Sequence-specific m⁶A demethylation in RNA by FTO fused to RCas9

KRISTINA RAU, LUKAS RÖSNER, and ANDREA RENTMEISTER

Institute of Biochemistry, Department of Chemistry, University of Münster, 48149 Münster, Germany

ABSTRACT

N⁶-methyladenosine (m⁶A) 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 m⁶A level; however, modifying distinct m⁶A sites has remained elusive. We genetically fused the dioxygenase FTO responsible for m⁶A 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 m⁶A level at a specific site and analyzed the effect of the PAM-to-m⁶A 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 m⁶A in RNA that can be readily adapted to any given RNA sequence and opens the door to studying the function of distinct m⁶A sites.

Keywords: RNA modification; N⁶-methyladenosine (m⁶A); 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, N⁶-methyladenosine (m⁶A) is the most abundant internal modification and was originally discovered in the early 1970s (Desrosiers et al. 1974). To date, m⁶A has been found in mRNA and a variety of noncoding RNAs including rRNA, tRNA, snRNA, miRNA, and lncRNA. The majority of m⁶A 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 m⁶A 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; Merkestein 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; Lesbirel et al. 2018; Tang et al. 2018). Consequently, m⁶A 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. 2018c) as well as tumor development (Lin et al. 2016; Barbieri et al. 2017; Li et al. 2017c). 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 m⁶A methyltransferase having a different RNA substrate specificity (Pendleton 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

Corresponding author: a.rentmeister@uni-muenster.de

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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 m^6A demethylation (Jia et al. 2011). Recently, Mauer et al. (2017) demonstrated FTO demethylation of $N^6,2'$ -*O*-dimethyladenosine (m^6A_m), which was expanded by a recent study of Wei et al. (2018). The latter reported demethylation of m^6A and m^6A_m with a clear preference for m^6A in cells. In the nucleus, m^6A_m demethylation by FTO is of high importance for snRNA biogenesis (Mauer et al. 2019). Additionally, FTO could demethylate N^1 -methyladenosine (m^1A) in tRNAs (Wei et al. 2018). Very recently, the crystal structure of FTO with the nucleobase N^6 -methyladenine was solved indicating a preference for this nucleobase compared to m^1A or 3-methylthymine. This study also confirmed preferential demethylation of m^6A in vitro and in vivo by FTO as well as reported selectivity of FTO for m^6A in a big loop of a stem-loop structure (Zhang et al. 2019). Additionally, FTO has an m^6A -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, m^6A can impact cellular processes, but most of the studies illustrate a global change of m^6A levels by overexpression of the “writer” proteins and knockdown or knockout of the “erasers,” respectively. The effect of a single m^6A site on the fate of the host RNA is still unknown and tools to manipulate m^6A in a sequence-specific manner are lacking. Therefore, we aimed to develop methodology to enable for sequence-specific “erasure” of m^6A sites. As FTO has no reported consensus motif, we decided to fuse an RNA-targeting protein that can be readily adjusted to bind any sequence of interest.

Although several families of sequence-specific RNA-binding proteins with high affinity exist (e.g., Pumilio [Murata and Wharton 1995; Zamore et al. 1997; Zhang et al. 1997; Wang et al. 2002; Abil et al. 2014] or pentatricopeptide repeat [PPR] proteins [Aubourg et al. 2000; Small and Peeters 2000; Barkan et al. 2012; Coquille et al. 2014]), engineering them to bind to a given target can be challenging. An alternative is RCas9, the nuclease-deficient RNA-targeting Cas9 of *Streptococcus pyogenes* (Jinek et al. 2012). Cas9 itself is a well-studied enzyme established for genome editing (Cong et al. 2013; Jinek et al. 2013; Mali et al. 2013) that binds to its target DNA via a single-guide RNA (sgRNA) and cleaves the DNA strands at defined positions (Jinek et al. 2012). Its nuclease-deficient form (dCas9) was used as a binding protein for specific DNA methylation and demethylation in many studies (Choudhury et al. 2016; Liu et al. 2016;

McDonald et al. 2016; Morita et al. 2016; Vojta et al. 2016; Xu et al. 2016). Recently, dCas9 was found to be suitable for targeting RNA if used in combination with a so-called PAMmer, which strengthens its interaction with the target RNA (O’Connell et al. 2014; Nelles et al. 2016; Rau and Rentmeister 2016). We chose RCas9 as the RNA-targeting protein for our study because it can be readily adjusted to target any given RNA using a suitable sgRNA and PAMmer. Thereby, the fusion of FTO to RCas9 should result in sequence-specific targeting and subsequent demethylation of m^6A sites.

RESULTS

Design of an RCas9-FTO fusion protein

FTO catalyzes the oxidative demethylation of m^6A , 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 (NΔ31), 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 m^6A -containing RNA (15 nt- m^6A RNA) by HPLC after degradation and dephosphorylation confirmed that RCas9-FTO was highly active ($57 \pm 4\%$ demethylation; Fig. 1C). Compared to FTO (Supplemental Fig. S1A), which showed $\sim 16\%$ demethylation under the same assay conditions, RCas9-FTO is considerably more active (Supplemental Fig. S2).

SCARLET analysis and production of longer m^6A RNAs

As demethylation of longer RNAs is hard to quantify by the HPLC-based assay due to the increasing A/ m^6A 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, m^6AMP and AMP are separated by TLC yielding the fraction of m^6A compared to

FIGURE 1. Concept for sequence-specific binding and demethylation. (A) The RCas9 fusion part is guided to the respective target RNA via a sgRNA (purple) and PAMmer (red). RCas9 is fused to FTO in order to recruit it close to the m^6A site for targeted demethylation. (B) SDS-polyacrylamide gel of purified RCas9-FTO. Expected size: 214 kDa. (C) Representative HPLC analysis of RCas9-FTO demethylation activity on 15 nt- m^6A RNA after digestion into single nucleosides A, C, G, U, and m^6A , respectively. After 1 h incubation (at 37°C), $57 \pm 4\%$ demethylation was observed ($n = 3$ independent biological replicates).

the A under investigation. To calibrate this method, we prepared mixtures containing defined ratios of synthetic RNAs with or without m^6A (m^6A -RNA and A-RNA), respectively. A coefficient of determination of 0.97 confirmed that SCARLET is suitable for quantitative assessment of m^6A levels at a given position (Supplemental Fig. S3). For subsequent SCARLET analysis, a mixture of the synthetic m^6A - 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^6A site (Fig. 2A). A short synthetic m^6A -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^6A -containing RNAs with the same m^6A site but different distances to the PAM (25–40 nt; Fig. 2D), allowing us to assess how the PAM-to- m^6A distance will affect demethylation activity. Varying the distance between PAM and m^6A 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^6A distance was tested for *REEP5*, an mRNA known to naturally contain a single m^6A (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^6A (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^6A 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^6A -distance (18 nt) resulted in the highest demethylation of $\sim 14\%$, whereas the longest distance (40 nt) resulted in the lowest activity ($\sim 5\%$), 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.

FIGURE 2. Effect of PAM-to- m^6A distance in *REEP5* RNA on demethylation activity of RCas9-FTO. (A) Splint ligation was used to produce twelve m^6A RNAs with m^6A in identical consensus motif, but varying PAM-to- m^6A distances. A synthetic m^6A RNA was ligated to RNAs from IVT containing 0–15 additional nucleotides at the 5' terminus, resulting in PAM-to- m^6A distances of 25–40 nt. (B) To analyze shorter distances, other sgRNAs and PAMmers are necessary, as shown for 18 nt (green) and 22 nt (light blue) binding to 25 nt *REEP5* RNA. (C) 10% denat. polyacrylamide gels of purified sgRNA1, sgRNA18, and sgRNA22. Expected sizes: 115/116 nt. (D) 15% denat. polyacrylamide gels of purified, splint ligated RNAs resulting in a PAM-to- m^6A distance of 25 nt to 40 nt. Expected sizes: 61–76 nt; ssDNA ladder. (E) Diagram of RCas9-FTO demethylation activity on different *REEP5* RNA constructs determined by SCARLET. Bars show average and standard deviation of three independent biological replicates.

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.

EMSA 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;

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* $62 \pm 5\%$ (blue) and for *M2515mut* $55 \pm 2\%$ (green) of complex IV were shifted to complex V ($n = 3$ independent biological replicates).

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 matching PAMmer and sgRNA, complex IV (target RNA with PAMmer and sgRNA) was formed in good yields, but more pronounced in case of the *REEP5* RNA. 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

(Fig. 3B,C; Supplemental Fig. S11). 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. S11A), 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 m^6A) 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 m^6A or the sequence context is preferred by FTO. Interestingly, vice versa, *M2515mut* demethylation was not affected by *REEP5* as the nontarget m^6A 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 m^6A -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),

FIGURE 4. Analysis and quantification of complex formation of RCas9-FTO and RCas9. (A) Representative EMSA with increasing concentrations of sgRNA (3, 25, and 100 nM). (B) Scheme illustrating binding of RCas9 with sgRNA and PAMmer to the target RNA (complex VI). (C,D) Evaluation of binding from EMSA shown in A. Comparison of RCas9 with and without fused FTO is shown in left and right panel, respectively. Results for *REEP5* (C) and *M2515mut* (D) RNAs are shown. All data were normalized to the bound complex IV ([$^{\circ}$] two independent biological $\times$ two independent technical replicates; [$^{\circ\circ}$] five independent technical out of two independent biological replicates).

FIGURE 5. Concept and analysis of sequence-specific demethylation influenced by various competitor RNAs. (A) *REEP5* (blue) is exclusively targeted by sgRNA1 and PAMmer1, whereas *M2515mut* (green) is targeted by sgRNA2 and PAMmer2. (B) Representative TLC indicating $m^6A/(m^6A + A)$ ratios of *REEP5* RNA influenced by addition of an unspecific competitor RNA (without m^6A ; 0, 5, and 10 μM). The control (C) indicates the position of m^6AMP and AMP spots. (C–E) Effect of addition of an unspecific competitor on demethylation activity of RCas9-FTO. (C) Effect of an unspecific RNA (without m^6A) on demethylation of *REEP5* RNA. (D) Effect of *M2515mut* RNA (10 μM) as the competitor on demethylation of *REEP5* RNA (10 μM). (E) Effect of *REEP5* RNA (10 μM) as the competitor on demethylation of *M2515mut* RNA (10 μM). Demethylation was determined by SCARLET ([3H] three independent biological replicates, 10 min incubation; [3H] two independent biological $\times$ two independent technical replicates, 30 min incubation).

whereas the nonmatching sgRNA and PAMmer were used for off-target demethylation analysis. Sequence-specific demethylation was then analyzed for m^6A in both *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., $\sim 8\%$ for *REEP5* and $\sim 11\%$ 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^6A in RNA. While FTO alone has a reported m^6A -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^6A distance on demethylation activity and found that—although very short distances (18 nt) are preferred suggesting a proximity effect—the RCas9-FTO with its flexible 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^6A -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^6A -containing target RNAs tested. In our experiments we used a short incubation time and naturally occurring m^6A 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^6A in a big loop of a stem-loop as well as another m^6A 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

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^6A 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. (**) $P < 0.01$; (***) $P < 0.001$.

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 unstable 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

(O'Connell et al. 2014; Nelles et al. 2016; Batra et al. 2017). 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 deoxyribozymes (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 (NΔ31) was cloned downstream from RCas9 with a linker region coding for GGGGS using restriction enzymes FastDigest XbaI and XhoI. The plasmid encoding FTO (NΔ31) was a gift from Chengqi Yi (Peking University; Zhu and Yi 2014). The plasmid pPlatTET-gRNA2 was a gift from Izuhito Hatada (Addgene plasmid #82559; Morita et al. 2016) and used for cloning of spacer sequences. At first, the plasmid was linearized with FastDigest AflIII and two BbsI restriction sites together with a sgRNA part were cloned via Gibson Assembly upstream of the existing sgRNA sequence part. 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' Polyphosphatase according to supplier's instructions (Biozym 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 Biomers 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 OD₆₀₀ 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 ÄKTApurifier 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,

5% glycerol and 1 mM TCEP. The SEC was performed as described above. The protein was concentrated to ~3 mg/mL. Aliquots were flash 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 ~10 mg/mL. Aliquots were flash frozen in liquid nitrogen and stored at –80°C.

All columns were ordered from GE Healthcare Life Sciences. Amino acid sequences can be found in Supplemental Figure S14.

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 U T4 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 Pyramid 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 is 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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