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

SHO NIINUMA, TAKASHI FUKAYA,¹ and YUKIHIDE TOMARI
Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan
Department of Computational Biology and Medical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan

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-Ortin 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. 2015). CCR4 belongs to the endonuclease-exonuclease-phosphatase (EEP) family of RNases (Dlakić 2000) and possesses the leucine-rich repeat (LRR) domain and the nuclelease 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 nuclelease domain of Saccharomyces cerevisiae CCR4 requires the neighboring LRR domain for its deadenylating activity, while the nuclelease domain of human CNOT6L (CCR4 homolog) retains its activity in the absence of the LRR domain (Clark et al. 2004; Wang et al. 2010).

¹Present address: Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA
Corresponding author: tomari@iam.u-tokyo.ac.jp
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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 miRNAs 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.

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-A30 with a 3′ exposed 20-nt poly(A) tail, Mini-let-7-A60C10 with the poly(A) tail internalized by the 5-nt post-poly(A) sequence, and Mini-let-7-A60C10 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-
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-N40A20C10 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...
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-A20C5; 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. If CCR4 and/or CAF1 removed the post-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...
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.
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 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-A60C10 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-A60C10 was deadenylated more effectively than noC-CG40A20C10. The graph shows means and standard deviations (n = 3).

**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.
Uridylation frequency shows a modest negative correlation with mRNA half-life, suggesting its role as a mark for mRNA decay. Recently, terminal uridylyl transferase 4 and 7 (TUT4/7) have been identified as the factors responsible for mRNA uridylation, and their activity is enhanced by a shorter poly(A) tail (Lim et al. 2014). In contrast, G is found mainly downstream from longer poly(A) tails (>40 nt) and potentially stabilizes mRNAs (Chang et al. 2014). Perhaps, the CCR4–NOT complex not only acts as a deadenaylase but also may serve as an eraser of such post-poly(A) sequences, adding another layer of complexity in mRNA stability control.

MATERIALS AND METHODS

Plasmid construction

pAWS

A PCR fragment containing the SBP-tag sequence was amplified with 5′-GTGAGCTCCGCGCACTAGTGAGAGACAGCACC-3′ and 5′-TACCTTCGCTTCAGGACGACGTC-3′ from Drosophila S2 cell cDNA using primers 5′-CACCCTTCCACTCACAGACGGAAAACACATCCGGTCAGACGCTGAGGACTCATCAGACCGGAAAACACATCCGGAGGCTCACCA-3′ by using InfusionHD (Takara). The Rluc-let-7-A50-HhR, pAWS-Rluc-let-7-A50N10-HhR

The Rluc-let-7-A50-HhR sequence was amplified by PCR from pUC57-Rluc-let-7-A114 (Fukaya and Tomari 2011) using the primers 5′-CCATCATGACATTTCAGGCGAGGTGTCG-3′ and 5′-CCATCATGACATTTCAGGCGAGGTGTCG-3′. The dsDNA fragment was then inserted into pENTR/D-TOPO (Invitrogen), followed by recombination into pAWS (Fukaya and Tomari 2012) and inserted into PCR-linearized pAWS with 5′-CACCCTTCCACTCACAGACGGAAAACACATCCGGTCAGACGCTGAGGACTCATCAGACCGGAAAACACATCCGGAGGCTCACCA-3′ by using InfusionHD (Takara).

pAWS-Rluc-let-7-A50N10-HhR

The Rluc-let-7-A50N10-HhR sequence was recombined from pAWS-Rluc-let-7-A50-HhR, two DNA fragments, 5′-TACCTTCGCTTCAGGACGACGTC-3′ and 5′-CCATCATGACATTTCAGGCGAGGTGTCG-3′, were phosphorylated using T4 polynucleotide kinase (Takara) and hybridized with the membrane using Perfecthyb Plus (Sigma) at 50°C.

Preparation of double-stranded RNAs for RNAi

Double-stranded RNAs (dsRNAs) corresponding to the open reading frame of GFP, Dcp2, CAF1, and CCR4 mRNAs were prepared as previously described (Temme et al. 2004; Forstemann et al. 2005; Rehwinke et al. 2005). All the dsRNAs were in vitro transcribed using T7-Scribe Standard RNA IVT Kit (CELLSCRIPT) and were purified by ethanol precipitation followed by ammonium acetate precipitation.

RNA interference and transfection

Dcp2 knockdown was performed as previously described (Rehwinke et al. 2005). After 1 d of soaking, the S2 cells were transfected with 5 µg pAWS-pri-let-7 (Fukaya and Tomari 2012) and 5 µg pAWS-Rluc-let-7-A50-HhR or pAWS-Rluc-let-7-A50C10-HhR using X-tremeGENE HP (Roche) and further cultured for 3 d. For CAF1 knockdown, S2 cells were seeded at a density of 1.0 × 10^6 cells/mL in 10-cm dishes, and 20 µg dsRNAs were added to the dishes. One day after soaking, S2 cells were transfected with 10 µg pAWS-Ago1 using X-tremeGENE HP (Roche) and further cultured for 3 d. For CCR4 knockdown, S2 cells in 10-cm dishes were soaked with 100 µg dsRNAs. After 3 d, 100 µg dsRNAs were added again, and the cells were cultured for 1 d. Then, the cells were diluted fivefold and transfected with pAWS-Ago1 similarly to CAF1 knockdown. Lysates from S2 cells were prepared as previously described (Fukaya and Tomari 2012).

Western blot analysis

Antibodies to FLAG (1:5000; Sigma), CAF1 (1:5000 [Temme et al. 2004]), CCR4 (1:5000 [Temme et al. 2004]), and Tubulin (1:1000; Sigma) were used as the primary antibodies. Chemiluminescence was induced by Luminata Forte Western HRP Substrate (Millipore) and images were acquired by LAS-3000 (Fujifilm Life Sciences).

Preparation of the target RNAs

Mini-let-7-A20 and Mini-let-7-A20N10 RNAs

DNA fragments were amplified by PCR from psiCHECK2-let-7 4× (Iwasaki et al. 2009) using primers 5′-GTAATACGACTCACTATAGGGAGCTCACGAGAAGGGTGGG-3′ and 5′-GTCAGAGACGAGAAGGGTGGG-3′, were phosphorylated using T4 polynucleotide kinase (Takara) and hybridized with the membrane using Perfecthyb Plus (Sigma) at 50°C.
noC-A20, noC-A20C5, noC-A20C10, noC-A60C10, and noC-A20C10 RNAs

DNA fragments were amplified by three-way overlapping PCR using primers 5′-GGTGAGAGTGAGTAGTATTGGGTGGTA-3′ and 5′-TACCACGCTGAAACGCGGTGGCCATGGCCAGCACATA GTG-3′ for CCAF1, 5′-CTGCTGCTGCTGCGTGCTTTCGCCTCCTG CTACCAGATTCA-3′ and 5′-TGAATCGGGTACGGAGCGCAA GGCACCGCACACGCAGCAG-3′ for CCR4.

Protein purification

The CCR4/CAF1 heterodimers were expressed in SF9 cells by using Bac-to-Bac Baculovirus expression system (Invitrogen). The heterodimers were purified by sequential affinity chromatography with His-trap FF crude (20–400 mM imidazole) and Mono S and Mono Q columns (20–1000 mM KCl), according to the manufacturer’s instruction (GE Healthcare). The heterodimers were then buffer-exchanged into 1× lysis buffer containing 10% glycerol and 1 mM DTT using NAP-5 (GE Healthcare).

Deadenylation assay in vitro

Preparation of 40× reaction mix (containing ATP, ATP regeneration system, GTP, and RNase inhibitor), lysis buffer [30 mM HEPES-KOH (pH 7.4), 100 mM KOAc and 2 mM Mg(OAc)₂], 2× proteinase K buffer, and formamide loading dye has been described in detail (Haley et al. 2003). The deadenylation assay with S2 cell lysate was previously described (Fukaya et al. 2014). Deadenylation reaction by recombinant CCR4/CAF1 heterodimers typically contained 6 µL of 1.5 µM recombinant CCR4/CAF1 heterodimer, 3 µL of 5× lysis buffer, 1.5 µL of 10 mM DTT, 0.3 µL of 40 U/µL RNasin plus (Promega), 2 µL of ~5 nM target RNA, and 2.2 µL of water and the mixture was incubated at 25°C. For 30-nt poly(N) RNAs (Fig. 4E), Na₃VO₄ was added at 10 mM in the deadenylation reaction. At each time point, 2.8 µL of the reaction mixture was taken and the equal volume of formamide loading dye was added. Then, the sample was electrophoresed on 5%, 6%, or 18% denaturing polyacrylamide gel and analyzed by PhosphoImager (Typhoon FLA 7000, GE Healthcare).

Thin layer chromatography

Typically, a polyethyleneimine-cellulose plate (MACHEREY-NAGEL) was pre-run with water for 2 h. Then, the plate was dried and 2 µL of reaction mixture in formamide loading dye was spotted on the plate. The plate was run with 450 mM ammonium sulfate for 1 h and dried. Finally, the plate was analyzed by Phospholmager (Typhoon FLA 7000, GE Healthcare).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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