CCR4 and CAF1 deadenylases have an intrinsic activity to remove the post-poly(A) sequence

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ABSTRACT

MicroRNAs (miRNAs) recruit the CCR4–NOT complex, which contains two deadenylases, CCR4 and CAF1, to promote shortening of the poly(A) tail. Although both CCR4 and CAF1 generally have a strong preference for poly(A) RNA substrates, it has been reported from yeast to humans that they can also remove non-A residues *in vitro* to various degrees. However, it remains unknown how CCR4 and CAF1 remove non-A sequences. Herein we show that *Drosophila* miRNAs can promote the removal of 3'-terminal non-A residues in an exonucleolytic manner, but only if an upstream poly(A) sequence exists. This non-A removing reaction is directly catalyzed by both CCR4 and CAF1 and depends on the balance between the length of the internal poly(A) sequence and that of the downstream non-A sequence. These results suggest that the CCR4–NOT complex has an intrinsic activity to remove the 3'-terminal non-A modifications downstream from the poly(A) tail.

Keywords: CCR4–NOT complex; deadenylase; deadenylation; microRNA; mRNA decay

INTRODUCTION

In eukaryotes, cytoplasmic mRNA decay pathways are generally initiated by shortening of the poly(A) tail or deadenylation. Deadenylated mRNAs are degraded by exosome from 3'-end or are decapped by the Dcp1–Dcp2 complex followed by degradation by Xrn1 from the 5'-end (Parker and Song 2004; Garneau et al. 2007; Houseley and Tollervey 2009). One of the well-known triggers of deadenylation is microRNA (miRNA) (Goldstrohm and Wickens 2008; Chen and Shyu 2011; Pérez-Ortín et al. 2013), which forms RNA-induced silencing complex (RISC) with Argonaute (Ago) proteins and guides Ago to their complementary target mRNAs (Bartel 2009; Kawamata and Tomari 2010; Hirose et al. 2014). In *Drosophila melanogaster*, miRNAs are loaded into Ago1 (Okamura et al. 2004; Forstemann et al. 2007; Tomari et al. 2007), which directly interacts with GW182 (also known as TNRC6 in vertebrates) (Behm-Ansmant et al. 2006; Eulalio et al. 2008). GW182 recruits two deadenylase complexes, the PAN2–PAN3 complex and the CCR4–NOT complex (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011), of which the CCR4–NOT complex provides a major contribution to miRNA-mediated deadenylation (Behm-Ansmant et al. 2006; Chen et al. 2009; Piao et al. 2010; Braun et al. 2011).

The CCR4–NOT complex is highly conserved in eukaryotes and its core components comprise eight subunits in flies (Collart and Panasenko 2012; Temme et al. 2014). Of these, CCR4 and CAF1 (also known as POP2) each possess an Mg²⁺-dependent deadenylase activity. While flies have only single orthologs each of CCR4 and CAF1, vertebrates such as humans have their multiple orthologs. CAF1 is a member of the DEDDh (Asp–Glu–Asp–Asp–His in the active site) family of RNases (Zuo and Deutscher 2001), and directly binds to the MIF4G fold of NOT1, the scaffold protein in the CCR4–NOT complex (Basquin et al. 2012; Petit et al. 2012). CCR4 belongs to the endonuclease-exonuclease-phosphatase (EEP) family of RNases (Dlakić 2000) and possesses the leucine-rich repeat (LRR) domain and the nuclease domain. The LRR domain of CCR4 binds to CAF1, thereby sandwiching CAF1 between CCR4 and NOT1 (Draper et al. 1995; Basquin et al. 2012). Interestingly, the nuclease domain of *Saccharomyces cerevisiae* CCR4 requires the neighboring LRR domain for its deadenylation activity, while the nuclease domain of human CNOT6L (CCR4 homolog) retains its activity in the absence of the LRR domain (Clark et al. 2004; Wang et al. 2010).

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.057679.116>.

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Although CCR4 and CAF1 generally prefer the poly(A) sequence, a number of reports suggest that they can remove non-A sequences at least in vitro (Chen et al. 2002; Thore et al. 2003; Viswanathan et al. 2004; Bianchin et al. 2005; Wang et al. 2010). For instance, recombinant yeast Pop2p (CAF1 homolog) exhibited robust RNase activity for poly(U) and poly(C) RNAs (Thore et al. 2003), although the competition assay revealed a modest preference toward poly(A) (Daugeron et al. 2001). Moreover, recombinant human CNOT7 (Caf1a) and CNOT8 (Caf1b/POP2) were able to degrade RNA substrates beyond their 3'-terminal poly(A) sequence (Bianchin et al. 2005). However, it remains unknown how CCR4 and CAF1 remove non-A sequences.

Herein we show that, in flies, miRNAs can promote the removal of 3'-terminal non-A residues, but only if an upstream poly(A) sequence is present. This activity is directly mediated by the CCR4–NOT complex and depends on the balance between the length of the internal poly(A) sequence and that of the downstream non-A sequence. Purified CCR4 or CAF1 alone shows such an internal poly(A)-dependent exonuclease activity, even though as previously reported they both strongly prefer poly(A) RNAs to other polyribonucleotides. The intrinsic property of the CCR4–NOT complex to remove 3'-terminal non-A modifications should not be restricted to miRNA triggers and may function to regulate mRNAs with widespread uridylation and guanylation downstream from the poly(A) tail.

RESULTS

miRNAs can induce the removal of a non-A sequence downstream from the poly(A) tail in S2 cells

We have previously reported that reporter RNAs bearing a 114-nucleotide (nt) poly(A) sequence internalized by a downstream 40-nt unrelated sequence (hereafter referred to as the post-poly(A) sequence), followed by the self-cleaving hammerhead ribozyme (HhR) to generate a defined 3' end, are refractory to miRNA-mediated deadenylation and decay in *Drosophila* S2 cells (Fukaya and Tomari 2012). However, in the course of our study, we noticed that a shorter post-poly(A) sequence cannot completely block miRNA-mediated deadenylation. Indeed, when we expressed reporter RNAs for *let-7* miRNA bearing a 50-nt poly(A) sequence with or without an unrelated 10-nt post-poly(A) sequence (Rluc-*let-7*-A₅₀ and Rluc-*let-7*-A₅₀C₁₀; Fig. 1A), they were both efficiently degraded upon ectopic *let-7* expression (Fig. 1B). Knockdown of Dcp2, an enzyme required for decapping, resulted in accumulation of deadenylated intermediates for both A₅₀ and A₅₀C₁₀ reporters, in a manner dependent on *let-7* expression (Fig. 1B). Thus, miRNAs can promote the removal of not only 3'-exposed poly(A) tails but also internalized poly(A) sequences with short post-poly(A) sequences in S2 cells.

FIGURE 1. miRNAs can induce the removal of a non-A sequence downstream from the poly(A) tail in S2 cells. (A) Schematic representation of Rluc-*let-7*-A₅₀-HhR and Rluc-*let-7*-A₅₀C₁₀-HhR. These reporter RNAs include *Renilla* luciferase (Rluc) ORF, eight *let-7* target sites, and hammerhead ribozyme. The subscripts show the length of an RNA sequence including the shown base. (B) Northern blot analysis of the reporter RNAs shown in A. Expression of *pri-let-7* induced degradation of the reporter RNAs in GFP knockdown (mock). In Dcp2 knockdown, deadenylated RNAs were detected dependently on expression of *pri-let-7*.

The balance between the poly(A) length and the downstream post-poly(A) length affects deadenylation efficiency

To analyze the effect of the post-poly(A) sequence on deadenylation more closely, we performed in vitro deadenylation assays. As reported previously, we prepared deadenylation-active lysate from S2 cells overexpressing Argonaute1 (Ago1) (Fukaya and Tomari 2011). We also constructed a series of reporter RNAs for *let-7* with increasing lengths of the post-poly(A) sequence: Mini-*let-7*-A₂₀ with a 3' exposed 20-nt poly(A) tail, Mini-*let-7*-A₂₀C₅ with the poly(A) tail internalized by the 5-nt post-poly(A) sequence, and Mini-*let-7*-A₂₀C₁₀ with the 10-nt post-poly(A) sequence (Fig. 2A). We then programmed Ago1-RISC by *let-7/let-7** duplex and measured the time course of reporter RNA deadenylation in vitro. As shown in Figure 2B and C, the length of the post-poly(A) sequence negatively correlated with the deadenylation efficiency. We then fixed the length of the post-poly(A) sequence at 10 nt and changed the length of the upstream poly(A) sequence from 20 nt to 60 nt. Intriguingly, the deadenylation efficiency was significantly improved by elongating the internal poly(A) sequence (Fig. 2B,C; Mini-*let-7*-A₂₀C₁₀ and Mini-*let-7*-A₆₀C₁₀). These results suggest that deadenylation is promoted by the length of the upstream poly(A) sequence and inhibited by the length of the downstream post-poly(A) sequence. To rule out the possibility that the entire length of the reporter RNAs is important, rather than the poly(A) length per se, we constructed a reporter RNA that has the same overall length as Mini-*let-7*-A₆₀C₁₀ but contains a 40-nt unrelated sequence and a 20-nt poly(A) sequence instead of the 60-nt poly(A) sequence, upstream of the 10-nt post-poly(A) sequence (Fig. 2A; Mini-*let-7*-

FIGURE 2. The balance between the poly(A) length and the downstream post-poly(A) length affects deadenylation efficiency. (A) Schematic representation of a series of Mini-let-7 reporter constructs. These reporter RNAs include four *let-7* target sites and were capped with [α -³²P] GTP. The subscripts show the length of an RNA sequence including the shown base. The asterisks indicate a radiolabel. (B) Deadenylation assay for Mini-let-7 reporter RNAs with S2 cell lysate overexpressed Ago1. (C) The signal intensity of the bands in B was quantified, and the ratio of the intensity from the lower band for the total intensity was calculated and plotted. Mini-let-7-A₂₀ effectively shifted to A₀. While the length of the post-poly(A) sequence negatively correlated with the deadenylation efficiency, elongating the internal poly(A) sequence improved. The graph shows means and standard deviations ($n = 3$). (D) Deadenylation assay for Mini-let-7-A₆₀C₁₀ and Mini-let-7-N₄₀A₂₀C₁₀ with S2 cell lysate overexpressed Ago1. (E) The signal intensity of the bands in D was quantified, and the ratio of the intensity from the lower band for the total intensity was calculated and plotted. Mini-let-7-A₆₀C₁₀ was deadenylated more effectively than Mini-let-7-N₄₀A₂₀C₁₀. The graph shows means and standard deviations ($n = 3$).

N₄₀A₂₀C₁₀). As shown in Figure 2D and E, deadenylation of Mini-let-7-N₄₀A₂₀C₁₀ was much slower than that of Mini-let-7-A₆₀C₁₀. Thus, the balance between the upstream poly(A) sequence and the downstream post-poly(A) sequence determines the deadenylation efficiency.

Deadenylation of the internalized poly(A) sequence involves the CCR4–NOT complex

It is well known that the CCR4–NOT deadenylase complex plays a major role in regular miRNA-directed deadenylation (Behm-Ansmant et al. 2006; Chen et al. 2009; Piao et al. 2010; Braun et al. 2011). Therefore, we asked whether deadenylation of the poly(A) sequence internalized by the post-poly(A) sequence is also mediated by the CCR4–NOT complex. To this end, we prepared lysate from S2 cells with either CCR4 or CAF1 knockdown together with Ago1 overexpression (Fig. 3A) and assayed deadenylation in vitro. Knockdown of CAF1 abolished deadenylation of Mini-let-7-A₂₀ with the 3′-exposed poly(A) tail (Fig. 3B), while knockdown of CCR4 impaired deadenylation only modestly (Fig. 3C,D). This is consistent with the configuration of the CCR4–NOT complex, in which CAF1 bridges CCR4 and NOT1;

CAF1 knockdown not only depletes CAF1 itself but also causes detachment of CCR4 from NOT1, while CAF1 is still functional on NOT1 in the absence of CCR4 (Temme et al. 2004). Importantly, both CAF1 knockdown and CCR4 knockdown inhibited deadenylation of Mini-let-7-A₂₀N₁₀ with the poly(A) sequence internalized by the post-poly(A) sequence, similarly to that of Mini-let-7-A₂₀ (Fig. 3B–D). These results suggest that the CCR4–NOT complex is involved in deadenylation of internalized poly(A) sequences with post-poly(A) sequences as well as normal 3′-exposed poly(A) tails.

CCR4 and CAF1 have an intrinsic activity to remove post-poly(A) sequences

To examine if the CCR4–NOT complex directly mediates removal of the post-poly(A) sequence, we prepared four types of recombinant *Drosophila* CCR4/CAF1 heterodimer using a baculovirus expression system; where both CCR4 and CAF1 are wild types (4W1W), either one of them is a catalytic mutant (4m1W and 4W1m), or both of them are catalytic mutants (4m1m) (Fig. 4A). We then constructed reporter RNAs without any miRNA target sites (Fig. 4B) and incubated them

FIGURE 3. Deadenylation of the internalized poly(A) sequence involves the CCR4–CAF1 complex. (A) Western blot analysis. CCR4 and CAF1 were significantly reduced by treatment of the corresponding dsRNA to S2 cells. (B) Deadenylation assay for Mini-let-7-A₂₀ and Mini-let-7-A₂₀N₁₀ with S2 cell lysate knocked down CAF1. While mock knockdown lysate effectively induced deadenylation for both reporter RNAs, CAF1 knockdown lysate failed to deadenylate. (C,D) Deadenylation assay for Mini-let-7-A₂₀ and Mini-let-7-A₂₀N₁₀ with S2 cell lysate knocked down CCR4. Compared to mock knockdown, deadenylation was retarded in CCR4 knockdown.

with the four types of the heterodimers. Except for 4m1m, the heterodimers showed deadenylation activity for the A₂₀ reporter. This suggests that, as expected, the heterodimer can mediate deadenylation as long as either CCR4 or CAF1 is intact (Fig. 4C). We then tested the four heterodimers for the A₂₀C₅ reporter with the internalized poly(A) sequence. Strikingly, as we observe with the normal A₂₀ reporter, the A₂₀C₅ reporter was also shortened by the heterodimers except when both CCR4 and CAF1 were catalytically mutated (Fig. 4D). These results indicate that *Drosophila* CCR4 and CAF1 have independent and intrinsic activity to remove the C₅ post-poly(A) sequences downstream from the A₂₀ poly(A) sequence. Nonetheless, when 30-nt poly(N) RNAs with each of the four different nucleotides were assayed, the CCR4/CAF1 heterodimer showed a strong preference for adenines as previously reported (Tucker et al. 2001; Viswanathan et al. 2003, 2004; Temme et al. 2004; Bianchin et al. 2005;

Wang et al. 2010) and the poly(C) RNA (C₃₀) remained completely intact (Fig. 4E). Moreover, Mini-let-7-A₂₀N₁₀, which contains all of four bases in the 3'-terminal N₁₀ sequence (UUUGUCUGAC), was deadenylated by the CCR4/CAF1 heterodimer, similarly to the noC-A₂₀C₅ reporter (Supplemental Fig. S1). These results support that CCR4 and CAF1 possess an activity of removing a stretch of non-A nucleotides only when an upstream poly(A) sequence exists.

The balance between the poly(A) length and the downstream post-poly(A) length affects the activity of CCR4 and CAF1

We next set out to ask if the recombinant CCR4/CAF1 heterodimer recapitulates the dependence of deadenylation on the balance between the poly(A) length and the downstream post-poly(A) sequence, which was observed in miRNA-mediated deadenylation in cell lysate. We prepared a series of reporter RNAs with different lengths of the internal poly(A) sequence and the downstream post-poly(A) sequence (Fig. 4B) and incubated them with the four types of CCR4/CAF1 heterodimers. As we had observed in the miRNA-mediated reaction (Fig. 2), deadenylation by the wild-type 4W1W heterodimer was inhibited by the length of the post-poly(A) sequence and promoted by the length of the internal poly(A) sequence (Fig. 5A,C) but not by the entire length (Fig. 5B, D). This trend was also true for the 4m1W and 4W1m heterodimers, where either CCR4 or CAF1 was mutated. Collectively, we concluded that CCR4 and CAF1 have an intrinsic ability to remove the post-poly(A) sequence in a manner dependent on the balance between the internal poly(A) length and the downstream post-poly(A) length.

CCR4 and CAF1 remove post-poly(A) sequences in an exonucleolytic manner

It has been reported that *Drosophila* CCR4 and CAF1 are 3'–5' exoribonucleases with a strong A preference (Temme et al. 2004). How do CCR4 and CAF1 remove the post-poly(A) sequence? To discriminate endonucleolytic and exonucleolytic reactions, we designed reporter RNAs with an internal label in the post-poly(A) sequence. Specifically, the RNAs contained no C in the 5' body region, while the 5-nt post-poly(A) sequence of "CGGGG" contained a single C internally (noC-A₂₀C₁G₄; Fig. 6A). We then transcribed the RNAs in the presence of [α -³²P] CTP so that the internal C in the post-poly(A) sequence was specifically radiolabeled. We also prepared a control reporter in which the internal 20-nt poly(A) sequence was replaced with UG repeats of 20 nt in total (noC-UG₂₀C₁G₄; Fig. 6A). If CCR4 and/or CAF1 removed the post-poly(A) sequence in an endonucleolytic manner before trimming the poly(A) sequence, a 5-nt radiolabeled fragment of *CGGGG should be detected. On the other hand, if the post-poly(A) sequence was removed in an exonucleolytic manner, radiolabeled CMP should be

FIGURE 4. CCR4 and CAF1 have an intrinsic activity to remove post-poly(A) sequences. (A) SDS-PAGE and Coomassie brilliant blue staining for four types of recombinant *Drosophila* CCR4/CAF1 heterodimer, in which both CCR4 and CAF1 are wild types, either one of them is catalytic mutant, and both of them are catalytic mutants. (W) Wild type, (m) catalytic mutant. (B) Schematic representation of a series of noC reporter constructs. These reporter RNAs were capped with [α -³²P] GTP. The subscripts show the length of an RNA sequence including the shown base. The asterisks indicate a radiolabel. (C) Deadenylation assay for noC-A₂₀ with four types of recombinant CCR4/CAF1 heterodimer. The heterodimers showed deadenylation activity for the A₂₀ reporter except for 4m1m. (4W1W) CCR4 and CAF1 are wild types, (4m1W) CCR4 is catalytic mutant and CAF1 is wild type, (4W1m) CCR4 is wild type, CAF1 is catalytic mutant, (4m1m) CCR4 and CAF1 are catalytic mutants. (D) Deadenylation assay for noC-A₂₀C₅ with four types of recombinant CCR4/CAF1 heterodimer. Despite the fact that the reporter RNA had C₅ following A₂₀, CCR4 and CAF1 could deadenylate. (E) 30 nt poly(A), (U), (G), and (C) RNAs were incubated with four types of recombinant CCR4/CAF1 heterodimer. Both CCR4 and CAF1 strongly preferred poly(A) as a substrate. Note that poly(U) was slightly degraded in all cases (even for the 4m1m double-mutant heterodimer), presumably due to contaminating nonspecific nuclease(s).

generated. Intriguingly, the catalytically active CCR4/CAF1 heterodimers (4W1W, 4m1W, and 4W1m) generated a 1-nt reaction product from noC-A₂₀C₁G₄, while the 1-nt signals from noC-UG₂₀C₁G₄ or from the 4m1m heterodimer

were markedly weaker (Fig. 6B). Thin-layer chromatography identified the reaction product as CMP (Fig. 6C). These results indicate that both CCR4 and CAF1 remove the post-poly(A) sequence in an exonucleolytic manner, as is the case with regular deadenylation.

DISCUSSION

In this study, we show that CCR4 and CAF1, the two deadenylases in the CCR4–NOT complex, can remove 3' terminal non-A residues in an exonucleolytic manner, if an upstream poly(A) sequence is present. This noncanonical but intrinsic activity of CCR4 and CAF1 depends on the balance between the length of the upstream poly(A) sequence and that of the post-poly(A) sequence. These observations suggest that CCR4 and CAF1 can remotely contact the internal poly(A) sequence, which then promotes exonucleolytic digestion of non-A residues at the 3' end.

Structural studies have shown that both CCR4 and CAF1 are capable of specifically recognizing an adenine adjacent to their catalytic cores (Andersen et al. 2009; Wang et al. 2010). For example, human CNOT6L (Ccr4b) recognizes an adenine by sandwiching the purine ring between the rings of Phe484 and Pro36. Additionally, the N6 group of the adenine base interacts with the carbonyl group of Asn412 (Wang et al. 2010). On the other hand, in *S. pombe* Pop2p (CAF1 homolog), Ser122 is thought to form a hydrogen bond to N3 of an adenine, with the side chain of Leu125 stabilizing the location of its purine ring (Andersen et al. 2009). In both cases, only a few specific interactions exist between the adenine base and the residues in the active sites, which may allow accommodation of a non-A residue for removal at the 3' end. However, it remains unknown how the internal poly(A) sequence can be recognized. One possibility is that nonproductive yet recurring binding of the internal poly(A) sequence

to the A-binding site simply increases the chance for the 3' non-A residue to be fortuitously accommodated in the catalytic core. Another possibility is that CCR4 and CAF1 have a second, as-yet-uncharacterized poly(A) recognition site.

FIGURE 5. The balance between the poly(A) length and the downstream post-poly(A) length affects the activity of CCR4 and CAF1. (A) Deadenylation of noC reporter RNAs with four types of recombinant CCR4/CAF1 heterodimer after 2 h incubation. (B) Deadenylation of noC-A₂₀C₁₀ and noC-CG₄₀A₂₀C₁₀ with four types of recombinant CCR4/CAF1 heterodimer after 2 h incubation. (C) The signal intensity of the bands in A was quantified, and the ratio of the intensity from the lower band for the total intensity was calculated and shown. As well as Figure 2C, noC-A₂₀ effectively shifted to A₀. While the length of the post-poly(A) sequence negatively correlated with the deadenylation efficiency, elongating the internal poly(A) sequence improved. The graph shows means and standard deviations ($n = 3$). (D) The signal intensity of the bands in B was quantified, and the ratio of the intensity from the lower band for the total intensity was calculated and shown. noC-A₂₀C₁₀ was deadenylated more effectively than noC-CG₄₀A₂₀C₁₀. The graph shows means and standard deviations ($n = 3$).

Intriguingly, a second AMP molecule was observed ~20 Å apart from the active site in the human CNOT6L–AMP complex structure (Wang et al. 2010); however, the adenine base is not specifically recognized there and it cannot be excluded that the second AMP site is a crystallization artifact.

It has been reported recently that human CCR4 and CAF1 work cooperatively in deadenylation; when either CNOT7 (Caf1a) or CNOT6L (Ccr4b) was mutated in the active site, the activity of recombinant BTG2–CNOT7–CNOT6L complex was abolished to the same extent as the double mutant (Maryati et al. 2015). In contrast, in our hands, the 4m1W and 4W1m fly CCR4/CAF1 heterodimers showed strong deadenylation activity compared to the 4m1m double mutant. Furthermore, 4m1W and 4W1m were able to remove post-poly(A) sequences in the same internal poly(A)-dependent manner as the 4W1W wild type. Future investigation is needed to address whether this apparent discrepancy results

from the difference in species origin, the configuration of the complex, or the experimental setting including substrate RNAs.

Although we here used artificially designed reporters to detect the post-poly(A) removal activity of CCR4 and CAF1, we speculate that this activity may function in vivo. Indeed, natural post-poly(A) sequences are observed broadly in mRNAs of yeast, plants and humans and are thought to regulate the stability of mRNAs (Rissland and Norbury 2009; Sement et al. 2013; Chang et al. 2014). In human cells, oligo U is the most frequent post-poly(A) sequence and is mainly found in <25-nt poly(A) tail (Chang et al. 2014).

FIGURE 6. CCR4 and CAF1 remove post-poly(A) sequences in an exonucleolytic manner. (A) Schematic representation of noC-A₂₀C₁G₄ and noC-UG₂₀C₁G₄. These reporter RNAs include no nucleotides with C besides the moiety of post-poly(A) sequence, whereby the only post-poly(A) sequence can be labeled by transcribing with NTP including [α -³²P] CTP. The asterisks indicate a radiolabel. (B) Deadenylation of noC-A₂₀C₁G₄ and noC-UG₂₀C₁G₄ with four types of recombinant CCR4/CAF1 heterodimer after 2 h incubation. Although CCR4 and CAF1 generated CMP from both reporter RNAs, the signal intensities from noC-A₂₀C₁G₄ in active CCR4/CAF1 heterodimers were higher than from noC-A₂₀C₁G₄ in inert CCR4/CAF1 heterodimer and from noC-UG₂₀C₁G₄. (C) Thin layer chromatography for the samples of B.

MATERIALS AND METHODS

Grant-in-Aid for Scientific Research (A) to Y.T. (grant number 15H02382) from the Japan Society for the Promotion of Science. S.N. is a recipient of JSPS Research Fellowships for Young Scientists (grant number 14J08195).

Author contributions: S.N., T.F., and Y.T. conceived and designed the study. S.N. performed the experiments and analyzed the data; T. F. started the research project and obtained initial data; Y.T. supervised the study; S.N. and Y.T. wrote the manuscript; all authors discussed the results and approved the manuscript.

Received May 25, 2016; accepted June 21, 2016.

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RNA 2016 22: 1550-1559 originally published online August 2, 2016

Access the most recent version at doi:[10.1261/rna.057679.116](https://doi.org/10.1261/rna.057679.116)

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