ABSTRACT
Wobble uridines (U34) are generally modified in all species. U34 modifications can be essential in metazoans but are not required for viability in fungi. In this review, we provide an overview on the types of modifications and how they affect the physico-chemical properties of wobble uridines. We describe the molecular machinery required to introduce these modifications into tRNA posttranscriptionally and discuss how posttranslational regulation may affect the activity of the modifying enzymes. We highlight the activity of anticodon specific RNases that target U34 containing tRNA. Finally, we discuss how defects in wobble uridine modifications lead to phenotypes in different species. Importantly, this review will mainly focus on the cytoplasmic tRNAs of eukaryotes. A recent review has extensively covered their bacterial and mitochondrial counterparts.1

Wobble uridine modifications are essential in evolution
Among the plethora of chemical posttranscriptional modifications that are found on tRNA, those of wobble uridine (U34) are peculiar, because U34 is almost invariably modified in any organism.2,3 This phenomenon implies a strong evolutionary pressure to maintain wobble uridines modified and is further affirmed by the analysis of minimal genomes. In Mollicutes species that have drastically reduced their genome size during evolution, uridine thiolation (s2U) is part of an essential core module of translation.4 Furthermore, recent attempts of synthetic biologists to generate a minimal genome based on Mycoplasma mycoides found modification systems of U34 to be required for rapid growth under laboratory conditions.5 Surprisingly however, the absence of U34 modification does not cause lethality in Caenorhabditis elegans and most yeasts but is essential in some strain backgrounds of Saccharomyces cerevisiae and in mice.6–12 Furthermore, several human diseases are linked to defects in U34 modifying enzymes.13–15 This apparent discrepancy between essentiality during evolution and variable effects in different organisms makes wobble uridines even more worth exploring.

We know a lot about U34 modifications from the research of many laboratories working mainly on baker’s yeast. Theoretically, 16 anticodons carry a uridine at their wobble position. UAA and UGA, 2 codons that would require U34-containing tRNAs for decoding are nonsense codons and therefore recognized by the Eukaryotic Release Factor 1 and 3 (eRF1 and eRF3) GTPase complex.16,17 However, the modification machinery is of such broad specificity that U34 in suppressor tRNA Ser UUA is modified.6 tRNA Arg UCG does not exist in yeast and tRNA Leu UAG is unmodified.18 tRNA Leu UAA carries a pseudouridine (Ψ34).18 The remaining 11 tRNA species are decorated by 4 types of modifications: First, 5-carbamoylmethyluridine (ncm5U34), the most abundant modification, is present on tRNA Val, tRNA Arg, tRNA Pro, tRNA Thr and tRNA Gla. Second, tRNA Leu UUA is further 2-O-methylated to 5-carbamoylmethyl-2-O-methyluridine (ncm5s2U34). Third, 5-methoxycarbonylmethyluridine (mcm5U34) is found on tRNA Arg and tRNA Glu. Finally, 3 tRNAs are further decorated by a 2-thio group to form 5-methoxycarbonylmethyl-2-thiouridine (mcm5s2U34): tRNA Glu, tRNA Lys and tRNA UCC (Fig. 1).20,21

The physicochemical properties of wobble uridine modifications
The multitude of modifications speaks for particular requirements in the U34 position. But what are the effects of these modifications on the chemical and structural properties of uridine? In his wobble hypothesis, Francis Crick proposed that U34 recognizes A and G in the third position of the codon.22 However, he could not account for the extent of modified nucleotides in tRNA as these modifications were not known. Later adaptations of the wobble hypothesis have attempted to integrate how modifications of U34 affect codon recognition.20,23–26

Uridines are structurally flexible and form only weak stacking interactions with neighboring nucleosides. This is because uridines—even when decorated by 5′-modifications-adopt a C(2′) endo conformation, which is relatively flexible.27,28 However, the presence of 2-thio modifications leads to the adaptation of a C(3′) endo, gauche plus [C(4′)-C(5′)], anti

CONTACT
Raffael Schaffrath  scheffra@uni-kassel.de  Institut für Biologie, Fachgebiet Mikrobiologie, Universität Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany; Sebastian A. Leidel  sebastian.leidel@mpi-muenster.mpg.de  Max Planck Research Group for RNA Biology, Max Planck Institute for Molecular Biomedicine, Von-Esmarch-Str. 54, 48149 Münster, Germany.
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structure.27-29 This conformation is hydrophobic, more restricted and appears to be best suited for anticodon base-stacking, therefore stabilizing the anticodon stem loop (ASL) and favoring the interaction with A in the codon.27,28 The conformational stability of s2U34 is similar to U34m, which also occurs mainly in its C(3′) endo form.30,31 While, s2U34 enhances the stability of the stacking of the triplet significantly over unmodified U34,32,33 the stabilizing effect of the C(3′) endo conformation appears to be more important for the codon-anticodon interaction than an improvement of base stacking.29,34 Furthermore, 2-thio modifications do not only restrict and stabilize the conformation of the nucleoside that carries s2 but also of a 3′-adjacent unmodified uridine.35,36 Importantly, a stabilization of the interactions between 2-thiolated anticodons with their codon partners has been observed in binding models and in the context of the ribosome.36-38 While the role of s2U34 modification is to restrict codon conformation than an improvement of base stacking,39,40 the role of xm5 modifications is to open up the rigidity to stabilize U/C15 G wobble pairings.39 In general, the ribosomal grip constrains the positioning of the mRNA more than that of the ASL. Therefore, G34•U3 wobble pairs can form in unmodified anticodons, while U34•G3 pairs are much less stable, if they form at all.36,40-42

Structural studies found that near-cognate tRNAs do not necessarily take up a wobble confirmation but that non-standard base pairing can retain Watson-Crick geometry.43 This structural arrangement is supported by the presence of U34 modifications. Indeed, mcm5’s s2U34 modifications change the physicochemical behavior of the base. While s2 of mcm5’s U34 is required for proper positioning of the nucleoside, the main function of the mcm5 modification is to modify the electron structure of the ring and to shift the keto-enol equilibrium toward enol thus enabling hydrogen bonding between U34•G pairs.44 Therefore, while s2U rigidifies the ASL structure thereby favoring an interaction with A-ending codons, the xm5 modifications relax the conformation and enable pairing with G-ending codons. However, when analyzing codon-translation speed by ribosome profiling the absence of both types of modifications seems to affect translation speed similarly.45 Nevertheless, also the context of the anticodon matters. For instance, N6-threonylcarbamoyladenosine (t6A37) synergizes with U34 modifications to order the ASL such that it will bind to the correct mRNA codons. Only when U34 and A37 are fully modified, will the ASL of tRNA34UUU bind to AAA and AAG programmed ribosomes41 and fulfill the function proposed by Crick.22 Therefore, U34 modifications contribute to the pre-structuring of the anticodon loop to achieve optimal translation.42

Wobble uridine modifications require multiple pathways

Two pathways are responsible for placing the two main classes of modifications on wobble uridine: The URM1 pathway is required for 2-thiolation and the Elongator complex needed for generating xm5U34 modifications (Fig. 2). Pseudouridine Synthase (Pus1) introduces pseudouridine at U34 in tRNA34UUU.36,47 Finally, during the 2′-O-methylation of tRNA34UUU tRNA Methyltransferase (Trm7) uses S-adenosylmethionine (SAM) as a methyl donor together with its cofactor Regulator of Ty1 Transposition 10 (Rtt10).48,49 However, the absence of 2′-O-
methylation in tRNA\textsubscript{Leu\_UAA} does not significantly contribute to phenotypes observed in \textit{trm7Δ} yeast since overexpression of tRNA\textsubscript{Phe\_GAA} is sufficient for rescue experiments, suggesting that the physiological role of this modification in U\textsubscript{34} is minor.\textsuperscript{49} Interestingly, x\textsubscript{m}U\textsubscript{34} formation is independent of the presence of s\textsubscript{3}U\textsubscript{34} while the reverse is not true. In \textit{S. cerevisiae} s\textsubscript{3}U\textsubscript{34} levels are reduced in the absence of mcm\textsubscript{5,11,50} In humans, mice and \textit{S. pombe} mcm\textsubscript{5} is even strictly required for s\textsubscript{3}U\textsubscript{34} formation.\textsuperscript{51,52}

The \textbf{URM1 pathway}:

Ubiquitin Related Modifier 1 (Urm1) is an ubiquitin like protein (Ubl) first described as a protein modifier in a process called urmylation.\textsuperscript{53-55} It was identified through its sequence homology to bacterial sulfur-carrier proteins\textsuperscript{53} and is considered to be a molecular fossil because it is the Ubl that most closely resembles the ancestors of this class of protein modifiers.\textsuperscript{56,57} Importantly, Urm1 is the only Ubl described as a protein modifier and at the same time to act in sulfur transfer, placing it at the evolutionary intersection of both pathways.\textsuperscript{50,58-67} Like every Ubl, Urm1 is activated by an E1-like enzyme Ubiquitin Activating 4 (Uba4).\textsuperscript{53} While the N-terminus of Uba4 contains an E1 domain, its C-terminal harbors a rhodanese homology domain (RHD).\textsuperscript{68,69} The RHD has been suggested to act as an E2 domain for protein conjugation of Urm1.\textsuperscript{70} However, there is no experimental support for this function and the mechanism of the domain rather points toward sulfur transfer. Two additional core members of the pathway are \textit{Needs GAA} to Survive 2 and 6 (Ncs2 and Ncs6),\textsuperscript{55,71} which form a complex in \textit{S. cerevisiae}, \textit{C. elegans} and \textit{S. pombe}.\textsuperscript{8,11} Difficulty in reconstituting the complex \textit{in vitro} has prevented its detailed analysis. It is clear that Ncs6, an iron-sulfur (Fe/S) cluster containing protein,\textsuperscript{72} and its worm homolog TUT-1 can bind to tRNA \textit{in vitro}\textsuperscript{11} and \textit{in vivo}.\textsuperscript{8} The protein carries 2 predicted zinc-finger domains and a P-loop.\textsuperscript{11,73,74} Interestingly, the number of tRNA species that bind to Ncs6 \textit{in vitro} exceeds the number of mcm\textsubscript{5}s\textsubscript{3}U\textsubscript{34} targets.\textsuperscript{11} Thus, Ncs2 may provide specificity to the complex. Alternatively, Ncs2 may stabilize or activate Ncs6. Ncs2 is the closest homolog of NCS6 in the yeast genome. However, the critical residues required for enzymatic function are mutated. The observation that only one catalytic subunit is required in \textit{S. cerevisiae} suggests a similar mechanism in enzymes that form a homo-dimer such as TuA.\textsuperscript{73} While URM1, UBA4, NCS2 and NCS6 are required for 2-thiolation, ThioUridine Modification 1 (TUM1), a gene coding for a protein with 2 RHDs, is not essential for thiolation. However, in the absence of Tum1, levels of s\textsubscript{3}U\textsubscript{34} are significantly reduced and the ratio between modified and unmodified tRNA changes.\textsuperscript{11,54,63,67}

In addition to this core set of proteins, there is another group that is essential for thiolation by more generally affecting sulfur pathways. NiFS like 1 (Nfs1), a cysteine desulfurase that converts cysteine into alanine using pyridoxal phosphate as a cofactor acts upstream of several cellular sulfur pathways.\textsuperscript{76} During this reaction, Nfs1 feeds sulfane sulfur to its acceptor protein. Uba4 uses the sulfur and transfers it into the downstream cascade for 2-thiolation.\textsuperscript{62} The exact mechanism of Tum1 in this reaction is unclear. Tum1 receives sulfur from Nfs1 and appears to stimulate its activity.\textsuperscript{63} This suggests that it acts as an enhancer of the transfer reaction or as a sulfur relay. Since Tum1 is not essential for 2-thiolation, the transfer step via Tum1 can be bypassed. Therefore, Tum1 may affect 2-thiolation through an indirect mechanism by rerouting sulfur pathways. Finally, proteins required for iron-sulfur (Fe/S) cluster biogenesis and assembly are essential for 2-thiolation. These are: Cytosolic Iron-sulfur protein Assembly 1 (Cia1), Nucleotide Binding Protein 35 (Nbp35) and Cytosolic Fe/S cluster Deficient 1 (Cfd1), which are all components of the CIA complex.\textsuperscript{77,78} The CIA complex works in conjunction with IscU homologs 1 and 2 (Isu1 and Isu2), 2 proteins that reside in the mitochondrial matrix and are required for Fe/S cluster generation.\textsuperscript{79} Additional proteins required for Fe/S cluster formation are, therefore, likely to affect 2-thiolation but have not been tested specifically. Whether Fe/S cluster formation affects s\textsubscript{3}U\textsubscript{34} formation directly or indirectly via the formation of mcm\textsubscript{5}s\textsubscript{3}U\textsubscript{34}.

**Figure 2.** Model of the \textit{URM1} pathway and the Elongator complex. Schematic representation of the 2 pathways cooperating in mcm\textsubscript{5}s\textsuperscript{3}U\textsubscript{34} formation. In the \textit{URM1} pathway (left), sulfur is mobilized by Nfs1 with the help of Tum1. Uba4 activates Urm1, leading to a thio-carboxylate at Urm1’s C-terminus, which acts as a sulfur carrier. Finally, the Ncs2•Ncs6 complex binds to and activates tRNA \textit{in the thiolation reaction and transfers the sulfur from Urm1 to uridine}. The Elongator complex consists of twice Elp1-Elp6. Elp1 dimerizes via its C-terminus and acts as a platform for Elp2 and Elp3 binding in a wing-like structure. A ring of Elp4-Elp6 binds to one of the wings (Handedness is only partially represented in this model).\textsuperscript{90,91}
will need to be determined.\textsuperscript{80} Recently, a Fe/S cluster was identified in Ncs6 and its archaeal homolog TtuC.\textsuperscript{72} However, its requirement for $s^2U_{34}$ formation remains to be demonstrated.

**The Elongator complex**

The Elongator complex is at the heart of $mcm^5$ and $ncm^5$ side chain formation at $U_{34}$. The complex has been reported to act in numerous cellular processes including transcription, DNA damage response, exocytosis, telomere gene silencing, DNA demethylation and wobble uridine modification.\textsuperscript{6,81-88} Importantly, all known phenotypes of Elongator-minus yeast, except for the defect in $U_{34}$ modification itself, can be rescued by over-expression of tRNA that are normally $mcm^5s^2U_{34}$ modified.\textsuperscript{12,45,51,86-95} Nevertheless, the question of whether different functions exist in other species is very persistent, in part due to the fact that tRNA overexpression experiments in metazoans are more difficult to perform than in yeast.

The Elongator complex consists of 6 subunits: Elp1-Elp6.\textsuperscript{81,96,97} Each is present twice in the holo complex, which can be divided into two sub-complexes: Elp1-Elp3 and Elp4-Elp6. The latter subunits, between 30–50 kDa in size, have very similar RecA folds and assemble into a heterohexameric ring structure that resembles RecA-like ATPase complexes.\textsuperscript{98} However, the subunits lack the P-loop motif, which is characteristic for ATPases.\textsuperscript{98} Importantly, the Elp4-Elp6 complex binds tRNA in an ATP dependent manner, where high levels of ATP decrease the affinity of tRNA for the complex. The Elp1-Elp3 subcomplex contains Elp3, which is the catalytic subunit of the complex.\textsuperscript{96,99} Elp3 carries an N-terminal radical SAM binding domain and a C-terminal histone acetyltransferase (HAT) domain.\textsuperscript{96,99} Interestingly, in the archaeon *Methanocaldococcus infernus* a homolog of Elp3 is sufficient for $U_{34}$ modification, while all other Elongator subunit genes are absent from its genome.\textsuperscript{101} Elp1 and Elp2 are both WD40 domain containing proteins. Two subunits of Elp1 dimerize via their C-terminal domains, while one subunit of Elp2 and Elp3 associates with each Elp1 subunit at either side.\textsuperscript{102,103} This generates a wing-like structure. Surprisingly, in the holo complex, a ring of Elp4-Elp6 is associated with the front of the left wing (Fig. 2) resulting in an asymmetric assembly, which is in contrast to previous models.\textsuperscript{98,102-105} The role of the asymmetry still needs to be determined as well as the position of the tRNA in the complex.

In addition to the Elongator complex, *Kluveromyces lactis* Toxin Insensitive 11–14 (Kti11-Kti14), Suppressor of Initiation of Transcription 4 (Sit4), Sit4 associated protein 185 and 190 (Sap185 and Sap190), RNA Methyltransferase 9 and 112 (Trm9 and Trm112) are required for $mcm^5U_{34}$ and $ncm^5U_{34}$ formation. Elp1-Elp6, Kti11, Kti12, Kti14, Sit4, Sap185 and Sap190 are essential for the formation of $cm^5U_{34}$, which is believed to be the precursor of $mcm^5$ and $nmc^5$.\textsuperscript{6,58,106} The lack of Kti13 leads to reduced levels of $xm^5$ formation.\textsuperscript{6,58} Finally, the Trm9-Trm112 complex uses SAM to synthesize $mcm^5U_{34}$.\textsuperscript{107} Whether $cm^5U_{34}$ or an intermediate precursor serves as the direct methylase substrate is not clear, yet. The observation that both *trm9* and *trm112* mutants accumulate $ncm^5U_{34}$ and $nmc^5s^2U_{34}$, however, suggests the existence of an enzyme required for formation of $ncm^5U_{34}$ (and $nmc^5s^2U_{34}$) from $cm^5U_{34}$.\textsuperscript{107,108} The identity of this activity is still not known, leading to contradicting ideas of how to rationalize these later steps of the $U_{34}$ modification pathway.\textsuperscript{106-110}

**Elongator regulation by phosphorylation**

Strikingly, tRNAs that carry Elongator-dependent $mcm^5s^2U_{34}$ can be cleaved by zymocin between anticodons position 34 and 35.\textsuperscript{111-113} Zymocin is a trimeric (α/β/γ) tRNase toxin complex produced from *K. lactis* that kills yeasts including *S. cerevisiae*.\textsuperscript{114-116} In line with this lethal mode of tRNase action, genetic studies have shown that mutations in Elongator genes trigger zymocin resistance and additional Elongator related factors (Kti11-Kti14, Sit4, Sap185 and Sap190) were genetically identified on the basis of zymocin survivor screens.\textsuperscript{58,112,117-136} Rather than affecting the assembly or the integrity of the Elongator complex, these proteins appear to be regulatory.

![Figure 3. Phosphomodification of Elongator subunit Elp1. (A) Elp1 electrophoretic mobility shifts based on anti-HA Western blots are diagnostic for Elongator de-/phosphorylation.\textsuperscript{130,134} In the *klt12* and the kinase-dead *hrr25/klt14* mutants, hypophosphorylated forms of Elp1-HA accumulate while *sit4* phosphatase mutants induce Elp1-HA hyperphosphorylation. Wild-type (wt) cells maintain both isoforms of Elp1-HA, which mediate sensitivity (S) to growth inhibition by the tRNase toxin zymocin (killer assay; lower panel; for details see text). Zymocin resistance (R) associates with Elp1 phosphorylation defects in *klt12*, *hrr25/klt14* and *sit4* mutants. (B) Elongator phosphorylation model. *Klt12* interacts with Elongator (and kinase *Hrr25*) thereby potentially activating Elp1 phosphorylation. In support of this, Elp1 is found to be hypophosphorylated in *klt12* and *hrr25/klt14* cells (see A). PPase: protein phosphatase (Sit4); Kinase: *Hrr25*/*Klt14*.](image-url)
Consistent with this, a casein kinase 1 (CK1) isozyme (Kti14, also called: Hrr25), type-2A protein phosphatases (Sit4•Sap185; Sit4•Sap190) and an Elongator interactor (Kti12) were all shown to affect the phosphorylation state of Elp1, which through dimerization assembles holo-Elongator. Elp1 is present as a hypophosphorylated isoform in kti12 and hrr25 mutants and is hyperphosphorylated in sit4 mutants, while wild-type cells maintain both forms (Fig. 3). This suggests that Elongator function may be phosphoregulated, which is in line with reports that tRNA modifications including mcm'-s''U34 can change in response to chemical stress and cell cycle progression.

Subsequently, phosphorylation sites on Elp1 and other Elongator subunits were identified using mass spectrometry. Among those mapped on Elp1, 2 (Ser-1198, Ser-1202) appear to be directly phosphorylated by Hrr25 confirming the genetic data that the CK1 isozyme has Elongator kinase activity. The analysis of phosphosite mutations revealed that Elp1 phosphorylation largely plays a positive role for Elongator activity. Accordingly, profiling modified U34 nucleosides in tRNAs from these phosphosite mutants by LC-MS/MS showed loss of mcm'-s''U34 and mcm'-U34 formation. The finding that normal phosphoforms of Elp1 at Ser-1209 were detectable in a kinase-dead hrr25 mutant implies that at least one additional Elongator kinase ought to exist. Furthermore, altered interaction between Elongator, Hrr25 and Kti12 was seen in several Elp1 phosphosite mutants, in line with data showing that hrr25 mutants unable to phosphorylate Elp1 affect Elongator association with Kti12. The data, therefore, suggest that normal Elongator interaction with Kti12 and proper tRNA modification are facilitated by phosphorylation of Elp1.

### Regulation of s''U34 formation

Whether the URM1 pathway is regulated is not clear. Most reports describing changes in mcm'-s''U34 levels in response to chemical stress do not separate the contribution of mcm'' and s'' formation. However, the analysis of 2-thiolation has shown that s''U34 levels are decreased in response to high temperatures or in growth media lacking a sulfur source. This is not a consequence of de-thiolation but depends on active transcription of tRNA by RNA polymerase III, since the use of inhibitors or temperature sensitive alleles of RNA polymerase III prevents the decrease in s''U34. Interestingly, the decrease in s''U34 is reversible when yeast is shifted back to ambient temperature, which seems like a prerequisite for active regulation. However, the kinetics for up- and downregulation of 2-thiolated tRNA are in the range of several hours.

This is in contrast to the idea that modification-specific changes to translation could provide a fast switch under stress conditions. The environmental stress response that elicits an extensive transcriptional change in response to various stress conditions peaks after 30 min. A translational rewiring by reducing 2-thiolation would therefore accompany or even follow the transcriptional response to high temperatures rather than being an active driver of such a cellular transition.

But what could be mechanisms for regulation? Different high-throughput analyses have identified phosphosites in Tum1, Ncs2 and Ncs6. However, to date none of these sites has been shown to affect s''U34 formation in vivo. Interestingly, Uba4 and Ncs6 were identified as targets for protein urmylation and similarly ATP3BP, the human Ncs6 homolog. However, these studies were performed using increased levels of Urm1 and oxidizing reagents. To show the in vivo relevance of urmylation it will be crucial to perform similar experiments under physiological conditions.

### The role of phosphorylation for U34 regulation

Although the precise role for Elp1 phosphorylation is unclear, two options can be envisaged. On the one hand, phosphorylation could act as an ‘on/off’ switch for Elongator’s U34 modifying activity, for example, in response to cellular stress. If translation of some mRNAs were dependent on U34 modification and hence tunable by Elongator, this raises the possibility that Elongator is part of a translational control mechanism that functions through its role as a U34 modifier. Such role is consistent with loss-of-function phenotypes associated with Elp1 phosphosite substitutions, kinase-dead hrr25 mutations and inhibition by ATP analogs of an analog-sensitive Hrr25-ITG2 kinase variant. All Hrr25 operates in many cellular functions, which complicates the analysis of Elp1 phosphorylation signals, its kinase activity is required for full functionality of ribosomes and U34 containing tRNAs. This is congruent with a role of the kinase in the regulation of a cell’s capacity for proper mRNA translation and protein synthesis. Finally, hrr25 and Elongator mutants are sensitive to DNA damaging mutagens.

Since efficient translation of the RNR1 message coding for Ribonucleotide Reductase subunit 1, involved in the DNA damage response (DDR) requires U34-modified tRNAs, Elp1 phosphorylation may link up to the known role the Hrr25 kinase plays in expression of other DDR genes, i.e. RNR2 and RNR3.

On the other hand, Elongator might require dynamic sequential phosphorylation and dephosphorylation cycles of Elp1 to carry out its tRNA modification reaction. It was shown that phosphates mutants like sit4 or sap185sap190 trapped Elp1 in a slower-migrating, hyperphosphorylated form whereas hrr25 kinase and kti12 mutants led to the presence of a fast-migrating, hypophosphorylated Elp1 isoform (Fig. 3). Both types of mutations cause loss-of-function phenotypes, suggesting that the functionality of Elongator requires sequential de-/phosphorylation of Elp1. Thus, dynamic de-/phosphorylation may impact on the catalytic activity of Elp3, its localization or even its ability to interact with accessory factors or substrate tRNAs. Importantly, the C-terminus of Elp1, which is phosphorylated, is required for dimerization and is adjacent to a basic region that is crucial for Elongator activity and tRNA binding. Thus, Elp1 phosphorylation may affect holo-Elongator dimerization as well as interaction with partner proteins or its tRNA substrates.

The dynamic model is further supported by the observation that the right balance between hypo- and hyperphosphorylated Elp1 isoforms appears critical for Elongator activity (Fig. 3). This may explain why the presence of exclusively one of the two isoforms results in Elongator loss of function (Fig. 3) and antagonistic de-/phosphorylation of Elp1 by phosphatase/
kinase activities may indeed control its activity.\textsuperscript{121,129-131,134} Although this contradicts the idea that Epl1 phosphorylation acts as an ‘on/off’ switch, data showing that loss of U\textsubscript{34} modifications is similar in epl1, hrr25, kti12, sit4 and sap185sap190 mutants with opposite Epl1 phosphorylation states (Fig. 3) agree with the dynamic phosphorylation model.\textsuperscript{58,131} However, this would predict that mimicking constitutive phosphorylation on at least some of the Epl1 phosphosites may inhibit Elongator function. Surprisingly, all of the phosphomimetic ELP1 alleles tested in zymocin assays conferred growth arrest by the tRNase indicating proper Elongator functioning in the U\textsubscript{34} modification pathway.\textsuperscript{142} However, whether these mutations are not fully phosphomimetic and thus allowed for residual Elongator activity, has not been analyzed. Thus, although removal of phosphorylation sites provides evidence that Epl1 phosphorylation acts positively on Elongator function, a requirement for dynamic de-/phosphorylation still needs to be shown. Furthermore, it is possible that inhibitory Epl1 phosphosites exist, which were not identified by Abdel-Fattah and colleagues.\textsuperscript{142} For example, although Epl1 phosphorylation at Thr-1212 was not detected by MS/MS, a substitution of this residue caused zymocin resistance suggesting that it represents a phosphosite in vivo.\textsuperscript{142} To conclude, while there is clearly more to learn about Elongator phosphorylation, experimental evidence demonstrates that the kinase Hrr25 affects Elongator’s tRNA modification function by phosphorylating (potentially reversible) phosphoacceptor sites in the Epl1 subunit.

**Elongator regulation through Kti11, Kti12 and Kti13 proteins**

Although the precise role of Kti12 is unclear, the yeast protein, its plant ortholog (DRL1/ELO4) and PSTK, a tRNA binding kinase, carry N-terminal P-loop motifs typical of nucleotide binding proteins.\textsuperscript{118,163-166} Consistent with a functional role for this domain, a P-loop truncation of Kti12 triggers defects typical of Elongator mutants.\textsuperscript{124} Importantly, Kti12 supports Elp1 phosphorylation and interacts with the Hrr25 kinase in an Elongator-dependent fashion.\textsuperscript{121,124,125,167} kti12 knockout abolishes Hrr25 interaction with Elongator, cause loss of U\textsubscript{34} modification and trigger the formation of hypophosphorylated Epl1 isoforms (Fig. 3) similar to a hrr25 kinase mutant.\textsuperscript{58,127,134} This led to the proposal that Kti12, through recruitment of Hrr25 to Elongator, positively acts on Elongator function.\textsuperscript{124} Intriguingly, KTI12 overexpression triggers the accumulation of hyperphosphorylated Epl1 isoforms and suppresses zymocin sensitivity, which is typical of sit4 phosphatase mutants (Fig. 3).\textsuperscript{118,130,133} This effect, however, is likely not caused by altered Elongator interactions in contrast to Epl1 phosphorylation defects, which enhance Kti12 association.\textsuperscript{134,142} Furthermore, zymocin suppression through excess Kti12 can be rescued by overexpression of SIT4.\textsuperscript{130} These genetic interactions suggest a negative role of Kti12 for sit4 phosphatase function. In support of this idea, multi-copy KTI12 was found to suppress the rapamycin-resistance of a mutant lacking Resistant to Rapamycin Deletion 1 (Rrd1), a Sit4 activator protein and TOR pathway component, which has not been reported to directly relate to Elongator function.\textsuperscript{92} It will be interesting to study whether this additional role for Kti12 can be separated from its ability to regulate Elongator phosphorylation.

Two additional Elongator regulatory factors are Kti11/Dph3 and Kti13/Ats1. Kti11 is a metal binder and electron transfer protein that copurifies with Fe/S cluster containing complexes including Elongator and Dph1\textbullet Dph2.\textsuperscript{126,168,169} Moreover, it forms a dimer with Kti13 shown to promote U\textsubscript{34} modification by Elongator and diphthamide synthesis by Dph1\textbullet Dph2.\textsuperscript{132,136,170,171} Diphthamide is an exotic modification of translation Elongation Factor 2 (EF2)\textsuperscript{142,172} which catalyzes ribosomal translocation during translation elongation and is, therefore, essential for protein synthesis and cell viability. Diphthamide-modified EF2 can be inhibited by cytotoxic ADP ribosylases\textsuperscript{173} including diphtheria toxin (DT)\textsuperscript{172,174} As a consequence, KTI11 mutations confer resistance against DT and the zymocin tRNase.\textsuperscript{123,132,172} Thus, Kti11 and Kti13 appear to partake in U\textsubscript{34} anticond modifications by Elongator and diphthamide synthesis on EF2\textsuperscript{174-176} presumably by providing electrons\textsuperscript{170,171} to the Fe/S clusters in Elp3\textsuperscript{100} and Dph1\textbullet Dph2.\textsuperscript{177} With the recent identification of a Kti11 reductase in yeast (Cbr1) that affects Elongator activity,\textsuperscript{178} it will be important to clarify the precise roles of Kti11 and Kti13 and study their potential to modulate the electron flow required for both the tRNA and the EF2 modification pathways.

**U\textsubscript{34} modification dependent tRNase ribotoxins**

Zymocin inhibits yeast growth through a complex mode of action that involves chitinolysis of the cell wall, tRNase (γ-toxin) uptake, anticodon cleavage and depletion of tRNAs, eventually resulting in cell death.\textsuperscript{111,112,114,115,117} Thus, zymocinity relies on the inhibition of mRNA translation and protein biosynthesis, reminiscent of bacterial anti-phage tRNase and colicin-type anticond nucleases.\textsuperscript{12,179-181} Importantly, up-regulating tRNA repair by overproducing the 2-component system Crr4 phage (Rnl1-Pnk1) or tRNA ligase from plants (AtTTL1) suppresses anticodon cleavage and depletion of tRNA\textsubscript{Glu}\textsubscript{UUG}.\textsuperscript{116} This suggested that the incision generated at the 3’ side of the modified wobble base is compatible with sealing and healing functions of heterologous ligases, and that tRNA repair can be an efficient antidote toward lethal anticond damage by microbial tRNases.\textsuperscript{116} This principle was further supported by findings that the inability of the yeast tRNA ligase Trl1 to repair the anticond damage can be rectified by shuffling genetically engineered ligase constructs. Interestingly, when the domains from AtTTL1 and Trl1 were swapped, the plant ligase domain plus the yeast healing domain rescued S. cerevisiae cells against depletion of tRNA\textsubscript{Glu}\textsubscript{UUC}.\textsuperscript{116} Thus, differences in the ligase component of the plant versus yeast enzymes likely account for tRNase anticond functions.\textsuperscript{116} Intriguingly, PaT, a zymocin-related tRNase ribotoxin complex from Pichia acaiae, exploits a second site resulting in nucleotide excision rather than incision.\textsuperscript{111,182} Hence, tRNA damage by PaT evades reconstitution of a functional ASL by the repair system, which is why plant AtTTL1 ligase fails to suppress PaT toxicity.\textsuperscript{182,183} Other than suppressing zymocin action through direct repair, tRNA protection can also be provided by higher-than-normal levels of tRNA\textsubscript{Glu}\textsubscript{UUG}, tRNA\textsubscript{Glu}\textsubscript{UUC} and tRNA\textsubscript{Lys}\textsubscript{UUA} which are
targeted for cleavage by the ribotoxin. In fact, a screen for zymocin insensitive mutants identified several tRNA\textsubscript{Gln\text{UUC}} loci as copy-dependent suppressors of the tRNase ribotoxin.\cite{118} Interestingly, this suppression can be efficiently countered by overexpressing the catalytic subunit of U\textsubscript{34} methylase (Trm\textsubscript{9}$\cdot$Trm112), suggesting that it is hypomodified tRNA\textsubscript{Gln\text{UUC}}, which in excess is able to bypass zymocicity.\cite{58,113} However, full resistance to zymocin is only conferred by mutations in ELP, KTI or URM1 pathway genes that trigger the loss of mcm\textsuperscript{5}s\textsuperscript{2}U\textsubscript{34} modifications in tRNA.

Since the U\textsubscript{34} modification pathways are conserved and elements of the Elongator pathway can be functionally exchanged between yeast and plants,\cite{135,184} it was obvious to apply the yeast tRNase toxins to metazoans. Preliminary findings indicate that tRNase expression not only inhibits yeast growth but also affects the viability of vertebrate cells (RS & SL, data not shown). This is consistent with HeLa cell growth inhibition as well as hypersensitivity reactions in response to transient tRNase induction and tRNA cleavage \textit{in planta}.\cite{185,186} Similarly, bacterial PrrC-type anticodon nucleases were found to be lethal when expressed in yeast.\cite{187} Importantly, using cytotoxic tRNase to study the formation of microbial biofilms suggests that tRNA cleavage may also be used in cell-cell communications.\cite{181,187} Thus, it is tempting to exploit tRNase ribotoxins as anti-proliferative agents for use in biomedical interventions against infections by microbial, fungal or viral pathogens or to prevent undesired growth of tumor cells whose proliferation heavily relies on protein synthesis and therefore, proper tRNA function.\cite{181,188}

**The origin of phenotypes of U\textsubscript{34} defects**

Exploiting U\textsubscript{34} modifications to kill competing yeasts is a curious strategy for pathogenicity. But what are the molecular mechanisms that underlie the pleiotropic phenotypes that we observe in organisms with inappropriate levels of U\textsubscript{34} modification? The absence of wobble uridine modifications is accompanied by increased sensitivity to biotic and abiotic stresses and defects in numerous cellular processes including transcription, DDR, exocytosis, telomere gene silencing and DNA demethylation.\cite{6,81-85,87,88} In prokaryotes, s\textsuperscript{2}U\textsubscript{34} deficient tRNAs are poor substrates for aminoacyl-tRNA synthetases.\cite{189} This however, does not appear to be the case in eukaryotes and can, therefore, not explain the observed phenotypes.\cite{20}

Most strikingly in yeast, except for the U\textsubscript{34} modification defects, all known phenotypes can be rescued by overexpression of tRNAs that would normally be decorated by mcm\textsuperscript{5}s\textsuperscript{2}U\textsubscript{34} (see above). In \textit{S. cerevisiae}, overexpression of tRNA\textsubscript{Gln\text{UUG}} and tRNA\textsubscript{Lys\text{UUC}} efficiently rescues the defects. This is in good agreement with ribosome-profiling experiments, which found that the codons CAA and AAA decoded by tRNA\textsubscript{Gln\text{UUG}} and tRNA\textsubscript{Lys\text{UUC}}, respectively, slow down during translation while a slow down at GAA (decoded by tRNA\textsubscript{Gln\text{UUG}}) was not detected consistently.\cite{35,94} Computational attempts to identify transcripts that are enriched in A-ending codons as well as several screens and proteomics studies have reported targets that are downregulated at the protein level in U\textsubscript{34} modification mutants.\cite{143,146,160,190} Interestingly, it is possible to rescue the levels of some of these target proteins by using engineered gene constructs that have AAA codons exchanged by AAG.\cite{51,191} However, in contrast to tRNA overexpression, none of these synthetic codon rescue experiments has suppressed the underlying phenotype. Failure to do so can have several reasons: First, the phenotypes may be triggered by the loss of function of a group of proteins rather than by the absence of individual proteins (Fig. 4A). Second, instead of a loss-of-function phenotype, the effects may be triggered by a cytotoxic gain-of-function (Fig. 4B). This concept is based on the recent findings that

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.png}
\caption{Two models to explain phenotypes of U\textsubscript{34} modification mutants. (A) Specific mRNA enriched in codons that depend on tRNA modifications are translated at lower rates. This leads to reduced levels of the protein encoded by this transcript triggering a loss-of-function phenotype. (B) Ribosomes slow down when translating codons that depend on tRNA modifications. The slowdown perturbs the optimized equilibrium between speed of protein synthesis and protein folding. The increased rate of protein stress leads to a systemic failure in protein homeostasis and the aggregation of endogenous proteins that associates with it in a toxic gain-of-function scenario. This can either affect viability of the cells directly or by changing cellular signaling (Street signs with “30” indicate slow speed of ribosomes).}
\end{figure}
U₃₄ modification mutants as well as other dysfunctional tRNA modification pathways are characterized by protein homeostasis defects. In yeast, these lead to the aggregation of endogenous metastable proteins in the cytoplasm. In mouse brains, the same modification defects induce the unfolded protein response in the endoplasmatic reticulum, which triggers differentiation defects in neuronal precursors, leading to microcephaly. Thus, proteotoxic stress provides an alternative mechanism (Fig. 4B) to explain the pleiotropic phenotypes. In this scenario, codon-specific translation defects perturb the equilibrium of mRNA-translation dynamics and peptide-chain folding that has been optimized during evolution to ensure accurate protein synthesis, folding and homeostasis. As a result, proteotoxic stress alone or in combination with the standard model that favors reduction of individual proteins (Fig. 4A), is likely the main trigger for pleiotropic defects by severely rearranging the cellular proteome or by interfering with downstream signaling of the affected cells.

Importantly, the underlying mechanism of U₃₄ defects has significant consequences for our options to remedy phenotypes, particularly in the context of human disease: The loss-of-function model suggests that the identification of undertranslated mRNAs will lead to treatment options by enhancing the activity of their encoded proteins. In contrast, the protein-homeostasis model suggests that instead of repairing individual proteins, our response rather has to focus on alleviating proteotoxicity or signaling output that is induced downstream of proteotoxic stress.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**ORCID**

Raffael Schaffrath [http://orcid.org/0000-0001-9484-5247](http://orcid.org/0000-0001-9484-5247)

Sebastian A. Leidel [http://orcid.org/0000-0002-0523-6325](http://orcid.org/0000-0002-0523-6325)

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