Biallelic variants in *HPDL* cause pure and complicated hereditary spastic paraplegia

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Abstract

Human 4-hydroxyphenylpyruvate dioxygenase-like (HPDL) is a putative iron-containing non-heme oxygenase of unknown specificity and biological significance. We report 19 families containing 28 individuals with neurologic disease associated with biallelic *HPDL* variants. Phenotypes ranged from juvenile-onset pure hereditary spastic paraplegia (HSP) to infantile-onset spasticity and global developmental delays, sometimes complicated by episodes of neurologic and respiratory decompensation. Variants included *bona fide* pathogenic truncating changes, although most were missense substitutions. Functionality of variants could not be determined directly as HPDL's enzymatic specificity is unknown; however, when *HPDL* missense substitutions were introduced into 4-hydroxyphenyl-pyruvate dioxygenase (HPPD, an HPDL orthologue), they impaired HPPD's ability to convert 4-hydroxyphenyl-pyruvate into homogentisate. Moreover, three additional sets of experiments provided evidence for a role HPDL in the nervous system and further supported its link to neurologic disease: (1) HPDL was expressed in the nervous system and expression increased during neural differentiation. (2) Knockdown of zebrafish *hpdl* led to abnormal motor behavior, replicating aspects of the human disease. (3) HPDL localized to mitochondria, consistent with mitochondrial disease that is often associated with neurologic manifestations. Our findings suggest that biallelic *HPDL* variants cause a syndrome varying from juvenile-onset pure HSP to infantile-onset spastic tetraplegia associated with global developmental delays.

Key Words: Hereditary spastic paraplegia, HSP, autosomal recessive, mitochondrial disorder, HPDL, 4-hydroxyphenylpyruvate dioxygenase-like

Introduction

Hereditary spastic paraplegias (HSP) are a group of inherited neurodegenerative conditions associated with progressive weakness, atrophy, and spasticity as a result of upper motor neuron dysfunction. “Pure” HSPs manifest with isolated lower limb spasticity, while “complicated” HSPs are associated with additional neurologic and non-neurologic features (e.g., upper limb involvement, cerebellar ataxia, intellectual disability, seizures, retinopathy, and liver disease) (Harding, 1983). Remarkable progress has been made in identifying many of the genetic causes of these syndromes; however, a substantial number of subjects without genetic diagnoses suggests the presence of further genetic heterogeneity (Hedera, 1993). Currently, there are over 80 different types of HSPs (Trummer *et al.*, 2018) that can be inherited in an autosomal dominant, recessive, or X-linked pattern and can involve a wide range of molecular and metabolic etiologies (Shribman *et al.*, 2019), that include metabolic dysfunction (e.g., *FA2H*/SPG35; *ALDH18A1*/SPG99), myelin abnormalities (e.g., *PLP1*/SPG2) and organellar dysfunction that can involve the endoplasmic reticulum (e.g., *ATL1*/SPG3; *REEP1*/SPG31), lysosomes (e.g., *SPG11*/SPG11; *ZFYVE26*/SPG15), as well as mitochondria (e.g., *SPG7*/SPG7; *FARS2*/SPG77). Spastic paraplegias due to mitochondrial dysfunction can present with a range of pure to complicated phenotypes, oftentimes involving the same gene (Casari *et al.*, 1998, Pierson *et al.*, 2011, Sahai *et al.*, 2018).

Here we report a series of subjects affected with autosomal recessive pure or complicated HSP associated with biallelic variants in the gene encoding a putative iron-containing oxygenase, HPDL. Furthermore, HPDL was expressed within the nervous system, was localized to mitochondria and played a role in motor function in zebrafish.

Materials and methods

Participants

In total, 28 affected subjects from 19 unrelated families with autosomal recessive HSP were included. All participants and their families consented to participation in the study and research protocols were approved by Institutional Review Boards at LMU Munich, University of Tübingen, University College London, and Cedars-Sinai Medical Center.

Methods

A comprehensive description of the methods used in this study is provided in the **Supplementary material**.

Data availability

Anonymized data will be shared by request from a qualified academic investigator for the sole purpose of replicating procedures and results presented in the article and as long as data transfer is in agreement with relevant legislation and decisions by Institutional Review Boards.

Results

Identification of *HPDL* as a new HSP gene

We performed genome-wide linkage analysis and exome sequencing (ES) on two unrelated consanguineous families with genetically undiagnosed juvenile-onset pure HSP. Linkage analysis using high-density single nucleotide polymorphism (SNP) arrays resulted in a single linkage peak on chromosome 1p34.1 in family B (logarithm-of-the-odds (LOD) score 3.56) that overlapped with one of four chromosomal regions that reached the maximum theoretical LOD score in family A (LOD score 1.81). ES on one affected individual from each family disclosed several rare (gnomAD minor allele frequencies (MAF) <0.01 (Lek *et al.*, 2016)) non-synonymous homozygous variants that were located in regions of interest as defined by linkage analysis; however, *HPDL* was the only gene residing in the linked region that carried different rare homozygous missense variants in both subjects (**Fig. 1A**, **Supplementary Table 1**). Subsequent data exchange with other research groups using informal enquiries and genetic collaboration platforms such as GeneMatcher (Sobreira *et al.*, 2015) and GENESIS (Gonzalez *et al.*, 2015) resulted in the identification of 17 additional index cases with spastic para- or tetraparesis associated with rare non-synonymous biallelic *HPDL* variants. In 16 subjects, previous analysis of ES data for all disease genes annotated in OMIM had failed to identify variants likely or possibly related to their phenotypes. In one subject with pure HSP (family ZC), *HPDL* was screened by Sanger sequencing after multi-gene-panel testing had not revealed convincing variants in > 300 genes associated with HSP, motor neuron diseases, peripheral neuropathies and spinocerebellar ataxias. Available clinical data and family information are shown in **Table 1** and **Supplementary Fig. 1**. Whenever tested, *HPDL* variants segregated in agreement with an autosomal recessive pattern of transmission (**Supplementary Fig. 1**).

Human *HPDL* is an 1.8-kb single-exon gene on chromosome 1p34.1, which is expressed as one transcript (RefSeq NM_032756.3). *HPDL* encodes a 371 amino acid protein named 4-hydroxyphenylpyruvate dioxygenase-like protein (HPDL; UniProt Q96IR7). This name is the result of its homology with the previously identified enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD). We used crystallographic structures of HPPDs from several species (available through PDB (Berman *et al.*, 2000)) to predict that the structure of HPDL may contain two open β-barrels. By further analogy to HPPD, the carboxy-terminal domain would possess the putative catalytic center, while the amino-terminal domain does not seem to have a direct catalytic function, but may play a role in dimer formation. In our study, we observed 25 distinct *HPDL* variants in our 19 families. Most variants were unique within single families, while three variants were shared between two or three kindreds (p.Gly50Asp, p.Ala86fs and p.Ile266Thr). Variants were distributed throughout the gene with most appearing within the putative carboxy-terminal catalytic domain (**Fig. 1B**). Variants were either loss-of-function (start-loss, frameshift or stop-gain) or missense substitutions involving residues that are conserved across several species (**Supplementary Fig. 2**, **Supplementary Table 2**). Further supporting the likely pathogenicity of these variants were the absence or extremely low MAF in public databases covering human genome variation (**Supplementary Table 3**) and the results from *in silico* algorithms for missense changes (**Supplementary Table 4**).

Phenotypic spectrum of subjects with biallelic *HPDL* variants

Specific phenotypic information was available for 28 affected individuals (**Table 1**). Spasticity was a consistent feature of all subjects; some individuals presented with pure spastic paraplegia, while others had a more complicated presentation (**Fig. 1C**, **Table 1**). Several subjects (individuals A2, F1, T1, T2, T3, ZF1, ZG1, CS1, CS3; mild phenotype) had normal motor development, but developed spastic paraparesis during late childhood or

teenage years. Some of these subjects also had upper limb involvement (3/9) that usually manifested with pyramidal tract signs. Another group of subjects (individuals A1, B1, B2, B3, C1, D1, ZA1, ZC1, ZE1; intermediate phenotype) also achieved independent walking (though delayed in 2/9), but had disease onset in early or middle childhood. Several subjects with this intermediate phenotype presented with additional manifestations including upper limb involvement (7/9), ataxia (6/9), and oculomotor abnormalities (6/9). A third group of subjects (individuals E1, ZB1, ZB2, ZD1, ZD2, ZH1, ZH2, ZH3, CS2, CS4; severe phenotype) had a much more serious condition consisting of global developmental delays and congenital spasticity, which affected all four limbs in all but one subject (individual E1). In this group, the disease was associated with variable combinations of additional manifestations including seizures (8/10) and encephalopathic episodes of acute neurological and respiratory decompensation (5/10). Of note, one subject with intermediate disease severity (individual C1) had a past medical history of a single episode of acute neurological deterioration with brain magnetic resonance imaging (MRI) indicating bilateral T2 hyperintensities of the inferior olive. This event had been diagnosed as acute disseminated encephalomyelitis (ADEM) and treated with corticosteroids leading to an incomplete clinical recovery.

Neuroimaging was performed in 23 subjects (**Fig. 1D**, **Table 1**, **Supplementary Fig. 3**). Brain MRI was normal in subjects within the mild phenotype cohort (6/6). Corpus callosal abnormalities were distributed across both the intermediate (4/8) and severe (7/9) phenotype cohorts; otherwise, the observed radiological features largely segregated with clinical severity. Cerebellar atrophy (5/8) and bilateral inferior olival hyperintensities on T2-weighted MRI images (5/8) were exclusively seen with intermediate presentations. Alternatively, generalized white matter changes (9/9), ventriculomegaly and global cerebral atrophy (3/9) as well as gyral abnormalities (3/9) were restricted to children with severe presentations. In one severely affected subject (individual CS4), brain MRI was indicative of Leigh syndrome and magnetic resonance spectroscopy (MRS) showed a high lactate peak in several locations.

Spinal MRI studies were normal in ten individuals for whom sufficient information could be retrieved from medical records (five subjects with mild, four with intermediate and one with severe disease). Overall, clinical and neuroimaging results suggested that individuals with HPDL-associated spastic paraplegia had a range of phenotypes consisting of severely-affected subjects with congenital diffuse cortical disease to more mildly-affected subjects with later-onset upper motor neuron dysfunction with later-onset.

Laboratory work-up for inborn errors of metabolism was either normal or yielded non-specific results that were not diagnostic (**Table 1**). In particular, lactate levels in blood (9/9) and cerebrospinal fluid (CSF; 2/2) samples as well as tyrosine levels in blood samples (12/12) were always within normal ranges. Urine organic acids were also normal or non-specifically altered whenever measured (10/10). Tyrosine derivatives 4-hydroxyphenylpyruvate (4-HPP), 4-hydroxyphenyllactate (4-HPL) and 4-hydroxyphenylacetate (4-HPA) were usually not explicitly mentioned in laboratory reports; however, detailed analysis in two subjects revealed normal values for all three metabolites except for borderline high 4-HPA in one subject (individual CS1).

Detection of *HPDL* mRNA and the encoded protein in the nervous system

Human RNA-seq data (GTEx Consortium, 2013) indicated that *HPDL* mRNA levels were generally low, with the highest expression observed within the nervous system (**Fig. 2A**). Using a specific anti-HPDL antibody (**Supplementary Fig. 4**), we confirmed presence of HPDL in adult mouse neural tissues including cerebellum and cerebral cortex (**Fig. 2B**). At the cellular level, neurons (Purkyně cells) in adult rat cerebellum had positive signals for HPDL (**Fig. 2B**). Analysis of *Hpdl* mRNA levels in murine neural tissues during late embryonic and early postnatal stages showed that expression was upregulated during nervous system maturation (**Fig. 2C**). In agreement with this observation, we noted a significant increase of HPDL levels during *in vitro* neuronal differentiation of murine neural cell line

NeuroA2 (**Fig. 2D**). Altogether, the spatiotemporal expression profile of HPDL was consistent with a continual requirement in the nervous system and its assumed etiological role in a neurological condition when mutated.

Localization of HPDL to the outer mitochondrial membrane

Several *in silico* algorithms predicted HPDL possessed an amino-terminal mitochondrial targeting signal (**Supplementary Table 5**). Separation of cellular compartments from HeLa cells, which had readily detectable levels of HPDL (**Supplementary Fig. 5**), showed endogenous HPDL resided in the mitochondrial fraction (**Fig. 2E**). Further confirmation was revealed with cytochemical colocalization of the HPDL signal with MitoTracker, a mitochondrial marker (**Fig. 2E**), while no major overlap was seen for HPDL and PDI or giantin (markers for the endoplasmic reticulum and Golgi apparatus, respectively, **Supplementary Fig. 6**). Similarly, forced expression of myc-tagged wild-type HPDL showed an identical pattern of localization; however, removal of the predicted mitochondrial targeting signal from myc-tagged HPDL resulted in a diffuse cytoplasmic signal (**Fig. 2E**). Subfractionation and proteinase K digestion of isolated intact mitochondria from HeLa cells indicated HPDL's preferential association with the outer mitochondrial membrane (**Fig. 2F**), consistent with *in silico* predictions of the amino-terminal portion of the protein possessing a single membrane-spanning domain (**Supplementary Table 6**).

Functional effects of decreased HPDL were not overt, as siRNA-mediated knockdown of HPDL did not alter levels of mitochondrial respiratory chain complex proteins (**Supplementary Fig. 7**) or have a detectable effect on cellular oxygen consumption (**Supplementary Fig. 8**) in HeLa cells. We also did not find evidence for a role of HPDL as a critical regulator of mitochondrial dynamics (fission and fusion): mitochondrial morphology in cells with decreased HPDL levels was largely indistinguishable from controls (**Supplementary Fig. 9**). Similarly, increasing HPDL levels by transfection of HeLa cells

with a plasmid expressing wild-type myc-tagged HPDL did not alter mitochondrial shape or numbers compared to control cells (**Supplementary Fig. 9**).

Interference of HPDL missense mutants with requirements for catalytic activity

Transfection studies with expression vectors for wild type and mutant HPDL proteins did not show any major effects of the variants on protein levels or localizations (**Supplementary Fig. 10**). The functionality of variants could not be determined directly as HPDL's enzymatic specificity is unknown; however, sequence and structural similarities between HPDL and its functionally well-characterized orthologue HPPD imply that these enzymes metabolize the same class of substrates (α-keto acids) and share a conserved reaction mechanism (Moran, 2005), even though they are probably not entirely functionally interchangeable (**Supplementary Fig. 11**). We thus adapted an assay that monitors HPPD's ability to convert 4-HPP into homogentisate via the detection of a brownish pigment (formed by further metabolism of homogentisate (Denoya *et al.*, 1994)) and tested the effect HPDL missense variants had on pigment production when placed in homologous sites of the HPPD protein. Mutants corresponding to HPDL missense variants (Leu217Pro, Thr263Arg, Ile266Thr, Tyr287His) affecting the carboxy-terminal domain all had drastically reduced pigment formation (**Fig. 3A**). According to the known crystal structure of human HPPD (Protein Data Bank (PDB) accession number 3ISQ), most of these residues were in proximity to the predicted catalytic center (**Fig. 3B**), consistent with the observed decrease in HPPD enzymatic activity. One variant (Gly140Arg) located in the amino-terminal domain, which most likely has no direct catalytic function, also affected the HPPD reaction, potentially through alteration of the overall protein structure or impaired dimerization. Another amino-terminal variant, Gly50Asp, had only a modest though still significant effect on HPPD activity (**Fig. 3A**).

Abnormal locomotor behavior in HPDL-deficient zebrafish

The zebrafish HPDL orthologue *hpdl* (NM_001109708) displays 40% sequence identity and 56% sequence similarity to the human protein (**Supplementary Fig. 12**). In a reverse genetic approach, we designed two separate morpholinos in order to silence *hpdl* in zebrafish larvae (**Supplementary Fig. 13**). Gross morphology of morphant zebrafish appeared normal with no detectable difference to control larvae (**Supplementary Fig. 14**). At five days post-fertilization, we compared the motor performance of *hpdl*-morphants and control fish using an automatized system that generates mechanical stimuli (vibration) and records the zebrafish's subsequent movements in a reproducible way (**Fig. 3C**). We found that *hpdl*-morphants responded less frequently to stimulation **(Fig. 3D)** and moved a shorter distance when responding (**Fig. 3E**). Alternatively, for larvae that responded to stimuli, the time from stimulus to maximum motor activity was similar in all three experimental groups (**Fig. 3F**), which implies an intact and functional sensory system that allowed appropriate detection of stimuli. Altogether, these results suggested that *hpdl*-morphant zebrafish had a primary motor impairment in general agreement with the findings in humans with biallelic *HPDL* variants.

Discussion

We report the genetic and clinical features of *HPDL*-associated spastic paraplegia. We have identified 28 individuals with spasticity being their most consistent clinical feature as a result of biallelic *HPDL* variants. This disorder has a range of phenotypic severity from severe early-infantile encephalopathy to milder adolescent-onset pure spastic paraparesis. Further supporting the role of HPDL in our subjects' neurological phenotypes was that HPDL was predominantly (though not exclusively) expressed in the nervous system and was upregulated in an *in vitro* model of neuronal differentiation. Moreover, silencing the zebrafish orthologue, *hpdl*, resulted in a motor phenotype, replicating aspects of the human disease. Database searches also revealed comparably high *HPDL* mRNA levels in colon and intestine; however, nothing is known about a function of HPDL in these organs and individuals with biallelic *HPDL* variants had no clinical manifestations related to the digestive system.

Identified disease-related *HPDL* variants included *bona fide* pathogenic truncating changes as well as missense variants. Protein levels and subcellular localizations of missense mutants were comparable to wild-type HPDL, suggesting that impaired enzyme function could be the relevant pathomechanism rather than premature degradation or mislocalization of the protein. This would be consistent with the finding that missense variants clustered within the predicted catalytic domain and often altered residues that were conserved or had similar chemical characteristics to the corresponding residues within the functionally well-characterized catalytic domain of HPDL's orthologue, HPPD (Moran, 2005). As HPDL's substrate specificity is unknown, effects of missense variants on enzyme function could not be examined directly; however, expression of HPPD possessing disease-associated substitutions resulted in significant impairment of enzyme activity in an established colorimetric assay in recombinant bacteria.

Human *HPPD* is related to two distinct human diseases, autosomal dominant hawkinsinuria (Tomoeda *et al.*, 2000) and autosomal recessive tyrosinemia type 3 (Endo *et al.*, 1983). Tyrosinemia type 3 is associated with variable and generally mild neurologic symptoms (Ellaway *et al.*, 2001), accumulation of tyrosine in bodily fluids and massive excretion of tyrosine derivatives 4-HPP, 4-HPL, and 4-HPA in the urine (Ruetschi *et al.*, 2000). Interestingly, subjects with biallelic *HPDL* variants had normal tyrosine levels in bodily fluids (including CSF) and normal levels of urine organic acids except for one subject (borderline high 4-HPA and normal 4-HPP and 4-HPL). It may thus be that HPDL does not catalyze the same reaction as HPPD, but may have oxygenase activity for another molecule, most likely another α-keto acid. The observation that substitution of wild-type HPDL for HPPD abolished pigment formation in the bacterial biosassay seems consistent with this hypothesis; however, interpretation of this finding requires some caution: Eukaryotic HPDL might be unable do degrade 4-HPP in bacteria due to technical constraints (perhaps as a result of impaired oligomerization or lack of specific cofactors in prokaryotes) but may display this activity under more physiological conditions.

Endogenous and overexpressed HPDL was localized at the outer mitochondrial membrane and HPDL-associated HSP shared clinical features with other known mitochondrial syndromes in terms of neurological issues, broad phenotypic spectrum and intermittent decompensation of chronic disease (Gorman *et al.*, 2016). Moreover, neuroimaging revealed abnormalities found with several other mitochondrial disorders including bilateral symmetric hyperintensities of the inferior olive (Bindu *et al.*, 2014) in five subjects as well as Leigh syndrome associated with elevated lactate on MRS in one subject. These observations prompted us to investigate a potential role for HPDL as a regulator of the properties and functions of mitochondria. Of note, we did not observe any altered bioenergetics or dynamics in HPDL-depleted cells, which suggested that either our assay techniques and non-neuronal cell models were not suitable for detecting such alterations or

that HPDL may regulate other structural or functional properties of mitochondria (Eisner *et al.*, 2018, Koenig, 2008, Spinelli & Haigis, 2018, Thorburn *et al.*, 2004). We noted that truncating *HPDL* variants tended to be more frequent in subjects with severe phenotypes (50% of chromosomes) compared to subjects with intermediate (28%) or mild disease (11%), which may suggest that overall residual protein activity may affect phenotypic severity similar to other syndromes related to abnormal nuclear-encoded mitochondrial proteins (e.g., *SARS2*; *FARS2*) (Linnankivi *et al.*, 2016, Sahai *et al.*, 2018).

In summary, our findings are consistent with the conclusion that biallelic *HPDL* variants are a previously unrecognized cause of neurologic disease and provide a reasonable basis for initiating in-depth analysis of the underlying disease mechanisms.

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Conflicts of interest statement

CBee is an employee of Centogene AG, Rostock, Germany, CBer is an employee of Limbach Genetics, Mainz, Germany and DG is an employee of genetikum, Neu-Ulm, Germany. Centogene AG, Limbach Genetics and genetikum are private companies which generate revenue from clinical genetic testing. The remaining authors declare no conflict of interest related to the content of this manuscript.

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Figure legends

Figure 1 Biallelic *HPDL* variants and associated phenotypes. **(A)** Genome-wide linkage analysis and exome sequencing (ES) in two consanguineous HSP families. SNP-based linkage analysis pinpointed four chromosomal regions where the maximum theoretical LOD score was obtained in family A (LOD 1.81) and a single linkage interval reaching genome-wide significance in family B (LOD 3.56). Blue circles indicate individuals included in linkage studies. ES on one subject from each family identified several rare non-synonymous homozygous variants. Variants located in regions of interest as defined by linkage analysis are printed in blue and variants outside these regions are printed in gray. *HPDL* was the only gene residing in the linked region on chromosome 1 that harbored rare homozygous missense variants in both families. Filled blue circles indicate individuals whose DNA was analyzed by ES. **(B)** Schematic representation of the human HPDL protein and distribution of variants. By analogy to the crystallographic structures of HPPD, the structure of HPDL is predicted to contain two open β-barrels. The carboxy-terminal domain (gray) would possess the putative catalytic center, while the amino-terminal domain (yellow) may not have a direct catalytic function, but may play a role in dimer formation. Amino acid numbering is shown along the bottom. Asterisks mark the position of residues predicted to coordinate an iron ion to form the active site (similar to HPPDs). **(C)** Variable severity of clinical presentation of individuals with HPDL variants. Subject A1 is able to walk without support, but with scissoring gait (age 16 years). Subject ZE1 can only ambulate with a walker (age 15 years). Subject ZB2 needs support to stand with legs crossed at age 6 years. Subject ZB1 cannot sit independently at age 2.5 years. **(D)** Brain MRI findings. Subject T1 (age 17 years) had mild juvenile-onset pure HSP and normal neuroimaging. Subjects ZA1 (age 15 years) and D1 (age 21 years) had earlier-onset, intermediate disease associated with cerebellar atrophy (red arrowheads, individual D1) and T2 hyperintensities (blue arrowheads) in the medulla oblongata

(individuals ZA1 and D1) and middle cerebellar peduncle (individual ZA1). Subjects ZD1 (age 5 years) and ZB1 (age 19 months) had severe disease associated with a hypoplastic corpus callosum (yellow arrowheads, individuals ZD1 and ZB1), generalized reduction of white matter volume (individuals ZD1 and ZB1) as well as ventriculomegaly, global cerebral atrophy, and a simplified gyral pattern (individual ZB1).

Figure 2 Expression and subcellular localization of HPDL. **(A)** Gene expression for *HPDL* across 24 human tissues. Expression values are shown as transcripts per million (TPM). Box plots represent median and 25th and 75th percentiles. Data were retrieved from the GTEx web resource (http://www.gtexportal.org/home/). The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data presented in this manuscript were obtained from the GTEx Portal on 20/06/2019. **(B)** Detection of the HPDL protein in young adult mice (P56) and rats (P56). Top panel: HPDL levels in mouse neural and non-neural tissues were analyzed by immunoblotting using an anti-HPDL antibody. Levels of the housekeeping protein GAPDH were determined to ensure equal loading of samples. Bottom panel: Immunofluorescence microscopy with an anti-HPD antibody demonstrated presence of HPDL in rat cerebellar neurons (Purkyně cells). Calbindin was used as a marker to visualize Purkyně cells. Merge represents the overlay of calbindin and HPDL signals. Sscale bar = 100 µm. **(C)** *Hpdl* expression during development of the mouse nervous system. *Hpdl* mRNA levels in embryonic and postnatal stages were determined by quantitative RT-PCR and normalized to *Actb* expression. Bars correspond to means of three biological and technical replicates and error bars represent standard deviations. **(D)** HPDL expression in an *in vitro* model of neural differentiation. HPDL protein levels in undifferentiated (control) and retinoic acid-differentiated (RA + serum removal) Neuro2a murine neuroblastoma cells were analyzed by immunoblotting. Bars represent the mean of

four independent experiments and error bars indicate standard deviations. The p value was calculated by Student's unpaired two-tailed t-test. Changes of cell morphology were recorded to document successful differentiation of cells. Scale bars = 50 µm. **(E)** Association of HPDL with mitochondria. Fractionation of HeLa cells using the Qproteome Mitochondria Isolation Kit (Qiagen) showed co-isolation of HPDL with mitochondrial marker Cox IV (upper panel). ER = endoplasmic reticulum, PM = plasma membrane. Immunofluorescence microscopy of HeLa cells showed co-localization of endogenous HPDL with the mitochondrial stain MitoTracker Red (top panel, scale bar = 10 µm). Myc-tagged HPDL showed an identical pattern of expression (middle panel, scale bar = 10 µm), while expression of an artificial myc-tagged mutant lacking the mitochondrial targeting signal (Δ-M-HPDL-myc) displayed a diffuse cytoplasmic signal (bottom panel, scale bar = 10 µm). **(F)** Submitochondrial localization of HPDL. Upon sub-fractionation of HeLa mitochondria, HPDL was found within the fraction containing the outer membrane (OM) and intermembrane space (IMS), but was not detectable in the fraction consisting of inner membrane (IM) and the mitochondrial matrix. Following proteinase K digestion of intact HeLa mitochondria, signals for HPDL and OM protein Tom20 but not for IMS protein cytochrome C (Cyt C) were lost. Using a high-resolution gel and short blotting time, a low molecular weight band was detected by the anti-HPDL antibody after proteinase K treatment (left panel), possibly corresponding to the region which is N-terminal of a predicted transmembrane domain and projects into the IMS.

Figure 3 Functional analysis of HPDL. **(A)** Enzymatic activity of recombinant *E. coli* expressing HPDL missense variants on a HPPD backbone. Catalytic activity of HPPD species was determined by formation of a brown pigment in growth medium (two upper panels). The variants tested included artificial variants known to render HPPD functionless (Gln375Asn, Arg378Lys (Lin *et al.*, 2013)) or affecting amino acid residues invariant in HPDL and HPPD amino-terminal (HPDL-Thr131 = HPDL-Thr145) and carboxy-terminal domains (HPDL-

Pro284 = HPPD-Pro292) as well as six amino acid changes corresponding to disease-related HPDL variants (HPDL-Gly50 = HPPD-Gly65, HPDL-Gly140 = HPPD-Gly154, HPDL-Leu217 = HPDD-Val229, HPDL-Thr263 = HPPD-Thr271, HPDL-Ile266 = HPPD-Ile274, and HPDL-Tyr287 = HPPD-Tyr295). Bars represent the mean of three independent experiments and error bars indicate standard deviations. The p values were calculated by one-way ANOVA followed by Tukey's post-hoc correction for multiple comparisons. Expression of HPPD species was confirmed by immunoblotting (lower panel, asterisk: protein purified from bacteria). Inhibitor = sulcotrione. **(B)** Structural model of human HPPD (PDB accession number 3ISQ) with bound 4-HPP. The protein folds into an amino-terminal (yellow) and a carboxy-terminal β-barrel (gray), which are represented as a ribbon backbone trace. Residues that bind to the substrate 4-HPP (green) and coordinate the iron ion within the catalytic center are displayed in ball and stick model format (gray). Residues affected by substitutions that were tested in the enzymatic assay (panel (A)) are drawn as CPK model (magenta). **(C)** Behavioral analysis of *hpdl* zebrafish morphants. MO2 and MO3 were morpholinos targeting zebrafish *hpdl*. The control MO was a random sequence not predicted to target any known gene. A single fish was placed in each well of the plate and movements were recorded automatically. The image shows representative traces (red color) of the entire path the animals swam during the whole experiment (20 min acclimation phase followed by 10 min experimental phase, 1 stimulus/min). **(D)** Average number of zebrafish's positive responses to stimulation. The maximum reachable number of responses was 10 responses to 10 stimuli. **(E)** Average distance zebrafish larvae swam in response to stimulation. Data were only included for measurements where fish responded to stimulation. **(F)** Representation of the dislocation of fish over time. The graph shows the Euclidian distance between the positions of fish in consecutive frames (50 frames /s). In all three experimental groups, maximum motor response is recorded at around 140 ms (time point 0 ms corresponds to the time of stimulation). Data were only included for measurements where fishes responded to

stimulation. Data points in panels (D) to (F) correspond to means from four independent experiments each involving 16 embryos per condition. Error bars represent standard errors of the mean. The p values were calculated by one-way ANOVA.

Table 1 Clinical, brain imaging and laboratory findings in individuals with biallelic *HPDL* variants

Family	A		B			C	D	E	F
Country of origin / Consanguinity	Syria / Yes		Turkey / Yes			Morocco / No	Italy / No	Saudi Arabia / Yes	Turkey / Yes
HPDL cDNA variant(s)	c.149G>A (hom.)		c.418G>A (hom.)			c.518C>A/ c.788C>G	c.797T>C/ c.256del	c.256del (hom.)	c.149G>A (hom.)
HPDL protein variant(s)	p.Gly50Asp (hom.)		p.Gly140Arg (hom.)			p.Ser173Tyr/ p.Thr263Arg	p.Ile266Thr/ p.Ala86fs	p.Ala86fs (hom.)	p.Gly50Asp (hom.)
Individual	A1	A2	B1	B2	B3	C1	D1	E1	F1
Sex / age at diagnosis / age at examination	F / 3y / 16 y	M / 12 y / 14 y	F / toddler age / 36 y	M / toddler age / 30 y	M / 5 y / 6 y	M / 6 y / 36 y	M / 3 y / 21 y	F / 12 m / n.a.	M / 13 y / 17 y
First symptom(s)	Spastic gait	Spastic gait	LL spasticity	LL spasticity	Spastic gait	Spastic gait	Stiffness in LL	n.a.	Gait problems
Disease severity / course	Intermediate / progressive	Mild / slowly progressive	Intermediate / progressive	Intermediate / progressive	Intermediate / n.e.	Intermediate / progressive	Intermediate / progressive	Severe / episodic deteriorations	Mild /slowly progressive
Motor delay / best motor ability reached	No / walking	No / walking	No / walking	Yes / walking	No / walking	No / walking	No / walking	Yes / n.a.	No / walking
Cognitive delay	No	No	No	Yes	No	No	No	Yes	No
Spastic gait	Yes	Yes	Yes	Yes	Yes	n.e. (wheelchair)	n.e. (wheelchair)	n.a.	Yes
UL pyramidal signs / spasticity / weakness	Yes / No / No	Yes / No / No	Yes / Yes / No	Yes / Yes / No	Yes / No / No	No / No / No	Yes / No / No	No / No / No	No / No / No
LL pyramidal signs / spasticity / weakness	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / n.a.	Yes / Yes / n.a.	Yes / Yes / n.a.	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / No	Yes / Yes / Yes
Pseudobulbar signs	No	No	No	No	No	Yes	Yes	No	No
Bladder dysfunction	No	No	Yes	Yes	No	n.a.	No	No	n.a.
Ataxia	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No
Cognitive decline	No	No	Yes	Yes	No	No	No	n.a.	No
Encephalopathic episodes	No	No	No	No	No	Yes (single attack diagnosed as ADEM at age 23 y)	No	Yes	No
Contractures	No	No	Yes	Yes	No	n.a.	Yes	Yes	Yes
Seizures	No	No	Yes	Yes	No	No	No	No	No
Oculomotor abnormalities	No	No	Yes	Yes	No	Yes	Yes	Yes	No
Brain MRI (age at examination)	Cerebellar atrophy (n.a.)	n.a.	Thinning of the CC (n.a.)	Thinning of the CC (n.a.)	n.a.	Dysplastic CC, cerebellar atrophy, T2 hyperintensities in the medulla oblongata (34 y)	Cerebellar atrophy, T2 hyperintensities in the medulla oblongata (21 y)	Agenesis of the CC, abnormal cortical gyration, periventricular leukomalacia (n.a.)	Normal (17 y)
Spinal MRI (age at examination)	n.a.	n.a.	n.a.	n.a.	n.a.	Normal (25 y)	n.d.	n.a.	Normal (17 y)
Muscle RC complex / blood / CSF lactate	n.a. / n.a. / n.a.	n.a. / n.a. / n.a.	n.a. / n.a. / n.a.	n.a. / n.a. / n.a.	n.a. / n.a. / n.a.	n.d. / n.d. / n.d.	Normal / Normal / n.d.	n.a. / Normal / n.d.	n.d. / n.d. / n.a.
Blood tyrosine / urine organic acids	n.a. / n.a.	n.a. / n.a.	n.a. / n.a.	n.a. / n.a.	n.a. / n.a.	n.d. / n.d.	Normal / Normal, including 4-HPP, 4-HPL and 4-HPA	Normal / Normal*	Normal / n.a.

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Table 1 Clinical, brain imaging and laboratory findings in individuals with biallelic *HPDL* variants (continued from previous page)

Family	T			ZA	ZB		ZC	ZD	
Country of origin / Consanguinity	Egypt / Yes			Japan / No	Pakistan / Yes		Czechia / No	China / No	
HPDL cDNA variant(s)	c.149G>A (hom.)			c.493A>C (hom.)	c.3G>C (hom.)		c.816_817del/ c.523_529del	c.995del/c.650T>C	
HPDL protein variant(s)	p.Gly50Asp (hom.)			p.Thr165Pro (hom.)	p.Met1? (hom.)		p.Val273fs/ p.Thr175fs	p.Thr332fs/p.Leu217Pro	
Individual	T1	T2	T3	ZA1	ZB1	ZB2	ZC1	ZD1	ZD2
Sex / age at diagnosis / age at examination	M / 15 y / 17 y	M / 14 y / 16 y	F / 11 y / 13 y	M / 6 y / 15 y	M / 7 m / 19 m	M / 4 m / 6 y	F / 7 y / 12 y	M / 1 m / 5 y	F / 6 m / 6 m (died at age 8 m)
First symptom(s)	Progressive LL weakness	Gait problems, LL stiffness, frequent falls	LL stiffness, frequent falls	Abnormal gait	Lethargy, recurrent vomiting, myo-clonic jerks	Lethargy, myo-clonic jerks	Abnormal gait	Increased muscle tone in LL	Infantile spasms, global develop-mental delay
Disease severity / course	Mild / n.e.	Mild / slowly progressive	Mild / slowly progressive	Intermediate / progressive	Severe / episodic deteriorations	Severe / episodic deteriorations	Intermediate / progressive	Severe / non-progressive	Fatal / episodic deteriorations
Motor delay / best motor ability reached	No / walking	No / walking	No / walking	Yes / walking	Yes / head control	Yes / sitting, crawling	No / walking	Yes / crawling	Yes / n.a.
Cognitive delay	Yes	Yes	No	No	Yes	Yes	No	Yes	Yes
Spastic gait	Yes	Yes	Yes	n.e. (wheelchair)	n.e. (no walking)	n.e. (no walking)	Yes	n.e. (no walking)	n.e. (too young)
UL pyramidal signs / spasticity / weakness	Yes / No / No	Yes / No / No	No / No / No	Yes / No / No	Yes / Yes / Yes	Yes / Yes / Yes	No / No / No	Yes / Yes / Yes	Yes / Yes / n.a.
LL pyramidal signs / spasticity / weakness	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / No	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / n.a.
Pseudobulbar signs	No	n.a.	n.a.	No	No	No	No	Yes	Yes
Bladder dysfunction	No	n.a.	n.a.	No	n.e. (too young)	Yes	No	n.a.	n.e. (too young)
Ataxia	No	n.a.	n.a.	No	No	No	Yes	Yes	Yes
Cognitive decline	No	No	No	No	n.e. (too young)	No	No	No	n.e. (too young)
Encephalopathic episodes	No	No	No	No	Yes	n.a.	No	No	Yes (causing death)
Contractures	No	No	No	Yes	Yes	Yes	Yes	Yes	No
Seizures	No	n.a.	n.a.	No	Yes	Yes	No	No	Yes
Oculomotor abnormalities	No	n.a.	n.a.	No	No	No	Yes	No	No
Brain MRI (age at examination)	Normal (17 y)	n.d.	Normal (13 y)	T2 hyperintensities in the right middle cerebellar peduncle & bilaterally in the medulla oblongata (15 y)	CC agenesis, global cerebral atrophy & ventriculomegaly, simplified gyral pattern, reduced white matter volume (19 m)	CC hypoplasia, global cerebral atrophy, simplified gyral pattern, reduced white matter volume (6 y)	Cerebellar atrophy, symmetric T2 hyperintensities in the medulla oblongata (12 y)	CC hypolplasia, generalized reduction of cerebral white matter volume (5 y)	n.a.
Spinal MRI (age at examination)	Normal (17 y)	n.a.	n.a.	Normal (15 y)	Normal (19 m)	n.d.	Normal (12 y)	n.a.	n.a.
Muscle RC complex / blood / CSF lactate	n.d. / n.d. / n.d.	n.d. / n.d. / n.d.	n.d. / n.d. / n.d.	n.d. / Normal / Normal	n.a. / n.a. / n.a.	n.a. / n.a. / n.a.	n.a. / Normal / Normal	n.a. / n.a. / n.a.	n.a. / n.a. / n.a.
Blood tyrosine / urine organic acids	n.d. / n.d.	n.d. / n.d.	n.d. / n.d.	n.d. / n.d.	Normal / Normal*	Normal / Normal*	Normal / Normal*	Normal / n.a.	n.a. / n.a.

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Table 1 Clinical, brain imaging and laboratory findings in individuals with biallelic *HPDL* variants (continued from previous page)

Family	ZE	ZF	ZG	ZH			CS1	CS2	CS3	CS4
Country of origin / Consanguinity	Iran / Yes	Ireland / No	UK / No	Italy / Yes			USA / No	USA / No	USA / No	USA / No
HPDL cDNA variant(s)	c.679del (hom.)	c.232G>A/ c.835C>T	c.692C>T/ c.529_530del	c.1072T>G (hom.)			c.859T>C/ c.847C>T	c.1013T>C/ c.769_771delinsTC	c.797T>C/ c.503G>A	c.27C>A/ c.569C>T
HPDL protein variant(s)	p.Thr227fs (hom.)	p.Ala78Thr/ p.Gln279*	p.Ala231Val/ p.Leu177fs	p.Trp358Gly (hom.)			p.Tyr287His/ p.Pro283Ser	p.Leu338Pro/ p.Gln257fs	p.Ile266Thr/ p.Cys168Tyr	p.Cys9*/ p.Pro190Leu
Individual	ZE1	ZF1	ZG1	ZH1	ZH2	ZH3	CS1	CS2	CS3	CS4
Sex / age at diagnosis / age at examination	F / 7 y / 15 y	M / 8 y / 8 y	M / 12 y / 12 y	F / infancy / 7 y	M / 3 m / 3 y	M / infancy / 3 y	F / 17 y / 18 y	F / 4 m / 4y	M / 17 y / 18 y	F / 10 m /11 m
First symptom(s)	Abnormal gait	Abnormal gait, stumbling, frequent falls	Walking with bent knees, not lifting his feet	Infantile spasms, hypsarrhythmia	Infantile spasms, hypsarrhythmia	Infantile spasms, hypsarrhythmia	Gait problems, LL stiffness, frequent falls	Global develop-mental delay, infantile spasms, hypsarrhythmia	Gait problems, LL stiffness, frequent falls	Global develop-mental delay, partial seizures, hypothermia
Disease severity / course	Intermediate / progressive	Mild / n.e.	Mild / n.e.	Severe / non-progressive	Severe / non-progressive	Severe / non-progressive	Mild / n.e.	Severe / non-progressive	Mild / n.e.	Severe / episodic deteriorations
Motor delay / best motor ability reached	No / walking	No / walking	No / walking	Yes / n.a.	Yes / n.a.	Yes / n.a.	No / walking	Yes / sitting	No / walking	Yes / head control
Cognitive delay	No	No	No	Yes	Yes	Yes	No	Yes	No	Yes
Spastic gait	Yes	Yes	Yes	n.a.	n.e. (no walking)	n.e. (no walking)	Yes	n.e. (not walking)	Yes	n.e. (not walking)
UL pyramidal signs / spasticity / weakness	Yes / Yes / No	n.a. / No / No	n.a. / No / No	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	No / No / No	Yes / Yes / Yes	No / No / No	Yes / Yes / Yes
LL pyramidal signs / spasticity / weakness	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes	Yes / Yes / Yes
Pseudobulbar signs	No	n.a.	No	Yes	n.a.	Yes	No	No	No	No
Bladder dysfunction	Yes	n.a.	No	n.a.	n.a.	n.a.	No	n.a.	No	n.a.
Ataxia	No	No	No	No	No	No	No	No	No	No
Cognitive decline	No	No	No	No	No	No	No	No	No	No
Encephalopathic episodes	No	No	No	No	No	No	No	No	No	Yes
Contractures	Yes	No	No	No	No	No	No	Yes	No	Yes
Seizures	No	No	No	Yes	Yes	Yes	No	Yes	No	Yes
Oculomotor abnormalities	Yes	No	No	No	No	No	No	Yes	No	n.a.
Brain MRI (age at examination)	Dysplastic CC, cerebellar atrophy, symmetric T2 hyperintensities in the medulla oblongata (8 y)	Normal (8 y)	n.a.	CC hypoplasia, hypomyelination particularly of the corticospinal tract (n.a.)	CC hypoplasia, hypomyelination particularly of the corticospinal tract (n.a.)	CC hypoplasia, hypomyelination particularly of the corticospinal tract (n.a.)	Normal (n.a.)	Mild supratentorial atrophy and hypo-myelination (n.a.)	Normal (n.a.)	Leigh syndrome, bilateral frontal white matter hypo-attenuation, MRS: lactate peak in multiple areas (n.a.)
Spinal MRI (age at examination)	Normal (8 y)	Normal (8 y)	n.a.	n.d.	n.d.	n.d.	Normal (n.a.)	n.d.	Normal (n.a.)	n.d.
Muscle RC complex / blood / CSF lactate	n.a. / Normal / n.a.	n.a. / Normal / n.a.	n.a. / n.a. / n.a.	n.d. / n.a. / n.a.	n.d. / n.a. / n.a.	n.d./ n.a. / n.a.	n.d. / Normal / n.d.	n.a. / Normal / n.d.	n.d. / Normal / n.d.	n.a. / n.a. / n.a.
Blood tyrosine / urine organic acids	n.a. / Normal*	Normal / n.a.	n.a. / n.a.	Normal / Normal*	Normal / Normal*	Normal / Normal*	Normal / mildly increased4-HPA but normal 4-HPP and 4-HPL	n.a. / n.a.	n.d. / n.d.	n.a. / n.a.

(continued on next page)

Table 1 Clinical, brain imaging and laboratory findings in individuals with biallelic *HPDL* variants (continued from previous page)

Legend to **Table 1**: ADEM = acute disseminated encephalomyelitis; CC = corpus callosum; CSF = cerebrospinal fluid; F = female; hom. = homozygous; LL = lower limbs; M = male; m = month(s); MRI = magnetic resonance imaging; MRS = magnetic resonance spectroscopy; n.a. = not available; n.d. = not determined; n.e. = not examinable (e.g., child too young to assess walking ability); RC = respiratory chain; UL = upper limbs; y = year(s). *Reports on urine organic acids profile did not explicitly refer to 4-HPP, 4-HPL and 4-HPA.