Sequence-specific m^6A demethylation in RNA by FTO fused to RCas9

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ABSTRACT

N\(^6\)-methyladenosine (m^6A) is the most common internal modification in eukaryotic mRNA and associated with numerous cellular processes in health and disease. Up- and down-regulation of its “writer” or “eraser” proteins alter the global m^6A level; however, modifying distinct m^6A sites has remained elusive. We genetically fused the dioxygenase FTO responsible for m^6A demethylation to RCas9 as an RNA-targeting module. The resulting RCas9-FTO retained demethylation activity and bound to RNA in a sequence-specific manner depending on the sgRNA and PAMmer. Using SCARLET analysis, we quantified the m^6A level at a specific site and analyzed the effect of the PAM-to-m^6A distance on activity. Sequence-specific demethylation by RCas9-FTO was tested on different RNA combinations and showed up to 15-fold sequence preference for target RNA compared to off-target RNA. Taken together, RCas9-FTO represents a new tool for sequence-specific demethylation of m^6A in RNA that can be readily adapted to any given RNA sequence and opens the door to studying the function of distinct m^6A sites.

Keywords: RNA modification; N\(^6\)-methyladenosine (m^6A); Cas9; FTO; demethylation; sgRNA; PAMmer

INTRODUCTION

A total of 160 RNA modifications have been reported to date with methylation being the most common one (Boccaletto et al. 2018). In eukaryotic mRNA, N\(^6\)-methyladenosine (m^6A) is the most abundant internal modification and was originally discovered in the early 1970s (Desrosiers et al. 1974). To date, m^6A has been found in mRNA and a variety of noncoding RNAs including tRNA, rRNA, snRNA, miRNA, and IncRNA. The majority of m^6A sites is present in a so-called DRACH motif (D = G/A/U, R = G/A, H = U/A/C) (Wei and Moss 1977; Harper et al. 1990; Dominissini et al. 2012; Meyer et al. 2012). In recent years, the importance of m^6A for the whole mRNA life cycle and many cellular processes has been elucidated, including RNA processing, nuclear mRNA export, RNA stability, and gene expression as well as RNA–protein interactions (Tuck et al. 1999; Zhong et al. 2008; Zheng et al. 2013; Merkstein et al. 2014; Wang et al. 2014a, 2015; Zhao et al. 2014; Liu et al. 2015; Zhou et al. 2015; Du et al. 2016; Bartosovic et al. 2017; Ke et al. 2017; Li et al. 2017a; Roundtree et al. 2017; Shi et al. 2017; Slobodin et al. 2017; Kretschmer et al. 2018; Lesbiel et al. 2018; Tang et al. 2018). Consequently, m^6A is found to play a key role in various developmental processes, like stem cell differentiation (Batista et al. 2014; Wang et al. 2014b; Geula et al. 2015), neurogenesis (Li et al. 2017b; Yoon et al. 2017; Ma et al. 2018), and tissue and organ development (He et al. 2017; Kudou et al. 2017; Wu et al. 2018a) as well as tumor development (Lin et al. 2016; Barbieri et al. 2017; Li et al. 2017b). The methyl group is installed by a methyltransferase complex (“writer”) containing METTL3 as the active methyltransferase (Bokar et al. 1994, 1997; Liu et al. 2014; Sledz and Jinek 2016; Wang et al. 2016a, b). Recently, METTL16 was identified as a second m^6A methyltransferase having a different RNA substrate specificity (Penention et al. 2017; Warda et al. 2017; Doxtader et al. 2018; Ruszkowska et al. 2018). The modification is recognized by various “reader” proteins and can be removed by the “eraser” proteins AlkBH5 (α-ketoglutarate-dependent dioxygenase AlkB homolog 5) or FTO (fat mass and obesity-associated protein), both nonheme iron- and α-ketoglutarate-dependent dioxygenases of the AlkB superfamily. The two display a distinct expression profile with AlkBH5 being mainly expressed in testis and necessary for spermatogenesis (Zheng et al. 2013; Tang et al. 2018). FTO is expressed in various tissues showing the highest...
expression in the brain (Frayling et al. 2007). It can be connected with infertility (Ding et al. 2018), but is mainly associated with obesity (Frayling et al. 2007; Fischer et al. 2009; Church et al. 2010) and adipogenesis (Zhao et al. 2014; Zhang et al. 2015; Wu et al. 2018a,b). FTO was first reported to demethylate 3-methylthymine and 3-methyluracil (Gerken et al. 2007; Jia et al. 2008; Han et al. 2010), but then was found to be more active in m6A demethylation (Jia et al. 2011). Recently, Maurer et al. (2017) demonstrated FTO demethylation of N6,2′-O-dimethyladenosine (m6Am), which was expanded by a recent study of Wei et al. (2018). The latter reported demethylation of m6A and m6Am, with a clear preference for m6A in cells. In the nucleus, m6Am demethylation by FTO is of high importance for snRNA biogenesis (Mauer et al. 2019). Additionally, FTO could demethylate N1-methyladenosine (m6A) in tRNAs (Wei et al. 2018). Very recently, the crystal structure of FTO with the nucleobase N6-methyladenine was solved indicating a preference for this nucleobase compared to m6A or 3-methylthymine. This study also confirmed preferential demethylation of m6A in vitro and in vivo by FTO as well as reported selectivity of FTO for m6A in a big loop of a stem–loop structure (Zhang et al. 2019). Additionally, FTO has an m6A-dependent sequence preference without a consensus sequence (Zou et al. 2016), whereas it shows some selectivity among various sequences of the reported DRACH motif (Zhang et al. 2019).

As described above, m6A can impact cellular processes, but most of the studies illustrate a global change of m6A levels by overexpression of the “writer” proteins and knockdown or knockout of the “erasers,” respectively. The effect of a single m6A site on the fate of the host RNA is still unknown and tools to manipulate m6A in a sequence-specific manner are lacking. Therefore, we aimed to develop methodology to enable for sequence-specific “erasure” of m6A sites. As FTO has no reported consensus motif, we decided to fuse an RNA-targeting protein that should induce specificity, a concept to render RNA-modifying enzymes sequence-specific that has been successfully realized in several ways (Wang et al. 2009; Cooke et al. 2011; Mackay et al. 2011; Choudhury et al. 2012; Campbell et al. 2014; Cao et al. 2014; Rentmeister and Kellermann 2016), including recent reports for editing (Stafforst and Schneider 2012; Wei and Wang 2015; Cox et al. 2017; Rauch et al. 2018; Vogel et al. 2018). We used an amino-terminally truncated version of FTO (Δ31), which was previously reported to show no catalytic defects (Han et al. 2010; Zhu and Yi 2014) and fused it to the carboxy terminus of RCas9 from Streptococcus pyogenes via a short linker sequence composed of glycine and serine residues that should be flexible and unstructured (Chen et al. 2013). The resulting fusion protein RCas9-FTO was recombinantly produced and tested for demethylation activity in vitro (Fig. 1A,B). Quantitative analysis of demethylation activity on a short m6A-containing RNA (15 nt-m6A RNA) by HPLC after degradation and dephosphorylation confirmed that RCas9-FTO was highly active (57 ± 4% demethylation; Fig. 1C). Compared to FTO (Supplemental Fig. S1A), which showed ~16% demethylation under the same assay conditions, RCas9-FTO is considerably more active (Supplemental Fig. S2).

SCARLET analysis and production of longer m6A RNAs

As demethylation of longer RNAs is hard to quantify by the HPLC-based assay due to the increasing A/m6A ratio, we used SCARLET (site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography), a multistep but sequence-specific assay (Liu et al. 2013), to investigate sequence-specific demethylation. In the last step, m6AMP and AMP are separated by TLC yielding the fraction of m6A compared to

RESULT

Design of an RCas9-FTO fusion protein

FTO catalyzes the oxidative demethylation of m6A, however, no consensus motif has been reported to date. We anticipated that the fusion of an RNA-targeting protein should induce specificity, a concept to render RNA-modifying enzymes sequence-specific that has been successfully realized in several ways (Wang et al. 2009; Cooke et al. 2011; Mackay et al. 2011; Choudhury et al. 2012; Campbell et al. 2014; Cao et al. 2014; Rentmeister and Kellermann 2016), including recent reports for editing (Stafforst and Schneider 2012; Wei and Wang 2015; Cox et al. 2017; Rauch et al. 2018; Vogel et al. 2018). We used an amino-terminally truncated version of FTO (Δ31), which was previously reported to show no catalytic defects (Han et al. 2010; Zhu and Yi 2014) and fused it to the carboxy terminus of RCas9 from Streptococcus pyogenes via a short linker sequence composed of glycine and serine residues that should be flexible and unstructured (Chen et al. 2013). The resulting fusion protein RCas9-FTO was recombinantly produced and tested for demethylation activity in vitro (Fig. 1A,B). Quantitative analysis of demethylation activity on a short m6A-containing RNA (15 nt-m6A RNA) by HPLC after degradation and dephosphorylation confirmed that RCas9-FTO was highly active (57 ± 4% demethylation; Fig. 1C). Compared to FTO (Supplemental Fig. S1A), which showed ~16% demethylation under the same assay conditions, RCas9-FTO is considerably more active (Supplemental Fig. S2).
the A under investigation. To calibrate this method, we prepared mixtures containing defined ratios of synthetic RNAs with or without m\(^6\)A (m\(^6\)A-RNA and A-RNA), respectively. A coefficient of determination of 0.97 confirmed that SCARLET is suitable for quantitative assessment of m\(^6\)A levels at a given position (Supplemental Fig. S3). For subsequent SCARLET analysis, a mixture of the synthetic m\(^6\)A- and A-RNA was analyzed as a control sample (C in TLCs).

We used splint ligation to generate longer RNAs with one specific internal m\(^6\)A site (Fig. 2A). A short synthetic m\(^6\)A-containing RNA (20 nt) was ligated at its 3′-end to a longer RNA produced by in vitro transcription (IVT; Supplemental Fig. S4A). Using this approach, we produced a set of longer m\(^6\)A-containing RNAs with the same m\(^6\)A site but different distances to the PAM (25–40 nt; Fig. 2D), allowing us to assess how the PAM-to-m\(^6\)A distance will affect demethylation activity. Varying the distance between PAM and m\(^6\)A also allowed us to use the same PAMmer and sgRNA in all but two cases, circumventing bias from different constructs. Specifically, the effect of PAM-to-m\(^6\)A distance was tested for REEP5, an mRNA known to naturally contain a single m\(^6\)A (Dominissini et al. 2012; Chen et al. 2015), in combination with PAMmer1 and sgRNA1 (Fig. 2A,C). PAMmer1 is a synthetic 27 nt oligomer, consisting of an 8 nt 5′-sequence complementary to the protospacer, followed by a 3 nt-long noncomplementary PAM and the 16 nt 3′-sequence (Supplemental Fig. S5), based on previous reports (O’Connell et al. 2014). Therefore, for PAMmer1 the shortest possible distance is 16 nt, which would end inside the DRACH consensus motif, directly next to m\(^6\)A (Supplemental Fig. S5). To avoid binding within this DRACH motif, we tested distances of 18–40 nt in 1–4 nt steps (Fig. 2E). To enable testing of the two shortest distances (18 nt and 22 nt, respectively), dedicated sgRNAs (sgRNA18, sgRNA22) and PAMmers (PAMmer18, PAMmer22) were designed matching the binding region within the 25 nt target RNA (Fig. 2B,C; Supplemental Fig. S5). Assessment of demethylation activity for various PAM-to-m\(^6\)A distances revealed that RCas9-FTO demethylates all RNAs tested (Fig. 2E; representative TLCs in Supplemental Fig. S6, normalized values in Supplemental Fig. S7). The shortest PAM-to-m\(^6\)A-distance (18 nt) resulted in the highest demethylation of ~14%, whereas the longest distance (40 nt) resulted in the lowest activity (~5%), suggesting that proximity fosters demethylation (Fig. 2E). PAMmer and target RNA are expected to form an A-form helix, which—in case of the 18 nt distance—would end directly downstream from the DRACH consensus sequence.
However, the activity trend from shorter to longer PAM-to-m^6A distances was not uniform and showed elevated relative activities in some cases, in particular for 27 nt (~10%) and 38 nt (~11%) distances (Fig. 2E; Supplemental Fig. S7). One could speculate that this 11 nt distance reflects preferred positioning making m^6A accessible to FTO because it would be in line with a full turn of the A-helix, but definite proof is missing and unanticipated interactions might also be the reason. Secondary structure effects can likely be excluded, because in our design all constructs contain the identical m^6A motif and sequence context and are therefore expected to form the same stem–loop structure with m^6A exposed on top of the loop as predicted by mfold (Zuker 2003). We therefore conclude that RCas9-FTO with its flexible GGGGS linker can adjust to demethylate a range of constructs with different PAM-to-m^6A distances.

EMS A analysis of sequence-specific target interactions

To assess sequence-specificity of RCas9-FTO binding, electrophoretic mobility shift assays (EMSAs) were performed using two different m^6A-containing RNAs and a PAM-to-m^6A distance of 38 nt. One RNA was the construct tested above, which is derived from REEP5 and the other one was based on the long noncoding RNA (lncRNA) MALAT1, which is known to be naturally modified at the 2515 position (Meyer et al. 2012; Liu et al. 2013; Linder et al. 2015). Interestingly, when we used the MALAT1 RNA sequence as substrate, named M2515 here (Supplemental Fig. S4B), FTO as well as RCas9-FTO did not exhibit demethylation activity—neither unspecific nor sequence-specific (Supplemental Figs. S8C, S9A), suggesting that m^6A site 2515 of MALAT1 is not a natural target of FTO. This would be in line with the reported secondary structure of MALAT1 (Supplemental Fig. S10) forming a stable hairpin with m^6A positioned in the stem (Liu et al. 2013, 2015; Zhou et al. 2016) and the fact that FTO has only been reported to act on single-stranded RNA (Han et al. 2010). To circumvent this issue and obtain two comparable RNA targets for sequence-specific demethylation, we introduced six mutations designed to avoid hairpin formation as calculated by mfold (Zuker 2003), but retain the sequence context of m^6A (Supplemental Fig. S10). The resulting construct M2515mut (Supplemental Fig. S4B) was a target of FTO and resulted in ~27% demethylation under the same conditions (Supplemental Fig. S8B). Importantly, the binding sites for sgRNA2 (Supplemental Fig. S4B) and PAMmer2 remained unchanged with respect to the natural MALAT1 sequence (Supplemental Fig. S5).

Both RNAs, REEP5 and M2515mut, were tested in EMSA experiments with stepwise addition of matching or non-matching PAMmer, sgRNA as well as RCas9-FTO (Fig. 3; Supplemental Fig. S11). Addition of matching PAMmer led to a complete shift to complex II (target RNA with PAMmer) for both RNAs (Fig. 3B; Supplemental Fig. S11). Similarly, addition of matching sgRNAs shifted both RNAs to the respective complex III (target RNA with sgRNA). However, additional bands were observed, suggesting formation of alternative secondary structures of the sgRNAs (Fig. 3B; Supplemental Fig. S11). In the presence of RCas9-FTO a large supershift was observed, indicating formation of complex V (target RNA with RCas9-FTO, sgRNA and PAMmer; Fig. 3B; Supplemental Fig. S11). Formation of complex V was more distinct in the case of REEP5 RNA compared to M2515mut, as deduced from quantification of independent experiments.

FIGURE 3. Sequence-specific binding of RCas9-FTO to REEP5 and M2515mut RNA (38 nt PAM-to-m^6A distance). (A) Scheme illustrating different complexes formed in binding assays. I = target RNA, II = I with PAMmer, III = I with sgRNA, IV = I with PAMmer and sgRNA, V = IV with RCas9-FTO. (B) Representative EMSA to analyze binding of RCas9-FTO to REEP5 and M2515mut with a sgRNA concentration of 250 nM. (C) Quantification of sequence-specific binding from EMSAs. The data were normalized to the bound complex IV. For REEP5 62 ± 5% (blue) and for M2515mut 55 ± 2% (green) of complex IV were shifted to complex V (n = 3 independent biological replicates).
Importantly, for both RNA constructs, all shifts were only observed in the case of matching sgRNA and PAMmer, whereas nonmatching sgRNA and PAMmer did not yield detectable shifted bands (Fig. 3B; Supplemental Fig. S11), indicating highly sequence-specific interactions.

To elucidate whether the fusion of FTO affected sequence-specific binding, we also performed EMSAs for RCas9 alone (Supplemental Fig. S1B) using identical conditions. Since the concentration of sgRNA was reported to be limiting for complex formation, we tested three different sgRNA concentrations (3, 25, and 100 nM) to have a range of concentrations for comparison (Fig. 4).

Consistent with results obtained for the fusion protein RCas9-FTO (complex V), RCas9 alone also showed more pronounced complex formation with REEP5 RNA than M2515mut (complex VI of RCas9, sgRNA and PAMmer bound to target RNA; lower shift than V because of the lower molecular weight of RCas9) (Fig. 4), indicating that fusion of FTO did not impair binding of RCas9.

**Sequence-specific demethylation of RCas9-FTO**

Based on this knowledge we then tested whether RCas9-FTO fusion proteins were able to preferentially demethylate a target RNA. We used the same RNA constructs, REEP5 and M2515mut, in combination with matching and nonmatching PAMmer and sgRNA (Figs. 5, 6). To rule out bias from preferential demethylation by FTO alone, we compared unspecific FTO demethylation on REEP5 and M2515mut in a quantitative HPLC analysis and found that both RNAs are efficiently demethylated with M2515mut (~27%) being slightly preferred to REEP5 (~23%; Supplemental Fig. S8).

Next, we tested sequence-specific demethylation by RCas9-FTO for REEP5 and M2515mut with matching sgRNAs and PAMmers. SCARLET analysis after a short reaction time (10 min to ensure linear product formation, i.e., initial velocities) showed ~11% demethylation of REEP5 and ~9% of M2515mut, indicating comparable demethylation activity for both REEP5 and M2515mut, respectively (Supplemental Fig. S9).

Furthermore, we tested how addition of a nonspecific or specific competitor RNA would affect sequence-specific demethylation (Fig. 5). Addition of equimolar amounts of unspecific competitor RNA slightly decreased the overall demethylation of REEP5 (<20% decrease; Fig. 5B,C; Supplemental Fig. S7). In comparison, the addition of heparin as an unspecific competitor has no influence on the activity of RCas9-FTO (Supplemental Fig. S12). The addition of equimolar amounts of M2515mut (containing m6A) as competitor led to a significant decrease of demethylation (~34% decrease; Fig. 5D; Supplemental Fig. S7). These data indicate that the presence of competitor RNAs can hamper RCas9-FTO. Since the effect is more pronounced in the case of an FTO target (M2515mut), it functions as a competitive inhibitor of RCas9-FTO, suggesting that m6A or the sequence context is preferred by FTO. Interestingly, vice versa, M2515mut demethylation was not affected by REEP5 as the nontarget m6A competitor (Fig. 5E; Supplemental Fig. S7), pointing to a sequence preference of FTO for M2515mut.

Importantly, the reduction of demethylation activity in the presence of a competitor does not contain information about specificity. To analyze sequence-specific demethylation, we mixed equimolar amounts of both m6A-containing RNAs, i.e., REEP5 and M2515mut. Sequence-specific demethylation was started by addition of RCas9-FTO in the presence of either sgRNA1 and PAMmer1 or sgRNA2 and PAMmer2, targeting either REEP5 or M2515mut (Fig. 6A),
whereas the nonmatching sgRNA and PAMmer were used for off-target demethylation analysis. Sequence-specific demethylation was then analyzed for m\textsuperscript{6}A in REEP5 and M2515mut using the SCARLET assay (Fig. 6D).

Evaluation of the SCARLET data showed that in the presence of sgRNA1 and PAMmer1, the desired demethylation of REEP5 was 15 times more efficient than its off-target demethylation, indicating high sequence-specificity (Fig. 6B; Supplemental Fig. S7). In the presence of sgRNA2 and PAMmer2, the intended demethylation of M2515mut was preferred, albeit to a lower degree (1.6-fold; Fig. 6C; Supplemental Fig. S7). This trend is in line with the EMSA results obtained for sequence-specific binding of RCas9-FTO to REEP5 and M2515mut, which also showed a higher degree of complex formation in the case of REEP5 (Fig. 3B; Supplemental Fig. S11).

To evaluate the potential of our approach for sequence-specific demethylation in cells, we isolated total RNA from HeLa cells and performed our assay in 0.5 µg total RNA per reaction. For both target RNAs, REEP5 and M2515mut, respectively, we obtained comparable demethylation as in the competition assays, i.e., ~8% for REEP5 and ~11% for M2515mut (Fig. 6E; Supplemental Fig. S7, representative TLC in Supplemental Fig. S13). This indicates that sequence-specificity is not compromised in the presence of the complex mixture of total RNAs, pointing toward applicability of this method in cells.

DISCUSSION

In this work we report for the first time an RCas9-FTO fusion protein for sequence-specific demethylation of m\textsuperscript{6}A in RNA. While FTO alone has a reported m\textsuperscript{6}A-dependent sequence preference as well as some substrate preference without a consensus motif (Zou et al. 2016; Zhang et al. 2019), RCas9 can be readily adapted to bind to any given target RNA by addition of a suitable PAMmer and sgRNA (O’Connell et al. 2014). We probed the effect of PAM-to-m\textsuperscript{6}A distance on demethylation activity and found that—although very short distances (18 nt) are preferred suggesting a proximity effect—the RCas9-FTO with its flex-ible linker is able to act on a broad range of distances, making it a versatile tool without the need to heavily optimize it for every given target. The observation that the trend in demethylation was not uniform can likely be attributed to subtle differences in the 3D-structural arrangement of protein to RNA, which may be favorable or unfavorable but are hard to predict. The secondary structure of the RNA itself should be similar for all constructs used (25–40 nt) and not be the reason for discrepancies in demethylation as they consist of the same m\textsuperscript{6}A-sequence part. Additionally, this trend confirms specific demethylation, because RCas9-FTO alone would presumably result in similar unspecific demethylation of all constructs. Especially, this would be the case for distances 18, 22, and 25 nt that are assayed with exactly the same target RNA (Fig. 2E; Supplemental Fig. S5). The longer distance of 38 nt was chosen for subsequent experiments, resulting in comparable demethylation of both m\textsuperscript{6}A-containing target RNAs tested. In our experiments we used a short incubation time and naturally occurring m\textsuperscript{6}A sequences. So, the observed levels of demethylation could be increased by longer incubation or optimization of substrates according to recently published FTO selectivities, including m\textsuperscript{6}A in a big loop of a stem–loop as well as another m\textsuperscript{6}A consensus motif (Zhang et al. 2019). Additionally, we encountered a lower activity of FTO caused by magnesium and TCEP in the assay reaction that are necessary for specific binding and stability of the
RCas9 part, respectively (data not shown). Optimizing those reaction conditions would improve the overall efficiency of demethylation but could lead to compromised selectivities.

In our study, we found out that FTO is not able to demethylate the natural m^6A site at position 2515 of MALAT1. We attributed this to the position of m^6A in a double-stranded region. This secondary structure was first reported by Liu et al. (2013) and confirmed by the six stem-destabilizing mutations that we introduced. We thus obtained M2515mut that retained the m^6A site and sequence context but became a target of FTO and was used for further studies. Our observation was based on in vitro experiments but may also be relevant in cells, suggesting that MALAT1 may not be a target of FTO or that additional proteins assist in secondary structure changes or demethylation of this MALAT1 m^6A site.

Using EMSAs we showed that RCas9-FTO, sgRNA and PAMmer form a complex that binds efficiently to the target RNAs, whereas no interaction with a nonmatching RNA was observed. Sequence-specific complex formation of RCas9-FTO was as efficient as in the case of RCas9 alone, indicating that FTO does not impair the binding properties of RCas9 in complex with sgRNA and PAMmer.

This sequence-specific binding enabled preferred demethylation of a specific m^6A-site in an equimolar mixture of target and nontarget m^6A-containing RNAs. As expected, the addition of a second RNA substrate as competitor generally lowers demethylation; however, this is not a measure for specificity. The use of heparin as an unspecific competitor, which inhibits unspecific interactions of the RCas9 part (O’Connell et al. 2014), did not impact demethylation negatively, indicating specificity.

In sequence-specific demethylation experiments, we could show for two target RNAs that addition of RCas9-FTO in combination with a suitable PAMmer and sgRNA leads to preferred demethylation at the intended site. However, the specificity varied between moderate (1.6-fold) and high (15-fold). As this reflected the trend observed in EMSAs, we think that optimization of RCas9-mediated binding is important for sequence-specificity. The observation that exclusive sequence-specificity is not obtained is most likely the result of unbound RCas9-FTO acting on all FTO targets or of the flexibility of the linker, allowing FTO to act on m^6A-containing RNAs in trans. This problem could be solved by further optimization of complex formation, e.g., using more sgRNA, as well as a short rigid linker of the fusion protein, although the latter could result in an instable RCas9-FTO or limit its activity to preferred PAM-to-m^6A distances.

Our approach presents an important step to manipulate individual m^6A sites and study whether they have a biological function or whether the general m^6A level is responsible for the functions of m^6A reported to date. To this end, our approach would have to be implemented in cells, which, based on our setup and previous work on Cas9-fusion proteins, should be possible (Choudhury et al. 2016; Liu et al. 2016; McDonald et al. 2016; Morita et al. 2016; Vojta et al. 2016; Xu et al. 2016). In cells, the high excess of sgRNA that is necessary for efficient RCas9-FTO complex formation and subsequent targeting can be achieved by using a U6 promoter for sgRNA production. Additionally, the sequence-specific demethylation could be adjusted to RNA-targeting without PAMmer addition, resulting in more convenient experiments in cells.

**FIGURE 6.** Concept and analysis of sequence-specific demethylation. (A) The sgRNA1 and PAMmer1 can exclusively anneal to REEP5 (blue) and not to M2515mut (green), which is only targeted by sgRNA2 and PAMmer2. (B–D) Representative TLC and analysis of on- and off-target effects of sequence-specific demethylation in a stoichiometric mixture of target and nontarget RNA for REEP5 (B) and M2515mut (C). For each assay, SCARLET analysis was performed for both RNAs (D). The control (C) indicates the position of m^6AMP and AMP spots. (E) Effect of sequence-specific demethylation of REEP5 (blue) and M2515mut (green) in cellular total RNA. Bars show average and standard deviation of two independent biological replicates, each performed in two independent technical replicates. Statistical significance was determined using two-tailed Student’s unpaired t-test. (**) $P < 0.01$; (***) $P < 0.001$. 
Evaluation of successful demethylation could be done by SCARLET (Liu et al. 2013), m6A-LAIC-seq (Molinie et al. 2016), SELECT (Xiao et al. 2018) or recently reported quantification methods using m6A-sensitive enzymes, such as T3 DNA ligase (Liu et al. 2018) and RNA-cleaving deoxyribonucleases (Sednev et al. 2018). The method could even be used to study other functions of FTO in a sequence-specific manner, e.g., specific demethylation of m1A in tRNA, which was recently identified to be demethylated by FTO (Wei et al. 2018). The analysis of off-target effects in cells is demanding and in this case could be performed by transcriptome-wide m6A-detection methods (Dominissini et al. 2012; Meyer et al. 2012; Schwartz et al. 2013; Chen et al. 2015; Ke et al. 2015; Linder et al. 2015; Molinie et al. 2016; Hartstock and Rentmeister 2018). Since it is hard to obtain information about the stoichiometry of m6A at a certain position, this could be backed up by testing the m6A-levels in mRNA by LC-MS/MS. Finally, our method is highly flexible and can be used to target any given RNA. In combination with multiple sgRNAs, it would be possible to study the effect of demethylation of select but multiple m6A sites at once. In summary, RCas9-FTO opens the door to sequence-specific targeting of m6A in RNA to gain more insights into the effects of those modifications or the combinations of different modifications.

**MATERIALS AND METHODS**

**Cloning**

The plasmid encoding RCas9 (pMJ841) was a gift from Jennifer Doudna (Addgene plasmid #39318; Jinek et al. 2012). Using site-directed mutagenesis the existing XbaI-site was removed and the stop codon of RCas9 was mutated to XbaI. In the next step, FTO (Na31) was cloned downstream from RCas9 with a linker region coding for GGGGS using restriction enzymes FastDigest XbaI and XhoI. The plasmid encoding FTO (Na31) was a gift from Chengqi Yi (Peking University; Zhu and Yi 2014). The plasmid pPlatTET-gRNA2 was a gift from Izuho Hatada (Addgene plasmid #82559; Morita et al. 2016) and used for cloning of spacer sequences. At first, the plasmid was linearized with FastDigest BbsI and two BbsI restriction sites together with a sgRNA part were cloned via Gibson Assembly upstream of the existing sgRNA sequence. Subsequently, all spacer sequences were cloned using BbsI digestion, which removes BbsI sites completely. The BbsI-part was ordered as gBlock from Integrated DNA Technologies and all DNA oligonucleotides were ordered from Biolegio. Restriction enzymes were ordered from Thermo Scientific: Plasmids and DNA oligonucleotide sequences used in this study can be found in Supplemental Tables S1, S2.

**RNA production**

The sgRNA templates were PCR amplified from the cloned plasmids to attach a T7 promoter. The purified templates were used for T7 in vitro transcription (IVT). The resulting sgRNAs were purified using denaturing polyacrylamide gel electrophoresis (PAGE, 10%, 1× TBE) and hybridized by heating at 95°C for 30 sec and slowly cooling down to room temperature.

For production of the remaining RNAs, synthetic DNA oligonucleotides (including the T7 promoter) were ordered from Biolegio. The respective forward and reverse strands were hybridized and used for T7 IVT. The resulting products were urea TBE PAGE purified and subsequently used for splint ligation with the respective m6A RNAs that were ordered from Dharmacon. At first, the γ- and β-phosphates of the T7 transcribed RNAs were removed by 5′ Polynucleotase according to supplier’s instructions (Biozyn Scientific GmbH). Then equimolar amounts of DNA splint and m6A RNA were hybridized by incubation at 95°C for 1 min followed by cooling for 3 min to room temperature and incubation on ice for 5 min. The ligation was performed using T4 DNA Ligase (0.15 U/pmol RNA, ThermoFisher Scientific) in ligation buffer (1× T4 PNK buffer A, 14.25% DMSO, 67.5 µM ATP) for 3.5 h at 37°C. The DNA splint was digested with Dnase I (0.022 U/pmol splint, ThermoFisher Scientific). The ligation product was purified via denaturing PAGE (15%, 1× TBE).

The SCARLET control A RNA and the competitor RNA were ordered from Bionars and the control m6A RNA as well as the 15 nt-m6A RNA for HPLC analysis from Purimex. RNA oligonucleotide sequences can be found in Supplemental Table S3. Structural predictions were calculated using mfold (Zuker 2003).

**Protein production**

*Escherichia coli* BL21 (DE3) cells transformed with the plasmid encoding RCas9-FTO were grown in LB medium at 37°C to an OD_{600} of 0.7. Expression was induced with 0.2 mM IPTG and performed at 18°C for 16 h. The protein was purified with an immobilized metal affinity (IMAC), anion exchange and size exclusion chromatography (SEC) using the AKTApurifier system. The sonication was performed in buffer containing 20 mM Tris pH 8, 500 mM NaCl, 1 mM TCEP, and 0.1 mM PMSF. The IMAC was performed with a 5 mL HisTrap FF column using buffer A (20 mM Tris pH 8, 500 mM NaCl) for injection and subsequent washing with 20 CV. The protein was eluted with a gradient of 20 CV to buffer B (20 mM Tris pH 8, 250 mM NaCl, 500 mM imidazole). The protein was dialyzed overnight at 4°C in 20 mM Tris pH 8.5, 150 mM NaCl, 1 mM TCEP and 10% glycerol containing additional TEV protease to cleave off the His6-MBP tag. The anion exchange chromatography was performed with a 5 mL HiTrap Q column. The protein was purified using a gradient from 100 mM to 1 M NaCl in a buffer with 20 mM Tris pH 8.5 and 1 mM TCEP. The resulting fractions were further applied to SEC using a Superdex 200 Increase 10/300 GL column with 20 mM Hepes pH 7.5, 150 mM KCl, 1 mM TCEP and 10% glycerol. The protein was concentrated to ~20–30 mg/mL. Aliquots were flash frozen in liquid nitrogen and stored at −80°C.

The expression of RCas9 for EMSA experiments was performed as reported by Jinek et al. (2012). The purification procedure was similar to RCas9-FTO with cation exchange instead of anion exchange chromatography. The IMAC buffers varied by using 20 mM Hepes pH 8 and additional 5% glycerol as well as 1 mM TCEP. For cation exchange chromatography a 5 mL HiTrap SP Sepharose column was used. The protein was eluted with a gradient from 100 mM to 1 M NaCl in 50 mM Hepes pH 7.5,
Amino acid sequences can be found in Supplemental Figure S14.

Frozen in liquid nitrogen and stored at ∼80°C. For FTO production, transformed E. coli BL21 (DE3) cells were cultivated in LB medium at 37°C to an OD600 of 1. Expression was induced with 1 mM IPTG and performed at 16°C overnight. The ÄKTApurifier system was used for purification with a 1 mL HisTrap FF column in the first step. The protein was eluted using a gradient of 20 CV from 50 mM to 500 mM imidazole and a buffer composed of 50 mM sodium phosphate pH 8 and 300 mM NaCl. As a next step, SEC was performed using the Superdex 200 Increase 10/300 GL column to remove RNases. For elution, the same buffer as for IMAC was used with additional 10% glycerol. The protein was concentrated to ∼3 mg/mL. Aliquots were flash frozen in liquid nitrogen and stored at ∼80°C.

All columns were ordered from GE Healthcare Life Sciences. Activity assay conditions

For activity assays, equimolar amounts of RCas9-FTO and sgRNA were preincubated in assay buffer (50 mM MES buffer pH 6.5, 283 μM (NH4)2Fe(SO4)2, 300 μM α-ketoglutarate, 2 mM L-ascorbic acid, 5 μg/mL BSA, 5 mM MgCl2 (Jia et al. 2008; Zhu and Yi 2014)) at 37°C for 10 min. The formed RNP was incubated with equimolar amounts (10 μM) of target RNA and PAMmer in assay buffer at 37°C for additional 10 min. To inactivate the reaction, the protein was denatured at 95°C for 5 min and 5 mM EDTA was added. Reactions with a competitor were performed in the same way using 5 or 10 μM competitor RNA and with 30 min incubation using 10 μM competitor m6A-RNA. Reactions with 50 ng/μL heparin as a competitor were incubated for 10 min and 30 min.

For sequence-specific reactions, the assay was supplemented by 10 μM off-target RNA and 50 ng/μL heparin and incubated at 37°C for 30 min. The cellular RNA assay was performed with standard assay conditions in 0.5 μg total RNA isolated from HeLa cells and incubated at 37°C for 1 h.

SCARLET

To analyze demethylation, the reported SCARLET assay (Liu et al. 2013) was used with the following changes. The amount of used activity assay was calculated to start with 5 pmol RNA. For RNase H cleavage, 5 pmol of chimera were hybridized in a total volume of 3 μL. The radioactive labeling was performed using 10 μT4 PNK and 4–8.5 μCi [γ-32P] ATP depending on the labeling efficiency. 6 pmol splint and 7 pmol ssDNA were hybridized for subsequent splint ligation. After addition of urea loading dye, 166.4 U RNase T1 and 2600 ng RNase A were used for overnight cleavage. The resulting products were purified by urea PAGE (10%, 1× TBE) and eluted with 0.4 mL 0.3 M sodium acetate buffer at 37°C for 2 h. After ethanol precipitation, P1 cleavage was performed in 30 mM sodium acetate/acetic acid pH 5.3. The TLC (TLC PEI Cellulose F plastic plates, Merck) was developed for ∼15 h. The signals were quantified using ImageJ. A mixture of control A- and m6A-RNA was used as a standard (C) in TLCs.

HPLC activity assay

To investigate general demethylation activities, activity assays were analyzed on HPLC. The activity assay was performed in the same assay buffer without MgCl2, sgRNA and PAMmer. 1 μM of RCas9-FTO or 1 μM/5 μM of FTO were incubated with 10 μM RNA at 37°C for 1 h. The enzyme was denatured at 95°C for 5 min and the RNA was digested overnight at 37°C with 0.1 U P1. After dephosphorylation using 1 U alkaline phosphatase at 37°C for 30 min, the nucleosides were separated on reversed-phase chromatography (NUCLEODUR C18 Pyramidal column, MACHEREY-NAGEL). Buffer A contained 50 mM ammonium acetate/acetic acid pH 6. Separation was achieved with a gradient to 30% buffer B (50:50 buffer A: acetonitrile).

EMSA

The preincubation of 1 μM RCas9-FTO/RCas9 with up to 250 nM sgRNA was done at 37°C for 10 min in activity assay buffer, which was supplemented with 5% glycerol and 10 μg/mL heparin. The latter was used to avoid unspecific binding of RCas9-FTO/RCas9 to the target RNA. The resulting RNP was incubated with 1 nM 5’-radiolabeled target RNA and 250 nM PAMmer at 37°C for additional 15 min. Separation was done by 6% native PAGE at 4°C (gel contains 0.5× TBE buffer and 1 mM MgCl2) whereupon labeled RNA was visualized by phosphorimaging and quantified with ImageJ.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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