The epitranscriptome in ageing and stress resistance: A systematic review

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ABSTRACT

Modifications of RNA, collectively called the “epitranscriptome”, might provide novel biomarkers and innovative targets for interventions in geroscience but are just beginning to be studied in the context of ageing and stress resistance. RNA modifications modulate gene expression by affecting translation initiation and speed, miRNA binding, RNA stability, and RNA degradation. Nonetheless, the precise underlying molecular mechanisms and physiological consequences of most alterations of the epitranscriptome are still only poorly understood. We here systematically review different types of modifications of rRNA, tRNA and mRNA, the methodology to analyze them, current challenges in the field, and human disease associations. Furthermore, we compiled evidence for a connection between individual enzymes, which install RNA modifications, and lifespan in yeast, worm and fly. We also included resistance to different stressors and competitive fitness as search criteria for genes potentially relevant to ageing. Promising candidates identified by this approach include RCM1/NSUN5, RRP8, and F33A8.4/ZCCHC4 that introduce base methylations in rRNA, the methyltransferases DNMT2 and TRM9/ALKBH8, as well as factors involved in the thiolation or A to I editing in tRNA, and finally the m^6^A machinery for mRNA.

1. Introduction

One of the most fundamental questions of life is how organisms age and how we might counteract the continuous increase of frailty in our society. The development of epigenetic “ageing clocks” based on the global mapping of DNA modifications in different human tissues significantly advanced the field (Horvath and Raj, 2018). Ageing clocks are important because they represent robust epigenetic biomarkers of ageing, which are helpful to objectively assess the individual risk of acquiring diseases and the outcome of therapeutic interventions. In contrast to DNA modifications, modifications of RNA, collectively called the “epitranscriptome”, are just beginning to be studied in this context and might provide novel biomarkers and innovative targets for interventions in geroscience.

Modifications of ribosomal RNA (rRNA), transfer RNA (tRNA), and messenger RNA (mRNA) all converge on protein synthesis by ribosomes. They modulate translation initiation and speed, miRNA binding, stability, and degradation (Peer et al., 2017). Remarkably, an increasing number of studies in the ageing field report significant discrepancies between the transcriptome and the proteome (Anisimova et al., 2020), which would indicate that profound changes in translational regulation of gene expression occur in ageing. Indeed, protein synthesis is considered one of the evolutionary best conserved and most promising targets to extend the healthy lifespan of a wide range of organisms, including vertebrates (Harrison et al., 2009; Selman et al., 2009). Depleting ribosomal proteins and other factors involved in translation extends the lifespan in Saccharomyces cerevisiae (Janssens et al., 2015; Steffen et al., 2008) and Caenorhabditis elegans (Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007). Interestingly, while global protein synthesis decreases, specific transcripts can be preferentially translated (Rogers et al., 2011). Similarly, reducing the activity of the mTOR pathway by dietary restriction, genetic manipulations, or rapamycin exposure affects translation at multiple levels (Liu and Sabatini, 2020).

In humans, the epitranscriptome is implicated in several human congenital diseases, premature ageing and age-related diseases, as recently reviewed by McMahon and colleagues (McMahon et al., 2021). Moreover, mutations in two A to I editing enzymes (Sebastiani et al., 2011) and one m^6^A reader (Cardelli et al., 2006) are associated with extreme longevity. A recent study demonstrated in mice that three of the most promising compounds promoting healthy ageing to date, all...
rapamycin, acarbose, and 17α-estradiol, all affect cap-independent translation, which m^6^A-modifications of mRNA can modulate. Indeed, METTL3 and METTL14, two enzymes involved in adding m^6^A, showed differential expression in ageing and upon exposure to these drugs (Shen et al., 2021). Although RNA modification levels change throughout the lifespan of mammals (McMahon et al., 2021), causal links between epitranscriptomic changes and human ageing are still lacking.

Apart from these data, our current knowledge of the role of RNA modifications in ageing relies exclusively on a few studies investigating single RNA modifying enzymes modulating simple model organisms longevity (Heissenberger et al., 2020; Liberman et al., 2020; Schosserer et al., 2015). Although a prerequisite for identifying robust biomarkers, an integrated view of the global landscape of RNA modifications utilising different cellular and organismal ageing models is still lacking. Moreover, the underlying molecular mechanisms and physiological consequences of most RNA modifications are only poorly understood (Table 1).

To facilitate future studies of the epitranscriptome in the context of ageing, we compiled in this review available data on RNA modifying enzymes that influence the lifespan of the model organisms *Saccharomyces cerevisiae* (Fig. 1), *Caenorhabditis elegans* (Fig. 2), and *Drosophila melanogaster* (Fig. 3). Increased resistance to stressors like oxidising agents, UV exposure, or heat shock (Holzenberger et al., 2003; Lithgow et al., 1995; Lithgow and Walker, 2002; Longo, 2003; Murakami and Johnson, 1996; Wang et al., 2003) is often associated with an increased healthy lifespan. To account for this connection, we included resistance to oxidative-, heat-, cold-, radiation-, starvation-stress, and competitive fitness as search criteria for genes potentially relevant to the ageing process. The primary database resources we used are the "Saccharomyces Genome Database" (Cherry et al., 2012), "Wormbase" (Harris et al., 2020) and "Flybase" (Larkin et al., 2021), as well as "GenAge: The Ageing Gene Database" (Tacutu et al., 2018). Thereby, we identified several genes catalysing the addition of epitranscriptomic marks (Fig. 4), which might modulate ageing and stress resistance across the different model organisms. In the following, we will discuss the different types of rRNA, tRNA, and mRNA modifications and current evidence for their involvement in ageing and stress resistance. We will also address human homologs of the identified genes and their human disease associations (Table 2). For a more detailed discussion of RNA modifications in human congenital and age-associated pathologies, we refer interested readers to the excellent review by McMahon and colleagues (McMahon et al., 2021).

### 2. Modifications of ribosomal RNAs

Approximately 2% of all nucleotides in rRNA are modified, rendering rRNA the second most modified RNA species after tRNA (Sloan et al., 2017). RNA modifications are introduced by either stand-alone enzymes or enzymes gaining specificity by interacting with small nucleolar RNAs (snRNAs) within box C/D and box H/ACA small nucleolar ribonucleoprotein particles (snoRNPs). Stand-alone enzymes predominantly catalyse methylation of RNA bases, such as m^6^C or m^6^A, and complex modifications. Box C/D snoRNPs introduce 2′-O-methylations (2′-O-Me) with yeast Nop1p (fibrillarin in mammals) acting as the catalytic entity (Kiss-Lázsló et al., 1996; Tollery et al., 1993), while box H/ACA snoRNPs introduce pseudouridines (Ψ) catalysed by Cbf5p (dyskerin in mammals) (Ganot et al., 1997; Lafontaine et al., 1998). Both 2′-O-Me and Ψ account for the majority of modifications in all four eukaryotic rRNAs, with approximately 100 modified bases each in humans, while only around one-tenth of all modifications are base modifications (Sloan et al., 2017).

Eveitranscriptomic marks of rRNA primarily reside in the regions necessary for ribosome function, like the decoding centre, tRNA-binding sites, the peptidyltransferase centre, and the intersubunit interface (Sloan et al., 2017). They stabilise the structure of the ribosome (Baxter-Roshek et al., 2007; Gigova et al., 2014; Helm, 2006; King et al., 2003) and are essential for translational function and fidelity (Baudin-Bailieu et al., 2009; King et al., 2003; Lafontaine et al., 1998; Liang et al., 2009, 2007; Schosserer et al., 2015). Consequently, altered ribosome performance potentially caused by changed levels of rRNA modifications can participate in the pathology of human diseases, for example, the Bowen-Conradi syndrome (Armistead et al., 2009), Williams-Beuren syndrome (Doll and Grzeschik, 2001), or X-linked dyskeratosis congenita (Heiss et al., 1998).

#### 2.1. m^5^C methyltransferases

While most of the information on RNA modifications in the context of ageing and stress resistance originates from high throughput reverse-genetic screens, the yeast m^5^C methyltransferase Rcm1p (NSUN5 in other organisms) and its *C. elegans*, *D. melanogaster*, *M. musculus* and *H. sapiens* homologs have been characterised on a single gene basis. Ribosomal RNAs in eukaryotes contain only two m^5^C modifications, both located in highly conserved regions of the large subunit. We and others demonstrated that Rcm1p and its homologs deposit one of the two m^5^C modifications at position C^3381^ in 25S (S. cerevisiae), C^3281^ in 26S (C. elegans), C^3438^ (M. musculus), and C^3492^ in 28S (H. sapiens) rRNA (Gigova et al., 2014; Heissenberger et al., 2019; Janin et al., 2019; Schosserer et al., 2015; Sharma et al., 2013). While NSUN5 is not an

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**Table 1**

| ID | Reference |
|----|-----------|
| 1  | (Garay et al., 2014) |
| 2  | (Brown et al., 2006) |
| 3  | (Deutschbauer et al., 2005) |
| 4  | (Breslow et al., 2008) |
| 5  | (Qian et al., 2012) |
| 6  | (Sinha et al., 2008) |
| 7  | (Gustavsson and Ronne, 2008) |
| 8  | (Auesukaree et al., 2009) |
| 9  | (Higgins et al., 2002) |
| 10 | (Endres et al., 2020) |
| 11 | (Li et al., 2009) |
| 12 | (Ruiz-Roig et al., 2010) |
| 13 | (Ben-Anya et al., 2008) |
| 14 | (Kadaba et al., 2004) |
| 15 | (Angelova et al., 2020) |
| 16 | (Moraru et al., 2017) |
| 17 | (Fabrizio et al., 2010) |
| 18 | (Davey et al., 2012) |
| 19 | (Marek and Korona, 2013) |
| 20 | (Figuero et al., 2012) |
| 21 | (Giaever et al., 2002) |
| 22 | (Hanway et al., 2002) |
| 23 | (Eisenberg et al., 2009) |
| 24 | (Jarolim et al., 2013) |
| 25 | (Honjo et al., 2016) |
| 26 | (Curran and Ruvkun, 2007) |
| 27 | (Peifer et al., 2013) |
| 28 | (Lapeyre and Purushothaman, 2004) |
| 29 | (Schosserer et al., 2015) |
| 30 | (Burner et al., 2011) |
| 31 | (Pintard, 2002) |
| 32 | (Sendinc et al., 2020) |
| 33 | (Liberman et al., 2020) |
| 34 | (Schafer et al., 2010) |
| 35 | (Liu et al., 2005) |
| 36 | (Ma et al., 2001) |
| 37 | (Khosnood et al., 2016) |
| 38 | (Tian et al., 2014) |
| 39 | (Lence et al., 2016) |
| 40 | (Kim and Sun, 2007) |
| 41 | (Navarro-Gonzalez et al., 2017) |
| 42 | (Garay et al., 2014) |
| 43 | (Sebastián et al., 2009) |
Fig. 1. The loss of RNA modifying enzymes influences the lifespan and stress resistance of *S. cerevisiae*. Yeast genes that show an ageing or stress-related phenotype upon knockout or knockdown are displayed in blue for an increase ("Increased") or red for a decrease ("Decreased") of the respective trait. Black indicates that both increases and decreases have been reported ("Both"). Phenotypes not yet described in the literature for the individual gene are shown in grey ("Nd"). Numbers correspond to the studies which reported the phenotypes (Table 1). CLS: chronological lifespan, RLS: replicative lifespan, CF: competitive fitness, OR: oxidative stress resistance, HR: heat resistance, CR: cold resistance, SR: starvation resistance, UR: UV radiation resistance. *For snR84, overexpression was tested and led to the observed phenotypes. **Additional sources for ISU2 CLS are "46" and "47", for ELP4 OR "61", for MTO1 CLS "47" and for DEG1 OR "64". # Trm112p interacts with Trm9p and Trm11p for the formation of the respective tRNA modifications and with Bud23p for the rRNA modification.

### tRNA modifications

| Gene      | CLS | RLS | OR  | CR  | SR  | UR  | CF  | mcm^5^U | mcm^2^U | mcm^5^U | mcm^2^U |
|-----------|-----|-----|-----|-----|-----|-----|-----|---------|---------|---------|---------|
| URM1      |     | 98  | 19  | 18  |     |     |     |         |         |         |         |
| UBA4      | 2   | 9   | 2   | 2   |     |     |     |         |         |         |         |
| TUM1      | 18  | 9   | 2   | 2   |     |     |     |         |         |         |         |
| TRM8      | 61  | 14  | 12  |     |     |     |     |         |         |         |         |
| ELPA15    | 62  | 14  | 2   | 2   | 2   | 2   | 2   |         |         |         |         |
| ELP4      | 58  | 20  | 12  |     |     |     |     |         |         |         |         |
| ELPA6     | 58  | 20  | 12  |     |     |     |     |         |         |         |         |
| MTO1      | 62  | 20  | 12  |     |     |     |     |         |         |         |         |
| MSS1      | 58  | 20  | 12  |     |     |     |     |         |         |         |         |
| TYW3      | 58  | 20  | 12  |     |     |     |     |         |         |         |         |
| TYW1      | 58  | 20  | 12  |     |     |     |     |         |         |         |         |
| TRM12     |     |     |     |     |     |     |     |         |         |         |         |
| PPM2      |     |     |     |     |     |     |     |         |         |         |         |

### mRNA modifications

| Gene      | CLS | RLS | OR  | CR  | SR  | UR  | CF  | sU   | cmcm^5^sU | m^5^A  |
|-----------|-----|-----|-----|-----|-----|-----|-----|------|-----------|--------|
| KRE33     |     |     |     |     |     |     |     |      | a^c^C     | m^5^A  |
| SLYZ1     |     |     |     |     |     |     |     |      | a^c^C     | m^5^A  |
| MUM2      | 46  | 44  | 44  |     |     |     |     |      | a^c^C     | m^5^A  |
| IME4      | 47  | 47  | 47  |     |     |     |     |      | a^c^C     | m^5^A  |
| PUS5      | 44  | 44  | 44  |     |     |     |     |      | a^c^C     | m^5^A  |
| PUS4      | 44  | 44  | 44  |     |     |     |     |      | a^c^C     | m^5^A  |
| PUS2      | 44  | 44  | 44  |     |     |     |     |      | a^c^C     | m^5^A  |
| PUS1      | 44  | 44  | 44  |     |     |     |     |      | a^c^C     | m^5^A  |
| DEG1**    |     |     |     |     |     |     |     |      | a^c^C     | m^5^A  |
| CFB5      |     |     |     |     |     |     |     |      | a^c^C     | m^5^A  |

### rRNA modifications

| Gene      | CLS | RLS | OR  | CR  | SR  | UR  | CF  | 2-O-m | mA  | a^c^C |
|-----------|-----|-----|-----|-----|-----|-----|-----|-------|-----|------|
| SPB1      |     |     |     |     |     |     |     |       |     |      |
| NOP56     |     |     |     |     |     |     |     |       |     |      |
| NOP1      |     |     |     |     |     |     |     |       |     |      |
| MRM2      |     |     |     |     |     |     |     |       |     |      |
| MRM1      |     |     |     |     |     |     |     |       |     |      |
| KRE33     |     |     |     |     |     |     |     |       |     |      |
| RRP8      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| BTM2      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| TSR3      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| EMG1      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| DIM1      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| BMT6      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| BMT5      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| RCM1      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| NOP2      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| BUD23     | 3   | 3   | 3   |     |     |     |     |       |     |      |
| SNR84     | 3   | 3   | 3   |     |     |     |     |       |     |      |
| PUS7      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| PUS5      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| NHP2      | 3   | 3   | 3   |     |     |     |     |       |     |      |
| CFB5      | 3   | 3   | 3   |     |     |     |     |       |     |      |

### Phenotype Qualifier

- Increased
- Decreased
- Both
- Nd
essential gene in any of the organisms tested, loss of the methyltransferase led to diet-dependent lifespan extension in *C. elegans* and *D. melanogaster* (Schosserer et al., 2015). Knockout also increased chronological life span (CLS) in *S. cerevisiae*. In the same study, the knockout of RCM1 decreased replicative life span (RLS), which agrees with other reports showing a frequent lack of correlation between CLS and RLS (Laun et al., 2006). In contrast, in a high-throughput study by Campos and colleagues, RCM1 knockout led to significantly decreased CLS under dietary restriction, induced by gamma-aminobutyric acid (GABA) as the sole nitrogen source (Campos et al., 2018). In terms of stress resistance, loss of the methyltransferase increased resistance to heat in flies and yeast and increased paraquat- and H$_2$O$_2$-tolerance in worms and yeast, respectively (Schosserer et al., 2015). Moreover, strains lacking Rcm1p together with snoRNAs guiding other methylations in the same region were outcompeted by the wild-type strain in growth competition assays, indicating that modifications in this region are necessary for overall cellular fitness (Gigova et al., 2014). In mammalian cell lines, deletion of *NSUN5* caused decreased cell size, slower proliferation, and reduced protein synthesis, while in *Nsun5* knockout mice, a reduction of lean body mass was observed (Heissenberger et al., 2019). In humans, *NSUN5* is among the genes deleted in Williams-Beuren syndrome (Doll and Grzeschik, 2001). If *NSUN5* depletion is sufficient to increase the healthspan of mice remains to be elucidated. Moreover, systematic studies of the connection between *NSUN5* function and the metabolic status of cells and organisms will be required for a better mechanistic understanding of the phenotypes caused by *NSUN5* loss.

Another NOP2/Sun RNA methyltransferase family member, *nsun-1* (also known as *nol-2*), is an essential gene, and its product catalyses the second m$^4$C modification in *C. elegans* 26S rRNA at position C$_{2982}$. (Heißenberger et al., 2020). Post-developmental RNA interference (RNAi)-mediated knockdown of *nsun-1* in an *eri-1* deficient worm strain increased lifespan in a high-throughput screen (Curran and Ruvkun, 2007). Compared to that, another study in *C. elegans* did not observe any changes in lifespan upon adult-onset knockdown of *nsun-1* in the N2 wild-type strain (Heißenberger et al., 2020). However, when *nsun-1* was depleted only in somatic tissues, lifespan was extended by approximately 10%. As predicted by the “dispensable soma theory” (Kirkwood et al., 1979), soma-specific knockdown further caused a reduction in brood size. Additionally, whole-animal RNAi targeting *nsun-1* increased resistance to heat and improved locomotor activity in aged animals (Heißenberger et al., 2020). The contradictory findings regarding *nsun-1* knockdown and *C. elegans* lifespan in the two studies might be due to differences in the spatial susceptibility to RNAi of the used *C. elegans* strains (*eri-1* and *ppw-1* in the case of Curran and Ruvkun (2007) and N2 wild-type in Heißenberger et al., 2020). The knockdown of *nsun-1* influenced translation, resulting in altered collagen production and decreased cuticle integrity. The loss of *NSUN1* further caused developmental defects, including lack of oocyte maturation, altered gonad morphology, and reduced animal size (Heißenberger et al., 2020). In contrast to *C. elegans*, deletion of the *S. cerevisiae* homolog NOPO has been associated with decreased heat tolerance (Jarolim et al., 2013). Finally, in mammals, *NSUN1/NOP2* plays a role in blastocyst development (Cui et al., 2016), tumour growth and cancer aggressiveness (Cheng et al., 2018; Saijo et al., 2001), and even HIV-1 proliferation (Kong et al., 2020), pointing at the broad importance of this gene.

### 2.2. m$^4$A methyltransferases

Another methylation prevalent on eukaryotic rRNA is that of adenine at the N$^6$ position (m$^4$A). In yeast, Rrp8p methylates the adenine at position A$_{445}$ in 25S rRNA (Peifer et al., 2013). In a high-throughput screen, loss of *RRP8* caused increased CLS. However, the lifespan-extending effect was not reproducible upon retesting the deletion on a single strain basis (Garay et al., 2014). In the high-throughput study by Campos and colleagues, deletion of *RRP8* led to increased CLS under dietary restriction (GABA as nitrogen source) and unrestricted (glutamine) conditions. Interestingly, the lifespan-extending effect was smaller under restricted than unrestricted conditions, leading the authors to conclude that Rrp8p might be partially involved in lifespan extension by dietary restriction (Campos et al., 2018). Yeast Rrp8p was further associated with increased resistance to oxidative stress by H$_2$O$_2$ treatment (Brown et al., 2006) and heat shock (Jarolim et al., 2013). Interestingly, *RRP8* mutants devoid of the methyltransferase activity or lacking the entire gene showed impaired growth at 19°C (Bouque-t-Antonelli et al., 2000; Peifer et al., 2013). Apart from yeast, *rrp-8* knockdown by postdevelopmental RNAi in *eri-1* mutated *C. elegans* increased the lifespan of the animals in a high-throughput screen (Curran and Ruvkun, 2007). Depending on the physiological context and the mode of depletion, (partial) loss of *RRP8* caused defects in the embryonic development of worms and mice (Murakami et al., 2018; Yokoyama et al., 2018), as well as a decreased ability to maintain

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**Table 2.** The loss of RNA modifying enzymes influences the lifespan and stress resistance of *C. elegans*. Worm genes that show an ageing or stress-related phenotype upon knockdown or knockdown are displayed in blue for an increase (“Increased”) or red for a decrease (“Decreased”) of the respective trait. Black indicates that both increases and decreases have been reported (“Both”). Phenotypes not yet described in the literature for the individual gene are shown in grey (“Nd”). Numbers correspond to the studies which reported the phenotypes (Table 1). LS: lifespan, OR: oxidative stress resistance, HR: heat resistance, CR: cold resistance, UR: UV radiation resistance. *adr-2;adr-1* double mutants and *adr-1* single mutants show decreased LS, while for *adr-2* both phenotypes have been observed. The increased LS was observed in the *mttu-2*;*mttu-1* double mutant and the

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![Fig. 2.](image_url)
2.3. m^A restriction, requiring further investigation. (Oie et al., 2014). Thus, the mechanistic connection of RRP8 with two in mice results in increased energy expenditure and activation of AMPK.

Phenotypes not yet described in the literature for the individual gene are shown transcription upon glucose deprivation (Yang et al., 2013). Loss of RRP8 Suv39h1 and together, they promote epigenetic silencing of rRNA as Nucleomethylin (NML) in humans and mice, interacts with SirT1 and cellular senescence (Yang et al., 2015). Interestingly, RRP8, also known as Nucleomethyltransferase F33A8.4, a homolog of human ZCCHC4, which catalyses the methylation of rRNA of the large ribosomal subunit (Ma et al., 2019; van Tran et al., 2019), was shown to be involved in C. elegans lifespan regulation (Sendice et al., 2020). A C. elegans mutant lacking F33A8.4 showed a significantly increased lifespan accompanied by broad transcriptional changes. Furthermore, RNAi targeting F33A8.4 led to decreased brood size of animals. In another recent study, Liberman and colleagues found that METL-5 (C38D4.9) places an m^A modification on 18S rRNA at position A_177 in C. elegans. The lifespan and brood size of metl-5 knockout worms were not significantly different from wild-type animals. However, increased resistance to oxidative, heat and cold stress, UV exposure, and hypoxia combined with mild heat stress was observed (Liberman et al., 2020). Sendine and colleagues also reported on METL-5 being an rRNA methyltransferase targeting 18S rRNA and confirmed the indistinguishable lifespan of C. elegans lacking METL-5 compared to wild-type. However, they did observe a significant decrease in brood size compared to the Liberman study (Sendine et al., 2020). In D. melanogaster, Metl5 is necessary for normal walking behaviour (Leismann et al., 2020). Mammalian METTL5, which requires interaction with TRMT112 for stability (van Tran et al., 2019), plays a role in development (Ignatova et al., 2020; Xing et al., 2020) and has been associated with autosomal-recessive intellectual disability and microcephaly (Richard et al., 2019) (Table 2).

2.4. Concluding remarks on rRNA modifications

While the mechanistic data on RNA modifications that influence organism lifespan are generally limited, rRNA is still the best-studied RNA species in this context. Especially for RCM1/NSUNS, the lifespan-extending effect has been shown in different organisms (Schosserer et al., 2015). Since most of the RNA modifications reside in functional regions of the ribosome (Fig. 4A), modulation of the translational regulation of gene expression caused by altered rRNA structure is likely the underlying molecular mechanism for improved lifespan and stress resistance. For example, in the case of RCM1/NSUNS, lack of the enzyme was associated with the recruitment of stress-specific mRNAs to the ribosome (Schosserer et al., 2015). Additionally, a moderate global decrease in protein synthesis caused by altered rRNA modifications might be beneficial and connected to an extended lifespan (Anisimova et al., 2018), while a severe translation dysfunction might have the opposite effect. Similarly, mild alterations in translation fidelity might activate stress response pathways and thereby increase stress resistance by hurnosis (Rattan, 2005), while a more severe disruption will cause proteotoxic stress and be detrimental to health.

Interestingly, while different types of modifying enzymes influence stress resistance, most of the rRNA modifying enzymes associated with a lifespan phenotype are stand-alone methyltransferases. The likely explanation for this observation is the lack of systematic studies on snoRNAs, which are necessary for the site-specific introduction of 2’-O-Me and Ψ. Furthermore, NOP1, CBFS, and other genes encoding components of the snoRNPs are essential genes in yeast, complicating systematic loss-of-function studies.

3. tRNA modifications

Transfer RNA is the most abundantly modified and, in the context of RNA modifications, the best-studied RNA species, remarkably with more genes encoding tRNA modifying enzymes than actual tRNAs (El Yacoubi et al., 2012). While relatively simple base or sugar modifications decorate other RNA species, tRNA modifications can be highly complex and require sophisticated protein complexes for their catalysis. These complex modifications are primarily present at position 34 (Huang et al., 2005), the wobble base position, and position 37 (Murphy et al., 2005; Noma et al. 2006) in the anticodon stem-loop (ASL) of various tRNAs (Fig. 4B). tRNA modifications in the ASL ensure translational fidelity by supporting correct decoding, reducing frameshifting, and maintaining the proper conformation of the ASL. Modifications outside of the ASL are

| rRNA modifications | LS | OR | HR | CR | SR |
|--------------------|----|----|----|----|----|
| Nsun5              |    |    |    |    | m^C |

| tRNA modifications | LS | OR | HR | CR | SR |
|-------------------|----|----|----|----|----|
| Trm7-34           | 15 |    |    |    | 2’-O-m |
| Trm7-32           | 15 |    |    |    | |
| Thada             |    |    | 16 | 16 | |
| Adar              |    | 36 |    | 36 | A to I |
| Mt2*              |    | 35 | 34,35 | 34 | m^C |
| Urm1              | 37 | 37 |    |    | |
| Uba4              |    | 37 |    |    | |
| IscU              |    |    | 38 |    | |
| Fid               |    |    |    | 25 | |

| mRNA modifications | LS | OR | HR | CR | SR |
|--------------------|----|----|----|----|----|
| Ythdf              | 70 |    |    |    | m^A |
| Ythdc1             | 70 |    |    |    | |
| Mettl3             | 39,70 |    |    |    | |

Fig. 3. The loss of RNA modifying enzymes influences the lifespan and stress resistance of D. melanogaster. Fly genes that show an ageing or stress-related phenotype upon knockout or knockdown are displayed in blue for an increase (‘Increased’) or red for a decrease (‘Decreased’) of the respective trait. Black indicates that both increases and decreases have been reported (‘Both’). Phenotypes not yet described in the literature for the individual gene are shown in grey (‘Nd’). Numbers correspond to the studies which reported the phenotypes (Table 1). LS: lifespan, OR: oxidative stress resistance, HR: heat resistance, CR: cold resistance, UR: UV radiation resistance. *For Mt2, overexpression was tested, leading to increased lifespan and elevated resistance to paraquat, while loss of Mt2 led to decreased oxidative and stress resistance. While the mechanistic data on RNA modifications that influence organism lifespan are generally limited, rRNA is still the best-studied RNA species in this context. Especially for RCM1/NSUNS, the lifespan-extending effect has been shown in different organisms (Schosserer et al., 2015). Since most of the RNA modifications reside in functional regions of the ribosome (Fig. 4A), modulation of the translational regulation of gene expression caused by altered rRNA structure is likely the underlying molecular mechanism for improved lifespan and stress resistance. For example, in the case of RCM1/NSUNS, lack of the enzyme was associated with the recruitment of stress-specific mRNAs to the ribosome (Schosserer et al., 2015). Additionally, a moderate global decrease in protein synthesis caused by altered rRNA modifications might be beneficial and connected to an extended lifespan (Anisimova et al., 2018), while a severe translation dysfunction might have the opposite effect. Similarly, mild alterations in translation fidelity might activate stress response pathways and thereby increase stress resistance by hormesis (Rattan, 2005), while a more severe disruption will cause proteotoxic stress and be detrimental to health.
mainly stabilising the tRNA molecule. The impact of tRNA modifications on their structure and function has been extensively reviewed elsewhere (Lorenz et al., 2017; Motorin and Helm, 2010; Vare et al., 2017). Mutations in enzymes responsible for tRNA modifications and consequently varying amounts of the corresponding modifications are associated with various human diseases, such as neurological disorders and diabetes (Table 2; reviewed in Pereira et al., 2018; Torres et al., 2014).

### 3.1. tRNA methyltransferases

In yeast, many conserved tRNA methyltransferases encoded by TRM genes are present, for which a lifespan phenotype has been reported. Among the CLS increasing mutants are TRM3, NCL1 (TRM4) and TRM11 knockout strains (Campos et al., 2018; Garay et al., 2014), whose protein products place a 2'-O-Me at position G18 (Trm3p) (Cavaillé et al., 1999), m5C at several positions (Ncl1p/Trm4p) (Motorin and Grosjean, 1999), or an m2G at position G10 (Trm11p in complex with Trm112p) (Purushothaman et al., 2005) (Fig. 4). Lack of TRM3, NCL1 and TRM11 rendered cells sensitive to various oxidative stressors (Brown et al., 2006; Endres et al., 2020; Helsen et al., 2020; Higgins et al., 2002) and, in the case of NCL1, also to elevated temperatures (Li et al., 2009; Ruiz-Roig et al., 2010). This sensitivity to oxidative stress is also relevant for NSUN2, the human homolog of NCL1, whose loss of function has been linked to neurological disorders caused by stress-induced cleavage of tRNAs (Abbasi-Moheb et al., 2012; Blanco et al., 2014; Khan et al., 2012; Martinez et al., 2012). Apart from these methyltransferases, the loss of most other Trm proteins and tRNA modifying enzymes causes a decreased lifespan and impairs resistance to primarily oxidative stressors (Figs. 1, 2 and 3). In the case of TRM7 and TRM1, those phenotypes were also observed in higher organisms or human cells (Agenlova et al., 2020; Dewe et al., 2017).

Usually, potential relations between genes and longevity are derived from gene deletion studies. However, in the case of Drosophila Mt2 (also known as DNMT2 in other organisms), overexpression increased lifespan and resistance to paraquat (Lin et al., 2005). Conversely, loss of Mt2 rendered the animals more sensitive to heat and oxidative stress (Schaefer et al., 2010). A recent study showed that lack of Mt2 leads to an age-dependent impairment of the Drosophila immune response and lipid metabolism (Abhyankar et al., 2018). Interestingly, while Mt2 and its homologs were initially assigned to be DNA methyltransferases, it was found that they also place an m5C modification at position C38 in several tRNAs (Goll et al., 2006; Jeltsch et al., 2006; Schaefer et al., 2010). In the absence of Mt2, its tRNA substrates were more prone to cleavage, and this effect was also observed in mouse embryonic fibroblasts (MEFs) (Schaefer et al., 2010). Recently DNMT2’s impact on cellular senescence was studied. In MEFs, it was reported that loss of DNMT2 led to increased senescence markers, altered telomere length and telomerase activity, increased levels of reactive oxygen species and genomic instability. Furthermore, the lack of DNMT2 caused increased sensitivity to hydrogen peroxide (Lewinska et al., 2017). The same group investigated DNMT2 depletion in human fibroblasts and reported similar effects as in mouse cells. In addition, RNAi targeting DNMT2 induced the expression of several antiproliferative microRNAs, which might contribute to the induction of a senescent phenotype, and fibroblasts at a high passage number showed increased cytosolic DNMT2 expression (Lewinska et al., 2018). Additionally, the role of DNMT2 in therapy-induced senescence was studied, with varying results depending on the cancer type and the drugs used (Bloniarz et al., 2021).

### 3.2. Other cytosolic tRNA modifying enzymes

Tad1p is an adenosine deaminase catalysing adenosine to inosine editing at position A37 in alanine-transporting tRNA in S. cerevisiae (Gerber et al., 1998). In the gene deletion screen by Powers and colleagues for CLS, TAD1 was among the 300 genes with the most extended lifespan (Powers et al., 2006). Yeast TAD1 is related to human adenosine...
In C. elegans, ADR-1 has a regulatory role in the editing process and the expression of edited genes (Ganem et al., 2019; Washburn et al., 2014). ADR-2 is the only active A to I editing enzyme in C. elegans, ADR-1 has a regulatory role in the editing process and the expression of edited genes (Ganem et al., 2019; Washburn et al., 2014).

### Table 1: Genes related to lifespan phenotype

| Yeast | Worm | Fly | Human | Main human disease association | Source |
|-------|------|-----|-------|--------------------------------|--------|
| TRM1  | trm-1| Trm1| Human | ARID                           | (Blaesi et al., 2018; Davarnia et al., 2015; Najmabadi et al., 2011) |
| TRM3  | T1484.1 | CG18596 | TARB1 | ADHD                           | (Wissi et al., 2021) |
| NCL1  | nun-2 | Nun2 | NSUN2 | ARID, Dubowitz-like syndrome   | (Abbas-Mohb et al., 2012; Khan et al., 2012; Martinez et al., 2012) |
| TRM7  | R74.7 | R7m-32/34 | FTS1 | Non-syndromic X-Linked ID      | (Guy et al., 2015; Nagayoshi et al., 2021) |
| RTT10 | Y54658.2 | CG33172 | WDR6 | Parkinson Disease              | (Kia et al., 2021) |
| TRM8  | mel-1 | CG4045 | METTL1 | Multiple sclerosis             | (Alcina et al., 2013; Hadjiegiorgiou et al., 2019; Mo et al., 2019) |
| TRM82 | wdr-4 | WDR4 | Galloway-Mowat syndrome, Microphallic primordial dwarfism | (Braun et al., 2018; Shaeen et al., 2015; Trimouille et al., 2018) |
| TRM10 | F25H8.1 | CG14618 | TRMT10A | Diabetes, ID, delayed development | (Cosentino et al., 2018; Igoillo-Esteve et al., 2013; Zung et al., 2015) |
| TYW3  | --   | --  | TYW3 | ALS, insulin resistance        | (Chen et al., 2016; Qi et al., 2012; Wei et al., 2019) |
| BUD32 | C272.4 | CG10903 | W85C22 | Williams-Beuren syndrome       | (Doll and Grzeschik, 2001) |
| RCM1  | nun-5 | Nun5 | NSUN5 | Williams-Beuren syndrome       | (Doll and Grzeschik, 2001) |
| JMD4  | tag-124 | CG3045 | PUS3 | Several neurological, cardiovascular, hepatic diseases | (Mathoux et al., 2021; Qin et al., 2020; Zhao et al., 2020) |
| DEG1  | tag-124 | CG3045 | PUS3 | Neurodevelopmental disorders   | (Abdelrahman et al., 2018; Froukh et al., 2020; Nestvik et al., 2021; Shaeen et al., 2016b) |
| PUS2  | pus-1 | Pus1 | PUS1 | MLASA syndrome                 | (Cao et al., 2016; Fernandez-Vizarza et al., 2007; Woods and Cederbaum, 2019) |
| PUS7  | B0024.11 | Pus7 | PUS7/PUSL | ID, developmental and behavioural changes, age-related macular degeneration | (de Broezer et al., 2018; Ratanapriya et al., 2020; Shaeen et al., 2019b) |
| KA1E  | Y71H2AM.1 | Tcs2 | OSGEP | Galloway-Mowat syndrome         | (Braun et al., 2017; Domingo-Gallego et al., 2019; Lin et al., 2018; Teng et al., 2021) |
| MSS1  | mtsu-1 | CG18528 | GTPBP3 | Cardiomyopathy, lactic acidosis, encephalopathy | (Rajaij et al., 2014) |
| MTG2  | mtsu-2 | CG4610 | MTO1 | Cardiomyopathy, lactic acidosis, cognitive impairment, optic neuropathy | (Baruffini et al., 2013; Charif et al., 2015; Ghezzi et al., 2012; O’Byrne et al., 2018) |
| SLM3  | mtsu-1 | CG3021 | MTU1/TRMU | MERFF, MELAS                   | (Yasuoka et al., 2000b, 2000a) |
| TAD1  | adr-2 | Ada11 | ADAR81/2, ADAT1 | Aicardi-Goutieres syndrome, microcephaly, ID, seizures, congenital heart defect, ALS, coronary artery disease | (Alfai et al., 2019; Gallo et al., 2017; Maroofian et al., 2021; Rice et al., 2012; Tan et al., 2020; Yamada et al., 2018) |
| MOD5  | gro-1 | CG31381 | TRIT1 | Mitochondrial dysfunction      | (Kernohan et al., 2017; Takeouchi et al., 2019; Yoo et al., 2021) |
| IK3   | efc-1 | Efp1 | IKBKAP | Familial dysautonomia           | (Nordcliffe-Kaufmann et al., 2017) |
| ELF2  | efc-2 | Elf2 | ELF2 | ID, ASD, frontotemporal dementia | (Cohen et al., 2015; Kojic et al., 2021; Taskesen et al., 2017) |
| ELF3  | efc-3 | Elf3 | ELP3 | ALS                           | (Bento-Abreu et al., 2018; Kwee et al., 2012; Simpson et al., 2009) |
| ELF4  | efc-4 | CG6907 | ELP4 | Rolandic epilepsy, ID          | (Adid et al., 2015; Gkampeta et al., 2014; Reinhaler et al., 2014; Strug et al., 2009) |
| TRM9  | alkb-8, C35D10.12 | CG17807, Fid | AELK88, TRMT98 | ID                          | (Maddirevula et al., 2021; Monies et al., 2019) |
| KT12  | Y57G11C.43 | CG5587 | KTI12 | Type 2 diabetes               | (Aga et al., 2020) |
| NCS2  | tus-2 | CG01189 | CTV2 | Type 2 diabetes               | (Shaeen et al., 2019a, 2016a) |
| UBA4  | moc-3 | Uba4 | MOCS3 | ID, autism, dysmorphic features, molybdenum cofactor deficiency | (Huijmans et al., 2017) |
| ISU2  | iscu-1 | Isu1 | ISCU | Iscu myopathy                 | (Kollberg et al., 2009; Mochel et al., 2008; Olsson et al., 2008) |
| NFS1  | nfs-1 | Nfs1 | Nfs1 | Mitochondrial dysfunction     | (Farhan et al., 2014; Hershkowitz et al., 2021) |
| TUM1  | mps-1/2/3/4/5/7 | -- | MPST, TST | Mercaptoacetate-cysteine disulfiduria | (Akabori et al., 2020) |
| --    | met-5   | Met5 | METTL5 | ARID, Microcephaly            | (Richard et al., 2019) |
| --    | F33A4.8 | CG12863 | ZCCHC4 | ADHD                          | (Zayas et al., 2016) |
| --    | Y92H12BL.1 | CG6550 | CDKAL1 | Type 2/insulin-dependent diabetes, Crohn’s disease, psoriasis | (Adami and Bottai, 2020; Powe and Kwak, 2020; Wei and Tomizawa, 2011) |

Deaminases acting on RNA (ADARs), which modify pre-mRNA (Gerber et al., 1998). Interestingly, mutations in human ADAR genes are associated with many human diseases (Galoo et al., 2017) (Table 2) and extremely old age, the latter leading Sebastiani and colleagues to investigate the lifespan of C. elegans devoid of adr-1 and adr-2 (Sebastiani et al., 2009). While ADR-2 is the only active A to I editing enzyme in C. elegans, ADR-1 has a regulatory role in the editing process and the expression of edited genes (Ganem et al., 2019; Washburn et al., 2014). adr-1/adr-2 double mutants and adr-1 single mutants showed significantly decreased lifespan, while for adr-2 single mutants, both decreased and substantially increased lifespan have been reported (Ganem et al., 2019; Sebastiani et al., 2009; Zhao et al., 2015). In D. melanogaster, loss of Adar (hypnos-2) decreased axonotrophic tolerance, heat resistance, and lifespan (Ma et al., 2001), whereas for Adat1, the tRNA specific enzyme, no relevant phenotypes have been reported. The tRNA specific A to I editing enzymes have not been studied in human ageing and disease except for one study in which mutations in the ADAT1 gene were associated with coronary artery disease (Yamada et al., 2018).
showed increased lifespan to varying degrees in two different C. elegans wild-type backgrounds (Lakowski and Hekimi, 1996; Lemieux et al., 2001), while the yeast homolog MOD5 decreased RLS upon deletion (Yu et al., 2021). Additionally, gro-1 mutants appear to be more resistant to heat shock, according to WormBase (Harris et al., 2020). Human TRT1 has been studied in the context of cancer (Chen et al., 2013; Spinolaouchi et al., 2019; Yoo et al., 2021) (Table 2).

Finally, Y92H12BL.1 is an uncharacterised C. elegans gene with predicted methylthiotransferase activity, which was among genes with extended lifespan when knocked down postdevelopmentally in the high throughputsudy by Curran and Ruvkun (Curran and Ruvkun, 2007). The human homolog, CDRAL1, is associated with type 2 and gestational diabetes (Adami and Bottai, 2020; Powe and Kwak, 2020; Wei and Tomizawa, 2011) (Table 2).

3.3. Enzymes catalysing complex tRNA hypermodifications

3.3.1. Wybutosine

Wybutosine is a hypermodified nucleoside at position 37 in phenylalanine tRNA (Fig. 4B), stabilising the codon-anticodon pairing (Nomai et al., 2006). Four genes denoted TYY1, TRM12 (TYY2), TYY3 and PPM2 (TYY4) are essential for forming this modification. Trm12p is a methyltransferase in the complex catalysing wybutosine formation (Kalhor et al., 2005). It is among genes with a putatively extended lifespan in C. elegans upon knockout (Garay et al., 2014), while TYY3 deletion led to decreased CLS under dietary restriction (Campos et al., 2018). In humans, TYY3 has been linked to neurological disorders and insulin resistance (Table 2).

3.3.2. Elongator complex / 5-methoxycarbonylmethyl-2-thioauridine (mcm3^5U)

Besides the wybutosine modification at position 37, the tRNA wobble base (position 34) in the ASL is highly modified in a complex array of reactions catalysed by several enzymes as well as accessory proteins (Fig. 4B) (Bjork et al., 2007; Huang et al., 2008, 2005; Kalhor et al., 2005; Krokan and Greenblatt, 2001; Leidel et al., 2009; Mazaric et al., 2010; Nakai et al., 2007; Otero et al., 1999; Witschiene et al., 1999). Lifespan phenotypes have been reported for many genes associated with the wobble base hypermodification. Among those, ELP2 (Fabrizio et al., 2010), ST4 (Barbosa et al., 2011; Pereira et al., 2020), TRM9 (Fabrizio et al., 2010), NCS6 and UBA4 (Burtner et al., 2011; Campos et al., 2018; Garay et al., 2014; Powers et al., 2006) showed increased CLS upon deletion. Additionally, loss of SAP185 and NCS2 was associated with increased RLS (McCormick et al., 2015) and those of IK13 (also known as ELP1) and ELP3 with increased survival during early chronological ageing (5–15 days) (Eisenberg et al., 2009). For URM1 and ISU2 depletion, contradictory results regarding CLS were reported (Campos et al., 2018; Fabrizio et al., 2010; Garay et al., 2014; Marek and Korona, 2013; Powers et al., 2006), as is the case for ELP4 in terms of RLS (Delaney et al., 2011; Managbanag et al., 2008; McCormick et al., 2015; Yu et al., 2021).

Recently it was shown that adult-onset RNAi targeting the ISU2 C. elegans homolog iscu-1 led to increased lifespan, and conversely, overexpression decreased the lifespan of worms. However, it did not affect oxidative stress resistance (Sheng et al., 2021). A similar lifespan-extending effect was reported for nfs-1 (Sheng et al., 2021), the homolog of yeast NFS1, which is also required for thiolation of tRNA at the wobble position in yeast (Nakai et al., 2007). Both S. cerevisiae Isu2p and Nfs1p are necessary to synthesise iron-sulphur cluster proteins (Gerber et al., 2004; Kispal et al., 1999) besides their role in forming tRNA thiolation, and it still needs to be determined whether the tRNA modification in C. elegans is also dependent on those enzymes. In the study on C. elegans iscu-1 mentioned above, the authors explained the lifespan phenotype by decreased reactive oxygen species levels and altered mitochondrial fission in iscu-1 knockout worms (Sheng et al., 2021).

Several stress phenotypes have been reported for wobble base hypermodification proteins. In yeast, loss of TRM9, SAP190, NCS6, KTI12 and ATS1 was associated with increased resistance to oxidative stress (Fig. 1). However, for most of the genes, either both phenotypes, depending on the oxidative stressor used, or decreased resistance to oxidative stress have been reported. Likewise, deletion of most of the wobble base modification proteins leads to increased sensitivity to elevated temperatures and heat shock (Fig. 1). In contrast to the oxidative stress sensitivity phenotype of URM1 and contradictory results for UBA4 deficient yeast (Fig. 1), knockout and knockdown of the D. melanogaster homologs Urm1 and Uba4 led to increased resistance to oxidative stress (Khoshnood et al., 2018). Additionally, loss of Urm1 was lethal in Drosophila, and the small fraction of survivors showed decreased fitness, reduced fecundity and a shortened lifespan (Khoshnood et al., 2016).
increased lifespan and increased resistance to the oxidative stress-inducing agent paraquat (Kim and Sun, 2007). In humans, variants of the Kae1p homolog OSGEP, as well as other proteins of the KEOPS complex, are associated with Galloway-Mowat syndrome, characterised by early-onset nephrotic-syndrome and microcephaly (Braun et al., 2017; Domingo-Gallego et al., 2019; Lin et al., 2018; Teng et al., 2021) (Table 2). Tcd1p and Tcd2p catalyse t^A-in a yeast (Miyashita et al., 2013). Interestingly, both gene deletions increased CLS (Campos et al., 2018; Powers et al., 2006), and TCD2 deletion also extended RLS (Yu et al., 2021). Additionally, TCD2 knockout was further associated with increased resistance to oxidative stress (Brown et al., 2006). Surprisingly, both TCD1 and TCD2 appear to lack homologs in worms, flies, and mammals.

3.4. Enzymes catalysing mitochondrial tRNA modifications

In the case of mt-tRNAs, loss of yeast MTO1, whose protein product is together with Ms1p involved in the initial steps of the mt-tRNA wobble base modification cmm^6^S^3^U (Umeda et al., 2005), contradictory results regarding lifespan were reported. Three high throughput screens identified MTO1 knockout to decrease CLS (Campos et al., 2018; Fabrizio et al., 2010; Garay et al., 2014), while another study found the opposite effect (Burgner et al., 2011). As with MTO1 deletion, also Mss1 deletion led to decreased CLS (Fabrizio et al., 2010; Garay et al., 2014). In contrast, increased RLS was observed for MTO1 knockout (Yu et al., 2021). mtcu-1 and mtcu-2 are the C. elegans orthologs of yeast Mss1 and MTO1, respectively. Navarro-Gonzalez and colleagues found that loss of mtcu-2 together with mtu-1 (SLM3 in yeast), which catalyses the thiolation of C2 at the same wobble nucleotide, is correlated with severe developmental and fertility defects. However, presumably maternally rescued, infertile double mutants showed substantially increased lifespan. Additionally, lifespan extension was also observed to a lesser extent in the mtu-1 single mutant (Navarro-González et al., 2017). Mutations in yeast and human proteins catalysing cmm^6^S^3^U and tm^6^S^3^U are associated with respiratory chain deficiencies and, specifically in humans, with hypertrophic cardiomyopathy and other pathologies (Baruffini et al., 2013; Ghezzi et al., 2012; Kopajtich et al., 2014; O’Byrne et al., 2018; Yasukawa et al., 2000a, 2000b) (Table 2).

S. cerevisiae has several genes coding for conserved pseudouridylases modifying cytosolic and mitochondrial tRNAs associated with various stress- and ageing-related phenotypes (Fig. 1). Interestingly, the two pseudouridylase genes increasing longevity when deleted, PUS2 and PUS9, act on mt-tRNAs (Behm-Ansma et al., 2007, 2004) (Fig. 4). Besides tRNA, Pus2p also modifies mRNA (Carlile et al., 2014). A PUS9 deletion strain was among the top 300 strains ranked according to increased CLS in a high throughput screen (Powers et al., 2006), and the deletion of PUS2 led to increased RLS (Yu et al., 2021). While human PUS9 mutations have not been associated with a specific disease, mutations in PUS1, the human homolog of PUS2, are common in patients with myopathy, lactic acidosis and sideroblastic anaemia (MLASA) (Cao et al., 2016; Fernandez-Vizarra et al., 2007; Woods and Cederbaum, 2019).

3.5. Concluding remarks on tRNA modifying enzymes

Interestingly, although modifications of tRNAs represent the majority of all RNA modifications by far, only a few were reported to extend the lifespan when deleted. Most notably, the loss of TRM9 increased CLS (Fabrizio et al., 2010). Mammalian cells lacking ALKBHB, a homolog of TRM9, concomitantly showed decreased translation of selenocysteine proteins and elevated reactive oxygen species levels (Eindres et al., 2015). Mechanistically, alterations of wobble base modifications can lead to a decrease in translational fidelity by misincorporation of wrong amino acids and proteotoxic stress (Patil et al., 2012a). Consequently, in the case of TRM9 mild activation of the unfolded protein response might induce hormesis, promote resistance to other stressors and increase lifespan.

Altered modification levels in regions distinct from the anticodon loop might influence stress responses and cellular fitness by other mechanisms. For example, a lack of m^6^A modifications by NSUN2 and DNM2 causes decreased stability of tRNAs by increased susceptibility to angiogenin cleavage (Blanco et al., 2014; Schaefer et al., 2010). The thereby generated RNA fragments play an important regulatory role in the oxidative stress response by dynamically repressing overall protein synthesis (Gkatza et al., 2019). In mammals, lack of DNM2 was associated with increased cellular senescence (Lewinska et al., 2018, 2017), while in Drosophila, overexpression of Mt2 (dDnm2) led to increased lifespan (Lin et al., 2005).

To summarise, while tRNA modifications are necessary for translational fidelity and tRNA stability, they do not directly modulate the translation of specific mRNAs, as modifications of rRNAs and mRNAs can do. However, their importance for generating tRNA fragments and the potential regulatory roles of these small RNAs are still only poorly understood. The significance of tRNA modifications for normal physiology is demonstrated by the fact that dysfunction of tRNA modifying enzymes occurs in several human neurological disorders, such as different forms of intellectual disability. In contrast, deficiencies in mt-tRNA modifications cause rare mitochondrial diseases (Table 2).

4. mRNA modifications

Besides the methylated guanosines forming the 5’-cap, mRNAs are also modified internally. m^A and m^P are most prevalent, with approximately 0.5% of the respective bases carrying modifications. Apart from those, also other modifications, like m^6^A, m^C, and to a lesser extent 2’-O-Me, decorate mRNA (Roundtree et al., 2017). mRNA modifications influence the fate of transcripts by modulating, for example, alternative splicing (Alarcon et al., 2015; Liu et al., 2017, 2015; Xiao et al., 2016), mRNA export (Yang et al., 2017), the efficiency of translation (Delatte et al., 2016; Li et al., 2017, 2016; Shi et al., 2017; Slobodin et al., 2017; Wang et al., 2015), as well as the stability of transcripts (Du et al., 2016; Shi et al., 2017; Wang et al., 2014).

m^A is currently the most studied epitranscriptomic mark because it was the first dynamic RNA modification described in the literature, introduced by m^A writers and removed by m^A eraser proteins. Depending on the position of the modification and the type of m^A reader protein interacting with the modified mRNA, the respective transcript can undergo increased translation or, on the contrary, show decreased stability and degradation (Hoernes and Erlacher, 2017; Shi et al., 2019). However, by now, there is evidence that also m^P (Schwartz et al., 2014) and m^A (Li et al., 2016) undergo dynamic regulation.

4.1. Writers, erasers and readers of m^6^A modifications

In S. cerevisiae, only one m^6^A writer acting on mRNA has been identified to date. The Mum2p-Ime4p-Slz1p (MIS) complex catalyses m^6^A modifications and thereby regulates the entry into meiosis and sporulation under nutrient-deprived conditions (Agarwala et al., 2012). Ime4p is the active methyltransferase of the complex (Clancy, 2002). While for IME4, no data on lifespan has been reported, deletion of the gene coding for Mum2p was associated with increased lifespan in two high throughput studies (Matecic et al., 2010; Powers et al., 2006). In contrast, depending on the study, SLZ1 knockout either decreased lifespan under normal conditions (Garay et al., 2014) or only when dietary restricted (Campos et al., 2018).

Flies lacking functional Mettl3, the D. melanogaster homolog of Ime4, and the two m^6^A readers Yhdf and Ythdc1 showed decreased lifespan and in the case of Mettl3 and Yhdf impaired neuronal functions, the latter relying heavily on a functioning m^6^A machinery in Drosophila (Kan et al., 2021; Lence et al., 2016).

The reports in mammals regarding the effects of m^6^A modifying enzymes in the context of ageing and senescence deviate. In long-lived
proteins involved in m^6_A modification, like the writers METTL3 and METTL14 and the readers YTHDF1 and YTHDF2, were increased compared to controls. This upregulation was associated with increased cap-independent translation (CIT), promoting stress resistance and healthy ageing (Ozkurede et al., 2019). Another study investigated common mechanisms of rapamycin, acarbose and 17α-estradiol, which all extend mouse lifespan. Shen and colleagues found that METTL3 levels increased in old compared to young mice, and all three pharmacological compounds blunted this increase. METTL14, on the contrary, showed a minimal but significant decrease with age, and again, this effect was reversed by drug treatment. Although CIT appears to be the converging mechanism for rapamycin, acarbose and 17α-estradiol, a causal role of METTL3 and METTL14 in modulating CIT and lifespan downstream of these drugs remains to be established (Shen et al., 2021).

Contrary to the age-related METTL3 increase in mice, levels of both m^6_A and METTL3 decreased in prematurely senescent human mesenchymal stem cells (hMSCs). Consequently, the knockout of METTL3 in young hMSC led to elevated senescence markers like reduced proliferative capacity and SA-β-Gal positivity, while the overexpression of METTL3 had the opposite effect (Wu et al., 2020). Similarly, m^6_A levels in human peripheral blood mononuclear cells and dermal fibroblasts showed an inverse correlation with donor age and population doublings, respectively (Min et al., 2018). In both studies, the underlying mechanism was the degradation of specific mRNAs due to their decreased m^6_A modification levels. The hypomodified and consequently degraded mRNAs were MSIS2, a gene regulating the cell cycle and senescence (Wu et al., 2020), and AGO2, involved in miRNA gene silencing (Min et al., 2018). m^6_A readers, such as IGF2BP2 for the MSIS2 mRNA (Wu et al., 2020), play an essential role in protecting the specific mRNA from degradation. Interestingly, a recent study reported that METTL3 and METTL14 modulate the SASP in an m^6_A-independent manner (Liu et al., 2021). Thus, also considering the non-canonical functions of RNA modifying enzymes is essential when interpreting high-throughput reverse genetic screens.

Finally, it is interesting to note that C. elegans lacks genes coding for the predominant mRNA m^6_A writer complex METTL3/METTL14 and although detection of m^6_A on mRNA has been reported, m^6_A levels appear to be extremely low (Liberman et al., 2020; Mendel et al., 2021; Sendinc et al., 2020). Nevertheless, it was shown that other m^6_A writers act on pre-mRNA, thereby regulating splicing, as well as small nuclear RNA and rRNA (Liberman et al., 2020; Mendel et al., 2021; Sendinc et al., 2020).

4.2. Concluding remarks on mRNA modifying enzymes

In summary, proteins involved in the dynamic regulation of the m^6_A modification in mRNA, especially the writers METTL3 and METTL14, seem to play a profound role in ageing and cellular senescence. However, which mRNAs and, more precisely, which of their specific nucleotides undergo modification in this context is just beginning to be explored. A larger body of data is already available for cancer (He et al., 2019; Hsu et al., 2017; Huang et al., 2020) and human age-associated diseases (Huang et al., 2020; Reitz et al., 2012), and it will be interesting to compare similarities and differences to the epitranscriptome of ageing and cellular senescence.

Besides m^6_A writers, the presence of eraser and reader proteins, like IGF2BP2, further determine the fate of methylated transcripts, rendering the study of m^6_A even more complex. For example, the m^6_A reader IGF2BP2 is highly expressed in Alzheimer’s disease, one of the most prevalent neurodegenerative diseases in the elderly population (Dong et al., 2021). Thus, it is not easy to attribute the m^6_A modification status of a single nucleotide to a particular phenotype due to its dynamic nature.

Surprisingly little is known about other mRNA modifications than m^6_A in ageing. The reason might be the relatively low abundance of mRNAs compared to rRNAs and tRNAs, which requires large amounts of total RNA for detection that is often difficult to obtain from aged organisms or senescent cells. In addition, mRNAs carry, in general, fewer modifications than other RNA species, and they might undergo dynamic regulation. These difficulties necessitate sensitive methods to locate and precisely quantify modifications at single-nucleotide resolution, which will be briefly introduced in the following chapter.

5. Detection methods for RNA modifications

The repertoire of methods for detecting RNA modifications is relatively new. Significant advances in next-generation sequencing and bioinformatics promoted their recent development. For a detailed review of current detection methods, see, for example, the review by Helm and Motorin (Helm and Motorin, 2017).

Global levels of a specific RNA modification can be accurately quantified by mass spectrometry (Wetzel and Limbach, 2015) or, if a suitable antibody is available, assessed semi-quantitatively by immunono-northern (Mishima et al., 2015) and dot blot techniques (Shen et al., 2017).

The discovery and continuous optimisation of several different approaches allowing transcriptome-wide mapping of RNA modifications revolutionised the field of epitranscriptomics. Some of these methods depend on the enrichment of modified RNAs by binding to an antibody for the respective modification and provide varying sequence-positional accuracy. The first generation of methods like MeRIP-seq (Meyer et al., 2012) detect only the approximate location of modifications like m^6_A, m^6_C and m^6_A. miCLIP, based on immunoprecipitation after an antibody-RNA crosslinking step, maps m^6_A modifications at single-nucleotide resolution by considering specific reverse transcription signatures introduced by the crosslinks (Linder et al., 2015).

Other primarily single-nucleotide mapping techniques use a similar chemical principle but do not require immunoprecipitation. For example, both A to I editing (Oakes et al., 2017) and m^6_A (Hauenschild et al., 2015) leave a characteristic misincorporation or truncation pattern in cDNA after reverse transcription, which can be detected by next-generation sequencing on a whole transcriptome scale, or by simple primer extension for known targets. Similarly, chemical derivatisation of modified RNA before reverse transcription and sequencing is applied to detect modifications, for example, A to I editing, Ψ and m^6_C (Sakurai et al., 2010; Schaefer et al., 2009; Schwartz et al., 2014). Besides base modifications, also ribose modifications can be detected at single-nucleotide precision either for specific sites by primer extension (Dong et al., 2012) or in a transcriptome-wide manner using RiboMeth-seq (Birkedal et al., 2015).

Finally, most of the available sequence-specific detection methods for RNA modifications comprise multiple complex library preparation steps, followed by next-generation sequencing and complicated bioinformatics analysis. For that reason, rigorous validation of detected modification sites by alternative methods and the inclusion of proper controls is a prerequisite for obtaining meaningful results. Moreover, the requirement for large amounts of input RNA and the lack of methods to detect several modification types simultaneously still considerably limit systematic research on the epitranscriptome. Current developments, such as the direct sequencing of modified RNA by Oxford Nanopore (Furlan et al., 2021) or further optimisation of existing protocols (Khoddami et al., 2019), are likely to circumvent these limitations and thereby promote further research in the field.

6. Conclusions and outlook

Ageing is a multifactorial and interconnected physiological process, which is still understood only to a limited degree. Even though the first RNA modifications were already described in the middle of the last century, their influence on human development, disease, and ageing has been recognised only recently.
Ageing research has been classically conducted in yeast, worm and fly models, owing to their shorter lifespan than most mammals. Moreover, in these organisms, the disruption of gene function is usually easily achieved by mutagenesis or RNAi, facilitating reverse-genetic screens. Especially in *S. cerevisiae*, a large body of high throughput studies explores the effects of gene knockout on lifespan and other traits related to fitness and stress response that we included in this review. These phenotypes were observed upon genetic interference with genes encoding RNA modifying enzymes. An important limitation of these studies is that the absence of the modification itself, which is catalysed by the respective enzyme, was verified only in a few cases. As this was not done in high-throughput genetic screens, direct causality between the presence of a particular modification and a phenotype cannot be established in most cases. Thus, changes in the phenotype might be due to other non-canonical functions of the targeted enzyme rather than the RNA modification itself.

Consequently, although we identified several genes coding for RNA modifying proteins that show lifespan, fitness, and stress response phenotypes upon genetic interference (Figs. 1, 2, 3), their precise molecular and physiological functions, as well as their conservation through evolution, remain to be characterised in more targeted experiments in different organisms. This aspect is also crucial in light of the false-positive rate of high throughput screens. Still, there are promising candidates for which some mechanistic data are already available. These proteins include RCM1/NSU5, RRP8, and F33A8.4/ZCCHC4 that introduce base methylations in rRNA, the methyltransferases DNMT2 and TRM9/ALKBH8, as well as factors involved in the thiolation or O to I editing in RNA, and finally the m6A machinery for mRNA.

Notably, only an increased lifespan upon genetic interference might indicate a significant potential for future therapeutic intervention. The reason is that inhibition of enzymatic functions is usually pharmacologically much more accessible than their activation. In addition, decreased lifespan by a specific mutation can simply be explained by the reduced fitness of that particular strain without direct causality to biological ageing (Powers et al., 2006).

Establishing direct functional relationships for single RNA modification sites is achieved by introducing point mutations in the RNA rendering a specific nucleotide non-modifiable. Unfortunately, this task is almost impossible for rRNAs and tRNAs with current CRISPR/Cas9-mediated genome editing technologies. The reason is that tRNAs and rRNAs are present in clusters of multiple, highly similar copies. Thus, until methods that allow editing of hundreds of sites simultaneously become available, researchers in the field have to rely on modifying the targeting entities, which participate in the addition of these modifications. This task is relatively straightforward for stand-alone protein-based enzymes, which are often included in public collections of mutant yeast, worm and fly strains, as well as in RNAi libraries. For snRNPs, in which snoRNAs guide the enzyme to the modification site, this is more complicated for several reasons: (i) snoRNAs are often not specific for a single target, (ii) snoRNAs are frequently encoded in introns of host genes, which might then also be affected by genetically interfering with the snoRNA, and (iii) only a few methods are currently available to specifically deplete (Liang et al., 2011) or overexpress snoRNAs (Darzacq et al., 2002). Thus, the function of more than 200 sites of mammalian rRNAs that are modified by either 2′-O-Me or 2′-O-SMe and catalysed by snRNPs still represent a mostly uncharted territory, especially in the context of ageing and cellular senescence.

Concluding, in gene expression, the study of the epitranscriptome is still in its infancy. It will be interesting to see to which degree the highly complex and dynamic RNA modification patterns change during cellular and organismal ageing or if alterations in the epitranscriptome directly influence the progression of ageing. Consequently, future research will determine if the epitranscriptome might serve as a potent biomarker for biological ageing or if novel discoveries might even promote the development of novel geroprotectors to increase resilience and delay the onset of age-associated diseases. If this should be the case, we propose to include “alterations of the epitranscriptome” as an additional pillar or hallmark of ageing (Kennedy et al., 2014; López-Otín et al., 2013).

CRediT authorship contribution statement

**Anja Wagner:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization.

**Markus Schosserer:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Competing interests

The authors have no competing interests to declare.

Data Availability

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

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