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ORIGINAL ARTICLE

Functional analysis of novel desert hedgehog gene variants improves the clinical interpretation of genomic data and provides a more accurate diagnosis for patients with 46,XY differences of sex development

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ABSTRACT

Background *Desert hedgehog* (*DHH*) gene variants are known to cause 46,XY differences/disorders of sex development (DSD). We have identified six patients with 46,XY DSD with seven novel *DHH* gene variants. Many of these variants were classified as variants of uncertain significance due to their heterozygosity or associated milder phenotype. To assess variant pathogenicity and to refine the spectrum of DSDs associated with this gene, we have carried out the first reported functional testing of *DHH* gene variant activity.

Methods A cell co-culture method was used to assess *DHH* variant induction of Hedgehog signalling in cultured Leydig cells. Protein expression and subcellular localisation were also assessed for *DHH* variants using western blot and immunofluorescence.

Results Our co-culture method provided a robust read-out of *DHH* gene variant activity, which correlated closely with patient phenotype severity. While biallelic *DHH* variants from patients with gonadal dysgenesis showed significant loss of activity, variants found as heterozygous in patients with milder phenotypes had no loss of activity when tested with a wild type allele. Taking these functional results into account improved clinical interpretation.

Conclusion Our findings suggest heterozygous *DHH* gene variants are unlikely to cause DSD, reaffirming that *DHH* is an autosomal recessive cause of 46,XY gonadal dysgenesis. Functional characterisation of novel *DHH* variants improves variant interpretation, leading to greater confidence in patient reporting and clinical management.

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INTRODUCTION

The highly conserved hedgehog family of signalling molecules plays a central role in the development and differentiation of numerous organs during embryogenesis (Franco:2012iz). Desert hedgehog (DHH), a member of this family, is one of three hedgehog homologues present in mammals, and plays a key role in the development of the gonads. In the XY male, differentiation of the bipotential gonads into the testis involves specification of the supporting cells, the Sertoli cells. A subset of these cells produce and secrete DHH from early in development (embryonic day 11.5 in the mouse). This secreted DHH binds the PTCH1 receptor in the adjacent Leydig cells, activating the hedgehog signalling pathway and triggering differentiation of these steroidogenic cells.[1–3] In the mouse, *Dhh* knockout female mice are viable and fertile but the majority of XY mice are externally female with a blind vagina.[4] In these mice, the testes lacked spermatogonia and had a severe reduction in Leydig cell numbers, and hence reduced testosterone which resulted in typical female appearance.[3] Work in mice has shown that Dhh and its signalling pathway are important for the upregulation of *Nr5a1 (encoding steroidogenic factor 1)*, one of the key genes involved in gonad development and steroidogenesis.[2]

Differences/disorders of sex development (DSD) in humans are defined as congenital conditions where the development of chromosomal, gonadal and anatomical sex is atypical (Chicago Consensus Meeting[5]). DSDs are a heterogeneous group, including 46,XY disorders of testicular development. These patients have a 46,XY karyotype and external genitalia that range from mild undervirilisation phenotypes such as microphallus or hypospadias to normal female appearance (complete sex reversal).[6] These conditions are most often caused by a breakdown in the genetic pathways required for testicular or ovarian differentiation and development. It is well established that pathogenic variants in *DHH* (OMIM 605423, chr12q13.12) are a rare cause of 46,XY DSD, in particular causing 46,XY partial or complete gonadal dysgenesis (OMIM 607080, OMIM 233420). To date, 12 different *DHH* pathogenic variants have been published in 17 cases of 46,XY DSD[7–16] (figure 1). In some cases the patients present with gonadal cancers and/or minifascicular neuropathies.[9 10 13] In 15 of the 17 reported cases the patients had *DHH* variants on both alleles (homozygous or compound heterozygous). Two patients reported to carry a heterozygous variant (p.Leu363Cysfs*4) also had

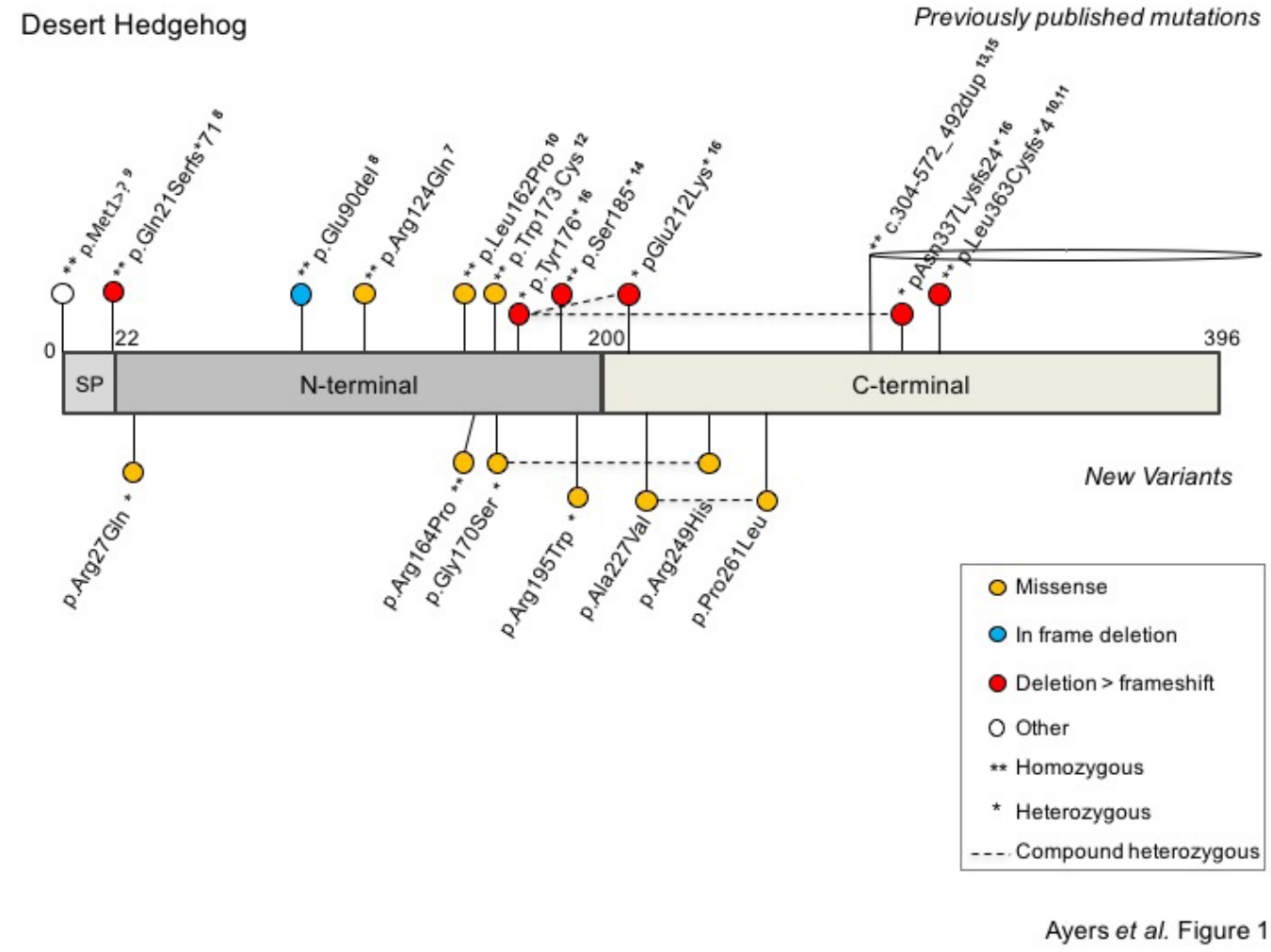

Figure 1 Previously reported and novel variants in desert hedgehog (DHH). A schematic figure showing the protein position of both previously reported variants in DHH (upperside), and the new ones reported in this study (underside). Publication references are in bold superscript after each variant. Yellow circles show missense variants, blue are in-frame deletions and red are deletions causing frameshifts. ** depicts those that were homozygous and * those that were heterozygous, and [?] where the zygosity is unknown. Compound heterozygous variants are depicted by a dotted line joining the two variants.

sex chromosome mosaicism (45,X/46,XY).[11] Based on these reports it could be concluded that when the sex chromosomes are normal, *DHH* variants act in a recessive manner to cause 46,XY complete or partial gonadal dysgenesis. Thus, it is still unclear whether carrying a variant in just one copy of *DHH* causes milder forms of DSD such as a 46,XY undervirilisation phenotype or hypospadias. In NCBI ClinVar there are 28 *DHH* variants reported associated with 46,XY DSD, 17 of which are listed as likely benign or of uncertain significance. Of those that are regarded as pathogenic and likely pathogenic (LP) just one is unpublished—c.T1027C.p.Cys343Arg (figure 1).

Previously, using massively parallel sequencing (MPS) on DNA from 278 patients with 46,XY DSD we reported novel homozygous, compound heterozygous and heterozygous variants in *DHH*.[17] Six patients carrying novel *DHH* variants presented with a wide spectrum of phenotypical severity. Here we detail patient phenotypes, and use the first published functional analysis to assess *DHH* variant activity. Our work highlights the importance of functional analysis for accurate variant curation and reporting in DSD, and refines the spectrum of disorders in which *DHH* likely plays a role.

MATERIALS AND METHODS

Study subjects and clinical evaluation

All patients were recruited by collaborating clinicians after written informed consent (as detailed in[17]).

Targeted gene capture, MPS and data analysis

Genomic analysis was carried out as previously described.[17] Annotations are based on DHH NM_021044.2. Annotations were initially created by our custom pipeline[18] and were also processed through Mutalyser.[19] Sequencing covered the *DHH* exons without gaps and often extended into the intron or UTRs by up to 100 bp (online supplementary figure S1).

Variant curation

Novel variants were curated using a detailed clinically accredited scheme used at the Victorian Clinical Genetics Services. This is based on the American College of Medical Genetics (ACMG) guidelines,[20] and allows classification of variants into one of five categories; pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB) or benign (B). This classification assigns scores based on type of variant, minor

allele frequencies (MAFs), Grantham scores, protein domains, existing classifications of variants (ie, ClinVar), segregation or inheritance, phenotype match and functional evidence. For functional evidence, two categories are considered. The first, which is scored the strongest (either as pathogenic or benign strong) is a functional test of activity where the endogenous tissue/patient cells are used. The second category (pathogenic moderate or benign supporting) is reserved for functional evidence from an assay using an exogenous cell line. VUS are categorised as one of three—VUS3a, VUS3b, VUS3c, based on whether conflicting evidence is sufficient to justify VUS classification. VUS3a (potentially pathogenic) is regarded as a variant that is unambiguously classifiable with predominantly pathogenic evidence, VUS3b is not unambiguously classifiable and VUS3c is not unambiguously classifiable with predominantly benign evidence.

Mutant DHH expression vectors

A human *DHH* expression construct was from OriGene Technologies (True ORF clone RC206715). The mutant *DHH* expression vectors (p.Arg124Gln, p.Leu363Cysfs*4, p.Glu90del p.Leu162Pro, p.Trp173Cys, p.Ala227Val, p.Pro261Leu, p.Gly170Ser, p.Arg249His, p.Gly170Ser, p.Arg195Trp, p.Arg27Gln, p.Arg164Pro) were created by site-directed mutagenesis (QuickChange II XL Site-directed Mutagenesis Kit, Agilent Technologies, Santa Clara, California, USA) according to the manufacturer's instructions (for primer sequences see online supplementary table 1). Sanger sequencing using vector primers was used to confirm the presence of the variant in the vectors.

DHH variant immunofluorescence

HEK293t cells were seeded on an eight-well chamber slide (Nunc), and transfected with DHH expression vectors (wild type [WT] and all mutants) or a PCMV (plasmid containing the cytomegalovirus constituitive promoter)-empty control using Lipofectamine 2000 (Invitrogen). Cells were processed as previously published 24 hours after transfection.[21] Cells were incubated overnight with a goat polyclonal DHH (N-19) antibody (1:300, Santa Cruz sc-1193) and/or a polyclonal rabbit anti-FLAG antibody (1in10000, Sigma). Secondary antibodies used were donkey antigoat Alexa488 (1:1000, Invitrogen) and donkey antirabbit Alexa594 (1:1500, Invitrogen). Nuclear counterstaining was performed with DAPI (**4′,6-diamidino-2-phenylindole**). Cells were imaged on a Zeiss lazer scanning microscope (LSM) 780 confocal microscope. Images were taken at 20× and 40x magnification.

Western blot

HEK293t cells were seeded at approximately 80% confluency on a 24-well plate (3.2×10^5 cells), and transfected with 0.5 ug of pCMV-Flag-DHH expression vector (WT or mutant) using lipofectamine 2000 (Invitrogen). After 24 hours cells were washed with PBS and lysed using Pierce Immuno-precipitation lysis buffer (25 mM Tris HCl pH7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol). Protein was run on a 10% Bis-Tris gel, transferred to polyvinylidene difluoride (PVDF) membrane, blocked using 5% skim milk powder/TBST (Tris-buffered saline with Tween) and incubated with rabbit anti-Flag antibody (1:10000 Sigma [F7425]) overnight at 4°C. After washing, the membrane was incubated with swine anti-rabbit Horseradish peroxidase (HRP) (1:10000 Dako P0399, Agilent) at room temperature for 2 hours. After blot washing the Amersham ECL (**Electrochemiluminescence**) Prime western blotting detection reagent was used and visualised with the GE Healthcare Life Sciences ImageQuant LAS 4000. Blots were washed and then incubated with anti-β tubulin loading control (HRP) (1:10000, Abcam-ab21058) and detected as mentioned above.

Co-culture activity assay

HEK293t cells were seeded at 70%–80% confluency on a 24-well plate (3.2×10^5 cells), and transfected with 0.5 ug of pCMV-DHH expression vector (WT or mutant) or pCMV-empty control using Lipofectamine 2000 (Invitrogen). After 24 hours cells were trypsinised using 0.025% trypsin PBS (phosphate buffered saline) and reseeded at 20% confluency (approx. 1.6×10^5 cells). Tm3 cells (mouse Leydig-like cells; 91060526 Cell Bank Australia) were also added at a 1:1 ratio (1.6×10^5) with the HEK293t cells. The co-culture was left for 48 hours, after which the cells were washed once with PBS and Trizol reagent (Invitrogen) was used for RNA extraction. DNAsing was carried out using the Promega Dnase free kit. RNA was converted to cDNA with the GoTaq reverse transcriptase kit and a mix of oligoDT and random primers (Promega). qPCR (GoTaq qPCR master mix, Promega) was carried out with the mouse *Gli1*, *Ptch1* genes as pathway read-outs, and the Tbp gene as a housekeeper (see supplementary table 1 for primers). Human *DHH* primers were used to assess the level of expression with the *hRPL32* housekeeper primers. The experiment was replicated three times. ΔΔCT (threshold cycle) values were calculated for each target and expressed as a ratio of the control (empty vector). Error bars are based on SEM and p values were calculated using one-way analysis of variance (ANOVA). Activity of each variant is calculated as a percentage of the WT, normalised to the empty, control.

RESULTS

DHH variants in a wide range of patients with DSD

We have previously published the use of a targeted MPS DSD gene panel to find causative variants in patients with DSD.[17] Here we have further characterised seven novel DHH gene variants identified in six patients (table 1). All novel *DHH* variants were rare alleles, with only three of the seven variants found in the online databases ExAC, gnomAD or Exome Variant Server (EVS), and none were found as homozygous on these databases. In gnomAD p.Ala227Val had a total MAF of 0.00005810, p.Arg249His MAF was 0.00003231 and p.Arg27Gln MAF was 0.0001402 (table 1). The patients had a wide range of phenotypes. Patient 1, previously the subject of a clinical report,[22] has a similar clinical presentation to those previously associated with *DHH* variants. This patient has a homozygous p.Arg164Pro variant and is 46,XY with female genitalia and bilateral dysgenetic testes. Variant curation using a clinically accredited scheme based on the ACMG guidelines (materials and methods) classified this DHH variant as LP (table 1). We also present two patients with compound heterozygous *DHH* variants (patients 2 and 3; table 1). Patient 2, a 46,XY female patient with clitoral hypertrophy, a blind ending vagina and no Müllerian duct derivatives, had two palpable gonads present in the labia majora with immature testicular tissue (table 1). This patient had the variants p.Ala227Val and p.Pro261Leu, and visualisation of sequencing reads showed these were present on different alleles (online supplementary figure S2). Each *DHH* variant is curated separately and both were considered VUS3a (table 1). In the second compound heterozygous case (patient 3), a 46,XY female patient with ambiguous genitalia, blind ending vagina and inguinal testis with an unknown histology, the variants p.Arg249His and p.Gly170Ser were identified. We were unable to obtain parental samples, but compound heterozygosity

Table 1 Novel *DHH* variants found in patients with 46,XY DSD

Patient ID	Karyotype	Sex of rearing	External genitalia	Gonadal location and histology (R/L)	Müllerian structures	Neuropathy	DHH variants		ExAC	gnomAD	EVS	Gratham score	Sift	Polyphen 2	Mutation taster	CADD	Clinvar	Curation before functional
1	46,XY	Female	Well-formed labia. Prominent clitorophallic structure. Normal vaginal opening with blind vagina.	L: intra-abdominal R: intra-abdominal. Histopathology: Bilateral dysgenetic testes. No evidence of gonado- blastoma or germ cell neoplasia	Left paragonadal biopsy showed Müllerian tissue resembling oviduct. Right paragonadal biopsy showed vasoepididymal tissue	No sign at 14yo.	NC_000012.11 :g.49484985C>G	NM_021044.2:c.491G>C :p.Arg164Pro	Homozygous	0	0	0	103	Damaging	Probably damaging	Disease causing	32	-	LP
2	46,XY	Female	Clitoral hypertrophy (1.5 cm). Otherwise female outer appearance, short blind ending vagina (2.5 cm)	L: Papable in labia majora. R: Palpable in labia majora. Histopathology: Immature testicular tissue with reduced spermatogenesis and tubuli without lumen.	Not present	No sign at 20yo.	NC_000012.11 :g.49484153G>A NC_000012.11 :g.49484051G>A	NM_021044.2:c.680C>T :p.Ala227Val NM_021044.2:c.782C>T :p.Pro261Leu	Compound heterozygote	0.0001803 0	0.00006461 0	0 0	64 98	Tolerated Tolerated	Possibly damaging Probably damaging	Disease causing Disease causing	23.5 24.6	- -	VUS-3a VUS-3a
3	46,XY	Male	Proximal hypospadias and chordee	L: scrotal R: scrotal. Histology unknown	Not present	Not reported	NC_000012.11 :g.49484087C>T NC_000012.11 :g.49484968C>T	NM_021044.2:c.746G>A :p.Arg249His NM_021044.2:c.508G>A :p.Gly170Ser	Compound heterozygote	0 0	0.00003231 0	0 0	29 56	Damaging Tolerated	Probably damaging Probably damaging	Disease causing Disease causing	28 32	- -	VUS-3a VUS-3a
4	46,XY	Male	Severe hypospadias	L: scrotal R: scrotal. Histology unknown	Not present	Not reported	NC_000012.11: g.49484968C>T	NM_021044.2:c.508G>A :p.Gly170Ser	Heterozygous	0	0	0	56	Tolerated	Probably damaging	Disease causing	32	-	VUS-3a
5	46,XY	Male	Severe hypospadias	L: inguinal, R: scrotal. Histology unknown	Potential remnant	Not reported	NC_000012.11: g.49484250G>A	NM_021044.2:c.583C>T :p.Arg195Trp	Heterozygous	0	0	0	101	Damaging	Probably damaging	Disease causing	32	-	VUS-3a
6	46,XY	Male	Severe hypospadias	L: scrotal R: scrotal. Histology unknown	Not present	Not reported	NC_000012.11: g.49488216C>T	NM_021044.2:c.80G>A :p.Arg27Gln	Heterozygous	0.00029	0.0001402	0	43	Damaging	Probably damaging	Disease causing	29.9	VUS (Invitae)	VUS-3a

Patient phenotypes are described along with the *DHH* variant(s) found. Minor allele frequencies of each variant are shown from the Exome Aggregation Consortium (ExAC), the Genome Aggregation Database (GnomAD)and Exome Variant Server (EVS) databases. Grantham Score and CADD (combined annotation dependent depletion) score are shown. In silico pathogenicity predictions from three programmes (Sift, polyphen and mutation taster) are shown. Variant curation following ACMG guidelines was performed prior to functional analysis.
ACMG, American College of Medical Genetics; DHH, desert hedgehog; DSD, differences/disorders of sex development; LP, likely pathogenic; VUS, variant of uncertain significance.

was confirmed by cloning the genomic fragment containing these two variants (online supplementary figure S3). Based on the existing data, both variants were classified as VUS3a, due to conflicting in silico predictions and low Grantham Scores as well as a lack of functional evidence to support pathogenicity (table 1). We also found three patients with heterozygous variants (table 1). Patient 4 was a 46,XY male patient with severe hypospadias, testes in the scrotum (p.Gly170Ser). Patient 5 (p.Arg195Trp) was a 46,XY male patient with one testis in the scrotal region and one inguinal gonad of unknown histology (table 1). Finally, a p.Arg27Gln change was found in patient 6, a 46,XY male patient with severe hypospadias (table 1). None of the patients had a reported peripheral neuropathy. The heterozygous variants found in patients 4, 5 and 6 were also classified as VUS3a (possibly pathogenic), in part due to the lack of evidence that *DHH* variants can cause DSD in an autosomal dominant manner (table 1).

Novel DSD-associated *DHH* gene variants are found throughout the protein

In humans, the *DHH* gene is autosomal (12q12.13) and comprises three exons encoding a 396 amino acid protein.[23] DHH, like all hedgehog proteins is produced as a 45 kDa precursor and the 25 kDa C-terminal drives autolytical cleavage assisted by the covalent addition of cholesterol moiety to the N terminus. This releases the 19 kDa N-terminal fragment, which acts as the active molecule. All of these features are thought to ensure proper secretion and efficient extracellular movement of HH proteins, allowing them to act as secreted morphogens (reviewed in[24]). All previously described *DHH* variants in patients with normal sex chromosome complement are homozygous or compound heterozygous (figure 1). While our biallelic variants fall in both the N-terminal and C-terminal regions, our heterozygous variants are all found within the N-terminal (figure 1). The *DHH* gene is highly conserved and shows constraint against both missense variants and loss of function (LOF) variants in ExAC (z=3.96 and probability of loss of function intolence/PLI=0.26). All of our variants affect highly conserved amino acids, in some cases conserved back to the single hedgehog protein in fruit flies (online supplementary figure S4).

Novel DHH variants do not alter DHH cellular localisation

To assess cellular localisation, the WT and mutant DHH alleles were transfected into HEK239t cells with an expression vector carrying a C-terminal FLAG tag. This allowed detection of the DHH protein with both an anti-DHH antibody (raised against an N-terminal peptide, detecting both cleaved N-terminal and full-length DHH) and an anti-FLAG antibody which will detect full-length DHH and C-terminal DHH. In WT DHH-FLAG overexpression we found that DHH was mostly expressed in the cytoplasm and at the cell membrane (figure 2A and online supplementary figure S5), with no nuclear staining. In general, good overlap was seen between the Flag and DHH antibodies (online supplementary figure S5), the former of which was more sensitive and stained some cells more strongly (online supplementary figure S5). This may be an indication that the majority of the DHH detected in the cells is uncleaved, consistent with previous reports.[25] The only DHH variant that had significantly reduced FLAG staining intensity was the previously reported p.Leu363Cysfs*4 variant in which deletion of a single nucleotide led to a frameshift and lack of a C-terminal Flag tag (online supplementary figure S5 and *data not shown*). Good overlap was shown for the remaining variants, and therefore we have shown only the anti-DHH staining henceforth. DHH staining revealed no clear differences in subcellular localisation for the previously published variants (figure 2B–E) or the novel variants reported here (figure 2F–L). The p.Arg164Pro variant appeared to have reduced overall number of cells showing expression compared with the WT (online supplementary figure S6F). Given that immunofluorescence is not quantitative, we assessed protein levels using western blot analysis. Anti-FLAG and anti-DHH staining showed just one band corresponding to uncleaved DHH at 43.5 kDa (online supplementary figure S5G,H). No full length protein was observed for the p.Leu363CysFs*4 variant, whereas the other variants showed WT protein levels (figure 3).

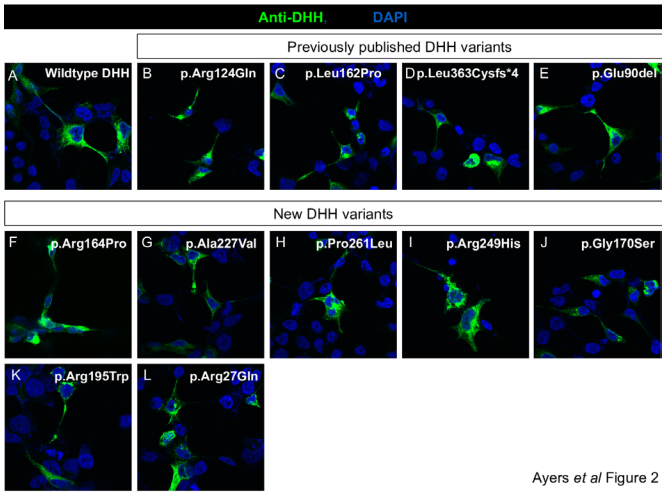

Figure 2 Variants in desert hedgehog (DHH) do not affect protein localisation. Immunofluorescence staining for HEK293t cells transfected with the different DHH variants, using a DHH antibody (green) and co-stained with DAPI (4′,6-diamidino-2-phenylindole) (blue). (A). Wild type DHH is expressed throughout the cytoplasm and at the plasma membrane. This staining is also seen for the four previously published pathogenic DHH variants (B–E). (F–L) Staining for the new DHH variants reported here. None of the variants appeared to significantly affect protein levels or localisation in this assay, yet the p.Arg164Pro variant had reduced overall number of cells with staining (see online supplementary figure S6).

A cell co-culture assay is a sensitive test of *DHH* gene variant activity

Functional assays can be a powerful tool in assessing variant pathogenicity. The ACMG guidelines for the interpretation of sequence variants[20] assigns a PS3 (pathogenic strong) to 'well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product'. Conversely, if this assay shows no effect on protein function, a BS3 (benign strong) score is assigned. We therefore sort a reliable in vitro assay to test *DHH* variant activity. Previous studies have shown that DHH is not effectively cleaved or secreted from cells, preferentially activating the hedgehog signalling pathway in adjacent cells via paracrine activity.[25] To test the paracrine activity of each DHH mutant compared with WT, we used a previously reported co-culture assay[25] (figure 4A,B). In this system, HEK293t cells are transfected with the DHH expression plasmids for 24 hours, after which these DHH-expressing cells are cultured with mouse Leydig-like cells (Tm3), which are responsive to the DHH signal (figure 4A,B). After 48 hours of co-culture, RNA is extracted and qPCR is used to assess the levels of expression of hedgehog pathway target genes *Ptch1* and *Gli1* in the Tm3

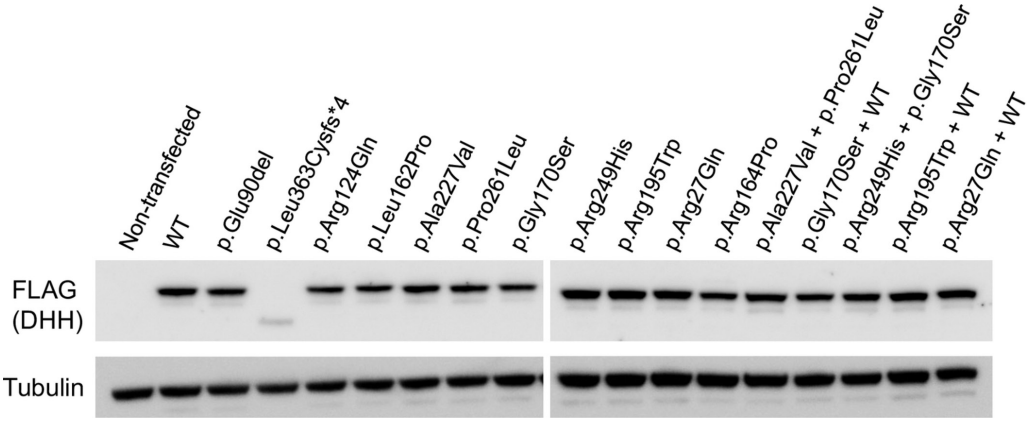

Figure 3 New desert hedgehog (DHH) gene variants do not significantly affect protein expression. Western blot analysis of protein from overexpression of DHH variants in HEK293t cells, using an anti-FLAG antibody. Tubulin is used as a control. Only the previously reported p.Leu363Cysfs*4, which causes a frameshift, caused a loss of DHH protein, seen as a smaller fragment due to the premature stop codon introduced. Staining with an anti-DHH antibody showed similar results (online supplementary figure S3 and data not shown).

cells, using mouse-specific primers. In this assay, we found that overexpressed WT human DHH induced *mGli1* expression 18-fold compared with an empty plasmid control. We found that *mPtch1* levels were less sensitive where WT DHH induced *mPtch1* expression twofold to threefold compared with the control. To validate the method, we tested four of the previously reported pathogenic variants and found that all of these had a significant reduction in activity compared with WT, observed for both *mGli1* (figure 4C) and *mPtch1* (figure 4D) expression levels. Of the four known DHH variants tested, three had almost complete loss of activity, (<10% of WT activity; p.Glu90del, p.Leu363Cysfs*4, p.Leu162Pro) whereas p.Arg124Glu retained around 30% of WT function (figure 4C,D). The three alleles with a complete LOF were found in 46,XY female patients with complete gonadal dysgenesis,[8 10 11] whereas the p.Arg124Glu variant was reported in[7] two 46,XY female sisters with the slightly milder phenotype of partial gonadal dysgenesis. Thus, DHH variant activity measured in this assay correlates with phenotype severity.

We next tested our seven novel variants. Using the *mGli1* read-out all variants but one (p.Arg27Gln) had reduced activity when tested individually (figure 4E). Those found in the most severely affected patients (46,XY female patients; patient 1 and patient 2) had the most reduced activity, with less than 2% of WT activity (figure 4E, table 2). Even when combined, the variants found in patient 2 had an activity of around 2% of the WT allele (figure 4E and table 2). The other patient with biallelic DHH variants (patient 3) who was less severely affected (46,XY male patient with hypospadias) had variants p.Gly170Ser and p.Arg249His, which showed a 26% and 47% activity, respectively, in this assay (figure 4E and table 2). Combined, these variants had an activity of 46% of the WT allele (table 2). Finally, two of the three heterozygous variants found in patients had a reduced activity (p.Gly170Ser, 47% and p.Arg195Trp, 16%, table 2). However, when these variants were tested in combination with the WT allele, to model the in vivo environment, they appeared to lose this inactivity, presenting activities close to WT (90% and 98%) (figure 4E and table 2). Finally, the heterozygous variant p.Arg27Gln found in patient 6 had no significant loss of activity compared with WT, either alone or in combination with WT DHH (figure 4E and table 2). *mPtch1* expression, although a less sensitive read-out, showed similar results (figure 4F and table 2), with variants present as homozygous or compound heterozygous tending towards LOF, whereas those present as heterozygotes show activity closer to WT DHH (figure 4F and table 2). Importantly, qPCR analysis found that DHH expression from all constructs was similar (online supplementary figure S7). Together, these data show that the co-culture assay is a sensitive read-out of *DHH* activity and variant pathogenicity.

Functional studies alter DHH variant curation and reporting

Having established that we can test DHH variant activity in vitro, the results of which are highly consistent with patient phenotypes and additional variant data, we now sought to assess whether functional outcomes would change variant curation. We performed a second curation taking into account the overall outcomes of the functional studies. For patient 1, inclusion of the functional data, showing LOF, did not give enough additional evidence to change the variant curation, which remains at LP (table 2). Patient 2 had two variants in trans, which were each considered VUS3a initially. When the LOF observed in the co-culture assay was taken into account, this additional evidence was sufficient to elevate these variants to LP. Patient 3 also had biallelic DHH variants, p.Arg249His and p.Gly170Ser, which were initially classified as VUS3a. Following our findings that each of these had a loss of activity, separately and combined, the p.Arg249His variant was elevated to LP, while the p.Gly170Ser variant remained at VUS3a (table 2), largely due to its conflicting in silico predictions (table 1). Interestingly, this variant was also found as a heterozygous variant in patient 4, where it was initially classified as VUS3a. Our functional findings suggested that when combined with the WT allele, as it would be in vivo, no significant loss of activity was observed—providing evidence for this variant being benign. Therefore this variant in this patient was downgraded to VUS3b (not unambiguously classifiable) (table 2). Patient 5 had a heterozygous p.Arg195Trp allele initially classified as VUS3a. Our data suggest that when alone, this allele shows a severe LOF (between 2% and 16% of WT activity), whereas when combined with the WT allele, no significant loss of activity is observed. This, combined with other data including the heterozygous state not previously shown to cause DSD, lead to a curation of VUS3b (not unambiguously classifiable). Finally, patient 6, also carrying a heterozygous DHH allele, p.Arg27Gln classified as VUS3a, was downgraded to VUS3c, due to the overwhelming evidence

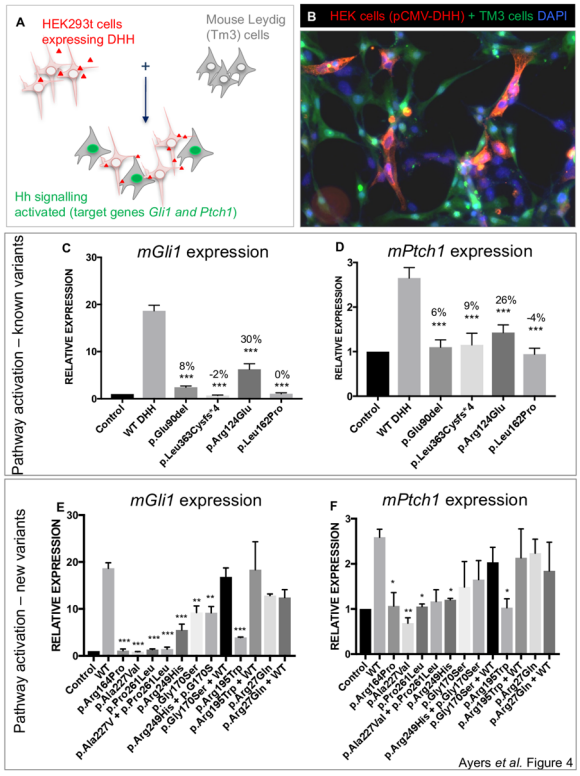

Figure 4 A co-culture assay for functional analysis of desert hedgehog (DHH) variant activity. (A) A schematic representation of the co-culture assay, where human embryonic kidney/HEK293t cells are transfected to express DHH (wild type [WT] or variant), and are co-cultured directly with mouse Leydig (Tm3) cells. DHH secreted by the HEK cells directly activates the Hedgehog signalling pathway in the adjacent Tm3 cells, assessed by qPCR analysis of pathway targets Gli1 and Ptch1. (B) Immunofluorescence staining of the co-culture, showing the HEK cells expressing DHH (red) growing in close proximity with the Tm3 cells (stained with Carboxyfluorescein succinimidyl ester (CSFE) green). DAPI stains nuclei (blue). (C) *mGli1* expression in the co-culture assayed by qPCR, for four previously reported *DHH* variants. (D) *mPtch1* expression in the co-culture assayed by qPCR, for four previously reported *DHH* variants. (E) *mGli1* expression in the co-culture assayed by qPCR, for novel *DHH* variants reported here, either alone or in combination (where found in compound heterozygous or heterozygous format). (F) *mGli1* expression in the co-culture assayed by qPCR, for novel *DHH* variants reported here, either alone or in combination. For all qPCRs the mouse *Tbp* gene is used as a housekeeper. Expression is relative to *Tbp* and the control (empty plasmid). Three biological replicates were performed and each qPCR was performed with technical triplicates. P values are calculated using one-way ANOVA. *p>0.05, **p>0.01 ***p>0.001. Activity is calculated as a percentage of WT DHH relative to the negative control.

that alone or with combination with the WT allele, this variant exhibits no LOF (table 2).

DISCUSSION

DSDs represent a major paediatric concern as they are estimated to affect 1.7% of live births.[26] We have previously shown that MPS can deliver a diagnosis rate of up to 43% in 46,XY DSD.[17] In the 52 patients with a disorder of testicular development, biallelic *DHH* variants were observed in four patients (contributing to 7.7%) and three patients carried a single *DHH* variant (heterozygous).[17]

The ACMG variant curation guidelines[20] use functional evidence from 'well-established functional assays' as strong evidence for or against variant pathogenicity (PS3 or BS3). The authors state that 'one must consider how closely the functional assay reflects the biological environment' and they give more weight to assays that use biopsied tissues. Unfortunately, access to biopsied gonadal tissue from 46,XY female patients is not often possible, and it is not clear that this dysgenic gonadal tissue would allow direct testing of DHH activity, which is important earlier in embryonic and fetal development. Thus a robust test of DHH variant activity using in vitro or cell culture methods is required. A recent review discussing the nuances of the ACMG guidelines suggests that any model for variant activity should be closely related to the disease aetiology and that a 'well-validated functional assay should provide variant level evidence of the effect on the gene or gene product'.[27] Here, we have used a co-culture cell method to functionally test *DHH* variants, specifically assessing their ability to activate the HH signalling pathway in Leydig cells, recapitulating the main function of DHH in the gonad. This is the first report of a functional test to assess the activity of DHH variants *in vitro*. Strande *et al* state that functional assays 'require benchmarking against multiple variants with definitive clinical interpretations as determined by genetics or other evidence'.[27] Testing of four previously reported pathogenic *DHH* variants in our co-culture method revealed that the functional activity of each variant in our assay correlated closely with gonadal phenotypical severity. Three of these variants; p.Leu363cysFS*, p.Arg124Glu and p.Leu162Pro, are reported in ClinVar as pathogenic, consistent with our functional studies which showed loss of activity, consistent with phenotype severity. Furthermore, one of patients had a homozygous missense variant—patient 1, who was a 46,XY female patient with complete gonadal dysgenesis. Consistent with this clinical phenotype, the variant found in this patient (p.Arg164Pro) demonstrated a complete lack of function in our assay. Our data suggest that our functional assay is a robust and reliable test for DHH variant gonadal activity. There are currently 11 non-synonymous DHH variants classified as VUS for 46,XY DSD in ClinVar, and we believe that out functional assay could be used to validate these variants.

While it is well established that biallelic *DHH* variants (in a homozygous state) cause DSD, this is just the second report of patients carrying two different *DHH* alleles (compound heterozygous), giving us the rare opportunity to functionally characterise each variant separately and in combination. Patient 2 was a 46,XY female patient carrying the variants p.Ala227Val and p.Pro269Leu which had an almost complete LOF, leading to a change in curation of the variants from VUS3a to LP. Patient 3 was also biallelic, carrying the p.Arg249His and p.Gly170Ser variants. This patient had the milder phenotype of 46,XY male with proximal hypospadias and chordee. Interestingly, both of these alleles had a milder reduction in function—between 13% and 50%. When tested in combination they had an activity around 45% of the WT DHH. These data confirm that *DHH* variants can cause DSD in a compound heterozygous inheritance pattern.

While it is well established that *DHH* variants lead to autosomal recessive DSD, it was unclear whether heterozygous *DHH* variants contribute to milder undervirilisation phenotypes such as proximal hypospadias or micropenis. Here we report three patients with normal sex chromosome complements and heterozygous *DHH* variants. These patients were all 46,XY male patients with severe hypospadias, and two patients had testis in the scrotum while the third had one undescended testis. All variants were initially curated as VUS. Our functional testing

Table 2 *DHH* gene variant functional activity and second curation

Patient ID	DHH variants		Curation before functional	% of WT activity (*mGli1*)	Adjusted p value (*mGli1*)	% of WT activity (*mPtch1*)	Adjusted p value (*mPtch1*)	Final curation
1	NM_021044.2:c.491G>C:p.Arg164Pro	Homozygous	LP	1%	<0.0001	4%	0.0398	LP
2	NM_021044.2:c.680C>T:p.Ala227Val	Compound	VUS-3a	−1%	<0.0001	−20%	0.0059	LP
	NM_021044.2:c.782C>T:p.Pro261Leu	heterozygote	VUS-3a	2%	<0.0001	4%	0.0377	LP
				Combined 2 %	*<0.0001*	*Combined 11 %*	*0.409*	
3	NM_021044.2:c.746G>A:p.Arg249His	Compound	VUS-3a	26%	0.0002	13%	0.0448	LP
	NM_021044.2:c.508G>A:p.Gly170Ser	heterozygote	VUS-3a	47%	0.0096	31%	0.2342	VUS-3a
				Combined 46 %	*0.0091*	*Combined 41 %*	*0.409*	
4	NM_021044.2:c.508G>A:p.Gly170Ser	Heterozygous	VUS-3a	47%	0.0096	31%	0.2343	VUS-3a
				Combined 90 %	*0.9956*	*Combined 65 %*	*0.9155*	
5	NM_021044.2:c.583C>T:p.Arg195Trp	Heterozygous	VUS-3a	16%	<0.0001	2%	0.0334	VUS-3a
				Combined 98 %	*0.9999*	*Combined 72 %*	*0.9777*	
6	NM_021044.2:c.80G>A:p.Arg27Gln	Heterozygous	VUS-3a	67%	0.9956	78%	0.9957	VUS-3c (potentially benign)
				Combined 65 %	*0.1674*	*Combined 53 %*	*0.6787*	

Results from co-culture functional assay are shown, including percentage of WT activity and p values for both *mGli1* and *mPtch1* read-outs. Where variants were found as compound heterozygous or heterozygous, functional read-outs for combined alleles are also shown. Curation outcomes for each variant taking these functional outcomes into account are also shown.
DHH, desert hedgehog; LP, likely pathogenic; VUS, variant of uncertain significance; WT, wild type.

revealed that p.Gly170Ser, present in patient 4, had a partial LOF when tested alone meaning that it may be functionally pathogenic, but when in combination with WT DHH (as it would be in the patient), it had no significant LOF. This leads us to conclude that this variant will only cause DSD when in combination with a second allele, as is seen in patient 3, and is therefore unlikely to be the definitive or sole cause of DSD in patient 4. Thus clinically, this variant remains a VUS3a. Similarly, the p.Arg195Trp allele (patient 5) had an almost complete LOF functionally, but when combined with the WT allele, no LOF was observed—again meaning that clinically it is unlikely to be considered pathogenic. Finally, the p.Arg27Gln allele found in patient 6, had no significant LOF either alone or with WT *DHH*, suggesting that unlike the others, this is a benign Single Nucleotide Variant (SNV) both functionally and clinically. This led us to reduce the curation from VUS3a to VUS3c, meaning this variant is unambiguously classifiable with predominantly benign evidence. Thus, taken together our data lead us to conclude that heterozygous *DHH* variants are unlikely to cause DSD alone. It must be pointed out that our genetic sequencing only covers the coding regions of the *DHH* gene, extending into the introns by about 100 bp. Therefore, we cannot discount the small possibility that we have missed a second variant in these patients that lies deep within the introns or the five or three prime regions. In addition, it is possible that a second hit in another gene within the DHH signalling pathway or testicular pathway could be contributing.

The molecular mechanism underlying the loss of activity in our variants is not known. Neither qPCR nor western blot suggested a significant loss of mRNA or protein for any of our novel *DHH* variants. No change in localisation was observed in immunofluorescence (IF), although p.Arg164Pro did show reduced number of cells with staining, HOPE analysis[28] suggests that this change (from a positive to a smaller neutral amino acid) will have severe effects on the structure of the protein as it lies within an α-helix, and forms a hydrogen bond with Val41 and E168. However, there did not appear to be reduced protein stability in western blot analysis. Thus, while our co-culture analysis provides an excellent read-out for DHH activity, which appears to correlate with gonadal phenotypical severity, further studies are required to pinpoint the exact disease mechanisms of each variant.

We had variants that fell in both the N-terminal and C-terminal domains of DHH. While it is well established that the N-terminal of DHH is the active molecule, the exact function of the C terminus is unclear. A recent study from the Werner Lab has found that, given the right conditions (addition of Dithiothreitol or β-mercaptoethanol), a cell-free in vitro transcribed and translated human fusion DHH protein does undergo cleavage.[16] In addition, they have shown that two variants found in patients within the C terminus lead to reduced or absent cleavage efficiency. Nevertheless, there is currently little evidence for DHH cleavage in testis cells, and studies have found that DHH C-terminal is less efficient in cleaving the Sonic Hedgehog (SHH) N-terminal, and that unlike SHH, DHH proteins function in cell-cellcontact-mediated juxtacrine signalling.[25] Indeed, like us, Pettigrew *et al* found that DHH expressed in HEK 293t cells was mostly uncleaved and yet it was still able to function in juxtacrine signalling. Tajouri *et al* suggest that DHH autoprocessing is necessary to mediate signalling from Sertoli cells to promote differentiation of the Leydig cells.[16] While this may be the case in vivo, our cell culture experiments show that while DHH expressed in this system is mostly uncleaved, variants that lie within the C-terminal domain can still have a significant effect on signalling activation in TM3 mouse Leydig cells. Interestingly, one of our C-terminal variants, p.Ala227Val, affects a residue important for autoprocessing in SHH.[29] Thus, it appears our assay is equally able to assess the pathogenicity of both N-terminal and C-terminal variants. As it is possible that a small amount of cleavage is occurring (undetectable in western blot), it would be interesting to test the cleavage efficiency of these C-terminal variants in the future.

Receiving a genetic diagnosis in DSD is important for many reasons—it can reduce stigma and blame, and increase acceptance. An early genetic finding can also reduce the 'diagnostic odyssey', including uninformative clinical tests patients may be subject to. As *DHH* gene variants are most often autosomal recessive, a genetic finding also informs family planning. Of the six patients reported here, four carried two affected *DHH* alleles raising the possibility of familial inheritance and importance of cascade/carrier testing. A DHH-associated DSD genetic diagnosis can also guide clinical management and patient monitoring, in particular, in relation to gonadal malignancies and neuropathies. Five patients with DHH pathogenic variants and gonadal cancers (including seminoma in situ, dysgerminoma and gonadoblastoma) have been reported.[7 10 11] Both homozygous missense variants and nonsense variants appear to cause these,

and our functional studies did not reveal any trend between the variants that caused malignancies and those that didn't. Importantly, a *DHH* genetic diagnosis alerts the clinicians to increase monitoring of these patients.

DHH is also expressed in the Schwann cells of the peripheral nervous system where it signals the formation of the connective tissue sheath around peripheral nerves and is key for their survival. In *Dhh* null mice, peripheral nerve defects are observed.[30] Seven of the 17 patients previously reported with DHH-associated DSD also had neuropathy, usually presenting between the ages of 20 years and 30 years.[7 9 13 14] While none of our patients reported any signs of peripheral neuropathy, in several cases the genetic diagnosis prompted clinicians to test for this, and continued monitoring will now be carried out. Tajouri *et al* hypothesised that C-terminal variants affecting autoprocessing are more likely to lead to gonadal dysgenesis without neuropathies, as even unprocessed DHH protein may induce peripheral nerve development.[16] Interestingly though, our patient with a homozygous variant in the N-terminal (patient 1; p.Arg164Pro) which showed a complete LOF has not reported neuropathies. We suggest that until larger cohorts of patients with DHH-associated DSD are followed longitudinally, all patients with DHH variants be monitored.

In conclusion, we report a functional assay that can assess DHH variant activity. Taking the results of this assay into account during variant curation improves the clinical interpretation of genomic data and provides a more accurate diagnosis for patients with 46,XY DSD.

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Contributors KA and JvdB carried out sequencing analysis and functional work. KA designed the study and wrote the manuscript with AS. GR carried out sequencing analysis and compiled patient data. NL carried out cloning/allelic segregation. JR, NL, IA, SR, KR, CC, SMHF consulted with and recruited patients and collected clinical information.

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