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Regulation and Function of RNA Pseudouridylation in Human Cells

Erin K. Borchardt[#,*,1], **Nicole M. Martinez**[#,*,1], **Wendy V. Gilbert**[1]

[1]Yale University, Yale School of Medicine, Department of Molecular Biophysics & Biochemistry, New Haven, CT 06520, USA.

[#] These authors contributed equally to this work.

Abstract

Recent advances in pseudouridine detection reveal a complex pseudouridine landscape that includes messenger RNA and diverse classes of non-coding 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 roles of non-coding RNA pseudouridylation in splicing and translation and point out potential effects of mRNA pseudouridylation on protein production including in the context of therapeutic mRNAs.

Keywords

pseudouridine; RNA modification; mRNA; tRNA; snRNA; translation; innate immunity

1. INTRODUCTION

Pseudouridine (Ψ) is an abundant RNA modification found in all domains of life [1–3]. Like other modifications – and there are 172 different modified nucleosides identified to date[4] – Ψ has distinct chemical properties that affect the structure of pseudouridylated RNA molecules [5–10]. and their interactions with protein and other RNAs[11–15]. Study of Ψ is experiencing a rapid expansion since the identification of many new Ψ sites in ncRNAs and the discovery of the presence of Ψ in yeast and human mRNAs by high-throughput sequencing approaches[16–19].

The biochemical roles of Ψ have been intensely investigated in a few cases and found to affect the function of tRNA and rRNA in translation[20–22] and the function of snRNA in

Correspondence to: wendy.gilbert@yale.edu.
*Equal contribution

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splicing [23,24]. However, it is currently difficult to predict the function of any individual Ψ. Genetic manipulation of pseudouridine synthases (PUS) impacts RNA metabolism and leads to cellular phenotypes [17,25,26], but whether and which Ψ mediate these effects are not well understood. The functions of endogenous Ψ in mRNA remain to be discovered. However, the striking effects of artificial RNA pseudouridylation on translation [27–29], mRNA processing [14] and innate immune sensing[30–32] demonstrate the potential for individual Ψ to broadly affect human gene expression.

In this review, we describe current knowledge of Ψ locations, production, and function in human RNAs. We begin by reviewing the methods that have revealed a more extensive Ψ landscape than 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 Ψ on RNA structure and RNA-protein interactions to speculate about the probable effects of Ψ in mRNA, with implications for mRNA therapeutics.

2. PSEUDOURIDINE DETECTION AND DISTRIBUTION

Bulk nucleoside fractionation by HPLC and detection by UV monitoring originally revealed Ψ as a very abundant component of yeast total RNA, “the fifth nucleoside” [33,34]. Today, two approaches dominate the study of the abundance and locations of Ψ: mass spectrometry on purified RNA[35,36] and high-throughput sequencing of transcriptomes[17,18,37,38]. As a result, Ψ has been mapped in most classes of RNA found in human cells including abundant non-coding RNAs such as tRNA, rRNA, snRNA, and snoRNA, as well as less abundant lncRNA and protein-coding mRNA.

2.1 High-throughput sequencing-based methods to identify pseudouridine locations

A breakthrough in the study of Ψ came in 1993 with the development of a method to map Ψ sites using primer extension [39]. Bakin and Ofengand showed that the stable adducts formed between Ψ and N-cyclohexyl-N’-β-(4-methylmorpholinium) ethylcarbodiimide p-tosylate (CMCT) block reverse transcriptase, producing truncated cDNA that could be sequenced to reveal the Ψ location with single-nucleotide resolution[39,40]. Three groups adapted this strategy to the Illumina sequencing platform to produce transcriptome-wide maps of Ψ [17,37,38], including in human mRNA[17,37]. A fourth synthesized a modified carbodiimide to permit chemical coupling of biotin to Ψ residues to enrich Ψ-containing RNA fragments for sequencing [18].

RBS-seq uses a distinct chemistry [41] to detect pseudouridines, in addition to other modified nucleosides [19]. An advantage of this approach, which detects Ψ as 1–2nt 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 Ψ sites from lowly expressed genes in Hela [37] and HEK293 [17,18] cells. These sequencing-based methods differ in subtle but nevertheless important ways. Biotinylation and enrichment of Ψ-containing RNA fragments before sequencing should give the most comprehensive detection of Ψ sites from the fewest sequencing reads [18], but has the potential disadvantage of detecting very low

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stoichiometry Ψ sites of uncertain biological significance. In contrast, transcriptome-wide RBS-seq detects mainly high-stoichiometry Ψ sites unless in very highly expressed genes[19]. A recent adaptation of CMC-dependent Ψ mapping offers targeted RNA Ψ quantitation and revealed Ψ stoichiometries ranging from ~30–80% in low-abundance human mRNAs[42].

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[43–45] and reveal regulation of RNA modification across tissues [46] and in response to environmental perturbations[47]. Analysis of RNA modifications by mass spectrometry usually targets purified RNA species. Ψ poses special challenges because it has the same mass as U. Ψ is only detectable by characteristic ions produced during fragmentation[35,36]. Direct sequencing by mass spectrometry produced a complete map of Ψ in human snRNAs from 293T cells [48] and determined absolute Ψ levels for human ribosomal RNA from TK6 cells [49].

Mass spectrometry has been used to analyze bulk mRNA Ψ content, revealing that Ψ is present at ~0.2–0.6% of total U in mRNA from human HEK293T cells[18]. This global abundance is on par with the abundance of m^6A[50]. Because Ψ 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 [18] actually reflect regulated modification of mRNA is to perform RNA-seq on the sample subjected to analysis by mass spectrometry[51].

2.2 Distribution of pseudouridine in mammalian RNAs

2.2.1 tRNA Ψ sites—Data for tRNAs are limited in most RNA sequencing studies, including pseudouridine profiling [17,18,37,38,41], due to problems synthesizing cDNA from these heavily modified RNAs. Pretreatment of RNA with demethylases improves tRNA coverage [52,53] and was recently combined with CMC-based pseudouridine profiling to produce a nearly comprehensive map of Ψ in human tRNAs from HEK293 cells [54], excluding positions close to the 3′ ends, which could not be mapped. Most Ψ were found at one of 17 positions that were previously known to be modified in human cytosolic tRNAs [4,55], 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 [54]. Several novel positions were reported (Figure 2), as well as new information about known positions in many tRNA isodecoders, tRNAs with the same anti-codon but different sequences for the tRNA body.

2.2.2 rRNA Ψ sites—Human ribosomes include 104 Ψ that have been experimentally validated by mass spectrometry in human TK6 cells [49], the majority of which were previously identified by primer extension and pseudouridine profiling [17,37,56]. These pseudouridines occur at positions predicted to base-pair with H/ACA snoRNA guide sequences[57]. Approximately half of human Ψ sites are conserved from budding yeast and all are located within conserved functional domains [49,58,59].

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2.2.3 snRNA Ψ sites—The mammalian U1, U2, U4, U5 and U6 snRNAs contain a combined 27 Ψ [48,60,61] (Table 1). U2 snRNA is the most highly modified with 14 reported Ψ, of which three are conserved across species (Ψ34, Ψ41 and Ψ43 in the branch site recognition region in vertebrates; Ψ35, Ψ42 and Ψ44 in the corresponding positions in budding yeast). U1 snRNA has two conserved Ψ at the 5′ end (Ψ5 and Ψ6) and U5 has one (Ψ46). Additional Ψ in human U4, U5 and U6 snRNAs have not been reported in budding yeast.

2.2.4 mRNA Ψ sites—Several groups discovered widespread pseudouridylation of mRNAs and mapped their locations in human cells using transcriptome-wide pseudouridine profiling methods [17–19,37]. 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[19,62]. Technical differences in sequencing-based approaches for modification detection could result in capture biases, which might explain some of these differences. Cell type-specific differences in mRNA abundance are likely the major contributor to observed differences in Ψ identification because current methods are limited to the most highly expressed genes. We have also identified instances of cell type specific mRNA Ψ in mRNAs expressed at sufficient levels for site calling in different human cell lines (Unpublished), consistent with regulation of mRNA pseudouridylation.

3. ENZYMES THAT CATALYZE RNA PSEUDOURIDYLATION

Pseudouridylation is carried out by a class of proteins called pseudouridine synthases (PUS). Here we focus on the 13 pseudouridine synthases found in humans and provide context from the more extensive literature on their *E. coli* and yeast counterparts (reviewed in[63,64]).

3.1 PUS protein domains

PUS proteins are classified into 6 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 PUS carry a common 8-stranded beta sheet catalytic fold and rely on a spatially conserved aspartate for catalysis despite sharing little primary sequence similarity [64]. Comparing the available structures of 5 human PUS (RPUSD1 5VBB; RPUSD4 5UBA; PUS1 4J37; PUS7 5KKP; PUS10 2V9K) to their *E. coli* counterparts reveals conservation of the core catalytic domain. However, human PUS proteins typically carry auxiliary domains 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 [65]. Human PUS1 carries a C-terminal extension comprised of three alpha helices that preclude the tRNA binding mode employed by bacterial TruA[66] and may constrain the RNA targets to be flexible as shown for yeast Pus1[67].

Flanking the catalytic cleft, PUS proteins are typically decorated with a series of alpha helices and loops. Two of these features, termed “forefinger” and “thumb” based on structures of *E.coli* PUS proteins complexed with target RNA, dock into the grooves of the target RNA stem, effectively “pinching it” [64–66,68].

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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 [69–71], but uncertainties remain (reviewed in[2]).

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 [66,68,72,73]. In *E. coli* PUS this residue is often critical, and mutation leads to loss of pseudouridylation activity despite retaining the ability to bind to target RNAs [68,73].

However, mutation of the residue responsible for base flipping in hPUS1 has different effects based on the substrate tested and the amino acid used for replacement [74]. One of the disruptive mutations is causative for Mitochondrial Myopathy and Sideroblastic Anemia (MLASA), and changes the arginine involved in base flipping to tryptophan [75]. The mutant PUS1 produced is inactive and the tryptophan replacing the arginine was hypothesized to sterically block the enzyme active site and not be able to intercalate into the substrate stem [74–76].

3.3 RPUSD3 lacks catalytic aspartate

RluA PUS family members are characterized by a conserved HRLD motif (Figure 4a). 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 [68]. 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).

3.4 Classes of RNA targeted by PUS

Most of the RNA targets of human PUS remain to be discovered and may include all classes of non-coding RNA as well as mRNA. Some tRNA targets of human PUS can be predicted based on homology to yeast PUS with known target sites (Figure 2). Several stand-alone

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PUS have been shown to modify human mRNA sequences including PUS1 [18,67], PUS7 [62,67], TRUB1[62,67], TRUB2 [62,67]and RPUSD2 [67]. The RNA-guided PUS, DKC1, targets snRNA and rRNA sites[3]. DKC1 is not known to have mRNA targets, but Ψ can be detected at computationally predicted target sites in human mRNA (unpublished) as shown in budding yeast [37]. 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 [77]. PUSL1, TRUB2, RPUSD3 and RPUSD4 have been detected in mitochondria [78–81], and PUS1, PUS7, TRUB1 and RPUSD4 have been shown to at least partially localize to the nucleus [62,77,82,83]. PUS10 is predominantly nuclear at steady-state and re-localizes to mitochondria in response to apoptotic signals [84].

4. SPECIFICITY OF RNA PSEUDOURIDYLATION

4.1 Stand-alone PUS targets and recognition

Of the 13 human PUS proteins, 12 are considered “stand-alone” pseudouridine synthases, meaning they recognize their targets without the use of accessory RNAs. With high-throughput assignment of Ψ 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 [62,67]. 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 [62,67]. Human PUS7, on the other hand, recognizes targets with a UNUAR motif [62,67]. This motif is not sufficient to direct pseudouridylation by PUS7 as the majority of motifs are not detectably pseudouridylated in human cells[17,18,37]. RNA structural features, as yet to be defined, are likely to be important for human PUS7 as they are for yeast PUS7[85] and yeast PUS1[67]. Both the TRUB1 and PUS7 motifs resemble motifs previously defined in yeast, and yeast Pus4 was capable of modifying many targets of human TRUB1 [67]. This finding is consistent with conservation of target features among evolutionary related PUS proteins.

Higher eukaryotes including humans have expanded PUS families that could support 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[18]. Likewise, depletion of TRUB1 eliminates pseudouridylation of mRNA targets in cells that express its paralog TRUB2[62]. Consistent with this lack of redundancy in cells, TRUB1 and TRUB2 modify distinct sets of mRNA targets in vitro[67]. 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 of which three contain the residues needed for catalytic activity (Figure 4).

4.2 RNA-guided pseudouridylation of rRNAs and snRNAs by H/ACA snoRNPs

The catalytic subunit of the snoRNPs, DKC1, is guided by base pairing interactions between box H/ACA snoRNAs and the target RNA, which provide the specificity for pseudouridylation of the substrate (reviewed in[3]). The conserved snoRNP complex contains DKC1, GAR1, NHP2, NOP10 and the guide RNA. Box H/ACA snoRNAs typically consist of two hairpins that contain an internal pseudouridylation pocket where the RNA target site

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is converted to Ψ and two conserved sequence elements, the H and ACA motifs, that recruit the protein components. H/ACA snoRNPs target pseudouridylation of nascent rRNA in the nucleolus (reviewed in[58]).

A subset of box H/ACA snoRNAs called small Cajal body specific RNAs (scaRNAs) are predicted to guide pseudouridylation of the snRNAs based on sequence complementarity [56,86–89]. (Table 1). This hypothesis is further supported by experiments showing that pseudouridylation of many snRNAs sites in human cell extracts is dependent on RNA [86,90,91] and that snoRNAs predicted to modify particular snRNAs specifically interact with the target snRNA in cells[92] (Table 1). Only one human scaRNA (SCARNA10) has been experimentally verified to direct pseudouridylation of its computationally predicted target, Ψ46 in human U5 snRNA [86]. Some snRNA Ψ lack a predicted guide snoRNA and might be modified by stand-alone PUS. Indeed, both RNA-dependent and independent mechanisms mediate snRNA pseudouridylation in budding yeast. The stand-alone PUS, Pus1 and Pus7, pseudouridylate yeast U2 snRNA at two positions[93,94]. 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 [95,96]. Micrococcal nuclease sensitivity of pseudouridylation *in vitro* indicates that some currently unassigned snRNA Ψ are targeted by RNA guides (Table 1). Recent studies have revealed that fewer base pairs (6–8) than the canonical (>10) base pairs and more mismatches are tolerated for pseudouridylation [97,98]. 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[99]. The scaRNAs that are predicted to guide snRNA modifications are enriched in CB due to a CB retention signal [100] and a protein anchor [101]. A mutation in U2 snRNA that accumulates in the cytoplasm, preventing re-entry of U2 snRNA into Cajal bodies, is deficient in pseudouridylation [88]. There is still uncertainty as to where in the cell U6 snRNA is pseudouridylated[101,102]. Furthermore, snRNAs are still pseudouridylated in cells lacking canonical Cajal bodies (e.g. cells lacking coilin) suggesting CBs aren't strictly required for snRNA pseudouridylation [61,88,103], 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 pseudouridylation of each snRNA takes place.

5. REGULATION OF RNA PSEUDOURIDYLATION

5.1 Regulation of pseudouridines in ncRNA

The Ψ content of mammalian (pork and mouse) tRNA differs between tissues[46], suggesting that tRNA pseudouridylation could be regulated in human cells. The complete tRNA Ψ landscape has been determined in only one human cell line[26]. Thus, it is an open question whether tRNA Ψ are regulated in humans, and if so, at which sites. Quantitative mass spectrometry identified positions with sub-stoichiometric Ψ in the human 80S ribosome[49], raising the possibility that these sites could be increased under some conditions. Consistent with this possibility, inhibition of the mammalian Target of Rapamycin (mTOR) slightly

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increased bulk Ψ levels in rRNA from mammalian (CHO) cells at sites that remain to be determined[104]. It is unclear whether Ψ sites in the human snRNAs are fully modified and whether the stoichiometry or the locations of Ψ 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 stresses affect both splicing and organismal growth [105–107].

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[37] and treatment with H_2O_2 alters mRNA Ψ in HEK293T cells [18]. Heat shock causes re-localization of yeast Pus7 from the nucleus to the cytoplasm and results in pseudouridylation of new targets in the cytoplasm[17]. Nutrient deprivation in yeast induces changes in mRNA pseudouridylation by multiple PUS[37] through mechanisms that may involve changes in mRNA structure[67].

Sequences that are non-optimal targets for a given PUS are likely to be sensitive to changes in PUS expression. For example, non-optimal 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 [62]. Tissue and cell type specific expression of the 13 human PUS provide a mechanism for regulated cell type specific pseudouridylation (Figure 5) and may contribute to differences in mRNA pseudouridines detected in different cell types [17–19,37]. 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 interrogation of highly expressed genes.

6. EFFECTS OF PSEUDOURIDINE ON RNA STRUCTURE AND FUNCTION

6.1 RNA structure

The conservation of Ψ in structured non-coding RNA likely reflects effects of Ψ on RNA conformation, thermodynamic stability and structural dynamics. Ψ forms Watson-Crick base pairs with A that have similar geometry but greater thermodynamic stability than U-A pairs[5,6]. Ψ also stabilizes single stranded RNA conformations in solution in addition to enhancing the formation of RNA duplexes[7]. These effects of Ψ are due to its preference for the C3′-*endo* sugar conformation and enhanced base stacking[5–7]. In addition, water bridges between adjacent nucleosides and the H1N that distinguishes Ψ from U have been visualized in crystal structures of tRNA[8] and modeled in molecular dynamics simulations[5]. Such water bridges may increase the rigidity of RNA backbones containing Ψ.

The functional consequences of changing a U to a Ψ are frequently summarized as ‘stabilizing RNA structure’ [2,3,108]. Although true, this simplification obscures context-dependent differences, which are large enough to have biological significance. Systematic comparison of RNA duplexes containing all 8 possible NΨ and ΨN neighbors with the corresponding NU and UN sequences measured sequence context differences in the

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stabilizing effect of Ψ versus U of >1 kcal/mol[9]. Sequence context also affects the stacking potential of Ψ in single-stranded RNA [5]. In addition to these differences that depend on local sequence context, the net effect of Ψ on RNA structure depends on the folding potential of the surrounding sequence and the position of the Ψ. For example, positioning a Ψ within the loop region can antagonize hairpin formation [10]. This is likely due to the ability of Ψ to stabilize the A-form helical conformation of single-stranded RNA[7], which would increase the energetic cost of loop closure thereby favoring the extended RNA conformation.

The biological effects of Ψ must originate in chemical differences between U and Ψ, which primarily affect RNA backbone conformation and the stability of base pairs. Because Ψ can form stable pairs with G, C and U in addition to A, it has been proposed as a "universal" base pairing partner[6]. Despite intensive study of the structural effects of Ψ 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 Ψ-containing duplexes is an important step towards accurate predictions[9]. 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[109]).

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, cytoplasmic RNA localization and/or stability. The cytoplasmic RNA-binding protein PUM2 recognizes an RNA sequence motif (UGUAR) that is frequently pseudouridylated in human cells, likely by PUS7[17,37,62,67]. Incorporation of Ψ into this motif (UNΨAR) reduced the affinity of PUM2 binding by ~3-fold *in vitro*[11]. Similarly, substitution of uridines with Ψ within CUG repeats, which are associated with myotonic dystrophy type 1, reduced binding of the splicing factor MBNL1 by ~4–20 fold *in vitro* [12,13]. Artificial pseudouridylation of a single position in a polypyrimidine tract strongly inhibited binding of the splicing factor U2AF2 [14]. In an another example, artificial pseudouridylation of an individual position in the Sm binding site of U7 snRNA inhibited snRNP assembly by 2-fold in xenopus oocytes [15]. Inhibition of U2AF binding and U7 snRNP assembly by Ψ were both attributed to the known effects of Ψ on RNA backbone conformation. Consistent with this interpretation, incorporation of a locked nucleic acid also inhibited binding of U2AF2[14]. 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 RNA-binding proteins are sensitive to pseudouridylation of their target RNAs. Given the preference of RNA-recognition motifs (RRMs) for nucleotides in the C2′-*endo* conformation at the binding interface[110], Ψ may broadly antagonize RBP binding by favoring the C3′-*endo* conformation. However, the observed effects of Ψ 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, binding of the yeast RNA helicase Prp5 to the branch site stem loop in the U2

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snRNA was stabilized in the presence of the two Ψ that are endogenously found in this region [111]. Structure probing revealed that these Ψ stabilized the stem structure suggesting that the effects on protein binding were indirectly mediated by changes in RNA secondary structure [111]. It will be interesting to see how binding of particular RBPs in specific contexts is affected by RNA pseudouridylation *in vivo*. This could be achieved by combining transcriptome-wide Ψ and RBP-binding profiles with genetic manipulation of PUS proteins to identify Ψ-sensitive binding sites in cells.

6.3 Non-catalytic functions of PUS proteins

Some functions of PUS are independent of their pseudouridine synthase activity. For example, bacterial TruB acts as a tRNA folding chaperone independent of catalysis and this activity is important for bacterial fitness [112]. Depletion of human PUS10 leads to a defect in miRNA precursor processing that can be rescued by catalytic null PUS10 [54]. PUS10 interacts with pri-miRNAs and the microprocessor complex suggesting a direct mechanism for this non-catalytic function in miRNA biogenesis. Depletion of human RPUSD3 results in decreased 16S rRNA levels and a reduction in mitochondrial translation [78,79]. 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 non-catalytic functions of PUS proteins in RNA metabolism.

7. BIOLOGICAL FUNCTIONS OF PSEUDOURIDINE IN STABLE NON-CODING 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[113]). For example, the presence of a single Ψ at position 39 in the anti-codon stem of tRNA-Lys increases the melting temperature by 5°C [114]. The fact that microorganisms lacking various tRNA modifying pseudouridine synthases are temperature sensitive for growth is consistent with the idea that Ψ stabilizes essential tRNA structure. Notably, not all tRNAs are equally sensitive to the lack of Ψ 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 over-expression of a single Pus3 target tRNA, tQ(UUG), was sufficient to rescue growth above 38°C [20]. 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, including charging by amino acyl tRNA synthetases (aaRS), 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 Ψ 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[115]. Unmodified tI(UAU) is charged with reduced efficiency compared to the properly modified anticodon,

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ΨAΨ[116]. The effects of deleting tRNA modifying PUS on charging in cells have been investigated in a few cases with negative results[20]. 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 [117] offer a sensitive and quantitative approach to investigate the effects of PUS depletion on 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 Ψ for tRNA function varies by tRNA in ways that are currently unpredictable. For example, only one 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[20]. 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[118].

Data on translation defects caused by 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[54], and to actually increase protein synthesis in the case of PUS7[25], the latter by a mechanism involving tRNA fragments (tRF) that is distinct from altered decoding by intact tRNAs. Ribosome footprint profiling[119] offers a potentially powerful approach to identify decoding events that are sensitive to tRNA modifications (see for example[120]). 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[54]. Similarly, ribosome profiling of seven PUS KOs in budding yeast revealed codon-specific changes in ribosome occupancy that were not easily explained by the known tRNA targets[121]. Improved ribosome profiling methods that better capture differences in decoding rate[122] may reveal stronger correlations between 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 KO cells.

The evidence that mammalian tRNA pseudouridines may be regulated to be tissue-specific[46] suggests that pseudouridine could contribute to 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 [123]).

7.2 tsRNA

Ψ affects the biogenesis and function of certain tRNA-derived small RNAs that regulate translation (translational control by tsRNAs reviewed in[124,125]). Knocking out PUS7 in human embryonic stem cells reduced the levels of specific 5′ tRNA fragments (tRF) shown to be pseudouridylated by PUS7 in cells[25]. Transduction of synthetic Ψ-containing 5′ tRFs, but not unmodified tRFs, led to a global reduction in protein synthesis[25] by a mechanism

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that may target the integrity of the cap binding complex, as shown previously for larger 5′ tRFs[126]. 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[127,128], which may be due to its conserved role in ribosome biogenesis (reviewed in[58,129]) rather than an absolute requirement for rRNA pseudouridylation. Yeast cells expressing catalytically inactive cbf5-D95A, the ortholog of DKC1, lack rRNA Ψ and are viable [130]. However, ribosomes isolated from these slow-growing mutants show biochemical defects in tRNA binding in vitro and altered translation fidelity in vivo [22]. Mammalian cells deficient in DKC1 likewise show defects in translation initiation and fidelity[21,22]. The mechanisms responsible for specific translation defects is unclear, but may arise from the effects of pseudouridines on RNA conformational dynamics, as demonstrated for one conserved pseudouridylated rRNA domain, helix 69[131–135].

Phenotypes caused by deletion or overexpression of individual H/ACA snoRNAs suggest important cellular functions for individual rRNA Ψ in human cells[136]. 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[137].

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 Ψ on RNA structure and RBP binding, these Ψ are predicted to be important for multiple steps in 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 [138,139]. Thermal melting experiments demonstrated the strengthened stability of Ψ-containing duplexes with U1 snRNA in a variety of 5′ splice site contexts [140,141]. U1 snRNA Ψ might be particularly important to promote interactions with weaker splice sites.

7.4.2 U2 snRNA—Spliceosome assembly and splicing of a pre-mRNA substrate was inhibited when U2 snRNA was lacking Ψ6, Ψ7 and Ψ15 in HeLa nuclear extracts. Chimeric snRNAs lacking individual Ψ gave reduced splicing efficiency, demonstrating that individual U2 snRNA Ψ contribute to pre-mRNA splicing [24].

Although the functions of Ψ in the branch site recognition region (BSRR) of U2 have not been interrogated in human cells, these Ψ were required for spliceosome assembly and splicing of reporters in Xenopus oocytes [23]. Consistent with stimulatory effects of Ψ in the

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BSRR in Xenopus oocytes, human Ψ35 stabilizes the U2 snRNA-pre-mRNA duplex *in vitro* [142]. NMR studies revealed that Ψ35 also altered the structure of this duplex; by changing the orientation of the branch site adenosine on the opposite strand, Ψ promoted bulging, perhaps poising it for more efficient splicing [142] (but see differences dependent on sequence context [143]). In yeast, loss of highly conserved Ψ35, Ψ42 and/or Ψ44 in the BSRR of U2 snRNA inhibited splicing and caused temperature sensitive growth [111]. 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 [111]. These Ψ alter the RNA secondary structure of the branchpoint-interacting stem loop in the U2 snRNA, which might facilitate the binding of Prp5 [111]. Two additional Ψ in human U2 are well-positioned to stabilize helix II of the U6/U2 duplex at the core of the spliceosome [144,145].

7.4.3 U4 snRNA—The functions of constitutive Ψ in human U4 and U6 snRNAs have not been investigated, but their positions suggest likely effects on spliceosome assembly and activation. Two Ψ 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 pre-loaded on U4 [146–148], stem III occludes the Brrr2 loading sequence on human U4 as seen in the human tri-snRNP and pre-B complex cryo-EM structures [149]. Ψ72 and Ψ79 are within the Brr2 helicase loading sequence which facilitates U4/U6 unwinding to allow U6 snRNA [150] to base pair with the pre-mRNA substrate 5′ splice site [144,145]. Therefore, Ψ 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 unwinding of U4. This Ψ 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) [151]. 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 [151]. Within the spliceosome Ψ86 is located at the end of a bulge immediately 5′ to the U2/U6 helix II [144,145], where it could play a stabilizing role.

7.4.5 U5 snRNA—U5 Ψ 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 [152]. Ψ43 and Ψ46 are within Loop I and Ψ53 is within the stem of Loop1, which could enhance internal U5 stem or U5-pre-mRNA pairing. Prp8 stabilizes the interactions between Loop I and the 5′ exon [153]. 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 [154,155]. Given that Ψ rigidify the RNA backbone, these Ψ might be important for Prp8 interactions with U5.

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Ψ 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 Ψ function will be greatly facilitated by identification of the enzymes/RNA guides that direct pseudouridylation at each position of the human snRNAs. Whether human snRNA Ψ affect alternative splicing or splicing efficiency of endogenous cellular pre-mRNAs is an open question. These questions could be answered by deleting the snoRNAs that target individual Ψ in snRNAs followed by RNA-seq and splicing analysis. To investigate the mechanism by which snRNA Ψ affect splicing one could perform psoralen crosslinking followed by snRNA-pull down to quantify snRNA-pre-mRNA or snRNA/RNA interaction in snoRNA deleted compared to wildtype cells. Similarly, the effect of snRNA Ψ on interactions with RNA-binding proteins throughout the splicing cycle could be investigated by profiling of RBP binding (e.g. CLIP-seq) in cells lacking individual snRNA Ψ.

8. POTENTIAL FUNCTIONS OF PSEUDOURIDINE IN mRNA

8.1 Translation Fidelity

The frequent occurrence of pseudouridines within mRNA [17–19,37] 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 read-through in rabbit reticulocyte lysate[27], with similarly efficient read-through observed in E.coli lysate[29], suggesting a conserved mechanism. Pseudouridylated stop codons were also read through at an undetermined efficiency *in vivo* in budding yeast cells[27,156], with different amino acids incorporated depending on the stop codon [27].

The mechanism by which Ψ promotes stop codon readthrough is incompletely understood. Ψ minimally affects peptide release by E. coli release factors in vitro[157,158] suggesting Ψ promotes readthrough by increasing mistranslation by near or non-cognate 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 accommodation of this non-cognate codon:anticodon pair in a manner that was similar to a non-pseudouridylated codon [159]. However, this structure did not illuminate how the chemical structure of Ψ allows for efficient mistranslation of stop codons. Pseudouridylated stop codons appear to be terminated correctly (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 Ψ[160]. To our knowledge, Ψ-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 Ψ, but the results from different experimental systems do not paint a consistent picture. Incorporation of single Ψ into UUU phenylalanine codons caused misincorporation in one reconstituted bacterial translation system[157] and decreased the yield of full-length peptide without producing detectable miscoded peptides in another[161]. Mechanistically, Ψ affected multiple steps in translation in

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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[157,162,163]. Fully pseudouridylated mRNAs transfected into human cells produce some amount of functional protein [30,157,160,164] although various Ψ-dependent mistranslated peptides were detected in one study[157]. The effect of Ψ on the yield of functional protein depends strongly on the specific codons used [157,164]. 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 (RIG-I), and protein kinase R (PKR), which detect foreign nucleic acid [165]. RNA modifications have been thought to provide a mechanism for discerning "self" RNA from non-self RNA, and indeed, incorporating RNA modifications, including pseudouridine, in foreign RNA allows for 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[166].

8.2.1 TLRs—Toll-Like Receptors (TLRs) are membrane-associated proteins which detect various pathogen associated molecular patterns (PAMPS) and subsequently stimulate production of proinflammatory cytokines. The RNA-sensing TLRs, TLR3, TLR7 and TLR8 reside within endosomal membranes. TLR3 recognizes 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 either TLRs and inclusion of Ψ in the RNA suppressed this response (most pronounced for TLR7 and TLR8) [167].

8.2.2 RIG-I—Retinoic Acid Inducible Protein (RIG-I) is a cytosolic innate immune sensor responsible for detecting short stretches of dsRNA or ssRNA with either a 5′-triphosphate or 5′-disphosphate group (a feature common to various RNA viruses). Activation of RIG-I relieves its autoinhibition, releasing its CARD domains to interact with MAVS and set off a signaling cascade that ultimately results in expression of immune factors. Inclusion of Ψ in a 5′-triphosphate capped RNA abolishes activation of RIG-I [168,169], providing another mechanism for pseudouridine-mediated suppression of innate immune activation. Further, the polyU/UC region of the HCV genome is also potent activator of RIG-I and complete replacement of U with Ψ in this RNA fully abrogates downstream IFN-beta induction, despite RIG-I still binding to the modified RNA [31,169], but with reduced affinity[169]. Durbin et al present biochemical evidence that RIG-I bound to pseudouridylated polyU/UC RNA fails to undergo the conformational changes necessary to activate downstream signaling[169].

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8.2.3 PKR—RNA-dependent Protein Kinase (PKR) is a cytosolic resident innate immune sensor. Upon detection of foreign RNA, PKR represses translation through phosphorylation of translation initiation factor eIF-2alpha. Molecules which activate PKR are varied, but include dsRNA formed intra- or inter-molecularly, and 5′ triphosphate groups. Inclusion of Ψ 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 Ψ (~30-fold reduction with Ψ) [170]. Ψ also modestly reduced PKR activity when this short RNA was annealed to a complementary unmodified RNA [170]. Likewise, in vitro transcribed, unmodified tRNA acted as much more potent activator of PKR than tRNAs transcribed with pseudouridine [171]. 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, transfection of an unmodified mRNA caused a greater reduction in overall cellular protein synthesis in cell culture compared to the same mRNA fully pseudouridylated [32]. Consistent with this result, fully pseudouridylated mRNA reduced PKR activation and subsequent phosphorylation of eIF-2alpha [32].

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 stimulation of the innate immune system in *in vitro* models. These findings also hold true *in vivo*. Indeed, while systemic injection of a uridine-containing in vitro transcribed reporter RNA triggered elevated INF-alpha levels in mice, use of fully pseudouridylated reporter dampened this response [30].

It is also important that therapeutic RNAs encoding proteins are 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[30,32,157,160,161]. Pseudouridine likely affects multiple facets of mRNA function, including reduced immune stimulation by several mechanisms[31,32,167–171], prolonged half-life of pseudouridine-containing RNA [30], as well as potentially deleterious effects of Ψ on translation fidelity and efficiency[27,157,160,161].

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[62,77,82,83] and PUS1, PUS7 and TRUB1 are known to pseudouridylate mRNAs[62,67]. Furthermore, PUS7 stably associates with chromatin and associates with DNA at active Pol II promoters and enhancers[82]. The nuclear localization and/or chromatin association of these PUS raise the possibility that they might act on nascent pre-mRNA. Given the many molecular effects of Ψ on RNA-protein and RNA-RNA interactions, if Ψ are indeed deposited in pre-mRNA they could function in splicing at multiple levels. The potential for Ψ to impact pre-mRNA splicing was shown: artificial pseudouridylation in the polypyrimidine tract of an adenoviral pre-mRNA substrate

inhibited binding of the 3′ splice site recognition factor U2AF2 and abolished splicing of the intron[14]. For a detailed discussion of the likely effects of Ψ and other RNA modifications on splicing see a recent review[172].

9. CONCLUSIONS AND PERSPECTIVES

Pseudouridine (Ψ) is the most abundant modified nucleoside in nature. Recent advances in Ψ detection have revealed a rich and dynamic landscape that includes regulated Ψ in tRNA, rRNA and snRNAs, where they were long known to occur, and identified many previously unknown Ψ in mRNA and additional non-coding RNAs. Despite intensive investigation of the structural and biochemical effects of Ψ in various systems, the biological roles of most endogenous Ψ remain unknown. With current technology[54], some important questions should soon be answered such as identifying the pseudouridine synthases (PUS) responsible for all sites of Ψ in human tRNA and snRNA. Other recent technical achievements establish promising approaches to elucidate the RNA sequence and structural features recognized by PUS to enable site-specific pseudouridylation[62,67]. The more challenging questions all relate to Ψ function in the context of cellular RNA metabolism. Why are some tRNAs more dependent on Ψ than others? How do individual Ψ in snRNAs affect the accuracy, efficiency and regulation of splicing? How does the ribosome decode Ψ (and why are the effects of Ψ on translation so context-dependent?) How do Ψ-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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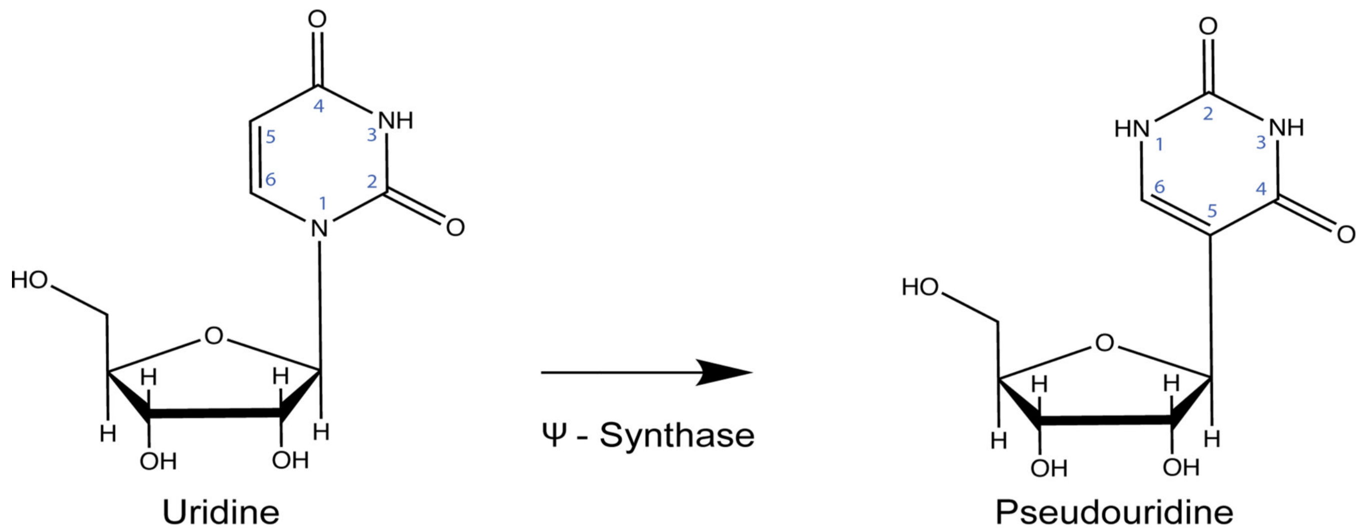

Figure 1. Chemical structures of uridine and pseudouridine

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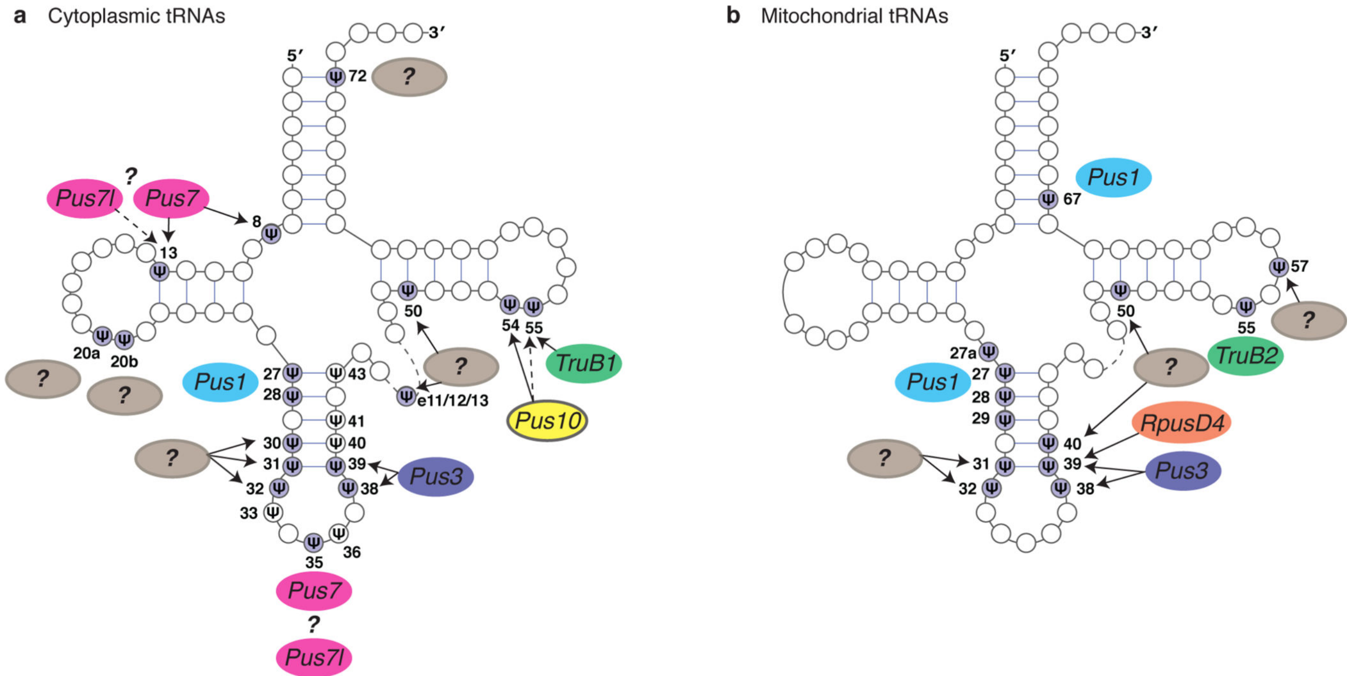

Figure 2. tRNA pseudouridylation.

Biochemically identified Ψ sites in cytoplasmic (A) and mitochondrial (B) 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.

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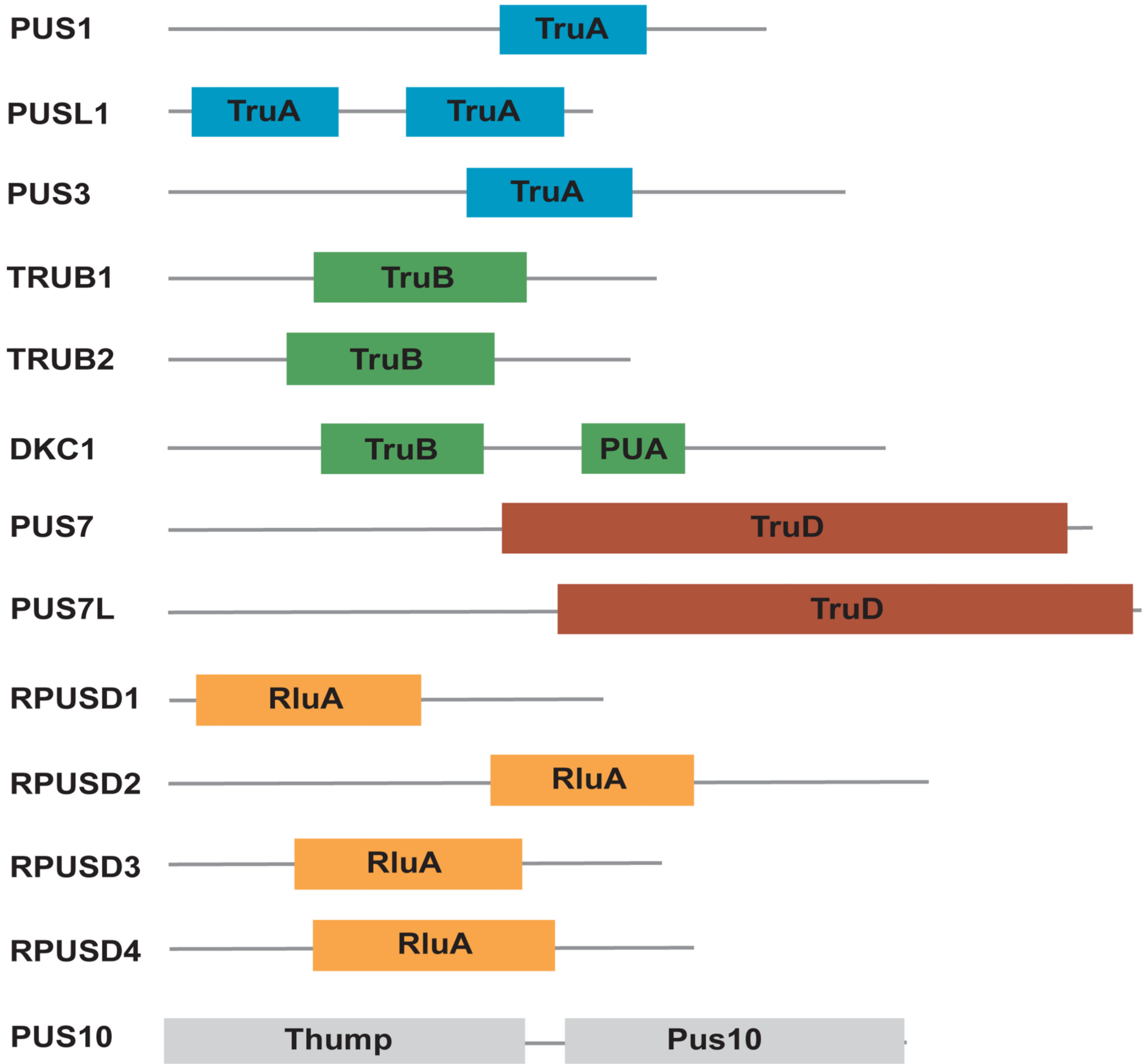

Figure 3. Pseudouridine is catalyzed by human PUS.

Domain schematic of human PUS grouped and color coded by family.

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                                                   *
SP|Q9UJJ7|RUSD1   48  FPELADPDTCYGFRFCHQLDFSTSGALCVALNKAAAGSAYRCF
SP|Q8IZ73|RUSD2  258  ---LGKEHQLKELHPLHRLDRLTSGVLMFAKTAAVSERIHEQV
SP|Q6P087|RUSD3  117  LSQ-SLGLREQELQVVRASGKESSGLVLLSSCPQTASRLQKYF
SP|Q96CM3|RUSD4  134  LAKMLHGHKAEPLHLCHRLDKETTGVMVLAWDKDMAHQVQELF
SP|P0AA37|RLUA_   54  YP---------QAESVHRLDMATSGVIVVALTKAAERELKRQF
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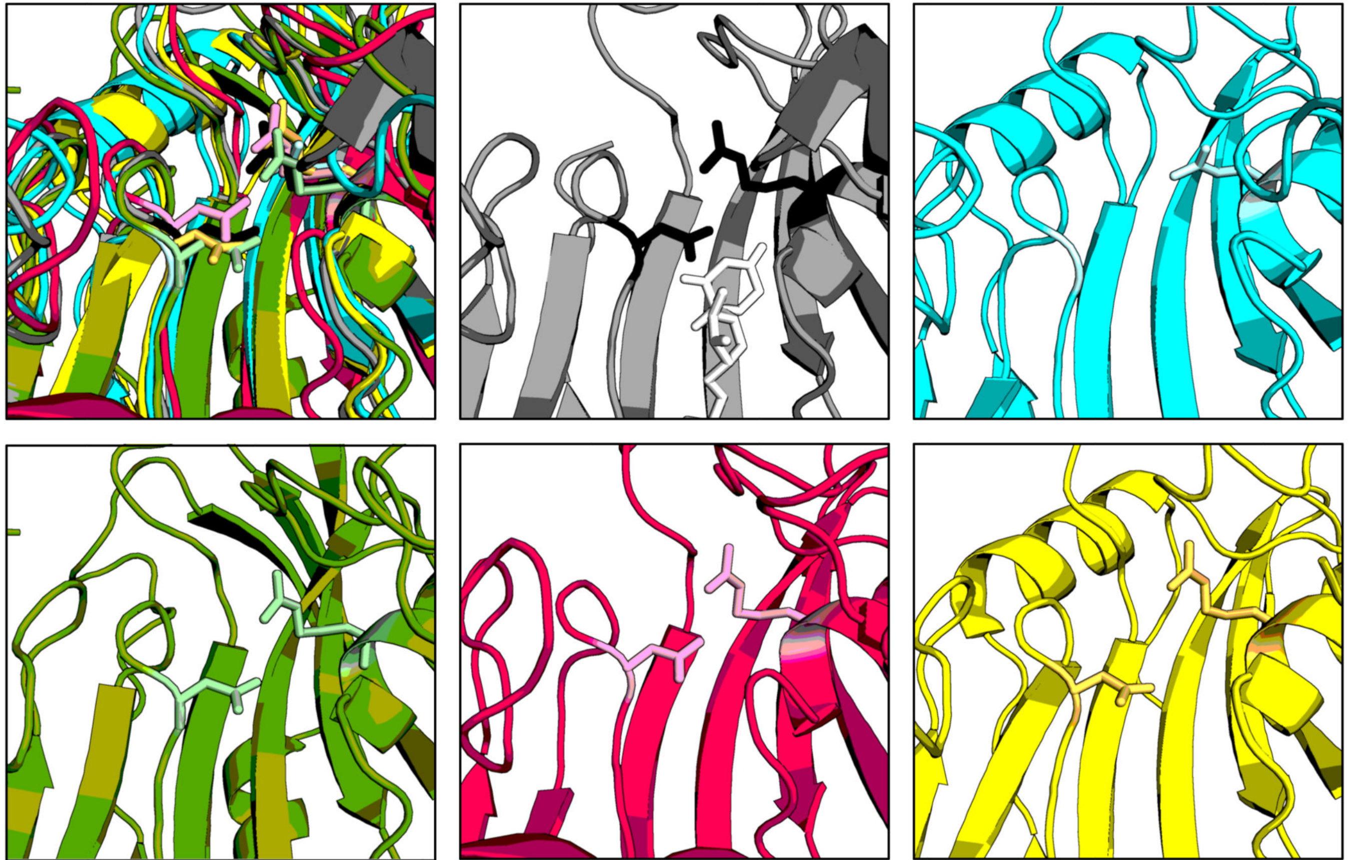

Figure 4. RPUSD3 lacks a catalytic aspartate:

(A) Multiple sequence alignment of human RluA family members and *E. 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 (B, PDB 2I82), human RPUSD3 (C, 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 SwissModel. The multiple sequence alignment was generated using ClustalOmega and formatted using BoxShade.

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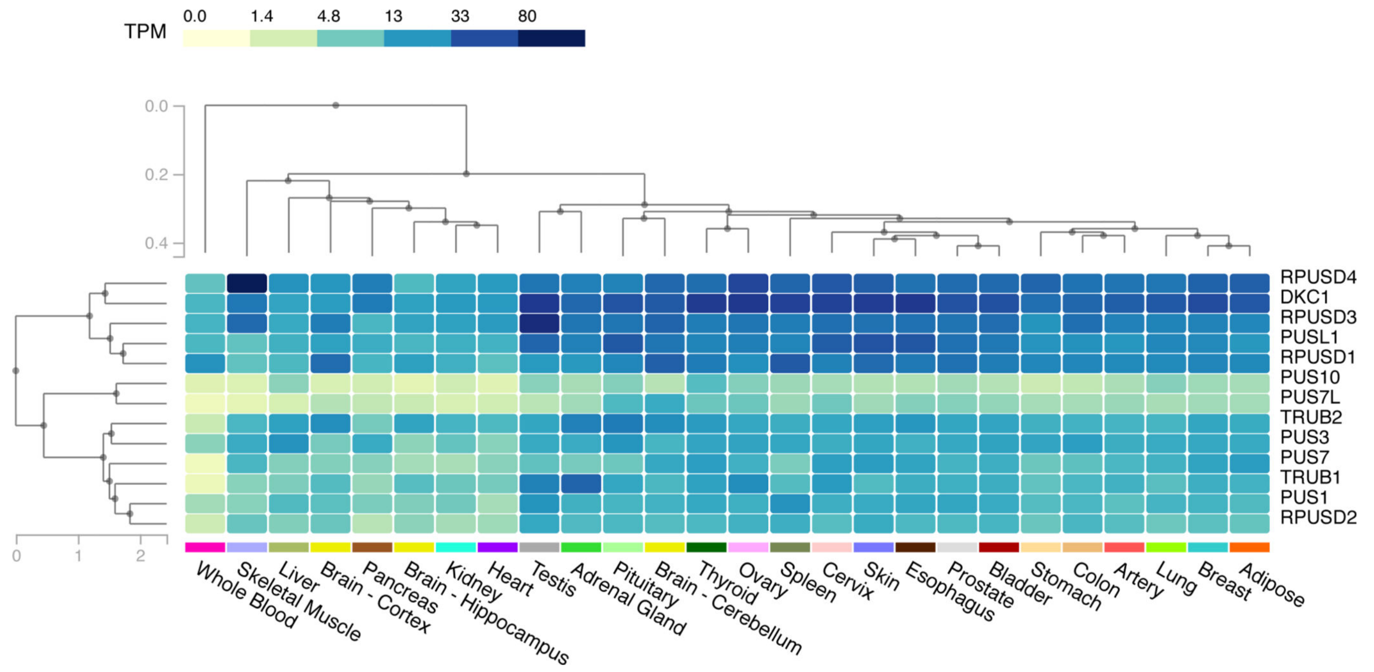

Figure 5. Expression of human PUS across human tissues.

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

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Table 1.

Locations and targeting of Ψ in human snRNAs by Box H/ACA snoRNAs.

Position of Ψ in human major and minor snRNAs. Ψ Synthase Box H/ACA snoRNA predicted or experimentally validated (bold) to target Interaction snoRNA/mRNA – experimental evidence for interaction between target snRNA and the predicted snoRNA guide. References are specific to the table as follows: [1] Reddy and Busch 1988, [2] Gong et al. 2017, [3] Kiss et. Al. 2004, [4] Gu et al. 2005, [5] Vitali et al. 2003, [6] Shattner et al. 2006, [7] Darzacq et al. 2002, [8] Deryusheva et al. 2017, [9] Kiss et al. 2002, [10] Deryusheva et al 2012, [11] Zerby et al. 1997, [12] Jorjani et al. 2016, [13] Yamauchi et al. 2016, [14] Jady et al. 2001, [15] Massenet et al. 1999, [16] Zerby et. Al. 1996

Human	Position	Ψ Synthase	MN sensitivity	Interaction snoRNA/snRNA	References
U1	Ψ5	SCARNA16		Yes	1, 2, 3
	Ψ6	SCARNA18		Yes	2, 4
U2	Ψ6				1
	Ψ7	SCARNA14			1, 2, 5, 6
	Ψ15				1
	Ψ34	SCARNA8		Yes	1, 2, 7
	Ψ37	SCARNA15		Yes	1, 2, 3
	Ψ39	SCARNA4		Yes	1, 2, 3
	Ψ41	SCARNA4		Yes	1, 2, 3
	Ψ43	SCARNA8		Yes	1, 2, 8
	Ψ44	SCARNA8		Yes	1, 7
	Ψ54	SCARNA13		Yes	1, 3
	Ψ60				10
	Ψ88				1
	Ψ89	SCARNA1		Yes	1, 2, 3
	Ψ91				1
U4	Ψ4		Yes		1, 11
	Ψ72		Yes		1, 11
	Ψ79	SCARNA26	Yes	Yes	1, 2, 11, 12
U5	Ψ11				13
	Ψ43	SCARNA11	Yes	Yes	1, 2, 14, 3
	Ψ46	**SCARNA10** SCARNA12	Yes	Yes	1, 2, 14, 7
	Ψ53	SCARNA13	No	Yes	1, 2, 14, 6
U6	Ψ9				13
	Ψ31	SNORA79	Yes	Yes	1, 2, 16, 6
	Ψ40	SCARNA23	Yes	Yes	1, 2, 16, 3
	Ψ86	SNORA79	Yes	Yes	1, 2, 16, 6

Human	Position	Ψ Synthase	MN sensitivity	Interaction snoRNA/snRNA	References
U12	Ψ19	SCARNA21		Yes	15, 6
	Ψ28	SCARNA20		Yes	15, 6
U4atac	Ψ12	SCARNA21			15
U6atac	Ψ83				15

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