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Regulation and Function of RNA Pseudouridylation in Human Cells

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Abstract

Recent advances in pseudouridine detection reveal a complex pseudouridine landscape that includes messenger RNA and diverse classes of noncoding RNA in human cells. The known molecular functions of pseudouridine, which include stabilizing RNA conformations and destabilizing interactions with varied RNA-binding proteins, suggest that RNA pseudouridylation could have widespread effects on RNA metabolism and gene expression. Here, we emphasize how much remains to be learned about the RNA targets of human pseudouridine synthases, their basis for recognizing distinct RNA sequences, and the mechanisms responsible for regulated RNA pseudouridylation. We also examine the roles of noncoding RNA pseudouridylation in splicing and translation and point out the potential effects of mRNA pseudouridylation on protein production, including in the context of therapeutic mRNAs.

1. INTRODUCTION

Pseudouridine (Ψ), an isomer of uridine, is an abundant RNA modification found in all domains of life (18, 49, 136) (**Figure 1**). Like other modifications—and there are 172 different modified nucleosides identified to date (10)—pseudouridine has distinct chemical properties that affect the structure of pseudouridylated RNA molecules (4, 26, 31, 64, 85, 106) and their interactions with proteins and other RNAs (19, 32, 33, 89, 147). The study of pseudouridine has experienced a rapid expansion since the identification of many new pseudouridine sites in noncoding RNAs (ncRNAs) and the discovery of the presence of pseudouridine in yeast and human messenger RNAs (mRNAs) by high-throughput sequencing approaches (15, 84, 93, 129).

The biochemical roles of pseudouridine have been intensely investigated in a few cases and found to affect the function of transfer RNA (tRNA) and ribosomal RNA (rRNA) in translation (55, 69, 157) and the function of small nuclear RNA (snRNA) in splicing (39, 169). However, it is currently difficult to predict the function of any individual pseudouridine. Genetic manipulation of the enzymes that catalyze pseudouridine formation, pseudouridine synthases (PUSs), impacts RNA metabolism and leads to cellular phenotypes (52, 129, 135), but whether and which pseudouridines mediate these effects are not well understood. The functions of endogenous pseudouridine in mRNA remain to be discovered. However, the striking effects of artificial RNA pseudouridylation on translation (44, 78, 153), mRNA processing (19), and innate immune sensing (2, 80, 146) demonstrate the potential for individual pseudouridines to broadly affect human gene expression.

In this review, we describe the current knowledge of pseudouridine locations, production, and function in human RNAs. We begin by reviewing the methods that have revealed a more extensive pseudouridine landscape than was previously known. Next, we summarize the confirmed and suspected RNA targets of the 13 human PUS proteins, the mechanisms that confer their substrate specificity, and the evidence that RNA pseudouridylation is regulated. Finally, we move from the established biochemical effects of pseudouridine on RNA structure and RNA–protein interactions to speculate about the probable effects of pseudouridine in mRNA, with implications for mRNA therapeutics.

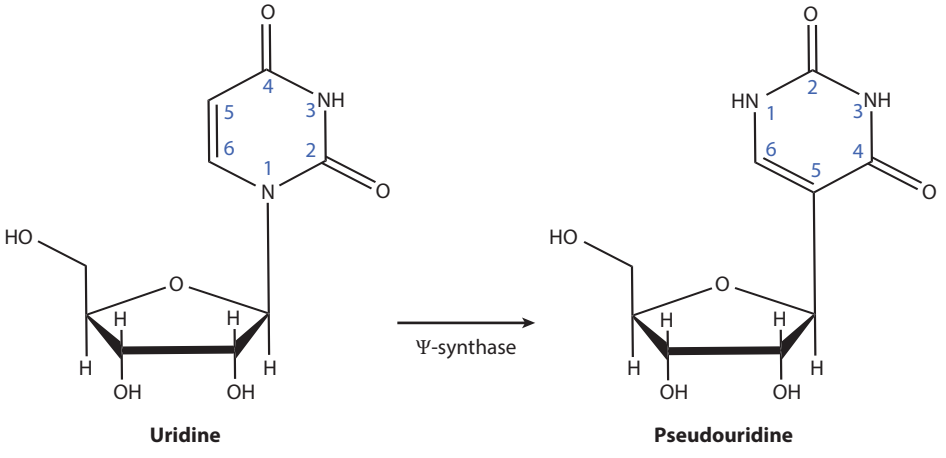

Figure 1

Chemical structures of uridine and pseudouridine. Pseudouridine synthase (Ψ-synthase) proteins catalyze the isomerization of uridine to pseudouridine. This isomerization reaction reorients the base such that it connects to the sugar via C5 rather than N1.

2. PSEUDOURIDINE DETECTION AND DISTRIBUTION

Bulk nucleoside fractionation by high-performance liquid chromatography and detection by ultraviolet monitoring originally revealed pseudouridine as a very abundant component of yeast total RNA, "the fifth nucleoside" (27, 158). Today, two approaches dominate the study of the abundance and locations of pseudouridine: high-throughput sequencing of transcriptomes (14, 15, 93, 95, 129) and mass spectrometry on purified RNA (1, 142). As a result, pseudouridine has been mapped in most classes of RNA found in human cells, including abundant noncoding RNAs such as tRNA, rRNA, snRNA, and small nucleolar RNA (snoRNA), as well as less abundant long noncoding RNA (lncRNA) and protein-coding mRNA.

2.1. High-Throughput Sequencing-Based Methods to Identify Pseudouridine Locations

A breakthrough in the study of pseudouridine came in 1993 with the development of a method to map pseudouridine sites using primer extension (6). Bakin & Ofengand (6) showed that the stable adducts formed between pseudouridine and N-cyclohexyl-N′-β-(4-methylmorpholinium) ethylcarbodiimide p-tosylate (CMCT) block reverse transcriptase, producing truncated complementary DNA (cDNA) that could be sequenced to reveal the pseudouridine location with single-nucleotide resolution (50). Three groups adapted this strategy to the Illumina sequencing platform to produce transcriptome-wide maps of pseudouridine (15, 95, 129), including in human mRNA (15, 129). A fourth synthesized a modified carbodiimide to permit the chemical coupling of biotin to pseudouridine residues to enrich pseudouridine-containing RNA fragments for sequencing (93).

A modified form of RNA bisulfite sequencing (RBS-seq) uses a distinct chemistry (46) to detect pseudouridines, in addition to other modified nucleosides (84). An advantage of this approach, which detects pseudouridine as 1–2-nt deletions in cDNA synthesized from bisulfite-treated RNA, is that it allows targeted sequencing to increase coverage of RNA sites of interest, such as validating mRNA pseudouridine sites from lowly expressed genes in HeLa (15) and HEK293 (93, 129) cells. These sequencing-based methods differ in subtle but nevertheless important ways. Biotinylation and enrichment of pseudouridine-containing RNA fragments before sequencing should give the most comprehensive detection of pseudouridine sites from the fewest sequencing reads (93) but have the potential disadvantage of detecting very low-stoichiometry pseudouridine sites of uncertain biological significance. In contrast, transcriptome-wide RBS-seq detects mainly high-stoichiometry pseudouridine sites unless in very highly expressed genes (84). A recent adaptation of CMC-dependent pseudouridine mapping offers targeted RNA pseudouridine quantitation and revealed pseudouridine stoichiometries ranging from ∼30% to 80% in low-abundance human mRNAs (166).

2.2. Mass Spectrometry–Based Methods to Map and Quantify Pseudouridine

Mass spectrometry is increasingly used to quantify the abundance of modified nucleosides in RNA (125, 126, 165) and reveal regulation of RNA modification across tissues (11) and in response to environmental perturbations (63). Analysis of RNA modifications by mass spectrometry usually targets purified RNA species. Pseudouridine poses special challenges because it has the same mass as uridine (U). Pseudouridine is only detectable by characteristic ions produced during fragmentation (1, 142). Direct sequencing by mass spectrometry produced a complete map of pseudouridine in human snRNAs from 293T cells (156) and determined absolute pseudouridine levels for human ribosomal RNA from TK6 cells (140).

Mass spectrometry has been used to analyze bulk mRNA pseudouridine content, revealing that pseudouridine is present at ~0.2–0.6% of total U in mRNA from human HEK293T cells (93). This global abundance is on par with the abundance of N^6-methyladenosine (74). Because pseudouridine is abundant in rRNA, which is a typical contaminant of poly(A)-selected mRNA, special care must be taken to obtain pure mRNA for analysis [e.g., multiple rounds of selection for poly(A), size selection to exclude tRNA, and depletion of rRNA]. Furthermore, rRNA contamination varies between experiments, which is evident from differences in rRNA reads in RNA-seq datasets. Therefore, the best assurance that apparent changes in bulk mRNA pseudouridylation (93) actually reflect the regulated modification of mRNA is to perform RNA-seq on the sample subjected to analysis by mass spectrometry (141).

2.3. Distribution of Pseudouridine in Mammalian RNAs

Pseudouridine has been detected in most classes of RNA examined to date. Here we briefly summarize the evidence for pseudouridine in tRNA, rRNA, snRNA and mRNA in human cells.

2.3.1. tRNA pseudouridine sites. Data for tRNAs are limited in most RNA-sequencing studies, including pseudouridine profiling (15, 46, 93, 95, 129), due to problems with synthesizing cDNA from these heavily modified RNAs. Pretreatment of RNA with demethylases improves tRNA coverage (23, 170) and was recently combined with CMC-based pseudouridine profiling to produce a nearly comprehensive map of pseudouridine in human tRNAs from HEK293 cells (135), excluding positions close to the 3′ ends, which could not be mapped. Most pseudouridines were found at one of 17 positions that were previously known to be modified in human cytosolic tRNAs (10, 29), with Ψ55 in the TΨC loop as the most frequently occurring, followed by positions on either side of the anticodon stem-loop, Ψ27, Ψ28, Ψ38, and Ψ39 (135). Several novel positions were reported (**Figure 2**), as well as new information about known positions in many tRNA isodecoders, tRNAs with the same anticodon but different sequences for the tRNA body.

2.3.2. rRNA pseudouridine sites. Human ribosomes include 104 pseudouridines that have been experimentally validated by mass spectrometry in human TK6 cells (140), the majority of which were previously identified by primer extension and pseudouridine profiling (15, 86, 129). These pseudouridines occur at positions predicted to base-pair with H/ACA snoRNA guide sequences (90). Approximately half of human pseudouridine sites are conserved from budding yeast, and all are located within conserved functional domains (56, 116, 140).

2.3.3. snRNA pseudouridine sites. The mammalian U1, U2, U4, U5, and U6 snRNAs contain a combined 27 pseudouridines (34, 119, 156) (**Table 1**). 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). Additional pseudouridines in human U4, U5, and U6 snRNAs have not been reported in budding yeast.

2.3.4. mRNA pseudouridine sites. Several groups discovered widespread pseudouridylation of mRNAs and mapped their locations in human cells using transcriptome-wide pseudouridine profiling methods (15, 84, 93, 129). Pseudouridines are present throughout mRNAs, including in 5′-UTR, 3′-UTR, and coding sequences, with no enrichment in any particular region. Differences in mRNA sites reported by different groups have been noted (84, 127). Technical differences in sequencing-based approaches for modification detection could result in capture biases, which

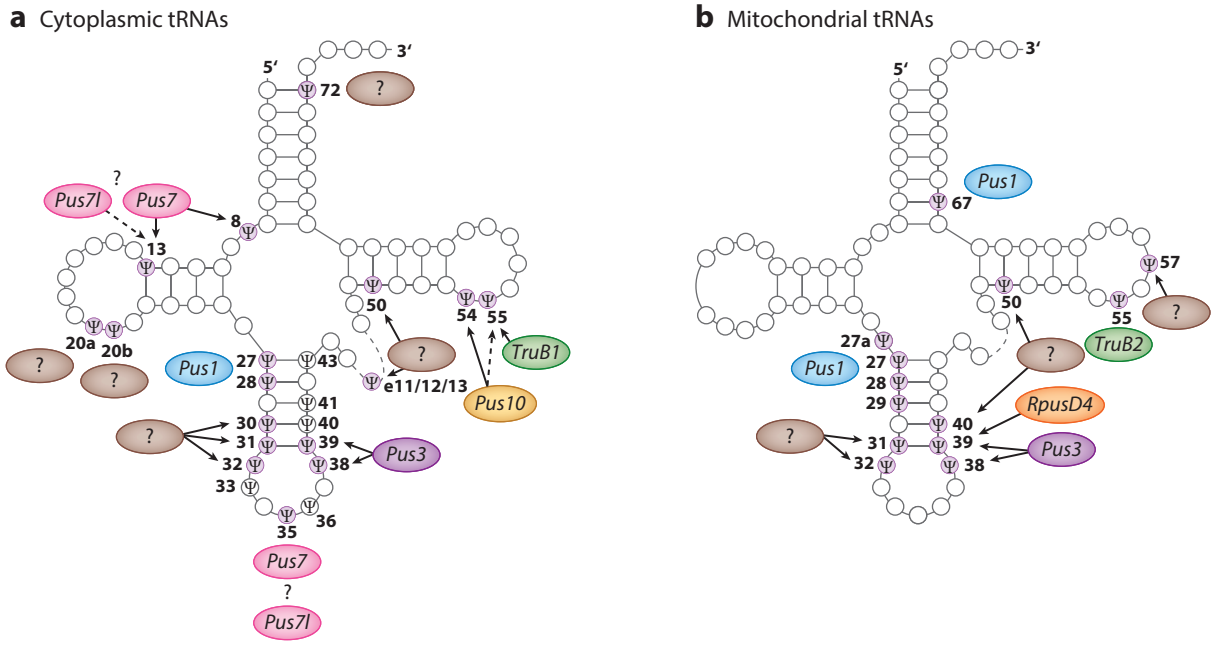

Figure 2

tRNA pseudouridylation. Biochemically identified Ψ sites in (*a*) cytoplasmic and (*b*) mitochondrial human tRNAs are marked by shaded circles with the PUS indicated where known. Sites identified exclusively by high-throughput sequencing are shown as open circles. Abbreviations: PUS, pseudouridine synthase; tRNA, transfer RNA; Ψ, pseudouridine.

might explain some of these differences. Cell type–specific differences in mRNA abundance are likely the major contributors to observed differences in pseudouridine identification because current methods are limited to the most highly expressed genes. We have also identified instances of cell type–specific mRNA pseudouridine in mRNAs expressed at sufficient levels for site calling in different human cell lines (N.M. Martinez & W.V. Gilbert, unpublished observations), consistent with the regulation of mRNA pseudouridylation.

3. ENZYMES THAT CATALYZE RNA PSEUDOURIDYLATION

Pseudouridylation is carried out by a class of proteins called pseudouridine synthases (PUSs). Here we focus on the 13 PUSs found in humans and provide context from the more extensive literature on their *Escherichia coli* and yeast counterparts (reviewed in 54, 122).

3.1. Pseudouridine Synthase Protein Domains

PUS proteins are classified into six families. The RluA, RsuA, TruA, TruB, and TruD families are named for their *E. coli* counterparts, while the PUS10 family is unique to some archaea and eukaryotes (**Figure 3**). All PUSs carry a common eight-stranded β-sheet catalytic fold and rely on a spatially conserved aspartate for catalysis despite sharing little primary sequence similarity (54). Comparing the available structures of 5 human PUSs (RPUSD1 5VBB, RPUSD4 5UBA, PUS1 4J37, PUS7 5KKP, and PUS10 2V9K) to their *E. coli* counterparts reveals conservation of the core catalytic domain. However, human PUS proteins typically carry auxiliary domains that are

Table 1 Locations and targeting of pseudouridine in human snRNAs by box H/ACA snoRNAs

Human	Position	Pseudouridine synthase	MN sensitivity	Interaction snoRNA/snRNA	References
U1	Ψ5	SCARNA16	ND	Yes	51, 86, 119
	Ψ6	SCARNA18	ND	Yes	51, 90
U2	Ψ6	ND	ND	ND	119
	Ψ7	SCARNA14	ND	ND	51, 90, 119
	Ψ99	ND	ND	ND	119
	Ψ34	SCARNA8	ND	Yes	25, 51, 119
	Ψ37	SCARNA15	ND	Yes	51, 86, 119
	Ψ39	SCARNA4	ND	Yes	51, 86, 119
	Ψ41	SCARNA4	ND	Yes	51, 86, 119
	Ψ43	SCARNA8	ND	Yes	36, 51, 119
	Ψ44	SCARNA8	ND	Yes	25, 119
	Ψ54	SCARNA13	ND	Yes	86, 119
	Ψ60	ND	ND	ND	34
	Ψ88	ND	ND	ND	119
	Ψ90	SCARNA1	ND	Yes	51, 86, 119
	Ψ91	ND	ND	ND	119
U4	Ψ4	ND	Yes	ND	119, 164
	Ψ72	ND	Yes	ND	119, 164
	Ψ79	SCARNA26	Yes	Yes	51, 77, 119, 164
U5	Ψ11	ND	ND	ND	156
	Ψ43	SCARNA11	Yes	Yes	51, 71, 86, 119
	Ψ46	**SCARNA10**	Yes	Yes	25, 51, 71, 119
		SCARNA12			
	Ψ53	SCARNA13	No	Yes	51, 71, 90, 119
U6	Ψ9	ND	ND	ND	156
	Ψ31	SNORA79	Yes	Yes	51, 90, 119, 163
	Ψ40	SCARNA23	Yes	Yes	51, 86, 119, 163
	Ψ86	SNORA79	Yes	Yes	51, 90, 119, 163
U12	Ψ19	SCARNA21	ND	Yes	90, 99
	Ψ28	SCARNA20	ND	Yes	90, 99
U4atac	Ψ12	SCARNA21	ND	ND	99
U6atac	Ψ83	ND	ND	ND	99

This table shows the position of pseudouridine in human major and minor snRNAs. Pseudouridine Synthase Box H/ACA snoRNA is predicted or experimentally validated (bold) to target the interaction between snoRNA and mRNA—experimental evidence for interaction between target snRNA and the predicted snoRNA guide.

Abbreviations: MN, micrococcal nuclease; mRNA, messenger RNA; ND, not determined; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; U, uridine; Ψ, pseudouridine.

not present in their *E. coli* counterparts, which may contribute to specific substrate recognition. For example, PUS10 carries an N-terminal THUMP domain, which is thought to be involved in binding tRNA substrates (102). Human PUS1 carries a C-terminal extension composed of three α-helices that preclude the tRNA-binding mode employed by bacterial TruA (24) and may constrain the RNA targets to be flexible, as shown for yeast Pus1 (13).

Flanking the catalytic cleft, PUS proteins are typically decorated with a series of α-helices and loops. Two of these features, termed forefinger and thumb based on structures of *E. coli* PUS

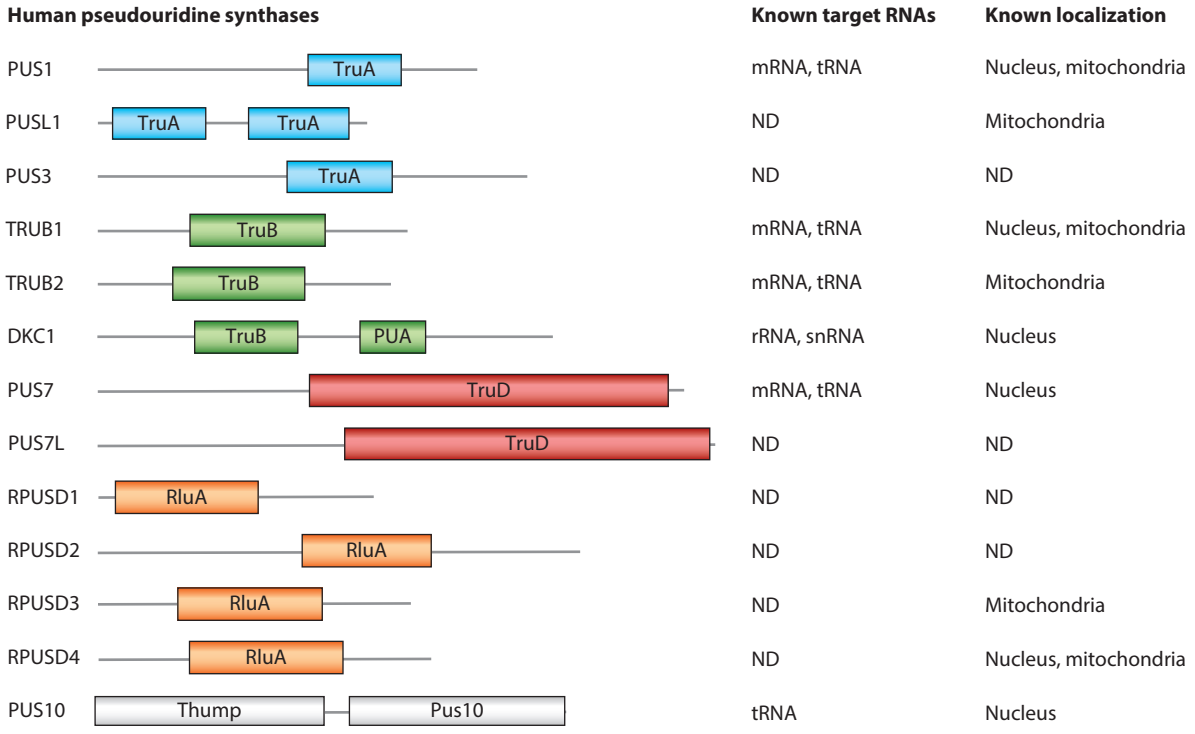

Figure 3

Pseudouridine is catalyzed by human PUSs. This domain schematic shows human PUSs grouped and color coded by family. TruA (PUS1, PUSL1, and PUS3), TruB (TRUB1, TRUB2, and DKC1), TruD (PUS7 and PUS7L), RluA (RPUSD1, RPUSD2, RPUSD3, and RPUSD4), and PUS10 human family homologs based on PUS domain are denoted. The RsuA family lacks human homologs. Known classes of target RNAs and localization for each PUS protein are listed. Abbreviations: mRNA, messenger RNA; ND, not determined; PUS, pseudouridine synthase; rRNA, ribosomal RNA; snRNA, small nuclear RNA; tRNA, transfer RNA.

proteins complexed with target RNA, dock into the grooves of the target RNA stem, effectively pinching it (24, 54, 57, 102).

3.2. Catalytic Mechanism

The isomerization of uridine to pseudouridine requires cleavage of the glycosidic bond between N1 and C1′, rotation of the base, and subsequent reattachment of the base to the ribose via C5 (**Figure 1**). PUS proteins are reliant on a highly conserved catalytic aspartate for this reaction, and mutation of this aspartate abolishes pseudouridylation activity. Proposed reaction mechanisms suggest aspartate acts as a nucleophile to achieve catalysis (62, 118, 148), but uncertainties remain (reviewed in 136).

Besides the catalytic aspartate, other amino acids within the catalytic cleft are well conserved within PUS families. Typically, there are five conserved amino acids within PUS protein active sites: the catalytic aspartate, a salt bridge partner for the catalytic aspartate, an aromatic amino acid to stack with the target uridine, a hydrophobic residue, and another residue providing nucleotide stacking interactions. Further, most PUS proteins also rely on an active site residue to facilitate base-flipping. While the exact identity of each of these amino acids, as well as other residues in the cleft, may vary between families, their functions are often similar.

Studies of *E. coli* PUS structure indicate a base-flipping mechanism to bring the target uridine residue into the catalytic pocket, though the amino acid used to achieve this flipping differs between PUS families (24, 57, 58, 65). In *E. coli* PUS, this residue is often critical, and mutation leads to loss of pseudouridylation activity while retaining the ability to bind to target RNAs (57, 65). However, mutation of the residue responsible for base-flipping in human PUS1 has different effects depending on the substrate tested and the amino acid used for replacement (133). One of the disruptive mutations is causative for mitochondrial myopathy and sideroblastic anemia and changes the arginine involved in base-flipping to tryptophan (12). The version of PUS1 that is produced is inactive, and the tryptophan replacing the arginine is hypothesized to sterically block the enzyme active site and not be able to intercalate into the substrate stem (12, 115, 133).

3.3. RPUSD3 Lacks Catalytic Aspartate

RluA PUS family members are characterized by a conserved HRLD motif (**Figure 4*a***). This motif contains the catalytic aspartate residue, as well as an arginine, which in RluA facilitates the flipping-out of the target uridine into the catalytic pocket (57). Alignment of the human RluA family members reveals a less conserved HRLD motif, with only RPUSD2 and RPUSD4 making a direct match. RPUSD1 is a near match, employing the residues HQLD, while RPUSD3 is highly divergent, having the residues RASG. Most notably, RPUSD3 carries a glycine at the conserved position for the catalytic aspartate, indicating that RPUSD3 is not an active PUS protein (**Figure 4**). Further biochemical study is necessary to interrogate the catalytic activity of RPUSD3.

3.4. Classes of RNA Targeted by Pseudouridine Synthases

Most of the RNA targets of human PUSs remain to be discovered and may include all classes of ncRNA as well as mRNA. Some tRNA targets of human PUSs can be predicted based on homology to yeast PUS with known target sites (**Figure 2**). Several stand-alone PUSs have been shown to modify human mRNA sequences including PUS1 (13, 93), PUS7 (13, 127), TRUB1 (13, 127), TRUB2 (13, 127), and RPUSD2 (13). The RNA-guided PUS, dyskerin pseudouridine synthase 1 (DKC1), targets snRNA and rRNA sites (49). DKC1 is not known to have mRNA targets, but pseudouridine can be detected at computationally predicted target sites in human mRNA (N.M. Martinez & W.V. Gilbert, unpublished observations) as shown in budding yeast (15). Localization of PUS proteins affects their access to potential RNA targets. For example, alternative isoforms of PUS1 localize to the nucleus and mitochondria where they modify distinct tRNA pools (45). PUSL1, TRUB2, RPUSD3, and RPUSD4 have been detected in mitochondria (3, 5, 120, 159), and PUS1, PUS7, TRUB1, and RPUSD4 have been shown to at least partially localize to the nucleus (45, 73, 127, 137). PUS10 is predominantly nuclear at steady state and relocalizes to mitochondria in response to apoptotic signals (72).

4. SPECIFICITY OF RNA PSEUDOURIDYLATION

4.1. Stand-Alone Pseudouridine Synthase Targets and Recognition

Of the 13 human PUS proteins, 12 are considered stand-alone, meaning they recognize their targets without the use of accessory RNAs. With high-throughput assignment of pseudouridine sites to specific PUS proteins, it is becoming clear that various PUS proteins rely on specific sequence and/or structural features to recognize their targets (13, 127). For example, human TRUB1 recognizes pseudouridines within the context of a GUUCNANYC sequence motif occurring in a stem-loop structure that resembles canonical TRUB family tRNA substrate (13, 127). Human PUS7, on the other hand, recognizes targets with a UNUAR motif (13, 127). This motif is not

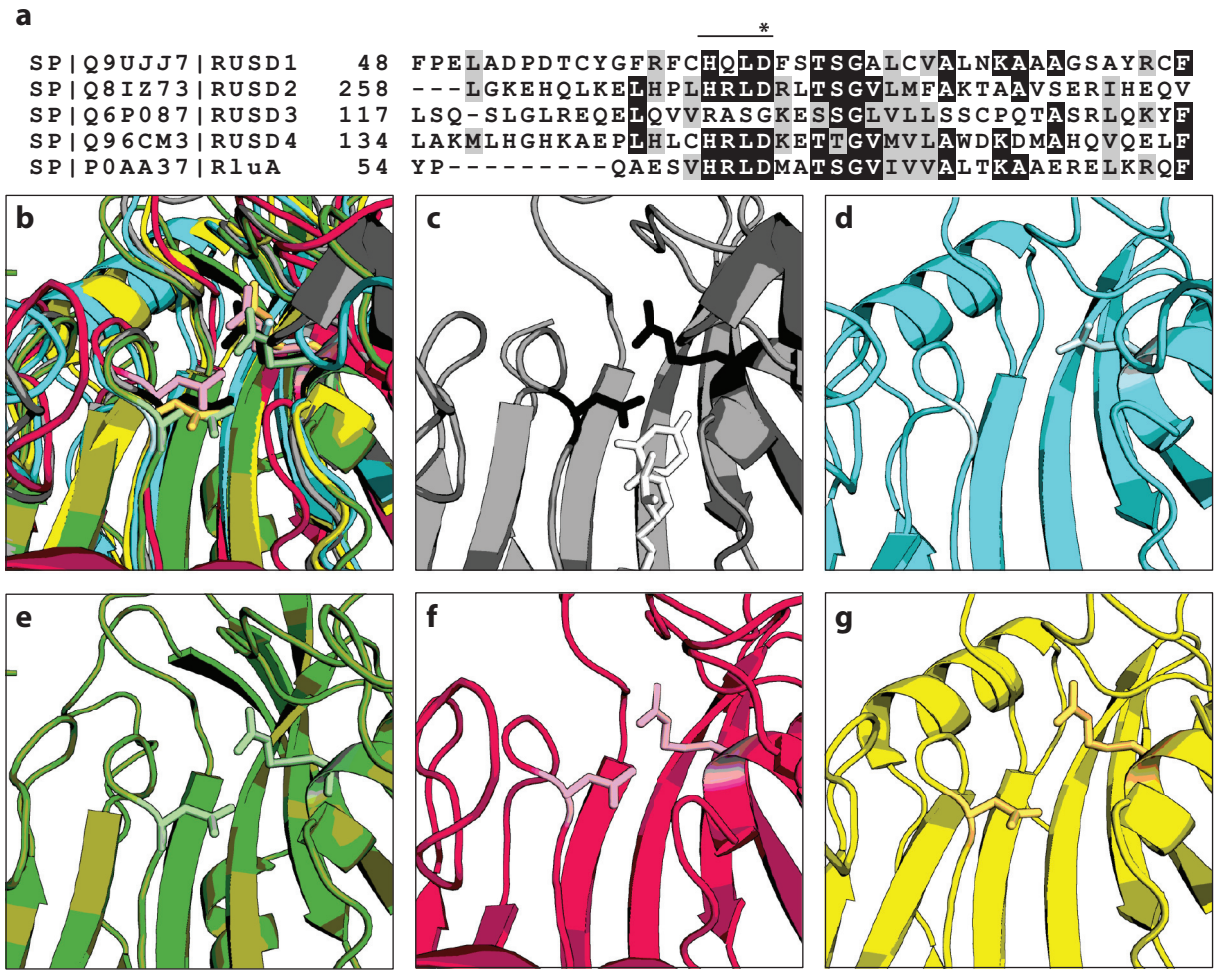

Figure 4

RPUSD3 lacks a catalytic aspartate. (*a*) Multiple sequence alignment of human RluA family members and *Escherichia coli* RluA. The HRLD motif is denoted by a line above the alignment and the position of the catalytic aspartate is indicated by the asterisk (*). (*b–g*) An overlay (*b*) or individual structures of the catalytic pocket of *E. coli* RluA (*c*, PDB 2I82), human RPUSD3 (*d*, model), RPUSD1 (*e*, PDB 5VBB), RPUSD2 (*f*, model), and RPUSD4 (*g*, PDB 5UBA). The residues at the catalytic and salt bridge positions are shown as sticks. The structure of *E. coli* RluA includes 5FU in position of the pseudouridylated residue. Models were generated using SWISS-MODEL. The multiple sequence alignment was generated using Clustal Omega and formatted using BoxShade.

sufficient to direct pseudouridylation by PUS7 as the majority of motifs are not detectably pseudouridylated in human cells (15, 93, 129). RNA structural features, yet to be defined, are likely to be important for human PUS7 as they are for yeast PUS7 (145) and yeast PUS1 (13). Both the TRUB1 and PUS7 motifs resemble motifs previously defined in yeast, and yeast Pus4 was found to be capable of modifying many targets of human TRUB1 (13). This finding is consistent with the conservation of target features among evolutionarily related PUS proteins.

Higher eukaryotes, including humans, have expanded PUS families that could support the pseudouridylation of more diverse RNA targets. PUS1 has a paralog PUSL1 that is predicted to be catalytically active but is not redundant with PUS1 for mRNA pseudouridylation in human

HEK293T cells (93). Likewise, depletion of TRUB1 eliminates the pseudouridylation of mRNA targets in cells that express its paralog TRUB2 (127). Consistent with this lack of redundancy in cells, TRUB1 and TRUB2 modify distinct sets of mRNA targets in vitro (13). Nothing is known about the targets of human PUS7L, which is also predicted to be an active PUS. Finally, the human RPUSD family includes four proteins, three of which contain the residues needed for catalytic activity (**Figure 4**).

4.2. RNA-Guided Pseudouridylation of rRNAs and snRNAs by H/ACA Small Nucleolar Ribonucleoproteins

The catalytic subunit of the small nucleolar ribonucleoproteins (snoRNPs), DKC1, is guided by base-pairing interactions between box H/ACA snoRNAs and the target RNA, which provide the specificity for the pseudouridylation of the substrate (reviewed in 49). The conserved snoRNP complex contains DKC1, GAR1, NHP2, NOP10, and the guide RNA. Box H/ACA snoRNAs typically consist of two hairpin loops that contain an internal pseudouridylation pocket where the RNA target site is converted to pseudouridine and two conserved sequence elements, the H and ACA motifs, that recruit the protein components. H/ACA snoRNPs target the pseudouridylation of nascent rRNA in the nucleolus (reviewed in 116).

Researchers predict that a subset of box H/ACA snoRNAs called small Cajal body–specific RNAs (scaRNAs) guide pseudouridylation of the snRNAs based on sequence complementarity (25, 70, 71, 77, 86, 87, 99) (**Table 1**). This hypothesis is further supported by experiments showing that the pseudouridylation of many snRNA sites in human cell extracts is dependent on RNA (71, 163, 164) and that snoRNAs, which are predicted to modify particular snRNAs, specifically interact with the target snRNA in cells (51) (**Table 1**). Only one human scaRNA (SCARNA10) has been experimentally verified to direct pseudouridylation of its computationally predicted target, Ψ46 in human U5 snRNA (71). Some snRNA pseudouridines lack a predicted guide snoRNA and might be modified by stand-alone PUSs. Indeed, both RNA-dependent and -independent mechanisms mediate snRNA pseudouridylation in budding yeast. The stand-alone PUSs, Pus1 and Pus7, pseudouridylate yeast U2 snRNA at two positions (96, 100). Pseudouridylation of these sites is not lost in mammalian cells lacking PUS1 and PUS7 activity, showing that distinct mechanisms modify conserved sites in different organisms (28, 36). The micrococcal nuclease sensitivity of pseudouridylation in vitro indicates that some currently unassigned snRNA pseudouridines are targeted by RNA guides (**Table 1**). Recent studies have revealed that fewer base pairs (eight) than the canonical number of base pairs (greater than ten) and more mismatches are tolerated for pseudouridylation (30, 82). Therefore, some of the orphan snoRNAs or snoRNAs that target other positions might target these sites.

Cajal bodies (CBs) are sites of snRNP maturation and assembly where snRNA pseudouridylation may occur (105). The scaRNAs that are predicted to guide snRNA modifications are enriched in CBs due to a CB retention signal (121) and a protein anchor (144). A mutation in U2 snRNA that accumulates in the cytoplasm, preventing reentry of U2 snRNA into CBs, is deficient in pseudouridylation (70). There is still uncertainty about where in the cell U6 snRNA is pseudouridylated (48, 144). Furthermore, snRNAs are still pseudouridylated in cells lacking canonical CBs (e.g., cells lacking coilin), suggesting that CBs are not strictly required for snRNA pseudouridylation (34, 35, 70), although residual Cajal-like bodies remain in cells lacking coilin and might still function as sites for pseudouridylation. Further studies are necessary to determine which snoRNAs pseudouridylate what positions in the snRNAs and where the pseudouridylation of each snRNA takes place.

5. REGULATION OF RNA PSEUDOURIDYLATION

5.1. Regulation of Pseudouridines in ncRNA

The pseudouridine content of mammalian (pig and mouse) tRNA differs between tissues (11), suggesting that tRNA pseudouridylation could be regulated in human cells. The complete tRNA pseudouridine landscape has been determined in only one human cell line (135). Thus, it is an open question whether tRNA pseudouridines are regulated in humans, and if so, at which sites. Quantitative mass spectrometry identified positions with substoichiometric pseudouridine in the human 80S ribosome (140), raising the possibility that these sites could be increased under some conditions. Consistent with this possibility, the inhibition of the mammalian target of rapamycin (mTOR) slightly increased bulk pseudouridine levels in rRNA from mammalian (Chinese hamster ovary) cells at sites that remain to be determined (22). It is unclear whether pseudouridine sites in the human snRNAs are fully modified and whether the stoichiometry or the locations of pseudouridine vary across cell types or other conditions, as demonstrated in budding yeast. Notably, pseudouridines in yeast U2 and U6 snRNAs that are induced during different stress conditions affect both splicing and organismal growth (8, 154, 155).

5.2. Regulation of Pseudouridines in mRNA

An exciting possibility is that regulated mRNA pseudouridylation controls mRNA metabolism in response to changing cellular conditions. Stress conditions induce changes in the expression of PUS proteins and/or the pseudouridine landscape in yeast and human cells. For example, serum starvation results in differential mRNA pseudouridylation in HeLa cells (15), and treatment with H_2O_2 alters mRNA pseudouridine in HEK293T cells (93). Heat shock causes the relocalization of yeast Pus7 from the nucleus to the cytoplasm and results in the pseudouridylation of new targets in the cytoplasm (129). Nutrient deprivation in yeast induces changes in mRNA pseudouridylation by multiple PUSs (15) through mechanisms that may involve changes in mRNA structure (13).

Sequences that are nonoptimal targets for a given PUS are likely to be sensitive to changes in PUS expression. For example, nonoptimal targets of human TRUB1, which show low levels of pseudouridylation with normal levels of TRUB1 expression, show the greatest increase in pseudouridylation levels upon overexpression of TRUB1 (127). Tissue- and cell type–specific expression of the 13 human PUSs provide a mechanism for regulated cell type–specific pseudouridylation (**Figure 5**) and may contribute to the differences in mRNA pseudouridines detected in different cell types (15, 84, 93, 129). The extent of tissue-specific and cell type–specific mRNA pseudouridylation in human cells is currently unclear since most pseudouridine profiling has been limited to the interrogation of highly expressed genes.

6. EFFECTS OF PSEUDOURIDINE ON RNA STRUCTURE AND FUNCTION

6.1. RNA Structure

The conservation of pseudouridine in structured ncRNA likely reflects effects of pseudouridine on RNA conformation, thermodynamic stability, and structural dynamics. Pseudouridine forms Watson-Crick base pairs with adenine (A) that have similar geometry but greater thermodynamic stability than U-A pairs (31, 85). Pseudouridine also stabilizes single-stranded RNA (ssRNA) conformations in solution, in addition to enhancing the formation of RNA duplexes (26). These effects of pseudouridine are due to its preference for the C3′-*endo* sugar conformation and enhanced base

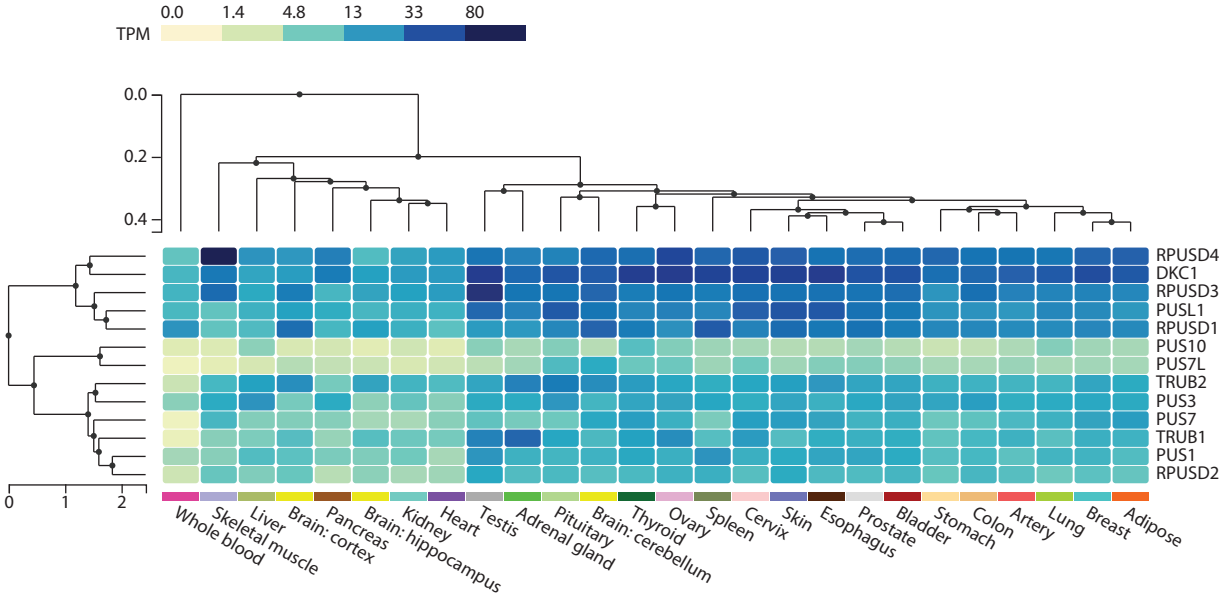

Figure 5

Expression of human pseudouridine synthases (PUSs) across human tissues. Gene expression in transcripts per million (TPM) of the 13 human PUSs across different tissue types. PUSs are clustered based on expression similarity. The figure was created using the Genotype-Tissue Expression (GTEx) Portal on 02/13/2020.

stacking (26, 31, 85). In addition, water bridges between adjacent nucleosides and the H1N that distinguishes pseudouridine from U have been visualized in crystal structures of tRNA (4) and modeled in molecular dynamics simulations (31). Such water bridges may increase the rigidity of RNA backbones containing pseudouridine.

The functional consequences of changing a U to a pseudouridine are frequently summarized as stabilizing RNA structure (49, 92, 136). Although true, this simplification obscures context-dependent differences, which are large enough to have biological significance. Systematic comparison of RNA duplexes containing all eight possible NΨ and ΨN neighbors with the corresponding NU and UN sequences measured sequence context differences in the stabilizing effect of pseudouridine versus U of >1 kcal/mol (64). Sequence context also affects the stacking potential of pseudouridine in ssRNA (31). In addition to these differences that depend on local sequence context, the net effect of pseudouridine on RNA structure depends on the folding potential of the surrounding sequence and the position of the pseudouridine. For example, positioning a pseudouridine within the loop region can antagonize hairpin formation (106). This is likely due to the ability of pseudouridine to stabilize the A-form helical conformation of ssRNA (26), which would increase the energetic cost of loop closure, thereby favoring the extended RNA conformation.

The biological effects of pseudouridine must originate in the chemical differences between it and U, which primarily affect RNA backbone conformation and the stability of base pairs. Because pseudouridine can form stable pairs with guanine (G), cytosine (C), and U in addition to A, it has been proposed as a universal base-pairing partner (85). Despite intensive study of the structural effects of pseudouridine on short, synthetic RNA oligos, it is currently impossible to predict the structural outcome of site-specific RNA pseudouridylation in longer RNAs. The systematic investigation of sequence–context effects on the stability of pseudouridine-containing duplexes is an important step toward accurate predictions (64). It will be important to determine the structural

consequences of RNA pseudouridylation in cells, which is possible using improved methods to probe RNA structure in vivo (reviewed in 107).

6.2. Effects of Pseudouridine on Interactions with RNA-Binding Proteins

Pseudouridine alters RNA–protein interactions for several RNA-binding proteins that regulate nuclear RNA processing and cytoplasmic RNA localization and/or stability. The cytoplasmic RNA-binding protein (RBP) PUM2 recognizes an RNA sequence motif (UGUAR) that is frequently pseudouridylated in human cells, likely by PUS7 (13, 15, 127, 129). The incorporation of pseudouridine into this motif (UNΨAR) reduced the affinity of PUM2 binding by approximately threefold in vitro (147). Similarly, the substitution of uridines with pseudouridines within CUG repeats, which are associated with myotonic dystrophy type 1, reduced binding of the splicing factor MBNL1 by ~4–20-fold in vitro (32, 33). The artificial pseudouridylation of a single position in a polypyrimidine tract strongly inhibited the binding of the splicing factor U2AF2 (19). In another example, the artificial pseudouridylation of an individual position in the Sm binding site of U7 snRNA inhibited snRNP assembly by twofold in *Xenopus* oocytes (89). Inhibition of U2AF2 binding and U7 snRNP assembly by pseudouridine were both attributed to its known effects on RNA backbone conformation. Consistent with this interpretation, the incorporation of a locked nucleic acid also inhibited the binding of U2AF2 (19). Likewise, substitution with 5′FU, which also favors the C3′-*endo* conformation and rigidifies the RNA backbone, inhibited snRNP assembly. These examples show that diverse RBPs are sensitive to the pseudouridylation of their target RNAs. Given the preference of RNA-recognition motifs for nucleotides in the C2′-*endo* conformation at the binding interface (88), pseudouridine may broadly antagonize RBPs binding by favoring the C3′-*endo* conformation. However, the observed effects of pseudouridine on RBP binding depend on sequence context and position relative to the binding motif, by mechanisms that remain to be explained.

Pseudouridines might also affect RBP binding indirectly by modulating RNA structure. As an example, the binding of the yeast RNA helicase Prp5 to the branch site stem-loop in the U2 snRNA was stabilized in the presence of the two pseudouridines that are endogenously found in this region (152). Structure probing revealed that these pseudouridines stabilized the stem structure, suggesting that the effects on protein binding were indirectly mediated by changes in RNA secondary structure (152). It will be interesting to see how the binding of particular RBPs in specific contexts is affected by RNA pseudouridylation in vivo. This could be achieved by combining transcriptome-wide pseudouridines and RBP-binding profiles with the genetic manipulation of PUS proteins to identify pseudouridine-sensitive binding sites in cells.

6.3. Noncatalytic Functions of Pseudouridine Synthase Proteins

Some functions of PUS are independent of their activity. For example, bacterial TruB acts as a tRNA-folding chaperone independent of catalysis, and this activity is important for bacterial fitness (81). The depletion of human PUS10 leads to a defect in microRNA (miRNA) precursor processing that can be rescued by catalytic null PUS10 (135). PUS10 interacts with primary miRNAs and the microprocessor complex, suggesting a direct mechanism for this noncatalytic function in miRNA biogenesis. The depletion of human RPUSD3 results in decreased 16S rRNA levels and a reduction in mitochondrial translation (3, 5). The mechanism is unknown and unlikely to be downstream of changes in RNA pseudouridylation because RPUSD3 lacks the conserved aspartate required for PUS catalytic activity (**Figure 4**). These examples highlight the possibility of additional noncatalytic functions of PUS proteins in RNA metabolism.

7. BIOLOGICAL FUNCTIONS OF PSEUDOURIDINE IN STABLE NONCODING RNA

7.1. tRNA

A general function of pseudouridine in tRNA is to stabilize the folded structure that is required for tRNAs to function in translation (reviewed in 94). For example, the presence of a single pseudouridine at position 39 in the anticodon stem of $tRNA^{Lys}$ increases the melting temperature by 5°C (40). The fact that microorganisms lacking various tRNA-modifying PUSs are temperature-sensitive for growth is consistent with the idea that pseudouridine stabilizes essential tRNA structure. Notably, not all tRNAs are equally sensitive to the lack of pseudouridine at a particular position. A thorough genetic investigation of the roles of yeast *PUS3*, which installs Ψ38 and Ψ39 in at least 19 tRNAs and is required for growth at elevated temperatures, made the surprising discovery that the overexpression of a single Pus3 target tRNA, tQ(UUG), was sufficient to rescue growth above 38°C (55). These results provide strong genetic evidence that the temperature-sensitive growth defect is due to compromised function of a tRNA target.

In principle, tRNA pseudouridylation could affect any aspect of tRNA biogenesis and function, such as charging by aminoacyl tRNA synthetases, decoding on the ribosome, degradation of the tRNA, or processing to produce tRNA-derived small RNAs (tsRNAs). We are not aware of evidence directly demonstrating a role for pseudouridine versus U in the fidelity of tRNA aminoacylation, but changing the nucleotide identity of a normally pseudouridylated position, Ψ35, from U to A increased misacylation in vitro (7). Unmodified tI(UAU) is charged with reduced efficiency compared to the properly modified anticodon, ΨAΨ (130). The effects of the deletion of tRNA-modifying PUS on charging in cells have been investigated in a few cases with negative results (55). Thus, it remains an open question whether tRNA pseudouridylation affects the efficiency of aminoacylation, particularly in human cells. New sequencing-based methods to determine the fraction of charged tRNA species (42) offer sensitive and quantitative approaches to investigate the effects of PUS depletion on the aminoacylation of specific tRNAs.

Pseudouridines affect the function of certain tRNAs in ribosome binding and decoding. Few studies have directly investigated human tRNAs, and the effects of pseudouridine on tRNA function may differ between isodecoders as well as between different tRNA families. The importance of pseudouridine for tRNA function varies by tRNA in ways that are currently unpredictable. For example, only one out of three tested yeast tRNAs required Ψ39 for nonsense suppressor tRNA activity, a difference between tRNAs that did not correlate with differences in the predicted stabilities of their anticodon stems (55). In some cases, the physiological and translational consequences of perturbed tRNA pseudouridylation might be compounded by coupled defects in additional tRNA modifications, as shown for bacterial tRNAs lacking Ψ55 due to inactivation of TruB (67).

Data on translation defects caused by a lack of tRNA pseudouridines in human cells are limited. Knocking out human PUS proteins has been reported to have no effect on global protein synthesis in the case of PUS10 (135) and to actually increase protein synthesis in the case of PUS7 (52), the latter by a mechanism involving tRNA fragments (tRF) that is distinct from altered decoding by intact tRNAs. Ribosome footprint profiling (66) offers a potentially powerful approach to identify decoding events that are sensitive to tRNA modifications (see for example 171). Using this approach, cells lacking PUS10-dependent pseudouridylation were observed to have slight changes in codon occupancy by ribosomes, but codons read by PUS10 target tRNAs were not preferentially affected (135). Similarly, ribosome profiling of seven PUS knockouts in budding yeast revealed codon-specific changes in ribosome occupancy that were not easily explained by the known tRNA targets (20). Improved ribosome profiling methods that better capture differences in decoding rate (151) may reveal stronger correlations between the loss of pseudouridines

in specific tRNAs and slowed translation of cognate codons. A complete census of PUS-dependent pseudouridylated sites in human tRNAs will be needed to interpret the results of ribosome profiling in PUS knockout cells.

The evidence that mammalian tRNA pseudouridines may be regulated to be tissue-specific (11) suggests that pseudouridine could contribute to the fine-tuning of tRNA function to correspond to the unique translatomes of different cell types. Coordination between mRNA expression and adaptive changes in tRNA modifications has been characterized for other modifications (reviewed in 16).

7.2. tsRNA

Pseudouridine affects the biogenesis and function of certain tRNA-derived small RNAs that regulate translation (translational control by tsRNAs is reviewed in 91, 132). Knocking out PUS7 in human embryonic stem cells reduced the levels of specific 5′ tRFs shown to be pseudouridylated by PUS7 in cells (52). Transduction of synthetic pseudouridine-containing 5′ tRFs, but not unmodified tRFs, led to a global reduction in protein synthesis (52) by a mechanism that may target the integrity of the cap-binding complex, as shown previously for larger 5′ tRFs (68). Given the diversity of functions ascribed to tsRNAs and the incomplete understanding of their biogenesis and regulation, it is likely that additional pseudouridine synthases may affect human gene expression via effects on tsRNAs.

7.3. rRNA

The rRNA-modifying pseudouridine synthase DKC1 is an essential gene in human cells (131, 150), which may be due to its conserved role in ribosome biogenesis (reviewed in 116, 134) rather than an absolute requirement for rRNA pseudouridylation. Yeast cells expressing catalytically inactive cbf5-D95A, the ortholog of DKC1, lack rRNA pseudouridine and are viable (162). However, ribosomes isolated from these slow-growing mutants show biochemical defects in tRNA binding in vitro and altered translation fidelity in vivo (69). Mammalian cells deficient in DKC1 likewise show defects in translation initiation and fidelity (69, 157). The mechanisms responsible for specific translation defects are unclear but may arise from the effects of pseudouridines on RNA conformational dynamics, as demonstrated for one conserved pseudouridylated rRNA domain, helix 69 (37, 75, 76, 128, 138).

Phenotypes caused by the deletion or overexpression of individual H/ACA snoRNAs suggest important cellular functions for individual rRNA pseudouridines in human cells (104). 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 (103).

7.4. snRNA

Mammalian snRNAs are heavily modified with pseudouridines that are concentrated in functionally important regions of RNA–RNA and protein–RNA interactions. Based on the known effects of pseudouridine on RNA structure and RBP binding, these pseudouridines are predicted to be important for multiple steps in precursor mRNA (pre-mRNA) splicing.

7.4.1. U1 snRNA. Ψ5 and Ψ6 in U1 snRNA are within the region that base-pairs with the pre-mRNA 5′ splice site, where they could stabilize base-pairing between U1 snRNA and the pre-mRNA and thereby affect 5′ splice site selection and, consequently, splicing. The G-Ψ base pairs between the 5′ end of U1 snRNA and the pre-mRNA are important for splice site selection and contribute to U1:5′ splice site stability (47, 124). Thermal melting experiments demonstrated

the strengthened stability of pseudouridine-containing duplexes with U1 snRNA in a variety of 5′ splice site contexts (53, 123). U1 snRNA pseudouridines might be particularly important for promoting interactions with weaker splice sites.

7.4.2. U2 snRNA. Spliceosome assembly and the splicing of a pre-mRNA substrate were inhibited when U2 snRNA was lacking Ψ6, Ψ7, and Ψ15 in HeLa nuclear extracts. Chimeric snRNAs lacking individual pseudouridines gave reduced splicing efficiency, demonstrating that individual U2 snRNA pseudouridines contribute to pre-mRNA splicing (39).

Although the functions of pseudouridines in the BSRR of U2 have not been interrogated in human cells, these pseudouridines were required for spliceosome assembly and splicing of reporters in *Xenopus* oocytes (169). Consistent with the stimulatory effects of pseudouridine in the BSRR in *Xenopus* oocytes, human Ψ35 stabilizes the U2 snRNA-pre-mRNA duplex in vitro (111). Nuclear magnetic resonance studies revealed that Ψ35 also altered the structure of this duplex. This study showed that pseudouridine changed the orientation of the branch site adenosine on the opposite strand by promoting bulging, which might position the branch site for more efficient splicing (111). However, others have shown that these effects might be dependent on sequence context (83). In yeast, loss of highly conserved Ψ35, Ψ42, and/or Ψ44 in the BSRR of U2 snRNA inhibited splicing and caused temperature-sensitive growth (152). Loss of Ψ42 and Ψ44 reduced the affinity and ATPase activity of Prp5 for U2, which resulted in a decrease in spliceosome assembly, providing a mechanistic basis for their effects on splicing (152). These pseudouridines alter the RNA secondary structure of the branchpoint-interacting stem-loop in the U2 snRNA, which might facilitate the binding of Prp5 (152). Two additional pseudouridines in human U2 are well positioned to stabilize helix II of the U6/U2 duplex at the core of the spliceosome (9, 168).

7.4.3. U4 snRNA. The functions of constitutive pseudouridine in human U4 and U6 snRNAs have not been investigated, but their positions suggest likely effects on spliceosome assembly and activation. Two pseudouridines in U4 snRNA are in the regions of base-pairing between U4 and U6 snRNA, Ψ4 in stem II and Ψ79 in the human-specific stem III, respectively. In contrast to yeast, where Brr2 is preloaded on U4 (113, 114, 149), stem III occludes the Brr2 loading sequence on human U4 as seen in the human tri-snRNP and pre-B complex cryogenic electron microscopy structures (17). Ψ72 and Ψ79 are within the Brr2 helicase loading sequence, which facilitates U4/U6 unwinding to allow U6 snRNA (117) to base-pair with the pre-mRNA substrate 5′ splice site (9, 168). Therefore, the pseudouridine in the U4 snRNA could influence the stability of the U4/U6 duplex and consequently affect di-snRNP or tri-snRNP assembly, helix unwinding, or Brr2 helicase activity—all of which could impact splicing.

7.4.4. U6 snRNA. Ψ31 in U6 snRNA is also within stem III of the U4/U6 duplex. Ψ40 is located in the nucleotide 5′ to the ACAGA-box, which pairs with the 5′ splice site in pre-mRNA following the unwinding of U4. This pseudouridine has the potential to affect splicing by influencing the stability of the interaction between U6 snRNA and the 5′ splice site of some pre-mRNAs. Ψ86 is located within the telestem of the U6 mono-snRNP, which is mutually exclusive with the U2/U6 helix II (part of the U2/U6 catalytic interaction network) (38). Stabilization of the telestem by Ψ86 may be important for U6 snRNP assembly or U4/U6 di-snRNP assembly since the factor SART3 binds to the telestem region and promotes U4/U6 annealing (38). Within the spliceosome, Ψ86 is located at the end of a bulge immediately 5′ to the U2/U6 helix II (9, 168), where it could play a stabilizing role.

7.4.5. U5 snRNA. U5 pseudouridines are poised to stabilize U5 secondary structure and base-pairing with pre-mRNA. The U5 snRNA holds the 5′ exon in place during the first and second steps of splicing and contacts the 3′ exon after the first step of splicing by base-pairing interaction between loop I in U5 and the pre-mRNA (112). Ψ43 and Ψ46 are within loop I, and Ψ53 is within the stem of loop I, which could enhance the internal U5 stem or U5-pre-mRNA base-pairing. Prp8 stabilizes the interactions between loop I and the 5′ exon (143). The structures of the human spliceosome show that Prp8 makes contacts with the backbone phosphates in the U5 loop I region (Ψ46) as it base-pairs with the 5′ exon (97, 167). Given that pseudouridines rigidify the RNA backbone, they might be important for Prp8 interactions with U5.

Pseudouridines in the snRNAs are predicted to impact numerous RNA–RNA and RNA–RBP interactions during the splicing cycle, but their individual functions have mostly not been interrogated. Studies to establish snRNA pseudouridine function will be greatly facilitated by the identification of the enzymes/RNA guides that direct pseudouridylation at each position of the human snRNAs. Whether human snRNA pseudouridines affect alternative splicing or the splicing efficiency of endogenous cellular pre-mRNAs is an open question. These questions could be answered by deleting the snoRNAs that target individual pseudouridines in snRNAs followed by RNA-seq and splicing analysis. To investigate the mechanism by which snRNA pseudouridines affect splicing, one could perform psoralen cross-linking followed by snRNA pull-down to quantify snRNA–pre-mRNA or snRNA-RNA interaction deleted in snoRNA compared to wild-type cells. Similarly, the effect of snRNA pseudouridine on interactions with RBPs throughout the splicing cycle could be investigated by the profiling of RBP binding (e.g., cross-linking immunoprecipitation sequencing) in cells lacking individual snRNA pseudouridines.

8. POTENTIAL FUNCTIONS OF PSEUDOURIDINE IN mRNA

8.1. Translation Fidelity

The frequent occurrence of pseudouridines within mRNA (15, 84, 93, 129) poses an important question: How does a ribosome decode a pseudouridylated codon? While the Watson-Crick face of the nucleotide is preserved between pseudouridine and uridine, it is clear that pseudouridine is not always treated as a uridine by the ribosome. Artificial pseudouridylation of stop codons caused >70% stop codon readthrough in rabbit reticulocyte lysate (78), with similarly efficient readthrough observed in *E. coli* lysate (44), suggesting a conserved mechanism. Pseudouridylated stop codons were also read through at an undetermined efficiency in vivo in budding yeast cells (78, 153), with different amino acids incorporated depending on the stop codon (78).

The mechanism by which pseudouridine promotes stop codon readthrough is incompletely understood. Pseudouridine minimally affects peptide release by *E. coli* release factors in vitro (43, 139), suggesting that pseudouridine promotes readthrough by increasing mistranslation by near or noncognate tRNAs. Consistent with this possibility, the structure of yeast $tRNA^{Ser}$ IGA anticodon complexed with a ΨAG stop codon in the context of the *Thermus thermophilus* 30S ribosomal subunit revealed the accommodation of this noncognate codon:anticodon pair in a manner that was similar to a nonpseudouridylated codon (44). However, this structure did not illuminate how the chemical structure of pseudouridine allows for the efficient mistranslation of stop codons. Pseudouridylated stop codons appear to be terminated correctly (i.e., produce full-length protein of the expected size) in human HEK293T cells in the context of synthetic mRNAs in which all U residues are replaced with pseudouridines (60). To our knowledge, pseudouridine-mediated nonsense suppression has been described exclusively in engineered systems, and there has been no characterization of readthrough at an endogenous pseudouridylated stop codon in cells.

Decoding of sense codons can also be affected by pseudouridine, but the results from different experimental systems do not paint a consistent picture. The incorporation of single pseudouridines into UUU phenylalanine codons caused misincorporation in one reconstituted bacterial translation system (43) and decreased the yield of full-length peptides without producing detectable miscoded peptides in another (59). Mechanistically, pseudouridine affected multiple steps in translation in vitro that could impact fidelity, including increasing the rate of misincorporation of valine by Val-tRNA^{Val} at a ΨUU phenylalanine codon and suppressing the surveillance mechanism that detects codon:anticodon mismatches in the ribosomal P site (43, 160, 161). Fully pseudouridylated mRNAs transfected into human cells produce some amount of functional protein (43, 60, 80, 101), although various pseudouridine-dependent mistranslated peptides were detected in one study (43). The effect of pseudouridine on the yield of functional protein depends strongly on the specific codons used (43, 101). The mechanisms underlying this sequence dependence are unknown, highlighting how much remains to be understood about the translational consequences of mRNA pseudouridylation in cells.

8.2. Innate Immunity

Cells are equipped with innate immune sensors, including various Toll-like receptors (TLRs), retinoic acid–inducible protein I (RIG-I), and protein kinase R (PKR), which detect foreign nucleic acid (21). RNA modifications have been thought to provide a mechanism for discerning self RNA from nonself RNA, and indeed, incorporating RNA modifications, including pseudouridine, into foreign RNA allows it to escape from innate immune detection. This makes RNA modification a powerful tool in the field of RNA therapeutics, where RNAs must make it into cells without triggering an immune response and remain stable long enough to achieve therapeutic goals. In addition, the presence of modified nucleosides in viral genomic RNA could contribute to immune evasion during infection (110).

8.2.1. Toll-like receptors. TLRs are membrane-associated proteins that detect various pathogen-associated molecular patterns and subsequently stimulate production of proinflammatory cytokines. The RNA-sensing TLRs, TLR3, TLR7, and TLR8, reside within endosomal membranes. TLR3 recognizes double-stranded RNA (dsRNA), while TLR7 and TLR8 recognize ssRNA. Upon target recognition, TLRs activate a signaling cascade that results in the expression of proinflammatory cytokines and interferon. In vitro transcribed RNA is immunostimulatory when transfected into HEK293 cells engineered to express TLR3, TLR7, or TLR8, and the inclusion of pseudouridine in the RNA suppressed this response (most pronounced for TLR7 and TLR8) (79).

8.2.2. Retinoic acid–inducible protein I. RIG-I is a cytosolic innate immune sensor responsible for detecting short stretches of dsRNA or ssRNA with either a 5′-triphosphate or 5′-diphosphate group (a feature common to various RNA viruses). Activation of RIG-I relieves its autoinhibition, releasing its caspase activation and recruitment domains to interact with mitochondrial antiviral signaling protein (MAVS) and set off a signaling cascade that ultimately results in the expression of immune factors. Inclusion of pseudouridine in a 5′-triphosphate-capped RNA abolishes the activation of RIG-I (41, 61), providing another mechanism for pseudouridine-mediated suppression of innate immune activation. Further, the polyU/UC region of the hepatitis C virus genome is also a potent activator of RIG-I, and the complete replacement of U with pseudouridine in this RNA fully abrogates downstream interferon-β induction, despite RIG-I still binding to the modified RNA (41, 146) but with reduced affinity (41). Durbin et al.

(41) present biochemical evidence that RIG-I bound to pseudouridylated polyU/UC RNA fails to undergo the conformational changes necessary to activate downstream signaling.

8.2.3. Protein kinase R. RNA-dependent PKR is a cytosolic resident innate immune sensor. Upon detection of foreign RNA, PKR represses translation through the phosphorylation of translation initiation factor eIF-2α. Molecules that activate PKR are varied but include dsRNA formed intra- or intermolecularly, and 5′-triphosphate groups. Inclusion of pseudouridine in various PKR substrates reduces PKR activation and downstream translation repression relative to unmodified RNAs. For example, a short 47-nt ssRNA potently activates PKR when synthesized with U but not with pseudouridine (an ∼30-fold reduction with pseudouridine) (108). Pseudouridine also modestly reduced PKR activity when this short RNA was annealed to a complementary unmodified RNA (108). Likewise, in vitro transcribed, unmodified tRNA acted as a much more potent activator of PKR than tRNAs transcribed with pseudouridine (109). It should be noted that it is unclear whether a fully pseudouridylated tRNA adopts canonical folding and what impact this may have on PKR recognition of this substrate. Finally, the transfection of an unmodified mRNA caused a greater reduction in overall cellular protein synthesis in cell culture compared to the transfection of the same fully pseudouridylated mRNA (2). Consistent with this result, fully pseudouridylated mRNA reduced PKR activation and the subsequent phosphorylation of eIF-2α (2).

8.3. Consequences for Therapeutic Applications

The success of mRNA therapeutics depends on the ability to synthesize functional protein from exogenously supplied mRNA and to deliver these RNAs without triggering an immune response. As described above, pseudouridylation of in vitro transcribed RNA is capable of reducing the stimulation of the innate immune system in in vitro models. These findings also hold true in vivo. Indeed, while the systemic injection of a uridine-containing in vitro–transcribed reporter RNA triggered elevated interferon-α levels in mice, use of fully pseudouridylated reporter dampened this response (80).

It is also important that therapeutic RNAs that encode proteins be translated well. Multiple studies have reported enhanced protein production from pseudouridylated reporter RNAs relative to their unmodified counterparts both in vitro and in vivo. However, the mechanisms are unclear, and the effect differs between studies and the specific mRNA sequence tested (2, 43, 59, 60, 80). Pseudouridine likely affects multiple facets of mRNA function, including reduced immune stimulation by several mechanisms (2, 41, 61, 79, 108, 109, 146), prolonged half-life of pseudouridine-containing RNA (80), as well as potentially deleterious effects of pseudouridine on translation fidelity and efficiency (43, 59, 60, 78).

8.4. Potential Effects of Pre-mRNA Pseudouridine on Pre-mRNA Processing

Human PUS1, PUS7, TRUB1 and RPUSD4 have been shown to at least partially localize to the nucleus in human cells (45, 73, 127, 137) and PUS1, PUS7, and TRUB1 are known to pseudouridylate mRNAs (13, 127). Furthermore, PUS7 stably associates with chromatin and associates with DNA at active RNA polymerase II promoters and enhancers (73). The nuclear localization and/or chromatin association of these PUSs raise the possibility that they might act on nascent pre-mRNA. Given the many molecular effects of pseudouridine on RNA–protein and RNA–RNA interactions, if pseudouridines are indeed deposited in pre-mRNA, they could function in splicing at multiple levels. The potential for pseudouridine to impact pre-mRNA splicing was shown: artificial pseudouridylation in the polypyrimidine tract of an adenoviral pre-mRNA substrate inhibited the binding of the 3′ splice site recognition factor U2AF2 and abolished the splicing of the

intron (19; for a detailed review of the likely effects of pseudouridine and other RNA modifications on splicing, see 98).

9. CONCLUSIONS AND PERSPECTIVES

Pseudouridine is the most abundant modified nucleoside in nature. Recent advances in pseudouridine detection have revealed a rich and dynamic landscape that includes regulated pseudouridines in tRNA, rRNA, and snRNA, where they were long known to occur, and identified many previously unknown pseudouridines in mRNA and additional ncRNAs. Despite intensive investigation of the structural and biochemical effects of pseudouridine in various systems, the biological roles of most endogenous pseudouridines remain unknown. With current technology (135), some important questions should soon be answered, such as identifying the PUSs responsible for all sites of pseudouridines in human tRNA and snRNA. Other recent technical achievements establish promising approaches to elucidate the RNA sequence and structural features recognized by PUSs to enable site-specific pseudouridylation (13, 127). The more challenging questions all relate to pseudouridine function in the context of cellular RNA metabolism. Why are some tRNAs more dependent on pseudouridine than others? How do individual pseudouridines in snRNAs affect the accuracy, efficiency, and regulation of splicing? How does the ribosome decode pseudouridines (and why are the effects of pseudouridines on translation so context-dependent)? How do pseudouridine-containing RNAs evade immune detection, and what are the implications of these mechanisms for naturally pseudouridylated viral RNAs and for therapeutic RNA applications? The future challenges are psi(Ψ)-zable indeed.

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Errata

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