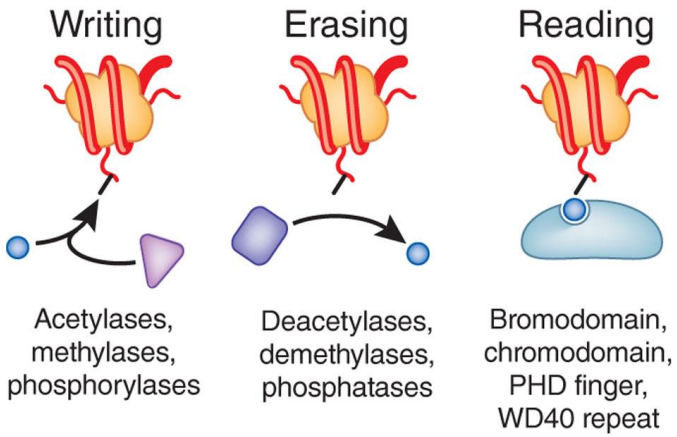
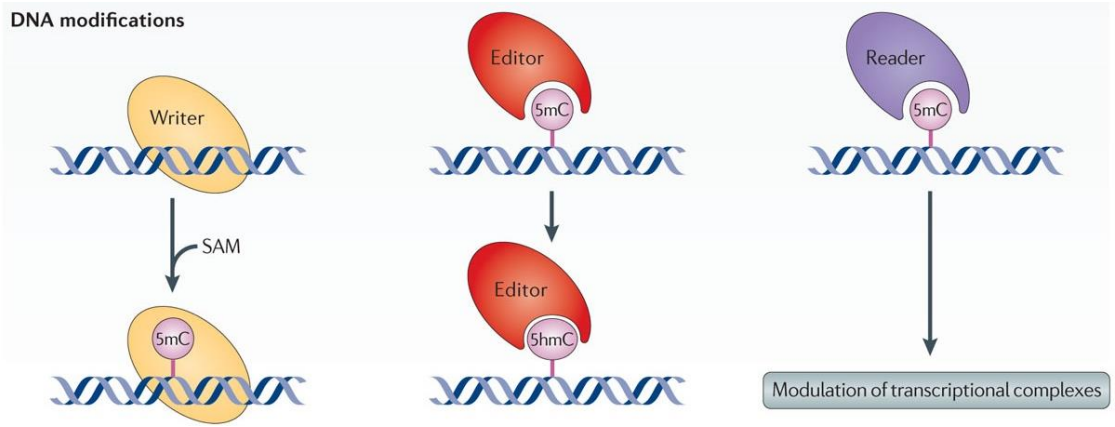
RNA chemical modifications

Dynamic and reversible chemical modifications control gene expression



Histones



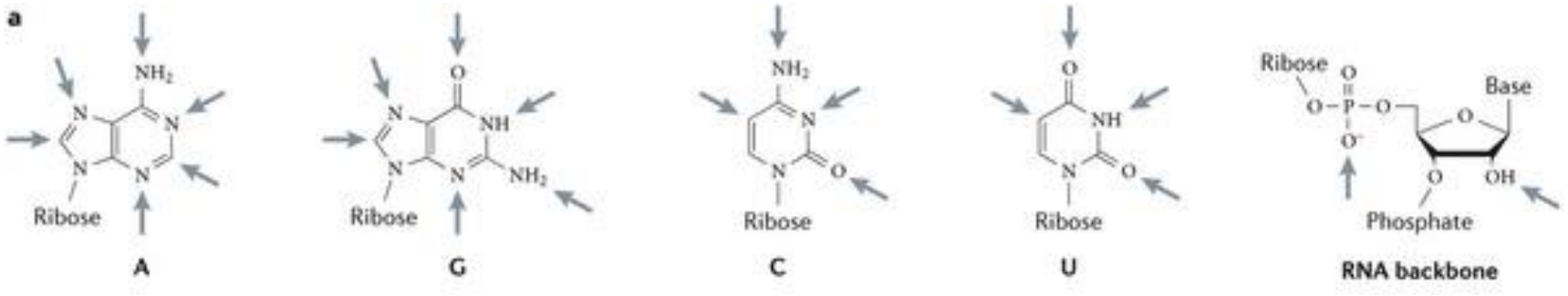
DNA

?

RNA

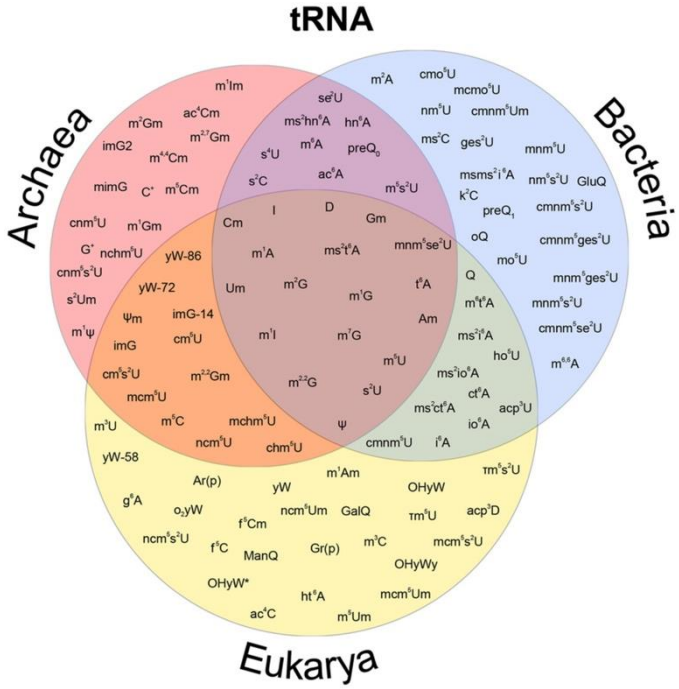
RNA chemical modifications

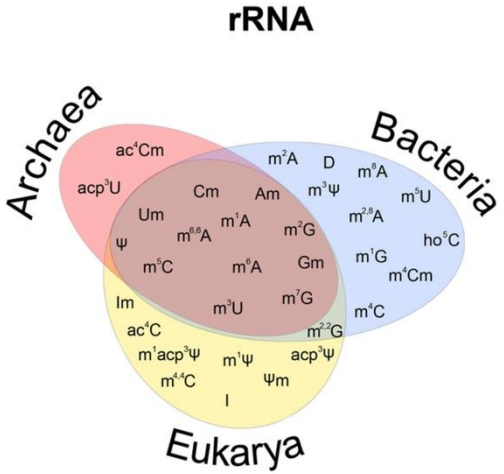
Cellular RNA species contain more than 160 chemical modifications with diverse properties. Chemical modifications of RNA can occur on the N1, N3, N7 and C8 atoms in both adenine and guanine; C2 and N6 in adenine; N2 and O6 in guanine; N1, O2, N3 and C5 in cytosine and uracil; N4 in cytosine and O4 in uracil; as well as on 2′-O of the ribose backbone and the OH group of the phosphate backbone. These modifications can modulate hydrophobicity, steric and electrostatic effects, and hydrogen-bonding abilities of RNA bases and backbones. The most common and simple RNA modification involves the methylation of bases and 2′-hydroxyls of RNA nucleotides.

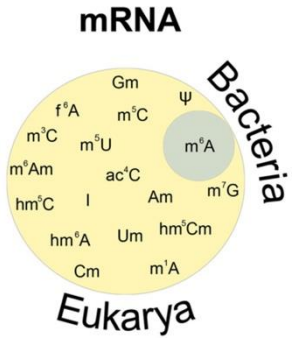


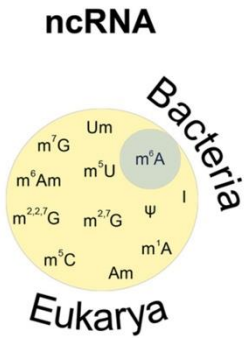
The enzymes responsible for each modification and the biological consequences of these modified RNAs are largely unknown.

RNA chemical modification
– *Epitranscriptomics* –









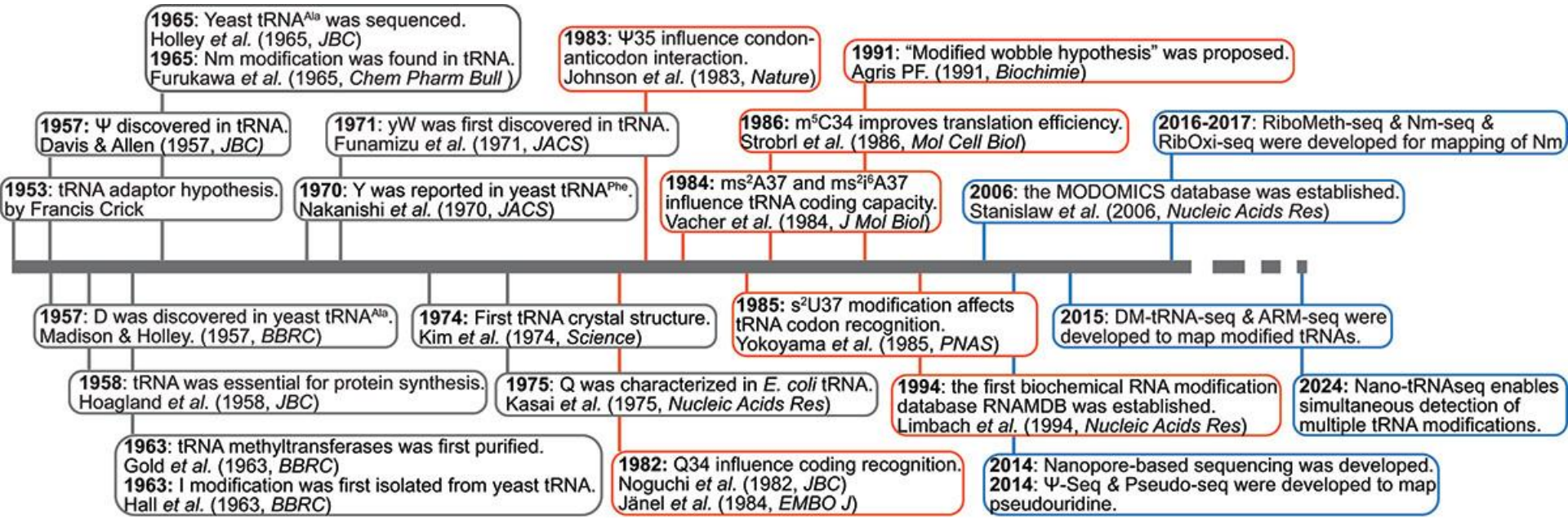
WIREs RNA, Volume: 11, Issue: 5, 2020, DOI: (10.1002/wrna.1595)

Timeline of significant discoveries in the field of tRNA modification

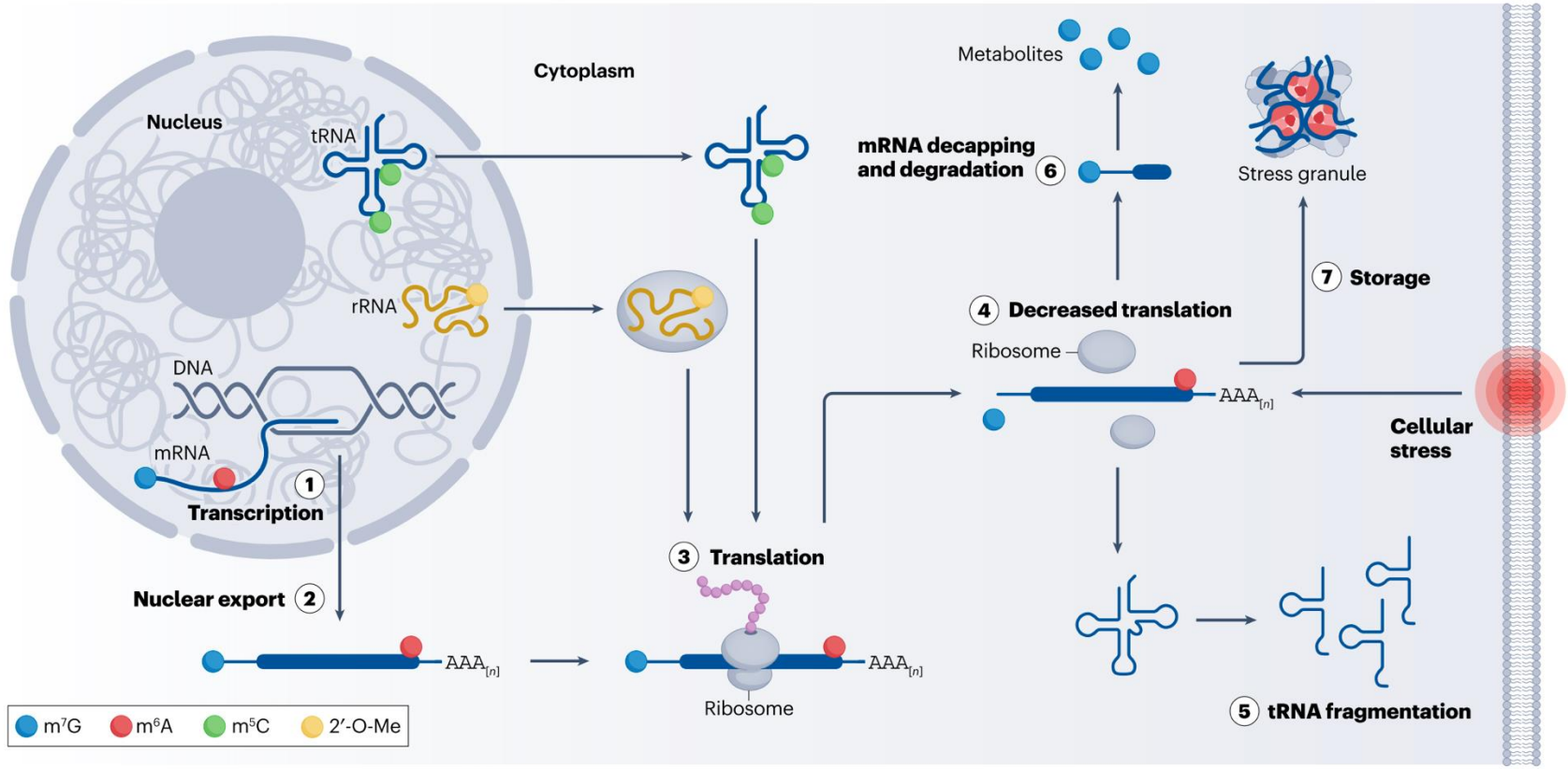
Before the 1980s

Between the 1980s and 1990s

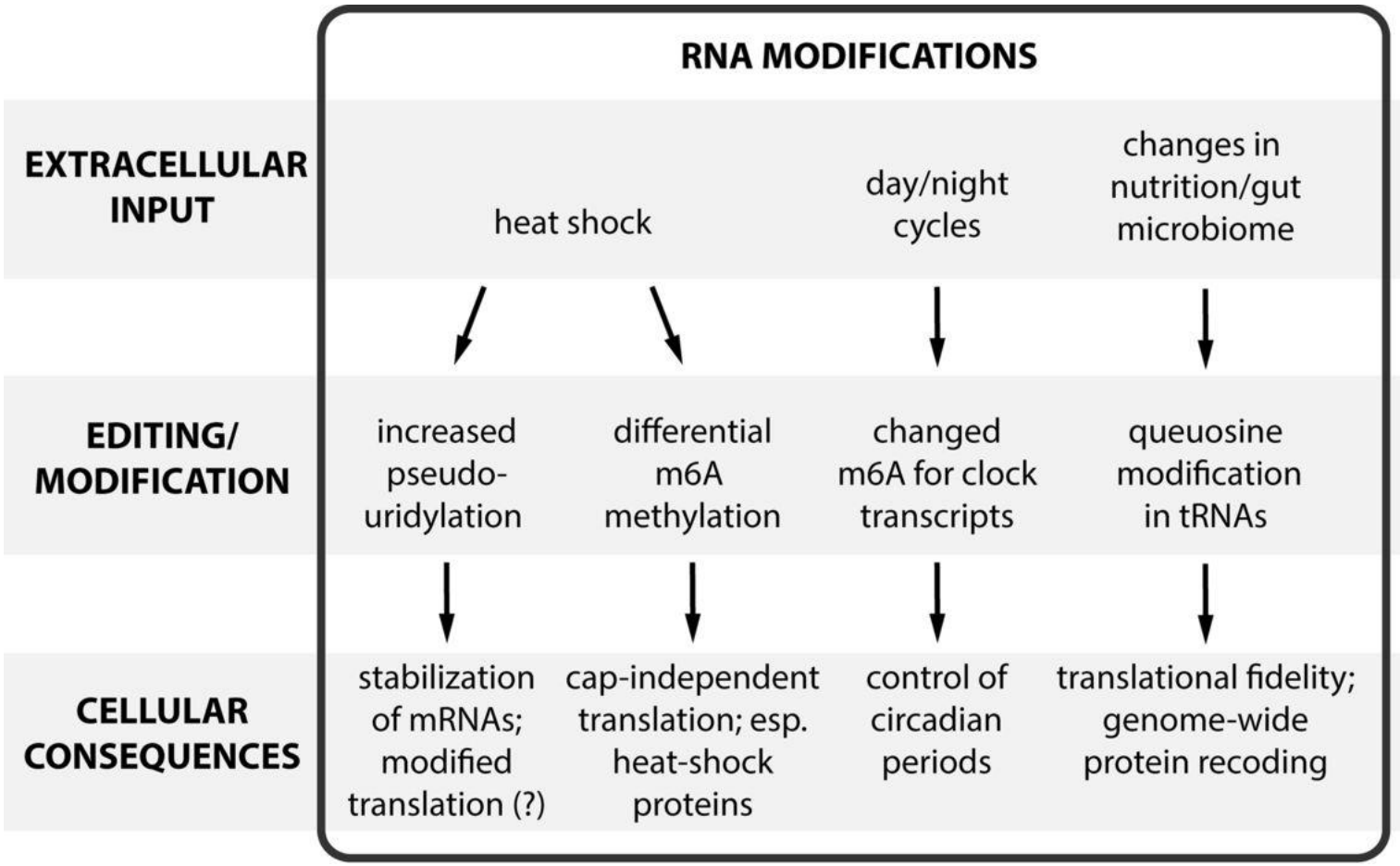
from 2000 to the present



Chemical RNA modifications can impact gene expression at different levels

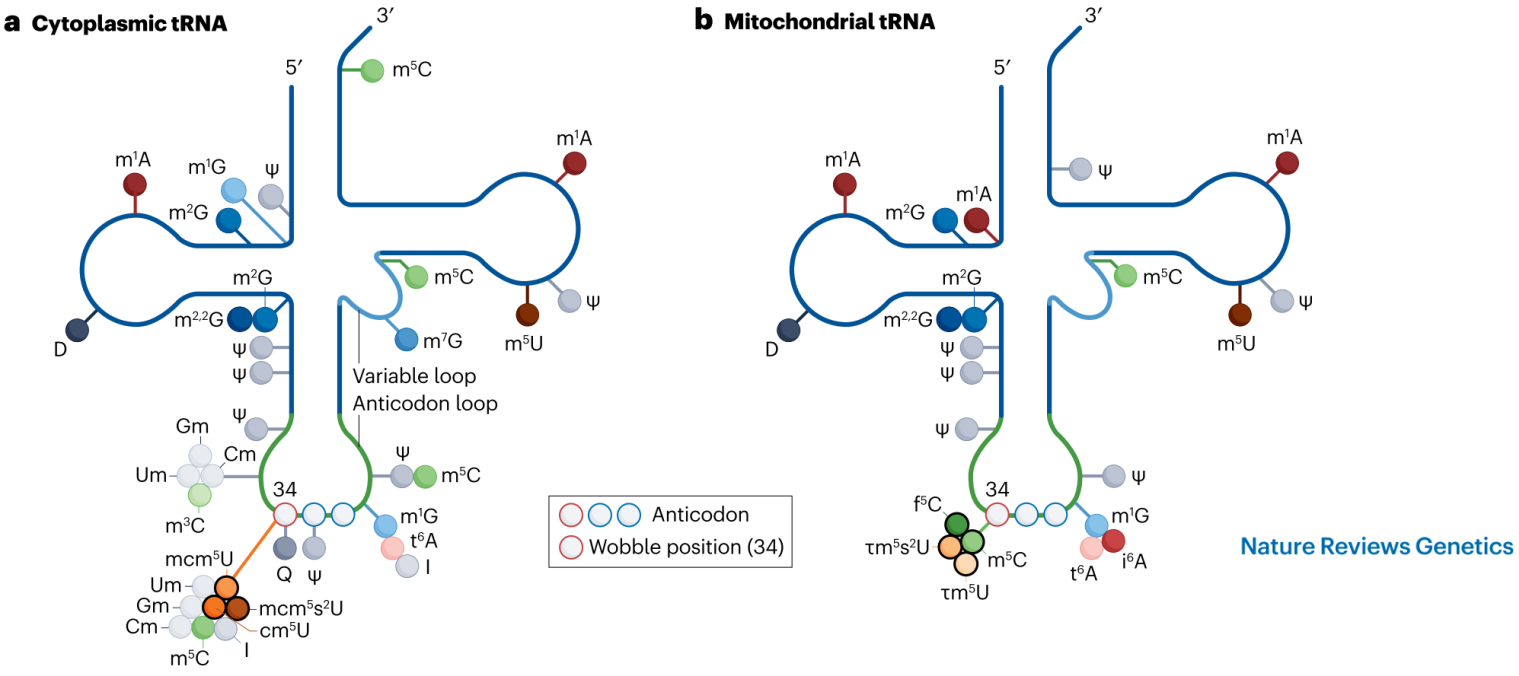


Rapid modifications of the epitranscriptome in response to extracellular inputs



Licth and Jantsch, 2016 JCB

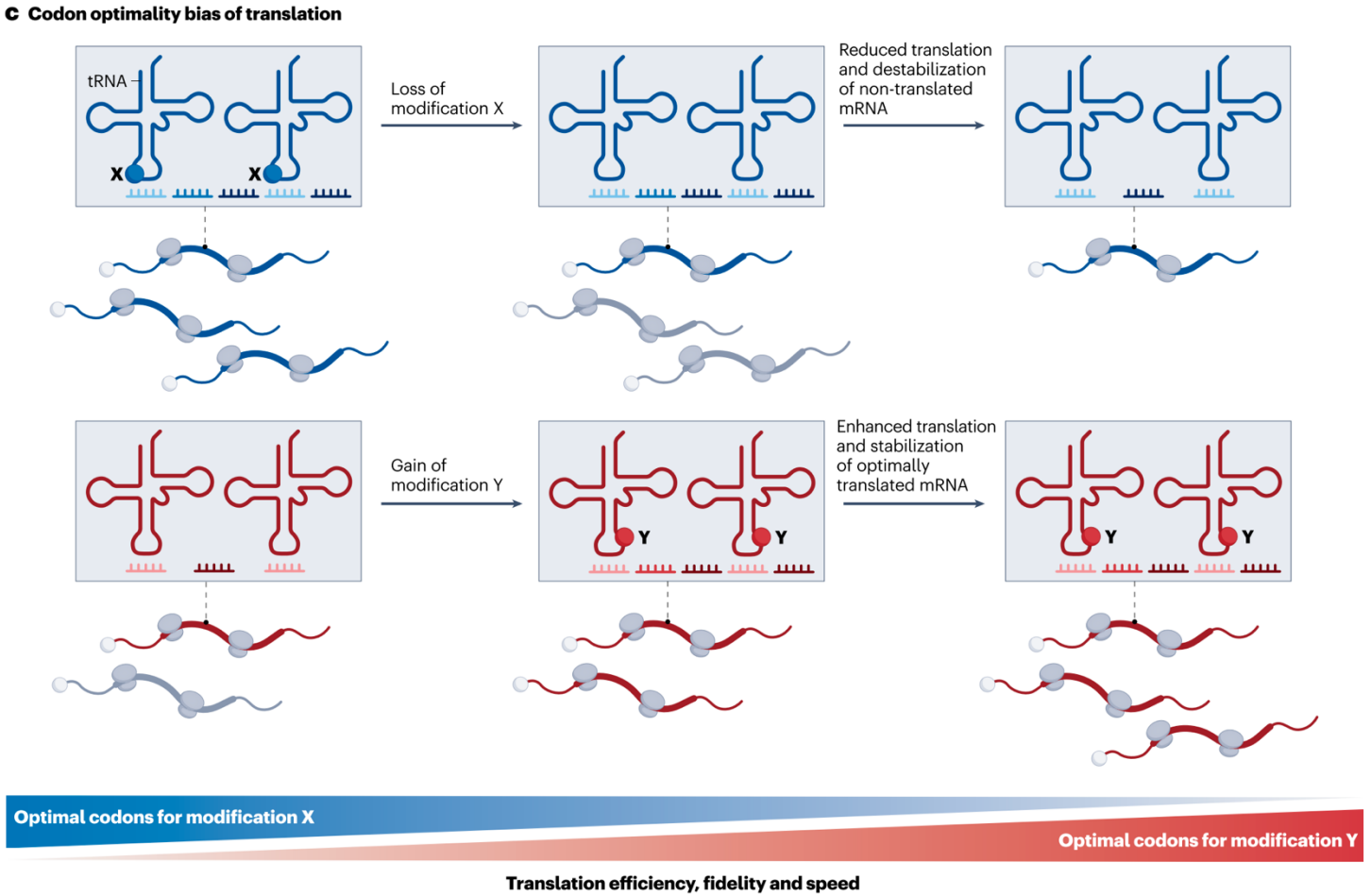
Nucleotide modifications in the anticodon sequence of tRNAs regulate efficient translation and allow optimal codon use



The **wobble position** refers to the **first nucleotide (5' end)** of the **anticodon** in tRNA, which pairs with the **third nucleotide (3' end)** of the codon on mRNA. It's called **"wobble"** because the base-pairing rules at this position are relaxed, allowing non-standard pairing. This flexibility helps one tRNA recognize multiple codons that code for the same amino acid.

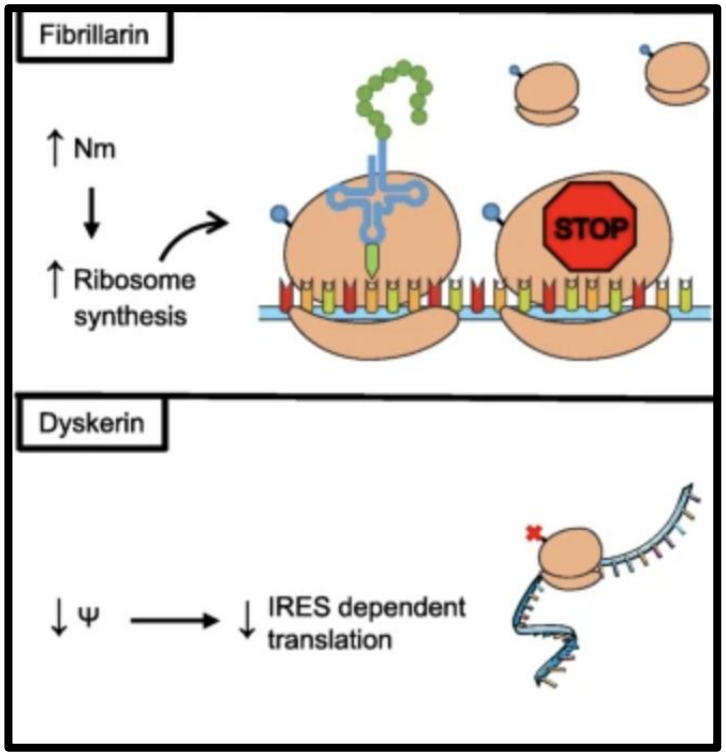
Nucleotide modifications in the anticodon sequence of tRNAs regulate efficient translation and allow optimal codon use

Re-balancing the mRNA translation speed and fidelity towards mRNAs of a different gene set (from blue to red) can rewire the translatome without requiring transcriptional changes in the nucleus. Transcript-specific enhanced translation can thereby determine the outcome of a cellular response.

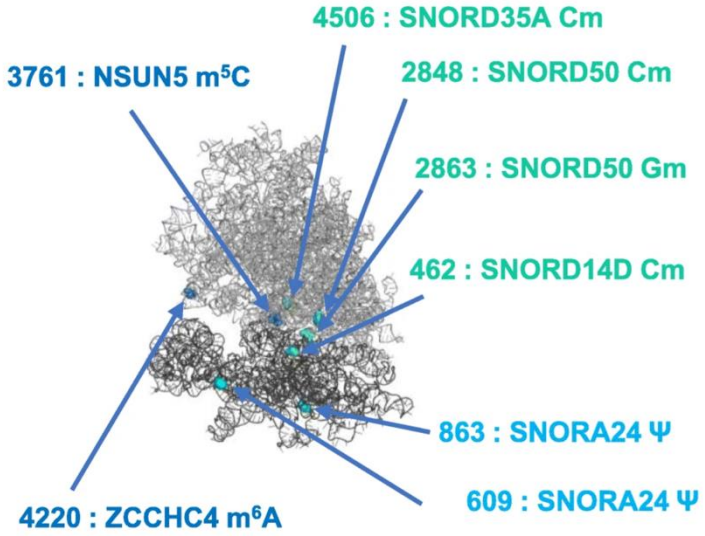


Changes in modifications of rRNAs can influence translational fidelity

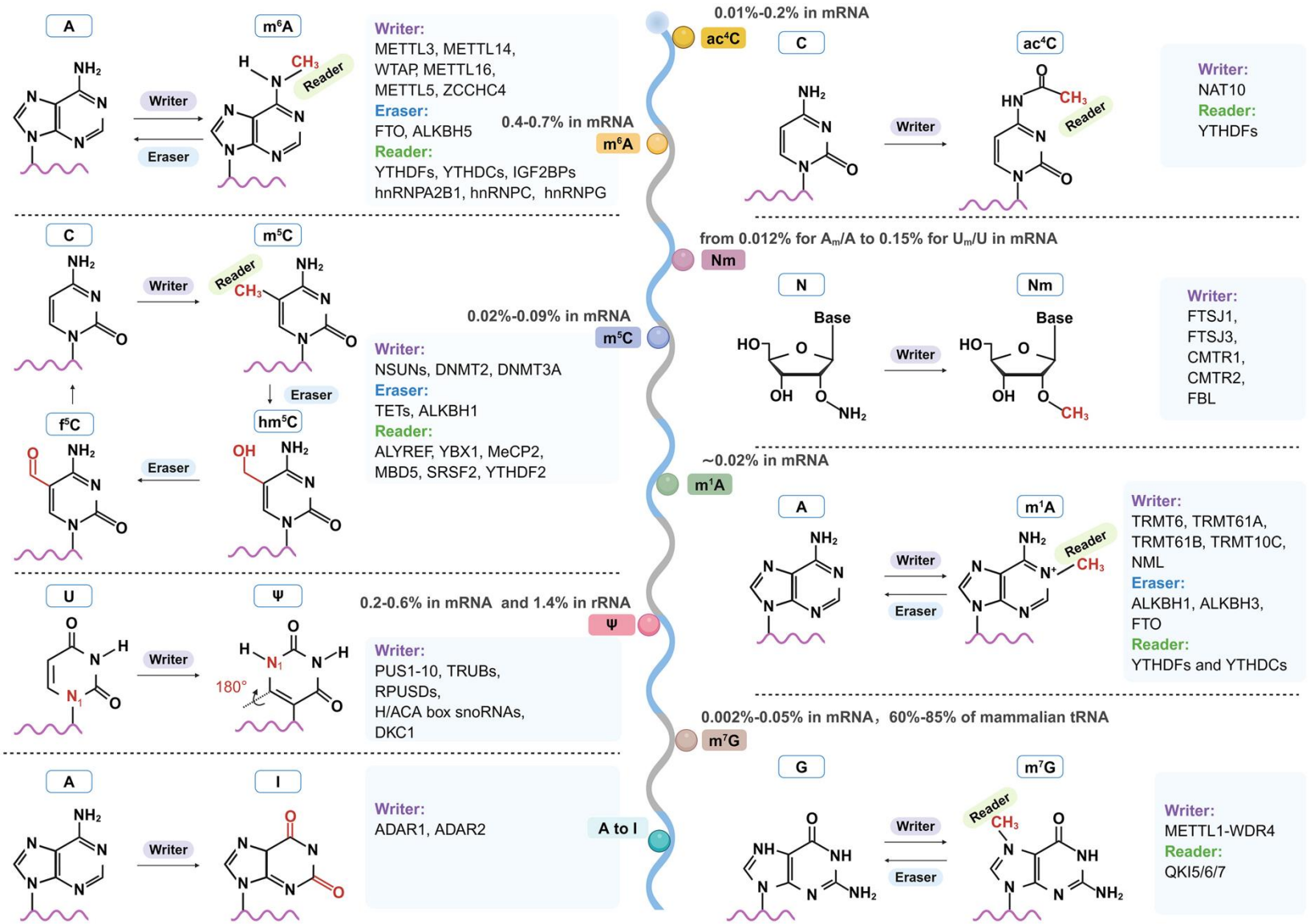
Global changes



Specific changes



Nucleotide modifications in mRNA



Chemical Modifications in Eukaryotic mRNA

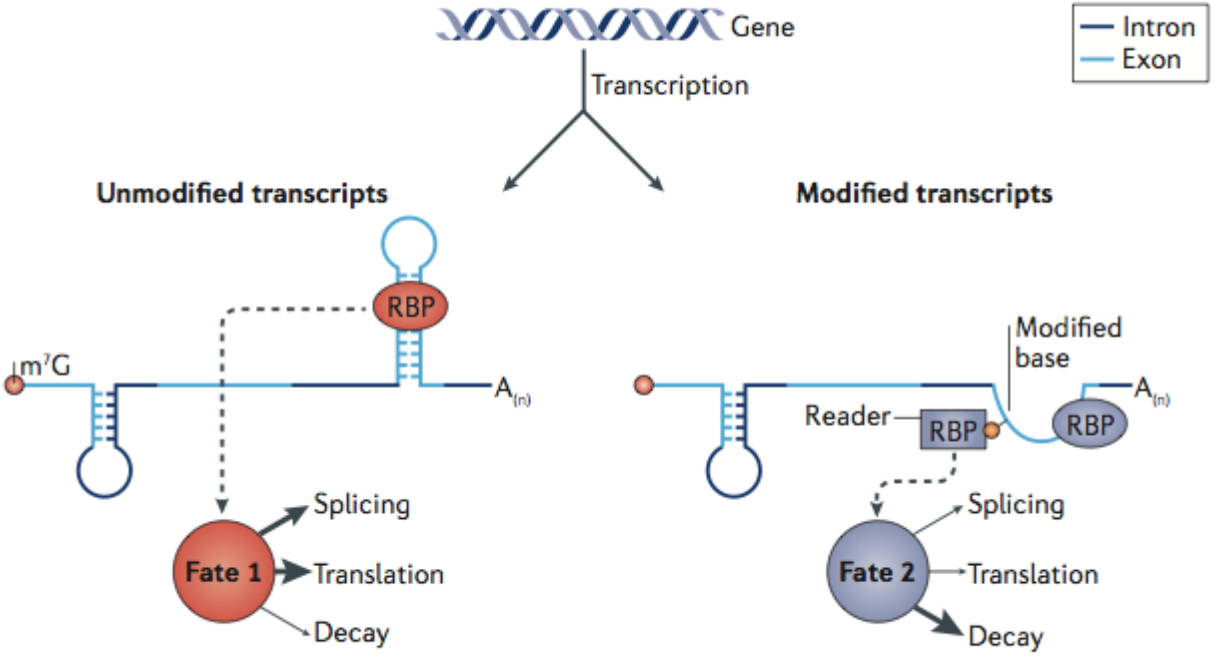
With the only exception of m^6A, chemical modifications lack a dedicated 'writer' machinery installing them on mRNA and are instead nearly invariably catalysed by the machineries installing the modifications on tRNA and/or rRNA. Modification in mRNA could be a consequence of fortuitously sharing sequence and/or structural similarity with 'canonical' targets of enzymes modifying tRNA or rRNA. Nonetheless, even under this scenario, the widespread activity of modification enzymes on mRNA can serve as an evolutionary playground, which in some cases may give rise to novel functions.

Whether RNA modifications are reversible — indeed whether there is even a need for active reversibility given the short half-life of RNAs — is under debate, even in the context of m^6A, where two demethylases were reported. Active removal of a modification may be the exception rather than a rule.

The dynamics observed are more likely to reflect global differences in the availability and/or function of the modifying enzymes across different systems and conditions.

Chemical Modifications in Eukaryotic mRNA

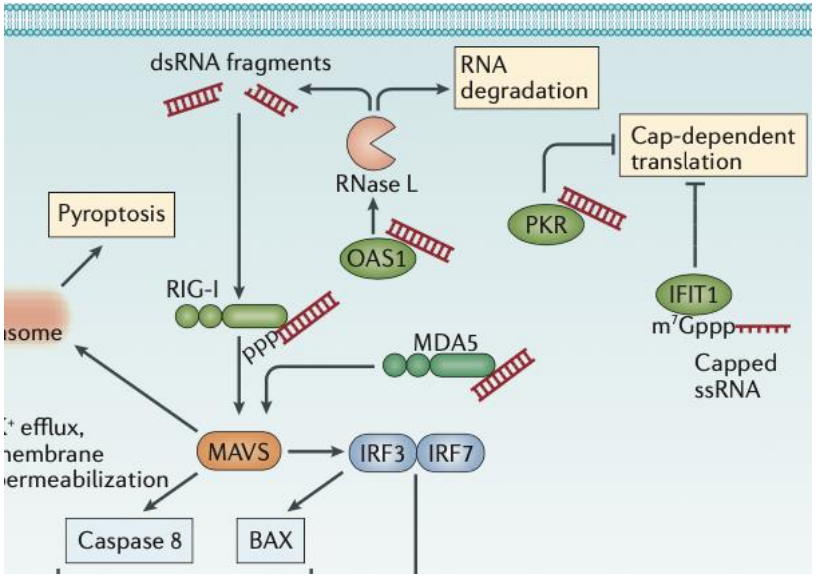
Chemical modifications in RNA affect the transcripts by altering charge, base-pairing potential, secondary structure, and protein-RNA interactions. These properties in turn shape the outcome of gene expression by modulating RNA processing, localization, translation, and eventual decay.



Cell state 1	90%	10%
Cell state 2	30%	70%

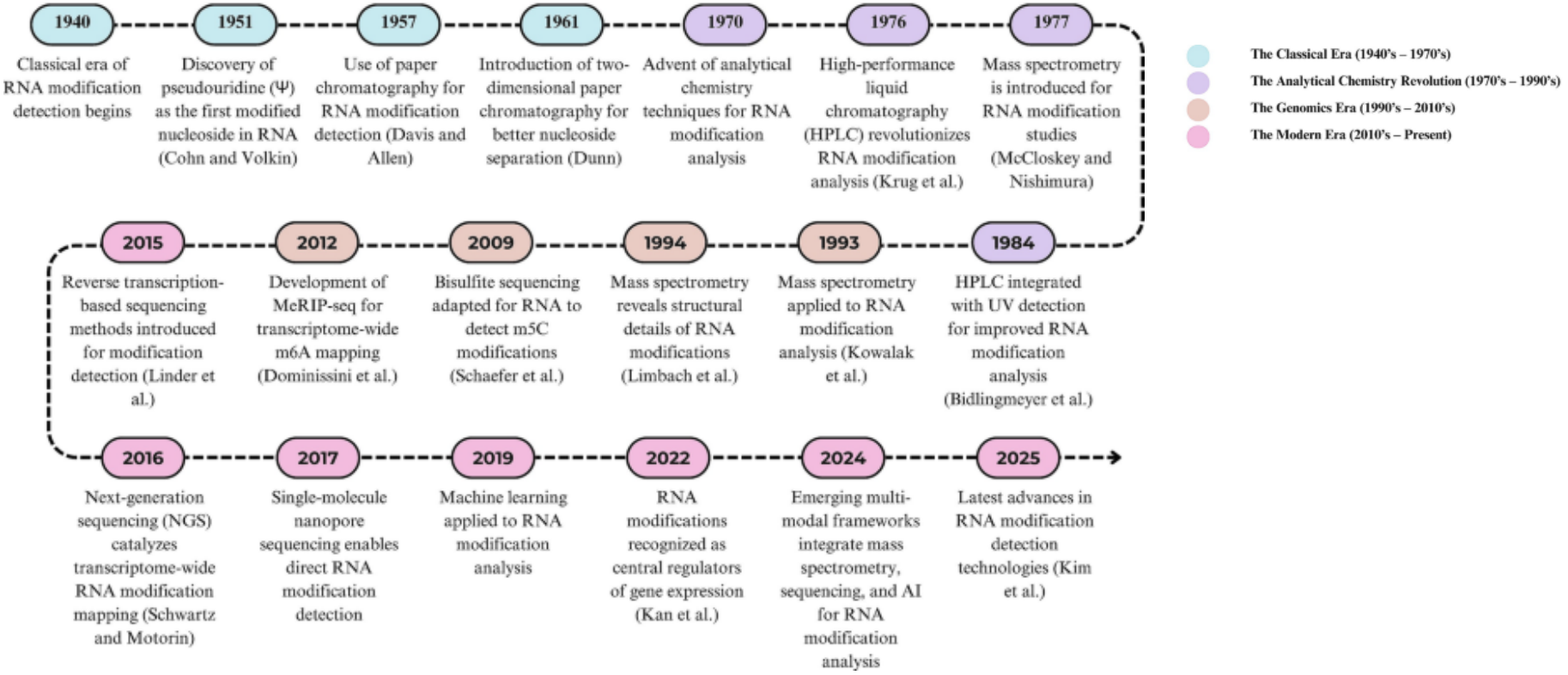
Modified RNAs inhibit antiviral signaling

RIG-I is in an auto-repressed conformation in the absence of ligand. 5-pppRNA binds the RIG-I CTD. RNA *m6A* binds RIG-I with lower affinity than unmodified RNA. The RIG-I helicase domain binds the RNA, triggering a protein conformational change. RNAs containing, Ψ or 5mC fail to induce RIG-I conformational change. Moreover, nucleoside modifications in RNA limit also the activation of 2′-5′ oligoadenylate synthetase (OAS1) and increase resistance to cleavage by RNase L .



RNA vaccines contain modified nucleotides, N^1-methylpseudouridine ($m^1\Psi$), to avoid activation of antiviral signalling.

Methodologies of detection

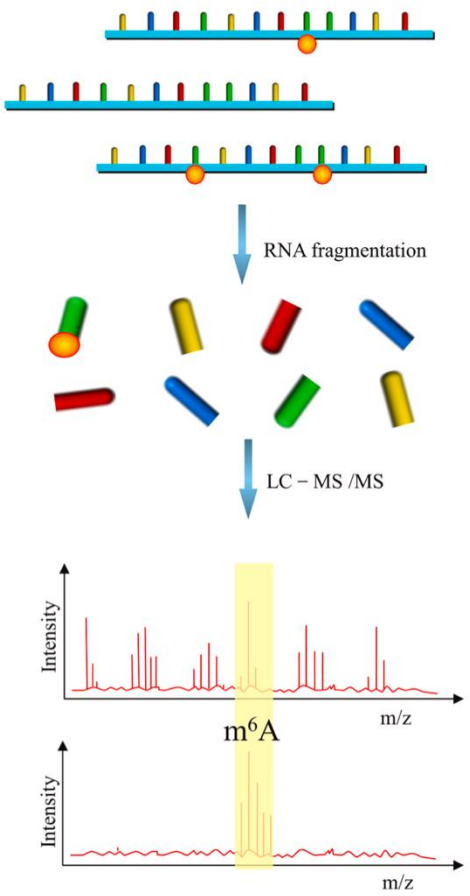


- Liquid chromatograph followed by mass spectrometry
- Sequencing-based approaches

Methodologies of detection

Liquid chromatography–mass spectrometry (LC-MS)

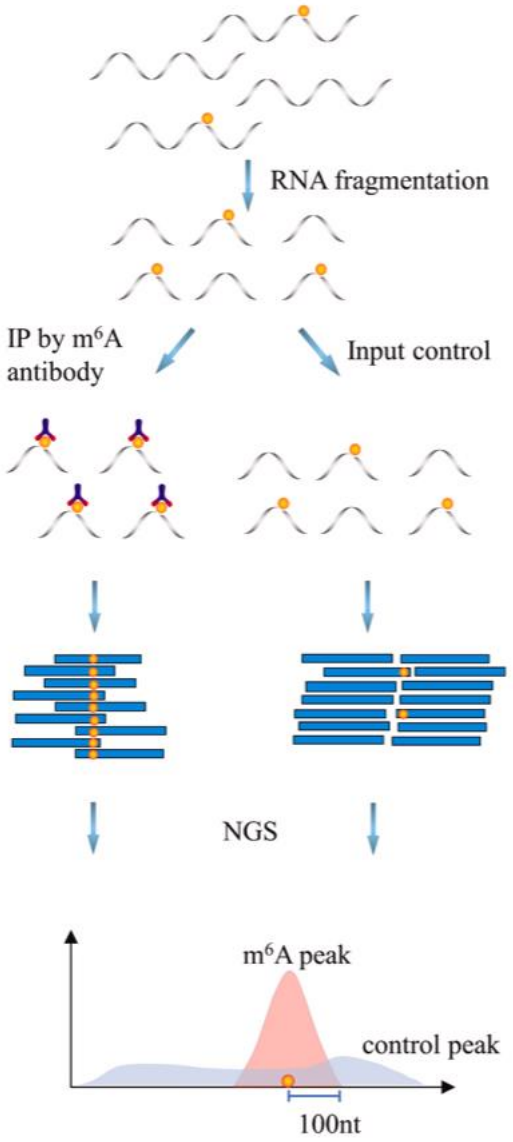
In the context of mRNA, LC-MS has been applied exclusively to fully digested nucleosides, which enables estimates of the 'bulk' levels of a modification in a sample but precludes assignment of this modification to individual sites. Moreover, it is difficult to interpret LC–MS results the lower the relative levels of the modification are in mRNA in comparison to tRNAs and rRNAs. The vast majority of chemical modifications of nucleotides are rarer in mRNA and more pervasive in both tRNA and rRNA. Even low levels of contamination from highly expressed tRNAs and rRNA (which can never be fully avoided) can lead to considerable overestimation of the modification levels in the mRNA fraction.



Methodologies of detection

Sequencing-based approaches

The identification of modifications using genomic approaches requires the development of a dedicated and unique workflow for each modification. The fundamental challenge that such approaches need to overcome is that the majority of modifications are 'invisible' in standard sequencing. For high abundance modification, such as m^6A, the primary detection approach relies on **antibodies, which are used to selectively immunoprecipitate short modified-RNA fragments**.

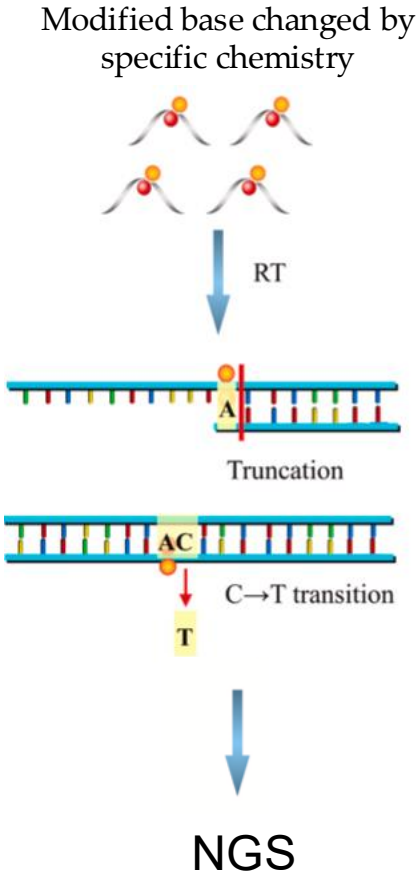


Methodologies of detection

Sequencing-based approaches

By contrast, modifications present at abundances orders of magnitude lower result in a dramatically increased ratio of non-specific to specific binding events.

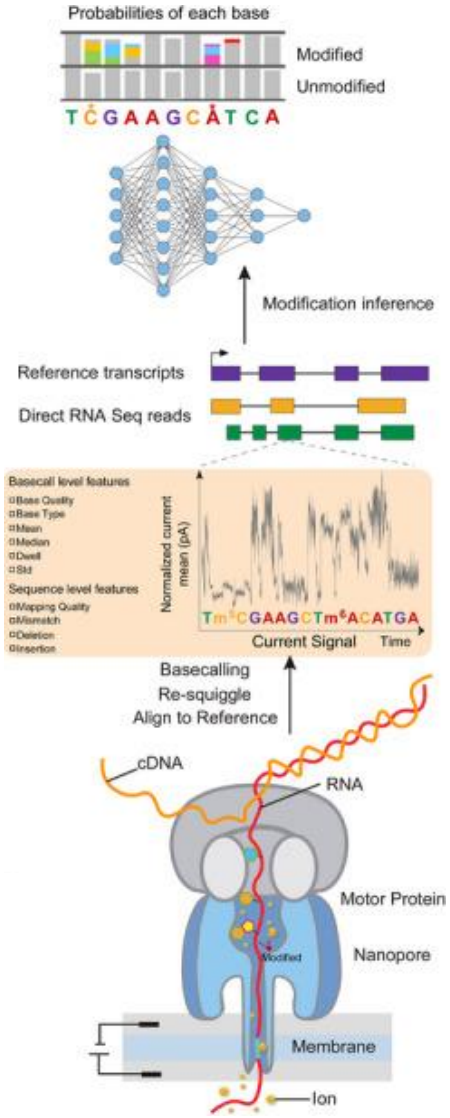
Thus, approaches have been developed by using **chemistries that alter modified bases to render them visible following reverse transcription** (result either in mismatches or deletions at the modified sites or in 'pile-ups' of reads that selectively begin or end at a specific position from which the presence of a modification can then be inferred). Key advantages of such chemical–genomic methods lie in their excellent, typically single-nucleotide, resolution and in their ability to provide relative and even absolute quantifications of modification levels.



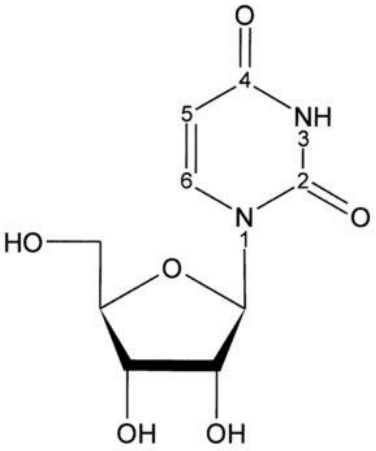
Methodologies of detection

Direct RNA sequencing

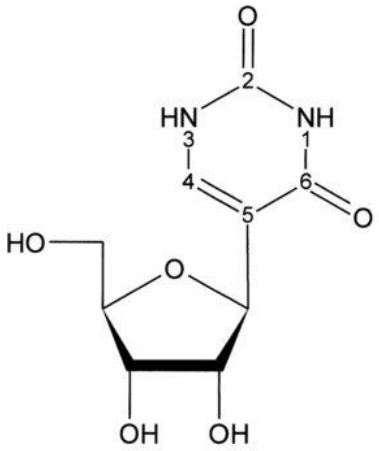
As RNA passes through the nanopore, an associated motor protein regulates the translocation speed, and ionic current changes are measured in real time. RNA modifications alter the local ionic current signal, which is recorded along with the nucleotide sequence. Computational analysis involves basecalling, signal re-squiggling, and alignment to a reference transcriptome, followed by feature extraction and machine learning–based prediction to infer the modification type and position.



Pseudouridine (Ψ)



Uridine



Pseudouridine

Pseudouridine (Ψ)

Ψ is likely the most frequently modified base reaching levels of ~0.3–0.4% of all uridines. It is highly abundant in rRNAs and tRNAs but also detected in snRNAs and mRNAs. Numerous techniques have been created for the detection and quantification of Ψ, including mass spectrometry (MS), N-cyclohexyl-N′-β-(4-methylmorpholinium) ethyl carbodiimide (CMC)-based next-generation sequencing technology (NGS), direct nanopore sequencing, high-performance liquid chromatography (HPLC), and bisulfite-induced deletion sequencing (BID-seq).

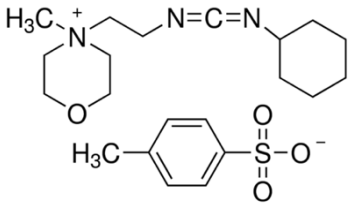
Table 1 The detection methods of pseudouridine

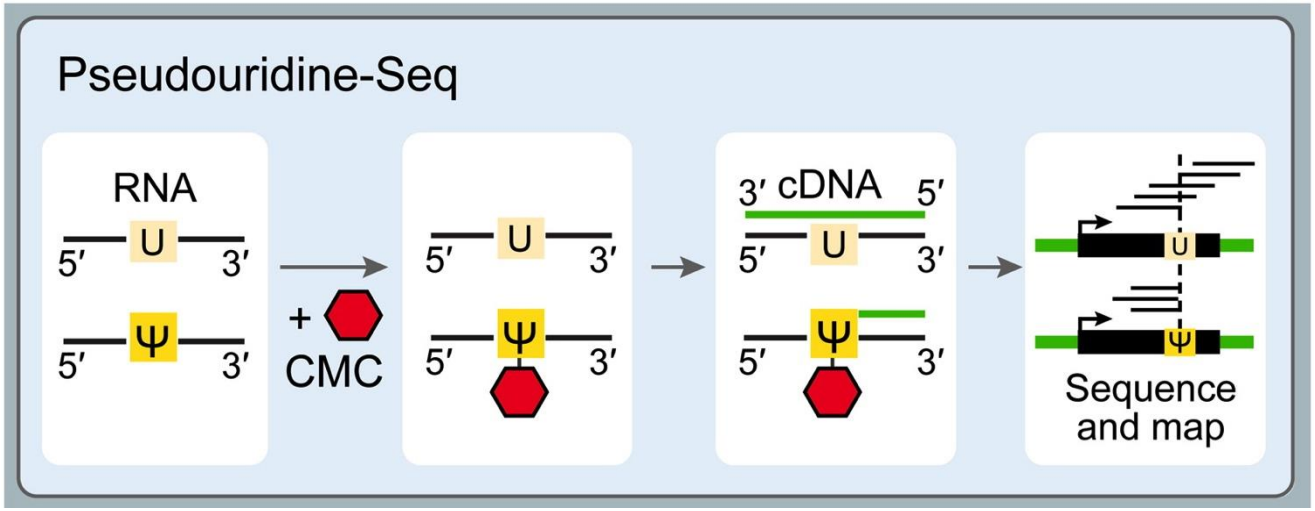
Methods	Advantages	Disadvantages	Turn-over time
MS	i. Determine the location and quantify the amount of Ψ in the RNA fragment ii. Sensitive and accurate	i. Time-consuming and sample requires cyanoethylation or deuterium labeling	1 h
HPLC	i. Free from labeling ii. Detect highly abundant modifications in RNAs iii. Can be combined with MS or other methods for further identification and quantification	i. Low detection sensitivity ii. Require a large amount of purified RNA iii. Require extensive chromatographic conditions	15–40 min
CMC-based NGS	i. Low sample amount ii. Economic iii. High-throughout iv. Sensitive and accurate	i. Bring in artifacts and false positives due to the non-specific reaction of CMC with RNA ii. Introduce biases owing to interference of surrounding modified nucleotides iii. Complex sample preparation process	2 days
Nanopore sequencing	i. Offer real-time analysis and allow rapid insights into RNA characteristics ii. Analyze native RNA fragments with any length	i. More powerful algorithm is required for combined analysis of multiple parameters and signal signatures	18 h
BID-seq	i. detects Ψ sites in mRNA at base resolution. ii. can be applied to low RNA input	i. bisulfite treatment can introduce DNA damage and affect RNA quality ii. is primarily applied for the Ψin mRNA	4 days

Pseudouridine (Ψ)

Psudouridine-Seq

Ψ can be selectively modified with a **carmodiimide** ((e.g. N-cyclohexyl-N9-(2-morpholinoethyl)- carbodiimide metho-p-toluenesulphonate, **CMC**)) to generate a block to reverse transcriptase one nucleotide 3′ to the pseudouridylated site. CMC is used to determine the locations of Ψ using next-generation sequencing. Mock-treated RNA fragments were processed in parallel to identify pseudouridine-independent reverse transcription stops. The carbodiimide can be coupled to biotin, allowing pre-enrichment for pseudouridylated RNAs prior to reverse transcription, potentially increasing sensitivity.

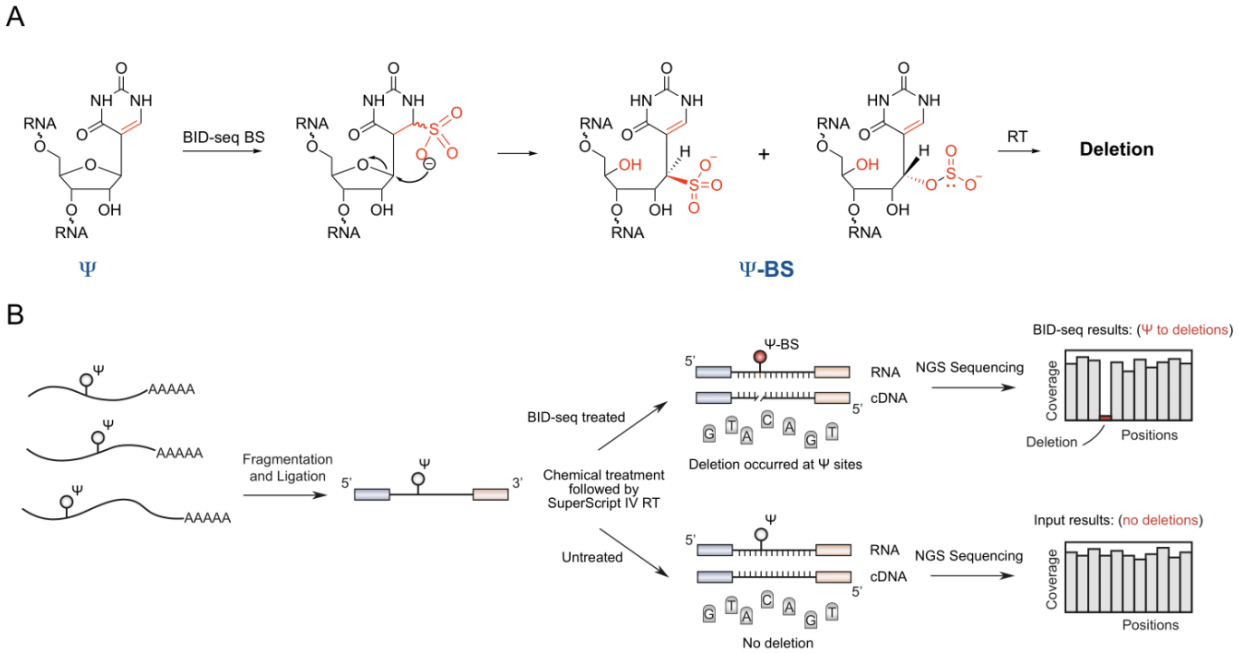




Pseudouridine (Ψ)

BS-induced deletion sequencing (BID seq)

A deletion sequencing technique induced by bisulphite (BS), enables high-throughput detection and precise single-base localisation of pseudouridylation. This method quantitatively converts Ψ into 'Ψ-BS adducts', resulting in base deletion signals at Ψ-containing sites following reverse transcription, thereby facilitating quantitative sequencing of Ψ modifications in RNA at single-base resolution.



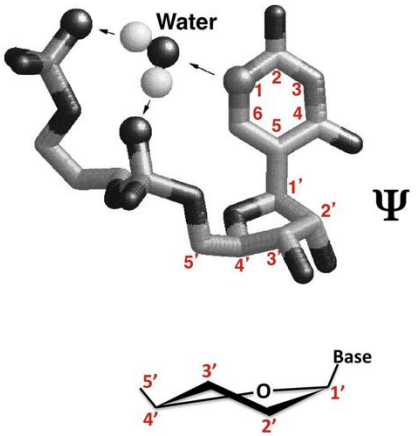
Dai et al., 2022. Nature Biotech.

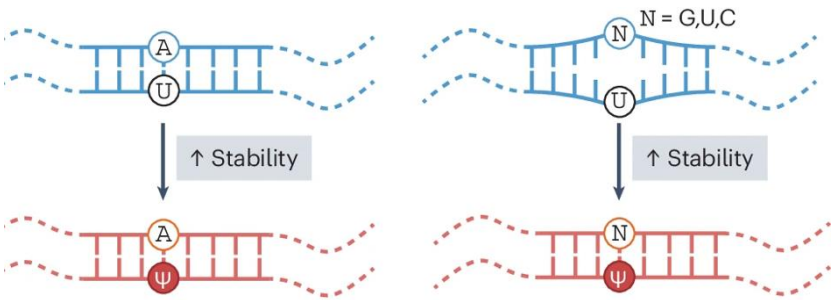
Effects of pseudouridine on RNA structure and function

Ψ can modulate the local secondary structure of RNA. Firstly, Ψ favours a C3′-endo sugar conformation. Secondly, this structural preference adds rigidity to both the backbone and the base, and stabilizes RNA duplexes Ψ can enhance the thermodynamic stability of RNA duplexes in synthesized oligonucleotide by stabilizing the canonical Ψ–A pairing and non-canonical Ψ–G, Ψ–U and Ψ–C pairings.

Pseudouridine also alters RNA–protein interactions, mainly by modulating RNA structure.

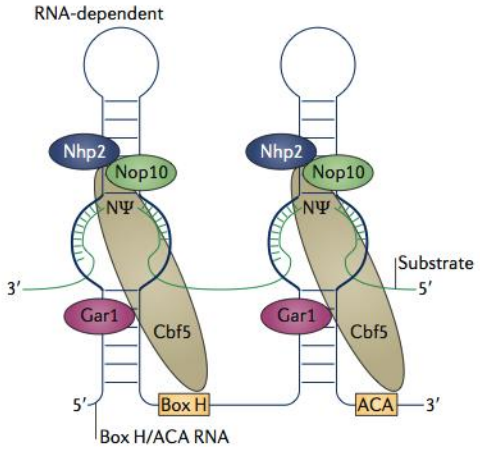
Pseudouridine is stable and irreversible, as no "erasers" have been identified to date.

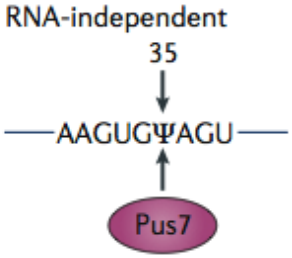




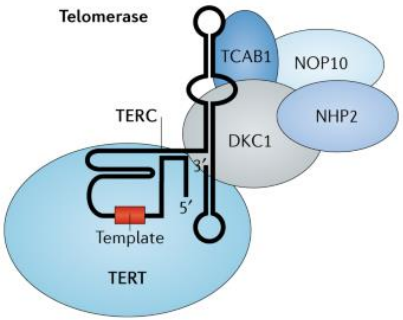
Pseudouridine (Ψ)

Pseudouridylation is catalysed by **pseudouridine synthases (PUSs)** and can be achieved through two distinct mechanisms, namely **RNA-independent pseudouridylation (PUSs)** and **RNA-dependent pseudouridylation (Cbf5)**.





Dyskerin pseudouridine synthase 1 (DKC1*/Dyskerin)** is the mammalian ***CBF5 ortholog. In mammals, DKC1 associates with and stabilize telomerase TERC RNA, which contains an H/ACA snoRNA. Thus, it controls telomerase activity.

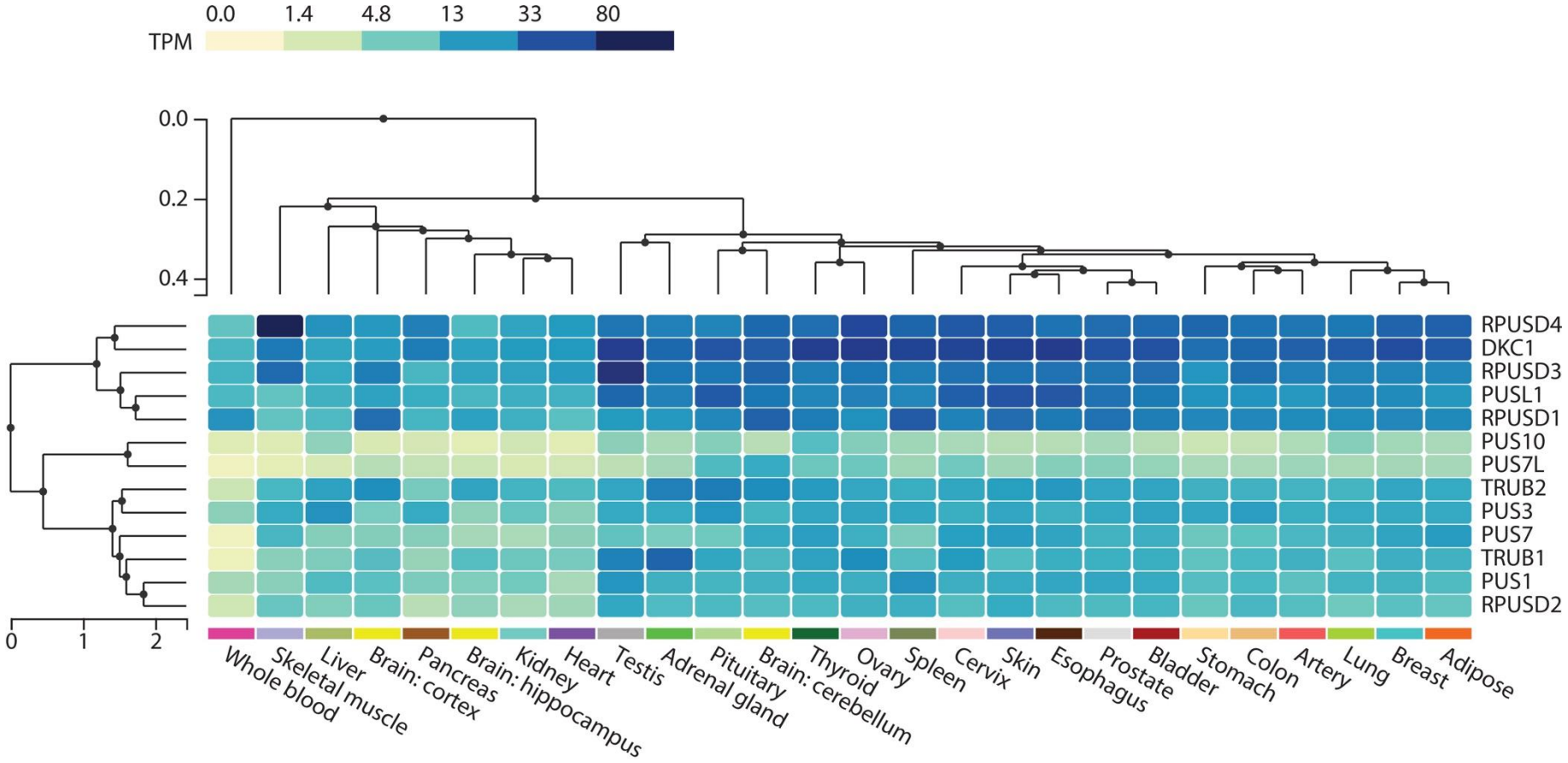


The Pseudouridine synthase (PUS) family

Thirteen putative PUS enzymes have been annotated in the human genome, with dyskerin pseudouridine synthase 1 (DKC1) known to rely on H/ACA snoRNAs to guide pseudouridine deposition.

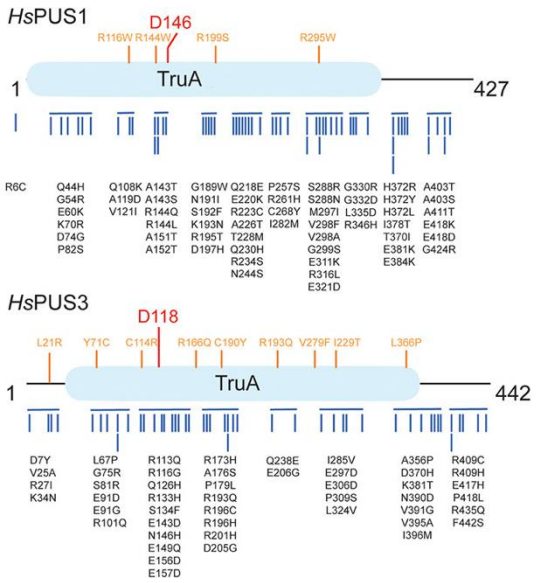
Enzyme	Known RNA Targets	Cellular Localization	Regulatory Conditions	Associated Functions
PUS1	tRNA, snRNA, mRNA	Nucleus, Mitochondria	Stress response, metabolic cues	Mitochondrial translation, RNA stability, splicing regulation
PUS3	tRNA (anticodon stem–loop)	Nucleus, Cytoplasm	Developmental regulation, neuronal contexts	Essential for tRNA modification; mutations linked to intellectual disability and neurodevelopmental disorders
PUS7	tRNA, snRNA, mRNA	Nucleus, Cytoplasm	Nutrient availability, stress signals	Translational control, stem cell differentiation, stress adaptation, splicing regulation, neurodevelopmental disorder
PUS7L	mRNA	Nucleus	Not well characterized	RNA pseudouridylation in transcripts
PUSL1	Mitochondrial tRNA	Mitochondria	Oxidative stress, mitochondrial function	Regulation of mitochondrial translation and RNA modification
PUS10	tRNA (Ψ at position 54, 55), snRNA	Cytoplasm, Nucleus, and Mitochondria	Apoptotic signals, immune activation	Apoptosis regulation, innate immune function
TRUB1	tRNA (Ψ55), mRNA	Nucleus, Cytoplasm	Growth conditions, differentiation cues	Translation fidelity, RNA structural stability
TRUB2	tRNA (mitochondrial), mRNA	Mitochondria	Mitochondrial stress, oxidative stress	Mitochondrial translation, respiratory function
DKC1	rRNA (28S, 18S), snRNA (H/ACA snoRNP-associated)	Nucleolus, Cajal bodies	Cell cycle, telomere maintenance	Ribosome biogenesis, telomerase RNA stabilization, splicing regulation, DKC-1 mutation related diseases
RPUSD1	rRNAs (mitochondrial), mRNA	Mitochondria, Cytoplasm	Energy demand and metabolic regulation	Maintains mitochondrial ribosome function and respiratory capacity, stabilize eIF4E mRNA via its RluA domain
RPUSD2	rRNA, mRNA	Nucleolus, Mitochondria	Developmental regulation	Ribosome assembly, mitochondrial gene expression
RPUSD3	rRNAs, mRNA (mitochondrial)	Mitochondria	OXPHOS regulation	Contributes to mitochondrial ribosome biogenesis and translation
RPUSD4	rRNAs (mitochondrial)	Mitochondria, Nucleus	Metabolic stress, hypoxia	Ensures proper mitochondrial ribosome assembly and function, splicing regulation

Expression of PUSs across human tissues



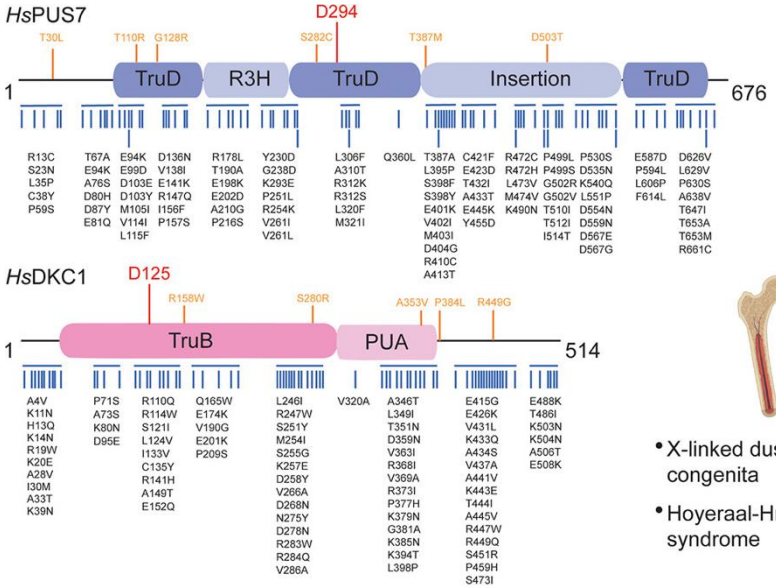
Borchardt EK, et al. 2020
Annu. Rev. Genet. 54:309–36

Summary of reported alterations of diseases relevant PUS



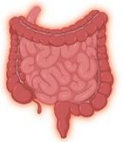
- Mitochondrial myopathy, lactic acidosis, and sideroblastic anemia

- Intellectual disorder



- Intellectual disorder

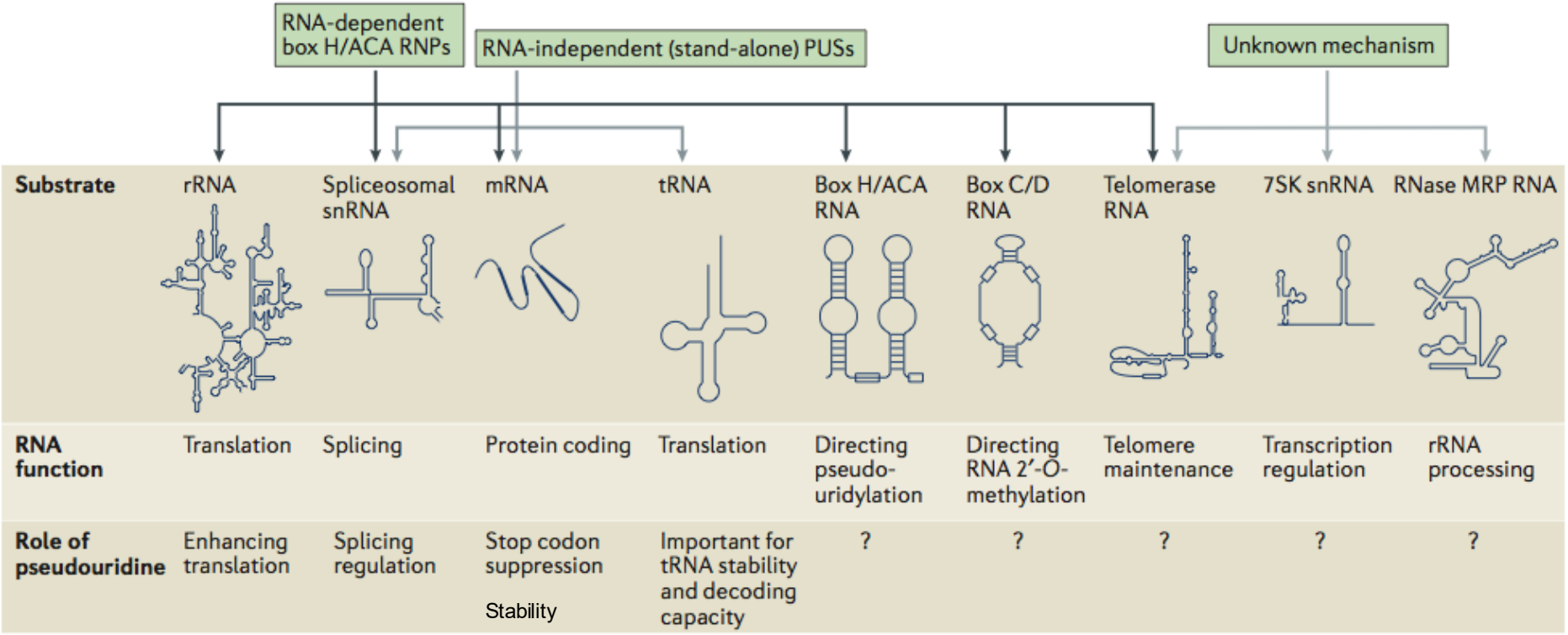
- X-linked duskeratosis congenita
- Hoyeraal-Hreidarsson syndrome



- At risk loci for Crohn's and celiac disease

Pseudouridine (Ψ)

Ψ can be found in different classes of cellular RNA. Ψ was found to undergo dynamic changes in response to heat shock and nutrient deprivation in yeast and in response to serum starvation, hydrogen peroxide and heat shock in mammalian cells.



Pseudouridine (Ψ) in snRNAs

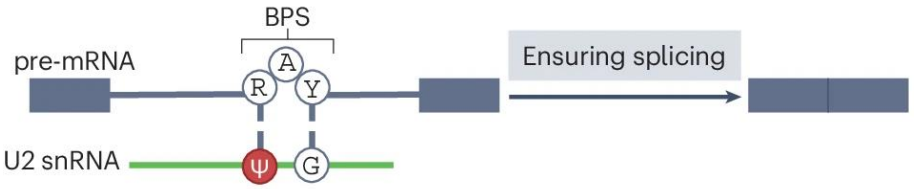
The mammalian U1, U2, U4, U5, and U6 snRNAs contain a combined 27 pseudouridines. U2 snRNA is the most highly modified with 14 reported pseudouridines, three of which are conserved across species [Ψ34, Ψ41, and Ψ43 in the branch site recognition region (BSRR) in vertebrates; Ψ35, Ψ42, and Ψ44 in the corresponding positions in budding yeast]. U1 snRNA has two conserved pseudouridines at the 5′ end (Ψ5 and Ψ6) and U5 has one (Ψ46).

A subset of box H/ACA snoRNAs called **small Cajal body–specific RNAs (scaRNAs)** guide pseudouridylation of the snRNAs based on sequence complementarity. Some snRNA pseudouridines lack a predicted guide snoRNA and might be modified by stand-alone PUSs.

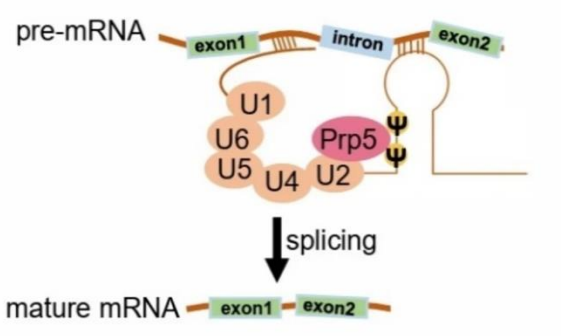
Human	Position	Pseudouridine synthase
U1	Ψ5	SCARNA16
	Ψ6	SCARNA18
U2	Ψ6	ND
	Ψ7	SCARNA14
	Ψ99	ND
	Ψ34	SCARNA8
	Ψ37	SCARNA15
	Ψ39	SCARNA4
	Ψ41	SCARNA4
	Ψ43	SCARNA8
	Ψ44	SCARNA8
	Ψ54	SCARNA13
	Ψ60	ND
	Ψ88	ND
	Ψ90	SCARNA1
	Ψ91	ND
U4	Ψ4	ND
	Ψ72	ND
	Ψ79	SCARNA26
U5	Ψ11	ND
	Ψ43	SCARNA11
	Ψ46	**SCARNA10**
		SCARNA12
	Ψ53	SCARNA13
U6	Ψ9	ND
	Ψ31	SNORA79
	Ψ40	SCARNA23
	Ψ86	SNORA79
U12	Ψ19	SCARNA21
	Ψ28	SCARNA20
U4atac	Ψ12	SCARNA21
U6atac	Ψ83	ND

The function of pseudouridine (Ψ) in snRNAs

In U2 snRNA, pseudouridylation helps the recognition of the branchpoint sequence by stabilizing the snRNA–pre-mRNA interaction. Specifically, all uridines in the U2 snRNA branch site recognition region are pseudouridylated, which increases the tolerance for recognition of a diverse branchpoint sequence. Additionally, when paired with the pre-mRNA branch site, Ψ34 of U2 snRNA helps maintain the bulge of the branch point nucleotide (A) for nucleophilic attack during splicing.

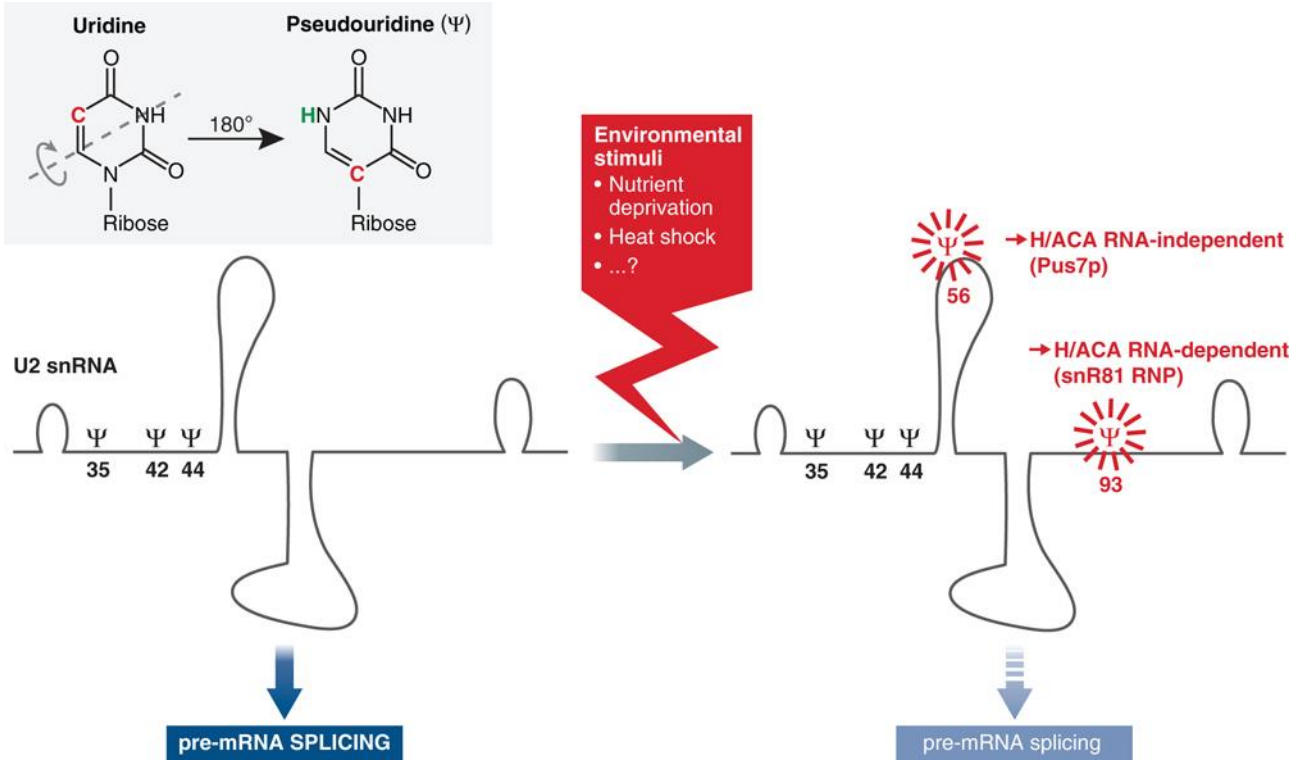


Ψs in U2 snRNA were also found to ensure RNA–protein binding in yeast through stabilizing local RNA structure: blocking the pseudouridylation of U42 and U44 altered the local structure of U2 snRNA and repressed the U2–Prp5 interaction, which is essential for pre-spliceosome assembly.



The function of pseudouridine (Ψ) in snRNAs

Pseudouridylation of *S cerevisiae* U2 snRNA can be conditionally induced. While only Ψ35, Ψ42 and Ψ44 are detected in U2 under normal conditions, nutrient deprivation leads to additional pseudouridylation at positions 56 and 93. Pseudouridylation at position 56 can also be induced by heat shock. The induced pseudouridylation of U2 results in less efficient pre-mRNA splicing.



Why would a cell *want* less efficient splicing during stress? It sounds counterproductive, but it is actually a highly coordinated survival strategy:

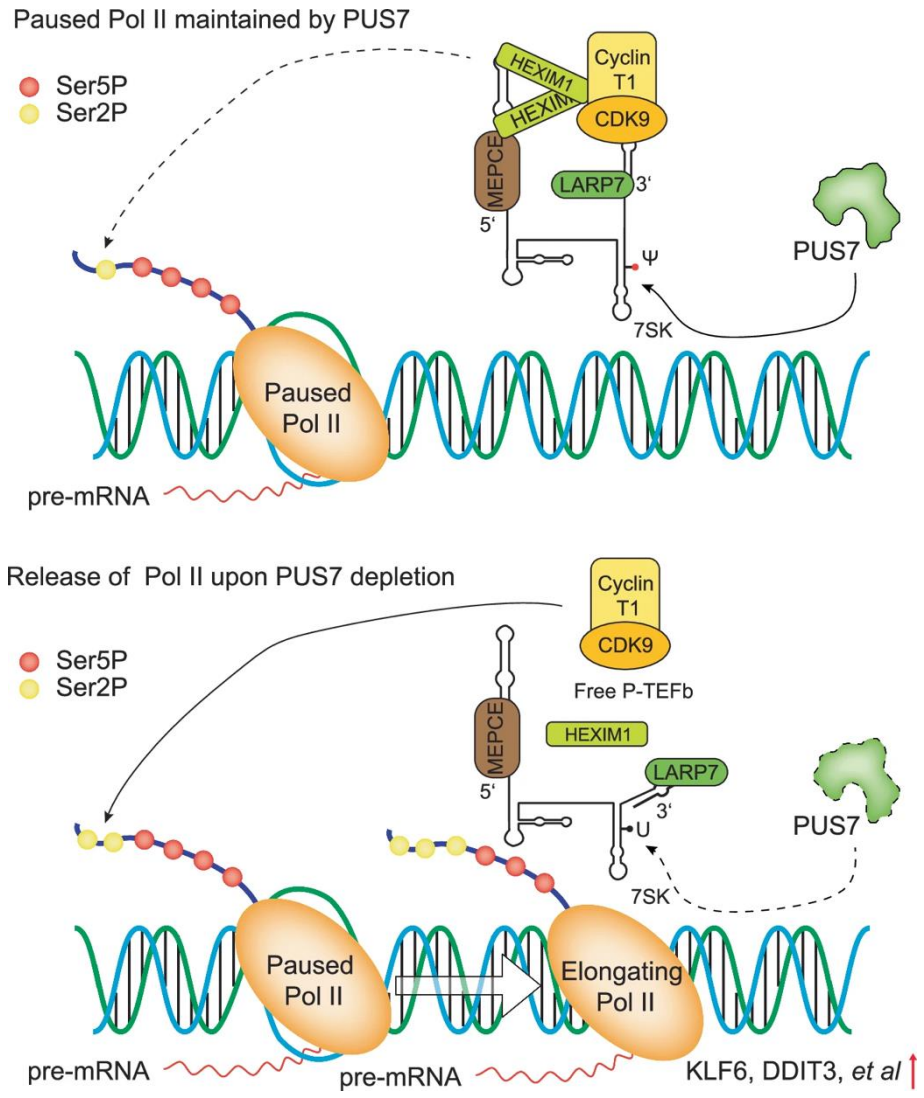
- **Splicing is incredibly expensive:** In yeast, a massive percentage of total cellular energy and RNA transcription goes into splicing pre-mRNAs—especially those coding for ribosomal proteins.
- **Slowing down production:** When food is scarce or temperatures are dangerously high, the cell needs to immediately stop growing and stop building new ribosomes.
- **The "Dimmer Switch":** Instead of completely shutting down transcription (which would kill the cell), the cell uses inducible pseudouridylation to selectively lower the efficiency of splicing. This serves as a global brake on protein synthesis, saving vital energy until the environmental stress passes.

When conditions return to normal, newly synthesized U2 snRNA will lack these modifications, restoring splicing to full speed.

Pseudouridylation of 7SK by PUS7 regulates Pol II transcription elongation

PUS7 installs 7SK Ψ250. PUS7 depletion results in hypo-peseudouridylation of 7SK and facilitates the dissociation of P-TEFb from 7SK. The released P-TEFb enhances transcription elongation by increasing phosphorylation of serine 2 (Ser2P) on carboxy terminal domain of RNA Polymerase II, thereby promoting transcription.

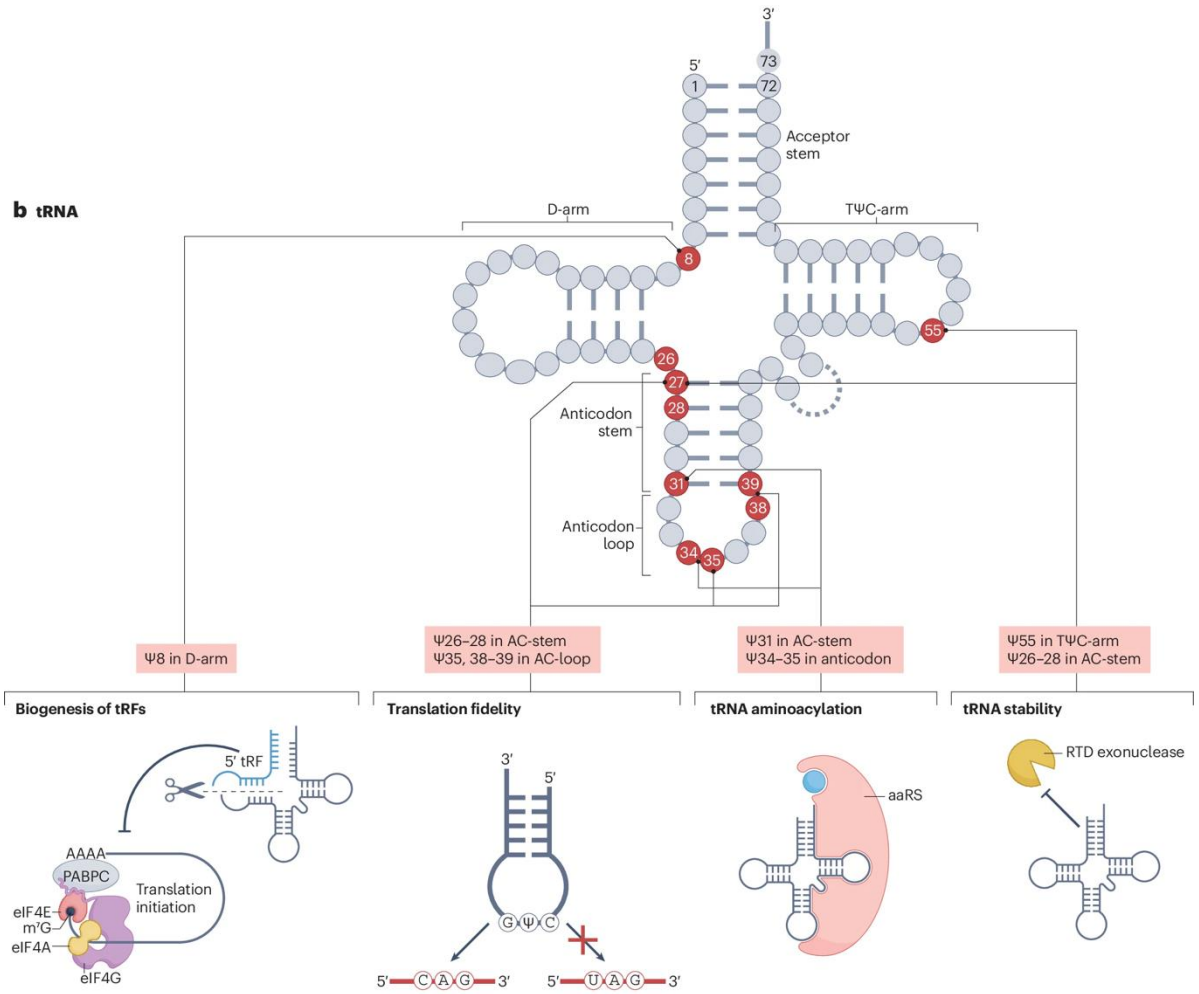
In colorectal cancer (CRC) cells, the Ψ level of 7SK can be modulated by PUS7, or by site-specifically targeted pseudouridylation through dCas13b-guided system. Hypo-pseudouridylation on 7SK upon PUS7 depletion promotes KLF6/DDIT3-mediated cell apoptosis and sensitizes CRC cells to 5-FU.

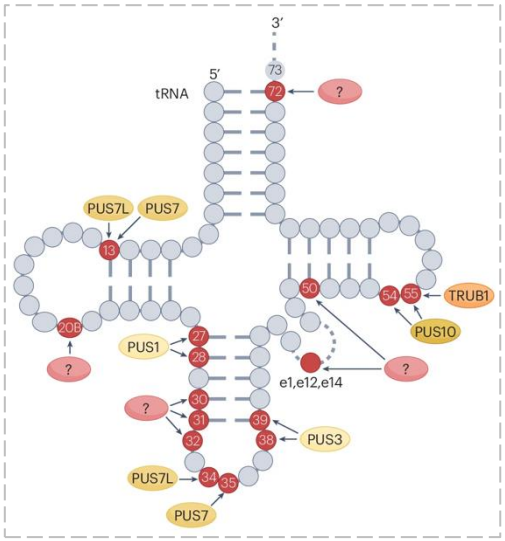


Zhao et al., 2025. Nature Comm.

The functions of Ψ modification in tRNA

tRNA pseudouridylation modulates various processes, including rapid tRNA decay (RTD), tRNA aminoacylation, translation fidelity and the biogenesis of tRNA-derived fragments (tRFs).

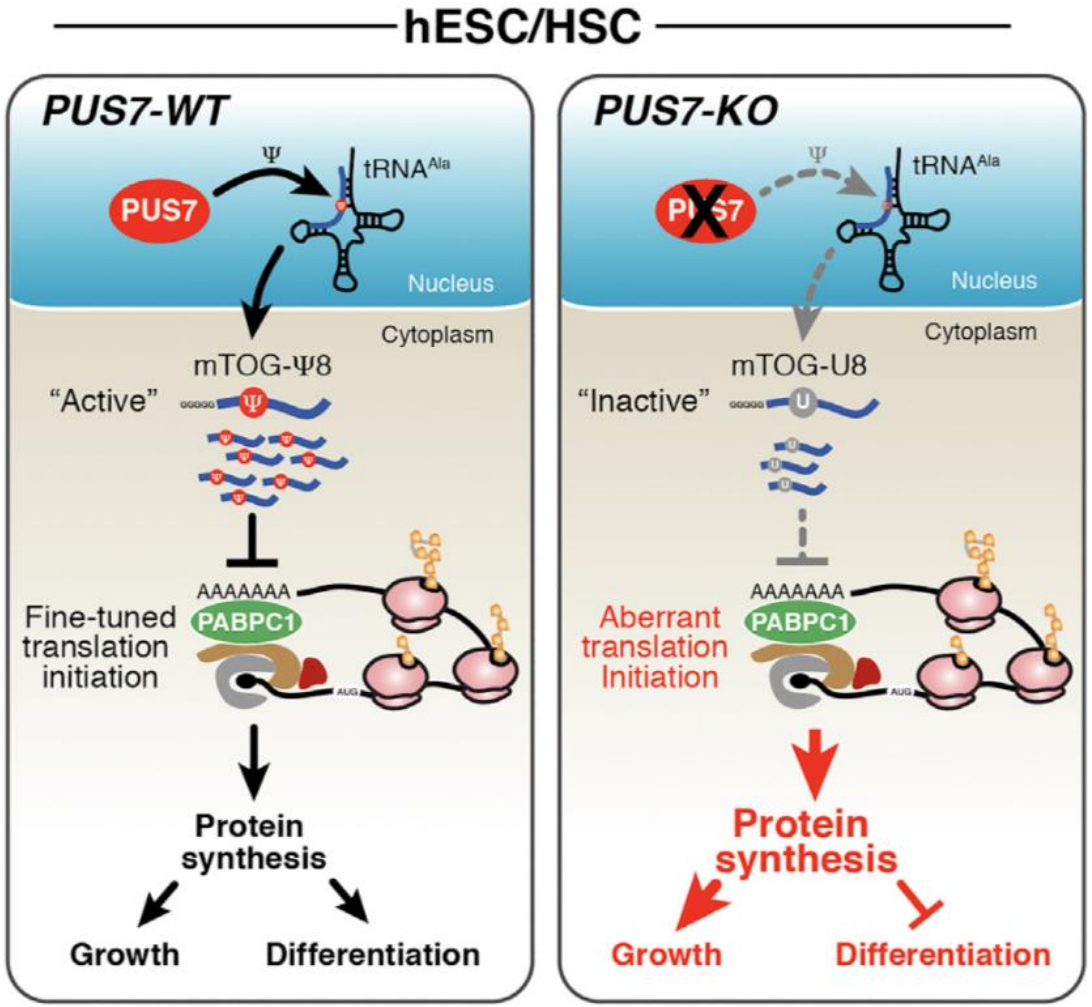




The functions of Ψ modification in tRNA

PUS7 binds distinct tRNAs and controls biogenesis of tRNA- derived fragments (tRFs) containing a 5′ terminal oli- goguanine (TOG). PUS7-mediated Ψ deploys specific tRFs (mTOGs) to repress translation in stem cells. PUS7 and mTOGs loss impairs early embryogenesis and impacts hematopoietic commitment.

RNA-Ala, tRNA-Cys, and tRNA-Val are examples of TOG tRNA

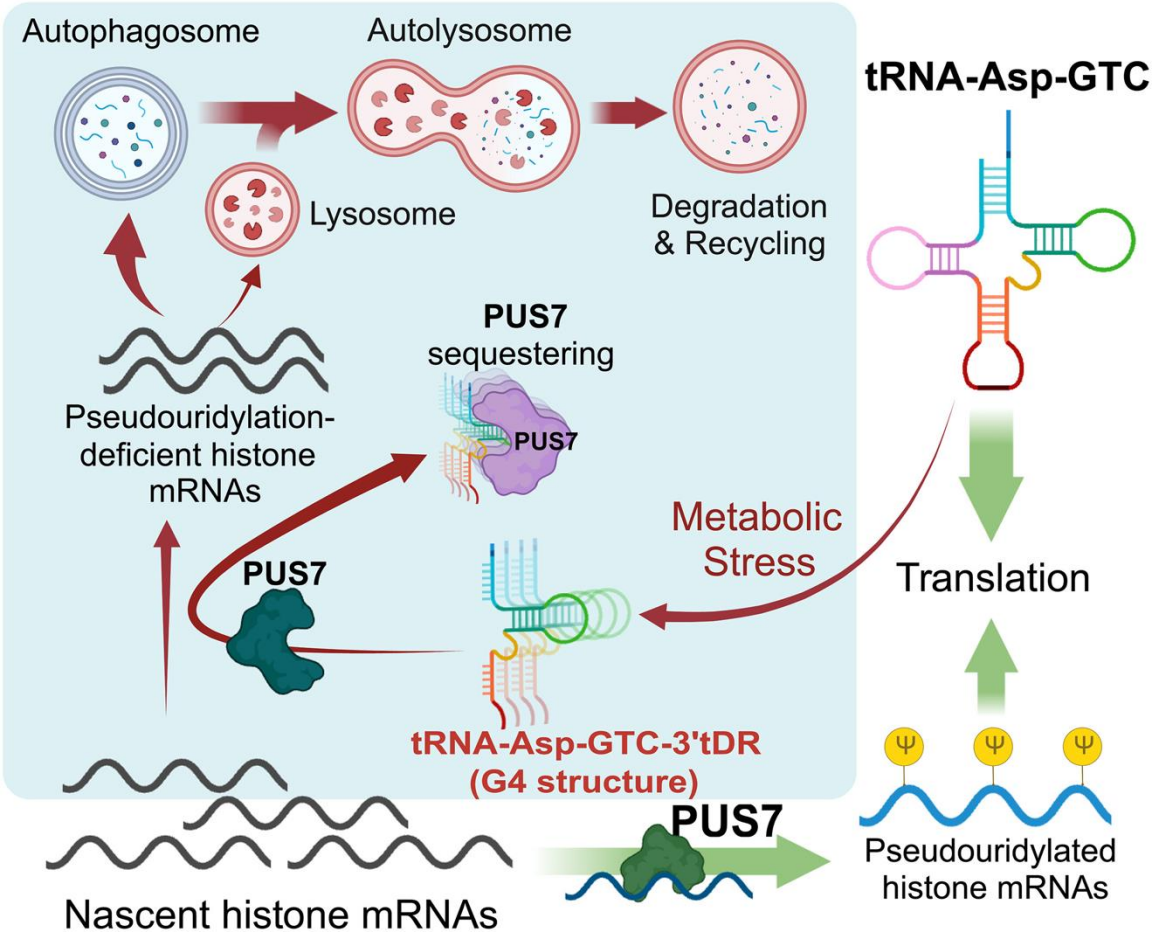


dysregulation of this circuitry is common to aggressive subtypes of human myelodysplastic syndromes.

Guzzi et al., 2018. Cell

The functions of Ψ modification in tRNA

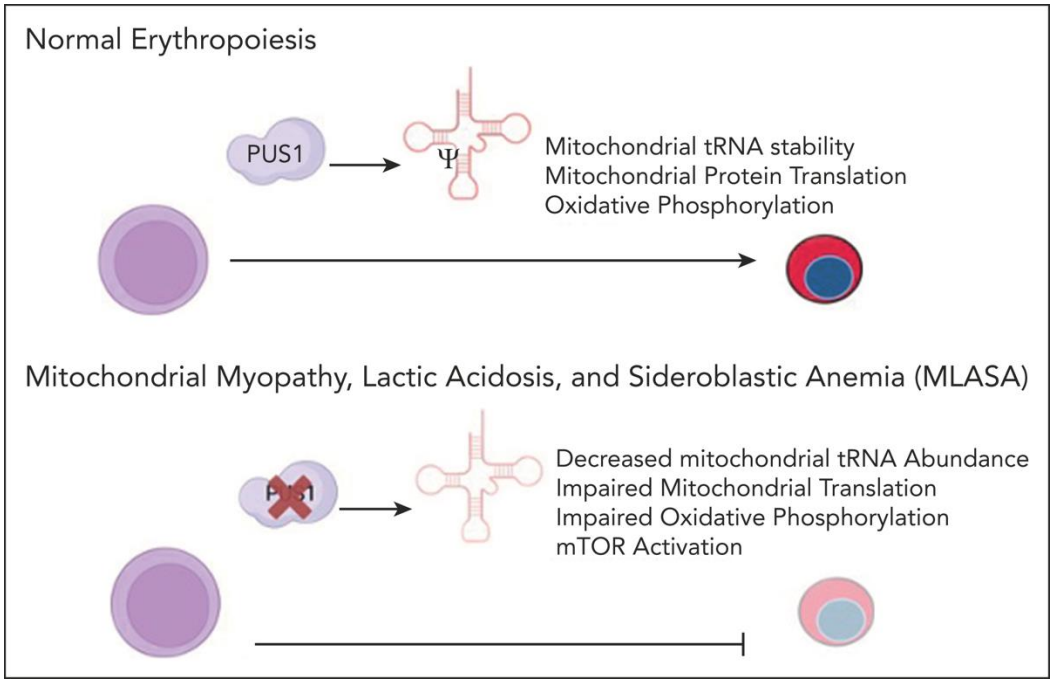
Under metabolic stress, kidney cells activate endonucleases such as angiogenin to cleave tRNA-Asp-GTC and generate tRNA-Asp-GTC-3′tDR. tRNA-Asp-GTC-3′tDRs form intermolecular G4 structures and sequester PUS7, thus preventing histone mRNA pseudouridylation. The resulting pseudouridine-deficient histone mRNAs entered the autophagosome-lysosome pathway, triggering RNA autophagy. This process serves as a protective stress response in kidney cells.



Li et al., 2025. Science

The functions of Ψ modification in tRNA

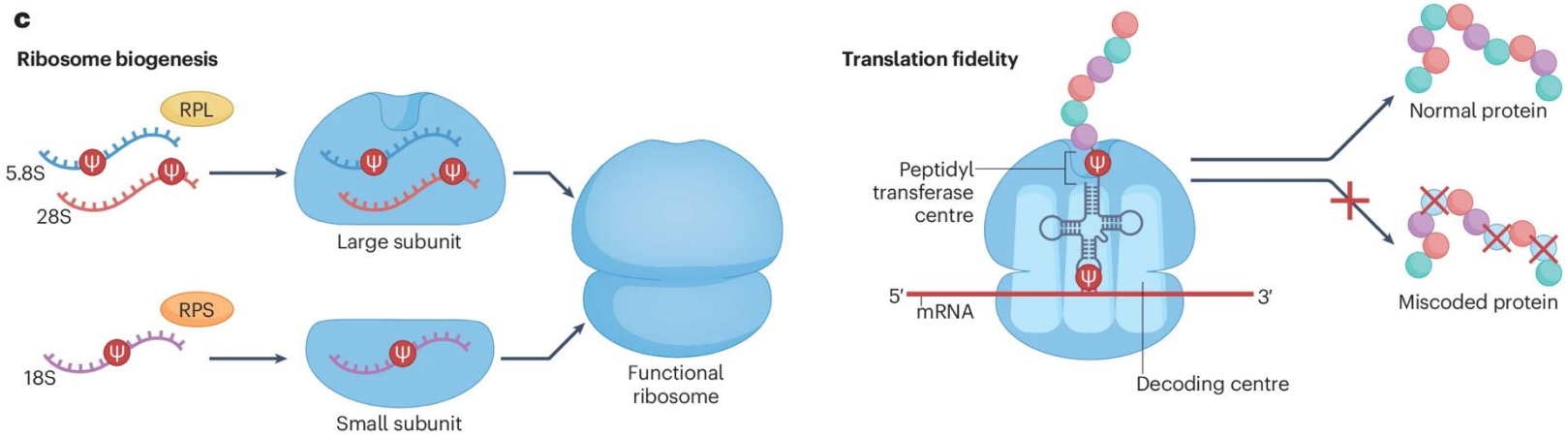
During normal erythropoiesis, the conversion of uridine to pseudouridine in tRNAs by PUS1 promotes tRNA stability and regulates protein synthesis rates and mitochondrial function. In inherited anemia known as **mitochondrial myopathy, lactic acidosis, and sideroblastic anemia (MLASA)**, mutations in PUS1 lead to loss of mitochondrial and cytoplasmic tRNA pseudouridylation and a subsequent decrease in tRNA abundance, which impairs erythropoiesis through dysregulated protein synthesis, disruption of oxidative phosphorylation, and activation of the mammalian target-of-rapamycin pathway.



Wang et al., 2024. Blood; Shi et al. 2024 iScience

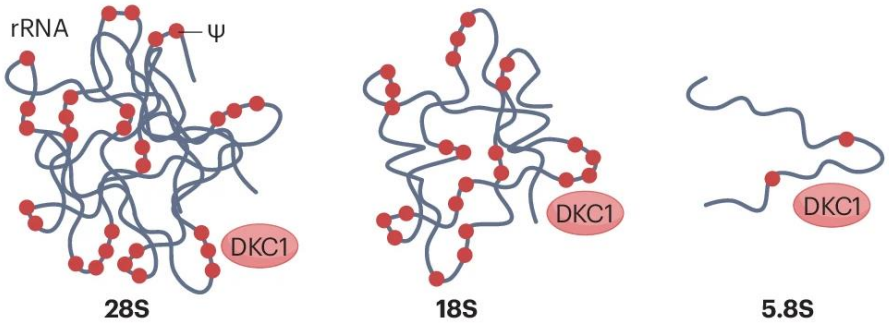
The functions of Ψ modification in rRNA

Ψ constitutes approximately 1.4% of all bases in human rRNAs (42, 60 and 2 Ψ sites identified in 18S, 28S and 5.8S, respectively). Overall, Ψs in rRNA are often clustered in functional regions of the ribosome, including the peptidyl transferase centre, the decoding centre and the inter-subunit interface, suggesting that pseudouridylation has an important role in regulating ribosome function. These modifications also affect the assembly of ribosomal proteins during ribosome biogenesis. Abnormal overall Ψ level severely impedes translocation and decoding, and alters ribosomal fidelity, leading to frameshift mutations and stop-codon readthrough.



The functions of Ψ modification in rRNA

- All Ψs in rRNAs are modified by H/ACA snoRNP/DKC1 (Cbf5 in yeast).



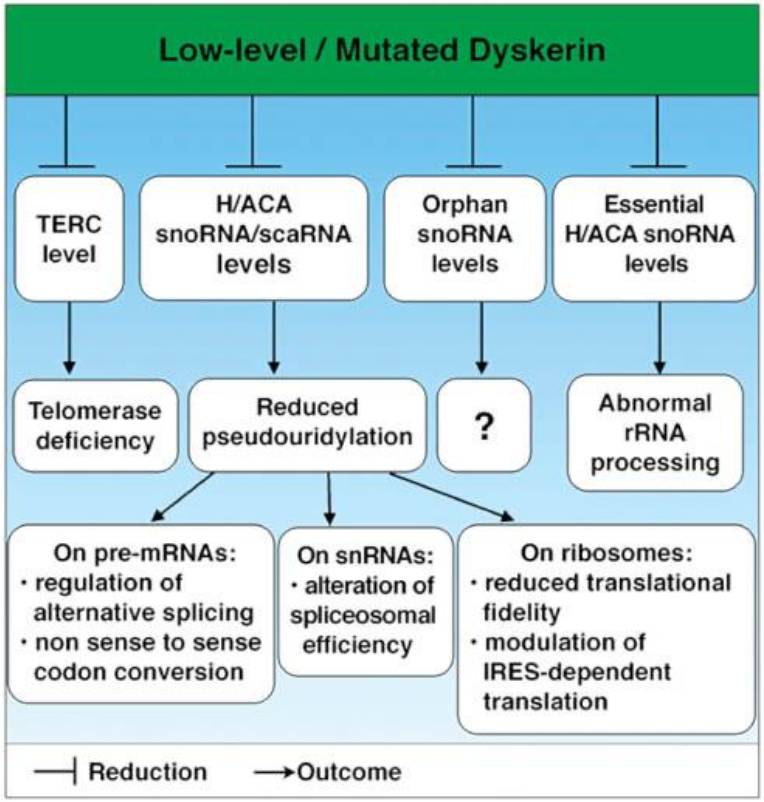
- Yeast cells expressing catalytically inactive Cbf5-D95A, the ortholog of DKC1, lack rRNA pseudouridine and are viable. However, ribosomes isolated from these slow-growing mutants show biochemical defects in tRNA binding *in vitro* and altered translation fidelity *in vivo* .

- DKC1 is an essential gene in human cells.

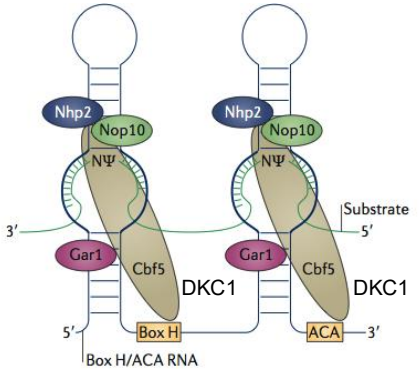
- Phenotypes caused by the deletion or overexpression of individual H/ACA snoRNAs suggest important cellular functions for individual rRNA pseudouridines in human cells. For example, ribosomes from cells lacking SNORA24, which guides pseudouridylation of two sites in 18S rRNA, show altered ribosome dynamics *in vitro* and translation fidelity defects *in vivo.*

The functions of Ψ modification in rRNA

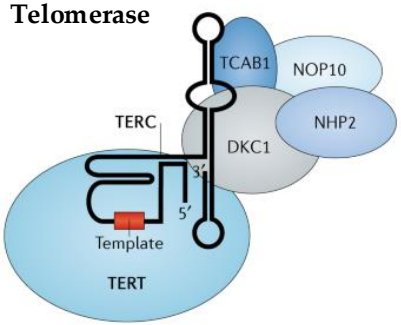
Human dyskerin participates in diverse nuclear complexes: the H/ACA snoRNPs, that control ribosome biogenesis, RNA pseudouridylation, and stability of H/ACA snoRNAs; the scaRNPs, that control pseudouridylation of snRNAs; and the telomerase active holoenzyme, which safeguards telomere integrity. The biological importance of dyskerin is further outlined by the fact that its deficiency causes the X-linked **dyskeratosis congenita disease**, characterized by mucocutaneous abnormalities and bone marrow failure.



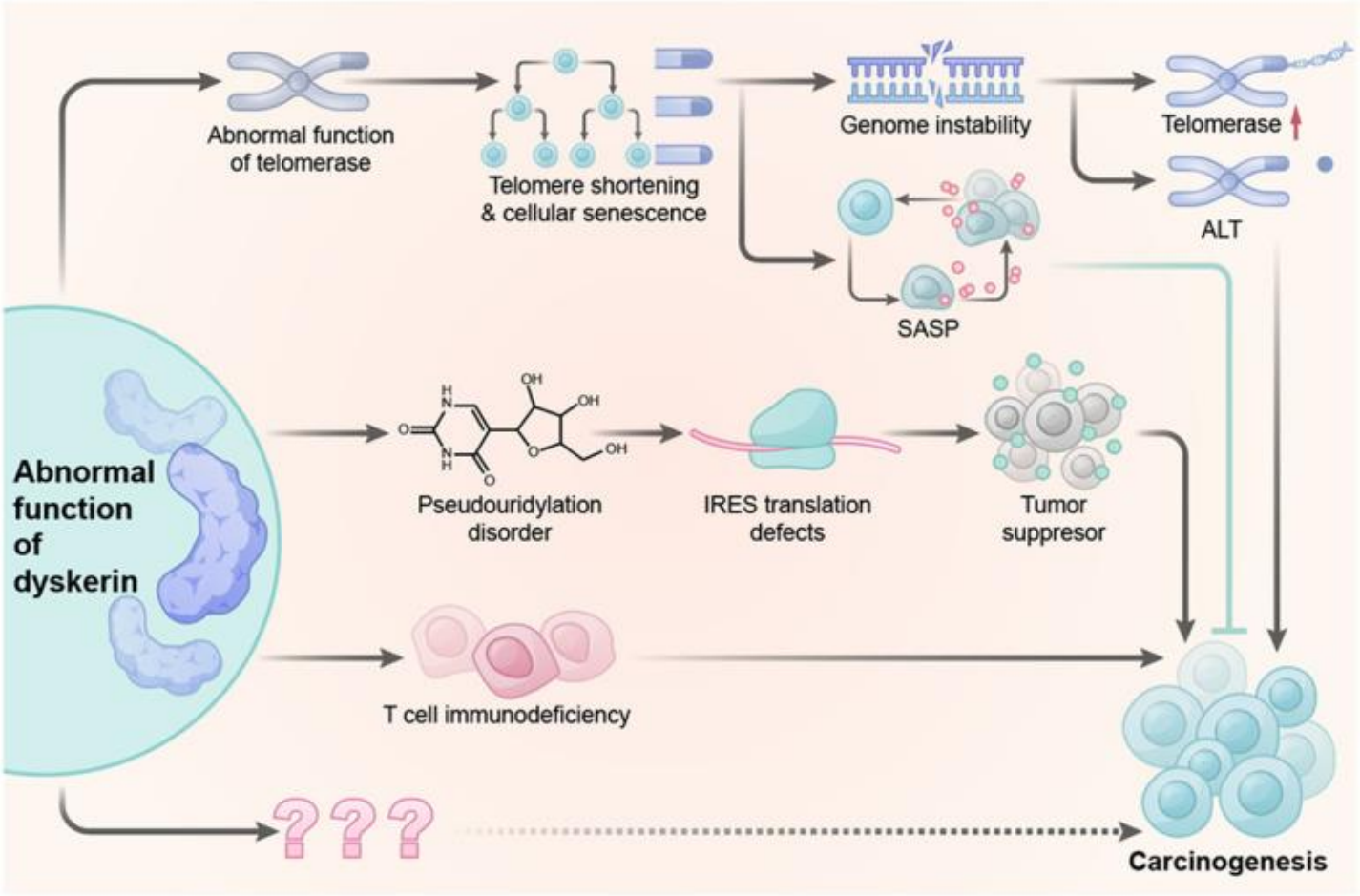
Box H/ACA snoRNPs



Telomerase

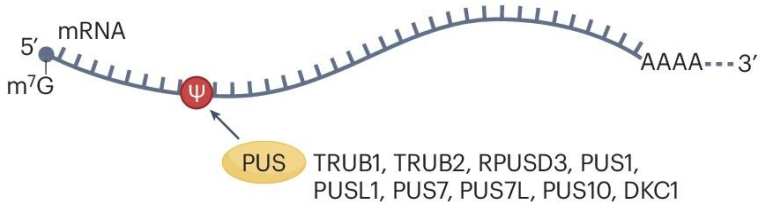


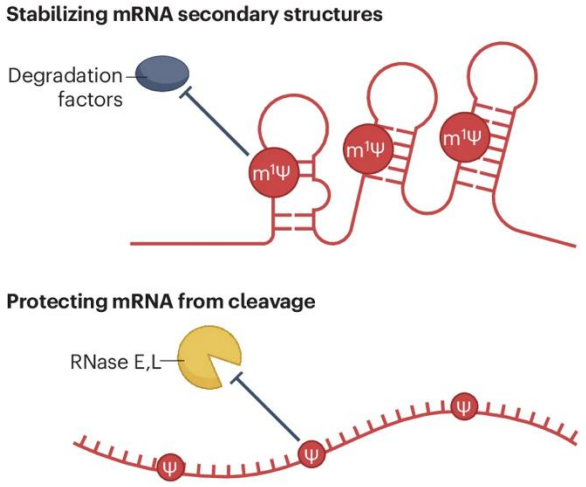
Carcinogenic mechanisms of dyskerin dysfunction



Pseudouridyne increases mRNA stability

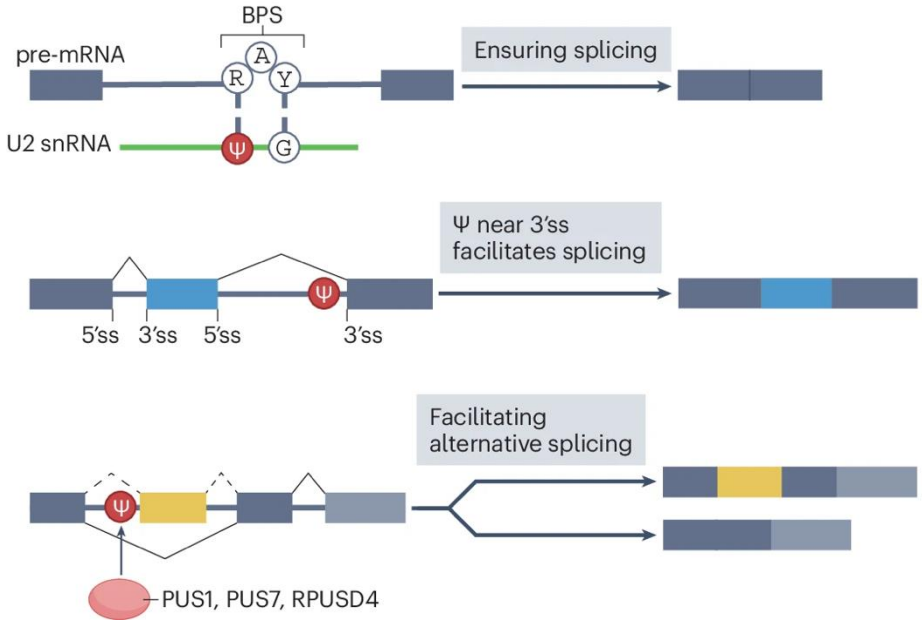
Ψ was recently reported to increase mRNA in-solution stability, possibly owing to the well-known effect of Ψ of enhancing the base stacking of RNA. Furthermore, the effect of Ψ on mRNA stability can also arise through alterations in secondary structure. For example, research has suggested that a Ψ derivative increased mRNA half-life by stabilizing a highly structured coding sequence. Finally, Ψ can protect mRNA from cleavage by ribonuclease (RNase) L and RNase E and hence increase the RNA stability.





The role of pseudouridyne in pre-mRNA splicing

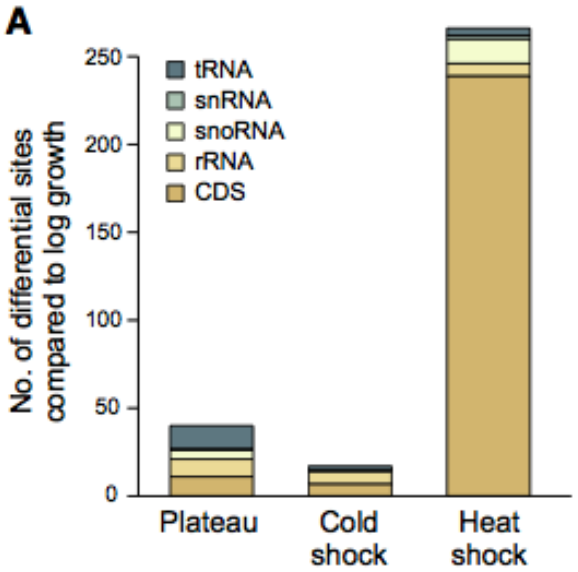
Pseudouridylation affects pre-mRNA splicing by ensuring splicing, facilitating pre-mRNA splicing and facilitating alternative splicing. Pseudouridylation in U2 small nuclear RNAs (snRNAs) helps in recognizing the branchpoint sequence (BPS) and maintaining the bulge of the branch point nucleotide (A) by stabilizing base pairing (top). A pseudouridine upstream of a 3′ splice site (3′ss) can directly facilitate splicing (middle). The pseudouridine synthases PUS1, PUS7 and RPUSD4 function in pre-mRNA splicing (alternative splicing in the coding sequence) and 3′-end processing (alternative splicing in 3′ untranslated region) (bottom).



Dynamic Pseudouridylation of mRNAs in Heat Shock

Comparsion of Ψ–seq profiles in log phase to those from yeast in growth saturation, cold shock, and heat shock showed that only a few positions selectively acquired Ψ in in stationary phase or cold shock while 265 Ψ sites were induced in heat shock.

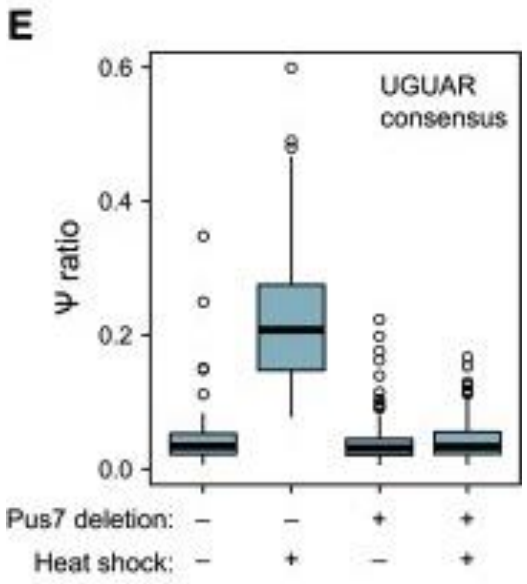
A few are in key heat-shock-induced genes, but most are in transcripts expressed at similar levels in heat shock and vegetative growth, indicating that they are differentially pseudouridylated rather than differentially expressed.



PUS7 is the major pseudouridine synthase for mRNAs in Heat Shock

- The majority of heat-shock-acquired sites (159/265, 60%) occur in a perfect "UG**U**AR" **Pus7p** consensus sequence

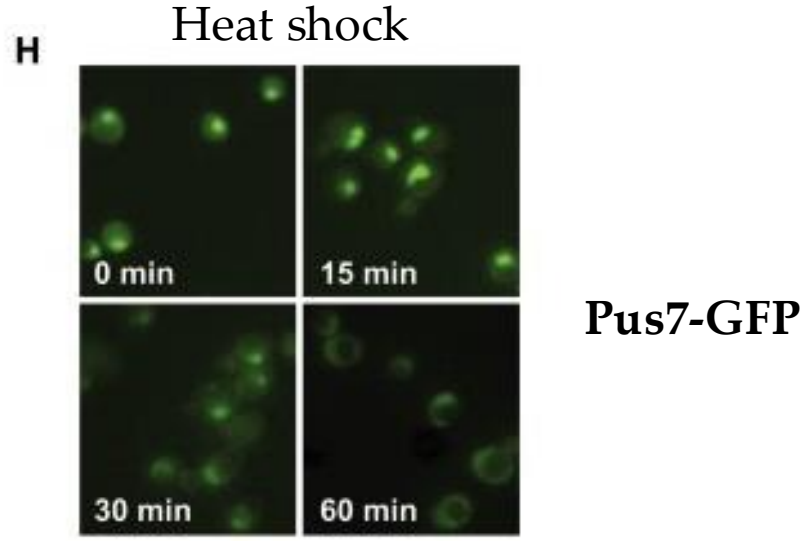
- Ψ sites harboring the Pus7p consensus were reduced to background levels in the Δ*pus7* strain in normal and heat shock conditions.



Pseudouridylation affects RNA stability

In heat shock, pseudouridylated genes were expressed at ~25% higher levels in WT strains than in Δ*pus7* strains, whereas non pseudouridylated genes were expressed at roughly equal levels in the two strains. In contrast, the two sets were comparably expressed in WT and Δ*pus7* at 30°C. Notably, Pus7 is predominantly nuclear in 30°C and primarily cytoplasmic in heat shock.

These differences in expression only in pseudouridylated genes and only in heat shock indicate that pseudouridylation may contribute to RNA stability.

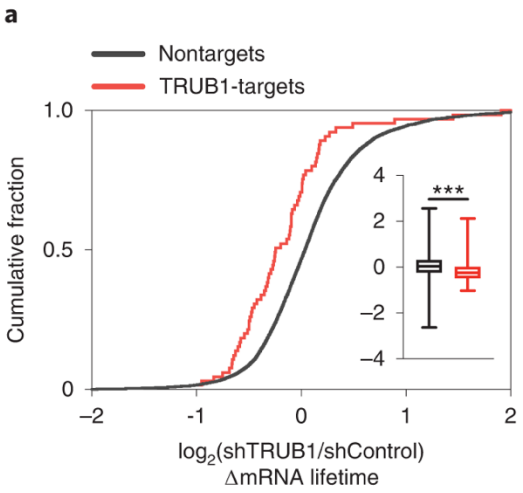


Pseudouridylation affects RNA stability

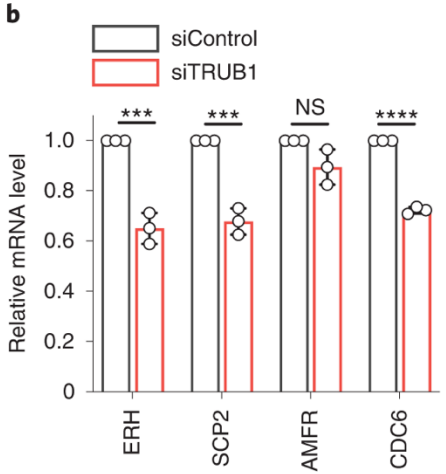
In HeLa cells, TRUB1 was identified as the main PUS enzyme that catalyzes Ψ deposition in mRNA (knockdown and BID-seq). The most frequent motifs are GUΨCN (N = A or C or G or U) and poly-U (UUUUU).

They performed TRUB1 knockdown and analysed its effects on transcript half-life by RNA-seq. TRUB1-targets, which carry TRUB1-modified Ψ in mRNA in shControl cells, displayed a shorter half-life upon TRUB1 knockdown, whereas the half-life of nontargets (without detectable Ψ) remained unchanged. Three of the four targets displayed notable reduced mRNA level after 72 h siTRUB1 knockdown compared with the control.

RNA-seq



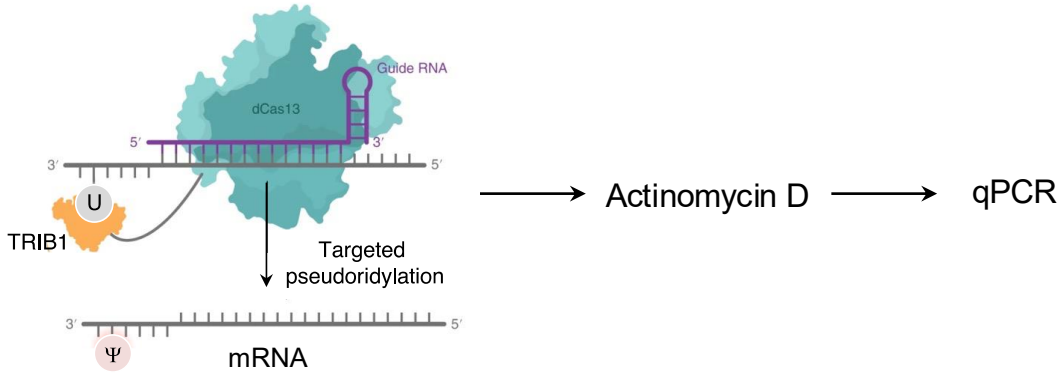
qPCR

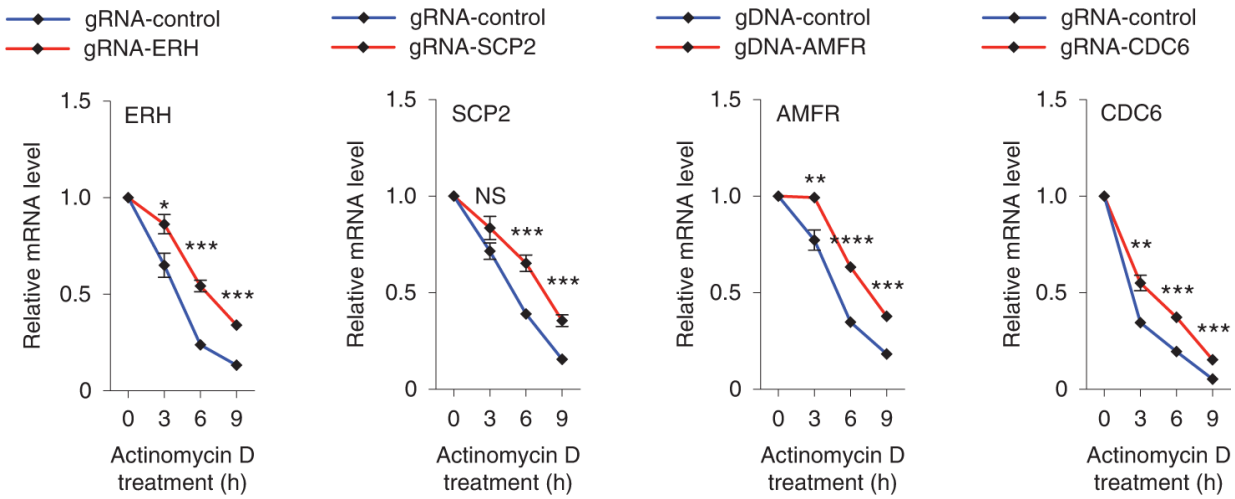


Dai et al., 2022. Nature Biotech.

Pseudouridylation affects RNA stability

To further validate the transcript stabilization role of TRUB1-regulated Ψ, they engineered a fused dCas13d-TRUB1 system—and confirmed that site-specific Ψ deposition could notably prolong mRNA lifetime.





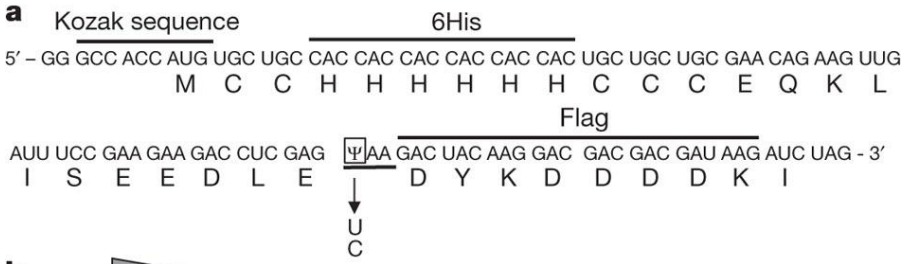
Dai et al., 2022. Nature Biotech.

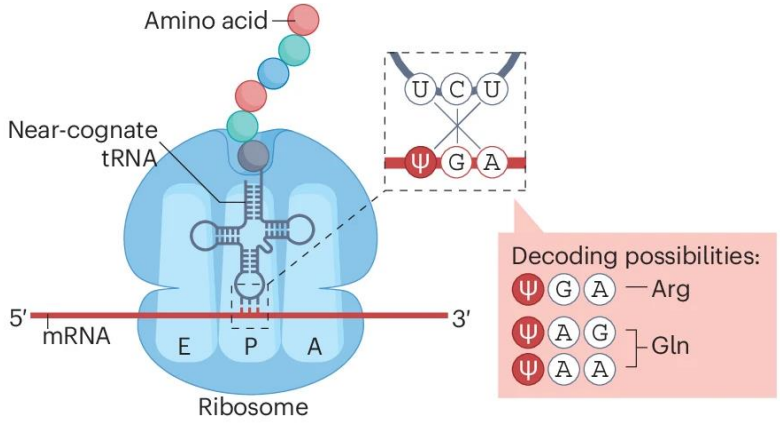
nature 2011

doi:10.1038/nature10165

Converting nonsense codons into sense codons by targeted pseudouridylation

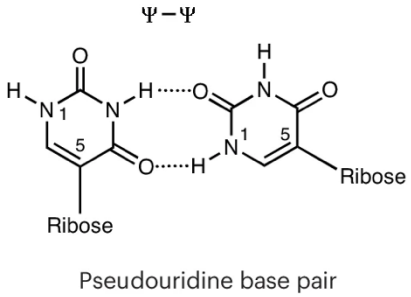
John Karijolich[1] & Yi-Tao Yu[1]



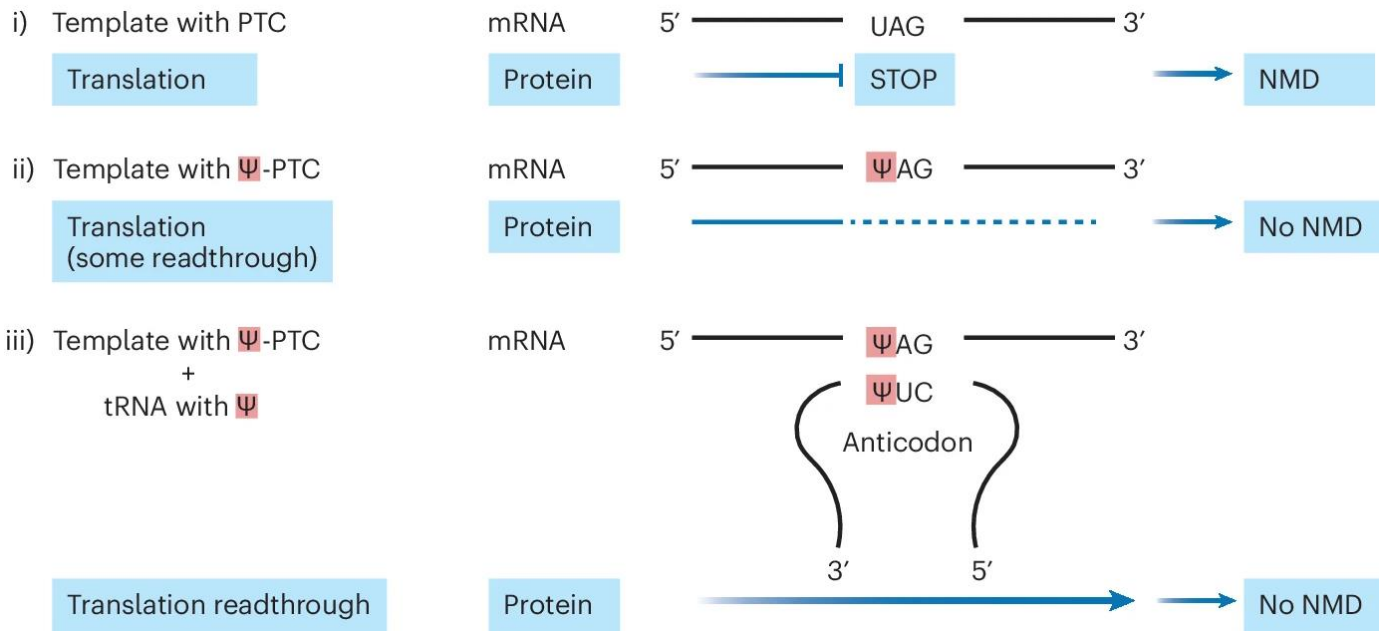


The effect of Ψ on readthrough of PTCs

A PTC arrests translation and solicits nonsense-mediated mRNA decay (NMD) (i). A PTC with a Ψ in its 5′ position results in some readthrough, avoiding NMD (ii). If the PTC in part ii is matched by a Ψ in the anticodon of its cognate tRNA, resulting in a Ψ–Ψ base pair, readthrough is efficient and NMD is avoided.

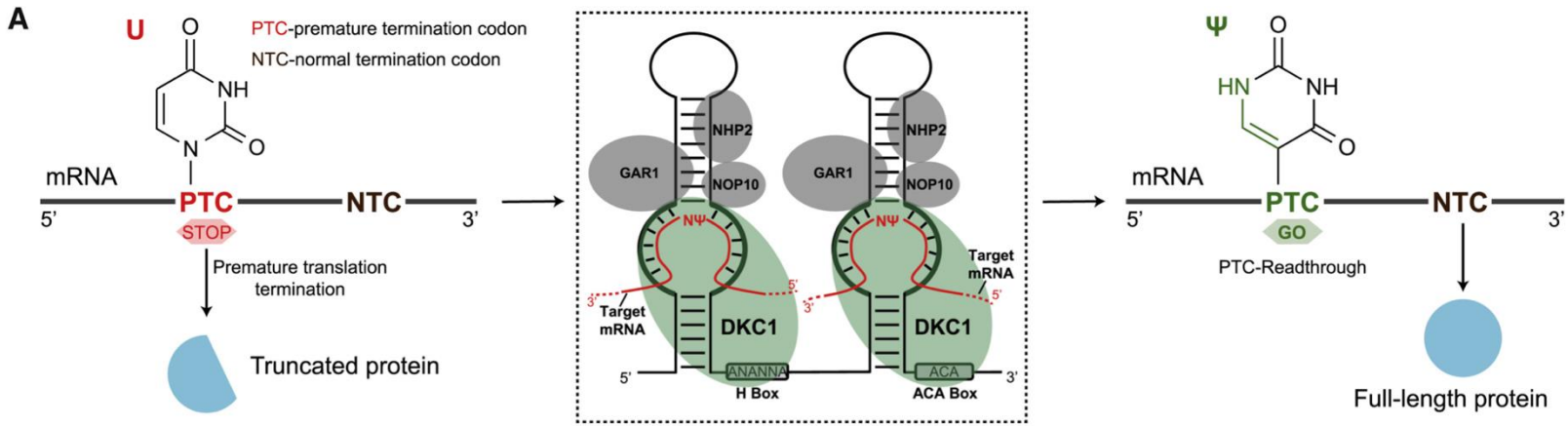


Pseudouridine base pair

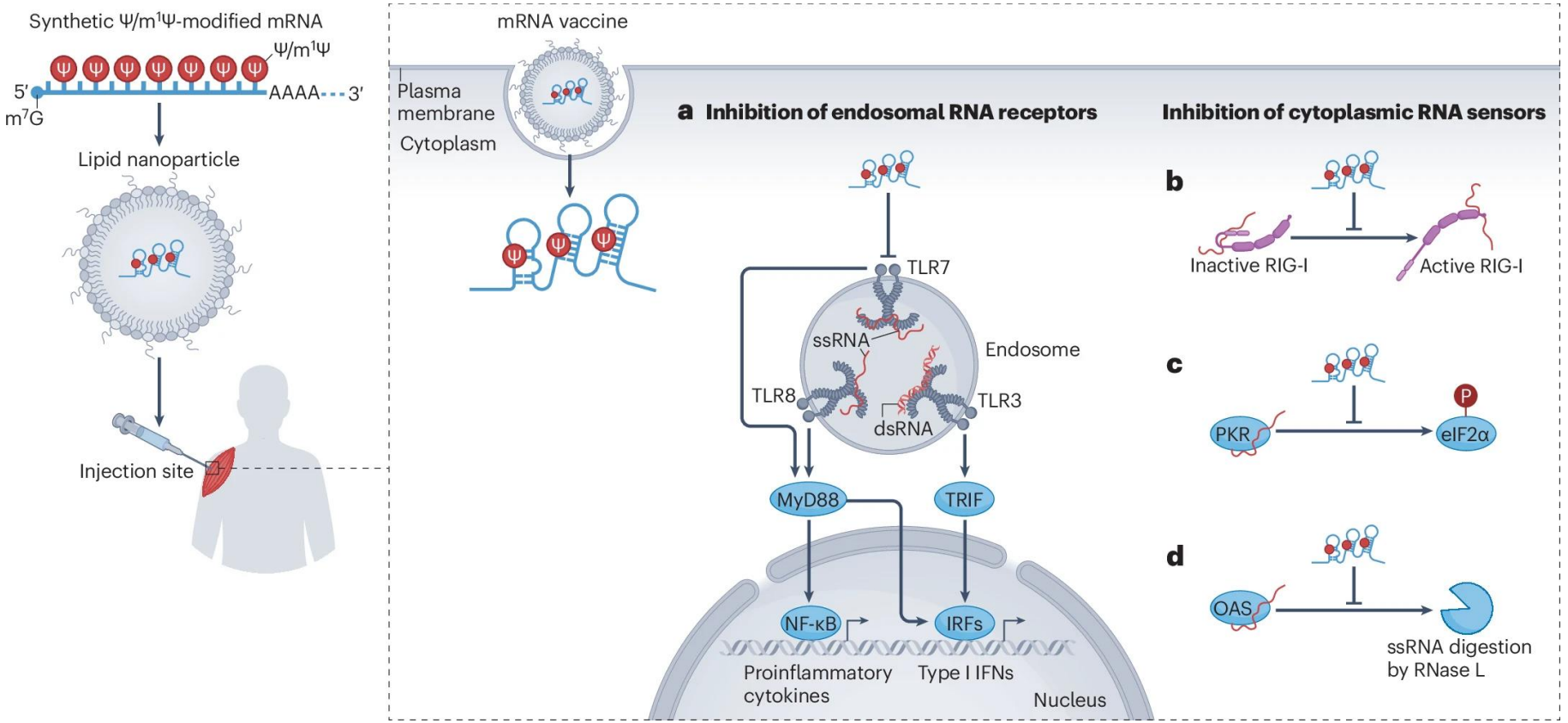


Targeted pseudouridylation to correct PTC-associated diseases

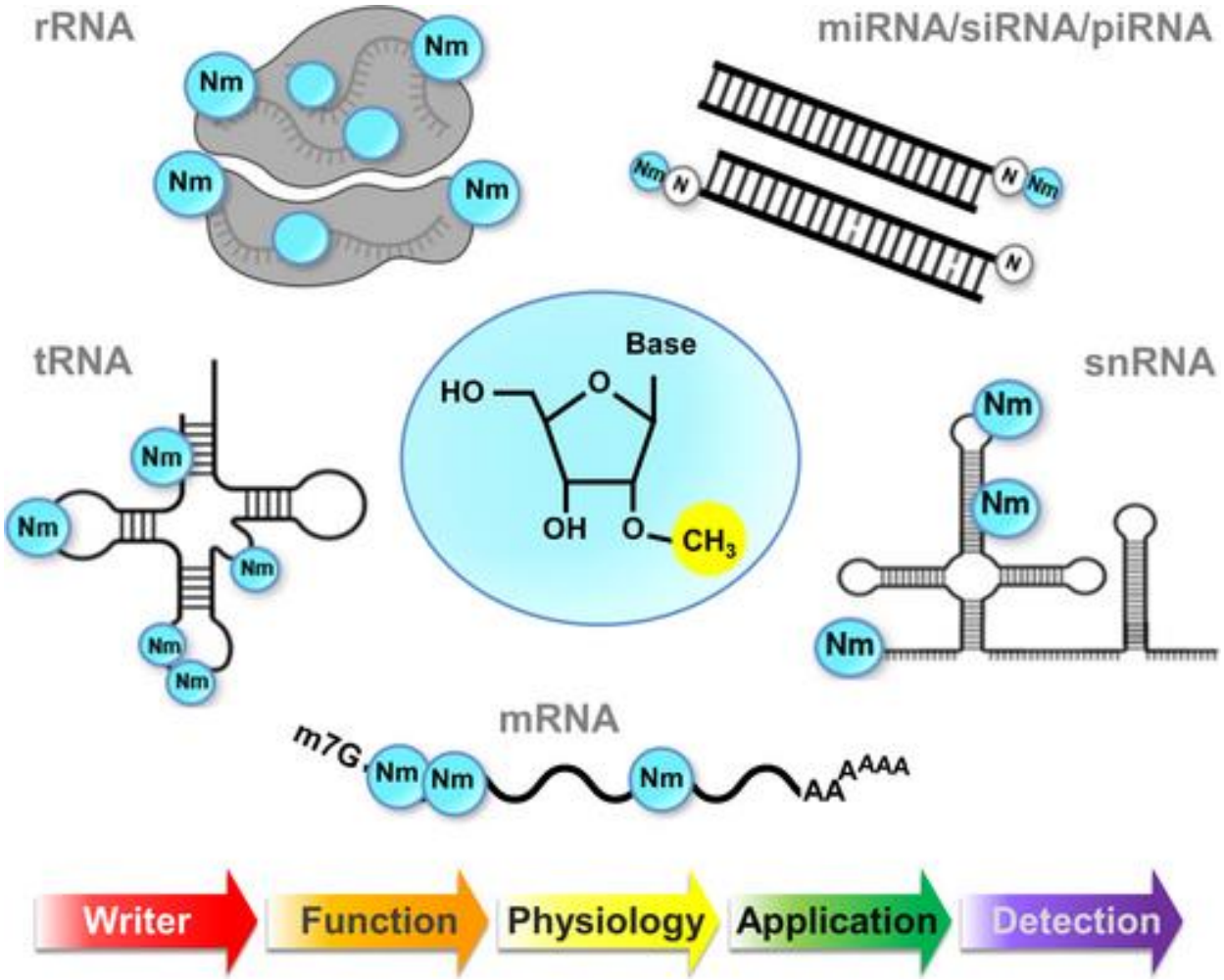
Adachi et al. (Mol Cell 2023) suppress the *CFTR G542X* nonsense mutation in a cystic fibrosis cell line, while Song et al. (Mol Cell 2023) achieve correction of the *Idua W392X* PTC in cells from a mouse model of Hurler syndrome.



Pseudouridylation in mRNA vaccines suppresses mRNA sensing by innate immune factors

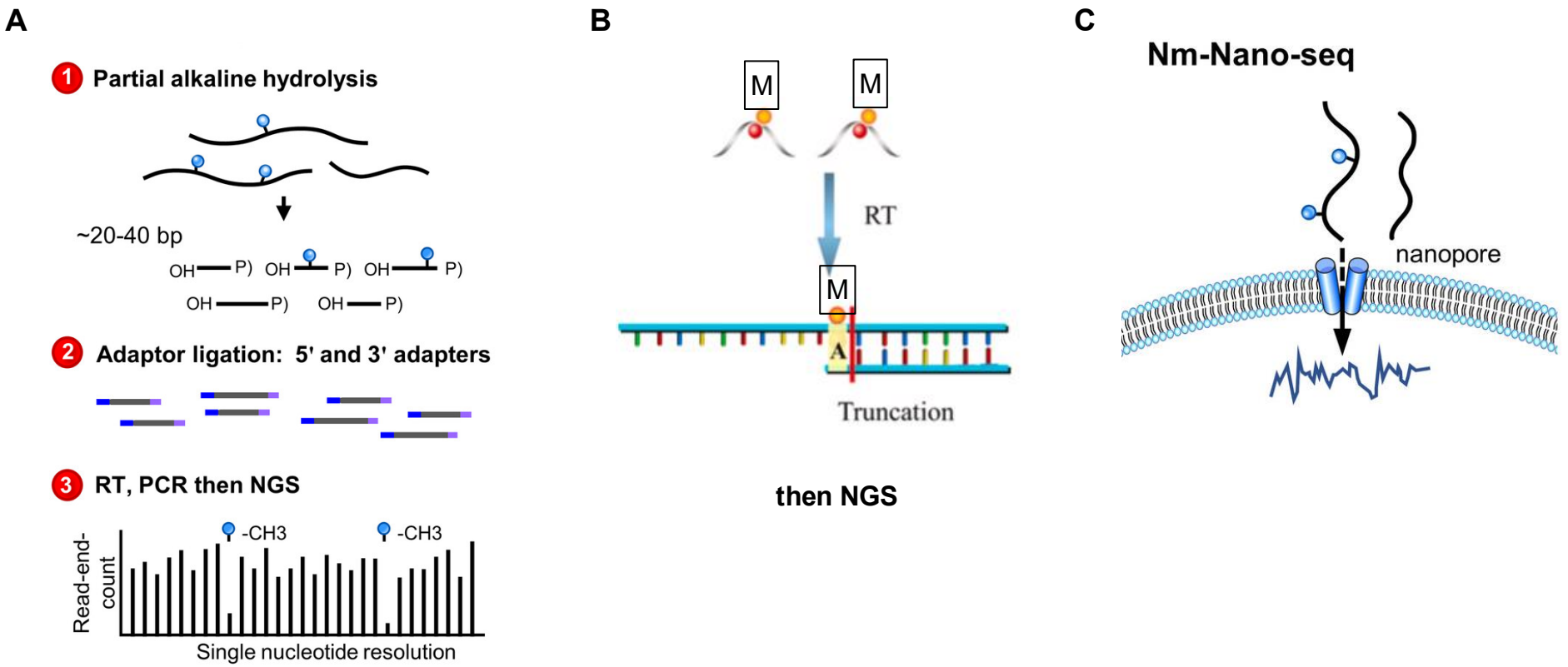


2′-*O*-Methylation



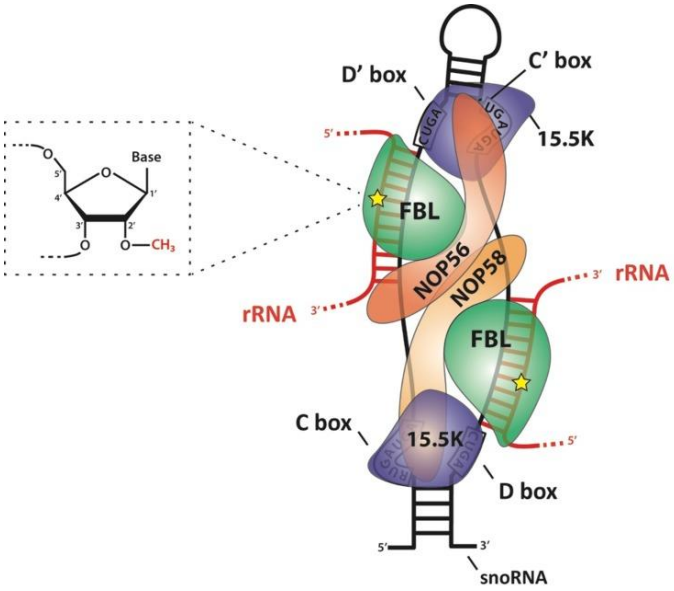
Methods for detecting 2′-O-methylation

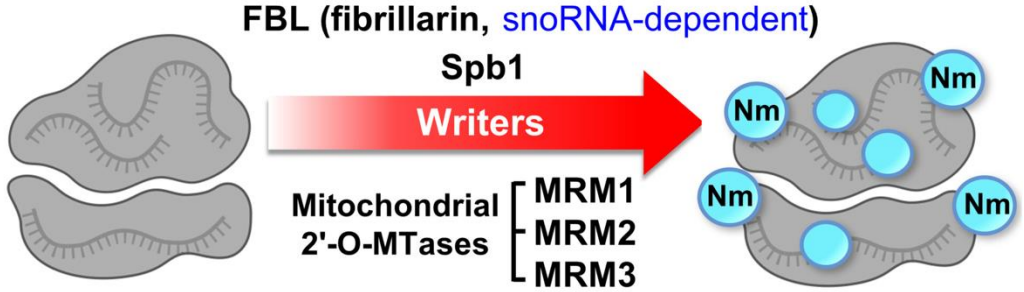
Detection of 2′-O-Me is based essentially on two properties of 2′-O- methylated RNA. First, 2′-O-Me replaces the 2′-OH group, thus rendering methylated RNA resistant to hydrolysis compared to unmethylated RNA (A). Second, 2′-O-Me tends to block retro-transcription of RNA into complementary DNA (cDNA) when the reaction is performed under non-optimal conditions (e.g., low deoxy-nucleoside triphosphate (dNTP) concentration) (B). Alternatively, 2′-O-Me can be detected by direct RNA sequencing with Nanopore (C).



2′-*O*-Methylation writers for ribosomal RNA

In eukaryotes, rRNA 2′-O-Me is primarily catalyzed by the MTase fibrillarin (FBL, also known as Nop1 in yeast). This process is guided by box C/D small nucleolar RNPs (snoRNPs). While less common, rRNA 2′-O-Me can occur independently of snoRNAs. Saccharomyces cerevisiae uses Spb1 to catalyze the 2′-O-Me of guanosine at position 2922 (G2922) in 27S pre-rRNA. Mitochondrial rRNAs also undergo site-specific 2′-O-Me by snoRNA-independent MTases, MRM1, MRM2, and MRM3, which are vital for mitoribosome biogenesis and mitochondrial respiration.





2′-O-Me appears to be relatively stable and is likely irreversible, as no demethylases (“erasers”) have been identified to date

2′-*O*-Methylation of ribosomal RNA

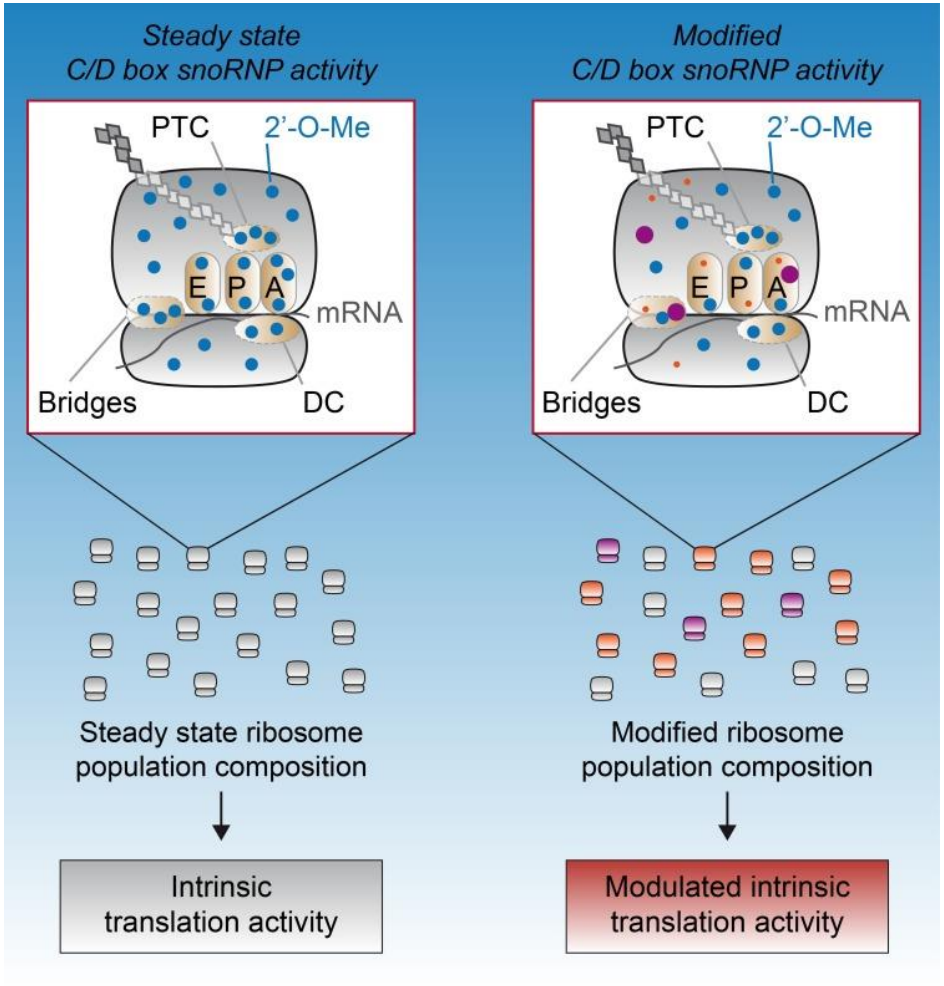
The 2′-O-Me of rRNA contributes to proper rRNA folding and stabilization, which is critical for ribosome assembly, nuclear export, and translation efficiency and fidelity.

2′-O-Me modifications are enriched in functional domains involved in key aspects of translation, such as the decoding site and the peptidyl transferase center. Downregulation of FBL or C/D box snoRNAs results in rRNA hypomethylation, affecting ribosome biogenesis and the efficiency of cap-dependent versus internal ribosome entry site (IRES)-dependent translation. Such hypomethylation also leads to defects in translational fidelity, including frameshifts and near-cognate start codon selection.

Most 2′-O-Me modifications are introduced during the early stages of ribosome biogenesis. These distinct 2′-O-Me patterns contribute significantly to ribosome heterogeneity, leading to the formation of specialized ribosomes that preferentially translate specific subsets of mRNAs.

2′-*O*-Methylation of ribosomal RNA

Methylation profiles can differ substantially between cell types suggests a role for 2′-O-me in regulating translation to meet the specific requirements of each cell type. Interestingly, it has been recently shown that site-dependent rRNA 2′-O-me levels can influence translation of mRNAs associated with functionally distinct categories. Importantly, this resulted in altered cellular phenotypes.



nature structural & molecular biology

ARTICLES

https://doi.org/10.1038/s41594-021-00669-4

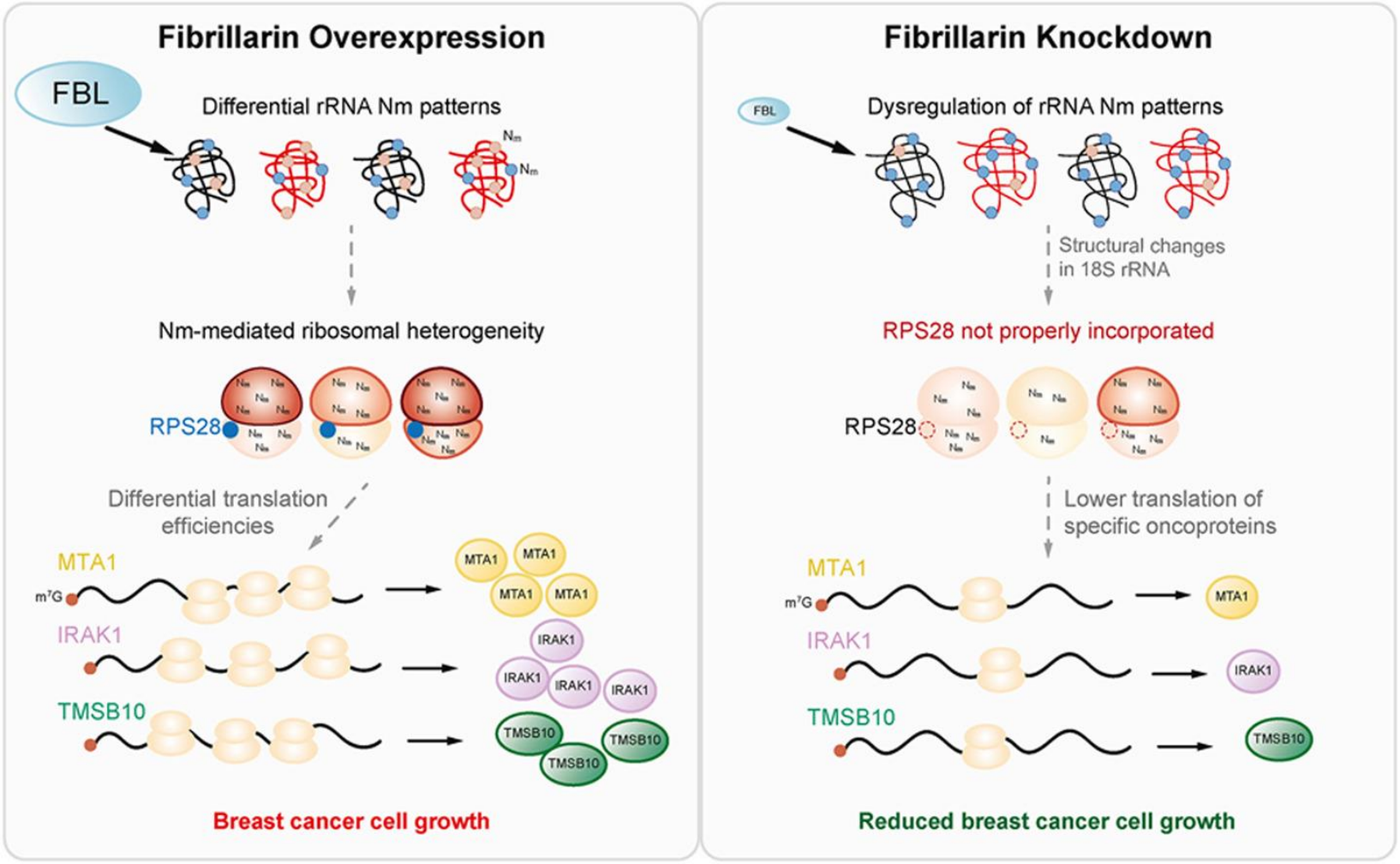
Check for updates

Regulation of translation by site-specific ribosomal RNA methylation

Martin D. Jansson[1]✉, Sophia J. Häfner[1], Kübra Altinel[1,5], Disa Tehler[1,5], Nicolai Krogh[2], Emil Jakobsen[3], Jens V. Andersen[3], Kasper L. Andersen[1], Erwin M. Schoof[4], Patrice Ménard[1], Henrik Nielsen[2] and Anders H. Lund[1]✉

Ribosomes are complex ribozymes that interpret genetic information by translating messenger RNA (mRNA) into proteins. Natural variation in ribosome composition has been documented in several organisms and can arise from several different sources. A key question is whether specific control over ribosome heterogeneity represents a mechanism by which translation can be regulated. We used RiboMeth-seq to demonstrate that differential 2′-O-methylation of ribosomal RNA (rRNA) represents a considerable source of ribosome heterogeneity in human cells, and that modification levels at distinct sites can change dynamically in response to upstream signaling pathways, such as *MYC* oncogene expression. Ablation of one prominent methylation resulted in altered translation of select mRNAs and corresponding changes in cellular phenotypes. Thus, differential rRNA 2′-O-methylation can give rise to ribosomes with specialized function. This suggests a broader mechanism where the specific regulation of rRNA modification patterns fine tunes translation.

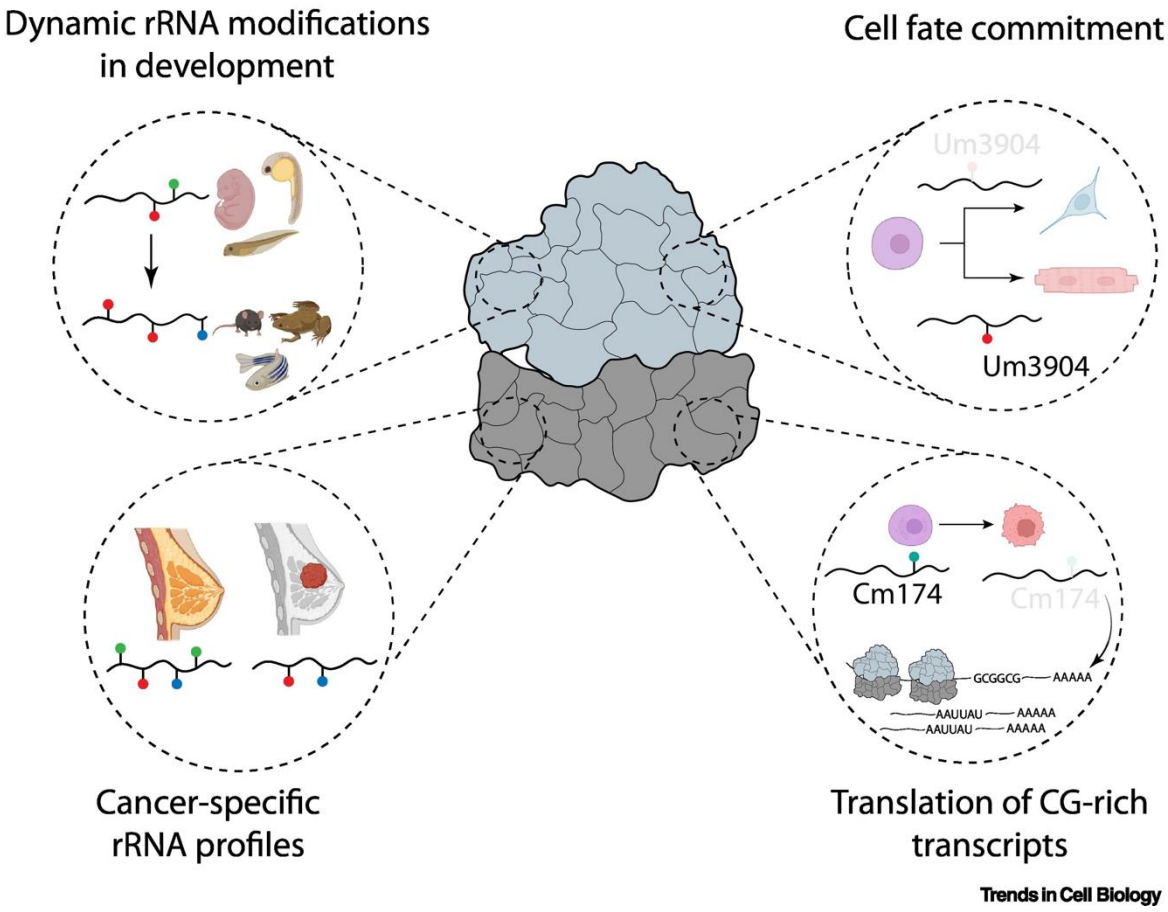
Fibrillarin supports oncogenic translation in breast cancer



Groza et al., 2026. Cancer Letters

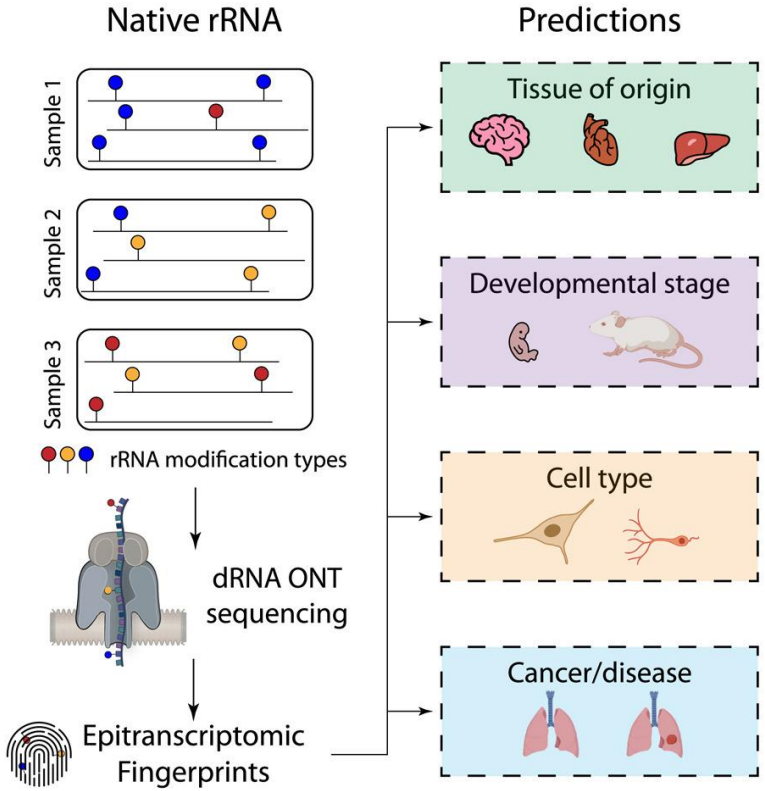
Dynamic rRNA modifications in health and disease

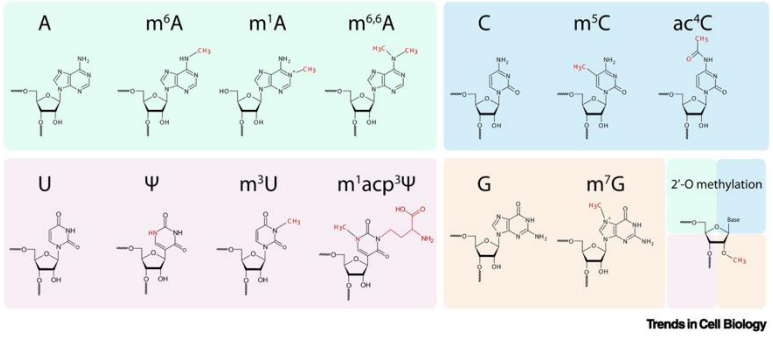
rRNA modification dynamics might play a role in disease onset and progress, influencing numerous cellular processes, and that this information may be exploited for diagnostic purposes in the near future.



Epitranscriptomic rRNA fingerprinting reveals tissue-of-origin and tumor-specific signatures

Direct RNA-seq by ONT reveals multiple rRNA sites that are differentially modified in a tissue- and/or developmental stage-specific manner. Analysis of rRNA modification patterns in normal-tumor matched samples from lung cancer patients demonstrated that epitranscriptomic fingerprinting accurately classifies clinical samples into normal and tumor groups from only 250 reads per sample, demonstrating the potential of rRNA modifications as diagnostic biomarkers.



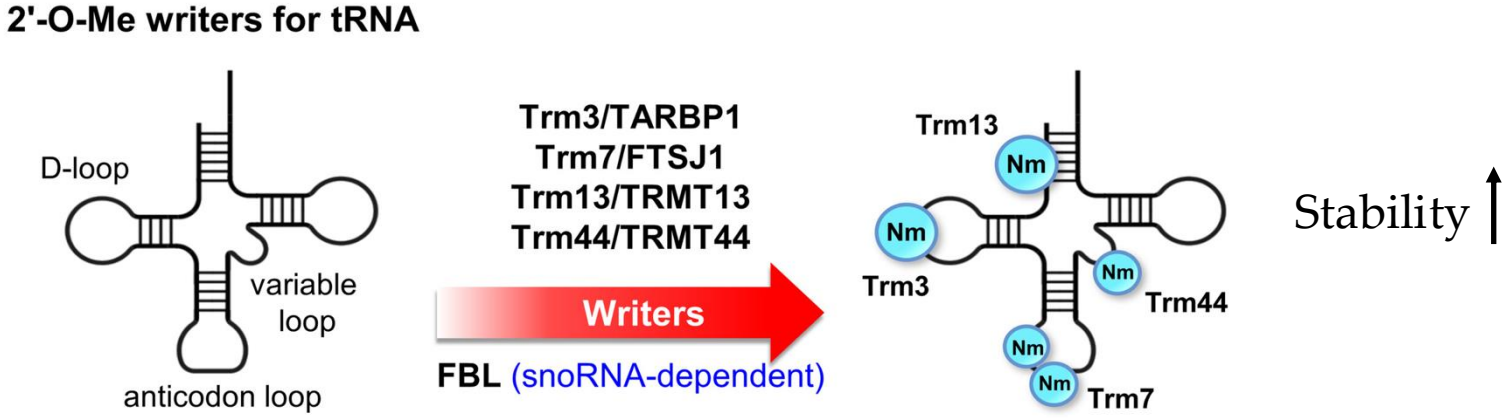


Human rRNA modifications

Milenkovic et al., 2025. Mol Cell

2′-*O*-Methylation of tRNA

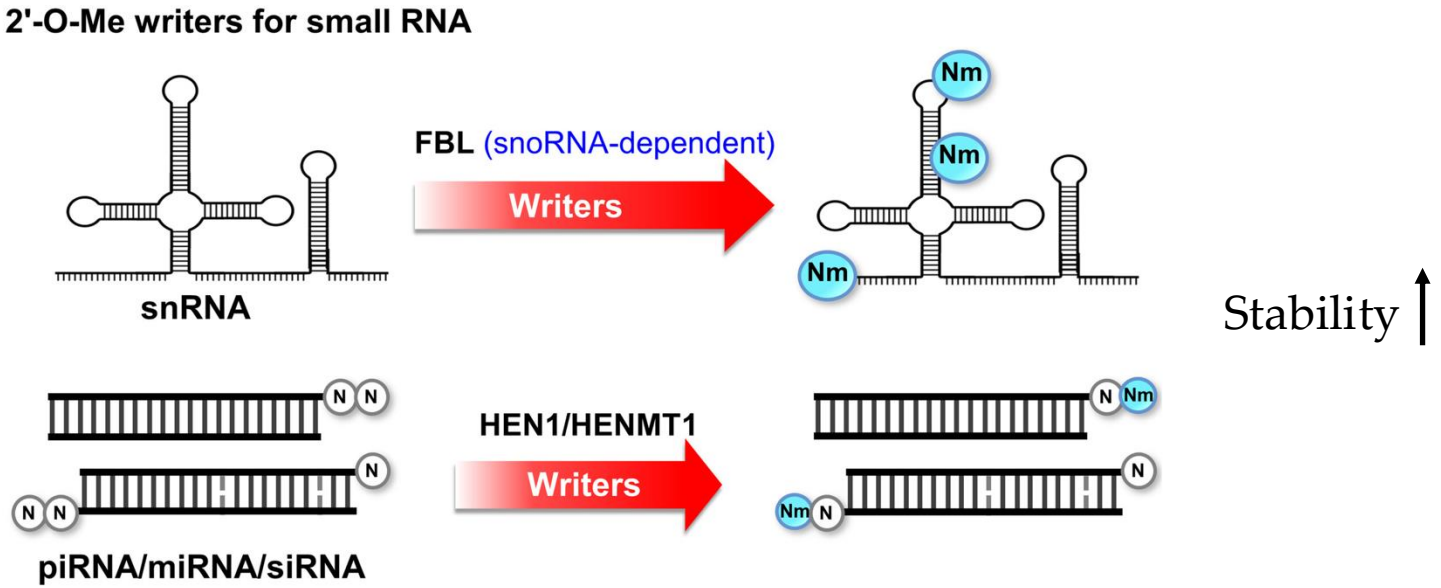
2′-O-Me in tRNA is critical for various physiological processes, including immune responses and translational efficiency. Similar to rRNA, 2′-O-Me in tRNA promotes thermal stability and facilitating correct processing and folding of tRNA. Methylation at specific sites prevents cleavage by endonucleases, thus preserving tRNA integrity. Knockdown of Trm7/FSTJ1 in 293T cells can affect the translation efficiency of specific codons at the wobble base.



2′-*O*-Methylation of small RNA

2′-O-Me is also found in small non-coding RNAs, including snRNA, miRNA, and piRNA. SnRNAs are integral to the spliceosome and can be 2′-O-methylated by the snoRNA/FBL complex . This modification is essential for their stability and proper splicing function. The loss of 2′-O-Me in U6 snRNA alters splicing fidelity, impairs spermatogenesis in mice, and contributes to the Alazami syndrome.

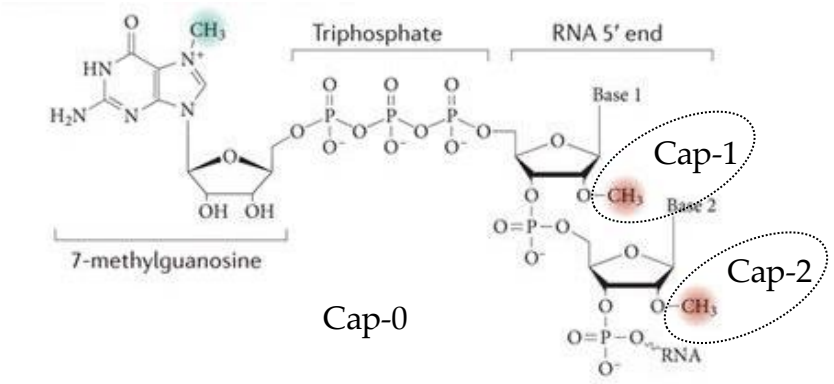
2′-O-Me protects miRNAs and siRNAs from degradation and ensures their proper function in gene-silencing pathways. In animals, HENMT1 is abundantly expressed in the testis and methylates the 3′-end of piRNAs to enhance their stability. This modification is vital for maintaining genome integrity, particularly in germ cells, by silencing transposable elements. Loss or dysfunction of HENMT1 leads to severe oligo-asthenoteratozoospermia in mice and severe azoospermia phenotypes in humans.

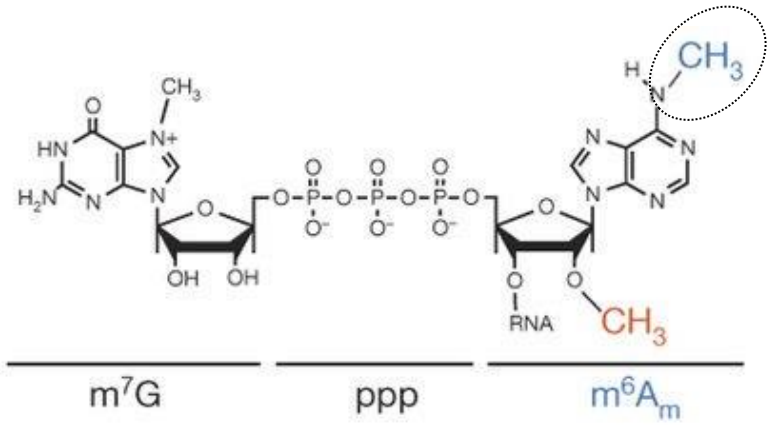


Cap-proximal mRNA methylation

5′ CAP: m7G(5′)ppp

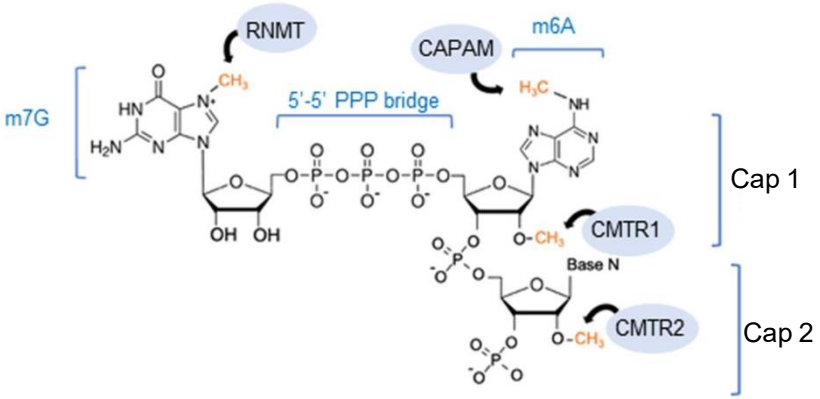
The mRNA cap consists of a **7-methylguanosine** linked to the 5′ nucleoside of the mRNA chain through a 5′–5′ triphosphate bridge **(Cap-0).** The methyl group at the N^7 position of the guanosine is shaded green, and the **2′-*O*-methyl groups** of the first and second nucleotide residues, forming the **Cap-1** and the **Cap-2** structures, respectively, are shaded red. In mammals, about 90% of mRNA initiating with adenosine has an additional modification, **N^6,2′-*O*-dimethyladenosine (m^6A_m).**

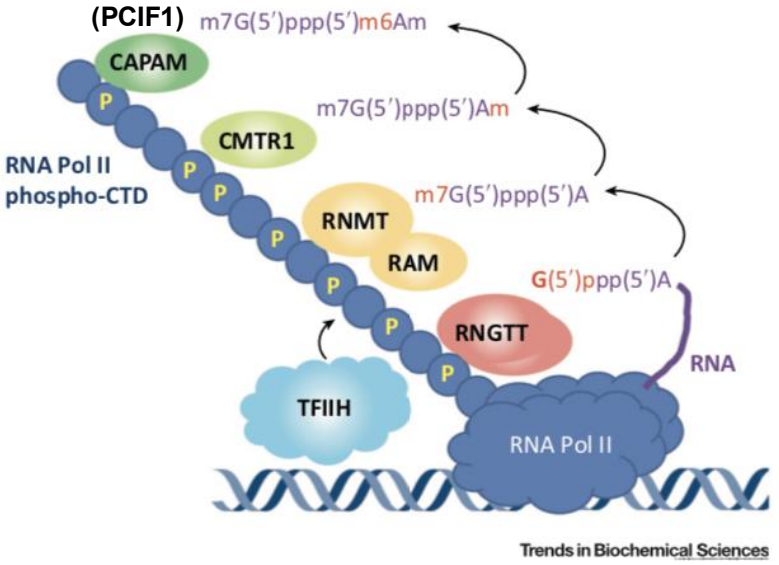




mRNA Capping Enzymes are recruited to RNA Pol II During Transcription

During the initial phase of transcription, TFIIH phosphorylates the Pol II CTD on serine 5. The capping enzymes RNGTT, RNMT–RAM, CMTR1/2, and CAPAM (PCIF1) are then recruited to the phosphorylated RNA Pol II. CMTR1 and PCIF1 methylate mRNA in a sequential manner in that PCIF1-mediated mRNA m^6A_m methylation occurs on the m7G cap-adjacent initial nucleoside only after it has already been 2′-O-methylated (Am) by CMTR1.





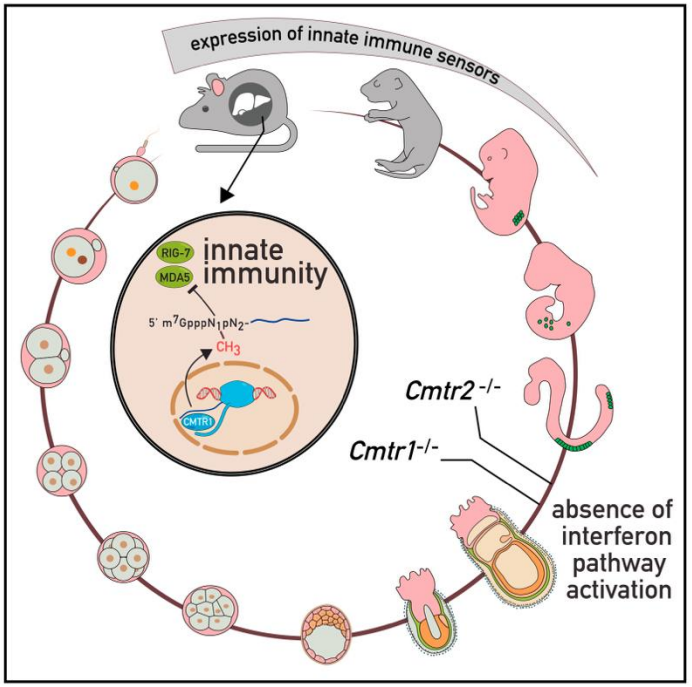
Cap1 -> Cap2 conversion occurs in the cytoplasm

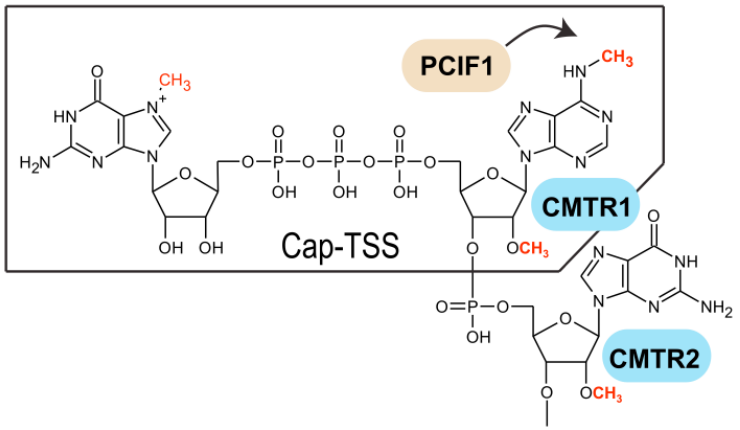
mRNA ageing shapes the Cap2 methylome in mammalian mRNA

mRNA caps exist in either the Cap1 or Cap2 form, depending on the presence of 2′-*O*-methylation on the first transcribed nucleotide or both the first and second transcribed nucleotides, respectively. Here we describe CLAM-Cap-seq, a method for transcriptome-wide mapping and quantification of Cap2. Unlike other epitranscriptomic modifications, Cap2 can occur on all mRNAs. Cap2 is formed through a slow continuous conversion of mRNAs from Cap1 to Cap2 as mRNAs age in the cytosol. As a result, Cap2 is enriched on long-lived mRNAs. Large increases in the abundance of Cap1 leads to activation of RIG-I, especially in conditions in which expression of RIG-I is increased. The methylation of Cap1 to Cap2 markedly reduces the ability of RNAs to bind to and activate RIG-I. The slow methylation rate of Cap2 allows Cap2 to accumulate on host mRNAs, yet ensures that low levels of Cap2 occur on newly expressed viral RNAs. Overall, these results reveal an immunostimulatory role for Cap1, and that Cap2 functions to reduce activation of the innate immune response.

Phenotypes of the mouse knockouts of the enzymes involved in Cap modifications

Loss of mouse Cmtr1 or Cmtr2 leads to embryonic lethality, with non-overlapping sets of transcripts being misregulated, but without activation of the interferon pathway. In contrast, Cmtr1 mutant adult mouse livers exhibit chronic activation of the interferon pathway, with multiple interferon-stimulated genes being expressed. Conditional deletion of Cmtr1 in the germline leads to infertility, while global translation is unaffected in the Cmtr1 mutant mouse liver and human cells. Thus, mammalian cap1 and cap2 modifications have essential roles in gene regulation beyond their role in helping cellular transcripts to evade the innate immune system.





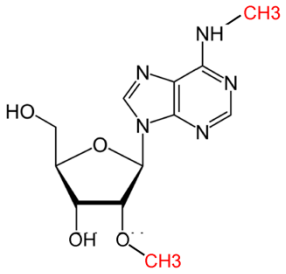
Dohnalkova et al. 2023 Mol Cell

N^6, 2′-*O*-dimethyladenosine (m^6Am)

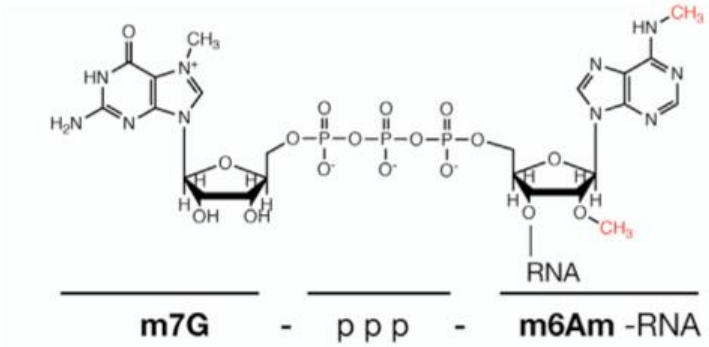
Cap-m^6Am is present only in mammalian mRNAs and snRNAs. It is formed by the base methylation of 2′-O-methyladenosine (Am) located at the first nucleotide of mRNAs, adjacent to the mRNA cap. It is also present as internal modification U2 snRNA.

m^6Am is similar to m^6A in that they both contain a N^6-methylated adenine ring. m^6A antibodies cross-react with m^6Am, preventing definitive identification of nucleotide identity.

m^6Am adjacent to the mRNA cap is installed by the methyltransferase **PCIF1** (**CAPAM**) and erased by the **FTO** demethylase, which can also demethylate internal m^6A. PCIF1 is recruited during transcription by Ser5-phosporilated CTD .



N^6,2′-O-dimethyladenosine
(m^6A_m)

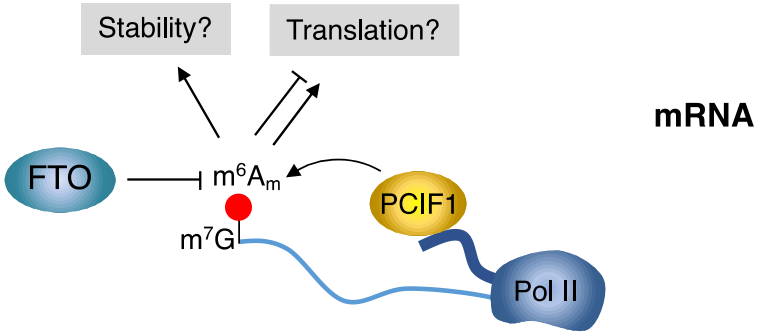


Function of m^{6}Am modification in mRNA

PCIF1 KO cells grew well under normal conditions (Akichika et al., 2019 Science). PCIF1 deletion in mice has no effects on viability and fertility. However, PCIF1 exerts an oncogenic role in the progression of several types of human cancers. Pcif1 deficiency retards tumor growth and enhances survival in multiple tumor models.

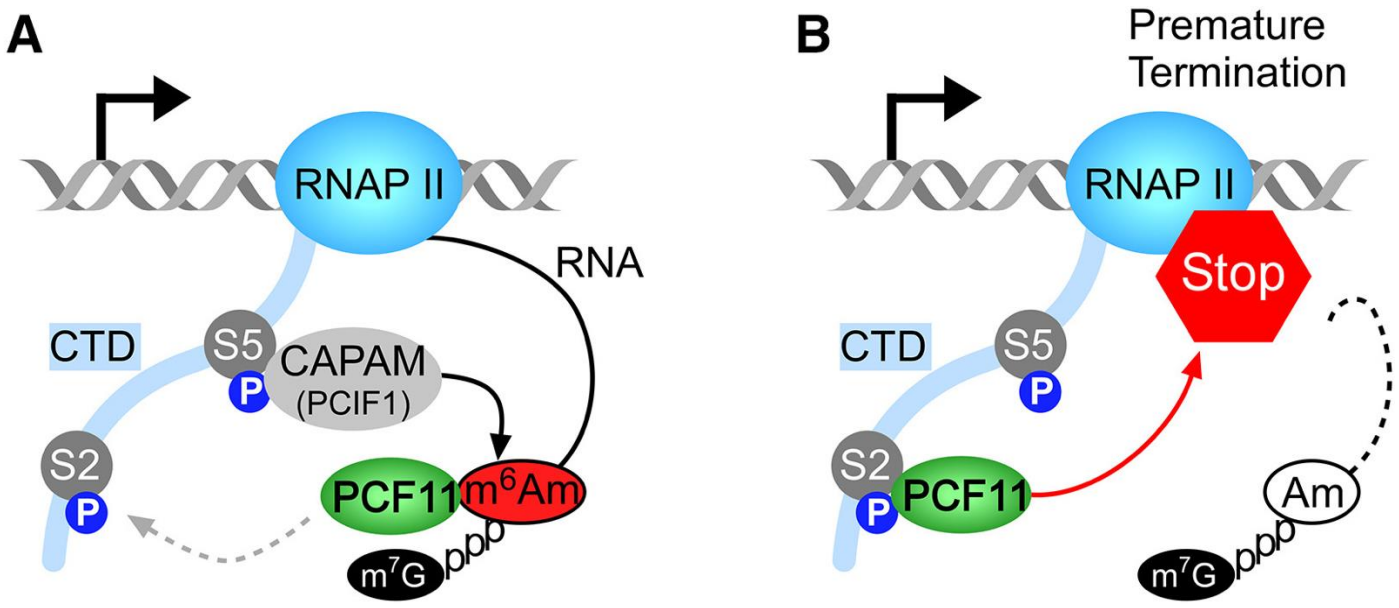
In *Drosophila* is present a catalytically dead PCIF1 that is unable to methylate RNA, but it is still able to bind Ser5-phosporilated CTD (Pandey 2020).

The role of m^{6}Am in **mRNA stability** and **cap-dependent translation** is still controversial.



Function of m^{6}Am modification in mRNA

A recent study demonstrated that m^{6}Am maintains transcriptional integrity of m^{6}Am-modified mRNAs. Specifically, they identified PCF11, a well-known transcriptional terminator, as a reader protein that specifically interacts with m^{6}Am modification. By sequestering PCF11 away from RNA polymerase II (RNAP II) at transcription start sites (TSSs), m^{6}Am prevents premature transcription termination, thereby promoting the full-length transcription of nascent m^{6}Am-modified mRNAs. This finding highlights a novel role for m^{6}Am as a transcriptional anti-terminator, a function distinct from its previously debated roles in mRNA stability and translation.



An et al., 2024. Mol Cell

Internal N^6, 2′-*O*-dimethyladenosine (m^6Am)

m^6Am sites have also been mapped to internal RNA sites, specifically at U2 snRNA position 30.

Internal m^6Am modification in snRNAs is installed by the methyltransferase **METTL4**.

Knocking out Mettl4 from cells results in the loss of U2 snRNA m^6Am and perturbs splicing of target mRNAs with distinctive features such as weak 3′ splice sites.