SUPPORTING INFORMATIONS OF:

Bi-allelic mutations in *HARS1* severely impair histidyl-tRNA synthetase expression and enzymatic activity causing a novel multi-system ataxic syndrome

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Materials and methods:

Quantitative real-time PCR:

RNA was obtained from cultured skin fibroblasts using High Pure RNA Isolation Kit (Roche, Basel, Switzerland), 1µg of total RNA was retrotranscripted using Transcriptor First Strand cDNA Synthesis Kit (Roche) following manufacturer's instructions. qPCR was performed using qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystems, Wayne, Pennsylvania) and 7500 Fast Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, Massachusetts). The ribosomal protein 18S was used as internal control. Gene expression levels were referred

to the internal control, the relative quantification was carried out by means of the ΔCt method and the results were expressed as relative mRNA expression. Primers are available upon request.

Western blotting:

About 30-50 µg of proteins were loaded in Novex™ 8-16% Tris-Glycine Mini Gels, WedgeWell™ format (Thermo Fisher Scientific) in reducing conditions. Blottings were performed using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories Inc, Berkeley, California, U.S.) and Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit (Bio-Rad Laboratories Inc). Membranes were blocked with TBS/0.1% Tween20 (TTBS) containing 5% non-fat dry milk. Primary antibodies were incubated overnight at 4°C in TTBS with 2.5% non-fat dry milk, whereas secondary antibody peroxidase-conjugated anti-mouse and anti-rabbit were used (Jackson ImmunoResearch, laboratories Inc.) for 1 hour at room temperature in the same buffer used for the primary antibodies (2.5% non-fat dry milk in TTBS). Bands were revealed using ChemiDoc™ Imaging System (Bio-Rad Laboratories Inc). ImageJ software (https://imagej.nih.gov/ij/) was used for densitometry analysis. Rabbit monoclonal anti-HARS1 antibody ab140640 (Abcam, Cambridge, UK) was used diluted 1:500, and mouse monoclonal Anti-GAPDH antibody ab8245 (Abcam) was used diluted 1:8000.

Yeast complementation assays:

Yeast complementation assays were performed as previously described (Vester et al., 2013; Abbot et al., 2018). The p.Asp206Tyr, p.Val244Cysfs*6, p.Leu305dup, and

p.Ile465Leu *HARS1* variants were modeled in the human wild-type *HARS1* open-reading frame (primers available upon request). Mutagenesis was performed using the Quickchange II XL Site-Directed Mutagenesis Kit, a sequence-validated wild-type *HARS1* pDONR221 construct, and mutation-specific primers. Reactions were transformed into *E. coli*, and plasmid DNA was purified from individual colonies and sequenced to confirm the presence of the mutation and the absence of PCR-induced errors. Sequence-validated constructs for wild-type and mutant *HARS1* were cloned into pYY1 (Chien et al., 2014) using Gateway cloning technology (Invitrogen, Carlsbad, California). The resulting reactions were transformed into *E. coli*, purified, and digested with *Bsr* GI to confirm proper recombination and the presence of an appropriately sized insert. To assess the ability of wild-type and mutant *HARS1* alleles to support cellular growth, a previously validated haploid yeast strain with the endogenous *HTS1* locus deleted and viability maintained via a pRS316 vector bearing wild-type *HTS1* (Vester et al., 2013; Abbot et al., 2018) was transformed with wild-type *HARS1*, mutant *HARS1*, or empty pYY1. Transformed yeast cells were selected for the presence of pYY1 by growth on solid media lacking leucine (pYY1 harbours the *LEU2* gene) and uracil (pRS316 harbours the *URA3* gene). Colonies were grown to saturation in 2 ml -leu-ura liquid medium at 30□C and 275 rpm for 48 hours. A 1 mL aliquot from each 2 ml culture was spun down at 10,000 rpm and re-suspended in 50 uL UltraPure RNase/DNase-free water. Undiluted cultures and dilutions of 1:10 and 1:100 were spotted on 0.1% 5-FOA complete solid medium (Teknova, Hollister, California) to select for cells that spontaneously lost the *URA3*- bearing maintenance vector (Boeke, Lacroute, & Fink, 1984). Yeast viability was assessed by visual

inspection after 5 days of incubation at 30□C. Two colonies per transformation were assayed.

Aminoacylation assay:

Aminoacylation assay was performed as previously described (Zhang et al., 2014). Whole cell extracts were prepared from control or patient-derived fibroblasts after washing twice with cold PBS by lysing cells in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM DTT, 0.5% Triton X-100, and protease inhibitor cocktail (Sigma, St. Louis, Missouri). Protein concentration was measured using standard Bradford assay. Aminoacylation assays were performed at 37 °C in 100 mM HEPES, pH 7.2, 30 mM KCl, 10 mM MgCl2 with 5 μM tRNAHis, 2 mM ATP, 50 μM [14C] histidine, and the reaction was initiated by adding protein extract to achieve a final concentration of 0.3 μg/μL of total protein. At three different time points over a 10-min interval, 5 μL aliquots were spotted onto 3MM Whatman filter papers presoaked with 5% TCA. The dried paper was washed three times with 5% TCA, once with 95% ethanol, and radioactivity was quantitated by liquid scintillation counting. To calculate the specific activity of each sample, aminoacylation rates (pmol aminoacylated tRNA/min) was calculated from linear fits of the progress curve data.

Supplemental figures:

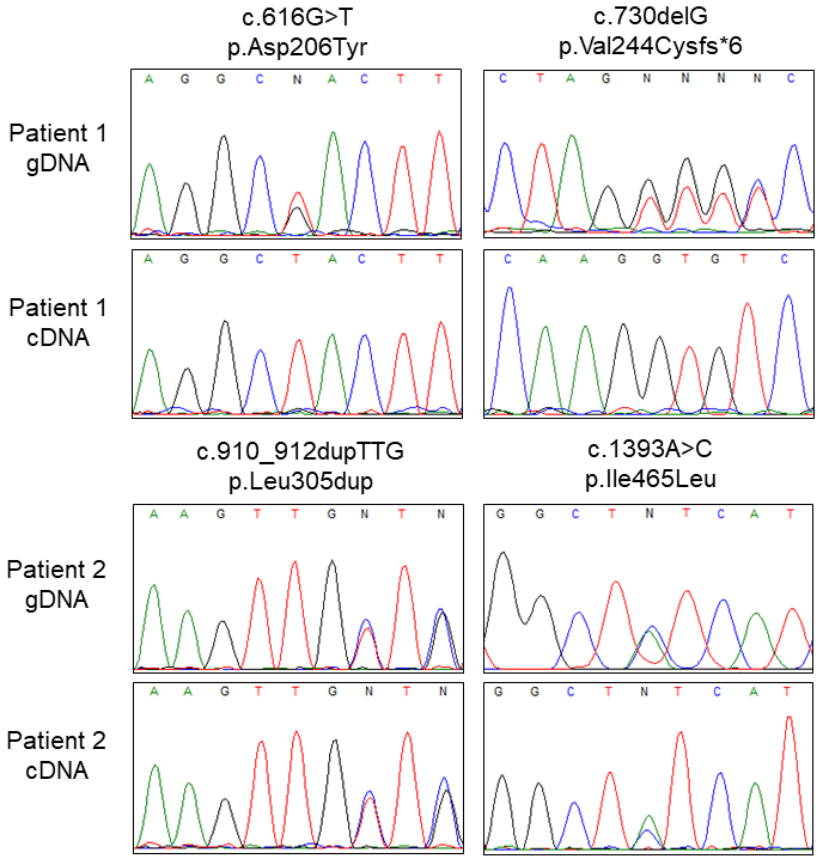

Figure S1. ***HARS1*** **variants confirmations and cDNA mutation analysis.** gDNA electropherograms show that all mutations are heterozygous. cDNA analysis in Patient 1 demonstrates that only mRNA bearing p.Asp206Tyr was expressed, thus suggesting that the mRNA carrying the p.Val244Cysfs*6 was unstable or subjected to nonsense-mediated decay, whereas both mRNA produced were stable in Patient 2.

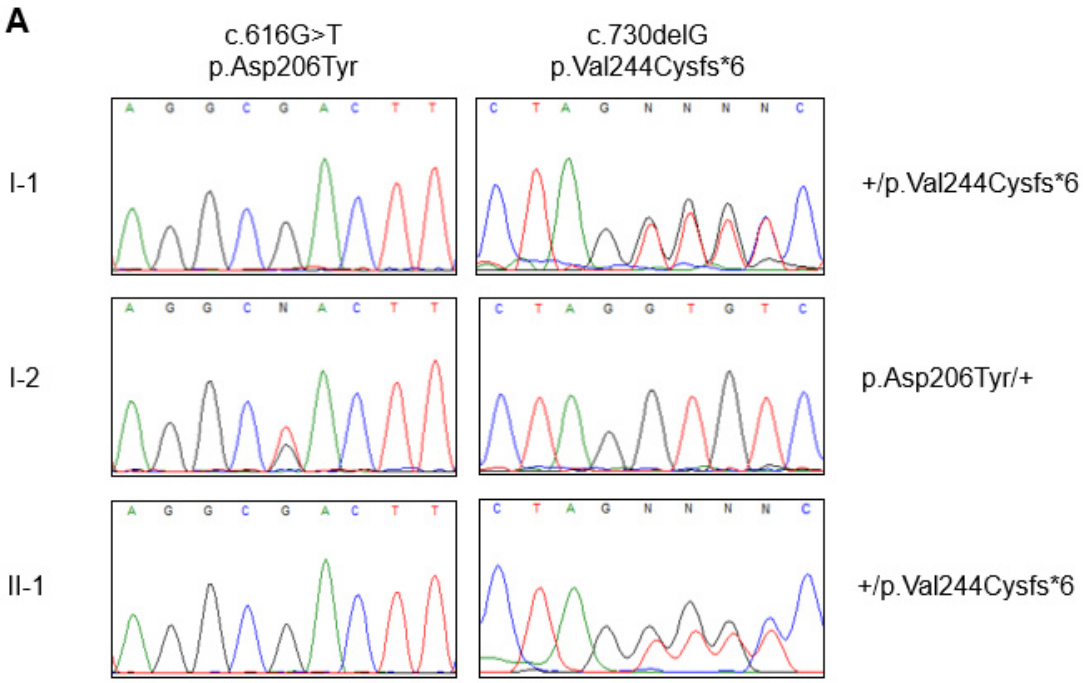

Figure S2. *HARS1* segregation analyses. (A) Patient's 1 family members electropherograms show that both healthy parents (I-1 and I-2) and healthy sister (II-1) harbour only one heterozygous mutation. (B) Patient's 2 healthy mother (I-2) sequences exhibit that she is carrier of one heterozygous mutation, whereas affected sister (II-2) bears the same genotype of the index case.

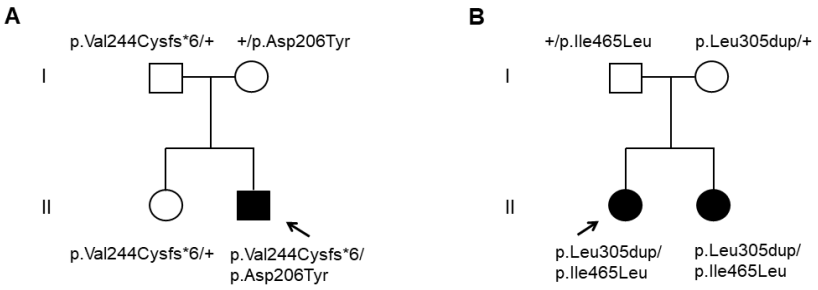

Figure S3. Patient's 1 and patient's 2 pedigrees. (A) The family pedigree of Patient 1 (II-2) shows that all healthy familiars harbored one heterozygous mutation. (B) The family pedigree of Patient 2 (II-1) indicates that the affected sister (II-2, Patient 3) had the same genotype of index case, whereas the healthy mother (I-2) carried only one heterozygous mutation. No biological samples were available for the healthy father (I-1), but segregation analysis suggests that he was carrier of the other heterozygous mutation.

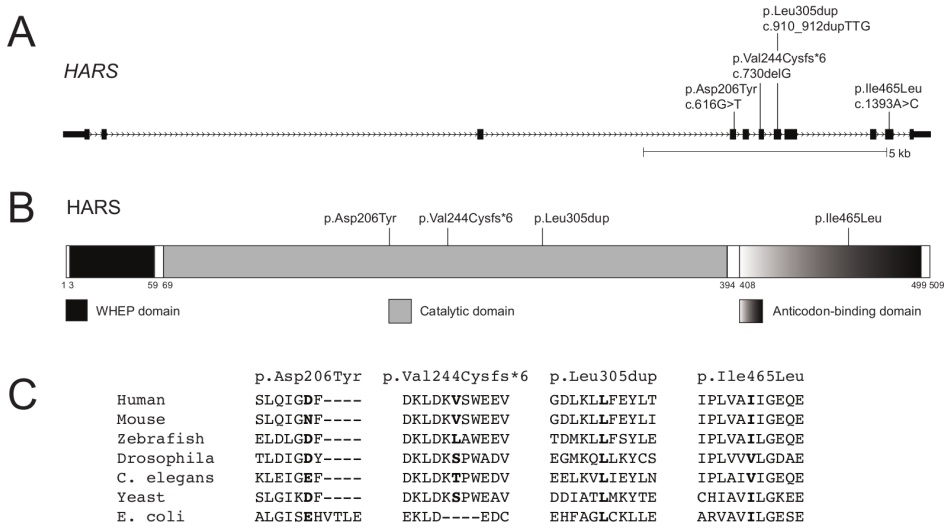

Figure S4. Variants localization and phylogenetic conservation. (A) Representation of *HARS1* gene (NM_002109.6) and position of variants identified. (B) Scheme of HARS1 protein (UniProt: P12081) shows that p.Asp206Tyr, p.Val244Cysfs*6 and Leu305dup are in the catalytic domain, whereas p.Ile465Leu is placed in the anticodon-binding domain. (C) Phylogenetic conservation of variants (indicated in bold) identified in this study.

Supplemental references:

Abbott, J. A., Meyer-Schuman, R., Lupo, V., Feely, S., Mademan, I., Oprescu, S. N., … Francklyn, C. (2018). Substrate interaction defects in histidyl-tRNA synthetase linked to dominant axonal peripheral neuropathy. *Human Mutation*, *39*(3), 415–432. https://doi.org/10.1002/humu.23380

Boeke, J. D., Lacroute, F., & Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Molecular & General Genetics*, *197*(2), 345–346. https://doi.org/10.1007/bf00330984

Chien, C., Chen, Y., Wu, Y., Chang, C., Wang, T., & Wang, C. (2014). Functional Substitution of a Eukaryotic Glycyl-tRNA Synthetase with an Evolutionarily Unrelated Bacterial Cognate Enzyme. *PLoS ONE*, *9*(4), e94659. https://doi.org/10.1371/journal.pone.0094659

Vester, A., Velez-Ruiz, G., McLaughlin, H. M., Nisc Comparative Sequencing Program, Lupski, J. R., Talbot, K., … Antonellis, A. (2013). A Loss-of-Function Variant in the Human Histidyl-tRNA Synthetase (HARS1) Gene is Neurotoxic In Vivo. *Human Mutation*, *34*(1), 191–199. https://doi.org/10.1002/humu.22210

Zhang, X., Ling, J., Barcia, G., Jing, L., Wu, J., Barry, B. J., … Nabbout, R. (2014). Mutations in QARS, encoding glutaminyl-tRNA synthetase, cause progressive microcephaly, cerebral-cerebellar atrophy, and intractable seizures. American Journal of Human *Genetics*, *94*(4), 547–558. https://doi.org/10.1016/j.ajhg.2014.03.003