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An Unconventional Human Ccr4-Caf1 Deadenylase Complex in Nuclear Cajal Bodies[▽]

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mRNA deadenylation is a key process in the regulation of translation and mRNA turnover. In *Saccharomyces cerevisiae*, deadenylation is primarily carried out by the Ccr4p and Caf1p/Pop2p subunits of the Ccr4-Not complex, which is conserved in eukaryotes including humans. Here we have identified an unconventional human Ccr4-Caf1 complex containing hCcr4d and hCaf1z, distant human homologs of yeast Ccr4p and Caf1p/Pop2p, respectively. The hCcr4d-hCaf1z complex differs from conventional Ccr4-Not deadenylase complexes, because (i) hCaf1z and hCcr4d concentrate in nuclear Cajal bodies and shuttle between the nucleus and cytoplasm and (ii) the hCaf1z subunit, in addition to rapid deadenylation, subjects substrate RNAs to slow exonucleolytic degradation from the 3′ end in vitro. Exogenously expressed hCaf1z shows both of those activities on reporter mRNAs in human HeLa cells and stimulates general mRNA decay when restricted to the cytoplasm by deletion of its nuclear localization signal. These observations suggest that the hCcr4d-hCaf1z complex may function either in the nucleus or in the cytoplasm after its nuclear export, to degrade polyadenylated RNAs, such as mRNAs, pre-mRNAs, or those RNAs that are polyadenylated prior to their degradation in the nucleus.

Deadenylation is a limiting step in mRNA turnover (34). In *Saccharomyces cerevisiae*, bulk mRNA deadenylation is carried out by the catalytic Ccr4p and Caf1p/Pop2p subunits of the Ccr4-Not deadenylase complex (8, 42, 43). Ccr4-Not complexes have also been identified in other eukaryotes, including humans, and possess deadenylase activity (4, 8, 41, 49). However, gene duplication and divergence have expanded this gene family in plants, flies, worms, and mammals (14). This raises the question of whether the newly duplicated genes have acquired novel functions or retained the ancestral function in mRNA deadenylation.

In addition to its role in cytoplasmic mRNA decay, deadenylation may also play a role in nuclear RNA turnover. For example, in yeast, inefficiently spliced mRNAs are retained in the nucleus where they are degraded (6). Also, fully processed mRNAs can be degraded in the nucleus in the absence of mRNA export (11, 25). Moreover, a nuclear polyadenylation complex was shown to target a variety of transcripts for degradation by the exosome in the nucleus (28, 44, 48). These all point to a nuclear quality control mechanism that could minimize cellular defects due to export of aberrant transcripts. The biological significance of these observations remains poorly understood, but many of these processes may involve unknown deadenylases.

The cell nucleus is highly structured, containing several distinct nuclear compartments. A predominant structure in the mammalian nucleus is the Cajal body (9). First noticed in 1904 by Santiago Ramon y Cajal, these subnuclear foci have remained enigmatic. Cajal bodies are believed to be sites of spliceosome assembly/reassembly, snRNA and snoRNA maturation, histone mRNA 3′-end formation, and telomerase biogenesis (5, 7, 23, 33, 35, 38, 46, 51). Highly dynamic in nature, Cajal bodies have been found associated with snRNA genes (17, 18, 22, 36, 39, 40), as well as snoRNA (19) and histone loci (37). Thus, a number of RNAs have been associated with Cajal bodies, none of which contain poly(A) tails.

Here, we identify an unconventional human Ccr4-Caf1 complex that concentrates in Cajal bodies and shuttles in and out of the nucleus. This Ccr4-Caf1 complex contains hCaf1z and hCcr4d, distant human homologs of yeast Caf1p/Pop2p and Ccr4p, respectively. Interestingly, hCaf1z catalyzes rapid deadenylation, which, in contrast to other known deadenylases, is followed by a much slower 3′-to-5′ exonucleolytic activity. These activities are observed both in vitro and when hCaf1z is exogenously expressed in human HeLa cells. Our observations suggest that the hCcr4d-hCaf1z complex functions in the nucleus, or in the cytoplasm after nuclear export, to activate degradation of as-yet-unidentified polyadenylated RNA substrates.

MATERIALS AND METHODS

Plasmids. The open reading frames encoding hCcr4a, hCcr4c, hCcr4d, hCcr4e, hCaf1a, hCaf1b, and hCaf1z (GenBank accession numbers are given in the legend to Fig. 1) and hNot2 were cloned into pcDNA3-FLAG and pcDNA3-Myc expression vectors (29), which are derivatives of pcDNA3 (Invitrogen), using BamHI and NotI sites. The hCcr4b open reading frame was cloned into pcDNA3-FLAG and pcDNA3-Myc using EcoRI and NotI sites. hCaf1z was cloned into BamHI and NotI sites of a pET-His plasmid for bacterial expression of hexahistidine-tagged hCaf1z. The D64A/E66A, C300A, and C309A mutant hCaf1z constructs were prepared using the QuikChange protocol (Stratagene). Expression plasmids for hnRNP A1 and hnRNP C and β-globin mRNA decay reporter transcripts have been described previously (16, 30–32). The reporter plasmid pPCβwt-Δ12 is similar to pPCβwt (expressing wild-type β-globin mRNA) but lacks introns 1 and 2. The plasmid pcDNA3-Myc-AT60, used to transcribe the deadenylation substrate, is a derivative of pcDNA3-Myc (29) in which a 60-nucleotide adenylate sequence flanked by MlyI and SapI sites was

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cloned into the XhoI and NotI sites. Plasmid sequences are available upon request.

Antibodies. Rabbit polyclonal sera were raised (Cocalico Biologicals) against hCaf1z (amino acids 378 to 510) and full-length hCaf1a fused to an N-terminal glutathione *S*-transferase tag. Rabbit polyclonal serum was raised against the hCcr4d peptide MNAEGDEPSSKRRK and affinity purified (Bethyl Laboratories).

Coimmunoprecipitation assays. Extracts of HEK293T cells derived from $\sim 2 \times 10^6$ transiently transfected cells were prepared as described earlier (32), RNase A treated (125 μg/ml), and incubated with 20 μl anti-FLAG M2 agarose (Sigma) at 4°C for 2 hours. Complexes were washed eight times with Net-2 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Igepal) and eluted with 1 mg/ml FLAG peptide (Sigma) in 25 μl Net-2 with gentle shaking at 4°C for 2 hours. Eluates were stored at −20°C. Proteins were detected after sodium dodecyl sulfate-polyacrylamide gel electrophoresis by immunoblotting with mouse monoclonal antibodies against Myc and FLAG epitopes per the manufacturer's recommendation (anti-Myc 9B11 from Cell Signaling; anti-FLAG M2 from Sigma).

Protein purification. FLAG-tagged hCaf1z fusion protein was expressed in transiently transfected HEK293T cells grown in Dulbecco modified Eagle medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen). Transfection was performed with TransIT-293 reagent per the manufacturer's protocol (Mirus). Affinity purification was performed as described above, except that five washes were done with Net-2 containing 300 mM NaCl and three washes were done with Net-2 containing 150 mM NaCl. Proteins were eluted using 200 μg/ml FLAG peptide in 40 μl Net-2 containing 10% glycerol for 20 min at 4°C, and eluates were stored at −80°C. His_6-tagged hCaf1z was produced from *Escherichia coli* BL21(DE3) cells and purified on nickel agarose using standard protocols (QIAGEN).

Preparation of deadenylation substrates. The deadenylation substrate and internal control RNAs were transcribed from pcDNA3-Myc-AT60 digested with SapI and pcDNA3-FLAG-hDcp1a (29) digested with BglII, respectively. In vitro transcription reactions were performed in the presence of 15 to 40 μCi [α-^{32}P]UTP, 1 mM ^{m7}GpppG cap analog, 0.5 mM ATP and CTP, 0.1 mM GTP, and 1 unit of T7 (pcDNA3-Myc-AT60) or SP6 (pcDNA3-FLAG-hDcp1a) RNA polymerase at 37°C for 1 h in 40 mM Tris-HCl (pH 8), 6 mM $MgCl_2$, and 5 mM dithiothreitol. Alternatively, transcription reactions were performed in the absence of radioactive UTP and visualized by UV shadowing. For the poly(U) substrate, a DNA template was created by hybridizing a 23-nucleotide oligonucleotide encoding T7 promoter (5′-TAATACGACTCACTATAGGGAGA-3′) with an 80-nucleotide oligonucleotide, A40-T7comp [5′-$A_{(40)}$TAGTCGAAAA CAAGGTATCTCCCTATAGTGAGTCGTATTA-3′], such that the T7 promoter sequence was double stranded and a 63-nucleotide RNA ending in 40 uridines was synthesized. The poly(U) RNA was transcribed with 1 unit of T7 RNA polymerase in the presence of 1 mM ATP, CTP, and GTP and 5 mM UTP at 37°C for 1 h in 40 mM Tris-HCl (pH 8), 6 mM $MgCl_2$, and 5 mM dithiothreitol. RNAs were precipitated with ethanol and separated in 6% polyacrylamide-6 M urea denaturing gels, visualized by autoradiography or by UV shadowing, excised, and eluted overnight at room temperature in 400 μl of 0.5% sodium dodecyl sulfate, 300 mM sodium acetate, 5 mM EDTA, and 400 μl phenol-chloroform-isoamyl alcohol. Following elution, RNAs were extracted, ethanol precipitated, and resuspended in water. Nonradioactive RNAs were subsequently cap labeled with [α-^{32}P]GTP using bacterially expressed vaccinia virus capping enzyme (construct kindly provided by Stewart Shuman) as described by Zhang et al. (50) and gel purified as described above.

In vitro deadenylation assays. Deadenylation assays were performed in 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 1 mM spermidine, 0.1% Igepal (NP-40; Sigma), 0.5 U/μl RNase-Out (Invitrogen), and 5 μg of yeast total RNA at 37°C for times indicated in each experiment, including 10,000 cpm of both control and substrate RNAs and 10 to 50 nM of FLAG-hCcr4 or FLAG-hCaf1 protein. RNAs were precipitated, separated on 6% polyacrylamide-6 M urea denaturing gels, and visualized by autoradiography. Removal of the poly(A) tail of the deadenylation substrate was done by incubating the RNA with 1 μM DNA oligonucleotide (5′-TTTTTTTTTTGACTCTC) in 10 mM Tris, pH 7.5, 2 mM $MgCl_2$ at 95°C for 2 min; annealing it at room temperature for 5 min; and adding 1.5 units of RNase H (Pierce) followed by 20 to 30 min of incubation at 37°C. This removes 7 nucleotides internal to the A_{60} sequence. Removal of the U tail of the poly(U) substrate was performed in a similar manner using oligo$(dA)_{21}$.

Immunofluorescence. HEK293T cells were transiently transfected using TransIT-293 reagent (Mirus) with plasmids expressing Myc-tagged hCaf1z or hCcr4d. One day posttransfection, cells were trypsinized and transferred to an eight-well chamber slide coated with polylysine. Two days posttransfection, the cells were fixed with 50% methanol-50% acetic acid and examined by indirect immunofluorescence using anti-Myc 9B11 antibody at 1:2,000 (Cell Signaling). Endogenous proteins were examined in HeLa cells using antibodies directed against hCaf1z (1:100), hCcr4d (1:100), and coilin/p80 (1:100) (BD Biosciences).

Heterokaryon assays. HeLa cells were transiently transfected with plasmids expressing either Myc-tagged hCaf1z or hCcr4d, together with green fluorescent protein (GFP)-hnRNP A1 or GFP-hnRNP C (30) using TransIT HeLaMonster reagent (Mirus). One day posttransfection, cells were transferred to an eight-well chamber slide and incubated overnight. Cells were cocultured with NIH 3T3 mouse cells in the presence of cycloheximide, and heterokaryons were formed and analyzed as previously described (15).

Pulse-chase mRNA decay assays. HeLa Tet-off cells (Clontech) were grown in Dulbecco modified Eagle medium-10% fetal bovine serum in 3.5-cm plates and transiently transfected in the presence of 50 ng/ml tetracycline using TransIT HeLaMonster transfection reagent (Mirus) per the manufacturer's protocol. A total of 2 μg of DNA was used per well, including 25 ng of internal control expression plasmid pPCβwt-GAP3UAC (producing β-GAP mRNA), 0.5 μg of mRNA reporter plasmid (pPCβwt, pPCβ39, or pPCβwt-AT granulocyte-macrophage colony-stimulating factor), or 1 μg of pPCβwt-Δ12 and empty vector or hCaf1z expression vectors to a total of 2 μg. Two days posttransfection, transcription was pulsed for 6 hours by removal of tetracycline and then shut off by addition of 1 μg/ml tetracycline. Cells were harvested at time points thereafter in TRIzol (Invitrogen). RNA was prepared and analyzed by Northern blotting as described previously (31).

RESULTS

Human homologs of yeast Ccr4p and Caf1p/Pop2p deadenylases. In *S. cerevisiae*, deadenylation is primarily carried out by the Ccr4p and Caf1p/Pop2p subunits of the Ccr4-Not complex (8, 42, 43). In accordance with a previous study (14), inspection of the expressed sequence tag and human genome databases revealed the presence of several Ccr4 homologs; for simplicity we refer to these here as hCcr4a to hCcr4e (Fig. 1A). All of the identified human Ccr4 proteins share a conserved Mg^{2+}-dependent nuclease domain that clusters with the AP endonuclease family (14). hCcr4a and hCcr4b contain leucine-rich repeats (LRRs) at their N termini and share 74% amino acid identity with each other. The LRR is present in yeast Ccr4p and is required for the interaction between Ccr4p and Caf1p/Pop2p (13). hCcr4c is the ortholog of the deadenylase nocturnin, which has been characterized in mouse and *Xenopus laevis*, where it is believed to play a role in circadian function (3). hCcr4c contains a putative leucine zipper motif in the N terminus (20). hCcr4d and hCcr4e are more divergent from yeast Ccr4p and contain no LRR or leucine zipper. All of the human Ccr4 proteins are smaller than their yeast homolog, which contains a glutamine-rich N terminus not found in the human Ccr4 proteins (13).

Inspection of the human genome also revealed that in addition to two close homologs of yeast Caf1p (here called hCaf1a and hCaf1b) (1), it encodes a more distant homolog that we call hCaf1z for its unique C_3H-type zinc finger domain (Fig. 1B; hCaf1z is also known as TOE1 [12]). hCaf1z also contains an arginine-rich predicted basic nuclear localization signal (NLS; Fig. 1B).

A distinct Ccr4-Caf1 complex containing hCcr4d and hCaf1z forms in human cell extracts. In yeast, the deadenylases Ccr4p and Caf1p/Pop2p exist together in a Ccr4-Not complex (42). To determine whether the various human Ccr4 and Caf1 proteins can form distinct complexes in human cells, we performed coimmunoprecipitation experiments with exogenously expressed hCcr4 and hCaf1 proteins. Transiently expressed Myc-tagged hCcr4 proteins were tested for their ability to coprecipitate with coexpressed FLAG-tagged hCaf1a,

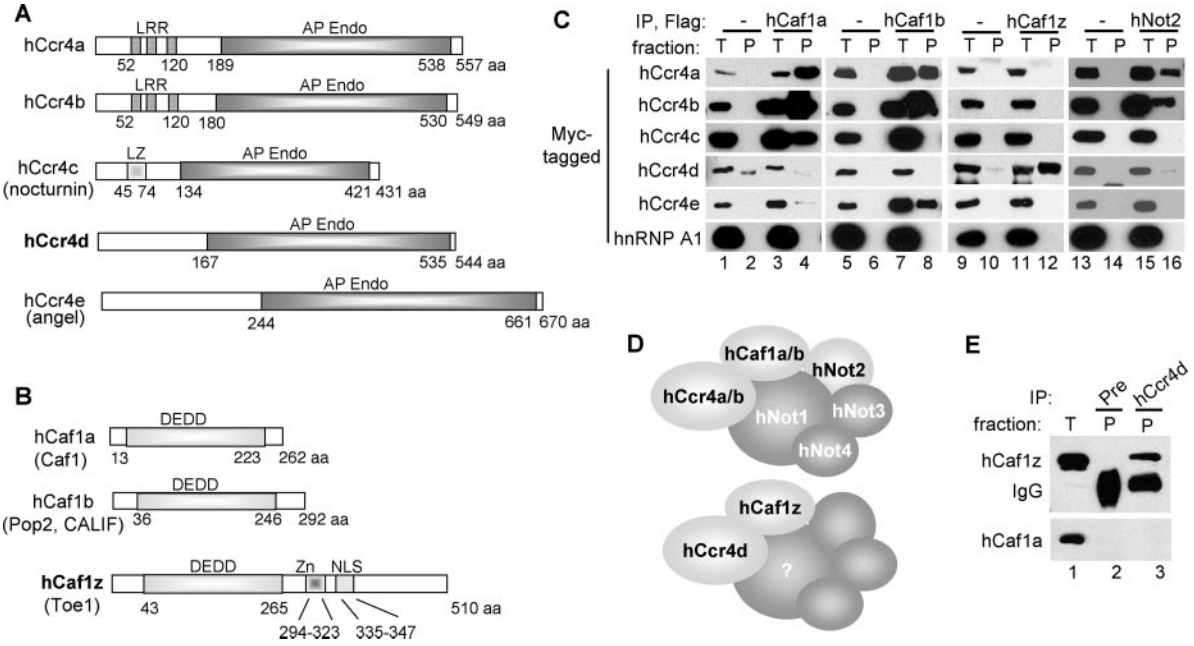

FIG. 1. hCaf1z forms a specific complex with hCcr4d. A. Schematic representation of the human Ccr4 homologs designated hCcr4a to hCcr4e. Each has a conserved nuclease domain (shown in gray) related to the Mg^{2+}-dependent apurinic/apyrimidinic endonucleases (AP Endo). Numbers refer to amino acid (aa) positions. hCcr4a and hCcr4b possess N-terminal LRRs, which are conserved in yeast Ccr4p. hCcr4c has a leucine zipper motif (LZ) and is the ortholog of the *Xenopus* protein nocturnin, while hCcr4e is related to the *Drosophila melanogaster* protein angel. GenBank accession numbers are as given: hCcr4a, AB033020; hCcr4b, XM_939929; hCcr4c, AF183961; hCcr4d, XM_034232; hCcr4e, AL137268. B. Schematic representation of human Caf1 proteins, hCaf1a, hCaf1b, and hCaf1z. The nuclease domains (shown in gray) of the Caf1 proteins are members of the DEDD superfamily of exonucleases. hCaf1z contains a C_3H-type zinc finger (Zn) and a basic NLS. GenBank accession numbers are as given: hCaf1a, NM_013354; hCaf1b, NM_004779; hCaf1z, NP_079353. C. Coimmunoprecipitation assays performed in RNase A-treated HEK293T extracts derived from cells coexpressing FLAG-tagged hCaf1a (lanes 3 and 4), hCaf1b (lanes 7 and 8), hCaf1z (lanes 11 and 12), or hNot2 (lanes 15 and 16) with Myc-tagged hCcr4a to hCcr4e and Myc-hnRNP A1 as indicated. FLAG fusion proteins were immunoprecipitated using anti-FLAG M2 agarose, and immunoprecipitates were subjected to immunoblotting with anti-Myc. Lanes 1, 2, 5, 6, 9, 10, 13, and 14 are negative immunoprecipitation controls from cells expressing no FLAG-tagged proteins. Myc-hnRNP A1 served as a negative coimmunoprecipitation control. Pellet (P) and 5% of total (T) fractions were loaded as indicated. D. Schematic of human Ccr4-Caf1 complexes based on the observations in panel C. The proteins tested in panel C are shown in light gray. E. Endogenous hCcr4d was immunoprecipitated from HeLa cell extracts and probed for the presence of endogenous hCaf1z and hCaf1a. Lane 1, 5% total HeLa extract; lane 2, preimmune serum immunoprecipitate; lane 3, anti-hCcr4d immunoprecipitate. IP, immunoprecipitation; IgG, immunoglobulin G.

hCaf1b, or hCaf1z from RNase-treated HEK293T cell extracts. hCcr4a was previously shown to interact with hCaf1a and hCaf1b in yeast two-hybrid assays (1). These interactions were confirmed in our assays, as Myc-tagged hCcr4a, as well as its close homolog hCcr4b, copurifies (~20%) with FLAG-tagged hCaf1a and hCaf1b (Fig. 1C). In addition, hCcr4c and hCcr4e may copurify weakly (<5%) with hCaf1a and hCaf1b, respectively. Interestingly, transiently expressed hCcr4d shows a specific interaction with hCaf1z (lane 12). This appears to be a unique hCcr4-hCaf1 complex, because none of the other human Ccr4 proteins copurify with hCaf1z (lane 12), and hCcr4d does not copurify with hCaf1a or hCaf1b (lanes 4 and 8). Importantly, the Myc-tagged hCcr4 proteins were not present in precipitates from cells expressing no FLAG-tagged proteins (lanes 2, 6, and 10) and the negative control protein Myc-hnRNP A1 (bottom panels) was not present in any of the precipitates, demonstrating the specificity of the observed interactions.

Since yeast Ccr4p is part of the Ccr4-Not complex, we also tested whether the human Not protein hNot2 copurifies with any of the human Ccr4 proteins. In coimmunoprecipitation assays similar to those described above, we found that only hCcr4a and hCcr4b copurify with hNot2 (Fig. 1C, lanes 13 to 16). This correlates with the presence of LRRs in these hCcr4 proteins (Fig. 1A). These observations suggest that alternative human Ccr4-Not complexes exist, which contain either hCcr4a or hCcr4b in complex with hCaf1a or hCaf1b (Fig. 1D). A distinct complex is formed between hCcr4d and hCaf1z, which does not appear to contain hNot2 (Fig. 1D). hCcr4c and hCcr4e may also exist in complex with hCaf1 proteins (Fig. 1C) but were not studied further here.

To test whether the complex between hCcr4d and hCaf1z is also observed between the endogenous proteins, we raised polyclonal antibodies. Figure 1E shows that hCaf1z is detected in anti-hCcr4d immunoprecipitates (lane 3) but not in the immunoprecipitates using the corresponding preimmune serum (lane 2). Furthermore, endogenous hCaf1a was not observed in complex with hCcr4d (lower panel). This confirms the specificity of the hCaf1z-hCcr4d interaction observed in Fig. 1C. We conclude that a unique human Ccr4-Caf1 complex exists that includes hCaf1z and hCcr4d (Fig. 1D). The hCaf1z-hCcr4d complex likely contains additional proteins (shown as dark gray in Fig. 1D), because gel filtration assays suggest that the hCcr4d and hCaf1z proteins, like Ccr4-Not complexes,

form a large (MDa sized) protein complex (A. Robitaille, E. Wagner, and J. Lykke-Andersen, unpublished results). However, the Not protein hNot2 does not appear to be part of the hCcr4d-hCaf1z complex (Fig. 1C).

hCaf1z catalyzes rapid deadenylation and slow 3′-to-5′ exonucleolytic decay. We next asked whether the unique hCcr4d-hCaf1z complex possesses deadenylase activity. FLAG-tagged hCaf1z protein affinity purified from transiently transfected HEK293T cells was incubated with a deadenylation substrate RNA containing a 60-nucleotide poly(A) tail and a control RNA lacking a poly(A) sequence. The result in Fig. 2A shows that FLAG-hCaf1z copurifies with deadenylase activity. Progressive deadenylation of the A_{60} substrate is observed (lanes 4 to 6), resulting in a fully deadenylated product after 5 min (lane 7). Importantly, the nonadenylated control transcript is unaffected during this stage (lanes 4 to 7, upper band). Unexpectedly, however, following deadenylation of the A_{60} substrate, an additional nuclease activity was observed, into the body of the deadenylated A_{60} substrate as well as the control transcript (lanes 7 to 13). This activity contrasts with that of hCaf1b (Fig. 2B) (47), as well as the hCcr4a and hCcr4b deadenylases (data not shown) (8), which do not degrade the same substrate beyond the poly(A) tail. The non-poly(A) nuclease activity shows much slower kinetics than the deadenylase activity (Fig. 2A; compare the first 5 min of the time course, lanes 4 to 7, with the remaining 55 min, lanes 7 to 13). In addition, the observation that specific RNA products accumulate and no loss of overall RNA signal is observed (Fig. 2A; compare lanes 13 and 4) suggests that specific sequences or structures may inhibit hCaf1z activity. The deadenylase activity of hCaf1z appears to be either distributive or processive but slow, as evidenced by the accumulation of deadenylated intermediates at the early time points (Fig. 2A, lanes 5 and 6). This is in contrast to the seemingly fast processive activity of hCaf1b (Fig. 2B, lanes 6 to 11) (47) and hCcr4a and hCcr4b (data not shown) (8), which when used at the same concentration as hCaf1z produce fully deadenylated RNAs at early time points. hCcr4d was tested for deadenylase activity in a manner similar to that for hCaf1z, but no activity was observed (data not shown).

Since the results in Fig. 2A are derived from hCaf1z protein purified from human cells, it was unclear whether the observed activity is due to hCaf1z itself or a copurifying factor (such as hCcr4d). To address this, we performed a similar experiment using a mutant version of hCaf1z in which two conserved acidic residues in the nuclease domain predicted to play a role in catalysis, D64 and E66, were mutated to alanines. The hCaf1z D64- and E66-to-alanine double mutant protein (hCaf1z DEAA) purified after exogenous expression in human cells shows little deadenylase activity in vitro (Fig. 2C). Point mutations in key cysteines in the unique zinc finger domain of hCaf1z were also tested (Fig. 2D). However, these mutants retained deadenylase activity (lanes 9 to 12).

Bacterially expressed hCaf1z is active. Even though the mutant hCaf1z DEAA protein was strongly impaired in deadenylation, a small fraction of the RNA substrate appears to be deadenylated at the latest time points (Fig. 2C). This suggests that the hCaf1z DEAA mutant protein either is not completely inactive or copurifies with trace amounts of endogenous active deadenylase activity. In order to distinguish between these two possibilities, we tested the ability of hexahistadine (His_6)-tagged hCaf1z and mutant hCaf1z DEAA proteins purified from *E. coli* to deadenylate RNA in vitro. Again, we observed that hCaf1z catalyzes rapid deadenylation of the substrate (Fig. 3A, lanes 5 to 7), followed by slower degradation into the RNA

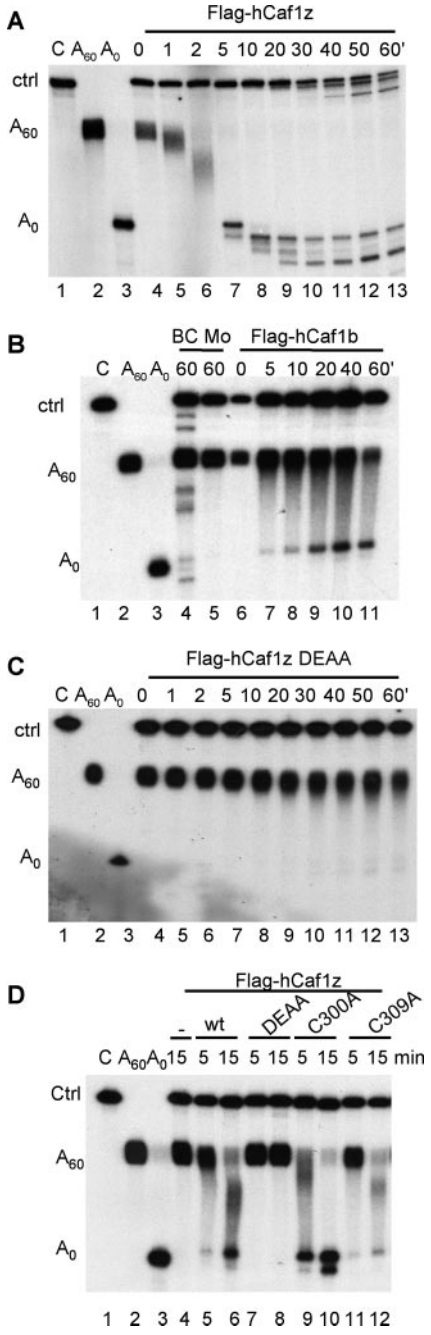

FIG. 2. hCaf1z copurifies with fast deadenylase and slow non-poly(A) nuclease activity. A. FLAG-hCaf1z deadenylation time course. The deadenylation substrate (A_{60}, lane 2) and the nonadenylated control (C, lane 1) were incubated with ~10 ng FLAG-hCaf1z for 0 to 60 min as indicated (lanes 4 to 13). Deadenylated substrate A_0, generated by RNase H/DNA oligonucleotide cleavage, is shown in lane 3. B. deadenylation time course with FLAG-hCaf1b, as described for panel A. BC, buffer control; Mo, "mock"-purified protein. C. Same as panel A but using a mutant hCaf1z DEAA (D64A, E66A) protein. D. hCaf1z zinc finger mutants C300A (lanes 9 and 10) and C309A (lanes 11 and 12) are active in deadenylation.

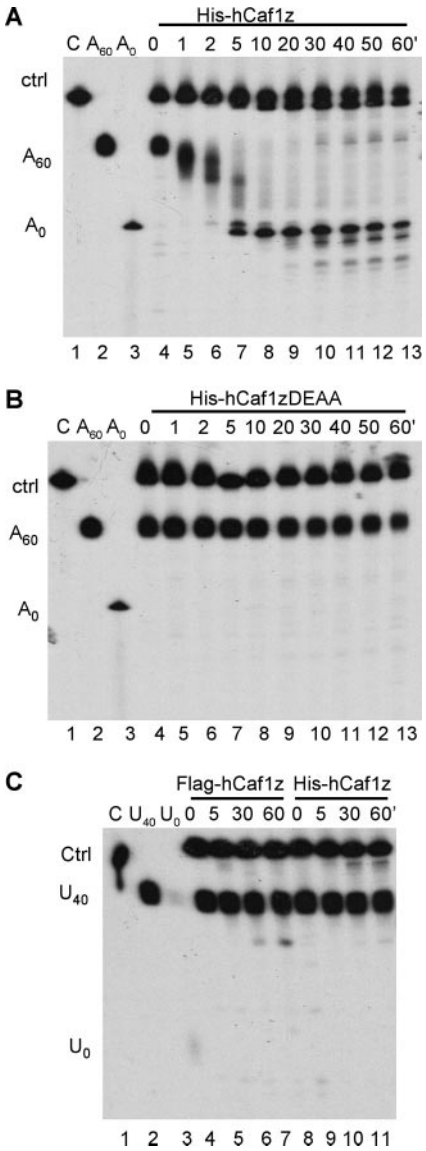

FIG. 3. hCaf1z is a poly(A)-specific nuclease. A. deadenylation time course as in Fig. 2 but using recombinant His_6-tagged hCaf1z purified from *E. coli*. B. deadenylation time course with mutant His_6-hCaf1z DEAA protein purified from *E. coli*. C. Incubation of poly(U) substrate with FLAG-hCaf1z or His_6-hCaf1z does not result in removal of the poly(U) tail. A polyuridylated substrate (U_{40}) and a nonuridylated/nonadenylated control RNA (C) were incubated with ~10 ng FLAG-hCaf1z (lanes 4 to 7) or His_6-hCaf1z (lanes 8 to 11) for 0 to 60 min as indicated. The migration of the U_0 product, generated by RNase H/oligo(dA) cleavage, is shown in lane 3.

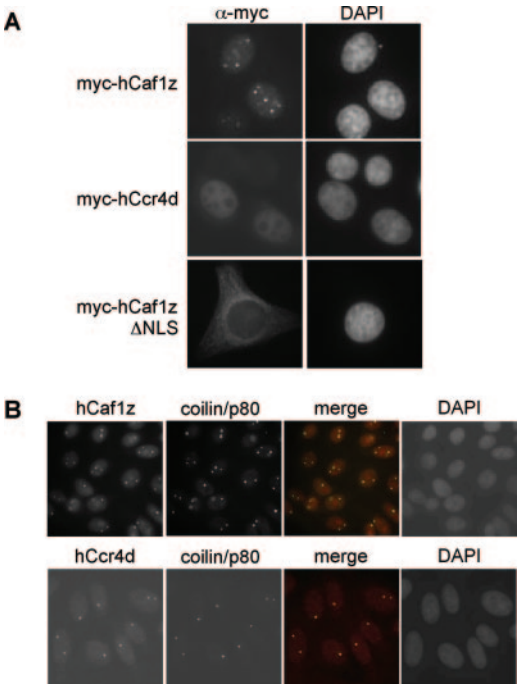

FIG. 4. Localization of human Ccr4d and Caf1z proteins in Cajal bodies. A. Myc-tagged hCaf1z, hCcr4d, and hCaf1z lacking the putative nuclear localization signal, hCaf1z ΔNLS, were transiently expressed in HeLa cells and visualized with anti-Myc antibody. Nuclei are stained with DAPI (4′,6′-diamidino-2-phenylindole). B. Endogenous hCaf1z and hCcr4d were stained with affinity-purified polyclonal antibodies and costained with the Cajal body marker protein coilin/p80. Merging of these panels reveals that both hCaf1z and hCcr4d (red) completely colocalize with coilin/p80 (green).

body (lanes 8 to 13). In contrast, the His_6-hCaf1z DEAA mutant protein is completely inactive in vitro (Fig. 3B). Thus, the weak deadenylase activity observed for the FLAG-tagged hCaf1z DEAA mutant protein (Fig. 2C) is likely due to a copurifying deadenylase. From the experiments in Fig. 2 and 3, it can also be concluded that the observed slow non-poly(A) exonuclease activity is catalyzed by hCaf1z itself, since this activity is abolished in the hCaf1z DEAA mutant protein (compare Fig. 2A with 2C and Fig. 3A with 3B).

hCaf1z shows little activity on poly(U) RNA. The experiments in Fig. 2 and 3 suggest a preference of hCaf1z for poly(A) RNA. However, from these experiments, it could not be ruled out that hCaf1z is in fact a 3′-to-5′ exonuclease with high activity on unstructured substrates, such as the homopolymeric poly(A) tail, but is inhibited by secondary structure, as may be formed in the body of the RNA substrate. In order to determine whether hCaf1z is active on homopolymeric sequences other than poly(A), we tested its activity on a substrate containing a 40-nucleotide poly(U) tail. As seen in Fig. 3C, although some "nibbling" activity is seen akin to that observed with the control RNA, neither FLAG-hCaf1z purified from HEK293T cells (lanes 4 to 7) nor His_6-hCaf1z purified from *E. coli* (lanes 8 to 11) efficiently catalyzes the removal of the poly(U) tail. We conclude that hCaf1z is a novel human deadenylase and that in addition to its activity on poly(A) sequences, this enzyme also displays low, but reproducible, activity towards non-poly(A) RNA.

hCaf1z and hCcr4d colocalize in Cajal bodies. We next examined the cellular localization of hCaf1z and hCcr4d. Other members of the hCcr4 and hCaf1 families have been observed to localize in the cytoplasm (2, 10, 49). However, the predicted basic NLS of hCaf1z (Fig. 1B) suggested that this protein might reside in the nucleus. Interestingly, both hCaf1z and hCcr4d reside in the nucleus when exogenously expressed, and hCaf1z was noted to be present in nuclear foci (Fig. 4A).

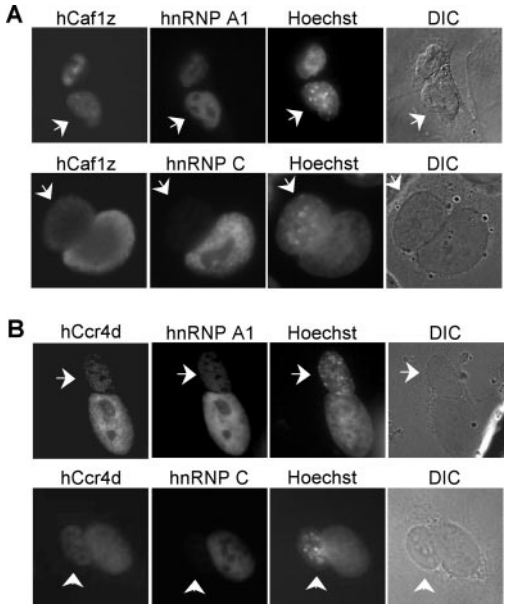

FIG. 5. hCaf1z and hCcr4d are nucleocytoplasmic shuttling proteins. A. HeLa cells coexpressing Myc-hCaf1z with GFP-hnRNP A1 or GFP-hnRNP C were fused with mouse NIH 3T3 cells. Nuclei are stained with Hoechst stain. Mouse nuclei are indicated by white arrows. B. Myc-hCcr4d was coexpressed with GFP-hnRNP A1 or GFP-hnRNP C in HeLa cells and fused to form heterokaryons with NIH 3T3 cells. DIC, differential interference contrast.

The nuclear localization of hCaf1z depends on its predicted NLS, because its deletion abolished nuclear staining and resulted in the cytoplasmic localization of the protein (hCaf1z ΔNLS; Fig. 4A). Using affinity-purified antibodies to hCaf1z and hCcr4d, we observed that endogenous hCaf1z and hCcr4d occur in nuclear foci (Fig. 4B, leftmost panels). These foci correspond to Cajal bodies, as evidenced by the complete colocalization with the Cajal body marker protein coilin/p80 (Fig. 4B). We conclude that the hCcr4d-hCaf1z deadenylase complex is a novel component of Cajal bodies. It is not clear why exogenously expressed hCcr4d is not observed in foci (Fig. 3A), but it is most likely due to its overexpression. hCaf1z was also observed to be distributed throughout the nucleus when exogenously expressed at high levels (data not shown).

hCaf1z and hCcr4d are nucleocytoplasmic shuttling proteins. Because the hCcr4d-hCaf1z complex resides in the nucleus but deadenylation may primarily occur in the cytoplasm, we wished to test whether hCaf1z and hCcr4d exist transiently in the cytoplasm. We therefore performed heterokaryon assays using HeLa cells exogenously expressing Myc-tagged hCaf1z or hCcr4d, along with either GFP-hnRNP A1 or GFP-hnRNP C as a positive or negative shuttling control, respectively. Heterokaryons were formed with mouse NIH 3T3 cells in the presence of cycloheximide to prevent protein synthesis subsequent to heterokaryon formation. The results of the heterokaryon assays in Fig. 5A and B show that both hCaf1z and hCcr4d are nucleocytoplasmic shuttling proteins, as they are detected in both human and mouse nuclei (mouse nuclei, indicated by arrows, are identified by speckled appearance when stained with Hoechst stain). Importantly, the negative shuttling control GFP-hnRNP C was restricted to the human nucleus, demonstrating nuclear integrity, whereas the positive control GFP-hnRNP A1 appeared in the mouse nuclei as expected. Note that due to overexpression, hCaf1z and hCcr4d are observed throughout the nucleus and not concentrated in Cajal bodies in these assays. The nucleocytoplasmic shuttling behavior of hCaf1z and hCcr4d suggests that they may have a function in cytoplasmic mRNA deadenylation.

Exogenously expressed hCaf1z can stimulate mRNA deadenylation in the cell. We next wished to test whether hCaf1z can affect mRNA turnover in human cells. Due to the many potentially redundant deadenylases in human cells (Fig. 1), and since the endogenous substrates of hCaf1z remain unknown, we tested the effect on the decay of reporter mRNAs upon hCaf1z overexpression. Overexpression of the human cytoplasmic deadenylase hCcr4a has been shown previously to accelerate deadenylation and decay of a stable reporter mRNA in human cells (49). Likewise, we have observed that overexpression of hCcr4b can accelerate the decay of both stable and unstable reporter transcripts in similar assays (data not shown). We therefore monitored the effect of exogenously expressed hCaf1z on the decay of β-globin reporter transcripts in HeLa Tet-off cells. The decay of a tetracycline-regulated stable β-globin mRNA (β-wt, Fig. 6A) was followed over time after transcriptional repression. A constitutively expressed β-GAP fusion mRNA served as an internal control (32). As seen in Fig. 6A, exogenously expressed hCaf1z appears to slightly impede β-globin mRNA decay, compared with a control in which transcript decay took place in the absence of exogenous protein (half-life [$t_{1/2}$], ≈600 versus ≈1,000 min). Interestingly, a shortened β-globin mRNA product (marked by an asterisk in Fig. 6A) consistently accumulated at low levels upon hCaf1z overexpression. Since hCaf1z shuttles in and out of the nucleus, we also tested the effect of expressing exogenous hCaf1z in the cytoplasm. As seen in Fig. 6A, when exogenous hCaf1z is placed in the cytoplasm though deletion of the NLS (hCaf1z ΔNLS), the β-globin mRNA decay rate is dramatically enhanced (≈6-fold; $t_{1/2}$, 110 min). Thus, hCaf1z can activate decay of a stable reporter mRNA when restricted to the cytoplasm.

We also tested whether exogenous hCaf1z can affect the degradation of unstable β-globin reporter mRNAs containing an AU-rich element (ARE) (Fig. 6B; β-ARE [32]) or a premature termination codon at position 39, which triggers nonsense-mediated decay (Fig. 6C, β-39 [31]). As observed for the stable β-globin mRNA, exogenous hCaf1z ΔNLS enhances the decay of these mRNAs, whereas the wild-type protein appears to impede mRNA decay (Fig. 6B and C) and cause low accumulation of a truncated β-globin mRNA product (marked by an asterisk). To test whether these effects were dependent on the catalytic activity of hCaf1z, we exogenously expressed the corresponding hCaf1z DEAA mutant proteins. As expected, the cytoplasmic hCaf1z DEAA ΔNLS mutant protein did not stimulate mRNA decay (Fig. 6B and C). Thus, the effect of cytoplasmic localized hCaf1z on decay of the reporter mRNAs is dependent on its catalytic activity. Somewhat surprisingly, hCaf1z exogenously expressed in the nucleus lost its ability to impair mRNA decay when catalytically inactive (hCaf1z DEAA; Fig. 6B and C). No strong dominant-negative effect of the hCaf1z DEAA mutant proteins was observed, possibly due

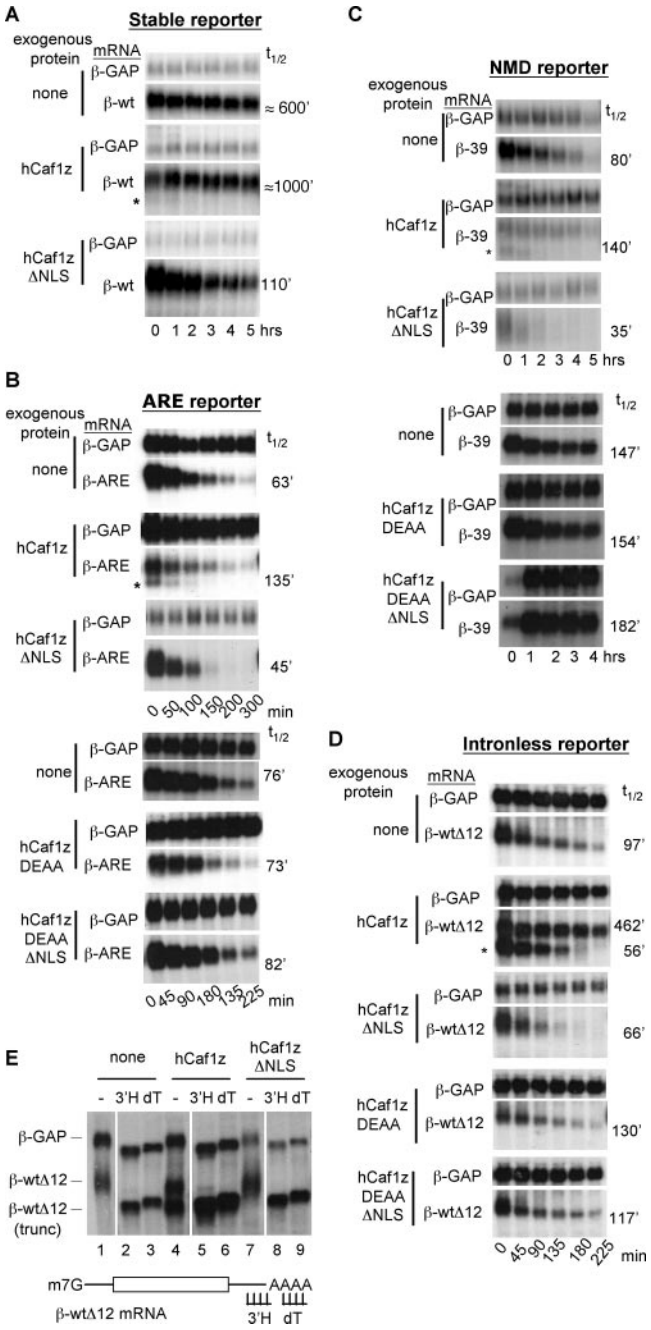

FIG. 6. Exogenously expressed hCaf1z can stimulate deadenylation and decay of reporter mRNAs in human HeLa cells. A. Transcriptional pulse-chase mRNA decay assays of a stable β-globin (β-wt) reporter transcript in the presence of no protein (none) or exogenously expressed wild-type or mutant hCaf1z ΔNLS proteins as indicated. Assays were performed in cells coexpressing a constitutive β-GAP control mRNA. After a 6-hour transcriptional pulse, tetracycline was used to stop reporter β-globin mRNA transcription, regulated by a tetracycline-repressible promoter, and total RNA was isolated from cells at intervals thereafter indicated below the panels and subjected to Northern blotting. Calculated mRNA $t_{1/2}$s are given on the right in minutes. B. Decay of an unstable β-globin transcript (β-ARE), which contains the granulocyte-macrophage colony-stimulating factor ARE in the 3′ untranslated region, in the presence of wild-type hCaf1z or mutant hCaf1z ΔNLS, hCaf1z DEAA, or hCaf1z DEAA ΔNLS proteins as indicated. C. Decay of the β-globin mRNA nonsense-mediated decay (NMD) substrate (β-39) in the presence of hCaf1z proteins. D. Decay of an intronless β-globin mRNA reporter (β-wtΔ12) in the presence of hCaf1z proteins. Asterisks in panels A to D indicate the shortened β-globin transcripts observed in the presence of exogenously expressed hCaf1z. E. Northern blotting of mRNAs isolated from cells transfected with the control (β-GAP) and intronless (β-wtΔ12) reporter constructs together with either an empty expression vector (lanes 1 to 3) or an expression vector encoding FLAG-hCaf1z (lanes 4 to 6) or FLAG-hCaf1z ΔNLS (lanes 7 to 9). Isolated RNA was treated with RNase H in the presence of oligo(dT) (lanes 3, 6, and 9) or a 3′-H oligonucleotide which hybridizes ~30 nucleotides upstream of the poly(A) site in both the β-GAP control and β-wtΔ12 mRNAs (lanes 2, 5, and 8; hybridization site illustrated below the panel). Lanes 1, 4, and 7 show RNase H treatment in the absence of DNA oligonucleotide.

to the existence of numerous redundant deadenylases in the human cell (Fig. 1).

Since hCaf1z is primarily nuclear at steady state, we also tested the effect of exogenous hCaf1z expression on an intronless β-globin mRNA (β-wtΔ12), predicted to be retained in the nucleus longer than the intron-containing β-globin mRNAs tested above. Similar to the other mRNA reporters, expression of exogenous hCaf1z caused stabilization, whereas hCaf1z ΔNLS triggered destabilization of the intronless β-globin mRNA (Fig. 6D). These effects were abolished when the mutant hCaf1z DEAA proteins were used (Fig. 6D). Interestingly, as seen in Fig. 6D, exogenous expression of hCaf1z resulted in strong accumulation of the truncated mRNA species, which was also observed at lower levels with the intron-containing mRNAs (marked by asterisks in Fig. 6A to D). For all mRNA reporters, the truncated product was highly unstable (Fig. 6A to D; $t_{1/2}$, <60 min). RNase H digestion in the presence of oligo(dT) or a DNA oligonucleotide (3′-H) hybridizing close to the poly(A) site of the transcript demonstrated that this shorter transcript is not only deadenylated but also further truncated at the 3′ end (Fig. 6E). Thus, both the deadenylation and the 3′-to-5′ exonucleolytic "nibbling" activity observed for hCaf1z in vitro (Fig. 2A and 3A) can be observed on a reporter mRNA in the cell.

While the endogenous substrates for hCaf1z remain unknown and the observations in Fig. 6 likely represent gain-of-function activities of exogenously overexpressed hCaf1z, they provide two important conclusions about the hCaf1z protein: (i) hCaf1z can stimulate cellular mRNA decay when expressed in the cytoplasm, and (ii) as evidenced by the accumulation of the truncated β-globin mRNA species, hCaf1z can deadenylate and "nibble" into the 3′ end of reporter mRNAs in the cell, as it can in vitro. These observations suggest that hCaf1z acts in the cell to degrade polyadenylated RNAs, either in the nucleus or in the cytoplasm following nuclear export. In contrast to hCaf1z, we observed no significant effects on mRNA turnover of exogenously expressing hCcr4d (data not shown).

DISCUSSION

The human cell expresses multiple hCcr4 and hCaf1 deadenylases. The human genome encodes a large family of human Ccr4 and Caf1 proteins (1, 14) (Fig. 1), a number of which function as mRNA deadenylases (8, 47, 49) (Fig. 2 and 3; also data not shown). In contrast to hCaf1a, hCaf1b, hCaf1z, hCcr4a, and hCcr4b (Fig. 2 and 3 and data not shown), we did

not observe in vitro deadenylase activity for FLAG-tagged hCcr4c, hCcr4d, or hCcr4e affinity purified from human cells (data not shown). However, these proteins may be deadenylases but fail to function under the conditions used in our assays. *Xenopus* nocturnin, an ortholog of hCcr4c with 66% amino acid identity, was previously shown to possess deadenylase activity in vitro (3).

The expansion of the Ccr4 and Caf1 gene families in multicellular organisms (14, 41) (Fig. 1) raises the question of whether these proteins have overlapping or distinct functions. Our observations suggest that multiple distinct complexes exist, which are comprised of different combinations of Ccr4-Caf1 proteins (Fig. 1D). The two human Ccr4 proteins that have retained the LRR motifs present in yeast Ccr4p, hCcr4a and hCcr4b (Fig. 1A), both copurify with hCaf1a and hCaf1b, as well as with hNot2 (Fig. 1C). This is in agreement with yeast two-hybrid studies showing that hCcr4a interacts with both hCaf1a, hCaf1b, and hNot proteins (1). In addition, a unique complex is observed between hCaf1z and hCcr4d, which does not appear to include hNot2 (Fig. 1C to E). This complex thus differs from previously studied Ccr4-Not complexes. hCcr4c and hCcr4e were also not observed in complex with hNot2 (Fig. 1C). We found no association between hCcr4 proteins and the human deadenylase PARN, a distant homolog of Caf1 proteins (data not shown). Together, our results suggest that multiple, distinct hCcr4-hCaf1 complexes can form in human cells, some in association with hNot proteins (Fig. 1D). An important goal for future studies is to understand why so many deadenylases exist in multicellular eukaryotes. They could conceivably have different temporal or spatial expression patterns or distinct substrate specificities.

The hCaf1z subunit of the hCcr4d-hCaf1z complex is an unconventional deadenylase. The catalytic activity of hCaf1z is distinct from that of conventional Ccr4-Not-complex deadenylases. First, it deadenylates RNA in what appears to be either a distributive or slow processive manner (Fig. 2A and 3A). This activity is specific for poly(A), as hCaf1z does not remove a poly(U) tail in similar assays (Fig. 3C). Second, after complete deadenylation, a "nibbling" of nuclease activity into the nonadenylate portion of the RNA occurs (Fig. 2A and 3A). This additional nuclease activity is also apparent on transcripts that have not been first deadenylated by hCaf1z [see control transcripts and poly(U) RNA in Fig. 2A, 3A, and 3C]. However, as observed in these time course experiments, hCaf1z has a clear preference for poly(A) sequences in vitro. We have not observed deadenylase activity for hCcr4d, the interaction partner of hCaf1z. However, it is possible that endogenous hCcr4d is responsible for the low deadenylase activity observed with affinity-purified FLAG-hCaf1z DEAA mutant protein in Fig. 2C. Further work is needed to understand the function of hCcr4d in the hCcr4d-hCaf1z complex.

Does the hCcr4d-hCaf1z complex participate in cellular mRNA decay? The mRNA decay assays in Fig. 6 revealed three specific effects of exogenously expressed hCaf1z on the tested reporter mRNAs: (i) hCaf1z expressed exogenously in the cytoplasm (hCaf1z ΔNLS) stimulates the degradation of several tested mRNA substrates, whereas hCaf1z exogenously expressed in the nucleus results in (ii) mRNA stabilization and (iii) accumulation of an unstable deadenylated and 3′-end-truncated mRNA species. The 3′-end-truncated mRNA product was particularly evident for the intronless β-globin reporter mRNA (Fig. 6D). Since the endogenous substrate of hCaf1z is unknown, these effects of hCaf1z on mRNA decay likely represent gain-of-function effects upon overexpression. Importantly, however, they reveal that hCaf1z can stimulate decay of polyadenylated mRNA in the cytoplasm and that the "nibbling" activity observed for hCaf1z in vitro can also take place in the cell, as evidenced by the accumulation of the 3′-end-truncated mRNA product. Based on these observations it is likely that hCaf1z, alone or in the hCcr4d-hCaf1z complex, serves a function in degradation of polyadenylated RNA in the cell.

The shuttling behavior of hCaf1z and hCcr4d (Fig. 5) and the observation that hCaf1z overexpressed in the cytoplasm activates decay of reporter mRNAs (Fig. 6) suggest that the hCcr4d-hCaf1z complex may have a function in cytoplasmic mRNA deadenylation and decay. Perhaps hCaf1z and hCcr4d are normally sequestered in the nucleus but can be exported to the cytoplasm under conditions where they are induced to degrade cytoplasmic mRNA. Future studies should reveal whether conditions exist for which the hCcr4d-hCaf1z complex is exported to the cytoplasm and whether under those conditions, it targets specific mRNA substrates for degradation.

An alternative possibility is that the hCcr4d-hCaf1z complex acts in degradation of nuclear mRNA or pre-mRNA. It has been shown that mRNAs that fail to undergo splicing are degraded in the nucleus (6, 27). It is possible that the hCaf1z-hCcr4d complex participates in such a process. However, this appears to be inconsistent with the observation that overexpression of hCaf1z, but not hCaf1z DEAA, results in inhibition of decay of an intronless β-globin reporter mRNA (Fig. 6D). The strong accumulation of the deadenylated, 3′-end-truncated form of this mRNA upon hCaf1z overexpression suggests that it is retained in the nucleus longer than those transcripts that undergo splicing and thereby becomes a better substrate for nuclear hCaf1z. It is unclear why hCaf1z overexpression results in mRNA stabilization and accumulation of 3′-end-truncated products and if these activities reflect real functions of endogenous hCaf1z or whether they are instead artifacts of overexpression. Future studies should reveal whether the endogenous hCcr4d-hCaf1z complex functions in nuclear mRNA/pre-mRNA degradation. We have been unsuccessful in efficiently depleting endogenous hCaf1z using small interfering RNAs (data not shown).

Does the hCcr4d-hCaf1z complex have a function in Cajal bodies? The hCcr4d-hCaf1z complex differs from conventional Ccr4-Caf1 complexes in its localization to nuclear Cajal bodies (Fig. 4B). Could the hCcr4d-hCaf1z complex have a function in RNA processing in Cajal bodies? A remarkable similarity between transcripts associated with Cajal bodies, which include snRNAs, snoRNAs, scaRNAs, telomerase RNA, and histone mRNAs (9), is that they all lack poly(A) tails. It is therefore mysterious why a deadenylase complex should localize there. Perhaps hCcr4d and hCaf1z are sequestered in Cajal bodies away from the nucleoplasm to avoid faulty deadenylation of nuclear mRNAs or pre-mRNAs. Alternatively, the hCcr4d-hCaf1z complex could serve to ensure that Cajal body RNAs do not acquire poly(A) tails by degrading spuriously added poly(A) tails. However, Northern blotting of oligo(dT)-selected RNA did not reveal any accumulation of polyadenylated

U2 snRNA or histone H2A mRNA upon overexpression of the hCaf1z DEAA mutant protein (data not shown). It also cannot be excluded that, despite its substrate preference, the hCcr4d-hCaf1z complex functions in cells as a 3′-to-5′ exonuclease rather than a deadenylase, for example, to trim Cajal body-associated RNAs.

Finally, the hCcr4d-hCaf1z complex could be involved in polyadenylation-mediated RNA degradation in the nucleus. Intriguingly, studies of yeast have shown that polyadenylated forms of tRNAs, rRNAs, snRNAs, and snoRNAs, as well as intergenic transcripts, can be detected in Rrp6 deletion mutants, in which the nuclear exosome is inactive (24, 26, 28, 45, 48). Furthermore, a nuclear polyadenylation complex was shown to adenylate these transcripts, which targets them for decay in the nucleus (21, 24, 44). It will be of great interest to determine whether this process occurs in human cells and, if so, whether the hCcr4d-hCaf1z complex functions in this pathway. The observed deadenylation followed by 3′-end "nibbling" activity of hCaf1z (Fig. 2A and 3A) may make it an ideal enzyme for such a process, as it would leave behind a 3′-end-shortened substrate for the nuclear exosome. An important goal of future studies is to establish whether the hCcr4d-hCaf1z deadenylase complex has a function in the nucleus and Cajal bodies or whether it functions instead in cytoplasmic mRNA decay following its nuclear export.

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