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Supplemental information

PLS3 missense variants affecting the actin-binding domains cause X-linked congenital diaphragmatic hernia and body-wall defects

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Supplemental note

Case reports

Family 7

The proband is a 16 year old Hispanic male with a c.617C>T, p.(A206V) [NM_005032.7] variant in *PLS3*. His family history was significant for his mother having 2 miscarriages, one with his father and one with a different partner. His mother took progesterone from 8-12 weeks gestation, was anemic during pregnancy, and had a UTI at 10 weeks which was treated with antibiotics. An 18-week ultrasound revealed a choroid plexus cyst and dilated renal pelvises. At 24 weeks, the cyst appeared to have resolved but the renal pelvises remained dilated. An ultrasound at 30 weeks showed malposition of the heart leading to a referral for a fetal echocardiogram. This study showed a mass in the thorax. A fetal MRI revealed bilateral diaphragmatic hernia with portions of the liver in the thorax on both sides. Amniocentesis was declined.

He was born at 38 weeks gestational via a forceps assisted, vaginal delivery. He was intubated almost immediately after birth and extubated on the second day of life. His OFC at birth was 36 cm (92nd centile) and his estimated weight was 3.5 kg (21st centile). On day of life four, he underwent a surgical repair of his diaphragmatic hernia. He was confirmed to have bilateral ventral diaphragmatic hernias with the right hernia measuring 5 cm X 4 cm and the left hernia measuring 4 cm X 3 cm. Both were covered by a hernial sac and a portion of the liver was herniated into both. A large epigastric, skin covered, abdominal wall defect measuring 5 cm X 5 cm that stretched from just below sternum to the umbilicus was also found. These hernias were closed primarily.

A head ultrasound revealed a tiny left side choroid plexus cyst but was otherwise unremarkable. His development was normal and he is doing well in school.

Other features noted at birth include a prominent forehead, hypertelorism, down slanting palpebral fissures, a broad flattened nasal bridge, anteverted nares, low set ears, micrognathia, a sacral dimple with a hair tuft, and hypoplastic first toenails bilaterally. He also had an undescended right testis and a right inguinal hernia, for which he ultimately underwent a right orchiopexy and hernia repair. He also had a membranous ventricular septal defect, an atrial septal defect, and bilateral hydronephrosis which resolved spontaneously. The latest measurements available were taken when he was 10 years, 10 months of age at which time he was 139 cm tall (29th centile) and weighed 33.3 kg (37th centile).

Family 8

The proband is a 15 year old white male with a maternally-inherited c.1054T>C, (p.Phe352Leu) [NM_005032.7] variant in *PLS3*. His family history was significant for 2 maternal half-brothers with hemidiaphragmatic left-sided congenital diaphragmatic hernias who died in infancy, and two maternal half-sisters, one of which has sensory integration disorder, inguinal hernia, and ADHD and the other who has a history of rheumatic fever and hypothyroidism. The father of these half-siblings also had hypothyroidism. His mother had ADHD. His mother also had at least 9 early miscarriages (≤ 6 weeks gestation) with four different partners, including 4 miscarriages with the father of the

proband, and 2 miscarriages with the father of his half-siblings. His mother also had one ovarian pregnancy with the proband's father that was treated with methotrexate.

The pregnancy was uncomplicated except for minor polyhydramnios. At the 20 week ultrasound a left-sided diaphragmatic hernia was detected by ultrasound. An amniocentesis was performed and chromosome and FISH analyses were negative. Also observed on the ultrasound were a two vessel chord and a cyst on the brain which resolved. A prenatal MRI at 22 and 2/7 weeks found the lung to head ratio to be 1:4.

He was born at 38 and 2/7 weeks gestation via a C-section. He was intubated immediately after birth but he failed conventional ventilation with NO and was switched to ECMO at 7 hours of life. His birth weight was 3.855 kg (91st centile). At 2 weeks of age he was taken off of ECMO and his diaphragmatic hernia was repaired at 2.5 weeks of age. At surgery the anterior portion of his large, left-sided defect was covered by a hernia sac. There was an approximately 1 cm thick strip of diaphragmatic muscle that crossed the defect about 1/3 of the way from the anterior chest wall. No diaphragm was found posteriorly. The stomach, spleen, most of the bowel and small rim of the left lobe of the liver were found in the defect. The hernia sac was excised and the strip of muscle traversing the defect was fixed to the anterior wall. A GORE-TEX patch used to repair the remainder of the hernia. A Meckel's diverticulum was found and excised. He had an unusual reaction to the GORE-TEX patch, and after two GORE-TEX patch failures, his defect was repaired with a biological patch.

Other features noted at birth include hypertelorism, low-set right ear, a short nose with a wide nasal root. An echocardiogram showed a structurally normal heart. A corneal pannus was found on his right. Shortly after birth he had a febrile UTI, and a renal ultrasound revealed left-sided grade 3 vesicoureteral reflux. He also had a small right hydrocele. He was also noted to have hypertelorism, down slanting palpebral fissures, and intermittent horizontal nystagmus. He had a gastrostomy tube placed. When discharged at four months of age, he was 58.4 cm in length (6th centile).

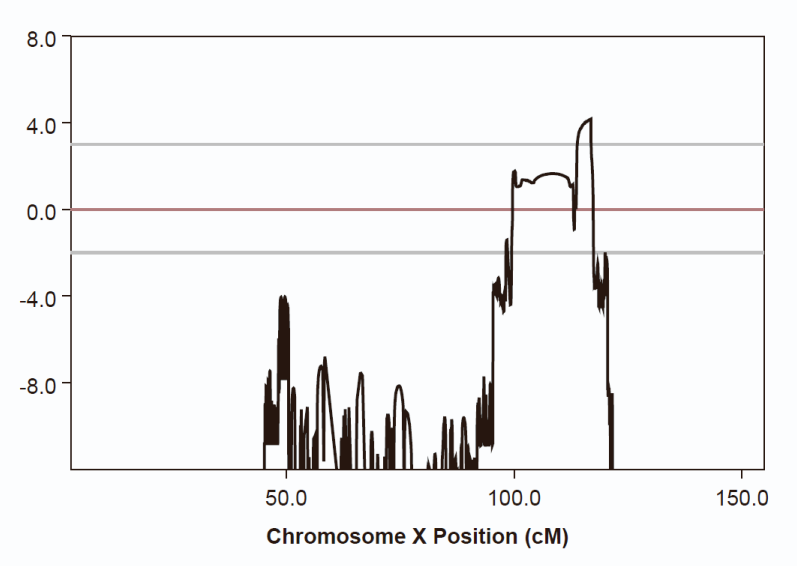
A brain MRI at 1 month found a marked increased amount of CSF fluid and enlarged lateral ventricles. Although he walked at 12 months, he was diagnosed with speech delay. He had been diagnosed with intellectual disability and autism spectrum disorder. Starting at 6 years of age, he had complex partial seizures. At the age of 9 he could follow commands but still communicated using 1-2 word phases. He was also diagnosed with sleep apnea and mild, bilateral sensory neural hearing loss.

Over time, he had significant gastrointestinal issues including anorexia, dysphagia, constipation, nausea, and failure to thrive. However, at 9 years 4 months of age he was 136.5 cm in height (58th centile) and 28.1 kg in weight (37th centile). Other physical features noted at included malocclusion, wide spaced teeth, and a high arched palate.

A chromosomal microarray analysis was normal. Exome sequencing showed two additional variants of uncertain significance including a non-maternally inherited c.G52C (p.Gly45Ser) variant in *SMARCA2*, and a maternally inherited c.G133A (p.Gly45Ser) in *SYP*. It is possible that these variants are also contributing to his phenotypes.

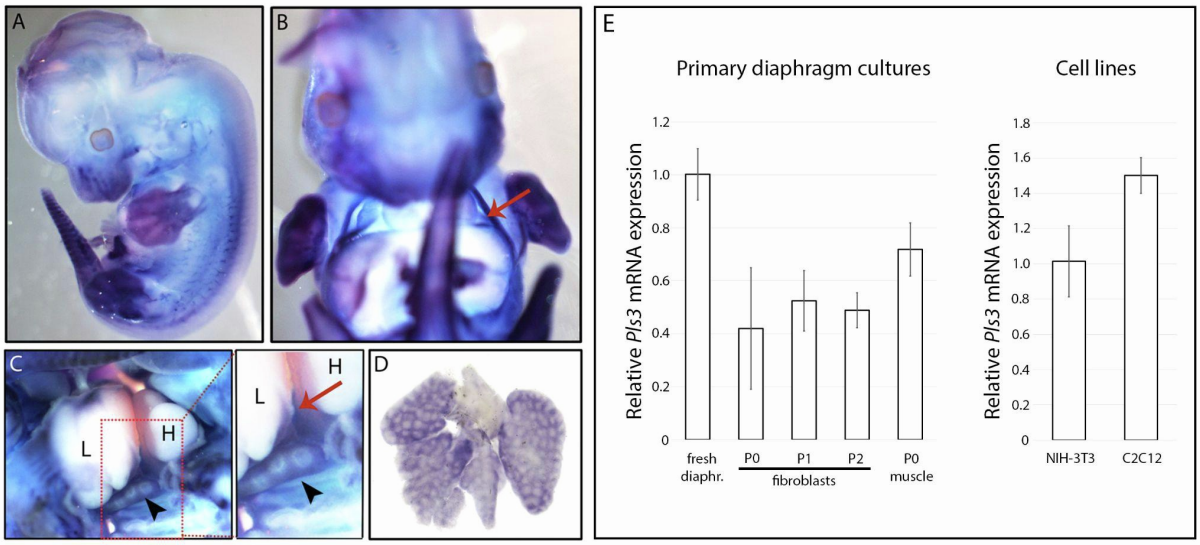
Supplemental figures

Figure S1. Linkage analysis in family 1



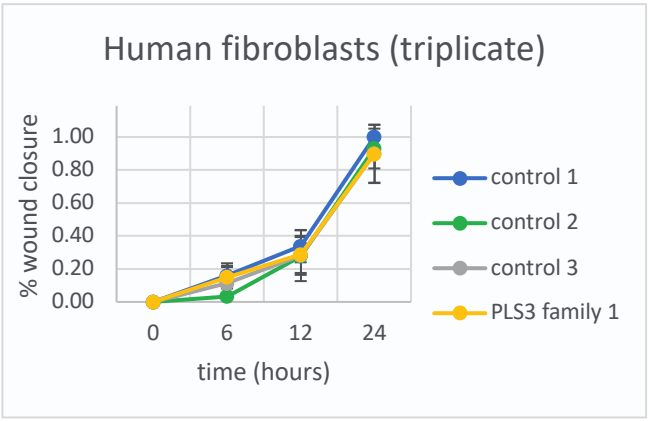
Linkage plot for the X-chromosome in Family 1. Lod-score >3 comprised between rs2192371 and rs2215785 (chrX:113,889,739-116,906,942 hg19).

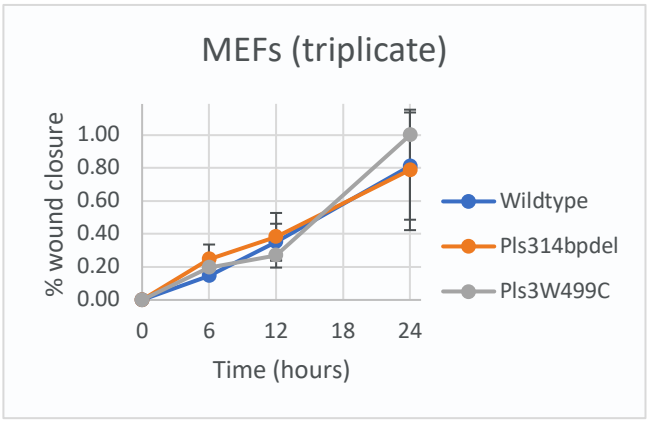
Figure S2. Pls3 developmental expression analyses



(A-D) Whole-mount in situ hybridization for Pls3 in mouse embryos at E12.5 (A-C) and E13.5(D). (A) Pls3 mRNA is observed in face, limbs, tail, and axial skeleton in a E12.5. (B) The ventral view shows Pls3 in symmetrical structures lateral to the midline (arrow) with a distribution compatible with edges of the anterior body wall. (C) Dissection of the lateral body wall shows that Pls3 mRNA is expressed in the developing lung (arrowhead) but not in the heart (H) or in the liver (L). The developing diaphragm, nested between the heart and the liver, shows Pls3 expression (red arrow in insert). (D) Microdissection of the lung shows Pls3 expression in the lung mesenchyme at E13.5. (E) Quantitative RT-PCR showed robust levels of Pls3 mRNA in mouse fresh diaphragm, mouse primary diaphragm cell cultures (left panel) and cell lines (right panel). Data represent the average of 3-6 replicates for each sample type. P0-P2 indicate passage number.

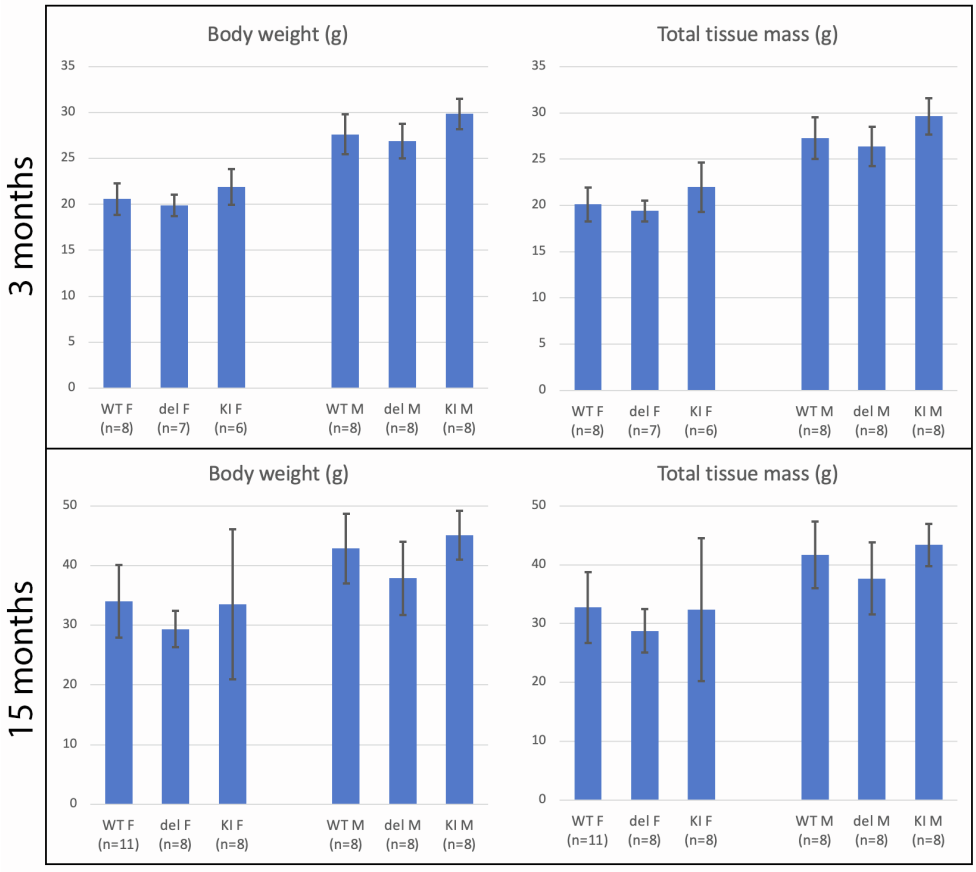
Figure S3. Wound healing assays





Wound closure assays for human fibroblasts (top panel) and mouse embryonic fibroblasts (MEFs) (bottom panel). Graphs represent experiments performed in triplicate, with error bars representing one standard deviation from the mean.

Figure S4. Body weight and total tissue mass of *Pls3*^{W499C} and *Pls3*^{14bpdel} mice



Body weight (left) and total tissue mass (right) values from wild-type (WT), Pls314bpdel (del) and Pls3W499C (KI) mice at 3 months (top) and 15 months (bottom). Error bars indicate one standard deviation. The values in both mutant mouse strains were not significantly different compared with the wild-types using a student's t-test.

Supplemental table

Table S1. Results of the bone densitometry performed for individual III-2 (affected male) at the age of 51 years old

DXA results summary					
Region	**Surface (cm^2)**	**BMC (g)**	**BMD (g/cm^2)**	**T-score**	**Z-score**
Femur :					
Neck	5.91	6.46	1.094	1.2	2.0
Trochanter	15.07	15.50	1.029	2.2	2.4
Intertrochanteric	27.99	41.92	1.497		
Total	48.97	63.88	1.304	1.8	2.2
de Ward	1.13	0.97	0.858		
Vertebrae :					
L1	16.56	26.50	1.600	5.4	5.7
L2	17.39	28.94	1.664	5.2	5.6
L3	18.82	32.37	1.720	5.6	6.0
L4	21.94	37.96	1.730	5.3	5.7
Total	**74.71**	**125.77**	**1.683**	**5.4**	**5.8**

BMC: bone mineral content (hydroxyapatite); BMD: bone mineral density (hydroxyapatite).

Supplemental material and methods

Linkage analysis for family 1: Single nucleotide polymorphism (SNP) data were collected on members of family 1 using the Infinium HumanOmniExpress-24 v1.0 (Illumina) platform. Parametric linkage analysis for X-linked traits was carried out using MINX in the Merlin software package20, assuming a disease allele with frequency 0.0001 in our model and an assumed penetrance of 100%.

Whole mount in situ hybridization: The mouse *Pls3* probe was generated using a 734 bp segment of the mouse transcript that was PCR amplified (PCR Master Mix, Promega, Madison, WI) with one set of exon-exon boundary overlapping primers (forward: gagctagcagcgtaggtcg and reverse: cattttgcagagcacgatccc). The purified PCR fragment was cloned into the pCR™II-TOPO® TA vector (TOPO® TA Cloning® Kit, Dual Promoter) (ThermoFisher, Life Technologies Corporation), and transformed into One Shot® TOP10 Chemically Competent Cells (ThermoFisher, Life Technologies Corporation). Sense and anti-sense Digoxigenin-11-UTP labeled probes (DIG RNA Labeling Mix, Sigma-Aldrich) were synthesized with SP6 and T7 RNA polymerases, respectively. E12.5 mouse embryos were collected in cold phosphate-buffered saline (PBS), fixed over night at 4°C with 4 % paraformaldehyde in PBS, and washed with PBT (PBS with 0.1% Tween 20) at 4°C. Whole-mount in situ hybridization was performed as described[30]. Embryos were bleached with 6% hydrogen peroxide in PBT for 1 hour, treated with 10μg/mL Proteinase K (Sigma-Aldrich) in PBT for 10 minutes, then incubated in pre-warmed hybridization buffer at 70^{O}C for 1 hour. DIG-labelled probe was added to fresh hybridization buffer at a concentration of 1μg/mL, and dissected embryos were incubated in this buffer at 70°C overnight. Embryos were then washed stringently and incubated overnight at 4°C in preabsorbed 1:2000 anti-DIG antibody (Roche) with 0.1% goat serum in TBST. Probe was visualized by incubating dissected embryos for 24-48 hours in BM-Purple (Roche). Embryos were then further dissected to isolate the lung for imaging using a Nikon AZ100 microscope.

Mouse diaphragm cultures and quantitative PCR: E13.5 developing diaphragms were micro-dissected from timed C57BL/6NJ (Jackson Laboratory JR#005304) *wildtype* embryos. Each collected diaphragm was placed in individual wells on a 96 well plate with 100 ⌈L of either fibroblast promoting media (Ham's F-12 Nutrient Mixture (Gibco), 10% Fetal Bovine Serum (FBS, Gibco), 50 μg/mL gentamycin (Gibco)) or muscle progenitor promoting media (DMEM/F-12 GlutaMAX (Invitrogen), 10% FBS, 50 μg/mL gentamycin). Cells were then cultured until confluent using methods previously described[31]. Once confluent, the cells were collected, lysed, and RNA was isolated. RNA was also collected from fresh diaphragms and cultured NIH-3T3 cells and C2C12 cells. qPCR methods are detailed in the main text.

Primers:

SYBR green qPCR primer sequences

Gene	Primer sequences (5' -> 3')

Actb	Primer 1- GATTACTGCTCTGGCTCCTAG Primer 2- GACTCATCGTACTCCTGCTTG
Gapdh	Primer 1 - CATCACTGCCACCCAGAAGACTG Primer 2 - ATGCCAGTGAGCTTCCCGTTCAG
Pls3 (first intron)	Primer 1- GCGACCACCCAGATTTCCAAA Primer 2- GCAGTGGCATATTAGCTTCCTTG
Pls3 (last intron)	Primer 1- ATATGCCCTCCCTGAAGACCT Primer 2- GCACCGGAGAGTAAGGTTGG

***Pls3* mouse genotyping information:**

The wild type and knock-in (KI) PCR products are 608 base pairs (bp) in length while the 14 bp del PCR product is 594 bp. The following primers were used:

Pls3 9064 5'-AGGGAACTCCATGAGAACATCTG-3'

Pls3 9062 5'-GCTTCTGGAGGAAAGAACTAGATC-3'.

Wound healing assays: Human or mouse fibroblast cells were grown to 100% confluency in 6-well tissue culture plates coated with 10 μg/ml fibronectin. A linear scratch was made in each well using a pipet tip, then cells were washed once with growth media and then covered with 2 ml of growth media. Images were collected using phase-contrast microscopy at the same location on the scratch at 0, 6, 12, and 24 hours. For each cell line, data were collected from 3 locations on the scratch in each of 3 separate wells. The distance between the margins of the scratch was measured using ImageJ software. Data are displayed as percent closure compared with the length of the measurement at 0 hours.