Title

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Journal

American Journal of Medical Genetics Part A, 176(12)

ISSN

1552-4825

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Publication Date

2018-12-01

DOI

10.1002/ajmg.a.40493

Peer reviewed

HHS Public Access

Author manuscript

Am J Med Genet A. Author manuscript; available in PMC 2019 December 01.

Published in final edited form as:

Am J Med Genet A. 2018 December ; 176(12): 2623–2629. doi:10.1002/ajmg.a.40493.

De Novo Variant in KIF26B isAssociated with Pontocerebellar Hypoplasia with Infantile Spinal Muscular Atrophy

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Abstract

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Conflicts of Interest:

Julie S. Cohen is a consultant to Invitae. Ali Fatemi has received personal compensation in form of consultant fees from Aevi Genomic Medicine, Vertex Pharmaceuticals, Stealth Biotherapeutics, BluebirdBio, and Calico Labs for services unrelated to the reported topic. Dan W Nowakowski is an employee and shareholder of N Molecular Systems, Inc.

KIF26B is a member of the kinesin superfamily with evolutionarily-conserved functions in controlling aspects of embryogenesis, including the development of the nervous system, though its function is incompletely understood. We describe an infant with progressive microcephaly, pontocerebellar hypoplasia and arthrogryposis secondary to the involvement of anterior horn cells and ventral (motor) nerves. We performed whole exome sequencing on the trio and identified a de novo *KIF26B* missense variant, p.Gly546Ser, in the proband. This variant alters a highly conserved amino acid residue that is part of the P-loop motif and motor-like domain and is deemed pathogenic by several in silico methods. Functional analysis of the variant protein in cultured cells revealed a reduction in the KIF26B protein's ability to promote cell adhesion, a defect that potentially contributes to its pathogenicity. Overall, KIF26B may play a critical role in brain development and, when mutated, cause pontocerebellar hypoplasia with arthrogryposis.

Keywords

pontocerebellar hypoplasia; KIF26B; kinesin; arthrogryposis; microcephaly

INTRODUCTION

Members of the kinesin superfamily (KIFs) are thought to play an important role in axonal transport, and pathogenic variants found in genes encoding certain members of this family have been associated with neurologic disease [Niwa, 2015]. KIF26B (MIM 614026) is one such kinesin whose biological function and relevance to human health are beginning to emerge in recent years. Genetic studies in *C. elegans* showed that the nematode orthologue of *KIF26B*, Vab-8, functions prominently in the developing nervous system to control processes such as neuronal migration, axonal polarity and guidance [Watari-Goshima, et al., 2007; Wolf, et al., 1998]. In the developing mouse embryo, *Kif26b* is found to be highly expressed in the mesenchymal cells of the limb buds, tail bud, nasal processes and branchial arches, and in somite derivatives, with the latter representation attributed to expression in muscle precursors [Marikawa, et al., 2004]. *Kif26b* has also been shown to be important for renal development, as *Kif26b*-null mice exhibit renal agenesis or other renal abnormalities [Uchiyama, et al., 2010; Terabayashi, et al., 2012]. More recently, a role for KIF26B in mammalian brain development has been proposed [Heinrich, et al., 2012]. Human *KIF26B* is located on chromosome one (1q44) and deletions encompassing this region have been shown to cause a developmental delay and intellectual disability syndrome with variable abnormalities of the corpus callosum and other brain malformations [Caliebe, et al., 2010; Raun, et al., 2017]. In mice, mRNA encoded by the closely-related gene *KIF26A* was recently reported to be transported by the syndromic autism protein FMPR in radial glia, and this dynamic process is believed to play an important role in mammalian brain development [Pilaz, et al., 2016]. *KIF26B* is highly expressed in human brain, especially in cerebellum, spinal cord, hypothalamus and basal ganglia and relatively less in other organs such as liver, kidneys, heart and skeletal muscles (gtexportal.org). Further, *KIF26B* has been proposed as a candidate gene for spinocerebellar ataxia, with a missense variant segregating within a family with an adult-onset form of this condition (Nibbeling et al. 2017). In this study, we present a patient with pontocerebellar hypoplasia with arthrogryposis due to anterior horn cell and motor nerve involvement (PCH, MIM PS607596) – arguing for a role of this kinesin

in the development of brain and brain stem, anterior horn cells and motor nerves in particular.

CASE REPORT

The patient was first evaluated shortly after birth due to lower extremity abnormalities and feeding issues. She was the 2.52 kg product of a 35 week gestation, delivered by vaginal delivery to a 23-year-old gravida 1 mother after a pregnancy notable for gestational diabetes requiring insulin, polyhydramnios, and reduced fetal movements. The family history was significant for two maternal first cousins with abnormal development of the corpus callosum and intellectual disability, though the proband's mother was apparently unaffected, as was the proband's younger brother, and the ancestry was South Asian (Indian). Postnatally, the infant was admitted to the neonatal intensive care unit and ultimately required placement of a gastrostomy tube for feeding due to aspiration. Her exam was notable for dysmorphic facial features including upslanting palpebral fissures, microcephaly, and camptodactyly in addition to congenital dislocations of both hips, dysplastic right femoral head, bilateral acetabular dysplasia, right knee subluxation, bilateral congenital vertical talus (rocker-bottom feet), and arthrogryposis of both upper extremities. A renal ultrasound was normal. The initial brain MRI performed at 3 days of life showed microcephaly, a right germinolytic cyst, a thinned corpus callosum, delayed myelination with abnormal increased T2 signal, most prominent in the temporal tips, and PCH (Figure 1). Spinal magnetic resonance imaging (MRI) performed at the same time showed thinning of the spinal cord from T1–2 to T11–12 as well as a small distal descending aorta, small renal arteries and a small distal inferior vena cava (Figure 2). Further neurologic investigation included an electromyogram (EMG) that revealed a pathologic process affecting the anterior horn cells and ventral nerve roots or motor nerves concerning for motor neuron disease. Creatine kinase was normal, muscle biopsy showed scattered atrophic fibers, and peripheral nerve biopsy showed loss of myelinated fibers with preserved unmyelinated nerve fibers concerning for a chronic axonopathy. Electroencephalography (EEG) showed myoclonic seizures which were treated with phenobarbital. Follow-up brain MRI at 4 months showed marked progressive cerebral, corpus callosum, pontine and cerebellar vermis volume loss as well as abnormal myelination. She was diagnosed with PCH with anterior horn cell disease and progressive microcephaly (similar to PCH Type 1) with head circumference Z-score of −5 standard deviations. Her respiratory status continued to deteriorate requiring noninvasive ventilation. Subsequent MRI at 5 months of age was similar to the MRI of at 4 months of age (Figure 1). Due to the poor prognosis, the infant was discharged home with hospice care and died at age 7 months.

Her clinical genetics workup consisted of a normal karyotype (with fluorescence in situ hybridization for chromosomes 13, 18, and 21) and whole-genome SNP chromosomal microarray (Integrated Genetics, Westborough, MA) performed on an Affymetrix Cytoscan HD platform (Santa Clara, CA) with 2,695,000 targets, in addition to negative sequencing of *TSEN54* (pontocerebellar hypoplasia) and *GCH1* (dopamine-responsive dystonia), and *SMN1* deletion testing (for spinal muscular atrophy). She also had a metabolic workup including urine glutaric and 3-OH glutaric acid, serum copper and ceruloplasmin that was unremarkable. Testing for congenital disorders of glycosylation via affinity chromatography-

mass spectrometry was unremarkable and mitochondrial respiratory chain enzyme analysis from skeletal muscle (Baylor Medical Genetics Laboratories, Houston, TX) did not show any deficiency of respiratory chain activity.

MATERIALS AND METHODS

Enrollment and Sequencing

The proband, an unaffected sibling, and parents were enrolled in a research study at the Manton Center for Orphan Disease Research at Boston Children's Hospital, approved by the Boston Children's Hospital Institutional Review Board. Whole exome sequencing (WES) was performed using methods that have been previously described [Joshi, et al., 2016]. FASTQs were filtered, aligned, and variants were filtered and annotated by Codified Genomics (proprietary algorithm, Houston, TX). The mean read depth of target regions was 52x and the median coverage of each PCH gene (listed below) was at least 10x. Likely pathogenic variants were selected to include nonsynonymous, splice site, and indel variants with an allele frequency <1% in the National Heart, Lung, and Blood institute (NHLBI) exome variant server database or 1000 Genomes Project and were evaluated in the Exome Aggregation Consortium (ExAC) [Lek, et al., 2016] and Genome Aggregation Database (gnomAD). The pathogenicity of the variants was evaluated in silico using Polyphen-2 [Adzhubei, et al., 2010], SIFT [Sim, et al., 2012], and MutationTaster [Schwarz, et al., 2014]. The candidate variant was confirmed by dideoxy (Sanger) sequencing using standard methods. This variant was added to GeneMatcher [Sobreira, et al., 2015] though, unfortunately, no additional individuals with a rare, de novo *KIF26B* variant and similar phenotype were found.

KIF26B expression and functional assay

The mouse *Kif26b* open reading frame [Susman, et al., 2017] was subcloned into the pCDNA5-FRT-TO vector (V6520–20, Thermo Fisher Scientific, Hanover Park, IL). The FLAG epitope (DYKDDDDK) was added to the amino terminus of KIF26B by PCR. The Gly546Ser variant was introduced by site-directed mutagenesis. The wild-type (WT) and Gly546Ser *Kif26b* open reading frames were confirmed by Sanger sequencing. To generate inducible KIF26B-expressing cell lines, the WT or Gly546Ser *Kif26b* constructs were co-transfected with an Flp recombinase-expressing plasmid (pOG44, V600520, Thermo Fisher Scientific) into Flp-In T-Rex HEK293 cells (R71007, Thermo Fisher). The Flp-In T-Rex HEK293 cells were purchased and used within two passages for the transfection experiments and were not independently re-authenticated. The Flp-In T-Rex HEK293 cells were tested and found to be negative for mycoplasma contamination using the Universal Mycoplasma Detection Kit (30–1012K, ATCC, Manassas, VA). Stable transformants were selected using 100ug/ml hygromycin B (10687010, Thermo Fisher Scientific). Expression of the KIF26B proteins was induced by treatment with 1ug/ml doxycycline (631311, Clontech/Takara, Mountain View, CA) for 2 days. Cell dissociation assays were performed as described previously [Uchiyama, et al., 2010]. Western analysis of FLAG-KIF26B induction was performed using the M2 mouse monoclonal anti-FLAG antibody (F3165, Sigma-Aldrich, St. Louis, MO). Protein loading control was performed using the DM1A mouse monoclonal anti-α-tubulin antibody (05–829, Sigma-Aldrich).

RESULTS

Identification of a de novo variant in KIF26B and molecular modeling

The WES data was evaluated for a comprehensive list of genes known to cause PCH including *AMPD2*, *CASK*, *CDK5*, *CHMP1A*, *CLP1*, *EXOSC3*, *PCLO*, *RARS2*, *SEPSECS*, *TBC1D23*, *TSEN2*, *TSEN15*, *TSEN34*, *TSEN54*, *TOE1*, *VPS53*, and *VRK1*, and no pathogenic, likely pathogenic or variant of unknown significance was identified. Analysis of the WES data by the methods specified above revealed a de novo missense variant in *KIF26B*, c.1636G>A, p.Gly546Ser (RefSeq NM_018012.4), which was confirmed absent from both parents and the proband's unaffected brother. This variant is absent from the ExAC and gnomAD databases, and is predicted to be "deleterious" by SIFT, "disease causing" by MutationTaster, and "probably damaging" by Polyphen-2. The variant occurs at a highly conserved amino acid residue (GERP score 5.73) within the motor domain (Fig. 3A, B); this kinesin motor domain shares over 40% amino acid sequence similarity with the kinesin 8 motor domain. Sequence alignment and homology modeling of the KIF26B motor domain based on the previously reported crystal structure of the kinesin 8 motor domain (Protein Data Bank code: 3LRE) [Peters, et al., 2010] suggests that the KIF26B Gly546Ser substitution occurs at the phosphate binding loop (P-loop) that interacts with the phosphate group of ADP (Fig. 3B, C). As the P-loop is known to play a critical role in the binding and hydrolysis of nucleotide triphosphate in a wide variety of nucleotide hydrolyzing enzymes, amino acid substitution within the P-loop of KIF26B is likely to affect the function of the protein.

The Gly546Ser variant reduces KIF26B's function to promote cell-cell adhesion

A documented function of the KIF26B protein is to regulate cell-cell adhesion [Uchiyama, et al., 2010]. The closely related protein KIF26A was previously shown to localize specifically to sites of cell-cell contact in cultured HeLa cells [Maliga, et al., 2013]. More recently it was demonstrated that overexpression of KIF26B increases cell-cell adhesion in human embryonic kidney 293 (HEK293) cells, resulting in a cell aggregation phenotype [Uchiyama, et al., 2010]. This assay provides a means to test the function of the KIF26B variant protein. We expressed WT KIF26B or the Gly546Ser variant in HEK293 cells under the control of a tetracycline-regulated promoter (Tet-On), and analyzed the resulting cell aggregation phenotypes. The Tet-On system allowed us to express KIF26B proteins in an acute manner, circumventing potential compensatory effects caused by chronic KIF26B expression. Replicating the previously reported finding, we observed that acute expression of WT KIF26B resulted in a marked increase in cell-cell adhesion and aggregation (Fig. 3D, F). In contrast, expression of the Gly546Ser KIF26B variant had little effect inducing cell-cell adhesion and aggregation (Fig. 3D, F). By immunoblot analysis, we showed that the Gly546Ser KIF26B variant was expressed at a similar level as the WT KIF26B protein, indicating that the inability of the Gly546Ser KIF26B variant to induce cell-cell adhesion is not due to alterations in protein expression level or stability (Fig. 3E). These findings provide direct evidence that the Gly546Ser variant causes a deficit in KIF26B function.

DISCUSSION

We report an infant with severe, congenital neurologic disease suspected to be caused by a de novo variant in *KIF26B*. This represents one of the first reports of neurologic disease in humans related to variants in this gene, though recent animal studies have demonstrated it to have a role in synaptogenesis [Heinrich, et al., 2012]. *Kif26B* was shown to be highly expressed in the cerebellum and hippocampus in a rat model and co-localized with microtubules. It was proposed that KIF26B shuttles post-synaptic protein Abi-1 as its cargo along microtubules; truncated KIF26B constructs in the rat model led to decreased presence of Abi-1[Heinrich, et al., 2012]. More recently, KIF26B has also been shown to affect the cell polarity of migrating endothelial cells during angiogenesis [Guillabert-Gourgues, et al., 2016] and has been shown to be expressed in the cerebellum in humans [Nibbeling et al. 2017]. While chromosomal microdeletions with partial deletions of *KIF26B* have been reported to cause intellectual disability, developmental delay, and variable microcephaly and abnormalities of the corpus callosum, it is unclear whether the phenotype in these patients is due to disruption of *KIF26B* or of neighboring genes such as *HNRNPU* [Caliebe, et al., 2010]. Haploinsufficiency of *KIF26B* may be related to the microcephaly and developmental phenotype seen in patients with chromosomal deletions of 1q44 [Raun, et al., 2017]. One such patient with a microdeletion including *KIF26B* had microcephaly in addition to dysmorphic features, developmental delay, intractable epilepsy, autonomic nervous system dysfunction, and a brain MRI that showed mild ventricular dilation and a thin corpus callosum, though *KIF26B* did not correlate with microcephaly across all patients [Raun, et al., 2017]. Further, a recent study to identify candidate genes in spinocerebellar ataxia presented a family with late adult-onset, dominant form in which a single missense *KIF26B* variant segregated with the disease [Nibbeling et al. 2017]. Their variant was present in the C-terminal tail region of *KIF26B* unlike our patient's variant in the motor domain that may explain the marked difference in the onset and severity of presentation. It is also interesting that affected individuals in that family had spasticity, and two members had mild atrophy of the cerebellum seen on brain MRI [Nibbeling et al. 2017]. Although *Kif26b*-null mice exhibit a renal phenotype [Uchiyama, et al., 2010; Terabayashi, et al., 2012], this has not been seen in all of the human studies cited above or in our patient, perhaps because these individuals did not have complete loss of KIF26B function. One patient with a 1q44 deletion containing *KIF26B* did have renal aplasia, though this finding is confounded by the presence of other genes within the deleted region [Caliebe, et al., 2010].

PCH is a rare neurodevelopmental disorder with structural brain anomalies causing global developmental delay and neurologic symptoms. Thus far, multiple genes have been associated with this disease in humans [Rudnik-Schoneborn, et al., 2014]. It is interesting to note that we propose an autosomal dominant mechanism of inheritance in our patient, as opposed to the previously-discovered PCH genes, which are inherited in an autosomal recessive fashion and generally thought to cause disease by a loss-of-function mechanism rather than the dominant-negative mechanism that we propose. However, there are prior reports of patients presenting with PCH who were found to have de novo variants in genes with an autosomal dominant mode of inheritance proposed [van Dijk, et al., 2017; Bacrot, et al., 2018]. Additionally, there are certainly other brain malformations caused by *de novo*

variants in genes, such as the tubulinopathies [Oegema, et al., 2015], that can function by this mechanism and it is likely that as gene discovery continues to progress, this list will expand. We hypothesize that the *KIF26B* variant found in our patient might be responsible for her PCH phenotype as Gly546 is located within the motor-like domain of KIF26B, which is highly conserved among kinesin superfamily members (Fig. 3A). In addition, Gly546 is part of the P-loop motif generally thought to be critical for ATP binding and hydrolysis (Fig. 3B, C). Substitution of the analogous glycine in other ATP-binding proteins, including motor proteins and kinases, has been shown to abolish or reduce the functions of these proteins [Cao, et al., 2017; Marzahn, et al., 2014; Nair, et al., 2016]. In this study, we used a previously established cell adhesion assay to demonstrate that the Gly546Ser substitution identified in this patient functionally compromises the KIF26B protein. Since cell adhesion is known to play a critical role in a wide range of neurodevelopmental processes [Wu, 2013; Togashi, et al., 2009], this finding suggests that defects in KIF26B-mediated cell-cell interactions may contribute to the widespread developmental abnormalities seen in this patient. It is also interesting to note that the cell-cell adhesion-promoting function of KIF26B was previously shown to require a C-terminal domain [Uchiyama, et al., 2010], whereas the Gly546Ser substitution of our patient is located within the motor-like domain outside of this C-terminal domain. These observations suggest that the motor domain, which is known to interact with microtubules, and the C-terminal domain, which is known to interact with non-muscle myosin IIB, may function in concert to mediate the function of KIF26B in cell adhesion [Uchiyama, et al., 2010; Terabayashi, et al., 2012]. It is also possible that the C-terminal domain, which contains coiled coil motifs, additionally functions to maintain the overall protein structure of KIF26B, such as dimerization [Vale, et al., 2000].

In the conventional paradigm of KIF function, the kinesin motors convert the energy of ATP hydrolysis into mechanical movement along the microtubules. However, a number of previous studies classified KIF26B and the closely related protein KIF26A as “unconventional” kinesins based on two criteria: 1) these proteins harbor substitutions in several critical regions of the motor domain, including the P-loop, and 2) unlike conventional KIFs, KIF26A and KIF26B do not dissociate from microtubules in an ATP-dependent manner [Zhou, et al., 2009; Terabayashi, et al., 2012]. Upon re-examination of the amino acid sequence of KIF26B, we noted that the motor domain of KIF26B does possess a P-loop motif that closely matches the consensus sequence (GxxxGKS/T) [Saraste, et al., 1990]. The Gly546Ser substitution disrupts the first glycine of this consensus, which is generally believed to be critical for P-loop function. Interestingly, disruption of the P-loop in other nucleotide-binding proteins, including the conventional kinesin Kif5a and the p21/Ras oncoprotein, is known to produce dominant-negative or dominant-active activities [Nakata and Hirokawa, 1995; Seeburg, et al., 2007]. Thus, it will be important in future studies to further investigate whether the KIF26B motor-like domain indeed harbors intrinsic ATP binding/hydrolysis activity and whether this activity is perturbed by the Gly546Ser substitution to produce a dominant-interfering effect.

In summary, we present further evidence linking *KIF26B* to neurologic disease in humans, suggesting its important role in the development of the brain.

Acknowledgements:

We acknowledge the enrolled family for their contribution to this research. We also acknowledge Brigette Jong for assistance with KIF26B mutagenesis.

Funding: This work was supported by the National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases (1R01AR068429–01 to PBA), the National Institute of Child Health and Human Development/National Human Genome Research Institute/National Institutes of Health (U19HD077671 to PBA), the National Institutes of Health/National Institute of General Medical Sciences (1R35GM119574–01 to HHH), and the National Institutes of Health (T32GM007748 for MHW).

Grant numbers:

- National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases (1R01AR068429–01)

- National Institute of Child Health and Human Development/National Human Genome Research Institute/National Institutes of Health (U19HD077671)

- National Institutes of Health/National Institute of General Medical Sciences (1R35GM119574–01)

- National Institutes of Health (T32GM007748)

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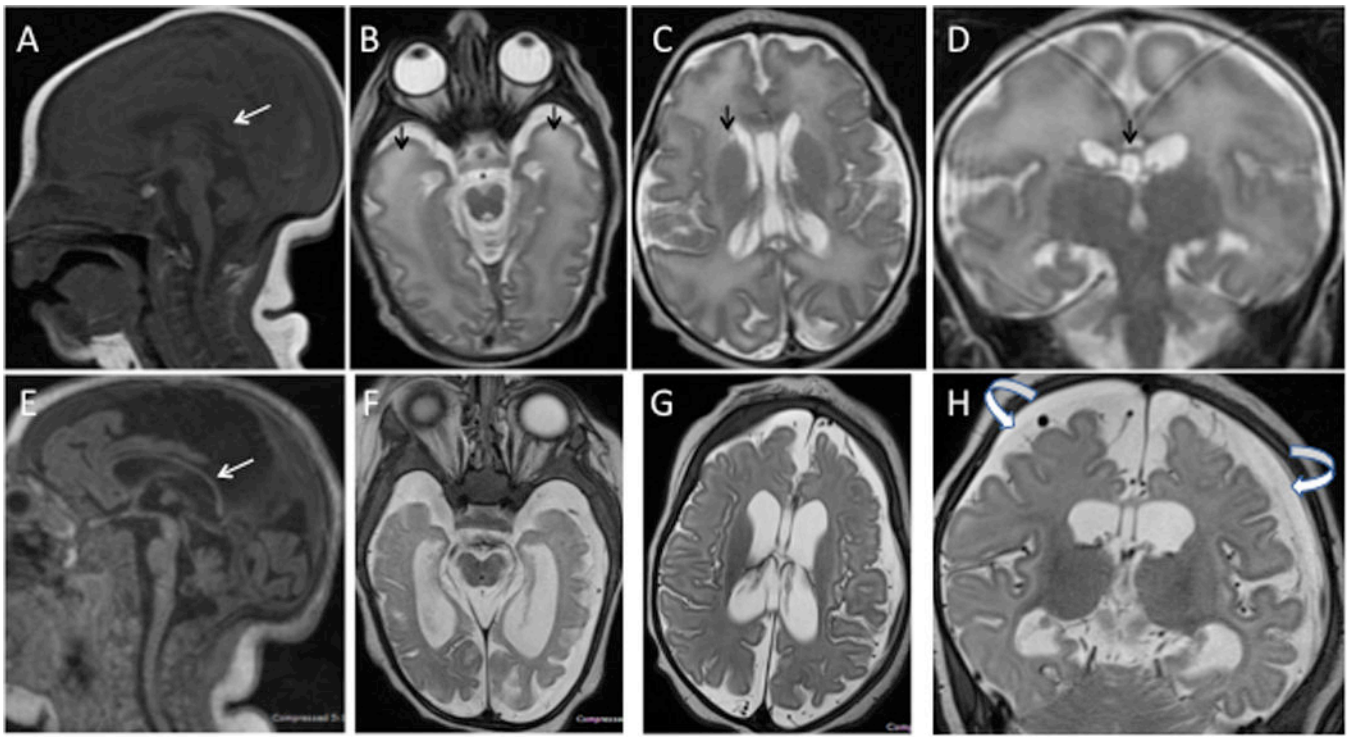

Fig. 1. Brain MRI of the proband demonstrating progressive microcephaly and pontocerebellar hypoplasia

(A) Sagittal T1, (B-D) axial T2-weighted images at 3 days of age show microcephaly, a small pons, a small cerebellum/vermis, a small corpus callosum (arrows in A and D), abnormal increased T2 signal most prominent at the temporal poles (arrows in B) and a right germinolytic cyst (arrow in C). (E) Sagittal T1, (F-H) and axial T2-weighted images at 5 months of age show a dramatic decrease in brain volume including the pons, cerebellum/vermis and corpus callosum (arrow in E), with secondary increases in ventricular and subarachnoid spaces as well as new bilateral holohemispheric subdural fluid collections (curved arrows).

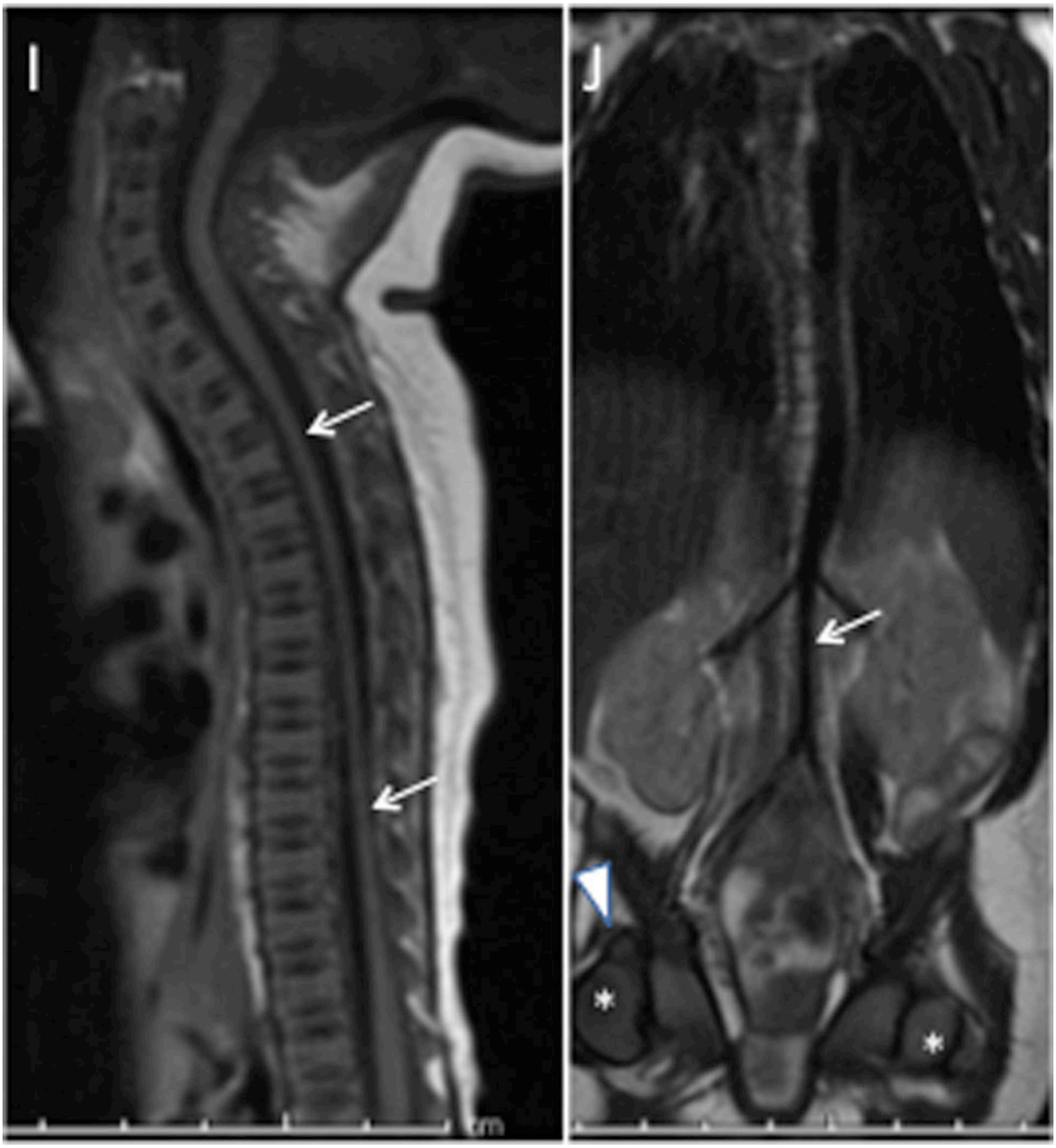

Fig. 2. Additional MRI findings in the proband

(A) Sagittal T1-weighted image through the cervicothoracic spine shows thinning of the spinal cord from T1–2 to T11–12. (B) Coronal T2-weighted image through the abdomen shows thinning of the descending aorta (arrow) with partial visualization of the bilateral hip dysplasia (*) and dysplastic right femoral head (arrowhead)

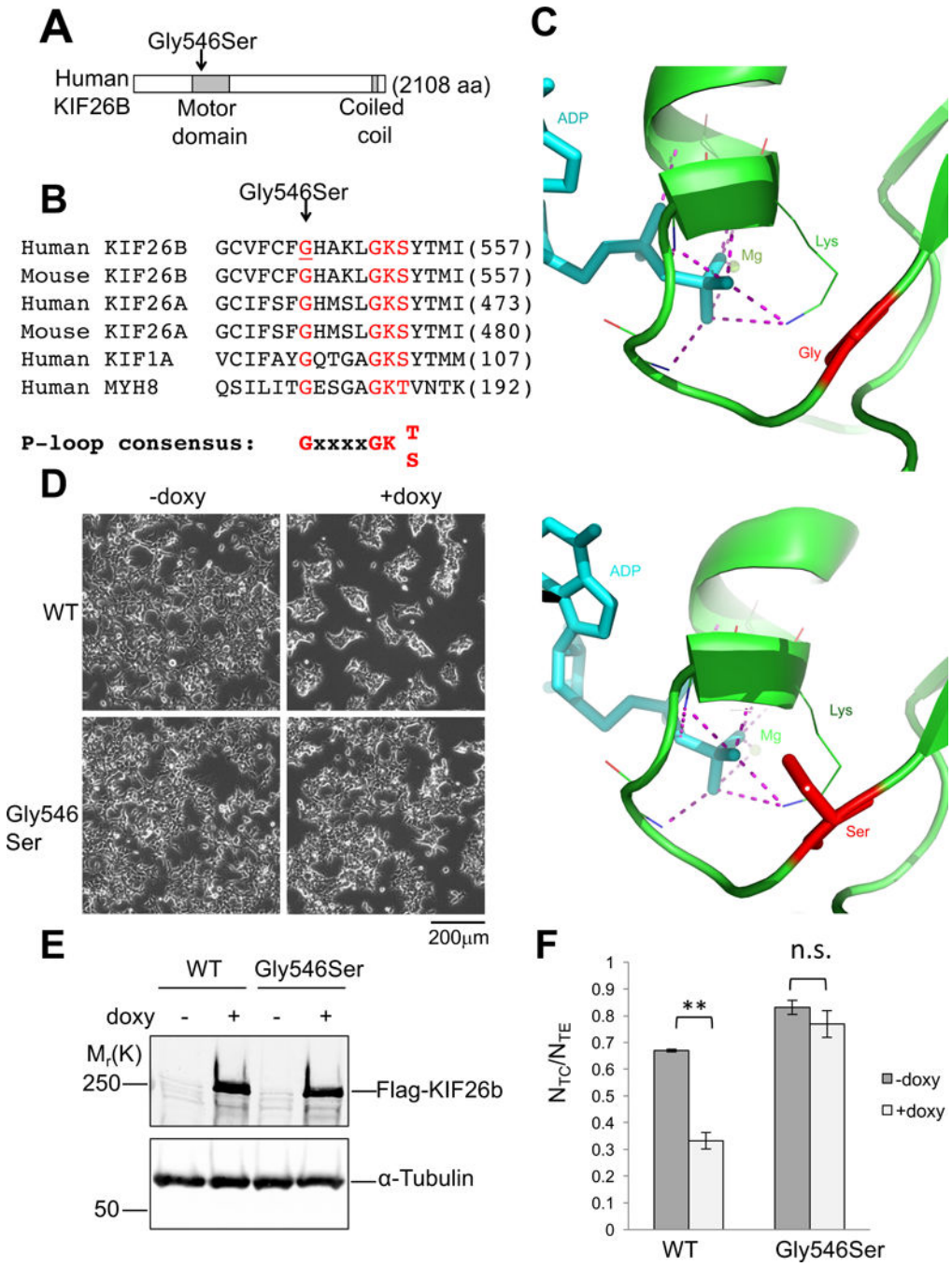

Fig. 3. The Gly546Ser variant compromises KIF26B's ability to induce cell-cell adhesion

(A) Schematic representation of the human KIF26B protein showing the motor domain and the coiled coil region. The relative position of the Gly546Ser substitution is indicated by the arrow. (B) Alignment of the amino acid sequences of P-loop regions of human KIF26B, mouse KIF26B, human KIF26A, mouse KIF26A, the human conventional kinesin (KIF1A) and the human skeletal muscle myosin heavy chain (MYO8). The P-loop consensus as previously defined [Saraste, et al., 1990] is shown in bold. Gly546 of human KIF26B is indicated by the arrow and underlined. (C) Molecular model demonstrating the site of the

KIF26B Gly546Ser variant, based on the previously described structure of the kinesin 8 motor domain. The protein is represented by a green ribbon and ADP is displayed in cyan. Gly546, or the corresponding Ser residue in the patient variant, is colored in red. Another conserved residue, Lys552 in the P-loop consensus sequence (GxxxGKS/T) is depicted by the thin green lines. The bonding interactions that coordinate the phosphate group of the ADP are shown in purple dashed lines. The small, light green ball represents a magnesium ion. (D) Representative images of HEK293 cells expressing the WT or Gly546Ser KIF26B proteins. Expression of KIF26B was driven by a tetracycline-regulated promoter and induced by treatment of the tetracycline analog doxycycline (doxy, 1ug/ml for 2 days). The formation of cell clumps or microislands, as observed in WT + doxy, signifies increased cell-cell adhesion. (E) Western blots (cropped) showing that the expression of KIF26B (WT or Gly546Ser variant) was induced to a similar level after doxycycline treatment. Anti-α-tubulin immunoblotting was used as the protein loading control. (F) Quantification of calcium-dependent cell-cell adhesion after doxycycline-induced KIF26B expression (WT or Gly546Ser variant). Cells were dissociated in the presence of calcium (TC) or EGTA (TE) and the numbers of cell clusters (N_{TC} or N_{TE}) were quantified. A lower N_{TC}/N_{TE} ratio indicates increased cell-cell adhesion. Error bars represent ±SEM calculated from four independent experiments. Two tailed *t*-tests were determined for the following comparisons: KIF26B WT –doxy vs +doxy, $p<0.01$ (**); KIF26B Gly546Ser –doxy vs +doxy, $p=0.38$, not significant (n.s.).