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Current Opinion in Chemical Biology

Pseudouridine: the fifth RNA nucleotide with renewed interests

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More than 100 different types of chemical modifications to RNA have been documented so far. Historically, most of these modifications were found in rRNA, tRNA and snRNA; recently, new methods aided by high-throughput sequencing have enabled identification of chemical modifications to mRNA, leading to the emerging field of 'RNA epigenetics'. One such example is pseudouridine, which has long been known as a RNA modification in abundant non-coding RNA (ncRNA) and has recently been found to be present in mRNAas well. This review first summarizes biogenesis and known functions of pseudouridine in ncRNAs. We then focus on progress in pseudouridine detection, especially the chemical-assisted, transcriptome-wide sequencing tools that revealed the dynamic nature of mRNA pseudouridylation. Such enabling tools are expected to facilitate future functional studies of pseudouridine.

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Current Opinion in Chemical Biology 2016, **33**:108–116

This review comes from a themed issue on **Chemical genetics and epigenetics**

Edited by **Danica G Fujimori** and **Stuart Conway**

For a complete overview see the Issue and the Editorial

Available online 24th June 2016

http://dx.doi.org/10.1016/j.cbpa.2016.06.014

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Chemical modifications of RNA

Modifications of DNA and proteins have been intensively explored, such as 5-methylcytosine in DNA and histone modifications that play regulatory roles in gene expression. In RNA, more than 100 types of chemical modifications have been found (http://mods.rna.albany.edu/mods/). While most of these modifications were found in abundant non-coding tRNA and rRNA, for a long time only a few chemical modifications were known to be present in eukaryotic mRNA: the cap-related 7-methylguanosine and 2′-O-methylation, and N^6-methyladenosine (m^6A), 5-methylcytosine and inosine which are internal to mRNA. Understanding the mechanism and regulation of these mRNA modifications (first termed 'RNA epigenetics' in 2012) will greatly expand our knowledge about RNA biology [1]. Recently, with the help of (bio)chemistry-assisted high-throughput sequencing tools, pseudouridine (ψ) and N^1-methyladenosine were found to be present in mRNA as well [2••,3••,4••,5••,6•,7•] (Figure 1), further expanding the alphabet of RNA epigenetics. While the biogenesis and functions of ψ in non-coding RNAs (ncRNA) have been extensively reviewed previously [8–11], in this review we focus on the recently discovered mRNA pseudouridylation events. In particular, we emphasize recently developed high-throughput sequencing tools that have revealed the dynamic and widespread nature of mRNA pseudouridylation.

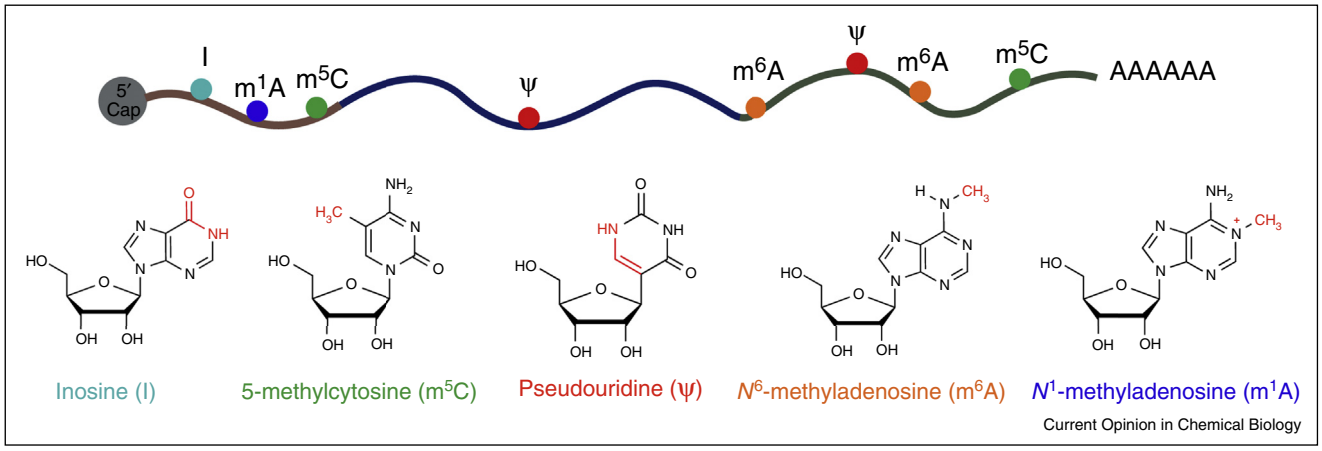
Pseudouridine: the first RNA modification discovered

Pseudouridine, also known as the 'fifth nucleotide' in RNA, was first discovered in 1951 and is overall the most abundant RNA modification (with an estimated ψ/U ratio of 7–9%) [12]. It has been known for decades that ψ is present in tRNA, rRNA and snRNAs [10]. In rRNA, Ψ is important for rRNA folding and for the control of translational fidelity [13–15]. The additional H-bond donor of Ψ (when compared to U) can enhance base stacking and thus influence rRNA folding [13,16]. Ψ is found to be clustered in functionally important regions of rRNA, including peptidyl transferase center region, an intersubunit bridge (helix 69), 'A-site finger' and the decoding center [17–20]. A lack of Ψs in any of these regions could impair translation and has been shown to lead to reduced growth rate in yeast. In tRNAs, Ψ38, Ψ39 and Ψ55 can stabilize the tRNA structure [21]. Ψ in the anticodon of tRNA could strengthen codon–anticodon base-pairing and affect translation rate and accuracy [22,23]. In snRNAs, Ψ is distributed in the conserved and functionally important regions. Ψs in U2 snRNA have effects in snRNP biogenesis and pre-mRNA splicing both in a mammalian cell free system and a *Xenopus oocyte* reconstitution system [24–28]. In summary, ψ plays essential roles in the biological functions of these ncRNAs.

Pseudouridine synthases

ψ is generated from isomerization of uridine, catalyzed by pseudouridine synthases (PUS) (Figure 2) [29]. Thirteen

Figure 1



Several chemical modifications are known to be present in eukaryotic mRNA, including the cap-related 7-methylguanosine and 2′-O-methylation, and internal modifications including N^6-methyladenosine (m^6A), 5-methylcytosine (m^5C), inosine (I), pseudouridine (Ψ) and N^1-methyladenosine (m^1A).

pseudouridine synthases exist in human cells. These can be categorized into two classes, namely RNA-dependent and RNA-independent PUSs. These PUSs and their RNA substrates are listed in Table 1. Most rRNA and snRNA pseudouridylation is catalyzed through the RNA-dependent mechanism, by the so-called box H/ACA ribonucleoproteins. Box H/ACA RNA can fold into a conserved hairpin–hinge–hairpin–tail structure and contain two loops complementary to the sequence of substrate RNA. In box H/ACA ribonucleoproteins, DKC1 (or Cbf5 in yeast) is the pseudouridine synthase that performs the isomerization of uridine. In the RNA-independent mechanism, pseudouridylation is carried out through a single PUS protein. Although sequence similarity between different PUSs is low, a similar fold consisting of an eight-stranded mixed β-sheet is shared by these PUSs, with a conserved active-site cleft flanked by several helices and loops (Figure 2) [30].

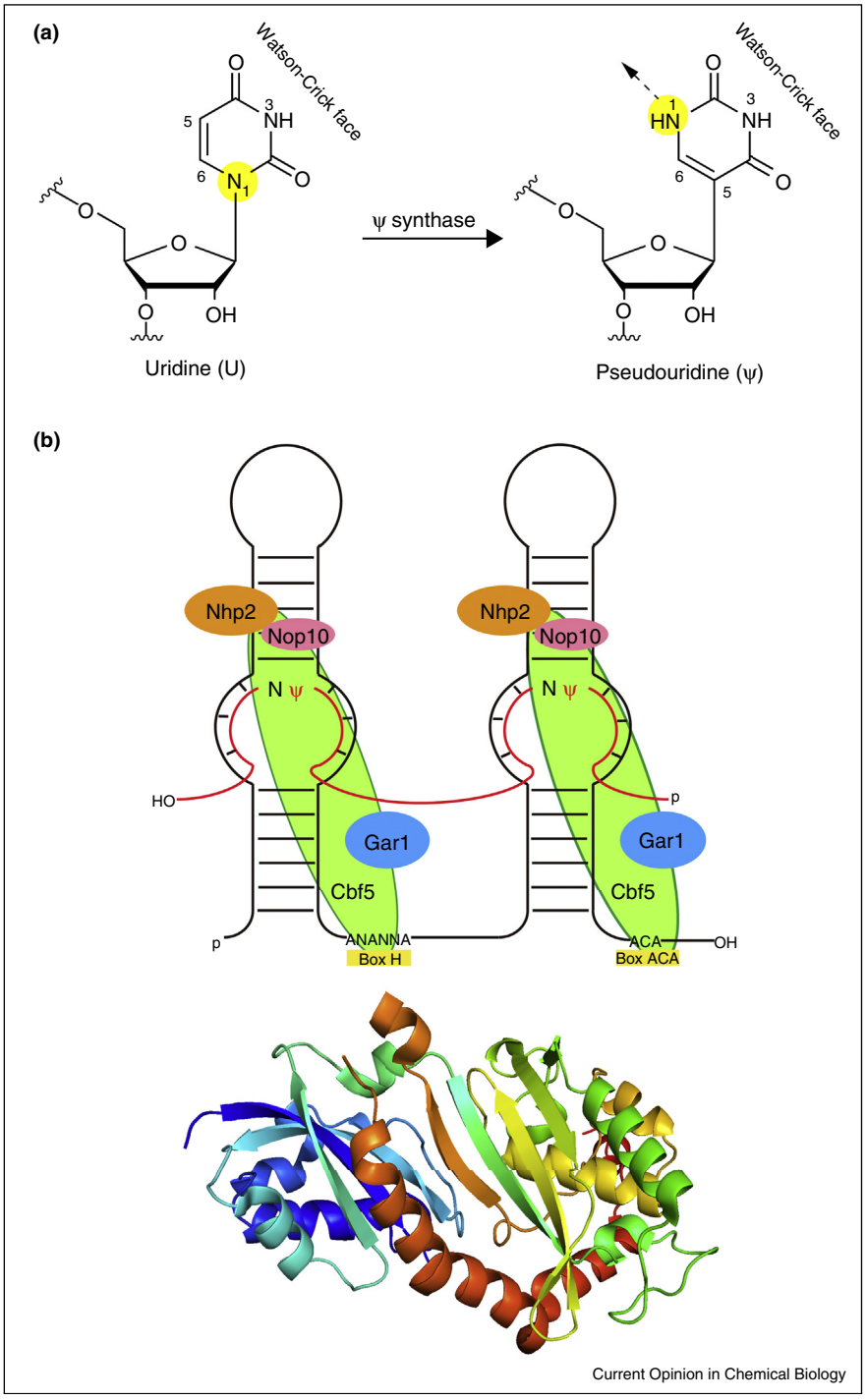
Defects in PUSs can lead to several diseases. When DKC1 is mutated, it leads to the multisystem disorder X-linked dyskeratosis congenita (X-DC) [31]. DKC1 mutations cause defects in rRNA pseudouridylation [32]; however, how defective ribosomes could lead to disease and cancer was unknown at the time of its initial discovery. Using an unbiased proteomics approach, the Ruggero lab discovered a specific defect in IRES (internal ribosome entry site)-dependent translation of mRNAs in the X-DC patients. Such a defect was found to cause impaired translation of IRES-containing mRNAs, some of which encode important anti-apoptotic factors and tumor suppressors [15,33,34]. Following this elegant study, Ruggero, Dinman and coworkers further revealed that DKC1 mutations decreased the rRNA binding affinity to IRES, thus impairing the IRES-dependent translation initiation. They also showed that tRNA exhibits decreased binding affinity to A and P sites of the impaired ribosome, which reduced the translation fidelity in both yeast and human cells [15]. In addition to X-DC, myopathy, lactic acidosis and sideroblastic anaemia (MLASA) is associated with a missense mutation (R116W) or a stop mutation (E220X) in PUS1 [35,36]. The missense mutation in PUS1 causes the absence of pseudouridine at PUS1 targeted sites in mitochondrial and cytoplasmic tRNAs, while the mutation does not appear to affect the distribution of PUS1 inside of the cell [37].

Site-specific detection methods for ψ

Functional investigations of ψ have been significantly facilitated by site-specific detection methods. The most frequently used method is to react RNA with cyclohexyl-N'-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMCT). CMCT can react with guanosine-like and uridine-like nucleotides, while an alkaline treatment (pH = 10.4) step hydrolyzes CMC adduct to G and U, leaving only CMC-ψ mono-adduct [38]. This reaction was first used in 1993 by the Ofengand group in a primer-extension assay to detect ψ sites in rRNA [39]. As the N3 position is on the Watson-Crick face, the reverse transcription will terminate one nucleotide 3′ to the CMC-ψ adduct, resulting in truncated cDNA. This property was used to achieve a single-base resolution detection of ψ in rRNA.

Becuase CMCT-based detection methods may not provide accurate modification fraction information due to the nature of the incomplete CMCT labeling reaction, alternative methods including the enzymatic ligation-based method or RNase H-based methods were developed [40–42]. In a more recently developed method, *s*ite-specific

Figure 2



Ψ is catalyzed by pseudouridine synthases (PUSs). **(a)** Ψ is the C-glycoside isomer of uridine and generated by pseudouridine synthases; **(b)** pseudouridine synthases in human are categorized into two classes, namely RNA-dependent and RNA-independent PUSs. Most rRNA and snRNA pseudouridines are catalyzed through the RNA-dependent mechanism, or box H/ACA ribonucleoproteins.

*c*leavage *a*nd *r*adioactive-labeling followed by *l*igation-assisted *e*xtraction and *t*hin-layer chromatography (SCARLET) was used to determine the precise location and modification fraction of RNA modification [41]. Using 2′-O-methyl RNA-DNA chimera probes, substrate RNA can be cleaved at the 5′ side of the target nucleotide. The cleaved RNA is then radio-labeled with [γ-^{32}P] ATP, further purified and digested with nuclease P1. The

Table 1

Thirteen pseudouridine synthases in human cells and their RNA substrates

Synthase in human	Putative RNA substrates
PUS1	tRNA, mRNA
PUS3	tRNA
PUS7	rRNA, mRNA
PUS10	tRNA
PUSL1	tRNA
PUS7L	rRNA
TRUB1	tRNA, mRNA
TRUB2	tRNA
RPUSD1	tRNA
RPUSD2	tRNA
RPUSD3	tRNA
RPUSD4	tRNA
DKC1	rRNA, snRNA, mRNA

digested nucleotides are finally resolved by TLC. Using SCARLET, three novel ψ sites in rRNA and one ψ site in EEF1A1 mRNA were demonstrated. The ψ modification level of U519 in EEF1A1 mRNA was determined to be ~56% [5••].

Mass spectrometry-based detection methods for ψ

Pseudouridine is the only known mass-silent modification, which means that it does not exhibit a mass shift compared with uridine. Despite this, pseudouridine can be chemically derivatized with CMCT or acrylonitrile, resulting in mass differences of 252 Da (CMCT-treated) or 53 Da (acrylonitrile) compared with untreated samples. These methods have allowed the detection of Ψ using mass spectrometry [43,44]. In a typical experiment, base-specific endoribonucleases were coupled to chemical derivatization to generate a mixture of oligoribonucleotides, which are amenable to accurate mass measurements by ESI or MALDI (matrix-assisted laser desorption/ionization) mass spectrometry. While these methods only partially digest the RNA molecules of interest, an SRM-based assay (*via* LC–MS/MS) utilizes fully digested nucleosides and the pseudouridine-specific fragmentation pattern to determine the degree of pseudouridylation [45].

Transcriptome-wide mapping of ψ

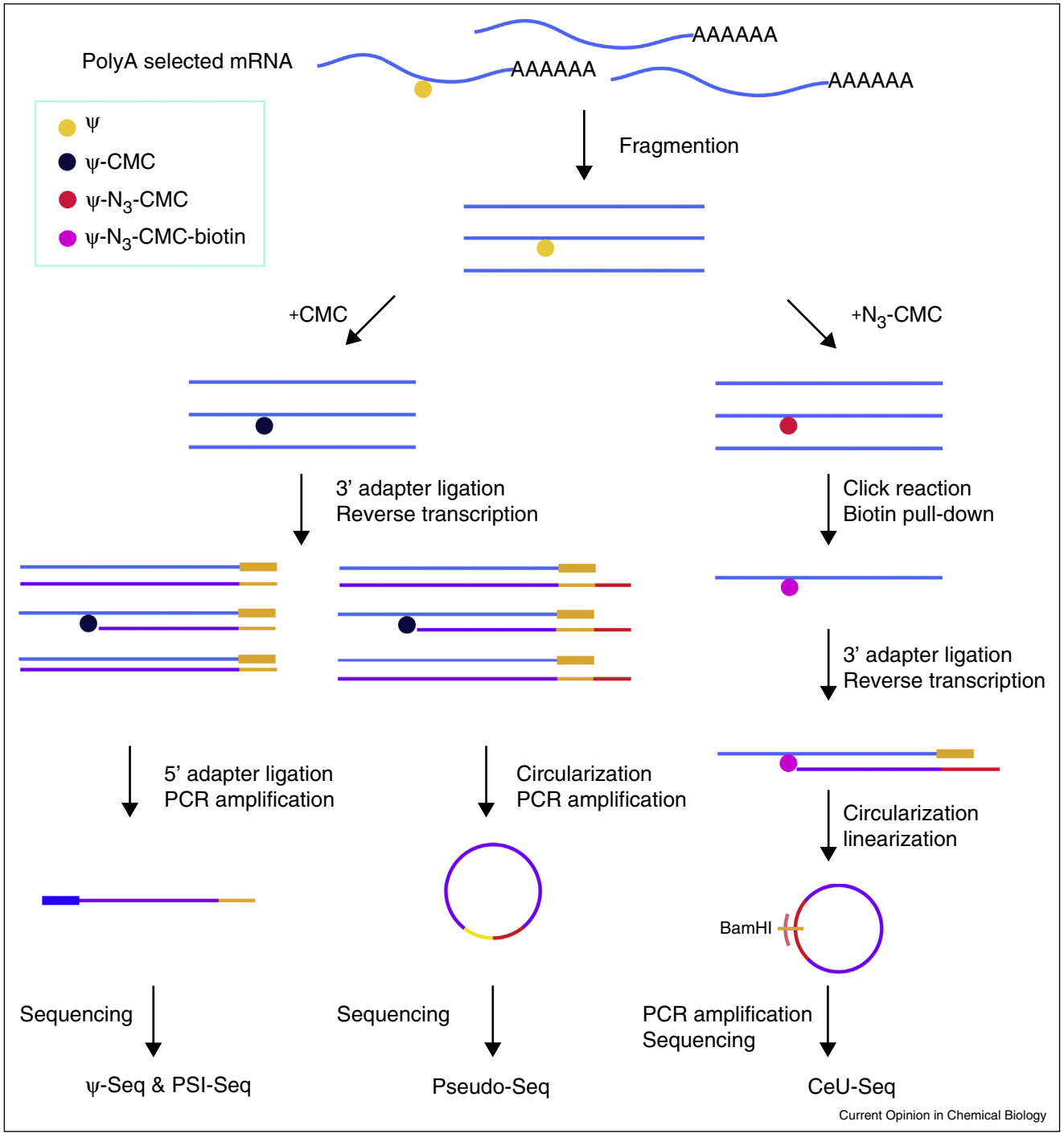
Coupling CMCT labeling and next generation sequencing, several transcriptome-wide sequencing methods have been developed and applied to yeast and mammalian samples (Figure 3) [2••,3••,4••,5••]. In Ψ-Seq, Pseudo-Seq and PSI-Seq, mRNAs were reacted directly with CMCT [2••,3••,4••]; CMC-Ψ adducts generate truncated cDNA during reverse transcription and these RT stops were utilized in next-generation sequencing for Ψ detection. In CeU-Seq (a homophone for 'see U seq'), an azido-modifed CMCT derivative was chemically synthesized and utilized to pre-enrich Ψ-containing RNAs *via* biotin pull-down [5••]. In CeU-Seq, RNA samples were first allowed to react with N_3-CMCT; the N_3-CMC-Ψ adduct was then coupled to biotin *via* click chemistry. Biotin pull-down results in the enrichment of Ψ-containing RNA fragments, which were then reverse transcribed and sequenced.

About 50–100 pseudouridine residues in yeast mRNA and ~100–400 sites in human mRNA were identified by Ψ-Seq, Pseudo-Seq and PSI-Seq; CeU-Seq identified more than 2,000 Ψ sites in human mRNA and ~1500–1700 Ψ sites in mouse tissue, which is more consistent with its 0.2–0.4% ψ/U ratio [2••,3••,4••,5••]. When using very similar sequencing depth (by randomly selecting similar numbers of sequencing reads from data GSE63655) and the same strict Ψ-calling criteria, CeU-Seq still identified over 1000 Ψ sites in the human transcriptome. Presumably the pre-enrichment feature of CeU-Seq enhanced the signal-to-noise ratio of the sequencing data. However, because of the pre-enrichment step, CeU-Seq cannot be used to quantify the modification level of Ψ sites of interests. Additionally, all the four methods appear to require relatively large amount of starting materials (5–10 μg mRNA) at their present stage, due to RNA degradation during the alkaline treatment step (pH ~10.4). This has limited the potential of pseudouridine sequencing to samples of limited amount (e.g. clinically related samples). Screening of new chemical compounds that could react with Ψ under milder conditions or preparation of specific IP-grade antibody could aid the development of future sequencing methods.

Inducible pseudouridylation under stress conditions

Besides the constitutive Ψ sites, Ψ can be induced by stress conditions. The Yu lab reported that nutrient deprivation can lead to novel pseudouridylation at positions 56 and 93 in *Saccharomyces cerevisiae* (*S. cerevisiae*) U2 snRNA, which has three constitutive Ψ sites at positions 35, 42 and 44. In addition to being induced by nutrient deprivation, Ψ56 can also be induced by heat shock [46]. Ψ56 and Ψ93 was found to be catalyzed by Pus7p or snR81 RNP, respectively [46]. The imperfect base pairing between U2 snRNA and snR81 is necessary for the inducible Ψ93; interestingly, when the sequence of U2 snRNA or snR81 was mutated to allow perfect base pairing, Ψ93 became constitutive [46]. More importantly, the induced Ψ93 reduced pre-mRNA splicing, indicating functional relevance of inducible pseudouridylation [46]. In another remarkable demonstration, the Query lab found that during filamentous growth of *S. cerevisiae*, Pus1p was upregulated and Ψ28 in U6 snRNA was inducibly catalyzed by Pus1p [47•]. Mutations to specific sites in U6 snRNA can lead to robust pseudouridylation at U28 and concomitant pseudohyphal growth phenotype. Moreover, site-specifically pseudouridylated U28 by modified snR81 RNP (which targets U28) can also activate pseudohyphal growth. Furthermore, utilizing the *ACT1-CUP1* reporter

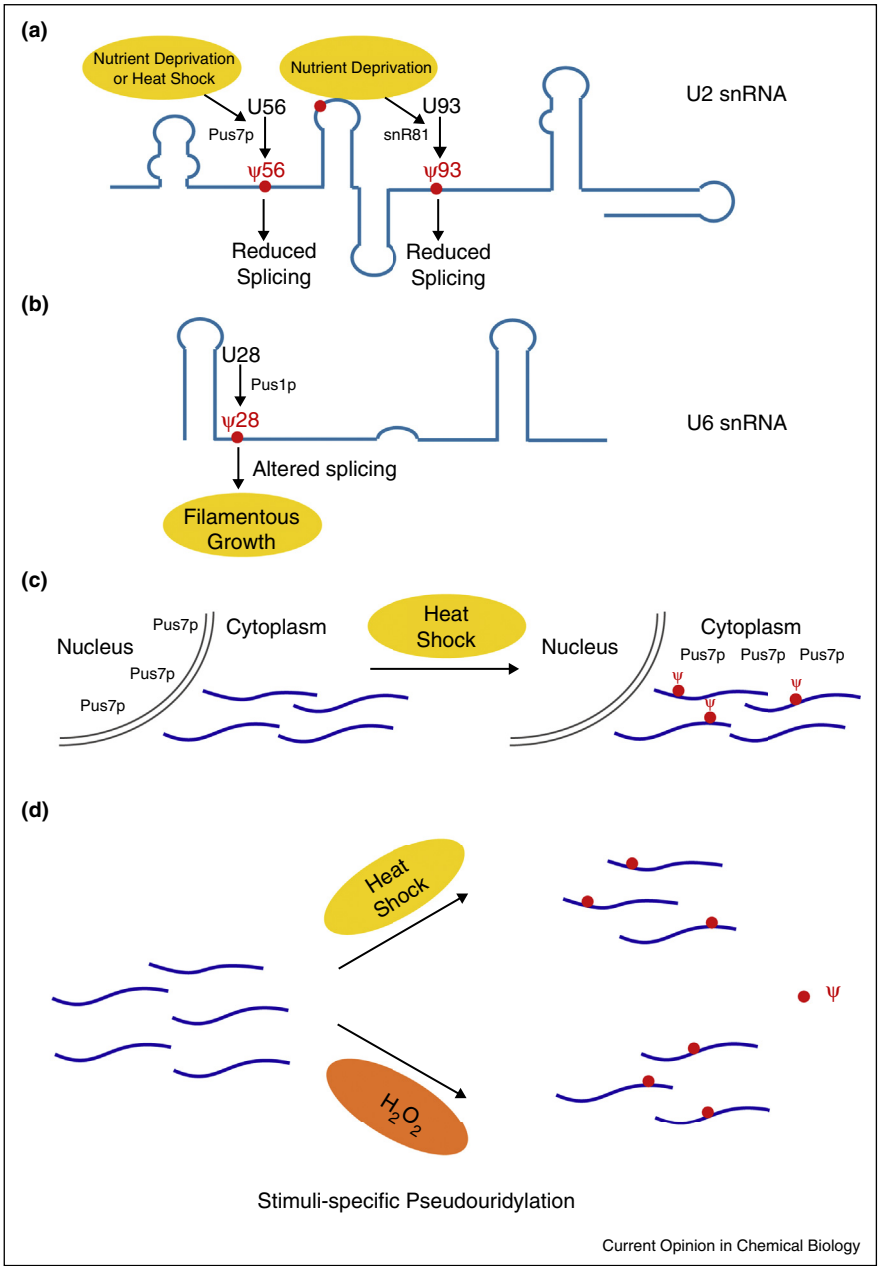
Figure 3



Transcriptome-wide mapping of Ψ in yeast and human. The flowchart of various transcriptome-wide Ψ sequencing methods, including Ψ-Seq, Pseudo-Seq, PSI-Seq and CeU-Seq, are shown. In Ψ-Seq, Pseudo-Seq and PSI-Seq, mRNAs were reacted directly with CMCT; in CeU-Seq, mRNAs were reacted with an azido-modified CMCT derivative, thus allowing pre-enrichment of Ψ-containing RNAs for sequencing. All these methods combine CMCT labeling and next generation sequencing to detect Ψ in the transcriptome at single base resolution.

system, the authors found that U6-Ψ28 could alter spliceosome function. Thus, the inducible U6-Ψ28 is also functionally important and can lead to the filamentous growth process (Figure 4) [47•]. In summary, these interesting results suggest that inducible Ψ can play important roles in gene regulation and cell physiology.

Figure 4



Inducible pseudouridylation under stress conditions. **(a)** Ψ56 in U2 snRNA in *Saccharomyces cerevisiae* can be induced by both nutrient deprivation and heat shock under the action of Pus7p. Ψ93 can be induced by nutrient deprivation under the action of snR81; **(b)** Ψ28 in U6 snRNA was inducibly catalyzed by Pus1p, leading to the filamentous growth process of *S. cerevisiae*; **(c)** upon heat shock, Pus7p was re-localized from nucleus to cytoplasm and the majority of inducible Ψ sites were found to be Pus7p-dependent; **(d)** in mammalian cells, inducible pseudouridylation in the transcriptome displays a stress-specific pattern.

Recently, *via* mapping pseudouridylation in the yeast and mammalian transcriptome, pseudouridylation in mRNA is also shown to be dynamically regulated upon stress conditions [2••,3••,5••]. In *S. cerevisiae* upon heat shock, 265 Ψ sites were induced and majority of them were found to be Pus7p dependent. Interestingly, the levels of Pus7p mRNA and protein were reduced in this process. It was found that Pus7p re-localized from nucleus to cytoplasm; thus the localization change of Pus7p may account for the induced Ψ sites (Figure 4) [2••]. In addition, the induced, Pus7-depedented pseudouridylated mRNAs appeared to be more stable, indicating a potential role of Ψ in mRNA stability [2••]. In mammalian cells, pseudouridylation in the transcriptome can also be dynamically

regulated and displays a stress-specific pattern [5••]. Both heat shock and H_2O_2 conditions were found to induce a similar increased pseudouridylation level (~1.5 fold), yet comparisons of the induced Ψ sites under the two stimuli revealed that the inducible Ψ sites displayed strong stimuli-specific patterns and were essentially non-overlapping [5••]. All of these observations indicate that Ψs in mRNA may have regulatory roles; the exact biological consequences of inducible pseudouridylation remain to be investigated.

Pseudouridine can influence mRNA translation *in vitro*

Via in vitro transcription, all the regular Us in a mRNA can be substituted with Ψs. When incubating the pseudouridylated mRNA with different *in vitro* translation systems, it was shown that Ψ could have different effects on mRNA translation [48]. In the rabbit reticulocyte system, the replacement of U with Ψ stimulated translation; in the wheat germ system, Ψ slightly repressed translation while in the *Escherichia coli* system Ψ almost blocked translation [48]. Recently, Ψ was site-specifically introduced into mRNA and translated in another *in vitro E. coli* system. This recent study showed that at each position of codon (UUU), Ψ can all repress translation although to different extent [49•]. In addition to Ψs within sense codons, the Yu lab found that Ψ could convert a nonsense codon into a sense codon, both in an *in vitro* nonsense suppression assay and in an *in vivo* targeted pseudouridylation system (by the newly derived H/ACA) [50]. Additional LC–MS/MS analysis showed that ΨAA and ΨAG directed serine and threonine incorporation while ΨGA directed tyrosine and phenylalanine incorporation (Figure 4) [50]. Following on from this work, the crystal structure of a ribosome complex with the anticodon stem loop of tRNA and a ΨAG containing mRNA showed non-canonical base pairing interactions between the anticodon and the ΨAG codon, indicating that the expansion of the genetic code is possible [51]. As Ψs have been shown to be abundantly present in CDS, whether Ψs can influence translation rate and accuracy *in vivo* is also worth future investigation.

Concluding remarks

Ψ is widely distributed in many types of ncRNAs; newly developed sequencing methods have also revealed its presence in mRNA and long non-coding RNA. However, many questions remain unanswered, even sixty years after its initial discovery. Firstly, 13 pseudouridine synthases are present in human cells; however, the substrate specificity, localization and biological functions of many synthases are poorly studied at present. CLIP or PAR-CLIP experiments of these PUSs and the existing ψ sequencing methods will, for sure, help us understand more about their biological roles and the mechanisms of related disease (if any). Secondly, although the existing ψ sequencing methods have opened many possibilities for ψ-related RNA research, for clinical samples or other samples which are difficult to obtain, more sensitive and milder methods are needed. Thirdly, the biological significance of many ψ sites remains unknown at this moment. Recent studies reveal that the m^6A modification is reversible, with direct and indirect reader proteins to recognize it for gene regulation. m^6A participates in many biological processes and developmental stages including yeast meiosis and circadian clock function [52–57]. As mRNA Ψ modifications are dynamic, it is tempting to speculate that Ψ may also participate in gene regulation. For instance, Ψ may affect mRNA secondary structures (by enhancing base pairing with adenine), leading to altered binding of RNA binding proteins. It is worth noting that during the revision of this review, the Yu group report that Ψs in U2 snRNA contribute to pre-mRNA splicing by altering the binding/ATPase activity of Prp5, representing the first example of Ψ directly influencing protein binding [58•]. Additionally, the presence of Ψ could alter the dynamics of translating ribosomes, as has been recently shown for m^6A [59]. Whether or not Ψ could affect the stability of mRNA is also open for future investigation; since under heat shock conditions an association of pseudouridylation and stability of mRNA transcripts has been reported [2••]. Although many questions remain, recent findings have attracted increasingly more attention to this long known modification; and we expect that future research on Ψ can further enhance our understanding of the emerging field of 'RNA epigenetics'.

Acknowledgements

The authors apologize to colleagues whose work was not cited owing to space limitation. This work was supported by the National Basic Research Foundation of China (no. 2014CB964900) and the National Natural Science Foundation of China (nos. 21472009 and 21522201).

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