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ORIGINAL ARTICLE

Drosophila as a platform to predict the pathogenicity of novel aminoacyl-tRNA synthetase mutations in CMT

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Abstract Charcot-Marie-Tooth disease (CMT) is the major form of inherited peripheral neuropathy in humans. CMT is clinically and genetically heterogeneous and four aminoacyl-tRNA synthetases have been implicated in disease etiology. Mutations in the *YARS* gene encoding a tyrosyl-tRNA synthetase (*TyrRS*) lead to Dominant Intermediate CMT type C (DI-CMTC). Three dominant *YARS* mutations were so far associated with DI-CMTC. To further expand the spectrum of CMT causing genetic defects in this tRNA synthetase, we performed DNA sequencing of *YARS* coding regions in a cohort of 181 patients with various types of peripheral neuropathy. We identified a novel K265N substitution that in contrast to all previously described mutations is located at the anticodon recognition domain of the enzyme. Further genetic analysis revealed that this variant represents a benign substitution. Using our recently developed DI-CMTC *Drosophila* model, we tested in vivo the pathogenicity of this new *YARS* variant. We demonstrated that the developmental and behavioral defects induced by all DI-CMTC causing mutations were not present upon ubiquitous or panneuronal TyrRS K265N expression. Thus, in line with our genetic studies, functional analysis confirmed that the K265N substitution does not induce toxicity signs in *Drosophila.* The consistency observed throughout this work underscores the robustness of our DI-CMTC animal model and identifies *Drosophila* as a valid read-out platform to ascertain the pathogenicity of novel mutations to be identified in the future.

Keywords CMT · *Drosophila* · YARS · Disease model · Mutation

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Introduction

Charcot-Marie-Tooth disease (CMT) is the most common inherited neuromuscular disorder with an estimated prevalence of 1/2500 individuals in the general population (Skre 1974). CMT affects the human peripheral nervous system (PNS) and is clinically characterized by distal muscle wasting, motor and sensory abnormalities and skeleton deformities (Dyck 1993). The systematic effort to unravel the molecular basis of CMT lead to the identification of more than 30 genes and loci associated with this disease (http://www.molgen.ua.ac.be/CMTMutations/). Based on neuropathological and electrophysiological parameters, CMT can be broadly divided in two main groups, a demyelinating form

(CMT1) and an axonal form (CMT2) of disease. In addition, the description of patients displaying both demyelination and axonal loss gave rise to a new entity named Dominant Intermediate CMT (DI-CMT) (Nicholson and Myers 2006). Our lab has mapped a locus on chromosome 1p34-p35 (Jordanova et al. 2003) and identified mutations in the *YARS* gene, coding for a tyrosyl-tRNA synthetase (TyrRS), as the cause of DI-CMT type C (DI-CMTC) (Jordanova et al. 2006). Besides *YARS*, mutations in three other aminoacyl-tRNA synthetase (aaRS) genes, namely *GARS* encoding a glycyl-tRNA synthetase (GlyRS) (Antonellis et al. 2003), *AARS* encoding an alanyl-tRNA synthetase (AlaRS) (Latour et al. 2010), and *KARS* encoding a lysyl-tRNA synthetase (LysRS) (McLaughlin et al. 2010), have been linked to CMT. So far, three *YARS* DI-CMTC associated mutations have been identified: two missense substitutions, G41R and E196K, and a 4 amino acid deletion, 153-156VKQVDel (Jordanova et al. 2006). All of them are located in the catalytic domain of the TyrRS protein (Fig. 1b).

By charging each tRNA with the respective amino acid, aminoacyl-tRNA synthetases are essential enzymes in the protein synthesis process. It is still intriguing how mutations in this group of ubiquitously expressed proteins lead to the strict PNS degeneration observed in CMT patients. Several strategies have been applied to identify the toxic properties that CMT associated mutations might confer to these proteins. The first attempts focused on determining how the aminoacylation activity is perturbed in the mutant enzymes. Independent studies have shown that, at least for TyrRS and GlyRS, loss of enzymatic activity is not a common feature to all mutations analyzed (Nangle et al. 2007; Storkebaum et al. 2009). In further work, the effects of *GARS* CMT associated mutations were modeled in yeast (Antonellis et al. 2006), neuronal cell cultures (Antonellis et al. 2006; Nangle et al. 2007) and *Drosophila* (Chihara et al. 2007). Additionally, two dominant mouse *GARS* alleles leading to peripheral neuropathy were identified and characterized (Achilli et al. 2009; Seburn et al. 2006). Our lab has recently developed a *Drosophila* model wherein the predominant DI-CMTC features can be recapitulated and the neuronal dysfunction induced by *YARS* CMT associated mutations be monitored (Storkebaum et al. 2009).

The identification of novel pathogenic mutations within a gene allows a constant refinement of the respective disease spectrum. On one hand, this can improve the genotype-phenotype correlations and enrich the set of pathogenic alterations of a particular protein; while on the other hand, it can challenge the current understanding of a disease and test the robustness of previously established disease models. In a screen for novel CMT-causing *YARS* mutations, we identified one patient carrying an alteration that in contrast with all previously identified mutations locates to a different domain of the TyrRS protein. Sequencing of control individuals revealed that this variant is most likely a benign polymorphism. Additionally, the same patient was identified to be a compound heterozygote for two CMT causing *FIG4* mutations (Chow et al. 2007). We have used this novel *YARS* mutation to challenge our DI-CMTC fly model (Storkebaum et al. 2009) and test its robustness. Importantly, we were able to exclude in vivo the pathogenicity of this *YARS* alteration. Our work identifies *Drosophila* as a platform to ascertain the pathogenicity of novel *YARS* sequence variants to be identified in future studies.

Materials and methods

CMT cohort

Our patient cohort consisted of 181 CMT patients with various types of peripheral neuropathy. Routine mutation screening of the common CMT genes was negative for the vast majority of patients. Patient BAB1161 developed the CMT disease at the age of five and has been previously diagnosed with demyelinating neuropathy and severely slowed nerve conduction velocities, (3,7 m/s for the ulnar nerve at the age of nine years). All patients gave their informed consent prior to their inclusion in the study. The Ethics Committees of the participating institutions approved this study.

Mutation screen

Human genomic DNA was extracted from peripheral blood samples using standard procedures. The coding regions and exon–intron boundaries of *YARS* were PCR amplified. Primer oligonucleotides design was assisted by SNPbox and can be provided upon request. PCR products were purified using the Exonuclease I–Shrimp Alkaline Phosphatase kit (USB, Cleveland, Ohio). Mutation screening was performed by direct DNA sequencing using the BigDyeTerminator v3.1 Cycle sequencing kit (Applied Biosystems, Fostercity, CA). Fragments were size separated on an ABI 3730 automated capillary DNA sequencer (Applied Biosystems). The resulting sequence traces were aligned according to the NM_003680.3 sequence (NCBI) and analyzed with SeqManII software (DNAstar Inc., Madison, WI).

DNA constructs and generation of transgenic flies

Full-length *dYARS* cDNA (clone LD21116) was obtained from the BDGP *Drosophila* Gold Collection. The K264N mutation in *dYARS* cDNA was created with the Quick Change mutagenesis kit (Stratagene). *dYARS* cDNA with the appropriate mutation was subcloned into the *Drosophila* pUAST transformation vector. All constructs

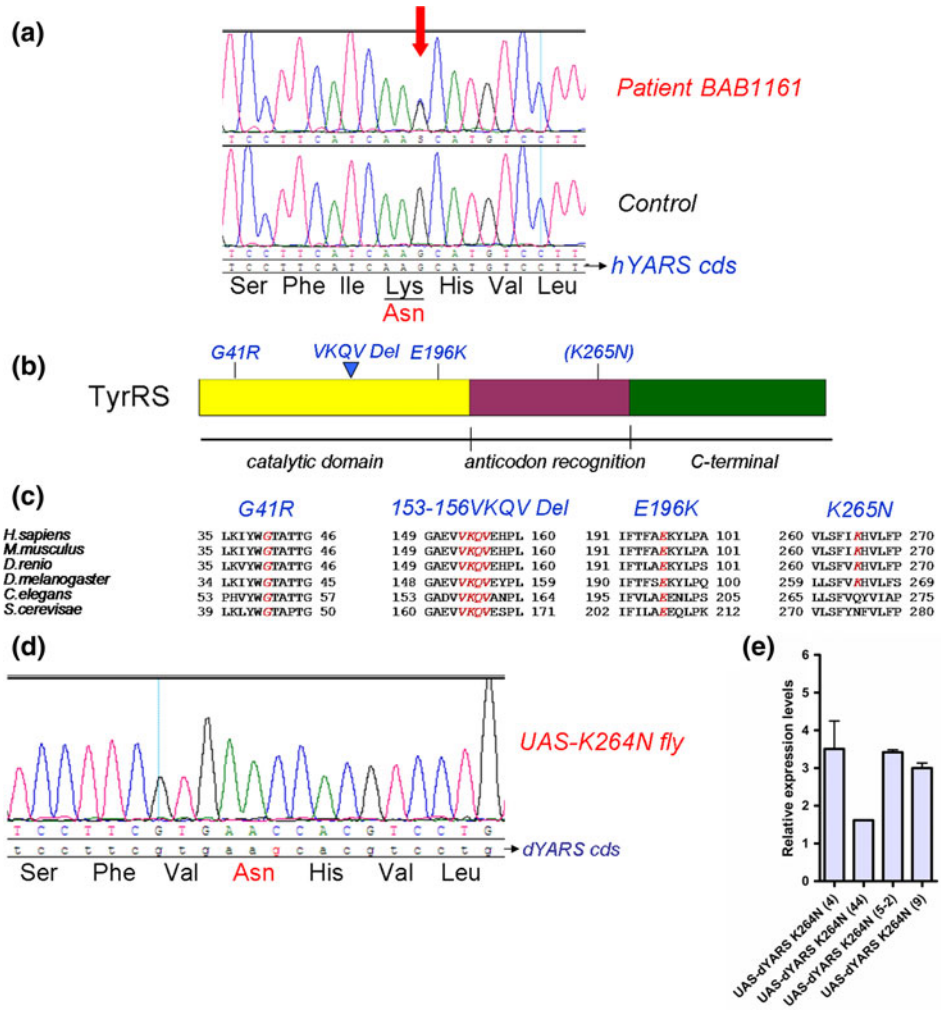

Fig. 1 Identification of the human Lys265Asn (K265N) YARS variant. **a** Sequence trace files of control individual and the BAB1161 patient with c.795G>C substitution, aligned to the hYARS cDNA sequence. **b** Distribution of YARS mutations within the TyrRS domains. Three domains can be distinguished, the *N*-terminal catalytic domain, the anticodon recognition domain and a *C*-terminal domain. All pathogenic *YARS* DI-CMTC mutated residues lay in the catalytic domain and the K265 residue locates in the anticodon recognition domain. **c** Protein sequence alignment of YARS orthologs. Amino acids mutated in DI-CMTC patients and BAB1161 are italicized. Note that K265 is not conserved in all eukaryotes. **d** Sequence trace file of a fly genomic DNA PCR product amplifying the UAS-*dYARS* K264N transgene. Human K265 residue corresponds to K264 in *Drosophila*. **e** Expression levels of four independent UAS-*dYARS* K264N transgenic lines (act5C wk > UAS-*dYARS* K264N). One insertion on the second chromosome (UAS-*dYARS* K264N 4) was further combined with two independent transgenes with similar expression levels on the third chromosome (UAS-*dYARS* K264N 5-2 and 9)

were sequence verified and transgenic flies were generated using standard procedures. Multiple independent transgenic lines were established.

Determination of transgene expression levels

Expression levels of independent pUAST_UAS-dYARS K264N transgenic lines were determined by real time qRT-PCR on RNA extracts from full bodies of actin5C-Gal4 wk > UAS-dYARS flies. RNA was extracted from three experimental (actin5C-Gal4 wk > UAS-dYARS) and three control (TM6B > UAS-dYARS) groups for each transgenic line. Real time qRT-PCR analysis using dYARS primers (forward: 5′-GAGAAGTACATCAACCGACTGC TAGA-3′; reverse: 5′-GTTTTTGCAGTTCTGGGTTTT CA-3′) and actin primers as an internal reference (forward: 5′-AGTCCGGCCCCTCCATT-3′; reverse: 5′-CTGATCCTC TTGCCCAGACAA-3′) was performed like previously described (Storkebaum et al. 2009).

Drosophila genetics

The two actin-Gal4 (actin5C-Gal4 st/Cyo and actin5C-Gal4 wk/TM6B) drivers were obtained from the Bloomington *Drosophila* Stock Center. B. Dickson kindly provided the nsyb-Gal4 stock. All UAS-*dYARS* stocks

corresponding to Wt, G40R, E195K and 152-155VKQV-Del were previously described (Storkebaum et al. 2009). Double UAS-*dYARS* transgene stocks were generated by combining single transgene insertions on second and third chromosomes in stable stocks. Stocks carrying UAS-*dYARS* K264N (4 and 5-2) and UAS-*dYARS* (4 and 9) correspond to 2×′K264N and 2×″K264N double transgene flies, respectively. For the determination of adult eclosion frequencies, the number of adult flies enclosing for each genotype/sex was counted.

Drosophila behavioral assays

To assay adult motor performance, negative geotaxis response of transgenic flies was analyzed. The assay was performed as described in (Strauss and Heisenberg 1993) with some slight modifications. Briefly, ten female flies with shortened wings were shaken down to the bottom of a cylindrical fly container (49 mm diameter) that was covered with a black cap. The experiment was carried out on a black surface under red light. Time needed for the first fly to climb a vertical wall and reach a mark at a height of 82 mm was measured. For each group of ten flies, the experiment was done ten times and the average of these ten walking speeds was calculated. For each genotype, ten groups of ten flies at 10 days of age were tested.

Statistics

We used GraphPad Prism 4 software to perform Chi-square analysis and One-Way ANOVA with Bonferroni′s Multiple Comparison Test.

Results

A mutation screen in CMT patients identifies a novel *YARS* variant located at the TyrRS anticodon recognition domain

We sequenced the 13 exons and exon–intron boundaries of the *YARS* gene in a cohort of 181 CMT patients. We identified one reported (rs699005/L406L) and two non-reported (c.391C>T/L130L and c.834A>C/L278L) single nucleotide substitutions leading to silent mutations. In addition, we found one heterozygous sequence variant, c.795G>C in exon 7, predicted to cause a missense K265N mutation (Fig. 1a). In contrast to all previously identified *YARS* mutations (Jordanova et al. 2006), this novel variant locates at the TyrRS anticodon recognition domain (Fig. 1b). After sequencing 368 control chromosomes, we identified the K265N substitution in a heterozygous state in one control individual, suggesting that this is a benign polymorphism. Despite the genetic findings, we decided to express the K265N *YARS* variant in *Drosophila*. This allows us to challenge our current DI-CMTC animal model and confirm the non-pathogenicity of this missense mutation.

A K264N YARS fly

As all the residues affected by pathogenic YARS mutations, the K265N mutation affects a residue conserved between the human (hTyrRS) and fly (dTyrRS) proteins (Fig. 1c). Human residues G41, VKQV153-156, E196 and K265 correspond to G40, VKQV152–155, E195 and K264 in *Drosophila*, respectively. We have previously shown that in *Drosophila,* phenotypes induced by DI-CMTC hTyrRS mutants can be mimicked by equivalent dTyrRS forms (Storkebaum et al. 2009). We generated transgenic flies where the equivalent dTyrRS variant (*dYARS* K264N) can be expressed under the control of a Gal4-inducible promoter (Fig. 1d). The UAS-Gal4 system allows performing misexpression studies in flies. Here, a particular transgene can be placed under the control of a UAS (upstream activating sequence) motif and be turned on by a Gal4 source in the spatial pattern associated with a particular Gal4 driver (Brand and Perrimon 1993). We have previously observed that double transgene expression of all DI-CMTC YARS mutants in *Drosophila* is sufficient to exert toxicity both with ubiquitous and neuronal specific Gal4 drivers (Storkebaum et al. 2009). Therefore, we built fly stocks with two copies of the UAS-*dYARS* K264N transgene. By real time qRT-PCR analysis, we selected lines with similar strong expression levels (Fig. 1e). In order to obtain double transgene stocks, we combined independent UAS-*dYARS* K264N insertions on the second and third chromosomes in single stable fly stocks.

No motor performance defects upon neuronal expression of the K264N variant in *Drosophila*

We have previously identified signs of toxicity upon restricted expression of *YARS* DI-CMTC mutants in the fly nervous system (Storkebaum et al. 2009). In this study, panneuronal transgene expression was achieved with the strong constitutive driver n-synaptobrevin-Gal4 (nsyb-Gal4) (Bushey et al. 2009; Pauli et al. 2008). In order to test for an equivalent dysfunction induced by the K264N variant, the motor performance of flies expressing this allele in neurons was quantified in a negative geotaxis assay. This paradigm evaluates *Drosophila* sensory-motor integration function and allows robust estimation of locomotion deficits (Strauss and Heisenberg 1993). Neuronal expression of all dTyrRS DI-CMTC mutants (G40R, VKQVDel and E195K) induced a climbing deficit

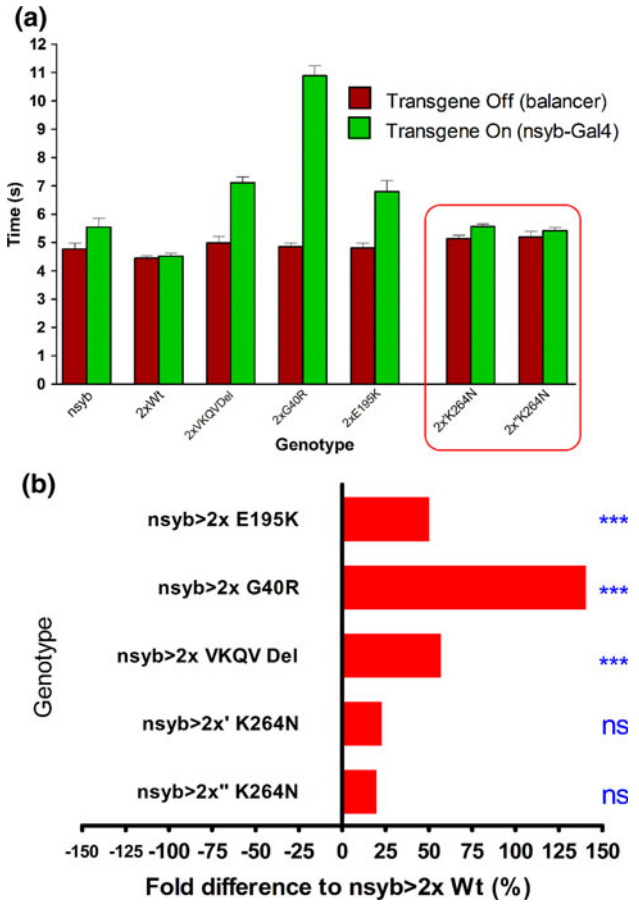

Fig. 2 Panneuronal expression of *dYARS* K264N does not reveal motor performance defects in a negative geotaxis assay. **a** The average time ten groups of 10 females per genotype needed to climb 82 mm is indicated. nsyb-Gal4 was used to drive expression of two copies of Wt, G40R, VKQVDel, E195K and K264N *dYARS* transgenes. Expression of all DI-CMTC *dYARS* mutations but not *dYARS* K264N induced significant differences. **b** Percentage difference between Wt *dYARS* (nsyb > 2× Wt) and mutant *dYARS* expressing flies (nsyb > 2× *dYARS*). *ns*, $P > 0.05$ and *** $P < 0.001$ indicates statistical differences as determined by one-way ANOVA with Bonferroni's Multiple Comparison Test

(Storkebaum et al. 2009) that was always significant when compared with Wt dTyrRS expressing flies (nsyb > 2× Wt) (Fig. 2a, b). In contrast, the climbing time of flies expressing two independent *dYARS* K264N transgene combinations was similar to Wt dTyrRs expressing flies indicating they do not display a motor deficit (Fig. 2a, b).

Ubiquitous expression of the K264N variant in *Drosophila* does not render developmental lethality or motor deficit

Besides the specific nervous system malfunction induced by all DI-CMTC mutations, we have previously reported that they are also toxic when expressed ubiquitously in the fly (Storkebaum et al. 2009). In order to identify a putative effect of the K264N mutation at whole organism level, we expressed independent combinations of two UAS-*dYARS* transgene copies with a strong actin-Gal4 driver (act5C-Gal4 st). Homozygous UAS-*dYARS* flies were crossed with an act5C-Gal4/Balancer stock. After flies eclosion, the deviation of the expected 50:50 ratio of act5C-Gal4 (transgene On) to Balancer (transgene Off) offspring was calculated. Upon ubiquitous expression with act5C-Gal4 st all *YARS* DI-CMTC alleles induced a significant lethality that ranged from 100 to 70% (Fig. 3a). This phenotype applied to the E195K mutation even when only one transgene copy is expressed with a weak actin-Gal4 driver (act5C-Gal4 wk). In contrast, the K264N variant did not induce any significant decrease in the percentage of eclosing adults expressing the transgenes (Fig. 3a). The same absence of adult eclosion phenotype was observed upon expression of Wt dTyrRS (Fig. 3a). This preadult lethality phenotype was prominent for males, where flies expressing either DI-CMTC mutants or Wt TyrRS raised two markedly distinguishable phenotypic groups (Fig. 3b). Importantly, eclosing males expressing K264N or Wt d*YARS* transgenes cluster in one group (Fig. 3b). Thus, in terms of eclosion frequencies, the expression of the dTyrRS K264N variant did not display any signs of pre-adult toxicity.

The existence of escapers ubiquitously expressing all *YARS* mutant alleles, allowed us the further characterization of adult-onset phenotypes. In a negative geotaxis assay, we compared the climbing abilities of these flies. Ubiquitous expression of all dTyrRS DI-CMTC mutants led to a significant climbing delay, ranging from 25 to 50% when compared with the Wt dTyrRS control (act5C-Gal4 st > 2× Wt) (Fig. 4a, b). In K264N expressing flies, we could not identify any significant impairment when compared with the same genetic control (Fig. 4a, b). Hence, in addition to the absence of developmental lethality, adult K264N flies do not display motor deficit.

Discussion

We performed DNA sequencing of the coding regions of the *YARS* gene in a cohort of 181 unrelated patients with various types of CMT disease and identified one novel missense K265N mutation. The same mutation was retrieved in 1/168 unrelated control individuals, suggesting this amino acid substitution is benign. In addition, a CMT causing genetic defect in *FIG4* was identified in the BAB1161 patient (Chow et al. 2007). Our study indicates that *YARS* mutations are a rare cause of CMT. This is in accordance with our previous observations where in a heterogeneous cohort of 255 CMT patients we identified only one pathogenic 153–156VKQV de novo deletion

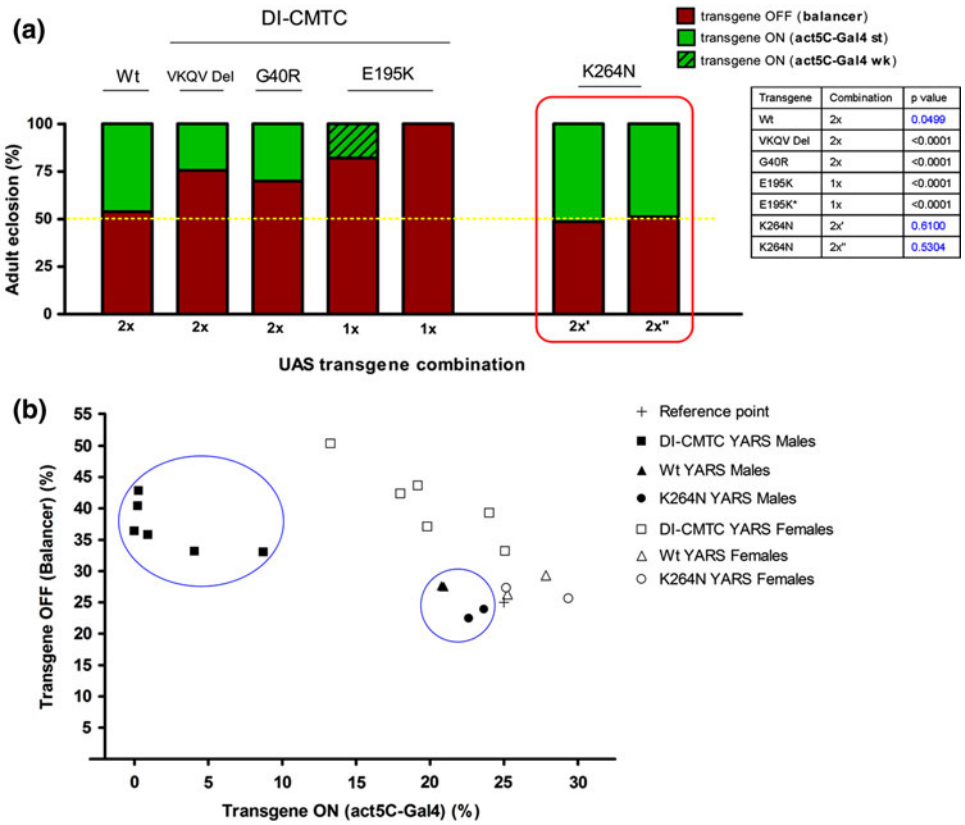

Fig. 3 Ubiquitous expression of *dYARS* K264N does not induce preadult lethality. **a** Double *dYARS* transgene combinations for Wt, VKQVDel, G40R and K264N (2×′ and 2×″) were crossed with act5C-Gal4 st/Balancer. Because E195K expression was fully lethal with act5C-Gal4 st, this mutant allele was crossed with act5C-Gal4 wk/Balancer. Percentages indicate the relative frequency of each genotype eclosing per cross. *Dashed line* marks the expected 50:50 genotype ratio (transgene On: transgene Off). Similar to Wt *dYARS* and differently from DI-CMTC *dYARS* mutants, expression of *dYARS* K264N does not induce significant deviation from the expected 50:50 ratio. The *P*-values determined after chi-square analysis are indicated in the table. "*" refers to act5C-Gal4 wk based experiments. **b** An overview of sex linked preadult lethality of flies ubiquitously expressing *dYARS* transgenes with act5C-Gal4. Percentages indicate the relative frequency of each genotype/sex eclosing per cross. "+" indicates the expected 25:25 genotype ratio for males or females. In contrast with the strong male preadult lethality induced by all DI-CMTC mutants, expression of K264N, like Wt *dYARS*, does not induce any sex specific lethality phenotype

(Jordanova et al. 2006). While all previously identified pathogenic *YARS* mutations locate at the catalytic domain of the protein and lead to an intermediate CMT phenotype (Jordanova et al. 2006), the K265N variant locates at the TyrRS anticodon recognition domain. In contrast, pathogenic *GARS* mutations are scattered in different domains and two of them lay in the anticodon recognition domain of GlyRS (reviewed in (Motley et al. 2010)). Animal models displaying signs of neurodegeneration due to dominant effects of either *YARS* or *GARS* mutations have only been established for mutations affecting residues laying within or in the proximity of the catalytic domain of both proteins (Seburn et al. 2006; Achilli et al. 2009). So far, the robustness of our DI-CMTC fly model was only tested upon Wt TyrRs overexpression (Storkebaum et al. 2009). The K265N variant allowed us further challenging the current DI-CMTC animal model and to confirm the non-pathogenicity of this missense mutation.

Our analysis of flies ubiquitously expressing different *YARS* alleles allowed us to monitor mutant *YARS* toxicity in different developmental stages and sexes. The predominant male lethality strictly correlated with the disease relevance of different *YARS* alterations. Moreover, the motor performance of female escapers ubiquitously expressing dTyrRS mutants indicates that all DI-CMTC mutations, but not the K265N variant or wild type dTyrRS expression, lead to a motor deficit. Together with the sex-linked preadult lethality and the previously unreported motor deficit upon dTyrRS ubiquitous expression, we underlined the absence of K265N pleiotropic effects. The motor performance analysis of flies specifically expressing this variant in fly neurons further confirmed the lack of cellular toxicity. Thus, in all parameters evaluated, flies expressing K265N did not differ from Wt YARS expressing flies. Therefore, K265N is unlikely to be a dominant cause of CMT. Furthermore, an overview at the

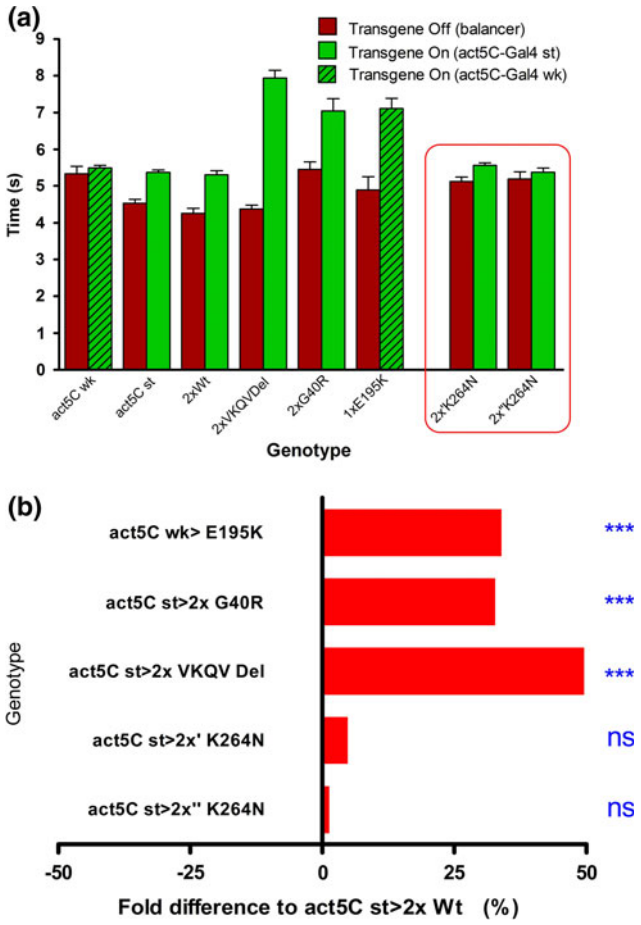

Fig. 4 Ubiquitous expression of *dYARS* K264N does not reveal motor performance defects in a negative geotaxis assay. **a** act5C-Gal4 st was used to drive expression of two copies of Wt, G40R, VKQVDel and K264N *dYARS* transgenes. act5C-Gal4 wk was used to drive a single copy of E195K *dYARS* transgene. Expression of all DI-CMTC *dYARS* mutants, but not *dYARS* K264N, induced significant differences. **b** Percentage difference between Wt *dYARS* (act5C st>2× Wt) and mutant *dYARS* expressing flies (act5C> *dYARS*). *ns*, $P > 0.05$ and *** $P < 0.001$ indicates statistical differences as determined by one-way ANOVA with Bonferroni's Multiple Comparison Test

phenotypes observed when DI-CMTC associated alleles are expressed in the fly reveals a clear distinction between a pathogenic and non-pathogenic *YARS* mutation.

The identification of missense mutations in disease-associated genes poses specific difficulties in interpreting their pathogenic nature. While tracing the segregation of a particular mutation in an extended pedigree can provide strong evidence for pathogenicity, mutations identified in isolated patients or nuclear families are more difficult to interpret. Screening cohorts of control individuals is a necessary further step. However, this is constrained by the fact that as rarer the disorder in question is, as higher the number of control individuals to be tested. With the advent of next generation sequencing technologies, it is expected that the number of sequence variants to be detected will increase enormously, thus requiring a reliable interpretation. This poses important challenges to human geneticists and requires the development of robust model systems and effective functional genomics analyses. Retrieving correct information about the pathogenicity of a missense mutation permits a reliable molecular genetics diagnosis, genetic counseling and further preventive strategies. This is exemplified by the tRNA synthetase genes, where the majority of CMT-causing defects are missense mutations and predicting their relevance to peripheral neuropathy is not straightforward.

Determining the effects aaRSs CMT mutations exert on the enzymatic aminoacylation activity seemed the logic approach to identify putative pathogenic effects leading to disease. However, we have recently quantified the enzymatic activity of all TyrRS mutants and shown that they range from completely inactive (G41R) to fully active (E196K) proteins (Storkebaum et al. 2009). Similar observations were reported for mutations in the *GARS* gene, causing axonal forms of CMT. At least three GlyRS mutations are fully active for aminoacylation (Nangle et al. 2007) and in addition, one P278KY *GARS* allele causes peripheral neuropathy despite normal enzymatic activity (Seburn et al. 2006). Taken together, these findings indicate that enzymatic function can be differently affected by CMT-causing mutations and therefore loss of aminoacylation activity is not a prerequisite for CMT to occur. Thus, additional functional studies are obligatory to evaluate the neuron-specific effects resulting from novel aaRSs mutations. We recently generated a *Drosophila* DI-CMTC model and demonstrated that despite the differential impact of DI-CMTC mutants on TyrRS aminoacylation activity, all mutants cell-autonomously induced signs of dysfunction when expressed in the fly nervous system (Storkebaum et al. 2009). Therefore, we anticipate that independently from its effect on TyrRS aminoacylation activity, toxic features of novel YARS variants would be pinpointed in our assays. In this study, we successfully demonstrated that the K265N variant does not induce functional deficits. Furthermore, in vivo exclusion of the K265N substitution as a disease-causing mutation in *Drosophila* was in accordance with our human genetic findings. This consistency underscores the robustness of our DI-CMTC model, establishing *Drosophila* as a reliable functional platform to study the effect of tRNA synthetases sequence variants in the context of a living organism.

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Conflict of interest None.

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