GCG TAG CCC CGG GAG GAA-Biotin-3'

Classification of mRNAs based on the first nucleotide

In experiments where we compared snRNAs to m⁶Am, Am, Cm, Gm, and Um-initiated mRNAs, we classified the mRNAs based on the nucleotide at the annotated transcription start site (TSS). Annotated TSS were extracted from UCSC table browser. A complete list of transcripts with their respective annotated transcription start site is found in the Supplementary Information Table S1.

Code availability

All custom code used in this study can be obtained upon request from the lead author (S.R.J: srj2003@med.cornell.edu).

Statistics and software

P-values were calculated with a two-tailed unpaired Student's *t*-test or, for the comparison of more than two groups, with a one- or two-way ANOVA followed by Tukey's post-test. *P*-values of 0.05 or less were considered significant.

Extended Data Figure 1 | FTO selectively demethylates small nuclear RNAs.

a, Expression levels of FTO in *Fto*^{-/-} liver and *FTO* knockout HEK293T cells. Upper panel shows a western blot analysis of FTO expression in wild-type (WT) and *Fto* knockout (*Fto*^{-/-}) mouse liver samples that were used for 6mA^m6A miCLIP in **Fig. 1**. FTO protein was absent in *Fto*^{-/-} livers. An antibody directed against β-ACTIN (ACTB) was used as a loading control. Lower panel shows a western blot analysis of FTO expression in wild-type (WT) and *FTO* knockout (*FTO*^{-/-}) HEK293 cells that were used for the experiments in **Figs. 2, 3** and **4**. FTO protein was absent in *FTO*^{-/-} HEK293 cells. An antibody directed against β-ACTIN (ACTB) was used as a loading control.

b, FTO deficiency leads to increased transcription-start nucleotide methylation of minor spliceosomal snRNAs. In **Fig. 1b**, we examined major spliceosomal snRNAs. Here we performed the same analysis on minor spliceosomal snRNAs. 6mA^m6A miCLIP reads were counted in a 20-nucleotide window surrounding the transcription-start nucleotide, normalized to input RNA-Seq in the same region and relative expression changes were calculated. In this analysis, no *P*-value cut-off was applied. Instead, the mean log₂ fold change in transcription-start nucleotide methylation of specific snRNA gene classes (U11, U12, U4atac and U6atac) in *Fto* knockout mouse liver (*Fto*^{-/-}) compared to wild-type liver (WT) is shown. Notably, Sm-class minor snRNA genes transcribed by RNA polymerase II increased methylation, whereas the U6atac snRNA gene, which is transcribed by RNA polymerase III and therefore does not acquire a m⁷G cap, shows no change in transcription-start nucleotide methylation upon FTO deficiency (Data represents the average from datasets of three independent biological replicates per genotype).

Extended Data Figure 2 | Read coverage tracks of minor spliceosomal small nuclear RNAs are markedly increased at transcription-start nucleotides in FTO-deficient cells.

a, U11 snRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U11 snRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. Input reads are shown in the lower panel. The transcription-start nucleotide (TSN) is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

b, U12 snRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U12 snRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. Input reads are shown in the lower panel. The transcription-start nucleotide (TSN) is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

c, U4atac snRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U4atac snRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. Input reads are shown in the lower panel. The transcription-start nucleotide (TSN) is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

d, U6atac snRNA transcription-start nucleotide methylation is not affected in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U6atac snRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. Input reads are shown in the lower panel. The transcription-start nucleotide (TSN) is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

Extended Data Figure 3 | Read coverage tracks of U3 and U8 snoRNAs and U7 snRNA are increased at the transcription-start nucleotide in FTO-deficient cells.

a, U3 snoRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U3 snoRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. Input reads are shown in the lower panel. The transcription-start nucleotide

(TSN) is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

b, U7 snRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U7 snRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. Input reads are shown in the lower panel. The transcription-start nucleotide (TSN) is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

c, U8 snoRNA shows increased transcription-start nucleotide methylation in FTO-deficient mouse liver. The grey tracks denote WT liver, whereas the blue track denotes *Fto*^{-/-} liver. A representative read coverage track for U8 snoRNA is shown. ~~6m~~A~~m~~⁶A miCLIP reads are shown in the upper panel. Input reads are shown in the lower panel. The transcription-start nucleotide (TSN) is indicated in red (RPM = reads per million mapped reads; data represents the combined tracks from datasets of three independent biological replicates per genotype).

Extended Data Figure 4 | FTO deficiency does not affect internal ~~6m~~A~~m~~⁶A residues in U6 and U1 snRNAs but regulates cap-adjacent N⁶,2'-O-dimethyladenosine (m⁶Am) in U2 snRNAs.

a, FTO deficiency does not lead to an increase in internal ~~6m~~A~~m~~⁶A in U6 snRNA. In **Fig. 1b**, we examined TSN methylation of major spliceosomal snRNAs. Here we performed the same analysis on internal ~~6m~~A~~m~~⁶A in U6 snRNA. ~~6m~~A~~m~~⁶A miCLIP reads were counted in a 4-nucleotide window surrounding position +43 of U6 snRNA, normalized to input RNA-Seq in the same region and relative expression changes were calculated. The mean log₂ fold change of U6 snRNA internal methylation in *Fto* knockout mouse liver (*Fto*^{-/-}) compared to wild-type liver (WT) is shown. The left panel depicts methylation changes of the five most highly expressed individual U6 transcripts, whereas the right panel shows the average change across all detectable U6 snRNA transcripts. Notably, we could not detect any significant changes in U6 snRNA internal methylation upon *Fto* knockout, indicating that Fto exclusively targets ~~6m~~A~~m~~⁶A residues at the TSN (Data represents the average from datasets of three independent biological replicates per genotype).

b, FTO deficiency does not lead to an increase in internal 6mAm⁶A in U1 snRNA. In **Fig. 1b**, we examined TSN methylation of major spliceosomal snRNAs. Here we performed the same analysis on internal 6mAm⁶A in U1 snRNA. 6mAm⁶A miCLIP reads were counted in a 4-nucleotide window surrounding position +70 of U1 snRNA, normalized to input RNA-Seq in the same region and relative expression changes were calculated. The mean log₂ fold change of U1 snRNA internal methylation in *Fto* knockout mouse liver (*Fto*^{-/-}) compared to wild-type liver (WT) is shown. The left panel depicts methylation changes of five highly expressed individual U1 transcripts, whereas the right panel shows the average change across all detectable U1 snRNA transcripts. Notably, we could not detect any significant changes in U1 snRNA internal methylation upon *Fto* knockout, indicating that Fto exclusively targets 6mAm⁶A residues at the TSN of U1 snRNA (Data represents the average from datasets of three independent biological replicates per genotype).

c, FTO deficiency leads to a marked increase in *N*⁶,2'-*O*-dimethyladenosine (m⁶Am) in U2 snRNA. The relative abundance of modified adenosines at the transcription-start nucleotide in U2 snRNA caps derived from wild-type (WT) and FTO-deficient (*FTO*^{-/-}) HEK293 cells was determined by thin layer chromatography. The left panel shows a TBE-Urea gel image stained with SYBR Gold, where the red dashed line indicates the U2-enriched fraction that was used for the analysis. The migration position of m⁶Am and 2'-*O*-methyladenosine (Am) is indicated by the dashed black circles. Additional spots are seen on the TLC chromatogram due to RNA fragmentation that occurs during RNA purification, resulting in 5' radiolabeling at internal sites²⁴. The right panel shows the quantification of the m⁶Am/Am ratio in the U2-enriched fraction (*n* = 3 independent biological replicates; mean ± s.d; unpaired student's *t*-test ***P* ≤ 0.001).

Extended Data Figure 5 | Development of an LC-MS/MS-based detection method for snRNA caps.

a, Schematic of sample preparation for LC-MS/MS analysis. RNA is digested with P1, which cleaves after each nucleotide and releases 5'-monophosphorylated nucleotides (p-N). However, since the triphosphate linker between the RNA cap and the first nucleotide is resistant to P1 nuclease cleavage, the intact extended cap is released as a cap-dinucleotide (p = phosphate; N = nucleotide; P1 = P1 nuclease; grey arrows indicate P1 cleavage sites).

b, Positive ion mode fragmentation at 50 V collision energy of a TMG-ppp- m^6 Am and a TMG-ppp-Am standard solution. Left panel: total ion current (TIC) of the product ion scan (m/z 50-850) of a (A) TMG-ppp-Am (precursor ion $[M+H]^+$ 843.1 and a (B) TMG-ppp- m^6 Am (precursor ion $[M+H]^+$ 829.1) standard solution. Right panel: MS/MS product ion spectra of the integrated areas of the peaks for (A) and (B). Chemical structures indicate the most prominent product ions for (A) 135.8 ($[\text{adenine}+H]^+$), 194.0 ($[\text{trimethylguanidine}+H]^+$) and 423.6 ($[\text{2'-}O\text{-methyladenosine-pp}+H]^+$); for (B) 150.1 ($[\text{N}^6\text{-methyladenine}+H]^+$), 193.9 ($[\text{trimethylguanidine}+H]^+$) and 438.1 ($[\text{N}^6,2'\text{-}O\text{-dimethyladenosine-pp}+H]^+$).

c, Standard curves for the ratio of the MRM transitions 843.2 \rightarrow 194.1 TMG-ppp- m^6 Am to 829.1 \rightarrow 194.1 for TMG-ppp-Am (MRM) recorded in positive ion mode. Standard dilutions (0.006-4 ng/ μ L) of TMG-ppp- m^6 Am (stock solution contained 400 ng/ μ l overall nucleotides in water, measured by nano-drop analysis) in a small RNA extract of wildtype HEK-cells (100 ng/ μ l overall nucleotide concentration in 80% methanol) in 80% methanol were subjected to LC-MS/MS analysis ($n = 3$, technical replicates). (left panel) A linear relationship of the MRM ratio of TMG-ppp- m^6 Am to TMG-ppp-Am over a nucleotide concentration range of 0.0125 to 4 ng/ μ l was determined. (right panel) After linear regression over a concentration range of 0.05 to 4 ng/ μ l with an R^2 of 0.9987, a second dilution series of 0.006 to 0.10 ng/ μ l was analyzed to determine an LOQ of 0.0125 ng/ μ l.

Extended Data Figure 6 | m_2 -snRNAs are differentially regulated in different cells and conditions.

a, FTO deficiency increases the relative abundance of m_2 -snRNA caps in HEK293 cells. Related to **Fig. 2c**: Small RNA from WT and *FTO*^{-/-} was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the abundance of m_2 -snRNA caps (m^6 Am) and m_1 -snRNA caps (Am) represented by the integrated peak area of the corresponding MRM transitions (recorded in positive ion mode) ($n=3$ independent biological replicates, mean \pm s.d.; unpaired Student's t-test, *** $p < 0.001$).

b, Differentiation increases relative abundance of m_2 -snRNA (cap-ppp- m^6 Am) caps in mouse embryonic stem cells (mESCs). Small RNA from naïve and differentiated (diff.) mESCs was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the ratio of m_2 -snRNA caps (m^6 Am) to m_1 -snRNA caps

(Am) represented by the integrated peak area ratio of the corresponding MRM transitions (recorded in positive ion mode). These data suggest that mESC differentiation induces a specific program that changes the abundance of m₂-snRNAs (n=3 independent biological replicates, mean ± s.d.; unpaired Student's t-test, ** p < 0.01).

c, Differentiation increases relative abundance of m₂-snRNA (cap-ppp-m⁶Am) caps in mouse embryonic stem cells (mESCs). Related to **Extended Data Fig. 6b**: Small RNA from naïve and differentiated (diff.) mESCs was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the abundance of m₂-snRNA caps (m⁶Am) and m₁-snRNA caps (Am) represented by the integrated peak area of the corresponding MRM transitions (recorded in positive ion mode). These data suggest that mESC differentiation induces a specific program that changes the abundance of m₂-snRNAs (n=3 independent biological replicates, mean ± s.d.; unpaired Student's t-test, ** p < 0.01).

d, m₂-snRNA are not detected in wild-type mouse liver and brain. Related to **Figs. 2d** and **3b**: Small RNA from wild-type (WT) and *Fto* knockout (*Fto*^{-/-}) mouse liver and brain tissue was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the abundance of m₂-snRNA caps (m⁶Am) and m₁-snRNA caps (Am) represented by the integrated peak area of the MRM transition (recorded in positive ion mode). (n.d.=not detected; n=3 independent biological replicates, mean ± s.d.; unpaired Student's t-test, ** p < 0.01).

e, Relative abundance of m₂-snRNA (cap-ppp-m⁶Am) caps in UOK262 and HT1080 cells compared to HEK293 cells. Small RNA was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the ratio of m₂-snRNA caps (m⁶Am) to m₁-snRNA caps (Am) represented by the integrated peak area ratio of the corresponding MRM transitions (recorded in positive ion mode) relative to HEK293 cells. These data suggest that m₂-snRNAs are differentially formed across different cell types (n=3 independent biological replicates, mean ± s.d.; unpaired Student's t-test, * p < 0.05).

Extended Data Figure 7 | m₂-snRNAs are reversibly regulated in oncometabolite-dependent cancer models.

a, Intracellular 2-hydroxyglutarate (2-HG) concentrations in mutant IDH1^{R132H} and IDH2^{R140Q} TF-1 cells ($n = 3$ independent biological replicates; mean \pm s.d.; one-way ANOVA with Tukey's post hoc test $**P \leq 0.001$).

b, Increased abundance of m₂-snRNA caps in oncometabolite-dependent cancer. Related to **Fig. 2e**: Small RNA from Ctrl and mutant IDH-expressing cells was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the abundance of m₂-snRNA caps (m⁶Am) and m₁-snRNA caps (Am) represented by the integrated peak area of the cap-specific MRM transitions (recorded in positive ion mode). IDH1^{R132H}- and IDH2^{R140Q}-expressing TF-1 cells have high levels of 2-hydroxyglutarate (2-HG). 2-HG is a natural inhibitor of FTO activity³⁸ and leads to increased abundance of m₂-snRNAs. Specific inhibition of the mutant IDH1 (AGI-5027) and IDH2 (AGI-6780) isoforms shows that these effects are readily reversible ($n=3$ independent biological replicates, mean \pm s.d.; one-way ANOVA with Tukey's post hoc test $*P \leq 0.05$, $**P \leq 0.01$).

c, Increased abundance of m⁶Am caps in U1 snRNA of oncometabolite-dependent cancer cells. Gel-extracted U1 snRNA from Ctrl and mutant IDH2^{R140Q} TF-1 cells was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and TLC. Shown is the ratio of cap-adjacent m⁶Am and cap-adjacent Am. IDH2^{R140Q}-expressing TF-1 cells have high levels of 2-hydroxyglutarate (2-HG). 2-HG is a natural inhibitor of FTO activity and leads to increased abundance of m⁶Am caps in snRNA. Specific inhibition of the mutant IDH2^{R140Q} isoform with AGI-6780 shows that these effects are readily reversible ($n=3$ independent biological replicates, mean \pm s.d.; unpaired Student's t-test, $*** p < 0.001$).

d, Dimethylfumarate does not alter the levels of m₂-snRNA caps in HEK293 cells. Small RNA was digested with nuclease P1 to specifically liberate the extended cap structure dinucleotide and analyzed by LC-MS/MS. Shown is the ratio of m₂-snRNA caps (m⁶Am) and m₁-snRNA caps (Am). Dimethylfumarate (DMF, 70 μ M) treatment of HEK293 cells did not lead to a significant increase of the m⁶Am/Am ratio. ($n=3$ independent biological replicates, mean \pm s.d.; one-way ANOVA with Tukey's post hoc test n.s. = not significant).

Extended Data Figure 8 | High levels of m₂-snRNAs are correlated with SRSF1-dependent exon inclusion.

a, Splicing regulator binding motif-enrichment in m₂-snRNA-regulated exons. Related to **Fig. 4b**: FIRE⁶⁰ analysis of exons preferentially included in FTO-deficient HEK293 cells. Binding motifs for U2AF2, HNRNPA1, HNRNPA2B1, SRSF2 and SRSF10 are not enriched in the included exons. These data suggest that changes of splicing regulator binding activity in the presence of m₂-snRNAs are restricted to SRSF1, HNRNPH2 and HNRNPK, which promotes specific splicing patterns (see **Fig. 4b**). For each motif, we indicate the mutual information (MI) value, Z-score associated with the MI value, and the robustness score ranging from 0/10 to 10/10. The enrichment score indicates over-representation of a motif; significant over-representation is indicated by red frames, whereas blue frames indicate under-representation.

b, m₂-snRNA-regulated exons show enrichment of specific splicing regulator binding motifs. To verify the experiments presented in **Fig. 4b** and **Extended Data Fig. 8a**), we performed FIRE⁶⁰ analysis on exons included in an independent FTO knockdown from HEK293T cells. We observed a similar specific enrichment of SRSF1, HNRNPH2 and HNRNPK motifs as in FTO knockout cells. For each motif, we indicate the mutual information (MI) value, Z-score associated with the MI value, and the robustness score ranging from 0/10 to 10/10. The enrichment score indicates over-representation of a motif; significant over-representation is indicated by red frames, whereas blue frames indicate under-representation.

Extended Data Figure 9 | DCP2-mediated decapping is not a primary mechanism to regulate m₁-snRNA levels.

a, DCP2 depletion does not affect m₁-snRNA expression in HEK293 cells. The snRNA pool in HEK293 cells consists predominately of the m₁-isoform. If m₁-snRNAs, similar to Am-mRNAs²², are subjected to DCP2-mediated decapping and degradation, we would expect snRNA levels to increase upon DCP2 depletion. We thus measured expression of expression of two representative snRNAs, U2 and U5, in wild-type and *DCP2* knockout (*DCP2*^{-/-}) HEK293 cells by northern blot. 5.8S rRNA was used as a loading control. However, we did not see any increase of snRNA expression in *DCP2*^{-/-} cells, indicating that m₁-snRNAs are not targeted by DCP2 under basal conditions (Two independent biological replicates are shown).

b, FTO deficiency increases the abundance of snRNAs. The MA plot shows the transcriptome-wide log₂ fold change of gene expression in *Fto* knockout mouse liver (*Fto*^{-/-}) compared to wild-type (WT) liver. Significantly regulated snRNAs are indicated in red. Notably, the significantly

changed snRNAs were exclusively upregulated in *Fto*^{-/-} liver. Since snRNAs largely exist in the m₂-form in *Fto*^{-/-} mice, these data suggest that m₂-snRNAs are intrinsically more stable than m₁-snRNAs that are predominately found in WT mice (Data represents the average from datasets of three independent biological replicates per genotype).

c, snRNA expression is broadly increased upon FTO deficiency in mice. Shown are log₂ fold changes of snRNA expression in FTO knockout (*Fto*^{-/-}) mouse liver. These data suggest that the outcome of FTO deficiency - i.e. elevated snRNA expression - is conserved between humans and mice. (Data represents the average from datasets of three independent biological replicates per genotype)

d, The MA plot of RNA-Seq analysis of total cellular RNA from previously published datasets²⁸ shows the transcriptome-wide log₂ fold change of gene expression in *FTO* knockout HEK293T cells (*FTO*^{-/-}) compared to wild-type HEK293T cells (WT). Here, we specifically plotted non-canonical snRNAs that also appear to exist as an m₂-isoform in *FTO* knockout cells (U3, U7, U8) to see whether they are similarly regulated as Sm- and Lsm-class snRNAs (see **Figs. 4a, b**). However, apart from a single U3 transcript (orange circle) we did not observe any significantly altered expression of these transcripts upon *FTO* knockout (Data represents the average of three independent biological replicates per genotype).

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