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## Impaired Function is a Common Feature of Neuropathy-Associated Glycyl-tRNA Synthetase Mutations

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### Abstract

Charcot-Marie-Tooth disease type 2D (CMT2D) is an autosomal dominant axonal peripheral neuropathy characterized by impaired motor and sensory function in the distal extremities. Mutations in the glycyl-tRNA synthetase (*GARS*) gene cause CMT2D. *GARS* is a member of the ubiquitously expressed aminoacyl-tRNA synthetase (ARS) family and is responsible for charging tRNA with glycine. To date, thirteen *GARS* mutations have been identified in patients with CMT disease. While functional studies have revealed loss-of-function characteristics, only four *GARS* mutations have been rigorously studied. Here, we report the functional evaluation of nine CMT-associated *GARS* mutations in tRNA charging, yeast complementation, and subcellular localization assays. Our results demonstrate that impaired function is a common characteristic of CMT-associated *GARS* mutations. Additionally, one mutation previously associated with CMT disease (p.Ser581Leu) does not demonstrate impaired function, was identified in the general population, and failed to segregate with disease in two newly identified families with CMT disease. Thus, we propose that this variant is not a disease-causing mutation. Together, our data indicate that impaired function is a key component of *GARS*-mediated CMT disease and emphasize the need for careful genetic and functional evaluation before implicating a variant in disease onset.

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## Keywords

Charcot-Marie-Tooth disease; aminoacyl-tRNA synthetase; peripheral neuropathy; glycyl-tRNA synthetase; GARS

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## INTRODUCTION

Charcot-Marie-Tooth (CMT) disease encompasses a clinically and genetically heterogeneous group of distal, symmetric polyneuropathies characterized by muscle weakness and loss of sensation in the extremities (Reilly et al., 2011; Millecamps and Julien, 2013). CMT disease is the most common inherited peripheral neuropathy and is estimated to affect one in 2,500 individuals worldwide (Skre, 1974). To date, over 80 loci have been associated with CMT disease (Timmerman et al., 2014), which is divided into two major subclasses based on electrophysiological studies. Patients with demyelinating CMT disease (CMT1) have decreased motor nerve conduction velocities (MNCVs) due to defects in myelinating Schwann cells (Dyck and Lambert, 1968). Not surprisingly, genes implicated in CMT1 have important roles in myelination including peripheral myelin protein 22 (*PMP22*) (Patel et al., 1992) and myelin protein zero (*MPZ*) (Hayasaka et al., 1993). Patients with axonal CMT disease (CMT2) have normal MNCVs but decreased amplitudes of evoked muscle action potentials due to axonal dysfunction (Dyck and Lambert, 1968). Also consistent with electrophysiological studies, the genes associated with CMT2 encode proteins critical for axon function including mitofusin 2 (*MFN2*) (Züchner et al., 2004) and neurofilament light chain (*NEFL*) (Mersiyanova et al., 2000). Interestingly, mutations in six genes encoding enzymes indispensable for protein translation have been implicated in CMT disease with an axonal pathology (Antonellis et al., 2003; Jordanova et al., 2006; Latour et al., 2010; McLaughlin et al., 2010; Vester et al., 2012; Gonzalez et al., 2013).

Aminoacyl-tRNA synthetases (ARSs) are ubiquitously expressed, essential enzymes responsible for charging tRNA molecules with cognate amino acids—the first step of protein translation—in the cytoplasm and mitochondria (Delarue, 1995). There are 37 ARS genes in the human nuclear genome that code for 17 cytoplasmic, 17 mitochondrial, and 3 bi-functional enzymes; the latter group charge tRNA in both cellular compartments (Antonellis and Green, 2008). Glycyl-tRNA synthetase (*GARS*; MIM# 600287) mutations have been identified in patients with CMT2D or distal spinal muscular atrophy type-V (dSMA-V), two autosomal dominant forms of axonal peripheral neuropathy characterized by distal muscle atrophy, which is typically more severe in the hands (Christodoulou et al., 1995; Ionasescu et al., 1996; Pericak-Vance et al., 1997; Sambuughin et al., 1998; Ellsworth et al., 1999; Antonellis et al., 2003). Subsequently, mutations in the tyrosyl- (*YARS*; MIM# 603623), alanyl- (*AARS*; MIM# 601065), histidyl- (*HARS*; MIM# 142810) and methionyl- (*MARS*; MIM# 156560) tRNA synthetase genes have been implicated in dominantly inherited CMT disease with an axonal pathology (Jordanova et al., 2006; Latour et al., 2010; Vester et al., 2012; Gonzalez et al., 2013). Finally, one individual with CMT disease plus multiple neurological and non-neurological sequelae was found to be compound heterozygous for two mutations in lysyl-tRNA synthetase (*KARS*; MIM# 601421)

(McLaughlin et al., 2010). The occurrence of mutations in multiple ARS family members suggests a common pathogenic mechanism of ARS-related axonal CMT disease.

While there is strong genetic evidence linking ARS mutations and CMT disease, the molecular pathology underlying the axonal phenotype remains unknown. A number of hypotheses have been presented regarding the mechanism of ARS-associated CMT disease, including both loss- and gain-of-function effects (Antonellis and Green, 2008; Motley et al., 2010; Wallen and Antonellis, 2013). Since ARSs are responsible for charging tRNA molecules with amino acids, one possibility is that ARS-associated CMT disease is caused by the failure to charge sufficient quantities of tRNA; mutations in ARS genes may inhibit tRNA charging by impairing enzyme activity or by altering proper cellular localization (Antonellis and Green, 2008). As a result, the translational machinery may not be able to meet the demand for protein synthesis in highly metabolic, terminally differentiated motor and sensory axons.

To date, functional studies performed on a subset of CMT-associated ARS mutations have revealed loss-of-function characteristics. All nine of the *YARS*, *KARS*, *AARS*, *HARS*, and *MARS* mutations tested to date cause a severe decrease in enzyme activity and/or an inability to rescue deletion of the endogenous ARS gene in yeast complementation assays (Jordanova et al., 2006; McLaughlin et al., 2010, 2011; Vester et al., 2012; Gonzalez et al., 2013). However, the majority of CMT-associated ARS mutations have been identified in *GARS* and there is conflicting data regarding the presence or absence of loss-of-function characteristics. The functional consequences of human *GARS* mutations have been assessed in enzyme activity assays, yeast and fly complementation assays, and localization studies in cultured neurons. While many of the mutations demonstrate a loss-of-function effect in at least one of these assays (Antonellis et al., 2006; Chihara et al., 2007; Nangle et al., 2007; Xie et al., 2007; Stum et al., 2011), nine of the thirteen identified *GARS* mutations have not been rigorously tested (Wallen and Antonellis, 2013). Indeed, this includes five mutations (p.Ala57Val, p.Asp146Asn, p.Ser211Phe, p.Pro244Leu and p.Ile280Phe (James et al., 2006; Rohkamm et al., 2007; Abe and Hayasaka 2009; Lee et al., 2012)) that have not been evaluated in any assay. Characterizing the full panel of *GARS* mutations will be necessary to determine if a loss-of-function mechanism is a component of CMT2D and dSMA-V. These data will be critical for a better understanding of the molecular pathology of ARS mutations and for developing effective therapies for patients with ARS-related CMT disease.

Here we describe genetic, enzyme kinetic, yeast complementation, and protein localization studies that reveal the functional consequences of disease-associated *GARS* mutations. Combined with previously reported data, our findings support the hypothesis that impaired *GARS* function plays a central role in CMT2D and dSMA-V. Additionally, further screening of patients for disease-associated *GARS* mutations revealed that a variant previously associated with neuropathy (p.Ser581Leu) does not segregate with disease. These findings illustrate the need for careful genetic and functional evaluation of variants identified in patient populations before reporting them as causative factors in human disease.

## MATERIALS AND METHODS

### Computational assessment of *GARS* mutations

*GARS* protein sequences were collected from the NCBI Protein Database (<http://www.ncbi.nlm.nih.gov/protein/>) for the indicated species using the following accession numbers: human (*Homo sapiens*, accession number AAA57001.1), mouse (*Mus musculus*, accession number AAH21747.1), zebrafish (*Danio rerio*, accession number XP\_692410.4), roundworm (*Caenorhabditis elegans*, accession number NP\_498093.1), and baker's yeast (*Saccharomyces cerevisiae*, accession number NP\_009679.2). Multiple-species amino acid sequence alignments were then generated using ClustalW2 software (Larkin et al., 2007).

### *GARS* and *GRS1* expression constructs

DNA constructs for aminoacylation, yeast complementation and localization studies were generated using Gateway cloning technology (Invitrogen, Carlsberg, CA). Briefly, the human *GARS* open reading frame was amplified from a human cDNA sample, and the *S. cerevisiae GRS1* locus was amplified from *S. cerevisiae* genomic DNA. Primers were designed with flanking Gateway sequences attB1 (forward) and attB2 (reverse; primer sequences available on request). Entry clones were generated by recombining PCR purified amplicons into the pDONR221 vector using BP clonase and the manufacturer's specifications. After transformation into *E. coli*, DNA from individual entry clones was isolated and subjected to DNA sequencing analysis to confirm the presence of the appropriate wild-type gene. For each mutation studied here, mutation-containing oligonucleotides were generated, and the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA) was used per the manufacturer's instructions. After transformation into *E. coli*, DNA from individual clones was purified and sequenced to confirm the presence of each mutation and the absence of any cycle-induced errors. Subsequently, validated entry clones were purified and recombined into the appropriate Gateway-compatible vector using LR clonase and the manufacturer's specifications. The vectors included pET-21a(+) (aminoacylation assays), pEGFP-N2 (for expressing *GARS* with a C-terminal EGFP tag), and pRS315 (yeast complementation assays). DNA from the resulting expression constructs was purified and digested with the restriction enzyme *BsrGI* (New England Biosystems, Ipswich, MA) to confirm the presence of the appropriate insert.

### ARS Aminoacylation Assays

Wild-type and mutant human *GARS* proteins (see above) were expressed in *E. coli* Rosetta 2 (DE3) pLys cells with a C-terminal in-frame His tag and purified with nickel affinity resin according to the manufacturer's protocol (Novagen, Rockland, MA). Based on the wild-type enzyme, the active fraction of human *GARS* in our preparations was ~20%. The T7 transcript of human tRNA<sup>Gly/UCU</sup> (UCU, anticodon) was prepared and purified as previously described (Hou et al., 1993) and was heat denatured at 85°C for 3 min and annealed at 37°C for 20 min before use. Steady-state aminoacylation assays were monitored at 37°C in 50 mM HEPES (pH 7.5), 20 mM KCl, 10 mM MgCl<sub>2</sub>, 4 mM DTT, 2 mM ATP, and 50 μM <sup>3</sup>H-glycine (Perkin Elmer, Waltham, MA) at a specific activity of 16,500 dpm/pmol. The reaction was initiated by mixing a *GARS* enzyme (20–600 nM) with varying concentrations of tRNA (0.3–20 μM). Aliquots of a reaction mixture were spotted on filter

papers, quenched by 5% trichloroacetic acid (TCA), washed, dried, and measured for radioactivity by a liquid scintillation counter (LS6000SC, Beckman Coulter Inc., Fullerton, CA). The amount of radioactivity retained on filter pads was corrected for quenching effects to determine the amount of synthesis of Gly-tRNA<sup>Gly</sup>. Steady-state kinetics were determined by fitting the initial rate of aminoacylation as a function of tRNA concentration to the Michaelis–Menten equation (Schreier and Schimmel, 1972).

### Yeast complementation assays

The RJT3/II-1 haploid yeast strain (MAT $\alpha$  *grs1::HIS3*, *his3 200*, *leu2 1*, *lys2 202*, *trp 63*, *ura3-52*, pTsscII-maint (*cen*, *GRS1*, *URA3*)) carrying a deleted endogenous *GRS1* allele and wild-type *GRS1* on a *URA3*-bearing pRS316 maintenance vector was previously reported (Turner et al., 2000; Antonellis et al., 2006). RJT3/II-1 was transformed with a *LEU2*-bearing pRS315 vector containing wild-type or mutant *GRS1* (described above) or pRS315 with no insert as previously reported (Antonellis et al., 2006). Each transformation was performed at least four times with at least three independent plasmid DNA preparations. Four colonies were selected from each transformation for additional analysis. Each colony was grown to saturation in –leu –ura selection medium for 48 hours. Next, 10  $\mu$ L of undiluted and diluted (1:10 and 1:100) samples from each culture were spotted on plates containing 0.1% 5-Fluoroorotic Acid (5-FOA) (Boeke et al., 1987) complete medium, yeast extract-peptone-glycerol (YPG) plus 0.1% 5-FOA medium, or SD –leu –ura growth medium (Teknova, Hollister, CA) and incubated at 30°C or 37°C for 72 hours. Yeast cell growth was determined by visual inspection.

### Cell culture and protein localization studies

The mouse motor neuron, neuroblastoma fusion cell line (MN-1) was cultured and transfected with constructs to express wild-type or mutant *GARS* in-frame with a C-terminal enhanced green fluorescent protein (EGFP) tag as previously described (Salazar-Gruesso et al., 1991; Antonellis et al., 2006). After 48 hours, growth medium was removed, and cells were washed in 1X PBS and then incubated in 1X PBS/0.4% paraformaldehyde for 10 min at room temperature. Cells were washed in 1X PBS, co-stained with 300 nM DAPI for 5 min, washed again in 1X PBS, and finally coated with Pro-Long antifade reagent (Invitrogen). Images were obtained with an IX71 Inverted Microscope using cellSens Standard image software (Olympus, Center Valley, PA).

### Population Screening and Segregation analysis

The presence of each *GARS* variant in the general population was assessed using the Exome Variant Server (snp.gs.washington.edu/EVS/), the 1000 Genomes Project database ([www.1000genomes.org/](http://www.1000genomes.org/)), and dbSNP ([www.ncbi.nlm.nih.gov/snp](http://www.ncbi.nlm.nih.gov/snp)). Additionally, a search of the GEM.app exome-sequencing database was performed for *GARS* variants in patients with CMT disease (genomics.med.miami.edu). For p.Ser581Leu *GARS* segregation studies, oligonucleotide primers were designed to amplify *GARS* exon 16, which harbors the 2<sup>nd</sup> and 3<sup>rd</sup> nucleotides of the serine 581 codon. Patient DNA samples were subjected to PCR amplification under standard conditions along with a ‘no DNA’ PCR-negative control reaction. Subsequently, PCR products were purified on Mini Spin Columns (Epoch Life Science, Inc., Missouri city, TX), eluted, and then subjected to Sanger DNA sequencing

analysis using the ‘forward’ and ‘reverse’ amplification primers in separate reactions (University of Michigan DNA Sequencing Core). The appropriate review boards of each participating institution approved these studies.

## RESULTS

### Localization and conservation of human *GARS* mutations

*GARS* mutations found in patients with CMT disease are distributed throughout the primary structure of the protein. Notably, the mutations affect conserved residues, which are often conserved between human and yeast, suggesting a critical role in protein function (Antonellis et al., 2003). To determine if newly reported *GARS* mutations demonstrate a similar level of conservation, we mapped the residues onto the human protein and generated an alignment with evolutionarily diverse organisms (Fig. 1A and B). Mutations were evenly distributed throughout the major functional domains of the protein (Xie et al., 2007)(Fig. 1A): (1) p.Ala57Val affects a residue in the disordered WHEP-TRS domain (residues 13–63; pfam00458 in the NCBI Conserved Domain Database), which is putatively involved in tRNA binding and interacting with other aminoacyl-tRNA synthetases in enzyme complexes (Ray et al., 2011), but that is not critical for GARS function (Xie et al., 2007); (2) p.Asp146Asn, p.Pro244Leu, and p.Ile280Phe affect amino acids in the catalytic core (residues 92–168, 241–305, and 350–418; pfam 00587) responsible for ligation of the amino acid to tRNA; (3) p.Ser211Phe, p.His418Arg and p.Asp500Asn affect amino acids within insertion domains of uncertain function; and (4) p.Ser581Leu and p.Gly598Ala reside in the anticodon-binding domain (residues 557–655; pfam03129) that binds to glycine-specific tRNAs.

The evolutionary conservation of mutated *GARS* residues varies greatly (Fig. 1B). Asp146, Pro244, Ile280, and His418 are identical between human and yeast, while Ser211 and Gly598 are conserved, but to a lesser degree (*e.g.*, between human and worm). In contrast, Ala57 is conserved among mammals and worm, but not zebrafish, and Asp500 and Ser581 are conserved only among vertebrates. Thus Asp146, Ser211, Pro244, Ile280, His418, and Gly598 are predicted to be critical for enzyme function, while Ala57, Asp500, and Ser581 are less likely to be essential for *GARS* activity.

### The majority of *GARS* mutations reduce aminoacylation activity

The primary function of ARS enzymes is to charge tRNA molecules with cognate amino acids (Delarue, 1995) in a two-step aminoacylation reaction: (i) binding and activation of the amino acid with ATP and (ii) conjugation of the activated amino acid to the 3' end of the appropriate tRNA (Antonellis and Green, 2008). The aminoacylation assay evaluates the combined reactions of both steps and has been used to establish if CMT-associated ARS mutations disrupt this critical canonical function (Nangle et al., 2007; McLaughlin et al., 2010, 2011; Froelich and First 2011). Here, we assessed previously untested *GARS* variants (p.Ala57Val, p.Asp146Asn, p.Ser211Phe, p.Pro244Leu, p.Ile280Phe, p.His418Arg and p.Gly598Ala) for the ability to charge human cytoplasmic tRNA<sup>Gly</sup> with tritium-labeled glycine. For comparison purposes, we included two *GARS* mutations previously shown to impair enzyme kinetics: p.Gly240Arg and p.Gly526Arg (Nangle et al., 2007). With regard

to the untested variants, p.Asp146Asn, p.Ser211Phe, p.Pro244Leu, p.Ile280Phe, p.His418Arg, and p.Gly598Ala GARS demonstrate less than 10% aminoacylation activity compared to wild-type GARS (Table 1). In contrast, p.Ala57Val displayed the ability to charge tRNA<sup>Gly</sup> molecules with a much higher efficiency than other mutant proteins, albeit with a 50% reduction relative to wild-type GARS.

### The majority of testable GARS mutations dramatically reduce yeast cell viability

Yeast complementation assays have been performed to determine the functional consequences of CMT-associated ARS mutations in the context of a living cell (Antonellis et al., 2006; Jordanova et al., 2006; McLaughlin et al., 2010, 2011; Stum et al., 2011; Vester et al., 2012; Gonzalez et al., 2013). Here, mutations are modeled in the *S. cerevisiae* GARS ortholog *GRS1*, and cellular growth is used as a proxy for enzyme function; this model employs a haploid yeast strain with the endogenous *GRS1* locus deleted and viability maintained via expression of wild-type *GRS1* on a *URA3*-bearing maintenance vector (Turner et al., 2000; Antonellis et al., 2006; Stum et al., 2011) (Fig. 2A). Based upon ClustalW alignments (Fig. 1B), we identified the orthologous *GRS1* amino acid of each previously untested GARS mutation and performed site-directed mutagenesis to model the human mutations in yeast. We were able to confidently model p.Asp146Asn, p.Pro244Leu, and p.Ile280Phe GARS in *GRS1* based on homology; p.Ala57Val, p.Ser211Phe, p.Asp500Asn and p.Ser581Leu are not conserved between human and yeast and thus could not be modeled in this assay. Based on protein alignments, GARS p.Asp146Asn, p.Pro244Leu, and p.Ile280Phe (GenBank accession number AAA57001.1) correspond to *GRS1* residues p.Asp96Asn, p.Pro227Leu, and p.Ile255Phe (GenBank accession number NP\_009679.2), respectively. *GRS1* mutations are referred to in Figure 2 based on the human mutation they model. Yeast cells were transformed with the experimental alleles on a *LEU2*-harboring vector and plated on solid growth medium containing 0.1% 5-Fluoroorotic Acid (5-FOA; Fig. 2A). 5-FOA is toxic to yeast cells expressing *URA3* and thus selects for cells that have spontaneously lost the maintenance vector (Boeke et al., 1987). Only yeast cells containing a functional *GRS1* allele on the *LEU2*-bearing experimental plasmid are able to complement the deleted endogenous *GRS1* allele and survive on 5-FOA plates. Our wild-type *GRS1* expression construct allowed robust yeast cell growth and our experimental vector without a *GRS1* gene ('Empty' in Fig. 2B) was unable to complement the null allele in yeast cells (Fig. 2B). These results are consistent with the construction of a functional *GRS1* experimental system and with *GRS1* being an essential gene, respectively (Turner et al., 2000; Antonellis et al., 2006). With regard to the modeled mutations, p.Ile280Phe *GRS1* was able to maintain yeast viability (Fig. 2B) in a comparable manner to wild-type *GRS1*. In contrast, p.Asp146Asn and p.Pro244Leu *GRS1* resulted in a severe reduction of yeast cell growth compared to wild-type *GRS1* (Fig. 2B).

We also evaluated yeast cells incubated at 37°C to assess for temperature-sensitive effects of the studied mutations. These analyses did not reveal any further depletion of growth associated with the modeled mutations compared to wild-type *GRS1* (Fig. 2C). Finally, since GARS is a bi-functional ARS enzyme responsible for charging tRNA in both the cytoplasm and mitochondria (Antonellis and Green, 2008), yeast were grown on medium containing glycerol and 5-FOA at 30°C to require yeast to rely solely on mitochondrial

respiration, which allows the detection of impaired mitochondrial function. Similar to the studies at 37°C, the relative pattern of viability remained consistent when comparing mutant and wild-type *GRS1* and therefore did not reveal any further mutation-dependent depletion in yeast cell growth (Fig. 2D). Thus, two of the three human *GARS* mutations modeled in *GRS1* dramatically decrease cell viability *in vivo* when expressed in isolation, consistent with a loss-of-function effect.

### The majority of *GARS* mutations reduce punctate localization in cultured neurons

The wild-type *GARS* protein localizes to ‘puncta’ within axons in the human peripheral nerve where it likely functions in local protein translation (Antonellis et al., 2006). Certain *GARS* mutations impair the punctate localization in cultured neurons, suggesting that reduced localization to axons is a component of CMT2D and dSMA-V disease pathogenesis (Antonellis et al., 2006). However, not all *GARS* mutations have been tested for an effect on puncta formation in neuronal cells. We therefore expressed wild-type and eight untested mutant *GARS*-EGFP fusion proteins in a mouse motor neuron, neuroblastoma fusion cell line (MN-1 cells) (Salazar-Grueso et al., 1991; Antonellis et al., 2006). Fluorescence microscopy revealed that wild-type, p.Ala57Val, p.Asp146Asn, p.Asp500Asn, and p.Ser581Leu *GARS*-EGFP all demonstrated a punctate localization pattern in MN-1 cells (Fig. 3A, B, C, G and H). In contrast, p.Ser211Phe, p.Pro244Leu, p.Ile280Phe, and p.Gly598Ala *GARS*-EGFP proteins did not associate with puncta in MN-1 cells (Fig. 3D, E, F, I). Importantly, for the mutant *GARS*-EGFP proteins that did form puncta, these structures were also observed in MN-1 neurite projections, consistent with previous observations of wild-type *GARS* localization (data not shown) (Antonellis et al., 2006; Fallini et al., 2011).

### p.Ser581Leu *GARS* does not segregate with disease in newly discovered pedigrees

The p.Ala57Val variant was identified in a single individual with weakness and muscle wasting in the upper extremities (Rohkamm et al., 2007) and p.Ser581Leu was identified in a single proband with an extensive family history of lower limb predominant axonal CMT disease (James et al., 2006). No further segregation analyses were performed on either mutation. Furthermore, ethnically-matched control populations were not screened for the absence of p.Ala57Val or p.Ser581Leu. Therefore, we searched genome variant databases for the occurrence of these two mutations. p.Ala57Val and p.Ser581Leu were both identified by the NHLBI GO Exome Sequencing Project at a low frequency of 1/11,842 (rs370531212:C>T) and 2/11,940 (rs201358272:C>T), respectively. The other 11 *GARS* mutations associated with disease have not been reported in control or general populations.

Our on-going mutation analyses revealed p.Ser581Leu *GARS* in two unrelated probands with dominant axonal CMT disease. To determine if this mutation is associated with disease in the two respective families, we performed segregation studies. DNA sequence analysis of additional affected individuals revealed that p.Ser581Leu *GARS* does not segregate with disease in either family (Fig. 4A and B). Specifically, clinically affected individuals in these two families do not carry p.Ser581Leu *GARS*. Combined with the identification of this variant in the general population, these data indicate that p.Ser581Leu *GARS* should not be considered a disease-causing mutation.



## DISCUSSION

Aminoacyl-tRNA synthetases (ARSs) are essential enzymes that charge tRNA molecules with cognate amino acids (Delarue, 1995). Interestingly, genes encoding ARSs have been implicated in a phenotypically diverse panel of dominant and recessive diseases (Wallen and Antonellis, 2013). While the majority of disease-associated alleles demonstrate a primary loss of function, only four of the 13 reported *GARS* mutations have been rigorously tested. In this study, we evaluated nine *GARS* mutations, and our results indicate that impaired function, either by reduced enzyme function or altered cellular localization, is an important component of CMT disease pathogenesis. We determined that p.Asp146Asn, p.Ile280Phe, p.Ser211Phe, p.Pro244Leu, p.His418Arg, and p.Gly598Ala *GARS* encode a protein product with a severe defect in tRNA charging. Additionally, p.Ser211Phe, p.Pro244Leu, p.Ile280Phe and p.Gly598Ala *GARS* proteins fail to form puncta characteristic of wild-type *GARS* (Antonellis et al., 2006; Fallini et al., 2011). Finally, our genetic analyses rule out p.Ser581Leu *GARS* as a pathogenic *GARS* mutation. Combined with previous reports, the data presented here reveal that 10 of the 12 disease-associated *GARS* mutations identified to date demonstrate loss-of-function characteristics (Antonellis et al., 2006; Chihara et al., 2007; Nangle et al., 2007; Xie et al., 2007; Stum et al., 2011). This study furthers our understanding of the allelic spectrum of *GARS*-associated CMT disease and suggests that impaired *GARS* function is an important component of the axonal peripheral neuropathy.

Aminoacylation and yeast growth assays are established systems for testing the function of ARS enzymes *in vitro* and *in vivo*, respectively. Importantly, all *GARS* mutations that fail to complement yeast cell growth also have a severe reduction in aminoacylation activity. However, certain mutations, such as p.Ile280Phe, show reduced aminoacylation activity *in vitro* but complement yeast cell growth in a manner similar to the wild-type enzyme. One possible explanation for this discrepancy is that bacterially produced proteins may lack post-translational modifications important for enzyme function in human cells or for proper activity in specific cellular contexts. Additionally, other cellular components may stabilize the mutant protein product in the yeast system, allowing for improved function and viability. The yeast assay could not be used to evaluate all *GARS* mutations due to a lack of conservation with human residues. Alternative assays (see below) should be explored to determine the *in vivo* consequences of such mutations.

While yeast offers insight into the functional consequences of *GARS* mutations *in vivo*, it does not reveal how *GARS* mutations affect axons. The function of *GARS* and other ARS enzymes in axons remains unclear; however, there is strong evidence that they provide tRNA charging for local protein translation (Ingoglia et al., 1983; Giuditta et al., 2002). Interestingly, wild-type endogenous *GARS* forms discrete puncta in peripheral neurons and axons from healthy human subjects (Antonellis et al., 2006). Data collected to date show that seven of the 12 disease-associated *GARS* mutations give rise to proteins that are unable to localize to puncta in cultured neurons (Table 2). Another study showed that mutant *GARS* proteins mislocalize in neurite projections (Nangle et al., 2007) but did not reveal discrete puncta, which is likely due to differential placement of the epitope tag. The loss of a punctate localization pattern observed for certain *GARS* mutant proteins may be due to altered protein structure and/or reduced binding to specific protein partners or RNA

molecules required for puncta formation. It will be critical to identify proteins and/or RNAs that GARS interacts with in these structures to further understand puncta function and how disruption of these structures may lead to CMT disease.

Although impaired function is associated with *GARS*-mediated CMT disease, three disease-associated mutations have not demonstrated impaired function in any of the assays used in this study: p.Ala57Val, p.Glu71Gly, and p.Asp500Asn (Table 2). The p.Glu71Gly *GARS* mutation segregates with disease in a large pedigree with upper limb predominant axonal neuropathy characteristic of CMT2D (Antonellis et al., 2003). Analysis of human p.Glu71Gly *GARS* in a fly model revealed that it cannot complement the loss of *gars* in neurons, and the authors conclude that p.Glu71Gly represents a loss-of-function allele (Chihara et al., 2007). Similar to p.Glu71Gly, p.Asp500Asn *GARS* segregates with disease in a large pedigree with dominantly inherited, upper limb predominate axonal neuropathy (Del Bo et al., 2006), providing strong genetic and phenotypic evidence supporting a role in CMT2D disease pathogenesis. In contrast, p.Ala57Val has weak genetic evidence for a role in CMT disease. First, this variant was identified in a single individual with upper limb predominant CMT disease and no additional family members were available for segregation studies (Rohkamm et al., 2007). Second, ethnically matched controls were not tested to determine the presence of this allele in appropriate populations. Finally, p.Ala57Val was identified by the NHLBI GO Exome Sequencing Project (rs370531212:C>T) at a low frequency; however, since individuals in the NHLBI study were not subjected to neurological exam and since CMT is often a late onset disease, inclusion in this database does not exclude p.Ala57Val from having a causative role in CMT disease. Evaluation of p.Asp500Asn and p.Ala57Val in the fly model will be important to determine if these variants have impaired function that cannot be detected by other assays.

To date, loss-of-function mutations at five *ARS* loci have been implicated in dominantly inherited CMT disease (Antonellis et al., 2003; Jordanova et al., 2006; Latour et al., 2010; Vester et al., 2012; Gonzalez et al., 2013). Thus, mechanisms of impaired function in dominant disease should be carefully evaluated, including haploinsufficiency, a dominant-negative effect, and a toxic gain of function. First, while mice heterozygous for loss-of-function missense *Gars* mutations (C201R and P234KY) demonstrate a dominant axonal neuropathy (Seburn et al., 2006; Achilli et al., 2009; Motley et al., 2011), mice heterozygous for a *Gars* null (gene-trap) allele have a wild-type phenotype (Seburn et al., 2006). In addition, *GARS* null alleles have not been identified in patients with CMT2D and dSMA-V. Combined, these findings argue against haploinsufficiency. Second, transgenic overexpression of wild-type human *GARS* in the two mouse models restored viability but did not rescue the neuropathy phenotype (Motley et al., 2011), arguing against a dominant-negative effect. However, overexpression of human GARS was unable to rescue the lethality associated with homozygosity for the *Gars* gene trap allele, indicating that the cDNA transgene may not function in all tissues and during all developmental periods (Motley et al., 2011). Thus, it will be important to assess the activity of wild-type:mutant GARS dimers and the effect of wild-type *GARS* overexpression in the context of mutations identified in human patients before excluding a dominant-negative mechanism (Wallen and Antonellis, 2013). Finally, impaired GARS function may be a prerequisite for a toxic gain-of-function effect. While mutant protein aggregates have not been reported (Antonellis et al., 2006;

Seburn et al., 2006; Nangle et al., 2007; Achilli et al., 2009; Motley et al., 2011; Stum et al., 2011), conformational changes of human mutant GARS proteins have been observed (He et al., 2011). These proteins should be assessed for mutant-specific binding to neuronal RNAs and/or proteins. If these experiments are informative, then therapies should be designed to reduce the expression of mutant *GARS* alleles in patients with CMT2D (Motley et al., 2011). Importantly, the three pathogenic mechanisms outlined here are not mutually exclusive; mutation-specific utilization of haploinsufficiency, dominant-negative, and/or toxic gain-of-function effects may explain the observed clinical spectrum of *GARS*-associated peripheral neuropathy.

The development of rapid, affordable technologies has allowed the use of DNA sequencing for research and diagnostic purposes. Importantly, once a gene is implicated in disease, patients with a similar phenotype can be rapidly screened for variants at that locus. However, rare variants identified in individual patients or small pedigrees are difficult to implicate in disease phenotypes due to the absence of strong genetic evidence (MacArthur et al., 2014). Here, we demonstrate that p.Ser581Leu *GARS*, a rare variant previously identified in patients with CMT disease, does not segregate with neuropathy in two newly identified families with dominant axonal CMT disease. This variant was originally identified in individuals with lower limb predominant CMT disease (James et al., 2006), which is uncharacteristic of *GARS*-associated CMT2D. Interestingly, p.Ser581Leu has normal aminoacylation activity (Nangle et al., 2007) and localization in neurons (Fig. 3). Thus, our data indicate that p.Ser581Leu is not a primary disease-causing mutation and should be removed from further studies to determine the pathogenic mechanism of *GARS* mutations.

In conclusion, our data indicate that impaired function is a common characteristic of CMT-associated *GARS* mutations and that this may be a necessary first step for disease pathogenesis. However, additional research is needed to implicate or refute a causative link between impaired tRNA charging and CMT2D disease. Importantly, determining the precise mechanism of mutant GARS toxicity in axons will be essential for designing effective therapies for patients with CMT2D.

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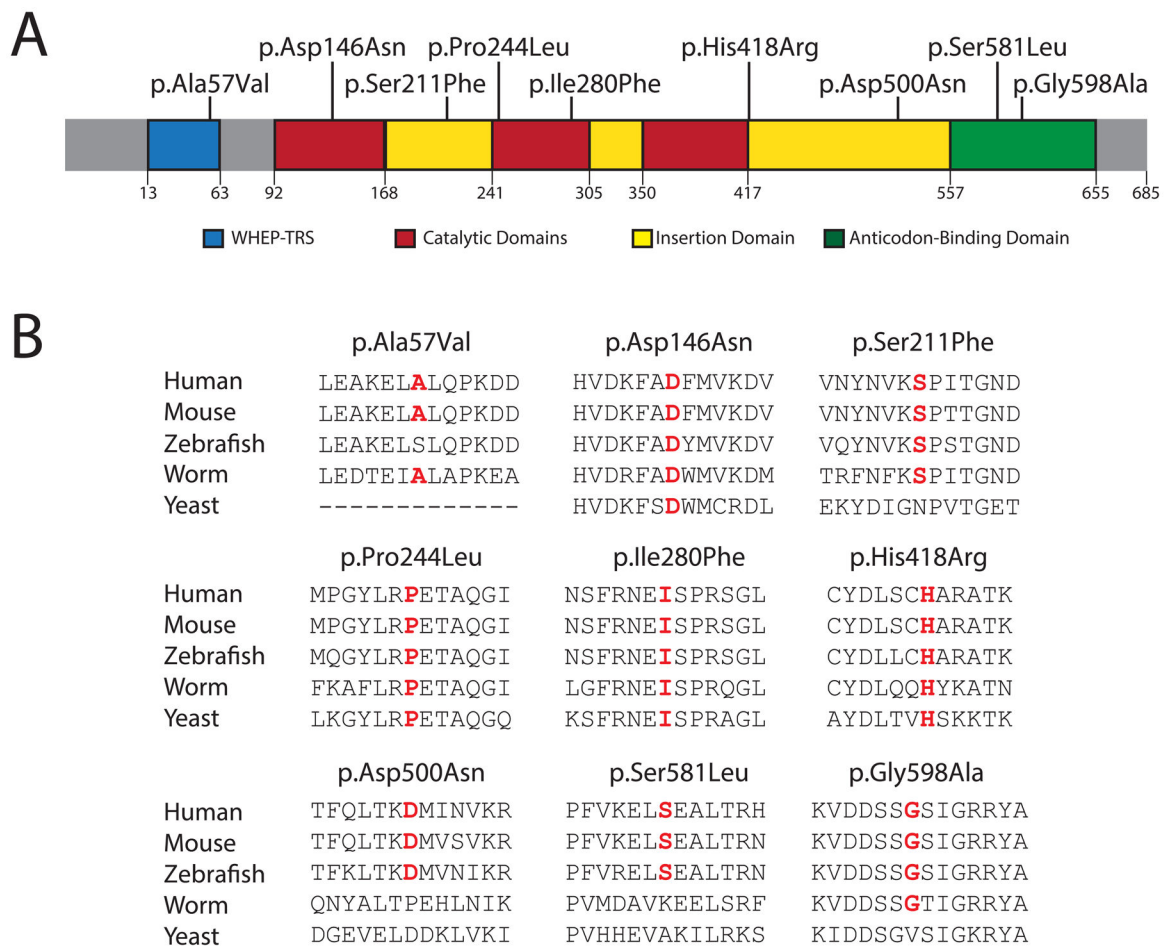
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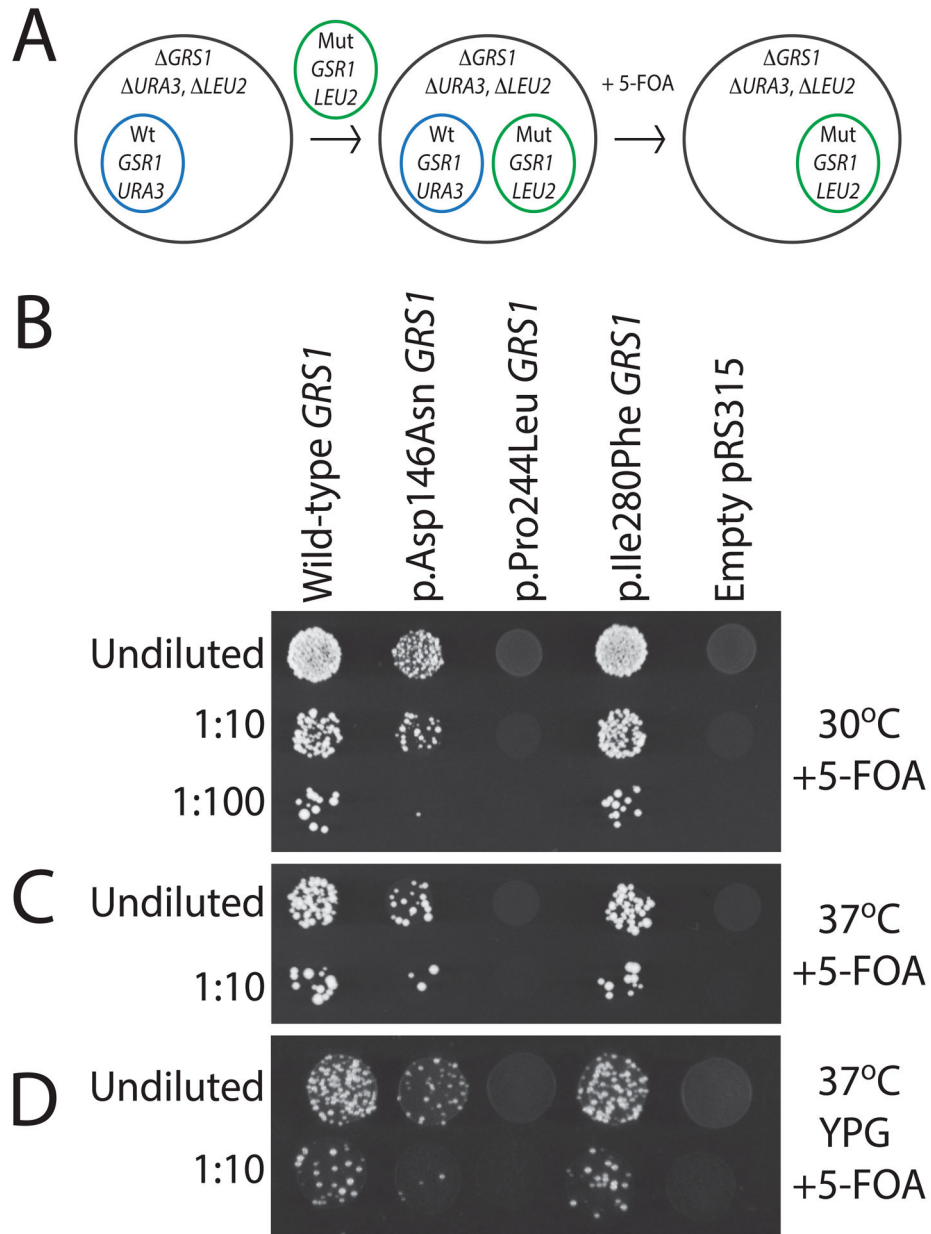
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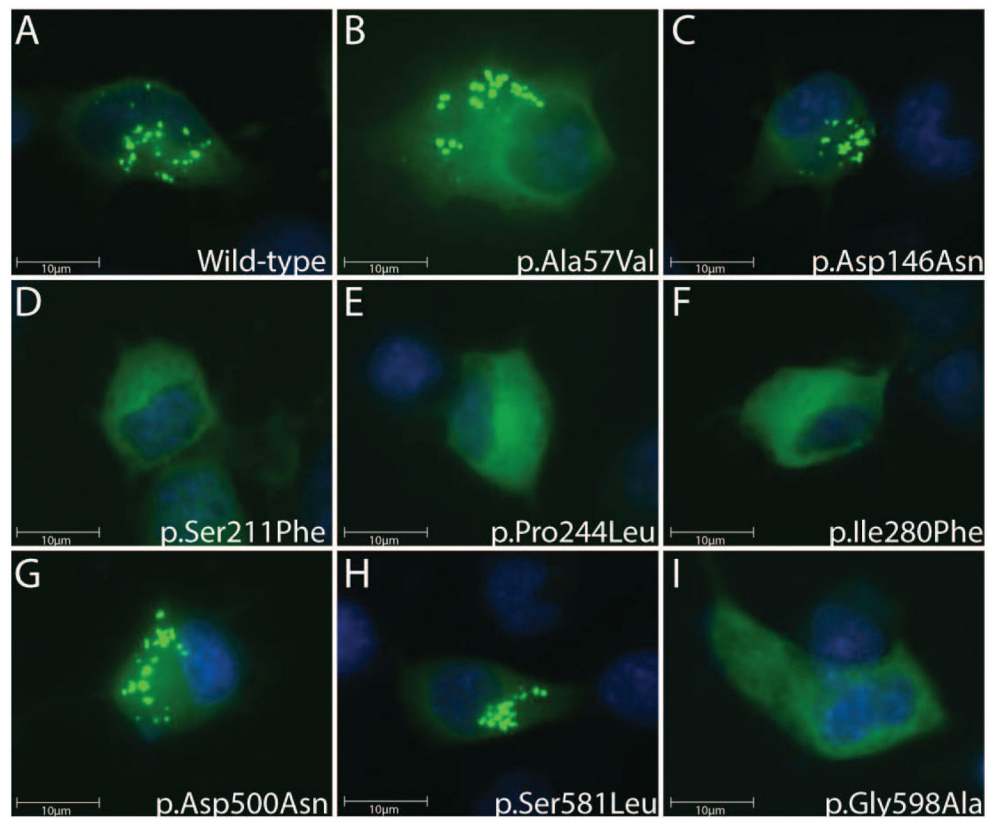
**Figure 1.**

Localization and conservation of *GARS* variants. **(A)** Mutations are depicted on a cartoon of the known functional and structural domains of *GARS* in blue (WHEP-TRS domain), red (catalytic domain), yellow (insertion domains), and green (anticodon-binding domain). The amino-acid positions for each domain are indicated below the cartoon. **(B)** *GARS* protein sequence alignments from multiple, evolutionarily diverse species are depicted. The amino acid change is listed at the top of each protein fragment. The affected amino acid is highlighted in red.



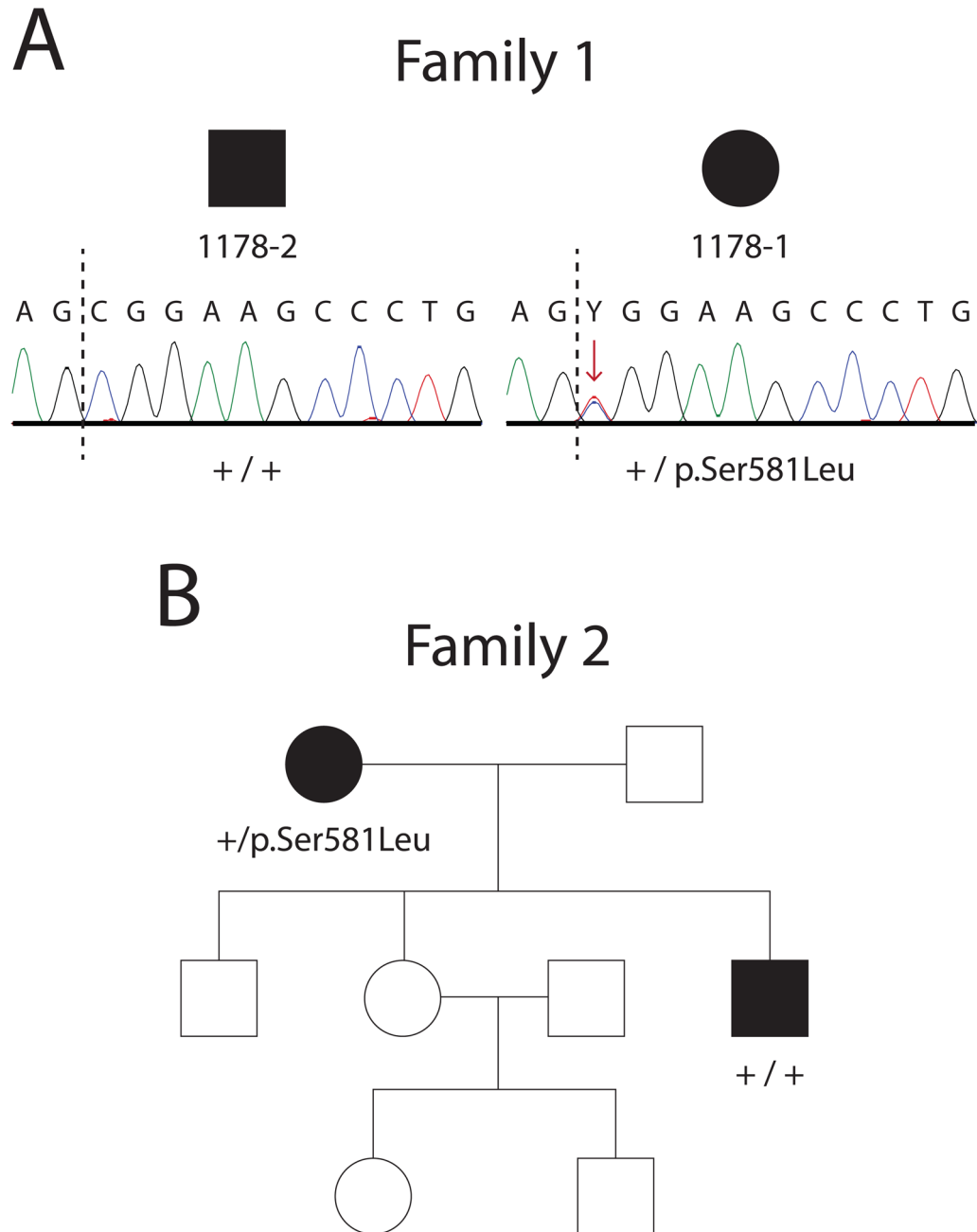


**Figure 2.** Characterization of yeast expressing wild-type and mutant *GRS1*. Cartoon illustrates yeast complementation strategy (A). Each yeast strain was transformed with a *LEU2*-bearing pRS315 vector containing wild-type *GRS1* (Wt), the indicated mutant form of *GRS1* (Mut), or no insert ('Empty'). Cultures for each strain (labeled along the top for each panel in B, C, and D) were grown for two days in liquid media and spotted on selective solid growth medium directly or after dilution as indicated. Strains were plated on medium containing 5-FOA to determine if the *GRS1* alleles complement loss of endogenous *GRS1* gene at 30°C (B) and 37°C (C). (D) Strains were plated on solid medium containing 5-FOA and glycerol (YPG) to assess for an effect on mitochondrial function.



**Figure 3.**

Expression of wild-type and mutant GARS in mouse motor neuron-derived cells. MN-1 cells expressing EGFP-tagged wild-type (A), p.Ala57Val (B), p.Asp146Asn (C), p.Ser211Phe (D), p.Pro244Leu (E), p.Ile280Phe (F), p.Asp500Asn (G), p.Ser581Leu (H), or p.Gly598Ala GARS (I) were evaluated for the presence or absence of puncta by fluorescence microscopy.



**Figure 4.** p.Ser581Leu *GARS* does not segregate with CMT disease. DNA samples from affected individuals from two presumably unrelated families with autosomal dominant, axonal CMT disease were genotyped for p.Ser581Leu *GARS*. Female patients are indicated with circles and male patients are indicated with squares. Filled symbols represent affected individuals with a diagnosis of dominant axonal CMT disease and empty symbols indicate unaffected individuals. Where applicable, an individual's genotype is indicated with + (for the wild-type allele) or p.Ser581Leu (for the variant allele). (A) Samples from individuals in Family 1 were anonymized resulting in loss of the pedigree structure for this family; however,

individuals 1178–1 and 1178–2 are directly related, both affected, and reside in a pedigree originally described as dominant axonal CMT disease. Representative chromatographs from sequencing analysis of genomic DNA from affected individuals are shown. The sequence includes the AG splice site acceptor at the 3' end of intron 15 followed by the first 12 nucleotides of exon 16 (intron/exon junction marked by dashed line). The p.Ser581 codon (TCG) is encoded by the last nucleotide of exon 15 (T; not shown) and the first two nucleotides (CG) of exon 16. Individual 1178–1 is heterozygous for a C>T mutation (red arrow) in the first base of exon 16 resulting in the p.Ser581Leu allele. 1178–2 is homozygous for the wild-type genotype at this nucleotide (C). **(B)** The pedigree for Family 2 illustrates the affected status of each family member and the p.Ser581Leu genotypes of two affected individuals with dominant axonal CMT disease.

**Table 1**

## Aminoacylation kinetics of GARS protein variants

<b>Variant</b>	<b><math>K_m</math> (<math>\mu\text{M}</math>)</b>	<b><math>k_{cat}</math> (<math>\text{s}^{-1}</math>)</b>	<b><math>k_{cat}/K_m</math> (<math>\mu\text{M}^{-1}\text{s}^{-1}</math>)</b>	<b>Relative to WT</b>
Wild-type	$1.03 \pm 0.03$	$0.14 \pm 0.02$	$0.14 \pm 0.02$	1
p.Ala57Val	$0.92 \pm 0.22$	$0.07 \pm 0.01$	$0.07 \pm 0.02$	1/2
p.Asp146Asn	$0.6 \pm 0.1$	$0.006 \pm 0.002$	$0.01 \pm 0.01$	1/14
p.Ser211Phe	-	-	$(3.2 \pm 1.5) \times 10^{-5}$	1/4,400
p.Gly240Arg	$3.2 \pm 0.6$	$(4.2 \pm 0.3) \times 10^{-3}$	$(1.3 \pm 0.4) \times 10^{-3}$	1/110
p.Pro244Leu	-	-	undetectable	undetectable
p.Ile280Phe	-	-	$(7.9 \pm 2.0) \times 10^{-5}$	1/1,700
p.His418Arg	$0.004 \pm 0.001$	$(3.9 \pm 0.1) \times 10^{-5}$	$0.01 \pm 0.01$	1/16
p.Gly526Arg	$7.1 \pm 1.3$	$(2.3 \pm 0.4) \times 10^{-5}$	$(3.2 \pm 2.0) \times 10^{-6}$	1/44,000
p.Gly598Ala	$1.4 \pm 0.3$	$(1.1 \pm 0.1) \times 10^{-3}$	$(7.9 \pm 3.0) \times 10^{-4}$	1/180

Amino-acid coordinates correspond to GenBank accession number AAA57001.1

$\pm$  indicates standard deviations

- indicates that  $k_{cat}$  and  $k_m$  are not directly measured; the value for  $k_{cat}/K_m$  (where applicable) was measured under an approximate condition where the substrate concentration is below an estimated  $K_m$

“Undetectable” indicates that values are below the limits of detection

**Table 2**Summary of functional studies on *GARS* variants

<u>Variant</u>	<u>Enzyme Activity</u>	<u>Yeast Viability</u>	<u>Puncta Formation</u>	<u>References</u>
Wild-type	Normal	Viable	Yes	Antonellis et al. 2006, and this study
p.Ala57Val	Normal <sup><i>l</i></sup>	NA	Yes	This study
p.Glu71Gly	Normal	Viable	Yes	Antonellis et al. 2006, Nangle et al. 2006
p.Leu129Pro	Reduced	Reduced	No	Antonellis et al. 2006, Nangle et al. 2006
p.Asp146Asn	Reduced	Reduced	Yes	This study
p.Ser211Phe	Reduced	NA	No	This study
p.Gly240Arg	Reduced	Viable	No	Antonellis et al. 2006, Nangle et al. 2006
p.Pro244Leu	Reduced	Lethal	No	This study
p.Ile280Phe	Reduced	Viable	No	This study
p.His418Arg	Reduced	Lethal	No	Antonellis et al. 2006, Nangle et al. 2006, and this study
p.Asp500Asn	Normal	NA	Yes	Nangle et al. 2006, and this study
p.Gly526Arg	Reduced	Lethal	Yes	Antonellis et al. 2006, Nangle et al. 2006, and Xie et al. 2007
p.Ser581Leu	Normal	NA	Yes	Nangle et al. 2006, Cader et al. 2007, and this study
p.Gly598Ala	Reduced	Viable	No	Stum et al. 2011, and this study

Amino-acid coordinates correspond to GenBank accession number AAA57001.1

Red text indicates a result consistent with a loss-of-function effect.

<sup>*l*</sup>This variant was associated with ~50% activity compared to wild-type GARS and we considered a >90% reduction in activity as dramatically 'Reduced'.

NA – not applicable; mutation could not be modeled in yeast ortholog