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([id345@cam.ac.uk](mailto:id345@cam.ac.uk), [rshaheen@kfshrc.edu.sa](mailto:rshaheen@kfshrc.edu.sa), [skgtevf@gmail.com](mailto:skgtevf@gmail.com), [msn27@cam.ac.uk](mailto:msn27@cam.ac.uk),  
[aalhashem@psmmc.med.sa](mailto:aalhashem@psmmc.med.sa), [steven.lisgo@newcastle.ac.uk](mailto:steven.lisgo@newcastle.ac.uk), [aferjaen@telefonica.net](mailto:aferjaen@telefonica.net),  
[hjs49@cam.ac.uk](mailto:hjs49@cam.ac.uk), [ks339@cam.ac.uk](mailto:ks339@cam.ac.uk), [dr.tlili@planet.tn](mailto:dr.tlili@planet.tn),  
[Sarah.Smithson@uhbristol.nhs.uk](mailto:Sarah.Smithson@uhbristol.nhs.uk), [susan.lindsay@newcastle.ac.uk](mailto:susan.lindsay@newcastle.ac.uk),  
[falkuraya@kfshrc.edu.sa](mailto:falkuraya@kfshrc.edu.sa), , [cw347@cam.ac.uk](mailto:cw347@cam.ac.uk))

Methodology if needed for subcellular localisation of mut v wt : Cellular localisation of the WT PLCH1 protein and mutant PLCH1 protein, showing a clear change in localisation to include in the nucleus in 1-7HB2 epithelial cell line, with PLCH1 being stained with antiFLAG (goat antirabbit 488) in green at 1:1000 and DAPI nuclear staining in blue. These were viewed using confocal microscopy at 100x.

# Mutations in Phospholipase C $\epsilon$ -1 (*PLCH1*) are associated with Holoprosencephaly

Ichrak Drissi<sup>1\*</sup>, Emily V Fletcher<sup>1\*</sup>, Ranad Shaheen<sup>2\*</sup>, Michael S Nahorski<sup>1</sup>, Amal M Alhashem<sup>3</sup>, Steve Lisgo<sup>4</sup>, Alberto Fernández Jaén<sup>5</sup>, Hayley Sharpe<sup>6</sup>, Katherine Schon<sup>1</sup>, Katlthoum Tlili-Graïess<sup>3</sup>, Sarah Smithson<sup>7</sup>, Susan Lindsay<sup>4</sup>, Fowzan Sami Alkuraya<sup>2,8</sup>, Christopher Geoffrey Woods<sup>1</sup>

<sup>1</sup> Cambridge Institute for Medical Research, Keith Peters Building, Addenbrookes Hospital, Hills Rd, Cambridge CB2 0QQ, UK

<sup>2</sup> Department of Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia.

<sup>3</sup> Neuroradiology Section, Department of Radiology, Prince Sultan Military Medical City, Riyadh, Saudi Arabia.

<sup>4</sup> Human Developmental Biology Resource, Newcastle Institute of Genetic Medicine, Newcastle University, NE1 3BZ, UK

<sup>5</sup> Especialista en Neurología Infantil, Hospital Universitario Quirónsalud de Madrid. Tlf. 914521900, Centro CADE, Spain.

<sup>6</sup> Babraham Hall, Babraham Research Campus, Cambridge, CB22 3AT, UK.

<sup>7</sup> Bristol Regional Genetics Service, St Michael's Hospital, Bristol, BS2 8EG, UK.

<sup>8</sup> Department of Anatomy and Cell Biology, College of Medicine, Alfaisal University, Riyadh, Saudi Arabia.

\* Joint first authors

Corresponding authors:

Fowzan Sami Alkuraya : [falkuraya@kfshrc.edu.sa](mailto:falkuraya@kfshrc.edu.sa), Tel 00966 11 464 7272, ORCID 0000-0003-4158-341X

Christopher Geoffrey Woods: [cw347@cam.ac.uk](mailto:cw347@cam.ac.uk), Tel 0044 1223 762322, ORCID 0000-0002-8077-2101

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## **ABSTRACT**

### **Background**

Holoprosencephaly is a spectrum of developmental disorder of the embryonic forebrain in which there is failed or incomplete separation of the prosencephalon into two cerebral hemispheres. To date, dominant mutations in sonic hedgehog (SHH) pathway genes are the predominant Mendelian causes, and have marked inter- and intrafamilial phenotypic variability.

### **Methods**

We describe two families in which offspring had holoprosencephaly spectrum and homozygous predicted-deleterious variants in *PLCH1*. Immunocytochemistry was used to examine the expression pattern of PLCH1 in human embryos. We used SHH as a marker of developmental stage and of early embryonic anatomy.

### **Results**

In the first family, two siblings had congenital hydrocephalus, significant developmental delay, and a mono-ventricle or fused thalami with a homozygous *PLCH1* c.2065C>T, p.(Arg689\*) variant. In the second family two siblings had alobar holoprosencephaly and cyclopia with a homozygous *PLCH1* c.4235delA, p.(Cys1079ValfsTer16) variant. All parents were healthy carriers, with no holoprosencephaly spectrum features. We found that the sub-cellular localisation of PLCH1 is cytoplasmic, but the p.(Cys1079ValfsTer16) variant was predominantly nuclear. Human embryo immunohistochemistry showed PLCH1 to be expressed in the notochord, developing spinal cord (in a ventral to dorsal gradient), dorsal root ganglia, cerebellum, and dermatomyotome; all tissues producing or responding to SHH. Furthermore, the embryonic sub-cellular localisation of PLCH1 was exclusively cytoplasmic, supporting protein mis-localisation contributing to the pathogenicity of the p.(Cys1079ValfsTer16) variant.

### **Conclusion**

Our data supports the contention that PLCH1 has a role in prenatal mammalian neurodevelopment, and deleterious variants cause a clinically variable holoprosencephaly spectrum phenotype.

## Introduction

Holoprosencephaly is a structural anomaly of the brain in which there is failed or incomplete separation of the embryonic prosencephalon into two cerebral hemispheres and associated bilateral structures. In humans, the defect causing holoprosencephaly arises between the third- and fifth-week post fertilisation [1]. The most severe forms of holoprosencephaly are classified as alobar, semilobar and lobar, and the greater the degree of holoprosencephaly the greater the degree of intellectual deficit and associated congenital anomalies [2]. These include facial anomalies (ranging from cyclopia and a proboscis, to a single upper incisor, to none), central (and bilateral) cleft lip and palate, congenital heart disease, pre-axial polydactyly, and endocrine deficiencies [2,3].

The prevalence of holoprosencephaly is estimated to be 1/250 in conceptuses and 1/16,000 live births [4]. The aetiology of human holoprosencephaly is varied; environmental (e.g. maternal diabetes gives a 1% risk to the foetus), chromosomal (the commonest overall cause, an example being trisomy 13), Mendelian, or unknown [2]. Mendelian causes are sub-classified into non-syndromic (which are inherited as dominant disorders) and syndromic (where there are additional distinctive features, and inheritance can be either dominant or recessive) [2]. Many of the 14 genes known to cause holoprosencephaly are involved in the Sonic Hedgehog (SHH) signalling pathway [5,6].

The study of families with genetic form of holoprosencephaly extended the spectrum of brain and facial anomalies to include the milder phenotypes. The “middle interhemispheric fusion variant” consists of syntelencephaly (where posterior, frontal and parietal areas lack midline separation, and/or lack of cleavage of the basal ganglia and thalami, and absence of the body of the corpus callosum). “Microform holoprosencephaly” is a much milder form where the brain is structurally normal, but there are subtle craniofacial anomalies with or without mild neurocognitive impairment [7-9].

A marked variability of phenotype is a constant finding in the Mendelian disorders associated with holoprosencephaly; and this variability is seen within families, and between individuals in different families with the same mutation [2,10]. The degree of phenotypic variability seen in dominant disorders that cause holoprosencephaly is unusually marked, and this variability is also seen in the recessive disorder Smith-Lemli-Opitz syndrome, where consistency of phenotype may have been expected [11]. The causes of this variability is unknown, but is considered related to three factors: the complexity of the developmental pathways in which

SHH participates (in consort with TGF- $\beta$  and FGF pathway signalling); that otherwise harmless polymorphisms in holoprosencephaly-related genes could become disease modifiers; and that the environmental/maternal factors such as alcohol, diabetes and cholesterol intake and metabolism can affect the intra- and inter-cellular environment resulting in altered SHH signalling [12-14].

We document two families in which children have severe developmental delay secondary to brain malformations within the holoprosencephaly spectrum. We were unable to find any known cause of their phenotypes but did discover homozygous, very rare, predicted deleterious variants in the *PLCH1* gene. Our clinical and molecular results suggest that bi-allelic mutations in *PLCH1* are a novel cause of holoprosencephaly.

## **METHODS**

### **Consent for clinical and scientific studies**

The consent of the family members to use their clinical information, or if children their parents, was obtained according to the Declaration of Helsinki. The studies we performed have been approved by the Research Advisory Council (RAC) at King Faisal Specialist Hospital and Research Centre (RAC# 2121053), and Cambridge East Research Ethics Committee (05\Q0108\402).

### **Clinical studies**

The authors ascertained families by direct referral from the family's Paediatricians' or Clinical Geneticists'. The family details and pedigrees were recorded by their physicians, informed consent taken for diagnostic genetic studies, and blood samples taken for DNA extraction. All clinical observations, studies and tests were performed to enable a diagnosis and guide treatment; none was performed solely for research purposes. Families and their physicians were informed of the research findings.

### **Molecular genetic analysis seeking pathogenic variants**

The two families were separately ascertained and clinically investigated. No further families were notified to us after a GeneMatcher request, gnomAD shows no homozygous loss of function variants, and there were no cases reported in the DECIPHER study.

In each family the affected individuals' genomic DNA was extracted from blood samples by standard methods – in Family one DNA was only available from the affected son and unaffected parents, in Family two DNA was available from both affected cases and their unaffected parents. Exome analysis was performed on the affected individual's DNA (SureSelect Human All Exon 50Mb Kit, Agilent Technologies). Potentially pathogenic mutations were identified by comparison with the GRCh37 reference human genome, filtering for changes with allele frequencies of < 1 in 500 in the ExAC database (now incorporated within the genomAD database), and focused on homozygous and bi-allelic changes (and concordance between siblings in Family two). Novel unreported variants and known SNPs were then sought that could be pathogenic by SIFT (missense variants) or by filtering (lost start and stop codons, premature nonsense variants, small insertions and deletions, and canonical splicing variants) in the exome results of the affected individuals of each family. A summary of the filtering process to discover potentially pathogenic variants in the two families is given in Supplementary Table 1. Separately, all variants and SNPs in known holoprosencephaly genes were reviewed by hand. Any potential pathogenic variants were confirmed and tested for expected recessive segregation by polymerase chain reaction (PCR) and Sanger sequencing of the parents and their affected offspring.

Microarray analysis seeking chromosome perturbations was performed in all four affected individuals. *PLCH1* has three isoforms reported. We used the commonest and longest transcript for our studies NM\_001130960, which contains both of the mutations we report.

### **Re-examination of family genetic data seeking changes in holoprosencephaly related genes**

The following genes implicated are in SHH signalling and holoprosencephaly; *BOC*, *CDON*, *DISP1*, *DHCR7*, *DLL1*, *FGF8*, *FOXH1*, *GAS1*, *GLI2*, *NODAL*, *PTCH1*, *SHH*, *SIX3*, *TDGF1*, *TGIF1*, *SUFU* and *ZIC2*. All exons were adequately covered in our exome analysis for base changes and small INDELS [15]. For these genes we sought both novel variants and known SNPs that would be predicted as potentially pathogenic in the exomes of each family's affected individuals. To find exon/gene scale deletions and duplications in these genes we both re-examined microarray analyses of the affected children and examined sequencing depth by comparative analysis of affected versus controls in the Integrated Genome Viewer.

### **Gene cloning, tagging and mutagenesis of *PLCH1***

Using the plasmid pFLAG-CMV2 wild type *PLCH1* was manufactured from IMAGE clone IRATp970F03110D (Source BioScience) of *PLCH1* isoform NM\_001130960; generating pFLAG-PLCH1. The p.Cys1079ValfsTer16 variant was introduced into pFLAG-PLCH1 using the QuikChange™ mutagenesis (Stratagene, USA) protocol, using the following primers: (forward) 5'CAGGAAAACCCCTTCCCAGCAAGTC and (reverse) 5'GACTTGCTGGGAAGGGGTTTTCTG.

### **Sub-cellular localisation studies of PLCH1 and PLCH1 p.(Cys1079ValfsTer16)**

HEK 293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10 % fetal calf serum, 1% L-Glutamine and 1% non-essential amino acids (Invitrogen). Cells were grown in a 95% air; 5% CO<sub>2</sub> atmosphere at 37°C. Prior to transfection cells were plated to 60-70% confluency on Poly-D-Lysine coated glass coverslips and transfected using Fugene HD (Promega) for 24 hours with four µg of pFLAG-PLCH1. All incubations were performed at room temperature and cells were analysed 72 hours post transfection. Transfected cells were washed with PBS before and after a 10-minute fixation with 4% paraformaldehyde and blocked in 10% donkey serum and 10% IHC blocking buffer (Sigma) diluted in PBS 0.1% Triton buffer (PBS-T) for 30 minutes. To detect PLCH1 variants tagged with FLAG, cells were incubated with rabbit anti-FLAG antibodies (Sigma) diluted 1:100 in PBS-T supplemented with 10% donkey serum for two hours. Cells were washed three times in PBS-T for 10 minutes before protein labelling with donkey anti-rabbit 488 antibodies (Invitrogen) diluted 1:1000 in PBS-T for 45 minutes. Coverslips were washed before mounting using Fluoromount-G DAPI (Invitrogen), and cells were viewed using the Zeiss LSM780 confocal microscope system.

### **Immunohistochemistry of PLCH1 expression in human embryos**

The CS10, CS12 and CS13 human embryonic sections were provided by the Human Developmental Biology Resource (HDBR) which is regulated by the Human Tissue Authority and operates in accordance with the relevant codes of practice. Embryos were collected from women undergoing social termination of pregnancy with appropriate maternal written consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee. The samples were staged using the guidelines of Bullen and Willson (1997), fixed in methacarn (60% absolute methanol, 30% chloroform, 10% glacial acetic acid) and processed through to paraffin wax (fibrowax, VWR) [16]. Sections were taken on a

microtome at seven micron thickness and mounted on Superfrost Plus microscope slides (Thermo Scientific).

Sections were dewaxed with Histo-Clear (National Diagnostics), rehydrated in graded ethanol and rinsed in distilled water. Antigen retrieval was performed by heating the sections to 95°C in sodium citrate buffer pH 6 for 20 mins. Cooled sections were rinsed in distilled water before two, five minute permeabilization washes in Tris-HCL 0.1% Triton buffer pH 10 (Tris-HCL-T buffer). To prevent non-specific staining, sections were blocked in 10% donkey serum and 10% IHC blocking buffer (Sigma) diluted in Tris-HCL-T buffer for 90 mins. Sections were incubated overnight at 4°C in a humidified chamber with rat polyclonal anti-sonic hedgehog antibody (Abcam, ab50515) and rabbit polyclonal anti-human PLCH1 (Genetex, GTX108612), both diluted 1:100 in Tris-HCL-T buffer containing 10% donkey serum. After washing with Tris-HCL-T buffer, sections were labelled for two hours at room temperature with donkey anti-rabbit IgG-488 (Invitrogen) and donkey anti-rat IgG-546 (Invitrogen). Slides were washed with Tris-HCL buffer and mounted using Fluoromount-G DAPI (Invitrogen). Specimens were visualized using the Zeiss LSM780 confocal microscope system.

## **RESULTS**

### **Clinical findings in the two families**

Inheritance pattern: in each family there were both affected and unaffected children; the parents were unaffected (no features of the holoprosencephaly spectrum, or other congenital anomalies or intellectual or neurological deficit); affected children were of both sex; parents were consanguineous; see Figure 1A. There were no known affected individuals in the wider pedigrees. From this we concluded that the most likely inheritance pattern of the phenotypes in both families was autosomal recessive.

Full clinical phenotype details are given in the Supplement and summarised here and in Figure 1B, C and D. Family one - the parents were Saudi first cousins and had three normal children. A five-year-old boy, and in retrospect his deceased sister, were both affected and born with macrocephaly secondary to hydrocephalus. In the girl postnatal brain MRI revealed: a large posterior fossa cyst with atrophy of the cerebellum and partial agenesis of the vermis; heterotopias in both cerebral hemispheres; posteriorly kinked midbrain; atrophic



pons; and a disfigured ventricular system giving the appearance of a mono-ventricle with absent septum pellucidum. The later findings were suggestive of holoprosencephaly, see Figure 1C. In the boy a postnatal brain MRI at one week revealed a subependymal cyst near the foramen of Monro and partial agenesis of the corpus callosum. Repeat MRI at age nine months showed partial thalamic fusion, evolving cerebral leukomalacia and apparent aqueduct stenosis, see Figure 1D. Both children developed tonic clonic epilepsy postoperatively and had gross motor and cognitive delay evident by two years. The girl also had a ventricular septal defect, cleft lip and palate (bilateral cleft lip and palate). In summary, both children had complex brain anomalies within the holoprosencephaly spectrum.

Family two - the parents were Pakistani first cousins with two normal children. Their first affected child was a male infant who had alobar holoprosencephaly detected by antenatal ultrasound at 20 weeks of gestation. He was born at term but died shortly after birth. He was confirmed to have alobar holoprosencephaly, and also cyclopia, a midline cleft lip, microcephaly (-3SD) and the cardiac anomalies (double outlet right ventricle, ventricular septal defect and possible aortic atresia). The parents declined post-mortem examination. A second pregnancy, a female, also had holoprosencephaly, with gross hydrocephalus. She also died a few hours after birth. She had no eyes but disrupted tissue beneath a proboscis, and her ears were joined together under her chin. The parents declined post-mortem examination. In summary, the babies both had a severe holoprosencephaly phenotype, and their diagnosis would include Young-Madders syndrome, Smith-Lemli-Opitz syndrome or any of the single gene causes of holoprosencephaly sequence – although the absence of polydactyly in both affected babies would be uncommon<sup>17</sup>.

### **Molecular genetic analysis seeking pathogenic variants**

In both families we analysed the exome data assuming a recessive mode of inheritance, seeking both bi-allelic and homozygous pathogenic variants, and that were concordant between the affected individuals in Family two. No potential mutations were found in any known holoprosencephaly gene. There were no potential bi-allelic mutations in either family. Microarray analysis of affected individuals were normal.

Figure 2A shows *PLCH1*, the PLCH1 protein, and the variants found in each family. In Family one we found a homozygous nonsense variant in the affected son, c.2065C>T in *PLCH1* which leads to a premature stop codon p.(Arg689\*) in exon 16 (of 22 exons), and

hence likely subject to nonsense mediated mRNA decay, see Figure 2B. In Family two a homozygous one base deletion in *PLCH1* c. 3235delT was found, predicted to result in p.(Cys1079ValfsTer16). The deletion was in the last exon on *PLCH1*, so would not be expected to be subject to nonsense mediated mRNA decay, but instead to yield a truncated protein with the C-terminal 623 amino acids (of 1693) deleted. Both of these mutations were confirmed as real, and to segregate as expected for a recessive genotype within families. These *PLCH1* variants have not been previously reported and are absent from gNOMAD.

### **Re-examination of family genetic data seeking changes in holoprosencephaly and SHH related genes**

In Family one and Family two we found no potential pathogenic variants in known SHH or holoprosencephaly related genes. In Family one, *PLCH1* variant was the only high quality novel homozygous coding variant with deleterious prediction was identified within the autozygome of the index. In Family two the following protein altering SNP genotypes were present in both affected babies: in *DISP1* heterozygous rs2609383 p.(Glu103Lys) with GMAF=0.1814, and heterozygous rs2789975 p.(Glu103Asp) with GMAF=0.1819; in *NODAL* homozygous rs1904589 p.(His165Arg) with GMAF=0.343; in *CDON* homozygous rs3740912 p.(Val75Ile) with GMAF=0.4525, heterozygous rs3740909 p.(Glu162Lys) with GMAF=0.1074, and homozygous rs684535 p.(Ile1221As) with GMAF=0.2002. In *PTCH1* rs357564 p.(Pro1315Leu) with GMAF=0.390 was homozygous in one and heterozygous in the other. None of these SNPs is reported to have functional effects.

### **Sub-cellular localisation studies of PLCH1 and the PLCH1 mutation p.(Cys1079ValfsTer16)**

We found that wild type *PLCH1* was localised solely to the cytoplasm, see Figure 2C. We had expected a nuclear localization as a bipartite nuclear localization motif was predicted to be present in *PLCH1*, see Figure 2A. The localization of the mutant p.(Cys1079ValfsTer16) *PLCH1* was either nuclear and cytoplasmic (25%), or only nuclear (75%), see Figure 2C. Therefore the pathogenic mechanism of the p.(Cys1079ValfsTer16) may be both of loss of the C-terminal domain, and also reduced protein availability at its site of action within critical cells. Furthermore, these results suggest the possibility that the *PLCH1* nuclear localization

signal can be functional, and that the C-terminus of PLCH1 is able to block/mask this motif, potentially enabling the protein to change sub-cellular localization. We found no evidence of PLCH1 expression in primary cilia using overexpression studies, in accord with published proteomics experiments; see Supplementary Fig 1 [18].

### **Determination of the human embryonic expression pattern of PLCH1**

Holoprosencephaly, and the other developmental structural brain anomalies seen within the holoprosencephaly spectrum brain, are thought to arise between human embryonic week three through to embryonic week five. This commences with human embryonic Carnegie stage 8, when the human midline notochord induces changes in the prechordal plate, which is anterior and lateral to the notochord (the prechordal plate is destined to become the pronencephalon, which ultimately giving rise to all forebrain structures). By embryonic week five, Carnegie stage 15, the prosencephalic structures, including the thalami, have formed, become distinct, and separated [1]. We did not attempt to perform a complete study of this developmental period, as the necessary embryo sections are unavailable.

The earliest embryo we were able to obtain was approximately Carnegie stage 10. SHH and PLCH1 both co-localise to the same small region of the embryo, both have a cytoplasmic sub-cellular localisation, but embryonic anatomical structures were impossible to accurately define, see Figure 3A and B.

Thereafter, the earliest embryo sections available were of Carnegie stage 12. We chose to examine a transverse sectioned Carnegie stage 12 embryo, a time-point when the spinal cord neural precursors are developing into different neuronal classes dependent upon a dorsal Bone Morphogenic Protein (BMP) gradient and a counter ventral SHH gradient. As expected SHH is expressed in the spinal cord, acting as a control for the embryo age and anatomy, see Figure 3C and D. We found PLCH1 was also expressed with a clear ventral-dorsal gradient (greatest nearest the notochord), see Figure 3C and D, and Figure 4A. Both SHH and PLCH1 are co-expressed in the developing dorsal root ganglia (identified by TRKA expression, results not shown), see Figure 3E. PLCH1, but not SHH (as expected [19]), is expressed in the laterally placed somites, see Figure 3C and 4A. We also found SHH and PLCH1 present together in the notochord in transverse sectioned Carnegie stage 12 embryo, Figure 4B. Consistently, PLCH1 had a cytoplasmic sub-cellular localisation and is not seen in the nucleus, as best shown in the hindbrain in sagittal sectioned Carnegie stage 12 embryo in

Figure 4C. The sections in Figure 3, and Figure 4A, B and C are each from different embryos.

These results show considerable co-localisation of PLCH1 with SHH in the human embryonic nervous system. The results suggest that PLCH1 has more than one role in human embryonic development. The gradient of PLCH1 in the developing spinal cord is compatible with a role in neural precursor/early neurons being able to respond to SHH (as does the presence of PLCH1 alone in somites). However, the presence of PLCH1 in the notochord supports a role in SHH secretion.

## DISCUSSION

We describe two families with a holoprosencephaly phenotype, one with extreme alobar holoprosencephaly and expected facial and cardiac malformations, the second with malformations restricted to the central nervous system. However, marked variability is common and expected in both dominant and recessive Mendelian diseases causing holoprosencephaly, and for this reason we pursued the finding of homozygous variants in *PLCH1* as being a possible novel Mendelian cause of the holoprosencephaly spectrum in these families [2,10,11].

We first compared our cases to those of the best known recessive diseases in which holoprosencephaly is a feature, Smith-Lemli-Opitz syndrome (SLOs) and Young Madders syndrome (also known as Pseudo-trisomy 13 syndrome), sporadic Trisomy 13, and the dominant SHH mutation phenotype [20], see Table. Our cases are similar to those with Young Madders syndrome and Trisomy 13, but do exhibit the phenotypic variability seen in recessive SLOs and dominant SHH mutations. Severely affected SLOs individuals have only a few possible differences to our cases, the presence of 2/3 toe syndactyly, ambiguous genitalia, and polydactyly - all of which are extra-CNS anomalies [11]. Young Madders syndrome has the same phenotype as Trisomy 13, with the karyotype distinguishing between the two [17,21]. The gene causing Young Madders syndrome has not been discovered (probably resulting in under-ascertainment of more milder affected cases), although the only report of a consanguineous child in which homozygosity mapping was performed revealed 21 regions of homozygosity - one of which included *PLCH1*<sup>23</sup> [22](an *a priori* 10% chance [23]); so *PLCH1* could be the cause of Young Madders syndrome. The gene (s) responsible

for Trisomy 13 phenotype are unclear, but poly-alanine expansions, deletions and a duplication of ZIC2 (on chromosome 13) have been reported in cases of holoprosencephaly without other known causes [24].

**TABLE**

	<b>Our cases<sup>2</sup></b>	<b>Smith-Lemli-Opitz syndrome</b>	<b>Young Madders syndrome</b>	<b>Trisomy 13</b>	<b>SHH mutation phenotype</b>
<b>Mode of inheritance</b>	recessive	recessive	recessive	Sporadic dominant	Autosomal dominant
<b>Holoprosencephaly</b>	4/4	5%	100% <sup>3</sup>	30%	36%
<b>Characteristic facies</b>	>90%	>90%	>90%	60%	>60%
<b>Cleft lip</b>	3/4	40%	53%	40%	20%
<b>Congenital heart disease</b>	3/4	50%	15%	80%	2%
<b>Microcephaly</b>	1/4	80%	12%	20%	10%
<b>Male ambiguous genitalia</b>	0/2	50%	60%	0%	5%
<b>Intellectual deficit range</b>	4/4 severe	Normal (>5%) to severe	100% severe	100% severe	Normal (30%) to severe
<b>Post-axial polydactyly</b>	0/4	33%	90% <sup>3</sup>	80%	<10%
<b>2/3 toe syndactyly</b>	0/4	>90%	10% <sup>4</sup>	0% <sup>4</sup>	0% <sup>4</sup>

The developmental processes that are considered to go awry in human holoprosencephaly occur between weeks three and six post-fertilisation. We therefore asked if there was evidence that PLCH1 was expressed in the human embryo during this period. Whilst PLCH1 was considered to be expressed exclusively in the central nervous system, there was no expression data available for this early period in human or murine embryos, and so we

generated this [25,26]. We used SHH as a marker of developmental stage and of early embryonic anatomy, and to see if PLCH1 and SHH were ever co-expressed. Results for a 4th weeks embryo were limited to showing coincidence of expression of cytoplasmic PLCH1 and SHH in a contiguous region of cells, but we were unable to define the anatomy of the section. In 5<sup>th</sup> week human embryo transverse sections of the upper thorax we found that PLCH1 and SHH were both expressed in the developing spinal cord, the notochord, and dorsal root ganglia. PLCH1 exhibited a ventral-dorsal gradient in the spinal cord, mimicking the known SHH morphogenic gradient, and the opposite of that of BMPs and WNTs. PLCH1 alone was detected in somites, which do not express SHH but respond SHH produced in the spinal cord. This data set is incomplete and most importantly we were not able to assess PLCH1 expression in the prechordal plate (beginning of 3<sup>rd</sup> week post-fertilisation), as human embryonic samples are not available. However, we have shown SHH expression to be anatomically and developmentally as expected in the 5<sup>th</sup> week post fertilisation (as shown in our Carnegie stage 12 and 13 results), and that PLCH1 and SHH have coincident expression in all neural tissues.

How could PLCH1 deficiency cause holoprosencephaly? Phospholipase C Eta-1 and Eta-2 (genes *PLCH1* and *PLCH2*) are members of the most recently discovered class of the phospholipase C family [27]. As with all members of the phospholipase C family, upon activation PLCH1 increase intracellular calcium levels. As increases of intracellular calcium are necessary for SHH signalling, we hypothesised that PLCH1 could be one of the mediators of the calcium intracellular signal generated by SHH binding to its receptors [28]. We speculate that PLCH1 has a role in the developmental responses to low levels of SHH, such as that generated by the spinal cord that then diffuse laterally to induce somite development. In holoprosencephaly the SHH produced by the centrally placed caudal end of the notochord induce differentiation in the large prechordal plate, which grows caudally in all directions away from the notochord; hence there will be a diminishing SHH gradient in outermost regions of the prechordal plate where we speculate PLCH1 to be expressed. Unfortunately, because of the unavailability of early human embryos (before Carnegie stage 10), we were unable to explore this. Taken together our data suggests that PLCH1 could be a novel cause of autosomal recessive holoprosencephaly spectrum.

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## **Competing interests**

The authors report no competing interests.

## **Declaration of authorship.**

EVF and ID conceived and performed experiments and were involved in writing the paper. RS, AFJ, SS, KS, KTG, FSA and CGW produced clinical and experimental data. SL and SL produced the human embryo sections used in the paper, and were involved in the immunohistochemistry analysis. MSN, HS, CGW and FSA conceived and planned aspects of the project, and were involved in writing the paper.

## **URLs**

Decipher – mapping the clinical genome at <https://decipher.sanger.ac.uk/>

gNOMAD at <https://gnomad.broadinstitute.org/about>

The MRC-Wellcome Trust Human Developmental Biology Resource (HBDR) at [www.hdbr.org](http://www.hdbr.org)

Human Genome Browser at <https://genome.ucsc.edu/>

IGV at <http://software.broadinstitute.org/software/igv/>

Nuclear export signal detection using NetNES at <http://www.cbs.dtu.dk/services/NetNES/>

Nuclear localisation signal mapper at [http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS\\_Mapper\\_form.cgi](http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi)

Primer3 at <http://primer3.ut.ee/>

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## Display items and legends

### Figure 1

Title: Family pedigrees, summary phenotype table, and brain scans of affected individuals

Legend: A: Pedigrees of the two families described in the paper. Proband is arrowed, affected individuals are filled in squares or circles and a red asterisk denotes people from whom we had a DNA sample. B: Key phenotypic features of the affected four children aligned; all other family members having no disease features. A minus sign designating the feature was absent, a plus sign it was present, and a triple plus that the features were severe.

C and D: MRI brain scans of the two affected children from Family one, detailed in the text. For each, the top pair of scans show results for one week of age, and the lower pairs for 39 weeks of age. C shows gross hydrocephalus and the later findings were suggestive of holoprosencephaly. In both right-hand axial T2 views a star shows the fused thalami, and two ventricular heteropias are best seen in the upper axial T2 view shown by arrow heads. D shows lesser hydrocephalus with partial thalamic fusion, again a feature of the holoprosencephaly spectrum

## Figure 2

Title: PLCH1 gene and protein, with position of mutations, and sub-cellular localisation of PLCH1 wild type and Family two mutation p.(Cys1079ValfsTer16).

Legend: A: uppermost is a cartoon of the structure of the *PLCH1* gene. The relative size of the exons, but not introns, is shown. Lowermost is a cartoon of the PLCH1 protein with the canonical PLCH domains in blue and named, a predicted nuclear localisation signal is shown in black, and predicted nuclear export signal in green. Between the gene and the protein, the Family one and Family two mutations are shown, with their respective positions in gene and protein indicated. B: shows an electrophoretogram of the Family one mutation, with upper homozygous wild type control and lower homozygous mutation. C: shows subcellular localisation of wild type PLCH1 on the left to be cytoplasmic in a transfected 1-7HB2 epithelial cell line, whereas the middle pane shows that the Family two mutation p. (Cys1079Valfs\*16) is also present in the nucleus. On the right a bar chart shows the nuclear signal depicted by pixel/area of PLCH1 covered in the nucleus of WT PLCH1 (red) and mutant PLCH1 (blue). A two tailed T-test performed on the data gave P value of 2.43116E-10, so  $P < 0.01$  and the mutant and WT PLCH1 nuclear signals are significantly different  $P < 0.01$  so mutant PLCH1 shows a statistically significant increase in nuclear signal.

Methodology if needed for subcellular localisation of mut v wt : Cellular localisation of the WT PLCH1 protein and mutant PLCH1 protein, showing a clear change in localisation to include in the nucleus in 1-7HB2 epithelial cell line, with PLCH1 being stained with antiFLAG (goat antirabbit 488) in green at 1:1000 and DAPI nuclear staining in blue. These were viewed using confocal microscopy at 100x.

## Figure 3

Title: Human embryo immunohistochemistry studies expression of PLCH1 and SHH in a Carnegie stage 10 sagittal section, and Carnegie stage 12 transverse section through the upper thorax.

Legend: Antibodies to human Sonic Hedgehog are shown in red, to PLCH1 in green, and DAPI in blue shows nuclei. A: is a near mid-line sagittal section of a CS10 embryo, with size bar 100  $\mu$ m. The boxed area contains cells with both SHH and PLCH1 expression, and is shown magnified in B. The green mass to the lower left is artefact autofluorescence. The orientation of the embryo is unclear, but probably the head is at the right top with the back at the bottom and abdominal area to the left side. B: is a magnification of the boxed area in A, size bar 40  $\mu$ m. The upper left panel is of SHH expression, upper right of PLCH1, lower left of DAPI and the composite is at the lower right and shows a collection of cells most of which express both SHH and PLCH1. C: is a transverse CS12 section through the upper thorax, with size bars 100  $\mu$ m. The upper left panel is of SHH expression, upper right of PLCH1, lower left of DAPI and the composite is at the lower right showing the spinal cord at the centre including a boxed area, developing dorsal root ganglia at either side – on the right indicated by an arrow, and laterally the somites stained prominently by PLCH1. D: is a magnification of the boxed area in C, size bar 25  $\mu$ m. The left panel is of SHH expression, middle of PLCH1, and the composite is at the right also including DAPI. It shows that some cells in the developing spinal cord express SHH or PLCH1, but rarely both. E: is a magnification of the arrowed area in C, size bar 10  $\mu$ m. The left panel is of SHH expression, middle of PLCH1, and the composite is at the right also including DAPI. This shows that the cells of the developing dorsal root ganglia predominantly express both SHH and PLCH1.

#### **Figure 4**

Title: Human embryo immunohistochemistry studies in Carnegie stage 12 transverse sections illustrating a PLCH1 ventro-dorsal gradient in the spinal cord, notocord expression, and that embryonic sub-cellular localisation PLCH1 is consistently cytoplasmic and not nuclear

Legend: Antibodies to human PLCH1 are shown in red, and DAPI in blue shows nuclei. A: is a transverse CS12 section through the upper thorax, with size bars 100  $\mu$ m. Leftmost is PLCH1 expression, middle DAPI, and the composite at right. This shows the ventro-dorsal gradient of PLCH1 expression in the developing spinal cord – mirroring a similar reported

SHH gradient. The laterally placed somites also express PLCH1. The dorsal root ganglia (between the somites and spinal cord) also express PLCH1 but are less well shown. This is likely a consequence of the somites and spinal cord being continuous, but the dorsal root ganglia being an intermittent chain of cell aggregates. B: is a transverse CS12 section through the upper thorax, with size bars 100  $\mu$ m; only staining for PLCH1 expression is shown. The white arrow shows PLCH1 expression in the notochord; the ventro-doral gradient of PLCH1 expression in the developing spinal cord is again shown. C: is a sagittal C12 section with size bar 10  $\mu$ m. Leftmost is PLCH1 expression, middle DAPI and the merge at right. This shows a cytoplasmic sub-cellular localisation of PLCH1 in the hindbrain.

### **Table**

Title: A comparison of the incidence of phenotype features of selected conditions in which Holoprosencephaly is common from literature review<sup>1</sup>

#### Key

1. From the references in the text.
2. As we describe four cases we give numbers not a percentage.
3. Presumably a bias introduced by the disease definition.
4. Presumably under-reported.