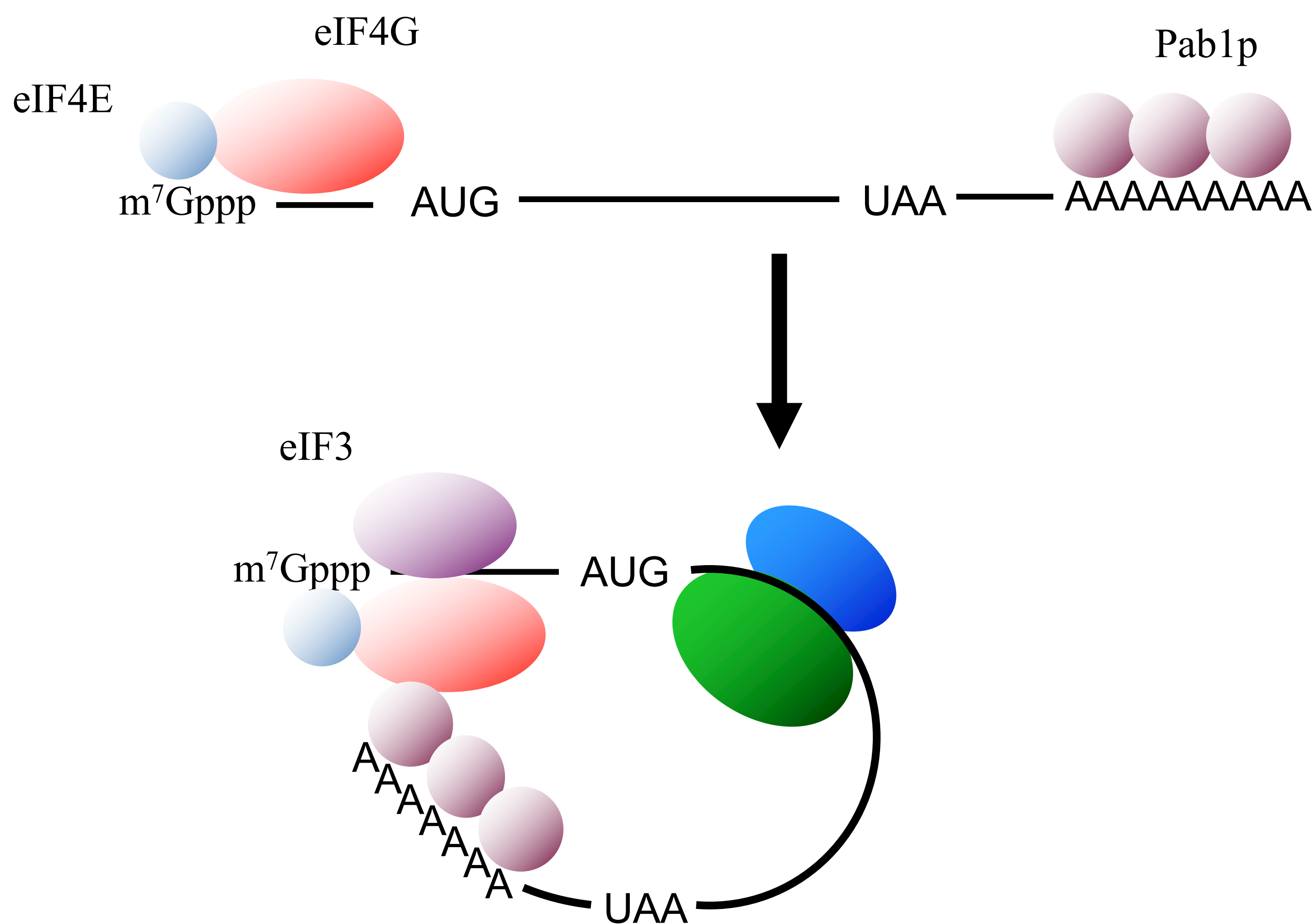
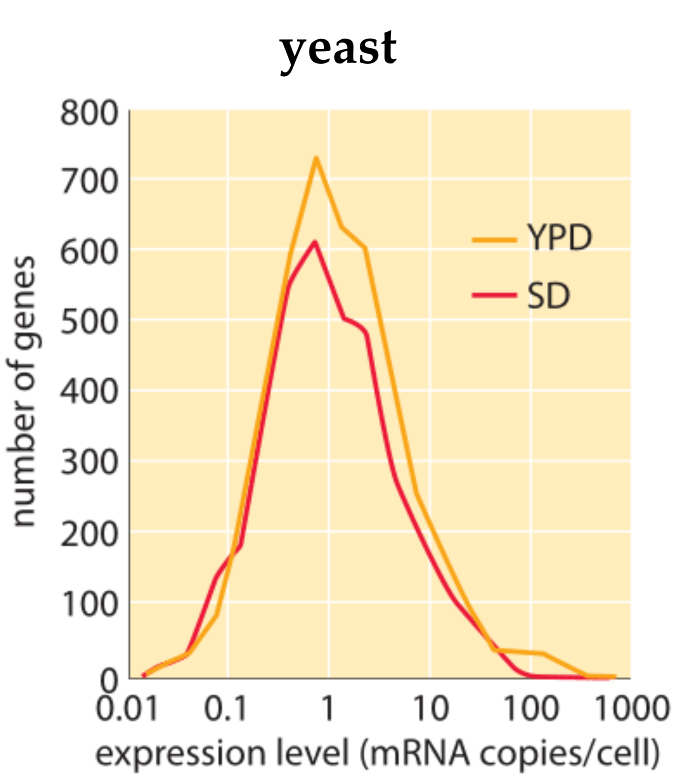
mRNA decay

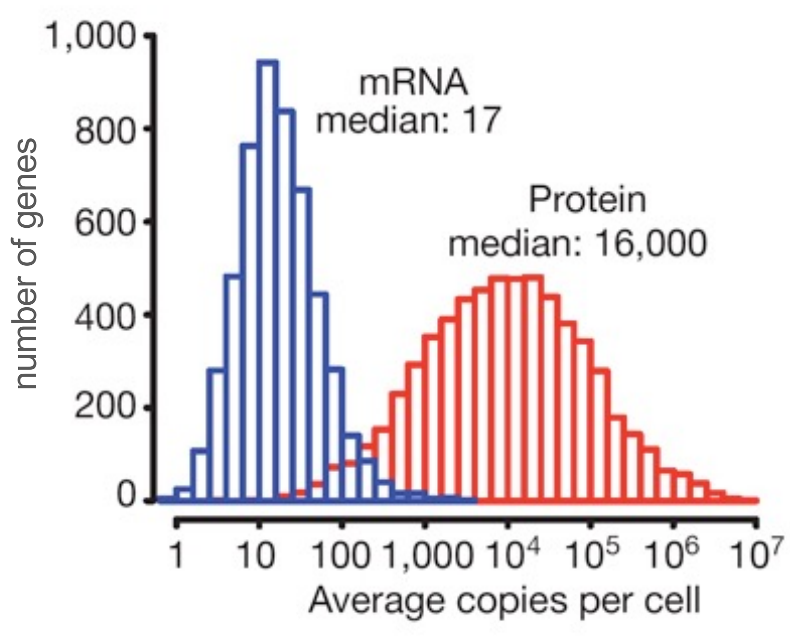
mRNA



mRNA levels in eukaryotic cells



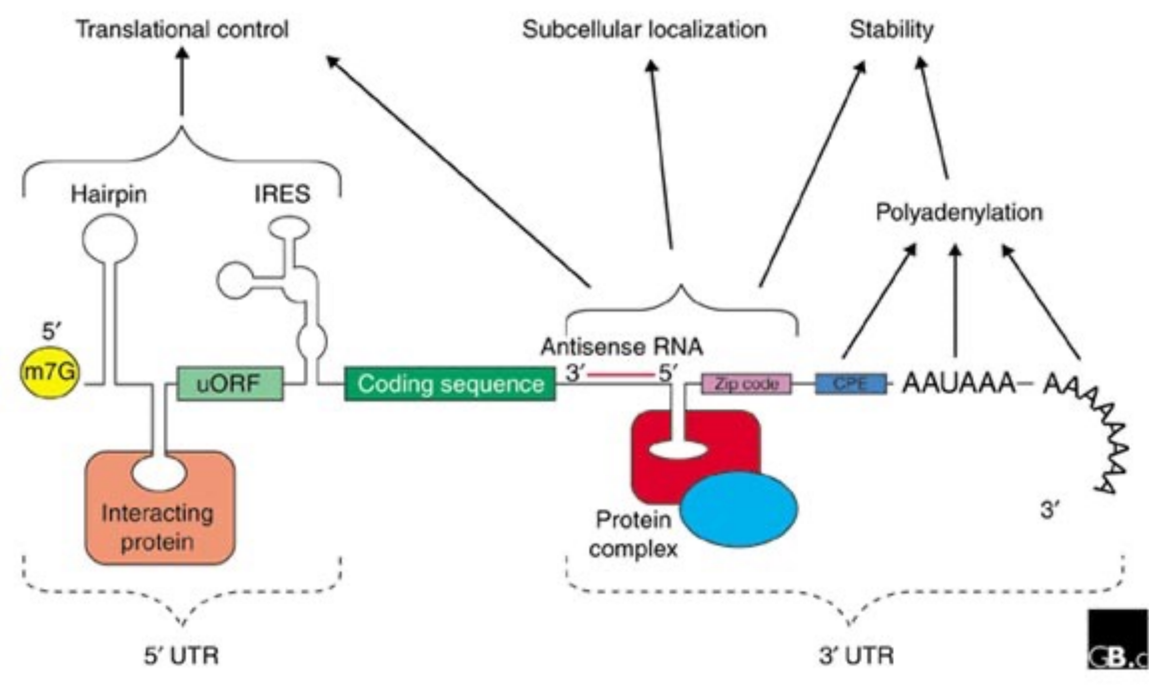
Marguerat et al., 2012 Cell



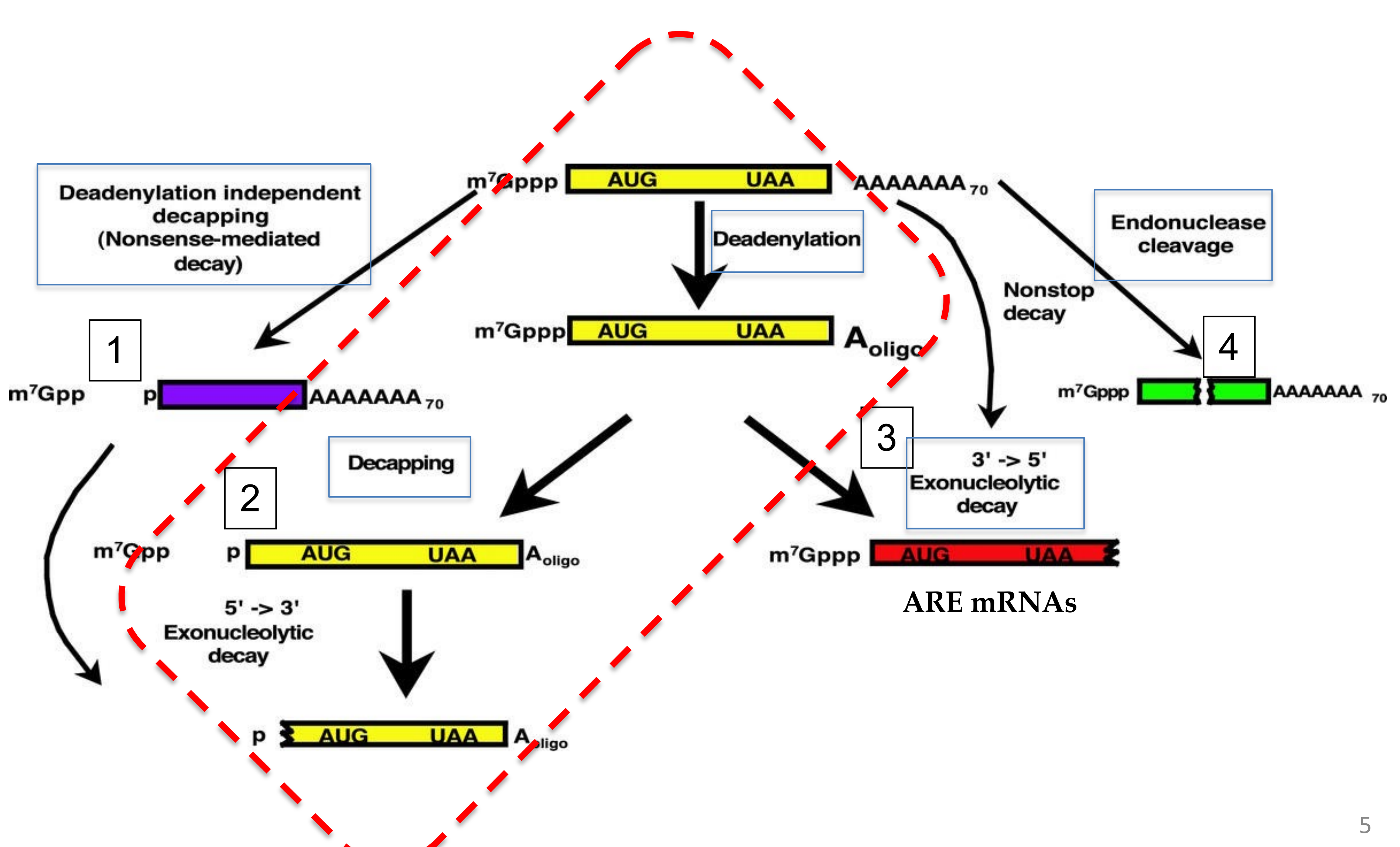
Schwanhäusser et al., 2011 Nature

Regulatory elements of mRNA

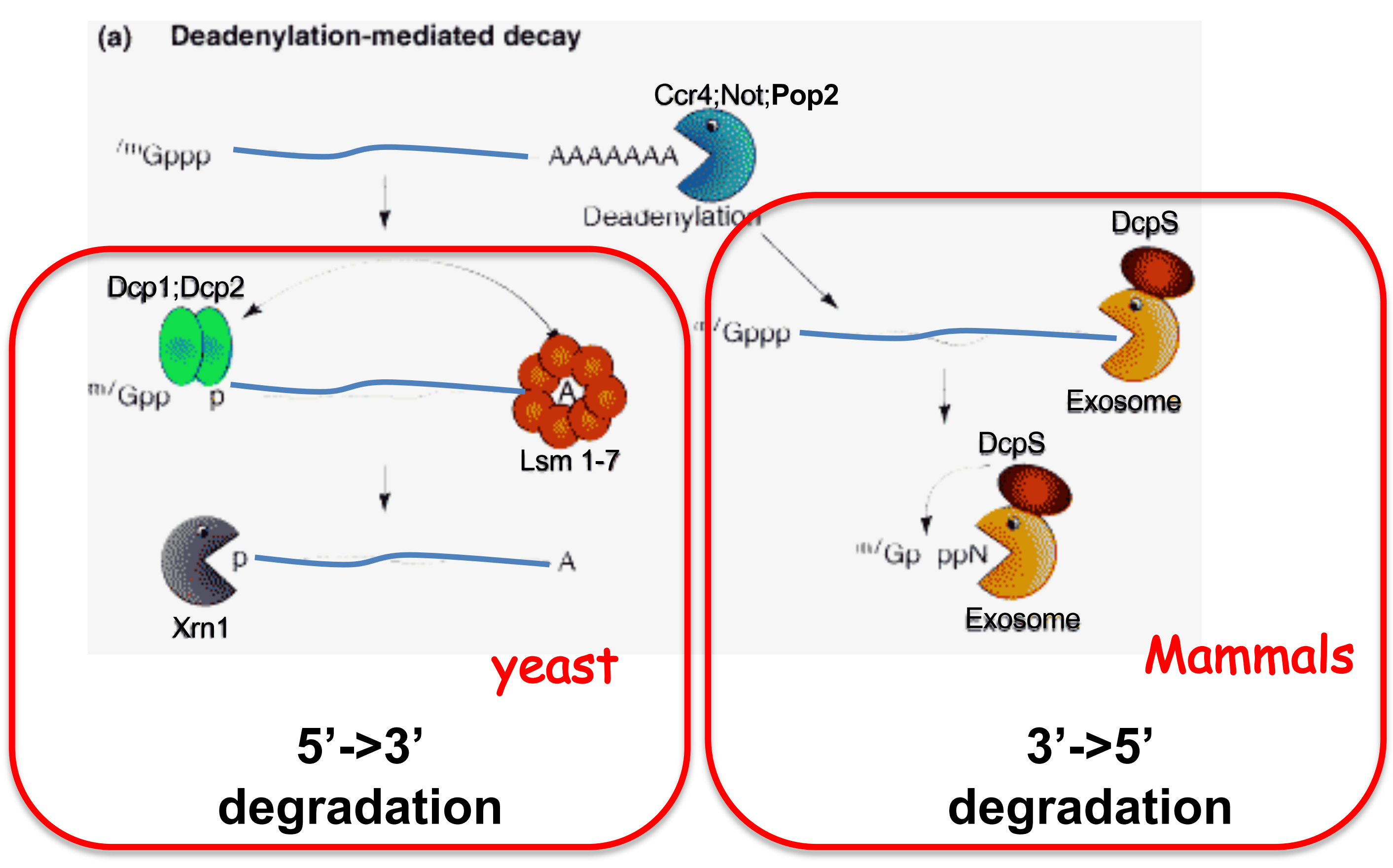
mRNAs contain several regulatory elements recognized by specific regulatory factors: **proteins** and **RNAs,** which allow fine-tuned regulation of gene expression.



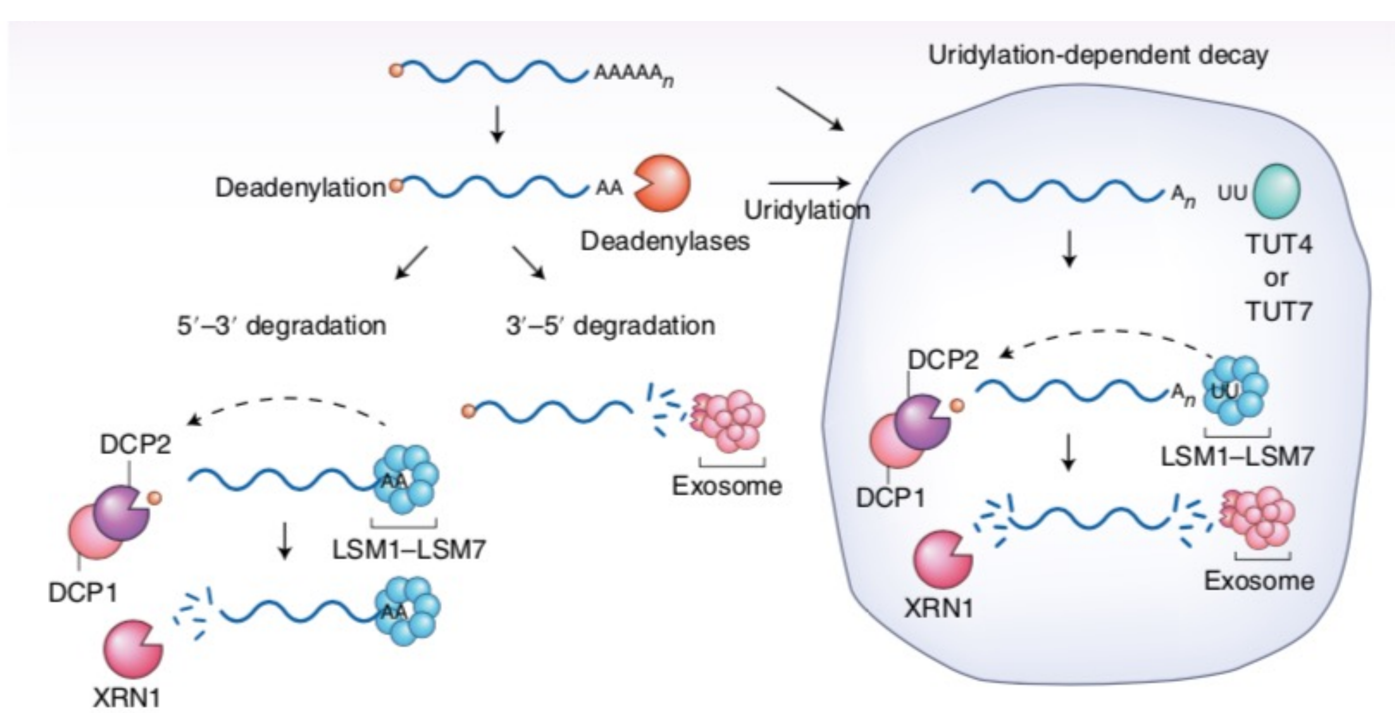
Pathways by which eukaryotic mRNAs are degraded



Different mRNA decays operate in yeast and mammals



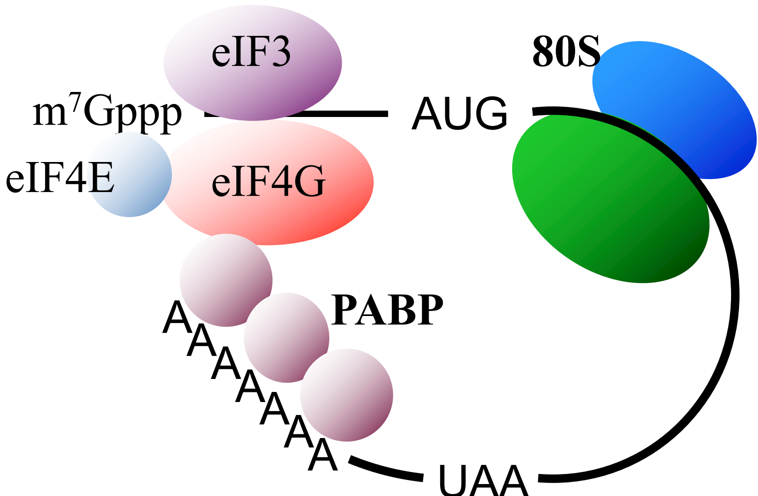
Degradation of polyadenylated mRNA in mammals



mRNA Deadenylation

The 3′ poly(A) tail

The **PAP mediated-3' poly(A) tail** is thought to be essential for the biological function of all eukaryotic mRNAs, with the exception of replication-dependent histone mRNAs in metazoans. The functionality of poly(A) tails is mediated by **PABPs**. They participate in many key aspects of mRNA biology, including export from the nucleus, protecting the otherwise vulnerable 3′ end of the mRNA from exonucleolytic degradation and promoting translation and mRNA circularization through interaction with the translation initiation factor **eIF4G.**

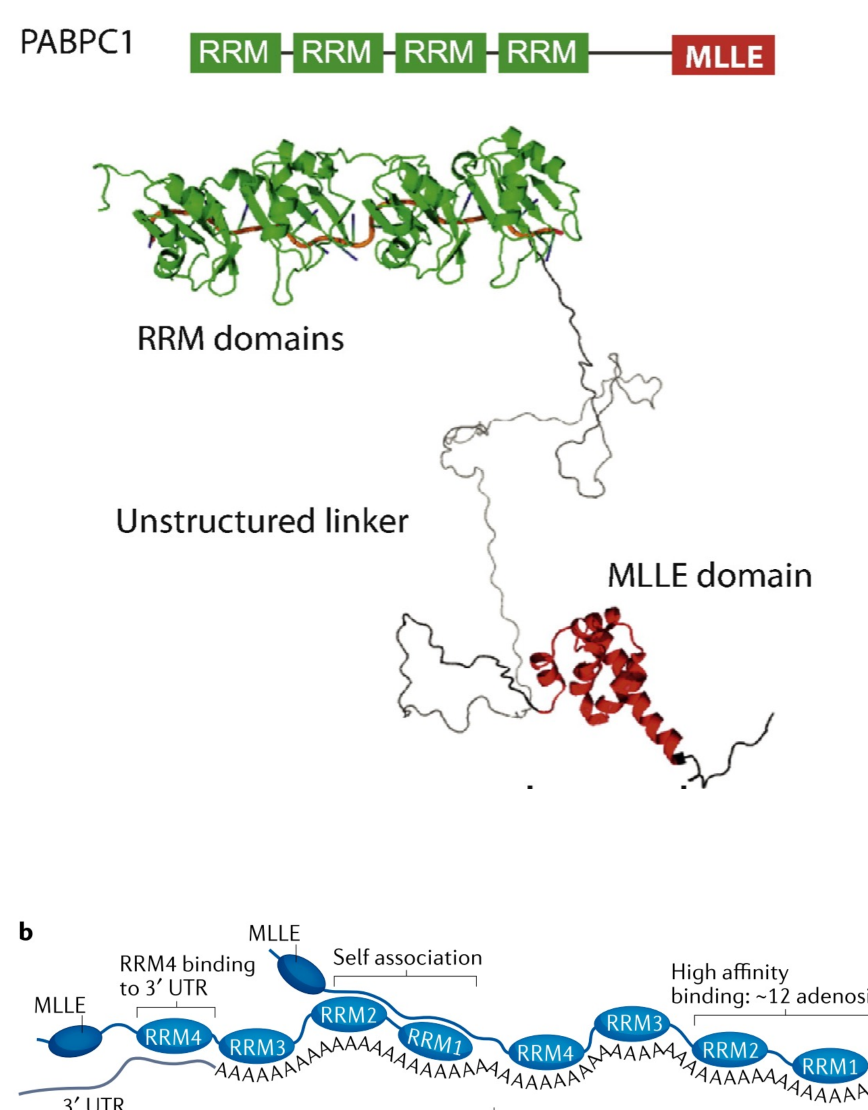


Polyadenylation binding protein (PABPC)

There is one PABC in yeast (Pab1) but multiple isoforms in mammals: **PABPC1** is the best studied mammalian isoform and likely the most abundant in most cell types. PABPC is highly conserved in eukaryotes and has four N-terminal RNA recognition motif (RRM) domains, which bind poly(A) RNA with a nanomolar affinity.

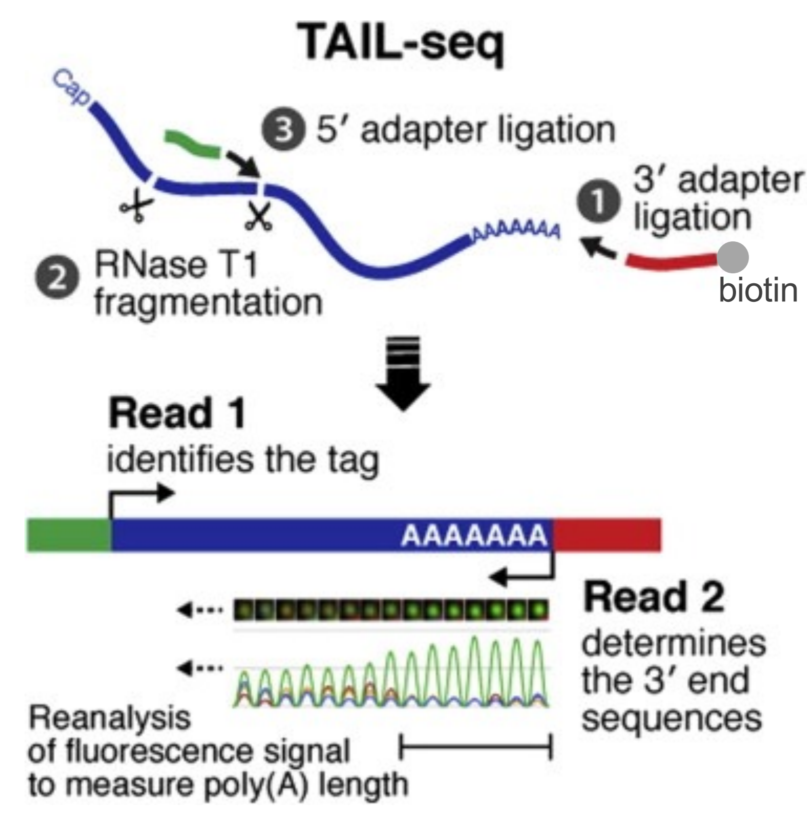
The RRMs are followed by a proline rich linker and a **C-terminal mademoiselle (MLLE)** domain. The MLLE domain recognizes a peptide motif called **poly(A) interacting motif 2 (PAM2)**, which is found in a number of PABPC partner proteins that regulate poly(A) tail dynamics.

PABPC requires about 12 adenosines for high affinity binding but physically covers about 25-30 nucleotides



TAIL-seq: Genome-wide Determination of Poly(A) Tail Length and 3′ End Modifications

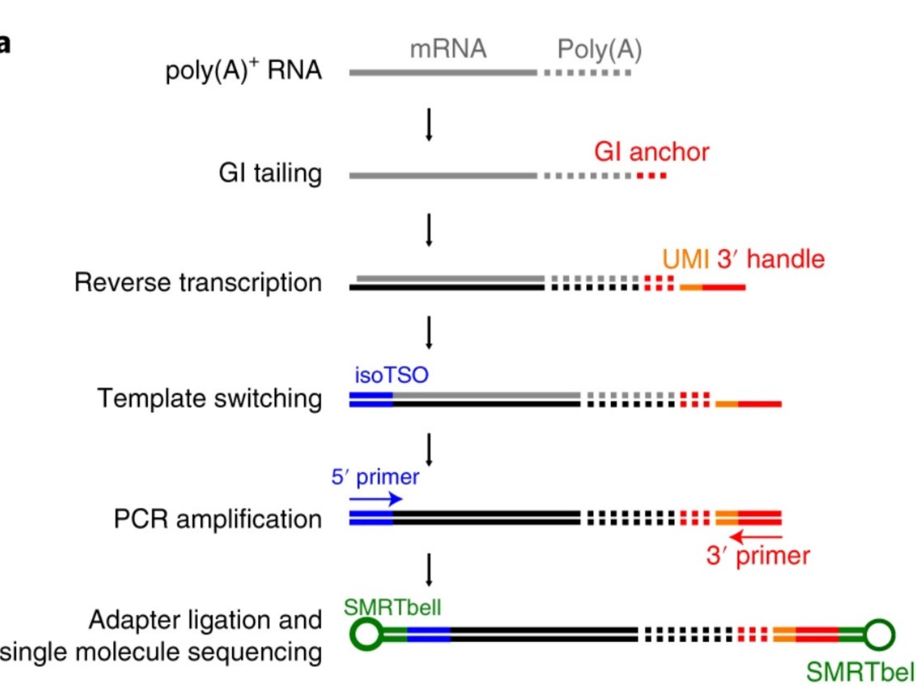
To investigate tail structures at the genomic scale, method called TAIL-seq that deep-sequences the 3′ most fragments of RNAs was developed.
The TAIL-seq protocol begins with removal of abundant noncoding RNAs such as rRNAs, tRNAs, snRNAs, and snoRNAs by affinity-based depletion and size fractionation. The resulting RNA sample enriched with mRNA is subsequently ligated to the 3′ adaptor that contains biotin residues. Following partial fragmentation, the 3′ most fragments are purified using streptavidin beads and sequenced.



Chang et al., 2014. Mol Cell

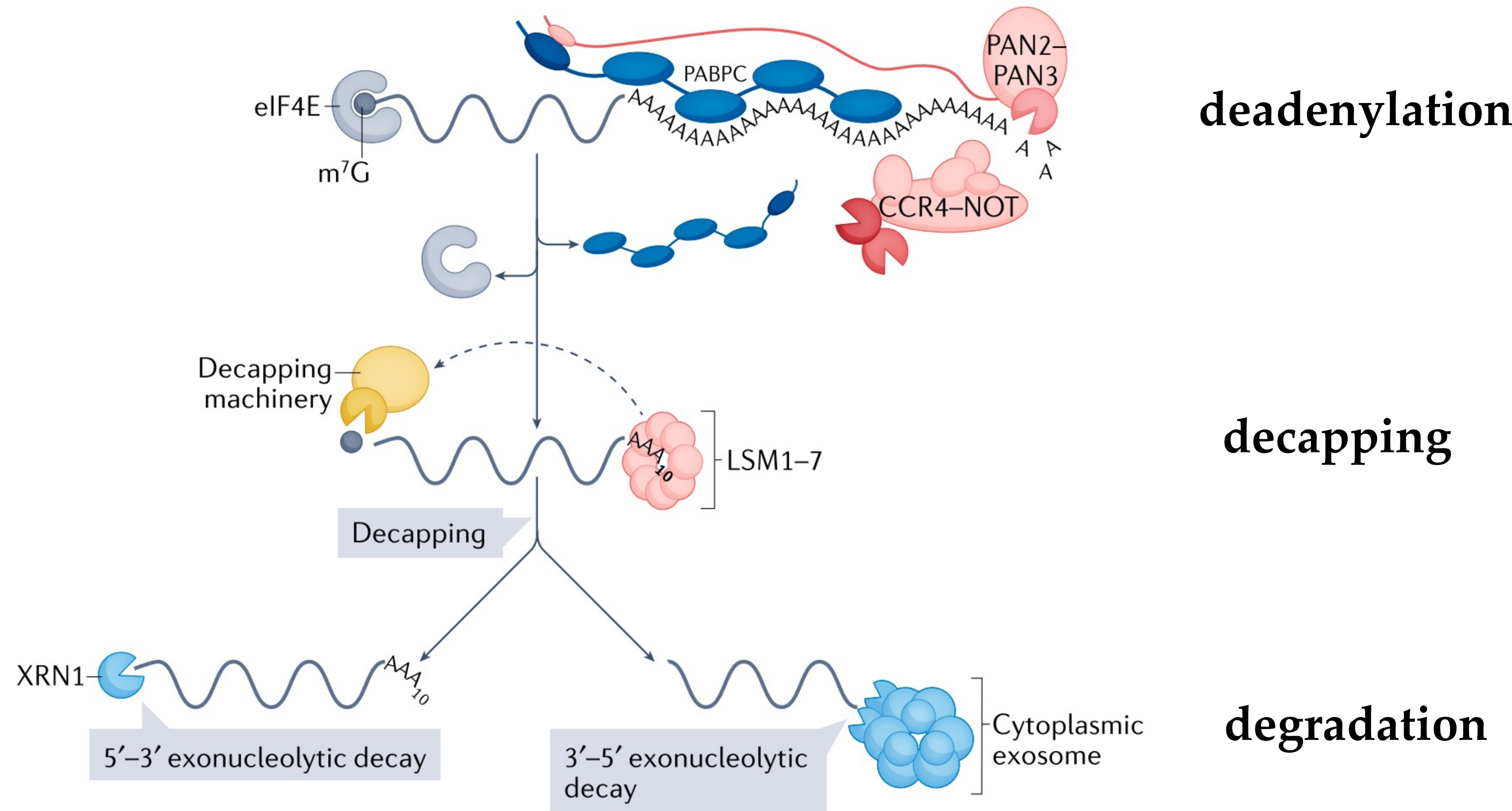
FLAM-seq: Full-Length mRNA Sequencing

A new method for sequencing of polyadenylated RNAs in their entirety, including the transcription start site (TSS), the splicing pattern, the 3′ end and the poly(A) tail. Poly(A)-selected RNA is enzymatically tailed with a short stretch of guanosines and inosines, which serves as a priming site for an anchored oligonucleotide carrying a unique molecular identifier (UMI) and a PCR handle. RNA is then reverse transcribed in combination with a chemically modified template switching oligo (iso-TSO) that allows tagging the RNA 5′-ends
with a second PCR handle. The resulting cDNA is amplified via PCR and subjected to long read sequencing with the PacBio Sequel System.



Legnini et al., 2019. Nature Methods

Eukaryotic mRNA decay



deadenylation

decapping

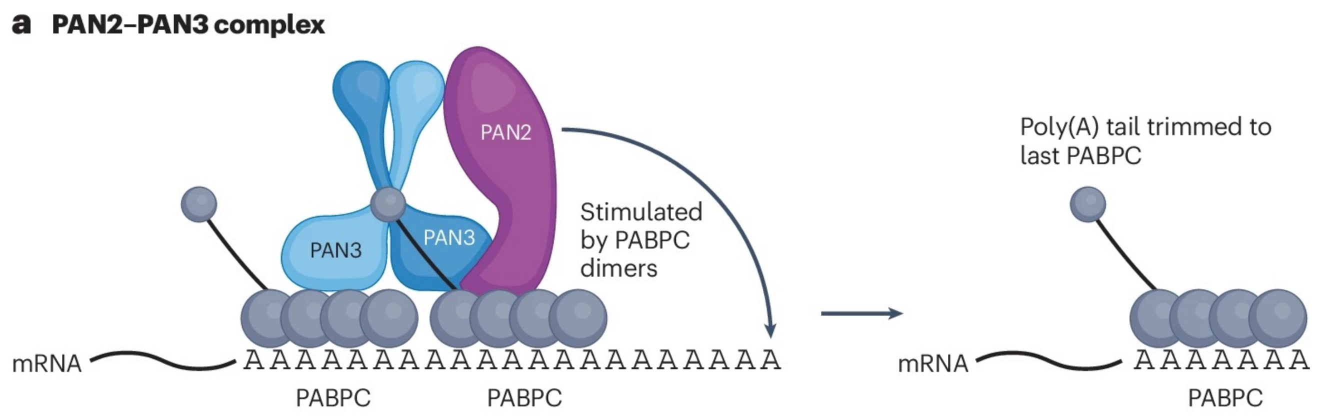
degradation

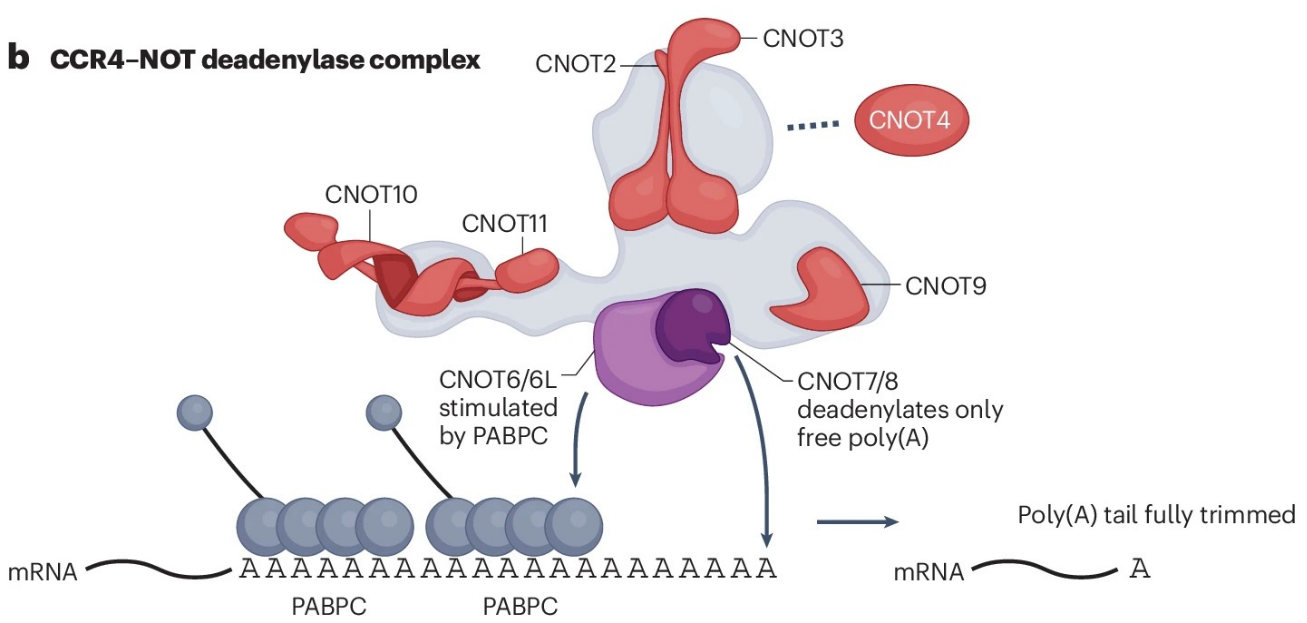
	Reaction	Enzymes: Yeast	Enzymes: Mammals	Effectors
I	deadenylation	• Pan2p/Pan3p • CCR4-NOT complex	• Pan2/Pan3 • CCR4-NOT complex • PARN	PABPC PABPC cap
IIa	decapping	• Dcp1p/Dcp2p	• Dcp1/Dcp2	Edc1p; Edc2p; Edc3p; Pat1p; Lsm1-7; Dhh1p; PABPC
IIb	cap hydrolysis	• Dcs1p	• DcpS	
III	5′-3′ exonucleolytic decay	• Xrn1p	• Xrn1	
IV	3′-5′ exonucleolytic decay	• exosome	• exosome	Ski2p; Ski3p; Ski7p; Ski8p

Major deadenylases in eukaryotes

Complex	Subunit name		Function
	Homo sapiens	*Saccharomyces cerevisiae*	
PAN2–PAN3	PAN2	Pan2	DEDD exonuclease (deadenylase)
	PAN3	Pan3	Binds PABPC dimers
CCR4–NOT	CNOT1	Not1	Scaffold
	CNOT2	Not2	NOT box scaffold
	CNOT3	Not3/5	NOT box scaffold/binds ribosome E-site
	CNOT4	Not4	RING E4 ligase
	CNOT6/6L	Ccr4	EEP exonuclease (deadenylase)
	CNOT7/8	Caf1	DEDD exonuclease (deadenylase)
	CNOT9	Caf40	Protein–protein interaction
	CNOT10	NA	RNA binding
	CNOT11	NA	RNA binding

Eukaryotic mRNA deadenylases





Yeast mRNA Deadenylase

In yeast, a **poly(A) nuclease**, referred to as **PAN**, has been identified biochemically and shown to be an enzyme consisting of the **Pan2** and **Pan3** proteins

The **Pan2p** subunit is the catalytic subunit since this protein is a member of the RNaseD family of 3′ to 5′ exonucleases

However, pan2Δ and pan3Δ yeast strains show minimal effects on deadenylation *in vivo* (required for initial shorthening-20nt)

Ccr4p and its associated factor **Pop2/Caf1p** have both nuclease domains (Mg^{2+} dep. endonuclease and RNaseD family)

Ccr4Δ and Pop2/Caf1Δ yeast strains show defects on deadenylation *in vivo*

Ccr4Δ/Pan2Δ strain is completely blocked for deadenylation

How to study mRNA decay

Transcription inhibition
(inducible promoter or drug)

↓

Time course

↓

RNA extraction

↓

RNA analysis
(Northern blot or real-time PCR)

Drugs to inhibit RNA trancription

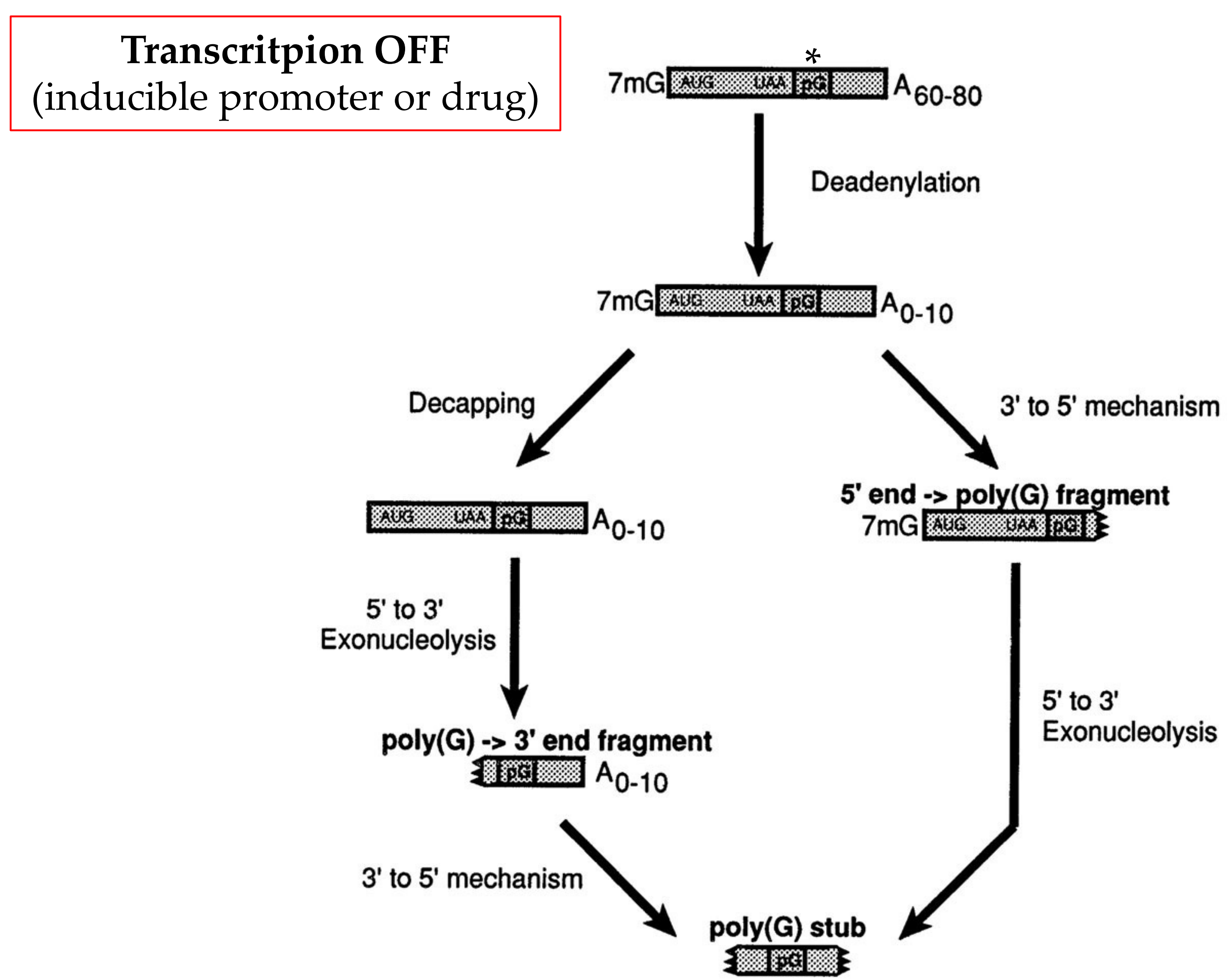
- **Actinomycin D** is a transcription inhibitor which intercalates into DNA. Actinomycin D forms a very stable complex with DNA, preventing the unwinding of the DNA double-helix, thus inhibiting the DNA-dependent RNA polymerase activity.

- ***α*-Amanitin** interacts directly with and inhibits the RNA polymerase II activity. Amanitin is an irreversible inhibitor because it triggers degradation of Rpb1, the largest RNAP II subunit.

- **5,6-dichloro-1β-1-ribofuranosylbenzimidazole (DRB)** inhibits the kinase subunit of P-TEFb, CDK9, thus blocking transcription elongation.

- The use of inducible promoters to control transient transcription have presented advantages over the potential cytotoxic effects of Actinomycin D or other transcription inhibitors in the analysis of mRNA decay. However, the advantage of Actinomycin D assay is that it does not require the construction and introduction of exogenous genes into cells, and provides a way of measuring stability changes of endogenous mRNAs.

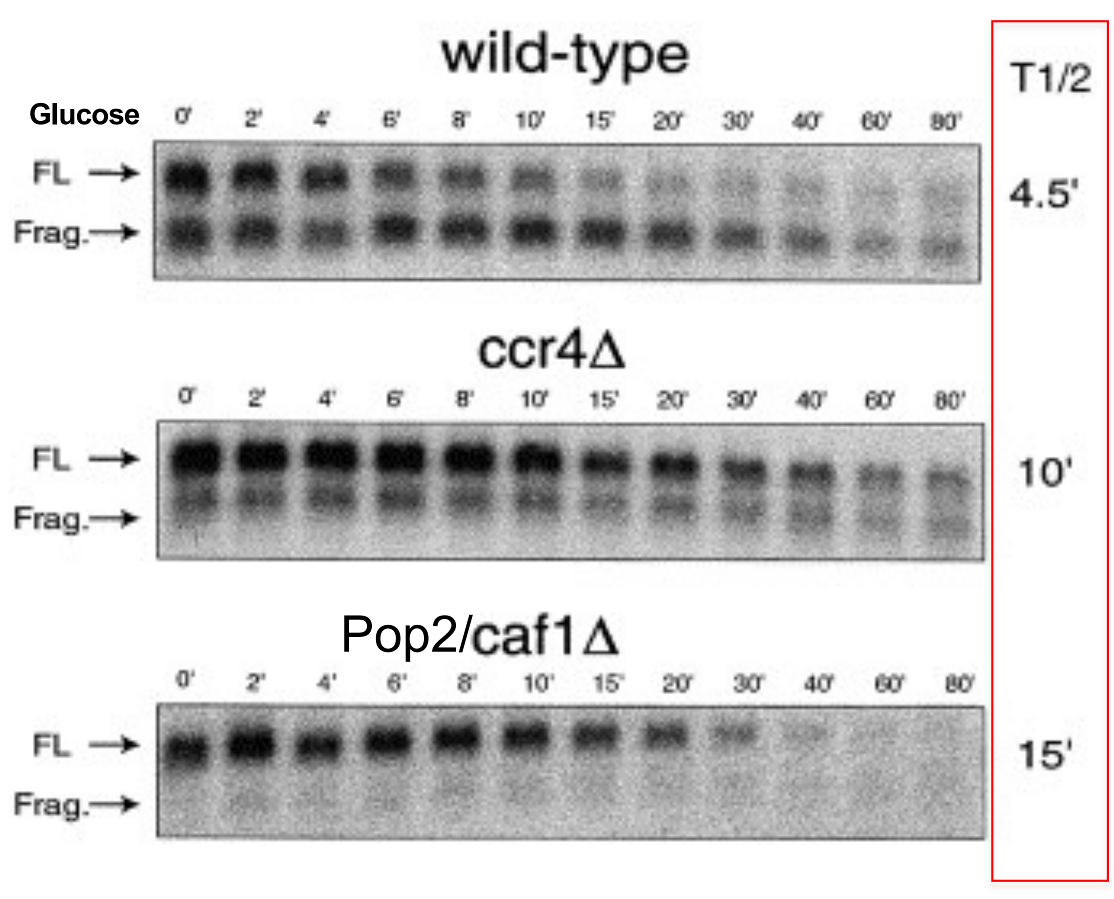
How to study mRNA decay in yeast



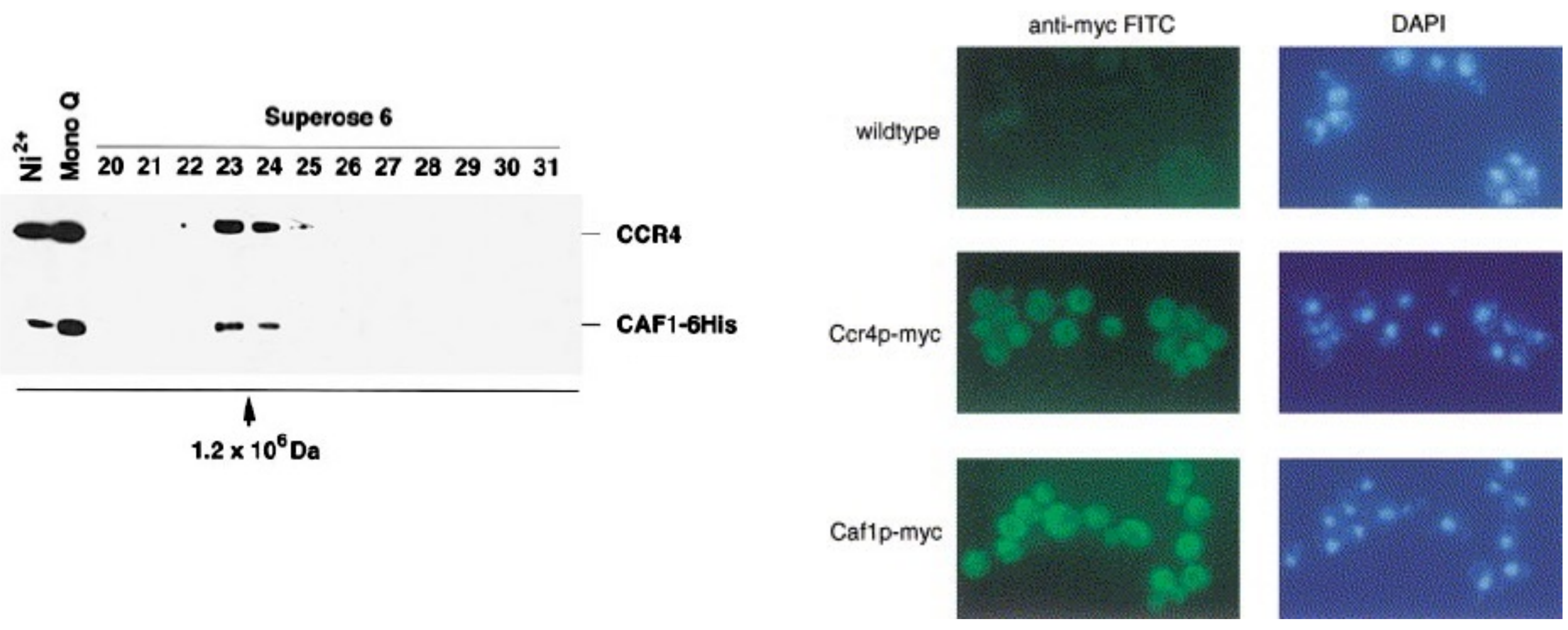
*The pG cassette blocks exonucleases

Ccr4p and Pop2/Caf1p Are Required for Normal Rates of mRNA Turnover

Measurement of the Decay Rate of the MFA2pG mRNA (GAL promoter):



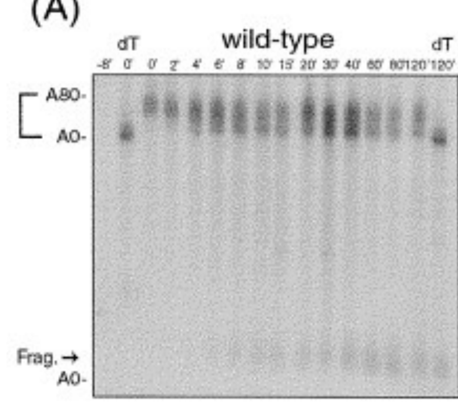
Ccr4p-Pop2(Caf1p) exist in a cytoplasmic complex

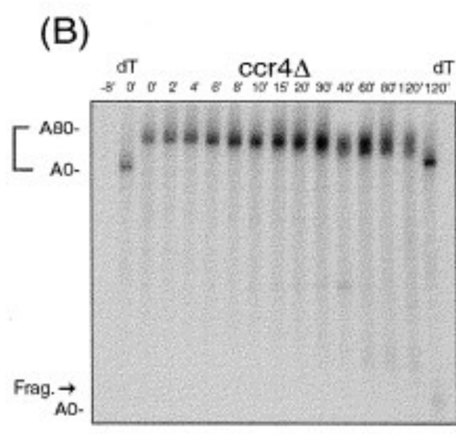


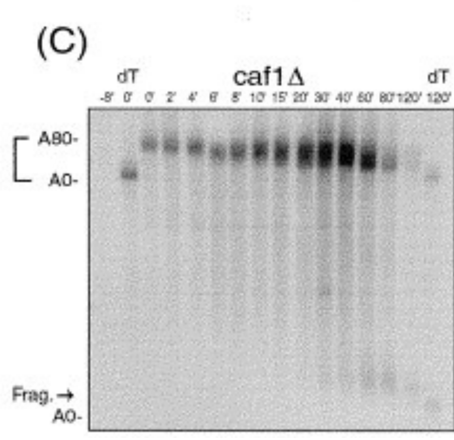
Ccr4p and Caf1p/Pop2 Are Required for mRNA Deadenylation

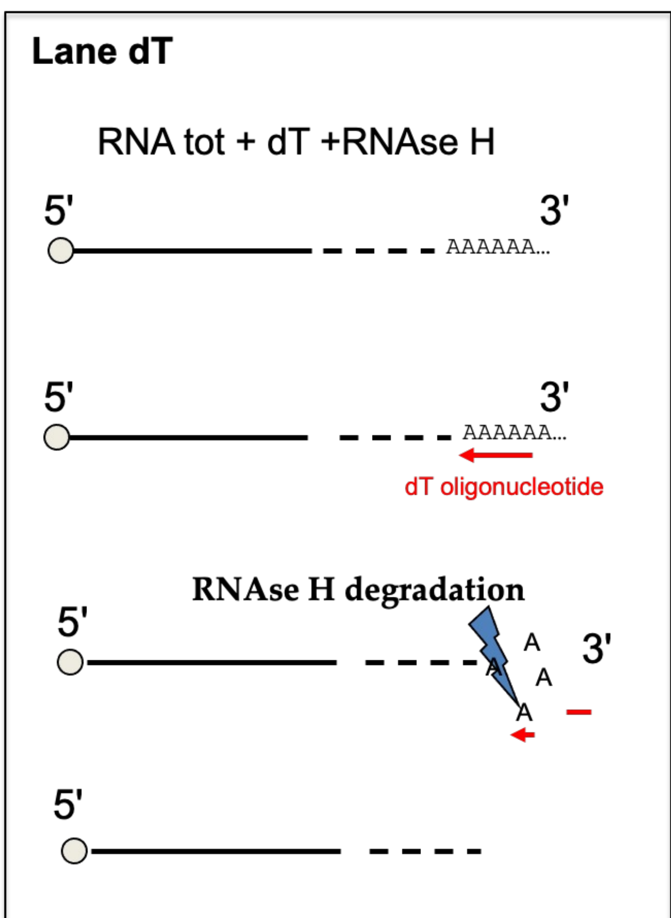
yeast

MFA2pG





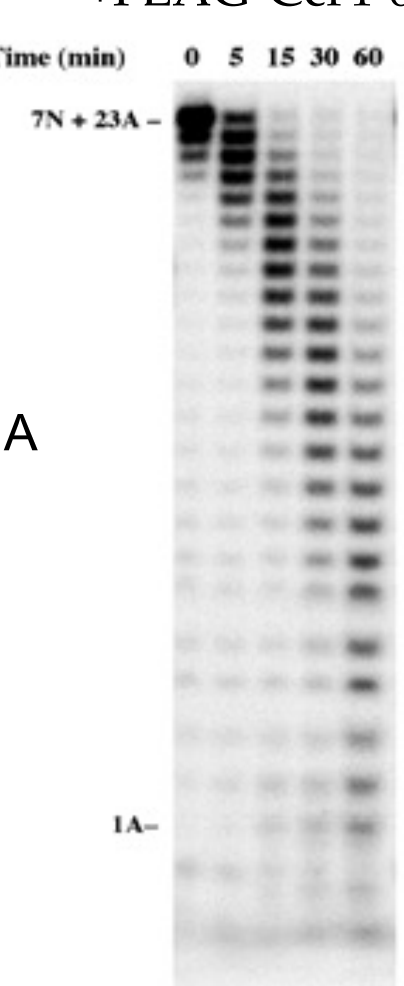




CCR4 and CAF1/POP2 are deadenylases

Both Ccr4 and Caf1 show independent deadenylase activity *in vitro*

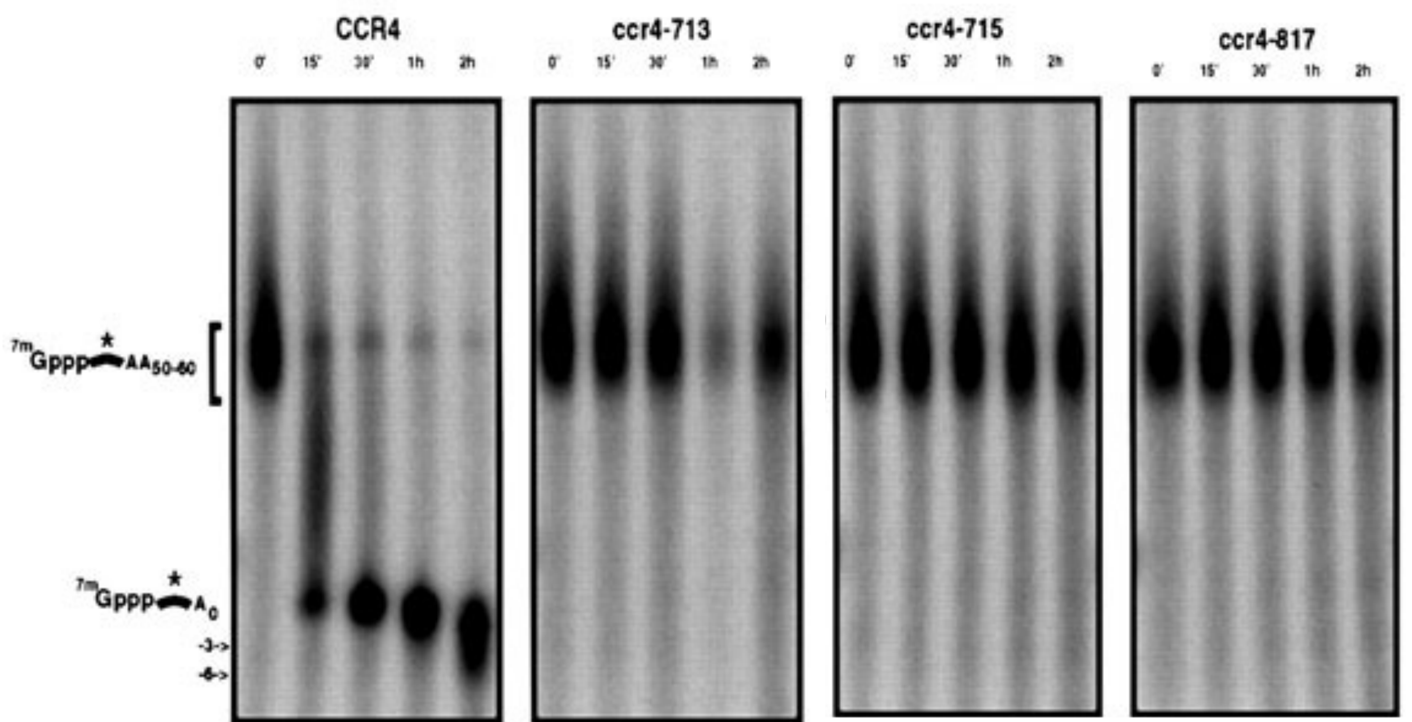
Purified Ccr4 + *in vitro* transcribed RNA



Caf1 is not required for Ccr4 activity *in vitro

Similar results are obtained with purified Caf1 protein

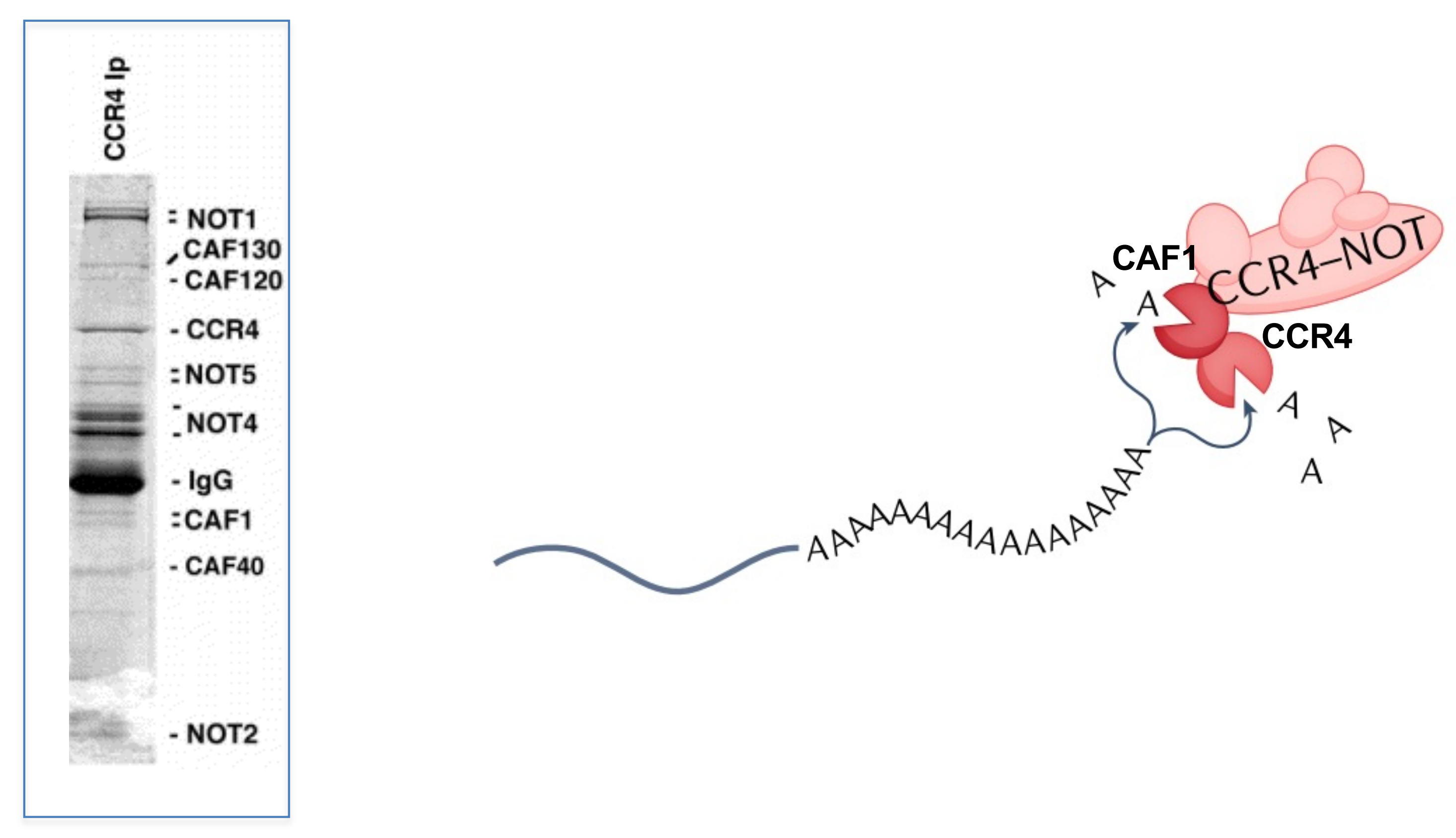
The Ccr4p nuclease motif is required for deadenylase activity



Analysis of deadenylation activity in Flag-Ccr4p purified fractions on a capped, polyadenylated substrate from a *ccr4 strain containing either a Flag-Ccr4p wild-type plasmid (pRP1045) or Flag-Ccr4p plasmids containing specific point mutants.*

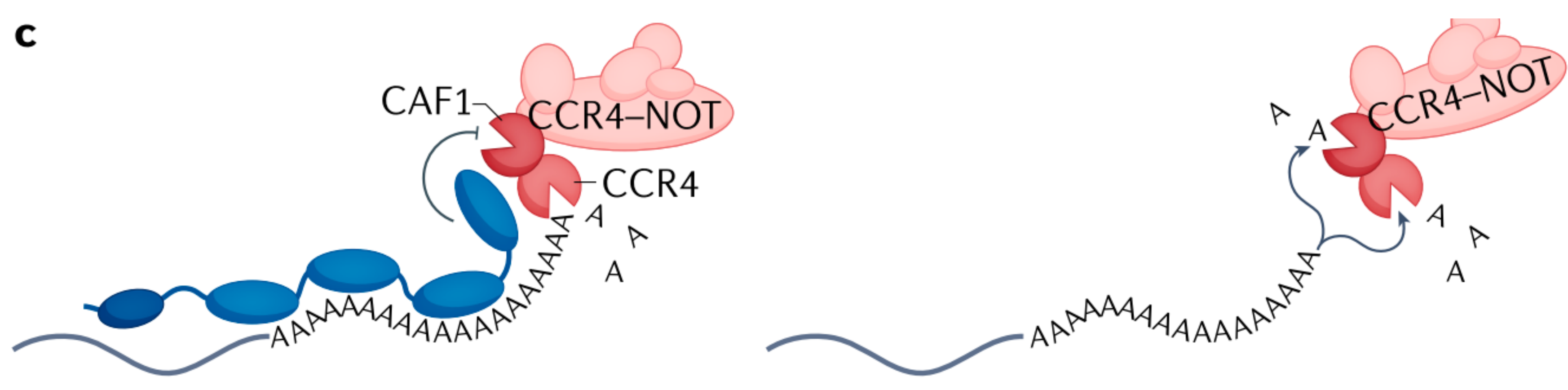
Deadenylation complex

- Not1-5 proteins and CAF40-130 were co-purified with the Ccr4-Caf1 complex
- Not and CAF proteins are not required for Ccr4 activity *in vitro* but affect deadenylation *in vivo*



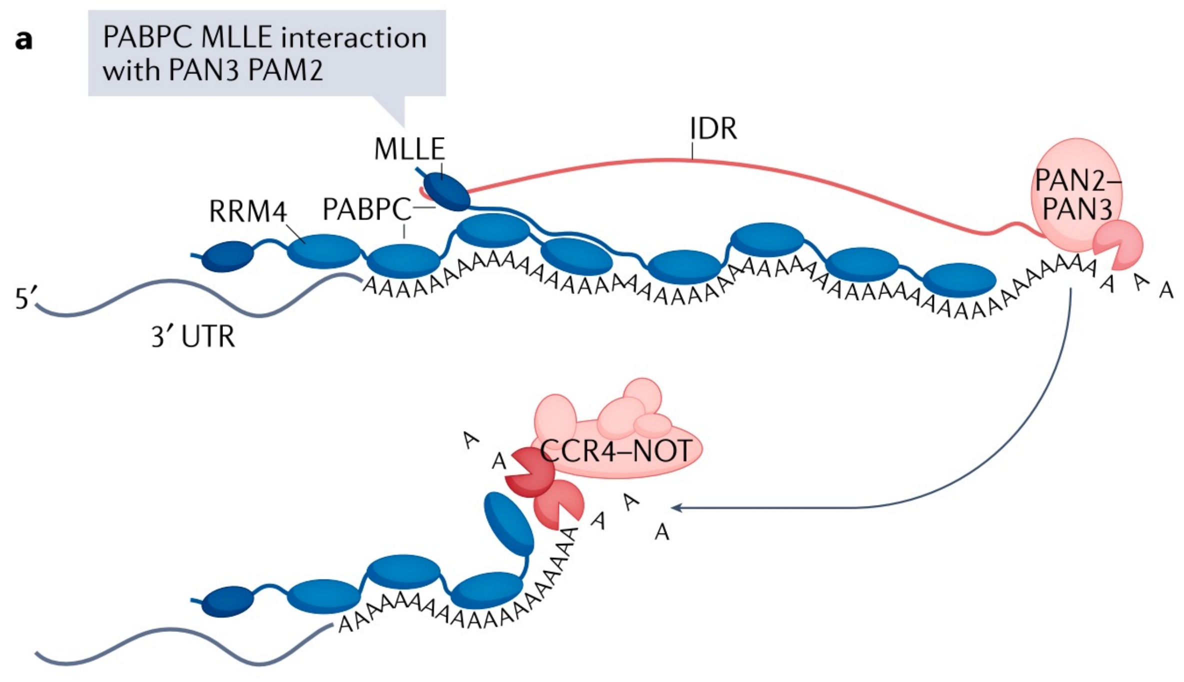
Deadenylation by Ccr4–Not complex

Caf1 did not deadenylate Pab1 bound RNA. By contrast, **Ccr4** binds Pab1 and can release it from poly(A) tails. The two nucleases in CCR4–NOT have different functions: CAF1 degrades naked poly(A) RNA and is blocked by PABPC, whereas CCR4 is able to release PABPC to deadenylate PABPC bound poly(A) RNA.



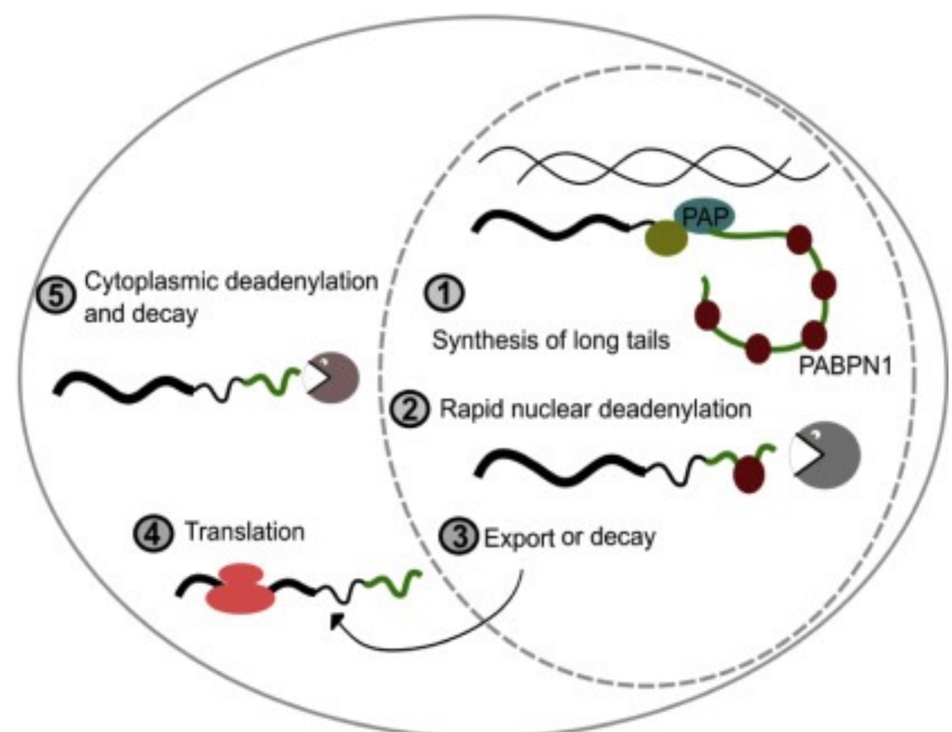
Models for mRNA Deadenylation

PAN2–PAN3 preferentially removes the distal part of the poly(A) tail. A cytoplasmic PABPC-interacting motif 2 (PAM2) within an intrinsically disordered region (IDR) of PAN3 interacts with MLLE domain of PABPC. Following PAN2–PAN3 function, CCR4–NOT removes the part of the poly(A) tail that is more proximal to the 3' untranslated region (3' UTR) of the mRNA.



Models for mRNA Deadenylation

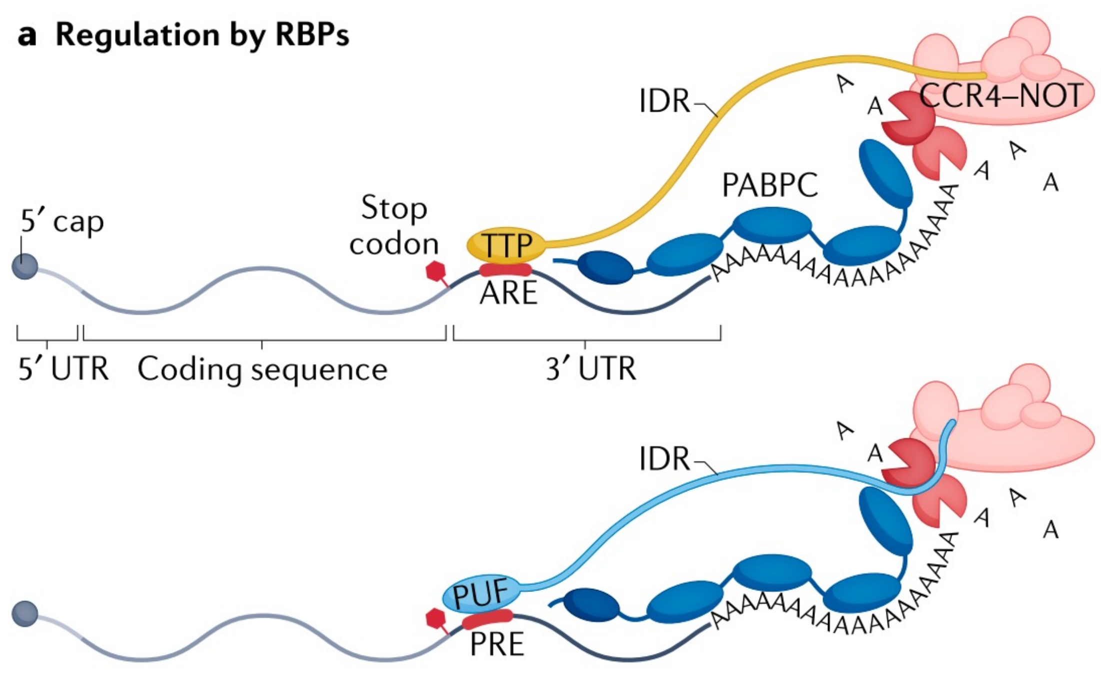
2023 model: mRNAs with long poly(A) tail are synthesized and rapidly deadenylated in the nucleus. mRNAs with shortened tails are exported to the cytoplasm where they are translated, until progressive deadenylation leaves mRNAs without poly(A) tails, which are decapped and degraded. An exception are lncRNAs, with more than 50% of nuclear lncRNAs not showing poly(A) tail shortening. Nuclear deadenylation has been proposed as a mechanism controlling RNA export.



Alles et al., 2023. iScience

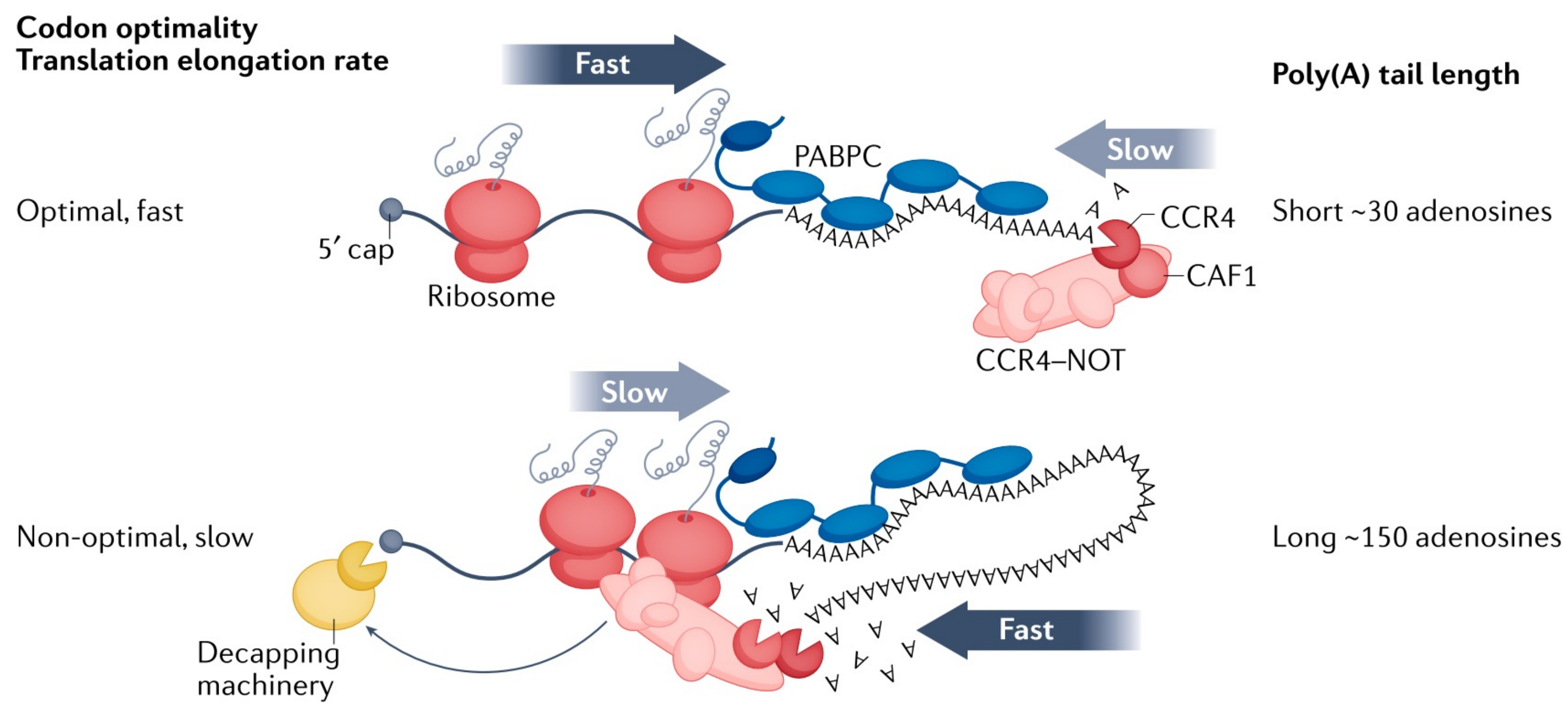
Factors that influence deadenylation rate

The deadenylation rates and half-lives of different transcripts can vary by more than a 1,000-fold. Specific sequences (often in the 3' UTR) are recognized by RBPs that recruit deadenylases to specific transcripts (such as GW182, TTP or Pumilio). RNA sequence affects the translation elongation rate, which is also a major determinant of mRNA half-life.



The relationship between translation and deadenylation rate

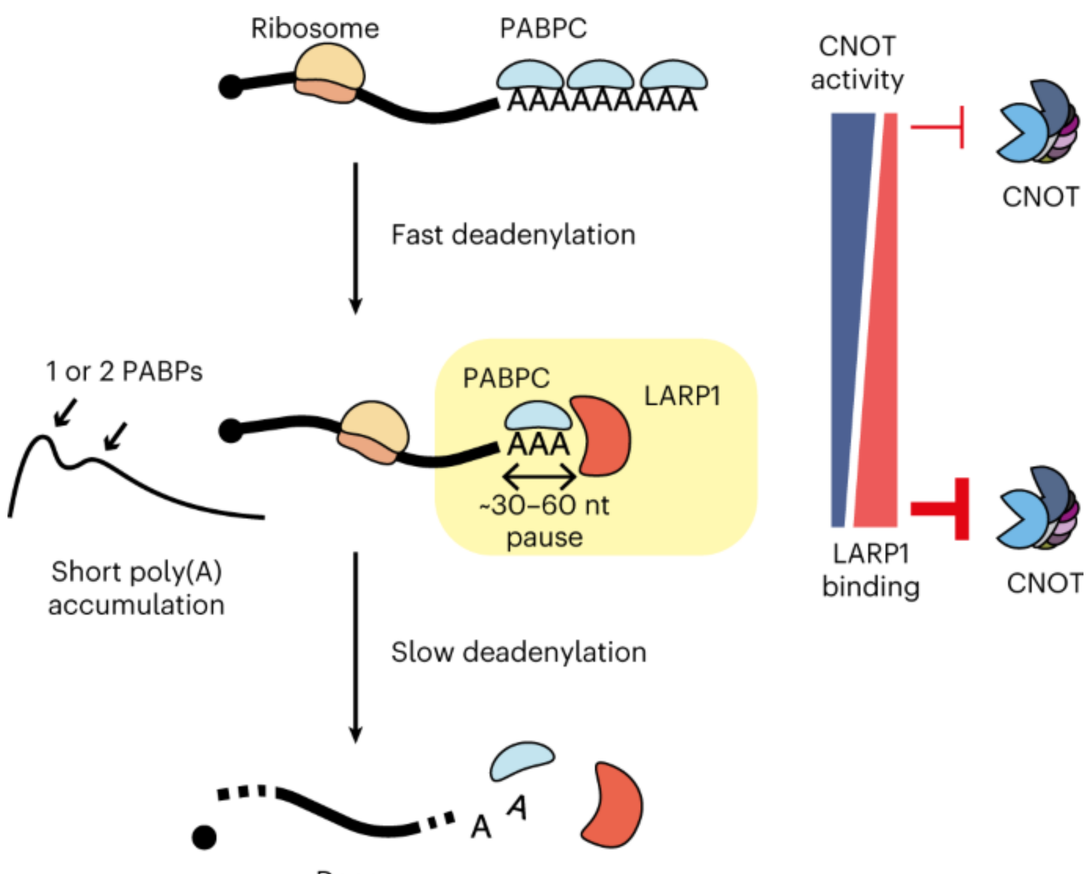
Multiple studies found that the length of poly(A) tails of highly translated, stable RNAs is relatively short (about 30 nt) at steady state. By contrast, poorly translated mRNAs have comparatively long poly(A) tails. CCR4–NOT may sense the rate of translation elongation by detecting ribosomes containing empty A and E sites and consequently recruit the decapping machinery . The concentration of cytoplasmic PABPC may also affect the role of poly(A) tails in gene expression.



LARP1 preferentially binds to and protects short poly(A) tails

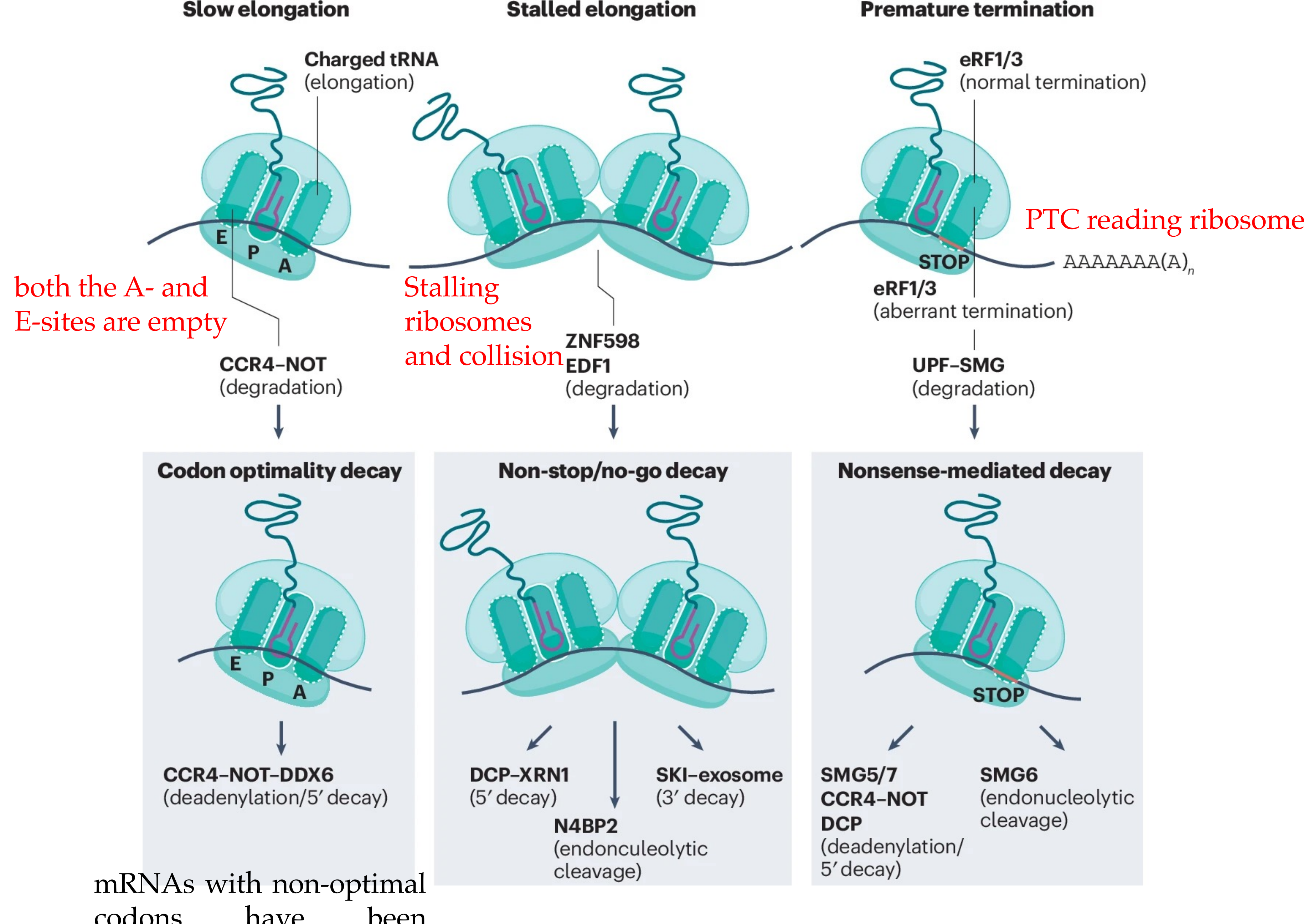
LARP1 protects short poly(A) tails (~30–60 nt) from CNOT deadenylation by forming a tertiary complex with PABPC and poly(A). LARP1-mediated deceleration of deadenylation results in the accumulation of short poly(A) tails, which may provide a buffering system preventing complete deadenylation (≲25 nt).

measuring the steady-state and pulse-chased distribution of poly(A)-tail length, it has been found that deadenylation slows down in the 30–60-nt range. LARP1 associates preferentially with short tails and its depletion results in accelerated deadenylation specifically in the 30–60-nt range.



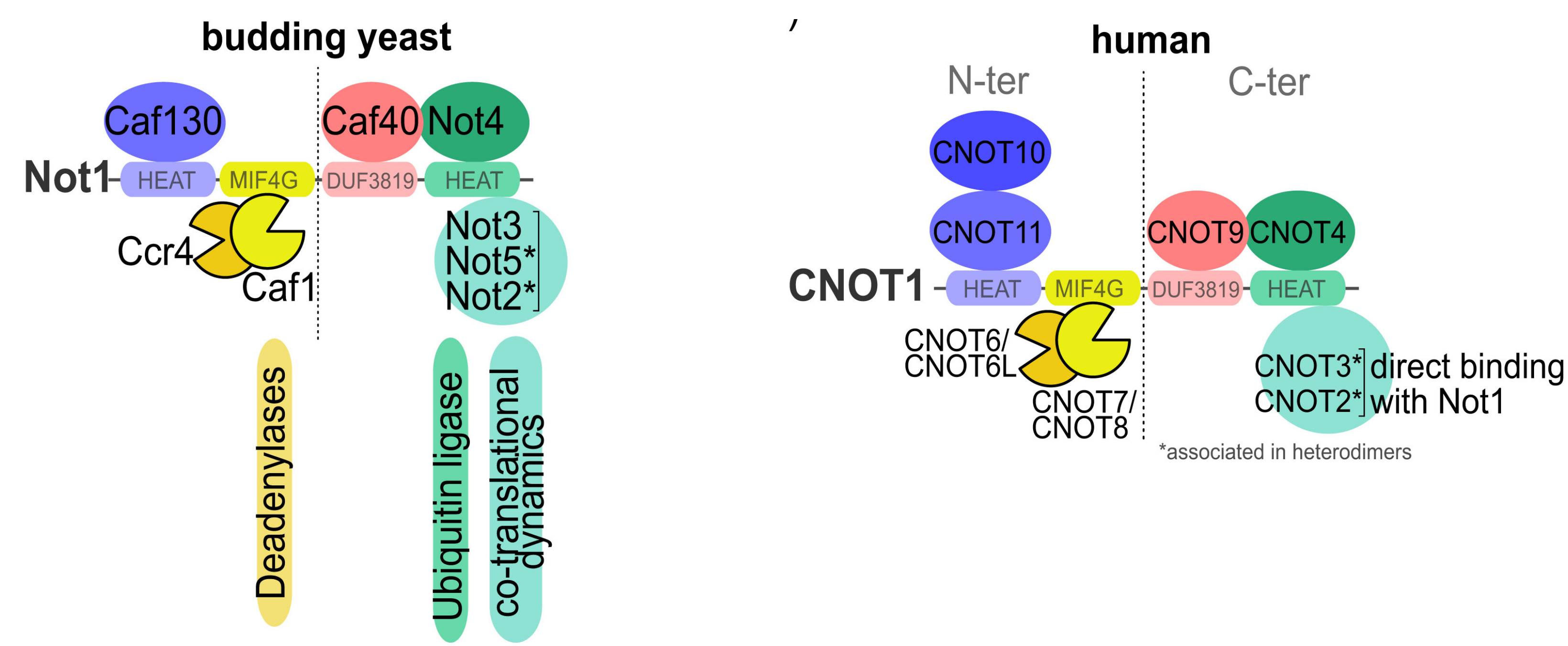
Park et al., 2023. at Nat Struct Mol Biol

mRNA decay governed by translation



mRNAs with non-optimal codons have been observed to undergo enhanced deadenylation

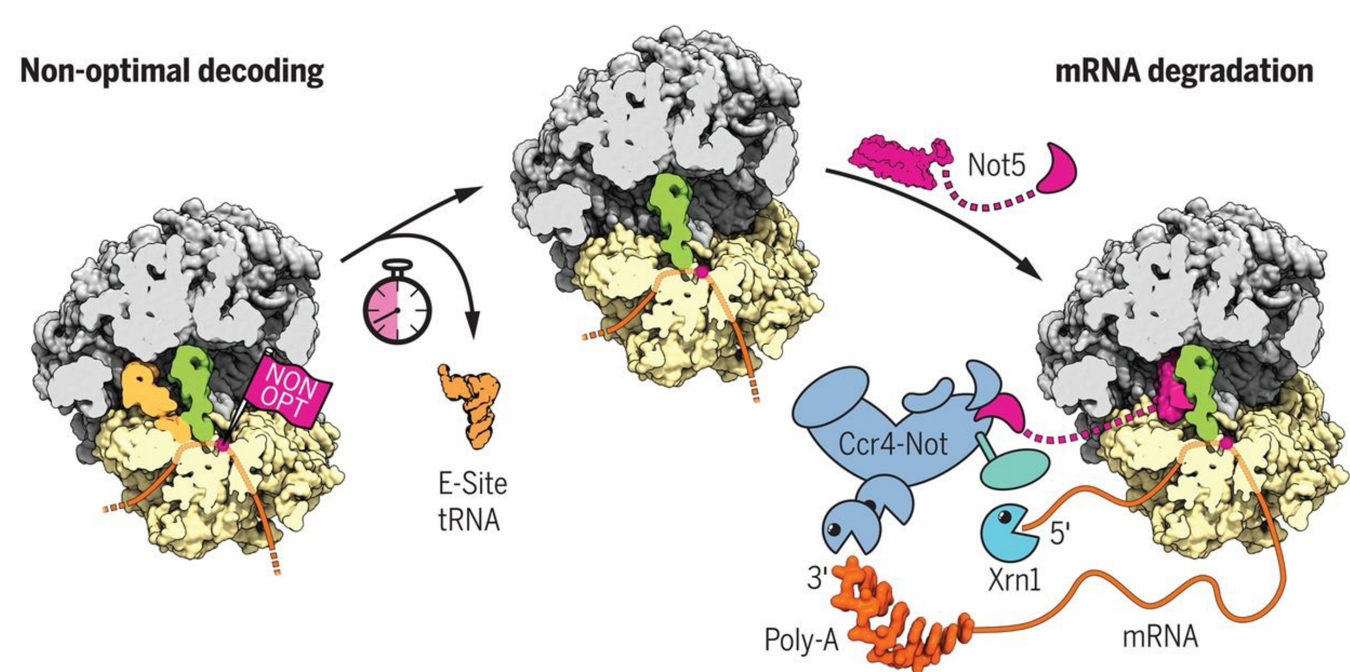
Deadenylation independent functions of the Ccr4-Not complexes



In yeast, deletion of the Not proteins was more detrimental to cell growth than deletion of the deadenylase subunits. Furthermore, mutations in the *NOT* genes, or their deletion, resulted in aggregation of newly synthesized proteins, and that this was not observed in cells lacking the deadenylase subunits. Not4 was then characterized as a RING E3 ubiquitin ligase and its first substrate was identified as a ribosome-associated chaperone, suggesting a role in translation regulation.

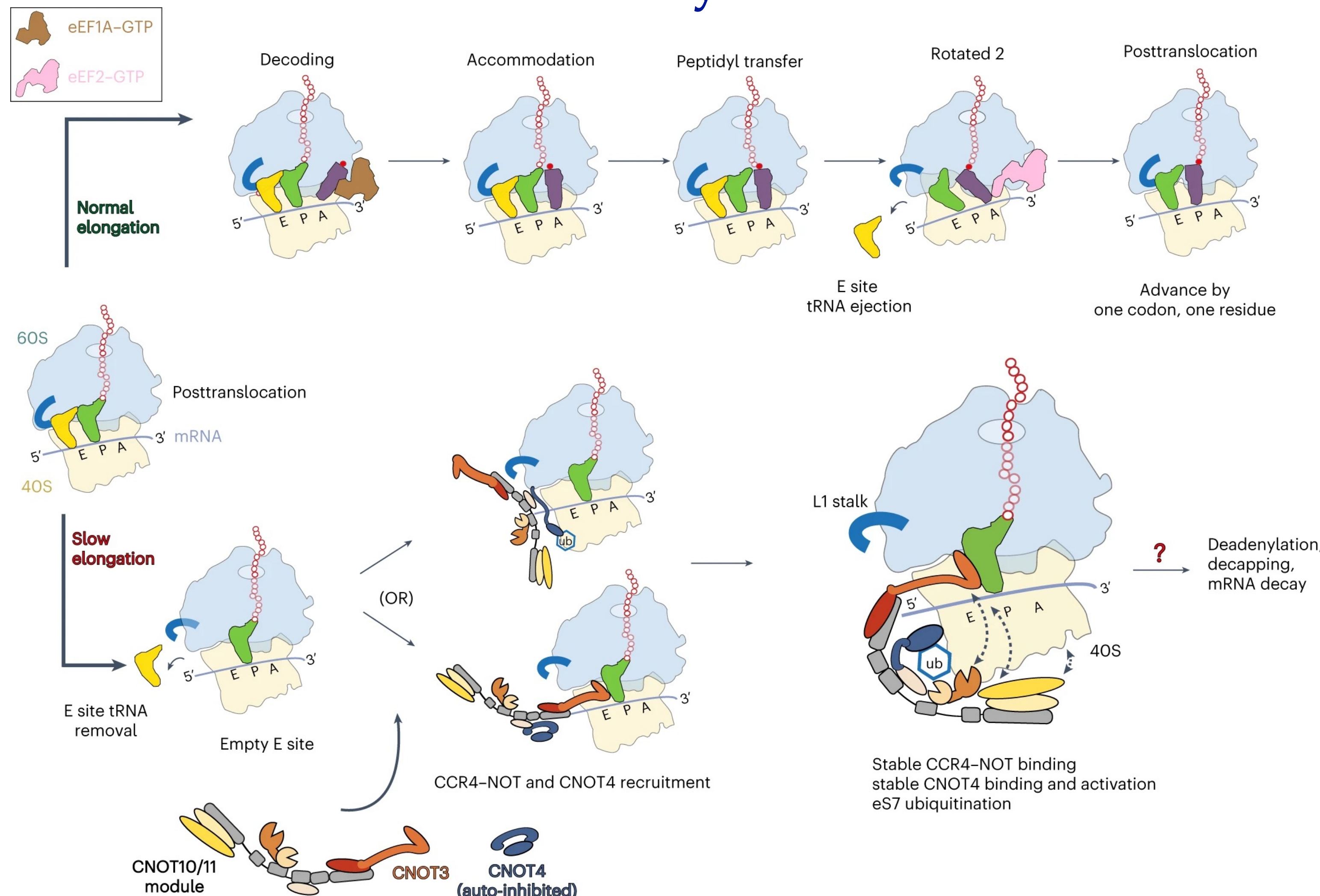
The yeast Ccr4-Not complex monitors the translating ribosome for codon optimality

When ribosomes encounter nonoptimal codons, low decoding efficiency leads to an increased likelihood of dissociation of the E-site tRNA before the cognate tRNA is accommodated in the A-site. Not5 interacts with the E-site of ribosomes in which both the A- and E-sites are empty recruiting the Ccr4-Not complex. As a result, the binding of the Ccr4-Not complex triggers mRNA degradation. This explains the shorter half-lives of transcripts enriched in nonoptimal codons.



Buschauer et al. Science 2020

hCCR4/NOT enforces ribosomal stalling to couple translation and mRNA decay

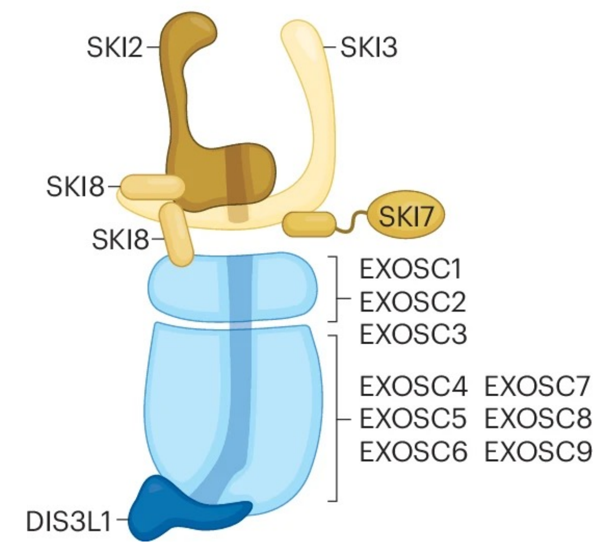


hCCR4–NOT is recruited by CNOT3 (yeast Not5) during slow elongation. CNOT4 (homologous of yeast Not4) mono-ubiquitinate eS7, which prevent the stalled ribosomes from participating in further rounds of translation. Binding reinforces translational stalling and may trigger poly(A) tail removal, decapping and mRNA decay.

The SKY complex

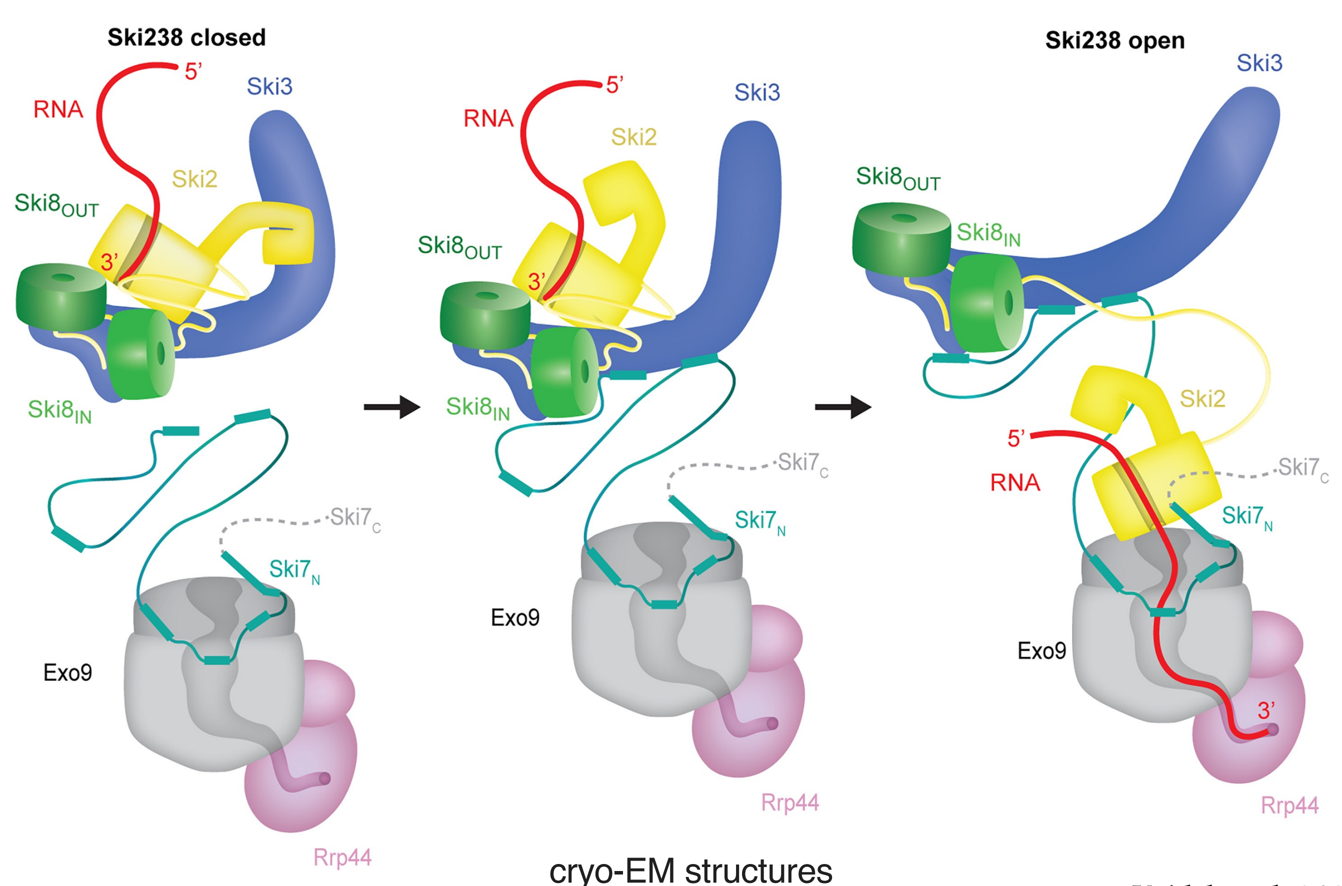
The **superkiller** (**SKI**) genes encoded in yeast nuclear genome were initially identified from mutations that cause overexpression of a killer toxin encoded by the endogenous double-stranded RNA.

The RNA helicase **SKI2** unwinds and feeds RNA substrates into the exosome core channel. The SKI2 helicase associates with co-factors SKI3 and SKI8 to form the cytoplasmic exosome-associated SKI complex, which is linked to the exosome core via another SKI protein, SKI7.



SKI7 is a GTP-binding protein consisting of two separate domains; one is the C-terminal region homologous to the GTP-binding elongation factor 1 (EF1a), and the other is an extra N-terminal domain that was not present in EF1. This domain structure is similar to that of the GTP-binding eukaryotic releasing factor 3 (eRF3).

Working model of the Ski2/3/8-Ski7-Exo10-mediated 3′-5' RNA decay in eukaryotes



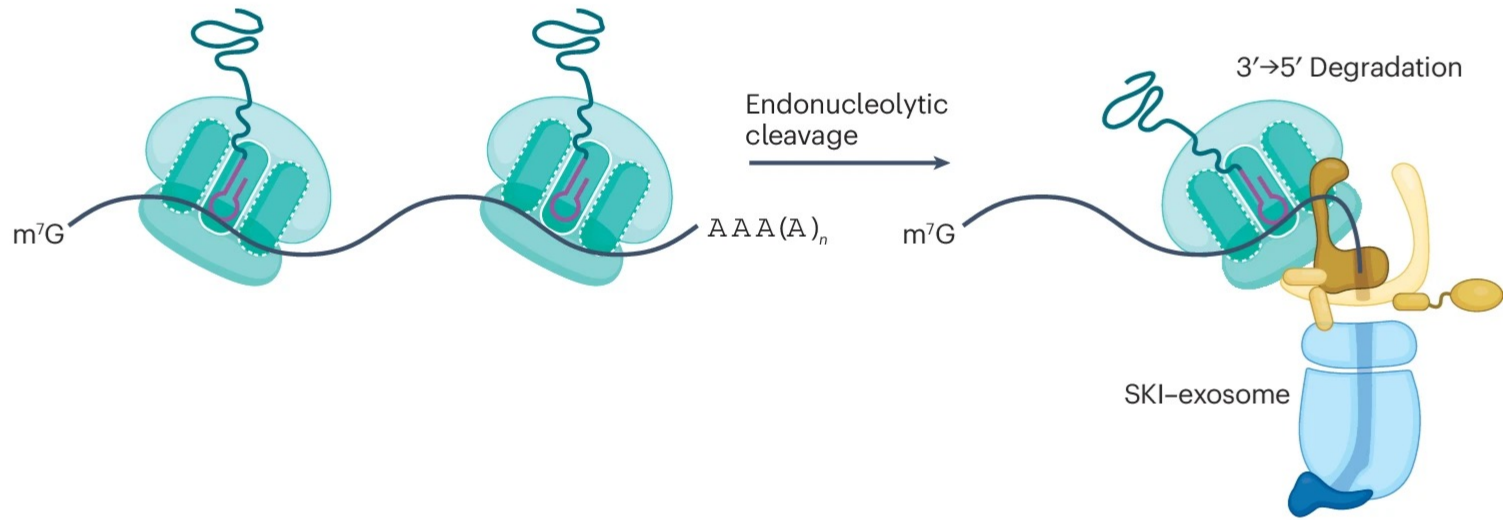
Keidel et al. 2023. Mol Cell

The closed-state Ski2/3/8 can bind the 3′ end of RNA but must undergo conformational changes to the open state to interact with Ski7-Exo10 in order to form a continuous channel that may be traversed by RNA.

The SKY complex

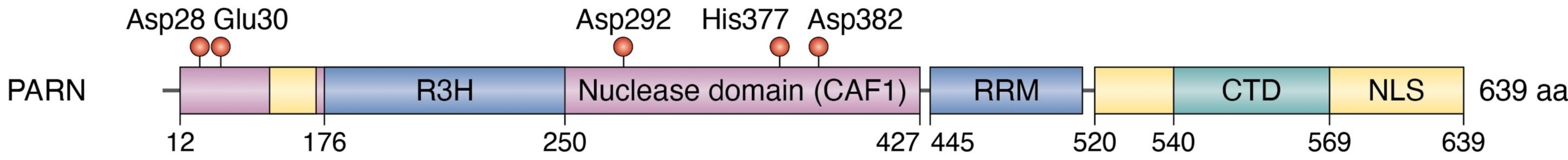
A primary function of the cytoplasmic SKI–exosome complex lies in the degradation of translating mRNAs that have undergone internal cleavage that causes, or results from, an aberrant translation event.

Structural studies have demonstrated a direct interaction of the SKI complex with the ribosome near the mRNA entry tunnel, and biochemical studies have demonstrated the ability of the SKI complex to extract RNA from stalled ribosomes



Human specific mRNA Deadenylase

A deadenylase activity in human cells has biochemically purified. Referred to as **PARN** (for poly[A] ribonuclease) or DAN (for deadenylating nuclease), this enzyme was first purified from *Xenopus* oocytes as a poly(A)-specific 3′→5′ exonuclease. Yeast lacks an obvious PARN homologue.

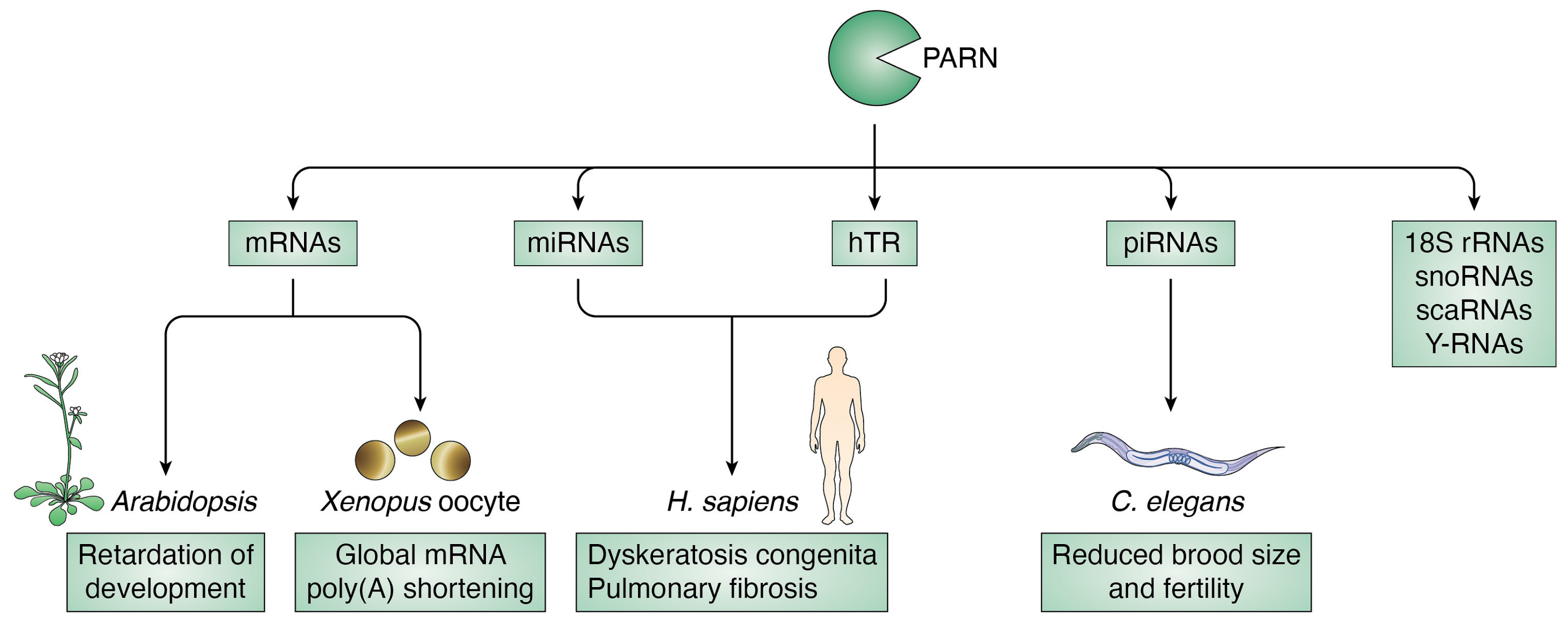


PARN consists of three domains: an RNA recognition motif domain (RRM), a nuclease domain, and an R3H domain. Mutational analyses showed that four conserved DEDD residues in PARN (Asp28, Glu30, Asp292, and Asp382) are essential for the catalytic activity of PARN and are required for the binding of divalent metal ions to PARN.

PARN

Among all deadenylases, PARN is unique as it can bind both the cap structure and the poly(A) tail during deadenylation . PARN recognizes and binds m7GpppG through Trp residue in the <u>RRM</u> domain while the R3H domain helps to stabilize PARN. PARN's activity was higher when processing RNA with 5′-cap structure compared to noncapped RNA substrates and the addition of free m7GpppG cap analog inhibited poly(A) degradation *in vitro*, suggesting that 3′-end poly(A) removal is linked to 5′ end cap structure of the RNA substrates.

Although PARN was suspected as a key regulator for mRNAs, PARN was shown to predominantly localize to the nucleolus and cytoplasmic foci and process ncRNAs in HeLa cells, such as 18S rRNAs, snoRNAs, hTR, scaRNAs, piRNAs, Y RNAs, and miRNAs



ARE-mediated decay (AMD)

•Several sequence elements within transcripts have been linked to the control of mRNA turnover. **AU-rich elements (ARE)** in 3'-UTR characterize most short-lived transcripts

•Destabilizing **RNA-binding proteins** have been shown to direct ARE-containing mRNA to the exosome

•RNA can be stabilized by proteins that compete for ARE binding and direct transcripts to the polysome for translation

•**AREs** are found in mRNAs involved in cellular responses to environmental and/or metabolic changes: cyclins, cytokines (GM-CSF, IL-3 etc.), oncogenes (c-myc, c-fos etc.)………

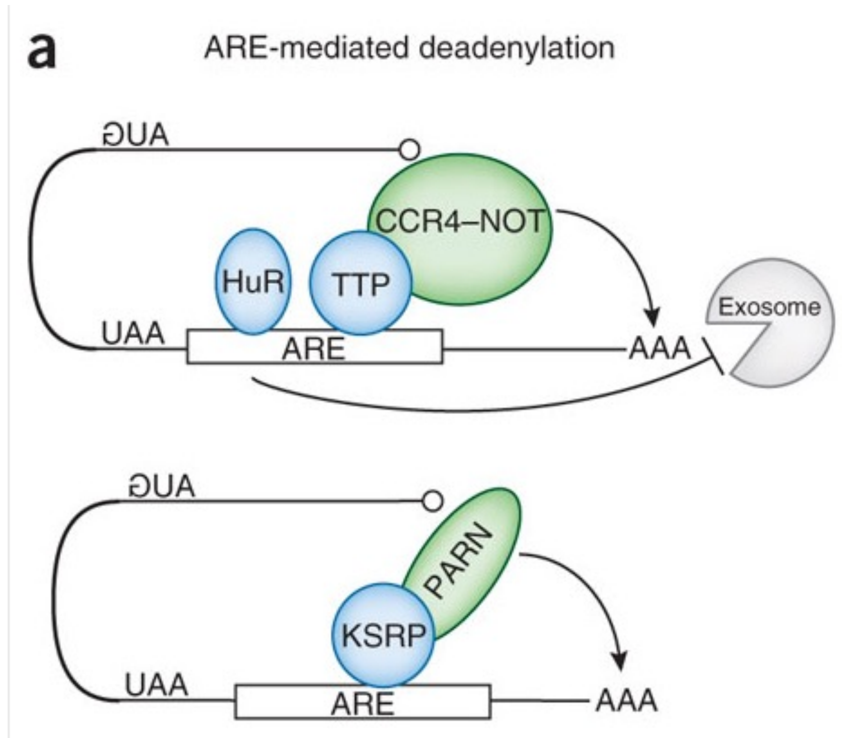
ARE binding proteins

Protein	Nucleus/Cytoplasm	Role in mRNA decay
AUF-1/hnRNP D 4 isoforms	p42, p45 nuclear, p37, p40 cytoplasmic	Destabilizing, stabilizing
HuR/HuA	Nuclear	Stabilizing
HuD, Hel-N1 /HuB, HuC	Nuclear	Stabilizing
TTP (tristetrapolin)	Nuclear, cytoplasmic	Destabilizing
KSRP	Nuclear, cytoplasmic	Destabilizing
Hsp70	Cytoplasmic	Destabilizing
TIA-1,TIAR	Nuclear, cytoplasmic	Destabilizing, translation
AUH	Cytoplasmic	Enzymatic activity ?
hnRNPA1	Nuclear	Destabilizing
hnRNPC	Nuclear	Destabilizing
GAPDH	Nuclear, cytoplasmic	Enzymatic activity ?
AUBF	Cytoplasmic	?
AU-A, AU-B, AU-C	AU-A nuclear, AU-B, AU-C cytoplasmic	Destabilizing

These proteins have been identified in cell extracts by ultraviolet (UV)-crosslinking and gel-shift assays

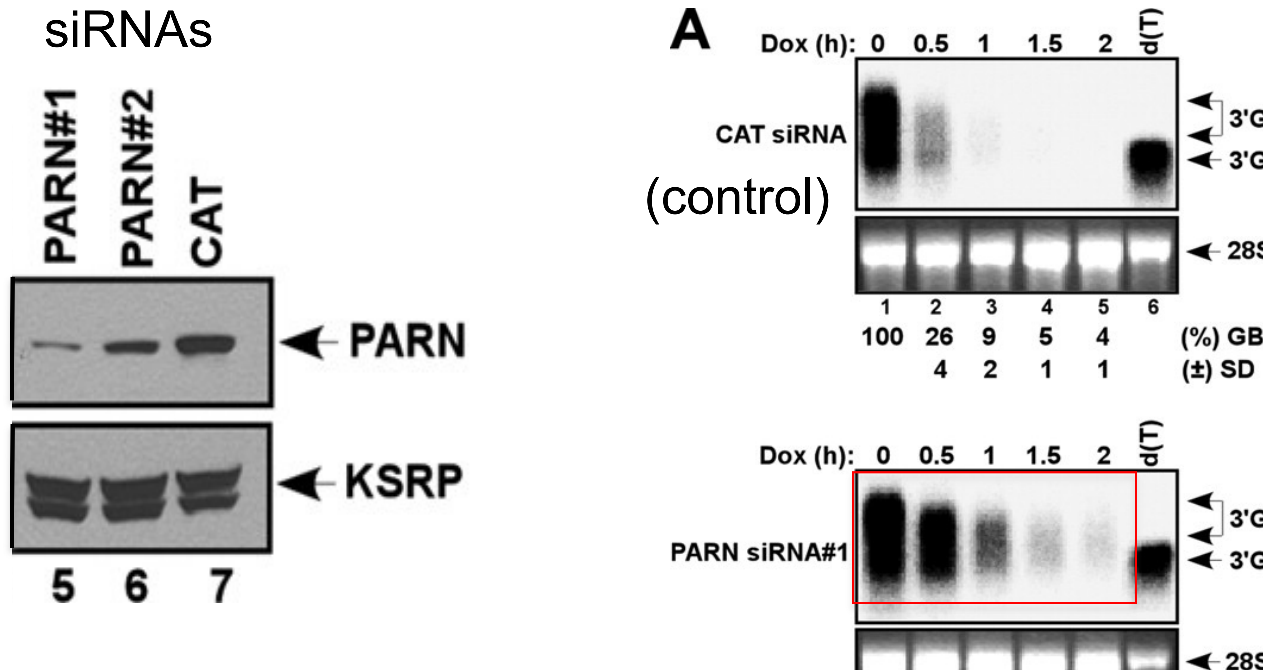
AREs drive mRNA deadenylation

The binding of the **TTP** and **KSRP** on AREs induces rapid mRNA deadenylation by the recruitment of the deadenylases CCR4–NOT complex and PARN, respectively. The binding of **HuR** stabilizes the transcript by inhibiting exosome recruitment.



Down-regulation of PARN inhibits deadenylation step of ARE-mediated Decay (AMD)

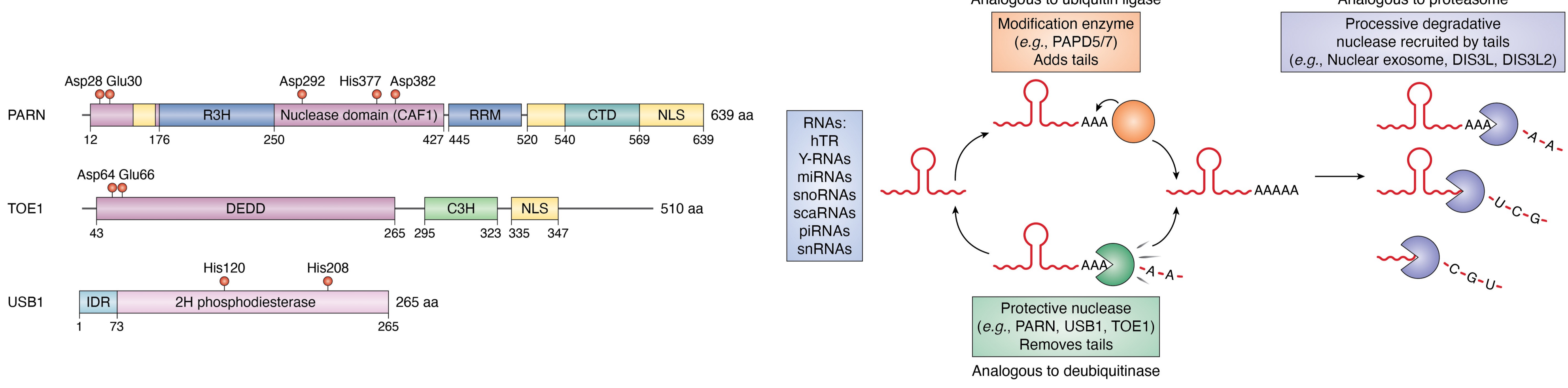
HeLa cells were transfected with a construct expressing beta globin (GB)-ARE^{GMCSF} under the control of a TetOFF promoter



Surprises in the 3′-end

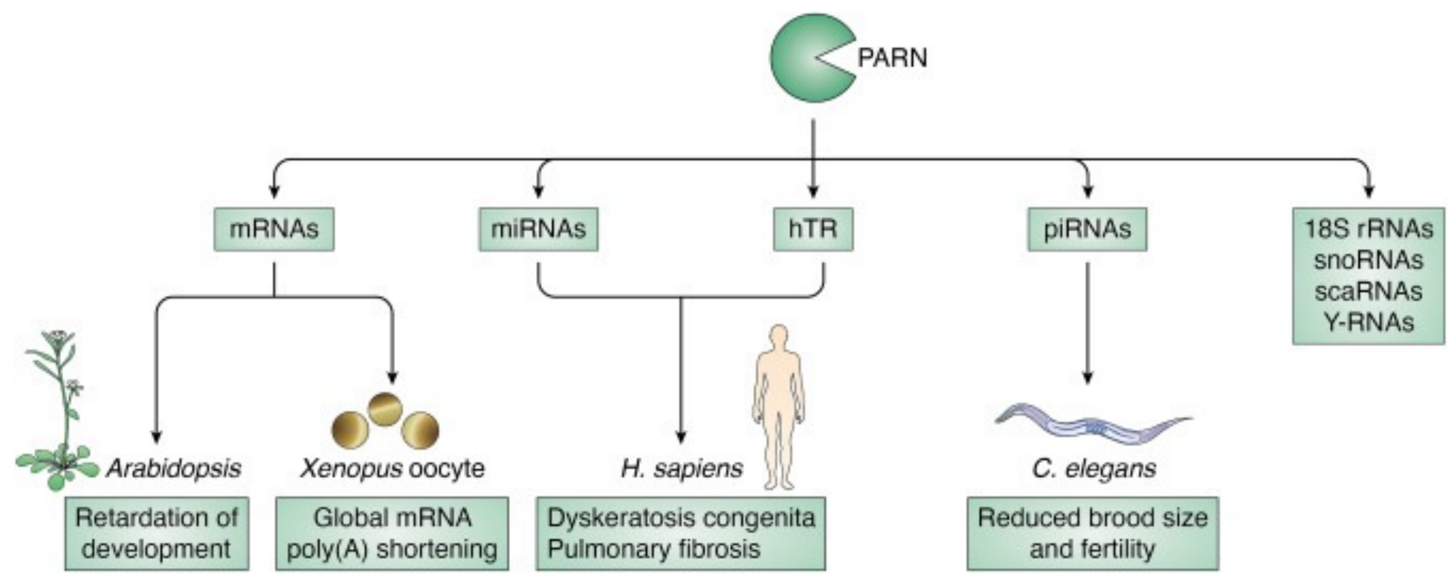
The USB1, PARN, and TOE1 enzymes

One mechanism for the degradation of ncRNAs involves the addition of oligo(A) tails by non-canonical poly(A) polymerases, which then recruit processive sequence-independent 3′ to 5′ exonucleases for RNA degradation. This pathway of decay is also regulated by three 3′ to 5′ exoribonucleases, USB1, PARN, and TOE1, which remove oligo(A) tails and thereby can protect ncRNAs from decay. Loss-of-function mutations in these genes lead to premature degradation of some ncRNAs and lead to specific human diseases such as Poikiloderma with Neutropenia for USB1, Dyskeratosis Congenita for PARN and Pontocerebellar Hypoplasia type 7 for TOE1.



PARN

PARN was shown to predominantly localize to the nucleolus and cytoplasmic foci and to remove poly(A) tails from mRNAs, miRNAs, hTR, piRNAs, 18S rRNAs, snoRNAs, scaRNAs, and Y RNAs. PARN mutants show dysregulation of these RNAs and lead to developmental defects in higher plants, zebrafish, *C.elegans*, and DC and pulmonary fibrosis in humans.

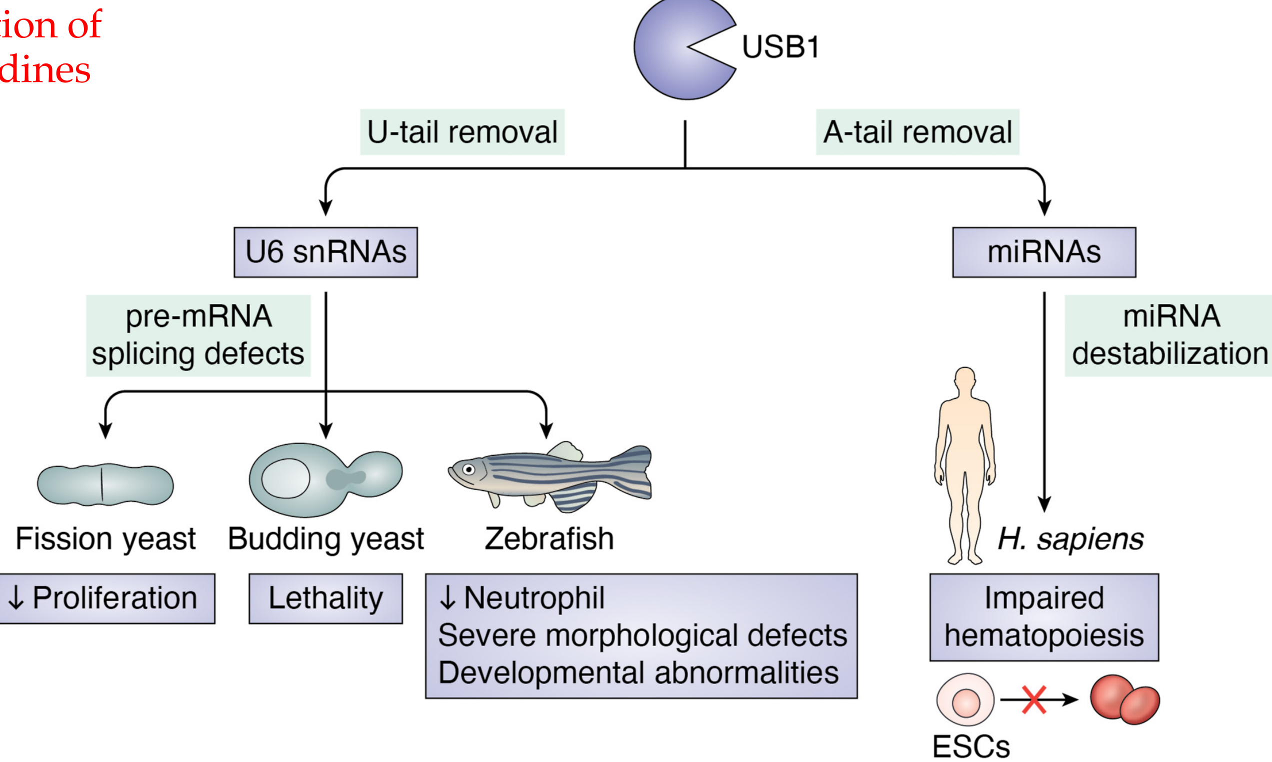


Among all deadenylases, **PARN is unique** as it can **bind both the cap structure and the poly(A) tail** during deadenylation. PARN's activity was higher when processing RNA with 5′-cap structure compared to noncapped RNA substrates and the addition of free m7GpppG cap analog inhibited poly(A) degradation *in vitro*, suggesting that 3′-end poly(A) removal is linked to 5′ end cap structure of the RNA substrates

USB1

USB1 removes poly(U) tails from U6 snRNAs and poly(A) tails from miRNAs. USB1-deficiency leads to pre-mRNA splicing defects and miRNA destabilization, resulting in developmental defects of various species and hematopoiesis in human.

Nascent human U6 snRNA transcripts are transcribed with a heterogeneous polyuridine 3′ end, owing to the stochastic nature of RNA polymerase III terminations. While TUT1 catalyzes 3′ polyuridylation of U6, USB1 removes 3′ uridines from U6



Biochemical roles of USB1

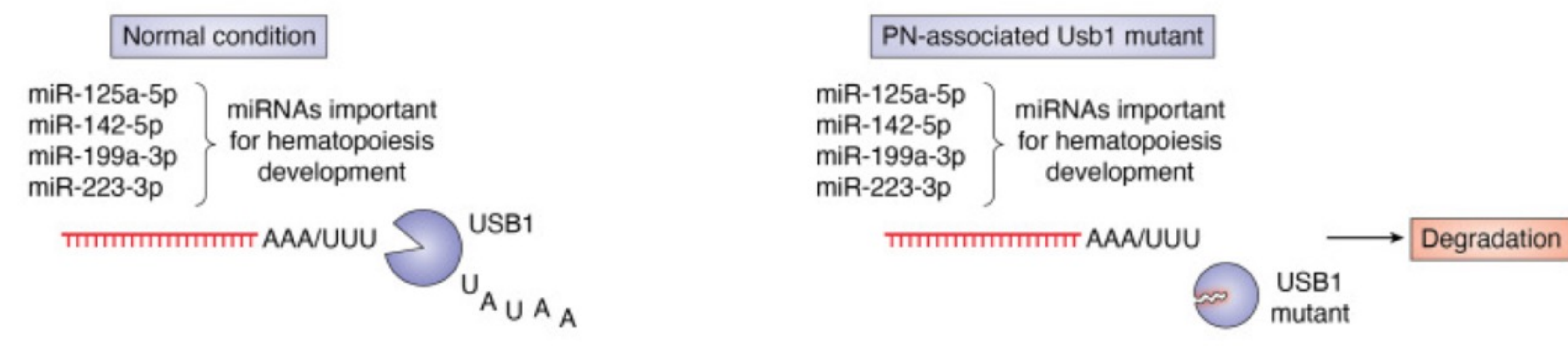
Crystallography studies showed that even with low similarity in sequence identity (<20%), humans and *S. cerevisiae* USB1 share highly similar structures

In vitro analyses showed that the human USB1 post-transcriptionally removes uridine and adenosine nucleosides from the 3′ ends of spliceosomal U6 snRNAs, and can catalyze terminal 2′, 3′-cyclic phosphate formation. USB1 measures the appropriate length of the U6 oligo(U) tail by reading the position of a key adenine nucleotide (A102) and pausing five uridine residues downstream.

In *S. cerevisiae*, an unbiased genetic screen revealed that the yeast ortholog of *USB1* is essential for U6 snRNA biogenesis and cell viability. In *S. cerevisiae*, USB1 mainly removes a single nucleotide from U6, leaving a 3′ monophosphate which strongly inhibits further processing

Trimming microRNA for blood development

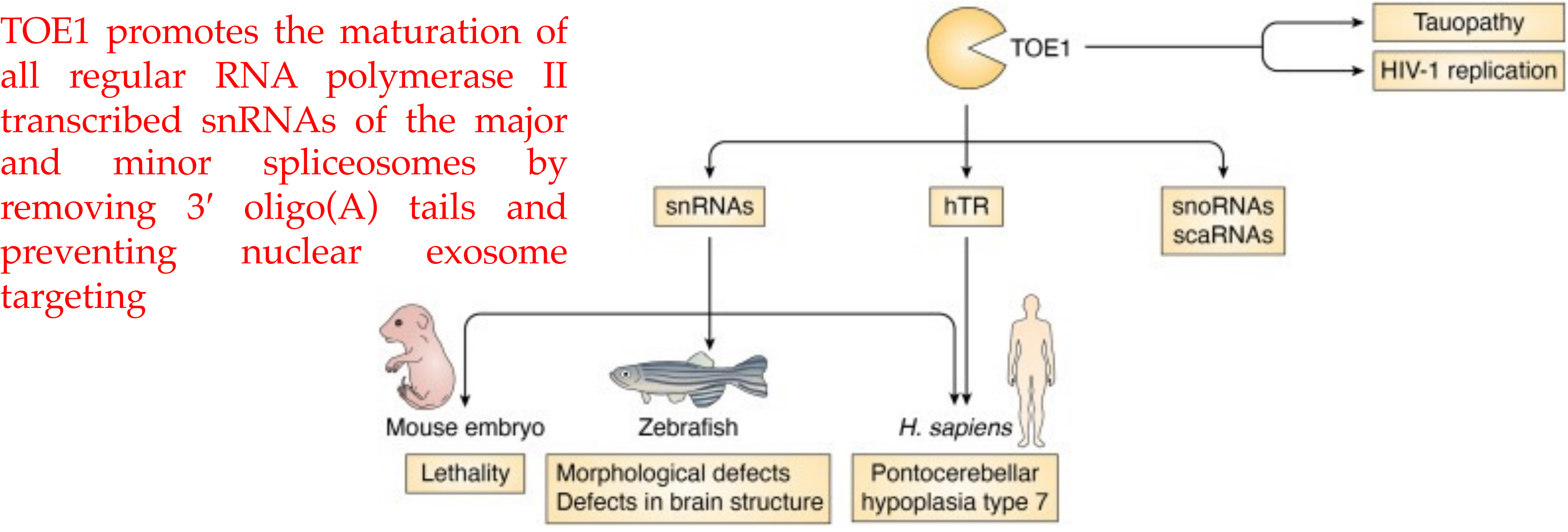
Mutations in the 3′ -> 5′ RNA exonuclease USB1 cause Poikiloderma with Neutropenia pediatric disease with defects in the production of blood cells. USB1 removes extra adenosines from the 3′ end of microRNAs, which if not removed, promote degradation of microRNAs that are necessary for blood development. Blocking the enzyme that adds extra adenosines to microRNAs also restores the production of blood cells in mutant settings.



TOE1

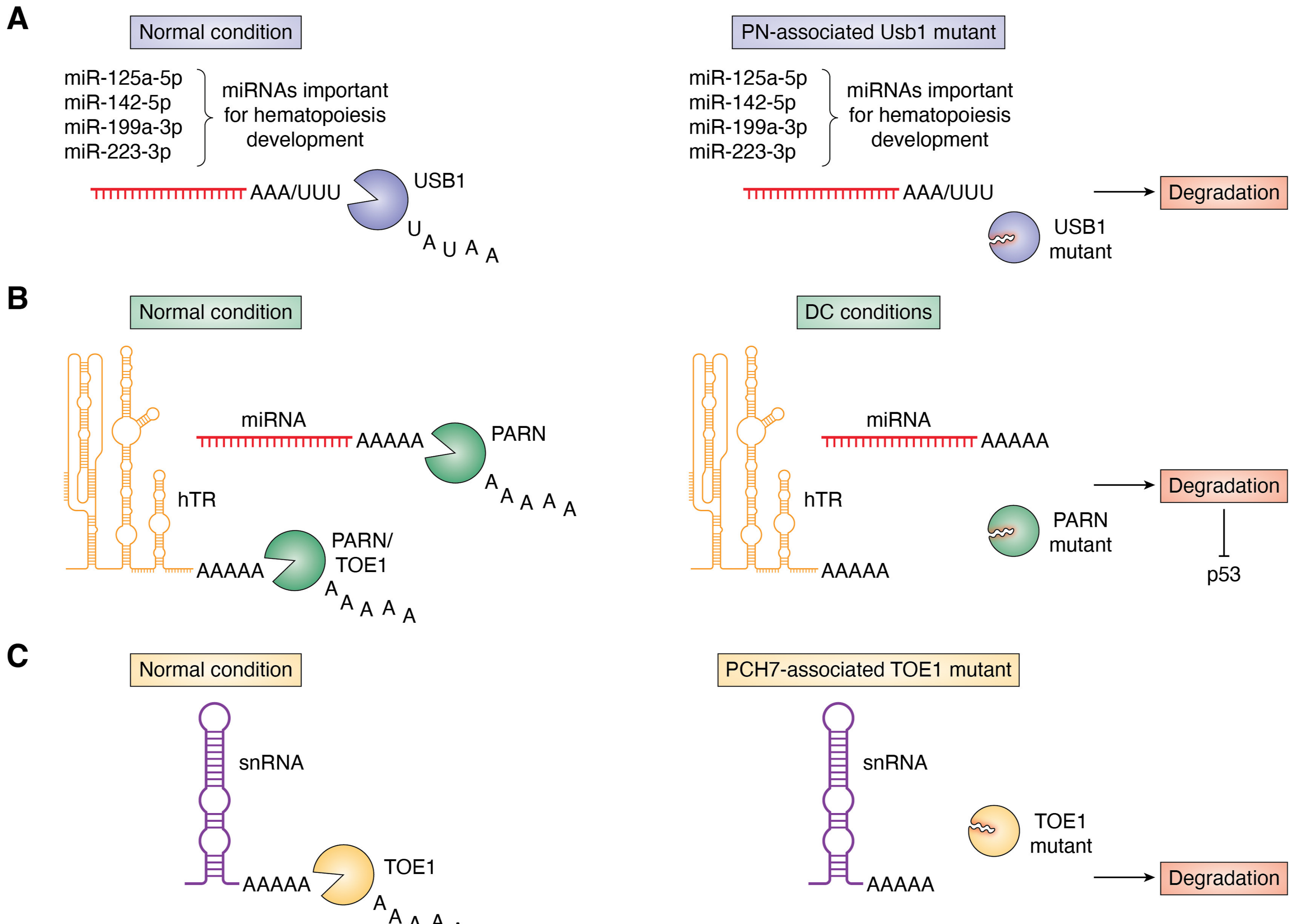
TOE1 removes poly(A) tails from snRNAs, hTR, snoRNAs, and scaRNAs. TOE1 mutants in PCH7 patients and PCH7-modeled mice and zebrafish lead to extended adenylated 3′ ends of hTR and snRNAs, resulting in developmental defects and defects in brain structures

TOE1 promotes the maturation of all regular RNA polymerase II transcribed snRNAs of the major and minor spliceosomes by removing 3′ oligo(A) tails and preventing nuclear exosome targeting



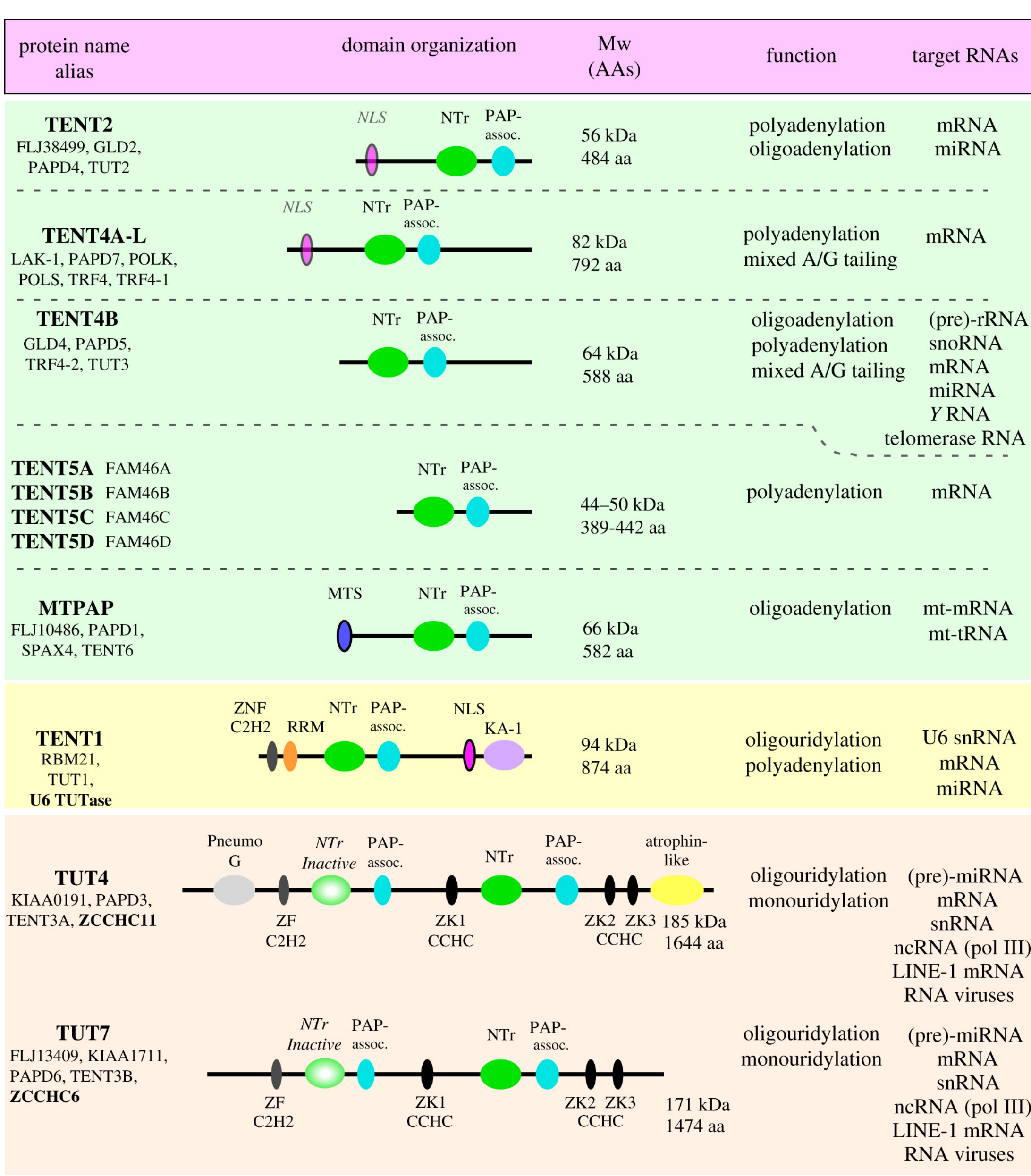
Since TOE1 shows high similarity to PARN through evolutionary distance analysis , but it does act redundantly with PARN in removing 3′ end tails of RNAs. In HeLa cells, TOE1 was shown to localize to Cajal bodies, while PARN is primarily in the nucleoli, indicating that TOE1 and PARN have distinct subcellular locations

non-coding RNA stability through ribonucleases



Terminal Nucleotidyl Transferases

In recent years, 11 TErminal NucleotidylTransferases (TENTs) have been discovered in human. Based on their substrate preference towards adenosine monophosphate (AMP) or uridine monophosphate (UMP) incorporation, human TENTs are divided into non-canonical poly(A) polymerases (ncPAPs) and terminal uridyl transferases (TUTases). TENTs are not restricted to the nucleus and have specific regulatory roles also in the cytoplasm and mitochondria.



TENT4A and 4B are human orthologues of the yeast Trf4/5 proteins

TENT2 (GLD2)

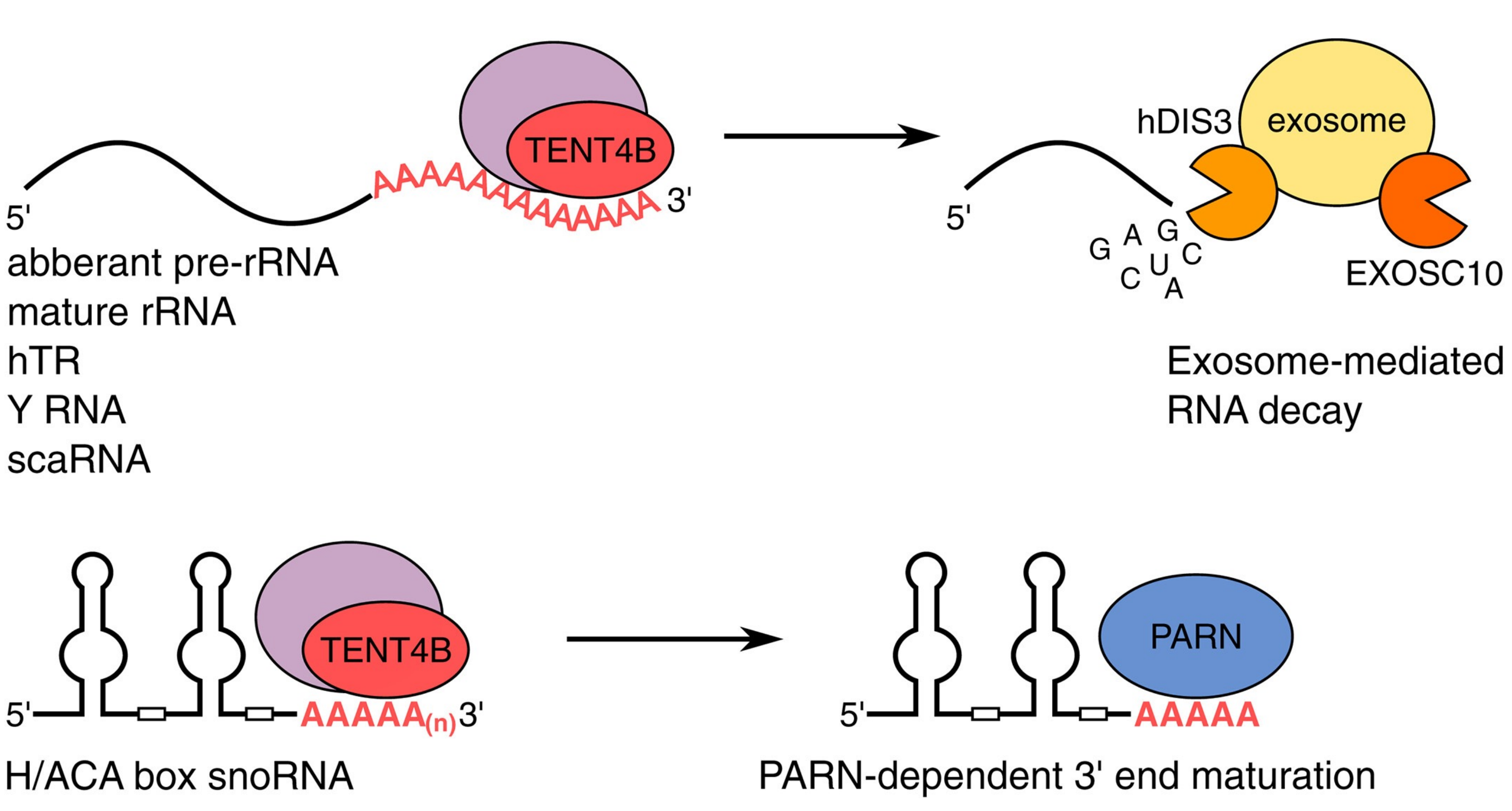
TENT2 plays as important role in gametogenesis and early development in non-mammalian species by acting as a ncPAP with a key role in translational activation of a subset of cytoplasmic mRNAs through elongation of their poly(A) tails. However, TENT2-deficient mice of both sexes are fertile and do not demonstrate any gross phenotype.

TENT2 activity may be necessary for long-term memory formation in mice as it is expressed in the hippocampus and co-localizes with proteins involved in synaptic plasticity.

TENT2 is responsible for monoadenylation of certain mature miRNAs like a liver-specific miRNA-122. Since in TENT2 knock-out mice the miRNA-122 level is significantly lower than in wild-type mice, it has been suggested that monoadenylation of miRNA by TENT2 enhances its stability.

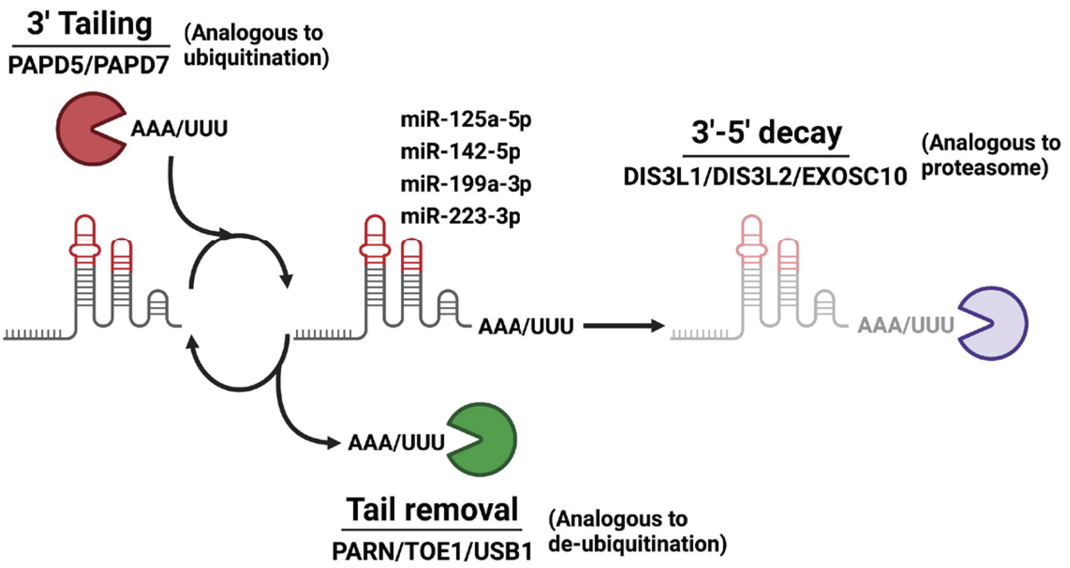
TENT4B/A

In the nucleus, polyadenylation by TENT4B/A, acting alone or in complex with other proteins, induces the exosome-mediated decay of various RNA species. TENT4B/A also cooperates with the poly(A)-specific ribonuclease PARN to promote H/ACA box snoRNA maturation.



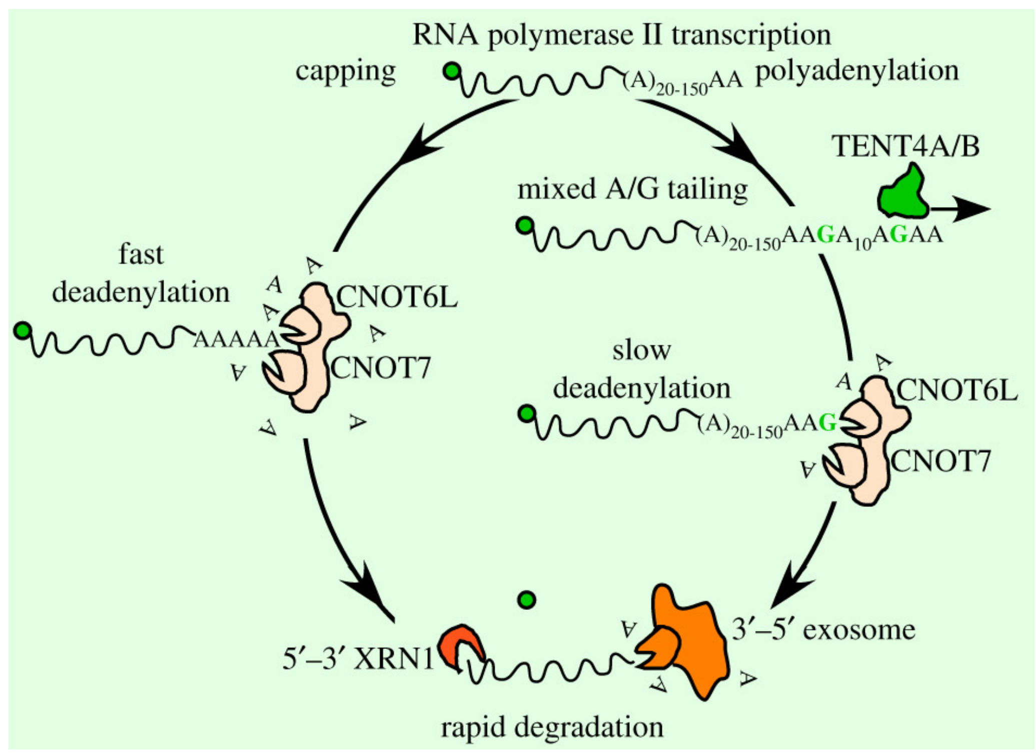
Regulation of noncoding RNA stability through competition between "Tailing" and "Tail removal" enzymes

This system is analogous to the ubiquitin-mediated proteasome degradation system, in which RNAs are tagged for degradation by 3′-end modification by enzymes such as TENT4A(PAPD5) and TENT4B(PAPD7), and protector exonucleases such as PARN and USB1 remove the posttranscriptional modifications to stabilize the RNA. In the absence of tail removal, the 3′-end–modified RNA would be degraded by 3′ to 5′ exonucleases



TENT4A and TENT4B generate specialized tails that protect mRNAs from active deadenylation

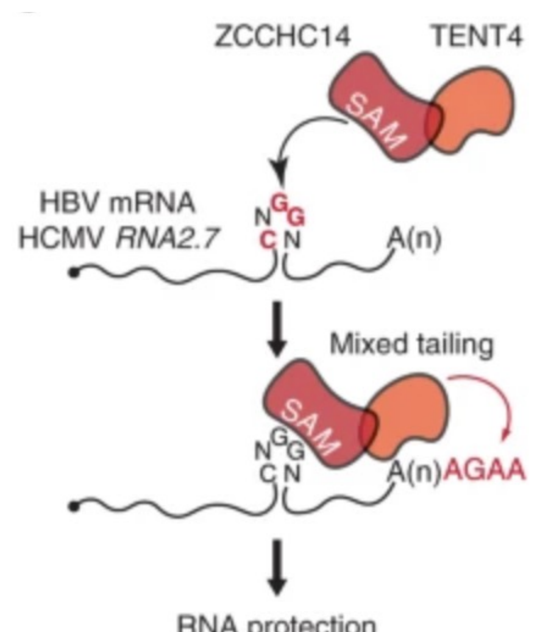
TENT4A and TENT4B extend mRNA poly(A) tails with intermittent nonadenosine residues, most commonly **guanosine**, to generate ‘mixed tails. A single guanosine residue is sufficient to impede the deadenylase CCR4-NOT complex. Consistently, depletion of TENT4A and TENT4B leads to a decrease in mRNA half-life and abundance in cells.



Lim et al., 2018 Science

Viral hijacking of the TENT4–ZCCHC14 complex protects viral RNAs via mixed tailing

HBV mRNAs and HCMV RNA2.7 recruit the TENT4–ZCCHC14 complex via the CNGGN pentaloop to induce targeted mixed tailing, which subsequently protects the viral RNAs from cellular decay factors.



SAM = sterile alpha motif

Kim et al. 2020 Nat Struct Mol Biol

TENT5 proteins

In stark contrast to the scarcity of studies describing TENT5 proteins' activity on a molecular level, there are plenty of reports linking mutations in TENT5 proteins to multiple less or more severe conditions.

TENT5A was first described as C6orf37 (Chromosome 6 open reading frame 37), a protein of unknown function with possible relation to human retinal diseases. Moreover, it has been shown that polymorphism in the second exon of TENT5A may be associated with an increased risk of large-joint osteoarthritis, which is consistent with severe skeletal abnormalities of TENT5A knock-out mice. Loss-of-function mutations in TENT5A have been reported in patients suffering from severe, autosomal recessive forms of osteogenesis imperfecta.

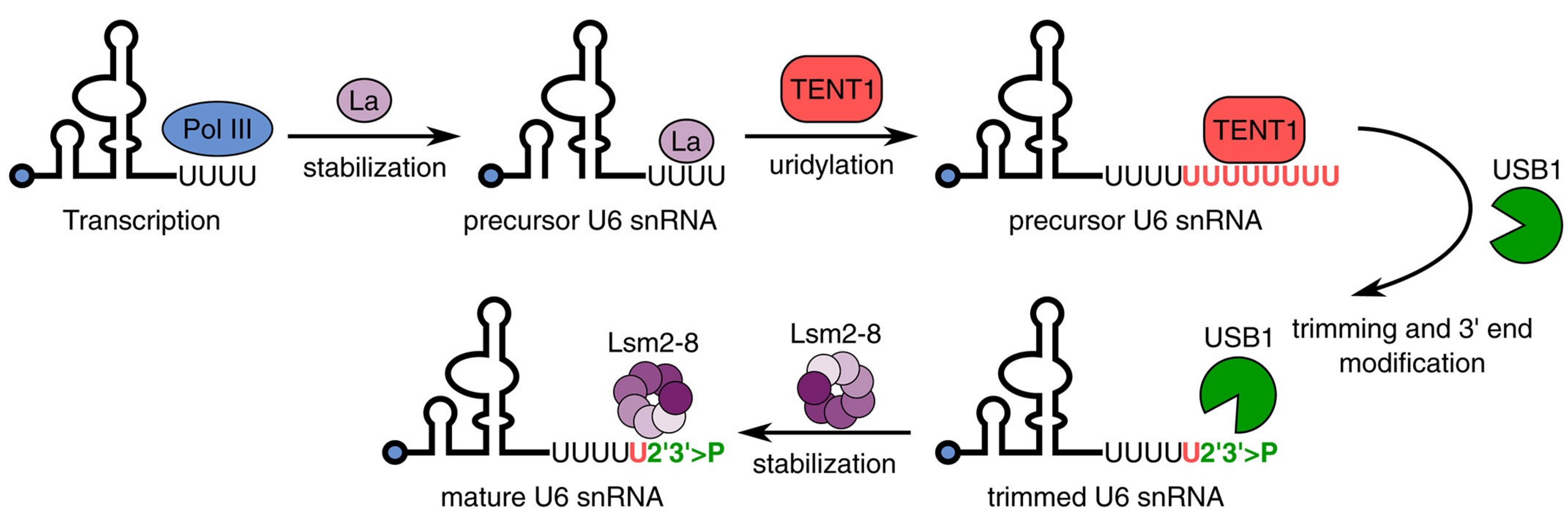
TENT5C is one of the most frequently mutated genes in a B-cell malignancy—multiple myeloma (MM). Besides MM, the TENT5C gene is suggested to play a role in the pathogenesis of other tumours.

TENT5D dysfunction might also be related to autism as TENT5D is overexpressed in the cerebral cortex of mice with autism-like behaviours.

U-tail

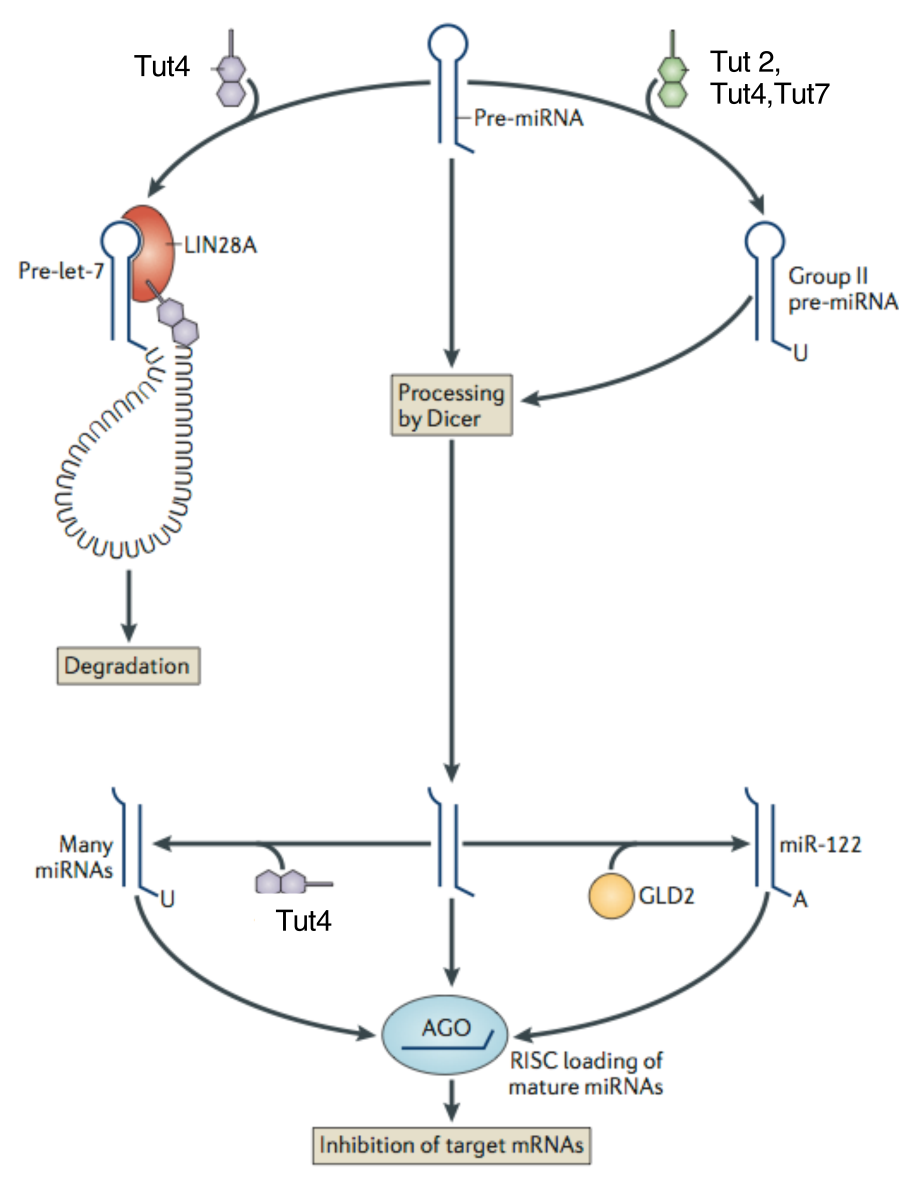
TENT1 is required for U6 snRNA maturation

Following transcription by Pol III, La protein protects the U6 snRNA precursor by binding the four uridines at the 3′ end. La protein is later replaced by TENT1, which uridylates the U6 snRNA 3′ end. The 3′-5′ exoribonuclease and phosphodiesterase USB1 removes uridines, leaving only five of them, and a terminal 2′,3′ cyclic phosphate (2′3′ > P). The U6 snRNA is further protected by recruitment of the Lsm2-8 protein complex.



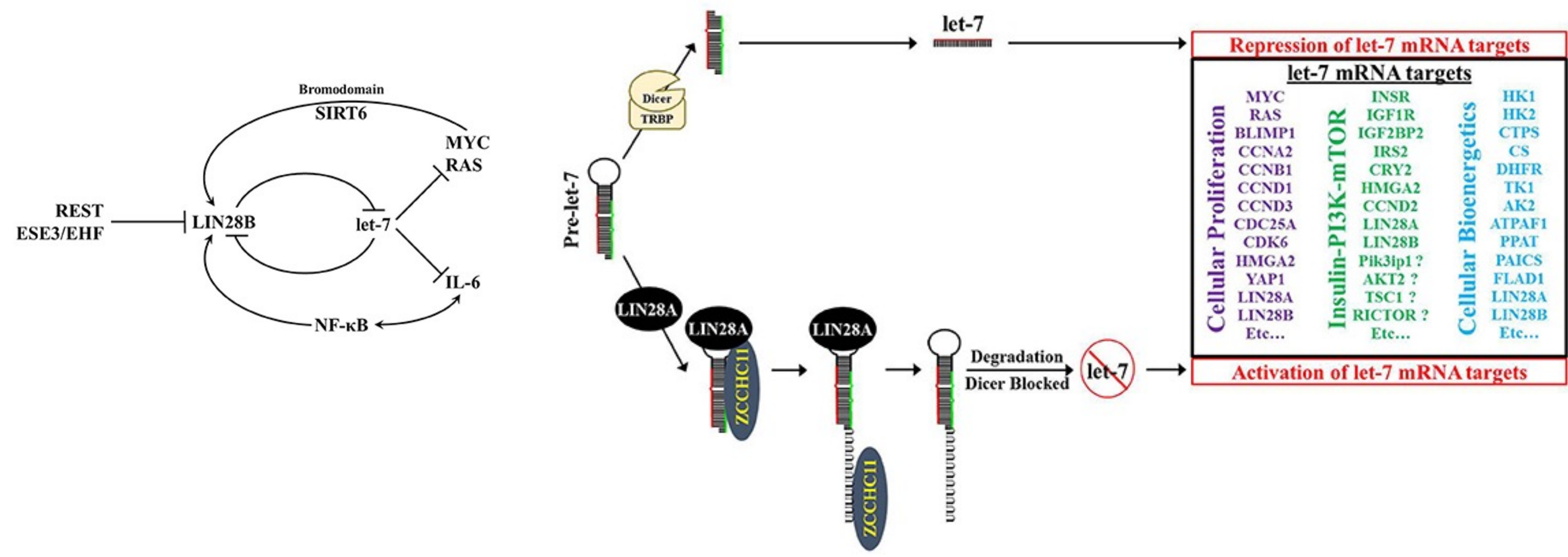
Addition of non-templated nucleotides to pre-microRNAs and microRNAs

In the presence of the RNA-binding protein **LIN28**A, pre-miRNAs of the let-7 family may be oligouridylated by **ZCCHC11/Tut4**, which targets them for degradation. By contrast, group II pre-microRNAs, which have a shorter 3' overhang (1 nt) that is suboptimal for Dicer processing, require monouridylation, carried out by Tuts (2, 4 and 7), before Dicer processing. Tut4 and GLD2 also act in a miRNA-specific manner to add single uridylyl or adenylyl residues, respectively, to miRNA 3' ends after Dicer cleavage. In the case of miR-122, terminal adenylation seems to be important for miRNA stability.



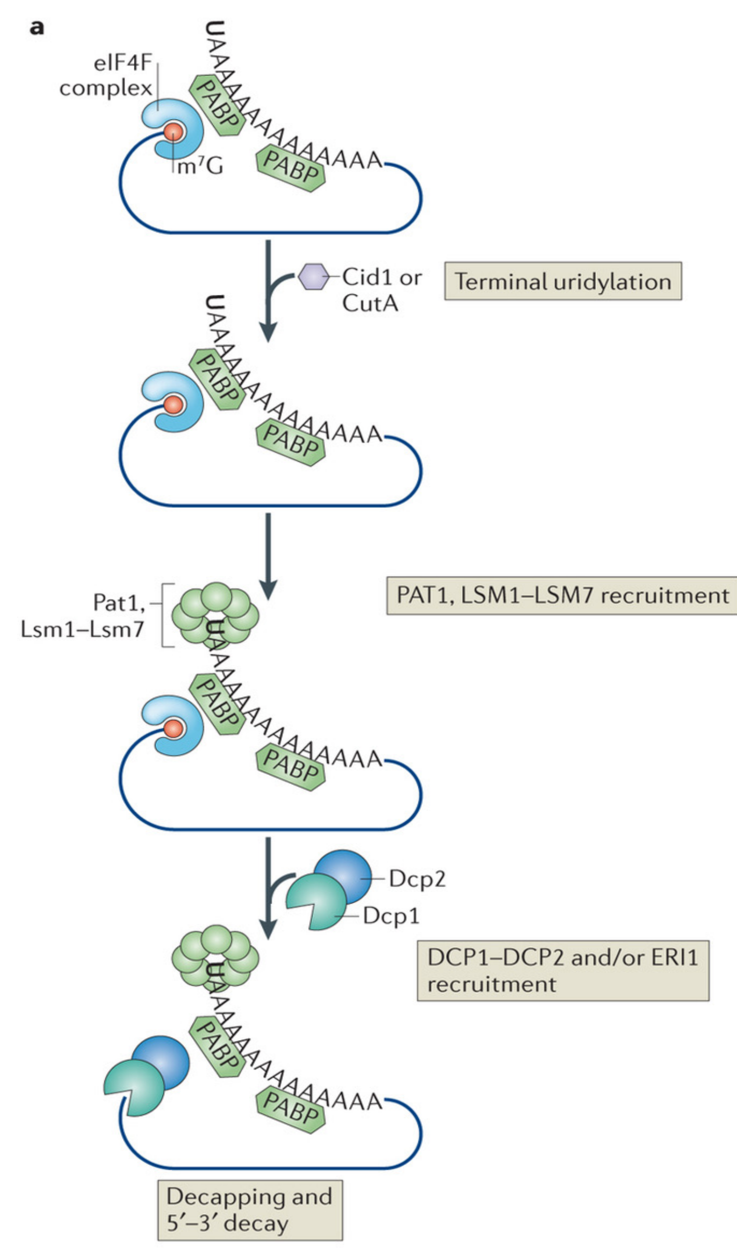
The LIN28/let-7 Pathway in Cancer

LIN28B is highly expressed during embryogenesis and as differentiation progresses, LIN28B expression is lost. In adult mammals, only a small subset of somatic cells exist where LIN28B expression occurs. However, LIN28B is upregulated in tumors. LIN28B recruits the TUTase ZCCHC11 to pre-let-7 where ZCCHC11 adds a short polyU tail to pre-let-7. Pre-let-7 is no longer a DICER substrate and is targeted for degradation, thereby blocking let-7 maturation into its functional tumor suppressor form. Loss of mature let-7 microRNAs causes overexpression of numerous oncogenes and bioenergetic genes.



Uridylation of cytoplasmic mRNAs in fungi

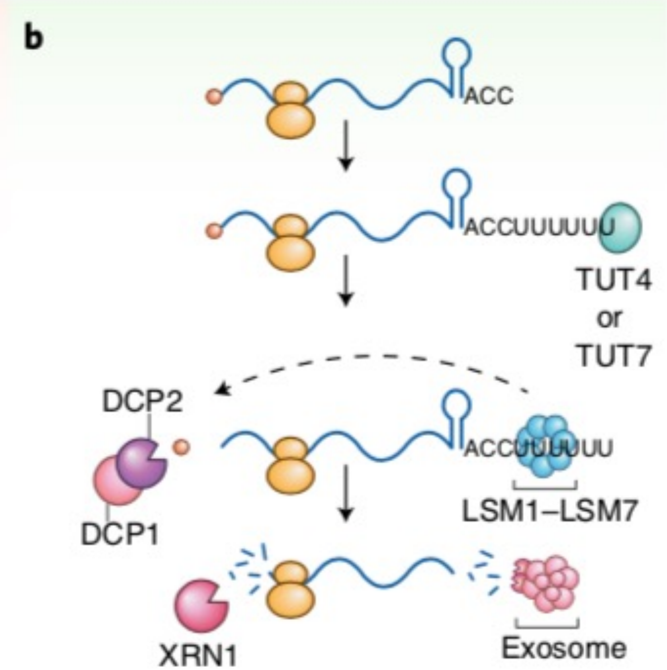
A polyadenylated cytoplasmic mRNA in *Schizosaccharomyces pombe* or *Aspergillus nidulans* is subject to 3′ uridylation by **Cid1** or **CutA**, respectively. The uridylated mRNA is a preferred binding target of the **Pat1** and **Lsm1–Lsm7** complex, which in turn recruits the decapping enzyme **Dcp1–Dcp2**. This leads to translational silencing of the mRNA and allows its degradation by 5′–3′ exonucleolysis.



Uridylation of histone mRNAs

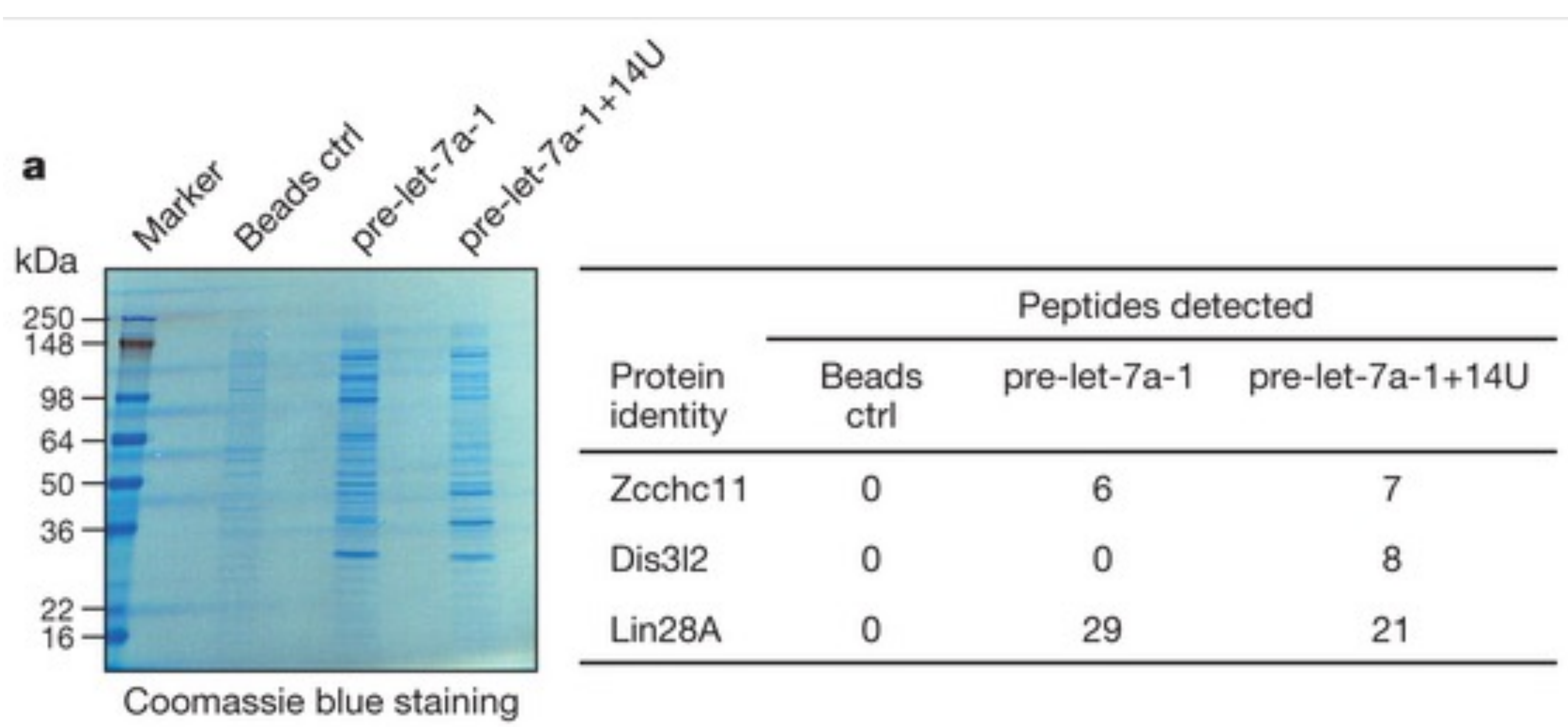
Uridylation of histone mRNAs is required for their rapid turnover following inhibition of DNA replication. It is important for the prevention of potentially toxic histone accumulation following the completion of chromosomal DNA replication.

- Degradation of replication-dependent histone mRNAs. At the end of S phase, poly(A)$^{-}$ histone mRNAs are uridylated by TUT4 and TUT7.
- Binding of the LSM1–LSM7 complex to the oligo(U) tail facilitates decapping and subsequent 5′-to-3′ decay.



Dis3L2 is associated with uridylated pre-let-7

RNA affinity pull-down with synthetic pre-let-7a-1 or pre-let-7a-1+14U was conjugated to and incubated with whole-cell extract to isolate factors that specifically associate with uridylated pre-let-7. This analysis showed that Lin28A and Zcchc11/TuT4 were associated with both pre-let-7a-1 and uridylated pre-let-7a-1 (pre-let-7+14U), whereas **Dis3L2,** a 3′->5′ exonuclease, was <u>specifically detected in the pre-let-7a-1+14U purification</u>.

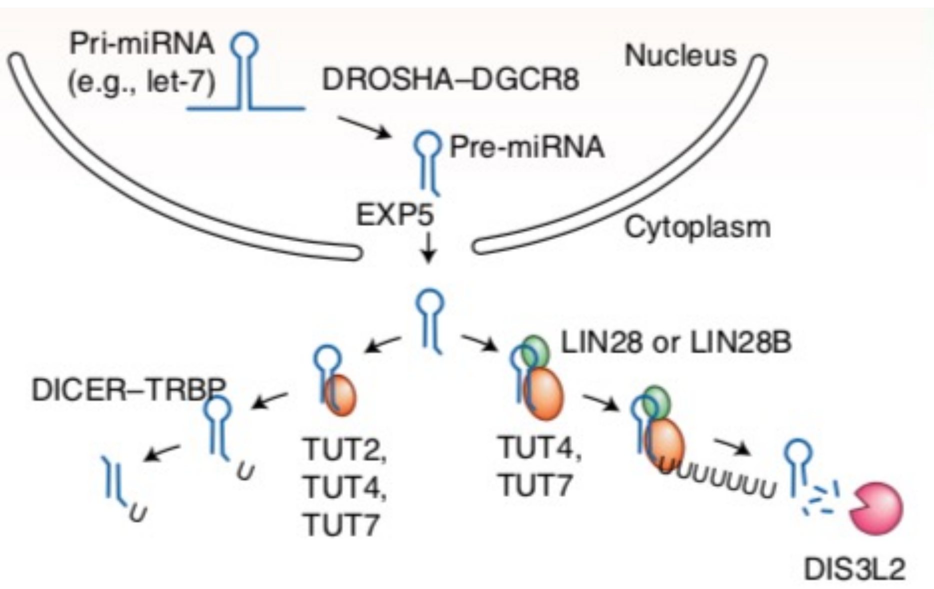


Protein identity	Peptides detected: Beads ctrl	pre-let-7a-1	pre-let-7a-1+14U
Zcchc11	0	6	7
Dis3l2	0	0	8
Lin28A	0	29	21

Chang et al. 2013. Nature

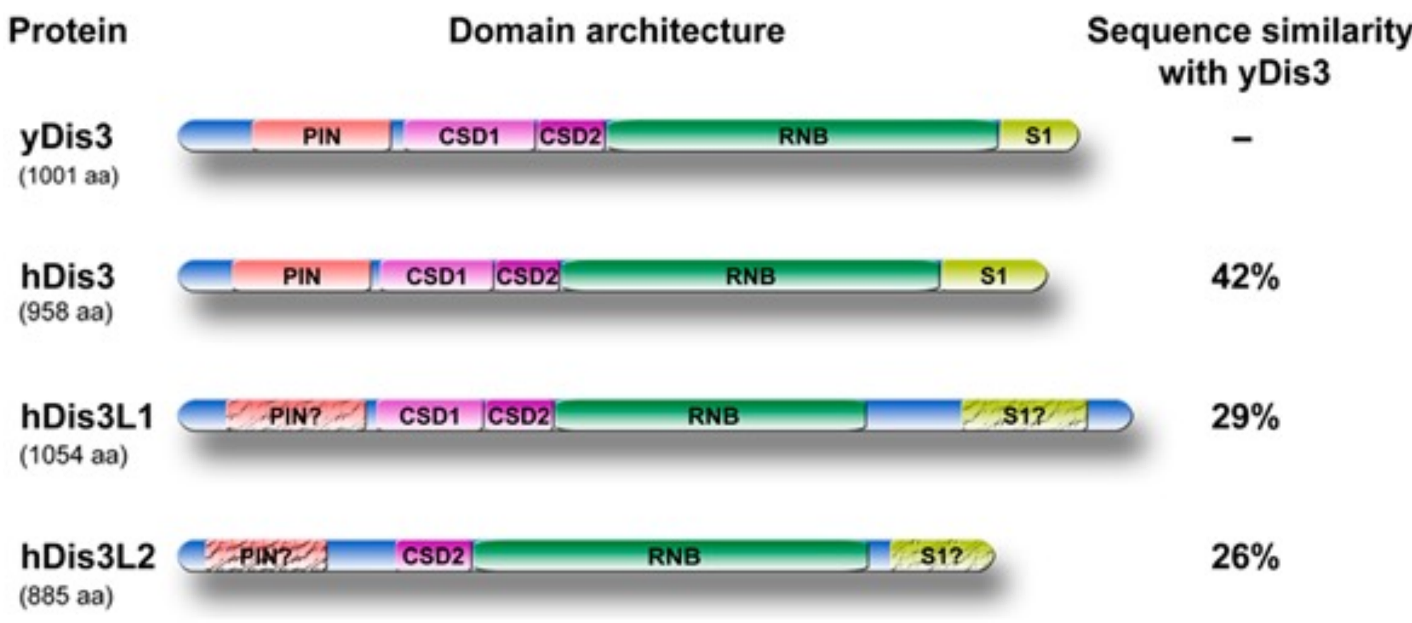
TUT-DIS3L2 regulates miRNA synthesis

The dual roles of precursor miRNA (uridylation. Primary miRNAs (pri-miRNAs) are processed by DROSHA and DICER to produce mature miRNA. Pre-let-7 is monouridylated by TUT2, TUT4 or TUT7, which enhances DICER processing. However, in embryonic stem cells and some cancer cells, LIN28 proteins bind to pre-let-7 and induce oligouridylation, which blocks processing and facilitates degradation by DIS3L2.



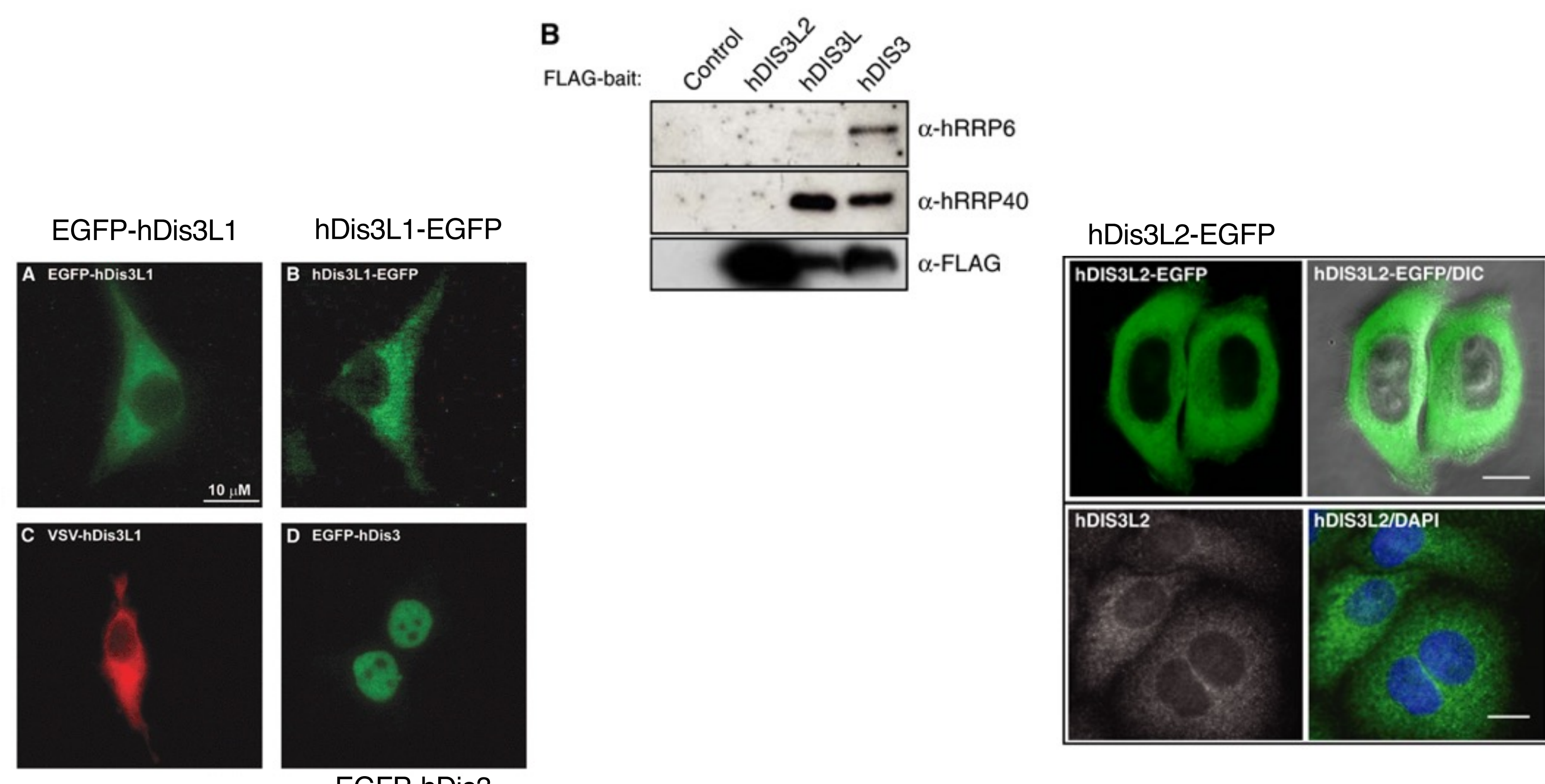
Human-specific Dis3 exonucleases

In the genome of human beings and other higher eukaryotes (including mice, zebrafish and frogs), in total three genes homologous to the yeast Dis3 protein are present: **Dis3**, **Dis3-like exonuclease 1** (**Dis3L1**) and **Dis3-like exonuclease 2** (**Dis3L2**).

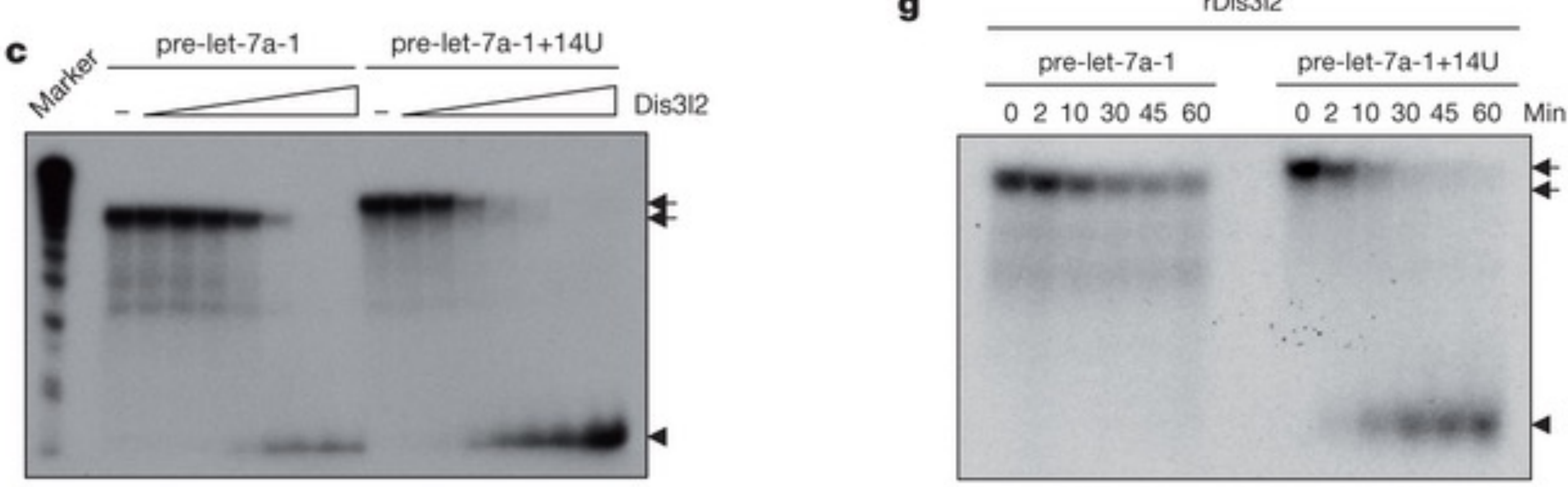


hDIS3L2 is an RNase II/R family-related and exosome-independent factor

Only hDis3 and hDis3L1 were found in the immunoaffinity-purified exosome fraction while hDis3L2 did not. hDis3 is mainly localised in the nucleus while hDis3L1 and hDis3L2 are mainly localised in the cytoplasm.

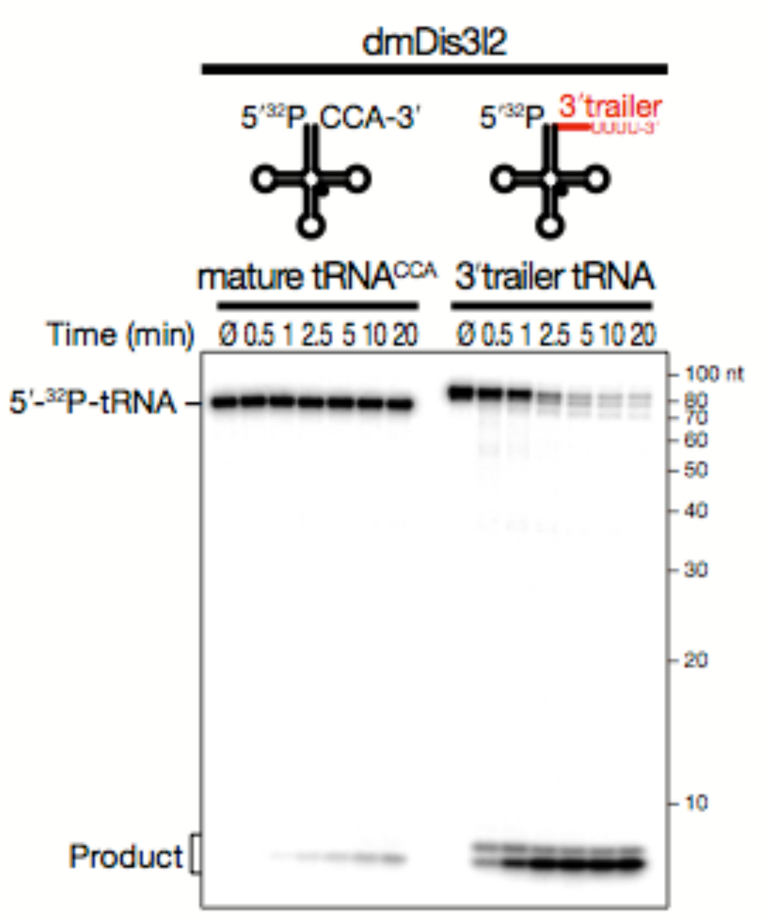


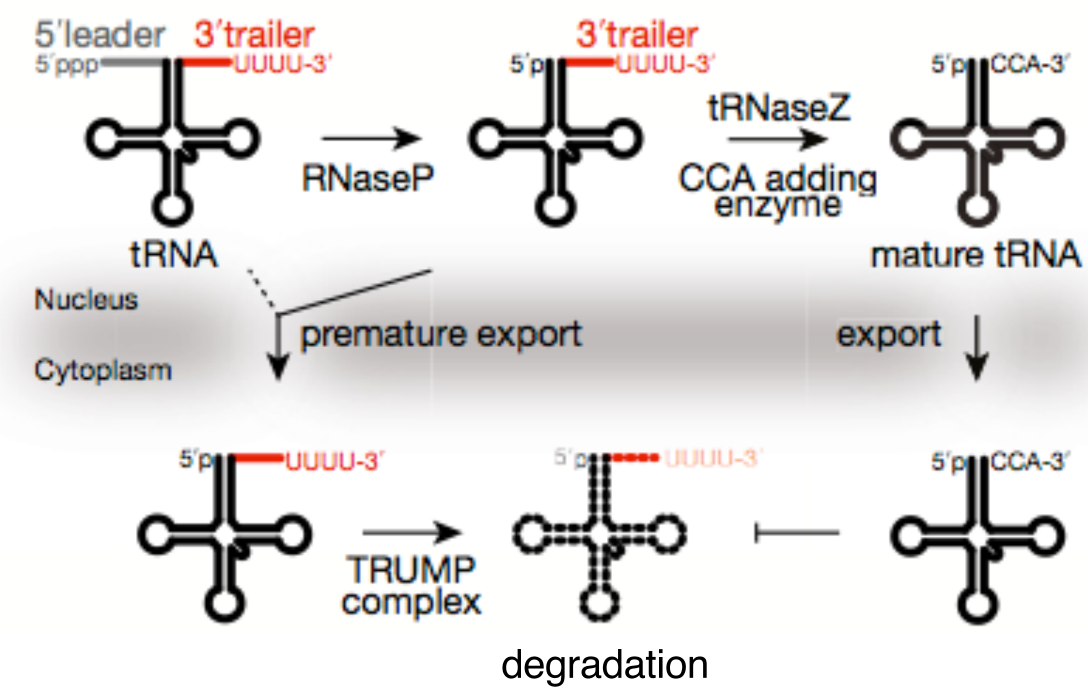
Dis3L2 preferentially degrades uridylated RNAs



RNA degradation assay with purified Flag–Dis3l2 (c) or recombinant His-Dis3L (g) incubated with different radiolabelled pre-miRNAs.

The Dis3L/TUT complex (TRUMP complex) targets unprocessed tRNA for decay.





TUT-DIS3L2 is a mammalian surveillance pathway for aberrant structured non-coding RNAs.

mRNA
snRNA
miRNA
Pol II
TUTase
DIS3L2
rRNA
Pol I
TUTase
DIS3L2
tRNA
5S rRNA
Y-RNA
Vault RNA
snoRNA
Pol III
TUTase
DIS3L2
NUCLEUS
CYTOPLASM

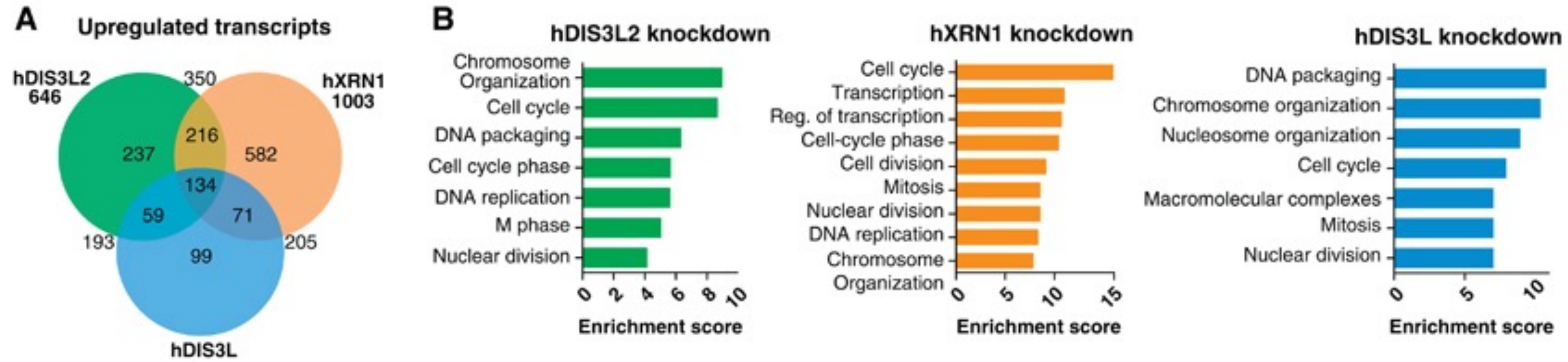
Germline mutations in *DIS3L2* cause the Perlman syndrome of overgrowth and Wilms tumor susceptibility

Dewi Astuti[1,9], Mark R Morris[1,2,9], Wendy N Cooper[1], Raymond H J Staals[3], Naomi C Wake[1], Graham A Fews[4], Harmeet Gill[1], Dean Gentle[1], Salwati Shuib[1], Christopher J Ricketts[1], Trevor Cole[4], Anthonie J van Essen[5], Richard A van Lingen[6], Giovanni Neri[7], John M Opitz[8], Patrick Rump[5], Irene Stolte-Dijkstra[5], Ferenc Müller[1], Ger J M Pruijn[3], Farida Latif[1] & Eamonn R Maher[1,4]

Perlman syndrome is a congenital overgrowth syndrome inherited in an autosomal recessive manner that is associated with tumor susceptibility. Affected children are large at birth, are hypotonic and show organomegaly, characteristic facial dysmorphisms, renal anomalies, frequent neurodevelopmental delay and high neonatal mortality.

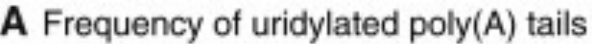
hDIS3L2 contributes to the maintenance of cellular RNA homeostasis

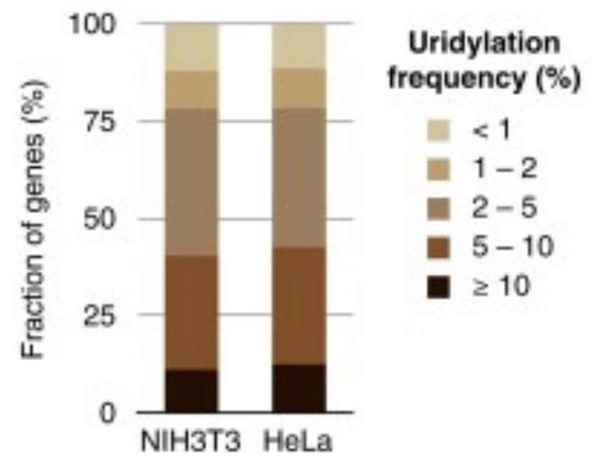
Genome-wide view of the effects of hDIS3L2 depletion on cellular RNA metabolism compared with the depletion of other cytoplasmic exonucleases (hDIS3L and hXrn1) showed up-regulation of specific mRNAs.



Human mRNAs are urydilated

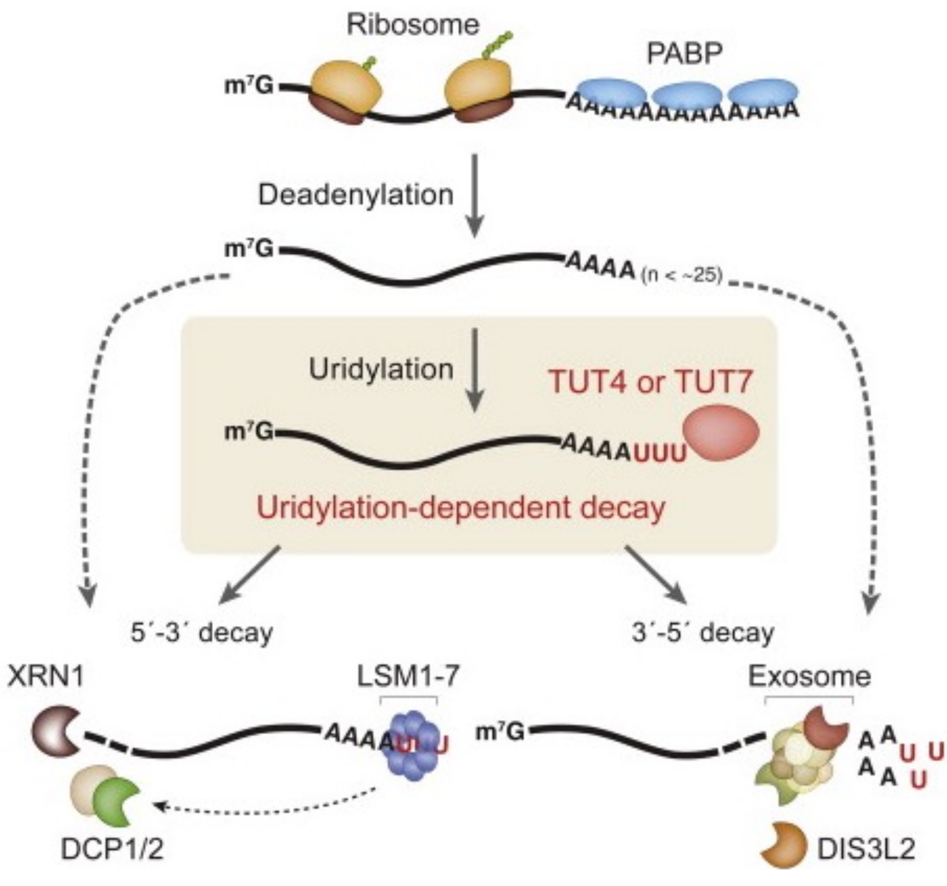
About half of mRNA species of human cell lines carry U-tails at more than 5% frequency, and ~80% of mRNA species are uridylated at a frequency higher than 2%. Some mRNAs such as encoding *suppressor of glucose autophagy associated 2* (*SOGA2*) and encoding *cytoplasmic poly(A) binding protein 4* (*PABPC4*) are frequently uridylated (41% and 24%, respectively), suggesting that uridylation may have biological importance. Interestingly, U-tails are found mainly on mRNAs with short A-tails (less than ~25 nt), indicating that uridylation may occur following deadenylation.





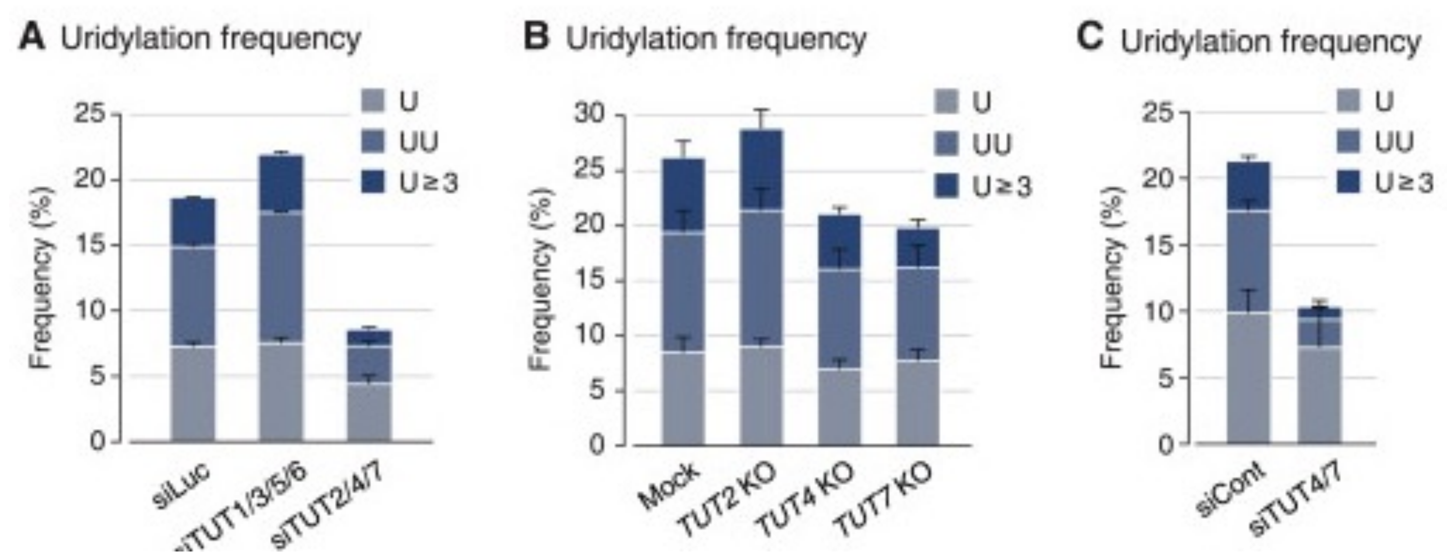
New Uridylation-Dependent mRNA Decay pathway in Humans

mRNA decay is generally initiated by deadenylation. PABP proteins are dissociated from mRNA as poly(A) tail becomes shorter (less than ≤25 nt). TUT4 and TUT7 act redundantly to uridylate mRNAs with a short A-tail. The U-tail is in turn recognized by the downstream decay factors. The LSM1–7 complex binds to the U-tail and facilitates decapping by the DCP1/2 complex. Decapped mRNAs are degraded by the 5′–3′ exonuclease XRN1. Alternatively, the U-tail is recognized by exosome or DIS3L2 that degrade mRNA exonucleolytically from the 3′ end.



TUT4 and TUT7 Catalyze mRNA Uridylation

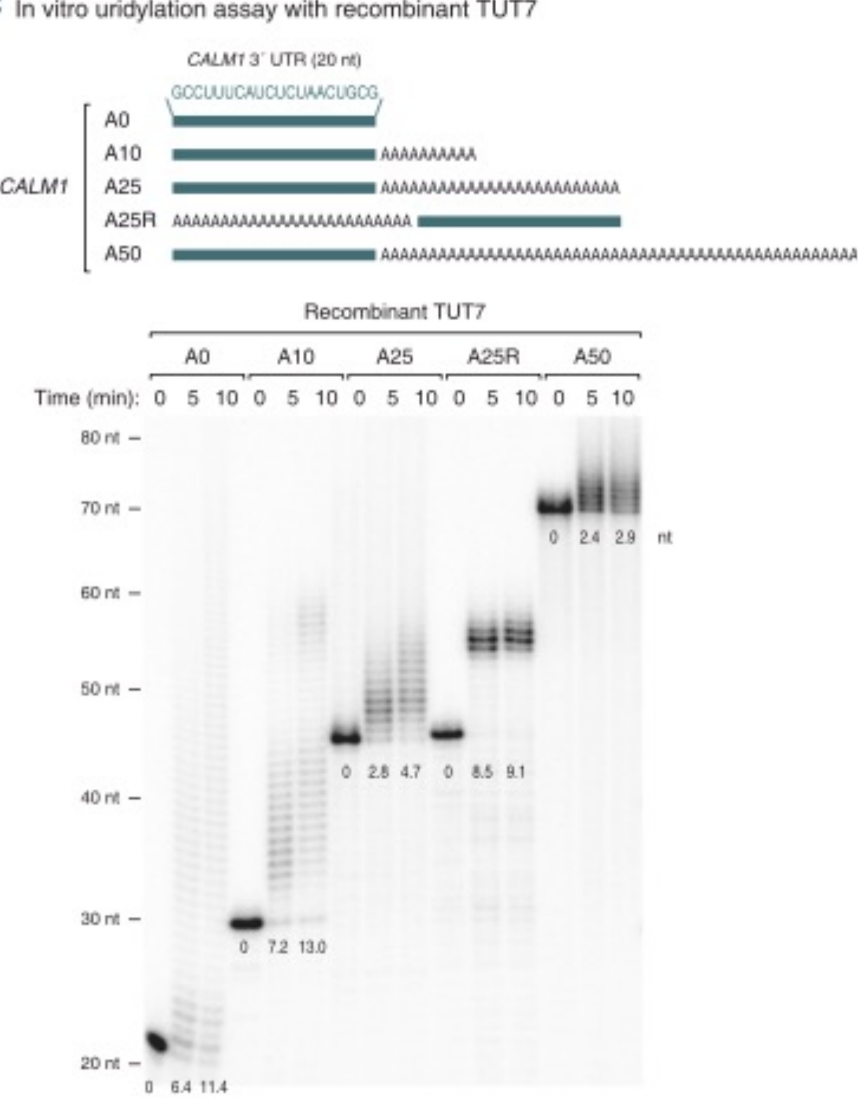
In order to identify enzyme(s) responsible for mRNA uridylation, they depleted the seven human TUTases by RNAi (kd) or by TALENs and carried out TAIL-seq. Interestingly, when TUT4/7 were depleted, terminal uridylation was significantly reduced while depletion of TUT1/2/3/5/6 did not affect uridylation.



Uridylation frequency measured by TAIL-seq

TUT4/7 Selectively Oligo-Uridylate mRNAs with Short A-Tails In Vivo and In Vitro

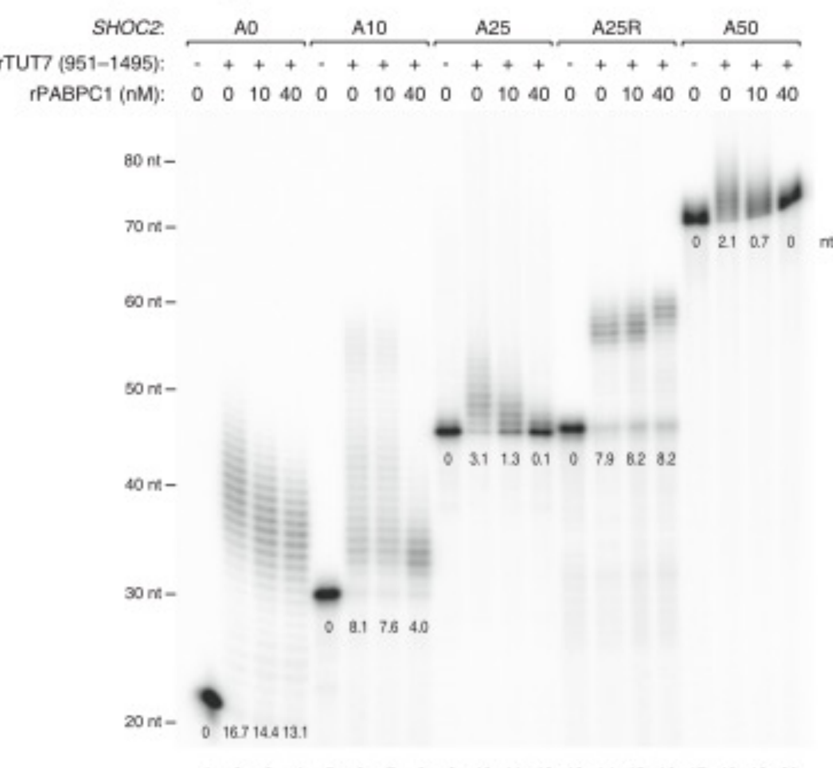
They also prepared recombinant TUT7 protein from *Escherichia coli* and used the protein for *in vitro* uridylation assay. The purified protein was fully capable of carrying out uridylation in an A-tail length-dependent manner with both RNAs. These results suggest that TUT4/7 possess an intrinsic ability to measure the 3′ terminal A length and avoid uridylation of long A-tails.



PABP Suppresses Uridylation of Poly(A)+ mRNA

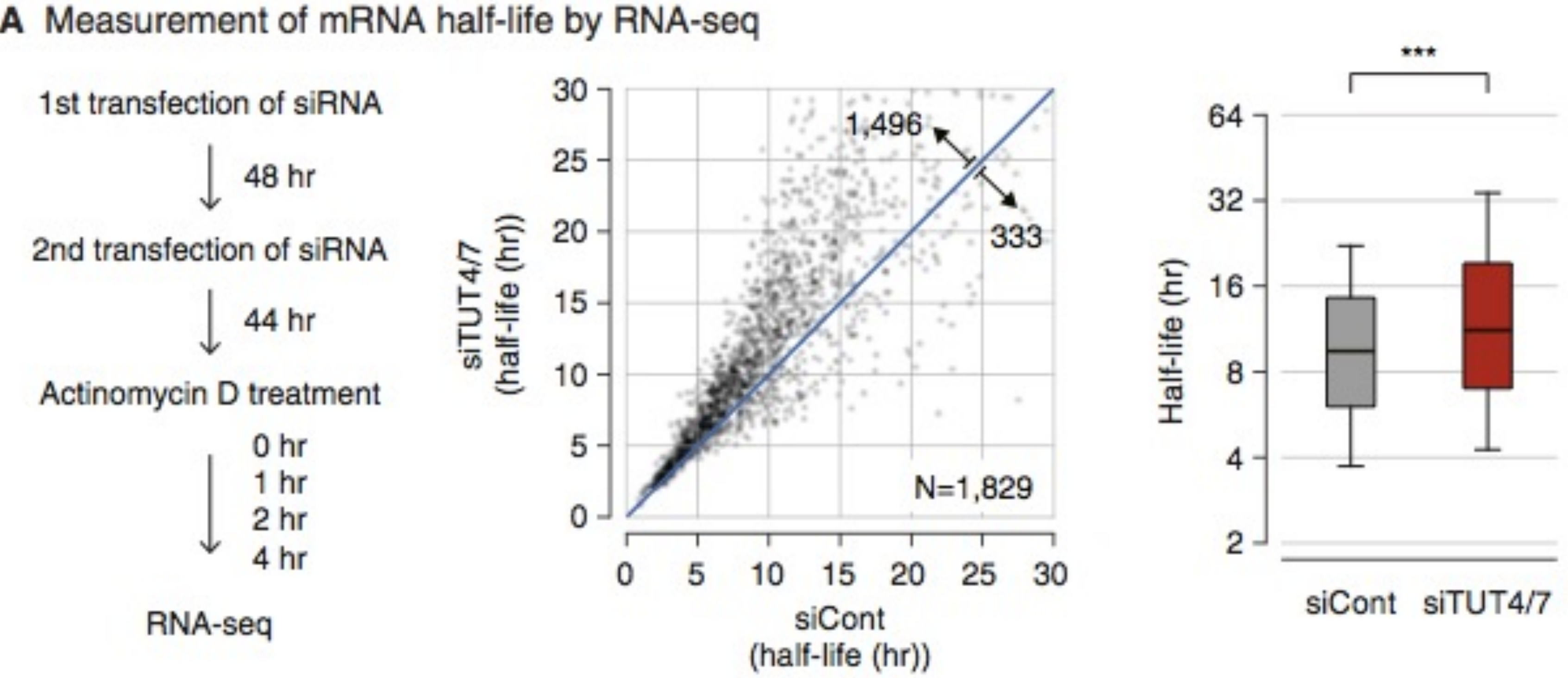
As poly(A)+ mRNAs are associated with **PABP** in cells, they asked if PABP has an influence on mRNA uridylation.

They carried out *in vitro* uridylation assays in the presence of recombinant PABPC1. Full-length PABP occupies an ~25 nt A-tail. When PABPC1 was added to RNA, uridylation of RNAs with long poly(A) tail (A25 and A50) was suppressed even at a low concentration of PABPC1 (10 nM) while those with no or short A-tail (A0, A10, and A25R) remained largely unaffected. This result suggests that PABPC1 binds preferentially to long poly(A) tails and protects them from TUT4/7 and thereby enhances the selectivity of uridylation according to poly(A) tail length.



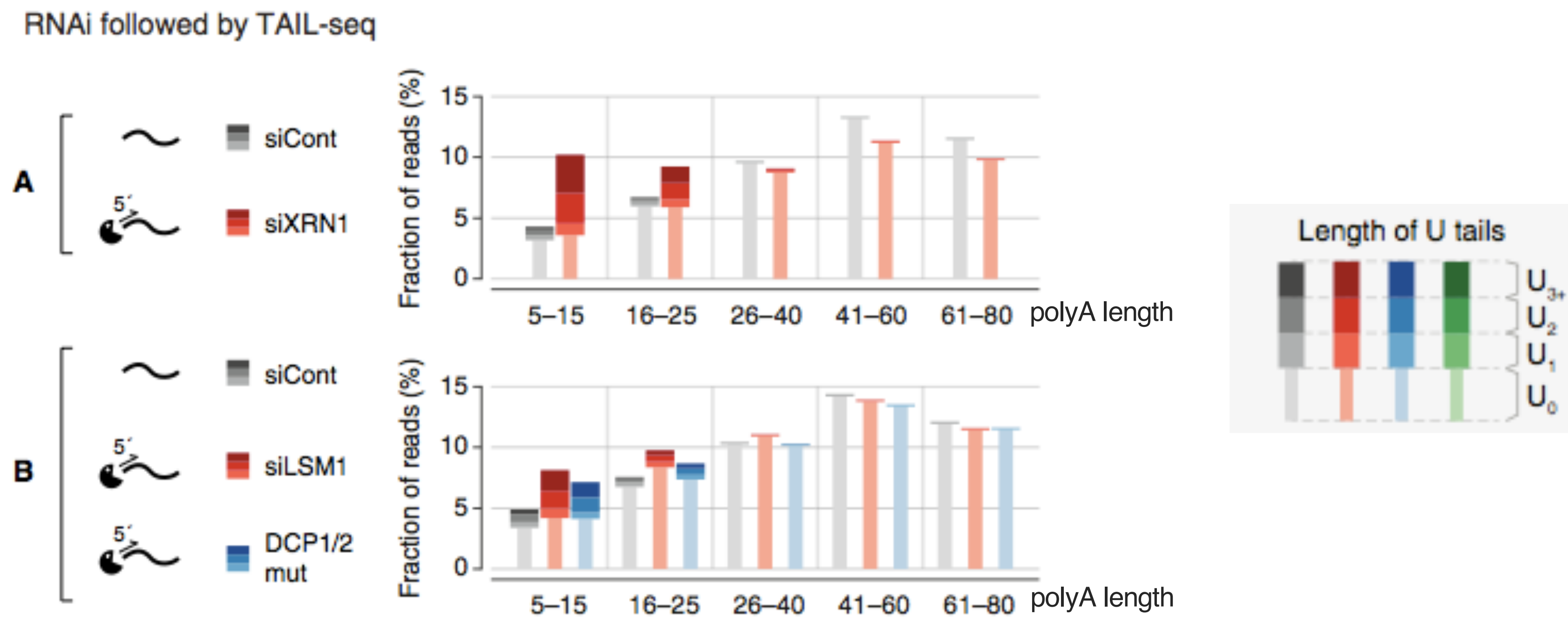
Uridylation Facilitates Global mRNA Decay

mRNA levels were determined by RNA-seq at 0, 1, 2, and 4 hr after actinomycin D treatment that blocks transcription. They could measure turnover rates of 1,829 mRNAs. In TUT4/7-depleted cells, the majority of mRNAs (1,426 out of 1,829 [78.0%]) showed increase stability. Half-lives were increased by ~30% on average, and median half-life was extended from 9.45 hr to 11.2 hr.



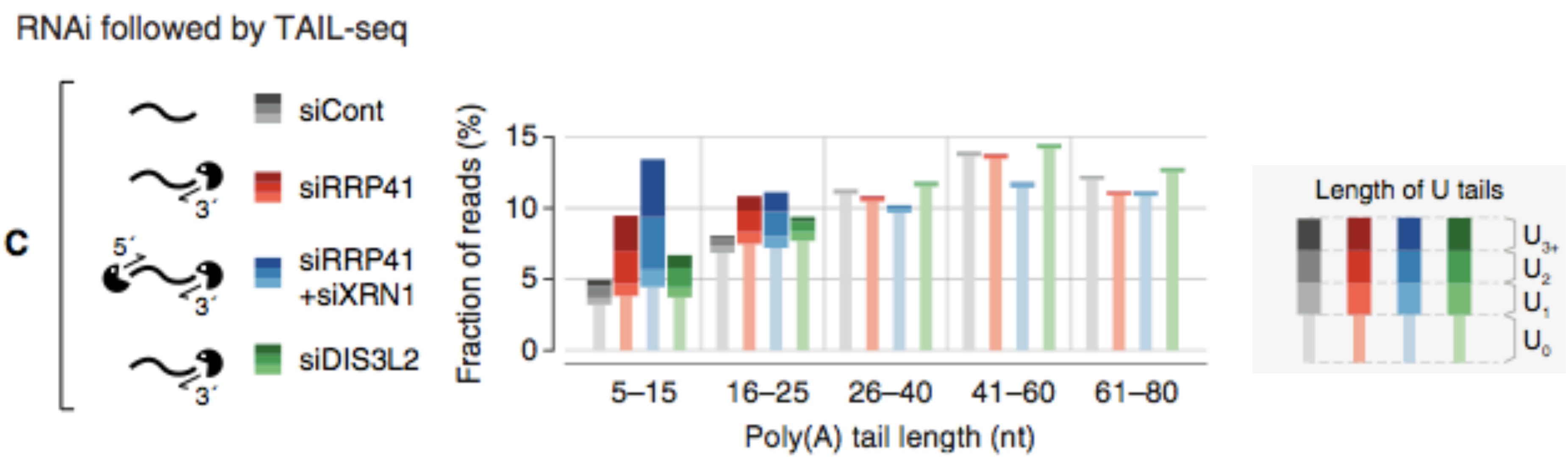
mRNA Decay Factors Remove Uridylated mRNAs

Interference of XRN1 resulted in a strong accumulation of uridylated mRNAs with short A-tails (≤25 nt). Additionally, when they depleted LSM1 or overexpressed dominant-negative mutants of the decapping complex (DCP1 and DCP2) they detected an increase of uridylation among short A-tailed mRNAs (≤25 nt). This result is consistent with a model that deadenylated, uridylated mRNAs are normally degraded rapidly by the 5′–>3′ decay factors while poly(A)+ mRNAs without U-tails are relatively stable. Thus, a short U-tail may first be recognized by the LSM1–7 complex which in turn facilitates decapping (by the DCP1/2 complex) and subsequent 5′–>3′ degradation (by XRN1).



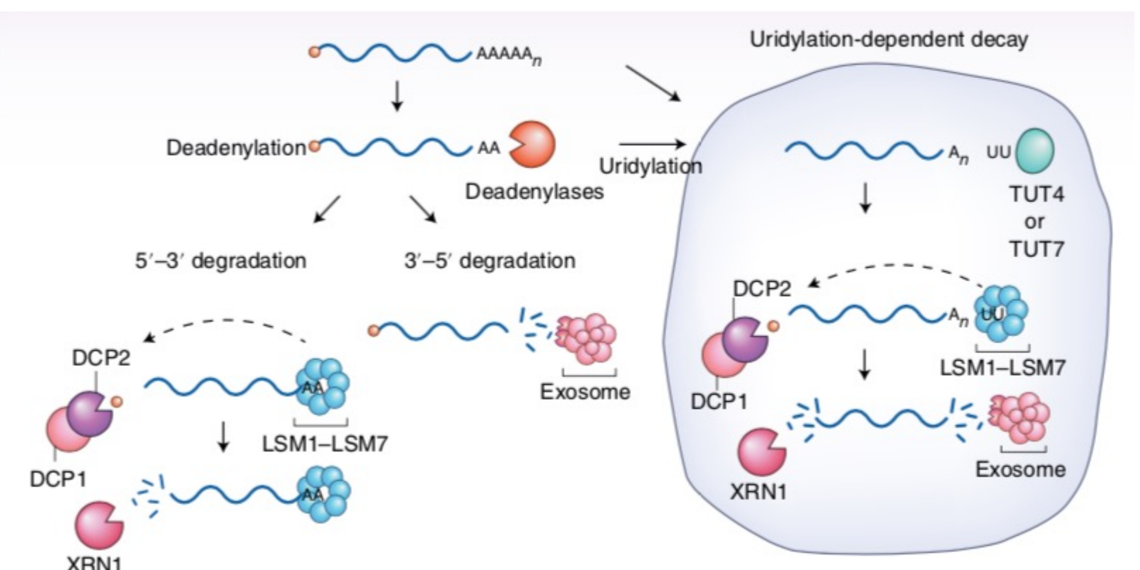
mRNA Decay Factors Remove Uridylated mRNAs

They also investigated the contribution of the 3′–>5′ decay pathway by depleting 3′ exonucleolytic factors. When they knocked-down RRP41 (exosome), they detected a substantial accumulation of uridylated mRNAs with short A-tails. Combinatorial knockdown of RRP41 and XRN1 resulted in a more pronounced increase of uridylation. Therefore, both decay pathways (5′–>3′ and 3′->5′) may act at the downstream of uridylation. They also tested DIS3L2 and in this case DIS3L2 depletion results in a modest accumulation of uridylated reads.

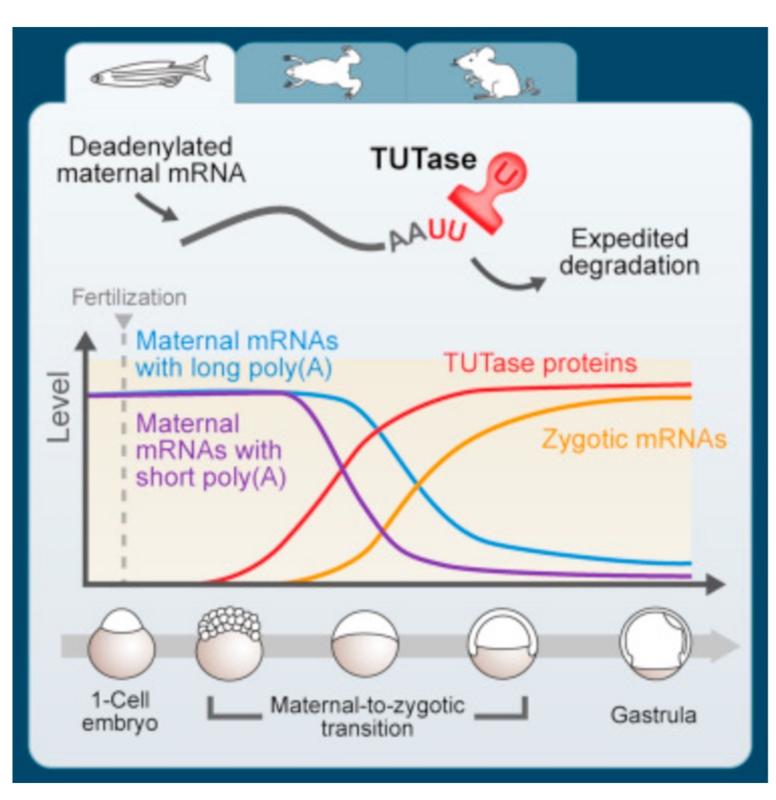


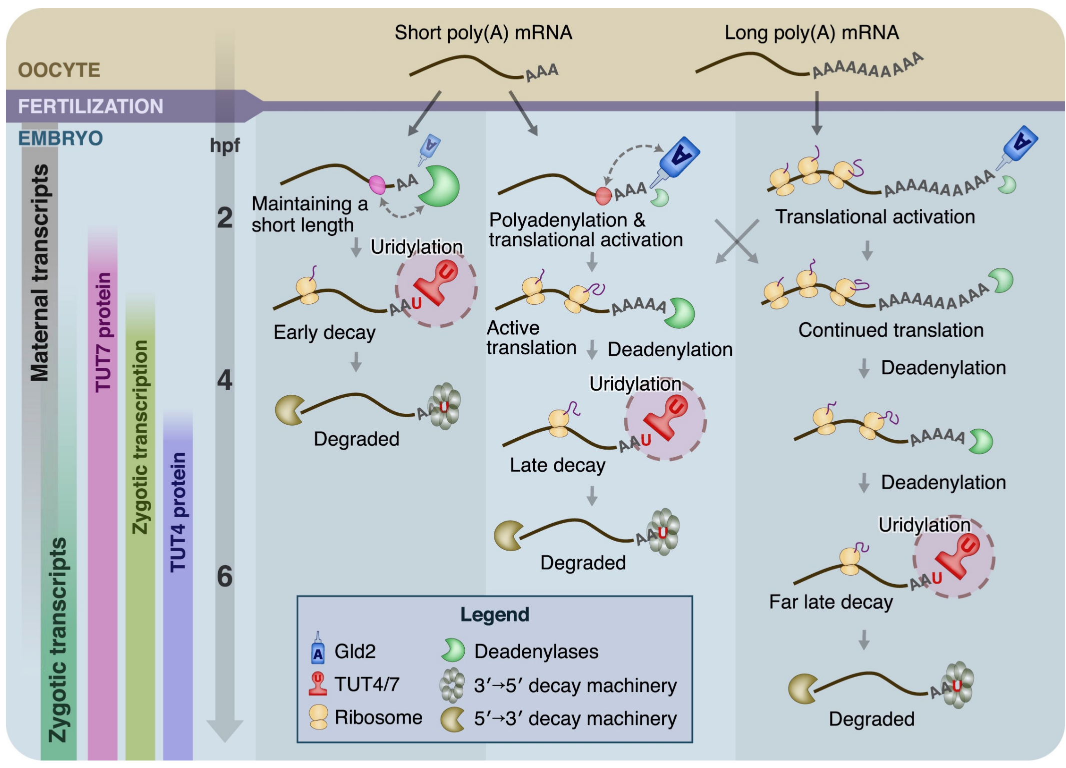
Degradation of polyadenylated mRNA

Following deadenylation, TUT4 and TUT7 uridylate mRNAs with a short poly(A) tail. U-tails are recognized by downstream decay factors, including LSM1–LSM7, decapping enzyme, Dis3L2 and the exosome.



Terminal Uridylyltransferases Execute Programmed Clearance of Maternal Transcriptome in Vertebrate Embryos

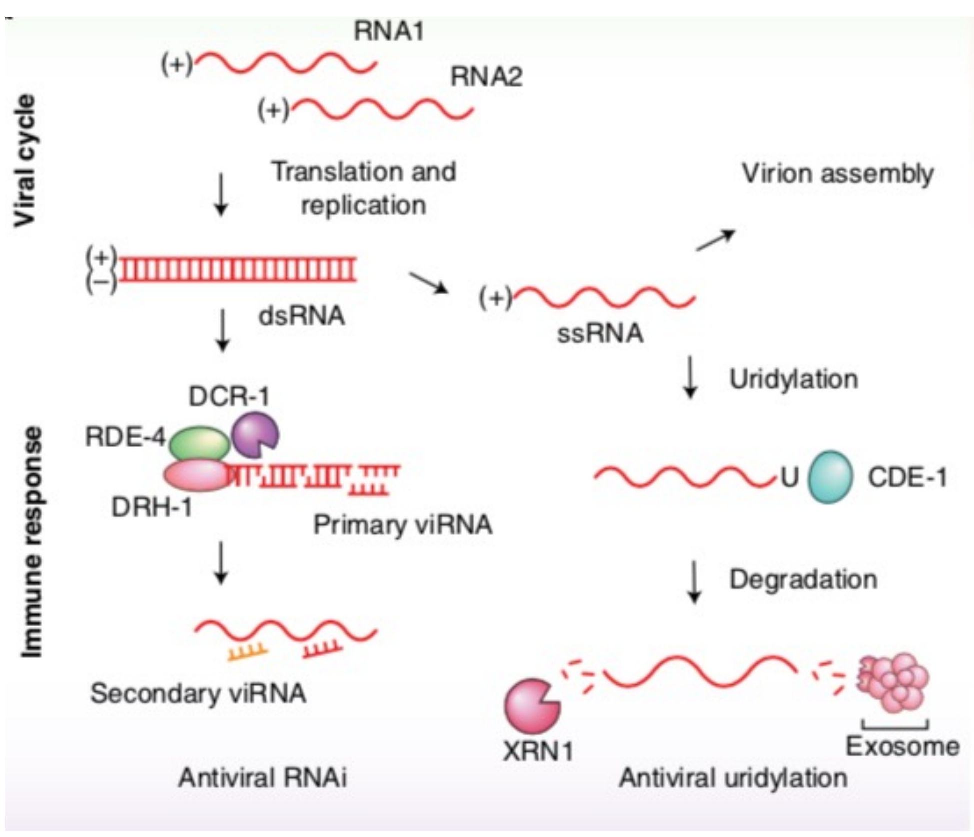




Chang et al., 2018. Mol Cell

The antiviral uridylation pathway functions in parallel with antiviral RNAi pathways in *C. elegans*

After *Orsay virus* infection, the antiviral RNAi response is initiated by the DCR-1–DRH-1–RDE-4 complex, which processes double-stranded viral RNAs into virus-derived siRNAs (primary viRNAs). Primary viRNAs in turn trigger the synthesis of secondary viRNAs. In parallel, viral RNAs are uridylated by the terminal uridylyltransferase CDE-1. The U-tail promotes both 5′-to-3′ decay by the exonuclease XRN1 and 3′ ->5′ decay by the exosome.



Restriction of L1 retrotransposition by uridylation

Following transcription in the nucleus, active L1 mRNA is translated in the cytoplasm. MOV10 binds to L1 mRNA and triggers its uridylation by TUT4 and TUT7. Although uridylated L1 mRNA can be imported to the nucleus, it cannot initiate efficient reverse transcription, presumably because the additional uracil residues prevent base pairing between the target and L1 mRNA, interfering with reverse transcription.