Deadenylation is prerequisite for P-body formation and mRNA decay in mammalian cells

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Deadenylation is the major step triggering mammalian mRNA decay. One consequence of deadenylation is the formation of nontranslatable messenger RNA (mRNA) protein complexes (messenger ribonucleoproteins [mRNPs]). Nontranslatable mRNPs may accumulate in P-bodies, which contain factors involved in translation repression, decapping, and 5′-to-3′ degradation. We demonstrate that deadenylation is required for mammalian P-body formation and mRNA decay. We identify Pan2, Pan3, and Caf1 deadenylases as new P-body components and show that Pan3 helps recruit Pan2, Ccr4, and Caf1 to P-bodies. Pan3 knockdown causes a reduction of P-bodies and has differential effects on mRNA decay. Knocking down Caf1 or overexpressing a Caf1 catalytically inactive mutant impairs deadenylation and mRNA decay. P-bodies are not detected when deadenylation is blocked and are restored when the blockage is released. When deadenylation is impaired, P-body formation is not restorable, even when mRNAs exit the translating pool. These results support a dynamic interplay among deadenylation, mRNP remodeling, and P-body formation in selective decay of mammalian mRNA.

Introduction

Regulation of mRNA turnover plays an essential role in modulating gene expression (Meyer et al., 2004; Parker and Song, 2004; Garneau et al., 2007). For all major paths of mRNA decay yet recognized in mammalian cells, including mRNA decay directed by AU-rich elements (AREs) in the 3′ untranslated region (Chen and Shyu, 1995), decay mediated by destabilizing elements in protein-coding regions (Grosset et al., 2000; Chang et al., 2004), nonsense-mediated decay (NMD; Chen and Shyu, 2003), decay directed by microRNAs (miRNAs; Wu et al., 2006), and decay of stable mRNAs such as β-globin mRNA (Yamashita et al., 2005), the first major step is deadenylation.

Mammalian deadenylation is mediated by the concerted action of two different poly(A) nuclease complexes (Yamashita et al., 2005). Poly(A) tails are first shortened to ∼110 nt by Pan2 in association with Pan3. In the second phase of deadenylation, a complex composed of Ccr4 and Caf1 catalyze further shortening of the poly(A) tail to oligo(A). Decapping by the Dcp1–Dcp2 complex (Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002; Piccirillo et al., 2003) may occur during and/or after the second phase of deadenylation (Yamashita et al., 2005). Although Pan3 and Caf1 associate with Pan2 and Ccr4 poly(A) nucleases, respectively (Brown et al., 1996; Albert et al., 2000; Tucker et al., 2001; Temme et al., 2004; Uchida et al., 2004), their in vivo roles in mammalian mRNA turnover remain unclear. In yeast, Pan3 does not exhibit poly(A) nuclease activity but its association with Pan2 is required for proper function of Pan2 (Brown et al., 1996; Mangus et al., 2004). In vitro experiments using recombinant human Pan2 and Pan3 proteins (Uchida et al., 2004) suggest that Pan3 plays a role in enhancing the poly(A) nuclease activity of Pan2 in mammalian cells. However, ectopic overexpression of Pan2 alone in mouse NIH3T3 cells results in highly rapid and processive deadenylation of an otherwise stable reporter mRNA or a premature translation-termination codon (PTC)–containing mRNA (Yamashita et al., 2005), indicating that Pan3 is not required for the nuclease activity of Pan2 for mammalian mRNA turnover. Instead, Pan3 may modulate the activity of Pan2 poly(A) nuclease or link deadenylation to subsequent decay of the mRNA body. Unlike Pan3, Caf1 exhibits poly(A) nuclease activity (Daugeron et al., 2001; Dupressoir et al., 2001; Temme et al., 2004; Bianchin et al., 2005; Molin and Puisieux, 2005). However, studies in yeast show that Caf1 poly(A) nuclease activity per se is not required for general deadenylation in vivo, although the presence of Caf1 is necessary for proper deadenylation by

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Ccr4 (Viswanathan et al., 2004). In contrast, the major poly(A) nuclease activity in *Drosophila melanogaster* resides in the Ccr4–Caf1 complex, with depletion of Caf1 exerting a stronger impeding effect than depletion of Ccr4 on deadenylation (Temme et al., 2004). Mammalian Caf1 has also been shown to display poly(A) nuclease activity in vitro (Viswanathan et al., 2004; Bianchin et al., 2005), but the role of Caf1 in mammalian mRNA turnover remains unclear.

Recent studies have revealed the existence of specific cytoplasmic foci, known as RNA P-bodies (processing bodies), that contain proteins with known functions in mRNA metabolism (for reviews see Eulalio et al., 2007b; Kedersha and Anderson, 2007; Parker and Sheth, 2007). These foci are also referred to as GW bodies because they carry GW182 proteins that are required for miRNA-mediated translation repression (for reviews see Ding and Han, 2007; Eulalio et al., 2007b). Translationally repressed mRNA protein complexes (mRNPs), factors which are involved in translational repression including miRNA-mediated translational silencing proteins (such as Argonaute proteins and Rck/p54), decapping enzymes, and a 5′-to-3′ exonuclease Xrn1, are also found in these foci (for reviews see Eulalio et al., 2007b; Kedersha and Anderson, 2007; Parker and Sheth, 2007). Thus, recent studies of P-bodies have mainly focused on the roles of decapping and translation repression in P-body formation. The relationship of these P-body–related machineries to deadenylation, the first step triggering mRNA decay, and the roles of deadenylation factors in P-body formation remain largely unclear. Besides, owing to the power of yeast and *D. melanogaster* genetics, the participating factors and their roles in eukaryotic P-body formation and mRNA turnover were largely identified by studies in yeast and *D. melanogaster*. Little was known about these important issues in mammalian cells.

In wild-type yeast strain under normal growth conditions, neither endogenous Ccr4–Caf1 (or Pop2) complex nor ectopically expressed Ccr4 form any discernable foci that colocalize with P-bodies (Sheth and Parker, 2003; Teixeira and Parker, 2007). Although Ccr4 and Pop2 can be localized in P-bodies in mutant yeast strains with xrn1 or dcp1 gene deleted (Teixeira and Parker, 2007), defects in Ccr4p and Pop2p do not significantly affect P-body formation and composition (Teixeira and Parker, 2007). In *D. melanogaster*, the Ccr4–Caf1 complex was also found in cytoplasmic foci (Temme et al., 2004), but whether those foci are P-bodies or not was not addressed. The only mammalian poly(A) nuclease that has been shown to localize in P-bodies is Ccr4 (Cougot et al., 2004; Andrei et al., 2005). Although knocking down Ccr4 in HeLa cells can lead to loss of P-bodies (Andrei et al., 2005), the effect of Ccr4 knockdown on deadenylation in these cells was not addressed. Thus, it is not clear as to whether P-body loss caused by knocking down Ccr4 is because of the impairment of deadenylation or simply because of the absence of the Ccr4 protein molecule that may be physically important for the structural integrity of P-bodies. Therefore, the role of deadenylation factors in P-body formation and the relationship between deadenylation and P-body formation in mammalian cells remain unknown. For example, are Pan2, Pan3, and Caf1 also present in P-bodies like Ccr4? Are these factors necessary for P-body formation, and are their functions in deadenylation crucial for P-body formation? Is deadenylation a prerequisite for P-body formation? In this paper, we address these questions by examining the subcellular localization and functions of poly(A) nucleases and their cofactors in relation to the formation of P-bodies and mRNA turnover in mammalian cells.

Results

Pan2 and Pan3 are components of P-bodies

We first determined whether Pan2 and Pan3, the factors involved in initiating mammalian mRNA deadenylation (Uchida et al., 2004; Yamashita et al., 2005), are found in P-bodies of mouse NIH3T3 fibroblasts (the cell line used for monitoring mRNA decay kinetics by the well-established transcriptional pulsing system; Xu et al., 1998; Chen et al., 2007). Dcp1a, a well-characterized component of P-bodies which is necessary for mRNA decapping in eukaryotes (Beelman et al., 1996; Lykke-Andersen, 2002; Cougot et al., 2004; Kedersha et al., 2005), was used as a marker to visualize P-bodies by immunofluorescence microscopy. Both endogenous Dcp1a and ectopically expressed Dcp1a fused with green fluorescent protein (GFP-Dcp1a) distributed in a focal pattern that disappeared upon translation blockage by cycloheximide in NIH3T3 cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1), as expected for P-bodies (Andrei et al., 2005; Brengues et al., 2005; Ferraiuolo et al., 2005). The results show that cytoplasmic foci detected by an anti-Pan2 antibody colocalize with P-bodies either marked by GFP- Dcp1a (Fig. 1 A) or detected by an anti-Dcp1a antibody (Fig. 1 B, top). Upon cycloheximide treatment, neither P-bodies nor Pan2 foci were detected (Fig. 1 B, bottom). These results indicate that Pan2 is a component of P-bodies.

We further substantiated this finding by showing that Pan2 foci colocalize with P-bodies in COS7 cells, using both Dcp1a and Xrn1 as markers to visualize P-bodies (Fig. 1 C, top and middle). Moreover, the endogenous Pan3 also distributes in cytoplasmic foci that colocalize with the Pan2 foci in COS7 cells (Fig. 1 C, bottom). Because of lack of an anti-Pan3 antibody suitable for immunofluorescence microscopy of mouse NIH3T3 cells, DNA coding for HA-Pan3 (HA-tagged Pan3) was delivered into NIH3T3 cells via transient transfection. Our data show that HA-Pan3 also distributes in a focal pattern colocalized with P-bodies (Fig. 1 D). It is worth noting that the number of P-bodies increased appreciably when HA-Pan3 was overexpressed (Fig. 1 D), suggesting a role of Pan3 in initiating or enhancing the formation of P-bodies. The HA-Pan3 foci are not stress granules, as they can be detected regardless of whether or not the cells were treated with arsenite (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1), a cellular stress inducer (Kedersha et al., 2002). Stress granules were only found in the arsenite-treated cells, as visualized by a stress granule marker G3BP1 (Fig. S2, bottom), and not in cells without arsenite treatment (Fig. S2, top). Importantly, when stress granules were induced, they did not colocalize with the HA-Pan3 foci (Fig. S2, bottom, right). Collectively, our results identify both Pan2 and Pan3 as being two new components of P-bodies in mammalian cells.
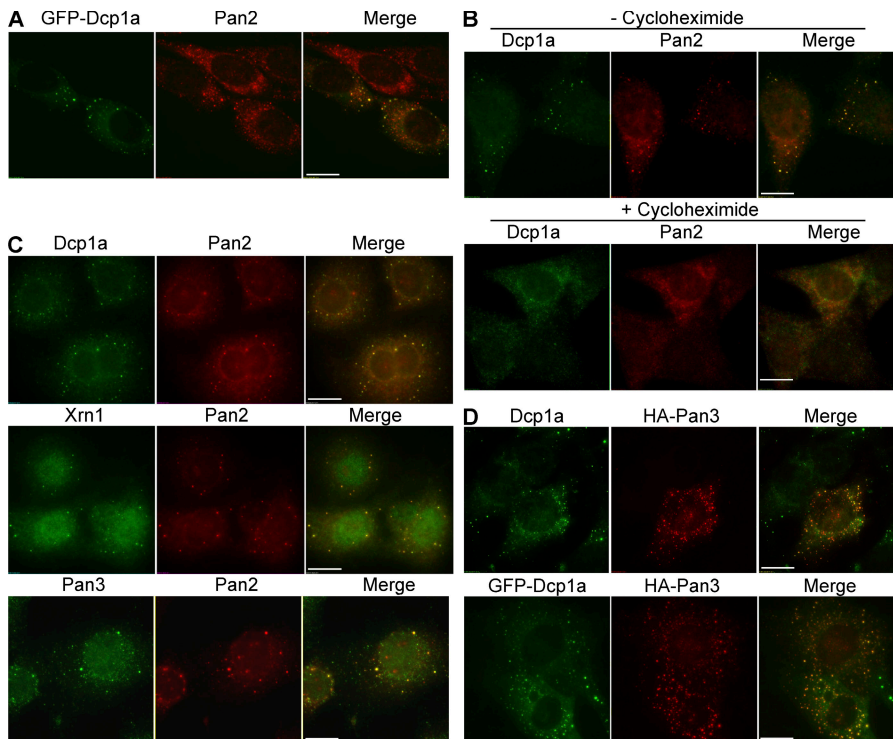
Figure 1. **Pan2 and Pan3 are P-body components.** (A and B) Pan2 foci colocalize with P-bodies in NIH3T3 cells (top) and are disassembled upon cycloheximide treatment (B, bottom, middle), as expected for P-bodies (B, bottom, left). (C) Pan2 foci colocalize with P-bodies detected with anti-Dcp1a (top) or anti-Xrn1 (middle) antibodies in monkey COS7 cells, in which Pan3 foci also colocalize with Pan2 foci (bottom). (D) HA-Pan3 foci colocalize with P-bodies in NIH3T3 cells. Immunofluorescence staining was performed as described in Materials and methods. The original blue color of HA-Pan3 staining was changed to red for easier visualization. Bars, 15 µm.

Pan3 helps Pan2, Ccr4, and Caf1 localize to P-bodies

Although Pan2 and Pan3 can form a complex in the cytoplasm, the finding that HA-Pan3 can be detected in P-bodies when it is overexpressed alone (Fig. 1 D) suggests that Pan3 can associate with P-bodies without a coordinated expression of Pan2. In contrast, ectopically expressed Pan2 did not distribute in a focal pattern when expressed alone (Fig. 2 A, top and middle) but did colocalize with P-bodies when coexpressed with HA-Pan3 (Fig. 2 A, bottom). In contrast, ectopically expressed myc-PABP, a non–P-body component (Kedersha et al., 2005) which has the ability to bind Pan3 (Brown et al., 1996; Mangus et al., 2004; Uchida et al., 2004), displayed a uniform distribution in the cytoplasm without forming foci even when coexpressed with HA-Pan3 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb .200801196/DC1). These results further corroborate our conclusion that HA-Pan3 foci correspond to P-bodies and indicate that Pan3 helps Pan2 localize to P-bodies.

As Ccr4 has been observed to colocalize with P-bodies in mammalian cells (Cougot et al., 2004; Andrei et al., 2005), we then tested whether Caf1 can also be found in mammalian P-bodies by examining subcellular localization of endogenous Caf1 in NIH3T3 cells (Fig. 2 B). Immunofluorescence microscopic results show a strong staining in both nuclei and cytoplasm of cells (with two to six foci colocalized with P-bodies marked by GW182 in each cell) when the anti-Caf1 antibody was used (Fig. 2 B, top), whereas only a weak and nonspecific background staining was detected when preimmune serum was used (Fig. 2 B, bottom). Thus, our data indicate that only a small portion of Caf1 colocalizes with P-bodies, which is similar to the staining patterns previously observed for Ccr4 (Cougot et al., 2004; Andrei et al., 2005). Although GFP-Ccr4 was observed to

colocalize with P-bodies in HEK293 cells (Cougot et al., 2004), ectopically expressed Ccr4 and Caf1 did not form foci that colocalized with P-bodies in NIH3T3 cells, regardless of whether they were overexpressed separately (Fig. 2, C [top and middle] and D [top, left]) or together (Fig. 2 C, bottom). Because Pan3 is able to enhance localization of Pan2 to P-bodies (Fig. 2 A), we then tested whether Pan3 also helps Ccr4 and Caf1 localize to P-bodies. After coexpressing HA-Pan3 with GFP-Ccr4 (Fig. 2 D, top, right) or Caf1-V5 (Fig. 2 D, bottom), we observed that both proteins distributed in a focal pattern colocalized with the P-bodies. These experiments show that Pan3 also enhances localization of Ccr4 and Caf1 to P-bodies.

Pan3 knockdown affects P-body formation and has differential effects on mRNA decay

The findings in the previous sections (Figs. 1 [D] and 2) suggest that Pan3 plays a critical role in mammalian P-body formation. We knocked down Pan3 in NIH3T3 cells using siRNAs and examined the effects on P-body formation (Fig. 3 A). Immunofluorescence microscopy revealed a reduction of P-bodies in cells treated with Pan3-specific siRNAs but not in cells treated with control siRNAs (Fig. 3 A, top and middle). Western blotting analysis indicated that an ∼80% knockdown efficiency was achieved (Fig. 3 A, bottom). These experiments demonstrate that Pan3 not only is an integral component of P-bodies but also is important for the formation of P-bodies.

We next examined the effect of Pan3 knockdown on mRNA deadenylation and decay in NIH3T3 cells. Decay of a nonsense-containing mRNA (BBB+PTC), a c-*fos* ARE-containing mRNA (BBB+ARE), and the stable β-globin (BBB) message, representing three distinct pathways, was tested. The transcripts were expressed under the control of an inducible Tet-off promoter
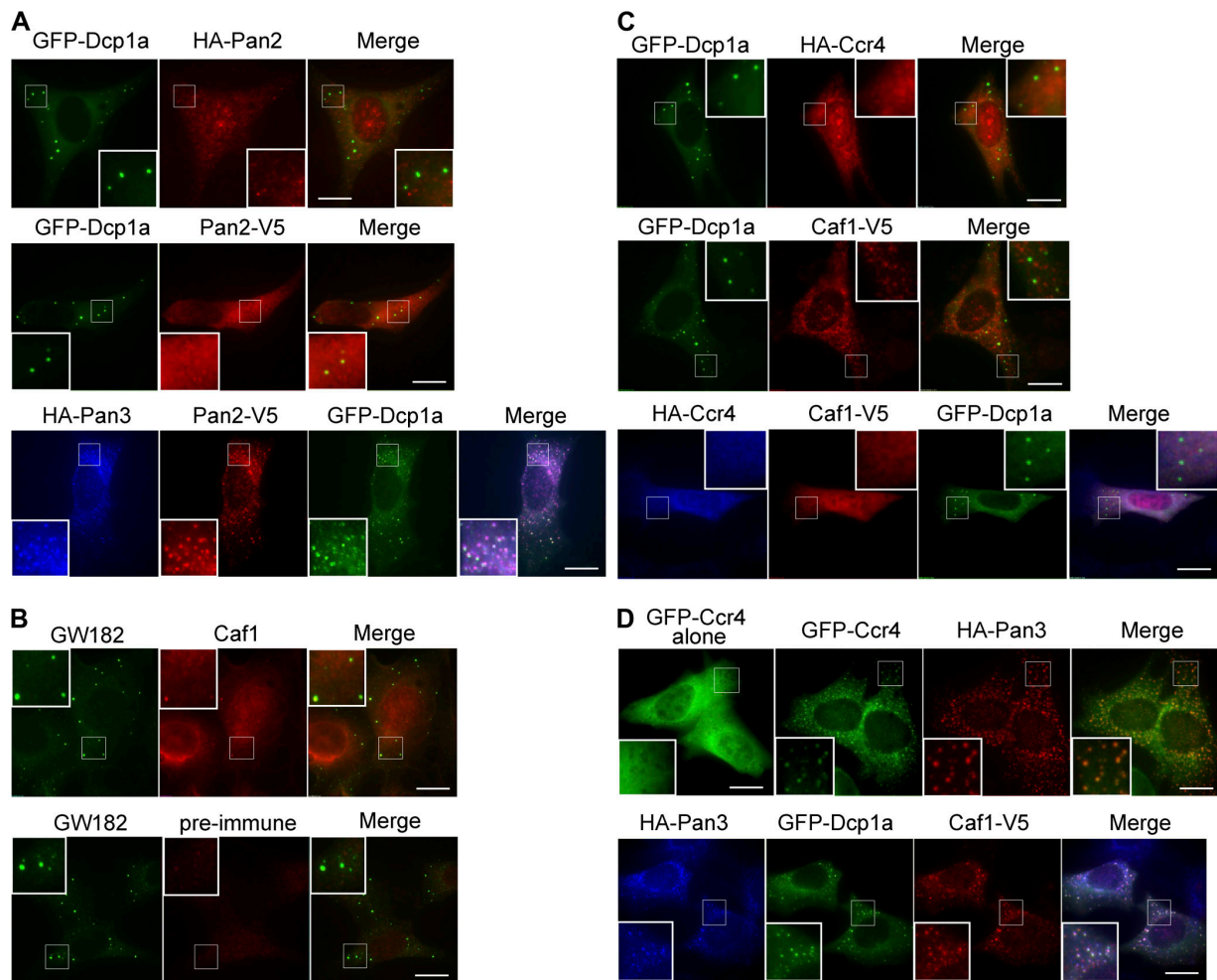
Figure 2. **Pan3 helps Pan2, Ccr4, and Caf1 localize to P-bodies.** (A) Ectopically expressed Pan2 proteins do not distribute in a focal pattern when expressed alone (top and middle) but can form foci that colocalize with P-bodies when coexpressed with HA-Pan3 (bottom). NIH3T3 cells were transfected with a plasmid encoding GFP-Dcp1a, either together with a plasmid encoding HA-Pan2 (top) or Pan2-V5 (middle) or with both a plasmid encoding HA-Pan3 and a plasmid encoding Pan2-V5 (bottom). Immunofluorescence staining was performed as described in Materials and methods. (B) Only a small portion of endogenous Caf1 can be found in foci that colocalize with P-bodies marked by GW182. Localization of endogenous GW182 or Caf1 was determined using human anti-GW182 serum (left) or rabbit anti-Caf1 antibody (top, middle). Rabbit preimmune serum (bottom, middle) was used as a control. (C) Ectopically expressed HA-Ccr4 and Caf1-V5 do not colocalize with P-bodies in NIH3T3 cells, regardless of whether they were overexpressed separately (top and middle) or together (bottom). (D) Ectopically expressed GFP-Ccr4 does not form foci that colocalize with P-bodies in NIH3T3 cells (top, left), but ectopically expressed Ccr4 and Caf1 can colocalize with P-bodies when coexpressed with HA-Pan3 (top [right] and bottom). Immunofluorescence staining was performed as described in Materials and methods. The original blue color of HA-Ccr4 staining in C (top) and HA-Pan3 staining in D (top, right) was changed to red for easier visualization. Insets show an enlarged view of the boxed regions to better depict the absence or presence of foci colocalized with P-bodies. Bars, 15 μm.

(Loflin et al., 1999b; Chen et al., 2007). This well-established promoter–reporter system allows a transcriptional pulsing for monitoring the deadenylation of a uniformly sized population of mRNA molecules, permitting evaluation of mRNA deadenylation and decay kinetics (Loflin et al., 1999b; Chen et al., 2007). α-Globin–GAPDH hybrid mRNA, transcribed from a cotransfected plasmid, was constitutively expressed from an SV40 early promoter to serve as internal standard for transfection efficiency and sample loading. Our results show that knocking down Pan3 had little effect on the first phase of deadenylation (between 0 and 1 h), but it slowed the second phase of deadenylation and increased the overall stability of the BBB+PTC mRNA (Fig. 3 B, top). In contrast, a faster decay of BBB and BBB+ARE mRNAs was observed (Fig. 3 B, middle and bottom). Our observations that Pan3 knockdown causes a reduction of

P-bodies (Fig. 3 A) and has differential effects on mRNA decay (Fig. 3 B) suggest that different roles of Pan3 and P-bodies in different mRNA decay pathways exist in mammalian cells (see Discussion).

Pan2–Pan3 and Ccr4–Caf1 complexes can interact with each other in vivo

The observations that Pan 3 helps recruit Pan2, Ccr4, and Caf1 to P-bodies (Fig. 2) suggest that the Pan2–Pan3 and Ccr4–Caf1 complexes can communicate with each other in vivo. To test this hypothesis, we performed a series of coimmunoprecipitation/Western blotting experiments using RNase A–treated lysates prepared from cells overexpressing the components of these complexes (Fig. 4). When HA-Pan3 was expressed alone, endogenous Pan2 and PABP were both coprecipitated along with
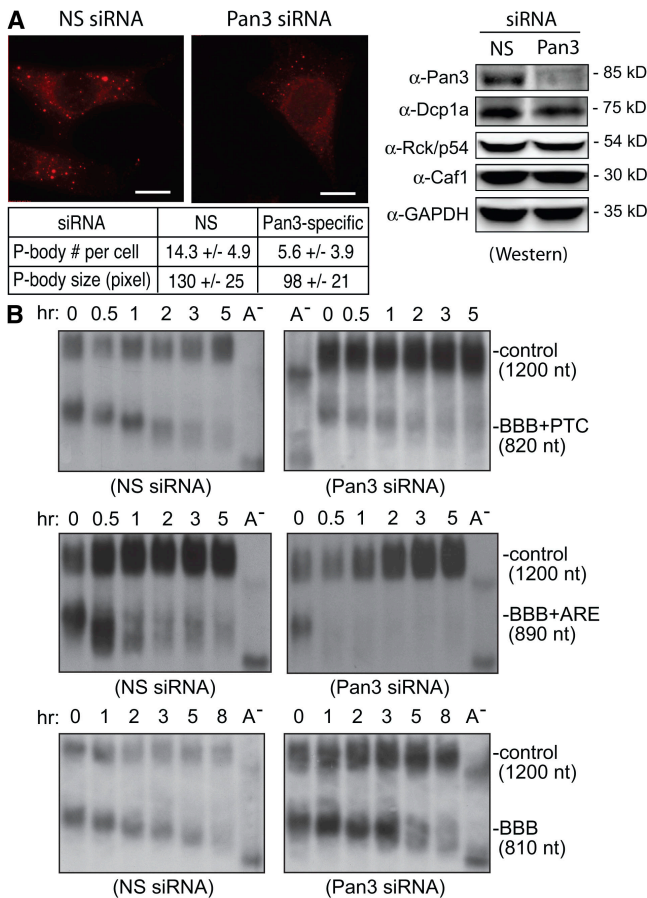
Figure 3 (left panels)

A

NS siRNA   Pan3 siRNA

siRNA
NS   Pan3

α-Pan3 — 85 kD
α-Dcp1a — 75 kD
α-Rck/p54 — 54 kD
α-Caf1 — 30 kD
α-GAPDH — 35 kD

(Western)

siRNA	NS	Pan3-specific
P-body # per cell	14.3 +/- 4.9	5.6 +/- 3.9
P-body size (pixel)	130 +/- 25	98 +/- 21

B

hr: 0 0.5 1 2 3 5 A⁻   A⁻ 0 0.5 1 2 3 5

-control (1200 nt)
-BBB+PTC (820 nt)

(NS siRNA)   (Pan3 siRNA)

hr: 0 0.5 1 2 3 5 A⁻   0 0.5 1 2 3 5 A⁻

-control (1200 nt)
-BBB+ARE (890 nt)

(NS siRNA)   (Pan3 siRNA)

hr: 0 1 2 3 5 8 A⁻   0 1 2 3 5 8 A⁻

-control (1200 nt)
-BBB (810 nt)

(NS siRNA)   (Pan3 siRNA)

Figure 3. Pan3 knockdown affects P-body formation and has differential effects on mRNA decay. (A, left) Immunofluorescence microscopy results showing a significant loss of P-bodies in cells transfected with the Pan3-specific siRNA (Pan3 siRNA) but not with the control nonspecific (NS) siRNA. Rabbit anti-Dcp1a antibody was used to detect P-bodies. (A, bottom) A summary of the changes in P-body number and size after Pan3 knockdown (see Materials and methods). (A, right) Western blots showing that the Pan3 siRNA efficiently knocked down the Pan3 expression but had little effect on the expression of the three other P-body components (Dcp1a, Rck/p54, and Caf1) and the control (GAPDH). (B) Northern blots showing the effects of Pan 3 knockdown on deadenylation and decay of BBB+PTC (top), BBB+ARE (middle), or BBB (bottom) mRNA. NIH3T3 B2A2 cells were transiently cotransfected with a Tet promoter–regulated plasmid encoding a reporter mRNA as indicated and either the nonspecific siRNA or Pan3 siRNA. A plasmid encoding constitutively expressed α-globin–GAPDH mRNA was also cotransfected to provide an internal standard for transfection efficiency and sample handling (control). Times correspond to hours after tetracycline addition. Poly(A)⁻ RNA was prepared in vitro by treating an RNA sample from an early time point with oligo(dT) and RNase H.

Figure 4 (right panels)

A

5% Input   α–HA IP

HA-Pan3- (86 kD)
Pan2- (120 kD)
PABP- (70 kD)
Caf1- (30 kD)
GAPDH- (35 kD)

B

5% Input   α–HA IP

HA-Pan2- (121 kD)
PABP- (70 kD)
Caf1- (30 kD)
GAPDH- (35 kD)

C

5% Input   α–HA IP

HA-Pan2- (121 kD)
Pan3-V5- (88 kD)
Caf1- (30 kD)
PABP- (70 kD)
GAPDH- (35 kD)

D

5% Input   α–V5 IP

Ccr4-V5- (67 kD)
HA-Pan3- (86 kD)
PABP- (70 kD)
Caf1- (30 kD)
Pan2- (120 kD)
GAPDH- (35 kD)

Figure 4. Pan2–Pan3 and Ccr4–Caf1 complexes can interact with each other in vivo. COS7 cells were transfected with plasmids encoding HA-Pan3 (A), HA Pan2 (B), HA-Pan2 and Pan3-V5 (C), or Ccr4-V5 and HA-Pan3 (D). RNase A–treated cell extracts were first subjected to immunoprecipitation (IP) using anti-HA antibody Affinity Matrix (A–C) or anti-V5 antibody conjugated to agarose beads (D). Immunoprecipitates were then separated by SDS-PAGE and analyzed by Western blotting using the various antibodies indicated to the left of each panel. Input: 5% of total lysate used for immunoprecipitation is shown for comparison.

HA-Pan3 (Fig. 4 A). These results are consistent with previous findings that Pan3 is a genuine PABP binding protein (Siddiqui et al., 2007) and that Pan2 and Pan3 can form complexes in vivo (Mangus et al., 2004). In contrast, endogenous Caf1 was not found in the HA-Pan3 precipitate (Fig. 4 A), indicating a lack of direct interaction between Pan3 and Caf1 or an interaction too weak to be detected. However, when HA-Pan2 was ectopically expressed alone (Fig. 4 B), endogenous Caf1, but not PABP, was coprecipitated with HA-Pan2, indicating a specific interaction between Pan2 and Caf1, either directly or indirectly. Therefore, Pan2–Pan3 and Ccr4–Caf1 complexes may communicate with each other in vivo via the interaction between Pan2 and Caf1.
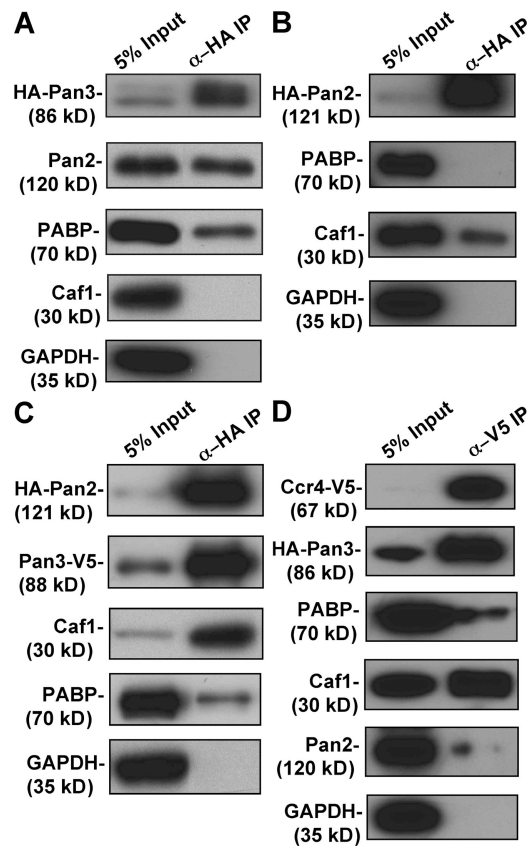
The notion that the Pan2–Pan3 and Ccr4–Caf1 complexes can associate with each other was further confirmed by the results showing that Pan3-V5, endogenous Caf1, and a modest amount of endogenous PABP were coprecipitated with HA-Pan2 by an anti-HA antibody when HA-Pan2 and Pan3-V5 were coexpressed (Fig. 4 C). The abundant GAPDH protein, which served as a negative control, was not found in the precipitates. The modest amount of endogenous PABP in the precipitate (Fig. 4 C) suggests an indirect association between HA-Pan2 and PABP, presumably mediated via Pan3-V5, as HA-Pan2 alone did not pull down PABP (Fig. 4 B). Because high titer Ccr4 and Pan3 antibodies for immunoprecipitation were unavailable, we coexpressed Ccr4-V5 and HA-Pan3 and used an anti-V5 antibody for coimmunoprecipitation experiments to test whether an interaction exists between Ccr4 and Pan3 (Fig. 4 D). Our data demonstrated a strong coprecipitation of endogenous Caf1 with Ccr4-V5 (Fig. 4 D). Moreover, a huge amount of HA-Pan3, as well as a modest amount of the endogenous Pan2 and PABP, was also coprecipitated with Ccr4-V5. As a control, no coimmunoprecipitation of GAPDH was detected. Collectively, our data support that Pan2–Pan3 and Ccr4–Caf1 poly(A)
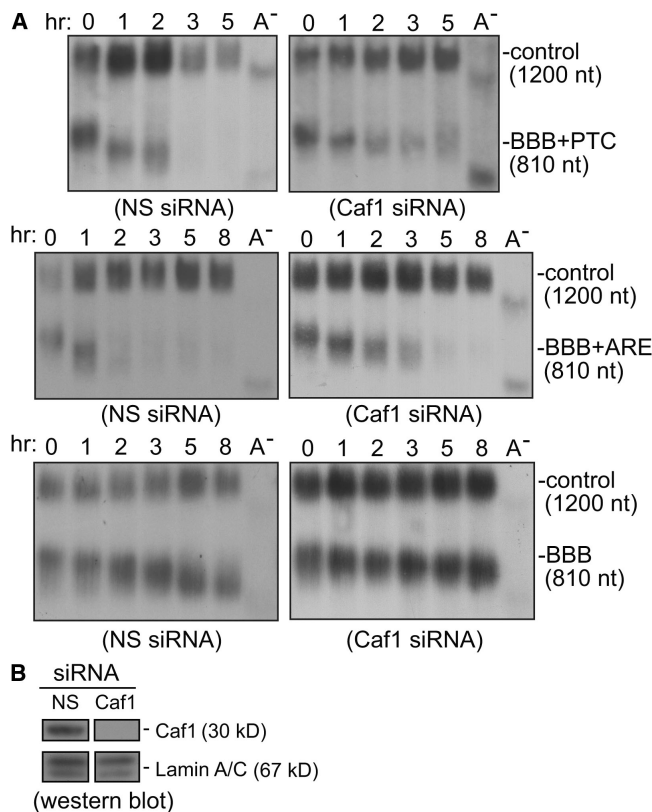
A

hr: 0 1 2 3 5 A⁻   0 1 2 3 5 A⁻

-control (1200 nt)

-BBB+PTC (810 nt)

(NS siRNA)   (Caf1 siRNA)

hr: 0 1 2 3 5 8 A⁻   0 1 2 3 5 8 A⁻

-control (1200 nt)

-BBB+ARE (810 nt)

(NS siRNA)   (Caf1 siRNA)

hr: 0 1 2 3 5 8 A⁻   0 1 2 3 5 8 A⁻

-control (1200 nt)

-BBB (810 nt)

(NS siRNA)   (Caf1 siRNA)

B siRNA

NS Caf1

- Caf1 (30 kD)

- Lamin A/C (67 kD)

(western blot)

Figure 5. **Caf1 is required for deadenylation.** (A) Northern blots showing the effects of Caf1 knockdown on deadenylation and decay of BBB+PTC (top), BBB+ARE (middle), or BBB (bottom) mRNA. NIH3T3 B2A2 cells were transiently cotransfected with a Tet promoter–regulated plasmid encoding a reporter mRNA, as indicated, and either the control siRNA (NS siRNA) or Caf1 siRNA. Times correspond to hours after tetracycline addition. The control mRNA (ctrl) and the preparation of Poly(A)⁻ RNA were as described in the Fig. 3 B legend. Black lines indicate that intervening lanes have been spliced out. (B) Western blot showing >90% efficiency of Caf1 knockdown.

nuclease complexes can associate with each other to form a super-complex in vivo.

Caf1 is required for deadenylation and can accelerate poly(A) shortening in mouse NIH3T3 cells

To address the role of Caf1 in mammalian mRNA turnover, we first determined whether mRNA deadenylation and decay requires Caf1. We knocked down endogenous Caf1 in NIH3T3 cells by siRNA (Fig. 5) and examined the effects on the deadenylation and decay kinetics of BBB+PTC, BBB+ARE, and BBB mRNAs. As shown in Fig. 5, Caf1 knockdown significantly impairs the deadenylation and decay of all the mRNAs tested, demonstrating that Caf1 plays a key role in mRNA deadenylation and decay in mouse NIH3T3 cells.

As Caf1 was shown to exhibit poly(A) nuclease activity in vitro (Viswanathan et al., 2004; Bianchin et al., 2005), we next tested whether Caf1 can accelerate deadenylation in vivo by examining the effects of overexpressing Caf1 on deadenylation of BBB+PTC, BBB+ARE, and BBB mRNAs in NIH3T3 cells (Fig. 6). Caf1 overexpression led to extremely rapid deadenylation of all the reporter mRNAs tested. Poly(A) shortening was detected as early as the 0-h time point (Fig. 6, A–C, top, right).

These results demonstrate that Caf1 exhibits a robust poly(A) shortening activity that enhances the deadenylation of mRNAs in the cytoplasm of mammalian cells. Interestingly, ectopically overexpressing both Ccr4 and Caf1 had an additive effect on accelerating poly(A) shortening (Fig. 6 C, bottom, left), indicating that coordinated expression of both proteins further promotes the deadenylation function of Ccr4–Caf1 poly(A) nuclease complex in NIH3T3 cells.

The poly(A) nuclease activities of Caf1 and Ccr4 have complementary roles in mammalian deadenylation

To better address the role of Caf1 in mammalian mRNA turnover, we took the approach of inhibiting endogenous Caf1 activity by ectopically expressing a catalytically inactive mutant of Caf1 and monitoring the effects on the decay of BBB+PTC, BBB+ARE, and BBB mRNAs in NIH3T3 cells (Fig. 6, A and B [bottom, left] and C [middle, middle]). We changed a critical aspartate residue, which is necessary for metal binding and Caf1 nuclease activity (Viswanathan et al., 2004), to alanine by site-directed mutagenesis to create the mutant Caf1. Northern blotting results show that the Caf1 mutant exhibits a profound dominant-negative effect, blocking deadenylation of all the reporter mRNAs tested and leading to drastic stabilization of these transcripts. It is worth noting that the broad bands normally detected for the internal control α-globin–GAPDH hybrid message constitutively transcribed from the SV40 early promoter also became tight and migrated much more slowly than the poly(A)⁻ band. These observations indicate that overexpression of mutant Caf1 has a general and very profound dominant-negative effect on deadenylation in mammalian cells.

As Caf1 and Ccr4 work as a complex (Tucker et al., 2002; Temme et al., 2004; Behm-Ansmant et al., 2006) and ectopically overexpressing both Ccr4 and Caf1 had an additive effect on accelerating poly(A) shortening (Fig. 6 C, bottom, left), we then tested whether coexpressing wild-type Ccr4 can release the deadenylation blockage caused by the Caf1 mutant. The results (Fig. 6 C, bottom, middle) show that the dominant-negative effect exerted by the Caf1 mutant was greatly diminished when wild-type Ccr4 was coexpressed. The deadenylation kinetics of BBB mRNA were now similar to those observed in cells coexpressing wild-type Caf1 and Ccr4 (Fig. 6 C, bottom, left). Thus, the poly(A)-shortening activity of Caf1 can be complemented by that of Ccr4 for deadenylation of the mRNA substrate. Previously, we had shown that overexpressing a dominant-negative catalytically inactive Ccr4 mutant slowed the deadenylation of BBB mRNA (Fig. 6 C, middle, left; Yamashita et al., 2005). We now tested whether the poly(A) nuclease activity of Caf1 can also complement that of Ccr4 by coexpressing wild-type Caf1 and the Ccr4 mutant. The results show that the deadenylation kinetics of BBB mRNA in the presence of both wild-type Caf1 and Ccr4 mutant (Fig. 6 C, bottom, right) are similar to those observed in cells coexpressing wild-type Ccr4 and Caf1 mutant (Fig. 6 C, bottom, middle) or coexpressing wild-type Ccr4 and Caf1 (Fig. 6 C, bottom, left). Collectively, we conclude that the poly(A) nuclease activities of Ccr4 and Caf1 in the Ccr4–Caf1 complex have complementary roles in mammalian deadenylation.
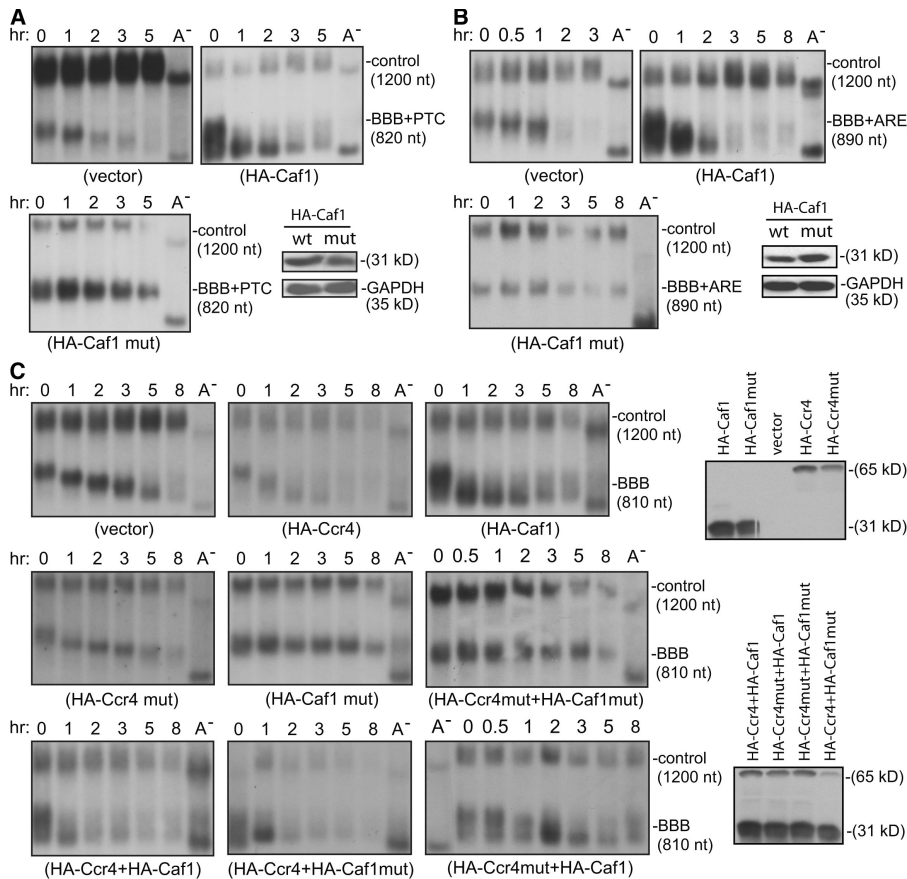
Figure 6. **The poly(A) nuclease activities of Caf1 and Ccr4 have complementary roles in deadenylation.** (A and B, top and bottom, left) Northern blots showing the effects of overexpressing wild-type or mutant (mut) Caf1 on deadenylation and decay of BBB+PTC (A) or BBB+ARE (B) mRNA. (A and B, bottom, right) Western blots showing the expression levels of the ectopically expressed wild-type (wt) or mutant HA-Caf1 proteins. (C) Northern blots showing the effects of overexpressing wild-type or mutant Ccr4 or Caf1 alone or different pairs of Ccr4 and Caf1 on deadenylation and decay of BBB mRNA. The expression levels of the ectopically expressed Caf1 or Ccr4 proteins were analyzed by Western blotting. NIH3T3 B2A2 cells were transiently cotransfected with a Tet promoter–regulated plasmid encoding a reporter mRNA and the plasmids encoding the wild-type or mutant Ccr4 or Caf1 as indicated. Times correspond to hours after tetracycline addition. The control mRNA (ctrl) and the preparation of Poly(A)⁻ RNA were as described in the Fig. 3 B legend. Black lines indicate that intervening lanes have been spliced out.

P-body formation requires active deadenylation

Our findings that expression of the dominant-negative HA-Caf1 mutant completely blocked deadenylation (Fig. 6 C, middle, middle) and coexpression of wild-type HA-Ccr4 released the deadenylation blockage (Fig. 6 C, bottom, middle) provided an approach to study how alteration of deadenylation may impact P-body formation without knocking down any deadenylase that may be physically required for the structural integrity of P-bodies. We performed immunofluorescence microscopy to test for the presence of P-bodies in cells overexpressing wild-type Caf1, mutant Caf1, or coexpressing the Caf1 mutant with wild-type or mutant Ccr4 (Fig. 7). It should be noted that without a coordinated expression of Pan3, ectopically expressed Ccr4 and Caf1 proteins did not form foci that colocalized with P-bodies (Fig. 2 C and Fig. 7, green staining). To test the presence of P-bodies, endogenous Pan2 and Dcp1a were used as markers to visualize P-bodies. The results show that P-bodies could hardly be detected in NIH3T3 cells overexpressing the Caf1 mutant (Fig. 7 A, right, dashed lines), whereas overexpressing wild-type Caf1 did not have any obvious effect (Fig. 7 A, left). Similar results were also observed in COS7 cells (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1). Remarkably, P-bodies were restored when wild-type Ccr4 was coexpressed with the Caf1 mutant (Fig. 7 B, left), a situation in which the negative effect of the Caf1 mutant on deadenylation was mitigated (Fig. 6 C, bottom, middle). Conversely, P bodies remained undetectable when cells were cotransfected with both mutant Ccr4 and mutant Caf1 (Fig. 7 B, right, dashed lines). Collectively, these results demonstrate that P-body formation is dependent on active deadenylation in mammalian cells. This conclusion was further substantiated by the observation that P-bodies were also undetectable when Caf1 was knocked down (Fig. 8 A, bottom, left), a condition that also severely impaired deadenylation (Fig. 5).

Several previous studies have shown that both the number and size of P-bodies are increased by treating eukaryotic cells with the translation inhibitor puromycin, which increases the amount of nontranslatable mRNPs (Cougot et al., 2004; Maroney et al., 2006; Eulalio et al., 2007a; Yang and Bloch, 2007). To further substantiate the finding that P-body formation is dependent on active deadenylation, we tested whether P-body formation can be induced by puromycin treatment of the cells in which deadenylation has already been impaired. As shown in Fig. 8, puromycin treatment increases the number and size of P-bodies when deadenylation is not blocked, namely in cells transfected with nonspecific siRNA (Fig. 8 A, top, right), overexpressing wild-type Caf1 (Fig. 8 B, top, right, dashed line), or nontransfected (Fig. 8 B, top and bottom, right, area surrounding the enclosed region). Strikingly, when deadenylation was blocked by knocking down Caf1 (Fig. 8 A, bottom) or by overexpressing the Caf1 dominant-negative mutant (Fig. 8 B, bottom, dashed line), puromycin treatment failed to induce P-body formation. These results demonstrate that P-bodies cannot form as long as deadenylation is impaired, even when mRNPs exit the translating pool.

Figure 7. P-body formation requires active deadenylation. (A and B) Overexpression of catalytic mutant (mut) Caf1 blocks formation of P-bodies (A, right, dashed lines), and the blocking effect of Caf1 mut can be reversed by coexpressing wild-type Ccr4 (B, left) but not by coexpressing a catalytically inactive mutant of Ccr4 (B, right, dashed lines). NIH3T3 cells were transfected with plasmids encoding either wild-type (A, left) or mutant (A, right) Caf1 alone or cotransfected with plasmids encoding mutant Caf1 and either wild-type (B, left) or mutant (B, right) Ccr4. Immunofluorescence staining was performed as described in Materials and methods. The original blue color of HA-tagged protein staining was changed to green for easier visualization. Endogenous Pan2 and Dcp1a were used as markers to visualize P-bodies. Bars, 15 µm. (C) Western blots showing the expression levels of the ectopically expressed Caf1 or Ccr4 as well as the levels of endogenous Pan2 and Dcp1a.
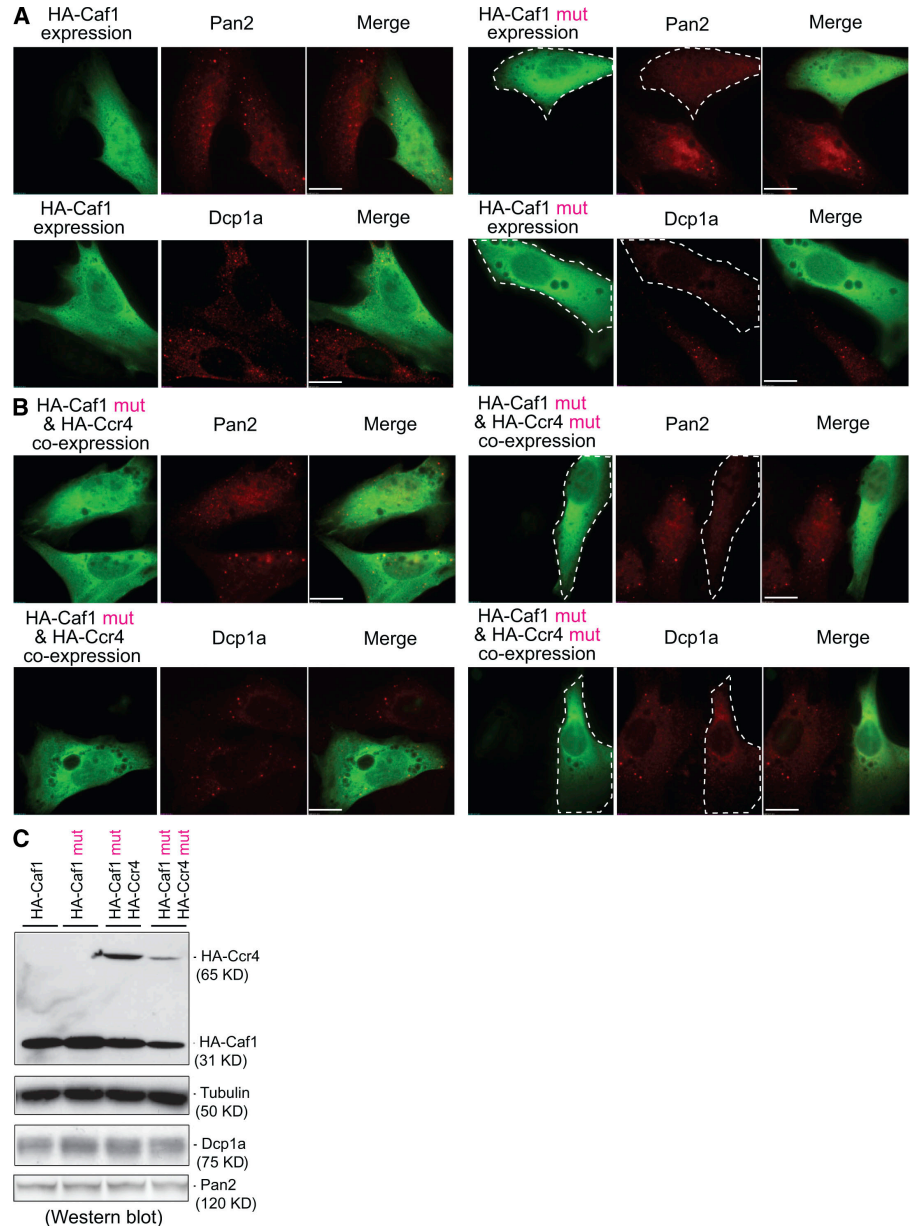
Discussion

Deadenylation is a necessary initial step in all major pathways of mammalian mRNA decay

Deadenylation is known as the first major step triggering general mRNA decay in mammalian cells (Meyer et al., 2004; Parker and Song, 2004). The importance of deadenylation for initiating NMD in mammalian cells is a recent insight (Chen and Shyu, 2003; Yamashita et al., 2005) because previous studies in yeast supported a decapping-dependent NMD pathway (Muhlrad and Parker, 1994). Several findings demonstrate that mammalian NMD occurs in the cytoplasm and is triggered by deadenylation but not by decapping. A PTC at codon 39 of β-globin (BBB+PTC) mRNA induces accelerated deadenylation and subsequent rapid decay of the RNA body. Blocking translation initiation stabilizes the transcript, confirming involvement of the NMD pathway in

the cytoplasm (Chen and Shyu, 2003). Moreover, knocking down Dcp2 does not have any appreciable stabilizing effect on a PTC-containing mRNA (Yamashita et al., 2005). In the present study, we further show that NMD, ARE-mediated mRNA decay, and the default decay pathway for a stable message are all impaired when deadenylation is inhibited either by knocking down Caf1 (Fig. 5) or by overexpressing a Caf1 dominant-negative mutant (Fig. 6). Thus, deadenylation is a necessary initial step for all major paths of mRNA decay yet recognized in mammalian cells, including NMD.

Deadenylation is a prerequisite and precursor for P-body formation

Several lines of evidence from this study demonstrate that deadenylation is a prerequisite and precursor for P-body formation in mammalian cells. First, impairment of deadenylation by knockdown of Caf1 (Fig. 5) leads to loss of P-bodies (Fig. 8 A).
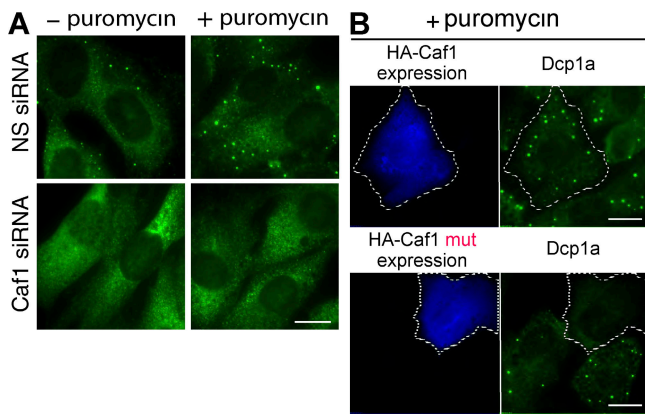
Figure 8. **Puromycin treatment does not induce P-body formation when deadenylation is impaired.** (A) Puromycin treatment enhances P-body formation (top, right) in NIH3T3 cells but does not induce P-body formation when Caf1 is knocked down (bottom, right). The cells transfected with either the control nonspecific siRNA (top) or Caf1 siRNA (bottom) were treated with (right) or without (left) puromycin for 1 h. Endogenous GW182 was used as a marker to visualize P-bodies. (B) Puromycin treatment does not induce P-body formation in cells overexpressing the mutant Caf1 (bottom, right, dashed line). NIH3T3 cells were transfected with plasmids encoding either wild-type (top) or mutant (bottom) Caf1 followed by puromycin treatment for 1 h. Immunofluorescence staining was performed as described in Materials and methods. Endogenous Dcp1a was used as a marker to visualize P-bodies. Bars, 15 μm.

In contrast, Pan3 knockdown results in P-body reduction (Fig. 3 A) but has little or no impairing effect on deadenylation (Fig. 3 B). These results show that blocking deadenylation impairs P-body formation but that the converse is not true, thereby establishing the cause–effect relationship between deadenylation blockage and P-body loss. Second, the inhibitory effects of a dominant-negative mutant of Caf1 on deadenylation and P-body formation (Figs. 6, 7, and S4) demonstrate that P-body loss caused by knocking down deadenylation factors is because of deadenylation blockage by itself and not simply because of the absence of the protein molecules that may be physically important for P-body integrity. Importantly, coexpression of wild-type Ccr4 with the Caf1 mutant, a situation in which deadenylation blockage is released (Fig. 6 C, bottom, middle), allows P-body formation (Fig. 7 B, left). Third, puromycin, which increases the pool of nontranslatable mRNAs, induces P-body formation only when deadenylation is not blocked (Fig. 8). Thus, it appears that mRNPs in P-bodies represent poly(A)-shortened mRNAs regardless of how they arrived in P-bodies or what their future fate is.

Deadenylation may induce mRNP remodeling required for P-body formation

Several observations in this study suggest that an mRNP containing a poly(A)-shortened mRNA undergoes remodeling before it appears in P-bodies. First, Pan2–Pan3 and Ccr4–Caf1 complexes can interact with each other in vivo (Fig. 4), suggesting that they form a super complex to direct the two consecutive phases of deadenylation in a concerted manner (Yamashita et al., 2005) and to elaborate subsequent mRNP remodeling. This notion is further supported by our observation that overexpression of the Caf1 mutant impaired both the first and the second phases of deadenylation (Fig. 6). It appears that the two

phases of deadenylation, although they take place sequentially, are functionally linked (Yamashita et al., 2005). Second, key components of both poly(A) nuclease complexes are all found in P-bodies (Figs. 1 and 2), suggesting a direct role for them in P-body formation. Third, although PABP can enhance Pan2 nuclease activity (Boeck et al., 1996; Mangus et al., 2004; Uchida et al., 2004) and can interact with Pan3 (Fig. 4), PABP does not colocalize with P-bodies (Fig. S3; Kedersha et al., 2005), suggesting that the first phase of deadenylation, as well as that of mRNP remodeling involving dissociation of PABPs, occurs before appearance of an mRNP in P-bodies.

Because PABPs are not found in P-bodies and one major change in mRNPs after deadenylation is the loss of PABPs, one important implication of our observations is that PABPs play an inhibitory role and prevent mRNPs from joining existing P-bodies or nucleating P-body formation in mammalian cells. It is plausible that the 3′ poly(A) tail of an mRNA must first be shortened, not only to permit dissociation of PABPs or factors that are important for efficient translation of the mRNP but also to allow joining of translation repressors or other P-body components to the mRNP.

P-bodies play differential roles in mRNA turnover and are not required for all mRNA decay pathways in mammalian cells

A central issue concerns whether all mRNA decay requires P-bodies. Our results (Fig. 3) show that knocking down Pan3, a manipulation that has little effect on deadenylation but significantly reduces P-bodies, slows decay of BBB+PTC mRNA but not BBB+ARE or BBB mRNA, supporting a differential involvement of P-bodies in mammalian mRNA decay. These results are consistent with observations that several NMD-required factors can accumulate in P-bodies in yeast and *D. melanogaster* (Barbee et al., 2006; Sheth and Parker, 2006; Eulalio et al., 2007a) and that knocking down GW182 effectively abolishes P-bodies but has little effect on ARE-mediated decay in some human cells (Stoecklin et al., 2006). Thus, even though decay of ARE-containing mRNAs could involve or occur in P-bodies (Ferraiuolo et al., 2005; Franks and Lykke-Andersen, 2007), ARE-mediated decay does not always require P-bodies.

Our finding that reduction of P-bodies by Pan3 knockdown enhances the decay of BBB and BBB+ARE mRNAs (Fig. 3) suggests a possible role for P-bodies in preventing mRNAs from degradation. In this case, P-bodies provide a storage site for mRNAs before they are degraded or reenter translatable pool (for reviews see Eulalio et al., 2007b; Kedersha and Anderson, 2007; Parker and Sheth, 2007). On the other hand, for aberrant mRNAs, such as PTC-containing transcripts, sequestration in P-bodies may provide a rapid means to prevent accidental translation before degradation. Collectively, our results indicate that P-bodies play differential roles in mammalian mRNA turnover and are not required for all mRNA decay pathways in mammalian cells.

Pan3 and Caf1 have distinct roles in mammalian mRNA turnover

Mammalian deadenylation is mediated by a concerted action of Pan2–Pan3 and Ccr4–Caf1 poly(A) nuclease complexes

Figure 9. **A model linking deadenylation, P-bodies, and mRNA decay.** See Discussion for details.
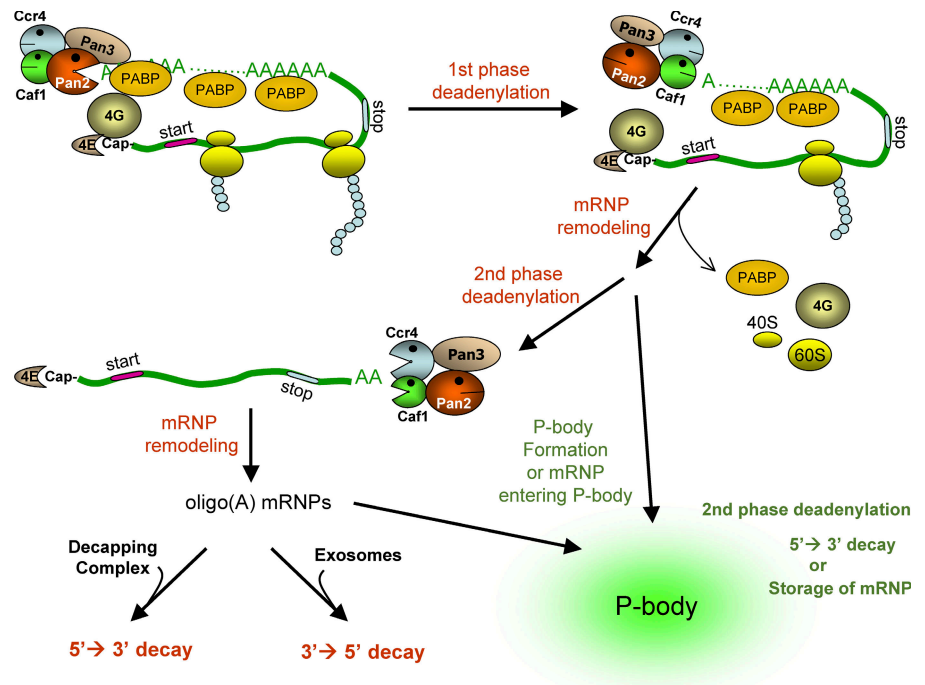
(Yamashita et al., 2005). Although knocking down Pan3 has little effect on the first phase of deadenylation (Fig. 3 B), Pan3 appears to play a role in modulating the activity of Pan2 or linking deadenylation to subsequent decay of the mRNA body. This notion is supported by the observations that ectopic overexpression of Pan2 alone results in highly rapid and processive poly(A) shortening of the otherwise stable BBB mRNA (Yamashita et al., 2005) and that the deadenylation and decay kinetics of a BBB transcript in cells coexpressing Pan2 and Pan3 are similar to those observed in cells not overexpressing these two factors (unpublished data). Moreover, we show in this paper that Pan3 knockdown enhances the decay of BBB and BBB+ARE mRNAs (Fig. 3 B, middle [right] and bottom [right]). Together with the newly discovered roles for Pan3 in P-body formation (Figs. 1, 2, and 3), our findings offer an explanation as to why this evolutionarily highly conserved protein is not required for deadenylation per se (Tucker et al., 2001; Temme et al., 2004; Molin and Puisieux, 2005).

Unlike Pan3, Caf1 is indispensable for mammalian deadenylation. Knocking down Caf1 or overexpressing a Caf1 dominant-negative mutant blocks deadenylation and stabilizes the transcripts (Figs. 5 and 6). Although Caf1 and Ccr4 each exhibit poly(A) nuclease activity, these two proteins associate with each other and function as a poly(A) nuclease complex in vivo (Tucker et al., 2002; Temme et al., 2004; Behm-Ansmant et al., 2006). Our data show that the poly(A) nuclease activities of Ccr4 and Caf1 in the Ccr4–Caf1 complex have complementary roles in mammalian deadenylation (Fig. 6 C). Overexpression of the Caf1 catalytically mutant alone or the Ccr4 mutant alone interferes with the normal deadenylation function of the Ccr4–Caf1 complex because these mutants have a dominant-negative effect that overrides the activity of endogenous deadenylase complexes. Overexpression of both HA-Ccr4 wild-type and HA-Caf1 mutant or overexpression of both HA-Caf1 wild-type and Ccr4

mutant promotes the formation of functional deadenylase complexes because the deadenylase activity of the wild-type protein can complement its partner in the same complex. In contrast, HA-Ccr4 mutant and HA-Caf1 mutant cannot form a functional deadenylase complex with each other, as both mutants are catalytically inactive. It is worth noting that although Caf1 knockdown severely impairs mRNA deadenylation and decay (Fig. 5), Ccr4 knockdown only modestly affects the second phase of deadenylation without significantly impairing overall mRNA decay (Yamashita et al., 2005). Therefore, the role of Caf1 in mammalian turnover is distinct from that of Ccr4 and is more significant than previously recognized.

A model linking deadenylation, P-bodies, and mRNA decay

Based on previous and the current findings, we envisage the following scenario for deadenylation, P-body formation, and differential mRNA decay in mammalian cells (Fig. 9). Pan2–Pan3 and Ccr4–Caf1 complexes first form a super complex on mRNAs in the cytoplasm. The 3′ poly(A) tail–PABP complex then stimulates poly(A) shortening by the Pan2–Pan3 in the super complex but inhibits the activity of Ccr4–Caf1 (Tucker et al., 2002), allowing the first phase of deadenylation to proceed. mRNP remodeling occurs during or after the first phase of deadenylation. Remodeling might involve dissociation of PABPs and some translation initiation factors and association of translation repressors (Coller and Parker, 2005; for reviews see Eulalio et al., 2007b; Parker and Sheth, 2007) to promote the transition of mRNPs to a nontranslatable state. A remodeled mRNP may associate with existing P-bodies or nucleate formation of new P-bodies, in which the second phase of deadenylation by Ccr4–Caf1 and/or decapping would proceed. For some mRNPs, such as a PTC-mRNP, this process may take place with the help of Pan3. Sequestration within P-bodies at this point may provide a

quick means of keeping aberrant mRNPs, such as PTC-mRNPs, from being translated before their poly(A) tail or cap is removed and the RNA body degraded. Alternatively, a remodeled mRNP may undergo the second phase of deadenylation outside of P-bodies, which could induce another mRNP remodeling that determines whether the oligo(A)-mRNP would be degraded inside or outside of P-bodies.

The observation that four component proteins of the two major poly(A) nuclease complexes can be found in mammalian P-bodies links all major mRNA decay factors except the 3′ exosome complex to P-bodies. The present results argue for a biological role for P-bodies in coupling deadenylation and mRNP remodeling to translation repression in an effective manner. One important conclusion from our findings in this study is that although P-bodies may be assembled via different mechanisms, deadenylation is always a necessary step that enables mRNPs to enter existing P-bodies or to initiate the formation of P-bodies. It will be interesting to see how knockdown of Pan3 or Caf1 or overexpression of the Caf1 dominant-negative mutant might impact different posttranscriptional mechanisms regulating mammalian mRNA expression, such as those mediated by miRNAs, whose functions were found to be linked to P-bodies (Jackson and Standart, 2007; Nilsen, 2007; Pillai et al., 2007; for reviews see Eulalio et al., 2007b; Parker and Sheth, 2007).

Materials and methods

Plasmids
To construct a plasmid encoding HA-tagged Pan3 or Caf1, a 2.2-kb Pan3L cDNA amplified by RT-PCR from human testis total RNA (Clontech Laboratories, Inc.) or an 855-bp fragment encoding Caf1 amplified from IMAGE clone 6207987 was inserted between the EcoRV and XhoI sites of pSRHi-sHA (gift from S. Ohno, Yokohama City University, Yokohama, Japan; Kashima et al., 2006). The Caf1 cDNA was inserted between the HindIII and XbaI sites of pcDNA6/V5-HisA (Invitrogen) to generate pcDNA6-Caf1-V5. A plasmid encoding catalytic inactive Caf1 mutant (D40A) was created using the QuikChange site-directed mutagenesis kit (Stratagene) with pSR-HA-Caf1 as the template. To construct pcDNA6-Pan2-V5, a 3.6-kb Pan2 cDNA was amplified from IMAGE clone 3357890 and inserted between the AflII and XbaI sites of pcDNA6/V5-HisA (Invitrogen). To generate myc-PABP, the coding region of PABP was amplified by PCR from GST-PABP (Chang et al., 2004) and inserted between the XmaI and XhoI sites of SRmyc (gift from S. Ohno). GFP-Dcp1a and GFP-Ccr4 (gifts from B. Seraphin, Centre de Génétique Moléculaire, Gif sur Yvette Cedex, France) were described previously (Eystathioy et al., 2003). Construction of all other plasmids used in this study has been described previously (Xu et al., 1998; Chen and Shyu, 1994, 2003; Loflin et al., 1999a; Chang et al., 2004; Yamashita et al., 2005).

Cell culture and transfection
Either Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche) was used for transient transfections. NIH3T3 B2A2 cells were split to a density of 2.6×10^6/10 cm dish 24 h before transfection in 100 ng/ml tetracycline. 40 µl Lipofectamine 2000 and 1.5 ml Opti-MEM were mixed well and incubated for 5 min at RT. DNA (1.3 µg of reporter plasmid and 1.3 µg of internal control plasmid, pSV–α-globin–GAPDH) and siRNA (4.86 µg of nonspecific siRNA or Pan3 siRNA, or 2.43 µg Caf1 siRNA plus 2.43 µg Pop2 siRNA [SMARTpool; Thermo Fisher Scientific]) were diluted into 1.5 ml Opti-MEM, added to the Lipofectamine 2000 mixture, and incubated at RT for 25 min. The final mixture was then added to the cells in a 10-cm dish and incubated at 37°C (5% CO_2). Cells were split 18 h later into 6-cm dishes (1.5×10^6/6 cm dish) and incubated at 37°C (8% CO_2) for 24 h. When using FuGENE 6, NIH3T3 B2A2 cells were split to a density of 0.65×10^6/6 cm dish 24 h before transfection in the presence of 50 ng/ml tetracycline. 6.9 µl FuGENE 6 was diluted in 100 µl DME and mixed with 2.3 µg

DNA containing 0.055 µg of reporter plasmid, 0.11 µg of internal control plasmid, and 2.13 µg of DNA encoding HA-tagged proteins. The mixture was incubated at RT for 25 min, added to the culture dish, and incubated at 37°C (8% CO_2) for 42 h. Time-course experiments using the Tet-off system for transcriptional pulsing were performed as described previously (Chen et al., 2007).

Preparation of RNA samples and Northern blot analysis
Isolation of cytoplasmic RNA, preparation of probes, and Northern blot analysis were conducted as described previously. In brief, at various time points after the transcription pulse driven by the Tet-off promoter of the reporter plasmid in transfected cells, cytoplasmic mRNA was isolated using the cytoplasmic lysis buffer RLN (50 mM Tris-HCl, pH 8, 140 mM NaCl, 1.5 mM $MgCl_2$ and 0.5% NP-40) and RNeasy Mini kit (QIAGEN). 15 µg RNAs were then separated using 1.4% formaldehyde agarose gel electrophoresis and then transferred to GeneScreen Hybridization Transfer membrane (PerkinElmer) and blotted with a ^{32}P-labeled gene-specific DNA probe using ULTRAhyb hybridization buffer (Ambion). Gene-specific DNA probes were prepared by the Rediprime II Random Prime Labeling System (GE Healthcare). The ^{32}P-labeled probes were produced by inclusion of α-[^{32}P]dCTP (>6000 Ci/mmol; PerkinElmer). RNase H treatment of cytoplasmic mRNA after annealing to Oligo dT (Invitrogen) was used to generate poly(A)$^-$ RNA as described previously (Shyu et al., 1991). All the time-course experiments were repeated at least twice.

Western blot analysis and immunoprecipitation
Cytoplasmic and nuclear lysates were prepared as described previously (Peng et al., 1998). Total cell lysates (5–40 µg) were resolved on a 7 or 10% SDS-polyacrylamide gel and analyzed using an ECL Western blotting kit (GE Healthcare). The PVDF blots were probed with specific antibodies as indicated in each figure and detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Membranes were incubated with one of the primary antibodies at the indicated dilution: HRP-conjugated monoclonal Anti-V5 antibody at 1:5,000 (Invitrogen); HRP-conjugated monoclonal anti-HA antibody at 1:1,000 (Roche); rat anti-HA monoclonal antibody at 1:4,000 (Roche); mouse anti–α-tubulin monoclonal antibody at 1:10,000 (Sigma-Aldrich); rabbit anti-Dcp1a serum at 1:4,000 (Bethyl Laboratories, Inc. or gift from S. Ohno); rabbit anti-Rck/p54 serum at 1:1,000 (Bethyl Laboratories, Inc.); mouse monoclonal antibody against GAPDH at 1:10,000 (Research Diagnostics, Inc.); and mouse antibody against lamin A/C at 1:1,000 (Santa Cruz Biotechnology, Inc.). To detect endogenous Caf1, Pan2, and Pan3 in mouse NIH3T3 or monkey COS7 cells, the corresponding polyclonal antibodies were generated in rabbits immunized with gene-specific peptides using the custom antibody service from Bethyl Laboratories, Inc. Affinity-purified anti-peptide antibodies were used at the following dilutions: rabbit anti-Pan3 peptide antibody at 1:2,000; rabbit anti-Caf1 peptide at 1:1,000; or rabbit anti-Pan2 peptide at 1:2,000. Rabbit anti-PABP antibody (gift from R. Lloyd, Baylor College of Medicine, Houston, TX) was used at a 1:4,000 dilution. HRP-conjugated donkey anti–rabbit IgG antibodies (1:4,000; GE Healthcare) or goat anti–mouse (1:5,000; Bio-Rad Laboratories) were used as secondary antibodies for detection with a chemiluminescence reagent (peroxide/luminol enhancer; Thermo Fisher Scientific).

For coimmunoprecipitation, COS7 cells expressing HA-Pan2, Ccr4-V5, HA-Pan3, or V5-Pan3 were lysed at 48 h after transfection by incubating for 10 min at 4°C in 600 µl of lysis buffer (20 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 1% NP-40, 1 mM Na orthovanadate, 1 mM Na pyrophosphate, and 1 mM NaF supplemented with a protease inhibitor cocktail [Roche]). 50 µl of cell lysate was preserved as input, and the remainder was incubated with a monoclonal anti–V5-agarose conjugate (Sigma-Aldrich) or a rat monoclonal anti-HA Affinity Matrix (Roche) in the presence of 0.1 µg/ml RNase A. Immunoprecipitations were performed at 4°C for 4 h. The beads were washed five times with the lysis buffer. Coprecipitated proteins were detected by Western blotting using the indicated antibodies. The protein samples were separated by 7–10% SDS-PAGE and analyzed by Western blot analysis as described in the previous paragraph.

Immunofluorescence microscopy
NIH3T3 cells were seeded in 6-well plates at a density of 0.4×10^6 cells per well, 24 h before transfection using Lipofectamine 2000 (Invitrogen). At 22–26 h after transfection, cells were reseeded to slide chambers (BD Biosciences) and incubated overnight. For cycloheximide, puromycin, or arsenite treatment, cells were incubated in media containing either

7.5 µg/ml cycloheximide (Sigma-Aldrich) for 2 h, 100 µg/ml puromycin (Sigma-Aldrich) for 1 h, or 0.3 mM sodium arsenite (Sigma-Aldrich) for 1 h before fixation. Cells in the slide chambers were fixed for 10 min each with 3.7% (wt/vol) PFA (Sigma-Aldrich) in PBS and then with cold methanol, followed by a 10-min incubation in 0.2% (vol/vol) Triton X-100 in PBS. Each microgram of Zenon-labeled antibodies (see third paragraph in this section) was diluted in 500 µl of 1% BSA in PBS and incubated with the fixed slides. After incubating for 1 h at RT and washing, the slides were fixed again in 3.7% PFA. For indirect immunofluorescence microscopy, all of the primary and secondary antibodies, except the mouse anti-myc serum, were diluted 1:1,000 with 1% (wt/vol) BSA in PBS. Endogenous Pan2 was detected using rabbit anti-Pan2 labeled with Zenon 555 rabbit IgG labeling reagent. Endogenous Dcp1a, Xrn1, or Pan3 was detected using rabbit anti-Dcp1a, anti-Xrn1, or anti-Pan3 labeled with Zenon 488 rabbit IgG labeling reagent. HA-tagged Pan3, Ccr4, or Caf1 was detected using rat anti-HA monoclonal antibody and Alexa Fluor 350 goat anti–rat IgG. HA-Pan2 or V5-tagged proteins were detected using rat monoclonal anti-HA or mouse monoclonal anti-V5 and Alexa Flour 555 goat anti–rat IgG. Rabbit anti-Xrn1 antibody was a gift from J. Lykke-Andersen (University of Colorado at Boulder, Boulder, CO), human anti-GW182 antiserum was a gift from M.J. Fritzler (University of Calgary, Calgary, Canada), rabbit anti-G3BP1 was a gift from R. Lloyd, and rabbit anti-Dcp1a antibody was a gift from S. Ohno. The mouse anti-myc monoclonal antibody from culture medium collected from anti-myc monoclonal antibody secreting hybridoma cells (American Type Culture Collection) was used at 1:50 dilution. After incubation with the indicated primary antibodies/sera, cells were washed three times in PBS for 5 min and incubated with fluorescently labeled secondary antibodies (Invitrogen) as indicated in the figures. Fluorescence mounting medium with or without DAPI was added.

Images were obtained at RT by optical z-sectioning (20 sections in total, 0.2-µm-space between sections) using an objective lens (100×/1.35 NA; Olympus) of a deconvolution microscope system (DeltaVision) containing an inverted microscope (IX70; Olympus). Immersion oil (n = 1.514) was from Applied Precision, LLC. Images were captured using a digital camera (Coolsnap HQ; Roper Scientific). Stacks of 20 images were projected as a single 2D picture using softWoRx Explorer (version 3.3.6; Applied Precision, LLC). The RGB colors of the resulting pictures were separated using Photoshop CS (Adobe).

When two different primary antibodies raised in rabbits were used together to visualize P-bodies, the following procedure was used. One µg of rabbit antibody was incubated for 5 min at RT with 5 µl Zenon rabbit IgG labeling reagent, either Alexa Fluor 488- or 555-labeled Fab fragment (Invitrogen). The labeled Fab fragment was bound to the Fc portion of the rabbit IgG, and excess Fab fragment was neutralized by the addition of 5 µl of nonspecific rabbit IgG, which prevented cross-labeling of the Fab fragment when two rabbit antibodies were used. After a 5-min incubation at RT, the labeled antibodies were used for staining in combination with each other or with labeled secondary antibodies specific for other species. To analyze the changes in P-body number and size after Pan3 knockdown, 87 cells transfected with control nonspecific siRNA and 91 cells transfected with Pan3 siRNA were analyzed using ImageJ software (pixel range for particle brightness between 40 and 215; National Institutes of Health). Dcp1a particles with values >50 square pixels were considered above background staining and were selected for P-body analysis.

Online supplemental material

Fig. S1 shows detection of P-bodies in NIH3T3 cells. Fig. S2 demonstrates that HA-Pan3 foci are P-bodies and not stress granules. Fig. S3 shows that Pan3 does not help PABP localize to P-bodies. Fig. S4 shows that overexpression of catalytically inactive mutant Caf1 blocks P-body formation in monkey COS7 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1.

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