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Disruption at the *PTCHD1* locus on Xp22.11 in autism spectrum disorder and intellectual disability

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Abstract

Autism is a common neurodevelopmental disorder with a complex mode of inheritance. It is one of the most highly heritable of the complex disorders, however, the underlying genetic factors remain largely unknown. Here, we report mutations in the X-chromosome *PTCHD1* (*patched*-related) gene, in seven families with autism spectrum disorder (ASD) and in three families with intellectual disability (ID). A 167 Kb microdeletion spanning exon 1 was found in two brothers, one with ASD the other with learning disability and ASD features, and a 90 Kb microdeletion spanning the entire gene was found in three males with ID in a second family. In 900 ASD and 208 ID male probands we identified seven different missense changes in eight probands, all male and inherited from unaffected mothers, and not found in controls. Two of the ASD individuals with missense changes also carried a *de novo* deletion at another ASD-susceptibility locus (*DPYD* and *DPP6*), suggesting complex genetic contributions. In additional males with ASD, we identified deletions in the 5' flanking region of *PTCHD1* disrupting a complex non-coding RNA and potential regulatory elements; equivalent changes were not found in male control individuals ($p=1.2 \times 10^{-5}$). Systematic screening at *PTCHD1* and 5'-flanking regions, suggests involvement of this locus in ~1% of ASD and ID individuals.

Introduction

Autism (MIM 209850) is a severe, lifelong neurodevelopmental disorder characterized by impairments in communication and socialization, and by repetitive behavior. Recent studies of sub-microscopic genomic copy number variation (CNV) have identified several loci associated with Autism Spectrum Disorder (ASD; MIM 209850) (1,2). *De novo* CNVs associated with ASD have been reported in ~7% of simplex families and ~2% of multiplex families (2,3). CNV studies have also led to the identification of autism candidate genes such as *SHANK3* (MIM 606230) and *NRXN1* (MIM 600565) (1,4,5). Intellectual disability (ID) is frequently associated with autism (in up to ~30% of cases for ASD, and ~67% for autism) (6). Moreover, mutations in several X-linked ID (XLID) genes (e.g. *NLGN4* and *IL1RAPL1*) have been shown to result in an autistic phenotype, which suggests that autism and ID may often share a common genetic etiology (3,7-10). We previously reported a 167 Kb microdeletion of exon 1 of *PTCHD1* (NM_ 173495.2) on chromosome Xp22.11 (3). *PTCHD1* has three exons spanning ~62 Kb and it is predicted to encode a protein of 888 amino acids. *In silico* analysis suggests that *PTCHD1* is a transmembrane protein containing a patched-related domain with

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twelve transmembrane helices, highly related to the Hedgehog (Hh) receptors PATCHED1 (PTCH1) and PTCH2 as well as to Niemann-Pick Type C1 protein (NPC1). Hh is one of the key signaling pathways involved in the formation of the neural tube and brain, specifically the differentiation of motor neurons ventrally and commissural interneurons dorsally (11,12). Mutations in Sonic Hedgehog, *SHH* (MIM 600725), have been reported in patients with developmental abnormalities, delay in speech acquisition and learning disabilities (13). Niemann-Pick disease type C1 also involves neurological and intellectual deficits (MIM 257220). This led us to investigate a possible role for *PTCHD1* as a candidate gene for ASD and ID.

Further to the initial CNV-screening ASD cohort (Marshall et al, 2008), we have now analyzed CNV screening data for a cohort of ID subjects, as well as cohorts of unaffected subjects, and, where CNVs have been identified at the *PTCHD1* locus, we have validated and characterized the CNVs and their inheritance in the families. This screening identified a second deletion at *PTCHD1*, segregating among males in a family with ID. This finding also prompted screening of additional ASD cohorts for CNVs at the *PTCHD1* locus. We also screened a proportion of the cases and controls for coding mutations within *PTCHD1* (see table S5 for details on cohorts studied). Preliminary functional evidence for the PTCHD1 protein is consistent with a role in Hh signaling.

Results

CNV Analysis of *PTCHD1*

We characterized the precise breakpoints of the 167 Kb deletion at *PTCHD1* identified in the male proband from Family 1. This CNV also disrupts long, spliced non-coding RNAs (ncRNAs) on the opposite strand, but no other coding genes were interrupted (Fig. 1). The deletion was validated in the family using both PCR and SYBR-Green I-based real-time quantitative PCR (qPCR) and was found to be transmitted from a heterozygous unaffected mother to two affected dizygotic twin sons, also to an unaffected daughter (Fig. 2). X-chromosome inactivation (XCI) analysis of the mother, carrier of the *PTCHD1* deletion, revealed a highly skewed allelic ratio of 94:6.

To assess the possible involvement of CNVs disrupting X-chromosomal genes in the etiology of ID, we initially screened 246 males with intellectual disability and probable X-linked inheritance using a custom-designed NimbleGen 385K array with probes targeting the X chromosome. A 90 Kb deletion encompassing the entire *PTCHD1* gene and 5’ exons of the ncRNAs (but no other known coding genes) was found in a male ID patient (Family 2). The deletion was validated using qPCR revealing that the deletion was maternally inherited in two affected brothers and their affected uncle (Fig. 2). XCI analysis revealed allele ratios of 51:49 and 75:25 from lymphocytes of 2 obligate female carriers.

Subsequent to the ascertainment of these cohorts, an additional case of ID with dysmorphic features was referred to us through cytogenetic services at the University of California, San Francisco, California, USA. CNV analysis with a custom designed 105K microarray identified a maternally inherited 146 kb deletion in this patient which spans *PTCHD1* exon 1 and upstream regions (chrX:23,146,927-23,293,273, hg18).

Mutation Screening of *PTCHD1*

In order to identify additional cases with *PTCHD1* mutations, we sequenced the coding regions in 900 (M=723; F=177) unrelated ASD cases and 225 unrelated male ID cases. Seven missense changes were identified in six unrelated ASD probands and two ID probands (Fig. 2; fig. S1 & fig. S2; table S1). All of these variants, which resulted in the substitution of highly conserved

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amino acids, were inherited from unaffected carrier mothers (fig. S1). In six of the eight families the missense variants appear to segregate with the phenotype, however in Family 6 L73F did not segregate, and in Family 7 the A470D did not segregate in different loops (not shown) of the extended pedigree (see Fig. 2 and table S1 for details).

We sequenced the entire coding region of *PTCHD1* in 700 control individuals (M=531 F=169), and none of the missense changes identified from among the ASD and ID patient cohorts has been detected. Only two missense changes have been identified: P252L from amongst our controls, and N497K reported in the SNP database (rs35880456, in 1 out of 39 screened; NCBI) (14), both in females who were heterozygotes. Altogether, absence of *PTCHD1* missense variants indicates that these variants are significantly enriched in the males with ASD (6/723 male ASD versus 0/531 male control: Fisher's exact test: p =0.042) and may contribute to the phenotype.

Additional controls were sequenced for the exons in which missense mutations were identified. We tested control chromosomes for the sequence underlying the I173V and V195I mutations (N=1100 chromosomes), the ML336_337II mutation (N=1193), and the L73F, E479G, A470D and H359R mutations (N=869) and detected none of these variants.

CNVs upstream of *PTCHD1* (*PTCHD1AS1/PTCHD1AS2* locus)

Additionally, from a study of 996 ASD families examined with the Illumina 1M BeadChip (15), we identified eight deletions in probands or affected siblings, and a ninth in a father with a diagnosis of Broad Autism Phenotype (BAP) (16,17), all occurring 5' of *PTCHD1* (Fig. 1). A tenth deletion at this upstream locus was identified in a patient from a CNV study of 167 unrelated attention deficit-hyperactivity disorder (ADHD) patients. The ADHD proband with the deletion also has a BAP diagnosis. These deletions were validated with qPCR and exact breakpoints were mapped (table S2). Additional CNV data for these 10 individuals are included in table S3.

We analyzed SNP microarray data from 10,246 control individuals (4,829 male; 5,417 female), for CNVs at *PTCHD1* and the upstream region. In a 1.4-Mb region spanning from *PTCHD1* to adjacent genes *PRDX4* (proximal) and *ZNF645* (proximal), we identified 15 CNVs (7 duplications and 8 deletions); however, it is notable that only 1 male control with a deletion was identified, which was 20.6 Kb in length and did not disrupt any known exons of any genes or non-coding RNAs, or any of the identified conserved or putative regulatory sequences. The remaining 7 deletions were all identified among female controls, consistent with the X-linked recessive inheritance observed for the *PTCHD1* mutations. Thus, *PTCHD1* and upstream deletions were not observed in 4,829 male controls, or in the Database of Genomic Variants (18), which suggests that the CNV directly disrupting *PTCHD1* and the 6 CNVs located just upstream in unrelated ASD probands are associated with autism (male ASD cases N=7, out of 1,185; male controls N=0 out of 4,829; Fisher's exact test: $p = 1.2\times10^{-5}$).

Subsequently, an additional case with ID and with a number of other clinical features was referred to us from Dr. A.E. Chudley, Children's Hospital, Winnipeg and Dr. Evica Rajcan-Separovic at Children's and Women's Health Centre of BC, Vancouver, BC. This family has a maternally inherited 112 kb deletion upstream of *PTCHD1*, including DDX53 and at least one exon of *PTCHD1AS1* (chrX:22,819,116-22,931,588, hg18).

Expression and Functional Studies of PTCHD1

Expression analysis for the *PTCHD1* and the ncRNA transcripts suggests that they are transcribed in brain regions, notably the cerebellum, as well as in other tissues (Fig. 3 and fig. S3. RNA *in situ* hybridization of *Ptchd1* in mouse showed widespread expression in the

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developing brain from E9.5/10.5 to P1 (Fig. 4a), as well as broad expression in the adult mouse brain (6 months), with highest density in the cerebellum (see Allen brain atlas online (19)).

To investigate its function, we studied the sub-cellular localization of PTCHD1 and found that a PTCHD1-GFP fusion protein predominantly localizes to the cell membrane (Fig. 4b). We further hypothesized that PTCHD1 may function in the Hh-signaling pathway and have similar functional attributes as PTCH1 and PTCH2. We performed a Gli-dependent transcription assay in Hh-responsive 10T1/2 cells to test whether PTCHD1 could interfere with Hh signaling. In 10T1/2 cells, overexpression of PTCH1 or PTCH2 inhibits transcription from a Gli-luciferase reporter containing multiple copies of the Gli protein-binding site in the presence of Smoothened agonist purmorphamine (20) (Fig. 4c) or Gli2 (fig. S4). Similar to PTCH proteins, PTCHD1 also exerted a statistically significant inhibitory effect in these assays suggesting that PTCHD1 functions in the Hedgehog signalling pathway.

Discussion

We have identified microdeletions that directly disrupt the *PTCHD1* gene in males in three families affected with either ASD, ID or learning disability. These deletions are maternally inherited and were not observed in more than 10,000 controls, which indicates that these alterations are associated with ASD and ID. We also report seven maternally inherited missense mutations in eight male probands. These variants were not seen in more than 500 controls, which further supports a possible role of this gene in autism and ID.

In addition, we have found another 11deletions that map to regions upstream of *PTCHD1*. The region 5' and distal to *PTCHD1* is relatively gene poor. Within this upstream region, a coding gene, *DDX53*, encoding DEAD Box 53, lies ~335 Kb 5' to *PTCHD1*. Five of the 11 upstream deletions span *DDX53*. However, based on the function of DDX53 protein and the expression pattern of this gene (which is restricted mainly to testis and tumor cells (21)), it is unlikely to contribute to the ASD or ID phenotype. Additionally, within the gene-poor region between *PTCHD1* and *DDX53*, there is a putative pseudogene of *FAM3C, FAM3C2*, which is disrupted by five of the 10 upstream deletions. *FAM3C*, a cytokine-like gene on 7q31.31, consists of 10 exons (22) whereas *FAM3C2*, although 99% identical, has no intron/exon structure and is interrupted by a short interspersed nuclear element (SINE). It appears to have inserted on Xp22 after human/chimp evolutionary divergence. Since no mRNA or EST matches exactly to *FAM3C2*, it is most likely an untranscribed processed pseudogene.

We examined the region just distal to *PTCHD1* in detail and identified a number of putative enhancer and promoter sequences, as well as conserved (and putative regulatory) elements (Fig. 1). We also identified several overlapping spliced long (>200nt) non-coding (nc) RNAs (*PTCHD1AS1* (from cDNA clone IMAGE:1560626; BX115199) and *PTCHD1AS2* (from cDNA clone BRSTN2000219; DA355362)), which map to the opposite strand and distal to *PTCHD1* (see Fig. 1). 5'RACE (Rapid Amplification of cDNA Ends) shows that a number of splice variants of these transcripts originate at the CpG island just upstream of *PTCHD1*, encompassing its putative promoter. Similar antisense transcripts are present at syntenic loci in other mammalian species, at least two exons of which appear to be conserved between rat, mouse and humans (Fig. 1).

Although these ncRNAs do not appear to encode protein, they may serve as regulators for other coding genes, particularly for *PTCHD1*, since the 5' exons are adjacent on opposite strands. Such ncRNAs may regulate expression of a coding transcript on the opposite strand through a number of mechanisms, including modification of chromatin, transcriptional regulation and post-transcriptional modification (23,24).

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All 11 of the upstream deletions as well as the three *PTCHD1* deletions (Families 1 and 2) disrupt conserved (and putative regulatory) sequences and/or exons of these ncRNAs (see Fig. 1). These deletions were not inherited by a subset of the affected family members; also, the missense variants do not segregate with disease in two families (Families 6 & 7) (Fig. 2). These findings are similar to other previously reported, major affect ASD loci such as 16p11.2 (25) and are also consistent with the complex, non-Mendelian inheritance believed to control the etiology of autism. As discussed in a recently proposed threshold model of relative contribution in ASD (26), it is anticipated that multiple common and rare variants may act in concert to generate the phenotype. For instance, under this model, some *de novo* CNVs may be solely sufficient to cause ASD. Conversely, other *de novo* CNVs may have weaker effects, requiring contributions from additional loci (for example additional risk haplotypes, or other CNVs), or environmental risk factors, for the burden of contributory factors to cross a risk threshold and result in an ASD phenotype. In three of the eight families (6 ASD and 2 ID) that carry putative *PTCHD1* missense mutations (Families 8, 9 and 10), we have identified other CNVs involving genes that may also contribute to the phenotype. In Family 9, in addition to the I173V substitution, we found a *de novo* ~1.0 Mb loss at 1p21.3 resulting in deletion of the entire *DPYD* gene (MIM 274270), encoding dihydropyrimidine dehydrogenase (DPD) (3). Complete DPD deficiency results in highly variable clinical outcomes, with convulsive disorders, motor retardation, and mental retardation being the most frequent manifestations, and autistic features occasionally reported (27). In this family, a balanced translocation, t(19;21)(p13.2; q22.12) is also present in the proband, but is inherited from the unaffected mother and shared with an unaffected sister (see Supplementary Materials). In Family 10, which shows the V195I substitution in *PTCHD1*, we have previously reported a 66 Kb *de novo* loss at 7q36.2 that results in deletion of the third exon of *DPP6* (MIM 126141) – previously reported as a positional and functional candidate gene for autism (3). In ID Family 8, we have identified a H359R substitution in *PTCHD1* and a 2 Kb deletion spanning the last exon of *SLC16A2*, both variants are maternally inherited. The phenotype in this family was severe ID compatible with Allan-Herndon-Dudley syndrome (MIM 300523) (28,29), for which mutations in *SLC16A2* have previously been reported.

Thus, in two ASD individuals we have evidence for the possible involvement of more than one locus in the disease, and these findings may support the threshold model of relative contribution in ASD described above (26) and polygenic inheritance in autism. As such, some *de novo* CNVs may be highly penetrant in causing ASD susceptibility (e.g. disruption of *PTCHD1* in Family 1). Conversely, other *de novo* CNVs (e.g. *DPP6* and *DPYD* deletions) may have more subtle effects, requiring contributions of additional loci (e.g. *PTCHD1* missense mutations in the case of Families 9 & 10) for ASD to be phenotypically evident. This scenario may also apply to the ID families with *PTCHD1* mutations, although for Family 8 the *PTCHD1* missense variant contribution is likely overwhelmed by the phenotypic effect of a whole exon deletion of *SLC16A2*.

PTCHD1 gene expression showed high correlation with expression of other cerebellar genes such as *ZIC1, CADPS2, EN2, CBLN1*, and with synaptic genes such as *PCLO, NRXN3, SNAP25, SYT2, DPP6* and *DPP10* (see table S4). Cerebellar abnormalities have frequently been linked to autism, including recent magnetic resonance imaging (MRI) studies showing significant decrease in cerebellar grey matter (30,31), and decreased cerebellar connectivity and activity (32).

PTCHD1 encodes a Patched-related protein with 12 transmembrane domains and a sterol-sensing domain, structurally similar to the Hh receptors PTCH1 and PTCH2, as well as the Niemann-Pick Type C1 protein (NPC1) and several others (fig. S5). Many Patched-related genes have been found in various organisms, from nematodes to humans, and they appear to play diverse biological functions, including cytokinesis, growth and pattern formation (33).

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For instance, there are just seven patched-related genes in humans (*PTCH1, PTCH2, PTCHD1, PTCHD2, PTCHD3, NPC1* and *c6orf138* (see fig. S5), whereas in *C. elegans* there are at least 26 patched-relaetd genes, with diverse roles in development in addition to Hh signaling, including cytokinesis, growth and pattern formation (33). In 10T1/2 cells, we have demonstrated an inhibitory effect of PTCHD1 on Gli-dependent transcription. Although these results suggest that PTCHD1 exhibits biochemical activity in Hh-dependent processes similar to that of PTCH1 and 2, other functions or roles for PTCHD1 cannot be excluded at this point.

In summary, our data indicate that mutations at the *PTCHD1* locus are highly penetrant and strongly associated with ASD (including BAP) and ID in ~1.1% and ~1.3% of the individuals analyzed, respectively (based on probands for whom comprehensive mutation screening, for both CNVs and sequence variants, has been performed (4 out of 353 ASD, and 3 out of 225 ID). Overall, our finding are reminiscent of genetic findings for several other X chromosome genes, including *NLGN4* (7,8) and *IL1RAPL1* (9,10,34), in that mutations can apparently cause either ASD or ID (or both), and thus *PTCHD1* may be a gene for both. *IL1RAPL1*, for example, was initially reported as a gene for non-syndromic X-linked ID (34), and then subsequently was also found to harbor mutations in ASD pedigrees (9,10). We have also identified two families in whom at least two loci may be contributing to the pathogenesis of ASD, and another seven families bearing upstream microdeletions that disrupt a complex non-coding RNA, providing possible genetic explanations for the clinical heterogeneity of these disorders. Finally, our results raise the possibility that Hh signaling may be perturbed in these conditions. This discovery may help provide possible targets for therapeutics in individuals with mutations at this locus.

Methods

Subjects

CNVs at the PTCHD1 locus were initially assessed in 427 ASD patients, as described (3). DNA samples from 900 individuals diagnosed with ASD were sequenced for PTCHD1 mutations. Among these, 400 samples were collected at three sites, namely The Hospital for Sick Children (HSC) in Toronto and child diagnostic centers in Hamilton, Ontario and St, John’s, Newfoundland. Details of these samples are published elsewhere (5). 420 ASD cases were recruited at Montreal, details of these samples are published elsewhere (35). Another 80 ASD probands from the Autism Genetic Resource Exchange (AGRE) were also included. The second cohort of 996 autism probands was recruited at different sites as a part of the Autism Genome Project (AGP); ascertainment is described elsewhere (15). 246 male patients with intellectual disability were recruited from the UK, United States, Australia, Europe and South Africa as the IGOLD study. A subset of 225 from this cohort were also used for sequence analysis of *PTCHD1*. Details of these samples are published elsewhere (36). 167 unrelated patients diagnosed with ADHD were recruited through the Department of Psychiatry at the Hospital for Sick Children, Toronto. Microarray data from controls included 1,123 (M=623, F=500) controls recruited from northern Germany as a part of the PopGen project, 1,234 (M=586, F=648) healthy controls of European origin recruited from the province of Ontario, Canada, 1,287 (M=383, F=904) controls from the Study of Addiction: Genetics and Environment (SAGE), 1,320 (M=589, F=1320) controls from Children’s Hospital of Philadelphia (CHOP), 4783 (M=2460, F=2323) controls were recruited by the Wellcome Trust Case Control Consortium, 440 (M=158, F=282) controls were recruited by The Centre of Addiction and Mental Health (CAMH) and GlaxoSmithKline (GSK), and 59 (M=30, F=29) from the Centre d’Etude Polymorphisme Humaine (CEPH) HapMap controls (total N=5,023). We sequenced more than 650 Ontario controls obtained from The Centre for Applied Genomics (TCAG) and The Centre for Addiction and Mental Health (CAMH). Details of all samples included in the study are summarized in table S5. Institutional ethical review board approval

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(CAMH, HSC, CHOP and all other collaborating institutions) was obtained for the study, and informed written consent was obtained for each family. Details of the clinical findings in families with *PTCHD1* mutations or CNVs are summarized in table S1.

Copy Number Variation Analysis

We used Affymetrix 500K SNP arrays to assess CNVs in a cohort of 427 ASD cases. Details on the methods of copy number analysis and complete results are published elsewhere (3). Only the CNV result at *PTCHD1* is described here. Another cohort of 996 autism probands was analyzed on 1M BeadChips (Illumina) (15). 246 male patients with ID were analyzed on a custom designed NimbleGen 385K array. Genomic DNA samples were sent to NimbleGen for the hybridizations to be performed. Each patient sample (Cy5-labelled) was co-hybridised with DNA from the reference sample NA10851 (Cy3-labelled; obtained from Coriell Cell Repository). After data normalisation, the ADM-1 algorithm (CGH Analytics 3.4, Agilent) was used for CNV discovery. The ADHD cohort was analyzed on Affymetrix 6.0 arrays. Three algorithms (Birdsuite, iPattern and Affymetrix Genotyping console (GTC)) were used to infer CNVs. The CEPH, PopGen and Ontario controls were analyzed on Affymetrix 6.0 arrays, SAGE controls were analyzed 1M BeadChips (Illumina) and Illumina 550K arrays were used for the CHOP and CAMH\GSK controls. Similar methods were used to infer CNVs in controls. The probe density of different microarray platforms at the *PTCHD1* locus is shown in fig. S6. Fisher's Exact Test was used to calculate the two-tailed p value.

DNA Sequencing and Mutation Screening

PCR primers were designed with Primer 3 (v. 0.3.0) to amplify all three exons and intron-exon boundaries. PCR were performed under standard conditions, and products were purified and sequenced directly with the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

X-Inactivation Studies

The X Chromosome Inactivation assay was performed on genomic DNA extracted from peripheral blood as described (37). Briefly, X Chromosome Inactivation was measured by the analysis of the (CAG)n repeat in the androgen receptor gene at Xq11-q12 before and after digestion with methylation sensitive restriction enzymes *Hha*I and *Hpa*II. Quantitative PCR amplification of androgen receptor gene repeat alleles was compared, with and without restriction digestion, to determine the ratio of X-active/inactive alleles.

Expression Analysis and Protein Localization

Expression analysis and tissue distribution for *PTCHD1, PTCHD1AS1* and *PTCHD1AS2* was performed by RT-PCR, with a multiple tissue panel of first strand cDNA. The housekeeping gene *G3PDH* was used as a control. Origene human adult brain tissue panel was used to check the expression of *PTCHD* mRNA in different regions of the brain. qRT-PCR was performed with TaqMan Gene Expression assay Hs00288486, and samples were pre-normalized to *GAPDH* expression. Northern blot analysis was performed with a six tissue mRNA blot (BioChain). The BioChain FastHyb solution was used to hybridize the probe according to manufacturer's instructions. RNA *in situ* hybridization was performed on paraffin sections and whole-mounted fetal mouse and adult mouse brain using a 411 bp (chrX: 152,008,934-152,009,344, UCSC Mouse July, 2007 (38)) digoxigenin-labeled mouse antisense probe (and sense probe as negative control), using standard methods. To examine cellular localization of PTCHD1 protein, full-length human fetal brain *PTCHD1* cDNA was PCR amplified and cloned into the pcDNA3.1/CT-GFP-TOPO expression vector (Invitrogen). After confirming the correct sequence and orientation of the insert, we transiently transfected COS-7 and SK-N-SH cells with 2 μg of purified construct DNA with SuperFect (Qiagen). 24

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hours after transfection, we visualized the PTCHD1-GFP fusion protein in transfected cells using a Zeiss Axioplan 2 imaging microscope, equipped with the LSM510 array confocal laser scanning system, and the Zeiss LSM510 version 3.2 SP2 software package.

Luciferase Assays

A luciferase assay was performed to compare the effect of PTCH1, PTCH2 and PTCHD1 on Gli-dependent transcription with a previously described method (39). Briefly, the 10T1/2 cells were transiently transfected with mixtures containing 0.1 μg β-galactosidase to normalize for transfection efficiency, 1 μg reporter plasmid (8xGlipro) encoding multimerized Gli binding sites fused to the luciferase gene and up to 1 μg of Gli2, PTCH1 or PTCH2 or PTCHD1. Gli-dependent transcription was measured and normalized by β-galactosidase. Data were replicated in independent experiments performed in triplicates. In another assay, 10T1/2 cells were transiently transfected with mixtures containing 0.1 μg β-galactosidase, 1 μg 8xGlipro reporter plasmid and purmorphamine, PTCH1 or PTCH2 or PTCHD1. The effect of PTCH1, PTCH2 and PTCHD1 on the endogenous Gli-dependent transcription was measured. Statistical significance was calculated as p below 0.05, using the Student's t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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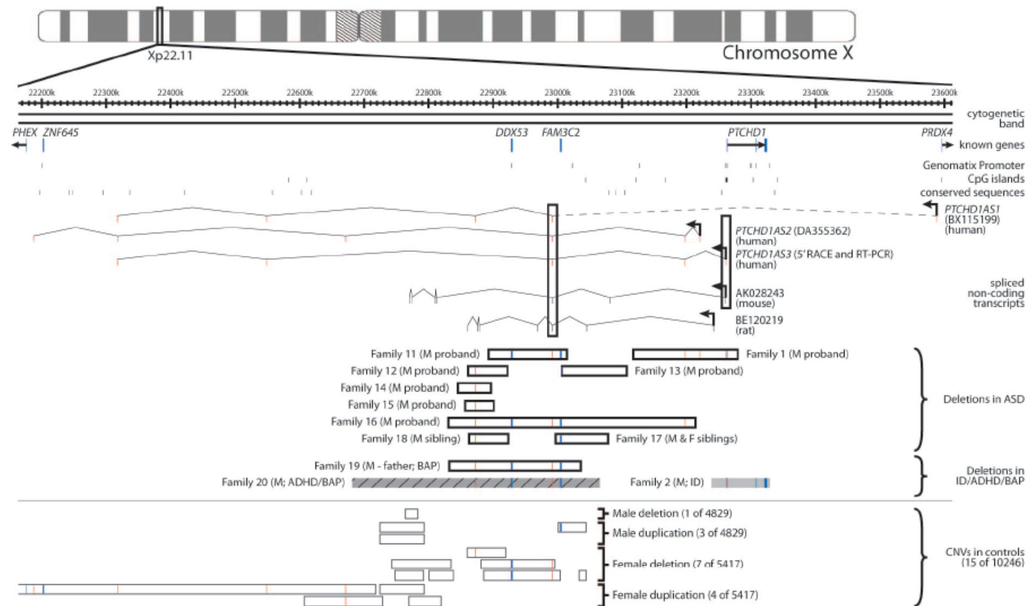

Fig. 1.

Detailed genomic organization of the *PTCHD1* locus. The known genes, predicted CpG islands (>300 bp), predicted promoters (ElDorado Suite from Genomatix) and conserved sequences (>75% identity with chicken, >90% identity with opossum or 100% identity with dog or horse) are shown. Putative non-coding RNA transcripts *PTCHD1AS1* (from cDNA clone IMAGE: 1560626; BX115199) and *PTCHD1AS2* (cDNA clone BRSTN2000219; DA355362) from human, mouse and rat genomes are also shown, with the transcripts assembled from RT-PCR and 5' RACE (*PTCHD1AS3*) (see Supplementary Material). The dotted line between the two exons in transcript *PTCHD1AS1* indicates that this is a putative exon, identified through clone sequencing. This exon is putative because, although this location represents its best genomic hit, it only partially matches the 5' end of the clone sequence. Black boxes within the spliced transcripts indicate homologous exons between the sequences. White bars with black borders indicate CNV losses within this locus that have been identified in patients with ASD and controls. Cross-hatched or grey bars indicate CNV losses identified in patients with ADHD and ID, respectively. Colored lines within these bars indicate overlap with exons of known transcripts (blue) or ncRNA (red). The breakpoints of the deletions for all families that are reported here were mapped by sequencing the junction (see table S2 for coordinates). Breakpoints for all CNVs in controls were mapped by using the physical positions of microarray probe fragments.

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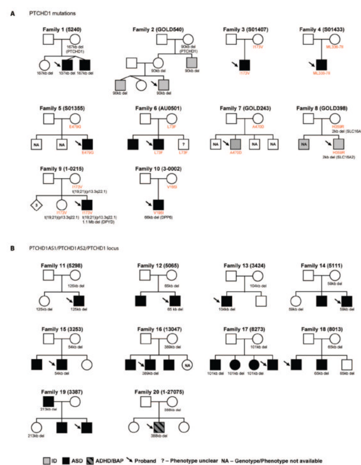

Fig. 2.

Pedigrees of families. (A) Pedigrees showing *PTCHD1* mutations. (B) Pedigrees showing deletions at the *PTCHD1/PTCHD1AS1-3* locus. The third male in Family 18 was assessed at age 4 and had speech and language problems, but was not available for further assessment. The father in Family 19 has a broader autism phenotype (BAP) (14,15). The proband in Family 20 (hatched) has ADHD plus BAP. A diamond symbol represents siblings who were not tested as part of the study, and with gender not indicated.

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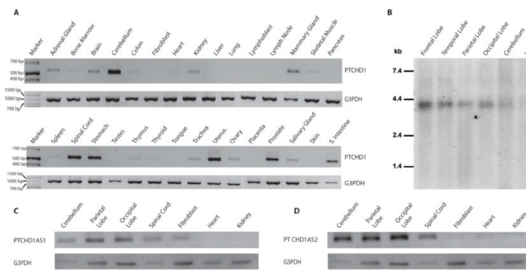

Fig. 3.

Transcription analysis. (A) RT-PCR expression analysis of *PTCHD1* transcript in 30 different adult tissues. The housekeeping gene *G3PDH* was used as a control. (B) Northern hybridization analysis of *PTCHD1* showing a ~4.1Kb band in all lanes. Current RefSeq annotation of *PTCHD1* describes a ~5.3Kb transcript; however, the only polyadenylation site predicted for the mRNA sequence (NM_173495) by POLYAH is at 4.379 bp. RT-PCR expression analysis of (C) *PTCHD1AS1* and (D) *PTCHD1AS2* expression in seven human tissues, also with *G3PDH* as a control. Northern analysis of the ncRNAs did not give sufficient signal for detection.

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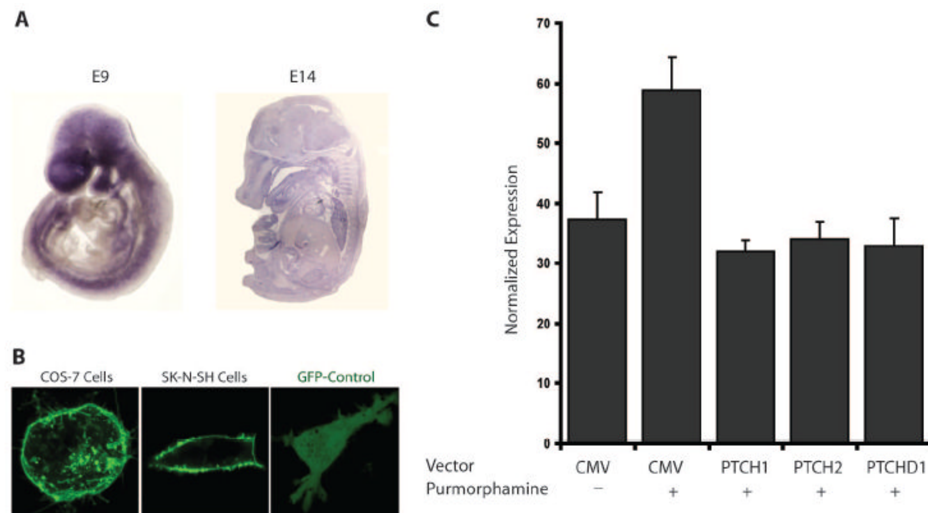

Fig. 4.

Expression and functional studies. (A) Whole-mount RNA *in situ* hybridization showing expression of *PTCHD1* in mouse embryo E9 and E14. (B) Localization of PTCHD1 protein in COS7, SK-N-SH and control cells shows that the PTCHD1-GFP protein is predominantly localized in the cell membrane. (C) PTCHD1 exerted a statistically significant inhibitory effect on endogenous Gli-dependent transcription, similar to PTCH1 and PTCH2, when transfected in Hedgehog-responsive 10T1/2 cells (PTCHD1: p= 0.0101; PTCH1: p= 0.0096; PTCH2: p= 0.0159). Statistical significance was calculated using the Student's t-test. Absolute expression of reporter gene normalized to β-gal expression is shown. Standard error bars are shown.