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Exploring pseudouridylation: dysregulation in disease and therapeutic potential

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Pseudouridine (Ψ), the most abundant RNA modification, plays a role in pre-mRNA splicing, RNA stability, protein translation efficiency, and cellular responses to environmental stress. Dysregulation of pseudouridylation is linked to human diseases. This review explores recent insights into the role of RNA pseudouridylation alterations in human disorders and the therapeutic potential of Ψ. We discuss the impact of the reduction of Ψ levels in ribosomal, messenger, and transfer RNA in RNA processing, protein translation, and consequently its role in neurodevelopmental diseases and cancer. Furthermore, we review the success of N1-methyl-Ψ messenger RNA vaccines against COVID-19 and the development of RNA-guided pseudouridylation enzymes for treating genetic diseases caused by premature stop codons.

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Introduction

Pseudouridine (Ψ) is the most prevalent RNA modification found in most types of RNA molecules, including ribosomal RNA (rRNA), messenger RNA (mRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and long noncoding RNA (ncRNA), across all kingdoms [1]. Ψ is a C5 glycoside isomer of uridine that contains a C1′-C5′ bond between the ribose sugar and uracil instead of the usual C1′-N1′ bond [2]. The enzymes responsible for pseudouridylation are called pseudouridine synthases (PUS), and they can be classified according to their dependence on snoRNA for their enzymatic action. In human, 13 enzymes have been identified and Dyskerin (DKC1) is the only snoRNA-guided one [1,3–5].

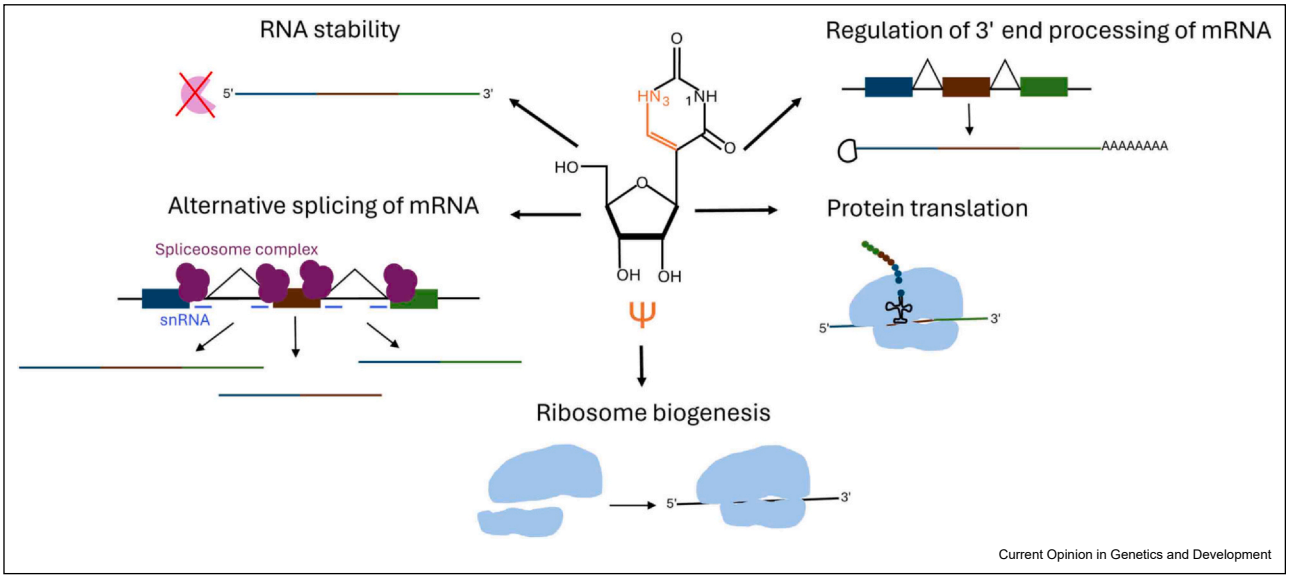
Ψ can impact the alternative splicing of pre-mRNA, 3′ end processing [6] stability of RNA molecules [7], the efficiency of protein translation [8], and ribosome biogenesis [9,10] (Figure 1). Moreover, Ψ is a dynamic modification as several Ψ sites in snRNA, ncRNA, and snoRNA have been observed to respond to environmental stress (oxidative and thermal) [11].

Owing to its prevalence and properties, there is a growing number of studies on the association of dysregulated Ψ levels and human diseases [Table 1]. This review focuses on the recent advances on the role of RNA pseudouridylation alterations in human diseases and its therapeutic potential.

Insights into dyskeratosis congenita: telomere dysfunction and pseudouridylation

Dyskeratosis congenita is an X-linked syndrome characterized by leukoplakia, reticulated skin pigmentation, nail dystrophy, cancer predisposition, and, if not detected early, bone marrow failure. It was previously thought that defective telomerase activity was the only cause of this pathology [20,21]. Mochizuki et al. [22] showed that in mice with *dkc1*$^{-}$ (A35V), one of the most frequent mutations found in patients, the stability of telomerase RNA was compromised, the telomerase activity was reduced, and the length of the telomeres was significantly decreasing through cell divisions. However, the study also showed that alterations in telomeres cannot solely explain this disease; dysregulated pseudouridylation also plays an important role. For instance, mice with *dkc1*$^{-}$ (G402E), a mutation identified in only a single family, have functional telomeres in terms of telomerase RNA stability, telomerase activity, and telomere's length but exhibit lower Ψ levels in rRNA and a decrease in the rate of pre-rRNA processing. The reduction of Ψ levels in rRNA was also reported in lymphoblasts and fibroblasts from patients with dyskeratosis congenita [23]. The zebrafish model generated by Balogh et al. [24] is a hypomorphic *dkc1* mutant since the homozygous loss of function mutant could not survive longer than 5 days after fertilization. The *dkc1*$^{-}$

Figure 1



Molecular functions of pseudouridine. Ψ is involved in stability of RNA molecules, efficiency of protein translation, alternative splicing of pre-mRNA via interaction with snRNA and spliceosome complex, regulation of 3′ end processing of mRNA and ribosome biogenesis.

Table 1

List of the human enzymes involved in pseudouridylation, their RNA substrates, and the diseases associated with their mutation.

Enzyme	RNA substrates	Associated diseases
Pseudouridine synthase 1 (PUS1)	tRNA, mRNA, snRNA [12]	MLASA [13]
Pseudouridine synthase 3 (PUS3)	tRNA, mRNA [12,14]	Neurodevelopmental disease [14,15]
Pseudouridine synthase 7 (PUS7)	tRNA, mRNA, rRNA, snRNA [12,16]	Lesch-Nyhan syndrome, autism spectrum disorder, neurodevelopmental disease [16,17]
Pseudouridine synthase 10 (PUS10)	tRNA, mRNA [12,18]	Crohn's disease, celiac disease [18]
Dyskerin	mRNA, rRNA, snRNA [12]	Dyskeratosis congenita [19]

zebrafishes showed microphthalmia and cataracts like the human patients with mutated Dyskerin, as well as altered Ψ levels in 18S rRNA, but not telomere attrition, even though telomere biogenesis is conserved between humans and zebrafish [25]. In line with this observation, seminal work from Ruggero et al. [26] showed that hypomorphic *Dkc1* mutant mice recapitulated the manifestations of dyskeratosis congenita and reduction of Ψ levels in rRNA started before the onset of the disease, in contrast to the shortening of telomere length that occurred later during the disease.

Thousands of Ψ sites have been found in mRNA, with TRUB1 being one of the primary writers [27]. Still, as shown in [Table 1], Dyskerin also targets mRNA, which suggests that mRNA pseudouridylation could be another player in dyskeratosis congenita. Pederiva et al. [28] performed genome-wide Dyskerin ChIP-seq in human osteosarcoma (U2OS) cells and showed enrichment of Dyskerin at expressed genes; the greatest enrichment was at the transcription end site, followed by 3′ UTR, introns, and 5′ UTR. Another study using BID-seq, an orthogonal method based on sulfite:bisulfite treatment that leads to deletions in the position of Ψ, also demonstrated this Ψ distribution along expressed genes in other human cell lines, mouse cell lines, and mouse tissues [12]. Pederiva et al. [28] observed a decrease in Ψ levels in rRNA and mRNA in U2OS with partially depleted *DKC1*, as well as in fibroblasts and lymphoblasts from patients with dyskeratosis congenita (L37del, K314R, and A386T). A puromycin assay in the *DCK1* knockdown (KD) U2OS cells demonstrated that there was a global increase in translation. Consistently, *in vitro* translation assay showed that lower Ψ levels in mRNA resulted in a higher amount of the corresponding protein. Nevertheless, the increase in global translation shown upon *DKC1* KD in U2OS cells was only observed in the first 48 hours, whereas the overall translation was reduced in the cells derived from the patients with dyskeratosis congenita. This discrepancy might be explained by the requirement of Ψ in ribosome biogenesis and the slow turnover of wildtype ribosomes in U2OS

DKC1-depleted cells. Another study showed no global increase in translation in hypomorphic *Dkc1* mice but only in specific mRNAs containing internal ribosome entry sites [29]. The Dkc1 dosage might then be a critical element that determines the ultimate impact of Dkc1 on translation.

Of note, Pederiva et al. [28] observed a decrease in Ψ levels in snRNA in U2OS KD cells. Given that snRNA pseudouridylation was previously shown to be important for pre-mRNA splicing [6], it would be interesting to decipher the impact of the Ψ loss on splicing in the context of dyskeratosis congenita.

Dysregulation of transfer RNA pseudouridylation in neurological and metabolic diseases

PUS3 and PUS7 mutations have been associated with neurological diseases. Patients with different *PUS3* and *PUS7* mutations shared defective phenotypes: facial dysmorphism, hyperactivity, developmental delay, microcephaly, intellectual disability, and speech delay [15,14,16]. Focusing on *PUS7*, de Brouwer et al. [17] showed that fibroblasts from *PUS7* mutant patients showed a decrease in *PUS7* mRNA and consequently a decrease in Ψ levels in tRNAs at position 13. In fibroblasts of other *PUS7* mutant patients, Han et al. [16] could demonstrate an increase in the translation rate. This observation is corroborated by Guzzi et al. [30], who reported an increase in translation in human embryonic stem cells (hESC) depleted of *PUS7*. In both cell types, the phosphorylation levels of 4EBP1 were the same in control and mutant indicating that increase in translation was not due to the activation of the mammalian target of rapamycin (mTOR) pathway [31]. Instead, in hESC, this was caused by the interference of pseudouridylated tRNA fragments with translation initiation. Hence, these different reports showed the relevance of PUS7 in regulating translation via pseudouridylation of tRNA and its role in development and disease.

Cui et al. [32] found greater expression levels of *PUS7* in glioblastoma compared to normal brain tissues, and this expression is associated with worse prognosis. In order to gain insight into the underlying molecular mechanisms, the authors knocked down *PUS7* in glioblastoma stem cells (GSCs), which inhibited self-renewal capacity and stem cell frequency. This effect was reversed when wild type (WT) *PUS7* was overexpressed but not with the overexpression of catalytically inactive PUS7. To evaluate the effect of PUS7 in tumorigenesis *in vivo*, immunodeficient NOD scid gamma mice were transplanted with control or *PUS7* KD GSCs. Mice transplanted with *PUS7* KD GSCs had a higher survival rate, and their tumor growth rate was reduced. This effect again appeared to be dependent on PUS7 activity, as the overexpression of catalytically inactive *PUS7* still inhibited tumor growth. Supporting this data, the authors found that compounds reducing PUS7 activity *in vitro* also inhibited tumor growth *in vivo* and increased the survival rate. Note that it remains to be firmly demonstrated whether these compounds reduce tumor growth through PUS7 inhibition or another activity. DM-Ψ-seq, Ψ mapping method for tRNA based on demethylation and N-cyclohexyl-N′-β-(4-methylmorpholinium)ethylcarbodiimide p-tosylate) (CMC) treatment [18], was performed in *PUS7* KO and control GSCs. It revealed 13 Ψ sites that are PUS7-dependent in 8 types of tRNA. Surprisingly, tRNA stability, tRNA levels, and the global translation rate were not affected by the loss of *PUS7*. Indeed, only the translation of proteins dependent on tRNA pseudouridylated by PUS7 was altered. This is in contrast to the general increase in protein translation observed by Guzzi et al. [30] upon *PUS7* KO, which suggests an effect that is cell type–dependent.

Other studies reported the relationship between higher *PUS7* expression levels and cancer development [33,34], which will open the door for future treatments and diagnostics.

Ψ mapping of mitochondrial RNA has gained relevance in recent years since it was reported to play a role in mitochondrial translation and biogenesis [35,36]. Genetic analyses have associated missense mutations of *PUS1*, one of the main Ψ writers in mitochondrial RNA [37], with mitochondrial myopathy and sideroblastic anemia (MLASA). The main manifestations of this autosomal recessive oxidative phosphorylation disorder are exercise intolerance and anemia [13]. In order to gain insight into the molecular mechanisms, a *pus1* mutant mouse model was generated by Mangum et al. [38]. *Pus1*$^{-/-}$ mice lost certain Ψ sites in cytoplasmic and mitochondrial tRNAs. Mutant mice partially recapitulated the MLASA phenotype, such as the reduction in exercise capacity. Analysis of the gene expression in muscle fibers from *pus1* KO mice showed higher levels of myosin heavy chain IIB and a decrease in IIA positive fiber. This type of muscle expression indicates a faster and more glycolytic metabolism, which correlates with higher lactic acid levels. Another *pus1* KO mouse model showed a similar mitochondrial defective phenotype, which was rescued by an inhibitor of CD38, a NADase that degrades NAD+ and thus regulates cellular NAD+ levels. The CD38 inhibitor was sufficient to rescue the decline in running endurance and reduce lactate production [38]. Future MLASA treatments could be developed to rescue PUS1 expression and consequently reestablish mitochondrial function. Of note, it has been reported that PUS1 also affects mRNA processing events including alternative splicing, alternative cleavage, and polyadenylation [6]; therefore, the potential contribution of these functions into MLASA development also needs to be investigated.

Finally, Song et al. [18] showed that the depletion of human PUS10 results in a reduction in the levels of mature miRNA and accumulation of unprocessed miRNA. Nevertheless, this effect seems to be independent of PUS10 catalytic activity. Contrarily, PUS10 modifies tRNA, and this activity is required for cell growth. Interestingly, a meta-analysis genome-wide association revealed PUS10 as a risk locus for Crohn's and celiac disease [39]. Further studies will tell if PUS10 plays a causative role in the disease and through which molecular activity.

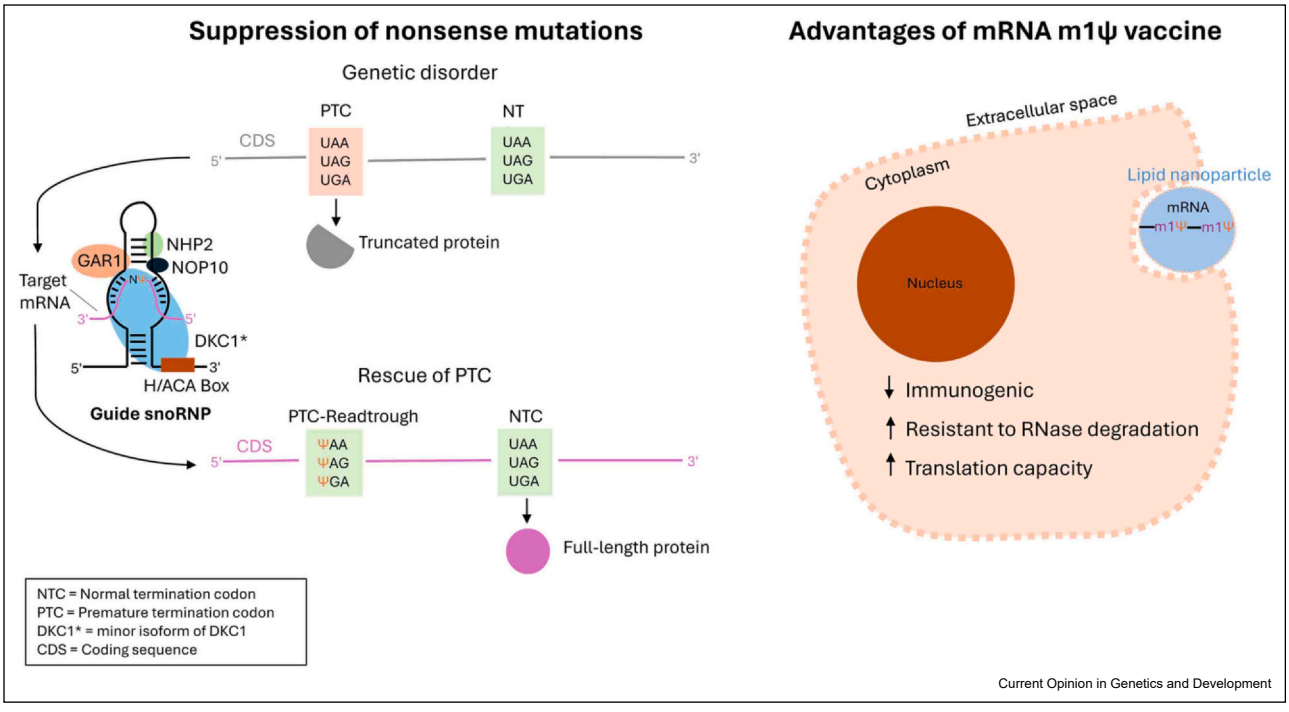
Exploring the therapeutic potential of pseudouridine

Ψ treatments for the diseases mentioned above have not been explored yet, but Ψ has a great potential for therapeutics for other diseases, as exemplified by the great success of Ψ mRNA vaccines against COVID-19. Why was the vaccine so efficient? The reasons are multifaceted: Ψ is less immunogenic than U, Ψ is more resistant to RNase degradation, and Ψ improves translational capacity. These beneficial features are further enhanced when Ψ is N^1-methylated (m1Ψ). This increase in efficiency with m1Ψ could be due to its higher fidelity ratio compared to Ψ [40–42] (Figure 2). Interestingly, recent work showed that the viral RNA is heavily modified, in particular with Ψ. These modifications appear to modulate the SARS-CoV-2 RNA interactome and consequently affect the recognition by the host immune system and the life cycle of the virus [43–45]. Thus, targeting the Ψ machinery or using RNA probes targeting the modified sequences can be alternative strategies to inhibit the replication of the virus.

The innovative mRNA technology for vaccine production holds extensive potential for clinical applications, including the treatment of autoimmune diseases like multiple sclerosis. Of note, Krienke et al. [46] recently developed an m1Ψ vaccine without adjuvant that delivers multiple sclerosis autoantigens into lymphoid dendritic cells conferring bystander tolerance. However, the development of vaccines for autoimmune diseases has not yet reached its full potential, which is largely due to the complexity of polyclonal autoimmune diseases and the variability in responses among different individuals [47].

The three termination codons share a uridine in the first position, and it was found that replacing this uridine with Ψ can result in the bypassing of these stop codons. Karijolich et al. [48] showed this conversion of nonsense codons into sense codons *in vitro* and *in vivo* (yeast

Figure 2



Therapeutical potential of pseudouridine. Left: genetic disorders caused by PTCs result in truncated proteins. Guide small nucleolar ribonucleoprotein (snoRNP) can target the PTC for pseudouridylation. This modification leads to the bypass of the premature stop codon and consequently to full-length proteins. Right: mRNA m1Ψ vaccines offer a wide range of advantages. Ψ is less immunogenic than U; Ψ is more resistant to RNase degradation, and Ψ increases translation capacity.

model via H/AC RNA pseudouridylation). Interestingly, this stop codon readthrough is sequence context–independent [49]. This discovery has therapeutic potential as 11% of genetic diseases are caused by premature stop codons (PTCs) [50]. Song et al. [51] and Adachi et al. [52] developed two different H/AC RNP complexes that pseudouridylate mRNA containing PTC (Figure 2). The guided pseudouridylated system from Song et al. [51], RESTART, takes advantage of a small isoform of DKC (without the C-terminal) that efficiently pseudouridylates PTC. It is not yet clear why the small isoform performs better, but this could be due to its cytoplasmic localization in contrast to the nuclear localization of the canonical isoform. Whereas Adachi et al. [52] engineered a H/AC RNP that in association with antibiotics enhances the readthrough PTC action. Both systems have great therapeutic potential, but they need further optimization as RESTART also pseudouridylates off-targets sequences [51], and the current efficiency of the Adachi et al. [52] system is still very low, 2–11%.

Considering the different therapeutic potentials of Ψ, one would wonder about the readthrough of Ψ in stop codons of mRNA vaccines, which could lead to the decrease of immunogenic proteins or the production of aberrant proteins. Thus, the design of strategies for the safe termination of protein translation is required to ensure more efficient m1Ψ vaccines.

Conclusions

In conclusion, alterations in Ψ levels contribute to the development of a wide range of diseases. In the case of dyskeratosis congenita, defective telomerase activity was initially determined as the only cause for the disease manifestation. However, as we discussed in the review, dysregulated pseudouridylation at rRNA, mRNA, and possibly snRNA levels also plays a role. The impact on translation is nevertheless inconsistent between different studies, and it remains to be determined what are the direct and indirect consequences of the DKC1 loss on RNA stability and protein production. Furthermore, studies on alterations in PUS7 levels in patients have unveiled the significance of tRNA pseudouridylation in neurodevelopmental diseases and cancer, again emphasizing the role of Ψ in the regulation of protein translation.

Advancements in mRNA therapeutics, exemplified by the success of Ψ mRNA vaccines against COVID-19, underscore the therapeutic potential of Ψ in treating various diseases, including autoimmune conditions and genetic disorders caused by premature stop codons.

Therefore, despite being the first discovered RNA modification, Ψ still lies at the forefront of RNA research for its critical role in disease and its unique properties in mRNA-based gene therapy.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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