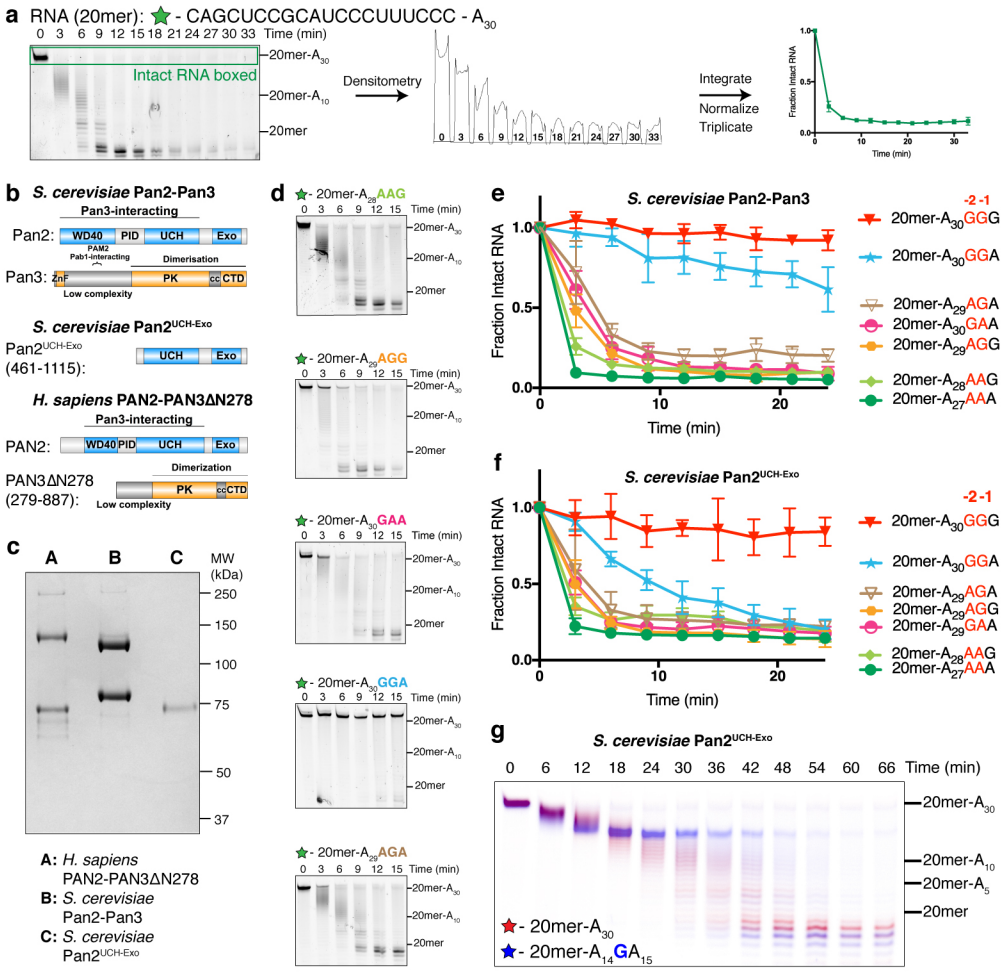
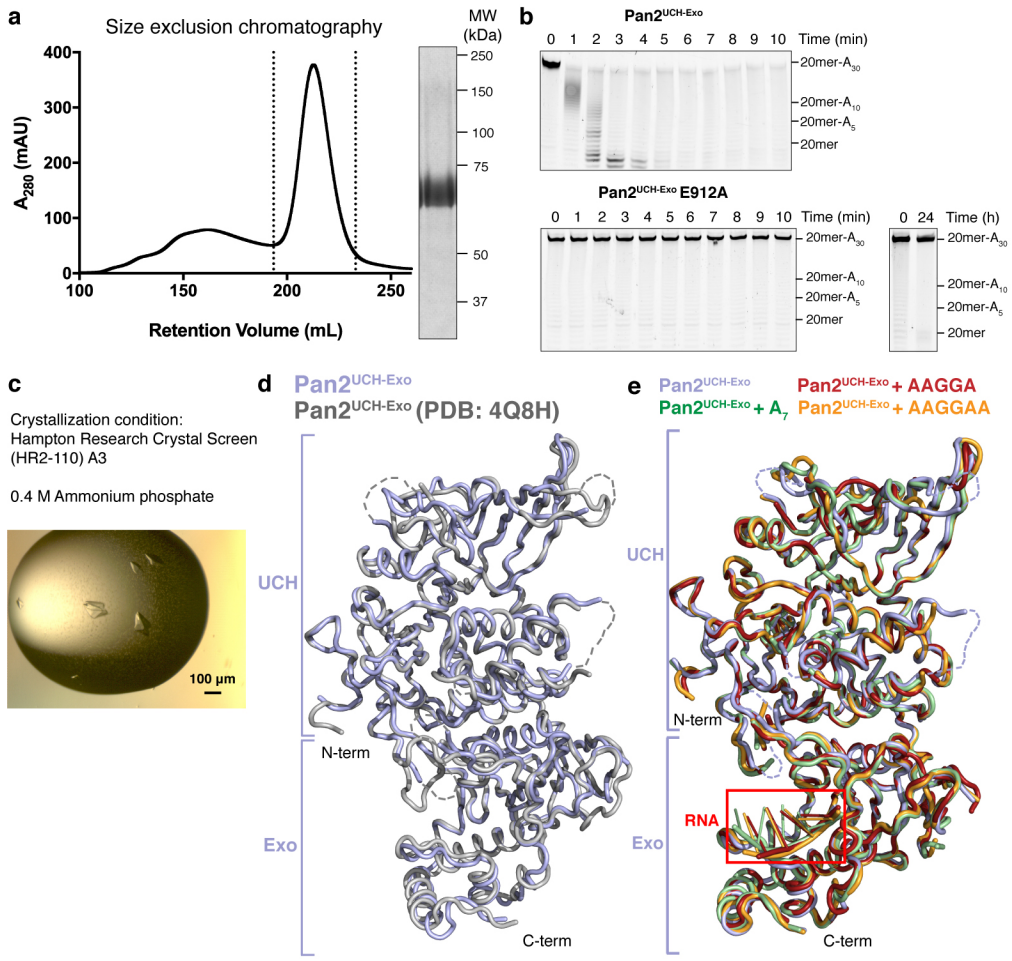


Supplementary Figure 1

Inhibition of Pan2 by 3′ guanosines depends on their number and position.

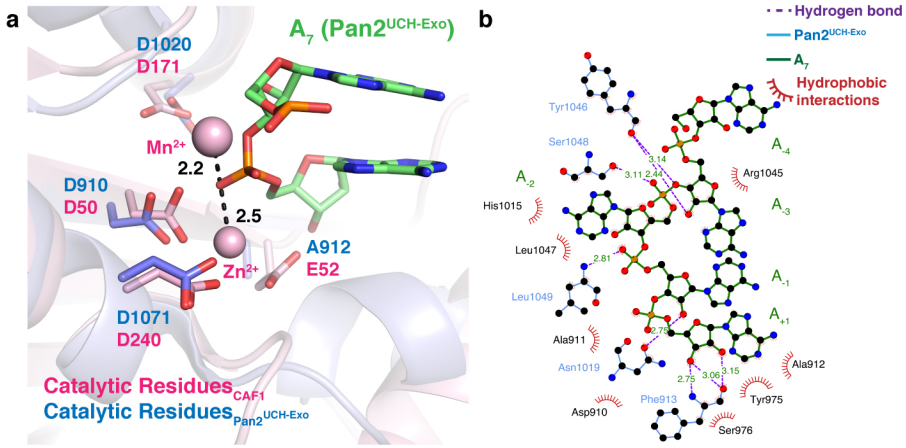
a, Method for quantitation of deadenylation assays. 5′ 6-FAM-labeled (green star) poly(A) RNA substrates were incubated with the deadenylase. Reactions were quenched at fixed time intervals and products were visualized on a denaturing 20% polyacrylamide gel. The intensity of the band corresponding to intact RNA was quantified by densitometry. Intensities were normalized to the signal at time = 0 and plotted against time. Straight lines connect each point for clarity. Assays were carried out in triplicate and error bars represent standard deviation. **b**, Domain diagrams of proteins or protein complexes used in deadenylation assays. The pseudo-ubiquitin C-terminal hydrolase (UCH) and exonuclease (Exo) domains of Pan2 form a single structural unit[12,13]. PID, Pan3-interacting domain; ZnF, zinc finger; PK, pseudokinase; CC, coiled coil; CTD, C-terminal domain. **c**, Coomassie-stained SDS-PAGE of purified protein components used in deadenylation assays. The experiment for this gel has been carried out once. **d**, Denaturing RNA gels showing deadenylation by recombinant *S. cerevisiae* Pan2-Pan3 on RNA substrates containing guanosines in different positions and in different numbers at the 3′ end following the 30-adenosine poly(A) tail. These gels are representative of identical experiments performed 3 times. **e-f**, Analysis of deadenylation by *S. cerevisiae* Pan2-Pan3 (**e**) and Pan2$^{UCH-Exo}$ (**f**) on RNA substrates containing guanosines in different positions and in different numbers at the 3′ end following the poly(A) tail. Reactions were performed in triplicate (n = 3 independent experiments), the data points shown represent the mean, and error bars represent standard deviation. **g,** Two-color deadenylation assay by 50 nM recombinant *S. cerevisiae* Pan2$^{UCH-Exo}$ on 100 nM RNA substrates, which are differentially labeled on the 5′ end (Alexa-647-20mer-A_{30}; 6-FAM-20mer-$A_{14}GA_{15}$). The experiment for this gel has been carried out once.



Supplementary Figure 2

Crystallization of Pan2$^{UCH-Exo}$ E912A in complex with RNA.

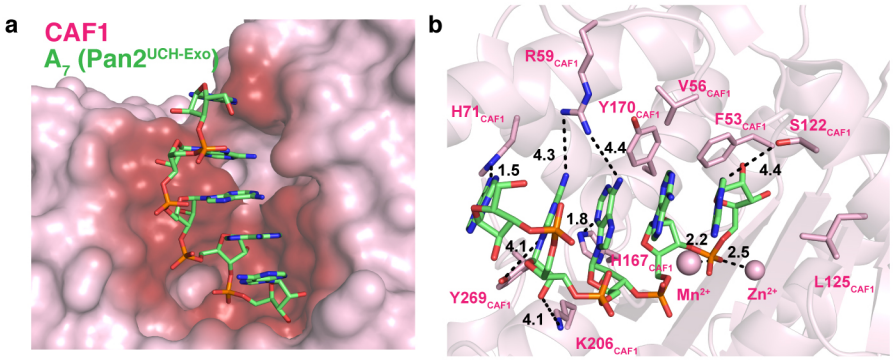
a, Size exclusion chromatography of purified Pan2$^{UCH-Exo}$ E912A. The fractions outlined by dotted lines on the chromatogram (left) were pooled, concentrated, and used in crystallization. A Coomassie-stained gel of Pan2$^{UCH-Exo}$ E912A used in crystallization is shown (right). The purification and SDS-PAGE analysis of fractions thereof are representative of experiments which have been carried out 6 times. **b**, Deadenylation assay comparing the activity of Pan2$^{UCH-Exo}$ to the catalytic mutant Pan2$^{UCH-Exo}$ E912A. The 5′ 6-FAM-labeled RNA substrate consists of a 20mer non-poly(A) sequence followed by a poly(A) stretch of 30 adenosines. Deadenylation reactions were quenched at one-minute intervals and reaction products were visualized on a denaturing 20% polyacrylamide gel. For Pan2$^{UCH-Exo}$ E912A, the reaction was incubated over 24 h to demonstrate the lack of activity over the timescale of RNA soaking. These gels are representative of identical experiments performed 2 times. **c**, Crystals of Pan2$^{UCH-Exo}$ E912A. Crystals were obtained by an initial sparse matrix crystallization screen. The best diffracting condition was further subjected to optimization. **d**, Superposition of apo Pan2$^{UCH-Exo}$ E912A (blue) with the previously determined structure of apo Pan2$^{UCH-Exo}$ (grey, PDB: 4Q8H[13]). **e**, Superposition of apo Pan2$^{UCH-Exo}$ E912A (blue) with crystal structures containing bound RNA, including Pan2$^{UCH-Exo}$ E912A-A$_7$ (green), Pan2$^{UCH-Exo}$ E912A-AAGGA (red), and Pan2$^{UCH-Exo}$ E912A-AAGGAA (orange). Superposition in (**d**) and (**f**) was carried out by the SSM tool in Coot[55]. Unmodeled loops are represented by dashed lines.



Supplementary Figure 3

Interactions of Pan2$^{UCH-Exo}$ E912A with RNA.

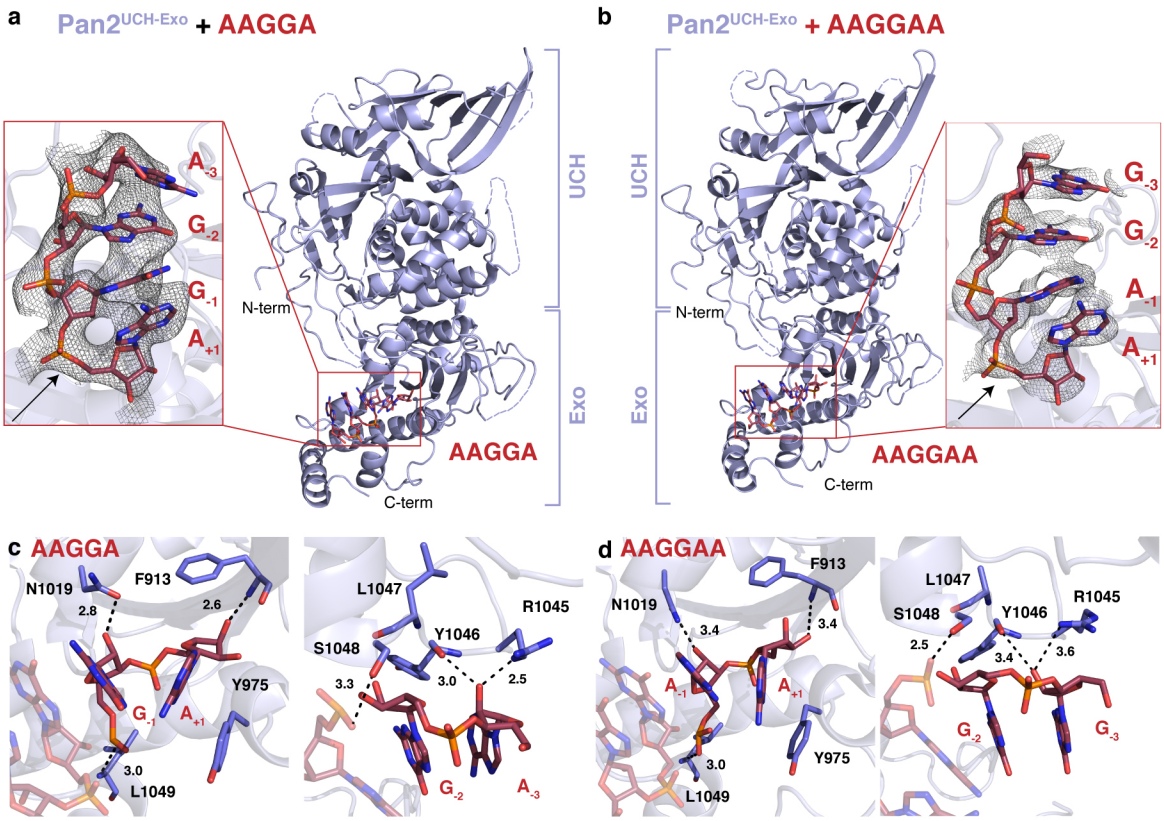
a, Superposition of Pan2$^{UCH-Exo}$-A_7 (protein: blue, RNA: green) with *H. sapiens* CAF1 (PDB: 3G0Z[20]; protein: pink). The structure of CAF1 contains two M^{2+} ions (pink) in the active site, which are shown as spheres. The numbers shown represent distance in Ångstroms. Relative to the scissile phosphate, the metal ions are approximately positioned for productive catalysis. **b**, Summary of interactions between Pan2$^{UCH-Exo}$ (blue) and A_7 RNA (green), visualized using LigPlot+[62].



Supplementary Figure 4

Docking of oligo(A) into CAF1 active site.

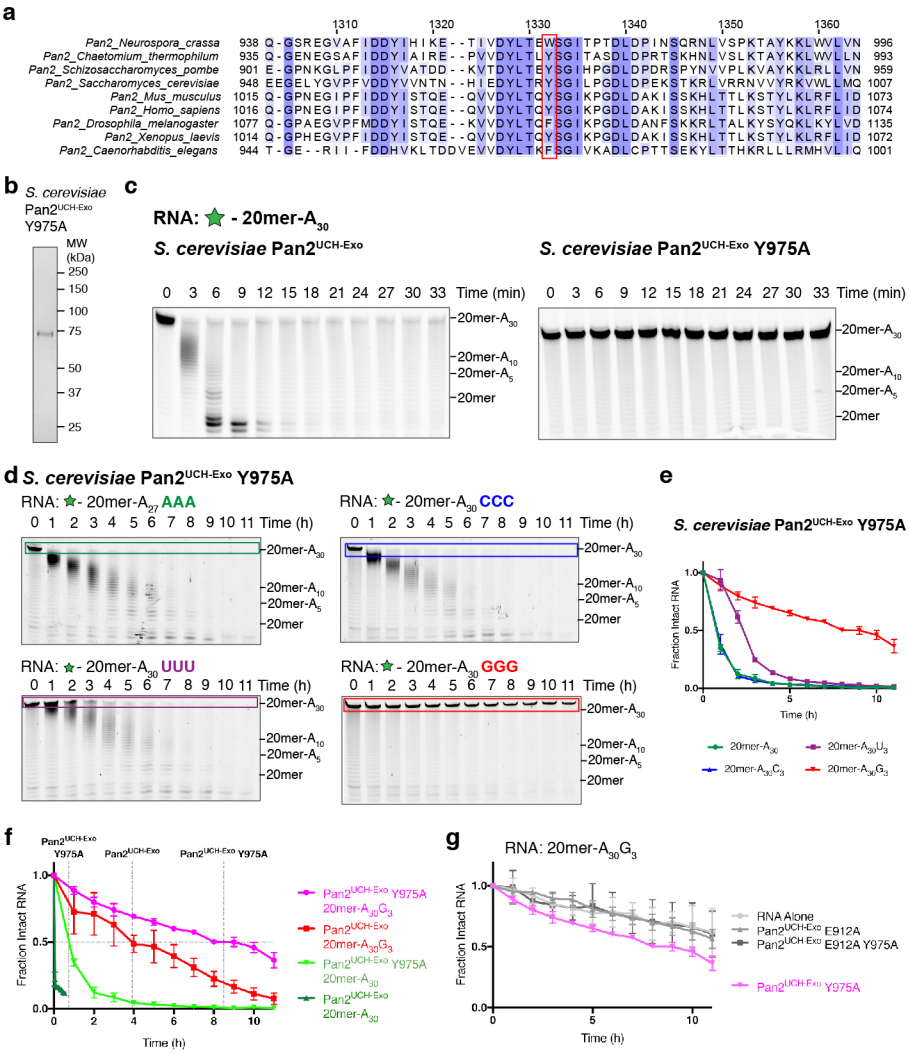
a, Surface representation of the CAF1 (PDB: 3G0Z[20], pink) active site with a modelled poly(A) substrate (green). The RNA was modelled by superposition of Pan2$^{UCH-Exo}$-A_7 with the structurally homologous CAF1 by Coot[55]. CAF1 is colored by proximity to A_7 from dark red (<3 Å) to pink (>7 Å). The CAF1 active site appears to contact both the ribophosphate backbone and adenine bases. **b**, CAF1 (pink) amino acid side chains which may contact the poly(A) substrate (green) in the docked model. Putative hydrogen bonds are indicated by dashed lines and interatomic distances are labeled in Ångstroms. The metal ions are shown as pink spheres. RNA placement was not refined and is therefore in a representative but likely non-optimal position.



Supplementary Figure 5

Guanosines disrupt the conformation of oligo(A) within the Pan2 active site.

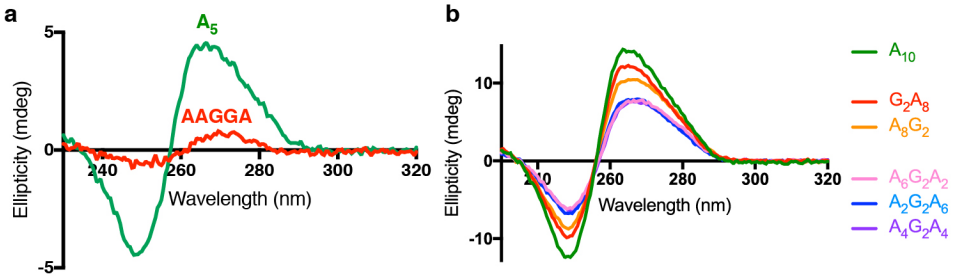
a-b, Crystal structures of Pan2$^{UCH-Exo}$ E912A (cartoon, blue) bound to AAGGA (**a**) or AAGGAA (**b**), where the RNA is represented by red sticks. Unmodeled loops are represented by dotted lines. Inset: detailed views of the Pan2 active site. The electron density represents a feature enhanced map (FEM)[56] contoured to 1.8σ. Nucleotides are numbered relative to the scissile phosphate bond, which is indicated by an arrow. **c-d**, Protein-RNA interactions between Pan2$^{UCH-Exo}$ E912A (blue) and AAGGA (**c**) or AAGGAA (**d**) (red). Hydrogen bonds are indicated by dashed lines and interatomic distances are labeled in Ångstroms.



Supplementary Figure 6

An aromatic residue at the base of the Pan2 active site is important for activity and specificity against guanosines.

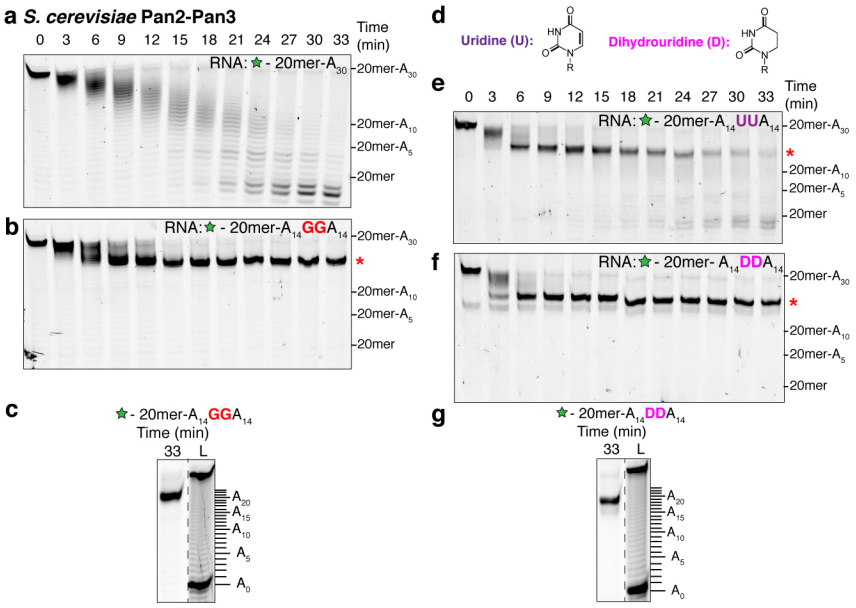
a, Pan2 sequence alignment from representative eukaryotes. Sequences were aligned using the T-Coffee package[59] and visualized using JalView[60]. Amino acids were colored by conservation using BLOSUM62. The conserved aromatic residue stacking against the RNA substrate is boxed in red. **b**, Coomassie-stained SDS-PAGE of Pan2$^{UCH\text{-}Exo}$ Y975A used in deadenylation assays. The experiment for this gel has been carried out once. **c**, Comparison of deadenylation by Pan2$^{UCH\text{-}Exo}$ with Pan2$^{UCH\text{-}Exo}$ Y975A on a 5′ 6-FAM-labeled (green star) RNA consisting of a 20mer non-poly(A) sequence followed by a poly(A) tail of 30 adenosines (20mer-A_{30}). The Y975A mutant is much less active compared to the wild type. These gels are representative of identical experiments performed 2 times. **d**, Deadenylation by Pan2$^{UCH\text{-}Exo}$ on 5′ 6-FAM-labeled (green star) 20mer-A_{30} RNAs with three additional non-A nucleotides at the 3′ end where indicated. These gels are representative of identical experiments performed 3 times. **e**, Quantitation of deadenylation by Pan2$^{UCH\text{-}Exo}$ Y975A on poly(A) RNA substrates with different 3′ nucleotides. **f**, Comparison of deadenylation by Pan2$^{UCH\text{-}Exo}$ and Pan2$^{UCH\text{-}Exo}$ Y975A on 20mer-A_{30} and 20mer-$A_{30}G_3$. The horizontal dotted line indicates when half of RNA has been deadenylated, and the vertical dotted lines indicate the time at which half of RNA has been deadenylated for each condition. Terminal guanosines inhibit Pan2$^{UCH\text{-}Exo}$ Y975A ~11-fold (8.5 h for 20mer-$A_{30}G_3$ compared to 0.8 h for 20mer-A_{30}), compared to a ~117-fold inhibition (3.9 h for 20mer-$A_{30}G_3$ compared to ~2 min for 20mer-A_{30}) of Pan2$^{UCH\text{-}Exo}$. **g**, Comparison of deadenylation by Pan2$^{UCH\text{-}Exo}$ Y975A and the catalytic mutants Pan2$^{UCH\text{-}Exo}$ E912A and Pan2$^{UCH\text{-}Exo}$ E912A Y975A, as well as RNA incubated alone. The negative control demonstrates that Pan2$^{UCH\text{-}Exo}$ Y975A retains deadenylase activity greater than background RNA degradation. Thus, the aromatic residue against which the substrate π-stacks is important for the activity of Pan2 and contributes to nucleotide specificity. For panels e-g, the experiments were carried out in triplicate (n = 3 independent experiments), the data points shown represent the mean, and the error bars represent standard deviation.



Supplementary Figure 7

The structure of oligo(A) is disrupted by guanosines in both 5′ and 3′ directions.

a, Circular dichroism spectra of 9.0 μM A_5 (green) and AAGGA RNAs as averages from 9 scans of the same sample obtained at 0.5 nm intervals. These spectra are representative of identical experiments performed 2 times. **b**, Circular dichroism spectra of G_2A_8 (red), A_8G_2 (orange), $A_6G_2A_2$ (pink), $A_2G_2A_6$ (blue), and $A_4G_2A_4$ (purple) RNAs, compared to A_{10} RNA (green). The RNAs are present at 25.0 μM. Each curve represents an average from 9 scans of the same sample obtained at 0.5 nm intervals. These spectra are representative of identical experiments performed 2 times.



Supplementary Figure 8

Poly(A) stacking is important for deadenylation by Pan2-Pan3.

a-b, Deadenylation by *S. cerevisiae* Pan2-Pan3 of 5′ 6-FAM-labeled (green star) RNAs consisting of a 20mer non-poly(A) sequence followed by a tail of the indicated sequence. RNAs either had no additional nucleotides (**a**) or guanosines (**b**) in the middle of the poly(A) tail. Red asterisks indicate the point of inhibition. **c,** Determination of site of inhibition of *S. cerevisiae* Pan2-Pan3. Deadenylation reactions of 5′ 6-FAM-labeled (green star) 20mer-$A_{14}GGA_{14}$ by *S. cerevisiae* Pan2-Pan3 were repeated and the point of inhibition determined by comparing the reaction at 33 minutes to an alkaline hydrolyzed RNA marker. Inhibition began when Pan2-Pan3 reached a 3′ sequence of –GGAAAA. Individual bases of the marker are indicated. The dashed line separates lanes which have different contrast adjustments to visualize the fainter bands in the marker lane. This gel is representative of identical experiments performed 2 times. **d,** The chemical structures of uridine and dihydrouridine. **e-f,** Deadenylation by *S. cerevisiae* Pan2-Pan3 of 5′ 6-FAM-labeled (green star) RNAs consisting of a 20mer non-poly(A) sequence followed by a tail of the indicated sequence. RNAs either had two uracils (**e**), or dihydrouracils (abbreviated D, panel **f**) in the middle of the poly(A) tail. Red asterisks indicate the point of inhibition. These gels are representative of identical experiments performed 2 times. **g,** Determination of site of inhibition of *S. cerevisiae* Pan2-Pan3. Deadenylation reactions of 5′ 6-FAM-labeled (green star) 20mer-$A_{14}DDA_{14}$ by *S. cerevisiae* Pan2-Pan3 were repeated and the point of inhibition determined by comparing the reaction at 33 minutes to an alkaline hydrolyzed RNA marker. Inhibition began when Pan2-Pan3 reached a 3′ sequence of –DDAAAA. Individual bases of the marker are indicated. The dashed line separates lanes which have different contrast adjustments to visualize the fainter bands in the marker lane. This gel is representative of identical experiments performed 2 times.