Aminoacyl-tRNA synthetases (aaRSs) are essential enzymes that catalyze the first reaction in protein biosynthesis, namely the charging of transfer RNAs (tRNAs) with their cognate amino acids. aaRSs have been increasingly implicated in dominantly and recessively inherited human diseases. The most common aaRS-associated monogenic disorder is the incurable neurodegenerative disease Charcot–Marie–Tooth neuropathy (CMT), caused by dominant mono-allelic mutations in aaRSs. With six currently known members (GlyRS, TyrRS, AlaRS, HisRS, TrpRS, and MetRS), aaRSs represent the largest protein family implicated in CMT etiology. After the initial discovery linking aaRSs to CMT, the field has progressed from understanding whether impaired tRNA charging is a critical component of this disease to elucidating the specific pathways affected by CMT-causing mutations in aaRSs. Although many aaRS CMT mutants result in loss of tRNA aminoacylation function, animal genetics studies demonstrated that dominant mutations in GlyRS cause CMT through toxic gain-of-function effects, which also may apply to other aaRS-linked CMT subtypes. The CMT-causing mechanism is likely to be multifactorial and involves multiple cellular compartments, including the nucleus and the extracellular space, where the normal WT enzymes also appear. Thus, the association of aaRSs with neuropathy is relevant to discoveries indicating that aaRSs also have nonenzymatic regulatory functions that coordinate protein synthesis with other biological processes. Through genetic, functional, and structural analyses, commonalities among different mutations and different aaRS-linked CMT subtypes have begun to emerge, providing insights into the nonenzymatic functions of aaRSs and the pathogenesis of aaRS-linked CMT to guide therapeutic development to treat this disease.

As an essential component of the translation machinery executing in the central dogma of molecular biology, aminoacyl-tRNA synthetases (aaRSs) in all three domains of life have been studied for decades (1). Regulatory functions of aaRSs beyond their classic enzymatic role in protein synthesis have also been widely discovered in recent years (2–4). With the rise of genomics in health care, aaRSs are increasingly implicated in human diseases. The focus of the review is on the neurodegenerative Charcot–Marie–Tooth disease (CMT), the first and the most common monogenic disorder associated with aaRSs. Before we go into the studies and considerations on the etiology, our review starts with a brief introduction of the enzymatic and the nonenzymatic roles of aaRSs, a synopsis on the disease association of aaRSs to highlight the uniqueness of the CMT-associated aaRS subset, and a list of the relevant mutations and established animal models. Despite some heterogeneities, clinical presentations of CMT patients with aaRS mutations are overall similar, implying shared disease mechanisms. The field is now at the point where loss of tRNA aminoacylation is thought unlikely to serve as the mechanism for aaRS-linked CMT and where new insights regarding commonality in pathogenesis among different aaRSs start to emerge.

Introduction of aminoacyl-tRNA synthetase

Aminoacyl-tRNA synthetases in the human genome

Protein synthesis requires aminoacylated transfer RNAs (tRNAs) to decode the mRNA with proper amino acids so that the genetic information can be translated into proteins. The job of creating the aminoacylated, or the “charged,” tRNAs is carried out by a family of enzymes called aminoacyl-tRNA synthetases (aaRSs). To charge the commonly used 20 proteinogenic amino acids onto their corresponding tRNAs, 20 members are included in the aaRS family.

In human cells, two sets of aaRSs exist for their respective use in cytosolic and mitochondrial protein synthesis. A total of 37 aaRS genes are encoded by the human nuclear genome, including 18 for cytoplasm only, 17 for mitochondria only, and 2 for both sites. GlyRS and LysRS are the two dual-localized aaRSs, with their mitochondrial targeting sequences included or excluded by alternative mRNA splicing or alternative sites of translation initiation (5, 6). Mitochondrial GlnRS is missing from the human genome, and the aminoacylation of mitochondria

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2 The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; CMT, Charcot–Marie–Tooth disease; PDB, Protein Data Bank; RMSD, root mean square deviation; SAXS, small angle X-ray scattering; HDX, hydrogen-deuterium exchange; VEGF, vascular endothelial growth factor; NCV, nerve conduction velocity; MSC, multisynthetase complex; Trk, tropomyosin receptor kinase.
Glutamyl-tRNA is achieved through an indirect pathway, where tRNA^Gln is first mischarged by mitochondrial GluRS with Glu, which is then modified to Gln by an aminoacyl-tRNA aminotransferase (GatCAB) (7). The 18 genes encoding for cytoplasmic-only aaRSs contain two separate genes for the two subunits of PheRS (PheRS-a and PheRS-b) and one gene for the fused GluRS and ProRS (GluProRS). We should note that a single-letter amino acid code for aaRS genes (e.g. YARS for cytoplasmic TyrRS and YARS2 for mitochondrial TyrRS) was recently adopted by the HUGO Gene Nomenclature Committee (HGNCl) database. In parallel, the 3-letter amino acid code, as the convention in the field, has been continuously used as a prefix to refer to the gene product for its easier and more explicit recognition as an amino acid. (For example, the name “AARS” can be confused between acronyms for “aminoacyl-tRNA synthetase” and for “alanyl-tRNA synthetase,” whereas “AlaRS” explicitly refers to “alanyl-tRNA synthetase.”) For this review, both naming systems are used; they are essentially interchangeable.

Two-step aminoacylation reaction, two classes of aaRSs and MSC

The evolutionarily conserved aminoacylation reaction is carried out in two steps. In the first step, aaRSs bind to amino acid and ATP to catalyze the formation of an enzyme-bound aminoacyl-adenylate, while liberating pyrophosphate (PP\(_2\)). In the second step, the activated amino acid subsequently reacts with a tRNA to yield the aminoacyl-tRNA.

Based on their unique active-site architecture and the corresponding conserved sequence motifs for ATP binding, aaRSs are evenly divided into two classes, with 10 members in each class of the human enzymes (8, 9). The catalytic domains of class I aaRSs are based on the Rossmann nucleotide binding fold composed of parallel \(\beta\)-sheets and connecting \(\alpha\)-helices, whereas the catalytic domains of class II aaRSs are made of anti-parallel \(\beta\)-sheets and flanking \(\alpha\)-helices. Class I aaRSs are monomeric, except for TyrRS and TrpRS, which are dimers. The dimerization is mediated through the catalytic domain and is required for their catalytic activity. Because of their capacity to function as homodimers, the genes encoding TyrRS and TrpRS are significantly smaller than other class I aaRS genes. The requirement of a dimer form (or tetramer [dimer of dimer] for PheRS) for catalytic function applies to most class II aaRSs, except for AlaRS (10).

An interesting feature of cytoplasmic aaRSs in higher eukaryotes (from flies to humans) is that nine aaRSs (from both class I and class II), together with three nonenzymatic factors, form a multisynthetase complex (MSC). The functional significance of the MSC is unclear, but two hypotheses have been proposed. One suggested that the complex could improve translation efficiency by channeling the charged tRNAs to the ribosome (11); the other proposed that the complex could serve as a “depot” to sequester the MSC components with regard to their diverse nonenzymatic, regulatory functions, only to be released in response to certain stimulations (12). Of course, these two hypotheses are not mutually exclusive.

Pleiotropic regulatory functions of cytoplasmic aaRSs

Coinciding with the emergence of the MSC, widespread regulatory functions of cytoplasmic aaRSs beyond their classic role in protein synthesis have been increasingly reported for both MSC and non-MSC components (2–4). The cytoplasmic aaRSs are not only found in the cytosol where protein synthesis occurs but are also frequently detected in the nucleus and in the extracellular space, such as in cell culture media and in the systemic circulations of humans and mice. The nonenzymatic functions of aaRSs regulate many physiological processes, such as angiogenesis, hematopoiesis, immune response, and stress responses, and are thought to provide a mechanism to coordinate protein synthesis with other biological processes (13). The functional expansion during evolution concurs with the incorporation of new domains and motifs. Almost all human cytoplasmic aaRSs have at least one new domain or sequence extension, added to the N or C terminus of the evolutionarily conserved catalytic core (14–16). Most of the extensions and new domains are dispensable for the aminoacylation activity, suggesting that they function to mediate or regulate the nonenzymatic roles of aaRSs, including facilitating the cellular translocation of human aaRSs from cytoplasm to other cellular compartments (17).

Disease association of aminoacyl-tRNA synthetases

aaRSs constitute the largest family of proteins implicated in CMT

The first human disease linked to mutations in aaRSs was a neurodegenerative disease that specifically affects the peripheral nervous system (Fig. 1) (18). It was named Charcot–Marie–Tooth disease (CMT), after the three physicians who first described the disease in 1886. The disease, also known as hereditary motor and sensory neuropathy, affects peripheral nerves in a length-dependent manner and is characterized by weakness and wasting of the distal limb muscles leading to progressive motor impairment, sensory loss, and skeletal deformities (19–21). Based on the predominant pathological features, CMT is divided into two major types: the demyelinating type 1, where abnormalities occur in the myelin sheath surrounding peripheral axons, and the axonal type 2, where the damage is within the axon itself, whereas intermediate forms also exist (19–21).

GlyRS (or GARS) was the first aaRS causally linked to CMT through dominant mono-allelic mutations (Fig. 1) (18, 22). The initial finding was a surprise to the field. How can a protein broadly required for protein synthesis in all cells be linked to a disease of extreme tissue specificity? However, the connection between aaRS and CMT was quickly reinforced by reports of additionally linked family members and by increasing numbers of aaRS mutations identified in CMT patients (23–25). Currently, with five members (i.e. GARS, YARS, AARS, HARS, and WARS) firmly linked and another one (MARS) possibly associated, aaRSs represent the largest family of proteins implicated in the etiology of CMT (Fig. 1). The various aaRS-linked CMT subtypes usually present as axonal peripheral neuropathies, and sometimes the nerve conduction velocity (NCV) can be in the range associated with demyelination (26, 27). The patients have...
predominantly motor deficits, with highly variable symptoms in terms of severity (27). Although the lower limbs are mainly affected by CMT, most patients with GlyRS mutations have upper limb predominance (22, 28). Despite these heterogeneities, clinical presentations of CMT patients with aaRS mutations are similar, implying shared disease mechanisms.

Mono-allelic versus bi-allelic mutations in aaRSs for disease association

Except for GlyRS being a dual-localized aaRS used for both cytoplasmic and mitochondrial protein synthesis (5), all CMT-linked aaRSs belong to the cytoplasmic set. Although dominant mono-allelic mutations in aaRSs exclusively affect the peripheral nerves system, recessive bi-allelic mutations in aaRSs—both cytoplasmic and mitochondrial—have been linked to a variety of syndromes, which affect multiple organ systems and are sometimes accompanied with developmental delays. These findings were reported in rapid succession in recent years. (Patients with bi-allelic mutations have a mutation (not necessarily the same mutation) in both alleles of a single gene (paternal and maternal), whereas patients with a mono-allelic mutation only have one of the two alleles mutated yet still exhibit a disease phenotype. Therefore, the mono-allelic mutation is considered “dominant” in disease presentation.) So far, bi-allelic, but not mono-allelic (except for the dual-localized GlyRS), mutations in every mitochondrial aaRS have been linked to human diseases, which mostly affected organs with high metabolic demand, such as the central nervous system and the heart (Fig. 1) (29). Trailing not too far behind, bi-allelic mutations in 16 cytoplasmic aaRSs have emerged as causative to various disease conditions, with an even broader range of organ systems affected than those by mutations in the mitochondrial aaRSs (Fig. 1) (23, 30). Because of the importance of protein synthesis in almost all cell types and other essential nonenzymatic roles of aaRSs, we would not be surprised if soon all cytoplasmic aaRSs are implicated in human diseases through bi-allelic mutations as well.

CMT is specifically linked to mono-allelic mutations in a selective set of cytoplasmic aaRSs

Although the broad disease phenotypes associated with bi-allelic aaRS mutations may sometimes include neuropathies (31), pure CMT and the related neuropathies are specifically associated with cytoplasmic aaRSs through mono-allelic mutations, indicating a special sensitivity and vulnerability of the peripheral nervous system toward mono-allelic aaRS mutations. It is possible that additional aaRSs may be linked; however, some characteristics that emerged from the existing list suggest a potential selectivity of certain aaRSs to be associated with CMT (Fig. 2).

The six aaRSs associated with CMT are evenly distributed into the two classes of aaRSs (TyrRS, TrpRS, and MetRS in class I; GlyRS, HisRS, and AlaRS in class II) (Fig. 2). However, CMT-associated aaRSs are mostly dimeric (5 out of 6, except for MetRS), are not associated with the MSC (5 out of 6, except for MetRS), and have a specific appended domain called the WHEP domain (4 out of 6, except for TyrRS and AlaRS) (Fig. 2). In fact, all single WHEP domain-containing aaRSs (GlyRS, HisRS, TrpRS, and MetRS) are CMT-associated (15).

3 M. Shy, IUBMB Focused Meeting on the Aminoacyl-tRNA Synthetases, Oct 29-Nov 2, 2017. p. 13, Clearwater, FL.
CMT-linked mutations in aaRS genes

A large number of dominant mutations in the six aforementioned aaRSs has been linked to CMT.

GlyRS (GARS)

Human GlyRS protein is composed of three domains: a metazoan-specific helix-turn-helix WHEP domain, the evolutionarily conserved class II catalytic domain, and the anticodon binding domain (Fig. 3). The catalytic domain of GlyRS, and of three other CMT-linked aaRS (i.e. TyrRS, HisRS, and TrpRS), mediates the dimerization of the synthetase, which is necessary for the catalytic activity of these synthetases and for providing a complete set of binding sites for their tRNA substrates. So far, at least 19 mono-allelic mutations in GlyRS with various degrees of genetic evidence have been linked to CMT subtype 2D (CMT2D/AD-CMTax-GARS) in patients (25, 32). Most of the mutations, especially the ones with the strongest genetic evidence and extensive family histories (e.g. E71G, L129P, G240R, E279D, H418R, D500N, and G526R), are located in the catalytic domain of the enzyme. Two anticodon binding domain mutations (i.e. S581L and G598A) lack strong genetic evidence but are recurrently identified in patients with neuropathy phenotypes (33–37).

Because GlyRS is a dual-localized aaRS and because two mutations in the Gars gene in mice were found to cause a CMT-like neuropathy (see below), it is important to note that the mutations are numbered according to the cytosolic form of the human protein and with the mitochondrial targeting sequence omitted in our review. In contrast, many reports numbered the same mutation sites with the mitochondrial targeting sequence included, and thereby resulted in an increase of 54 in amino acid residue numbers. (The mitochondrial targeting sequence is supposedly deleted after protein importation to the mitochondria.)

TyrRS (YARS)

Human TyrRS protein is composed of three domains: the evolutionarily conserved catalytic (class I) and anticodon binding domains and a C-terminal EMAP-II–like domain (from insects to humans) (Fig. 3). Five dominant mutations with different degrees of genetic association have been linked to an intermediate form of CMT with both demyelinating and axonal features (DI-CMTC/AD-CMT in–YARS) (26, 38, 39). All mutations are located in the catalytic domain. Three (G41R, E196K, and E196Q) segregated with the disease in large families, whereas D81I and H9004153–156(VKQV) are de novo mutations, and each was found in a single patient. Nevertheless, through transgenic overexpression, the pathogenicity of H9004153–156, as well as that of G41R and E196K, has been recapitulated in Droso phila models (40, 41).

HisRS (HARS)

HisRS has an identical domain structure as that of GlyRS, including the WHEP domain, the class II catalytic domain, and the C-terminal anticodon binding domain (Fig. 3). So far, eight...
mutations have been linked to CMT subtype 2W (CMT2W/AD-CMTax-HARS), and five of them (T132I, P134H, V155G, D175E, and D364Y) exhibit clear segregation with disease in large families (42, 43). All mutations are located in the catalytic domain. Through transgenic overexpression, the toxicity of D364Y and R137Q mutations has been recapitulated in Caenorhabditis elegans neurons (42, 44).

TrpRS (WARS)

TrpRS also has a N-terminal WHEP domain, followed by a class I catalytic domain and the anticodon binding domain (Fig. 3). Although only one mutation (H257Q, located in the catalytic domain) in WARS has been linked to CMT, it is recurrently identified in multiple families with a clear disease segregation (45).

AlaRS (AARS)

Human AlaRS is the only cytoplasmic tRNA synthetase that has not acquired any new domain during evolution (15). AlaRS is also unique among the CMT-linked aaRSs in that it does not have an anticodon binding domain, but has an evolutionarily conserved editing domain and a C-terminal domain designated as C-Ala (Fig. 3). The tRNA recognition of AlaRS does not involve the anticodon and is entirely based on a G3:U70 bp in the acceptor stem of the tRNA (46). At the same time, due to structural similarities between alanine and some other amino acids (e.g. serine), AlaRS can misactivate or mischarge noncanonical amino acids and therefore requires a hydrolytic editing function to ensure the accuracy of the tRNA aminoacylation reaction (47). Although the C-Ala domain is conserved from prokaryotes to humans, its sequence and function have evolved from enhancing tRNA binding in prokaryotes (48) to engendering new roles outside of aminoacylation in humans (10). So far, nine mutations, five of which are located in the catalytic domain (N71Y, G102R, R326W, R329R, and E337K), two in the editing domain (S627L and E688G), and two others in the C-Ala domain (E778A and D893N), have been linked to CMT subtype 2N (CMT2N/AD-CMTax-AARS) (Fig. 3) (27, 49–54). All mutations segregate with disease. In particular, the R329H mutation in the catalytic domain has been recurrently identified in multiple families (51–53). Although AlaRS-linked CMT is designated as type 2, some CMT2N patients (i.e. some...
patients carrying the E337K mutation) also exhibit demyelinating features (27).

**MetRS (MARS)**

The conserved class I catalytic domain and anticodon binding domain of human MetRS are sandwiched between an N-terminal appended GST domain to anchor it to the MSC and a C-terminal WHEP domain with unknown function (Fig. 3). MetRS is the only CMT-linked aaRS that does not form a dimer (Fig. 2). Three mutations, all in the anticodon binding domain, have been linked to CMT type 2U (CMT2U/AD-CMTax). The R618C mutation was identified in two CMT patients within a family, with incomplete penetrance (55). The same mutation was found in the seemingly unaffected father of a patient with the R618C allele compounded with another mutant allele in MARS, causing recessive interstitial lung and liver disease (56). The P800T mutation has been recurrently identified as the human disease, recapitulating the dominant trait of the disease.

### Table 1

**Existing animal models for aaRS-induced CMT through dominant mutations**

| Gene/CMT subtype | Animal                | Mutation | Genetic manipulation | Phenotype severity | Refs. |
|------------------|-----------------------|----------|----------------------|--------------------|-------|
| GARS/CMT2D       | Mouse                 | C157R    | Knockin              | P234KY > C157R     | 61    |
|                  | Drosophila            | E71G     | Transgenic overexpression | P234KY > E71G | 60    |
|                  |                       | G240R    | Transgenic overexpression | G240R > E71G | 64    |
|                  |                       | G526R    | Transgenic overexpression | G526R > E71G | 61    |
| YARS/DI-CMTC     | Drosophila            | G41R     | Transgenic overexpression | E196K > G41R   | 40    |
|                  |                       | Δ153–156 | Transgenic overexpression | Δ153–156 | 40    |
|                  | Zebrafish             | R326W    | mRNA injection of embryos | Varies among experiments | 27    |
|                  |                       | E337K    | mRNA injection of embryos | Varies among experiments | 27    |
|                  |                       | S627L    | mRNA injection of embryos | Varies among experiments | 27    |
| HARS/CMT2W       | C. elegans            | R137Q    | Transgenic overexpression | Not reported | 44    |
|                  |                       | D364Y    | Transgenic overexpression | Not reported | 42    |

**Fly model**

Expressing the cytosolic form of GlyRS mutants (GlyRS<sup>E71G</sup>, GlyRS<sup>P234KY</sup>, GlyRS<sup>G240R</sup>, and GlyRS<sup>G526R</sup> (41, 64, 65) and of TyrRS mutants (TyrRS<sup>G41R</sup>, TyrRS<sup>E196K</sup>, and TyrRS<sup>Δ153–156</sup>) (40) induced neuronal phenotypes that successfully recapitulated some of the hallmarks of the human disease, including progressive motor performance deficits, electrophysiological evidence of neuronal dysfunction, and terminal axonal degeneration (Table 1). Expression of the *Drosophila* TyrRS gene containing CMT-associated mutations induced similar defects as the human TyrRS mutants, suggesting evolutionary conservation between the *Drosophila* and human orthologs (40). The mouse mutation P234KY induced greater toxicity in fly than the human mutation G240R, consistent with the severity of the mouse model (64). Among the human GlyRS mutations tested, the order of phenotypic strength ranks as G240R > G526R > E71G, whereas the phenotypic severity rank for TyrRS mutations is E196K > G41R > Δ153–156 based on their ability to induce motor performance deficits (Table 1) (40, 41). Importantly, overexpression of a benign variant TyrRS<sup>K265N</sup> did not induce signs of toxicity, validating *Drosophila* as a readout platform to evaluate the pathogenicity of CMT mutations (66).

**Worm model**

*C. elegans* is another organism successfully used to recapitulate neurotoxicity of CMT mutations in a dominant manner. Transgenic overexpression of the *C. elegans* HisRS gene containing the mutation equivalent to R137Q or D364Y in human HisRS, but not of the WT gene, in GABAergic neurons (a subclass
of motor neurons in nematodes) caused morphological neurotoxicity denoted by dorsal and ventral nerve gaps, axonal blebbing, and severely aberrant axonal processes (Table 1) (42, 44).

**Fish model**

Most recently, zebrafish have been used successfully to demonstrate the dominant toxicity of CMT mutants (Table 1). Injection of mRNAs of three different AlaRS CMT mutants (R326W, E337K, and S627L) produced neural developmental toxicity in the embryos, whereas the same amount of WT mRNA did not (27).

**Studies on disease mechanism**

**Gain-of-function rather than loss-of-function mechanism demonstrated for CMT2D**

The mono-allelic nature of the CMT-causing mutations in aaRSs and the fact that heterozygous null mice (Gars+/−) do not develop neuropathy (60) suggest a gain-of-function rather than a loss-of-function disease mechanism. Nevertheless, genetic experiments have been carried out in mouse and fly models to specifically address this question (41, 67). The strategy was to overexpress the WT GlyRS in the CMT2D animal model (Fig. 4). If the disease is caused by a loss of function (either through haplo-insufficiency or a dominant-negative effect), then the overexpression would suppress the phenotypes; otherwise, a toxic gain of function by the mutation would be the cause of the disease phenotypes (Fig. 4). For both GarsP234KY/+ and GarsC157R/+ mice, despite a very high level (10-fold more than the endogenous level) of overexpression of the WT GlyRS, neuropathy phenotypes did not improve (67), thus supporting strongly toxic gain-of-function effects for both mutations (Fig. 4). The same conclusion on the human CMT2D mutation (G240R) was reached by another study using the Drosophila CMT model (Fig. 4). The G240R mutation per se causes a loss of aminoacylation activity as shown by in vitro assays and induces strong phenotypes in the fly model (41, 68). However, overexpression of the WT gene in the mutant fly did not provide any phenotypic rescue (41). Thus, genetic studies conclusively and consistently have demonstrated that dominant mutations in GlyRS cause CMT through toxic gain-of-function effects.

**CMT2D mutations may have loss-of-function properties but they are not the cause of CMT**

Although the genetic experiments in mice and flies have ruled out loss of function as the mechanism for CMT2D, the mutations per se do cause loss-of-function effects as manifested in phenotypes beyond neuropathy in the homozygous GlyRS

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**Figure 4. Genetic experiments used to clarify whether CMT2D is caused by a gain- or loss-of-function mechanism in mouse and fly models.** CMT-like neuropathy phenotypes cannot be rescued by overexpressing WT GlyRS, suggesting toxic gain-of-function effects by the mutations as the cause of CMT2D. In contrast, phenotypes beyond CMT2D and caused by recessive bi-allelic mutations can be rescued by the WT GlyRS overexpression, suggesting some mutations do have loss-of-function properties, although they are not the cause of CMT.
mutant mice. For example, Gars\(^{P234KY/P234KY}\) mice are not viable; Gars\(^{C157R/C157R}\) mice can be born, but they show reduced viability and early death (60, 61). Compared with heterozygous Gars\(^{C157R/+}\) mice, Gars\(^{C157R/C157R}\) mouse have more severe neuropathies and also present abnormalities in the central nervous system, possibly related to the central nervous system phenotypes observed in patients with bi-allelic GlyRS mutations (35, 37, 69). Importantly, overexpression of WT GARS was able to rescue the perinatal death, but not the neuropathy phenotypes of the homozygous Gars\(^{C157R/C157R}\) mice, again supporting that the neuropathy phenotype is caused by a toxic gain-of-function effect, whereas the phenotypes beyond neuropathy are caused by a loss of function, which may include enzymatic and/or nonenzymatic activities (Fig. 4). Similarly, Chihara et al. (70) showed that a homozygous mutation in the GlyRS gene (P98L) causes preferential loss of dendritic and axonal terminal arborization in Drosophila olfactory projection neurons, and this defect can be fully rescued by transgenic expression of WT GlyRS, demonstrating that a loss of function (undefined) can cause developmental defects in the nervous system (Fig. 4) (70). However, the defect cannot be rescued or fully rescued by CMT2D mutants (L129P and E71G, respectively), suggesting that the mutants do have some undefined loss-of-function properties, albeit they are not the cause of the neuropathy.

**Loss of function in aminoacylation is not a shared property of CMT-causing mutations**

Because the aminoacylation function is an essential activity of aaRS, and because the CMT-causing mutations are predominantly located in the catalytic domains, the mutational effects on tRNA aminoacylation have been extensively characterized through in vitro aminoacylation assays and in vivo genetic complementation assays using yeast (25, 71). Although many mutations per se do affect the aminoacylation function, it became clear that loss of aminoacylation is not a shared property of CMT-causing mutations. For example, GlyRS\(^{E71G}\) (68, 72) and TyrRS\(^{E196K}\) (40, 73), both of which segregate with CMT in large families, exhibit no defect in these assays. In addition, the level of defect in aminoacylation does not correlate with the severity of the disease phenotype. For example, flies expressing the enzymatically intact TyrRS\(^{E196K}\) mutant show a stronger defect in motor performance than flies expressing the aminoacylation-compromised mutants TyrRS\(^{G41R}\) and TyrRS\(^{A153−156}\) (40).

**CMT patients and animal models are unlikely to be deficient in tRNA aminoacylation**

With regard to the aminoacylation activity, it is important to consider the presence of the WT allele in the CMT patients. If a defect caused by a mono-allelic mutation at the molecular level can be suppressed by the presence of the WT protein, and therefore is not manifested at the level of cells and tissues, this defect is unlikely to be disease-causing. Studies using the two CMT2D mouse models discussed above provided results consistent with this scenario. No significant decrease in enzymatic activity was found in tissues of Gars\(^{P234KY/+}\) and Gars\(^{157R/+}\) mice compared with the WT animals (60, 61). Consistently, Northern blot analysis revealed that the endogenous aminoacylation levels of tRNA\(^{Gly}\) in Drosophila were not altered by transgenic expression of WT or mutant GlyRS (i.e., E71G, G240R, and G526R), regardless of the effect of the mutations on the aminoacylation activity of GlyRS per se (41).

**Mutant GlyRS may cause CMT through cell-autonomous and noncell-autonomous mechanisms**

The ubiquitously expressed mutant tRNA synthetases may give rise to CMT phenotypes through cell-autonomous and/or noncell-autonomous manners. In the case of a neurological disease, such as CMT, if the expression of a mutant protein in neuronal cells alone is able to induce a disease phenotype, the effect is cell-autonomous. Conversely, a noncell-autonomous effect is one in which the neuronal phenotype is caused the mutant protein expressed from non-neuronal cells. This question has been addressed in the Drosophila model using the GAL4 overexpression system with different cell-type–specific promoters. Both ubiquitous (actin-GAL4) and neuron-specific (Nsyb-GAL4) expression of TyrRS mutants, but not muscle-specific expression (MHC-GAL4), induced motor performance deficits, thereby indicating an intrinsic toxicity of TyrRS mutants to neurons (40). However, for GlyRS mutants, both cell-autonomous and noncell-autonomous mechanisms were observed. By using Nsyb-GAL4 (pan-neuronal) to drive the expression of GlyRS\(^{P234KY}\) or GlyRS\(^{G240R}\), and the OK371-GAL4 (motor neuron–specific) to drive the expression of GlyRS\(^{E71G}\), GlyRS\(^{G240R}\), or GlyRS\(^{G526R}\), the two studies each showed that neuronal expression of GlyRS mutants induce motor performance deficits that are cell-autonomous (41, 64). The GlyRSCMT mutations do not affect the localization of GlyRS within neuronal cells, including neuromuscular junctions (41, 65, 74). In contrast, Grice et al. (65) suggested that the neuronal toxicity of GlyRS\(^{P234KY}\) is, at least in part, noncell-autonomous, as expression of the mutant GlyRS in mesoderm or muscle alone results in motor deficits and progressive deterioration at the neuromuscular junction.

Supporting the noncell-autonomous mechanism, the secretion of GlyRS has been demonstrated in many cell types, including neuronal and muscle cells (65, 75, 76). In the fly model, Grice et al. (65) showed that muscle-expressed GlyRS\(^{P234KY}\), but not GlyRS\(^{Wt}\), accumulates outside muscle cells and on the pre-synaptic membrane of axon terminals. Interestingly, at least in Cos-7 cells, GlyRS\(^{P234KY}\) and GlyRS\(^{Wt}\) have similar levels of secretion (76).

**Distribution of CMT-associated residues on aaRS structures**

Crystal structures have been solved for all the WT CMT-linked aaRSs (10, 77–81) and thus provide a structural platform to understand the CMT-associated mutations. There is an apparent concentration of CMT mutants near the dimer interface of GlyRS, TyrRS, TrpRS, and HisRS (Fig. 5). For example, at least nine CMT-associated residues in GlyRS are located in the immediate vicinity of the dimer interface; also, the only reported CMT-associated residue in TrpRS (His-257) is adjacent to the dimer interface. However, not all CMT mutations in GlyRS, TyrRS, and HisRS are located near the dimer interface, and the effect of different mutations on dimer formation varies: some mutations (e.g., G526R and S81L in GlyRS) strengthen,
and others (e.g., L129P and G240R in GlyRS and E196K in TyrRS) significantly weaken the dimers (68, 82, 83). It is worth noting again that most, if not all, CMT mutations in GlyRS, TyrRS, TrpRS, and HisRS are located in the catalytic domain (Fig. 3) and that the catalytic domain mediates dimer formation for these four enzymes. In contrast, when the catalytic domain is not involved in dimerization, as in the case of AlaRS, the CMT mutations are no longer concentrated in the catalytic domain (Fig. 3). Although AlaRS can dimerize through the C-Ala domain (Fig. 5), the dimerization, as well as the C-Ala domain itself, does not contribute to the catalytic activity (10). The same idea applies to MetRS. MetRS does not form dimers, and all of the putative CMT-associated residues are located in the anticodon binding domain (Fig. 3). Which aspect, being in the catalytic domain or being at the dimer interface, is more fundamentally connected to disease, or is neither?

**Potential connection with tRNA**

In addition to the quasi-concentrations of CMT-associated residues at the dimer interface and the catalytic domain, the tRNA-binding surface is another possible consideration. The tRNAs are large molecules compared with the amino acid and ATP substrates, being about 76 nucleotides with a molecular mass of 25 kilodaltons. They fold into L-shaped structures and occupy large binding surfaces on aaRSs, which span from the catalytic domain to the anticodon domain of GlyRS, TyrRS, HisRS, TrpRS, and MetRS or, in the unique case of AlaRS, from the catalytic to the editing and the C-Ala domains (Fig. 6). The CMT-associated residues are mapped onto the co-crystal structures or structure models of the aaRS/tRNA complexes in Fig. 6. Although some CMT-linked residues are closer than others to the tRNA contact sites, the large areas of aaRSs involved in tRNA binding make it possible to imagine a tRNA connection.

The binding between an aaRS and a tRNA is strongly contributed by electrostatic interactions between the positively-charged basic residues on the protein and the negatively-charged phosphate groups on the tRNA. Interestingly, CMT-associated mutations in aaRSs predominately result in a net increase of positive charges, suggesting a potential enhancement of the aaRS/tRNA interaction (Fig. 3). For example, although none of the CMT2D-associated mutations in GlyRS introduce a negatively charged residue, 12 mutations, including those with the strongest evidence for pathogenicity (e.g., E71G, C157R, P234KY, G240R, and G526R), cause a net increase in the positive charge. Except for the deletion mutation Δ153–156(VKQV), the same is true for DI-CMTC–linked TyrRS mutations; all mutations (i.e., G41R, D81I, E196K, and E196Q) lead to a net increase of positive charges (Fig. 3). The only reported CMT-associated residue in TrpRS (i.e., H257R) also introduces a positive charge, whereas six out of nine CMT2N mutations in AlaRS cause a net increase in positive charge (Fig. 3). In contrast, CMT-associated mutations in HisRS and MetRS do not show this tendency. It would be interesting to study the effect of CMT-causing mutations on tRNA binding. Any potential effects, however, are unlikely to be linked to CMT through affecting the status of tRNA aminoacylation. Insights into the potential tRNA connection to CMT may be inspired by recent findings on the roles of tRNA and tRNA fragments in multiple regulatory networks and that link tRNA to neurodegeneration (84–86).

**Conformational opening induced by CMT mutations**

Although several CMT mutants have been successfully crystallized, their crystal structures revealed little conformational change compared with their corresponding WT enzymes. These include GlyRS<sub>G526R</sub> (77), GlyRS<sub>S581L</sub> (87), and GlyRS<sub>L129P</sub> (88) and also TyrRS<sub>G41R</sub> and TyrRS<sub>E196K</sub> (83). In addition, we found that those mutants of GlyRS associated with a weakened dimer (e.g., GlyRS<sub>L129P</sub> and GlyRS<sub>G240R</sub>) (68, 82) were refractory to crystallization, suggesting a potential mutation-induced conformational change that might affect the ability of these mutants to be crystallized. Although conformational changes might also exist in the mutants that were successfully crystallized, crystal-packing interactions can suppress the conformational change and prevent it from being revealed.

These considerations directed us to use solution-based methods, such as hydrogen-deuterium exchange (HDX) and small-angle X-ray scattering (SAXS), to study the structures and the dynamics of the CMT mutants. HDX monitored by MS determines which areas on the protein become more or less exposed to solvent due to a mutation. This information can be modeled in 3D using the structure template provided by the crystal structure of the WT protein. Although SAXS does not provide high-resolution information, it is useful for detecting a global conformational change. A combined use of these structural methods has so far provided significant insight into the conformational changes in GlyRS and TyrRS induced by the CMT mutations (Fig. 7) (82, 83).

Five different human CMT2D mutants have been studied by HDX (82). These mutations were selected based on their distinct effects on dimerization: L129P and G240R significantly weaken the GlyRS dimer formation, whereas G526R, S581L, and G598A slightly strengthen the dimer. Despite having different effects on dimerization, all five mutations induce conformational openings of various degrees. Eight hot spots were consensus areas shared by each of the mutants, which partially overlap with the dimerization interface. The mouse mutation P234KY also induces a similar conformational opening in GlyRS (Fig. 7) (76).

A similar conclusion was reached for CMT mutations in TyrRS (83). We focused on the three mutations (G41R, E196K, and Δ153–156) that have been validated for their pathogenicity in the fly–CMT model (40). The G41R and Δ153–156 mutations have no significant effect on dimerization, whereas E196K substantially weakens dimer formation (83). Despite the different effects of G41R and E196K on dimerization, they share a similar conformational opening effect on the catalytic domain in an area that is near the dimer interface (Fig. 7). In contrast, the deletion mutation (Δ153–156) does not induce a global conformational change. However, the area that is opened up by G41R and E196K sits right behind the loop containing 153-VKVQ. Thus, the same area is also opened up by the deletion mutation (Fig. 7) (83).
Figure 5. Distribution of CMT-linked mutation sites in relationship to the dimer interface of aaRSs. A, GlyRS; B, TyrRS; C, HisRS; D, TrpRS; E, AlaRS. For clarity, aaRS dimers are shown in ribbon representation for one subunit and in space-filling model for the second subunit. CMT mutation sites directly located at the dimerization interface are colored in red; CMT mutation sites near but not immediately at the dimerization interface are labeled in green; CMT mutation sites far away from the dimerization interface are colored in dark blue. CMT-linked residues from the space-filling subunits are shown in italic type with /H11032. The crystal structures of human GlyRS (A), TyrRS (B) (without the C-terminal EMAP-II domain), and TrpRS (D) dimers are provided by PDB entries 2PME, 1N3L, and 1R6T, respectively. The structure of human HisRS dimer (C) is obtained by re-processing the deposited data of PDB 4X5O to reveal the WHEP domain (B. Kuhle, personal communication). The full-length human AlaRS dimer (E) is modeled based on PDB entries 3WQY, 5KNN, and 5T5S. The AlaRS editing domain from Archaeoglobus fulgidus (PDB 3WQY) was docked onto human AlaRS catalytic domain (PDB 5KNN) by superimposing the catalytic domains (RMSD 1.903 Å). Human C-Ala domain (PDB 5T5S) was further docked onto the model by following the domain arrangement of human AlaRS based on SAXS analysis in solution (10). The crystal structure of human C-Ala dimer (PDB 5T5S) provided the dimer interface.
Figure 6. Distribution of CMT-linked mutation sites in relationship to tRNA-binding sites on aaRSs. A dimeric aaRS is shown for GlyRS (A), TyrRS (B), HisRS (C), and TrpRS (D) because the dimer form is required to provide the complete binding sites for a single tRNA and is necessary for catalysis. In contrast, monomeric AlaRS (E) and MetRS (F) are sufficient for tRNA aminoacylation. In the dimeric cases, one subunit of the dimer is in ribbon representation, and the other subunit is in a space-filling model. CMT mutation sites are indicated as orange-red spheres. Mutations from surface presentation subunits are labeled in italic type with \( /H_{11032}\).

A, human GlyRS/tRNAGly complex (PDB 5E6M). Insertion III (residues 423–518) was modeled by superimposing PDB 5E6M with another human GlyRS/tRNAGly structure (PDB 4QEI) with an RMSD of 0.803 Å, which indicates high accuracy of the structure model. B, human TyrRS/tRNATyr complex model. The complex was modeled by superimposing archaeal TyrRS/tRNATyr complex (PDB 1J1U) with human TyrRS catalytic and anticodon domains (PDB 1N3L) with an RMSD of 1.833 Å. In this model, two 3'-nucleotides of the tRNA are missing. C, human HisRS/tRNAHis complex model. The complex was modeled by superimposing the Thermus thermophilus HisRS/tRNAHis complex (PDB 4RDX) with human HisRS structure (PDB 4X5O), with an RMSD of 3.815 Å. Although the large RMSD indicates potential inaccuracy of the model, the tRNA fits well on the structure of human HisRS. D, human TrpRS/tRNATrp complex (PDB 2DR2). The WHEP domain was docked in by superimposing the complex with human TrpRS structure (PDB 1R6T) with a small RMSD of 0.6 Å. E, human AlaRS/tRNAAla complex model. The AlaRS and tRNAAla complex from A. fulgidus (PDB ID: 3WQY) was superimposed with the human AlaRS catalytic domain (PDB 5KNN) through the catalytic domains (RMSD 1.903 Å). The distances from the CMT mutation sites in C-Ala to tRNA may not be reliable, as the C-Ala domain may undergo structural re-arrangement upon tRNA binding. F, human MetRS/tRNAMet complex model. The Aquifex aeolicus MetRS/tRNAMet complex (PDB 2CSX) was superimposed with a truncated human MetRS structure (PDB SGL7) with an RMSD of 1.67 Å.
**CMT-causing mutations in aaRSs are unlikely to cause misfolding-induced aggregation**

The shared conformational impact among different CMT mutations in GlyRS and TyrRS provided a conceptually unifying molecular framework to consider the disease etiology. However, the structural change is different from the usual concept of protein misfolding, which is often associated with exposure of hydrophobic residues, reduction of protein stability, and formation of aggregates as an underlying mechanism for the development of many neurological diseases (83). The conformational change induced by the CMT mutations in GlyRS and TyrRS does not necessarily affect protein stability. In fact, many mutants, such as TyrRSG41R and TyrRS<sub>153–156</sub>, as well as GlyRSG526R and GlyRSS581L, are more stable than their WT counterparts as purified proteins. Even for the mutants (e.g. GlyRSP234KY) that are less stable than the WT protein as purified proteins, the reduced in vitro stability does not translate into reduced stability in vivo. No aggregation or ubiquitin-positive inclusion was detected in neural tissues of the GarsP234KY/H11001 mouse model (74). These observations suggest protein misfolding and aggregation do not underlie the development of aaRS-linked CMT (83). Rather, we hypothesized that the conformational changes induced by CMT-linked aaRS mutations allow the mutant proteins to make specific aberrant interactions with other molecules (Fig. 7) (82, 83).

**GlyRSCMT mutants interact with Nrp1/Plexin, Trk, and HDAC6**

The concept that CMT mutants make aberrant interactions through the neomorphic surfaces has been well validated. So far, at least three aberrant interaction partners have been identified for mutant GlyRS and a separate one for mutant TyrRS (Fig. 7). The first was Neuropilin 1 (Nrp1), identified to interact with GlyRSCMT mutants but not with GlyRSS<sub>WT</sub> (76). The aberrant GlyRS–Nrp1 interaction can also be detected in the neural tissue of the GarsP234KY/+ mouse and in the lymphocytes of CMT2D patients carrying the L129P mutation (76).

Nrp1 is a cell-surface receptor expressed in motor neurons, endothelial cells, and other cell types. Through binding to signaling proteins such as the semaphorins and vascular endothelial growth factor (VEGF), Nrp1 regulates both the nervous and vascular systems. The secreted mutant GlyRS interacts with the extracellular B1 domain of Nrp1, which is also the high-affinity binding site for both VEGF and semaphorins (89). Mutant GlyRS competes with VEGF for Nrp1 binding and thereby inhibits the neurotrophic VEGF–Nrp1 signaling, which in turn leads to the progressive motor neuron degeneration of CMT2D (Fig. 8) (76). This model is supported by a genetic interaction between GarsP234KY+/+ and Nrp1<sup>+/−</sup> mice, and by the rescue of motor performance deficits of the CMT2D mice through VEGF overexpression. Interestingly, CMT2D mice do not exhibit defects in the vasculature system (76, 90).

The impact of mutant GlyRS on semaphorin binding to Nrp1 has not yet been studied, because the neurological impact of semaphorin–Nrp1 signaling is thought to be mediated through the A1 domain of Nrp1 (91, 92), which is not the binding site of mutant GlyRS. However, it was recently reported that, in the fly–CMT model where GlyRSP234KY<sup>+</sup> is expressed, the pre-synaptic localized mutant GlyRS interferes with plexin B signaling (93). Plexin B binds to Semaphorin-2A (Sema2a) and is a functional homolog of Nrp1 for semaphorin signaling in Drosophila. Plexin B co-localizes with mutant GlyRS at the neuromuscular junction, and plexin B levels modify association of mutant GlyRS with the presynaptic membrane. Furthermore, increasing availability of the Sema2a alleviates the pathology and the build up of mutant GlyRS, suggesting that mutant GlyRS competes with Sema2a for binding to plexin B, which contributes to...
the CMT phenotypes in the fly model (Fig. 8) (93). It is interesting to note that in the SOD1<sup>G93A</sup> mouse model of ALS, semaphorin-3A signaling through Nrp1 was shown to be an early trigger for distal axonopathy (94). These studies highlight the importance of understanding the impact of GlyR<sup>CMT</sup> on semaphorin-Nrp1 signaling.

Tropomyosin receptor kinase (Trk) receptors were also shown to aberrantly interact with GlyR<sup>CMT</sup> and have been suggested to explain the sensory involvement seen in CMT2D (Fig. 8) (95). Trk signaling is essential for sensory neuron differentiation and development. Mutant GlyRS binds and misactivates multiple Trk receptors, thereby subverting sensory neuron differentiation and/or survival during early stages of development. In contrast to the progressive motor neuron deficits, the sensory defect is developmental and nonprogressive in CMT2D mice. The developmental nature of the sensory defect might give rise to a binary presentation of sensory involvement in CMT2D patients and may explain the absence of sensory defect in some patients with GlyRS mutations (classified as dSMA-V or dHMN-V) (95).

Recently, HDAC6 was also identified to aberrantly interact with all CMT2D mutants tested, including GlyRS<sup>E71G</sup>, GlyRS<sup>L120P</sup>, GlyRS<sup>L211F</sup>, GlyRS<sup>C240R</sup>, GlyRS<sup>E279D</sup>, GlyRS<sup>H418R</sup>, GlyRS<sup>G526R</sup>, GlyRS<sup>S581L</sup>, and GlyRS<sup>G598A</sup> (96, 97). A main target of the HDAC6 deacetylase is α-tubulin, a critical component of the microtubule, which provides the tracks along which long-distance axonal transport occurs. Acetylation of α-tubulin facilitates axonal transport by promoting the binding of motor proteins to the microtubule. The aberrant GlyRS–HDAC6 interaction enhances the activity of the deacetylase, resulting in a decreased acetylation level of α-tubulin and leading to axonal transport defects (Fig. 8) (96). Importantly, the decreased α-tubulin acetylation was only found in peripheral nerves but not in brain and spinal cord of the P234KY mouse model. Because the
decrease in acetylation and the axonal transport deficits were found in advance of the onset of CMT symptoms in the mouse model, these defects appear not to be secondary to axonal degeneration (96).

It is worth noting that the two anticodon binding domain mutations S581L and G598A induce much stronger aberrant interactions with HDAC6 than other patient mutations in the catalytic domain of GlyRS (96). The S581L and G598A patients have more severe distal weakness and wasting in the lower limbs (33–37), in contrast to the upper limb predominance found in other CMT2D patients (22, 28). Thus, the aberrant GlyRS–HDAC6 interaction appears to correlate with the divergent clinical presentation among CMT2D patients. Moreover, the G598A mutation can induce strong aberrant interactions of GlyRS with both Nrp1 and HDAC6, potentially explaining the severe, early-onset clinical symptoms of patients carrying this mutation (33, 36). Although the aberrant Nrp1–Plexin and Trk interactions may be responsible for the motor and sensory neuron specificity of the disease, respectively, the aberrant HDAC6 interaction helps explain the length-dependent vulnerability of axons in CMT2D.

**TyrRS**

TyrRS mutants aberrantly interact with TRIM28

TRIM28 was identified to interact with TyrRS WT through an interactome study of nuclear TyrRS (98). TRIM28 is a scaffolding protein, which forms a complex with the deacetylase HDAC1 to suppress acetylation and activity of transcription factors such as E2F1. The TyrRS–TRIM28 interaction sequesters TRIM28 and HDAC1 and thereby activates E2F1. We found that all three validated DI-CMTC–causing mutations (G41R, E196K, and Δ153–156) caused an enhanced interaction with TRIM28 (83), presumably through the exposed neomorphic surface near the dimer interface (Fig. 7), leading to E2F1 hyperacetylation and overactivation4 (Fig. 8). The aberrant interaction and E2F1 hyperactivation could be verified in patient-derived lymphocytes, suggesting transcriptional dysregulation is associated DI-CMTC. In fact, a broad transcriptional dysregulation network was identified with neuronal tissues of Drosophila expressing TyrRS E196K versus TyrRS WT, indicating additional transcription regulators that could be aberrantly interacted and dysregulated by mutant TyrRS. Remarkably, pharmacological inhibition of TyrRS nuclear entry reduced, whereas genetic nuclear exclusion of mutant TyrRS completely rescued, hallmark phenotypes of CMT in the Drosophila model, uncovering the importance of a nucleus-localized aaRS for CMT. The CMT-causing mutations do not appear to affect the nuclear localization of TyrRS.4

**WHEP domain**

Except for TyrRS and AlaRS, all CMT-associated aaRSs contain a WHEP domain, either at the N terminus (GlyRS, HisRS, and TrpRS) or at the C terminus (MetRS) (Fig. 3). The only other aaRSs that contain WHEP domains are the bi-functional EPRS, which is a component of the MSC and has three consecutive WHEP domains between the fused GluRS and ProRS. WHEP domains adopt a helix-turn-helix structure and have the capacities to bind to proteins and nucleic acids, including RNA and DNA (99, 100). Although they do not significantly affect the tRNA-binding affinity and the aminoacylation activity of their host aaRSs (101–103), the WHEP domains were found to regulate or mediate interactions of aaRSs with other proteins or nucleic acids for nonenzymatic functions. For example, the WHEP domains in EPRS are essential for the role of the bi-functional synthetase in translationally silencing specific mRNAs associated with the inflammatory response (104–106). Also, the WHEP domain of HisRS is the main epitope for the anti-Jo-1 antibodies in inflammatory myositis patients (107, 108). The WHEP domain alone (HisRSWHEP) can be produced as a splice variant, and the expression of HisRSWHEP is up-regulated in the myositis patients (108). Removal of the WHEP domain in TrpRS, either by proteolysis or alternative splicing, activates the anti-angiogenic activity of the synthetase by exposing its active site for interaction with the extracellular domain of the endothelial adhesion molecule VE-cadherin (102, 109). The WHEP domain of TrpRS also mediates direct interactions with DNA-PK and PARP-1 in the nucleus to activate p53 (110). Interestingly, deletion of the WHEP domain from GlyRS creates a similar conformational opening as seen in the GlyRS-CMT mutants (82).

The exceptional prevalence of WHEP domains in CMT-associated aaRSs suggests a potential relevance of this appended domain for CMT. Indeed, Cader and co-workers (65) demonstrated that the toxicity of GlyRS P234K-Y in the fly model is WHEP domain-dependent. Deletion of the WHEP domain abrogated the toxicity of GlyRS P234K-Y through either ubiquitous or muscle-specific expression, although the mechanism underlying the rescue is not yet understood. Further investigations on the role of the WHEP domain in CMT will be of great interest.

**Site of lesion** of aaRSs in CMT

GlyRS is one of the two dual-localized aaRSs, functioning for both cytoplasmic and mitochondrial protein synthesis. Therefore, the initial link of GlyRS to CMT immediately triggered the question of which subcellular site–cytoplasm or mitochondria—is relevant to the disease. The subsequently identified aaRSs all encode enzymes specifically used in the cytoplasm but not in the mitochondria, suggesting that the mitochondrial site may not have a strong relevance for aaRS-linked CMT. Consistently, transgenic expression of the cytoplasmic version of the GlyRS-CMT mutants successfully induced phenotypes that recapitulate the hallmarks of the human diseases (41, 64, 65). However, it became clear that these supposedly cytoplasmic-restricted enzymes also have the potential to be multilocalized in the mitochondria and nucleus and to be secreted (111).

A neuron-specific mitochondrial defect was detected in the induced neuronal progenitor cells of a CMT2D patient carrying a dominant mono-allelic mutation H162R GlyRS (reported as H216R due to the inclusion of the mitochondrial targeting sequence) (35). The defects include reduced levels of both mtDNA and nucleus-encoded mitochondrial respiratory chain

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complexes and decreased mitochondrial respiration and a reduced level of vesicle-associated membrane protein-associated protein B (as part of the mitochondria-associated endoplasmic reticulum membrane complex) and its downstream signaling, including mitochondrial calcium uptake and autophagy. As calcium uptake regulates synaptic vesicles at the neuromuscular junction, this study may provide part of the explanation for the presynaptic defects of neuromuscular transmission in CMT2D mice (62).

As discussed above, GlyRSCMT could be secreted and interfere with proper signaling in motor and sensory neurons through aberrant interactions with ectodomains of membrane receptors (e.g. Nrp1 and Trk) (76, 95). Mutant GlyRSCMT also causes an axonal transport defect due to an aberrant interaction with intracellular protein HDAC6 (96, 97), presumably in the cytoplasm. Moreover, the relevance of the nucleus in TyrRSLINKED CMT has been demonstrated using the Drosophila model. Therefore, the “site of lesion” of CMT-linked aaRSs should not be restricted to the cytosol and/or the mitochondria and is likely to involve multiple compartments.

Concluding remarks and future directions

Peripheral neuropathy was the first human disease linked to aaRSs. The number of CMT-linked aaRSs has now expanded to a total of six family members (Figs. 1 and 3). Although this number may be further increased, we speculate that not all aaRSs have the potential to be linked to CMT through dominant mutations. Certain molecular features, such as the capacity for dimerization, the lack of association to the large MSC, and the possession of a helix-turn-helix WHEP domain, are prevalent in CMT-linked aaRS members (Fig. 2), suggesting selectivity.

Although CMT-causing mutations have loss-of-function properties, genetic studies have clearly demonstrated that dominant mutations in GlyRS cause CMT2D through toxic gain-of-function effects (Fig. 4), and this may apply to other aaRS-linked CMT subtypes. It is important to differentiate the disease-causing features of a mutation from its many other possible effects. This is particularly important for understanding dominantly transmitted diseases in which the functional effect of a mutation could be different in the absence or the presence of the WT protein.

The most important future direction for the field is to understand the commonality in pathogenesis among different aaRS-linked CMT forms. The disease-causing mechanism of aaRS-linked CMT is likely to be multifactorial and to involve multiple cellular compartments, including the extracellular space. At the same time, some common pathogenic mechanisms among different aaRSs are expected to contribute to their overall similar clinical phenotypes. Commonalities have emerged from the genetic (64), functional (41), and structural studies (Figs. 5–7) (82, 83). For example, common genetic modifiers with nuclear localization were found in fly-based screens for the CMT-associated mutants GlyRS and TyrRS (64). Expression of either the GlyRS or TyrRS mutant in Drosophila impairs protein synthesis through an unknown mechanism that is independent of tRNA aminoacylation (41). Different CMT-linked mutations in different aaRSs (e.g. GlyRS and TyrRS) cause shared conformational openings that expose new protein surfaces for potential aberrant interactions with other proteins, nucleic acids, or small molecules (Fig. 7). This conceptual framework has guided the discovery of many aberrant interaction partners of CMT-linked aaRSs, including Nrp1–Plexin, Trk, HDAC6, and TRIM28. These interactions were identified through a combination of serendipity or hypothesis-based investigations. For future studies, unbiased, systematic approaches are necessary to further reveal the global aberrant interactome of aaRS mutants, from which commonality among different aaRS-linked CMT forms may emerge.

Understanding the commonality in pathogenesis among different aaRS-linked CMT subtypes is key for therapeutic development. Although gene therapies have emerged strongly for monogenic disease such as CMT (112), the large number of different mutations involved and the small number of patients affected by each mutation render classic gene therapy onerous for aaRS-linked CMT. Identifying a causal treatment strategy applicable to different mutations in several genes would therefore be the most attractive therapeutic approach.

Several treatment strategies have been tested in animal models for proof-of-concept. For CMT2D, overexpressing VEGF in hindlimb muscles to overcome the competition of GlyRSP234KY for Nrp1 binding improved the motor performance of GarSP234KY/H11001 mice (76); administration of the HDAC6 inhibitor tubastatin A to block the aberrant HDAC6–GlyRS interaction and the resulting HDAC6 hyperactivation also improved motor performance of both GarSP234KY/H11001 and GarSC157R/H11001 mice (96, 97). A symptomatic treatment using the postsynaptic-acting cholinesterase inhibitor physostigmine to overcome presynaptic defects at neuromuscular junctions also showed benefit (62). For DI-CMTC, administration of the p300/CBP-associated factor inhibitor embelin to impede the nuclear entry of TyrRS improved the viability and motor performance of TyrRSE196K-expressing Drosophila (4). Although these function-based strategies are likely to benefit patients with different mutations, they may have limited efficacy as a monotherapy, because they target only one of many aspects of the pathology. If the mutation-induced, shared conformational opening is indeed a fundamental cause of aaRS-linked CMT forms, targeting the opening site on each aaRS to prevent aberrant interactions may be a more effective strategy.

Another priority for the field is to develop additional mouse models based on human mutations and on aaRS-linked CMT subtypes beyond CMT2D. They are much needed to facilitate the study of common mechanisms and therapeutic development. Progress in recent years has benefited tremendously from the availability of animal models, especially the mouse models. These animal models also provide opportunities to understand the physiological significance of aaRSs with their enzymatic and nonenzymatic regulatory functions. In this regard, the neurodegenerative CMT disease phenotypes might represent a dysregulated state of the normal homeostatic, regulatory network made of a selective group of aaRSs.

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