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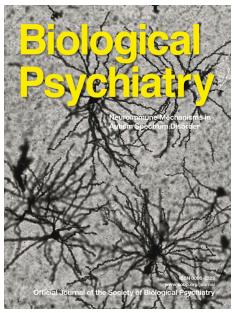

Partial loss of USP9X function leads to a male neurodevelopmental and behavioural disorder converging on TGFβ signalling

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TITLE

Partial loss of USP9X function leads to a male neurodevelopmental and behavioural disorder converging on TGFβ signalling.

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KEY WORDS: USP9X, Deubiquitylating Enzyme, TGFβ, Hippocampus, Brain Malformation, Neurodevelopmental Disorder

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17 Supplemental Figures

7 Supplemental Tables

1 Supplemental Clinical Description

ABSTRACT:

BACKGROUND: The X-chromosome gene *USP9X* encodes a deubiquitylating enzyme that has been associated with neurodevelopmental disorders (NDDs) primarily in females. *USP9X* escapes X-inactivation, and in females *de novo* heterozygous copy number loss or truncating mutations cause haploinsufficiency culminating in a recognisable syndrome with intellectual disability (ID), signature brain and congenital abnormalities. In contrast, the involvement of *USP9X* in male NDDs remains tentative.

METHODS: We collected and interrogated the pathogenicity of 44 male-ascertained *USP9X* variants associated with NDDs using clinically recommended guidelines. Functional studies in patient derived cell lines and mice were used to determine mechanisms of pathology.

RESULTS: Twelve missense variants showed strong evidence of pathogenicity. We define a characteristic phenotype of the CNS (white matter disturbances, thin corpus callosum and widened ventricles), global delay with significant alteration of speech, language and behaviour, hypotonia, joint hypermobility, visual system defects and other common congenital and dysmorphic features. Comparison of *in silico* and phenotypical features align additional variants of unknown significance with likely pathogenicity. In support of partial loss-of-function mechanisms, using patient derived cell lines we show loss of only specific USP9X substrates which regulate neurodevelopmental signalling pathways and a united defect in TGF signalling. In addition, we find correlates of the male phenotype in *Usp9x* brain-specific knockout mice, and further resolve loss of hippocampal dependent learning and memory.

CONCLUSION: Our data demonstrate the involvement of *USP9X* variants in a distinctive neurodevelopmental and behavioural syndrome in males and identify plausible mechanisms of pathogenesis centred on disrupted TGFβ signalling and hippocampal function.

INTRODUCTION

USP9X is a highly conserved X-chromosome gene encoding a substrate specific deubiquitylating enzyme (1). Complete *Usp9x* loss of function (LOF) is embryonic lethal in mouse (2), and homo- or hemizygous complete LOF germline mutations have never been identified in human. We previously reported the identification of 17 females with neurodevelopmental disorders (NDDs) due to *USP9X de novo* heterozygous complete LOF mutations (predominately early frame shift / stop gain mutations) (3). *USP9X* escapes X-inactivation and in these subjects the mRNA and protein levels are significantly reduced. The phenotype is recognisable, and involves intellectual disability (ID), structural brain abnormalities, characteristic facial features, and distinctive congenital malformations (3). We also reported two missense variants and a truncating frame shift variant (escaping NMD) associated with male intellectual disability (4). These variant proteins retained core enzymatic activity, and instead impaired specific USP9X 'brain functions' including neuronal migration and growth (4). Two additional novel missense variants were also implicated in epilepsy, one *de novo* and likely pathogenic, and another of unknown significance (5). Thus the involvement of *USP9X* remains only tentatively associated to nonspecific male NDDs.

USP9X has a central deubiquitylating catalytic domain and long N- and C-terminal extensions used to mediate substrate recognition (1). USP9X interacts with at least 53 proteins, each in a tissue and context dependent manner. USP9X deubiquitylates substrates, typically antagonising their proteasomal degradation and as such stabilising their levels (1). In brain, many USP9X substrates are encoded by NDD-associated genes (1), whilst others regulate neurodevelopmental signalling pathways including TGFβ, Notch, Wnt and mTOR (6-15). Conditional deletion of *Usp9x* in the embryonic forebrain alters these signalling pathways, and causes defective neural progenitor cell function, neuronal cell growth and maturation (4, 7, 12, 16-18). Prominent anatomical features of these mice include agenesis of the corpus collosum, and loss of post-natal hippocampal growth (17, 18). Establishing behavioural phenotypes of these mice is critical to establish models of human NDDs involving *USP9X*.

Here we interrogate 44 additional *USP9X* missense variants in males with NDDs, establish a characteristic clinical phenotype and resolve key features in knockout mice. We use patient derived cell lines to discover molecular mechanisms involving neurodevelopmental signalling pathways. Our data underscore the relevance of partial LOF effect of human *USP9X* variants and point to a loss of TGFβ signalling and hippocampal function as major contributors to pathology.

METHODS

Subjects

This study was approved by the Women's and Children's Health Network Human Research Ethics Committee, South Australia, Australia (HREC786-07-2020). All subject information was provided following informed parental consent (Table S7).

Cell Culture

Primary fibroblasts were maintained as previously described (3). TGFβ luciferase assays were conducted as previously described (18) in biological quadruplicate using 20ng/ml TGFβ (R&D Systems, In Vitro Technologies, Australia). Scratch migration assays were conducted as previously described (19). Number of cells migrating into the scratch were quantified using ImageJ (NIH, USA). Assays were blinded to genotype, and biological triplicates were assayed in technical triplicate. TGFβ-SMAD4 localisation assay were performed by incubating cells without serum for 8 hours prior to addition of 20ng/ml TGFβ. Assay was conducted blinded to genotype and conducted across 5 experiments.

Immunofluorescence

Immunofluorescence was performed as previously described (20). List of antibodies is provided in Table S6.

Biochemical Analysis

Protein isolation and western blots were performed as previously described (3). List of antibodies is provided in Table S6. RNA isolation and qPCR was described previously (3).

Proteomics

Immunoprecipitation was as previously described (4). Rabbit IgG or anti-USP9X antibody (5 µg/treatment; A301-350A, Bethyl Laboratories, USA) were used. Proteins were identified and quantified using tandem-mass-tag 1D-liquid-chromatography Electron-Spray-Ionisation tandem-mass-spectrometry by Australian Proteome Analysis Facility, Sydney Australia. Raw data was searched using Proteome Discoverer v2.1 to identify proteins. Raw quantitative values were mean normalized to handle batch effects and log transformed. Paired t-tests identified proteins significantly enriched (adjusted $p<0.05$ and fold change>0.5) in USP9X (wildtype and mutant) IPs compared to IgG controls.

Mouse husbandry

Usp9x$^{LoxP/LoxP}$ female mice (129SvJ / C57Bl6 mixed background) and Emx1-*Cre* male mice (C57Bl6 background) were crossed as previously described (17, 18). As Usp9x is located on the X chromosome, male offspring that inherit the Emx1-Cre allele lacked Usp9x in the telencephalon and derived cortex and hippocampal structures (referred to as Usp9x$^{-/Y}$; Emx1-Cre or simply knockout mice). Cre-negative males were used as controls (referred to as Usp9x$^{loxP/Y}$ or simply wildtype). Female mice were not analysed.

Open Field Test

Locomotor behaviour was assessed in adult mice as previously described (21). Ethovision XT software (Noldus Information Technology, NLD) recorded distance travelled over a 30 minute test period and data were assessed in six 5 minute time-bins.

Primary SHIRPA screen

Adult mice were screened for gross neurological deficits using a primary SHIRPA (SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St. Mary's; Royal London Hospital, St. Bartholomew's and the Royal London School of Medicine; Phenotype Assessment) screen (22). Mice were observed in a cylindrical viewing jar for 5 min, transferred to an arena (45 cm × 45 cm), followed by a series of anatomical and neurological measures, including assessments of muscular, spinocerebellar, sensory, neuropsychiatric and autonomic functions.

Active place avoidance task

Adult animals included (6-7 months) *Usp9x $^{-/Y}$; Emx1-Cre* (n = 17) knockout and *Usp9x$^{loxP/Y}$* control (n=16) mice. Littermates were raised together regardless of genotype. Test mice were placed onto a rotating platform arena within a room marked by visual cues (23). Upon entering the stationary shock zone, mice received electric shocks (0.5 milliamps at 15 ms intervals) until they exited. Habituation consisted of exploration without shock. The following 5 consecutive days, mice were placed on the rotating platform for 10 minutes with active shocks. Data was acquired using Ethovision™XT software. Testing and analysis was performed blind to genotype. Two-way ANOVA was performed involving two independent variables, with repeated measures if applicable. Multiple comparisons were adjusted (Bonferroni correction). Statistical significance was set at $p<0.05$.

Histology

Histology and immunofluorescence was conducted as previously described (17).

RESULTS

Identification of de novo and inherited USP9X missense variants in affected males

Targeting males with NDDs, we discovered 48 cases with 44 unique *USP9X* variants (3 were recurrent), primarily through trio-based exome sequencing (Table S1). Two of these subjects were obtained via DECIPHER (UK1 = Decipher Patient: 260068; and Netherlands 2 = Decipher Patient: 323395; (24)). The clinical history of each case is provided in Supplemental Information. We classified each variant's pathogenicity in accordance with the American College of Medical Genetics and Genomics (ACMG) guidelines (Figure S1); (25). Eleven variants (13 families) were classified as likely benign (Table S1). The remaining 33 variants (in 35 families) were considered the most plausible genetic cause, either being the sole genetic finding, or prioritised over other variants of unknown/unlikely significance (Table S2). Nine *de novo* variants were classified as likely pathogenic (Table S1 and Figures S1-S2). Three of these located in the catalytic domain, however structural homology modelling mapped these variants to positions outside of the core catalytic site (Figure S3 and S4); (26). The remaining 24 variants (26 families) were maternally inherited, of which segregation beyond trio analysis was able to be performed in 11 cases (Table S1). We conducted functional studies to provide evidence of pathogenicity of three such inherited variants (see below) (Figure S2). Another variant p.Ala2481fs*17 was recurrent in 3 unrelated cases (Table S1). However, this variant impacted only the longer of two alternative coding *USP9X* isoforms, and was intronic in the short (Figure S5). Interpretation was further complicated by the presence of four hemizygous alleles in gnomAD (Table S1). Preferential isoform usage could underlie a variable penetrance, and rare SNPs (dbSNP and gnomAD) affecting the 5' donor splice sites exist, but we were unable to classify its involvement beyond VUS (Figure S5). Thus from our collection of 44 variants, 12 were likely pathogenic (9 *de novo*, 3 maternally inherited), 11 likely benign, and 21 VUS (Table S1, Figure S1). The likely pathogenic variants altered highly conserved residues and were distributed throughout the protein (Figure S2).

USP9X variants are associated with a spectrum of neurodevelopmental features in males.

We collated clinical information of subjects with the 12 likely pathogenic variants and from the four previously published male likely pathogenic variants (Table S3); (4, 5). Global developmental delay / ID was reported in all cases varying from mild to severe (Figure 1A). Speech and language problems and motor disability were also found in all cases (where reported). Subjects also presented with behavioural issues, predominantly autistic and obsessive behaviours, but also attention deficit hyperactivity disorder (ADHD), anxiety and aggression (Figure 1A). Ophthalmic abnormalities, in particular strabismus, were also prevalent. In all cases where neuro-imaging was performed, brain malformations were present that included (but not limited to) white matter disturbances, hypoplastic corpus callosum, widened ventricles and cerebellar defects (Figure 1A-B). Outside of the brain, the affected individuals' most common features included joint hypermobility, a range of gastroenterological problems (feeding difficulties, reflux and constipation in particular) and growth defects of pre- and post-natal onset (Figure 1A). All affected males presented with dysmorphic facial features, although variable in nature across the cohort (Figure 1C). Digital defects were also frequently reported, mainly tapered and pointed fingers (Figure 1D). Collectively, we associate several anomalies of the central nervous system, global delay with significant alteration of speech, language and behaviour, hypotonia, joint hypermobility, strabismus and some common dysmorphic features with missense *USP9X* variants in males. The prominent neurological features reported in female USP9X subjects (3) are frequent in this male cohort, however other major congenital features of females are infrequent or absent in males (Figure S6).

USP9X variants of unknown significance exhibit features of pathogenicity

We leveraged our resources of likely pathogenic and benign variants (Table S1) and common *USP9X* variants (gnomAD hemizygous variants with allele frequency > 1:100,000) to comparatively assess VUS. There was no clear difference in spatial distribution of variant types across the protein (Figure 2A). Three VUS located in the catalytic domain were also shown to lie outside of the catalytic site (Figure S4). All variants were then compared using ANNOVAR predictive tools (27) to discover algorithms with discriminatory power for *USP9X*. Eight predictive tools validated for which (1) common and benign variant scores were similar, and (2) common and pathogenic variants score were significantly different (Figure 2B). These validated tools aligned VUS more closely with likely pathogenic variants (Figure 2B). CADD and MutPred2 tools employ independent algorithms (28, 29), and respective variant scores were moderately correlated (Figure 2C). Combining these scores to predict pathogenicity (CADD >25 and MutPred2 >0.7) enriched for likely pathogenic variants (80% of

all likely pathogenic variants), and were accompanied by ~half of VUS (Figure 2C). Similar results were obtained by combining CADD and PROVEAN scores (Figure S7).

We also compared the prevalence of the characteristic clinical features in subjects with VUS. Developmental delay, speech and language problems and behavioural problems were frequent, whilst other features including motor problems, brain malformation and gastroenterological problems (among others) were observed at reduced frequency (Figure S7 and Table S4). In aggregate, these data reveal *in silico* and clinical overlap between *USP9X* VUS with likely pathogenic variants.

USP9X missense variants affect levels of USP9X and its substrates.

For four maternally inherited variants, we generated patient derived skin fibroblast cell lines and performed functional studies: USA 6 (p.Ile79Val); France 2 (p.Ala696Val); Portugal 1 (p.Ser2233Pro); and the recurrent frameshift variant from Netherlands 3 (p.Ala2481fs*17). Studies on the p.Ala2481fs*17 variant were uninformative, as the long isoform was barely expressed (Figure S5). We investigated the steady-state levels of *USP9X* mRNA and protein (Figure 3). The p.Ser2233Pro variant line showed a significant (~50%) reduction of USP9X protein level (Figures 3A-B, Figure S8). Subcellular localisation of USP9X variants was not overtly affected (Figure 3C and Figure S9).

As complete *Usp9x* LOF is embryonic lethal, we hypothesised a molecular mechanism of *USP9X* missense variants consisting of disruption of only specific subsets of USP9X protein-protein interactions, rather than all. To test this, we immunoprecipitated (IP) USP9X and interacting proteins from control and variant fibroblast cell lines, and subjected them to Tandem-Mass-Tag based quantitative proteomic analysis (Figure 3D-E and Figure S10). We identified 6 proteins (HMGN2, DLAT, ROCK2, KCTD9, FNBP1L and RPS7) in addition to USP9X statistically enriched in control USP9X IPs over IgG (Figure S10). Of these interactors, only KCTD9 was significantly depleted (by 20%) in the p.Ile79Val IPs; and RPS7 was significantly depleted (~40%) in the p.Ala697Val IPs (Figure 3E). We conclude that the variants did not overtly impact the majority of USP9X interactions detectable by IP coupled proteomics.

As a deubiquitylating enzyme, USP9X-substrate interactions are rapid and transient, which can render vigorous detection of interactions refractory to IP. We therefore took a targeted western-blot approach to study the protein expression levels of USP9X substrates. We studied substrates specifically involved in neurodevelopmental signalling pathways (Figure 3F-G and Figure

S8); (1, 4, 7, 12). All *USP9X* missense variant cell lines had reduced levels of substrates SMURF1, a regulator of TGFβ signalling (30), and the activated (hypo-phosphorylated) form of CTNNB1 (aka β-CATENIN), a regulator of Wnt signalling (31). In addition, total β-CATENIN was significantly reduced in the p.Ile79Val and p.Ser2233Pro cell lines (Figure 3 F-G and Figure S8). We also found significant reduction of RAPTOR (mTOR pathway) and MCL1 (apoptotic pathway) levels in the p.Ala696Val cell lines (n=2 brothers) (32, 33). Other substrates including ITCH, MINDBOMB, and SMAD4 (regulators of EGF, NOTCH and TGFβ pathways respectively) were unchanged (Figure 3F and Figure S8). Ubiquitylation can also direct the nuclear localisation of SMAD4 and β-CATENIN, but we failed to identify any major difference in localisation under standard culture conditions (Figures S11 and S12). Taken together, USP9X missense variants lead to reduced levels of substrates specifically involved in neurodevelopmental signalling pathways, whilst other (more stable / robust interactions) identified via immunoprecipitation were largely unaffected.

USP9X missense variants lead to a loss of TGFβ signalling.

As SMURF1 levels were reduced across *USP9X* variant fibroblast lines, we assessed TGFβ signalling capacity. Basal levels of signalling assessed by TGFβ luciferase reporter assays were not affected (Figure 4A and Figure S13). The addition of TGFβ resulted in ~8-fold increase in luciferase activity in control cells, and in the p.Ala2481fs*17 cell line in which the variant isoform is barely expressed (Figure 4B and Figure S5 and S13). In contrast, only a 2-4 fold induction observed was observed in the remaining inherited variant cell lines. A similar result was obtained from a cell line derived from subject USA1 harbouring the *de-novo* likely pathogenic variant p.Val1868Glu (Figure 4B and Figure S13). We tested TGFβ signalling further using the inherited variant cell lines. SMAD4 is translocated into the nucleus during TGFβ signalling, and an ~8-fold increase in nuclear SMAD4 was identified in control cells following TGFβ stimulation, significantly greater than in the variant cell lines tested (Figure 4C-D and Figure S14). Lastly, we conducted a scratch migration assay to determine if variant cell lines are induced to migrate in response to TGFβ (19, 34). The TGFβ stimulated migration in control cells (~20% increase) was not observed in variant cells (Figure 4 E-F and Figure S15). In addition, we tested mTOR signalling capacity specifically in the p.Ala696Val cell lines (n=2 brothers) in which RAPTOR levels were reduced (Figure 4 C-D and Figure S8). Across two independent assays involving either a standard or serum stimulated cell culture protocol, the p.Ala696Val variant cell lines displayed evidence of a reduced mTOR response as assessed by reduced phospho-S6 (pS6) levels and reduced pS6:S6 ratio (Figure S16). We await additional cell lines with this variant/phenotype for more rigorous testing.

Collectively, these data provide functional support for pathogenicity of three inherited USP9X missense variants p.Ile79Val, p.Ala696Val and p.Ser2233Pro, and reveal the strongest impact of these variants was on neurodevelopmental signalling pathways.

Loss of Usp9x function causes learning and memory deficits in mice

Our collective data on USP9X variants to date in males and females to date suggest partial LOF as the initial molecular driver of the associated pathology. To support this hypothesis, we interrogated the phenotypic consequence of *Usp9x* deficiency in mice. We mated floxed *Usp9x* allelic mice with *Emx1-Cre* driver mice to delete *Usp9x* in the embryonic forebrain as previously described (17, 18). Unlike in human, *Usp9x* is subjected to X-inactivation in mouse (35), and as such we forwent studies in heterozygous female offspring and studied hemizygous deletion in males (compared to wildtype male littermates). These mice, herein referred to as knockout mice, survive and provide opportunity to study behaviour. At postnatal day 60, we subjected knockout mice to a broad modified SHIRPA phenotype screen (See Methods, Figure 5A-B and Table S5); (22). Knockout mice displayed a significant increase in distance travelled in an open field test (Figure 5A), and exhibited deficits in weight, gait, grip strength, and visual placing (Figure 5B). Body position was also altered (less likely to be rearing or jumping), whilst no significant difference between control and knockout mice was identified for all other tests (Table S5).

Next we interrogated hippocampal-dependent cognitive function using the Active Place Avoidance (APA) test (23). This test assesses the capacity to learn and remember the position of a fixed shock zone within a rotating platform, using visual cues (Figure 5C). No significant differences in behaviour were observed during the APA habituation Phase (Figure S17). Total distance travelled, and average speed were also comparable over the test period (Figure S17B, C). Knockout mice did however display significantly reduced performance across a variety of test parameters in the APA task, including number of entrances into the shock zone (Figure 5D), total number of shocks (Figure 5E), latency to first shock (a measure of long-term memory; Figure 5F), latency to second entry to the shock zone (a measure of short-term memory; Figure 5G), maximum time and path avoiding the shock zone (Figure 5H-I). Moreover, intra-genotype analyses revealed that wild-type mice showed significant improvements in learning the avoidance task, whilst knockouts did not (Figure S17D-I). As the APA test is highly dependent on the CA1 region of the hippocampus, we assessed CA1 cellular architecture. Analysis revealed reduced total numbers of CA1 neurons in knockout mice, albeit at equivalent density (Figure 5J-L). Collectively, these data show that complete Usp9x LOF severely

impacts hippocampal-dependent learning and memory, together with additional (CNS derived) motor, muscular and visual defects in adult mice.

DISCUSSION

Our study redefines the molecular and clinical effect of rare, predicted to be deleterious *USP9X* variants in males. Through integrated studies of patient-derived cell lines, and with evidence of learning and memory deficits of knockout mice, we conclude that DNA variation in *USP9X* leading to (partial) LOF has detrimental effects on normal brain development.

Prior to this study, only four male USP9X variants had been associated with pathogenicity in subjects with limited clinical information (4, 5). Here we report an additional 12 likely pathogenic cases. Although further clinically actionable information cannot be solely provided by *in silico* predictive tools, we discovered and utilised the best *USP9X*-centric tools to provide support of pathogenicity to around half of our cohort of VUS. This proportion aligned with the prevalence of the clinical attributes as defined by our likely pathogenic cohort. Taken together, our work provides incentive and framework for ongoing clinical and genetic studies towards resolving these cases.

We characterise the *USP9X* clinical presentation in males. Some features overlap with the more clearly defined female *USP9X* syndrome, including global developmental delay, ID, hypotonia, motor and speech delay and brain abnormalities including thin corpus callosum and cerebellar defects (3); however, consistent congenital features found in females were rare or absent in males. This difference is likely due to differing molecular consequences acting downstream of the mutation type: In females, mutations cause loss of *USP9X* dosage (with potential to impact all substrates) compared to missense mutations in males (including inherited through apparently asymptomatic mothers), which involve disruption to particular subsets of substrates (see below). The shared core neurological features between male and female cases do however suggest a convergent mechanism of pathology despite the differing mutation types. Whilst it remains to be tested in females, we speculate disruption to TGFβ as a prime candidate, whereby either reduced USP9X dosage (females) or missense mutation (males) may both culminate in a loss of TGFβ signalling in brain, stemming from a loss of key USP9X substrate(s) involved in signal transduction. Disrupted TGFβ has been implicated in several NDDs (36), and is involved in multiple aspects of brain development and function (37-40). *USP9X* joins an emerging group of X-linked genes including *DDX3X*, *IQSEC2*, *KDM5C*, *SMC1A*, *ALG13* and *OFD1* which (1) escape X-inactivation (2) feature *de novo* heterozygous LOF

mutations in female NDDs, and (3) feature missense variants with milder allelic impact (e.g. partial LOF) in male NDDs, often maternally inherited (41-46).

The hippocampus plays significant roles in learning and memory, and human ID and NDDs, and we discovered hippocampal-dependent learning and memory deficits in *Usp9x* knockout mice (47-50). Reductions in grip strength, body tone, gait and visual placement also phenocopies hypotonia, motor deficits and visual defects seen in humans. Previous studies revealed several brain malformations in the mouse, which we now show are frequent in male subjects, including agenesis of the corpus callosum, dilated ventricles (ventriculomegaly) and other brain malformations (18). The remarkable phenotypic similarities between human and mouse models have two implications. Firstly they align the mechanism of *USP9X* missense variation in males with partial LOF. We appreciate that the mouse is a complete LOF model with a comparatively severe phenotype, and analogous germline compete LOF mutations in humans (i.e. hemizygous or homozygous) are not likely compatible with life (2). Nevertheless, the similarities between human and the knockout mice suggest the variants hinder specific *USP9X* brain functions. Secondly, because a brain LOF mechanism is suggested, we speculate that the cellular and molecular mechanisms resolved in knockout mice may be indeed relevant to human pathology.

Using this same Usp9x knockout mice model, we previously established that loss of Usp9x results in decreased TGFβ-mediated axonogenesis (18), decreased mTOR-mediated neural stem cell proliferation (7) and differentiation defects associated with defective Wnt and Notch signalling (12). As *USP9X* variant cell lines were refractory to TGFβ stimulation, it's plausible that loss of axonal tracts (e.g. agenesis of the corpus callosum) stems in part from defective TGFβ signalling. USP9X has several substrates involved in regulating the TGFβ pathway, including SMURF1, SMAD4 and PJA1 (6, 9, 15). In our male subjects, we observed evidence of both down regulation of SMURF1 and loss of nuclear localisation of SMAD4. Both phenomena are consistent with a loss of USP9X interaction and may drive defective TGFβ signalling, but they may alternatively reflect loss of TGFβ signalling stemming from other molecular calamities downstream of USP9X. Indeed, it is intriguing that the variants tested in these assays are located in divergent regions of the protein that are predicted to mediate distinct protein-protein interactions. Thus whilst all tested variants caused a loss of TGFβ signalling, the key substrates driving this effect may be different. We provide evidence that different variants can uniquely impact various USP9X substrate interactions. For example, only the p.Ala696Val variant cell lines had reduced RAPTOR and MCL1 levels, with loss of RAPTOR correlating with evidence of reduced mTOR activity. Thus the p.Ala696Val variant resulted in both defective TGFβ and mTOR signalling, and was associated with the two most severely affected subjects. USP9X has also other substrates that are encoded by genes whose LOF are associated with NDDs (*CTNNB1,*

ITCH, NUAK1, PEX5, SMAD4, SMURF1, DCX, MIB1, SOX2, HERC2, NONO, RPGRIP1L, PRICKLE 1, PRICKLE 2, MTORC1; (1)). Importantly, USP9X functions upstream of all these substrates by maintaining their stability (and hence function) via deubiquitylation, and therefore, any loss of interaction between USP9X has potential to cause the neurodevelopmental pathology associated with that substrate.

USP9X sits at the "hub" of a protein interactome network enriched with NDD genes. It is also known that USP9X regulates processes relevant to NDDs through this NDD network, including neurogenesis, migration, neurite growth and synaptogenesis. Resolving the molecular, cellular and developmental pathologies underpinning *USP9X* variants is likely to converge on pathologies of NDDs of diverse genetic origins and potentially offer a point for intervention.

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FOOTNOTE

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FIGURE LEGENDS

Figure 1. Likely pathogenic *USP9X* variants cause a characteristic NDD in males. A. Constellation and penetrance of defining clinical features. *n* = the number of subjects whose information contributed to the data. B. Magnetic Resonance Imaging of the brains of individuals with likely pathogenic *USP9X* variants. Examples highlight evidence of white matter loss and ventricular widening in all, and in particular peri-ventricular leukomalacia (p.Ile79Val), loss of myelination / gliosis of posterior peri-ventricular white matter (p.Asn971Ser), cerebellar vermis hypoplasia (p.Arg2085His) and hypoplastic corpus callosum (p.Ser2233Pro). C-D. Photographs of individuals with *USP9X* variants. Note short, tapered fingers.

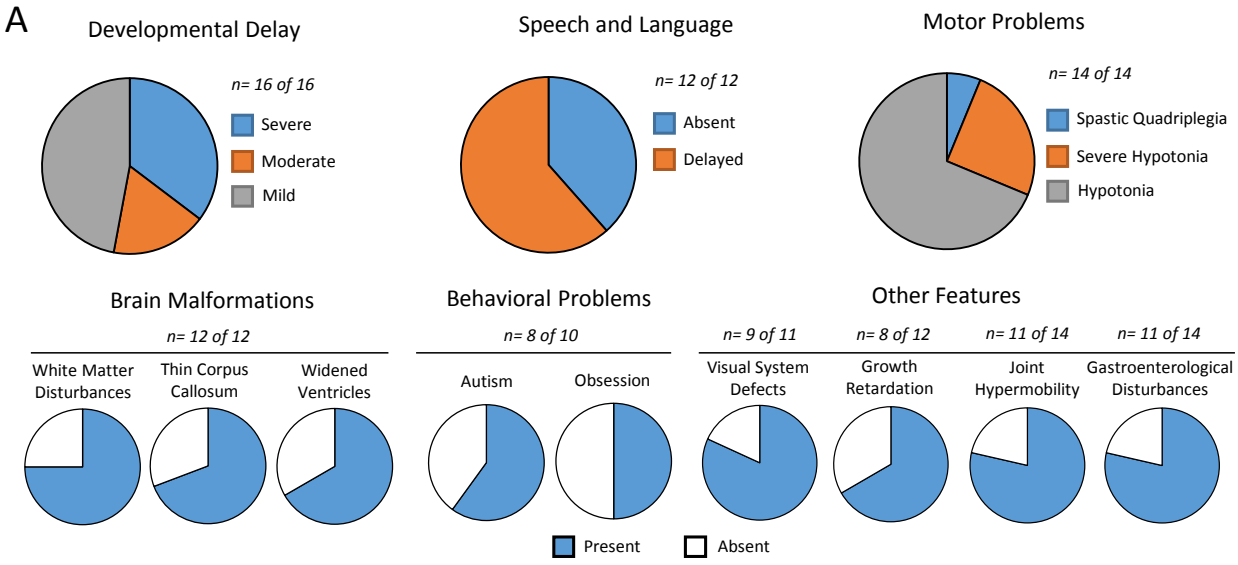
Figure 2. *USP9X* VUS share *in silico* signatures with likely pathogenic variants. A. Protein location of USP9X variants and common variants extracted from gnomAD data base. B. Bulk comparison of common, benign, likely pathogenic and variants of unknown significance by a suite of *in silico* prediction tools. *significantly different from common variants $p<0.05$ by Student's t-test. C. Comparison of CADD and MUT_PRED2 scores reveal clustering of variants of unknown significance with likely pathogenic variants in upper-right quadrant consistent with pathogenicity (CADD >25, MUT_PRED >0.7). Scores are significantly correlated (Pearson's correlation given). Colour scheme as in A and B. Inset identifies each variant in the 'pathogenic quadrant'. Graphs show percent of each type of variant, and the overall composition of variant types within the pathogenic quadrant.

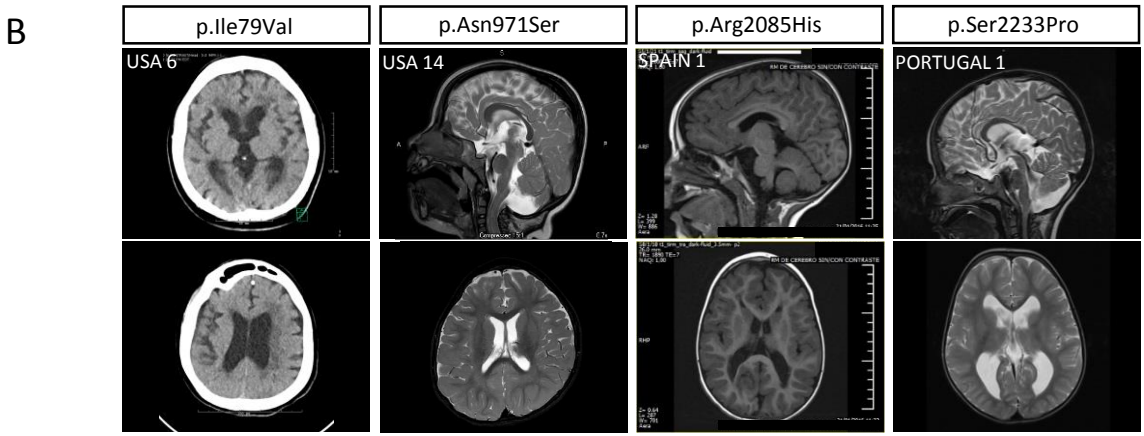
Figure 3. USP9X variants impact substrates that regulate neurodevelopmental signalling pathways. A. qRT-PCR of *USP9X* mRNA expression in male control and patient derived fibroblasts. B. Quantitation of n=3 western blot experiments analysing USP9X protein expression (See Figure S8). *$p<0.05$ Student's t-test. C. Representative immunofluorescence images from control and *USP9X* variant fibroblast cell lines. D. Western-blot of representative USP9X immunoprecipitation (IP) experiment from control and *USP9X* variant fibroblast lysates. Immunoprecipitated proteins from n=3 independent experiments (See Figure S10) were analysed by tandem mass tag mass spectroscopy for quantitation. E. Relative protein quantities of significantly enriched USP9X interactors (enriched in USP9X IPs compared to IgG IPs in control cells) in variant USP9X IP experiments. *$p<0.05$ paired Student's t-test. F. Representative western blot analysis of USP9X substrates implicated in neurodevelopmental signalling pathways in control and variant USP9X fibroblast cell lines. G. Quantitation of western-blots in C and replicates experiments (Figure S8; n=3

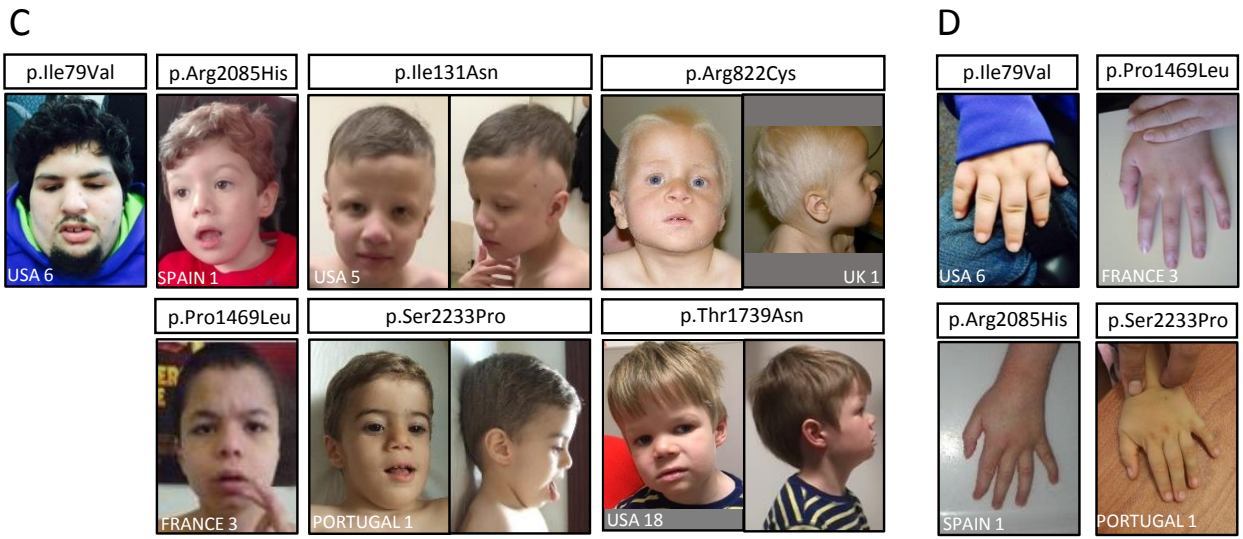
experiments). Values represent relative abundance compared to controls (n=3 cell lines); values underlined are significantly reduced (p<0.05 Student's t-test).

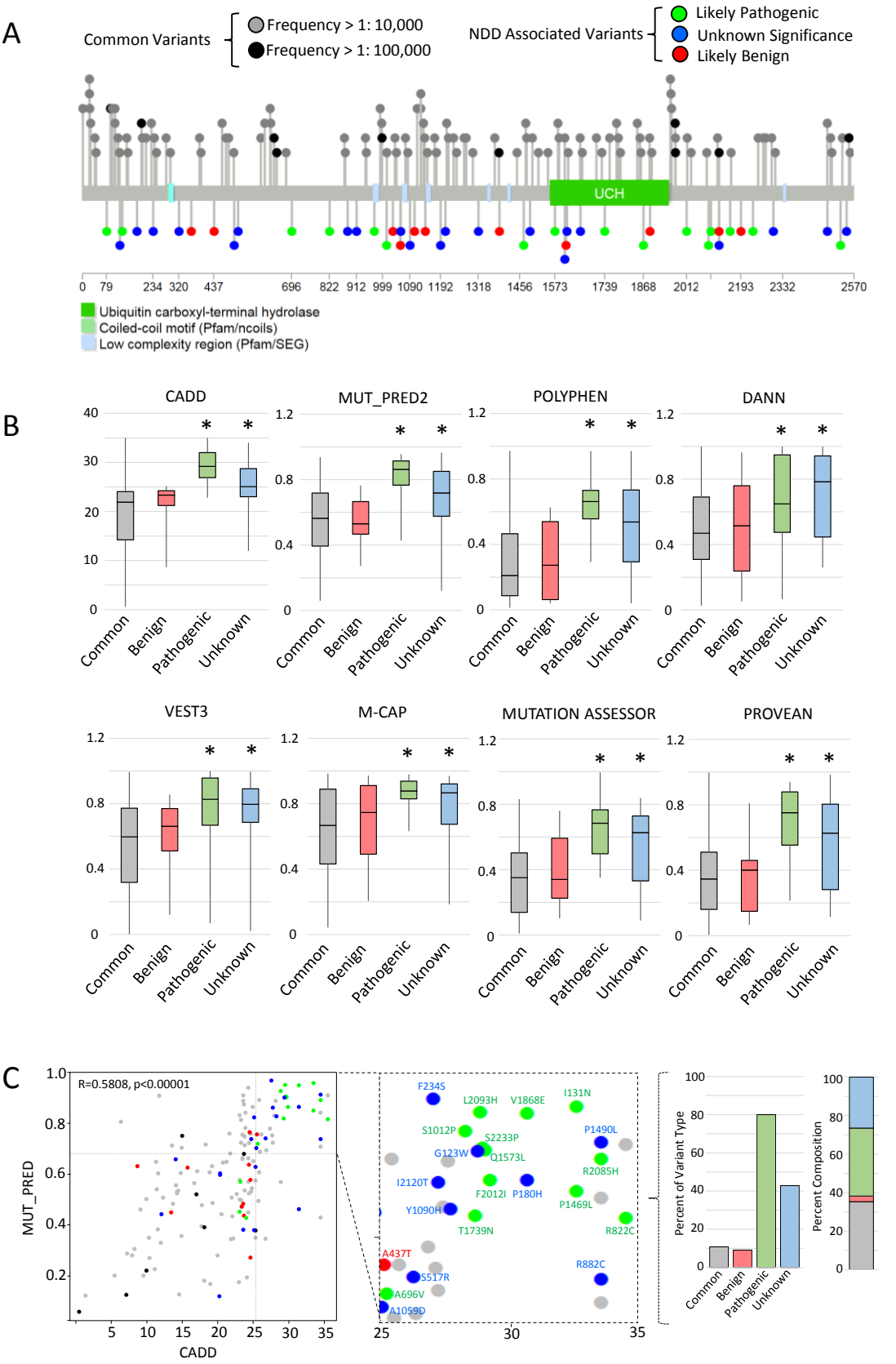
Figure 4. TGFβ signalling is disrupted in USP9X variant fibroblast cell lines. Cells were serum starved (0.2% serum) for 16 hours prior to addition of TGFβ and assayed 24 hours later. A. In the absence of added TGFβ, cells display similar basal levels of signalling as assessed by TGFβ luciferase reporter assay. B. Relative increase of TGFβ signalling following addition of ligand as assessed by TGFβ luciferase reporter assays. Experiment done in quadruplicate. C. Representative immunofluorescent images of SMAD4 localisation before (time = 0 hr) and after (time = 24 hours) addition of TGFβ. Arrow heads indicate nuclear localisation. D. Quantitation of SMAD4 nuclear translocation following addition of TGFβ. n=5 replicates. E. Representative images of scratch migration assay. F. Quantitation of the relative stimulation of migration of cells into the scratch area following addition of TGFβ. n=3 technical x 3 biological replicates. * statistical difference between +/- TGFβ. # statistical difference between controls and USP9X variant cell lines. n.s.: non-significant difference between controls and USP9X variant cell lines. #* p<0.05 Student's t-test.

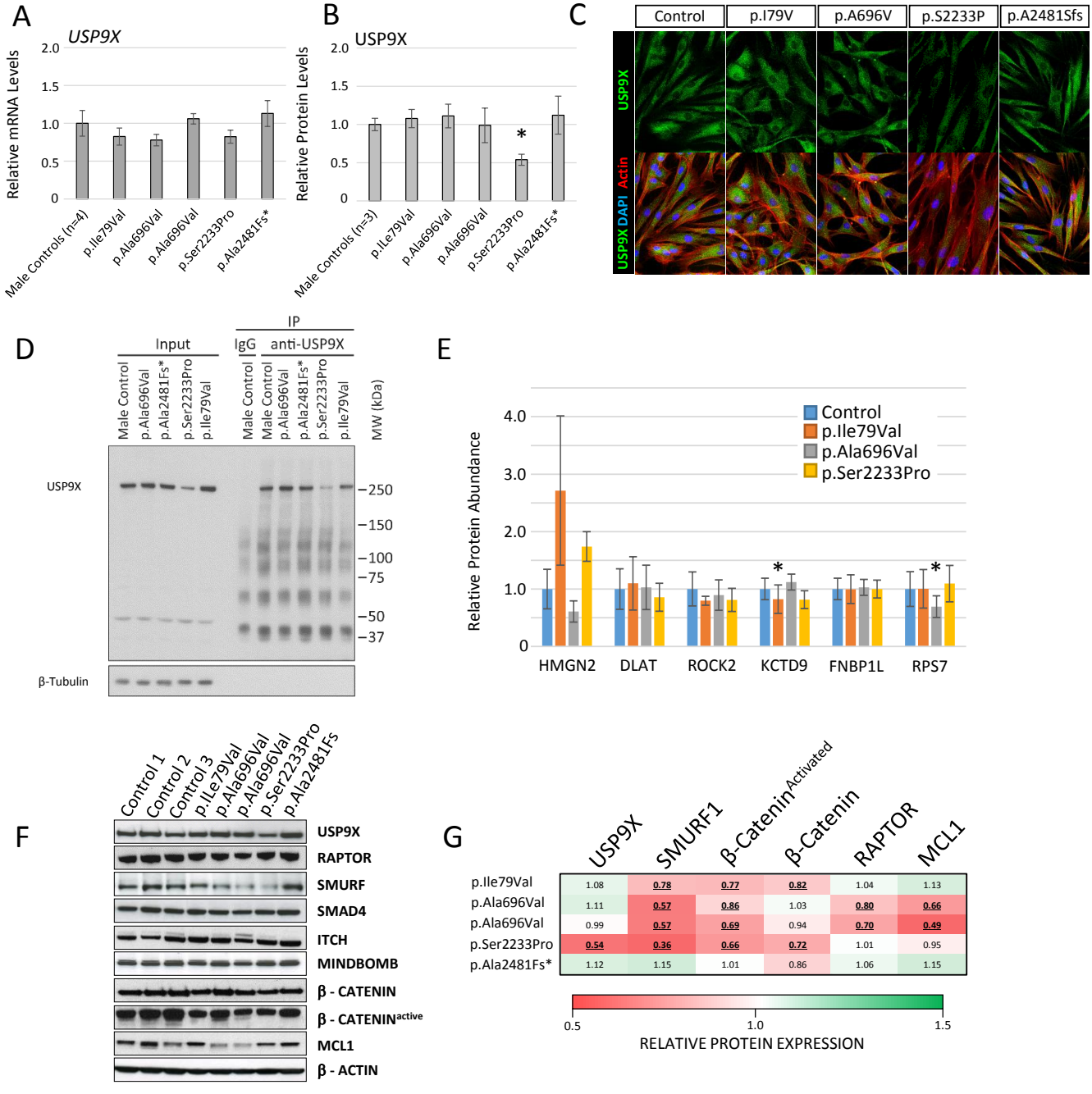
Figure 5. Behavioral deficits in *Usp9x* knockout mice. A. Adult *Usp9x* forebrain-specific knockout mice (*Usp9x*$^{-/Y}$*; Emx1-Cre*) travel further than wildtype littermate controls (*Usp9x*$^{LoxP/Y}$) in an open field test. B. Knockout mice also exhibited significant differences in various parameters of the modified SHIRPA neurological screening protocol (also see Supplementary Table 4); * $p < 0.05$; 2-tailed unpaired t test. C. Schematic of the active place avoidance (APA) arena. D-I. Knockout mice exhibited significantly reduced performance on different aspects of the APA task. Statistics relate to comparisons between wild-type and knockout animals on individual days of the five day test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two way ANOVA (also see Figure S16). J-L. Coronal sections of adult wild-type (left 2 panels) and mutant (right two panels) at the level of the hippocampus. OCT6 (red) was used a marker for CA1 hippocampal neurons, and DAPI (blue) was used to label nuclei. Whereas the density of OCT6-expressing neurons was not different between control and mutant animals (K), the total number of OCT6-expressing neurons per CA1 region was reduced within the hippocampus of mutant animals. * $p < 0.05$; t test. Scale bars in J; 250 μm in low magnification images; 30 μm in high magnification images.

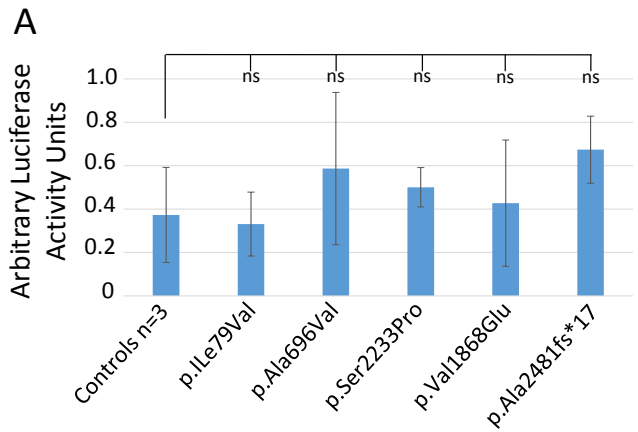

A
Developmental Delay
n= 16 of 16
Severe
Moderate
Mild
Speech and Language
n= 12 of 12
Absent
Delayed
Motor Problems
n= 14 of 14
Spastic Quadriplegia
Severe Hypotonia
Hypotonia
Brain Malformations
n= 12 of 12
White Matter Disturbances
Thin Corpus Callosum
Widened Ventricles
Behavioral Problems
n= 8 of 10
Autism
Obsession
Other Features
n= 9 of 11
Visual System Defects
n= 8 of 12
Growth Retardation
n= 11 of 14
Joint Hypermobility
n= 11 of 14
Gastroenterological Disturbances
Present
Absent

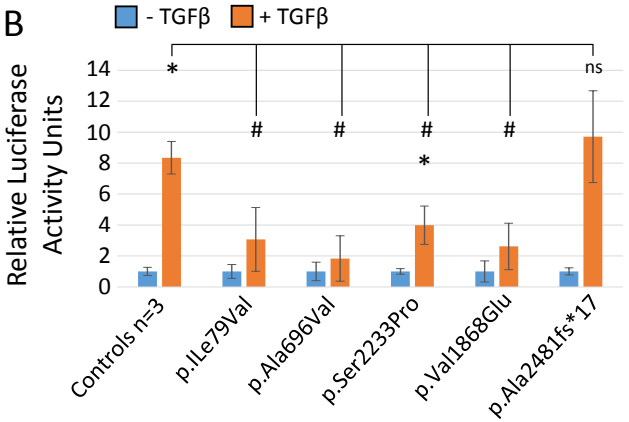

B
p.Ile79Val
p.Asn971Ser
p.Arg2085His
p.Ser2233Pro
USA 6
USA 14
SPAIN 1
PORTUGAL 1

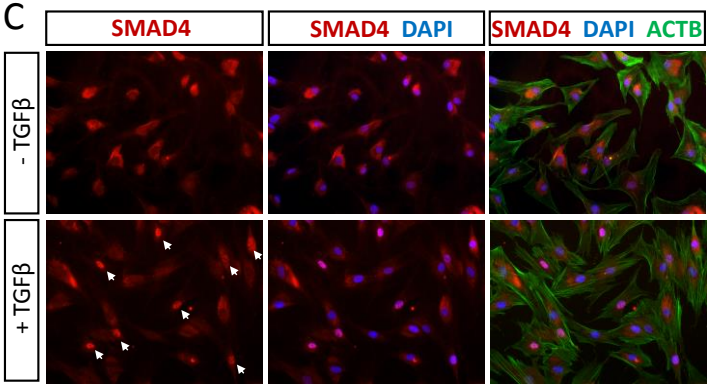

C
p.Ile79Val
p.Arg2085His
p.Ile131Asn
p.Arg822Cys
USA 6
SPAIN 1
USA 5
UK 1
p.Pro1469Leu
p.Ser2233Pro
p.Thr1739Asn
FRANCE 3
PORTUGAL 1
USA 18
D
p.Ile79Val
p.Pro1469Leu
USA 6
FRANCE 3
p.Arg2085His
p.Ser2233Pro
SPAIN 1
PORTUGAL 1

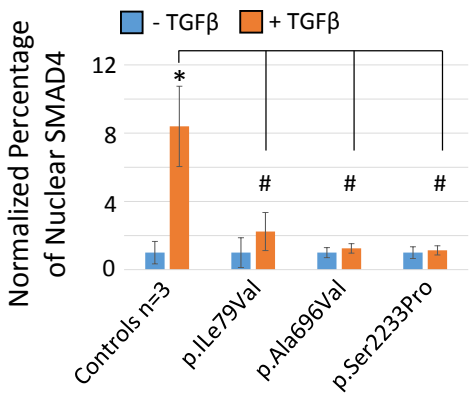

A
Common Variants
Frequency > 1: 10,000
Frequency > 1: 100,000
NDD Associated Variants
Likely Pathogenic
Unknown Significance
Likely Benign
UCH
0 79 234 320 437 696 822 912 999 1090 1192 1318 1456 1573 1739 1868 2012 2193 2332 2570
Ubiquitin carboxyl-terminal hydrolase
Coiled-coil motif (Pfam/ncoils)
Low complexity region (Pfam/SEG)
B
CADD
MUT_PRED2
POLYPHEN
DANN
VEST3
M-CAP
MUTATION ASSESSOR
PROVEAN
Common
Benign
Pathogenic
Unknown
C
R=0.5808, p<0.00001
MUT_PRED
CADD
F234S
L2093H
V1868E
I131N
S1012P
S2233P
P1490L
G123W
Q1573L
R2085H
I2120T
F2012I
P180H
P1469L
Y1090H
T1739N
R822C
A437T
R882C
S517R
A696V
A1059D
Percent of Variant Type
Percent Composition

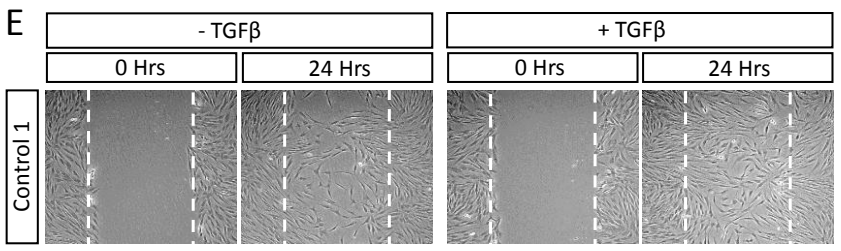

A
USP9X
Relative mRNA Levels
2.0
1.5
1.0
0.5
0
Male Controls (n=4)
p.Ile79Val
p.Ala696Val
p.Ala696Val
p.Ser2233Pro
p.Ala2481Fs*
B
USP9X
Relative Protein Levels
2.0
1.5
1.0
0.5
0
*
Male Controls (n=3)
p.Ile79Val
p.Ala696Val
p.Ala696Val
p.Ser2233Pro
p.Ala2481Fs*
C
Control
p.I79V
p.A696V
p.S2233P
p.A2481Sfs
USP9X
USP9X DAPI Actin
D
IP
Input
IgG
anti-USP9X
Male Control
p.Ala696Val
p.Ala2481Fs*
p.Ser2233Pro
p.Ile79Val
Male Control
Male Control
p.Ala696Val
p.Ala2481Fs*
p.Ser2233Pro
p.Ile79Val
MW (kDa)
USP9X
250
150
100
75
50
37
β-Tubulin
E
Relative Protein Abundance
4.0
3.0
2.0
1.0
0
Control
p.Ile79Val
p.Ala696Val
p.Ser2233Pro
*
*
HMGN2
DLAT
ROCK2
KCTD9
FNBP1L
RPS7
F
Control 1
Control 2
Control 3
p.Ile79Val
p.Ala696Val
p.Ala696Val
p.Ser2233Pro
p.Ala2481Fs
USP9X
RAPTOR
SMURF
SMAD4
ITCH
MINDBOMB
β - CATENIN
β - CATENINactive
MCL1
β - ACTIN
G
USP9X
SMURF1
β-CateninActivated
β-Catenin
RAPTOR
MCL1
p.Ile79Val 1.08 0.78 0.77 0.82 1.04 1.13
p.Ala696Val 1.11 0.57 0.86 1.03 0.80 0.66
p.Ala696Val 0.99 0.57 0.69 0.94 0.70 0.49
p.Ser2233Pro 0.54 0.36 0.66 0.72 1.01 0.95
p.Ala2481Fs* 1.12 1.15 1.01 0.86 1.06 1.15
0.5
1.0
1.5
RELATIVE PROTEIN EXPRESSION

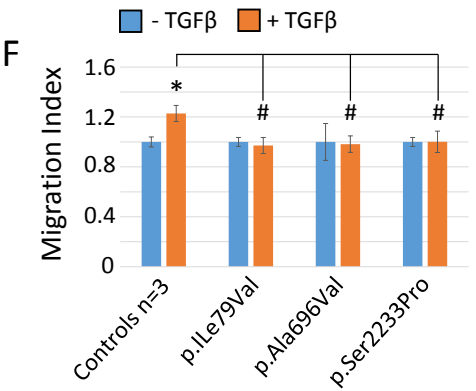

A
Arbitrary Luciferase Activity Units
1.0
0.8
0.6
0.4
0.2
0
ns
ns
ns
ns
ns
Controls n=3
p.Ile79Val
p.Ala696Val
p.Ser2233Pro
p.Val1868Glu
p.Ala2481fs*17


B
- TGFβ
+ TGFβ
Relative Luciferase Activity Units
14
12
10
8
6
4
2
0
*
#
#
*
#
ns
Controls n=3
p.Ile79Val
p.Ala696Val
p.Ser2233Pro
p.Val1868Glu
p.Ala2481fs*17


C
SMAD4
SMAD4 DAPI
SMAD4 DAPI ACTB
- TGFβ
+ TGFβ


D
- TGFβ
+ TGFβ
Normalized Percentage of Nuclear SMAD4
12
8
4
0
*
#
#
#
Controls n=3
p.Ile79Val
p.Ala696Val
p.Ser2233Pro


E
- TGFβ
+ TGFβ
0 Hrs
24 Hrs
0 Hrs
24 Hrs
Control 1


F
- TGFβ
+ TGFβ
Migration Index
1.6
1.2
0.8
0.4
0
*
#
#
#
Controls n=3
p.Ile79Val
p.Ala696Val
p.Ser2233Pro

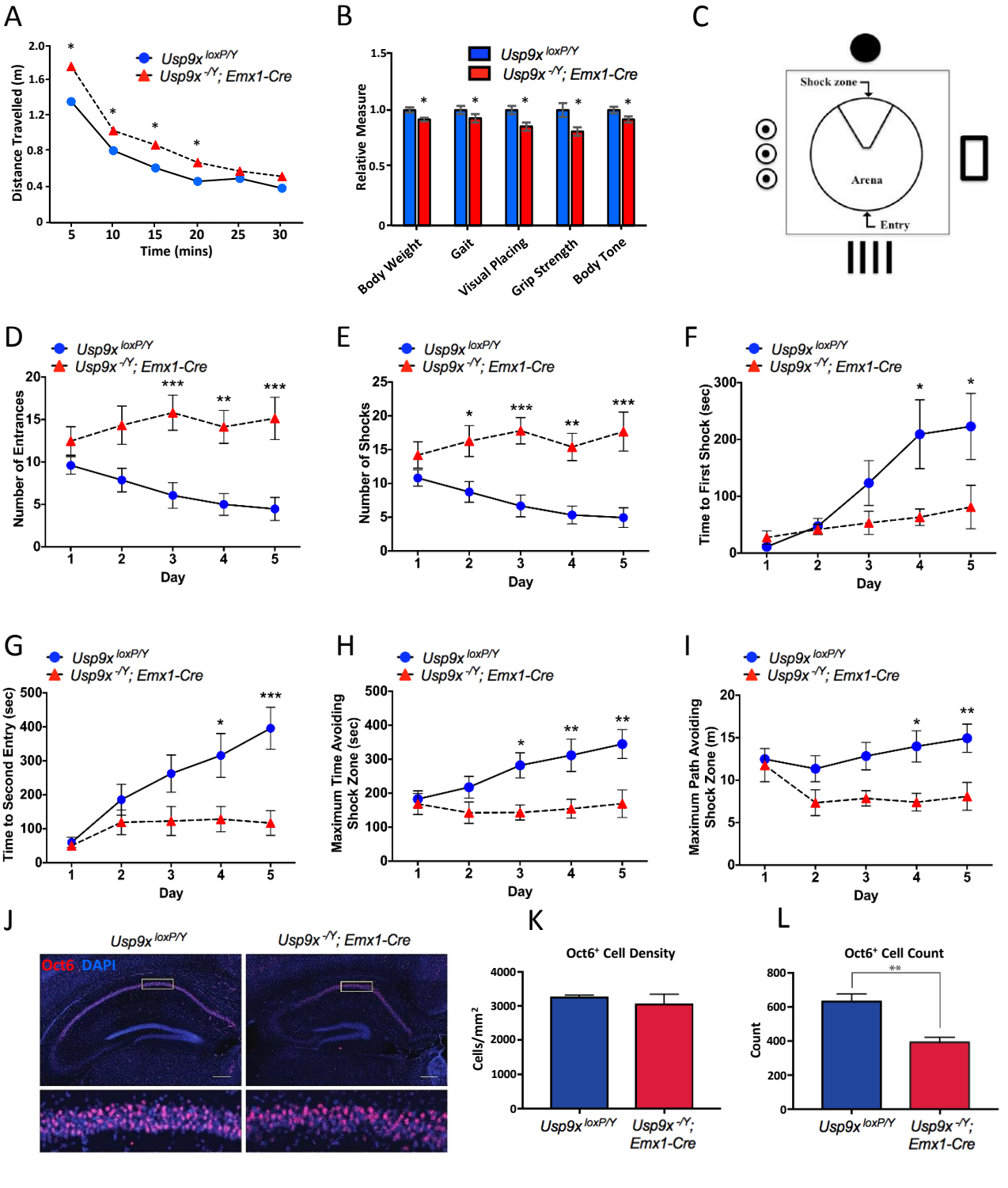

A
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Distance Travelled (m)
Time (mins)
B
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Relative Measure
Body Weight
Gait
Visual Placing
Grip Strength
Body Tone
C
Shock zone
Arena
Entry
D
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Number of Entrances
Day
E
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Number of Shocks
Day
F
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Time to First Shock (sec)
Day
G
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Time to Second Entry (sec)
Day
H
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Maximum Time Avoiding Shock Zone (sec)
Day
I
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Maximum Path Avoiding Shock Zone (m)
Day
J
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
Oct6 DAPI
K
Oct6+ Cell Density
Cells/mm2
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre
L
Oct6+ Cell Count
Count
Usp9x loxP/Y
Usp9x -/Y; Emx1-Cre

Table S3: Phenotypic features of male patients with likely pathogenic varinats in the *USP9X* gene

Patient ID			USA 6	USA 5	France 2		UK 1	USA 14	France 3	USA 13	USA 18	USA 1	USA 19	Spain 1	Portugal 1	Homan *et al* 1	Homan *et al* 2	Homan *et al* 3	Paemka *et al* 1
***USP9X* genetic variant**	**Nomenclature**	**gDNA (chrX, *hg19*)**	g.40988391A>G	g.40994047T>A	g.41025226C>T		g.41027299C>T	g.41029757A>G	g.41057806C>T	g.41060427A>T	g.41073847C>A	g.41075423T>A	g.41075854T>A	g.41077669G>A	g.41082601T>C	g.41077693T>A	g.41078388C>A	g.4108948delA	g.41031097T>C
		cDNA (NM_001039590.2)	c.235A>G	c.392T>A	c.2087C>T		c.2464C>T	c.2912A>G	c.4406C>T	c.4718A>T	c.5216C>A	c.5603T>A	c.6034T>A	c.6254G>A	c.6697T>C	c.6278T>A	c.6469C>A	c.7574delA	c.3034T>C
		Protein (NP_001034679.2)	p.Ile79Val	p.Ile131Asn	p.Ala696Val		p.Arg822Cys	p.Asn971Ser	p.Pro1469Leu	p.Gln1573Leu	p.Thr1739Gln	p.Val1868Glu	p.Phe2012Ile	p.Arg2085His	p.Ser2233Pro	p.Leu2093His	p.Leu2157Ile	p.Gln2525fs*18	p.Ser1012Pro
	Inheritance		Maternal	*de novo*	Maternal		*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	Maternal	n.a.	Maternal	Maternal	*de novov*
	Neurological	**Developmental delay**	Severe	Moderate	Severe	Severe	Moderate / severe	Severe	Severe	Mild	Moderate	Mild	Severe	Severe	Severe	Mild	Mild	Mild / moderate	n/a
		Speech / Language	Absent	Apraxia	n/a	n/a	Delayed	Delayed	Only few words	Delayed	Delayed	Delayed	Absent	Delayed	Absent	n/a	Absent	n/a	n/a
		Seizures	Febrile	No	n/a	n/a	No	No	No	No	No	Febrile and absence	Partial complex siezures	Mild seizures during the sleep	no	n/a	n/a	n/a	Epilepsy
		Behavior	n/a	Autism, obsessions	n/a	n/a	Normal	Normal	Autism, obsession, anxiety	n/a	Autism, temper tantrums	Anxiety, fearful, overstimulated, tearful	n/a	n/a	Autism, obsessions, self-injury	Agression	Autism, obsessions	Autism, obsessions	n/a
		Motor problems	Severe hypotonia (non-ambulatory)	Hypotonia	n/a	n/a	Hypotonia, uses standing frame	Spastic quadriparesis (non ambulatory)	Hypotonia, drooling, broad based gait	Hypotonia	Hypotonia (pseudoataxic gait)	Hypotonia	Severe hypotonia	Hypotonia, drooling	Severe hypotonia (first steps at 7, waddling)	Hypotonia	Hypotonia	Hypotonia	n/a
		Visual deficits	Optic atrophy, cataracts	Exotropia	n/a	n/a	Duane's anomaly left eye	No	No	Myopia	Alternating exophoria	Cortical visual impairment	Mild right astigmatism; amblyopia	Myopia and strabismus	Myopia	n/a	n/a	n/a	n/a
	Growth	**Prenatal**	Normal	normal	IUGR	IUGR	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	n/a	IUGR	n/a	n/a
		Postnatal	Severe retardation (-9 SDS)	Mild retardation (-1.5 SDS)	n/a	n/a	Mild retardation secondary to severe scoliosis	Retardation requiring injections of growth hormone	Retardation (-3 SDS)	Normal	Normal	Normal	Retardation (AGA (38wks))	Normal	Normal	n/a	Retardation	n/a	n/a
		Head circumference	Microcephaly (-3.7 SDS)	Normal	n/a	n/a	Microcephaly (-2 SDS)	Micro-brachycephaly	Normal	Microcephaly (-1.96SD)	Normal	Normal	Normal	Normal	Normal	Relative macrocephaly		n/a	n/a
	Malformations	**Brain**	Progressive volume loss, widened ventricles, white matter loss, lacunar infart in left basal ganglia, periventricular leucomalacia	Arachnoid cyst or mega cisterna magna, low white matter volume, ventriculomegaly, thinning of corpus callosum	Pachygyria, subcortical band heterotopia, thinning of corpus callosum	Pachygyria, subcortical band heterotopia, thinning of corpus callosum	Reduced normally myelinated white matter bulk, ventriculomegaly	Possibly delayed myelination or gliosis in the posterior periventricular white matter, widened ventricles, mild thinning of corpus callosum	Ventriculomegaly	n/a	Periventricular leukomalacia, periatrial small cysts bilaterally, colpocephaly	Thin corpus callosum, colpocephaly of the lateral ventricles, bilateral polymicrogyria, bilateral hippocampal malrotation, bilateral hypoplastic olfactory bulb, left middle cranial fossa arachnoid cyst	Multiple cortical infarcts, cerebellar hypoplasia, ventriculomegaly, hypoplastic corpus callosum, pyriform aperture stenosis, pituitary hypoplasia	Cerebellar vermis hypoplasia, Dandy-Walker malformation, widened ventricles, mild thinning of corpus callosum	Peripheral white matter hyperintensities, ventriculomegaly, widened ventricles, hypoplastic corpus callosum	n/a	n/a	n/a	n/a
		Dysmophic features	Blepharophimosis, full eyebrows, large incisors	Broad forehead, epicanthic folds, flat nasal bridge, broad nasal tip, sacral dimple	n/a	n/a	Deep set eyes	Flat nasal bridge, broad depressed nasal tip, high and narrow palate, hypertelorism	Broad nasal tip, downslanting palpebral fissures	Upslanting palpebral fissures, low set ears with attached lobes, broad nasal tip, short philtrum	Deep set eyes, full eyebrows, hypertelorism, epicanthic folds, flat nasal bridge	Epicanthic folds, flat nasal bridge, small malformed and posteriorly rotated ears	Micrognathia	Blepharophimosis, epicanthic folds, ear fissures, flat nasal bridge, broad nasal tip	Low frontal hairline, broad nasal tip	n/a	n/a	n/a	n/a
		Skeletal	Short hands; short, trident shaped fingers	Clubfeet, pectus carinatum, joint hypermobility	Punctate epiphyses	Punctate epiphyses	Severe scoliosis, overlapping 2nd & 3rd toes bilaterally, joint hypermobility	Bi lateral hip dysplasia	Broad thumb, joint hypermobility	Joint hypermobility	Calcaneovalgus feet, Achilles contracture, joint hypermobility	Joint hypermobility	Irregular long bone metaphyses, joint hypermobility, severe genu recurvatum, clubfeet. Bilateral type B postaxial polydactyly, also in father and paternal great uncle	Kyphosis, clubfeet, joint hypermobility	Broad toes, clinodactyly 4th & 5th toes, joint hypermobility	Broad thumbs, great toes, joint hypermobility	Broad thumbs	n/a	n/a
	Additional features	**Gastrointestinal problems**	Feeding difficulties, G-E reflux	Feeding difficulties, constipation	Meconium Ileus	Meconium Ileus	No	Feeding difficulties, G-E reflux, constipation	Feeding difficulties, G-E reflux	Feeding difficulties	No	Normal	Feeding difficulties, G-E reflux	G-E reflux, intrahepatic cholestasis/giant cell hepatitis	Feeding difficulties	Constipation	Feeding difficulties, G-E reflux, traceomalacia	n/a	n/a
		Skin and anexes	Hirsutism	Thin hair, eczema, pitting of nails	n/a	n/a	Coarse and very fair hair	Thick hair,	Normal	Normal	Normal	Normal	Eczema	Seborrheic dermatitis, angioma in forehead	Small ovoid scalp aplasia cutis (secondary to birth trauma?)	n/a	n/a	n/a	n/a
		Analytical-metabolic findings	Blood cytopenia, lactic acidosis in newborn period which ultimately	No	Blood cytopenia	Blood cytopenia	No	Hypoglycemia	Hypoglycemia	n/a	Low free carnitine, high lactate, low thyroine	Hypoglycemia	n.a	No	Hypoglycemia	n/a	n/a	n/a	n/a
		Miscellaneous	Sleep apnea, diabetes type 2, hypertension, adrenal insufficiency, chronic kidney disease	Sleep apnea	Early post-natal lethality	Early post-natal lethality	No	Cryptochidism	No	No	No	Displays occasional chorea and dystonic posturing of hands but not when in use. Ankle dystonia when walking. Displays some flapping / stereotypies	Anterior piriform stenosis; laryngeal cleft, type 1, small umbilical hernia, large right inguinal hernia	Inguinal hernias	Bilateral cryptorchidism	n/a	n/a	n/a	n/a
		Prenatal Findings	n/a	Clubfeet, ventriculomegaly (U/S), mat. low platelets	n/a	n/a	n/a	Twin gestation - born at 31 weeks	n/a	n/a	n/a	n/a	Ventriculomegaly, clubfeet, bilateral polydactyly, unusual facies (U/S). Born at 38w	clubfeet	Transient placental abruption (2nd trimester), oligohydramnios after amniocentesis	n/a	n/a	n/a	n/a

Table S4: Phenotypic features of male patients with variants of unknown significance in the USP9X gene.

	Patient ID		USA 7	Netherlands 11		USA 9	USA 3	Netherlands 9	Netherlands 12	USA 12		Canada 2	Netherlands 10	Netherlands 1	Netherlands 7	USA 21	USA 11	USA 22	Spain 2	USA 17	USA 20	Canada 6	USA 28	Netherlands 3		Canada 1		Canada 3	Netherlands 5
USP9X genetic variant	Nomenclature	gDNA (chrX, hg19)	40994033G>T	g.40981693C>A		g.40999993T>C	g.41000408A>G	g.41007713C>T	g.41007713C>T	g.41009255C>T		g.41032634A>G	g.41063278C>A	Epilepsy, febrile seizures	g.41043788A>G	g.41045833C>T	g.41048703C>G	g.41053788G>T	g.41060327C>A	g.41064156G>A	g.41077747T>C	g.41084144A>C	g.41085041dupA		g.41085041dupA		g.41085041dupA		g.41089037C>T
		cDNA (NM_001039590.2)	c.3870G>T	c.3230C>A		c.7017T>C	c.3698A>G	c.1511C>T	c.1548A>C	c.2644C>T		c.2724A>G	c.3119G>A	c.3268T>C	c.3375A>G	c.3422 C>T	c.3364C>G	c.4489C>T	c.4883C>A	c.4837C>A	c.4973G>A	c.6334T>C	c.6900A>C	c.7445dupA		c.7445dupA		c.7445dupA		c.7833C>T
		Protein (NP_001034659.2)	p.Gly1237p	p.Pro1900Ha		p.Pro2345er	p.Arg3232gly	p.Ala5040Val	p.Ser5174Arg	p.Arg8821Cys		p.Ser9121Val	p. Ala10354sep	p.Tyr10909Ha	p.Hsr11024rg	p.Pro11208Ser	p.Asp1219Gly	p.Pro1490Leu	p.Asp1659Gly	p.Pro1637thr	p.Arg1638Gln	p.Ile2126Thr	p.Lys2331Gln	p.Asp2481fsr17		p.Asp2481fsr17		p.Asp2481fsr17		p.Pro2545Ser
	Inheritance		Maternal	Maternal		Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal		Maternal		Maternal	Maternal
Phenotype	Neurological	Developmental delay	Severe	Moderate / severe	Severe	Moderate	Moderate	Severe	Mild	Mild	Mild	Mild	Mild	Severe	Severe	Moderate	Mild	Mild	Mild	Mild	Moderate	Moderate	Moderate	Mild	Mild	Moderate	Moderate	Mild	Mild
		Speech / Language	Only few words	Only few words	Only few words	Delayed	Delayed	Non verbal	Delayed	Delayed	Normal	Delayed	Delayed	Absent	Only few words	Only few words	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Slow speech		Delayed	Delayed
		Seizures	Epilepsy, partial idiopathic	Epilepsy	No	No clinical seizures, EEG @4 yo: spiking during sleep	Epilepsy	No	No	No	No	No	Yes, onset 3y	Yes	No	Many febrile seizures from 19-28 months of age. Mild diffuse slowing on EEG.	No	Yes	No	No	Epilepsy	Refractory, generalized tonic clonic, atonic, myoclonic, absence.	No	No	No	No	Febrile	Epileptiform activity on EEG but no clinical seizure'	No
		Behavior	Autism and obsessive repetative behaviours, irritable, non agressive	n/a	n/a	Autism, some obsessive behaviours in past	Autism	Anxiety, obsession, agression, automutilation	No	ADHD	ADHD	ADHD	Anxiety (shy)	No	Autism, anxiety,	No	Autism, ADHD	Autism	No	No	No	No	n/a	Autism	Autism				Autism, some obsessions, Anxiety	Autism
		Motor problems	Hypotonia	Hypotonia, spasticity	Non-ambulatory	Hypotonia, ataxia	Hypotonia	Hypotonia	Extrapyramidal movements, broad based walking pattern, foot elevator weakness.	No	No	No	Delay	Delayed	Alexia, wheel chair for long distances	Severe generalised hypotonia, ataxia	No	No	n/a	Delayed, mild hypotonia	Hypotonia, unsteady	Fine motor delay, ataxia	Hypotonia, tremor (possible cerebellar ataxia), global developmental delay	n/a	n/a	Central hypotonia	Hypotonia, cerebellar ataxia	Delayed	Hypotonic as an infant
		Visual deficits	No	n/a	n/a	No but has slow EOMI, mild intermittent left esotropia	n/a	No	No	No	No	No	No	No	No	Intermittent downbeat nystagmus	No	n/a	No	Left esotropia, glasses	hyperopia, esophoria	No	No	No	No	Strabismus	n/a	No	No
	Growth	Prenatal	Normal	n/a	n/a	Normal	n/a	Normal	Normal	Normal	Normal	Normal	IUGR	Normal		IUGR	Normal	Normal	Normal	Normal	Normal	Normal	Normal	IUGR	IUGR	n/a	n/a	Normal	IUGR
		Postnatal	Normal	n/a	n/a	Retardation	n/a	Normal	Normal	Retardation (5-10% height @10 yrs)	Normal	Normal	Normal	Normal		Retardation	Normal	Normal	Retardation	Retardation (Height 5%)	Normal	Retardation (Height 3%)	Normal			n/a	n/a	Normal	Normal
		Head circumference	Normal	n/a	n/a	Normal	n/a	Normal	n/a	Normal	Normal	Normal	Normal	Normal	Normal	Microcephaly	Normal	Normal	Microcephaly	Normal	Normal	No	Normal	No	No	n/a	n/a	Macrocephaly	No
	Malformations	Brain	Delayed maturation of white matter, small pituitary gland, posterior positioning of the cerebral arteries, thin corpus colosum	Thin corpus colosum	n/a	Normal	n/a	n/a	n/a	n/a	n/a	n/a	Normal	Delayed myelination , absent splenium of the corpus callosum	n/a	Possible T2 signal hyperintensity involving the preventricular WM in the frontal lobes at 3 years old. Mild prominence of subc. in the frontal poles bilaterally at 14 months. Possible mildly delayed myelination at 3 years old.	Normal	Non-specific white matter abnormalities	Cochlear Nerve Hypoplasia	Normal	Normal	Well-defined FLAIR hyperintense nodular cortical/subcortical lesion in left frontal white matter from the age of 6 years, likely low grade DNET or glioma	Brain MRI reported as unremarkable	n/a	n/a	Cerebellum Vermis atrophy, thick corpus colosum	Cerebellum Vermis atrophy, thick corpus colosum	n/a	Normal
		Dysmorphic features	Turricephalic, tall forehead, arched eyebrows, downslanted palpebral fissures, long nose, hypoplastic alae, small mouth, slightly posteriorly rotated ears,	Sacral dimple, broad forehead, deep set eyes, full upper eyelids, broad base to the nose, broad mouth, small posteriorly rotated ears	n/a	Broad forehead, small nose, overhanging columella, upslant of palbebral fissures, high palate, low set and posterior rotated ears, prognathism of lower jaw, overcrowded upper teeth		Lateral flaring of the eyebrows, deep set eyes, large ears, small high nasal bridge, broad chin	Frontal bossing, bitemporal narrowing, Deepset eyes, deep philtrum, low nasal bridge, almond shaped eyes. Inverted nipples. Hypoplastic toe nails and finger nails. Fetal pads fingers.	Large and tall forehead,	Large block shaped head, prominent forehead,	Broad forehead, 5th finger brachytactyly, dimpled chin	Broad mouth, flat nasal bridge, full upper eyelids, high forehead	Fracial Dysmorphism, hypertelorism, macrostomia, full eyebrows, low frontal hairline, brachycephaly, spaced teeth	Facial dysmorphism including macrostomia, large ears, small high nasal bridge,	Upslanting palpebral fissures, almond shaped and mildly wide spaced eyes, round and cupped ears with simple helices	No	Full eyebrows with mild medial flaring, spaced teeth	Flat nasal bridge otherwise normal	No	Flat nasal bridge, full eyebrows,	No	None	n/a	n/a	n/a	n/a	Sacral dimple, borderline brachytactyly, very mild asymmetry of face,	No
		Skeletal	Pectus carinatum, bilateral coxa valga, hyperelastibility of joints, flat foot, persistent finger pads, 5th finger clinodactyly	Hip dysplasia, large hands with long fingers, Thumbs cannot be fully extended	Postacial polydactyly, scoliosis	Mild neuromuscular scoliosis, very mild pectus carinatum, hyperextensible dislocatable thumbs, long bulbous toes	n/a	Scoliosis, Pes planus	Medial malleolus positioning ankels	No	No	No	n/a	Scoliosis, broad feet	n/a	Osteopenia with recurrent bone fractures,	No	No	n/a	No	Clinodactyly of bilateral 3rd and 4th toe, joint hypermobility, tapered fingers with fingertip like pads	No	None	Possibly broad thumbs	Possibly broad thumbs	n/a	n/a	Hyperelastensitie MCP joints, genu valgum	No
	Additional features	Gastrointestinal problems	Milk intolerance and frequent vomiting when ill	n/a	n/a	Constipation, frequent vomniting during infancy, occesional mucous like bowel movements	n/a	No	Constipation	Constipation	Constipation	No	No	No	Constipation	Constipation, early feeding difficulties due to prematurity	Frequent vomiting independent of food intake	No	No	Cyclical vomiting, GE reflux (Crohns genetic marker)	Feeding difficulties, G-E reflux, constipation,	Lacks appetite and restricted food choices	None	GE-reflux	Feeding difficulties	n/a	n/a	Post natal tube feedings	No
		Skin and anexes	Normal	n/a	n/a	Normal	n/a	Normal	Dry skin arm pits and inside elbows with millar vesicles.	Keratitis on palms/soles	Keratitis, rough skin	Normal	2 hyperpigmented spots , 1 hypopigmentation	Thin Hair, Skin Blechnlines (hyperpigmented on right side of body), hypogtastic toenails	n/a	Normal	Normal	Hypertrichosis	Thin hair, skin blackhollnes	Normal	Eczema	Normal	Normal	Normal	Normal	n/a	n/a	Normal	Café latte spot
		Analytical-metabolic findings	Hypoglycemic at times, epileptic of low plasma serine	n/a	n/a	Low cysteine, mildly elevated asparagine, mildly elevated lactate, low 'free' carnitine	n/a	No	No	No	No	mild to moderate elevation of branched chain amino acids, elevated lysosine	No	No	n/a	n/a	No	No	n/a	Hypoglycemic and ketotic hypoglycemic episodes	Low hemoglobin with microcytosis	Normal	n/a	Hypoglycemic after birth	Hypoglycemic after birth	n/a	n/a	Normal	n/a
		Miscellaneous	Cryptochidism, fight heal cords,	Surgery for inguinal hernia, abnormal shape of the thorax with low set nipples. He has pes equinas, hammer toes and calf muscle atrophy	Pregnancy was complicated by HELLP syndrome	Obstructive sleep apnea, fight heal cords	n/a	Bruxism	Frequent upper-airway infections.	n/a	n/a	n/a	Polyhydramnion in pregnancy. Surgery for velopharyngeal insufficiency	n/a	n/a	n/a	Hearing impaired (normalized after surgery)		Unilateral hearing impairment (hypoplasia of cochlear nerve)	n/a	Obstructive sleep apnea	n/a	secondhand report of burr cells on CBC smear	n/a	n/a	n/a	n/a	n/a	n/a
		Prenatal Findings	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Partial Loss of USP9X Function Leads to a Male Neurodevelopmental and Behavioral Disorder Converging on Transforming Growth Factor β Signaling

SUPPLEMENTAL INFORMATION

This file contains 17 Figures (Figure S1-S17), 7 Tables (Table S1-S7) and 1 Clinical Data Description

SUPPLEMENTAL FIGURES S1-S17

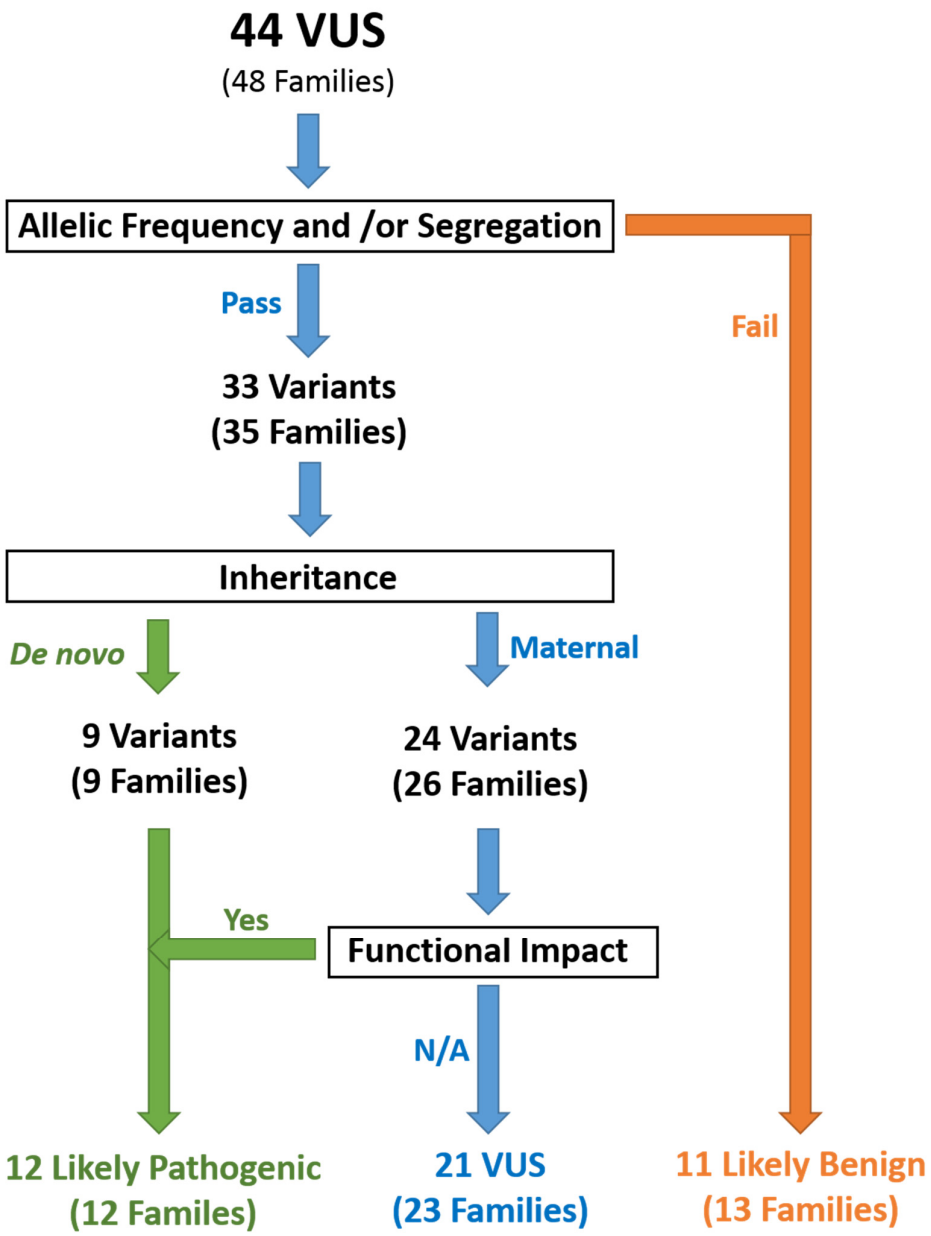


Figure S1. Overview of classification of *USP9X* male variants. Fail at 'Allelic Frequency and/or Segregation' based on either being found in > 1: 10000 alleles, or having >4 alleles found in hemizygous state in gnomAD (genome and exome) data base, or being discovered in a healthy male relative.

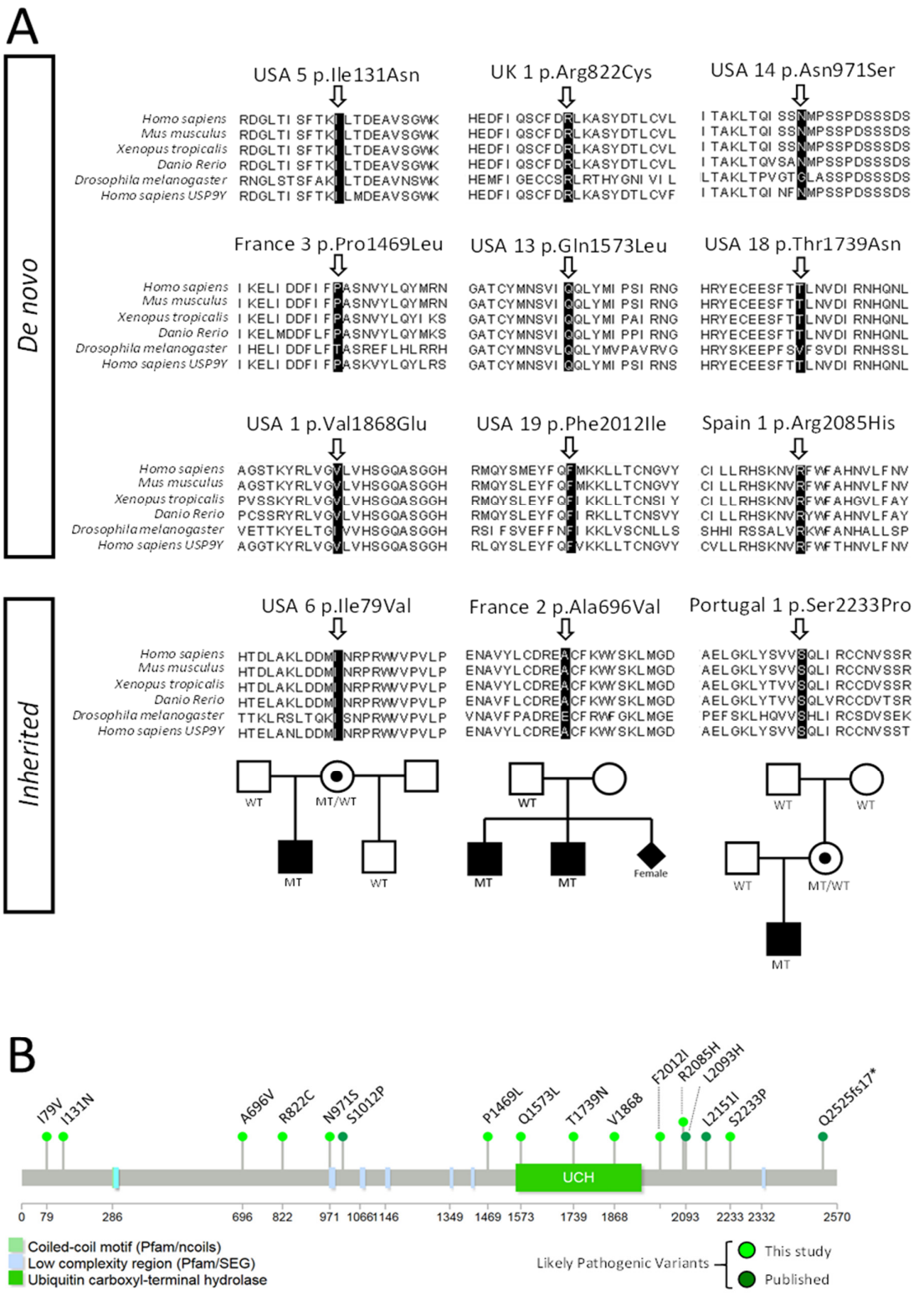


Figure S2. Conservation and protein location likely pathogenic USP9X variants. A. Cross species protein alignment of USP9X showing conservation of altered amino acid residues. Pedigrees are shown for inherited cases. UK1 has Decipher ID: 260068. B. Location of likely pathogenic variants on the USP9X protein structure.

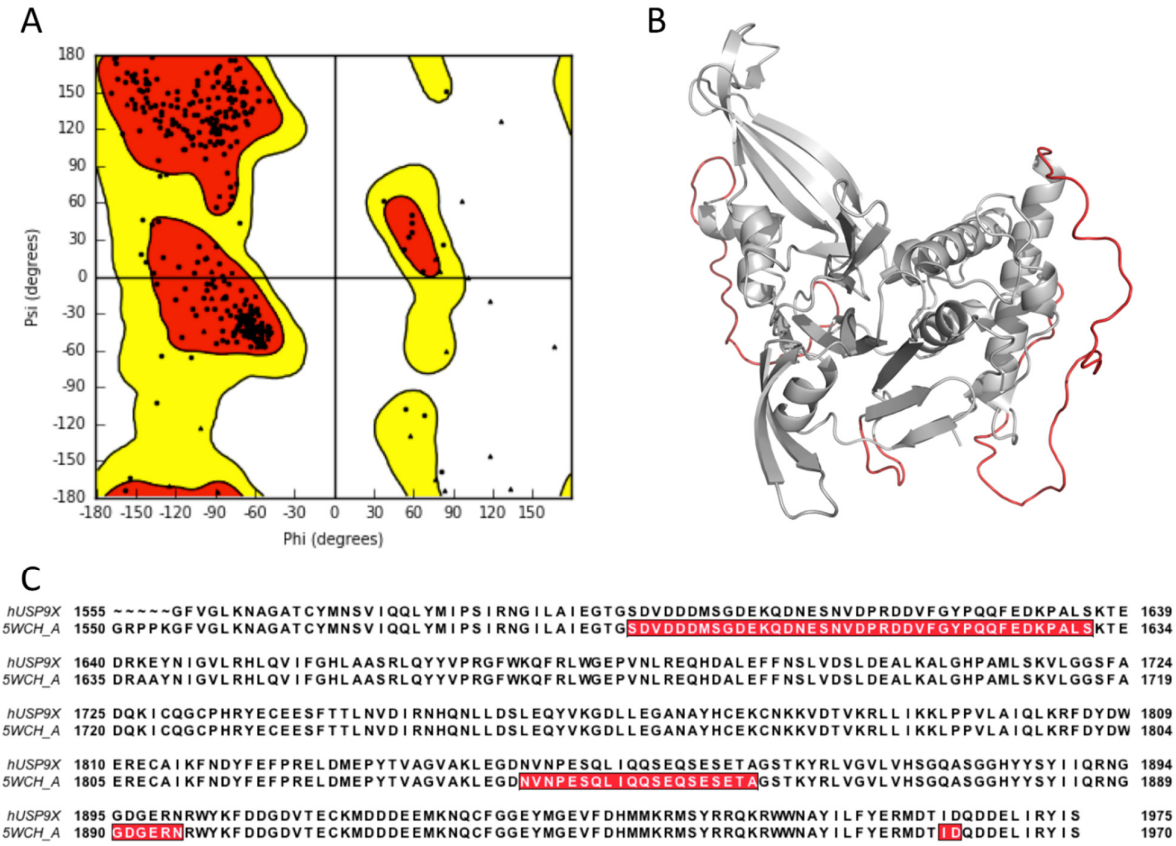


Figure S3. Ramachandran plot of USP9X catalytic domain homology model. Human USP9X/1555-1975 was aligned to crystal structure 5WCH using Maestro multiple sequence viewer (Schrödinger). Homology model was generated using Maestro Bioluminate (Schrödinger) using energy minimisation to model flexible loops absent from 5WCH. A. Ramachandran plot of USP9X homology model. Images were prepared using PyMol (Schrödinger). >97% amino acids are within accepted torsion angles. B. Homology model showing regions present within the crystal structure 5WCH (Zhang et al (1), grey), with absent and likely flexible loops modeled by energy minimization (red). C. Alignment showing the primary sequence positions of flexible loops (red) and sequence alignment used for homology modeling.

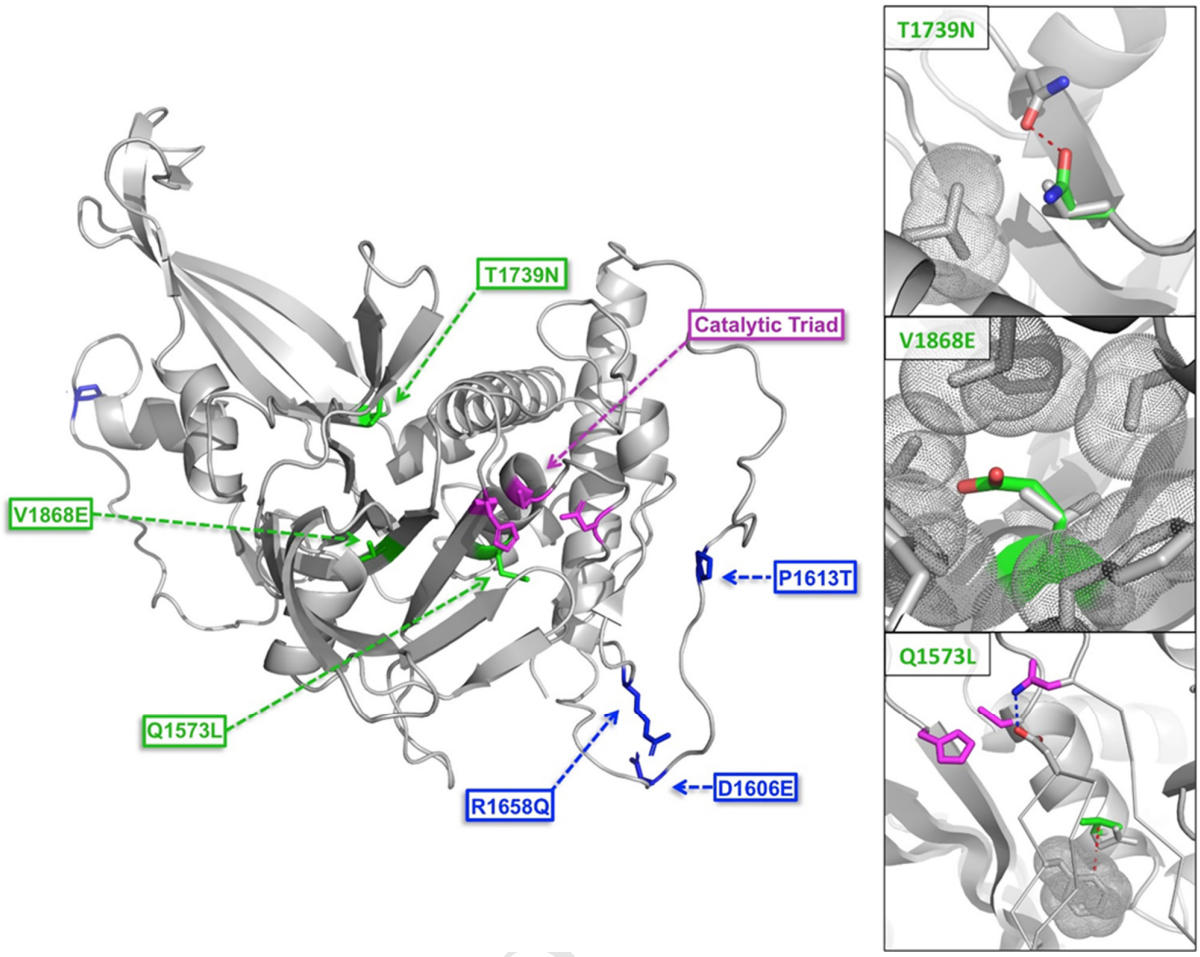


Figure S4. Structural characterization of USP9X variants located in the catalytic domain. Homology model of USP9X (grey) with catalytic site (magenta), likely pathogenic variants (green) and variants of unknown significance (blue) indicated. Likely pathogenic variants are positioned in regions of well-ordered secondary structure which are peripheral to the core catalytic site. Variants of unknown significance occur within two loops of predicted (absent from crystal structure) disordered regions, distal to the catalytic site. Variants within this region are less likely to drastically alter protein stability or catalytic activity, but may produce more nuanced alterations in intra/intermolecular interactions. Insets indicate local structural effects of indicated likely pathogenic variants. All native amino acid side chains are represented as grey sticks, with the position of variants / catalytic residues indicated by colored sticks. Charged atoms are indicated in blue (positive) and red (negative). Hydrophobic van der vaals radii are indicated by dots. T1739N may result in charge-charge repulsion with N1741, indicated by a red dashed line, and steric clash with L1687 altering local secondary structure. V1868 lies within a tight hydrophobic core (composted of L1865, W1897, M1916, Y1883, F1818 and C1920). Change of Valine to the bulky, highly polar glutamate is likely to disrupt hydrophobic packing and produce steric clash with adjacent residues, altering local secondary. Q1573 lies upon an alpha helix adjacent to the catalytic site. Glutamate to Leucine substitution may alter hydrophobic packing (with F1900), inducing conformational change within this loop, and potentially altering the position of D1902. This residue forms charge-charge interactions with the catalytic N1561, indicated by a blue dashed line. Alterations in the positioning of D1902 are likely to affect the availability or efficiency of N1561 during proteolytic activity.

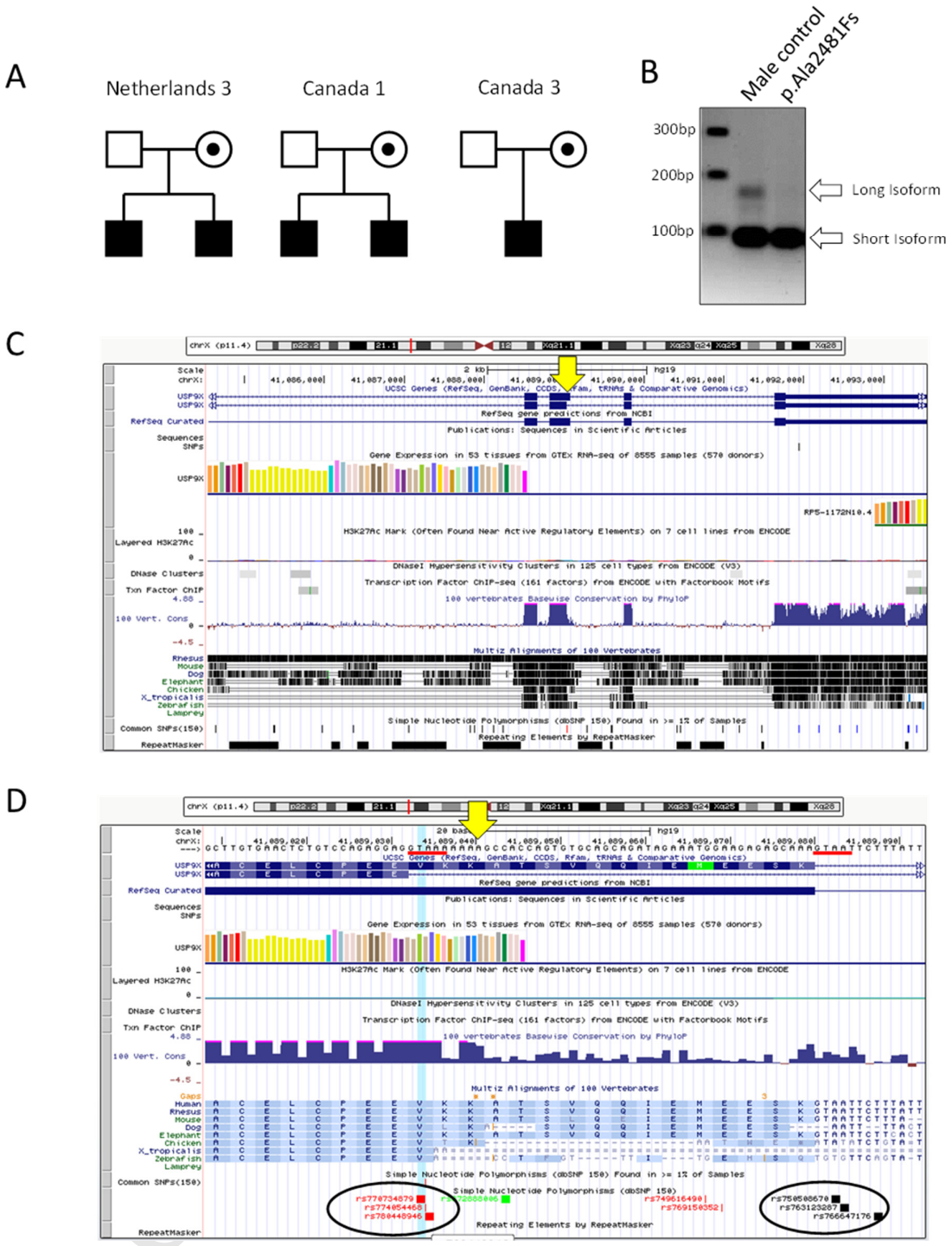


Figure S5. A recurrent variant with deleterious or benign impact depending on USP9X isoform usage. p.Ala2481Fs*17 variant impacts the long *USP9X* isoform (NM_001039590.2:c. c.7440dupA, NP_001034679.2:p.ALa2481Serfs*17) but is intronic in the short isoform (NM_001039591.2;c.[0]. NP_001034680.2 p.[0]). A. Pedigrees of the three independent families with this variant. B. Analysis of mRNA species in fibroblast cell lines from control individual and individual with p.Ala2481fs*17 variant (from family Netherlands 3). Note that long isoform is poorly expressed in control, but absent in the variant cell line (suggestive of non-sense mediated mRNA decay). C-D. UCSC Genome Browser views of the relevant genomic region. Yellow arrows represent site of variant (dup A). Red underlines in D represent splice sites. Black circles highlight SNPs in splice sites.

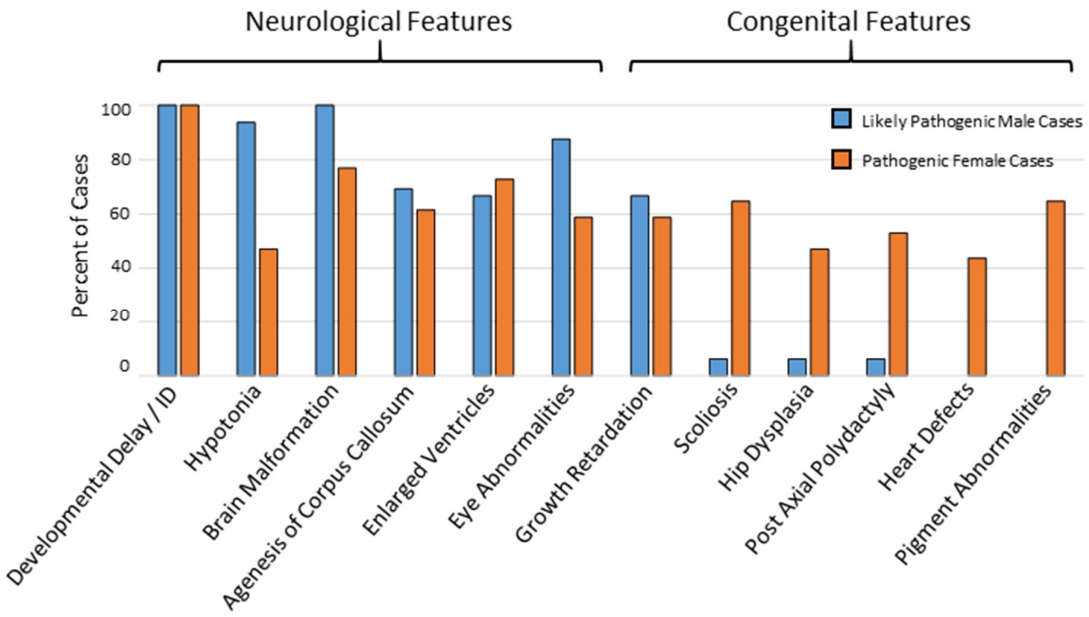


Figure S6. Comparison of the major neurological and congenital features observed in female subjects with male subjects. Most frequent features of female subjects with pathogenic heterozygous loss of function mutations as reported in Reijnders *et al* (2). Assessed against the cohort of males with likely pathogenic missense variants. Note the major neurological findings in females are frequently observed in males, but the congenital features are not.

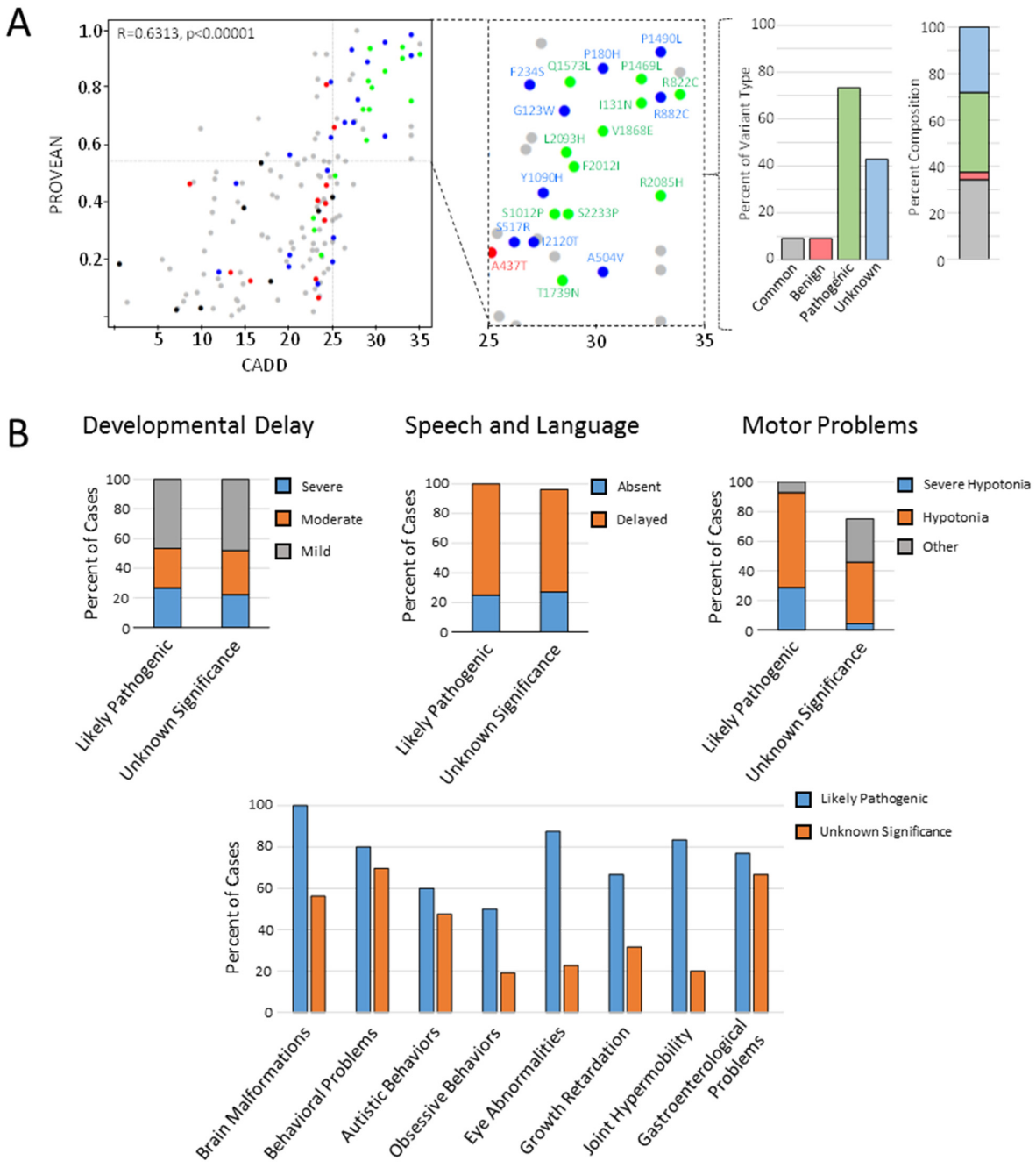


Figure S7. Comparison variants of unknown significance with likely pathogenic variants. A. CADD and PROVEAN scores reveal clustering of subsets of variants of unknown significance with likely pathogenic variants in upper-right quadrant consistent with pathogenicity. Scores are significantly correlated (Pearson's correlation given). Inset identifies variants in the 'pathogenic quadrant'. Graphs show percent of each type of variant, and the overall composition of variant types within the pathogenic quadrant. B Distinctive clinical features of individuals with USP9X likely pathogenic variants are observed also in many individuals with USP9X variants of unknown significance.

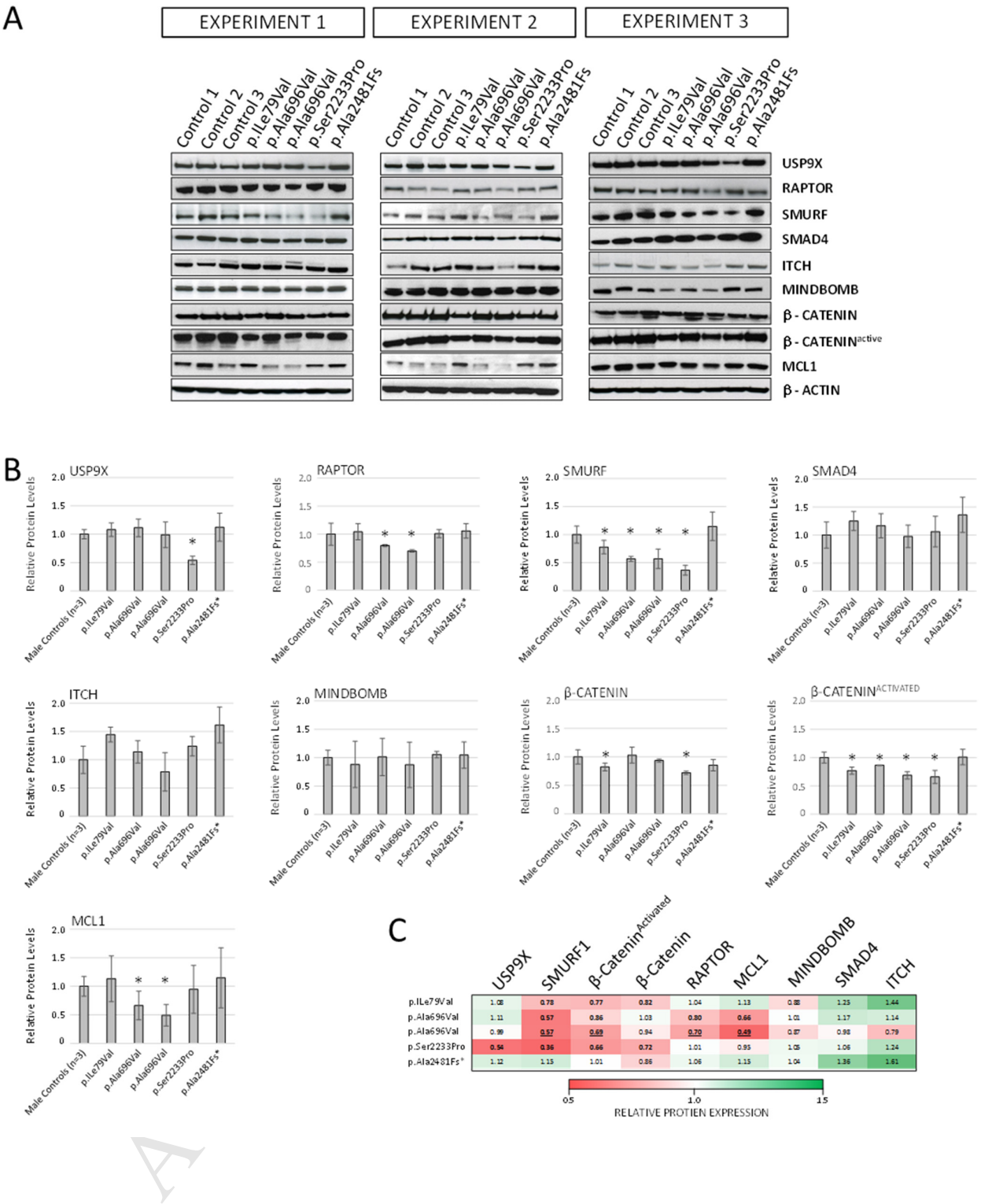


Figure S8. Protein expression of USP9X and its substrates in fibroblast cell lines derived from individuals with USP9X variants. A. Western blot analysis of n=3 independent experiments. B-C. Quantitation of western blots in A. *significantly different to controls, p<0.05 by Student's t-test. In C, significant values are in bold and underlined.

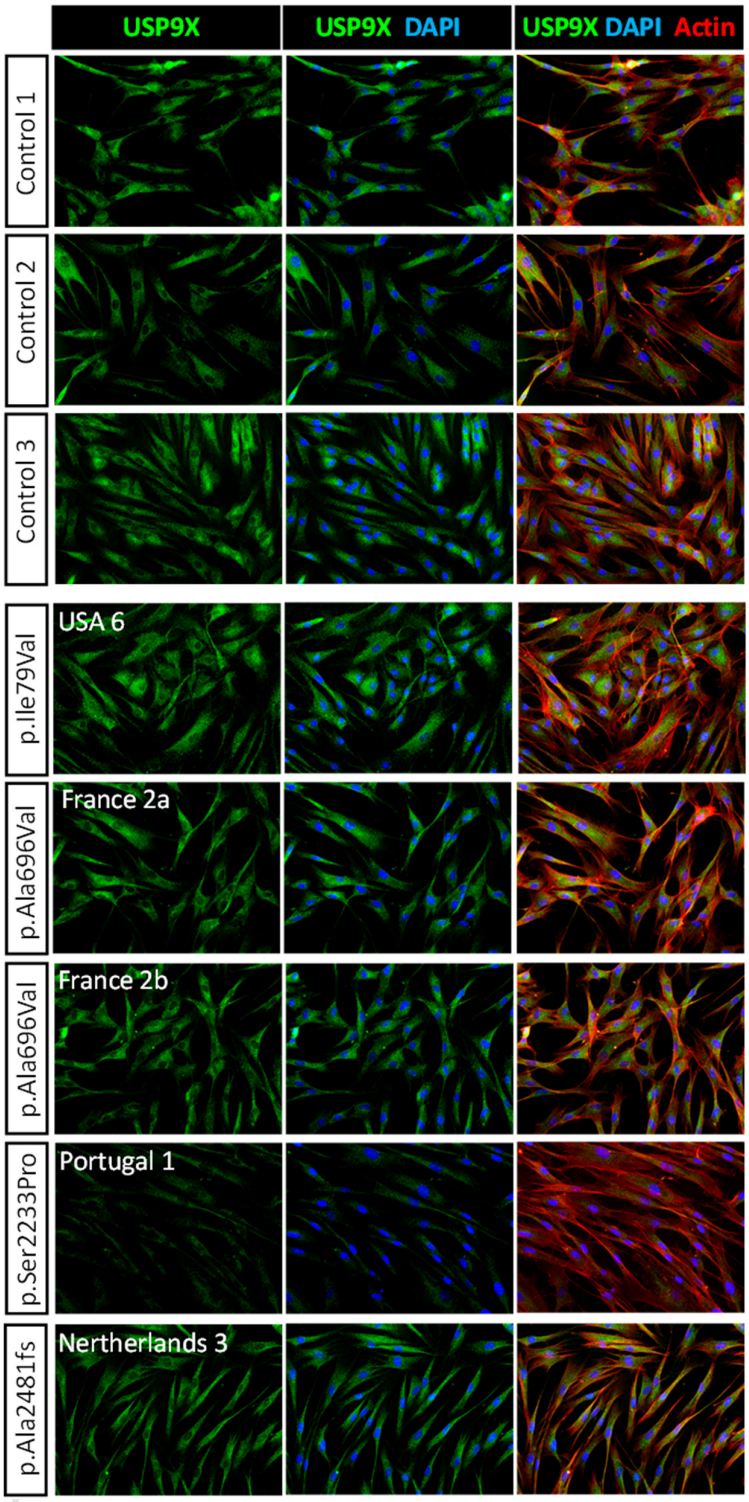


Figure S9. Representative immunofluorescent images of fibroblast cell lines derived from control individuals, and individuals with USP9X variants. Cells strained with antibodies against USP9X (Green), and counterstained with DAPI (Nuclei, Blue) and Phalloidin (Filamentous actin cytoskeleton, Red).

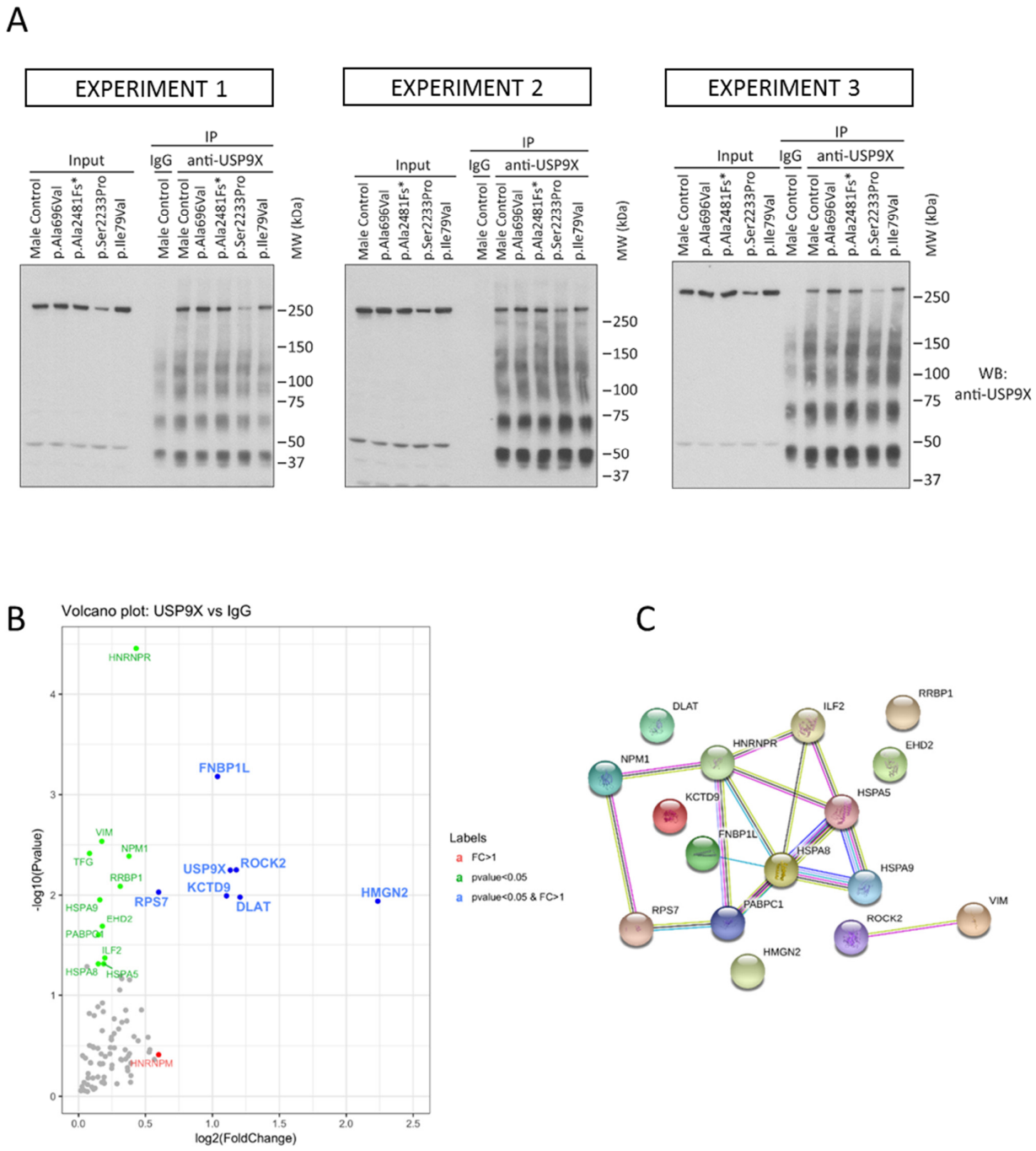


Figure S10. Identification of USP9X interactors in fibroblast cell lines derived from control individuals and individuals with USP9X variants. A. Western blot analysis of USP9X immunoprecipitation experiments. IgG immunoprecipitations served as negative control samples in subsequent proteomics. USP9X and IgG control immunoprecipitated samples from each experiment subsequently analysed using TMT-EIS-MS/MS identification and quantification. B. Volcano plot of proteins immunoprecipitated with USP9X from control fibroblasts. Results derived from 3 independent immunoprecipitation experiments in A. Fold change and statistical values represents comparison to proteins immunoprecipitated with control IgG. p values derived from adjusted Students paired t-test. C. STRING analysis of significantly enriched proteins in B. Statistical analysis reveals a well-connected network (Protein-Protein Interaction enrichment p-value: 0.000589).

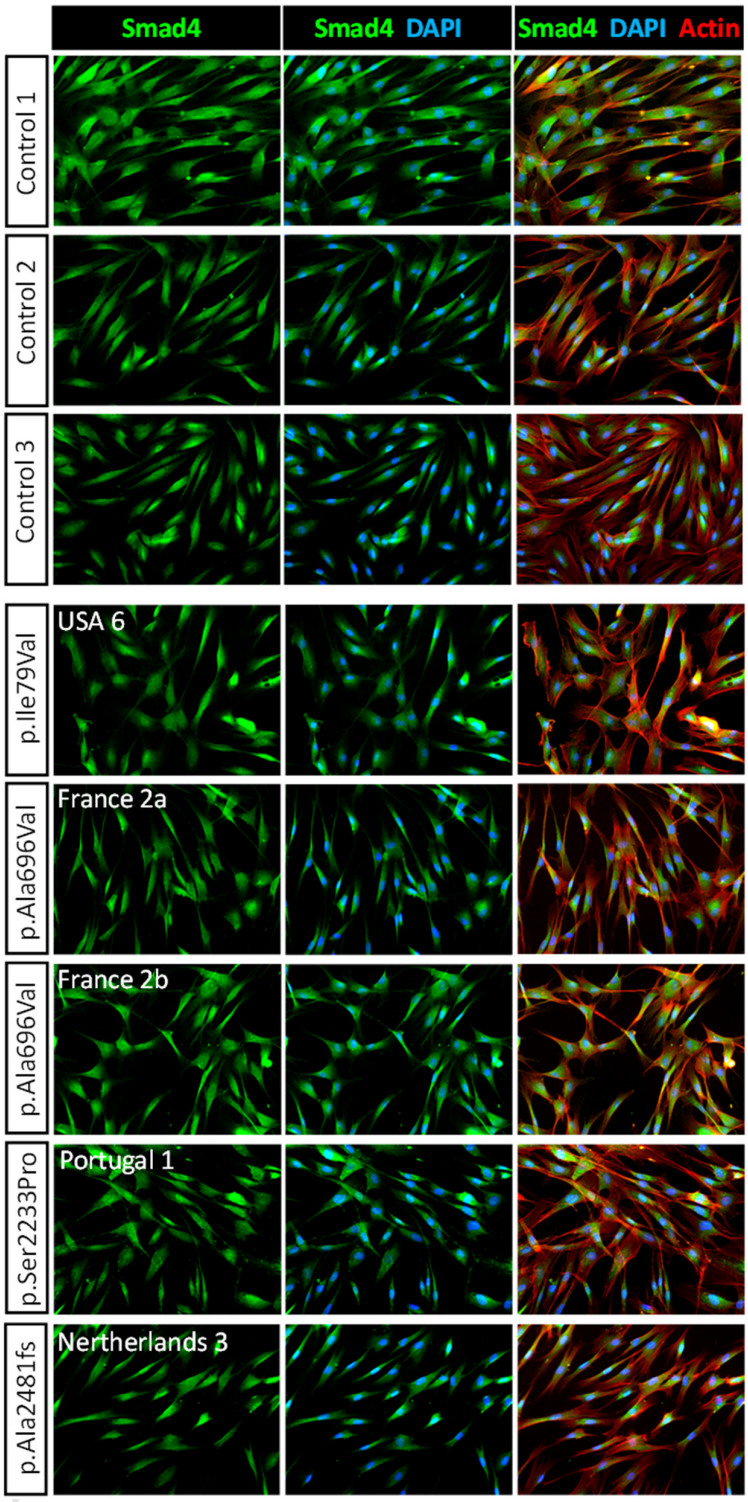


Figure S11. Expression and localisation of SMAD4 in fibroblast cell lines derived from control individuals, and individuals with USP9X variants. Representative immunofluorescent images. Cells strained with antibodies against SMAD4 (Green), and counterstained with DAPI (Nuclei, Blue) and Phalloidin (Filamentous actin cytoskeleton, Red).

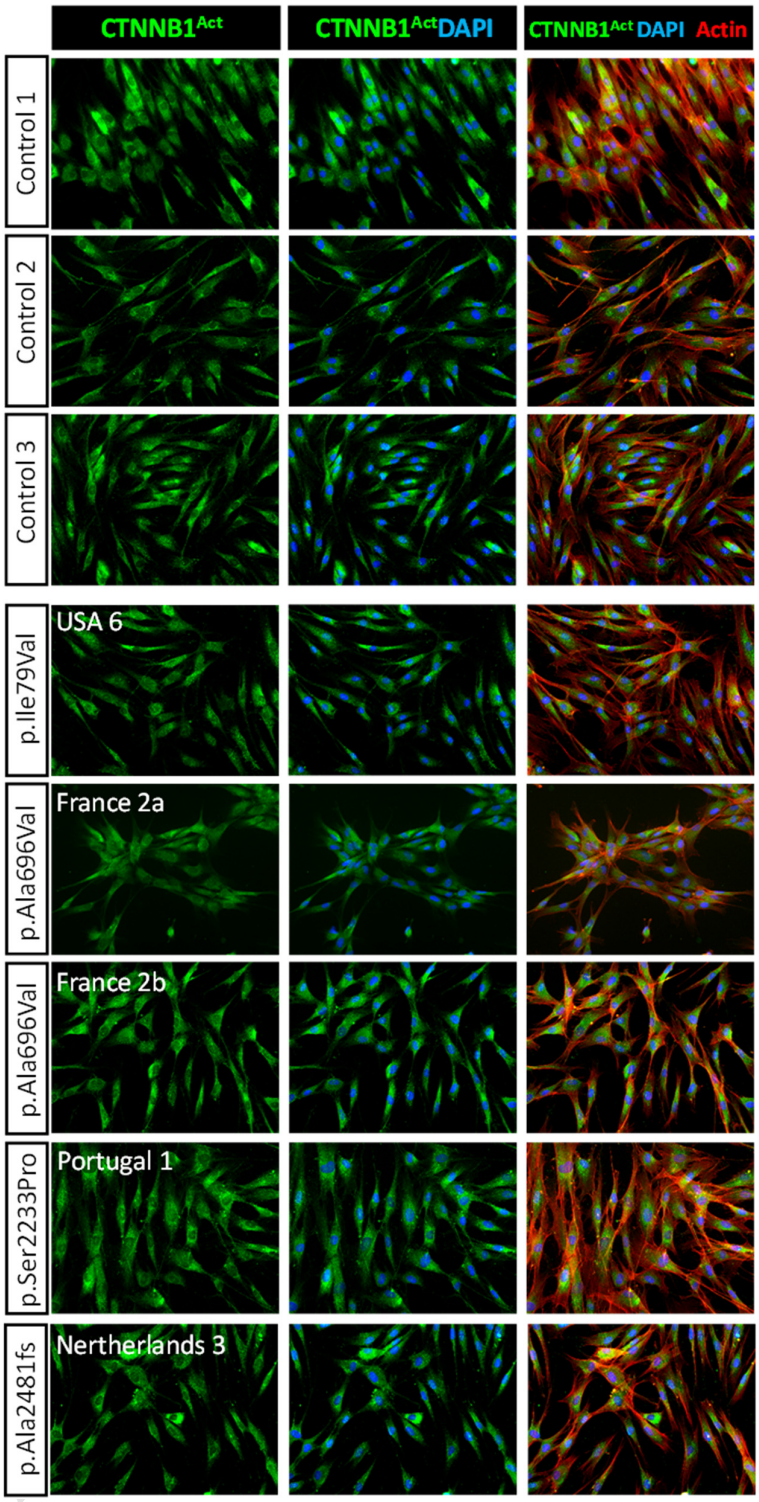


Figure S12. Expression and localisation of CTNNB1 in fibroblast cell lines derived from control individuals, and individuals with USP9X variants. Representative immunofluorescent images. Cells stained with antibodies against activated CTNNB1 (aka β-catenin, Green), and counterstained with DAPI (Nuclei, Blue) and Phalloidin (Filamentous actin cytoskeleton, Red).

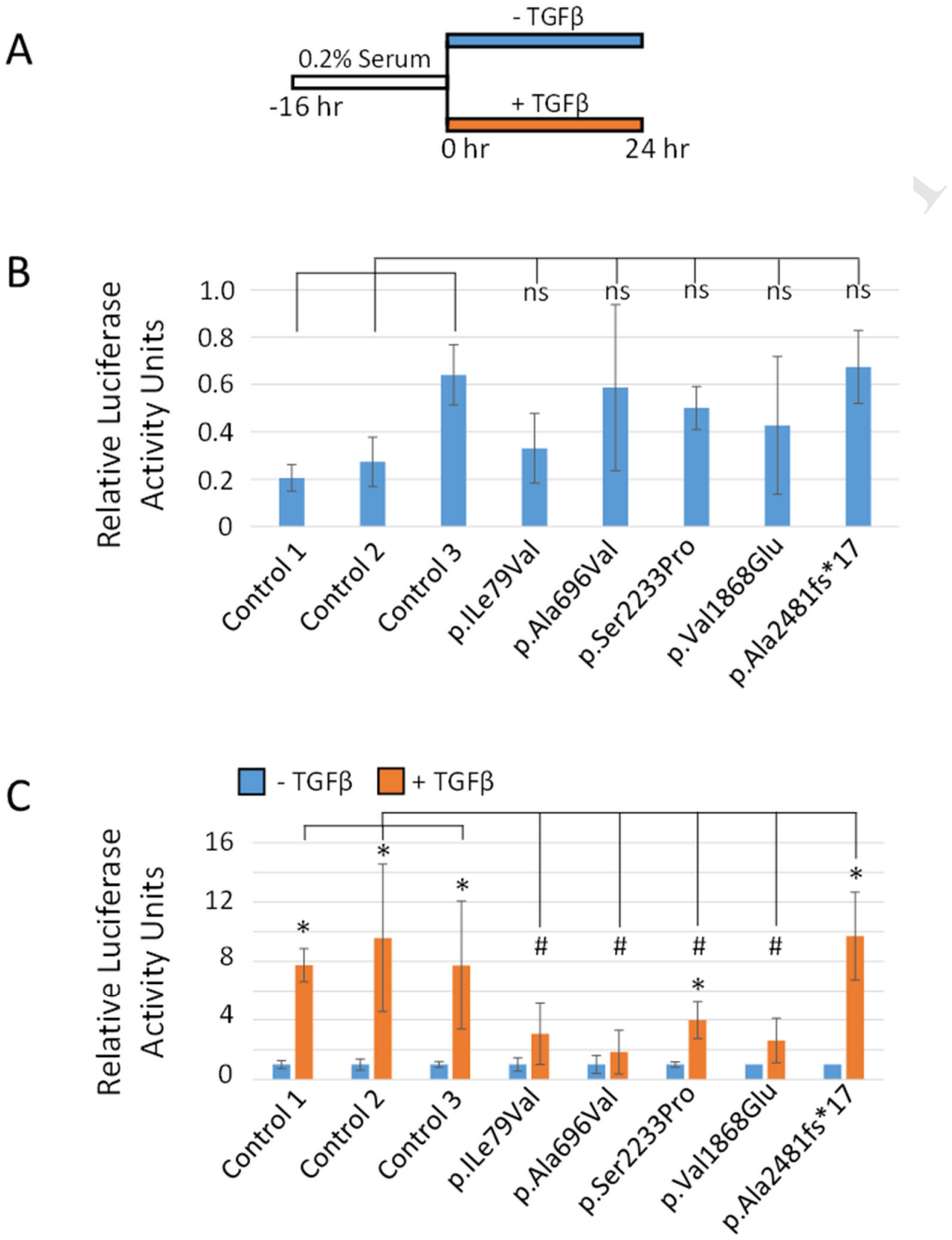


Figure S13. USP9X variants disrupt TGFβ signalling in fibroblast cell line derived from individuals with USP9X variants. A. Pictorial view of the experimental approach. Cells were first serum starved (0.2% serum) for 16 hrs prior to addition of TGFβ and assayed 24 hours later. A. In the absence of added TGFβ, cells display similar basal levels of signalling as assessed by TGFβ luciferase reporter assay. B. Relative increase of TGFβ signalling following addition of ligand as assessed by TGFβ luciferase reporter assays. Experiment done in quadruplicate. * statistical difference between +/- TGFβ. # statistical difference between controls and USP9X variant cell lines. *#p<0.05 Student's t-test. N.B. p.Ala2481fs*17 variant effects only the long USP9X isoform which is barely expressed in fibroblasts.

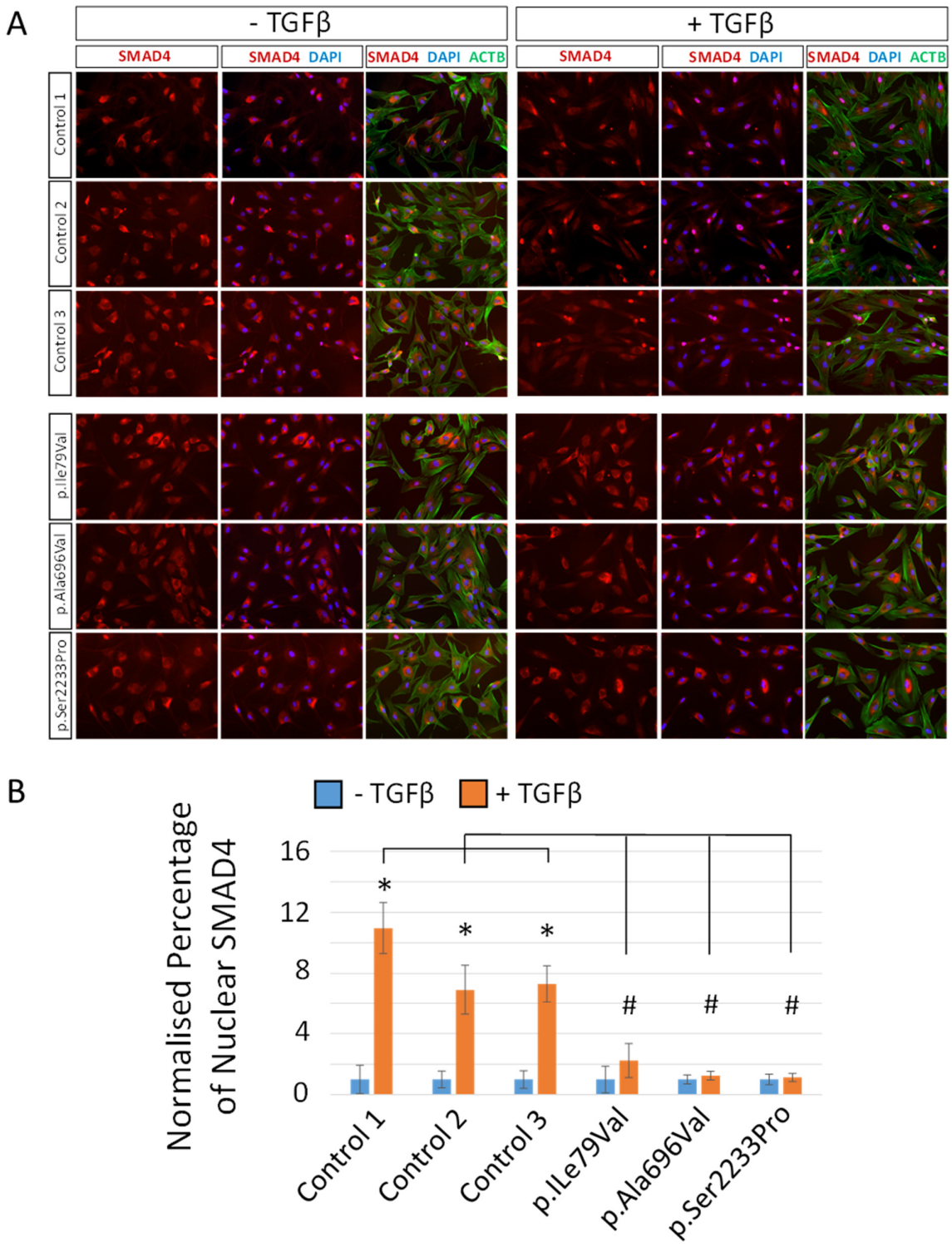


Figure S14. SMAD4 nuclear enrichment is deficient in fibroblast cell lines derived from individuals with USP9X variants in response to TGFβ stimulation. A. Representative immunofluorescent images of SMAD4 localisation before (time = 0 hr) and after (time = 24 hours) addition of TGFβ. Cells stained with antibodies against SMAD4 (Red), and counterstained with DAPI (Nuclei, Blue) and Phalloidin (Filamentous actin cytoskeleton, Green). B. Quantitation of SMAD4 enriched nuclei following addition of TGFβ. * statistical difference between +/- TGFβ. # statistical difference between controls and USP9X variant cell lines. $p<0.05$ Student's t-test; n=5 replicates.

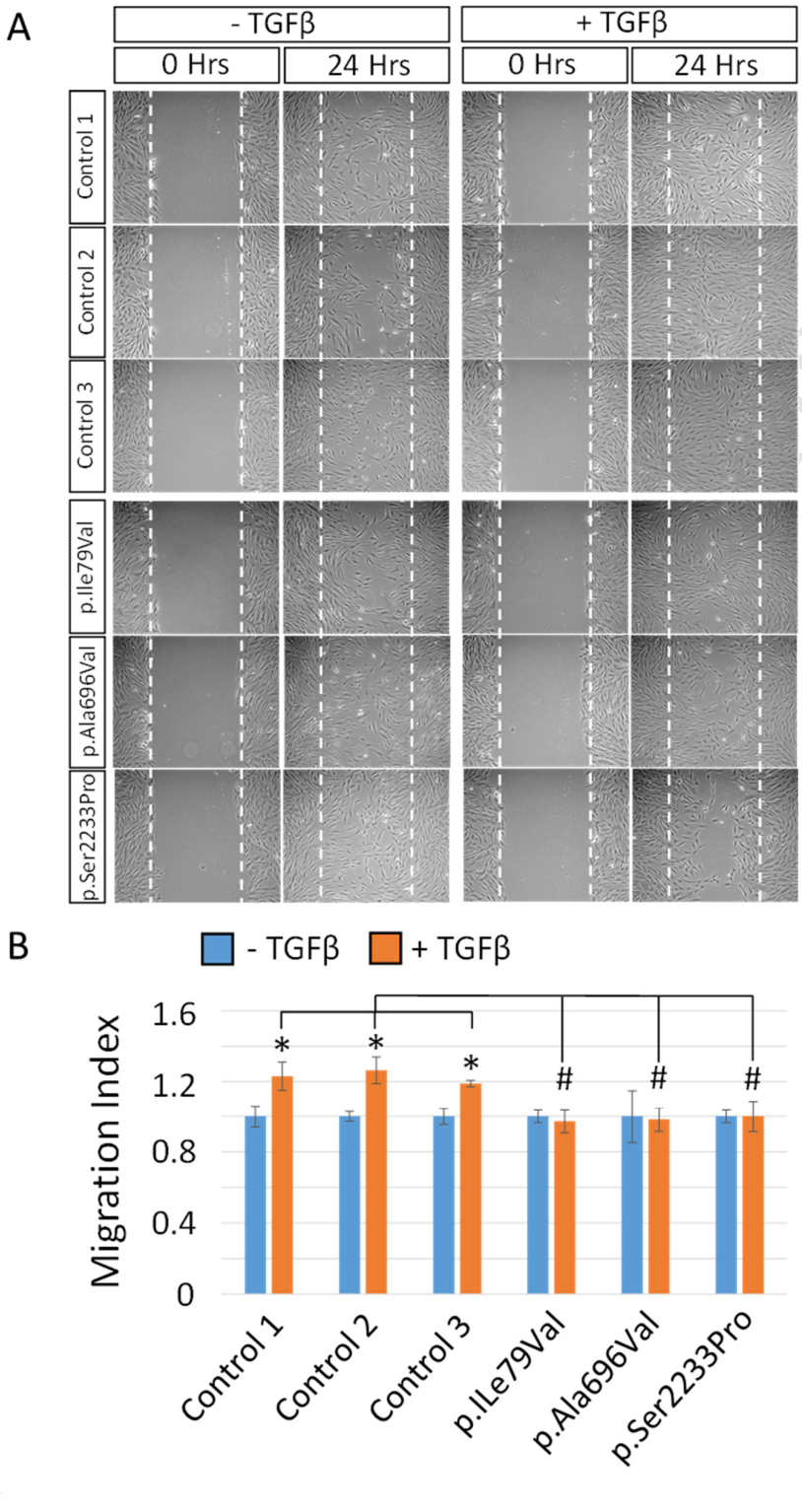


Figure S15. TGFβ-stimulated cell migration is defective in fibroblast cell lines derived from individuals with USP9X variants. A. Representative phase-contrast images of scratch migration assays. B. Quantitation of TGFβ-stimulated migration of cells into the scratch area. n=9 replicates (3 biological x 3 technical (scratches) analysed). * statistical difference between +/- TGFβ. # statistical difference between controls and USP9X variant cell lines. #* $p<0.05$ Student's t-test.

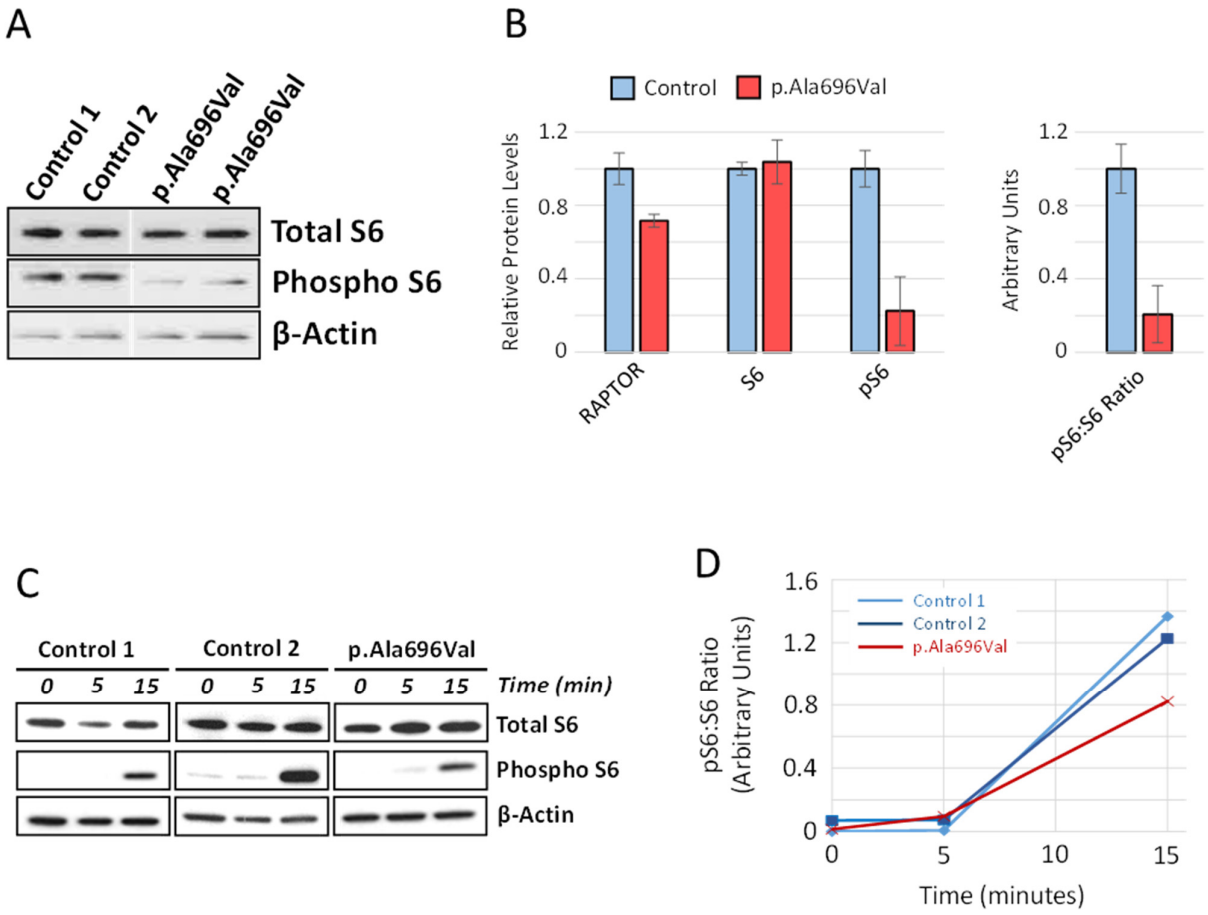


Figure S16. Fibroblast cell lines harbouring p.Ala696Val variant show defective mTOR signalling. A-B. Cells grown in presence of 10% foetal calf serum and protein harvested for analysis. A. Western-blot analysis of control and p.Ala696Val cell lines (from 2 unique individuals, brothers). B. Quantification of blots in A and calculation of ratio of phosphorylated S6 (pS6) to total S6 levels. Loss of pS6:S6 ratio indicates loss of mTOR signalling. C-D. Cells grown for 16 hours in absence of serum. Serum was returned and protein lysates collected over 15 minute time course. C. Western blot analysis showing kinetics of pS6 induction. D. Quantitation of western-blots in C.

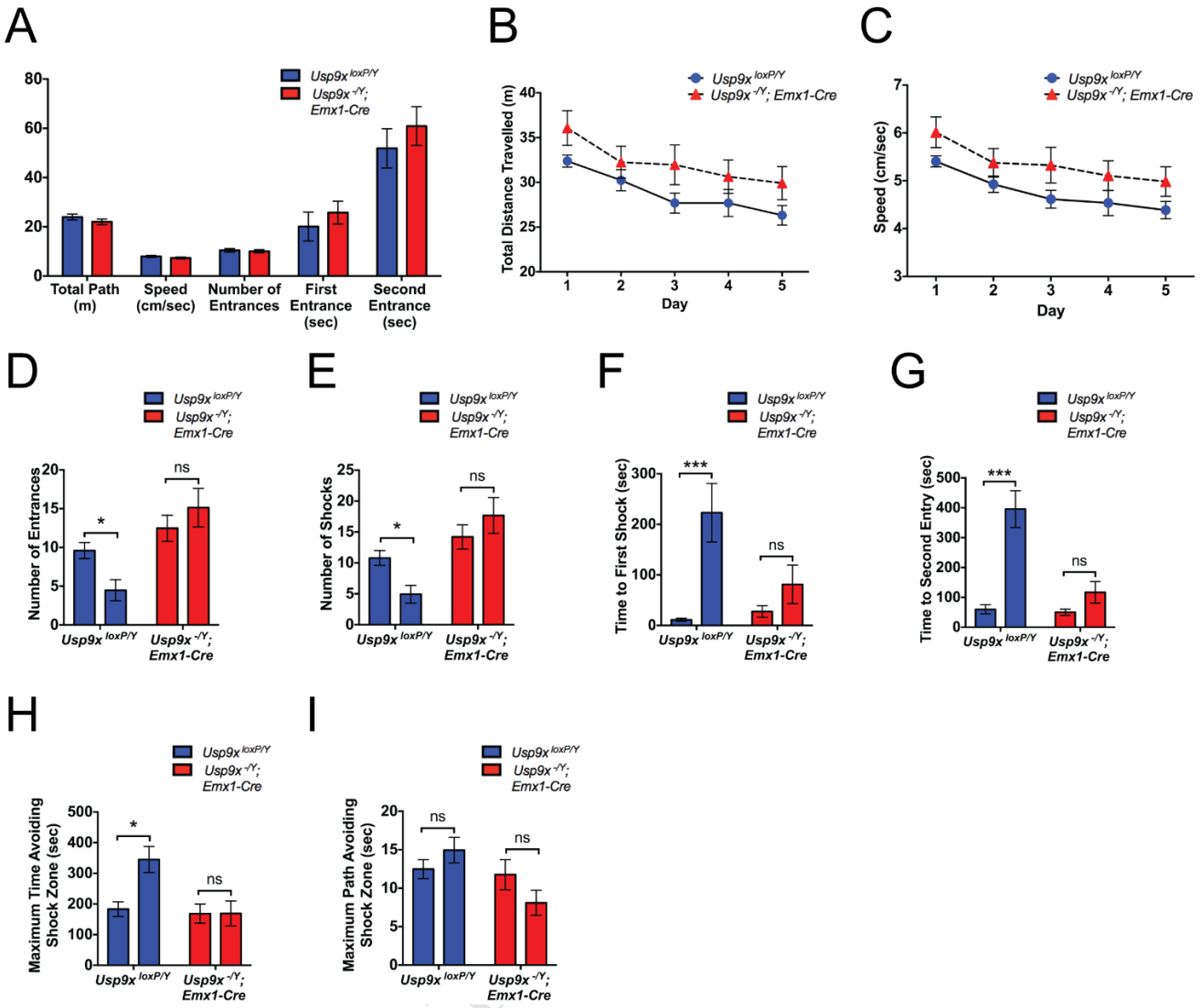


Figure S17. A. *Usp9x* knockout mice do not show altered locomotory behaviour within the APA test arena, and do not learn within the APA task. A. Analysis of mouse movement patterns within the habituation phase of the APA test revealed no significant difference between wild-type (blue bars) and *Usp9x* knockout (red bars) mice. Similarly, there were no significant differences in the total distance travelled (B) and speed (C) between control and knockout animals over the five days of the APA protocol. D-I. Intra-genotype analysis revealed that, whereas wild-type mice performed better on day 5 than day 1, indicative of learning within the APA task, knockout mice did not perform better on day 5 versus day 1 of the task. * $p < 0.05$; *** $p < 0.001$, two way ANOVA.

SUPPLEMENTAL TABLES S1-S7

	Family	Subjects	Variant Details			Platform	Segg#	Number and Frequency in gnomAD			Prediction Algorithms				
			Chromosome X GRCh37(hg19)	cDNA NM_001039590.2	Protein NP_001034679.2			Het	Hem	Frequency	Polyphen2 Score	Pred	PROVEAN Score	Pred	CADD Score
Likely Pathogenic	USA 6	1	40988391A>G	c.235A>G	p.Ile79Val	tWES	Yes				0.837	P	-0.77	N	23.7
	USA 5	1	40994047T>A	c.392T>A	p.Ile131Asn	WES	De novo				0.999	D	-6.13	D	33
	France 2	2	41025226C>T	c.2087C>T	p.Ala696Val	WES	n/a				0.287	B	-2.18	N	25.3
	UK1	1	41027299C>T	c.2464C>T	p.Arg822Cys	tWES	De novo				0.991	D	-6.47	D	35
	USA 14	1	41029757A>G	c. 2912A>G	p.Asn971Ser	tWES	De novo				0.999	D	-6.99	D	29.3
	France 3	1	41057806C>T	c.4406C>T	p.Pro1469Leu	tWES	De novo				0.963	D	-7.13	D	33
	USA 13	1	41060427A>T	c.4718A>T	p.Gln1573Leu	tWES	De novo				0.102	B	-1.39	N	22.8
	USA 18	1	41073847C>A	c.5216C>A	p.Thr1739Asn	tWES	De novo				0.83	P	-2.95	D	28.9
	USA 1	1	41075423T>A	c.5603T>A	p.Val1868Glu	tWES	De novo				0.732	P	-5.48	D	31
	USA 19	1	41075854T>A	c.6034T>A	p.Phe2012Ile	tWES	De novo				0.961	D	-4.71	D	29.5
	Spain 1	1	41077669G>A	c.6254G>A	p.Arg2085His	tWES	De novo				0.974	D	-4.14	D	34
	Portugal 1	1	41082601T>C	c.6697T>C	p.Ser2233Pro	DES	Yes				0.918	D	-3.85	D	29.2
Unknown Significance	USA 7	1	40994022G>T	c.367G>T	p.Gly123Trp	tWES	n/a				0.996	D	-6.18	D	32
	Netherlands 11	2	40996160C>A	c. 539C>A	p.Pro180His	tWES	Yes				0.999	D	-7.76	D	31
	USA 9	1	40999955T>C	c.701T>C	p.Phe234Ser	tWES	n/a				0.999	D	-6.86	D	27.2
	USA 3	1	41000406A>G	c.958A>G	p.Arg320Gly	WES	n/a				0.903	P	-4.95	D	24.8
	Netherlands 9	1	41007713C>T	c.1511C>T	p.Ala504Val	tWES	Yes				0.955	D	-3.05	D	31
	Netherlands 12	1	41007751A>C	c.1549A>C	p.Ser517Arg	tWES	Yes				0.982	D	-3.46	D	26.4
	USA 12	2	41029255C>T	c.2644 C>T	p.Arg882Cys	tWES	Yes				1	D	-6.37	D	34
	Canada 2	1	41029345A>G	c.2734A>G	p.Ile912Val	tWES	n/a	1	0	5.60E-06	0.001	B	-0.49	N	11.99
	Netherlands 10	1	41043278C>A	c.3176C>A	p. Ala1059Asp	tWES	Yes				0.186	B	-1.05	N	25.1
	Netherlands 1	1	41043370T>C	c.3268T>C	p.Tyr1090His	tWES	Yes				0.999	D	-4.2	D	27.9
	Netherlands 7	1	41045786A>G	c.3575A>G	p.His1192Arg	tWES	n/a				0.039	B	-2.02	N	13.96
	USA 21	1	41045833C>T	c.3622 C>T	p.Pro1208Ser	tWES	n/a				0.05	B	-3.01	D	24.8
	USA 11	1	41048705C>G	c.3954C>G	p.Asp1318Glu	tWES	Yes				0.083	B	-2.63	D	20.1
	USA 22	1	41057869C>T	c.4469C>T	p.Pro1490Leu	tWES	n/a				0.973	D	-9.26	D	34
	Spain 2	1	41060527C>A	c.4818C>A	p.Asp1606Glu	tWGS	n/a				0.003	B	-0.58	N	20
	USA 17	1	41064568C>A	c.4837C>A	p.Pro1613Tyr	tWES	n/a				0.014	B	-0.66	N	25
	USA 26	2	41064704G>A	c.4973G>A	p.Arg1658Gln	tWES	Yes	3	2	1.70E-05	0.004	B	-0.27	N	23.3
	Canada 6	1	41077774T>C	c.6359T>C	p.Ile2120Thr	WES	n/a	1	0	5.59E-06	0.474	P	-3.46	D	27.4
	USA 28	1	41084144A>C	c.6901A>C	p.Lys2301Gln	tWGS	n/a				0.36	B	-2.29	N	24.4
	Netherlands 3	2	41089041dupA	c.7440dupA	p.Ala2481fs*17	WES	Yes	7	4	6.21E-05	n/a	n/a	n/a	n/a	n/a
	Canada 1	1	41089041dupA	c.7440dupA	p.Ala2481fs*17	DES	Yes	7	4	6.21E-05	n/a	n/a	n/a	n/a	n/a
	Canada 3	2	41089041dupA	c.7440dupA	p.Ala2481fs*17	tWES	n/a	7	4	6.21E-05	n/a	n/a	n/a	n/a	n/a
	Netherlands 5	1	41091697C>T	c.7633C>T	p.Pro2545Ser	WES	n/a	0	1	5.60E-06	0.986	D	-0.7	N	20.1
Likely Benign	Netherlands 2	1	41000604G>C	c.1081G>C	p.Val361Leu	WES	No				0.739	P	-1.99	N	24.3
	Netherlands 6	1	41002691G>A	c.1309G>A	p.Ala437Thr	WES	No				0.69	P	-3.32	D	25.2
	Swiss 1	1	41031160A>G	c.3097A>G	p.Met1033Val	WES	No				0.001	B	-0.48	N	13.33
	Norway 1	1	41043275G>A	c.3173G>A	p.Arg1058Lys	tDES	No				0.002	B	0.03	N	23.4
	Canada 5	1	41043684C>A	c.3314C>A	p.Pro1105His	WES	No				0.906	P	-4.83	D	24.3
	Netherlands 4	1	41043792T>C	c.3422T>C	p.Met1141Thr	WES	No	1	1	1.12E-05	0.001	B	-2.01	N	8.661
	Netherlands 8	1	41055921A>G	c.4163A>G	p.Asn1388Ser	tWES	n/a	16	4	1.03E-04	0.013	B	-0.36	N	23.1
	Belgium 1	1	41064560A>G	c.4829A>G	p.Asn1610Ser	DES	n/a				0.137	B	-1.71	N	23.3
	France 1	1	41075489G>A	c.5669G>A	p.Gly1890Glu	tWES	No	2	0	1.12E-05	0.002	B	-0.33	N	15.6
	USA 4	1	41075489G>A	c.5669G>A	p.Gly1890Glu	WES	No*	2	0	1.12E-05	0.002	B	-0.33	N	15.6
	USA 29	1	41077775A>G	c.6360A>G	p.Ile2120Met	tWES	No*	17	6	1.15E-04	0.754	P	-1.65	N	24.2
	Germany 1	1	41077775A>G	c.6360A>G	p.Ile2120Met	WES	No	17	6	1.15E-04	0.754	P	-1.65	N	24.2
	Denmark 1	1	41082482A>G	c.6578A>G	p.Lys2193Arg	WES	No				0.078	B	-1.35	N	24.1
Reported	Paemka 1	1	41031097T>C	c.3034T>C	p.Ser1012Pro	WES	De novo				0.943	D	-3.84	D	28.5
	Homan 1	1	41077693T>A	c.6278T>A	p.Leu2093His	XES	De novo				0.984	D	-4.99	D	29.1
	Homan 2	2	41078388C>A	c.6469C>A	p.Leu2157Ile	XES	n/a	1	0	5.68E-06	0.245	B	-1.18	N	22.9
	Homan 3	3	41089848delA	c.7574delA	p.Gln2525fs*18	XES	Yes				n/a	n/a	n/a	n/a	n/a

Table S1. USP9X variants associated with NDDs. Likelihood of pathogenicity assigned using American College of Medical Genetics and Genomics Guidelines (ACMG). Cases refers to number of affected individuals in the family. Subject UK 1 has Decipher ID: 260068. Subject Netherlands 2 has Decipher ID: 323395. Subject Homan 1 has Decipher ID: 318087. Platform refers to sequencing approach: WES, Whole Exome sequencing; WGS, Whole Genome Sequencing; DES Disease gene panel Exome sequencing; XES, X-chromosome Exome Sequencing; prefix t is for trio-based approach. Segg#: segregation studies: yes: segregates beyond trio analysis; n/a: not conducted beyond trio analysis; No, found in healthy male relative (*recurrent variant found in a healthy male relative in an unrelated family). Results of prediction algorithms colour coded: Red, deleterious/pathogenic; pink potentially deleterious / likely pathogenic.

	Gene	Variant	Inheritance	Genotype	ACMG	Relevant Associated Genetic Disease (MIM)	Notes
Netherlands 1	ARID1B	Chr6 g.157528688T>G; NM_020732.3:c.6413T>G; p.Leu2138Arg	De novo	Heterozygous	VUS	Coffin-Siris Syndrome (135900)	Pathogenicity stems from LOF mutations. No missense mutations reported in OMIM. Pateint not displying hallmark Coffin-Siris Syndromic features (e.g. 5th finger abnormalities). Varinat absent in gnomAD.
Netherlands 5	FBXO28	Chr1 g.224345411_224345414delCTCT; NM_015176.3:c.1070_1073delCTCT; p.Ser357Leufs*28	De novo	Heterozygous	LB	n/a	4x heterozygous LOF alleles in gnomAD
USA 5	PHKA1	ChrX g.71864208T>C; NM_002637.3:c.1459+4A>G (IVS14+4A>G)	Maternally Inherited	Hemizygous	LB	Muscle glycogenosis (300559)	Phenotypes do not match. Variant found 1x heterozgous in gnomAD; 4 other varaints affecting same splice site found 4x hemizygous in gnomAD; 4x hemizygous LOF alleles found in gnomAD.
USA 9	POLG1	Chr15 g.89870178C>A; NM_002693.2:c.1550G>T; p.Gly517Val	De novo	Heterozygous	LB	Progressive external ophthalmoplegia (157640)	Phenotypes do not match. 30 LOF heterozygous alleles in GnomAD. Varaint absent in gnomAD.
USA 11	CDK11A	Chr1 g.1650770C>T; NM_024011.3:c.352G>A; p.Gly118Arg Chr1 g.1636016C>T; NM_024011.3:c.1537G>A; p.Glu513Lys	Inherited	Compound Heterozygous	LB	n/a	10 LOF homozygous alleles found in gnomAD. Both variants absent in gnomAD
USA 13	MECP2	ChrX g.153296048G>A; NM_004992.3:c.1231C>T; p.Pro411Ser	Maternally Inherited	Hemizygous	VUS	Severe congenital encephalopathy with early death (300673) and Mental retardation with spasticity and other features (300055)	Missense varinats have been associated with male neurodevelopmental disorders. Pateint without characteristic Rett Syndromic features (e.g. epilepsy, spastcity). Varinat absent in gnomAD.
	KDM2B	Chr12 g.121947486C>T; NM_032590.4:c.1531G>A; p.Glu511Lys Chr12 g.121947542T>C ;NM_032590.4:c.1475A>G; p.Lys492Arg	Inherited Denovo	Compound Heterozygous	LB	n/a	p.Glu511Lys allele found 4x heterozygous in gnomAD. p.Lys492Arg is absent in gnomAD. >800 LOF alleles in heterozygous state, and 4 LOF alleles in homozygous state in gnomAD
USA 17	SORCS1	Chr10 g.108432707C>A; NM_001206572.1:c.1977G>T; p.Glu659His	De novo	Heterozygous	LB	Alzheimer disease 6 (605526)	Phenotypes do not match. Varaints absent in gnomAD. 55 LOF heterozygous alleles in gnomAD.
	Duplication 9q21.2	525.93kb region spanning 5 genes.	De novo	Heterozygous	VUS	n/a	n/a
USA 22	RPS6KA3	ChrX g.20179844G>A; NM_004586.2:c.1877C>T; p.Pro626Leu	Maternally inherited	Hemizygous	VUS	Coffin-Lowry syndrome (303600) and XLID (300844)	Varaint has not been reported in these disorders. Variant absent in gnomAD. Pateint not displaying hallmark Coffin-Lowry Syndomic features (e.g. short stature, skeletal abnormalitites, hearing deficit, digital features); but may overlap with milder non-syndromic XLID also reported for missense variants.

Table S2. Additional genetic variants of note in USP9X cohort.

Table S3. Clinical features of individuals with likely pathogenic USP9X missense variants. Please see accompanying Microsoft Excel File.

Table S4. Clinical features of individuals with USP9X missense variants of unknown significance. Please see accompanying Microsoft Excel File.

Test	Genotype						Statistical Analysis		
	Usp9x+/Y			Usp9x-/Y			Levene's Test	t-test	
	Mean	SEM	N	Mean	SEM	N		Equal Variance Assumed	Equal Variance Not Assumed
of.1	1366.34	104.84	27	1760.75	111.58	19	0.464	0.015	0.014
of.2	814.28	75.39	27	1038.33	67.84	19	0.444	0.041	0.032
of.3	619.72	71.51	27	878.23	58.38	19	0.222	0.012	0.008
of.4	468.7	59.87	27	680.38	74.67	19	0.398	0.031	0.033
of.5	501.01	64.19	27	583.86	86.68	19	0.286	0.436	0.447
of.6	391.69	57.57	27	524.01	79.49	19	0.306	0.173	0.186
Body Length	3.7	0.1	30	3.4	0.1	26	0.088	0.037	0.039
Spont	2.5	0.1	30	2.6	0.1	26	0.692	0.599	0.598
Respiration	2	0	30	2	0	26	N/A	N/A	N/A
Tremor	2	0	30	2	0	26	N/A	N/A	N/A
Urination in Jar	0.3	0.1	30	0.5	0.1	26	0.062	0.263	0.277
Defecation in jar	0.8	0.2	30	0.9	0.2	26	0.842	0.767	0.765
Weight	26.6	0.6	30	24.4	0.4	26	0.193	0.004	0.003
Transfer.arousal.	3.8	0.2	30	3.5	0.2	26	0.274	0.2	0.204
Locomotor Activity	21.5	1.1	30	19.1	1.3	26	0.247	0.169	0.173
Palpebral Closure	2	0	30	2	0	26	0.029	0.287	0.327
Piloerection.	1	0	30	1	0	26	0.029	0.287	0.327
Gait	2.8	0.1	30	2.6	0.1	26	0	0.039	0.044
Pelvic Elevation	2	0	30	2	0	26	0.029	0.287	0.327
Tail Elevation	1	0	30	1	0	26	0.029	0.287	0.327
Touch Escape	2.5	0.1	30	2.2	0.1	26	0.177	0.07	0.077
Positional Passivity	4	0	30	4	0	26	N/A	N/A	N/A
Trunk.Curl	1	0	30	1	0	26	0.007	0.196	0.169
Limb Grasping	1	0	30	1	0	26	0.059	0.357	0.326
Visual Placing	2.8	0.1	30	2.4	0.1	26	0.358	0.024	0.025
Grip Strength	2.9	0.1	30	2.8	0.1	26	0.044	0.359	0.38
Body Tone	1.1	0	30	1.1	0.1	26	0.771	0.884	0.885
Pinna.Reflex	0.1	0.1	30	0.1	0.1	26	0.807	0.944	0.943
Corneal Reflex	1	0	30	1	0	26	0.306	0.137	0.163
Toe.Pinch	2.7	0.1	30	2.7	0.1	26	0.899	0.831	0.831
Skin Colour	1.6	0.1	30	1.4	0.1	26	0.176	0.083	0.084
Heart Rate	1.5	0.1	30	1.6	0.1	26	0.078	0.287	0.285
Limb Tone	2	0.1	30	1.9	0.2	26	0.466	0.485	0.487
Abdominal Tone	1	0	30	1	0	26	0.059	0.357	0.326
Lacrimation	1	0	30	1	0	26	0.84	0.92	0.92
Salivation	1.8	0.1	30	1.8	0.1	26	0.412	0.985	0.985
Provoked Biting	0.2	0.1	30	0.2	0.1	26	0.972	0.821	0.821
Righting Reflex	3	0	30	3	0	26	0.059	0.357	0.326
Negative Geotaxis	3.7	0.1	30	3.3	0.2	26	0.033	0.136	0.147
Wire Manoeuvr	3.9	0.1	30	3.8	0.1	26	0.649	0.723	0.72
Contact Righting.	1	0	30	1	0	26	N/A	N/A	N/A
Fear	0.4	0.1	30	0.3	0.1	26	0.98	0.951	0.951
Irritability	0.9	0	30	0.9	0	26	0.691	0.843	0.842
Aggression.	0.7	0.1	30	0.7	0.1	26	0.819	0.806	0.805
Vocalization	0.6	0.1	30	0.8	0.1	26	0.023	0.09	0.087
Grip Strength 1	1.34	0.08	30	1.09	0.05	26	0.026	0.011	0.009
Grip Strength 2	1.29	0.06	30	1.02	0.03	26	0	0	0
Grip Strength 3	1.2	0.06	30	1.06	0.04	26	0.022	0.079	0.07
Average Grip Strength	1.28	0.06	30	1.06	0.04	26	0.001	0.005	0.004
Hot Plate 1	7.7	1.3	3	9	1.2	6	0.685	0.522	0.487
Hot Plate 2	6.9	1.2	3	12.9	2.1	6	0.258	0.095	0.039
Hot Plate 3	7.4	1.2	3	11.9	1.8	6	0.345	0.142	0.073
Avergae Hot Plate	7.3	0.94	3	11.24	1.41	6	0.336	0.11	0.053

Table S5. Results of SHIRPA neurological screen of mice lacking USP9X in the developing and adult forebrain structures. Tests highlighted in green identify statistical difference.

Antibodies for Western Blot

Antigen	Species	Dilution	Source
USP9X	rabbit	1:500	Bethyl Laboratories, USA
Raptor	rabbit	1:1000	Cell Signaling Technology, USA
SMURF1	mouse	1:250	Abcam
Phospho-S6	rabbit	1:1000	Cell Signaling Technology
S6	rabbit	1:1000	Cell Signaling Technology
SMAD4	mouse	1:200	Santa Cruz Biotechnology
ITCH	rabbit	1:1000	Cell Signaling Technology
MIB1	rabbit	1:1000	Abcam
CTNNB1	mouse	1:1000	BD Transduction Laboratories, Australia
activated CTNNB1	mouse	1:300	Millipore, Merck, Australia
MCL1	mouse	1:250	BD Pharmingen, Australia
ACTB	mouse	1:20000	Sigma-Aldridge

Antibodies for Immunofluorescence

Antigen	Species	Dilution	Source
USP9X	rabbit	1:500	Bethyl Laboratories, USA
SMAD4	mouse	1:200	Santa Cruz Biotechnology, USA
activated CTNNB1	mouse	1:500	Millipore, Merck, Australia

Table S6. Antibodies used in this study.

Family	Approving Institutional Review Board
USA 6	Women's and Children's Health Network Human Research Ethics Committee, South Australia.
USA 5	University of Michigan Review Board, MI, USA
France 2	French Law on Genetic Tests (Law of Bioethics)
UK1	UK Research Ethics Committee approvals 10/H0305/83 and GEN/284/12.
USA 14	Western Institutional Review Board, Puyallup, WA, USA
France 3	French law on genetic tests (law of Bioethics)
USA 13	Women's and Children's Health Network Human Research Ethics Committee, South Australia, Australia
USA 18	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
USA 1	UPMC Children's Hospital of Pittsburgh, PA, USA
USA 19	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
Spain 1	Women's and Children's Health Network Human Research Ethics Committee, South Australia.
Portugal 1	Medical Genetics Unit, Hospital Pediátrico, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal.
USA 7	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
Netherlands 11	Clinical Exome Sequencing study University Medical Center Utrecht, The Netherlands
USA 9	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
USA 3	Cook Children's Medical Center IRB, TX, USA
Netherlands 9	Clinical Diagnostic Exome Sequencing Study, Radboud University Medical Center Hospital, The Netherlands
Netherlands 12	Clinical Diagnostic Exome Sequencing Study, Erasmus University Medical Center Hospital, The Netherlands
USA 12	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
Canada 2	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
Netherlands 10	Clinical Diagnostic Exome Sequencing Study, Utrecht University Medical Center Hospital, The Netherlands
Netherlands 1	Clinical Diagnostic Exome Sequencing Study, Radboud University Medical Center Hospital, The Netherlands
Netherlands 7	Clinical Diagnostic Exome Sequencing Study, Radboud University Medical Center Hospital, The Netherlands
USA 21	National Human Genome Research Institute, USA
USA 11	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
USA 22	Mayo Clinic Institutional Review Board, MN, USA
Spain 2	Hospital Universitario Quirónsalud de Madrid Ethics Board, Spain.
USA 17	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
USA 26	Duke University Medical Center, NC, USA
Canada 6	The Hospital for Sick Children Research Ethics Board (REB#1000054798), Canada.
USA 28	Nationwide Children's Hospital Ethics Board (IRB11-00215), OH, USA
Netherlands 3	Clinical Diagnostic Exome Sequencing Study, Utrecht University Medical Center Hospital, The Netherlands
Canada 1	Children's Hospital of Eastern Ontario, Canada
Canada 3	Cedars Sinai Institutional Review Board (Protocol #00046408), CA, USA
Netherlands 5	Clinical Diagnostic Exome Sequencing Study, VU University Medical Center, The Netherlands
Netherlands 2	Clinical Diagnostic Exome Sequencing Study, Radboud University Medical Center Hospital, The Netherlands
Netherlands 6	Clinical Diagnostic Exome Sequencing Study, Radboud University Medical Center Hospital, The Netherlands
Swiss 1	Cantonal Ethical Board Zurich, Switzerland.
Norway 1	University Hospital of North Norway, Tromsø, Norway
Canada 5	Children's Hospital of Eastern Ontario, Canada
Netherlands 4	Clinical Diagnostic Exome Sequencing Study, Radboud University Medical Center Hospital, The Netherlands
Netherlands 8	Clinical Diagnostic Exome Sequencing Study, Radboud University Medical Center Hospital, The Netherlands
Belgium 1	French Law on Genetic Tests (Law of Bioethics)
France 1	French Law on Genetic Tests (Law of Bioethics)
USA 4	The University of Texas Health Science Center at Houston, HSC-MS-09-0057, TX, USA
USA 29	Nationwide Children's Hospital IRB, OH, USA
Germany 1	Ethics Board of the Medical Faculty of the University of Heidelberg, Germany
Denmark 1	University Hospital Copenhagen, Denmark

Table S7. Patient consent protocols.

SUPPLEMENTAL CLINICAL DATA DESCRIPTION

USA 6

Variant: ChrX GRCh37(hg19) g.40988391A>G; NM_001039590.2 c.235A>G, NP_001034679.2 p.Ile79Val.

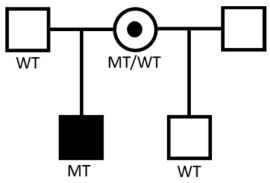
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely Pathogenic

Authors: Tyler Pierson, Elizabeth Bhoj, Stephanie Byers

Pedigree:



Clinical Notes:

Patient now 19 years old has intellectual disability, developmental delay, absent speech, seizures, hypotonia, severe motor disability (non-ambulatory), short stature, relative macrocephaly. Patient uses gastric tube for feeding and has gastroesophageal reflux. Facial dysmorphisms include short palpebral fissures, large incisors, full eyebrows. Fingers are short and trident-shaped.

Brain MRI revealed progressive cerebral and cerebellar volume loss, hypodensity in the left basal ganglia, unchanged and consistent with a lacune infarct (remote). There is a less conspicuous area of hypodensity on the contralateral side. There are hypodense white matter changes along the periventricular white matter and bilateral centrum semiovale.

USA 5

Variant: ChrX GRCh37(hg19) g.40994047T>A; NM_001039590.2 c.392T>A; NP_001034679.2 p.Ile131Asn.

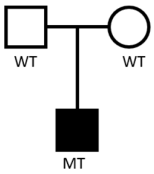
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No; Variant of unknown significance in PHKA1 [c.1459+4A>G (IVS14+4A>G)]

ACMG Classification: Likely Pathogenic

Authors: Catherine E. Keegan

Pedigree:



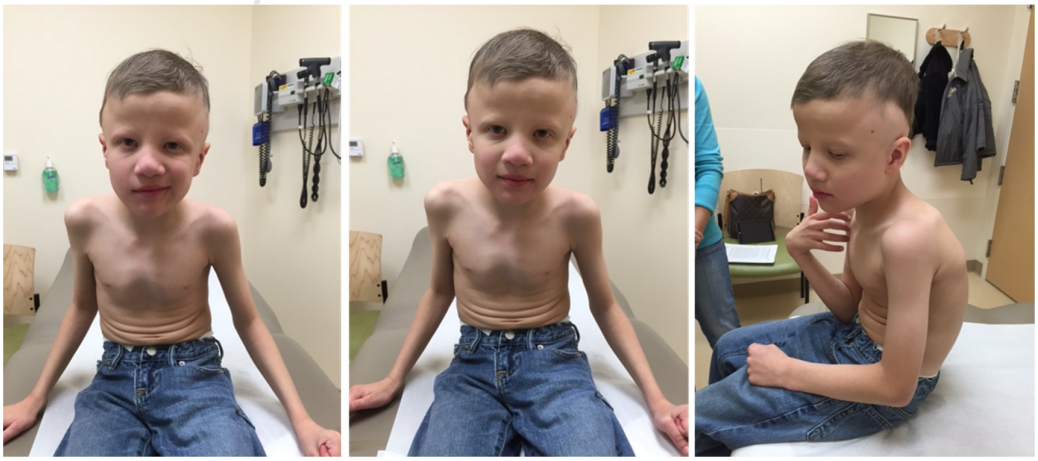
Clinical Notes:

A 9 year old male initially referred for evaluation at 9 months of age due to a history of hypotonia and left clubfoot requiring casting. He was the product of an uncomplicated pregnancy, delivered at 39 weeks of gestation, and weighing 8 lbs 3 oz. The clubfoot was identified while in utero. Following delivery, it was noted that he had hypotonia and feeding problems due to a poor suck. A brain MRI revealed a thin posterior corpus callosum and midbrain at the lower limits of normal in size. Physical findings on exam were a prominent anterior fontanelle and right-sided posterior plagiocephaly, mild frontal bossing, somewhat low-set ears that were normally rotated. He had epicanthal folds and inferior orbital creasing, a flat nasal bridge, wide nose, a sacral dimple with a visualized base, small toenails, and significant head lag on pull to sit.

The patient was referred for re-evaluation at age 9 after having been lost to follow up due to persistent hypotonia, generalized weakness, speech apraxia and dysarthia. A recent brain MRI redemonstrated mildly enlarged trigones with thinning of the posterior aspect of the corpus callosum and superimposed generalized low white matter volume. An arachnoid cyst versus a cisterna magna in the posterior fossa was also identified. He had a neurological evaluation for possible absence-type seizures; his EEG was normal. He had had a normal cardiology evaluation including a normal EKG and echo. He was evaluated by Pediatric Ophthalmology where he was noted to have a mild astigmatism and exotropia. He had been diagnosed with obstructive sleep apnea and subsequently had a tonsilloadenoidectomy. Other past medical history included constipation, eczema, alopecia involving his posterior scalp, pitted and ridged nails, and hypodontia (two missing teeth).

His examination at age 9 revealed a weight of 20.4 kg (0.8th percentile), a height of 122.5 centimeters (4.5th percentile), and a head circumference of 51.9 centimeters (38th percentile), consistent with relative macrocephaly. He had a tall, broad forehead. He had mild ptosis. Skin exam was unremarkable. He had ridging of his fingernails. He had reduced muscle bulk in both the upper and lower extremities and hypotonia. He had joint laxity notable at the fingers, wrists, elbows and knees, as well as pes planus.

The patient was reported to have a significant history of developmental delay in both motor and speech and language, although he has continued to make slow developmental progress. His motor milestones include sitting at 10 months, crawling at 12 to 13 months, and walking independently at 3 years of age. He has apraxia of speech. His receptive language was reported to be more advanced than his expressive language. He is presently in a moderately cognitively impaired program receiving multiple services and therapies.



FRANCE 2

Variant: ChrX GRCh37 (hg19) g.41025226C>T; NM_001039590.2 c.2087C>T; NP_001034679.2 p.Ala696Val

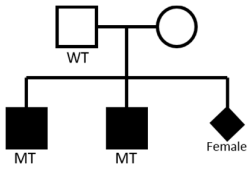
Discovery Platform: Whole exome sequencing.

Other variants of significance: No

ACMG Classification: Likely Pathogenic

Authors: Martine Raynaud, Laurent Pasquier

Pedigree:



Clinical Notes:

The clinical picture was very severe and was identical in two brothers separated by two years, who died very early after birth. Prenatally they displayed intrauterine growth restriction and increased echogenicity of the fetal bowel. Postnatally they had meconium ileus, pancytopenia, punctuata epiphysis. MRI revealed malformation of cortical development (reduced gyration with thickening of the cerebral cortex, thin corpus callosum, features of double cortex on temporal lobes) Extended metabolic explorations were normal.

UK1

Variant: ChrX GRCh37(hg19) g.41027299 C>T; NM_001039590.2 c.2464C>T; NP_001034679.2 pArg822Cys

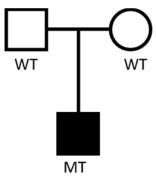
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely Pathogenic

Authors: Henrietta Lefroy, Usha Kini.

Pedigree:



Clinical Notes:

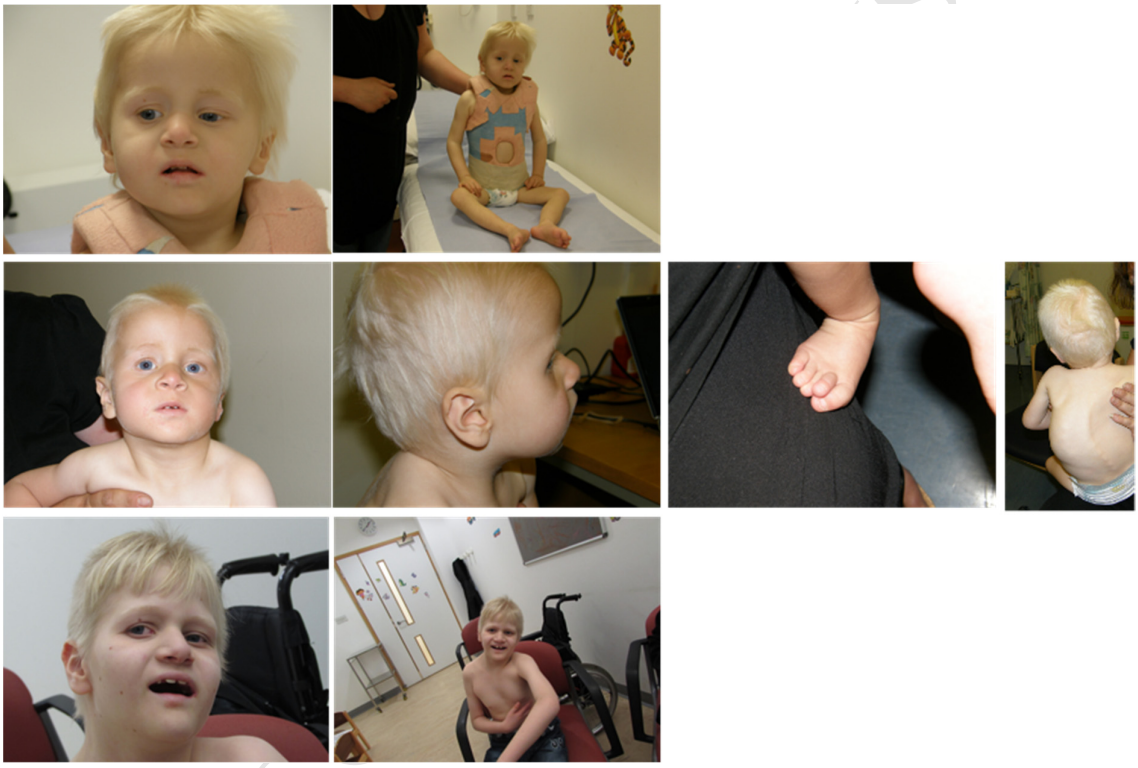
9 year old boy referred due to developmental delay, thoracolumbar scoliosis, hypotonia and microcephaly. He is the fourth child of unrelated parents. He has three older brothers, the oldest of whom has speech and language difficulties.

His parents became concerned at 6 months as they felt he was quite floppy. His motor milestones were delayed; sitting unsupported at 11 months and standing with support at 13 months. He is unable to walk at 9 years of age but can use a standing frame and walker. He is also able to mobilise via bottom shuffling. In terms of his speech, he began making sounds such as 'mmm' by 2.5 years. At 9 years he has no words but makes non-specific noises. His understanding is limited but he does recognise familiar people and objects.

He was diagnosed with significant thoracolumbar scoliosis from 6 months which was treated with a spinal plaster cast and subsequently magec rods from T2 to L4. He had positional plagiocephaly. He was found to have a Duane anomaly affecting his left eye. He had grommets inserted for glue ear. He has a persistent defect in his tympanic membrane and ongoing discharge.

On examination he has striking coarse blonde hair and blue eyes. He has microcephaly (< 0.4th centile, -2 SD). He has facial asymmetry with deep set eyes and broad anteverted nares. He has overlapping 2/3rd toes bilaterally and thin arms and legs.

His investigations include an MRI brain which revealed mildly dilated, mildly dysmorphic ventricles and an 'impression' of reduced white matter which was normally myelinated. His genetic testing included an array-CGH which showed a paternally inherited 7q31 deletion. This only contained one gene; IMMP2 and was felt less likely to be significant. He underwent testing for Prader-Willi syndrome which did not find an abnormality. He was then enrolled into the DDD study which revealed a de novo USP9X mutation.



USA 14

Variant: ChrX GRCh37(hg19) g. 41029757A>G; NM_001039590.2 c. 2912A>G; NP_001034679.2 p.Asn971Ser

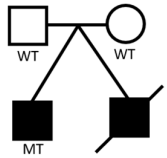
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No.

ACMG Classification: Likely Pathogenic.

Authors: Keri Ramsey

Pedigree:



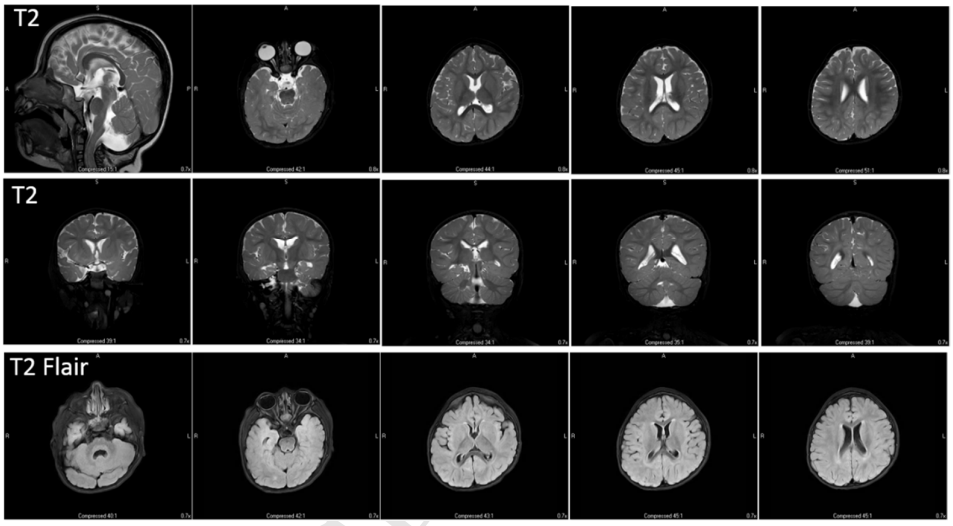
Clinical Notes:

Patient has Global developmental delay, severe intellectual disability, speech delay (babbling) spastic quadriparesis (non-weight bearing; tight abductors in his legs, non ambulatory), hypotonia (truncal, head lag, slips through at shoulders, joint hypermobility). Brain MRI shows possibly delayed myelination or gliosis in the posterior periventricular white matter.

Has severe growth retardation (receives growth hormone), and is of short stature. Brachycephaly, prominence of the temples (on either side of the orbits), shallow orbits, mid face hypoplasia, flat nasal bridge, prominence of midline forehead resembles slightly the Kleeblattshadel deformity, intermittent nystagmus with lateral gaze. Had Bi-lateral hip dysplasia requiring surgery. Fed completely by G-tube because of aspiration. Is hypoglycemic.

Had a twin brother that died at 1 year of respiratory illness. Twins thought to be monozygotic because they were in the same sac. Twin diagnosed with congenital heart disease.

MRI at 20 months



France 3

Variant: ChrX GRCh37(hg19) g.41057806C>T; NM_001039590.2 c.4406C>T; NP_001034679.2 p.Pro1469Leu

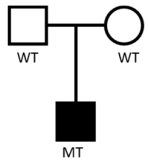
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No.

ACMG Classification: Likely Pathogenic.

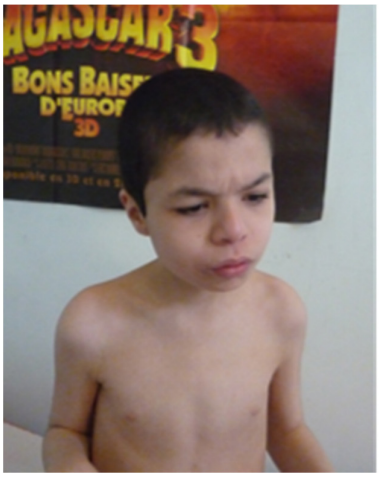
Authors: Boris Keren, Alexandra Afenjar, Thierry Billette de Villemeur

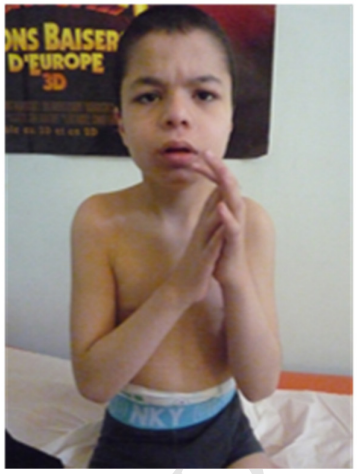
Pedigree:

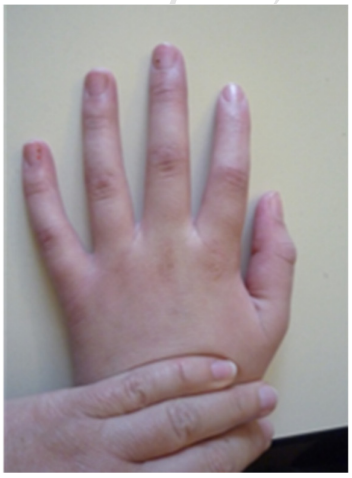


Clinical Notes:

Severe developmental delay and intellectual disability. Started walking at 6 years old. Has hypotonia and motor disability and broad based gait. Has autistic, obsessive and aggressive behaviors. Displayed growth retardation and is of short stature. Has joint hypermobility. Dysmorphisms include broad thumbs, broad nasal tip, palpebral fissure oblique down. Has feeding difficulties and gastro-esophageal reflux. Is hyperglycemic.







USA 13

Variant: ChrX GRCh37(hg19) g.41060427A>T; NM_001039590.2 c.4718A>T; NP_001034679.2 p.Gln1573Leu

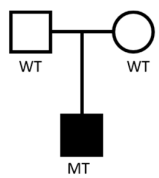
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No. *MECP2* maternally inherited (c.1231C>T; p.Pro411Ser) – previously reported in hemizygous state in 2 individuals in gnomAD; *KDM2B* – 2 heterozygous variants (c.1531G>A;p.Glu511Lys – maternally inherited) and (c.1475A>G;p.Lys492Arg – de novo) – of unknown significance.

ACMG Classification: Likely Pathogenic.

Authors: Carey Mcdougall, Elaine Zackai

Pedigree:



Clinical Notes:

Global developmental delay, speech delay, hypotonia and motor disability. Slight myopia. Facial dysmorphisms include mild upslant to palpebral fissures, mildly low set ears, short philtrum and bulbous nasal tip. Has slow weight gain and requires soft foods. Has hyperextensibility of joints and skin.

USA 18

Variant: ChrX GRCh37(hg19) g.41073847C>A; NM_001039590.2 c.5216C>A; NP_001034679.2 p.T1739N

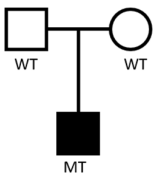
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely pathogenic

Authors: Tyler Pierson, Naomi Yachelevich

Pedigree:



Clinical Notes:

Subject is a former full-term 5yo boy with a history of a diagnosis of autistic spectrum disorder. He has also been found to have diffusely low tone. He had normal growth parameters at births and was note to have mild neonatal jaundice. He spent some time in the NICU to gain some extra weight, as well as having some difficulty regulating his temperature, which corrected over the next few days. He had significant infantile hypotonia with subsequent delayed motor milestones. He started walking at 2 years of age. Has wide based gait and pseudoataxia in gait may be due to low tone - wears SMOs, has external rotation of lower extremities with excessive pronation of both feet. Parents state that he is generally happy and eats well, and is affectionate. He has challenges with communication and sensory issues. He has some self-stimulatory behaviour, and often clenches his hands and gets very excited by anything to do with numbers or clocks. He tends to have certain interests such as exit signs and red lights, as well as anything with numbers. He has alternating exophoria. They feel he can follow 2-step commands, but anything more is quite challenging for him. He knows his letters and numbers, and can count up to 100. He has a little wide-based gait as well. He had some mildly diffuse low tone, which involved both his axial and appendicular musculature. His bulk was normal. His strength was within normal limits. Deep tendon reflexes were hypoactive at 1/5 throughout. Plantar responses were flexor bilaterally. Mildly decreased sensation with regard to light touch, temperature and vibratory sense, but that could be secondary to his cooperation waning at this point. He had an MRI, which suggested some mild colpocephaly. Brain MRI shows Periventricular Leukomalacia; Periventricular Leukomalacia; 'mild FLAIR hyperintensity along the periventricular white matter most prominent in parietal regions bilaterally which show mild thinning'; also small cystic foci in periatrial white matter bilaterally. Dysmorphisms include deep set eyes, bilateral epicanthus, flat nasal bridge, mild hypertelorism, full eyebrows. Has calcaneovalgus feet, joint hypermobility, tight heel cords. Metabolic testing revealed low free carnitine, high lactate, low thyroxine.

USA 1

Variant: ChrX GRCh37(hg19) g.41075423T>A; NM_001039590.2 c.5603T>A; NP_001034679.2 p.V1868E

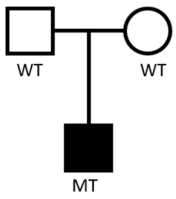
Discovery Platform: Trio-based whole exome sequencing.

Other variants of significance: No

ACMG Classification: Likely Pathogenic

Authors: Elena Infante, Amy Goldstein, Suneeta Madan-Khetarpal

Pedigree:



Clinical Notes:

At 6 years, patient with intellectual disability, global delay (non-verbal, cannot walk independently), cortical visual impairment and hypotonia (Tone decreased centrally). Displays occasional chorea and dystonic posturing of hands but not when in use. Ankle dystonia when walking. Displays some flapping/stereotypies. Has a history of febrile seizures with normal EEG.

Has some dysmorphic facial features including ears which are very small, malformed, and posteriorly rotated.

Repeated Brain MRI was stable, showing a thin corpus callosum with associated colpocephaly of the lateral ventricles, a suggestion of bilateral polymicrogyria along the sylvian fissures, bilateral hippocampal malrotation, stable, bilateral hypoplastic olfactory bulb, stable, and a left middle cranial fossa arachnoid cyst, which is stable.

At 7 years, walks independently with very wide-based gait. Has major issues with anxiety/overstimulation. He gets very fearful, tearful and stiff. Seizures present. A recoded event revealed Epileptiform discharges noted as generalized spike and slow wave discharges. Electroclinical seizures were recorded as numerous brief typical absences recorded, clinically presenting with staring, unresponsiveness and eye flutter, and associated with 3 Hz generalized spike and slow wave activity on the EEG, lasting approximately 12-16 seconds each. Treatment initiated with Depakote.

USA 19

Variant: ChrX GRCh37(hg19) g.41075854T>A; NM_001039590.2 c.6034T>A; NP_001034679.2 p.F2012I

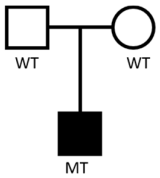
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely pathogenic

Authors: Tyler Pierson, Angela E. Lin, MD, Marcie A. Steeves, Mohammed Ali Almuqbil

Pedigree:



Clinical Notes:

Prenatal US suggested trisomy 13 or 18 with ventriculomegaly, bilateral clubfeet, bilateral polydactyly, unusual facial features.

Patient now 3 ½ years.

Global developmental delay, severe ID, non-verbal, chronic epilepsy, hypotonia, motor disability, growth restriction (weight <3rd, height 3rd (OFC not measured)). Anomalies have included: Laryngeal cleft, type 1, postaxial polydactly type B of hands, Striking diffuse joint hypermobility, retroflexed hips, clubbed feet bilaterally and feeding difficulties (needs G-tube).

Brain MRI #1 at birth showed evidence of infarcts in bilateral temporal, occipital, parietal lobes, partial agenesis of corpus collosum, cerebellar hypoplasia, ventriculomegaly, piriforma apetrure stenosis and probable hypoplasia of the pituitary gland. MRI #2 at 15 months: Unchanged appearance of callosal dysgenesis, left cerebellar hypoplasia and probable hypoplasia of the pituitary gland. Unchanged size and configuration of the dilated ventricles.

5 ½ year old sister has absence seizures, normal brain MRI.

Spain 1

Variant: ChrX GRCh37(hg19) g.41077669G>A; NM_001039590.2 c.6254G>A; NP_001034679.2 p.R2085H

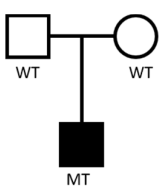
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely Pathogenic.

Authors: Carlos López-Otín, Olaya Santiago-Fernández

Pedigree:



Clinical Notes:

Patient has intellectual disability, developmental delay, speech delay, hypotonia, motor disability, visual impairment (myopia, strabismus). Brain MRI revealed hypoplasia of cerebellum vermis and Dandy Walker malformation, dysplasia of corpus callosum, ventriculomegaly, and white matter loss. Dysmorphisms including Bilateral epicanthus, Flat Nasal Bridge, clinodactyly IIIth to Vth toes, ear fissure, bilateral equinovarus feet and hyperextensibility of joints and skin.

Portugal 1

Variant: ChrX GRCh37 (hg19) g.41082601T>C; NM_001039590.2 c.6697T>C; NP_001034679.2 p.Ser2233Pro

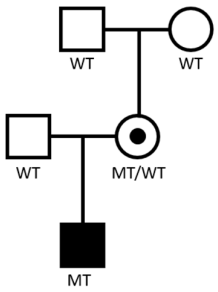
Discovery Platform: TrusightOne disease exome.

Other variants of significance: No

ACMG Classification: Likely Pathogenic

Authors: Cláudia Falcão-Reis, Joaquim Sá

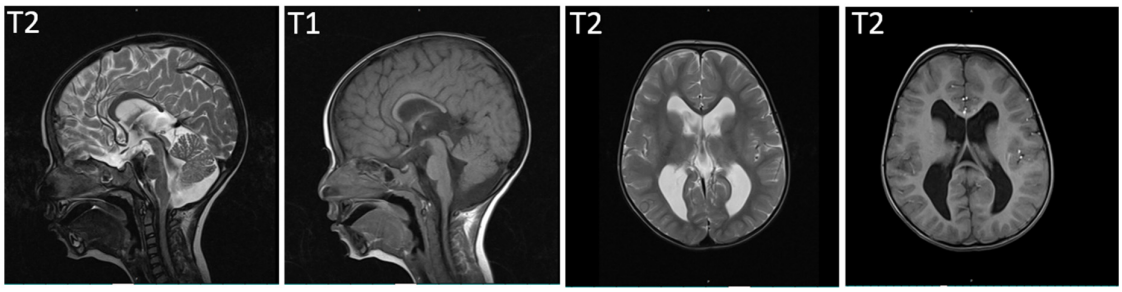
Pedigree:



Clinical Notes:

Patient delivered at 39 weeks with normal growth parameters, APGAR score 6,8,9. During gestation: transient placental abruption during 2nd trimester and oligohydramnios after amniocentesis (advanced maternal age – 36YO; prenatal karyoptype 46,XY), no abnormalities noted on ultrasound. Sent to the NICU after birth because of suspected sepsis (10 days duration), bilateral hip dysplasia and cryptorchidism were noted and managed.

At 3 years of age patient presented with severe developmental delay (non-verbal) with global hypotonia (non-ambulant) and sometimes auto-aggression (very slow progress since age 3, no regression). Dysmorphic facial features include low anterior hairline, synophrys, broad and depressed nasal tip, spaced teeth. Patient has broad and large great toes, hyperextensibility of joints and skin, small ovoid scalp aplasia cutis (secondary to birth trauma?). Growth has been normal (weight, stature and OFC between 25th and 50th centile). Brain MRI revealed lateral ventriculomegaly, peripheral T2 hypersignal areas, hypoplasia of corpus callosum.



USA 7

Variant: ChrX GRCh37(hg19) g.40994022G>T; NM_001039590.2 c.367G>T; NP_001034679.2 p.G123W

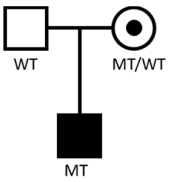
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance.

Authors: Tyler Pierson, Yezmin Perilla-Young, Laurie Smith

Pedigree:



Clinical Notes:

Patient with hypotonia, profound developmental and intellectual impairment and seizures of unknown etiology. Has speech delay (communicates by picture book, some sign language. uses only two words), autism, partial idiopathic epilepsy, hypotonia, motor disability, repetitive behaviors hand flapping and is irritable. Has motor disability, with waddling gait and requires ankle-foot orthosis for walking. Displays macrocephaly, pectus carinatum, and bilateral coxa valga. Brain MRI shows delayed maturation of white matter, thinning corpus collosum, small pituitary gland, and posterior positioning of the cerebral arteries. Dysmorphisms include turricephalic, tall forehead, arched eyebrows, down slanted palpebral fissures, slightly posteriorly rotated ears with small cartilaginous nodule on posterior caudal aspect, flat foot, persistent finger pads, 5th finger clinodactyly, cryptorchidism.

Netherlands 11

Variant: ChrX GRCh37(hg19) g.40996160C>A; NM_001039590.2 c.539C>A; NP_001034679.2 p.Pro180His

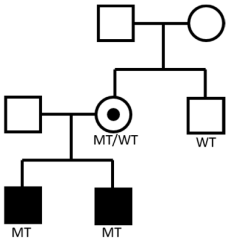
Discovery Platform: whole exome sequencing

Other variants of significance: ?.

ACMG Classification: Unknown significance

Authors: Renske Oegema, Bert van der Zwaag, E. van Binsbergen

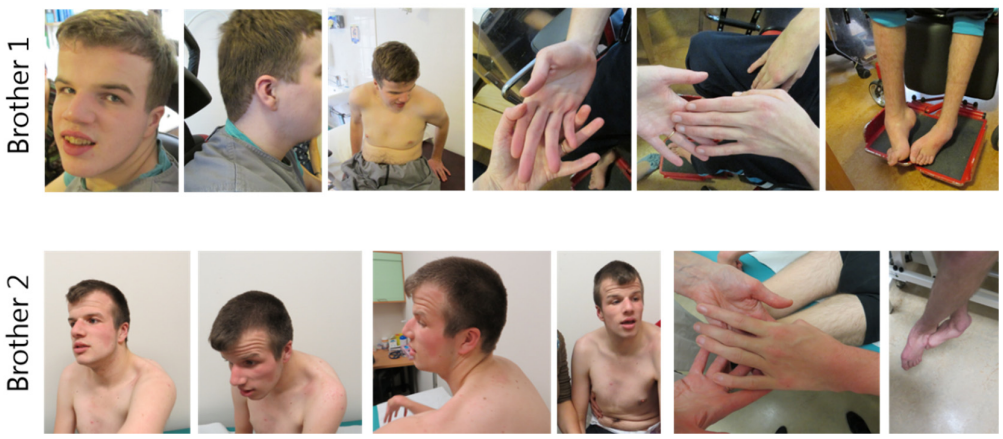
Pedigree:

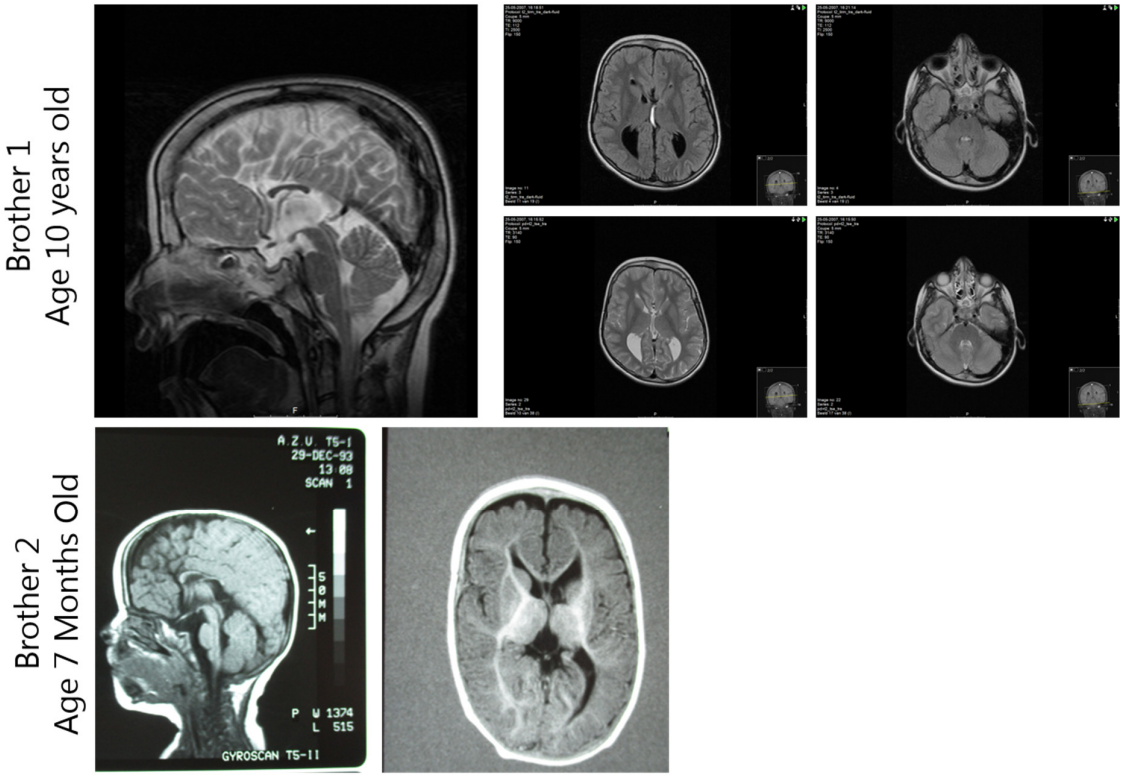


Clinical Notes:

Proband is a 21-year old male with moderate to severe ID, epilepsy and spasticity, and on brain MRI thin corpus callosum. He has a sacral dimple, hip dysplasia. In childhood he had hypotonia and recurrent upper respiratory tract infections. He was born at 37 weeks of gestation, in breech position. He underwent surgery for bilateral inguinal hernia at 6 weeks of age. His development was delayed from birth. He started crawling after his second birthday and walking at 4 years of age. Epilepsy was diagnosed at age 3. Generally he has a friendly and cheerful personality. Occasionally he has temper tantrums. Clinical genetic examination: speaks a few words, makes noises. OFC 59 cms (+0.75 SDS). He has a broad forehead, deep set eyes with full upper eyelids. Broad base to the nose, broad mouth. Caries dentition. Small, posteriorly rotated ears. Large hands with long fingers (hand 21.5 cm, third finger 9.5 cm). Thumbs cannot be fully extended. There is an abnormal shape of the thorax with low set nipples. He has pes equines, hammer toes and calf muscle atrophy.

His brother is more severely disabled, he never gained independent walking and his behaviour is more challenging. He can crawl and ride a (special) bicycle. He speaks 7-8 words and used speech computer and signing. He is not diagnosed with epilepsy. He was born after 33 weeks of gestations with postaxial polydactyly. Pregnancy was complicated by HELLP syndrome. BW 1880 grams (normal), Apgar 4/8. He was admitted to the neonatal care unit, and treated for respiratory insufficiency and hyperbilirubinemia. He suffers from constipation. He has scoliosis. OFC 56.7 cm (- 0.5 SD).





USA 9

Variant: ChrX GRCh37(hg19) g.40999955T>C; NM_001039590.2 c.701 T>C; NP_001034679.2 p.F234S

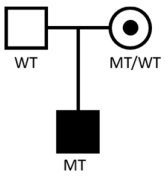
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No. De-novo Heterozygous *POLG1* c1550 G>T; pGly517Val of unknown significance

ACMG Classification: Unknown significance.

Authors: Tyler Pierson, John M. Graham, Christine Shieh.

Pedigree:



Clinical Notes:

Patient with intellectual disability, developmental delay, speech delay, autistic and obsessive behavior. Has hypotonia, athetoid movements, and is ataxic with a wide based gait. He wears ankle-foot orthoses and pronates badly when he walks. Sleep is disturbed with frequent awakenings. Has mild scoliosis. Has growth retardation and short stature. Dysmorphisms include broad forehead, flat nasal bridge, small nose, overhanging columella, up-slanted palpebral fissures, high palate, hyperextensible dislocatable thumbs, overcrowded upper teeth, low set and posterior rotated ears, prognathic lower jaw. Metabolic testing revealed low cysteine, mildly elevated asparagine, mildly elevated lactate, low 'free' carnitine.

USA 3

Variant: ChrX GRCh37(hg19) g.41000406A>G; NM_001039590.2 c.958A>G; NP_001034679.2 p.R320G

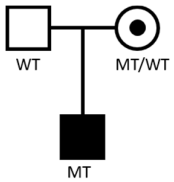
Discovery Platform: Whole exome sequencing.

Other variants of significance: No

ACMG Classification: Unknown Significance

Authors: Scott Perry

Pedigree:



Clinical Notes:

Patient with global developmental delay, speech delay, epilepsy, autistic mannerisms, and hypotonia.

Netherlands 9

Variant: ChrX GRCh37(hg19) g.41007713C>T; NM_001039590.2 c.1511C>T; NP_001034679.2 p.Ala504Val

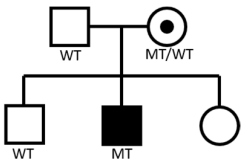
Discovery Platform: Whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance

Authors: David Koolen, Tjitske Kleefstra

Pedigree:



Clinical Notes:

Severe ID, Non verbal, Automutilation, Scoliosis

Netherlands 12

Variant: ChrX GRCh37(hg19) g.41007751A>C; NM_001039590.2 c.1549A>C; NP_001034679.2 p.Ser517Arg

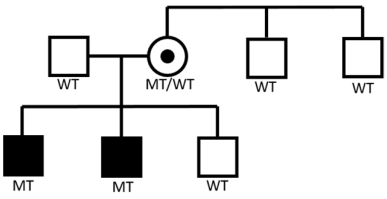
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance

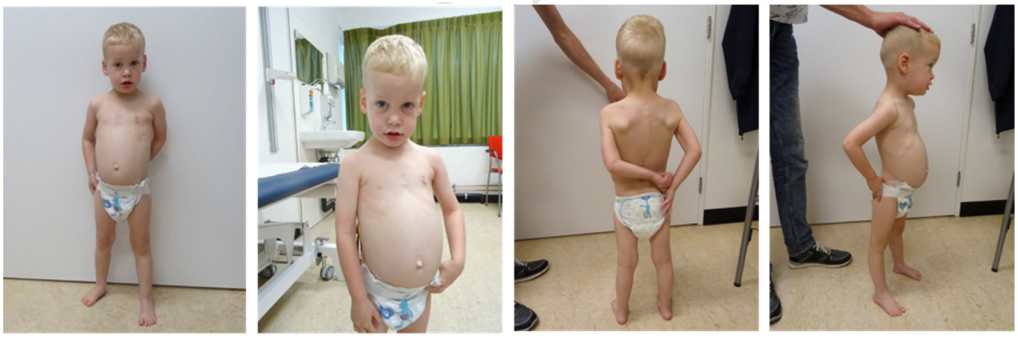
Authors: Serwet Demirdas

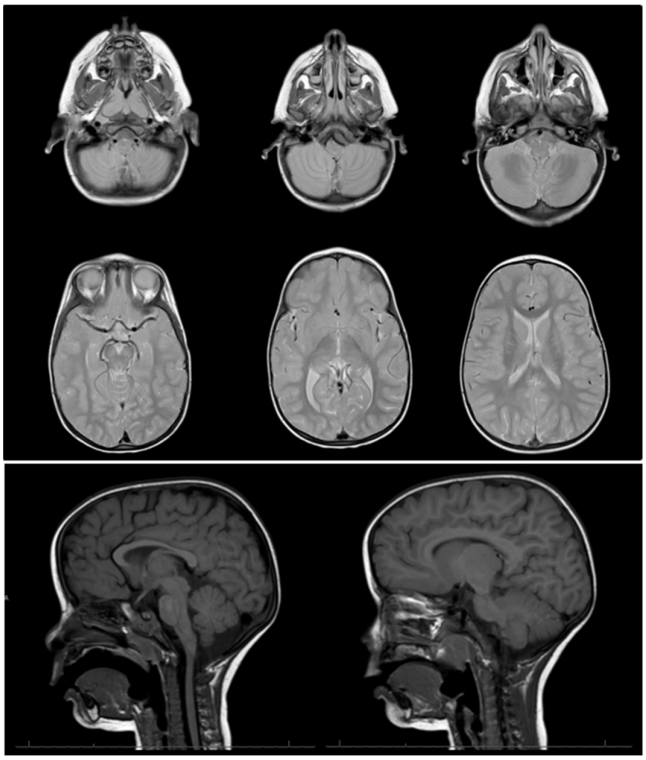
Pedigree:



Clinical Notes:

2-year-old boy with developmental delay, specifically a speech-/language delay and walking difficulties. The walking difficulties are clinically difficult to distinguish between peripheral or central problem. His walking is broad-based with overstretching, foot lifters weakness and he has some extrapyramidal. Dysmorphic features are apparent: deepset eyes, frontal bossing, hypoplastic nails, inverted nipples etc. His growth parameters are within range. MRI of both the brain and the spine were unremarkable. Metabolic screening in plasma and urine, array results and sequencing of CGG-repeats in the FMR1 gene were normal. WES of a panel of 1174 genes contributing to intellectual disability showed a VOUS class 3 in the USPX9 gene, inherited from his healthy mother. The patient has a brother with autism whom also carries the variant, and another brother and two healthy maternal uncles who do not carry the variant.





USA 12

Variant: ChrX GRCh37(hg19) g.41029255C>T; NM_001039590.2 c.2644 C>T; NP_001034679.2 p.R882C

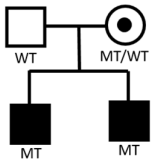
Discovery Platform: Quartet based whole exome sequencing

Other variants of significance: No.

ACMG Classification: Unknown significance.

Authors: Tyler Pierson, Bradley Schaefer, Noelle R Danylchuk.

Pedigree:



Clinical Notes:

Patient 1. Developmental delay, intellectual disability, speech delay but responded to therapy, obsessive about food, has ADHD, mild growth retardation and short stature. Has keratitis' on palms/soles.

Patient 2. Developmental delay, intellectual disability, obsessive about food, has ADHD. Has keratosis.

Mother with learning disability and psychiatric disorder.

Canada 2

Variant: ChrX GRCh37(hg19) g.41029345A>G; NM_001039590.2 c.2734A>G; NP_001034679.2 p.Ile912Val

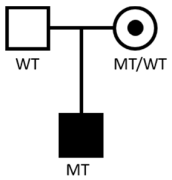
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance

Authors: Tyler Pierson, Margot Van Allen, Tracy Oh.

Pedigree:



Clinical Notes:

Patient with global developmental delay, intellectual disability, speech delay and regression of language skills, autism, ADHD and has frequent tantrums. Dysmorphisms include broad forehead, dimpled chin and 5th finger brachydactyly.

Netherlands 10

Variant: ChrX GRCh37(hg19) g.41043278C>A; NM_001039590.2 c.3176C>A; NP_001034679.2 p.Ala1059Asp

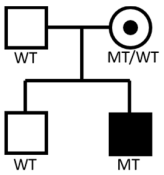
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No. Maternally inherited variant in the RANBP2 gene (RANBP2 (NM_006267.4): c.[7418A>G];[=] p.[(Glu2473Gly)];[(=)] (Chr2(GRCh37):g.[109384413A>G];[=]) of unknown significance

ACMG Classification: Unknown significance

Authors: Renske Oegema, Bert van der Zwaag, E. van Binsbergen

Pedigree:



Clinical Notes:

This boy was born with cesarean section at 37 weeks of gestation. The last week of the pregnancy were complicated by preeclampsia and polyhydramnios. Birth weight 2400 grams. He had feeding difficulties during the first months with nasal

regurgitation. There was excessive drooling till 8 months. His motor development was delayed, he walked independently at 23 months. He also had a speech delay, started speaking at almost 3 years of age, with hypernasality and articulation difficulties. Intensive speech therapy was beneficial. He is very shy in communication with strangers. At 3 years of age he was diagnosed with velopharyngeal insufficiency. He was successfully operated at 4 years. He is now 5 years of age and attends regular education.

Clinical genetic examination (age 4): OFC -1 sds, height -1 sds, weight +1 SDS. A boy with a prominent forehead, full upper eyelids, low nasal bridge, broad mouth. He has broad thumbs.

At 3years 10 months he presented with an epileptic seizure - generalized tonic-clonic. Two weeks later he presented with status epilepticus and was started on anti-epileptic drug. Brain MRI was normal. SNP array.

Netherlands 1

Variant: ChrX GRCh37(hg19) g.41043370T>C; NM_001039590.2 c.3268T>C; NP_001034679.2 p.Tyr1090His

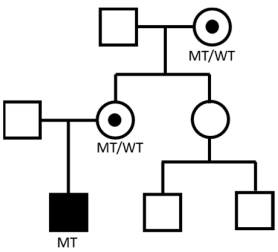
Discovery Platform: whole exome sequencing.

Other variants of significance: ARID1B. Chr6 (GRCh37): g.157528688T>G; NM_020732.3: c.6413T>G p.Leu2138Arg (de-novo)

ACMG Classification: Unknown Significance

Authors: Tjitske Kleefstra and Margot Reijnders

Pedigree:



Clinical Notes:

Patient has ID, epilepsy, no speech, scoliosis (not progressive), thin hair, facial dysmorphisms: hypertelorism, macrostomia, full eyebrows, low frontal hairline. Skin abnormalities in Blaschkolines. MRI: absent distal part corpus callosum. N.B. There is no skewed X-inactivation present in mother and grandmother. Mother has epilepsy.

Netherlands 7

Variant: ChrX GRCh37(hg19) g.41045786A>G; NM_001039590.2 c.3575A>G; NP_001034679.2 p.His1192Arg

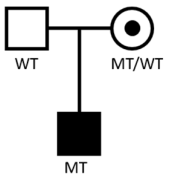
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance.

Authors: Tjitske Kleefstra, Margot Reijnders

Pedigree:



Clinical Notes:

Patient with severe intellectual disability, global developmental delay, speech delay (only few words), autistic behaviour, motor disability (wheelchair for long distances), ataxia and anxiety. Patient has microcephaly. Facial dysmorphisms including macrostomia, large ears, small and high nasal bridge. Patient has constipations.

USA 21

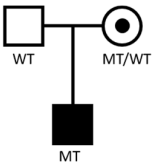
Variant: ChrX GRCh37(hg19) g.41045833C>T; NM_001039590.2 c.3622 C>T; NP_001034679.2 p.Pro1208Ser

Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance

Authors: Kelly Schoch, Loren Pena, Undiagnosed Disease Network
Pedigree:



Clinical Notes:

4 year old Hispanic male with global developmental delay, moderate intellectual disability, speech delay (only a few words), many febrile seizures from 19-28 months of age (mild diffuse slowing on EEG), hypotonia, motor disability (assisted walking), ataxia, visual impairment (intermittent downbeat nystagmus). Patient had intrauterine growth restriction and post-nataly had growth retardation, short stature and microcephaly. Brain MRI showed mild prominance of sulci in the frontal poles bilaterally at 14 months and possible T2 signal hyperintensity involving the periventricular WM in the frontal lobes and possible mildly delayed myelination at 3 years old. Dysmorphisms include upslanting palpebral fissures, almond shaped and mildly wide spaced eyes, sacral dimple, round and cupped ears with simple helices and mild hypertelorism. Has constipation, and has osteopenia with recurrent bone fractures (may be due to rickets of prematurity).

USA 11

Variant: ChrX GRCh37(hg19) g.41048705C>G; NM_001039590.2 c.3954 C>G; NP_001034679.2 p.D1318E

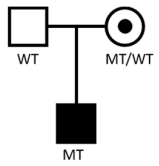
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No. Compound heterozygous CDK11A variants (both inherited) E513K, G118R. Unknown significance.

ACMG Classification: Unknown significance.

Authors: Tyler Pierson, Kamer Tezcan.

Pedigree:



Clinical Notes:

Developmental delay and regression, intellectual disability, speech delay, autism and ADHD, impaired hearing (corrected by surgery). Brain MRI was normal. Frequent vomiting independent of food intake.

USA 22

Variant: ChrX GRCh37(hg19) g.41057869C>T; NM_001039590.2 c.4469C>T; NP_001034679.2 p.Pro1490Leu

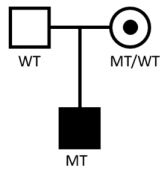
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No; ChrX(GRCh37):g.20179844G>A; NM_004586.2(RPS6KA3):c.1877C>T; p.(Pro626Leu) of unknown significance.

ACMG Classification: Unknown significance

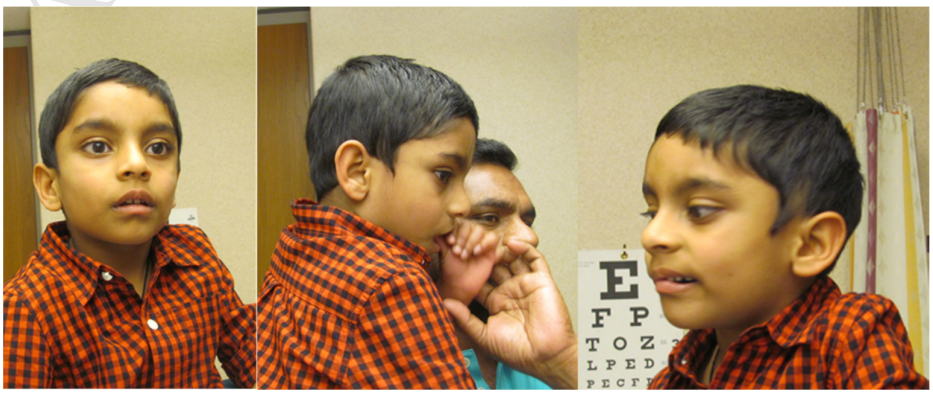
Authors: Filippo Pinto e Vairo, Pavel N. Pichurin, Sarah A. Ewing, Sarah S. Barnett, Eric W. Klee

Pedigree:



Clinical Notes:

6-year-old male with a history of autism, speech delay, abnormal EEG and Focal versus generalized epilepsy of unknown etiology. Also, his HC is at p5, he has mild medial flaring of eyebrows, nonspecific white matter abnormalities, hypertrichosis, and generally nondysmorphic. EEG showed mild diffuse slowing, bifrontal spikes and sharp waves, and intermixed bursts of generalized atypical spike and wave discharges occurring with the bifrontal discharges.



Spain 2

Variant: ChrX GRCh37(hg19) g.41060527C>A; NM_001039590.2 c.4818C>A; NP_001034679.2 p.Asp1606Glu

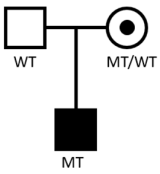
Discovery Platform: Trio based whole genome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance.

Authors: Alberto Fernandez-Jaén.

Pedigree:



Clinical Notes:

Patient clinical features include unilateral deafness (hypoplasia of cochlear nerve), microcephaly, short stature, mild intellectual disability, speech delay, and no dysmorphic features. Other studies (metabolic, serologies, ocular, muscular) were normal.

USA 17

Variant: ChrX GRCh37(hg19) g.41064568C>A; NM_001039590.2 c.4837C>A; NP_001034679.2 p.P1613T.

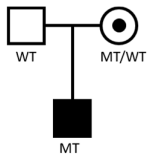
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: SORC21 c.1977G>T p.Q659H (de novo; may contribute). Duplication 9q21.2; 525.93kb, 5 genes – unknown significance.

ACMG Classification: Unknown significance.

Authors: Tyler Pierson, Alexander Asamoah, Kelly Jackson.

Pedigree:



Clinical Notes:

Subject is a 6yo male former full-term child intrauterine growth retardation, short stature with macrocephaly. He has a history of motor delay and speech delays, episodes of hypoglycemia, and cyclic vomiting. The hypoglycemia/vomiting episodes have resolved for the most part, except when he is ill and he can get hypoglycemic quite easily. He has had GERD as well and is thought to possess a genetic marker for Crohn disease (he does not have it). He is non-dysmorphic and has left exotropia. No family history of childhood onset neurological/developmental disease. Family is non-consanguineous.

At ~2yoa he had several episodes that seemed like staring spells/absence seizures, but EEG was normal and he has not had any further episodes since. He had an MRI brain, that father thought was normal, but did not provide a report. He was a late walker at 18mos, but currently does not exhibit any motor issues. He was also a late talker and still has some minor cognitive and speech issues.

USA 26

Variant: ChrX GRCh37(hg19) g.41064704G>A; NM_001039590.2 c.4973G>A; NP_001034679.2 p.Arg1658Gln

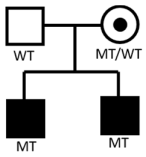
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance

Authors: Vandana Shashi, Jennifer A. Sullivan, Ioana Cutcutache

Pedigree:



Clinical Notes:

6 year old male with epilepsy with history of prolonged status epilepticus, acquired microcephaly, and global developmental delays. Seizure control is good with phenobarbital. Walked independently at 4.5 years. Profound Speech delay with Expressive > receptive. He is functionally non- verbal. 9 year old full brother is significantly delayed with reading skills and mildly delayed with math skills. His developmental milestones were normal except for a mild speech delay. He has ADHD. Father required special education in school.

Canada 6

Variant: ChrX GRCh37(hg19) g.41077774T>C; NM_001039590.2 c.6359T>C; NP_001034679.2 p.I2120T

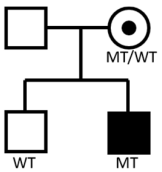
Discovery Platform: Whole exome sequencing.

Other variants of significance: No

ACMG Classification: Unknown Significance

Authors: Saadet Mercimek-Andrews

Pedigree:



Clinical Notes:

At age of 9 ¾ years phenotype consisted of ID, hypotonia from his first year, fine motor delay, speech language delay, history of developmental regression, febrile seizures (onset 21 months) and after age 3 refractory epilepsy (of different types, generalized tonic clonic, atonic, myoclonic, absence) refractory to multiple anti-epileptic medications (Phenobarb, valproate, clobazam, lamotrigine, keppra) and the ketogenic diet. Non dysmorphic apperance. Brain MRI was normal at the age of 2 years, whereas showed increased T2 signal intensity in the left middle frontal gyrus, raising the question of low-grade glioma at the age of 6 years old. His neuropsychological assessment showed moderate ID with markedly restricted functions for speaking, walking and mental functions necessary for everyday life.

USA 28

Variant: ChrX GRCh37(hg19) g.41084144A>C; NM_001039590.2 c.6901A>C; NP_001034679.2 p.Lys2301Gln

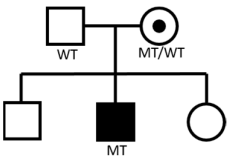
Discovery Platform: Trio based whole genome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance

Authors: Daniel Koboldt

Pedigree:



Clinical Notes:

Patient is a currently 4-year-old male who was born at full term after an uncomplicated pregnancy weighing 6lbs, 9oz. He had some issues with feeding in the neonatal period, including poor latching for breastfeeding. His general health was good, and he first came to attention at age 9 months when concern was raised about hypotonia and motor delay. At age 4, he still cannot walk independently though has been cruising since about 18 months. He knows 2-3 single words and a few signs. Other features include chronic drooling and tremor that has been described by his pediatric neurologist as a cerebellar tremor. Cranial MRI obtained at 3 years was unremarkable. Acanthocytes were reportedly seen on CBC smear, but a repeat smear was normal. *MTTP* gene sequencing and deletion/duplication testing was negative. Whole exome sequencing was performed and 3 variants of unknown significance were identified: paternally inherited variants in *CACNA1A* and *COL6A2*, and maternally inherited *USP9X* variant. Deletion/duplication testing for *COL6A2* was performed and negative. Whole genome sequencing was performed on a research basis, and the patient was found to carry other variants of unknown significance in several genes. It is unclear at this time whether or not any of these could be contributing to this patient's phenotype.

Netherlands 3

Variant: ChrX GRCh37(hg19); g.41089041dup;

Long isoform: NM_001039590.2 c.7440dup; NP_001034679.2 p.Ala2481fs
Short isoform: NM_001039590 c.[0]; NP_001034679 p.[0]

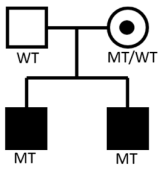
Discovery Platform: whole exome sequencing (both affected brothers and both parents)

Other variants of significance: No

ACMG Classification: Unknown Significance

Authors: Peter VanHasselt

Pedigree:



Clinical Notes:

Both patients displayed intrauterine growth restriction. Both present with global developmental delay, intellectual disability, autistic behaviour. Both had microcephaly. Both had transient (after birth) hypoglycaemia and elevated tyrosine. Both have feeding difficulties and one diagnosed with gastro-esophageal reflux.

Canada 1

Variant: ChrX GRCh37(hg19); g.41089041dup;

Long isoform: NM_001039590.2 c.7440dup; NP_001034679.2 p.Ala2481fs
Short isoform: NM_001039590 c.[0]; NP_001034679 p.[0]

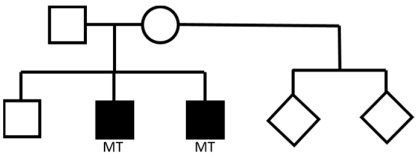
Discovery Platform: Whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown Significance

Authors: Mathew Lines

Pedigree:



Clinical Notes:

Family History

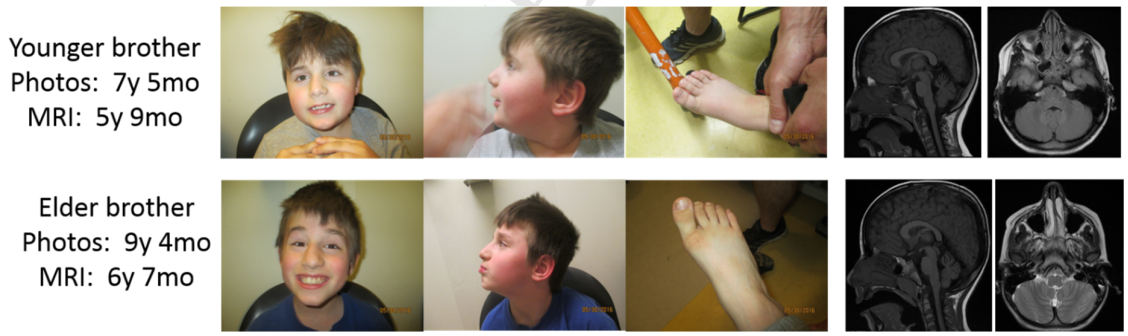
Paternal family history: (non-contributory) aortic valve surgery (for aortic regurgitation). Cerebellar stroke. Attention deficit. Brother (paternal uncle to probands) with cerebral palsy and had severe developmental delay ("toddler level" according to family). Father's ethnic background is 'Caucasian' (paternal) and Austrian/Hungarian (maternal). Maternal family history: Mother denies learning problems; did have seizures as a child, but these resolved at age seven, and she has required no medication since. Mother has several family members with bipolar disorder. She is of mixed French Canadian/Italian/Irish/First Nations ancestry.

Younger Brother

Born at term weighing 6 lbs 12 oz. Mother was a 30 year old G5P4A1 at the time of delivery. He was delivered by repeat C-section and had an uncomplicated neonatal course. Boy with Global developmental delay. (1) Gross motor: Sat unsupported at 16 months; Prone ('commando') crawl at 2 years. At 5 ½ years: Pull to stand, ambulate with walker, eat with spoon, scribble with fist grip, no writing, some speech (dysarthric), not toilet trained. (2) Cerebellar ataxia (truncal, postural, extremities) – needs walker for ambulation (3) Hypotonia (4) Generalized tonic-clonic seizures (six episodes, always in context of febrile illness) requiring no medications. Physical Exam: Growth (age 7 years); Height: 118 cm (24 %, Z = -0.70, Source: WHO Growth Chart for Canada); Weight: 30 kg (97 %, Z = 1.86, Source: WHO Growth Chart for Canada); Head circumference: 52.8 cm (62nd centile). Appearance is nondysmorphic and in keeping with parents. The major exam finding is marked limb and gait ataxia, which expressed itself both in terms of dramatic gait instability, marked dysmetria and dysdiadochokinesis, and dysarthria. He was able to sit on the edge of the exam table unassisted. Extraocular movements were full. Tone was reduced with no ankle clonus; reflexes were normal; plantar responses were flexor. Brain MRI: Enlarged vermian fissures and CSF between cerebellar lobes, in keeping with atrophy of the anterior vermis (region from the lingula to the tuber) without evidence of hypoplasia. The cerebellar hemispheres are within normal limits. The corpus callosum has a slightly thick appearance. All of the following were normal: Microarray (SNP), SCA panel (repeat enumeration SCAs 1-3, 6-8, and 17), Transferrin isoelectric focusing, AFP, VLCFAs, Monogenic ataxia NGS panel (U. Chicago) (negative for mutations in any of the 346 genes included in the panel) (*done as an exome slice, see below). The USP9X diagnosis was made upon inspection of the remaining (off-panel) exome variants from the U. Chicago ataxia panel.

Elder Brother

Born at term by (repeat) Caesarean section, weighing 6 lbs 12 oz. Mother was a 30 year old G5P4A1 at the time of delivery. Neonatal course was uncomplicated. Boy with (1) Global developmental delay, (2) central hypotonia, (3) Cerebellar ataxia, (4) Strabismus (s/p surgery) No seizures. Development: Delays recognized ~6mo. Is considered to be less cognitively affected than his brother. Roll over 14mo. Sit unsupported 2.5 years. At 7y 11mo: Attends a modified Grade 3 program. Can walk a few independent steps, or for long distances in his walker. He is able to feed himself with a knife, fork, or spoon, and can write his name with some errors. His speech consists of complete sentences, including pronouns and plurals, and his speech is slow but relatively understandable. He exhibits normal eye contact and is toilet-trained. Growth (7y 11mo): Height: 127.1 cm (21 %, Z = -0.80, Source: WHO Growth Chart for Canada); Weight: 29 kg (61 %, Z = 0.29, Source: WHO Growth Chart for Canada); Head circumference: 53.1 cm (58th centile). The main findings on exam are marked gait and limb ataxia. Reflexes are normal. Plantar responses are down-going. Investigations: EMG / Nerve conduction studies normal; Brain MRI findings similar to those seen in brother (anterior vermis atrophy; thick corpus callosum); Microarray, TIEF, AFP, VLCFAs, monogenic ataxia panel (U. Chicago) – all normal.



Canada 3

Variant: ChrX GRCh37(hg19); g.41089041dup;

Long isoform: NM_001039590.2 c.7440dup; NP_001034679.2 p.Ala2481fs
Short isoform: NM_001039590 c.[0]; NP_001034679 p.[0]

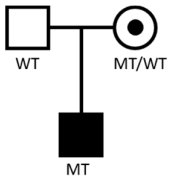
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Unknown significance

Authors: Tyler Pierson, Christine Shieh.

Pedigree:



Clinical Notes:

Patient with global developmental delay, intellectual disability, speech delay, autism, primary generalized epileptiform activity on EEG but no clinical seizures. Also with anxiety and some obsessions. Has macrocephaly. Dysmorphisms include borderline brachydactyly, sacral dimple, broad forehead, dimpled chin, very mild asymmetry of face. Also has hyperextensibility of metacarpophalangeal joints displays and genu valgum. Has feeding difficulties requiring tube feeding.

Netherlands 5

Variant: ChrX GRCh37(hg19) g.41091697C>T; NM_001039590.2 c.7633C>T; NP_001034679.2 p.P2545S

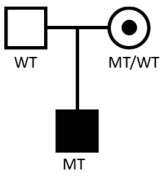
Discovery Platform: Whole exome sequencing.

Other variants of significance: No. Variant of unknown significance NM_015176.3 FBXO28 c.1070_1073delCTCT p.(S357Lfs*28) de novo and heterozygous.

ACMG Classification: Unknown Significance

Authors: Janneke Weiss, Petra Zwijnenburg

Pedigree:



Clinical Notes:

Patient had intrauterine growth restriction. Postnataly, growth within target height range, with no dysmorphisms, global developmental delay, absent speech and autism. Brain MRI revealed delayed myelinisation, otherwise no abnormalities.

Netherlands 2

Variant: ChrX GRCh37(hg19) g.41000604G>C; NM_001039590.2 c.1081G>C; NP_001034679.2 p.Val361Leu

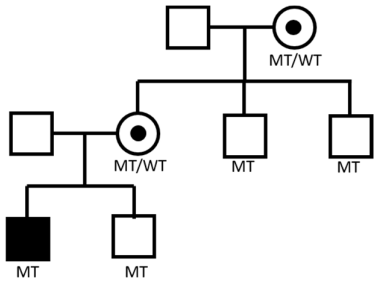
Discovery Platform: whole exome sequencing.

Other variants of significance: No

ACMG Classification: Likely Benign – Found in healthy male relative

Authors: Tjitske Kleefstra and Margot Reijnders

Pedigree:



Netherlands 6

Variant: ChrX GRCh37 (hg19) g.41002691G>A; NM_001039590.2 c.1309G>A; NP_001034679.2 p.Ala437Thr

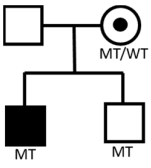
Discovery Platform: Whole exome sequencing.

Other variants of significance: No

ACMG Classification: Likely Benign – Found in healthy male relative (maternal uncle – not shown)

Authors: Tjitske Kleefstra and Margot Reijnders

Pedigree:



Swiss 1

Variant: ChrX GRCh37(hg19) g.41031160A>G; NM_001039590.2 c.3097A>G; NP_001034679.2 p.Met1033Val

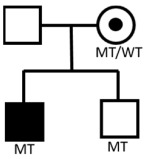
Discovery Platform: Whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely Benign – Found in healthy brother

Authors: Pascal Joset

Pedigree:



Severe global DD, Dandy Walker formation and hypoplasia of cerebellum.

Norway 1

Variant: ChrX GRCh37(hg19) g.41043275G>A; NM_001039590.2 c.3173G>A; NP_001034679.2 p.Arg1058Lys

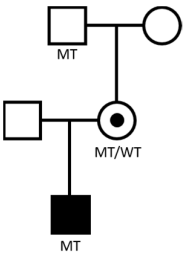
Discovery Platform: Trio based Disease exome sequencing, (4813 gene Illumina TruSightOne "Mendeliome" panel).

Other variants of significance: No

ACMG Classification: Likely Benign (variant found in healthy male relative)

Authors: Marie Falkenberg Smeland

Pedigree:



Canada 5

Variant: ChrX GRCh37(hg19) g.41043684C>A; NM_001039590.2; c.3314C>A; NP_001034679.2 p.Pro1105His

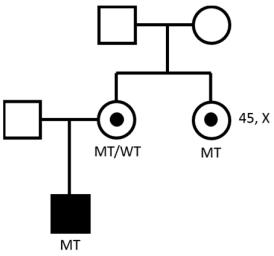
Discovery Platform: Whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely Benign (Found in unaffected maternal aunt who has Turner syndrome)

Authors: Mathew Lines

Pedigree:



Netherlands 4

Variant: ChrX GRCh37(hg19) g.41043792T>C; NM-001039590.2 c.3422T>C; NP_001034679.2 p.M1141T

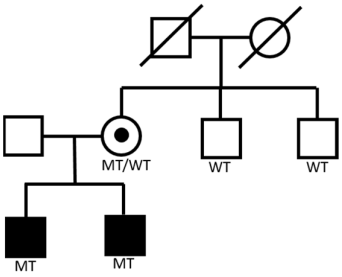
Discovery Platform: whole exome sequencing.

Other variants of significance: No

ACMG Classification: Likely Benign – Found in healthy male relative

Authors: Tjitske Kleefstra and Margot Reijnders

Pedigree:



Netherlands 8

Variant: ChrX GRCh37(hg19) g.41055921A>G; NM_001039590.2 c.4163A>G; NP_001034679.2 p.Asn1388Ser

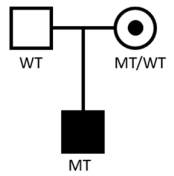
Discovery Platform: Trio based whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely Benign. –Hemizygous alleles found in gnomAD.

Authors: Tjitske Kleefstra, Margot Reijnders

Pedigree:



Belgium 1

Variant: ChrX GRCh37(hg19) g.41064560A>G; NM_001039590.2 c.4829A>G; NP_001034679.2 p.Asn1610Ser

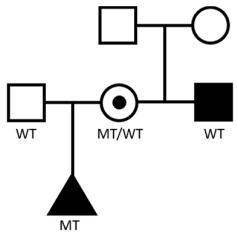
Discovery Platform: Trio based Disease exome sequencing (3989 genes, SeqCap EZ Choice XL, NimbleGen Roche)

Other variants of significance: No.

ACMG Classification: Likely benign

Authors: Lionel Van Maldergem, Julie Désir, Martina Marangoni

Pedigree:



FRANCE 1

Variant: ChrX GRCh37 (hg19) g.41075489G>A; NM_001039590.2 c.5669G>A; NP_001034679.2 p.Gly1890Glu

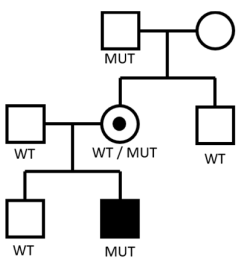
Discovery Platform: Trio-based whole exome sequencing.

Other variants of significance: Missense change in *H3F3A* [Chr1(GRCh37):g.226259121G>C NM_002107.4:c.352G>C p.(Val118Leu)] considered likely pathogenic.

ACMG Classification: Likely Benign – Found in healthy male relative.

Authors: Sebastien Kury, Sandra Mercier

Pedigree:



USA 4

Variant: ChrX GRCh37(hg19) g.41075489G>A; NM_001039590.2 c.5669G>A; NP_001034679.2 p.Gly1890Glu

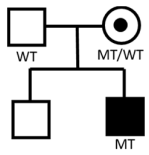
Discovery Platform: Whole exome sequencing

Other variants of significance: No

ACMG Classification: Likely Benign (Found in healthy male in case France 1)

Authors: Mary Kay Koenig

Pedigree:



Germany 1

Variant: ChrX GRCh37(hg19) g.41077775A>G; NM_001039590.2 c.6360A>G; NP_001034679.2 p.Ile2120Met

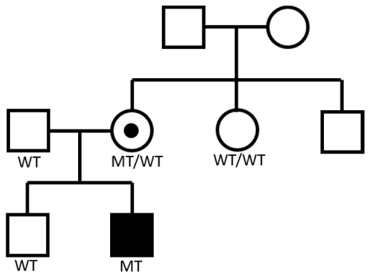
Discovery Platform: whole exome sequencing.

Other variants of significance: No

ACMG Classification: Likely Benign – Variant found in healthy male relative, Hemizygous alleles found in gnomAD

Authors: Nicola Dikow, Ute Moog

Pedigree:



USA 29

Variant: ChrX GRCh37(hg19) g.4107775A>G; NM_001039590.2 c.6360A>G; NP_001034679.2 p.Ile2120Met

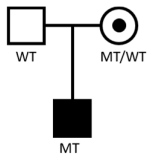
Discovery Platform: Trio based whole genome sequencing

Other variants of significance: No.

ACMG Classification: Likely Benign. Variant found in healthy a male in family Germany 1, Hemizygous alleles found in gnomAD

Authors: Scott E. Hickey

Pedigree:



Denmark 1

Variant: ChrX GRCh37(hg19) g.41082482A>G; NM_001039590.2 c.6578A>G; NP_001034679.2 p.Lys2193Arg

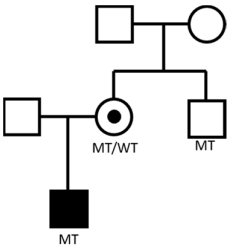
Discovery Platform: whole exome sequencing.

Other variants of significance: No

ACMG Classification: Likely Benign – Found in healthy male relative

Authors: Sabine Grønborg

Pedigree:



Supplemental References

1. Zhang Q, Dong A, Walker JR, Bountra C, Arrowsmith CH, Edwards AM, et al. (2018): *Crystal structure of a peptidase*. http://www.rcsb.org/structure/5WCH.
2. Reijnders MR, Zachariadis V, Latour B, Jolly L, Mancini GM, Pfundt R, et al. (2016): De Novo Loss-of-Function Mutations in USP9X Cause a Female-Specific Recognizable Syndrome with Developmental Delay and Congenital Malformations. *Am J Hum Genet*. 98:373-381.