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A loss-of-function homozygous mutation in DDX59 implicates a conserved DEAD-box RNA helicase in nervous system development and function

Vincenzo Salpietro1 | Stephanie Efthymiou1 | Andreea Manole1
Bhawana Maurya2 | Sarah Wiethoff1 | Balasubramaniem Ashokkumar1,3
Maria Concetta Cutrupi4 | Valeria Dipasquale4 | Sara Mantì4 | Juan A. Botia1,5
Mina Ryten1 | Jana Vandrovcova1 | Oscar D. Bello6 | Conceicao Bettencourt1,6
Kshitij Mankad7 | Ashim Mukherjee2 | Mousumi Mutsuddi2 | Henry Houlden1

1Department of Molecular Neuroscience, Institute of Neurology, University College London, London, UK
2Department of Molecular and Human Genetics, Banaras Hindu University, Varanasi, India
3Department of Genetic Engineering, School of Biotechnology, Madurai Kamaraj University, Madurai, India
4Department of Paediatrics, University of Messina, Messina, Italy
5Department of Information and Communications Engineering, University of Murcia University of Murcia, Murcia, Spain
6Department of Clinical and Experimental Epilepsy, Institute of Neurology, University College London, London, UK
7Department of Neuroradiology, Great Ormond Street Hospital for Children, London, UK

Correspondence
Mousumi Mutsuddi, Department of Molecular and Human Genetics, Banaras Hindu University, Varanasi 221005 India.
Email: mousumi.mutsuddi@yahoo.com
Henry Houlden, Department of Molecular Neuroscience, UCL Institute of Neurology, London WC1N 3BG, UK.
Email: h.houlden@ucl.ac.uk

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Abstract
We report on a homozygous frameshift deletion in DDX59 (c.185del: p.Phe62fs*13) in a family presenting with orofaciodigital syndrome phenotype associated with a broad neurological involvement characterized by microcephaly, intellectual disability, epilepsy, and white matter signal abnormalities associated with cortical and subcortical ischemic events. DDX59 encodes a DEAD-box RNA helicase and its role in brain function and neurological diseases is unclear. We showed a reduction of mutant cDNA and perturbation of SHH signaling from patient-derived cell lines; furthermore, analysis of human brain gene expression provides evidence that DDX59 is enriched in oligodendrocytes and might act within pathways of leukoencephalopathies-associated genes. We also characterized the neuronal phenotype of the Drosophila model using mutant mahe, the homolog of human DDX59, and showed that mahe loss-of-function mutant embryos exhibit impaired development of peripheral and central nervous system. Taken together, our results support a conserved role of this DEAD-box RNA helicase in neurological function.

KEYWORDS
DEAD-box RNA Helicase, DDX59, mahe, leukoencephalopathy, NOTCH signaling, Sonic Hedgehog signaling

Postaxial polydactyly (PAP) is the occurrence of a supernumerary sixth digit of the hand and/or the feet, and can be observed in the context of several clinically and molecularly heterogeneous genetic disorders (Malik, 2014). The occurrence of PAP and intellectual disability (ID) is often due to mutations in genes involved in ciliogenesis (e.g., BBS2, WDPCP, KIAA0586, TCTN1, TCTN2, MKS1, TMEM67, and CC2D2A) and most of the times these phenotypes are associated with a wide array of multiple, variable (e.g., skeletal,
FIGURE 1  Family tree, Sanger sequencing, and DDX59 mutation analysis. A: Pedigree from the family. B: Electropherograms of one carrier parent (I.1) and one index case (II.1) with the heterozygous and homozygous c.185delT DDX59 variant, respectively. C: Reverse transcription PCR amplifying the mutant cDNA transcript from mRNA extracted from the immortalized lymphoblastoid cell lines of the two affected siblings and a wild-type (age-matched) control (CTRL). D: Analysis of the semiquantitative PCR using the densitometry software ImageJ after normalization relative to a housekeeping gene (GAPDH) and calculation using a relative relationship method. E: Ddx59 protein representative. F: Multiple-sequence alignment showing complete conservation of DEAD-BOX RNA Helicase active domains (PTRELA, TPGR, and DEAD) sequence across DDX59, Mahe and the other Mahe homologs (DDX5, DDX17, and DDX43). G: Patient II.1 at the age of 19 years, not the prominent, thick eyebrows, malocclusion, high-arched palate, and rounded and prominent jaw. H: Patient II.1 at the age of 29 years. I: Patient II.1, note the subtle midline defect. J: Patient II.2 at the age of 13 years, note the distinctive facial features similar to her elder brother. K: Patient II.2 at the age of 24 years. L: Left hand postaxial polydactyly and camptodactyly of Patient II.2 at the age of 13 years. M: Skeletal X-ray of the hands of Patient II.2 at the age of 13 years. N: Brain MRI of Patient II.2, note in the coronal scan the diffuse white matter hyperintensities. O, P, and Q: Magnetic resonance imaging (MRI) of the brain of Patient II.1 at the age of 27 after a stroke-like episode; note in the axial scans the diffuse subcortical infarction in the right hemisphere mainly involving frontoparietal lobes; also note the diffuse white matter hyperintensities.
ophthalmological, hepatic, renal, and genitourinary) abnormalities (Bretu et al., 2017; Castilla, Lugarinho, da Graça Dutra, & Salgado, 1998). However, the combination of autosomal recessive PAP and neurological involvement in the absence of other symptoms is very rare, with few families reported in the literature as Oliver syndrome (OS; MIM# 258200), a rare distinct clinical phenotype with no causative gene yet identified (Oliver, 1940; Stevenson & Wilkes, 1983, Salpietro et al., 2005). Using a whole-exome sequencing (WES) approach we investigated an Italian family previously reported as having OS (Salpietro et al., 2005). The two probands presented a typical orofaciodigital syndrome phenotype with PAP, subtle midline anomalies (i.e., cleft lip) and distinctive facial features. In addition, they also had an heterogeneous neurological involvement that included delay of development milestones, ID, infantile-onset seizures, lower limbs weakness and neuropathy, and adult-onset white matter signal abnormalities associated with episodes of ischemic strokes (Figure 1A and G–Q). There was no history of previous neurological or genetic diseases in the family and the pedigree suggested an autosomal recessive inheritance (Figure 1A). After institutional review board approval of this study and informed consent from the family, we collected blood samples from the patients and their parents, and extracted DNA using standard procedures. To investigate the genetic cause of the disease, WES was performed in both the affected siblings (Figure 1A, II-1 and II-2). Nextera Rapid Capture Enrichment kit (Illumina, San Diego, California, USA) was used according to the manufacturer instructions. Libraries were sequenced on an Illumina HiSeq3000 using a 100-bp paired-end reads protocol. Sequence alignment to the human reference genome (UCSC hg19), and variants calling and annotation was performed as described elsewhere (Mencacci et al., 2016). In total, 83,572,847 (II-1) and 81,527,162 (II-2) unique reads were generated. Only indels and non-synonymous exonic/splicing variants shared by the two probands were kept and further filtered. In accordance with the pedigree and phenotype, priority was given to rare variants [-c1% in public databases, including 1000 Genomes project, NHLBI Exome Variant Server, Complete Genomics 69, and Exome Aggregation Consortium (ExAC v0.2)] that were fitting a recessive model (i.e., homozygous or compound heterozygous), and/or located in genes previously associated with neurological phenotypes or PAP (Deng H et al., 2015; Verma PK & El-Harouni AA., 2015). After applying the above filtering criteria, no plausible shared compound heterozygous variants were identified by WES; there were however three genes carrying rare (likely) damaging variants, according to guidelines for variants interpretation (Richards et al., 2015), which were homozygous in both probands (Supp. Table S1). Two out of these three variants were missense changes not consistent with the phenotype and also identified in additional (non-affected) individuals from our in-house exome database, containing over 4,000 exomes. A homozygous frameshift deletion in DDX59 (NM_001031725.4: c.185del: p.Phe62fs*13) emerged as the most likely explanation for the disease pathogenesis; this is also supported by a more severe impact of the mutation on protein function (truncating vs. missense) and an existing report previously linking DDX59 (MIM# 615464) to a similar (albeit milder) phenotype of Orofaciodigital syndrome type V (OFD5; MIM# 174300) with PAP and ID (Shamseldin et al., 2013). Also, that study shows expression patterns and indicates a likely important role of this gene in midline development and the nervous system (Shamseldin et al., 2013). Segregation analysis performed by traditional Sanger sequencing confirmed the mutation homozygous in the two affected siblings and heterozygous in both their parents. The identified DDX59 homozygous variant (NM_001031725.4: c.185del: p.Phe62fs*13) was submitted to the Leiden Open Variation Database (www.lovd.nl/; variant ID #0000221973). Patient 1 (Figure 1A, II-1) was the first born from healthy parents, non-consanguineous for their account. Family history was unremarkable, except for three prior spontaneous miscarriages. The pregnancy was complicated by intrauterine growth retardation. Delivery at term was normal, with a weight at birth of 2,350 g (<3rd centile), length of 47 cm (3rd centile), and occipital–frontal circumference of 32 cm (5th centile), APGAR scores were 6 and 9 at 1 and 5 min, respectively. He had bilateral postaxial extra-digits on his hands that were surgically removed in his late childhood. He also had bilateral cutaneous syndactyly of fingers 2–5, clinodactyly of the fifth fingers, and fingertip pads. His lower limbs were normal. Since the first months of life, he developed generalized seizures, which were controlled by anticonvulsant drugs. Developmental milestones were delayed and the patient showed cognitive difficulties during childhood, with an I.Q. of 70 (Terman-Merrill scale) measured at the age of 9 years. For these reasons, he has undergone developmental and speech therapies since the age of 3 years. At the age of 17 years, his height was 165 cm (3rd centile), weight was 67 kg (50th centile), and head circumference 53 cm (10th centile). He had distinctive facial features, including prominent thick eyebrows, malocclusion, high-arched palate, and rounded prominent jaw (Figure 1G and H). A cerebral magnetic resonance imaging (MRI) disclosed thinning of the cerebral cortex in front of the ventricular collateral trionge (not shown). Wakefulness electroencephalograms showed diffuse high amplitude slow waves intermingled with sharp waves or spikes. Since early adulthood he started to complain of migraine. As part of his neurological presentation, he also presented lower limbs weakness and some walking difficulties. At the age of 30, after an episode of loss of consciousness associated with generalized seizures, he underwent a follow-up brain MRI scan, which showed diffuse white matter signal abnormalities and multifocal cortical–subcortical infarcts involving both cerebral hemispheres (Figure 1P and Q). Extensive metabolic and genetic investigations, which included array comparative genome hybridization (array-CGH) and panel sequencing for 22 leukoencephalopathies-associated genes, were performed and fully reported as normal. Patient 2 was the younger sister of Patient 1 (Figure 1A, II-2). Pregnancy was uncomplicated, and delivery at term was normal. Her birth weight was 2,850 g (10th centile), length was 47 cm (3rd centile), and occipital–frontal circumference was 33 cm (10th centile). Her APGAR scores were 8 and 9, at 1 and 5 min, respectively. Since 4 months of age she developed generalized tonic–clonic seizures, which were controlled by anticonvulsant drugs. She had PAP of the left hand, with a camptodactylyous extra digit, bilateral clinodactyly of the fifth fingers, cutaneous syndactyly of fingers 2–5, and prominent fingertip pads (Figure 1L and M). PAP was also present on the right foot, with bilateral brachydactyly of toes 3–5. She also had malocclusion, high-arched palate, and thoracic right convex lateral scoliosis. Similarly to her brother, the psychomotor development was delayed, with an I.Q. of 68
(Terman-Merril scale) at the age of 7 years. She also had speech difficulties. At the age of 13 years, her height was 137 cm (3rd centile), weight was 34 kg (10th centile), and occipital–frontal circumference was 50 cm (3rd centile). Extensive laboratory tests, including metabolic studies, karyotype, array-CGH, and FRAXA analyses were normal. A cerebral MRI showed thinning of the cerebral cortex in front of the ventricular collateral trigone (not shown). A follow-up MRI performed at the age of 21 years showed signal abnormalities in the subcortical and deep white matter of both cerebral hemispheres, with more focal cortical–subcortical gliosis in the right frontal lobe (Figure 1N and O). She presented with weakness of the lower limbs and motor nerve conduction studies showed a mild reduction of motor conduction velocities with peroneal amplitude of 2.4 mV, and conduction velocity of 42.9 m/sec [low limits (3rd per age and height): amplitude (mV) 2.6, conduction velocity (m/s) 43], suggesting a mild axonal neuropathy. To investigate the functional impact of the identified truncating mutation in DDX59 we performed a reverse-transcriptase PCR (RT-PCR) using lymphoblastoid cell lines derived from patients and an age-matched control. Semiquantitative PCR (semi-qPCR) was performed in 50-µl reaction volume prepared by combining the cDNA template, gene-specific primers (F 5'-GAGTTCCCGTGTAGCTGT-3' and R 5'-GAGCTTATTCGAGAGAAAAC-3'), nuclease-free water, and SYBR Green Master Mix. The PCR reaction conditions were: one cycle of 94°C for 4 min, followed by 37 cycles of 94°C for 45 sec, 54°C for 45 sec, and 72°C for 50 sec. In semi-qPCR experiments, all measurements were made in triplicate, and GAPDH was used as an endogenous reference gene, with amplification under the same conditions. The PCR products were then loaded in a 1% agarose gel, and densitometry analysis was carried out. According to in silico predictions, the homozygous single base deletion (c.185delT; p.Phe62fs*13) in DDX59 would either lead to an early truncation of the protein or cause nonsense mRNA decay (NMD). The semi-qPCR from patients and an age-matched control's lymphoblastoid cell lines did not show complete NMD, although there was a reduction of the mutant cDNA complete NMD, although there was a reduction of the mutant cDNA. According to in silico predictions, the homozygous single base deletion (c.185delT; p.Phe62fs*13) in DDX59 would either lead to an early truncation of the protein or cause nonsense mRNA decay (NMD). The semi-qPCR from patients and an age-matched control's lymphoblastoid cell lines did not show complete NMD, although there was a reduction of the mutant cDNA complete NMD, although there was a reduction of the mutant cDNA.

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or incomplete ventral cord along with gaps observed in mahe null embryos reflects similar brain malformation phenotype like that of ciliopathy-associated syndromes. Unlike mahe mutants that have iso-
genized genetic background, human patients may have additional vari-
ants in genes which could modify the neurological phenotype associ-
ated with biallelic loss-of-function mutations in human.

Of importance, RNA helicases from the DEAD-box family are found in almost all organisms and have crucial roles in many aspects of RNA metabolism, including transcription, pre-mRNA splicing, translation initiation, RNA transport and decay (Jarmoskaite & Russell, 2014; Rocak S & Linder P, 2004). Despite their importance in cellular processes and involvement in several molecular pathways, only relatively few examples of mutations in DEAD-box RNA helicase genes have been reported so far in association to monogenic human diseases and none of them with biallelic truncating mutations (Jang et al., 2015; Shamseldin et al., 2013). In our family the two patients presented autosomal recessive PAP associated with a complex neurological involve-
ment. They had global neurodevelopmental delay and episodes of gen-
eralized seizures with onset in the first months of life. They both presented small OFC since birth, similarly to the patients recently reported by Faily et al. (2017). Proband II.1 presented since early adulthood diffuse white matter signal abnormalities associated with sub-
cortical ischemic changes, Proband II.2 showed brain MRI features similar to her brother and EMG features of mild peripheral (axonal) neuropathy. Notably, the adult-onset abnormal findings on brain imag-
ing observed in the two patients resemble those reported in CADASIL syndrome, caused by mutations in the NOTCH3 gene, although the white matter involvement in the latter consist in a more confluent leukoencephalopathy pattern, which is associated with episodes of recurrent ischemic strokes often progressing to subcortical dementia (Di Donato et al., 2017). Importantly, our findings further strengthened the association of DDX59 biallelic variants with OFD syndrome characterized by the variable combination of digital/midline abnormalities, distinctive facial features, and ID. However, the homozygous mutations reported by Shamseldin et al. (2013) were missense vari-
ants (c.1100T > G: p.Val367Gly; c.1600G > A: p.Gly534Arg) and the mutation recently described by Faily et al. (2017) affected a stop codon (c.1859G > T: p."620Leuext*22") extending the protein product, whereas the homozygous mutation we identified in the present study lead to an early truncation of the protein, likely explaining the more severe phenotype including the complex heterogeneous neurological involvement. Interestingly, our functional analysis of the Drosophila model clearly showed that mahe mutation reduces lifespan and also displays neuronal defects. Unlike embryos reflects similar brain malformation phenotype like that of sub-
cortical ischemic changes, and also shows shortened lifespan. In conclusion, our findings further strengthen the association of DDX59 biallelic truncating mutations in humans. Further work remains to be done in fully understanding the role of this gene as well as other conserved DEAD-box RNA helicases in brain development and func-
tion, and human neurological diseases.

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**DISCLOSURE STATEMENT**

The authors declare no conflict of interest.

**ORCID**

Vincenzo Salpietro http://orcid.org/0000-0003-0132-7921

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Additional Supporting Information may be found online in the support- ing information tab for this article.

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