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Predicting the Pathogenicity of Aminoacyl-tRNA Synthetase Mutations

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Abstract

Aminoacyl-tRNA synthetases (ARSs) are ubiquitously expressed, essential enzymes responsible for charging tRNA with cognate amino acids—the first step in protein synthesis. ARSs are required for protein translation in the cytoplasm and mitochondria of all cells. Surprisingly, mutations in 28 of the 37 nuclear-encoded human ARS genes have been linked to a variety of recessive and dominant tissue-specific disorders. Current data sustains that impaired enzyme function is a robust predictor of the pathogenicity of ARS mutations. However, experimental model systems that distinguish between pathogenic and non-pathogenic ARS variants are required for implicating newly identified ARS mutations in disease. Here, we outline strategies to assist in predicting the pathogenicity of ARS variants and urge cautious evaluation of genetic and functional data prior to linking an ARS mutation to a human disease phenotype.

Mutations in nuclear-encoded ARS enzymes cause human inherited disease

Aminoacyl-tRNA synthetases (ARSs) are ubiquitously expressed, essential enzymes that charge tRNA molecules with cognate amino acids in the cytoplasm and mitochondria. The human nuclear genome harbors 37 ARS loci: 17 that encode a cytoplasmic enzyme, 17 that encode a mitochondrial enzyme, and three that encode a bi-functional enzyme that charges tRNA for both cytoplasmic and mitochondrial protein translation [1]. One of the more interesting, albeit perplexing, findings in ARS research is that mutations in many of the genes encoding these enzymes cause myriad tissue-specific human diseases. As such, determining the genetic heterogeneity of ARS-related disease and the pathogenic

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mechanism of each disease-associated ARS mutation will be important for patient diagnosis, prognosis, and treatment. As the number of ARS mutations implicated in human disease rises, it is important to reflect on our current knowledge of the consequences of ARS mutations so that the pathogenicity of newly identified alleles can be carefully evaluated.

To date, mutations in 28 aminoacyl-tRNA synthetase (ARS) genes have been implicated in a spectrum of inherited, single-gene (Mendelian) human disorders (Table 1)[2]. Genes encoding mitochondrial ARSs have been implicated in recessive syndromes, while those encoding cytoplasmic ARSs have been implicated in both recessive and dominant disorders. Not surprisingly, mutations in genes encoding mitochondrial ARS enzymes often cause phenotypes in tissues with high metabolic demands such as the brain, muscle, and liver, similar to pathogenic mutations in mitochondrial genes and in other nuclear genes that encode mitochondrial proteins. For example, mutations in mitochondrial glutamyl- (*EARS2*) and aspartyl-tRNA synthetase (*DARS2*) cause autosomal recessive leukoencephalopathy [3,4], and mutations in mitochondrial phenylalanyl-tRNA synthetase (*FARS2*) have been associated with liver disease, encephalopathy, and lactic acidosis [5]. However, mutations in other mitochondrial ARS enzymes appear to cause tissue-restricted phenotypes; for example, mutations in mitochondrial histidyl-tRNA synthetase (*HARS2*) cause autosomal recessive ovarian dysgenesis and sensorineural hearing loss [6].

Similar to mitochondrial ARSs, mutations in genes encoding cytoplasmic ARS enzymes cause a spectrum of recessive syndromes, many of which include neurological phenotypes (Table 1). For example, arginyl- (*RARS*) and aspartyl-tRNA synthetase (*DARS*) mutations cause autosomal recessive hypomyelination in the central nervous system [7,8], and homozygosity for Y454S histidyl-tRNA synthetase (*HARS*) has been implicated in Usher syndrome [9]. Interestingly, only a single dominant phenotype has been associated with heterozygosity for mutations in a cytoplasmic ARS; mutations in glycyl- (*GARS*), tyrosyl- (*YARS*), alanyl- (*AARS*), and histidyl-tRNA synthetase (*HARS*) cause autosomal dominant peripheral neuropathy [9–14] (Table 1). Also referred to as Charcot-Marie-Tooth (CMT) disease, peripheral neuropathies compose a heterogeneous class of neurodegenerative diseases characterized by muscle weakness and sensory loss in the distal extremities [15]. CMT disease is divided into two major subgroups based on the primary cell type affected, with CMT1 referring to demyelinating neuropathy caused by a defect in Schwann cells and CMT2 referring to axonal neuropathy caused by impaired axon function [16]. To date, CMT-causing ARS mutations have been primarily associated with dominantly inherited CMT2 indicating that the mutations are particularly detrimental to the long axons of the peripheral nervous system.

Since 28 of the 37 human ARS loci have been implicated in disease phenotypes, it is reasonable to predict that mutations in the remaining nine ARS genes may also cause human disease. Furthermore, newly discovered mutations in previously implicated ARS loci are being reported at a rapid rate. As such, each newly identified ARS gene and allele must be carefully evaluated to distinguish *bona fide* disease-causing mutations from rare, non-pathogenic variants coincidentally identified in a patient with a relevant phenotype. To achieve this goal, we need to utilize all of the current knowledge of well-characterized, disease-associated ARS mutations. Here, we present strategies for assessing the

pathogenicity of ARS variants identified in patients with inherited disease and provide considerations for careful interpretation of the data generated from each genetic and functional approach.

ARS variant identification and validation

Linkage analysis

To identify a genetic locus involved in Mendelian disease pathogenesis, investigators must first ensure that the phenotype of interest is both monogenetic (*i.e.*, caused by a single gene) and measurable [17]. When feasible, generating a pedigree that illustrates the familial structure and phenotypic information for each individual allows for interrogation of the inheritance pattern (*e.g.*, dominant, recessive, or X-linked). Clinical data must be carefully collected to ensure that factors such as age of onset, incomplete penetrance, variable expressivity, and phenocopy do not confound the analysis. Furthermore, detailed clinical evaluations are critical for identifying families with similar phenotypes that may have disease-causing mutations in the same gene. For example, CMT-associated *YARS* mutations were identified through the analysis of two families with a similar dominant, intermediate form of CMT disease [11,18] and *GARS* mutations were identified through the analysis of unrelated families with a unique, upper-limb predominant form of CMT disease [10,19–23]. Once family and clinical data have been collected, various approaches can be used to identify disease-associated mutations including linkage, candidate gene, and whole-exome sequence (WES) analyses.

Linkage analysis is used to identify loci harboring a pathogenic mutation [24] by assessing for the co-segregation of genetic loci (*e.g.*, polymorphic markers) and the disease phenotype (*i.e.*, a marker of the disease-causing mutation) within pedigrees [17,24]. To perform linkage, affected and unaffected individuals are genotyped using genetic markers (*e.g.*, SNPs or microsatellite repeats) at known locations throughout the genome. Subsequently, a logarithm of the odds of linkage (LOD) score [25] is calculated— $\log_{10}[(1-\theta)^{NR*0R}/(0.5)^{NR+R}]$ —where θ is the recombination fraction, ‘NR’ is the number of non-recombinant offspring, and ‘R’ is the number of recombinant offspring. A LOD score of +3 is required for linkage with a 5% error rate [26]; however, a LOD score of +3.3 is required to correct for multiple testing in genome-wide analyses [26]. LOD scores are additive across pedigrees, which underscores the importance of having multiple, well-phenotyped families in order to identify a disease-associated locus. Limitations of linkage studies include errors in genotyping and phenotyping individuals (*e.g.*, misdiagnosis, phenocopy, late onset, and incomplete penetrance) and the limited resolution of genotyping platforms (*i.e.* the number of genetic markers assessed). Once a region of the genome has been linked to a disease phenotype, each gene (as well as non-coding sequences) can be interrogated for causative mutations.

Linkage analysis in multiple, large pedigrees remains the most convincing approach for identifying single-gene disease loci; note that this includes linkage analysis using genome-wide sequencing data [27]. For example, R329H *AARS* was identified via linkage analysis of 17 affected individuals from a five-generation family with autosomal dominant axonal CMT disease (Figure 1A)[12]. This revealed a region on chromosome 16—containing the

alanyl-tRNA synthetase (*AARS*) gene—with a maximum LOD score of 4.77. Since *GARS* and *YARS* mutations were already reported to cause CMT disease [10,11], subsequent mutation screening focused on *AARS*, which revealed that all affected individuals carry the R329H missense mutation (Figure 1A). Genetic data that further supported the pathogenicity of R329H *AARS* in CMT disease include: (1) an additional small family with CMT disease carrying this mutation [12]; (2) an additional large, four-generation family with eight affected individuals with CMT disease that carry this mutation [13]; and (3) experimental data showing that R329H *AARS* is a recurrent mutation that has occurred on multiple, independent haplotypes [13]. Thus, there is strong genetic evidence that implicates R329H *AARS* in human disease (*i.e.*, dominant CMT2). We will therefore use this mutation as an example in the approaches outlined below.

Candidate gene studies

Linkage analysis is often not possible due to, for example, limited access to DNA samples or small pedigree sizes. In these cases, a candidate gene approach can be pursued where genes or gene families known or predicted to cause the observed phenotype are directly assessed for disease-causing mutations. For example, since mutations in three ARS enzymes (*GARS*, *YARS*, and *AARS*) had been implicated in dominantly inherited CMT disease [10–13], it was proposed that mutations in other ARS loci may lead to a similar phenotype [14]. To test this hypothesis, 355 patients with CMT disease and no known disease-causing mutation were screened for variants in the protein-coding sequences of all 37 ARS enzymes by PCR amplification and DNA sequence analysis. The results of these studies included a histidyl-tRNA synthetase variant (R137Q *HARS*) identified in a single individual with peripheral neuropathy [14]. While functional studies supported the pathogenicity of R137Q *HARS* (see below), the genetic evidence was insufficient to implicate *HARS* in the pathogenesis of peripheral neuropathy (demonstrating an important limitation to candidate gene approaches). However, these findings made *HARS* an attractive candidate gene for further genetic studies in individuals with peripheral neuropathy. Indeed, linkage analysis and whole-exome sequencing subsequently revealed four disease-associated *HARS* mutations in four families with peripheral neuropathy, which provided the necessary genetic evidence to implicate *HARS* in CMT disease [9].

Whole-exome sequencing analysis

As the cost of whole-exome (WES) and whole-genome (WGS) sequencing decreases, the use of these technologies to identify disease-causing mutations is becoming more common [28–30]. A major strength of WES and WGS is the ability to directly scrutinize all (or most) relevant nucleotides in patient DNA samples, where WES focuses on protein-coding sequences and WGS provides data from both coding and non-coding sequences (*e.g.*, to include transcriptional regulatory elements). While variant filtering is often employed to prioritize mutations for follow up studies [31], WES and WGS can be combined with linkage analyses to build a statistical argument for pathogenicity [27]. Currently, WES is more commonly used due to the lower cost and the rationale that disease-associated mutations are likely to reside in and around protein-coding sequences. For example WES was used to identify aspartyl-tRNA synthetase (*DARS*) mutations in patients with recessive hypomyelination of the central nervous system [8]. In this study, the authors computationally

prioritized all of the identified variants, which revealed *DARS* as the sole candidate gene for the disease phenotype. Caveats to WES include an inability to assess non-coding regions of the genome and an inability to capture 100% of protein-coding sequences in the genome during sequence library preparation [31]. However, the nature of the mutations and the identification of multiple *DARS* mutations in unrelated individuals with a nearly identical recessive phenotype provided strong genetic evidence to implicate *DARS* in human disease [8].

Considerations for building a genetic argument for pathogenicity

As discussed above, recent advances have increased our ability to identify disease-causing alleles in human populations. These include sequencing technologies that allow rapid and affordable data acquisition as well as a deeper knowledge of human disease genetics, which permits efficient mutation analysis in research and clinical settings. However, two major issues face the field of human genetics. First, erroneous assignment of a gene as disease-associated can occur if rigorous validation and statistical analyses are not performed [32]. Second, once a gene is implicated in disease based on a strong genetic argument, newly identified variants can be erroneously attributed as ‘pathogenic’, which can be overlooked if the variant was coincidentally identified in a patient with the correct disease phenotype. For example, S581L *GARS* was identified in a patient with peripheral neuropathy and was therefore classified as pathogenic [33]. However, genetic studies revealed that S581L *GARS* occurs in the general population (see below) and, more importantly, that this mutation does not segregate with CMT disease in two unrelated families, where *affected* individuals *did not* carry the variant [34]. These data strongly argue against a pathogenic role for S581L *GARS* in dominant CMT disease and illustrate the importance of strong genetic evidence before implicating newly identified alleles in a disease phenotype.

To initially assess the pathogenicity of a Mendelian disease-causing variant, three basic approaches have been employed. First, genotyping is performed on all available family members to ensure that the variant segregates with disease with respect to the inheritance pattern (*i.e.*, all affected individuals have the expected genotype while all unaffected individuals do not). For example, all affected individuals in the pedigree with *AARS*-associated dominant CMT disease are heterozygous for R329H *AARS* and all unaffected individuals do not carry this mutation (Figure 1A) [12]. In contrast, S581L *GARS* does not segregate with disease in multiple families (see above) [34], which excludes it as a pathogenic mutation in these cases. There are, however, important caveats to this approach including phenocopy (*e.g.*, certain individuals do not carry the mutation but have a phenotype similar to other family members that is due to non-genetic factors), incomplete penetrance (*e.g.*, certain individuals carry the mutation but never express the disease phenotype, possibly due to environmental or genetic modifiers), and age of onset (*e.g.*, certain individuals carry the mutation but have not reach an age required for disease manifestation).

Second, genotyping is performed on unaffected control individuals (*i.e.*, individuals at the appropriate age that have been clinically assessed for the studied disease) and databases are scrutinized to rule out the presence of the variant in the general population. For example,

R329H *AARS* was not detected in 1,086 chromosomes from individuals without neurological disease and has not been detected (as of the preparation of this manuscript) in the Exome Aggregation Consortium (ExAC) database (<http://exac.broadinstitute.org>) (doi: <http://dx.doi.org/10.1101/030338>) nor in the overlapping and updated genome Aggregate Database (gnomAD; <http://gnomad.broadinstitute.org>). In contrast, S581L *GARS* is present in both ExAC (16 out of 119,342 alleles) and gnomAD (54 out of 282,216 alleles); note that S581L is equivalent to S635L on an alternate protein isoform. Furthermore, 39 of the 54 S581L *GARS* alleles in gnomAD are from 10,144 alleles from individuals of Ashkenazi Jewish decent, underscoring the importance of employing ethnically matched control populations. Combined, these data are consistent with S581L *GARS* being a non-pathogenic allele. There are also caveats to relying on this approach. In addition to the issues discussed for segregation analysis, the presence of a variant in the general population may not exclude it from being pathogenic especially if the phenotype is characterized by a late onset or if the variant is observed in non-disease-associated genotypes (*e.g.*, a heterozygous carrier for a mutation associated with a recessive disease). Indeed, G240R *GARS* has now been detected in gnomAD (1 out of 252,112 alleles; Table 2) and this mutation was identified via linkage analysis. To adjust for these possibilities, the population frequency of the variant should be compared to the frequency of that variant in patients with the disease phenotype.

Finally, conservation analysis is performed to determine if the disease-associated variant occurs at a nucleotide, or affects an amino-acid residue, that is conserved among evolutionarily diverse species. For example, R329H *AARS* affects an arginine that is conserved between human and bacteria, suggesting that this residue is important for enzyme function and consistent with pathogenicity (Figure 1B) [12]. In contrast, S581L *GARS* is only conserved among vertebrate species [34] indicating that this residue may not be essential for enzyme function. While this approach can be useful for mutations that impair gene function, conservation may not be expected for mutations that cause a gain-of-function effect; however, in this case one would not predict to identify the disease-associated change in another species. Of course, assessing the cis-genomic context at individual loci will provide a more accurate assessment of conservation as it relates to mutation pathogenicity [35].

Functional studies to predict the pathogenicity of ARS variants

Linkage, statistical, and validation studies are required for implicating a mutation in any Mendelian disease. However, functional studies can also be used to assist in predicting, but not proving, pathogenicity. These studies are most useful when the functional consequences of a newly identified variant can be compared to the functional consequences of variants that have been implicated in disease via strong genetic data (*e.g.*, R329H *AARS*). As mentioned, mutations in genes encoding ARS enzymes have been implicated in recessive and dominant disease phenotypes. In the case of recessive diseases, the inheritance patterns and phenotypes indicate a loss-of-function effect (Table 1). In the case of dominant CMT disease caused by ARS mutations, however, the pathogenic mechanism is less clear. While the majority (if not all) of CMT-causing variants impair ARS function (Table 2) it is not clear how this relates to the CMT disease phenotype [1,36,37]. In this section, we review model systems that have been employed to validate the pathogenicity of ARS mutations implicated

in recessive and dominant disease phenotypes. We argue that, regardless of the downstream effect of the mutations, impaired ARS function is a valuable predictor of pathogenicity.

Biochemical studies: pyrophosphate release and aminoacylation assays

Two approaches have been used to study the effects of ARS mutations on enzyme function: *in vitro* enzyme kinetic assays and *in vivo* yeast complementation assays. ARS enzymes charge tRNA with cognate amino acids via a two-step reaction (Figure 2) [38]. The first step involves binding of the ARS to the amino acid and ATP to form the amino-adenylate intermediate, which results in pyrophosphate release. In the second step, the amino-adenylate intermediate recognizes and binds to the appropriate tRNA, which is then covalently linked to the amino acid. Pyrophosphate release and aminoacylation assays are used to determine the ability of mutant ARS enzymes to charge tRNA *in vitro* compared to the wild-type enzyme [39] with pyrophosphate release assays testing for completion of the first step of the reaction [40,41]. Briefly, purified recombinant ARS enzyme (mutant or wild-type) is incubated with tRNA, ATP, cognate amino acid, and inorganic pyrophosphatase. Pyrophosphate is released upon formation of the amino intermediate, which can be measured by the hydrolysis of PPi by pyrophosphatases. Addition of a colorimetric reagent is used to quantitate the amount of free phosphates and absorbance is proportional to the amount of amino-adenylate intermediate formed. Assays to evaluate the first step of aminoacylation were used to determine the effects of *YARS* mutations (E196K and G41R) implicated in dominant CMT disease, and *HARS2* (L200V and V368L) and *FARS2* (I329T) mutations implicated in recessive disease phenotypes [5,6,11]. These efforts revealed that all five mutant proteins have reduced activity compared to the respective wild-type enzyme; however, another study showed that E196K *YARS* completes the first step of aminoacylation in a manner similar to wild-type *YARS* (please see below) [42].

Aminoacylation assays test the completion of both steps of the charging reaction and have been more commonly used to assess the effect of human mutations on ARS function. Briefly, purified recombinant ARS enzyme (wild-type or mutant) is incubated with tRNA, ATP, and radiolabeled amino acid. Aliquots of the reaction mixture are collected, spotted on filter paper, and the tRNA is precipitated using TCA to remove any unincorporated amino acid. Radioactivity levels are then assessed to determine the amount of amino acid ligated to tRNA molecules. Steady-state kinetics are calculated by fitting the initial rate of aminoacylation as a function of tRNA concentration (or the concentration of another substrate) to the Michaelis–Menten equation [43]. Aminoacylation assays have been used to demonstrate a loss-of-function effect on tRNA charging for mutations in 15 genes implicated in recessive disease (*AARS*, *CARS2*, *DARS2*, *FARS2*, *IARS*, *KARS*, *LARS2*, *MARS*, *MARS2*, *QARS*, *RARS2*, *SARS2*, *TARS2*, *VAR2*, and *YARS2*) [4,44–59]. Interestingly, aminoacylation assays have also revealed a loss-of-function effect of *GARS*, *YARS*, and *AARS* mutations that cause dominant peripheral neuropathy (Table 2); *HARS* mutations associated with dominant CMT disease have not been tested. For example, R329H *AARS* has 1/50 the charging capacity compared to the wild-type *AARS* protein (Figure 1C) [13]; the disease-associated mutant protein N71Y *AARS* also has dramatically reduced activity (Figure 1C). In contrast, the rare, non-pathogenic variants E778A *AARS* and S581L *GARS* do not affect enzyme activity (Figure 1C and Table 2). These data indicate that tRNA

charging assays should be employed to predict the pathogenicity of all newly identified ARS variants, with impaired function supporting a pathogenic role. However, additional functional analysis of non-pathogenic alleles is required to confirm that this assay can distinguish between disease-causing mutations and rare, non-pathogenic ARS variants.

Aminoacylation and pyrophosphate assays are limited in that they may not reflect the activity of ARS enzymes in living cells; for example, post-translational modifications or stabilizing cellular molecules may impact the function of the ARS enzyme *in vivo* [34]. Furthermore, there are a few notable discrepancies in the literature that question the rationale for using reduced enzyme activity as a predictor of ARS mutation pathogenicity, specifically in dominant CMT disease. For example, two studies assessed the effect of three CMT-associated *YARS* mutations (G41R, 153-156delVKQV, and E196K) on enzyme function. In one study, G41R and E196K had reduced activity in pyrophosphate exchange assays; 156delVKQV was not evaluated [11]. In the other study, G41R and 156delVKQV had reduced activity while E196K displayed activity similar to wild-type *YARS* [42]. Based on the normal activity of E196K *YARS*, the authors of the second study concluded that *YARS*-associated CMT disease is not caused by altered enzyme function. However, two studies reported that E196K *YARS* has reduced function when studied *in vivo* using yeast complementation assays (see below) [11,60] and another loss-of-function variant at this residue (E196Q) has been identified in a family with dominant CMT disease [60]; together, these data support an important role for E196 in *YARS* enzyme function.

Yeast complementation assays

Baker's yeast (*Saccharomyces cerevisiae*) is a robust, single-celled eukaryotic organism that is genetically tractable [61] and that shares certain biological features with mammalian cells, including ARS function. The effect of ARS mutations on gene function has been assessed via yeast complementation assays, which evaluate the ability of yeast cells to survive and grow in the presence of only the mutant ARS allele (Figure 3). Here, haploid yeast strains are developed with a non-functional (*e.g.*, deleted) ARS gene. Since ARS proteins are essential for cell survival, a wild-type copy of the ARS gene under study is expressed from a maintenance vector that also encodes the yeast *URA3* gene. *URA3* is an enzyme required for the biosynthesis of pyrimidines and, importantly, is lethal to yeast cells in the presence of 5-fluoroorotic acid (5-FOA); *URA3* converts 5-FOA to a toxic compound. Mutant ARS alleles are then modeled in either the yeast or human ARS ortholog and expressed from a second, experimental vector [62]. This system (Figure 3) allows the use of 5-FOA to select against the presence of the maintenance vector (containing the wild-type ARS locus) and to test for the ability of yeast cells to grow in the presence of only the experimental ARS allele (*e.g.*, wild-type or mutant). Thus, a functional allele on the experimental vector will result in yeast cell growth while a non-functional ARS allele will result in no (or reduced) yeast cell growth; here, cellular growth is used as a proxy for enzyme function.

Yeast complementation assays have been used to demonstrate a loss-of-function effect on ARS function for mutations in eight genes implicated in recessive diseases (*AARS*, *AARS2*, *HARS2*, *IARS*, *KARS*, *LARS2*, *MARS*, *VAR2*) [6,44,47,48,52,57,63,64]. Similar to aminoacylation assays, *in vivo* studies in yeast have revealed a loss-of-function effect for

GARS, *YARS*, *AARS*, and *HARS* mutations that cause dominant CMT disease (Table 2). For example, R329H *AARS* modeled in the yeast ortholog *ALAI* does not allow yeast cell growth consistent with a loss of enzyme function (Figure 1D). Similarly, the disease-associated N71Y *ALAI* allele does not support yeast cell growth while the non-pathogenic E778A *ALAI* allele allows growth similar to wild-type *ALAI* (Figure 1D) [13]. Likewise, the T132I *HARS* mutation associated with dominant CMT disease impairs yeast viability while the rare population variant T132S *HARS* allele sustains viability [9]. Together, these data indicate that yeast complementation assays can be employed to predict the pathogenicity of newly identified ARS variants, with reduced yeast cell growth supporting a pathogenic role. As with aminoacylation assays, the inclusion of non-pathogenic alleles is essential for assessing the efficacy of yeast to predict pathogenicity. Indeed, the results from yeast complementation studies of *AARS* variants are consistent with the biochemical studies (Figure 1C and 1D) [13] and suggest that these assays can distinguish between disease-causing and benign *AARS* alleles; however, many more disease-associated mutations and non-pathogenic variants need to be assessed.

There are clear limitations to the utility of yeast complementation studies for assessing the consequences of ARS mutations. First, while all mutations that demonstrate decreased yeast cell growth also show impaired function in aminoacylation assays, the converse is not true (Table 2). For example, G240R *GARS* has dramatically reduced enzyme activity *in vitro* but supports yeast cell growth *in vivo* when modeled in the yeast ortholog *GRS1* [34,65]. Explanations for this type of discrepancy include: (1) an inability of the yeast assay to resolve subtle differences in cell growth; (2) the growth of yeast at 30°C compared to the physiological environment afforded the human enzymes *in vivo* and the temperature at which aminoacylation assays are conducted (37°C); and (3) differences between the human and yeast ARS proteins that may lead to differential effects of mutations on the function of the two orthologs. Some of the above issues have been addressed: growth curves have been assessed to detect more subtle differences in yeast cell growth [11], certain *GARS* mutations modeled in *GRS1* grow the same at 30°C and 37°C [34], and human *YARS* has been used to show a loss-of-function effect of *YARS* mutations in yeast [11]. However, the discrepancies outlined above warrant careful interpretation when studying human ARS mutations in a yeast model system. Second, yeast studies (similar to the kinetic assays) evaluate individual alleles in isolation, which is appropriate for assessing impaired function but does not allow an assessment of interactions between the two alleles that exist in a human patient. Finally, yeast are small, single-celled organisms, which do not represent differentiated human cells within a multicellular environment. Indeed, one mutation with strong genetic evidence that has yet to show reduced function in enzyme kinetic or yeast complementation assays (E71G *GARS*) [34,65] shows a loss-of-function effect in a fly model (see below) [66], warranting a more detailed characterization of the enzyme activity of the mutant protein. In summary, while caution is required when interpreting the data, yeast complementation assays are currently the most effective and efficient *in vivo* test for predicting the pathogenicity of ARS mutations [67].

Animal models to predict the pathogenicity of ARS variants

Assessing the functional consequences of ARS variants in multi-cellular organisms is important for validating the pathogenicity of ARS variants in human disease. *Drosophila* is a powerful model organism due to the short life cycle and complex neural network that recapitulates many aspects of the mammalian nervous system. Two fly models were developed to assess the pathogenicity of *GARS* and *YARS* mutations identified in patients with dominant CMT disease [66,68]. The mosaic analysis with a repressible marker (MARCM) system in combination with a forward genetic screen revealed a mutation in the *Drosophila gars* gene (P98L) that causes axonal and dendritic morphological defects in olfactory projection neurons [66]. Neurons homozygous for the P98L *gars* mutation had normal growth and guidance of axonal and dendritic stalks but were unable to properly arborize the distal axons and dendrites. To determine if the P98L mutation defects were due to loss of GARS function, a neuron-specific *gars* null allele was generated; neurons homozygous for the *gars* null allele had an identical phenotype to neurons homozygous for P98L. This model was then used to test the functional effects of human disease-associated *GARS* mutations (E71G and L129P) on neurons. Specifically, expression of wild-type human GARS rescued the *gars*^{-/-} neuronal phenotype, while L129P did not and E71G only partially rescued axon growth. This study provided the first *Drosophila* model of ARS-mediated disease and supported a loss-of-function effect as part of the pathogenic mechanism of *GARS*-associated CMT disease [66]; however, non-pathogenic variants have yet to be evaluated in this system.

A second *Drosophila* model was developed to assess *YARS* mutations implicated in CMT disease for dominant toxicity (Figure 4) [69]. Here, mutant human alleles modeled in the fly ortholog were expressed in neurons from an inducible transgene. All three CMT-associated mutations tested (G41R, 153-156delVKQV, and E196K) caused a dominant climbing defect while wild-type *YARS* and a non-pathogenic variant (K265N) did not give rise to this phenotype. These data are critical for two reasons: (1) they reveal the fly model as effective in distinguishing between pathogenic alleles and rare, non-pathogenic variants; and (2) they demonstrate a dominant, toxic effect of *YARS* mutations consistent with the autosomal dominant patient phenotype and previous studies in yeast demonstrating a dominant-negative effect. In summary, informative fly models are available—and should be further employed—to evaluate the pathogenicity of newly identified ARS alleles.

C. elegans is a nematode (roundworm) that has also proven to be a relevant model to test ARS variants for dominant toxicity. *C. elegans* has characteristics that make it particularly useful for studying neuron biology [70]. First, it has a short lifespan and can be synchronized to specific developmental stages, which allows the evaluation of gene mutations associated with late-onset diseases. Second, *C. elegans* is transparent and has a fully defined, non-myelinated nervous system that permits easy visualization of axonal defects *in vivo*. The worm GABAergic nervous system consists of 26 neurons, including 19 inhibitory (D-type) motor neurons that project commissural axons from nerve cell bodies located in the ventral nerve cord to the dorsal nerve cord [71]. These morphologically simple and largely invariant axonal projections innervate the body wall muscles of the worm and are critical for proper locomotion. Functional or morphological alterations in these axonal

projections can lead to abnormal locomotion, which can be assessed using a battery of behavioral assays. For example, the thrash assay quantifies the number of lateral swimming movements an animal makes over time (thrashes min^{-1}) and can be used to examine the fidelity of GABA nervous system connectivity and function [72]. Typically, animals that have abnormal D-type GABA motor neuron function display irregular body bending and a reduction in the number of body bends over time.

A *C. elegans* model has been developed to study the effects of loss-of-function, CMT-associated missense ARS mutations on neurons (Figure 5) [9,14]. Briefly, wild-type or mutant (R137Q and D364Y) *C. elegans HARS* cDNA was cloned downstream of a GABAergic neuron-specific promoter (*Punc-25*) and injected into a *C. elegans* strain that stably expresses GFP under the GABA-specific vesicular transporter gene (*Punc-47*) promoter. Offspring were synchronized to the fourth larval stage and aged for 1 to 4 days before axons were visualized using fluorescence microscopy to assess for nervous system abnormalities. Confocal imaging revealed abnormal axonal branching and blebbing, failure of axonal commissures to extend to the dorsal nerve cord, and large dorsal nerve cord gaps that progressed with age in animals expressing mutant but not wild type *HARS*. Decreased locomotion in thrash assays correlated with abnormal axonal morphology that also progressed with age in animals expressing the mutant proteins [9,14]. Similarly, overexpression of R329H *AARS* leads to axonal defects in worm, which is not observed upon overexpression of wild-type *AARS* (Figure 1E and 1F) (Antonellis and Beg, unpublished data). These results have implications similar to the *YARS* studies in fly. Specifically, they show that: (1) the worm may be useful for distinguishing between pathogenic ARS alleles and non-pathogenic variants; and (2) loss-of-function missense mutations are dominantly toxic in overexpression studies, consistent with the patient phenotype. We therefore suggest that the complementary fly and worm model systems should be further used to predict the pathogenicity of ARS alleles identified in patients with dominantly inherited CMT disease. Importantly, data sets from a broader panel of disease-causing and benign ARS alleles will provide key information on the utility of these model systems.

Models to study the mechanism of ARS mutations in human disease

The functional strategies outlined above are useful for predicting the pathogenicity of newly identified ARS variants. Interestingly, the majority of previous studies employing these approaches revealed loss-of-function effects for ARS mutations implicated in recessive and dominant disease phenotypes. A loss-of-function effect is clearly the predominant hypothesis for the molecular pathology of ARS-associated recessive diseases, which is supported by functional analyses and the types of alleles identified (*e.g.*, many patients are compound heterozygous for one frameshift or nonsense allele and one hypomorphic missense allele). However, even though many (if not all) ARS mutations implicated in dominant peripheral neuropathy (*i.e.*, CMT disease) cause impaired enzyme function (Table 2), the precise mechanism of disease has not been defined; multiple gain- and loss-of-function hypotheses have been presented [2,36,73], and none of these mechanisms are mutually exclusive. For example, the loss-of-function characteristics of missense mutations identified in patients with dominant ARS-associated neuropathy in addition to the lack of

complete null alleles (nonsense, frameshift, etc.) raises the possibility of a dominant-negative effect. Here, the mutant protein may interfere with, and reduce the function of, the remaining wild-type protein. As such, it is important to briefly consider the models that have advanced our understanding of the pathogenic mechanism of ARS-related disease toward developing accurate assays to test newly identified variants.

In addition to directly showing that ARS mutations impair enzyme kinetics, molecular and cellular biology techniques have been useful in teasing out the mechanism of ARS-associated disease. For example, mutations in mitochondrial ARS enzymes that cause recessive diseases can directly affect protein solubility [74] or impair mitochondrial protein translation [4,5,46,50,56–59,63,75–79], consistent with a loss-of-function effect. With respect to ARS mutations that cause dominant peripheral neuropathy, structural analyses have revealed that the majority of *GARS* mutations affect residues within the dimer interface [80]. Subsequent molecular studies have shown that these mutations alter the dimer interface, which may mediate a gain-of-function effect via the acquisition of protein partners (see below) [81,82]; one caveat of these latter studies is that this effect was also observed for S581L *GARS* [81], which is a non-pathogenic variant [34]. Finally, studies in cultured neurons have shown that certain *GARS* and *YARS* mutations alter the sub-cellular localization of the protein suggesting a localized loss-of-function effect [11,34].

Animal models have also been useful in determining how ARS mutations cause recessive and dominant human disease phenotypes. For example, gene knock-down studies showed that reduced glutaminyI-(*gars*) and isoleucyl-tRNA synthetase (*iards*) function in zebrafish cause, respectively: (i) reduced eye size and pigmentation, smaller brains, and lack of coordination; and (ii) growth retardation and brain deformity [47,54]. These data support a loss-of-function effect of *QARS* and *IARS* mutations in the associated autosomal recessive disease phenotypes (Table 1). Furthermore, the zebrafish models provide an *in vivo* system to test the pathogenicity of newly identified *QARS* and *IARS* alleles through determining if they are able to rescue the fish phenotype. The pathogenic role of ARS mutations in dominant CMT disease has also been studied in animal models including fly, zebrafish, and mouse. For example, analysis of three disease-associated *GARS* mutations and three disease-associated *YARS* mutations in flies revealed that each resulted in reduced protein translation compared to the respective wild-type proteins; however, this effect was independent of impaired tRNA charging and a gain-of-function mechanism was therefore proposed [83]. In contrast, studies in zebrafish have shown that loss-of-function *gars* alleles impair neuromuscular junction morphology (consistent with an axonal CMT disease phenotype) and that dimerization is required for missense mutations to have this effect [84]. These data support the dominant-negative mechanism associated with *YARS* mutations [11]; however, data directly implicating a dominant-negative effect of *GARS* mutations have not been reported. Finally, three mouse models of *Gars* mutations support a gain-of-function mechanism. These studies revealed that: (1) missense mutations in *Gars* cause dominant neurodegeneration in mouse [85,86]; (2) over-expression of wild-type human *GARS* does not rescue the mouse phenotypes [87]; (3) *GARS* mutations act via inappropriate binding to neuropilin-1 [81]; (4) impaired neuromuscular junction development followed by pre-synaptic defects are part of the neuropathy phenotype [88,89]; and (5) carnitine is decreased

in the spinal cord of mutant *Gars* mice, and treatment with carnitine improves motor performance but had no effect on axon number, axon size, or neuromuscular junction innervation, deeming the relevance of these findings unclear [90]. Combined, extensive studies on neuropathy-associated ARS mutations suggest that both gain- and loss-of-function mechanisms may be at play. Interestingly, an additional mouse model revealed that homozygosity for a mutation in *Aars* that impairs editing of misacylated tRNAs causes recessive cerebellar Purkinje cell loss and ataxia, but no peripheral neuropathy [91,92]). While such mutations have not been identified in human populations, it may eventually be important to assess ARS editing activity when evaluating newly identified variants [13]. For all of the studies presented in this section, a more complete set of pathogenic and non-pathogenic alleles must be tested to validate the findings and to determine if the results are broadly applicable to ARS mutations and the respective patient phenotypes.

Moving forward: New disease-associated ARS loci and alleles

It is important to emphasize that building a strong genetic argument is essential for implicating any newly identified ARS locus or allele in human Mendelian disease. Specifically, the mode of inheritance (autosomal recessive or autosomal dominant; all ARS loci are encoded on autosomes) must be considered in the context of factors such as the disease prevalence, the frequency of the implicated variant in control populations, and the genotypes of individuals in control populations that carry the variant. A major issue facing human geneticists is the ease at which a newly identified variant in a known disease-causing gene can be erroneously reported as pathogenic; conclusive genetic data should also be required in these cases and we feel that S581L *GARS* serves as a cautionary tale [34].

In addition to genetic evidence for pathogenicity, we argue that our current understanding of disease-causing ARS mutations warrants the careful employment of functional studies to assist in predicting the pathogenicity of newly identified ARS alleles. For variants identified in patients with recessive phenotypes, testing for impaired ARS function using *in vitro* and *in vivo* model systems is rather straightforward; a loss-of-function mechanism is the leading hypothesis in these diseases. However, while the downstream effect on patient phenotypes is not clear, reduced gene function is also useful for predicting the pathogenicity of ARS missense variants identified in patients with dominant CMT disease. This argument is supported by studies showing that most, if not all, CMT-associated ARS missense mutations impair enzyme function (Table 2) and that loss-of-function missense mutations in *GARS*, *YARS*, *AARS*, and *HARS* have not, to date, been reported in unaffected individuals or in the general population. Multiple animal models have also been developed to test loss-of-function missense mutations for dominant toxicity and these models should be used to complement assays that test for the impaired function of ARS variants identified in patients with dominant disease.

Careful design and interpretation of functional studies is required to assess the consequences of ARS variants identified in patients with inherited diseases. First and foremost, positive and negative results should be interpreted in the context of the molecular, cellular, and tissue environment of the respective assay. For example, reduced enzyme activity should be tested in the appropriate tissue when feasible and substrate-specific effects (*e.g.*, amino acid,

tRNA, ATP) on enzyme activity should be considered before ruling out a loss-of-function mechanism. When performing yeast complementation assays, the human gene should be utilized if possible to rule out any species-specific effects of certain ARS mutations, and correlations between yeast data and *in vitro* tRNA charging data should be evaluated. Yeast assays also provide a rapid, tractable system to screen for molecules that can improve ARS function. Indeed, supplementation with methionine improved the *in vivo* function of mutant methionyl-tRNA synthetase (*MARS*) alleles that cause pulmonary alveolar proteinosis [52]. Finally, current worm and fly models rely on over-expression of the mutant allele to test for pathogenicity. Models should be developed to express ARS variants from the endogenous locus—similar to the mouse models of *Gars* mutations [85,86]—and should include the analysis of non-pathogenic control alleles.

ARS variants are being identified in patients with inherited disease at a rapid rate and databases such as gnomAD (*i.e.*, ExAC) are expanding the number of known ARS variants in the general population. As such, it is becoming impractical to test the functional consequences of individual variants as they are identified. Screens are needed to assess the functional consequences of all possible ARS variants toward developing a dataset to allow rapid predictions of pathogenicity. For example, massively parallel mutagenesis [93] of the human GARS open-reading frame followed by growth assays in allelically heterogeneous yeast cultures would provide a quantitative assessment of allele function. If all possible *GARS* missense mutations were tested in such a system, the resulting data could be used by clinical geneticists to build an argument for or against the pathogenicity of a newly identified *GARS* allele; however, we caution that these data alone would not be sufficient to determine the role of a variant in disease. Tools are also available to test the pathogenic potential of mutations in any ARS locus. This is particularly important because, while it is clear that mutations in any ARS gene could lead to a severe recessive phenotype, it is not clear if mutations in any ARS could lead to dominant CMT disease or if there is something unique about the four loci implicated to date—*GARS*, *YARS*, *AARS*, and *HARS*. For example, potentially pathogenic missense variants in an ARS locus not yet associated with CMT disease could be predicted via conservation analysis. Subsequently, the missense variants could be tested for a loss-of-function effect in enzyme kinetic and yeast assays. Loss-of-function missense mutations could then be evaluated for dominant toxicity in fly or worm, with any toxic alleles modeled in mouse to test for a peripheral neuropathy phenotype. These experiments would inform the allelic and locus heterogeneity of ARS-associated CMT disease, provide additional models to study disease pathogenesis, and direct future patient screening. Importantly, non-pathogenic control alleles need to be included in the above experiments to confirm the ability to distinguish between pathogenic and benign variants, and the suggestions presented here will likely be modified as we gain more information on the pathological mechanisms of ARS-associated disease.

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Highlights

1. Aminoacyl-tRNA synthetases (ARSs) are ubiquitously expressed, essential enzymes that ligate tRNA molecules to cognate amino acids
2. Genes encoding ARSs are associated with a spectrum of human inherited diseases
3. Implicating an ARS locus or allele in genetic disease requires strong genetic evidence
4. The majority of ARS mutations implicated in human disease cause impaired enzyme function
5. Functional studies should be carefully employed to predict the pathogenicity of newly identified ARS alleles

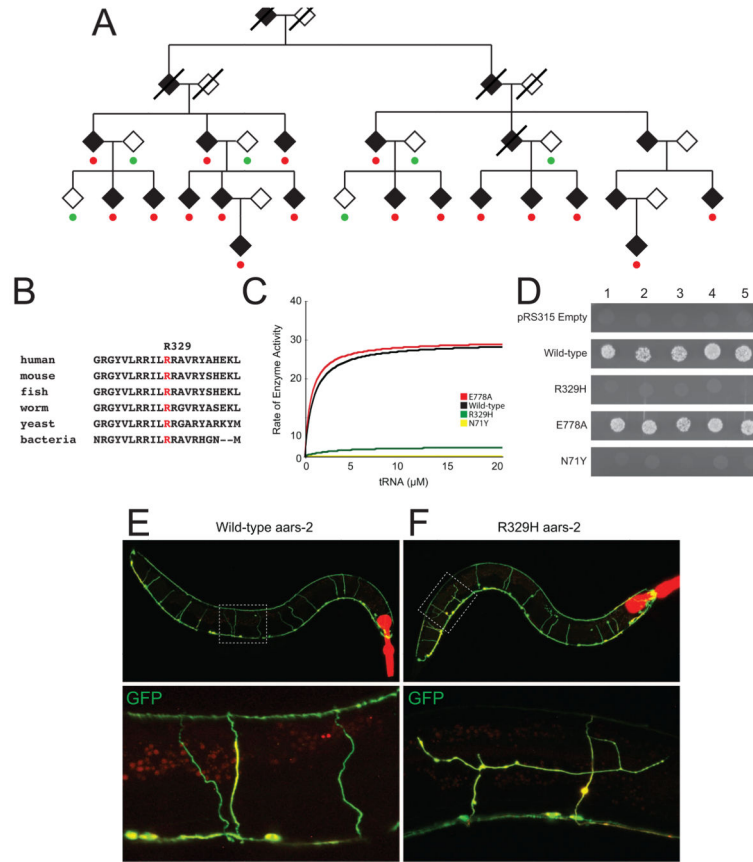


Figure 1. Characterization of the R329H AARS allele associated with dominant CMT disease (A) R329H AARS segregates with dominant CMT disease in a large pedigree. Filled symbols represent affected individuals, empty symbols represent unaffected individuals, and deceased individuals are crossed out. Red circles denote heterozygosity for the R329H AARS allele and green circles represent homozygosity for wild-type AARS. Diamonds were used to protect the identity of the family. (B) A multiple-species sequence alignment at the R329 amino-acid residue illustrates conservation among diverse species. The affected residue is indicated in red. (C) Aminoacylation results for tRNA^{ALA} charging for wild-type (red), E778A (black), R329H (green), and N71Y AARS (yellow). The rate of enzyme activity (pmol/min/pmol enzyme) is plotted against tRNA concentration. (D) Yeast complementation results for wild-type, N71Y, R329H, and E778A AARS modeled in the yeast ortholog, *ALAI*. Five independent cultures for each indicated genotype were grown on solid medium containing 5-FOA. (E and F) Confocal, fluorescence microscopy of animals over-expressing the wild-type (E) or mutant (R329H; F) worm AARS ortholog (*aars-2*, tagged with TagRFP). In each panel, merged images of GFP-filled GABA motor neurons (*Punc-47::GFP*) and the TagRFP::*aars-2* fusion protein are shown. The bottom frames are high magnification images and boxes in the top frames indicate the zoomed in area. Panel A was adapted from Latour *et al.*, 2010, and Panels C and D were adapted from McLaughlin *et al.*, 2014.

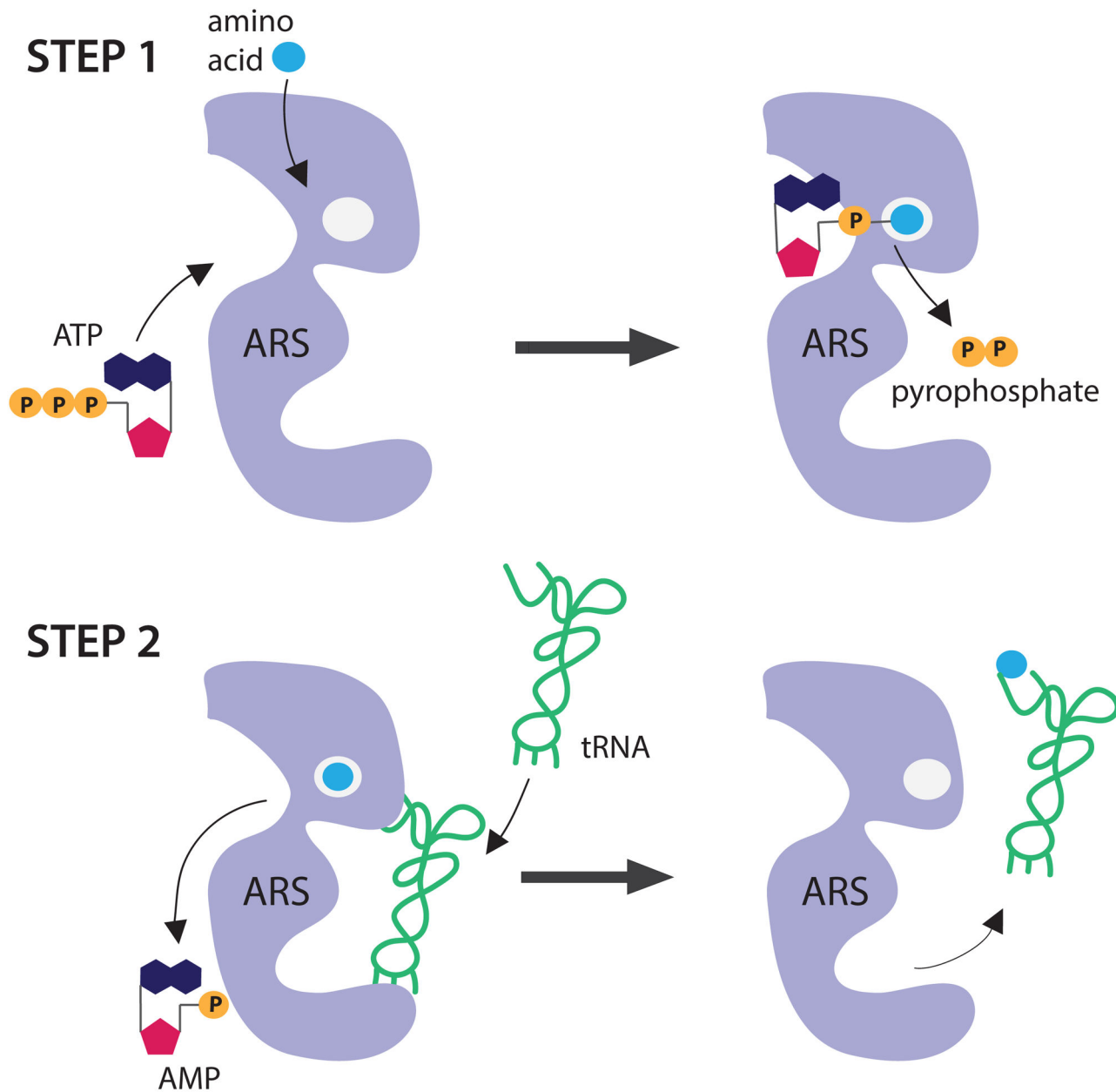


Figure 2. Schematic of the two-step aminoacylation reaction

The aminoacyl-tRNA synthetase (ARS; purple), amino acid (blue), ATP, AMP, pyrophosphate (orange), and tRNA (green) are all indicated. The reaction occurs in two steps, as depicted. First, the ARS binds the amino acid and ATP to form the amino-adenylate intermediate, which releases pyrophosphate. In the second step, the ARS binds the cognate tRNA to facilitate transfer of the amino acid to the tRNA. The tRNA is then released for protein synthesis. The image represents a monomeric enzyme; however, the reaction proceeds similarly for oligomeric enzymes.

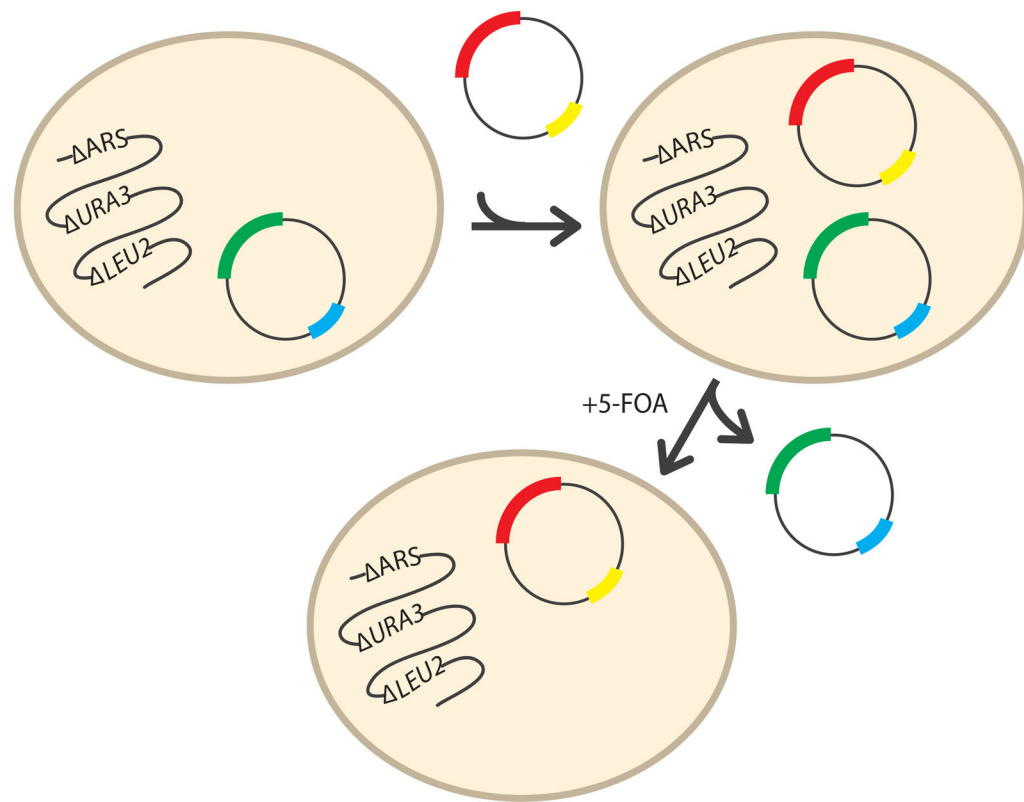


Figure 3. Overview of the yeast complementation assay

Haploid yeast disrupted for the endogenous ARS (Δ ARS) locus of interest are maintained viable via a maintenance vector bearing a wild-type copy of the respective ARS locus (green) and the *URA3* gene (blue) for selection. Wild-type or mutant alleles (red) are introduced via a *LEU2*-bearing (yellow) vector and resulting strains are selected for in medium lacking uracil and leucine. Subsequent growth on medium containing 5-FOA allows for the selection of cells that have spontaneously lost the maintenance vector and thus growth is correlated to the function of the ARS allele (red) on the experimental vector.

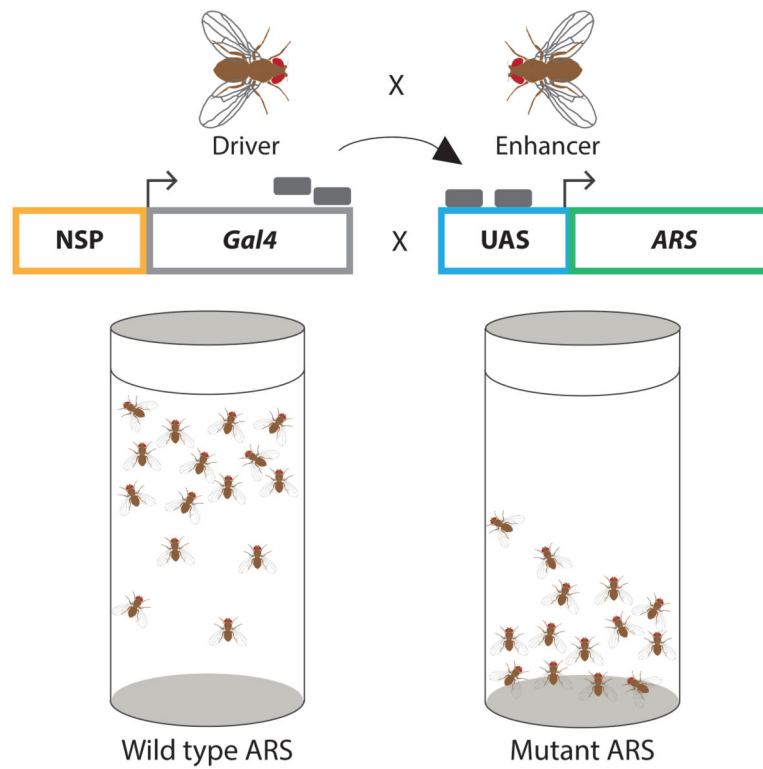


Figure 4. Studying ARS toxicity in *Drosophila*

Flies expressing Gal4 (grey rectangles) from a neuron-specific promoter (NSP) are crossed with flies bearing a transgene with the ARS cDNA of interest under control of an upstream activation sequence (UAS). In the progeny, Gal4 binds to the UAS to activate transcription of the ARS cDNA of interest in a neuron-specific fashion. Flies are then evaluated for their ability to climb using a negative geotaxis-climbing assay. Motor neuron function is quantified by the average climbing height of each cohort. The cartoon illustrates climbing defects observed in transgenic animals expressing a mutant (right), but not wild-type (left), ARS.

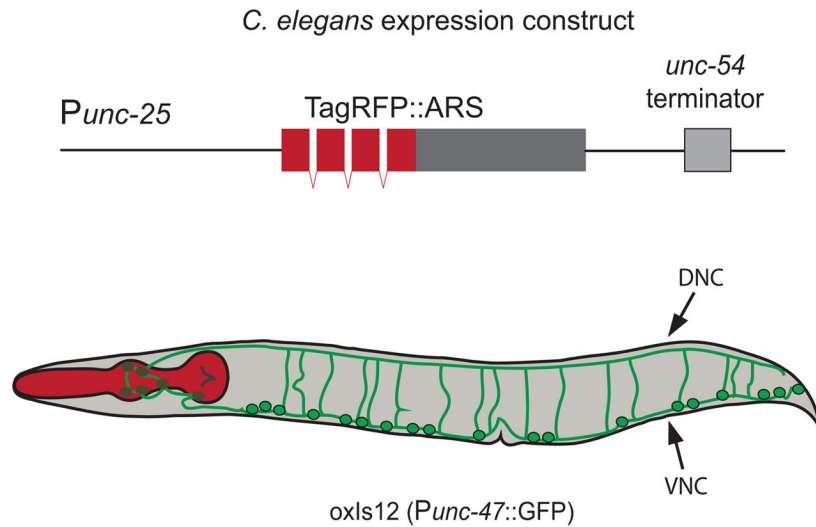


Figure 5. Studying ARS toxicity in *C. elegans*

GABA-neuron-specific expression of a fluorescently tagged ARS protein (TagRFP::ARS) is achieved via a GABA-specific promoter (*Punc-25*). The construct is injected into the worm strain *oxIs12*, which stably expresses GFP in GABAergic neurons via the *unc-47* promoter (*Punc-47*). Axons (green) extend ventrally from the cell bodies (green, black outline). The dorsal (DNC) and ventral (VNC) nerve cords are indicated. Fluorescent microscopy is used to assess for abnormalities in axonal morphology (*e.g.*, Figure 1F) and thrash assays are used to quantify motor neuron function.

Table 1

ARS Loci Implicated in Human Disease

Gene	Locus	Location of Protein Function	Mode of Inheritance	Disease Phenotype(s)	References
<i>AARS</i>	16q22	Cytoplasm	Autosomal dominant Autosomal recessive	CMT2N EEIE29	[12,34,94]
<i>AARS2</i>	6p21.1	Mitochondria	Autosomal recessive	Mitochondrial Infantile CMP Leukoencephalopathy with ovarian failure	[63,75]
<i>CARS2</i>	13q34	Mitochondria	Autosomal recessive	COXPD27	[45]
<i>DARS</i>	2q21.3	Cytoplasm	Autosomal recessive	HBSL	[8]
<i>DARS2</i>	1q25.1	Mitochondria	Autosomal recessive	LBSL	[4]
<i>EARS2</i>	16p12.2	Mitochondria	Autosomal recessive	LTBL COXPD12	[3]
<i>FARS2</i>	6p25.1	Mitochondria	Autosomal recessive	COXPD14 SPG77	[5,95,96]
<i>GARS</i>	7p15	Cytoplasm & Mitochondria	Autosomal dominant Autosomal recessive	CMT2D dSMA-V Myalgia, CMP	[10,97]
<i>HARS</i>	5q31.3	Cytoplasm	Autosomal dominant Autosomal recessive	CMT2W Usher Syndrome 3B	[14,98]
<i>HARS2</i>	5q31.3	Mitochondria	Autosomal recessive	Perrault Syndrome 2	[6]
<i>IARS</i>	9q22.31	Cytoplasm	Autosomal recessive	Intellectual disability, growth retardation, muscular hypotonia	[47]
<i>IARS2</i>	1q41	Mitochondria	Autosomal recessive	CAGSSS; Leigh Syndrome	[77]
<i>KARS</i>	16q23.1	Cytoplasm & Mitochondria	Autosomal recessive	RI-CMTB DFNB89 Visual impairment, microcephaly, DD, seizures	[48,99,100]
<i>LARS</i>	5q32	Cytoplasm	Autosomal recessive	Infantile hepatopathy	[101]
<i>LARS2</i>	3p21.31	Mitochondria	Autosomal recessive	Perrault syndrome 4 HLASA	[50,64]
<i>MARS</i>	12q13.3	Cytoplasm	Autosomal dominant Autosomal recessive	CMT2U / ILLD	[52,102]
<i>MARS2</i>	2q33.1	Mitochondria	Autosomal recessive	Spastic Ataxia 3 COXPD25	[53,103]
<i>NARS2</i>	11q14.1	Mitochondria	Autosomal recessive	COXPD24	[79]
<i>PARS2</i>	3p21.31	Mitochondria	Autosomal recessive	Alpers syndrome	[78]

Gene	Locus	Location of Protein Function	Mode of Inheritance	Disease Phenotype(s)	References
<i>QARS</i>	3p21.31	Cytoplasm & Mitochondria	Autosomal recessive	MSCCA	[54]
<i>RARS</i>	5q34	Cytoplasm	Autosomal recessive	HLD9	[7]
<i>RARS2</i>	6q16.1	Mitochondria	Autosomal recessive	PCH6	[55]
<i>SARS2</i>	19q13.2	Mitochondria	Autosomal recessive	HUPRA Syndrome	[56]
<i>TARS2</i>	1q21.2	Mitochondria	Autosomal recessive	COXPD21	[57]
<i>VARS</i>	6p21.33	Cytoplasm	Autosomal recessive	Severe DD, microcephaly, seizures	[104]
<i>VAR2</i>	6p21.33	Mitochondria	Autosomal recessive	COXPD20 Encephaloaridiomyopathy	[57,105,106]
<i>YARS</i>	1p35.1	Cytoplasm	Autosomal dominant Autosomal recessive	DI-CMTC Multi-system disease, DD, FTT	[18,107]
<i>YARS2</i>	12p11.21	Mitochondria	Autosomal recessive	MLASA2	[58]

Notes: CAGSSS: Cataracts, growth hormone deficiency, sensory neuropathy, sensorineural hearing loss, and skeletal dysplasia; CMP: Cardiomyopathy; CMT2D: Charcot Marie Tooth disease type 2D; CMT2N: Charcot Marie Tooth disease type 2N; CMT2U: Charcot Marie Tooth disease type 2U; CMT2W: Charcot Marie Tooth disease type 2W; COXPD12: Combined oxidative phosphorylation deficiency 12; COXPD20: Combined oxidative phosphorylation deficiency 20; COXPD21: Combined oxidative phosphorylation deficiency 21; COXPD24: Combined oxidative phosphorylation deficiency 24; COXPD25: Combined oxidative phosphorylation deficiency 25; DD: Developmental delay; DI-CMT: Dominant-intermediate Charcot Marie Tooth disease; dSMA-V: distal spinal muscular atrophy type V; EEIE29: Epileptic encephalopathy, early infantile, 29; FTT: failure to thrive; HBSL: Hypomyelination with brainstem and spinal cord involvement and leg spasticity; HLASA: Hydrops, lactic acidosis and sideroblastic anemia; HLD9: hypomyelinating leukodystrophy 9; HUPRA: Hyperuricemia, pulmonary hypertension, renal failure, and alkalosis; ILLD: Interstitial lung and liver disease; LBSL: Hypomyelination with brainstem and spinal cord involvement and elevated lactate; LTBL: Leukoencephalopathy with thalamus and brainstem involvement and high lactate; MLASA2: myopathy, lactic acidosis, and sideroblastic anemia; MSCCA: Progressive microcephaly, intractable seizures, and cerebral and cerebellar atrophy; PCH6: pontocerebellar hypoplasia type 6; RI-CMTB: Recessive-intermediate Charcot Marie Tooth disease type B; SPG77: spastic paraplegia 77.

¹While missense variants in *MARS* have been identified in patients with CMT, the genetic evidence is not strong enough to conclude, at this point, that this gene is associated with CMT disease.

Table 2

Characteristics of ARS variants identified in patients with dominantly inherited disease

Gene	Variant	Method of Discovery	Enzyme Activity	Yeast Viability	Detection in gnomAD	References
<i>AARS</i>	N71Y	Candidate gene sequencing	Reduced	Lethal	Not present	[13,108]
	G102R	Candidate gene sequencing	NA	Lethal	Not present	[67]
	R326H	Linkage analysis	Reduced	Lethal	Not present	[12,13]
	D893N	Candidate gene sequencing	NA	NA	Not present	[109]
<i>GARS</i>	A57V	Candidate gene sequencing	Reduced	NA	Not present	[34,110]
	E71G	Linkage analysis	Normal	Viable	Not present	[10,65,80]
	L129P	Linkage analysis	Reduced	Reduced	Not present	[10,65,80]
	D146N	Whole-exome sequencing	Reduced	Reduced	Not present	[34,111]
	S211F	Whole-exome sequencing	Reduced	NA	Not present	[34,111]
	G240R	Linkage analysis	Reduced	Viable	1/252,112	[10,65,80]
	P244L	Candidate gene sequencing	Reduced	Lethal	Not present	[34,112]
	I280F	Candidate gene sequencing	Reduced	Viable	Not present	[33,34]
	H418R	Candidate gene sequencing	Reduced	Lethal	Not present	[34,65,80,113]
	D500N	Candidate gene sequencing	Normal	NA	7/281,982	[80,114]
	G526R	Candidate gene sequencing	Reduced	Lethal	Not present	[10,65,80,115]
	S581L/	Candidate gene sequencing	Normal	NA	54/282,216	[33,80]
	G598A	Candidate gene sequencing	Reduced	Viable	Not present	[33,34,92]
	T132I	Linkage analysis	NA	Lethal	Not present	[9]
	P134H	Linkage analysis and candidate gene sequencing	NA	Lethal	Not present	[9]
	R137Q	Candidate gene sequencing	NA	Lethal	20/252,310	[14]
	D175E	Whole-exome sequencing	NA	Reduced	Not present	[9]
	D364Y	Linkage analysis and whole-exome sequencing	NA	Lethal	Not present	[9]
	R618C	Whole-exome sequencing	NA	Lethal	3/282,684	[102]
<i>MARS³</i>	P800T	Whole-exome sequencing	NA	NA	11/252,290	[116]
	G41R	Linkage analysis	Reduced	Lethal	Not present	[11,18,42]
<i>YARS</i>	153–156	Candidate gene sequencing	Reduced	NA	Not present	[11,42]
	VKQV	Candidate gene sequencing	Reduced	NA	Not present	[11,42]

Gene	Variant	Method of Discovery	Enzyme Activity	Yeast Viability	Detection in gnomAD	References
	E196K	Linkage analysis	Reduced ²	Reduced	Not present	[11,18,42]
	E196Q	Whole-exome sequencing	NA	Reduced	Not present	[60]

¹ S581L *GARS* was identified in a patient with axonal neuropathy via candidate gene screening and it is unclear if segregation analysis was performed in the family. Subsequent studies showed that S581L *GARS* does not segregate with neuropathy in two additional families. Please see text for details.

² E196K *YARS* was reported as having reduced enzyme activity in one study [17] and as having normal activity in another study [41].

³ While missense variants in *MARS* have been identified in patients with CMT, the genetic evidence is not strong enough to conclude, at this point, that this gene is associated with CMT disease.