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Yeast models for Charcot-Marie-Tooth disease-causing aminoacyl-tRNA synthetase alleles reveal the cellular basis of disease

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Abstract

Charcot-Marie-Tooth disease (CMT) is a genetically diverse hereditary disorder that affects the motor and sensory nerves, impacting about 1 in 2500 people. It can be inherited through autosomal dominant (AD), autosomal recessive (AR), or X-linked genetic patterns. CMT2, one of the primary subtypes, is characterized by axonal degeneration and commonly presents with muscle weakness, atrophy, foot deformities, and sensory loss. Aminoacyl-tRNA synthetases (aaRSs) play an important role in the genetic underpinnings of CMT2, with more than 60 disease-causing alleles identified across eight different aaRSs, including alanyl-, asparaginyl-, histidyl-, glycyl-, methionyl-, tryptophanyl-, seryl-, and tyrosyl-tRNA synthetases. Mutations in aaRS genes can lead to destabilization of the enzyme, reduced aminoacylation, and aberrant protein complex formation. Yeast as a simple organism provides a robust model system to study the pathogenic effects of aaRS CMT mutations. In this review, we discuss the advantages and limitations of the yeast model systems for CMT2-causative mutations in aaRS.

KEYWORDS

aminoacyl-tRNA synthetase, Charcot-Marie-Tooth disease, humanized yeast, Inherited peripheral neuropathy, protein synthesis, yeast

1 | INTRODUCTION

1.1 | Charcot-Marie-Tooth disease

Charcot-Marie-Tooth disease (CMT) is a hereditary motor and sensory neuropathy and affects one in 2500 people. As a genetically heterogeneous disorder, it is associated with autosomal dominant (AD), autosomal recessive (AR), and X-linked inheritance patterns.[1] Mutations in over 100 different genes have been linked to CMT. The four most common types include CMT1 (demyelinating form), CMT2 (axonal form), CMT4 (autosomal recessive form), and CMTX (X-linked form).[2] A dominant-intermediate CMT (DI-CMT) type, with

histological evidence of both demyelinating and axonal features, can also occur. Although the age of onset varies, most patients present similar classical CMT phenotypes characterized by muscle weakness in the feet manifesting as hammertoes and pes cavus (high arch), atrophy, nerve pain, paralysis of the foot and lower leg muscles, balance problems, as well as loss of sensation to pain and temperature.[3]

At present, there is no disease-specific treatment available for CMT. Therefore, treatment is limited to alleviating symptoms through pain medication, physical and occupational therapy, braces or splints to support the feet and ankles, or surgery to correct joint deformities.[4] CMT2, the focus of this review, is characterized by progressive chronic degeneration of peripheral axons and is typically inherited in an AD manner.[1] Axonal dysfunction in CMT2 is linked to mutations in many different genes.[4] One such family of genes implicated in CMT2 is the enzymes known as the aminoacyl-tRNA synthetases (aaRSs). Over 60 CMT disease-causing alleles have been found in eight different aaRSs including glycyl-, tyrosyl-, alanyl-, histidyl-, methionyl-, asparaginyl-, seryl-, and tryptophanyl-tRNA synthetases (GlyRS, TyrRS, AlaRS, HisRS, MetRS, AsnRS, SerRS, and TrpRS).[5–11] Thus, aaRSs are the largest family of enzymes implicated in CMT2, emphasizing their crucial role in the development of this disease.

1.2 | Aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases are highly conserved, essential enzymes that play a vital role in protein synthesis by catalyzing the transesterification of transfer RNA (tRNA) molecules with their corresponding amino acids.[12] This crucial step, essential for accurate protein synthesis, ensures that each tRNA is charged with the correct amino acid to produce aminoacyl-tRNA complexes for translation. The ribosome decodes the genetic information of the codon triplets of the messenger RNA (mRNA) by base pairing codons with the tRNA anticodon, ligating the amino acids to form polypeptides.[12,13] aaRSs are the guardians of the proteome, physically linking the nucleic acid triplet anticodon with the corresponding amino acid. aaRS accuracy is critical for protein synthesis, and aaRS must accurately distinguish their cognate amino acid and tRNA substrates from the large pool of similar non-cognate substrates in the cell.[14] Each of the 20 proteinogenic amino acids has at least one designated aaRS enzyme. In addition, noncanonical aaRSs such as pyrrolysyl-tRNA synthetase and phosphoseryl-tRNA synthetase are also found in nature, but their occurrence is limited to certain clades of the phylogenetic tree.[12]

Based on their diverse structures, aaRSs are generally grouped together as either class I or class II synthetases. In class I synthetases, the catalytic domain consists of a Rossmann fold featuring five-stranded parallel β-sheets connected by α-helices. The Rossmann fold also contains highly conserved HIGH and KMSKS motifs. Meanwhile, the structure of class II synthetases is usually arranged in the form of seven-stranded β-sheets flanked by α-helices.[13] The two classes of synthetases show differences in substrate recognition. Class I aaRSs bind the minor groove of the tRNA acceptor stem (except for TrpRS and TyrRS) and ligate the amino acid onto the 2′OH of the ribose of base A76. In contrast, class II aaRSs bind the major groove and the amino acid is ligated onto the 3′OH (except for phenylalanyl-tRNA synthetase [PheRS]).[12,15] Both aaRS classes charge the tRNAs in a two-step reaction (Figure 1). First, the amino acid is activated as it binds the catalytic site of aaRS along with an ATP molecule, resulting in the formation of an aminoacyl-adenylate intermediary complex and the release of pyrophosphate (PPi). Then, in a second step, the aaRS ligates the activated amino acid to the 3′ end of the tRNA, yielding the final aminoacyl-tRNA substrate ready for ribosomal engagement[16] (Figure 1).

Mutations in the aaRS enzymes are associated with many disease phenotypes, including cancer, and a multitude of developmental and neurological disorders.[17,18] Disease-causing mutations can arise in diverse regions, including the active site as well as the tRNA-binding domain of these enzymes.[19,20] In general, loss-of-function mutations lead to reduced aminoacyl-tRNA availability in the cell. Reduced synthetase activity is often caused by reduced aaRS stability or deficient aminoacylation activity. In addition, structural changes can impair dimerization or structural flexibility of the enzyme. In gain-of-function mutations, relaxed enzyme specificity may allow the binding of non-cognate amino acid to aaRS, leading to mistranslation across the proteome since the ribosome cannot distinguish a correctly charged tRNA from a mischarged tRNA.[21] In some cases, aaRS mutants have been found to gain a new function, where they interact with additional proteins, such as neuropilin-1 (Nrp1).[22] To develop allele-specific treatment for CMT causative aaRS alleles, it is imperative to define the underlying molecular and cellular mechanism of disease. While many model systems have been developed to study aaRS, including higher eukaryotes like zebrafish and mice[23,24] yeast have emerged as a versatile model system to study the proteomic and cellular consequences of aaRS-related diseases.

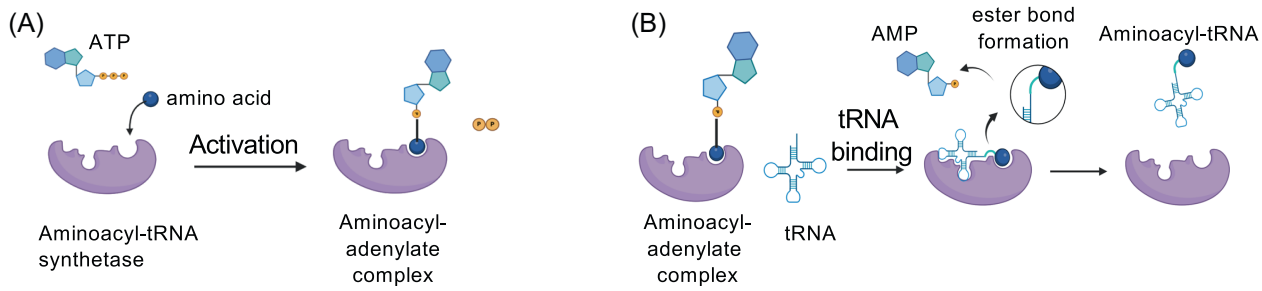

FIGURE 1 Aminoacylation of tRNA by aminoacyl-tRNA synthetases (aaRSs). aaRSs charge their cognate tRNA in a two-step mechanism. (A) The amino acid binds to the enzyme's active site and undergoes activation through ATP cleavage. (B) The amino acid is ligated to the cognate tRNA through the formation of the aminoacyl-ester bond. The resulting aminoacyl-tRNA product is then released from the aaRS.

1.3 | Yeast models of disease

In recent years, great strides have been made toward discovering the molecular mechanisms and pathways that contribute to the degeneration of nerve cells in the brain and peripheral nervous system. Model organisms offer a simplified system to study the regulation of complex biological processes such as neurological function. As these organisms have many genetic and physiological similarities with humans, much can be learned regarding the underlying mechanisms of the disease. *Saccharomyces cerevisiae* (*S. cerevisiae*) is an established model system to investigate impaired translation and its effect on the proteome and cellular viability.[25] *S. cerevisiae* is a single-celled eukaryotic organism characterized by rapid growth, genetic tractability, and a relatively small genome. Basic processes such as the machinery for gene transcription and translation, DNA replication and repair, metabolic pathways, intracellular transport, and organization are highly conserved between yeast and higher-level eukaryotes. Yeast homologues exist for around half of the genes linked to heritable human illnesses.[26]

S. cerevisiae has proven to be a powerful research tool in understanding the underlying mechanism of neurodegenerative diseases. Hallmarks of neurodegenerative diseases, including protein aggregation, mitochondrial dysfunction, and oxidative stress responses, have been studied within the simple yeast model system and have contributed to our understanding of the pathogenesis of neurodegenerative diseases.[27,28] In studying the effects of human disease-causing mutations in yeast, one of two approaches has been taken: utilizing the human gene to complement a deletion of the homologous yeast gene or introducing corresponding mutations into the homologous yeast gene. Furthermore, yeast cells that have been genetically altered to express human genes are known as "humanized yeast," which enables the examination of the disease-causing mutations in a native genetic context. The process of introducing human genes into yeast involves either inserting the human gene into a yeast expression vector or directly into the yeast chromosome. The resultant protein interacts with many yeast cellular constituents (lipids, proteins, RNAs, etc.), altering cellular physiology and resulting in identifiable phenotypes.[29] For example, the pathogenic alleles of the human gene HTT (huntingtin) lead to protein aggregation and cellular dysfunction in yeast, very similar to the aggregation of HTT in human cells.[30] Expression of other human proteins prone to aggregation in *S. cerevisiae*, such as α-synuclein (Parkinson's) or amyloid-β (Alzheimer's), has shown the importance of changes in normal protein homeostasis in this group of diseases as well.[31] The expression of human APP (amyloid precursor protein) or its cleavage products (amyloid-β) in yeast accumulates and leads to cellular toxicity, reminiscent of amyloid plaque formation in those with Alzheimer's disease.[28]

By using the conserved mechanisms between yeast and human cells, genes and pathways that are involved in the pathology of neurodegenerative diseases can be identified and their roles in disease progression determined. With yeast's rapid growth and the feasibility of simple genetic manipulation, compounds that show promise within yeast cells can be further studied, tested, and modified to develop novel therapeutics. Overall, yeast is a potent model organism for understanding neurological disease processes and a powerful system for developing treatments.

This review will focus on the pathogenic aaRS alleles causative for CMT and the application of yeast model systems in CMT2 research. Recently, Seryl-tRNA synthetase (SerRS) has been associated with CMT.[32] As of today, no yeast model has been published for SerRS-related CMT, and we have therefore omitted SerRS from this review.

For consistency, proteins will be referred to by the three-letter nomenclature (e.g. HisRS for histidyl-tRNA synthetase). Human genes are referred to by the one-letter code (HARS) whereas we will use the yeast-specific acronym for yeast, for example, *HTS1* for histidyl-tRNA synthetase. For yeast, dominant alleles are usually capitalized and recessive alleles are in lower case. As the dominant/recessive nature of the human CMT-causing mutations is not known in yeast, we will use uppercase for yeast genes, except for yeast gene deletions which are indicated with a Δ following the gene name, for example, *hts1Δ*. Mitochondrial aaRS alleles are denoted by the number 2 (e.g. *AARS2*), while the cytosolic variants are denoted by the number 1 (e.g. *AARS1*). All CMT-associated variants occur in the cytosolic aaRS.

1.4 | Alanyl-tRNA synthetase

The human cytosolic *AARS1* gene is located on chromosome 16 and encodes AlaRS, which belongs to the family of class II synthetases. AlaRS is a 968 amino acid long polypeptide with a molecular mass of ~107 kDa.[33] Its structure consists of an N-terminal catalytic domain, followed by an editing domain, and a C-terminal C-Ala domain (Figure 2). The catalytic domain containing the enzyme's active site and the tRNA-binding site is necessary and sufficient for charging $tRNA^{Ala}$ with its cognate amino acid, alanine.[22] In yeast, AlaRS is a 107-kDa protein encoded by the gene *ALA1*. It is notable to mention that yeast encodes a single AlaRS, and deletion of the endogenous enzyme abolishes function in both mitochondria and the cytosol. Furthermore, it is to be expected that only the cytosolic function is rescued by the human cytosolic AlaRS.

AlaRS is unique in several ways as compared with other synthetases. It recognizes its cognate tRNA through a single conserved identity element, the G3:U70 wobble base pair in the acceptor helix of $tRNA^{Ala}$ through key residues present in its active site.[35] This feature contrasts with other aaRSs, such as TyrRS and GlyRS, that require both the acceptor stem and the anticodon for tRNA recognition. Human AlaRS is predominantly present and active as a monomer under normal cellular conditions, unlike most other class II aaRSs, which are only functional in their dimeric form.[36] Due to the structural similarity of alanine with other amino acids, the catalytic active site of AlaRS alone is not sufficient to select for alanine with high specificity. This is evident in nature as AlaRS has been found to mischarge $tRNA^{Ala}$ with both the smaller glycine and the relatively larger serine, yielding $Ser\text{-}tRNA^{Ala}$ and $Gly\text{-}tRNA^{Ala}$ products.[37] Thus, to prevent the release of mis-aminoacylated $tRNA^{Ala}$, AlaRS has evolved additional mechanisms that enable proofreading and correction. The first line of defense is the editing domain itself, while the second layer of protection comes from the free-standing homologs of the editing domain, known as AlaXps, which further ensures accuracy in aminoacylation.[37,38]

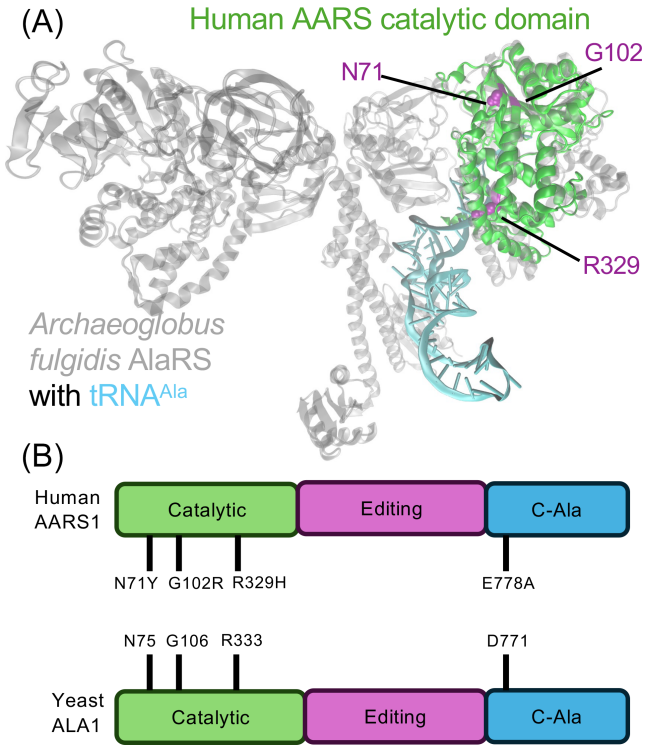

FIGURE 2 Structure and domains of alanyl-tRNA synthetase (AlaRS). (A). The crystal structure of the human AlaRS catalytic domain (green, PDB ID# 4XEM) was structurally aligned with the structure of *Archaeoglobus fulgidis* AlaRS in complex with $tRNA^{Ala}$ (grey & blue, PDB ID# 3WQY), using Visual Molecular Dynamics (VMD).[34] The alignment shows that the catalytic core of the enzyme is conserved between both species. The van der Waals radius of amino acids corresponding to Charcot-Marie-Tooth disease (CMT) causative amino acids is depicted in purple. (B) The domain organization of human *AARS1* and yeast *ALA1*. Both human *AARS1* and the yeast *ALA1* contain N-terminal aminoacylation domains, an editing domain, and the C-terminal C-Ala domain. The amino acid sequences were aligned using protein blast which showed that residues N71, G102, and R329 are conserved between human and yeast enzymes, whereas human E778A corresponds to yeast D796.

Mutations in the human *AARS1* gene are known to cause CMT-subtype 2 N (CMT2N). Genetic screening of the *AARS1* gene in patients exhibiting symptoms of peripheral neuropathy has revealed several AlaRS variants associated with this disease. McLaughlin et al. inserted three CMT2N-associated *AARS1* variants, including R329H and N71Y in the catalytic domain as well as E778A in the C-Ala domain, in the yeast ortholog,

ALA1.[39] A heterozygous diploid deletion strain, *ala1Δ*, was transformed with a plasmid containing wildtype *ALA1*. Sporulation and subsequent tetrad dissection were performed to obtain a haploid *ala1Δ* strain containing the wildtype *ALA1* maintenance vector. Afterwards, the haploid *ala1Δ* with the maintenance vector was transformed with the *LEU2*-bearing pRS315 test vector containing either the wildtype or mutant *ALA1*. Plasmid shuffling was carried out by counter-selecting against the maintenance plasmid with 5-fluoroorotic acid (5-FOA), which is toxic for cells bearing a plasmid with a *URA3* gene. It is to be noted that in this model system, yeast exclusively relies on the pathogenic alleles modeled by mutating the corresponding allele in the yeast *ALA1*, while in humans these alleles are dominant with a second wildtype *AARS1* in the genome. The study found that the E778A *ALA1* allele did not show any significant growth defects when compared with the wildtype *ALA1*, demonstrating that not all alleles that are pathogenic in humans show a phenotype in yeast. In contrast, the N71Y and R329H *ALA1* alleles both failed to support growth in the *ala1Δ* yeast, indicating a loss-of-function phenotype.[39] Similarly, Motley et al. (2015) analyzed a G102R *AARS1* mutation present in the catalytic domain of AlaRS in *ALA1* by using the same method described by McLaughlin et al. (2011). Results showed that the G102R *ALA1* also failed to complement the *ala1Δ*, highlighting its disease-causing capacity.[40]

Interestingly, R329H and G102R *AARS1* alleles not only show loss-of-function properties, but also dominant-negative phenotypes, where the pathogenic alleles interfere and disrupt the function of wildtype AlaRS protein. To assess dominant-negative phenotypes, both wildtype and pathogenic alleles were co-expressed in a humanized yeast model system using the ptetO7-*ALA1* strain.[41] In this strain, endogenous *ALA1* is placed under a doxycycline-repressible promoter. The strain was then transformed with two compatible plasmids, containing the constitutively expressed wildtype *AARS1* allele, and a galactose-inducible second copy of either wildtype or mutant *AARS1*. As a control, a loss-of-function phenotype *AARS1* was used (*AARS1* with a premature stop codon, G757*, located at the junction between the AlaRS editing and C-Ala domain). In this system, it is to be expected that only the cytosolic function is rescued by the human cytosolic AlaRS. The data showed that co-expression of two wildtype *AARS1*, as well as co-expression of wildtype and G757* *AARS1* plasmids supported robust yeast growth. However, co-expression of wildtype *AARS1* with either R329H or G102R significantly reduced growth.[41] The data demonstrate that both a loss of function, as well as a dominant-negative effect can be recapitulated in a yeast model for AlaRS disease.

1.5 | Asparaginyl-tRNA synthetase

The AsnRS is a highly conserved and ubiquitously expressed cytoplasmic tRNA synthetase. The human gene *NARS1* is localized to chromosome 18, encodes AsnRS, a class II aaRS which links $tRNA^{Asn}$ to the cognate amino acid, asparagine. In humans, AsnRS is comprised of a unique N-terminal extension domain, a tRNA-binding domain, and a C-terminal catalytic domain which contains the motif permitting dimerization and forms the active site of the enzyme (Figure 3). This domain layout is paralleled in the yeast ortholog *DED81*; however, only 51% of the residues share identity between the human and yeast synthetase

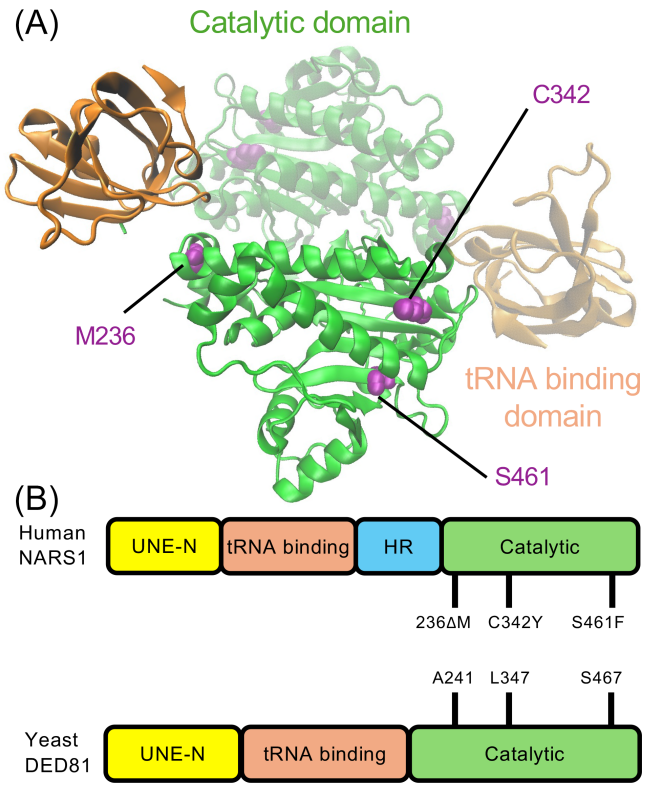

FIGURE 3 Structure and domains of asparaginyl-tRNA synthetase (AsnRS). (A). The crystal structure of truncated human AsnRS lacking the UNE-E (PDB ID# 8TC8) was visualized in VMD.[34] The catalytic domain is depicted in green, and the tRNA-binding domain is in orange. The hinge region (HR) linker motif is not resolved in the structure. The Van der Waals radius of amino acids corresponding to Charcot-Marie-Tooth disease (CMT) causative amino acids is depicted in purple. (B). The domain organization of the human *NARS1* and yeast *DED81*. Both enzymes contain the unique N-terminal extension domain (UNE-N), tRNA-binding domain, and the C-terminal catalytic domain. The hinge region (HR) is absent in the yeast enzyme. Protein blast alignment showed that CMT-causing residues M236 and C342 are not conserved between the two species and correspond to A241 and L347, whereas S461 is conserved and corresponds to yeast S467.

(Figure 3). Both yeast and human genomes encode two copies of the synthetase for mitochondrial and cytoplasmic function. Pathogenic alleles in *NARS1* are mostly associated with a multisystem disorder, and the literature describes patients with clinical characteristics including neurodevelopmental disorder with microcephaly, language delay, and gait abnormalities.[42] Recently, *NARS1* alleles 236ΔM, C342Y, and S461F were associated with a sensorimotor axonal neuropathy phenotype, consistent with dominant axonal CMT.[43] M236, C342, and S461 residues are found in the C-terminal catalytic domain of AsnRS. The S461 residue is conserved between humans and yeast. A *NARS1* yeast model was generated similarly to the model for *AARS1* described above, where a yeast haploid *ded81Δ* strain was complemented with wildtype or pathogenic *NARS1* variants. In contrast to the model above, the mutations were not inserted into the yeast *DED81*; instead, the human *NARS1* was used to generate a humanized *NARS1* yeast model. This complementation assay showed that the 236ΔM and S461F variants do not support yeast growth, whereas C342Y leads to reduced yeast growth.[43] This phenotype in yeast is consistent with a loss-of-function pathogenicity and is supported by the loss-of-function effects observed in yeast for other neuropathy-associated aaRS variants.[44] These data demonstrate that *NARS1* pathogenicity can be modeled in yeast.

1.6 | Histidyl-tRNA synthetase

Histidyl-tRNA synthetase (HisRS) is a class II aaRS and catalyzes the ligation of $tRNA^{His}$ to the cognate amino acid, histidine. Almost all HisRS enzymes in the three kingdoms of life use a unique identity element to distinguish cognate $tRNA^{His}$ from non-cognate tRNAs: a G-1 residue at the 5′ end of the $tRNA^{His}$.[45,46] In all eukaryotes, a specialized enzyme adds the G-1 residue to the $tRNA^{His}$, establishing identity.[47,48] HisRS consists of an N-terminal WHEP domain, a catalytic domain, and a C-terminal tRNA-binding domain (Figure 4). We recently reviewed mechanisms and potential treatments of disease-causing HisRS mutations.[49] Humans encode two HisRS copies, one localizing to mitochondria (*HARS2*) and one to the cytosol (*HARS1*), while yeast encodes a single copy (*HTS1*) with cytosolic and mitochondrial forms translated from two different translation start sites.[50] Interestingly, abolishing the mitochondrial *HTS1* by deleting the upstream ATG codon of *HTS1* for the mitochondrial targeting sequence results in a respiratory deficient phenotype but does not reduce viability.[50]

For HisRS, both humanized yeast models and models relying on inserting mutations in yeast *HTS1* have been established and used to characterize CMT2-causative mutations. In the yeast *HTS1* system, most assays have focused on characterizing mutations based on their ability to complement the yeast deletion (*hts1Δ*) strain. For example, CMT2-causative mutations V155G, Y330C, and S356N in the catalytic domain were inserted in yeast *HTS1* and showed a reduced growth phenotype in the *hts1Δ* strain. Similarly, T132I, P134H, D175E, and D364Y, also located in the catalytic domain, fail to complement *hts1Δ*.[8,51]

The humanized yeast model for HisRS disease was recently used by us to characterize CMT2-causative mutations in more detail. We found that four mutations located in the HisRS active site lead to a reduced growth phenotype and protein aggregation in humanized yeast.[52] Here, alleles V155G and S356N lead to insoluble protein aggregation and mistranslation in yeast with glutamine and threonine at histidine codons, indicating reduced amino acid specificity of the mutants. It is likely that these *HARS1* alleles do not sufficiently discriminate cognate from non-cognate amino acids and, therefore, mis-aminoacylate $tRNA^{His}$ with glutamine and threonine. Mistranslation could be rescued by the addition of excess histidine to the media, indicating a potential treatment for these patients. For two other alleles, V133F and Y330C, histidine had an opposing effect where it exacerbated proteome aggregation.[52] Interestingly, these alleles could be rescued by supplementation of $tRNA^{His}$, which alleviated mistranslation, opening possibilities for future tRNA therapeutics applications.[53,54] These data indicate a toxic gain-of-function phenotype. In another study, we showed that an inherited *HARS1* Y138H mutation, which is localized to the dimer interface, leads to a reduced growth phenotype but no protein aggregation or mistranslation.[55] Biochemical data confirmed a reduced dimer formation. Interestingly, the phenotype persisted in a heterozygous yeast model, where both human wildtype and Y138H alleles were provided on separate plasmids, indicating a dominant mechanism. Nonetheless, we were able to rescue the growth defect by adding histidine, providing excess substrate to promote sufficient His-$tRNA^{His}$ formation.[55] These data clearly demonstrate that while complementation assays are exceedingly useful in proving reduced function of a suspected pathogenic allele, they can also be used to determine the underlying cause, such as loss of function or toxic gain offunction due to mistranslation. In addition, yeast models provide a tractable phenotype to test potential treatment for disease, such as amino acid supplementation.

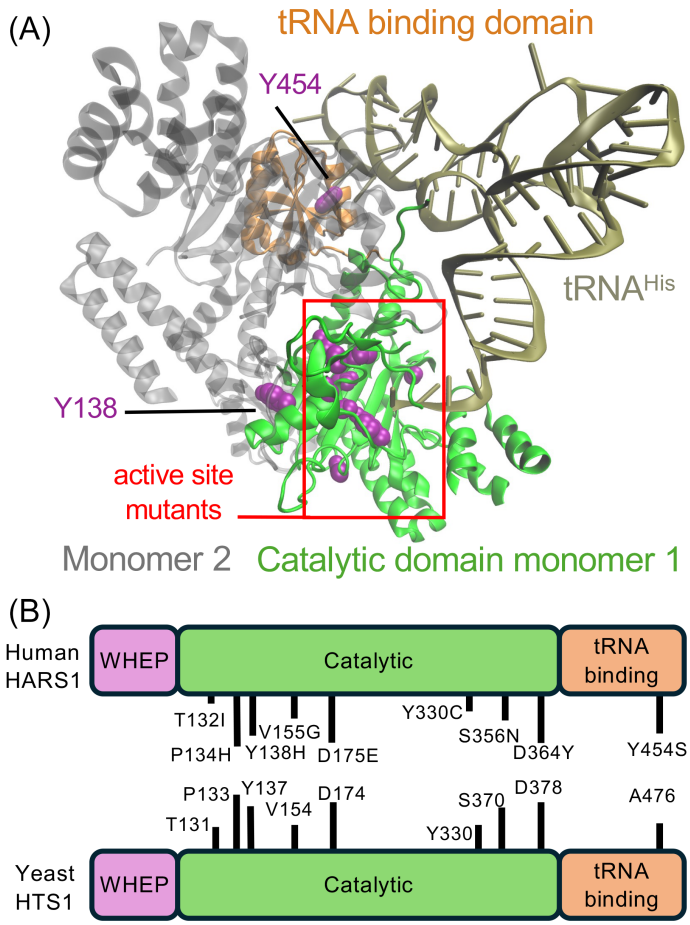

FIGURE 4 Structure and domains of histidyl-tRNA synthetase. (A). The crystal structure of human HisRS without the WHEP domain (green & grey, PDB ID# 6O76) was structurally aligned with the structure of *Thermus thermophilus* HisRS in complex with $tRNA^{His}$ (PDB ID# 4RDX), using VMD.[34] Protein residues are shown from the human structure and the tRNA from the *T. thermophilus* structure is depicted, whereas the protein structure of *T. thermophilus* was omitted for clarity. The Van der Waals radius of amino acids corresponding to Charcot-Marie-Tooth disease (CMT) causative amino acids is depicted in purple. (B) Domain organization of human *HARS1* and yeast *HTS1*. Both human and yeast enzymes share identical structures as they contain an N-terminal WHEP domain, the catalytic domain, and C-terminal tRNA-binding domain. Protein blast analysis showed that the CMT2-causative residues, T132, V133, P134, Y138H, V155, D175, Y330, and D364 are conserved between both species, whereas residues S356 and Y454 are not conserved. Most CMT2-causative mutations are localized to the active site, whereas Y138 is localized to the dimer interface, and the Usher syndrome associated residue Y454 is localized to the tRNA-binding domain.

1.7 | Glycyl-tRNA synthetase

Cytoplasmic GlyRS is classified as a class II aaRS, characterized by a conserved catalytic domain (Figure 5)[18] that catalyzes the ligation of glycine to $tRNA^{Gly}$. Human *GARS1* encodes an N-terminal WHEP domain, a catalytic domain, and a C-terminal anticodon binding domain (Figure 5). GlyRS is encoded as a bifunctional protein that is transcribed from the same gene by alternative translation initiation.[56] As such, the GlyRS gene encodes a mitochondrial targeting sequence. Yeast also encodes a single GlyRS that is localized to both cellular compartments. The first reported dominant-missense mutation in GlyRS associated with CMT2D and distal hereditary motor neuropathy type VA (HMN5A) dates back to 2003.[57] Since then, over 20 missense variants associated with CMT2-causative mutations on *GARS1* have been identified.[11] The majority of these mutations are located within the catalytic domain, with a few exceptions.[58] Notably, *GARS1* A57V has been identified in the WHEP domain, while S581L and G598A are localized in the anticodon binding domain.[59] Interestingly, *GARS1* G526R, which is located in the catalytic domain and is found at the dimer interface, increases dimer formation.[60] Additionally, this GlyRS variant exhibits a stronger affinity for tRNA binding and fails to release the tRNA into the cytosol, resulting in abolished aminoacylation activity.[60]

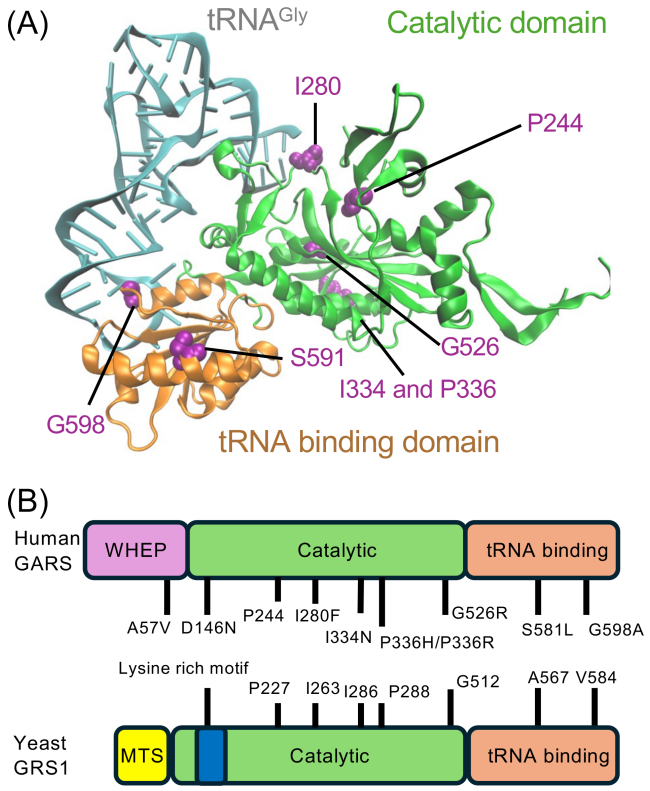

FIGURE 5 Structure and domains of glycyl-tRNA synthetase. (A). The crystal structure of human GlyRS without the WHEP domain in complex with $tRNA^{Gly}$ (light blue) (PDB ID# 4KR2) was visualized using VMD.[34] The tRNA-binding domain is depicted in orange, the aminoacylation domain in green. The Van der Waals radius of amino acids corresponding to Charcot-Marie-Tooth disease (CMT) causative amino acids is depicted in purple. (B) The domains of human *GARS1* and yeast *GRS1*. The human enzyme encodes an N-terminal WHEP domain, while the yeast dual function enzyme encodes an N-terminal mitochondrial targeting sequence (MTS). The catalytic domain of yeast *GRS1* encodes an additional Lysine rich domain not found in the human variant. The tRNA-binding domain is localized to the C-terminus. Various CMT2-causing mutations are indicated across the linear structure.

Several studies have utilized a yeast complementation assay to demonstrate the pathogenicity of *GARS1* alleles found in patients with CMT2. Most of these assays are based on whether an allele identified in patients complements the yeast deletion strain, and alleles not supporting growth or leading to a growth phenotype are classified as pathogenic. For GlyRS, different yeast model systems have been developed, based on studying both human mutations inserted in yeast *GRS1* and *GARS1* humanized yeast. Griffin et al. characterized nine CMT2-causative *GARS1* mutations. Of these, three residues that are also conserved in yeast *GRS1*, and the human pathogenic alleles were modeled onto yeast *GRS1*. Complementation assays showed no phenotype for I280F, while D146N showed reduced growth, and P244L did not support growth.[5] The remaining six mutations are not conserved, and were not tested in yeast, which demonstrates some of the drawbacks of creating human pathogenic mutations in yeast orthologs.

More recently, the humanized yeast model was used to study pathogenic *GARS1* mutations. This model relies on the expression of a truncated *GARS1* in yeast, lacking both the mitochondrial targeting sequence (MTS) and the WHEP domain of human GlyRS. Full-length human *GARS1* complemented the yeast *grs1Δ* strain, but only allowed for minimal cell growth, while *GARS1* lacking the MTS (ΔMTS) allowed for more efficient complementation. *GARS1* with both the MTS and the WHEP domain deleted (ΔMTSΔWHEP) fully supports yeast growth.[61] This model has been used in complementation assays studying mutations in patients with multisystem disorder[62] and most recently CMT mutations. Here, *GARS1* P336H and P336R were identified in two unrelated patients with symptoms of CMT. This mutation is highly conserved across several organisms, particularly in yeast and *E. coli*, and is in the catalytic domain of the protein.[63] The humanized yeast system was used to evaluate the functional impact of the P336H and P336R mutations, and neither allele supported yeast growth, whereas the wildtype successfully complemented growth.[63] Another study demonstrated a similar effect, where the *GARS1* I334N mutation failed to complement yeast growth.[64] This suggests that these mutations may lead to a loss off-unction. The studies highlighted above demonstrate how yeast can be employed as a rapid and reliable model for ortholog studies, but also demonstrate that the sequence and structural differences of human and yeast GlyRS limit some studies of pathogenic human alleles.

1.8 | Methionyl-tRNA synthetase

The human cytosolic MetRS is a 900 amino acid long polypeptide with a molecular mass of ~101 kDa which is encoded by the *MARS1* gene located on chromosome 12. MetRS is responsible for ligating methionine to $tRNA^{Met}$ which is utilized by the ribosomes during both initiation and the elongation steps of protein synthesis.[65] In eukaryotes, MetRS exists as part of a multi-synthetase complex (MSC) which includes at least eight other aaRSs in addition to proteins that act as cofactors.[66,67] MetRS is part of the class I family of synthetases. Its conserved catalytic main body consists of a Rossmann fold catalytic domain, the connecting peptide and stem contact fold domains, as well as the helical anticodon binding domain (Figure 6). Furthermore, in contrast to other organisms, the human MetRS protein has additional domains such as the N-terminal GST-like domain that interacts with AARS-

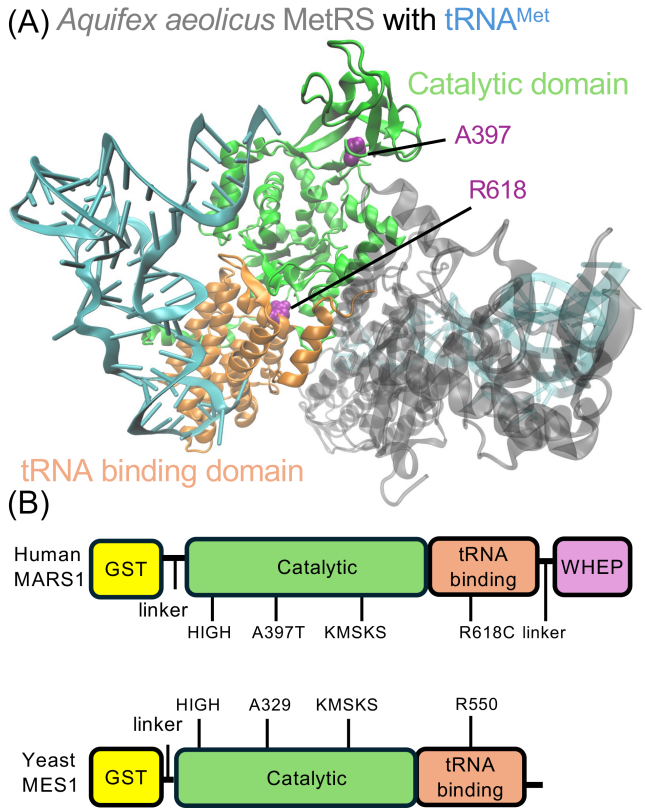

FIGURE 6 Structure and domains of methionyl-tRNA synthetase (MetRS). (A). The crystal structure of truncated human MetRS lacking the WHEP and GST domains (PDB ID# 5GOY). The tRNA-binding domain is depicted in orange, the aminoacylation domain in green. The human crystal structure was structurally aligned with structure of *Aquifex aeolicus* MetRS in complex with $tRNA^{Met}$ (protein in grey & tRNA in blue, PDB ID# 2CSX), using VMD.[34] The Van der Waals radius of amino acids corresponding to Charcot-Marie-Tooth disease (CMT) causative amino acids is depicted in purple. (B) The domains of human *MARS1* and yeast *MES1*. The human and yeast enzymes both contain the N-terminal GST domain, connecting peptide (CP), catalytic domain (CD), stem contact fold (SCF), and the tRNA-binding domain. The human C-terminal WHEP domain is absent in the yeast MES1. The linker peptide between the GST domain and CD is smaller in yeast when compared with the human enzyme. Both CMT-causing residues, A397 and R618, are conserved between both species when analyzed using protein blast alignment.

interacting multifunctional protein 3 (AIMP3) and the C-terminus WHEP domain that aids in tRNA binding and sequestering.[65]

Mutations within the *MARS1* gene are linked to the CMT-subtype 2 U (CMT2U). *MARS1* was first attributed as a novel candidate for this disease by analyzing a late-onset pathogenic R618C *MARS1* variant in the yeast ortholog, *MES1*.[9] Researchers carried out yeast viability assays by using similar methodology as described above in order to determine whether R618C mapped into yeast *MES1* could complement the deletion of endogenous *MES1* (*mes1Δ*) in the yeast strain. Their findings showed that the wildtype *MES1* construct was able to fully complement *mes1Δ*; however, R618C *MES1* failed to rescue *mes1Δ* allele, thus representing a loss-of-function phenotype in vivo.[9] Another novel *MARS1* mutation, A397T, was reported in a patient with an early-onset progressive motor neuropathy.[33] For this mutation, a humanized yeast was generated with *MARS1*. Yeast *MES1* does not encode the WHEP domain that is found in human MetRS, but nonetheless, the authors showed that full-length *MARS1* complements a yeast *mes1Δ* strain. Subsequent yeast viability assays revealed that while wildtype human *MARS1* complemented *mes1Δ*, *MARS1* A397T only supported minimal cell growth, consistent with a loss-of-function allele.[33] These data demonstrate that, in contrast to GlyRS, structural differences between the human and the yeast MetRS do not impact MetRS functionality in yeast, allowing for the analysis of mutations throughout the entire protein.

1.9 | Tryptophanyl-tRNA synthetase

The human *WARS1* gene encodes the cytoplasmic TrpRS. Human TrpRS, which is part of the family of class I synthetases, is composed of three main domains: the WHEP domain, a catalytic domain, and an anticodon binding domain (Figure 7).[11] TrpRS charges $tRNA^{Trp}$ with its cognate amino acid, tryptophan. Human *WARS1* and the yeast homolog *WRS1* show a 46% sequence identity,[68] and both genomes encode a second copy for mitochondrial function. Human TrpRS contains an additional 100 amino acids at the N-terminus that do not have a homologous sequence in the yeast protein, and the two proteins vary in the C-terminal sequence, while the catalytic core is largely conserved. To explore the potential of yeast as a model for human *WARS1*, a humanized yeast model was generated as outlined above, replacing endogenous *WRS1* with a plasmid-encoded human *WARS1* gene. While limited studies on *WRS1* in yeast are published as of right now,[69] yeast models of disease have confirmed changes of function of CMT2-causative *WARS1* alleles.

There is contradicting evidence on whether human *WARS1* can complement a yeast *WRS1* deletion strain (*wrs1Δ*). It was shown that full-length human *WARS1* can complement yeast *wrs1Δ* with growth rates identical to the endogenous protein.[68] In this case, human *WARS1* was provided on a 2 micron high-copy plasmid under a constitutive gal promoter. However, in a second study, full-length human wild-type *WARS1* failed to complement the yeast *wrs1Δ* strain when expressed with the

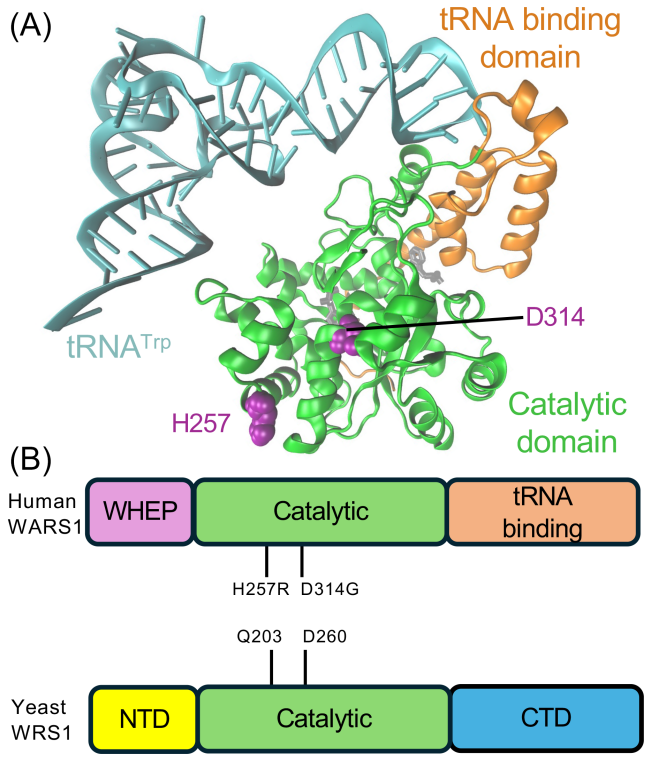

FIGURE 7 Structure and domains of tryptophanyl-tRNA synthetase (TrpRS). (A). The crystal structure of human TrpRS lacking the WHEP domain in complex with tRNA (blue) (PDB ID# 2AZX) was visualized using VMD.[34] The tRNA-binding domain is depicted in orange, the aminoacylation domain in green. The Van der Waals radius of amino acids corresponding to Charcot-Marie-Tooth disease (CMT) causative amino acids is depicted in purple. (B) The domains of human *WARS1* and yeast *WRS1*. The human enzyme encodes a N-terminal WHEP domain, the catalytic domain, and the C-terminus tRNA-binding domain. The yeast enzyme contains the N-terminal domain, the catalytic domain, and the C-terminal domain. Protein blast analysis showed that CMT-causing residue H257 is not conserved, corresponding to Q203, while residue D314 is conserved.

native *WRS1* promoter from low-copy centromeric plasmid.[70] Here, the authors studied a pathogenic *WARS1* allele; a missense variant was reported based on two unrelated individuals with a heterozygous *WARS1* H257R missense mutation.[70] *WARS1* H257R is located in the catalytic domain of the TrpRS protein[11] and follows an autosomal dominant inheritance pattern. The human H257R variant of *WARS1* showed a significantly enhanced ability to complement the yeast *wrs1Δ* strain compared with the wild type. Additionally, this variant demonstrates increased aminoacylation activity with yeast tRNA.[70] While these data, combined with the biochemical analysis, provided insight into the relaxed specificity of the pathogenic *WARS1* allele which eventually supported growth in yeast, it also demonstrated a caveat in humanized aaRS model systems: the subtle differences in yeast and human tRNA substrates that may conceal differences in substrate recognition in yeast and may account for why some pathogenic alleles do not lead to a phenotype in humanized yeast.

1.10 | Tyrosyl-tRNA synthetase

Tyrosyl-tRNA synthetase (TyrRS) was the second aaRS that was implicated in CMT. The human *YARS1* gene is orthologous to the *S. cerevisiae TYS1* gene. In yeast, *TYS1* encodes a 44-kDa protein, while in humans the *YARS1* encodes a 58-kDa protein.[71] TyrRS functions as a homodimer in both humans and yeast.[72] Human TyrRS is composed of an N-terminal catalytic domain, an anticodon binding domain, and a C-terminal endothelial monocyte-activating polypeptide II (EMAPII). The N-terminal catalytic domain and anticodon binding domains are homologous between yeast and humans, while the EMAPII domain, which is not required for aminoacylation activity in humans, is absent from yeast.[73]

Dominant mutations in the *YARS1* gene cause DI-CMT. Five variations in the *YARS1* gene have been found to lead to CMT2, each differing in their effects on the activity of the TyrRS protein (Figure 8).[11,74,75] The TyrRS G41R and Δ153-156 variants are unable to activate tyrosine, whereas the TyrRS E196K variant increases the overall catalytic activity to a rate that is higher than what is observed for the wild type. This indicates that the activity of the synthetases can be altered in a multitude of ways, leading to a loss of activity or an increase in function depending on where the mutation occurs.

In order to observe the effects of these mutations in a living organism, complementation experiments were carried out in yeast to determine whether the cells could be rescued through a plasmid. *TYS1* G41R and E196K mutations, corresponding to G45R and E200K in *YARS1*, were introduced in the yeast native *TYS1*.[6] As in the yeast models above, endogenous *TYS1* was deleted (*tys1Δ*), and growth relied on the plasmid-encoded alleles. The E200K protein somewhat compensated for the lack of TyrRS protein, but the G45R protein did not support growth.[6] When placed under the *ADH1* promoter, *TYS1* wild type and E200K variant both were able to rescue the growth of the *tys1Δ*, but the G45R variant did not. However, expression of the G45R variant under the GAL1 promoter partially complemented the *tys1Δ*.[6]

TyrRS was the first aaRS to be studied in yeast that showed an indication of a dominant-negative effect in a heterozygous *TYS1* deletion strain (*TYS1*/*tys1Δ*). In the *TYS1*/*tys1Δ* background, when the E200K and G45R *TYS1* variant alleles were expressed, a decline in growth rate as compared with the wild type was observed.[6] When

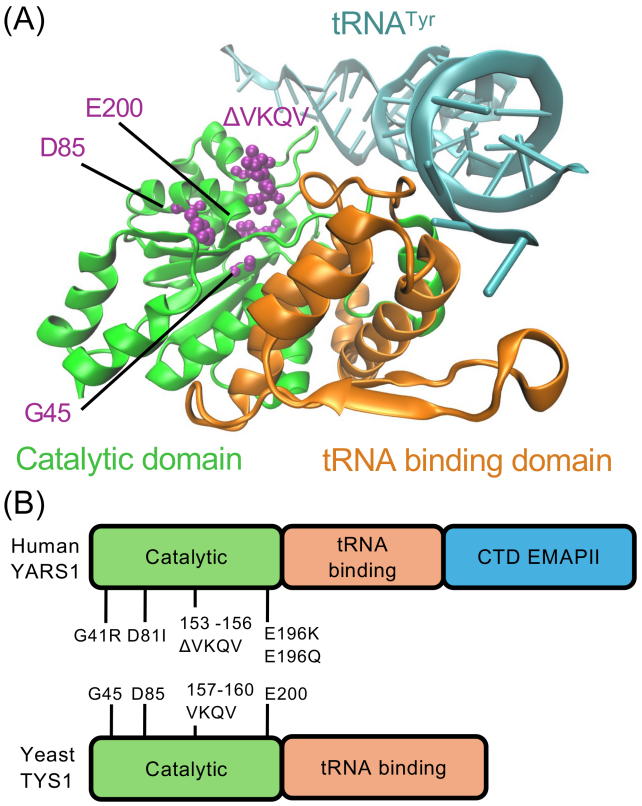

FIGURE 8 Structure and domains of tyrosyl-tRNA synthetase (TyrRS). (A) The crystal structure of yeast TyrRS in complex with $tRNA^{Tyr}$ (blue) (PDB ID# 2DLC) was visualized using VMD.[34] The tRNA-binding domain is depicted in orange, the aminoacylation domain in green. The Van der Waals radius of amino acids corresponding to Charcot-Marie-Tooth disease (CMT) causative amino acids is depicted in purple. (B) The domains of human *YARS1* and yeast *TYS1*. Both human and yeast enzymes encode an N-terminal catalytic domain and a tRNA-binding domain. At the C-terminus, the human gene encodes the cleavable C-terminal EMAPII domain. Protein blast alignment showed that the CMT-causing residues, G41, D81, and E196 are conserved between both species.

the human *YARS1* gene was expressed in the *TYS1*/*tys1Δ* background, the G45R variant showed decreased cell growth, whereas the E200K variant grew similarly to the wild type. The dominant-negative effect was shown to be due to the dimerization of the wild type and mutant subunits causing decreased enzymatic activity.[57]

2 | CONCLUSION

Yeast has become an important model system for the investigation of the molecular and cellular basis of pathogenic aaRS CMT mutations. Yeast models have been used to assess the biochemical consequences of mutations in aaRSs found in patients with CMT, test the cellular effects of aaRS CMT mutations, and learn the pathogenic inheritance patterns associated with these mutations. The powerful genetic system of yeast has enabled researchers to overcome challenges in understanding CMT-causing aaRS mutations by allowing them to interpret the pathogenicity of these variants.[6,41,76] Looking forward, this simple system can permit systematic screening of variants deposited in clinical genomic databases such as ClinVar, to provide valuable information on the pathogenicity as well as the underlying molecular mechanism of CMT mutations. Furthermore, yeast offers the exciting possibility of developing large libraries of random mutations to create a catalogue of mutations that induce hallmarks of CMT. An exciting area of work is already emerging where researchers have been able to develop and screen therapies for CMT by using yeast as a model system.[49]

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CONFLICT OF INTEREST STATEMENT

These authors declare no conflicts of interest.

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