Taylor & Francis
Taylor & Francis Group

REVIEW

The Dihydrouridine landscape from tRNA to mRNA: a perspective on synthesis, structural impact and function

Olivier Finet, Carlo Yague-Sanz, Florian Marchand, and Damien Hermand [ORCID]

URPHYM-GEMO, The University of Namur, Namur, Belgium

ABSTRACT

The universal dihydrouridine (D) epitranscriptomic mark results from a reduction of uridine by the Dus family of NADPH-dependent reductases and is typically found within the eponym D-loop of tRNAs. Despite its apparent simplicity, D is structurally unique, with the potential to deeply affect the RNA backbone and many, if not all, RNA-connected processes. The first landscape of its occupancy within the tRNAome was reported 20 years ago. Its potential biological significance was highlighted by observations ranging from a strong bias in its ecological distribution to the predictive nature of Dus enzymes overexpression for worse cancer patient outcomes. The exquisite specificity of the Dus enzymes revealed by a structure-function analyses and accumulating clues that the D distribution may expand beyond tRNAs recently led to the development of new high-resolution mapping methods, including Rho-seq that established the presence of D within mRNAs and led to the demonstration of its critical physiological relevance.

The RNA backbone is a succession of covalently bound ribonucleotides whose nucleobase, ribose or 5'-extremity can be modified. To date, there are more than 150 known RNA chemical modifications, spanning the three domains of life and viruses. RNA modifying enzymes can work as (cofactor-dependent) stand-alone proteins, be part of a protein complex that is required for modification or are guided by small nucleolar RNAs (snoRNAs). tRNAs are the most heavily modified RNA species with up to 25% (in eukaryotes) and 15% (in prokaryotes) of their ribonucleotides being modified [1]. It is estimated that one-fifth of all known tRNA modifications is spread across all domains of life [2]. Ribosomal RNAs are also widely modified yet to a lesser extent with up to 2% of modified positions [3]. The abundance of rRNAs and tRNAs facilitated their study and the modification status of their building blocks. However, other RNA species have been known to carry post-transcriptional modifications (PTMs) for decades. Besides the well-characterized eukaryotic mRNA 5'-cap, internal modifications are found in coding RNAs, such as the highly abundant m⁶A, which was detected in mRNAs more than forty years ago [4]. In Eukarya, snoRNAs and snRNAs carry 5'-end and internal modifications but have a narrower range of PTMs [5]. Over the past decade, a still-increasing set of modifications including m^6A, m^6Am, m^5C, hm^5C, ψ, m^1A or 2'-O-Me was mapped at transcriptome-scale, which has been largely discussed elsewhere, for example [6,7].

1. Dihydrouridine is structurally unique

The dihydrouridine (D, sometimes DHU) RNA modification is a modified pyrimidine nucleoside whose corresponding nucleobase is 5,6-dihydrouracil. D is synthesized from uridine (U) by hydrogenation [8] (Fig. 1A). Reduction of the uridine C5-C6 bond

generates a saturated nonplanar and nonaromatic nucleobase that is a landmark of dihydrouridine (Fig. 1B). Although the chemical synthesis of dihydrouracil was already reported in 1896, its first detection in a biological sample dates back from 1952 when it was isolated from the beef spleen [9]. Using *in vitro* approaches, dihydrouridine monophosphate (dihydro-UMP) was shown to be efficiently introduced into RNA molecules, but whether D was a genuine component of cellular RNA was still to be determined [10,11]. D was then reported as a naturally occurring component of yeast $tRNA^{Ala}$ and included in the first published structure of a ribonucleic acid [12,13] (Fig. 1C). Simultaneously to the publication of the tRNA sequence, Visser and colleagues were already discussing the possibility that 'non-random distribution of hydrogenated pyrimidine [dihydrouridine] may be explained more readily by a process of enzymatic hydrogenation at the polynucleotide level' ([11], p. 297).

In the next decades, optical studies along with X-ray and NMR crystallographic analyses elucidated the biochemical properties of dihydrouridine. The structures of the nucleobase, the nucleotide, of D-containing tRNAs and D-containing oligoribonucleotides led to the conclusions that; (I) the carbon 6 of the nucleobase (C6) is out of the plane after the C5-C6 double bond reduction, (II) the deviation from the planar nature of the pyrimidine results in the loss of the stacking ability with neighboring nucleobases, (III) the C2'-*endo* conformation is adopted by the ribose moiety whereas canonical ribonucleotides prefers the C3'-*endo* conformation (Fig. 1D) and (IV) the C2'-*endo* pucker is propagated to the 5'-nucleotide (references and comments in Table 1). Therefore, the complete destacking of the bases and the unusual adoption of C2'-*endo* ribose pucker make dihydrouridine a unique modification [14]. The structural properties of dihydrouridine include the potential destabilization of
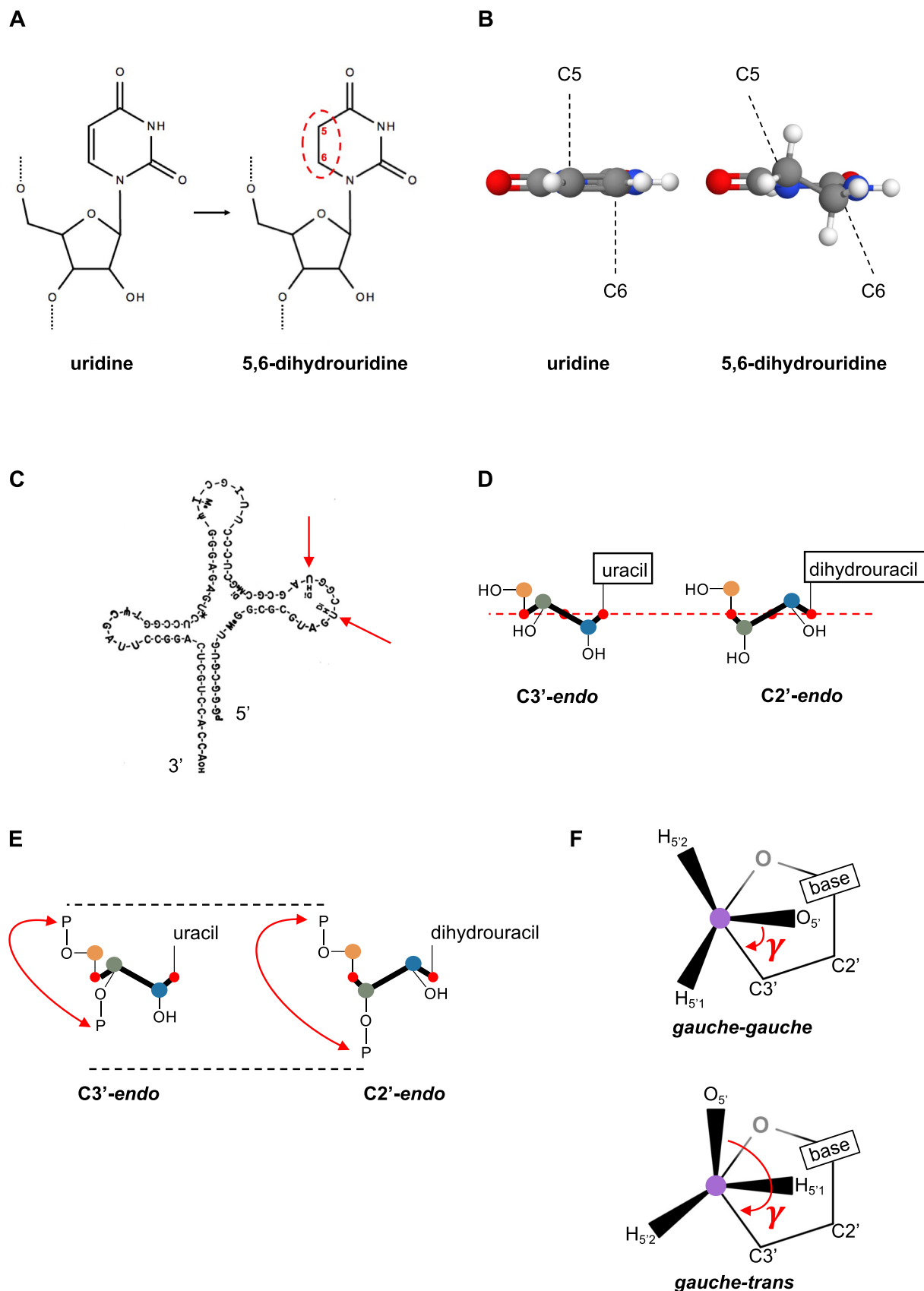

Figure 1. Biochemistry of dihydrouridine.

A Reduction of uridine into dihydrouridine. **B** Dihydrouracil is a nonplanar nucleobase (carbon in gray, nitrogen in blue, oxygen in red and hydrogen in white). **C** First published structure of a ribonucleic acid (yeast tRNA[Ala]) where D (red arrows) are shown in a loop at the 5'-end [12]. **D** Schematic representation of ribose pucker. C5' (orange dot) is considered as being *above* the C4'-O'-C1' plane (red dashed line and red dots). **Left panel**: C3'-*endo* has the C3' (green dot) above the plane. **Right panel**: C2'-*endo* has the C2' (blue dot) above the plane. **E** C2'-*endo* pucker produces a longer 5'-phosphate/3'-phosphate distance and therefore spans the polynucleotide [147]. **F** Schematic representation of ribose *gauche-gauche* and *gauche-trans* conformations. C5' (purple dot) adopts different torsion angles (γ) that modulate the positioning of C5'-bound atoms (H$_{5'1}$,H$_{5'2}$ and O$_{5'}$).

Table 1. Seminal studies on optical and structural properties of dihydro-uracil/-uridine.

Structural and optical studies	Comments	References
dihydrouracil	C5-C6 and N1 out of the base plane	[140]
	expectation for Watson-Crick interaction impairment	
dihydrouridine	deviation from planarity	[143]
	anti orientation	
	C2'-*endo* conformation	
	anti orientation	[149]
	favoured *gauche-trans* and *trans-gauche* conformations	
	deviation from planarity	[85]
	anti orientation	
	preference for C3'-*exo* and C2'-*endo* conformations	
	preference for *gauche-gauche* conformation	
	deviation from planarity	[142]
	anti orientation	
	C2'-*endo* conformation	
	favoured *gauche-trans* and *trans-gauche* conformations	
	C6 out of the base plane	[141]
	no base stacking and *anti* orientation	
	C2'-*endo* conformation	
	favoured *gauche-trans* and *trans-gauche* conformations	
dinucleoside phosphate:	DpA stacking	[137]
DpA, ApD, GpD	ApD and GpD low stacking	
yeast or *E. coli* tRNAs	formation of a D-loop with increased hydrophobicity	[135,138,139,144–146]
	D provides extra flexibility	
	interaction of D-loop and TψC-loop	
trinucleoside phosphate: Dp(acp^{3}U)pA	C2'-*endo* conformation and upstream propagation	150
trinucleoside phosphate:	C6 out of the base plane	[134,, 136]
ApDpA	no base stacking	
	enhanced C2'-*endo* conformation at low temperature (5°C) and downstream propagation	

The table is divided into three columns; (I) studied chemical entities, (II) main conclusions relative to the nucleobase (green writing) or to the ribose (blue writing) and (III) references. C (carbon), N (nitrogen), A (adenosine), D (dihydrouridine), G (guanosine), p (5'-3' phosphodiester bond), acp^{3}U (3-(3-amino-3-carboxypropyl) uridine).

the RNA structure (by promoting C2'-*endo* conformation) and molecular flexibility (by spanning the sugar-phosphate backbone, Fig. 1E). In parallel, the D crystal structure revealed the adoption of *gauche-trans* or *trans-gauche* conformations around the C4'-C5' ribose bond, rather than the common *gauche-gauche* rotamer (Fig. 1F, references in Table 1). In addition, the dihydrouracil nucleobase was shown to be in *anti* orientation in respect to the ribose moiety (i.e. with C2 = O pointing away from the sugar) (**Movie 1**, references in Table 1). More recently, the contribution of D in folding of the D loop was investigated by NMR, which revealed that the absence of D results in the stable stem-loop hairpin to adopt several undefined interconverting conformations in solution [15].

To sum up, D is the sole known non-aromatic modified nucleotide, a feature that promotes noncanonical ribose conformation and hinders proper base stacking. These features have potential deep consequences on many if not all aspects of processes implicating RNA. Indeed, the RNA 'structurome' affects splicing, translation or stability [16,17]. In the context of the epitranscriptomic landscape and its readers, it is worth mentioning that the C2'-*endo* conformation promoted by D is bound by RNA recognition motifs (RRM) and that D may therefore enforce the binding of the large repertoire of RNA binding proteins [18].

2. Detection of dihydrouridine

Since the discovery of D, several methods to detect this modified nucleotide were developed based on its physico-chemical properties. Their recent adaptations to high-throughput sequencing are discussed in a separate section.

2.1 Sodium borohydride and alkaline treatments

Distinguishing the RNA modification from its canonical nucleotide is challenging. Chemical treatments affecting D are summarized in Table 2. The dihydrouridine undergoes ring opening upon sodium borohydride (NaBH$_4$) or alkaline (OH$^-$) treatments, resulting in the formation of an ureido-group (NH$_2$CONH) linked to an alcohol or a carboxylic acid, respectively (Fig. 2) [19–21]. The accumulation of ureido-groups can be quantified by a colorimetric assay [22]. The ribosylureidopropanol (D + NaBH$_4$) can be used for labelling of RNA with a fluorescent dye or for the cleavage of ureidopropanol upon acid conditions (H$^+$) [23–30]. The ribosylureidopropionic acid (D + OH$^-$) is used as a semi-quantitative tool following the breakdown between ribose and ureidopropionic acid, this latter being decomposed to β-alanine that serves as a substrate for a colorimetric assay with ninhydrin [31,32]. The D-ring disruption upon OH$^-$ condition was also shown to generate an RT (reverse transcription) termination assessed by primer extension [33]. Both treatments, NaBH$_4$ or OH$^-$ result in an abasic site (nucleobase-free ribose) that leads to the cleavage of the RNA chain with aniline treatment [34,35]. These chemical reactivities of D have been recently exploited to develop transcriptome-wide mapping of D occupancy (see below).

Thin-layer chromatography was first used to spot ^{3}H-labelled D on a cellulose membrane after oxidation with an inorganic salt and reduction with borohydride [36]. In reverse-phase high performance liquid chromatography (HPLC), D is the earliest eluting nucleoside, sometimes barely distinguished from contaminants with short retention times. However, its detection is possible at 254 nm [37] and detectability is improved by

Table 2. Chemical reactions and techniques specifically applicable to D.

Principles	tRNA	Comments	References
alkaline hydrolysis	/	sodium hydroxide → cleavage at the N3-C4 linkage of dihydrouracil	[19]
β-alanine detection	Y	sodium hydroxide → D-ring opening → partial β-alanine formation → ninhydrin colorimetric assay	[32]
sodium borohydride reduction	Y	sodium hydroxide → cleavage at the N3-C4 linkage of dihydrouridine	[20]
hydrochloric acid hydrolysis	B, Y, M	sodium borohydride → D-ring opening → hydrochloric acid → cleavage of the glycosodic bond	[24,and b; 26]
loss of absorbance	Y	loss of absorbance at 265 nm upon mild sodium hydroxide treatment	[132]
cleavage at D position	Y	sodium borohydride → D-ring opening → aniline treatment → RNA chain cleavage	[34]
ureido-group detection	B, Y, M	sodium hydroxide → D-ring opening → solution neutralization → colorimetric assay after iron chloride addition (550 nm)	[22]
replacement of D by proflavine or EtBr	Y	sodium borohydride → D-ring opening → incubation with the dye	[29]
microarray	Y	differential fluorescent labeling of tRNA from WT and dus strains → annealing on a microchip → quantification of A-U vs A-D interaction	151
primer extension	B, Y	sodium hydroxide → D-ring opening → assessment of RT termination by primer extension	[23,33]
fluorescent labeling with rhodamine 110	B	*in vitro* tRNA → *in vitro* dihydrouridylation → sodium borohydride → incubation with the dye	[23]
replacement of D by Cy3 or 5	B, Y	sodium borohydride → D-ring opening → incubation with the dye	[27]
benzoyhydrazide addition	B	sodium borohydride → THU formation → incubation with benzohydrazide	[42]
predictive modelling	B, Y	jackknife-based test to predict modified sites	[113,115]
nanopore sequencing	B	RNA through a nanopore scale → specific ionic signature	[116]

The table is divided into four columns; (I) list of principles, (II) phylogenetic origin of the studied tRNA; B (bacterial), Y (yeast), M (mammalian), (III) general comments on the principle. EtBr (ethidium bromide), N (nitrogen), C (carbon), RT (reverse transcription), WT (wild type), dus (dihydrouridine synthase gene), A-U (adenine-uridine Watson-Crick interaction), A-D (adenine-dihydrouridine Watson-Crick interaction), Cy3/5 (cyanines 3 or 5), THU (tetrahydrouridine) and (IV) references. Only the seminal works are cited although most of the techniques were implemented in other studies. Chromatographic applications are not listed here.

lowering the wavelength at 210 or 230 nm [38]. Accurate determination of D is readily obtained by liquid chromatography coupled to mass spectrometry (LC-MS) [37].

2.2 Effects on double strand formation

The comparison of binding properties of polyU- *vs* polyD-nucleotides with polyA revealed a reduced ability of polyD to interact with polyA although the reduction of uridine occurs at Hoogsteen edge (C5 = C6) of the nucleobase whereas the hydrogen bonds of the Watson-Crick interactions are formed from positions 3 and 4 (N3-C4 = O) [39]. The decreased binding capacity is nevertheless too weak to induce a clear RT termination in a primer extension context [40]. Nevertheless, Phizicky and colleagues took advantage of the decreased D-A binding to implement a microarray-based technology to monitor the presence of D at specific tRNA positions [33,41].

2.3 Labeling with a fluorescent dye

As stated above, the treatment of D by sodium borohydride is a prerequisite for the subsequent incorporation of fluorescent molecules, and more specifically of NH_2-dyes (*e.g.* proflavine, rhodamine, cyanine hydrazide). Fig. 2 shows that the D-ring opening forms ribosylureidopropanol in a $NaBH_4$-dependent manner. However, this commonly accepted N3-C4 cleavage has been recently challenged [42]. Cooperman and colleagues induced tRNA reduction with sodium borohydride and performed labelling with NH_2-containing fluorophores nucleophiles. By combining TLC (thin layer chromatography) and mass spectrometry, they detected tetrahydrouridine (THU) instead of the expected ribosylureidopropanol. Based on their results, they proposed that the dihydrouridine C4 carbonyl group is reduced by the H^- donor $NaBH_4$ to THU. Upon

the addition of an NH_2-dye in acid conditions, a nucleophilic substitution occurs on the C4 hydroxyl group by formation of a Schiff base-bearing intermediate called tetrahydrocytidine (THC) and consecutive fluorophore binding. Fig. 3 summarizes both mechanisms in the context of the addition of the rhodamine 110 fluorophore.

3. Seminal landscape of the distribution of Dihydrouridine

Based on 602 tRNA sequences from viral, prokaryotic and eukaryotic species, D is the second most prevalent tRNA modification (925 counts) after pseudouridine (1,164 counts) [43]. The dihydrouridylated positions include the canonical D_{16}, D_{17}, D_{20}, D_{20a}, D_{20b} and D_{47} (Fig. 4A) and the rare non-canonical D_{14}, D_{17a}, D_{21} and D_{48}. Among the most frequent modified positions are D_{20} and D_{16} both positioned in the eponym *D-loop* that has a pivotal role in the establishment of secondary and tertiary structures of tRNAs [15,44]. The biochemical specificities of D play a role in the cloverleaf-related tRNA secondary structure and in the L-shaped tRNA tertiary structure that is achieved through D- and T-loops interaction (reviewed in [45]). Although the most important residues for the kissing D/T loops are not dihydrouridines, a compilation of crystal structures highlighted a set of base pairing events where D is involved through various types of interactions (*cis* or *trans* interactions between Watson-Crick, Hoogsteen and sugar edges) [46,47].

3.1 Dihydrouridine in eukaryotes

All eukaryotic tRNA species have been described with at least one dihydrouridine, with the exception of tRNA^{selenocysteine}. Particularly, the 18 described cytoplasmic tRNA$_i^{Met}$ only have
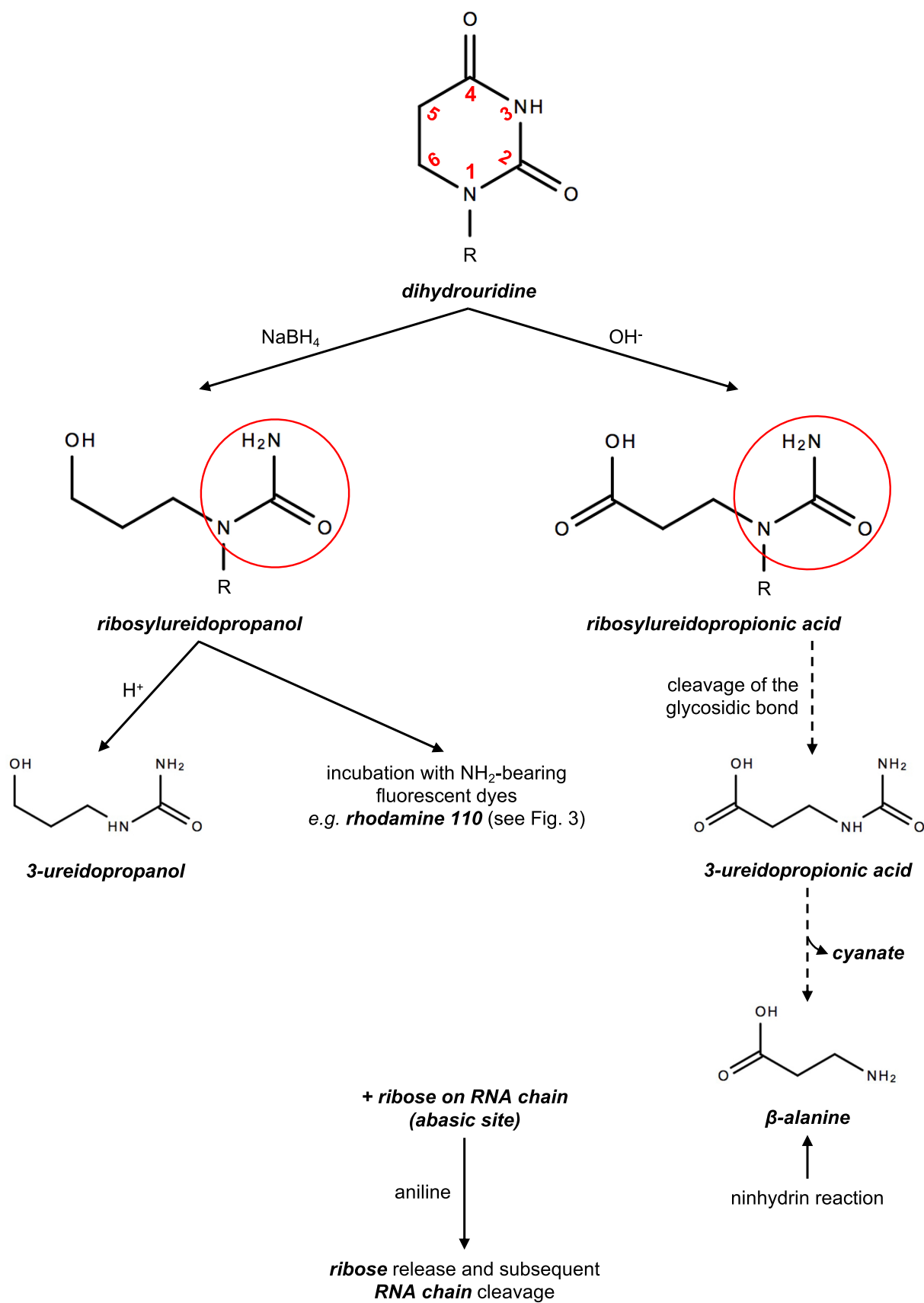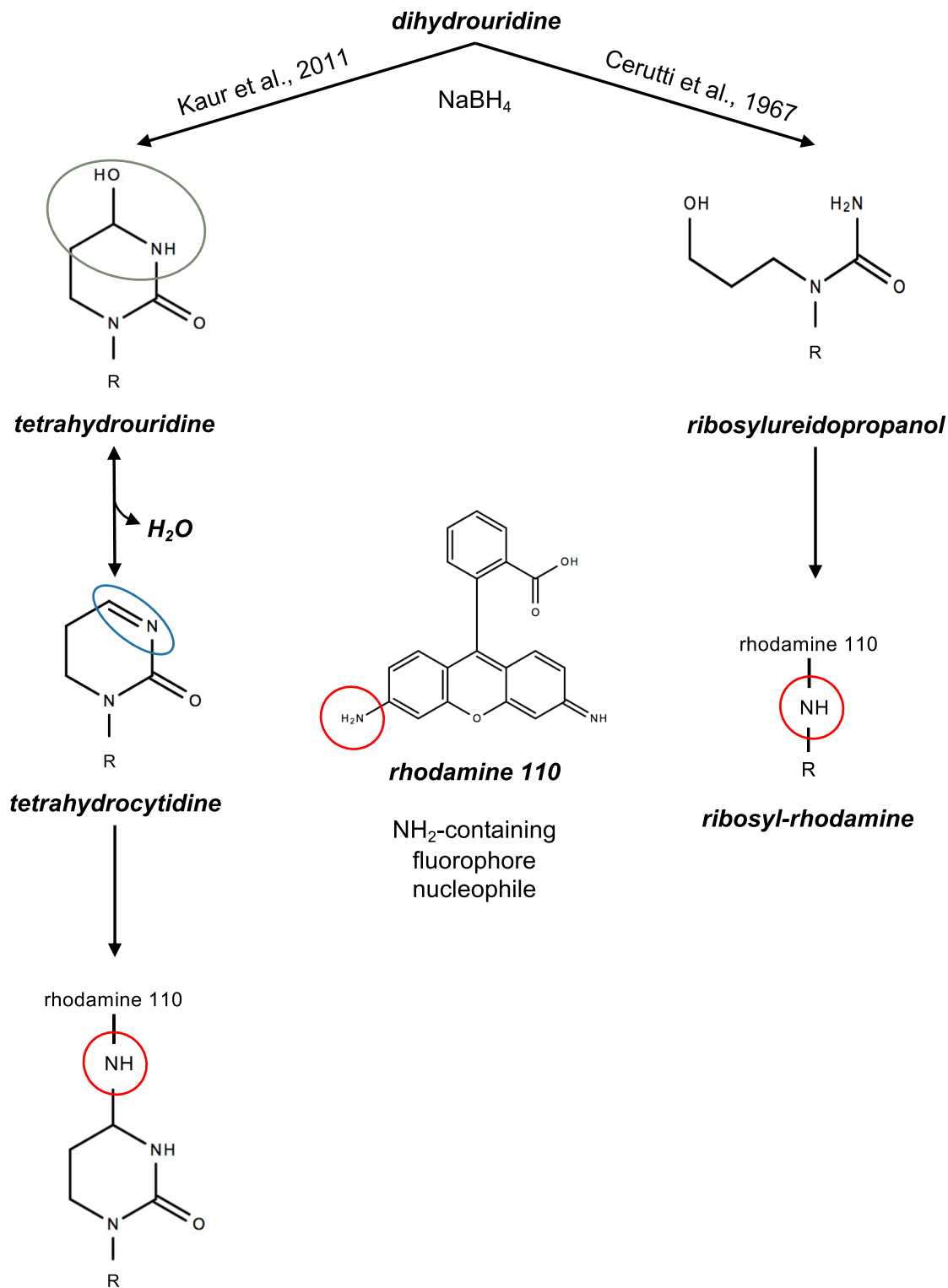
Figure 2. Molecular fate of D upon sodium borohydride (NaBH$_4$) or alkaline (OH$^-$) treatments.
R stands for ribose attached to the RNA chain. Red circles highlight ureido-groups.H$^+$ stands for acid conditions. Details in the text.

Figure 3. Proposed mechanism of D rhodamine labeling following sodium borohydride (NaBH₄) reduction.

Left panel, dihydrouridine is reduced to tetrahydrouridine that is characterized by an electrophilic carbonilamine (green circle), including the C4 hydroxyl group (C-OH). Upon addition of the nucleophilic rhodamine 110 in acid conditions, the tetrahydrocytidine intermediate is formed with its reactive Schiff base (blue circle). Covalent binding of rhodamine 110 occurs by substitution of the C4 hydroxyl group. **Right panel**, ureidopropanol generated by D-ring opening is replaced by rhodamine 110. R stands for ribose attached to the RNA chain.
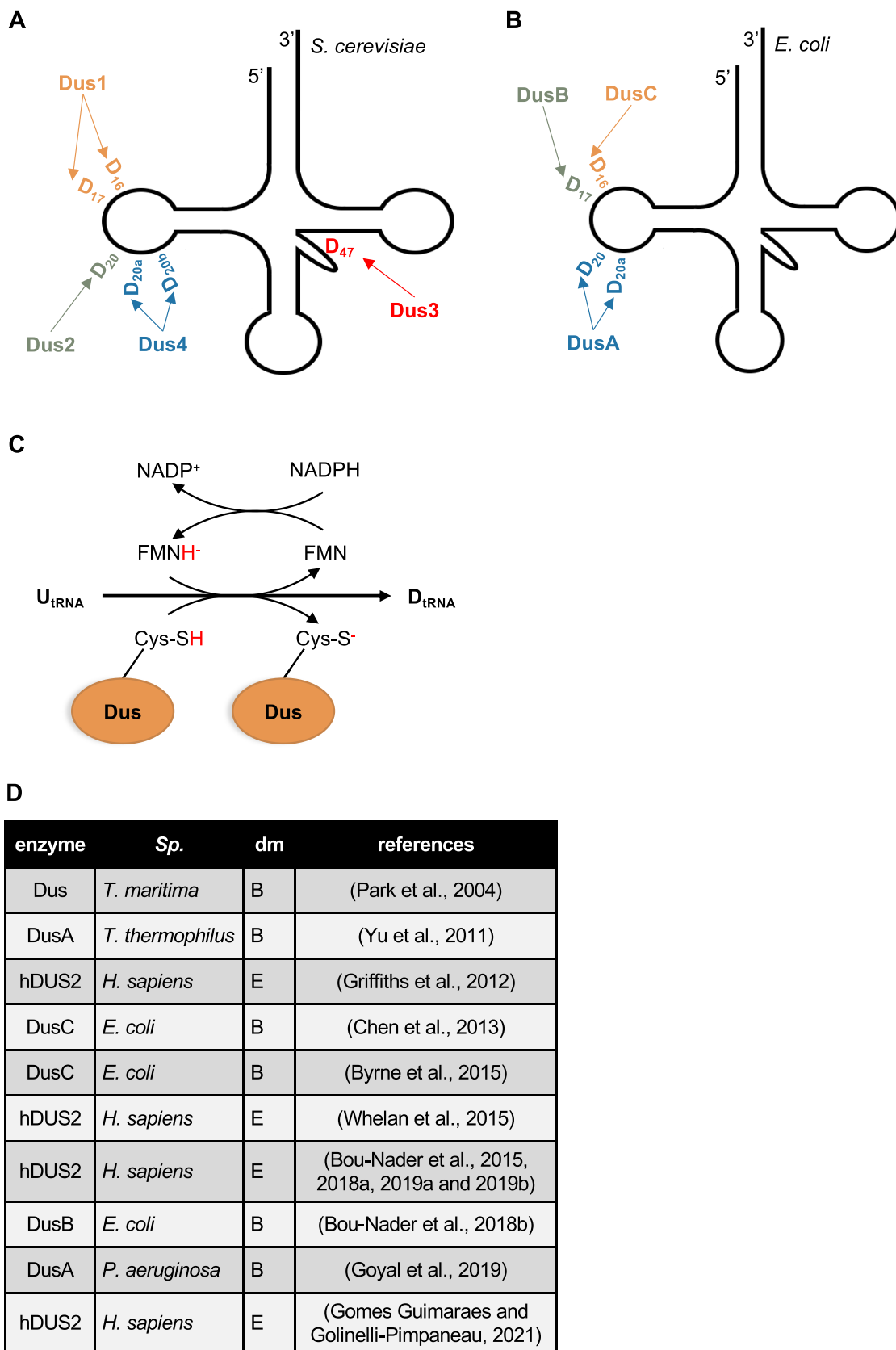
Figure 4. The biology of dihydrouridine.
A Specificity of yeast Dus enzymes for cytoplasmic tRNAs.**B** Specificity of *E. coli* Dus enzymes for tRNAs.**C** Putative enzymatic mechanism of Dus enzymes. Details in the text.**D** Currently available structures of Dus enzymes in different species (*Sp.*) from different domains (dm; B for Bacteria and E for Eukarya). The references are indicated in the right column.

D_{16} but no D_{17}, D_{20}, D_{20a}, or D_{20b}. In mitochondrial tRNAs (mt-tRNAs), all six canonical D are found, except D_{20b} [43]. Importantly, there are dihydrouridylated mt-tRNAs that are encoded by the mitochondrial genome; at least three mammalian mt-tRNAs are known to have a D_{20} and accordingly, the putative cognate enzyme was shown to localize in human and murine mitochondria [48,49]. It has been shown that a set of mammalian mt-tRNAs have a truncated cloverleaf structure by lacking the D-loop [50,51]. Surprisingly, these tRNAs seem to adopt a functional tertiary structure by establishing unique interactions in a Mg^{2+}-dependent manner [52,53].

Phizicky and colleagues identified the first dihydrouridine synthase Dus1 in 2002 in *S. cerevisiae* [54] based on a dihydrouridylation assay using a collection of GST-ORF fusion proteins. Homology searches highlighted three related enzymes encoded in the yeast genome. The specific modification of tRNA^{Phe} by Dus1 and tRNA^{Leu} by Dus2 supported that Dus are substrate-specific enzymes, which was confirmed and expanded in an elegant microarray-based experiment relying on the D-dependent alteration of strands interaction. The resulting tRNA D landscape was U_{16} and U_{17} targeted by Dus1, U_{20} by Dus2, U_{20a} and U_{20b} by Dus4 and the extra D-loop U_{47} by Dus3 (Fig. 4A). The budding yeast quadruple *dus* mutant was shown to be viable and to lack any detectable dihydrouridine [33].

Early on, the D landscape was suspected to expand beyond tRNAs as D was found associated with plant and mammalian histone-bound RNAs [55,56] and detected on an enzymatic digest of rat U5 snRNA [57]. However, this was not further analyzed until recently (see below).

3.2 Dihydrouridine in prokaryotes

All canonical D residues (D_{16}, D_{17}, D_{20}, D_{20a}, D_{20b}) are found in Bacteria. D_{47} is a scarce modification with a unique occurrence described so far (tRNA^{Met} of *B. subtilis*), although the position 47 in bacterial tRNAs is a U in almost 90% of the 134 known sequences. D_{20b} is also very uncommon and found on a cyanobacterial tRNA^{Glu} [43]. A peculiarity of the bacterial dihydrouridine landscape is the presence of a unique D on the 23S rRNA in Gram-negative (*E. coli*) and -positive (*M. hominis*, *C. sporogenes*) bacteria [58–60]. In *E. coli*, D is located at position 2449, a residue located in the highly conserved 23S rRNA central loop of domain V [59–61]. Remarkably, this region is part of the peptidyltransferase center that is also one of the sites of interaction with antibiotics targeting ribosomal activity. However, D_{2449} is dispensable in *E. coli* [62]. The Gram-positive *C. acetobutylicum* is the only known organism to have a D on its 16S rRNA. The modification occurs on position 1211 or 1212 but its function is still unknown [63].

Another type of non-coding RNA known to be dihydrouridylated is the Y RNA (a non-coding RNA involved in RNA degradation) from the γ-proteobacterium *S. typhimurium*. It has a DusA-dependent D and folds in a tRNA-like manner [64].

The de Crécy-Lagard laboratory implemented an *in silico* comparative genomic screen in order to find bacterial *dus*

genes. Because no D was ever detected in *P. furiosus*, they assumed that the genome of this organism should not contain any *dus* gene, in contrast to other microorganisms such as *E. coli* or *S. cerevisiae*. By doing so, they found ortholog genes absent in the D-free *P. furiosus* species but present in other D-containing species [65]. This approach led to the identification of three *E. coli* Dus enzymes referred to as DusA, DusB and DusC that have non-redundant activities on tRNAs (Fig. 4B). *E. coli* DusB and DusC are mono-specific proteins that target U_{17} and U_{16}, respectively while DusA substrates include U_{20} and U_{20a} [66]. Importantly, this conclusion is not valid for the whole bacterial world as the DusB protein from *Mycoplasma capricolum* was shown to modify U17, U20 and U20a, making it the only known Dus enzyme to modify three different tRNA sites [67].

4. First insights into the biological relevance of Dihydrouridine

4.1 Implications in human health and development

The isolated tRNA^{Phe} from malignant human tissues was reported to contain more dihydrouridines [68]. D is also present in urine samples and more significantly abundant in the urine of lymphoma patients [69,70]. This is in line with the idea that tumour tissues undergo high turnover of tRNAs that can be quantified and used as a noninvasive biomarker for diagnosis and treatment of cancer [38,71,72]. More generally, D can be considered as a metabolic modulator for a large set of pathological conditions; D is upregulated in the serum of patients with the major form of oral cancer in the world (oral squamous cell carcinoma) [73] and downregulated in the serum of mice bearing breast cancer [74]. D is associated with lethal prostate cancer [75].

At the molecular level, the human DUS2 protein (hDUS2) was shown to act as an inhibitory factor of the interferon-induced protein kinase PKR – whose kinase activity is enhanced in melanomas and colorectal cancers [76]. Similarly, the anti-cancer ginsenoside compound was shown to repress the expression of *hDUS2* in human colorectal cancers cells [77].

To date, the most comprehensive study linking D with cancer was provided by Nakamura and colleagues [78]. *hDUS2* showed a 3-fold overexpression in non-small cell lung carcinomas (NSCLCs) compared to healthy samples. The hDUS2 protein followed the same pattern, localized at endoplasmic reticulum and harbored a C-terminal double-stranded RNA binding motif (DSRM or dsRBD). In addition, an interaction between hDUS2 and the glutamyl-prolyl tRNA synthetase EPRS was reported. Phenotypically, the suppression of the tumor cell growth was observed after siRNA-dependent *hDUS2* depletion and the NSCLCs patients with high levels of hDUS2 showed worse prognostics. The subsequent model was that overexpression of hDUS2 led to the hypermodification of tRNAs and consecutive increase of conformational flexibility. Because hDUS2 interacted with EPRS, they hypothesized that the tRNAs were more promptly charged in NSCLCs, which globally resulted in a more efficient translation although this

remains to be established. In addition, increased D level was associated with worse outcomes in several cancers [79,80].

hDUS3 was also shown to be an inhibitor of the regenerative ability of the central nervous system [81]. Strikingly, the dihydrouridine was more abundantly detected during neural development in human embryonic stem cells [82].

4.2 Implications in prokaryotic growth

The quantitative detection of dihydrouridine led to the conclusion that psychrophilic bacteria have up to 70% more D on tRNAs than their mesophilic counterparts, which is in contradiction with the general observation that tRNAs of psychrophiles tend to be hypomodified [83]. The psychrophilic organisms that grow between 0°C and 20°C – with an optimum at 15°C – have the necessity to cope with low environmental temperatures, unlike the mesophiles bacteria that live above 20°C. It has been established by NMR that low temperatures tend to stabilize the C2'-*endo* conformation of the dihydrouridine ribose moiety [84,85]. The accumulation of D in psychrophilic prokaryotes could therefore constitute an evolutive adaptation to allow these organisms to maintain the conserved L-shaped conformation of tRNA, despite a growth at very low temperatures that otherwise could be detrimental for tRNA structure and function. More generally, the set of modifications present on a tRNA depends on environmental cues, such as the temperature. In agreement with this idea, the *in vitro* synthesis of dihydrouridine in the hyperthermophilic bacterium *T. thermophilus* is possible on an unmodified tRNA^{Phe} at 60°C but not at 80°C where the tRNA substrate needs to carry other modified nucleosides [86]. At the transcriptional level, the gene coding for the mesophilic *Clostridium botulinum* DusB homolog is downregulated during a heat shock stress at 45°C. In line with the above principles, the bacterium would require less D at high temperatures and would therefore decrease the expression of its cognate enzyme [87]. DusC is also differentially regulated in response to the growth temperature in the thermophilic *Bacillus manusensis* bacterium [88].

An interesting case is found in the Archaea *Methanococcoides burtonii* that has a thermal niche around 2°C and an *in vitro* optimal growth at 23°C. It was noticed that despite its low percentage of modified tRNA nucleotides – only 2%, which is one of the lowest in the living world – this psychrotolerant archaeon has, on average, more than one D residue per tRNA [89]. The fact that an archaeon living at low temperatures and having a limited tRNA epitranscriptome possesses D residues is another clue that D impacts the flexibility of tRNAs.

Finally, D was also shown to be dramatically affected when the *Lactobacillus agilis* gastrointestinal bacterium is grown on an alternative energy source, underlying again the dynamic regulation of this modification [90].

5. Structure-function analyses of the Dus enzymes

Dus are flavin-dependent enzymes that function similarly to the dihydroorotate and dihydropyrimidine dehydrogenases. Based on kinetics and structural data, an FMN- and NADP-dependent enzymatic mechanism has been proposed [91–93]. The reductive half-reaction is initiated with NADPH binding to the Dus enzyme. The NADPH transfers a hydride to the Dus-bound flavin prosthetic group (FMN to FMNH$^-$), which reduces the enzyme. The dissociation of NADP results in a free reduced enzyme that binds the tRNA. In the oxidative half reaction, the nucleophilic C6 of uridine is reduced. The second hydride transfer to C5 likely occurs through the oxidation of a highly conserved Dus cysteine residue (Cys) that argues in favour of an evolutionary conserved mechanism (Fig. 4C) [94,95]. Interestingly, the reduced Dus enzyme has a very slow oxidative half-reaction when an *in vitro* transcribed tRNA is used as a substrate, contrary to a purified tRNA (from a *dus-* mutant). This strongly suggests that a tRNA already bearing modifications is the genuine substrate and that an ordering of modifications may exist *in vivo* [92].

To date, six Dus enzymes structures have been published (Fig. 4D). The seminal crystallographic structure of an unknown FMN-binding protein in *T. maritima* revealed an oxidoreductase enzyme with two domains; an N-terminal TIM barrel and a C-terminal helical domain [96]. Later, this enzyme was referred as a dihydrouridine synthase. The *T. thermophilus* DusA crystal highlighted the same general structure [93]. Moreover, the FMN cofactor (flavin mononucleotide) was captured in a positively charged groove at the center of the N-terminal domain corresponding to the catalytic site. DusA-tRNA^{Phe} complex revealed that DusA interacts with the D-stem loop, the anticodon stem loop and the T-stem loop of tRNA and that the D-loop but not the D-stem is strongly distorted when DusA is bound. The third published bacterial Dus structure was DusC from *E. coli* that also displays a two-domain conformation with an N-terminal catalytic domain and a C-terminal RNA binding domain [97,98]. The structural similarities between *T. thermophilus* DusA and *E. coli* DusC led to the hypothesis that they share the same catalytic mechanism. Remarkably, notable structural dissimilarities were discovered by comparing the bacterial DusA (targeting tRNA-U$_{20}$ and $_{20a}$) and DusC (targeting tRNA-U$_{16}$). Both enzymes adopted the same general fold – while having different substrate specificities – but bound and recognized the tRNA in different orientations. The tRNA binding differed by a 160° rotation that resulted in the proper integration of the targeted uridine in the catalytic pocket. This trademark way of catalyzing a reaction is unique in RNA enzymology and is achieved through specific *binding signatures*. According to its target, each Dus enzyme has a cluster of amino acids – that is phylogenetically conserved in Bacteria – that defines the docking of tRNA to allow the reduction of a specific uridine [97]. The missing DusB structure was provided by Hamdane and colleagues [66]. Even though the crystal was incomplete, it was concluded that *E. coli* DusB adopted the same overall structure with an N-terminal TIM barrel fold carrying the catalytic function and a C-terminal helical domain. The tRNA docking in DusB was similar to the one of DusC, which makes sense since DusB and C modify neighboring nucleotides (17 and 16). However, a major difference between DusB and C relied on the positioning of the nucleobase into the catalytic center. Reversed polar and nonpolar amino acids in the catalytic

pocket of DusB led to the 180 degrees rotation of the nucleobase that is targeted for reduction. In conclusion, diversification of bacterial Dus specificities was made possible through two astonishing strategies; nucleobase rotation or tRNA docking rotation (Fig. 5). More recently, the DusA structure from *P. aeruginosa* was resolved by combining *in vitro* and *in silico* methods [99].

The only available eukaryotic Dus structure is the human DUS2. hDUS2 is particularly important because it is associated with pulmonary carcinogenesis and, unlike other Dus enzymes, harbors a dsRBD. This domain turns out to be conserved in animals (mammals, amphibia, flatworms, nematodes, insects) [94]. The sequence similarity is quite low between hDUS2 and bacterial Dus or even yeast Dus2, suggesting a potential novel strategy for the substrate recognition [100]. The analogy between bacterial Dus and hDUS2 includes an N-terminal catalytic domain folded in a TIM barrel, an interaction of the catalytic domain with FMN, a high sequence conservation of the residues required in the active site – including the cysteine as an H⁻ donor and the presence of a central helical domain. The C-terminal dsRBD domain was shown to be necessary but not sufficient for D_{20} synthesis on yeast tRNA extracted from a Δ*dus2* strain. Furthermore, this domain was suggested to serve as primary tRNA binding site, before the canonical helical domain [101]. Using a dsRBD for tRNA recognition was never reported before, making hDUS2 the only known tRNA modifying enzyme to adopt this strategy. This unexpected feature was recently clarified by showing that the helical domain of hDus2 was less electropositive than its yeast orthologue partly because of the absence of two lysine residues, which played a role in the emergence of a new tRNA binding mode [102,103].

6. New high-resolution methods to decipher the transcriptome-wide distribution of Dihydrouridine

As indicated above, $NaBH_4$ or OH^- treatments result in an abasic site (nucleobase-free ribose) leading to the cleavage of the RNA chain with aniline treatment [34,35]. This led to the development of AlkAniline-Seq, a method relying on the chemistry-based enrichment of sequencing libraries with fragments containing certain modifications including D [104,105]. While AlkAniline-Seq responded to the presence of D, the signal strength was considerably lower than for other marks including m⁷G. This is likely due to the incomplete formation of abasic sites at D position following limited alkaline hydrolysis.

On a completely different basis, RNA-mediated activity-based protein profiling (RNABPP) relies on metabolic RNA labelling, mRNA interactome capture and quantitative proteomics. In this approach, a 5-halopyrimidine (typically 5-FUrd, 5-fluorouridine for DUS3L) is incorporated in RNA and form stable RNA-protein adducts with the modifying enzyme, allowing RNA interactome capture [106]. These experiments confirmed that U46-48 in the tRNA variable loop is the major substrate of DUS3L. Notably, the approach also revealed DUS3L crosslinking peaks on non-tRNA substrates including mature mRNAs [106]. These data are reminiscent of a previous work reporting that in cardiomyocytes, the human DUS3 homolog was shown to interact with polyA⁺ RNAs, raising the possibility of mRNAs hDUS3-specific modification in this specialized cell type [107].

We recently introduced Rho-seq to globally detect the presence of D residues on RNA [108]. In a two-step protocol, total RNA is first incubated with $NaBH_4$ and then covalently bound by the rhodamine fluorophore (Rho). From there, the Rho-seq protocol unfolds as follows; (I) RNA extraction from WT and Δ4 strains (lacking the four *dus* genes), (II) Rho+ and mock-treatments, (III) ribodepletion, (IV) cDNA synthesis and library preparation, (V) strand-specific deep-sequencing, (VI) data analysis by implementation of a multifactorial analysis. The detailed protocol is available in a separate publication [109].

Rho-seq provided the first transcriptome-wide Dus1 to 4-specific repertoire of the distribution of D and highlighted that D is an integral component of yeast and human mRNAs, though at low level. These data also provided a framework for the previous findings that DUS1 and DUS3 cross-link to mRNAs in both yeast and human [110,111].

The absence of detected D-sites on the bacterial coding transcriptome suggests that mRNA dihydrouridylation may be a eukaryotic-specific mechanism although this requires additional support [108].

A set of *in silico* tools were developed to predict the presence of D on various RNA types. These computational predictions need however to consider a larger set of proven D-containing sequences to build more objective tools [112–115].

Finally, the detection of modified nucleotides with the Oxford Nanopore Technologies is still in its infancy but further developments will likely confirm the seminal detection of D on *E. coli* tRNAs through the nanoscale pore [116].

7. Is dihydrouridine an adaptative mark regulating translation?

Similarly to other mRNA modifications, the detected D-sites could be relevant in a specific physiological context. Two previously reported cases are illustrated in the literature: induced pseudouridylation upon heat shock and widespread methylation during the meiotic programme [117,118]. We found that the deletion of *dus3* in fission yeast specifically affects meiotic chromosome segregation and the translation of a set of proteins including tubulins while the mitotic cell cycle is barely affected [108].

Ribosome profiling and proteomics revealed that a dihydrouridylated mRNA can be translated but that the modification affects this process, which may result in modulation of the translational speed and ribosome stalling [108]. In the late sixties, several teams investigated the *in vitro* coding properties of D-containing oligoribonucleotides. Despite the technical boundaries encountered at that time, they concluded that the presence of D resulted in the loss of coding ability. Rottman and Cerutti showed that a ribopolymer carrying 4.2% of D (and 95.8% of U) lost up to 60% of Phe(UUU) residues incorporation into the protein [119]. Another study highlighted the complete loss of ability for GUD, GDU and GDD trinucleotides to code for Val (GUU)
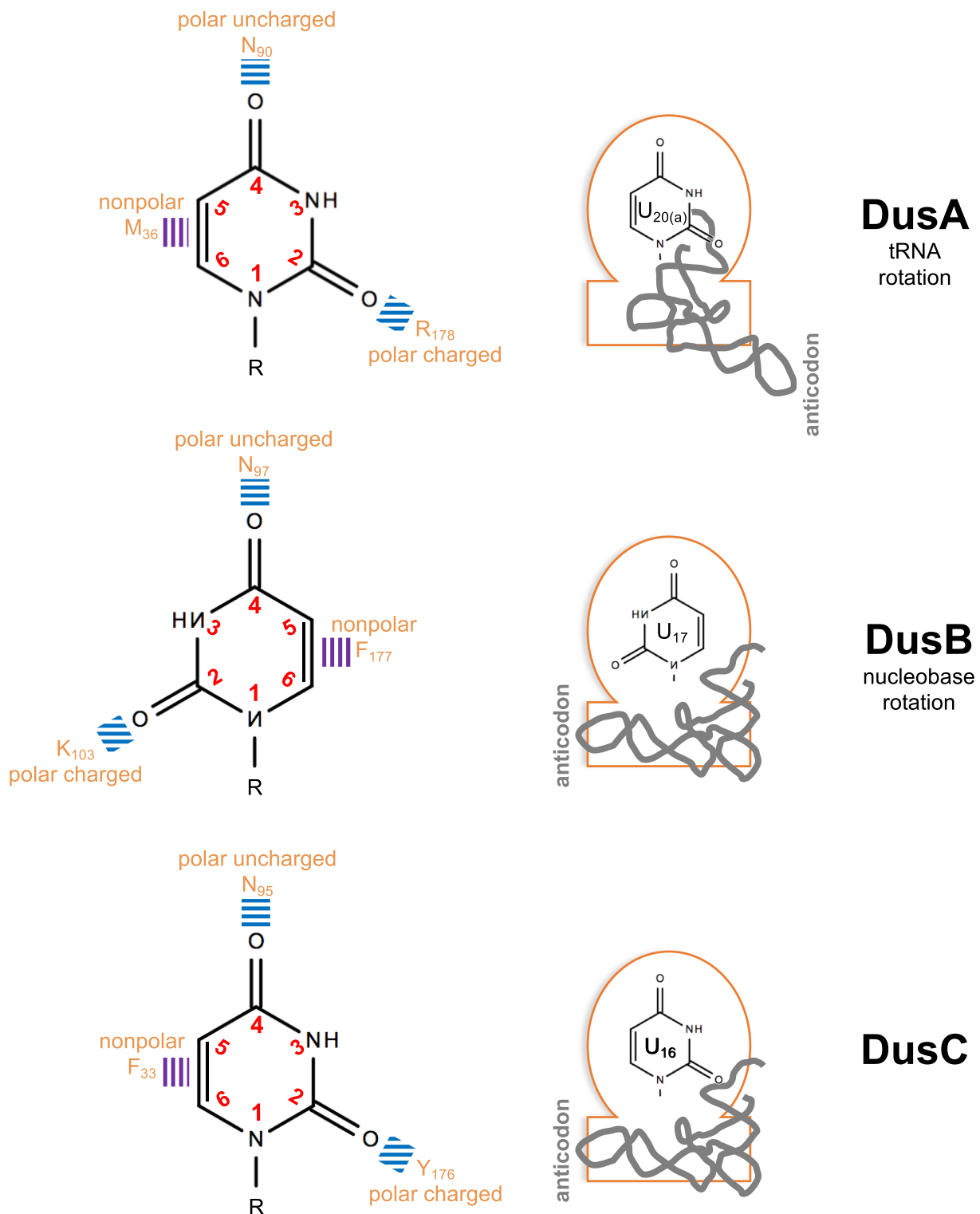
Figure 5. Molecular strategies of bacterial Dus enzymes for substrate specificities.
Left panel, interaction of the U nucleobase (in the context of the RNA chain (R)) with Dus amino acids (orange: F for Phe, N for Asn, K for Lys and Y for Tyr) through ionic (blue lines) or hydrophobic (purple lines) interactions. In the DusB catalytic pocket, the rotation of the U nucleobase (180°) is observed in comparison to the DusA and C counterparts. **Right panel**, schematic representations of the Dus enzymes (orange line) with the N-terminal nucleobase-containing catalytic domain (round) and the C-terminal RNA binding domain (rectangle). The tRNA (gray line) docking is similar in DusB and C but differs by a rotation (160°) in DusA. These strategies (tRNA or nucleobase rotations) allow the unvariable targeting of a specific uridine.

[120]. The conclusion was the same for the dihydrouridyla-tion of the AUG (Met) codon [121,122]. These were the first data supporting that D-containing mRNAs could be transla-tionally repressed.

The mRNAs encoding several subunits of the CCT (cha-peronin-containing tailless complex polypeptide 1) complex, which is required for the folding of newly synthesized tubulin and actin proteins [123], contain D. A possibility to

investigate is that the D modification modulates the speed of translation to allow co-translational folding. Interestingly, Dus2 was shown to help in the detoxification of the amyloid-β peptides which form aggregates in the Alzheimer's disease [124].

As stated above, the alterations of the expression level of some Dus enzymes and the D landscape in cancers will be the topic of additional investigations to understand if translation is affected and to test if this is mediated by modified mRNAs and/or tRNAs.

Perspectives

It took almost 40 years from the first detection of dihydrouridine within tRNAs to the discovery of Dus enzymes, and 20 more years to expand the D landscape to the coding RNA world. Recent advances open up several interesting research directions.

A peculiarity of *E. coli* is the 23S-D_{2449} deposition is independent of the *dusA-B-C* genes [108]. To our knowledge, this is the first experimental evidence suggesting that D could be deposited by a *non-canonical* dihydrouridine synthase. In Bacteria, uracil can be reduced to dihydrouracil by the dihydropyrimidine dehydrogenase complex formed by PreA and PreT [125]. Noticeably, the dihydropyrimidine dehydrogenase is of clinical importance in human because deficiency in this enzyme (DPYD in *H. sapiens*) leads to a severe sensitivity to the administration of 5-fluorouracil, an agent used widely to treat cancer [126].

The catalytic activity of the DPYD enzyme is reversible, suggesting the immediate possibility that Dus enzymes may also function backward as D eraser to restore uracil from D in some circumstances. A proof of concept supporting this possibility was already reported [92].

It will also be of interest to explore the D landscape in plants where it is predicted to be present on mRNAs as well [127]. Interestingly, D could be a developmentally regulated modification in *A. thaliana* where the *DUS* orthologs are expressed at low level in rosette leaves and apex tissues, unlike other RNA modifying enzyme genes [128].

Deciphering the synthesis of a carboxypropylated dihydrouridine (acp^{3}D) from D in *T. brucei* is another important perspective to unravel the biology of this complex modification [129].

Another interesting possibility discussed above is the capacity for D to alter the recognition of RNA by interacting proteins. While the possibility of D readers will be investigated by classical unbiased approaches, a pilot study predicted 20 RNA binding-proteins (RBPs) to interact with D [114]. Interestingly, these RBPs are enriched for the alternative mRNA splicing process, including the SRSF9, SFPQ and ESRP2 splicing factors.

The abundant, simple yet fascinating D modification is only about to reveal its importance.

Acknowledgments

Disclosure statement

Funding

ORCID

Damien Hermand http://orcid.org/0000-0002-1029-5848

References

[1] Grosjean H. Nucleic acids are not boring long polymers of only four types of nucleotides: a guided tour. DNA and RNA modification enzymes: structure, mechanism, function and evolution. Landes Bioscience. 2009: 1–18.

[2] Lorenz C, Lunse CE, Morl M. tRNA modifications: impact on structure and thermal adaptation. Biomolecules. 2017;71:35.

[3] Sloan KE, Warda AS, Sharma S, et al. Tuning the ribosome: the influence of rRNA modification on eukaryotic ribosome biogenesis and function. RNA Biol. 2017;14:1138–1152.

[4] Desrosiers R, Friderici K, Rottman F. Identification of methylated nucleosides in messenger RNA from novikoff hepatoma cells. Proc Natl Acad Sci U S A. 1974;71:3971–3975.

[5] Boccaletto P, Stefaniak F, Ray A, et al. MODOMICS: a database of RNA modification pathways. 2021 update. Nucleic Acids Res. 2022;50:D231–D235.

[6] Helm M, Schmidt-Dengler MC, Weber M, et al. General principles for the detection of modified nucleotides in rna by specific reagents. 2021;Adv Biol (Weinh). 5:e2100866. 10.1002/adbi.202100866

[7] Roundtree IA, Evans ME, Pan T, et al. Dynamic RNA modifications in gene expression regulation. Cell. 2017;169:1187–1200.

[8] Green M, Cohen SS. Studies on the biosynthesis of bacterial and viral pyrimidines. II. Dihydrouracil and dihydrothymine nucleosides. J Biol Chem. 1957;225:397–407.

[9] Ehrlich A, Funk C, Merritt AJ. The isolation of hydrouracil from beef spleen. Arch Biochem Biophys. 1952;35:468–469.

[10] Carr DO, Grisolia S. Incorporation of Dihydrouridine monophosphate and uridine monophosphate into liver and brain ribonucleic acid. J Biol Chem. 1964;239:160–166.

[11] Royburman P, Royburman S, Visser DW. Incorporation of 5,6-dihydrouridine triphosphate into ribonucleic acid by DNA-dependent RNA polymerase. Biochem Biophys Res Commun. 1965;20:291–297.

[12] Holley RW, Apgar J, Everett GA, et al. Structure of a ribonucleic acid. Science. 1965;147:1462–1465.

[13] Madison JT, Holley RW. The presence of 5,6-Dihydrouridylic acid in yeast "soluble" ribonucleic acid. Biochem Biophys Res Commun. 1965;18:153–157.

[14] Davis DR. Biophysical and conformational properties of modified nucleotides in RNA. modification and editing of RNA. Washington (DC): American Society for Microbiology Press; 1998. p. 85–102.

[15] Dyubankova N, Sochacka E, Kraszewska K, et al. Contribution of dihydrouridine in folding of the D-arm in tRNA. Org Biomol Chem. 2015;13:4960–4966.

[16] Kwok CK. Dawn of the in vivo RNA structurome and interactome. Biochem Soc Trans. 2016;44:1395–1410.

[17] Kwok CK, Tang Y, Assmann SM, et al. The RNA structurome: transcriptome-wide structure probing with next-generation sequencing. Trends Biochem Sci. 2015;40:221–232.

[18] Kligun E, Mandel-Gutfreund Y. The role of RNA conformation in RNA-protein recognition. RNA Biol. 2015;12:720–727.

[19] Batt RD, J.k M, J.m P, et al. Chemistry of dihydropyrimidines. Ultraviolet spectra and alkaline decomposition. J Am Chem Soc. 1954;76:3663–3665.

[20] Cerutti P, Miller N. Selective reduction of yeast transfer ribonucleic acid with sodium borohydride. J Mol Biol. 1967;26:55–66.

[21] Igo-Kemenes T, Zachau HG. On the specificity of the reduction of transfer ribonucleic acids with sodium borohydride. Eur J Biochem. 1969;10:549–556.

[22] Jacobson M, Hedgcoth C. Determination of 5,6-dihydrouridine in ribonucleic acid. Anal Biochem. 1970;34:459–469.

[23] Betteridge T, Liu H, Gamper H, et al. Fluorescent labeling of tRNAs for dynamics experiments. RNA. 2007;13:1594–1601.

[24] Cerutti P, Holt JW, Miller N. Detection and determination of 5,6-dihydrouridine and 4-thiouridine in transfer ribonucleic acid from different sources. J Mol Biol. 1968a;34:505–518.

[25] Cerutti P, Kondo Y, Landis WR, et al. Photoreduction of uridine and reduction of dihydrouridine with sodium borohydride. J Am Chem Soc. 1968b;90:771–775.

[26] Cohn WE, Doherty DG. The catalytic hydrogenation of pyrimidine nucleosides and nucleotides and the isolation of their ribose and respective ribose phosphates. J Am Chem Soc. 1955;78:2863–2866.

[27] Pan D, Qin H, Cooperman BS. Synthesis and functional activity of tRNAs labeled with fluorescent hydrazides in the D-loop. RNA. 2009;15:346–354.

[28] Wintermeyer W, Schleich HG, Zachau HG. Incorporation of amines or hydrazines into tRNA replacing wybutine or dihydrouracil. Methods Enzymol. 1979;59:110–121.

[29] Wintermeyer W, Zachau HG. Replacement of Y base, dihydrouracil, and 7-methylguanine in tRNA by artificial odd bases. FEBS Lett. 1971;18:214–218.

[30] Wintermeyer W, Zachau HG. Replacement of odd bases in tRNA by fluorescent dyes. Methods Enzymol. 1974;29:667–673.

[31] House CH, Miller SL. Hydrolysis of dihydrouridine and related compounds. Biochemistry. 1996;35:315–320.

[32] Magrath DI, Shaw DC. The occurrence and source of beta-alanine in alkaline hydrolysates of sRNA: a sensitive method for the detection and assay of 5,6-dihydrouracil residues in RNA. Biochem Biophys Res Commun. 1967;26:32–37.

[33] Xing F, Hiley SL, Hughes TR, et al. The specificities of four yeast dihydrouridine synthases for cytoplasmic tRNAs. J Biol Chem. 2004;279:17850–17860.

[34] Beltchev B, Grunberg-Manago M. Preparation of a pG-fragment from tRNA(Phe)(yeast) by chemical scission at the dihydrouracil, and inhibition of tRNA(Phe)(yeast) charging by this fragment when combined with the -CCA half of this tRNA. FEBS Lett. 1970;12:24–26.

[35] Wintermeyer W, Zachau HG. A specific chemical chain scission of tRNA at 7-methylguanosine. FEBS Lett. 1970;11:160–164.

[36] Randerath K, Gupta RC, Randerath E. 3H and 32P derivative methods for base composition and sequence analysis of RNA. Methods Enzymol. 1980;65:638–680.

[37] Pomerantz S, McCloskey J. Analysis of RNA hydrolyzates by liquid chromatography-mass spectrometry. In: McCloskey JA, editor. Methods enzymol. Academic Press, Inc; 1990. p. 796–824.

[38] Topp H, Duden R, Schoch G. 5,6-Dihydrouridine: a marker ribonucleoside for determining whole body degradation rates of transfer RNA in man and rats. Clin Chim Acta. 1993;218:73–82.

[39] Cerutti P, Miles HT, Frazier J. Interaction of partially reduced polyuridylic acid with polyadenylic acid. Biochem Biophys Res Commun. 1966;22:466–472.

[40] Motorin Y, Muller S, Behm-Ansmant I, et al. Identification of modified residues in RNAs by reverse transcription-based methods. Methods Enzymol. 2007;425:21–53.

[41] Hiley SL, Jackman J, Babak T, et al. Detection and discovery of RNA modifications using microarrays. Nucleic Acids Res. 2005;33:e2.

[42] Kaur J, Raj M, Cooperman BS. Fluorescent labeling of tRNA dihydrouridine residues: mechanism and distribution. RNA. 2011;17:1393–1400.

[43] Machnicka MA, Olchowik A, Grosjean H, et al. Distribution and frequencies of post-transcriptional modifications in tRNAs. RNA Biol. 2014;11:1619–1629.

[44] Grosjean H, Houssier C, Romby P, et al. Modulation role of modified nucleotides in RNA loop-loop interaction. Washington (DC): American Society for Microbiology Press; 1998. p. 113–134.

[45] Vare VY, Eruysal ER, Narendran A, et al. Chemical and conformational diversity of modified nucleosides affects tRNA structure and function. Biomolecules. 2017;7 (1):29.

[46] Chawla M, Oliva R, Bujnicki JM, et al. An atlas of RNA base pairs involving modified nucleobases with optimal geometries and accurate energies. Nucleic Acids Res. 2015;43:6714–6729.

[47] Seelam PP, Sharma P, Mitra A. Structural landscape of base pairs containing post-transcriptional modifications in RNA. RNA. 2017;23:847–859.

[48] Calvo SE, Clauser KR, Mootha VK. MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. Nucleic Acids Res. 2016;44:D1251–1257.

[49] Suzuki T, Suzuki T. A complete landscape of post-transcriptional modifications in mammalian mitochondrial tRNAs. Nucleic Acids Res. 2014;42:7346–7357.

[50] Arcari P, Brownlee GG. The nucleotide sequence of a small (3S) seryl-tRNA (anticodon GCU) from beef heart mitochondria. Nucleic Acids Res. 1980;8:5207–5212.

[51] de Bruijn MH, Schreier PH, Eperon IC, et al. A mammalian mitochondrial serine transfer RNA lacking the "dihydrouridine" loop and stem. Nucleic Acids Res. 1980;8:5213–5222.

[52] de Bruijn MH, Klug A. A model for the tertiary structure of mammalian mitochondrial transfer RNAs lacking the entire 'dihydrouridine' loop and stem. EMBO J. 1983;2:1309–1321.

[53] Jones CI, Spencer AC, Hsu JL, et al. A counterintuitive Mg2+-dependent and modification-assisted functional folding of mitochondrial tRNAs. J Mol Biol. 2006;362:771–786.

[54] Xing F, Martzen MR, Phizicky EM. A conserved family of saccharomyces cerevisiae synthases effects dihydrouridine modification of tRNA. RNA. 2002;8:370–381.

[55] Huang RC, Bonner J. Histone-bound RNA, a component of native nucleohistone. Proc Natl Acad Sci U S A. 1965;54:960–967.

[56] Shih TY, Bonner J. Chromosomal RNA of calf thymus chromatin. Biochim Biophys Acta. 1969;182:30–35.

[57] Krol A, Gallinaro H, Lazar E, et al. The nuclear 5S RNAs from chicken, rat and man. U5 RNAs are encoded by multiple genes. Nucleic Acids Res. 1981;9:769–787.

[58] Johnson JD, Horowitz J. Characterization of ribosomes and RNAs from mycoplasma hominis. Biochim Biophys Acta. 1971;247:262–279.

[59] Kirpekar F, Hansen LH, Mundus J, et al. Mapping of ribosomal 23S ribosomal RNA modifications in Clostridium sporogenes. RNA Biol. 2018;15:1060–1070.

[60] Kowalak JA, Bruenger E, McCloskey JA. Posttranscriptional modification of the central loop of domain V in Escherichia coli 23S ribosomal RNA. J Biol Chem. 1995;270:17758–17764.

[61] Popova AM, Williamson JR. Quantitative analysis of rRNA modifications using stable isotope labeling and mass spectrometry. J Am Chem Soc. 2014;136:2058–2069.

[62] O'Connor M, Lee WM, Mankad A, et al. Mutagenesis of the peptidyltransferase center of 23S rRNA: the invariant U2449 is dispensable. Nucleic Acids Res. 2001;29:710–715.

[63] Emmerechts G, Barbe S, Herdewijn P, et al. Post-transcriptional modification mapping in the Clostridium acetobutylicum 16S rRNA by mass spectrometry and reverse transcriptase assays. Nucleic Acids Res. 2007;35:3494–3503.

[64] Chen X, Sim S, Wurtmann EJ, et al. Bacterial noncoding Y RNAs are widespread and mimic tRNAs. RNA. 2014;20:1715–1724.

[65] Bishop AC, Xu J, Johnson RC, et al. Identification of the tRNA-dihydrouridine synthase family. J Biol Chem. 2002;277:25090–25095.

[66] Bou-Nader C, Montemont H, Guerineau V, et al. Unveiling structural and functional divergences of bacterial tRNA

dihydrouridine synthases: perspectives on the evolution scenario. Nucleic Acids Res. 2018b;46:1386–1394.

[67] Faivre B, Lombard M, Fakroun S, et al. Dihydrouridine synthesis in tRNAs is under reductive evolution in mollicutes. RNA Biol. 2021;18:2278–2289.

[68] Kuchino Y, Borek E. Tumour-specific phenylalanine tRNA contains two supernumerary methylated bases. Nature. 1978;271:126–129.

[69] Jiang HP, Chu JM, Lan MD, et al. Comprehensive profiling of ribonucleosides modification by affinity zirconium oxide-silica composite monolithic column online solid-phase microextraction - mass spectrometry analysis. J Chromatogr A. 2016;1462:90–99.

[70] Reimer ML, Schram KH, Nakano K, et al. The identification of 5,6-dihydrouridine in normal human urine by combined gas chromatography/mass spectrometry. Anal Biochem. 1989;181:302–308.

[71] Borek E, Baliga BS, Gehrke CW, et al. High turnover rate of transfer RNA in tumor tissue. Cancer Res. 1977;37:3362–3366.

[72] Dudley E, Bond L. Mass spectrometry analysis of nucleosides and nucleotides. Mass Spectrom Rev. 2014;33:302–331.

[73] Sridharan G, Ramani P, Patankar S. Serum metabolomics in oral leukoplakia and oral squamous cell carcinoma. J Cancer Res Ther. 2017;13:556–561.

[74] Licha D, Vidali S, Aminzadeh-Gohari S, et al. Untargeted metabolomics reveals molecular effects of ketogenic diet on healthy and tumor xenograft mouse models. Int J Mol Sci. 2019;20 (16):3873.

[75] Huang J, Mondul AM, Weinstein SJ, et al. Prospective serum metabolomic profiling of lethal prostate cancer. Int J Cancer. 2019;145:3231–3243.

[76] Mittelstadt M, Frump A, Khuu T, et al. Interaction of human tRNA-dihydrouridine synthase-2 with interferon-induced protein kinase PKR. Nucleic Acids Res. 2008;36:998–1008.

[77] Luo X, Wang CZ, Chen J, et al. Characterization of gene expression regulated by American ginseng and ginsenoside Rg3 in human colorectal cancer cells. Int J Oncol. 2008;32:975–983.

[78] Kato T, Daigo Y, Hayama S, et al. A novel human tRNA-dihydrouridine synthase involved in pulmonary carcinogenesis. Cancer Res. 2005;65:5638–5646.

[79] Cancer genome atlas research N. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature. 2013;499:43–49.

[80] Cancer genome atlas research N, Linehan WM, Spellman PT, et al. Comprehensive molecular characterization of papillary renal-cell carcinoma. N Engl J Med. 2016;374:135–145.

[81] Buchser WJ, Smith RP, Pardinas JR, et al. Peripheral nervous system genes expressed in central neurons induce growth on inhibitory substrates. PLoS One. 2012;7:e38101.

[82] Basanta-Sanchez M, Temple S, Ansari SA, et al. Attomole quantification and global profile of RNA modifications: epitranscriptome of human neural stem cells. Nucleic Acids Res. 2016;44:e26.

[83] Dalluge JJ, Hamamoto T, Horikoshi K, et al. Posttranscriptional modification of tRNA in psychrophilic bacteria. J Bacteriol. 1997;179:1918–1923.

[84] Dalluge JJ, Hashizume T, Sopchik AE, et al. Conformational flexibility in RNA: the role of dihydrouridine. Nucleic Acids Res. 1996b;24:1073–1079.

[85] Deslauriers R, Lapper RD, Smith IC. A proton magnetic resonance study of the molecular conformation of a modified nucleoside from transfer RNA. Dihydrouridine. Can J Biochem. 1971;49:1279–1284.

[86] Kusuba H, Yoshida T, Iwasaki E, et al. In vitro dihydrouridine formation by tRNA dihydrouridine synthase from thermus thermophilus, an extreme-thermophilic eubacterium. J Biochem. 2015;158:513–521.

[87] Liang WD, Bi YT, Wang HY, et al. Gene expression profiling of Clostridium botulinum under heat shock stress. Biomed Res Int. 2013;2013:760904.

[88] Sun QL, Sun YY, Zhang J, et al. High temperature-induced proteomic and metabolomic profiles of a thermophilic bacillus manusensis isolated from the deep-sea hydrothermal field of manus basin. J Proteomics. 2019;203:103380.

[89] Noon KR, Guymon R, Crain PF, et al. Influence of temperature on tRNA modification in archaea: methanococcoides burtonii (optimum growth temperature [Topt], 23 degrees C) and Stetteria hydrogenophila (Topt, 95 degrees C). J Bacteriol. 2003;185:5483–5490.

[90] Wang H, Simpson JH, Kotra ME, et al. Epitranscriptomic profile of Lactobacillus agilis and its adaptation to growth on inulin. BMC Res Notes. 2021;14:154.

[91] Lombard M, Hamdane D. Flavin-dependent epitranscriptomic world. Arch Biochem Biophys. 2017;632:28–40.

[92] Rider LW, Ottosen MB, Gattis SG, et al. Mechanism of dihydrouridine synthase 2 from yeast and the importance of modifications for efficient tRNA reduction. J Biol Chem. 2009;284:10324–10333.

[93] Yu F, Tanaka Y, Yamashita K, et al. Molecular basis of dihydrouridine formation on tRNA. Proc Natl Acad Sci U S A. 2011;108:19593–19598.

[94] Kasprzak JM, Czerwoniec A, Bujnicki JM. Molecular evolution of dihydrouridine synthases. BMC Bioinformatics. 2012;13:153.

[95] Savage DF, de Crecy-Lagard V, Bishop AC. Molecular determinants of dihydrouridine synthase activity. FEBS Lett. 2006;580:5198–5202.

[96] Park F, Gajiwala K, Noland B, et al. The 1.59 A resolution crystal structure of TM0096, a flavin mononucleotide binding protein from Thermotoga maritima. Proteins. 2004;55:772–774.

[97] Byrne RT, Jenkins HT, Peters DT, et al. Major reorientation of tRNA substrates defines specificity of dihydrouridine synthases. Proc Natl Acad Sci U S A. 2015;112:6033–6037.

[98] Chen M, Yu J, Tanaka Y, et al. Structure of dihydrouridine synthase C (DusC) from Escherichia coli. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2013;69:834–838.

[99] Goyal N, Chandra A, Qamar I, et al. Structural studies on dihydrouridine synthase A (DusA) from Pseudomonas aeruginosa. Int J Biol Macromol. 2019;132:254–264.

[100] Griffiths S, Byrne RT, Antson AA, et al. Crystallization and preliminary X-ray crystallographic analysis of the catalytic domain of human dihydrouridine synthase. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2012;68:333–336.

[101] Bou-Nader C, Pecqueur L, Bregeon D, et al. An extended dsRBD is required for post-transcriptional modification in human tRNAs. Nucleic Acids Res. 2015;43:9446–9456.

[102] Bou-Nader C, Barraud P, Pecqueur L, et al. Molecular basis for transfer RNA recognition by the double-stranded RNA-binding domain of human dihydrouridine synthase 2. Nucleic Acids Res. 2019a;47:3117–3126.

[103] Bou-Nader C, Bregeon D, Pecqueur L, et al. Electrostatic potential in the trna binding evolution of Dihydrouridine synthases. Biochemistry. 2018a;57:5407–5414.

[104] Marchand V, Ayadi L, Ernst FGM, et al. AlkAniline-seq: profiling of m(7) G and m(3) C RNA modifications at single nucleotide resolution. Angew Chem Int Ed Engl. 2018;57:16785–16790.

[105] Marchand V, Bourguignon-Igel V, Helm M, et al. Mapping of 7-methylguanosine (m(7)G), 3-methylcytidine (m(3)C), dihydrouridine (D) and 5-hydroxycytidine (ho(5)C) RNA modifications by AlkAniline-Seq. Methods Enzymol. 2021;658:25–47.

[106] Dai W, Li A, Yu NJ, et al. Activity-based RNA-modifying enzyme probing reveals DUS3L-mediated dihydrouridylation. Nat Chem Biol. 2021;17:1178–1187.

[107] Liao Y, Castello A, Fischer B, et al. The Cardiomyocyte RNA-binding proteome: links to intermediary metabolism and heart disease. Cell Rep. 2016;16:1456–1469.

[108] Finet O, Yague-Sanz C, Kruger LK, et al. Transcription-wide mapping of dihydrouridine reveals that mRNA dihydrouridylation is required for meiotic chromosome segregation. Mol Cell. 2022b;82:404–419 e409.

[109] Finet O, Yague-Sanz C, Hermand D Epitranscriptomic mapping of RNA modifications at single-nucleotide resolution using rhodamine sequencing (Rho-seq). STAR Protoc. 2022;3(2):101369 . DOI:10.1016/j.xpro.2022.101369

[110] Beckmann BM, Horos R, Fischer B, et al. The RNA-binding proteomes from yeast to man harbour conserved enigmRBPs. Nat Commun. 2015;6:10127.

[111] Mitchell SF, Jain S, She M, et al. Global analysis of yeast mRNPs. Nat Struct Mol Biol. 2013;20:127–133.

[112] Dou L, Zhou W, Zhang L, et al. Accurate identification of RNA D modification using multiple features. RNA Biol. 2021;18:2236–2246.

[113] Feng P, Xu Z, Yang H, et al. Identification of D modification sites by integrating heterogeneous features in saccharomyces cerevisiae. Molecules. 2019;24(3):380.

[114] Salekin S, Mostavi M, Chiu YC, et al. Predicting sites of epitranscriptome modifications using unsupervised representation learning based on generative adversarial networks. Front Phys. 2020;8. DOI:10.3389/fphy.2020.00196

[115] Xu ZC, Feng PM, Yang H, et al. iRNAD: a computational tool for identifying D modification sites in RNA sequence. Bioinformatics. 2019;35:4922–4929.

[116] Thomas NK, Poodari VC, Jain M, et al. Direct nanopore sequencing of individual full length tRNA strands. ACS Nano. 2021;15:16642–16653.

[117] Schwartz S, Agarwala SD, Mumbach MR, et al. High-resolution mapping reveals a conserved, widespread, dynamic mRNA methylation program in yeast meiosis. Cell. 2013;155:1409–1421.

[118] Schwartz S, Bernstein DA, Mumbach MR, et al. Transcriptome-wide mapping reveals widespread dynamic-regulated pseudouridylation of ncRNA and mRNA. Cell. 2014;159:148–162.

[119] Rottman F, Cerutti P. Template activity of uridylic acid-dihydrouridylic acid copolymers. Proc Natl Acad Sci U S A. 1966;55:960–966.

[120] Smrt J, Skoda J, Lisy V, et al. Loss of coding properties of the trinucleotide guanylyl-uridylyl-uridine on replacement of uridylic by dihydrouridylic acid. Biochim Biophys Acta. 1966;129:210–211.

[121] Lee S, Brown GL, Kosinski Z. Loss of coding properties of the oligonucleotide adenylyluridylylguanosine after photoreduction or hydration. Biochem J. 1967;103:25C–27C.

[122] Smrt J, Kemper W, Caskey T, et al. Template activity of modified terminator codons. J Biol Chem. 1970;245:2753–2757.

[123] Vallin J, Grantham J. The role of the molecular chaperone CCT in protein folding and mediation of cytoskeleton-associated processes: implications for cancer cell biology. Cell Stress Chaperones. 2019;24:17–27.

[124] Chen X, Ji B, Hao X, et al. FMN reduces Amyloid-beta toxicity in yeast by regulating redox status and cellular metabolism. Nat Commun. 2020;11:867.

[125] Hidese R, Mihara H, Kurihara T, et al. Escherichia coli dihydropyrimidine dehydrogenase is a novel NAD-dependent heterotetramer essential for the production of 5,6-dihydrouracil. J Bacteriol. 2011;193:989–993.

[126] Schnackerz KD, Dobritzsch D, Lindqvist Y, et al. Dihydropyrimidine dehydrogenase: a flavoprotein with four iron-sulfur clusters. Biochim Biophys Acta. 2004;1701:61–74.

[127] Vandivier LE, Campos R, Kuksa PP, et al. Chemical modifications mark alternatively spliced and Uncapped messenger RNAs in arabidopsis. Plant Cell. 2015;27:3024–3037.

[128] Chen P, Jager G, Zheng B. Transfer RNA modifications and genes for modifying enzymes in arabidopsis thaliana. BMC Plant Biol. 2010;10:201.

[129] Krog JS, Espanol Y, Giessing AM, et al. 3-(3-amino-3-carboxy-propyl)-5,6-dihydrouridine is one of two novel post-transcriptional modifications in tRNALys(UUU) from Trypanosoma brucei. FEBS J. 2011;278:4782–4796.

[130] Bou-Nader C, Pecqueur L, Barraud P, et al. Conformational stability adaptation of a double-stranded rna-binding domain to transfer RNA ligand. Biochemistry. 2019b;58:2463–2473.

[131] Guimaraes BG, Golinelli-Pimpaneau B. De novo crystal structure determination of double stranded RNA binding domain using only the sulfur anomalous diffraction in SAD phasing. 2021; Curr Res Struct Biol. 3:112–120. 10.1016/j.crstbi.2021.05.002

[132] Molinaro M, Sheiner LB, Neelon FA, et al. Effect of chemical modification of dihydrouridine in yeast transfer ribonucleic acid on amino acid acceptor activity and ribosomal binding. J Biol Chem. 1968;243:1277–1282.

[133] Whelan F, Jenkins HT, Griffiths SC, et al. From bacterial to human dihydrouridine synthase: automated structure determination. Acta Crystallogr D Biol Crystallogr. 2015;71:1564–1571.

[134] Dalluge JJ, Hashizume T, McCloskey JA. Quantitative measurement of dihydrouridine in RNA using isotope dilution liquid chromatography-mass spectrometry (LC/MS). Nucleic Acids Res. 1996a;24:3242–3245.

[135] Davis DR, Griffey RH, Yamaizumi Z, et al. 15N-labeled tRNA. Identification of dihydrouridine in Escherichia coli tRNAfMet, tRNALys, and tRNAPhe by 1H-15N two-dimensional NMR. J Biol Chem. 1986;261:3584–3587.

[136] Deb I, Sarzynska J, Nilsson L, et al. Rapid communication capturing the destabilizing effect of dihydrouridine through molecular simulations. Biopolymers. 2014;101:985–991.

[137] Formoso C, Tinoco I Jr. Minor nucleosides in RNA: optical studies of dinucleoside phosphates containing dihydrouridine. Biopolymers. 1971;10:1533–1541.

[138] Jack A, Ladner JE, Klug A. Crystallographic refinement of yeast phenylalanine transfer RNA at 2-5A resolution. J Mol Biol. 1976;108:619–649.

[139] Quigley GJ, Rich A. Structural domains of transfer RNA molecules. Science. 1976;194:796–806.

[140] Rohrer DC, Sundaralingam M. Stereochemistry of nucleic acids and their constituents. VI. The crystal structure and conformation of dihydrouracil: a minor base of transfer-ribonucleic acid. Acta Crystallogr B. 1970;26:546–553.

[141] Suck D, Saenger W, Hobbs J. Molecular and crystal structure of 2'-chloro-2'-deoxyuridine. Biochim Biophys Acta. 1972;259:157–163.

[142] Suck D, Saenger W, Zechmeister K. Conformation of the tRNA minor constituent dihydrouridine. FEBS Lett. 1971;12:257–259.

[143] Sundaralingam M, Rao ST, Abola J. Molecular conformation of dihydrouridine: puckered base nucleoside of transfer RNA. Science. 1971a;172:725–727.

[144] Westhof E, Dumas P, Moras D. Crystallographic refinement of yeast aspartic acid transfer RNA. J Mol Biol. 1985;184:119–145.

[145] Westhof E, Sundaralingam M. Restrained refinement of the monoclinic form of yeast phenylalanine transfer RNA. Temperature factors and dynamics, coordinated waters, and base-pair propeller twist angles. Biochemistry. 1986;25:4868–4878.

[146] Woo NH, Roe BA, Rich A. Three-dimensional structure of Escherichia coli initiator tRNAfMet. Nature. 1980;286:346–351.

[147] Moreau C, Ashamu GA, Bailey VC, et al. Synthesis of cyclic adenosine 5'-diphosphate ribose analogues: a C2'endo/syn "southern" ribose conformation underlies activity at the sea urchin cADPR receptor. Org Biomol Chem. 2011;9:278–290.

[148] Sehnal D, Bittrich S, Deshpande M, et al. Mol* viewer: modern web app for 3D visualization and analysis of large biomolecular structures. Nucleic Acids Res. 2021;49:W431–W437.

[149] Sundaralingam M, Rao ST, Abola J. Stereochemistry of nucleic acids and their constituents. 23. Crystal and molecular structure of dihydrouridine "hemihydrate", a rare nucleoside with a saturated base occurring in the dihydrouridine loop of transfer ribonucleic acids. J Am Chem Soc. 1971b;93:7055–7062.

[150] Stuart JW, Basti MM, Smith WS, et al. Structure of the trinucleotide D-acp3u-A with coordinated Mg2+ demonstrates that modified nucleosides contribute to regional conformations of RNA. Nucl Nucl. 1996;15:1009–1028.

[151] Peng WT, Robinson MD, Mnaimneh Set al. A panoramic view of yeast noncoding RNA processing. Cell. 2003;113:919–933.

Additional references from Figures:

Bou-Nader et al. [130], Guimaraes and Golinelli-Pimpaneau [131], Molinaro et al. [132], Whelan et al. [133].

Dalluge et al. [134], Davis et al. [135], Deb et al. [136], Deslauriers et al. [85], Formoso and Tinoco, Jack et al. [137], Quigley and Rich [138], Rohrer and Sundaralingam [139], Suck et al. [140], Suck et al. [141], Sundaralingam et al. [142], Westhof et al. [143], Westhof and Sundaralingam [144], Woo et al. [145], [146].

Movie 1. The contribution of D in folding of the D loop.

Predicted non-covalent interactions are indicated by dashed lines: hydrogen bonds in blue, others in green. Dihydrouridine is colored red and is pointing away from the loop. The stem loop sequence from tRNA$^{\text{Met}}$ is GGAGAG**D**GGAACUCC (https://www.rcsb.org/structure/2MN0). The animation was created with Mol* [148].