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TITLE PAGE

Wnt/ β -catenin pathway and cell adhesion deregulation in *CSDE1*-related intellectual disability and autism spectrum disorders

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ABSTRACT

Among the genetic factors playing a key role in the etiology of intellectual disabilities (ID) and autism spectrum disorders (ASD), several encode RNA binding proteins (RBPs). In this study, we deciphered the molecular and cellular bases of ID-ASD in a patient followed from birth to the age of 21, in whom we identified a *de novo* *CSDE1* (*Cold Shock Domain-containing E1*) nonsense variation. *CSDE1* encodes an RBP that regulates multiple cellular pathways by monitoring the translation and abundance of target transcripts. Analyses performed on the patient's primary fibroblasts showed that the identified *CSDE1* variation leads to haploinsufficiency. We identified through RNA-seq assays the Wnt/ β -catenin signaling and cellular adhesion as two major deregulated pathways. These results were further confirmed by functional studies involving Wnt-specific luciferase and substrate adhesion assays. Additional data support a disease model involving APC Down-Regulated-1 (APCDD1) and cadherin-2 (CDH2), two components of the Wnt/ β -catenin pathway, CDH2 being also pivotal for cellular adhesion. Our study, which relies on both the deep phenotyping and long-term follow-up of a patient with *CSDE1* haploinsufficiency and on *ex-vivo* studies, sheds new light on the *CSDE1*-dependent deregulated pathways in ID-ASD.

INTRODUCTION

Intellectual disability (ID) is a neurodevelopmental condition characterized by significantly impaired intellectual functioning affecting the ability to learn, reasoning and problem solving and by deficits in adaptive behavior, hindering day-to-day social and practical skills (1). ID is usually associated with other co-occurring neurodevelopmental disorders and a large majority of patients with ID also present with features of autism spectrum disorders (ASD). The co-occurrence of these two disease conditions is accounted for by their common molecular and genetic origin as several genes have been shown to be involved in both ID and ASD (1,2). Many of the identified mutations are responsible for dysfunctions of specific key signaling pathways, such as the TGF- β /BMP (Transforming growth factor-beta/bone morphogenetic protein), the Wnt/ β -catenin, the SHH (Sonic Hedgehog), and the retinoic acid signaling pathways (reviewed in (3)). For instance, ID-ASD-causing mutations have been reported in genes encoding proteins involved in several steps of the Wnt/ β -catenin pathway, like the upstream ligands *WNT1* and *WNT2* (4–7), the downstream transcription factor *TCF7L2* (8,9) or the β -catenin (*CTNNB1*), the core element of this pathway (10–13). Other major players involved in the onset of ID-ASD are RNA-metabolism regulators and RNA-binding proteins (RBP). This is for instance the case for the *FMR1* gene responsible for the Fragile X syndrome (FXS), the most common heritable form of ID-ASD (14), but also several other genes involved in ID-ASD (e.g. *UPF3A* and *B*, *SMG6*, *EIF4A3*, *RNPS1*, *PQBP1*, and *VCX-A*) (15–19). However, despite these advances in identifying ID-ASD-causing mutations, the molecular diagnosis of these conditions is challenging owing to the high clinical and genetic heterogeneity. Furthermore, whole genome sequencing or whole exome sequencing approaches are constrained by the identification of huge amount of variants in each patient and by the large number of patients that have to be studied considering the ID-ASD prevalence (1 to 3% of the general population) (20–22). It is therefore crucial to conduct molecular studies relying on a precise phenotype that could be associated with mutations in a given gene or set of genes.

Here, we describe the case of a patient followed from birth to the age of 21, presenting with severe ID and features resembling the Pitt-Hopkins syndrome (PTHS, MIM #610954) a neurodevelopmental disorder characterized by recognizable facial features and behavioral abnormalities, among which some overlap with ID-ASD. The patient carries no pathogenic variation in *TCF4* (Transcription factor 4, MIM#602272), the gene involved in PTHS (23,24), a result that led us to perform an *in trio* exome sequencing in the patient and his healthy parents. This analysis identified a *de novo* heterozygous nonsense variation in *CSDE1* (Cold Shock Domain-containing E1 - also known as *UNR*: Upstream of NRAS, MIM #191510) in the patient. *CSDE1*, a ubiquitously expressed RBP with high affinity for

mRNAs containing A/G-rich binding motifs (25,26), binds to regulatory regions of its targets transcripts, i.e. the 5'UTR and 3'UTR (untranslated regions) or the IRESs (Internal Ribosomal Entry Sites) to control their translation (26–29) and abundance (25,30,31). CSDE1 is involved in a multitude of biological processes, namely cell cycle and migration, apoptosis as well as embryonic development, neurogenesis and neuronal differentiation (27–32). The diverse CSDE1 targets and their associated biological processes could account for its involvement in the occurrence of multiple disease conditions (reviewed in (33)) as diverse as cancers, Diamond-Blackfan anemia or ASD. *CSDE1* is one of the genes mentioned in ASD large scale studies (34,35), and has recently been reported in patients with neurodevelopmental disabilities (36). However, the CSDE1-related pathways involved in the pathophysiology of ID-ASD have not been explored in patient-derived cells so far.

The identification of a nonsense *CDSE1* mutation in our patient, whose disease phenotype has been accurately described over two decades, prompted us to perform diverse functional *ex-vivo* studies with the aim to decipher the CSDE1-dependent biological pathways involved in the pathophysiology of ID-ASD.

MATERIALS AND METHODS

The affected patient and the healthy individuals – Informed consents were obtained from patient's parents and from the healthy individuals participating in the study for genetic tests, skin biopsies, data sharing and publication of the patient's photographs according to French legislation and the principles of the Declaration of Helsinki.

Whole exome sequencing – Genomic DNA was extracted from a peripheral blood sample and captured with the SureSelect HumanAll Exon V2 kit (Agilent, Santa Clara, CA). Whole-exome sequencing was performed (Integragen SA, Evry France) on a HiSeq 2000 (Illumina, San Diego, CA) according to the manufacturer's recommendations. Alignment was carried out as previously described (37).

Sanger sequencing and semi-quantitative PCR – for the *TCF4* and *CSDE1* genes were performed as previously reported (38).

Fibroblasts' culture – Skin biopsies were performed in the patient at the age of 14 and in five healthy donors aged between 10 and 33 years (three adults and two children - males and females). Fibroblast cultures were handled as previously described (39) and used for RNA-seq analysis and for functional studies.

RNA extraction and quantitative RT-PCR assay – Total RNA from fibroblasts was isolated using RNAeasy Mini Kit (Qiagen, Germany) and 1 µg of RNA was subjected to reverse transcription using Reverse Transcriptase kit (Roche, Switzerland) according to the manufacturers' instructions. cDNA was amplified using the Mesa Blue qPCR MasterMix Plus (Eurogentec, Belgium) in the Light Cycler LC480 (Roche). Primers are provided in Supplementary Information.

RNA-seq – Total RNA was captured using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina, San Diego, CA) sequencing was performed (Integragen SA, Evry France) on a HiSeq4000 (Illumina, San Diego, CA) according to the manufacturer's recommendations. RNA-Seq data analysis was performed by GenoSplice (www.genosplice.com). Sequencing, data quality, reads repartition, and insert size estimation were performed using FastQC, Picard-Tools, Samtools and rseqc. Reads were mapped using STARv2.4.0 (40) on the hg19 Human genome assembly. Gene expression regulation study was performed as previously described (41). Further information is provided in Supplementary Information. Pathway and process enrichment analysis was performed by Metascape (42) as

described in Supplementary Information. Data from three healthy individuals were compared to those obtained from the ID-ASD patient.

Nonsense-mediated mRNA decay assessment – Fibroblasts were treated with 30 µg/mL cycloheximide (Sigma Aldrich, MO, USA) or DMSO for 4 h. Total RNA was extracted and subjected to reverse transcription. PCR amplification was carried out using the Q5 (New England Biolabs, MA, USA) PCR products were purified using ExoSap (Thermo Fisher Scientific, MA, USA) before being sequenced.

Western blot analysis – Protein expression analyses in fibroblasts were performed using a standard Western blot protocol and specific antibodies as described in Supplementary Information. Proteins were detected with Amersham ECL Select Western Blotting Detection Reagent (GE healthcare, Ill, USA) according to the manufacturer's recommendations and BioRad ChemiDoc Imaging Systems (BioRad, CA, USA) was used for detection. The ImageJ software was used for signal quantification.

Luciferase assay – Fibroblasts were transiently transfected with 2 µg of the β-catenin-responsive firefly luciferase reporter plasmid *TOPFlash* (Millipore, Ma, USA) or the negative control *FOPFlash* (Millipore) by a double transfection using Lipofectamine LTX (Thermo Fisher Scientific) and Fugene (Promega, Wis, USA) at equal amounts according to the manufacturer's instructions. 24 hours after transfection, cells were treated with 20 mM LiCl for 6 hours then lysed with Passive Lysis Buffer (Promega). Samples were incubated for 30 min at 4°C and then centrifuged for 1 min at 13 000 g at 4°C. Supernatants were collected and firefly luciferase activity was measured according to the manufacturer's instructions. The firefly luciferase activity was normalized against protein amounts and relative *TOPFlash/FOPFlash* activity was reported.

Adhesion assay – Fibroblasts were deprived of serum for 8 hours and then detached using 10 mM EDTA in DMEM for 10 min, washed twice with DMEM (Thermo Fisher Scientific) and resuspended in 2 mL of DMEM with 0.1% BSA. 100 µL of cell suspension was added by well of a 96-well plate precoated for 12 hours with 40 µg/mL Collagen I (Corning, NY, USA) in PBS. The plate was incubated 20 min at 37°C to allow cell adhesion. Cells were then washed and cultured for 4 hours in DMEM supplemented with 10% FBS to enable recovery. Cells were then counted using MTT cell proliferation assay kit (ATCC, VA, USA).

Statistical analyses - Statistical analyses and graph representation were performed using GraphPad Prism 5.0 software (GraphPad Software Inc.). Unpaired two-tailed Student's t tests were used for

comparisons of means of two groups. The means of data provided by sets of independent experiments carried out on samples from the patient were compared to the means of data provided by sets of independent experiments carried out on samples from three healthy individuals. Data are presented as means \pm SEM and individual data points are represented when scatter plots are used.

RESULTS

Case history and patient's phenotype

The medical history of the patient (individual II.1 in Figure 1A & 1B) from birth to the age of 21 is the following: the patient was born to non-consanguineous healthy parents; increased fetal nuchal skin-fold thickness was observed during the 2nd trimester of pregnancy. Birth was uneventful and birth measurements were normal. He presents no malformation or Hirschsprung disease. He was first referred for genetic counseling at the age of 5 years for hypotonia and developmental delay. The diagnosis of Pitt-Hopkins syndrome was suspected at the age of 10 based on facial features, smiling appearance, ataxic gait, and repetitive behavior, associated with severe ID and absence of speech. Motor milestones were delayed as he walked at the age of 8. He also experiences generalized tonic-clonic seizures with a frequency of one seizure every 2 months. Facial features comprise long thin eyebrows, long eyelashes, narrow palpebral fissures, epicanthic folds, divergent squint, a short nose with broad nasal bridge, macrostomia with thick lips and small spaced teeth. He also presents pectus excavatum, genu flexum, amyotrophy, flat feet, a brachydactyly of the third toe and mild camptodactyly (Figure 1A and Table 1). Brain MRI and muscle biopsy showed no abnormalities. Other features include scoliosis, lymphomatoid papulosis, testicular ectopia and recurrent alternation of constipation/diarrhea (Table 1). At the age of 21, the patient is 1m69 tall, he weighs 47 kg and has a head circumference of 60.5 cm (within the normal range). He still lacks speech capacities and exhibits a friendly behavior with no signs of aggressivity. He eats alone but cannot dress himself and is not toilet trained. He has sleep disturbance.

Identification of a *CSDE1* c.362C>A p.(Ser121*) heterozygous *de novo* variation in the patient with severe ID

As no molecular abnormalities were found in *TCF4*, the gene involved in the Pitt-Hopkins syndrome, whole-exome sequencing was performed and identified the *de novo* heterozygous nonsense variation c.362C>A, p.(Ser121*) in *CSDE1* (NM_001242891). The identified variation has not been described in sequence-variant databases, such as the genome aggregation database (gnomAD). Sanger sequencing confirmed that the c.362C>A variation is carried by the patient and absent in his parents (Figure 1B). Another *de novo* missense variation c.616C>T, p.(Leu206Phe) was identified in the *GNB2L1* gene (NM_006098). Taking into account the previously reported involvement of *CSDE1* in ID-ASD (36) and the nature of the identified variation (nonsense), we sought to further study the functional impact of this gene variation. As the identified variation leads to a premature termination codon (PTC) in the 4th exon of *CSDE1*, which contains 21 exons, it is expected to result either in the production of a truncated protein or in the absence of protein production through activation of the

nonsense-mediated mRNA decay (NMD) pathway. Indeed, mutant PTC-containing mRNAs are expected to be degraded through the NMD signaling pathway, unless the PTC is located more than 50–55 nucleotides upstream of the last exon-exon junction (43,44). To discriminate between these two possibilities, we sequenced the *CSDE1* transcripts obtained from the patient's fibroblasts before and after treatment with cycloheximide, an inhibitor of the NMD pathway. The patient's fibroblasts expressed only the normal *CSDE1* allele, whereas cycloheximide led to accumulation of the mutated *CSDE1* transcripts (Figure 1C). These data indicate that NMD plays an active role in the degradation of the mutated *CSDE1* transcripts that contain a premature stop codon. Consistent with this observation, we found a ~50% decrease of *CSDE1* transcripts from total RNA extracted from the patient's skin fibroblasts as compared to healthy individuals (Figure 1D), leading to a ~40% decrease in *CSDE1* protein levels (Figure 1E). Taken together, these data demonstrate the deleterious character of the identified c.362C>A variation, which leads to *CSDE1* haploinsufficiency.

Recurrent phenotypic features in patients upon partial loss of *CSDE1*

With the aim to identify the main phenotypic features associated with a partial loss of *CSDE1*, we analyzed the phenotype of our patient and compared it to the phenotypes of the previously reported individuals carrying a deletion encompassing *CSDE1* (located on the short arm of chromosome 1, 1p13.2) (45,46) and the recently reported ASD patients carrying truncating *CSDE1* mutations (36). All phenotypic features are summarized in Table 1. Noticeably, the patients share some common features among which the most recurrent clinical symptoms are language impairment (20/20), motor developmental delays (18/20), intellectual disabilities (17/19), and autistic features (12/16).

Deregulation of major cellular pathways upon partial loss of *CSDE1* in the patient's fibroblasts

CSDE1 interacts with a large number of transcripts to regulate multiple cellular pathways. In order to identify the pathways hindered upon partial loss of *CSDE1* in the patient, we performed an RNA-Seq transcriptomic analysis on fibroblasts obtained from the patient and three healthy individuals. This analysis showed that the steady-state of 1,131 transcripts is significantly modified in the patient's fibroblasts compared to the healthy individuals (545 upregulated genes and 586 down-regulated genes), with a cut-off of 0.05 for the unadjusted p-value and a minimum fold change of 1.5 (Table S1 – Data deposited at Gene Expression Omnibus GEO, Accession code GSE162954). Gene ontology (GO) biological process term analysis was performed to identify the key pathways related to the differentially expressed genes using Metascape (<http://metascape.org>, (42)). The statistically enriched terms identified were hierarchically clustered into a tree based on the similarities among their gene memberships. The GO enrichment analysis revealed that the most significantly up-regulated genes were associated with the regulation of the cell cycle and cell division (e.g. *CDC25A*,

CDC20, *CHEK1*, *E2F1*) (Figure 2A – left panel and Table S1). Noteworthy, the down-regulated genes were essentially involved in the homophilic cell adhesion *via* plasma membrane adhesion molecules, mainly belonging to the cadherin and protocadherins family e.g. *CDH4*, *CDH6*, *CDH10*, *PCDH7*, *PCDHA9*, *PCDHB5* (Figure 2A – right panel and Table S1).

In order to further characterize the specific contribution of the *CSDE1* loss-of-function mutation in these transcriptomic data, we focused our analyses on genes containing *CSDE1* binding motifs. To this end, we conducted a comparative study of our results with those obtained by Ju Lee *et al.* in their transcriptomic analysis of genes containing at least one consensus *CSDE1* binding motif upon *CSDE1* knockdown in human embryonic stem cells (31). Furthermore, the identification of deregulated genes common to fibroblasts and embryonic stem cells allowed us to palliate the bias linked to a cell-type specific analysis. The comparison of the list of deregulated genes detected in our study with the list of Ju Lee *et al.* (531 up-regulated genes and 676 down-regulated genes retained on the basis of a log₂-fold change and at a False Discovery Rate (FDR) < 0.05) identified 30 and 33 common genes that are up- and down-regulated, respectively (Figure 2B & 2C, top panel). We conducted GO enrichment analyses on these genes and all statistically enriched terms were converted into a network layout (Figure 2B & 2C, bottom panel). We observed a significant upregulation of genes involved in the negative regulation of Wnt signaling (*CDH2*, *GRB10*, *DACT1*, *APCDD1*, *APCDD1L*, *E2F1*), cell-substrate adhesion (*COL8A1*, *ITGA5*, *LGALS1*, *SERPINE1*, *ZYX*, *ACTA2*, *CDH2*, *TGFBI*), smooth muscle contraction (*ACTA2*, *ADRA1D*, *CNN1*, *MSN*, *CDC42EP1*), p53 downstream pathway (*E2F1*, *SERPINE1*, *STEAP3*), regulation of cell shape (*MSN*, *CDC42EP1*, *SHROOM3*, *ACTA2*, *CDH2*, *SERPINE1*), tissue morphogenesis (*ACTA2*, *ITGA5*, *MSN*, *DACT1*, *SHROOM3*, *COL8A1*, *LGALS1*), actin cytoskeleton organization (*CNN1*, *INPPL1*, *ZYX*, *CDC42EP1*, *SHROOM3*, *NEFM*), and cell junction organization (*CDH2*, *ITGA5*, *KIFC3*) (Figure 2B, bottom panel). Downregulated genes belong to five main pathways: regulation of reproductive process (*BMP4*, *GJA1*, *MSX2*, *SERPINF1*, *CSDE1*, *FZD5*, *CD9*, *PRKAR2B*, *CDA*, *CREG1*), cellular response to hormone stimulus (*BMP4*, *GJA1*, *MSX2*, *SERPINF1*, *PRKAR2B*, *PCSK9*, *KLF15*, *KCNS3*), cell-cell junction assembly (*CD9*, *GJA1*, *FZD5*, *MSX2*, *PRKAR2B*, *KCNS3*, *GNPTAB*, *SERPINF1*), ECM (extracellular matrix) regulators (*SERPINF1*, *SERPINB9*, *PAMR1*) and cellular carbohydrate metabolism process (*FAM3C*, *GNPTAB*, *PPP1R3B*) (Figure 2C, bottom panel). Overall, these transcriptomic analyses unveil that decreasing *CSDE1* levels lead to a deregulated expression of genes involved in several pathways, among which, most importantly, the Wnt/ β -catenin pathway and cellular adhesion exhibit the lowest p-values.

Inhibition of the Wnt/ β -catenin pathway in the fibroblasts of the patient with *CSDE1* haploinsufficiency

Our RNA-seq analysis revealed that *APCDD1* (APC Down-Regulated 1, MIM #607479), a component of the Wnt/ β -catenin pathway is upregulated in the patient's fibroblasts as compared to three healthy individuals (Table S1). As shown in Figure 3A, this significant increase in the expression levels of *APCDD1* was confirmed by RT-qPCR. Western blot analysis on protein samples obtained from fibroblasts of the patient and healthy individuals showed a 2-fold increase of the APCDD1 protein in the patient (Figure 3B). *APCDD1* is a direct target of the Wnt/ β -catenin pathway and the corresponding protein is an essential inhibitor of this pathway, thus exerting a negative feedback control (47,48).

In order to evaluate the state of the Wnt/ β -catenin signaling in the patient's fibroblasts, we first assessed through western blot analysis the expression levels of β -catenin and showed a ~25% decrease in the β -catenin levels in the patient as compared to healthy individuals (Figure 3C), whereas *β -catenin* transcripts were unchanged compared to healthy individuals as shown by our RNA-seq analysis. We further quantified the Wnt/ β -catenin signaling in fibroblasts from the patient and from healthy individuals using a *TOPFlash/FOPFlash* luciferase reporter assay that provides a precise measurement of the canonical Wnt-specific transcriptional activity (Figure 3D). Results of this assay showed a significant inhibition of this pathway in the patient, in keeping with the identified decrease of β -catenin protein levels. Altogether, these data show an increase of *APCDD1* transcripts and protein levels and a subsequent inhibition of the Wnt/ β -catenin pathway in the patient carrying the *CSDE1* null-mutation.

Enhanced substrate adhesion faculties of the fibroblasts of the patient with *CSDE1* haploinsufficiency

As our RNA-seq analysis suggested an alteration of the overall adhesion capacities of the cells of the patient through deregulation of the expression of cadherins and integrins (Figure 2B & 2C and Table S1), we sought to assess cellular adhesion in the patient's fibroblasts. We first confirmed by RT-qPCR the significant increase, in the patient's fibroblasts, of the expression levels of *CDH2* (*cadherin 2* or *n-cadherin*, MIM #114020), a pivotal actor in cell-cell adhesion as well as of *SERPINE1* (*Serpin Family E Member 1*, MIM #173360) and *ITGA5* (*Integrin Alpha-5*, MIM #135620), two genes involved in cell-substrate adhesion (Figure 3E). Noteworthy, CDH2 is also a regulator of the Wnt/ β -catenin pathway. To further investigate the cell-substrate adhesion abilities of the fibroblasts obtained from the patient, we conducted a cell-substrate adhesion assay using a collagen I - coated surface. We observed significant enhanced adhesion abilities of the fibroblasts of the patient as compared to

those from healthy individuals (Figure 3F). Taken together, these data reveal enhanced cell-substrate adhesion faculties directly through the modulation of CSDE1 target genes.

DISCUSSION

In this study, we provide a thorough description of the disease phenotype of a patient with severe ID-ASD carrying a heterozygous *de novo* nonsense variation in *CSDE1* and followed from birth to the age of 21 years. We performed functional studies aiming at deciphering the *CSDE1*-dependent pathways involved in the pathophysiology of this highly heterogeneous disease condition. The study of the patient's primary fibroblasts, through a combination of various approaches, indeed revealed impairment of the Wnt/ β -catenin pathway and of the cellular adhesion abilities that result from a deregulated expression of two specific *CSDE1* targets, namely *APCDD1* and *CDH2*. Overall, these data support a pathophysiological model of the disease in which reduced levels of *CSDE1* impair the regulation of two of its target transcripts (*APCDD1* and *CDH2*) leading to abnormal levels of the corresponding proteins and therefore deregulation of the associated pathways (Figure 4).

***CSDE1* and ID-ASD** – The in-depth description of the patient's phenotype, which includes severe developmental delay with no malformations, cognitive impairment, seizures and distinctive morphological characteristics at different stages of his life, represents a major asset for the diagnosis of other *CSDE1*-related ID-ASD cases. By reviewing the phenotype of the patients so-far reported with a *CSDE1* loss-of-function variant, we noticed a variable expressivity of this autosomal dominant disorder, with severity ranging from isolated language impairment in some parents of the patients carrying an inherited *CSDE1* mutation to severe ID (36). Such large phenotypic spectrum suggests a potential role of environmental or modifying factors. In this regard, our patient carries another *de novo* missense variation c.616C>T p.(Leu206Phe) in the *GNB2L1* gene. Despite the fact that *GNB2L1* is not involved in human ID-ASD pathology, we cannot exclude the possibility that this *GNB2L1* variation may have an impact on disease expressivity in our patient. Implication of the homolog of *GNB2L1* (*Rack1*) was suggested in axon guidance in *C.elegans* (49), in neurite extension of neuronally differentiated rat cells (50), and in axon outgrowth of mouse primary cortical neurons (51).

Among the commonly used patient-derived cellular models for studying neurological disorders (e.g. human dermal fibroblasts, induced pluripotent stem cells, neural cells induced from iPSC or directly from postmitotic somatic cells, immortalized lymphocytes and peripheral leukocytes), we have chosen to conduct our investigations using dermal fibroblasts derived from the patients as they represent a relevant patient-derived cellular model for the study of ID-ASD. Indeed, they have been widely used as patient-derived cellular models for studying several disease conditions affecting the central nervous system (CNS) function as they share molecular similarities with CNS cells and express molecular factors that are highly similar to cells of neuro-ectodermal origin (52,53). Our study establishes the role of *CSDE1* in the onset of this ID-ASD in humans based on data obtained from the

patient's fibroblasts. So far, the involvement of *Csde1* in neurodevelopment has been previously suggested by two studies performed in mouse and drosophila models. *Csde1* has been implicated in neuronal and synapse development including synapse transmission (36) and has been shown to be crucial for the proper control of the migration of precerebellar neurons (54). Furthermore, our study highlights the role of CSDE1 in human neuronal development as our transcriptomic analysis reveals a significant deregulation of the expression of NEFM (Neurofilament medium polypeptide) in the patient's fibroblasts, a protein belonging to GO: actin cytoskeleton organization and involved in neuron projection development.

CSDE1 is a key regulator of the Wnt/ β -catenin pathway - Our study shows that the Wnt/ β -catenin pathway is deregulated upon partial loss of CSDE1 through a mechanism at least partly explained by the modulation of *APCDD1* and *CDH2* expression by CSDE1. Several of our data strongly support a downregulation of the Wnt/ β -catenin pathway in the patient's cells. First, we show that among the diverse CSDE1 direct transcript targets that belong to the Wnt/ β -catenin network, *APCDD1* and *CDH2* are significantly upregulated, *APCDD1* being the transcript with the most deregulated expression as compared to healthy individuals. *APCDD1*, a target of the Wnt/ β -catenin pathway also acts as an inhibitor of this pathway by preventing the formation of the Wnt-Fzd-Lrp5/6 (LDL Receptor Related Protein 5/6) receptor complex, thus creating a negative feedback loop on Wnt/ β -catenin signaling (47,48). *CDH2* regulates the availability of key factors of the Wnt/ β -catenin signaling like the core protein β -catenin or the Wnt co-receptor Lrp6 (55,56). Second, our data show decreased levels of the β -catenin protein in the patient's fibroblasts although the levels of *β -catenin* transcripts are not deregulated, thereby supporting a posttranslational role of CSDE1 in the regulation of β -catenin. Accordingly, decreased levels of the β -catenin protein with no deregulation of the *β -catenin* transcripts was observed in *Csde1* knock-out mice (36) as well as after *CSDE1* silencing in human melanoma cells (30). The discrepancy we observe between the decreased levels of β -catenin protein and the unchanged levels of the corresponding transcripts is in accordance with the well-established posttranslational regulation of the β -catenin protein through modulation of its phosphorylation and ubiquitination states (reviewed in (57)). Third, the observed decreased levels of β -catenin are indeed followed by an overall inhibition of the pathway as shown by our luciferase assay. Overall, CSDE1, which acts as an upstream modulator of the expression of individual effectors of the Wnt/ β -catenin pathway, plays therefore a pivotal role in the regulation of this pathway.

Alteration of the fine-tuning of the Wnt/ β -catenin pathway in ID-ASD – The spatiotemporal regulation of the evolutionarily conserved Wnt/ β -catenin pathway is crucial for correct neurodevelopmental and post-neurodevelopmental processes; actors of this pathway are involved in

regionalization of nervous system, differentiation of neural progenitor cells, axon guidance, dendritic development, as well as neuronal migration and plasticity (reviewed in (3,58)). In consistence with these functions, our study highlights the involvement in ID-ASD pathophysiology of three key players of the Wnt/ β -catenin signaling pathway: the β -catenin core element and two modulators of the pathway, APCDD1 and CDH2. Accordingly, specific gene mutations in β -catenin (*CTNNB1*) (10,11,59) or *CDH2* (60) have been reported in ID. Furthermore, APCDD1 has been involved in neurological disorders through its roles in oligodendrocyte differentiation and regenerative myelination in the central nervous system (61) as well as in the generation of neurons from progenitors (47). Whereas our data show that the Wnt/ β -catenin pathway is downregulated in the ID-ASD patient, both hyper- and hypoactivity of this pathway have been shown to alter cognitive functions (reviewed in (62)). Indeed, the deregulated expression of the β -catenin protein illustrates this duality: several patients with cognitive impairments including ID and ASD present β -catenin haploinsufficiency (10,11) whereas others exhibit increased β -catenin levels due to the deletion of the gene encoding the APC (Adenomatous polyposis coli) protein that mediates β -catenin degradation (63). Modulation of the Wnt/ β -catenin pathway can also occur in a cell-specific manner as illustrated by the regulation mediated by CHD8 (chromodomain helicase DNA binding protein 8), a protein involved in ASD pathogenesis (64) which acts as a positive regulator of the Wnt β -catenin pathway in neural progenitor cells (65), and as a negative regulator of this pathway in non-neuronal cell lines.

Overall, these data raise the larger question of the mechanisms by which the altered spatiotemporal fine-tuning of the Wnt/ β -catenin pathway contributes to the occurrence of ID-ASD. Several non-exclusive answers can be put forward based on studies performed in mouse and rat models: (i) the Wnt/ β -catenin pathway plays a key role in the regulation of neuronal polarity and axonal outgrowth through the ligand Wnt3a (66); (ii) it is also important for dendritic morphogenesis *via* the β -catenin protein (67); (iii) the Wnt/ β -catenin pathway is also pivotal for the proper functioning of the neuronal network through the modulation of the neural glucose metabolism. Indeed, by means of a mechanism dependent on the ligand Wnt3a and the metabolic sensor Akt, the Wnt/ β -catenin pathway regulates the glucose intake in neurons as well as the activity of the hexokinase and the glycolytic rate (68). In keeping with this impact of the Wnt/ β -catenin pathway on metabolism, our RNA-seq analysis shows a deregulated expression of genes involved in the carbohydrate metabolism.

Enhanced cellular adhesion in a neurodevelopmental disease – Our results unveil a role for CSDE1 in enhanced cellular adhesion in the context of ID-ASD. The implication for CSDE1 in cellular adhesion has so far been reported in the context of cancer progression in melanoma cell models (30), but not in the context of ID-ASD. Most importantly, our transcriptomic analysis performed in patient's

primary fibroblasts shows the modulation of the expression of genes belonging to the cadherin/protocadherin superfamily and integrin families that control both cell-cell and cell-matrix adhesion. On the one hand, cell-cell adhesion - essentially ensured by molecules belonging to the cadherin and protocadherin superfamily - regulates the formation of both adherens and synaptic junctions in the central nervous system (69). *CDH2*, a gene known to be essential for proper axon guidance (60), is one of the cell-cell adhesion genes whose expression was found deregulated in our patient. On the other hand, cell-matrix adhesion - under the control of proteins mainly belonging to the integrin family - affects cell migration, axon guidance and synapse formation in the central nervous systems (70–72). Among the integrins, *ITGA5* - significantly deregulated in the fibroblasts of our patient - is known to contribute to the survival and proliferation of glial progenitor cells (73). Moreover, the integrin-mediated cell interactions are tightly regulated by plasminogen activators like *SERPINE1* (74) that is significantly upregulated in the fibroblasts of our patient. Neurological impairments can result from deregulation of cell-cell or cell-substrate adhesion (reviewed in (69,75)). Overall, our data support a key role for *CSDE1* in the regulation of these two cell adhesion types, a result that is in line with the wide-ranging neurological phenotype observed in the patient (epilepsy and cognitive, motor, behavioral impairments).

In summary, our work identifies the Wnt/ β -catenin signaling and cellular adhesion as two major deregulated pathways in an ID-ASD patient carrying a monoallelic null mutation in *CSDE1* through mechanisms relying on *APCDD1* and *CDH2*. In addition, by providing an in-depth description of the phenotype of the patient at different stages of his life, our study should help identifying patients with ID-ASD who could benefit from a screening for *CSDE1* mutations.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

Figure 1. The patient's clinical features and the molecular bases of the CSDE1 haploinsufficiency

A. Phenotypic features of the patient - Front and profile view of individual II.1 at 5 (a,b) and 21 years (c,d) of age showing the distinct facial features. Photos of the patient at age 21 showing the (e) pectus excavatum, (f) his hand and (g,h) his foot.

B. Genealogical tree of the patient with ID-ASD (Individual II.1) and his parents (Individuals I.1 and I.2). Electropherograms from Sanger sequencing showing the heterozygous c.362C>A p.(Ser121*) mutation only in individual II.1 and not in his parents.

C. Sanger sequencing of cDNA obtained from fibroblasts of healthy individuals and individual II.1 upon cycloheximide treatment (30 µg/ml, for 4 hours) showing accumulation of the mutated *CSDE1* transcripts.

D. Quantification of *CSDE1* transcripts from fibroblasts of three healthy individuals and individual II.1 by RT-qPCR. The transcript levels of *CSDE1* were normalized to the housekeeping gene *ERCC3*. The results are shown as the mean of three independent experiments ± SEM and plotted relative to the healthy controls conditions.

E. *CSDE1* protein expression in fibroblasts of healthy individuals and individual II.1 - Equal amounts of protein extracts were subjected to SDS-PAGE and immunoblotted with *CSDE1* antibody. The *CSDE1* signal was quantified with ImageJ software and normalized to the amount of GAPDH used as a loading control. The results are shown as the mean of three independent experiments and are plotted as a percentage of the healthy controls condition.

For all experiments, asterisks indicate that the mean is significantly different; *: t test p value < 0.05, **: t test p value < 0.01, ***: t test p value < 0.001.

Figure 2. Transcriptomic analysis in the patient's fibroblasts

A. Enrichment analysis of RNA-seq data sets - Bar graph of enriched terms across input gene lists, colored by p-values and showing biological processes enrichment analysis of up-regulated genes (left panel) and for down-regulated genes (right panel).

B. Analysis of the common upregulated genes from our transcriptomic study and Ju Lee et al. (31).

C. Analysis of the common downregulated genes from our transcriptomic study and Ju Lee et al. (31). Network of enriched terms colored by cluster identity, where nodes that share the same cluster ID are typically close to each other. The p-value corresponding to each enriched term is indicated in brackets; terms containing more genes tend to have a more significant p-value. All graphs were generated by Metascape (<http://metascape.org>, (42)) and were modified graphically only.

Figure 3. Deregulation of the Wnt/ β -catenin pathway and cellular adhesion in the patient

A. Quantification of *APCDD1* transcripts from fibroblasts of three healthy individuals and individual II.1 by RT-qPCR. The transcript levels of *APCDD1* were normalized to the housekeeping gene *ERCC3*. The results are shown as the mean of three independent experiments \pm SEM and plotted relative to the healthy controls conditions.

B. *APCDD1* protein expression in fibroblasts of three healthy individuals and individual II.1 - Equal amounts of protein extracts were subjected to SDS-PAGE and immunoblotted with the indicated antibody. The *APCDD1* signal was quantified with ImageJ software and normalized to the amount of GAPDH used as a loading control. The results are shown as the mean of three independent experiments and are plotted as a percentage of the healthy controls condition. Note: Samples from figure 1E and 3B were loaded on the same gel and different parts of the membrane were revealed with different antibodies, therefore, the GAPDH loading control is the same for figure 1E and 3B.

C. β -catenin protein expression in fibroblasts of three healthy individuals and individual II.1. - Equal amounts of protein extracts were subjected to SDS-PAGE and immunoblotted with the indicated antibody. β -catenin signal was quantified with ImageJ software and normalized to the amount of β -tubulin used as a loading control. The results are shown as the mean of three independent experiments and are plotted as a percentage of the healthy controls condition.

D. Quantification of the Wnt/ β -catenin signaling in fibroblasts from the patient and from healthy individuals using a *TOPFlash/FOPFlash* luciferase reporter assay - Fibroblasts of healthy individuals and individual II.1 were transfected with TOP or FOP plasmids then 20 hours later treated with 20 mM LiCl for 6 hours. Luciferase activity was normalized to total protein amounts and plotted as 4 independent experiments.

E. Quantification of *CDH2*, *SERPINE1* and *ITGA5* transcripts from fibroblasts of three healthy individuals and individual II.1 by RT-qPCR. The transcript levels of the mentioned genes were normalized to the housekeeping gene *ERCC3*. The results are shown as the mean of three independent experiments \pm SEM and are plotted relative to the healthy controls conditions.

F. Cell-substrate adhesion assay in fibroblasts of healthy individuals and individual II.1 – Fibroblasts were harvested on Collagen-I coated plates and the number of cells was quantified by MTT assay. The results are shown as the ratio of number of adherent cells on the number of total cells seeded and are plotted as five independent experiments.

For all experiments, asterisks indicate that the mean is significantly different; *: t test p value < 0.05, **: t test p value < 0.01, ***: t test p value < 0.001.

Figure 4. Pathophysiological model of the CSDE1-haploinsufficiency-related disease

Upper panel - In healthy individuals the levels of *CSDE1* mRNA and its corresponding proteins are normal. CSDE1 proteins interact with the IRES, the 5'UTR or the 3'UTR of their target transcripts to regulate their translation or their stability. In the case of *APCDD1* and *CDH2*, this interaction would lead to the regulation of the transcripts abundance. Normal amounts of APCDD1 and CDH2 proteins control the homeostasis of specific cellular pathways.

Lower panel - In the ID-ASD patient, the heterozygous nonsense mutation in *CSDE1* leads to mRNA decay of the transcripts that carry it. This results in CSDE1 protein haploinsufficiency which will compromise the regulation of the target transcripts (e.g. *APCDD1* and *CDH2*) and consequently the levels of the corresponding proteins. Increased levels of APCDD1 and CDH2 proteins lead to the downregulation of the Wnt/ β -catenin pathway and the increase of cellular adhesion.

TABLE LEGEND

Phenotype and features of the individual II.I carrying the c.362C>A, p.(Ser121*) variation in *CSDE1* compared to patients lacking the chromosomal region that contains *CSDE1* (del 1p13.1p13.2, (46) and del 1p13.1p21.1 (45)) and patients presenting mutations in *CSDE1* (7 frameshift, 6 stopgain, 3 splicing mutations (36)); n.d. : not defined; [§]: The molecular anomalies are present at the heterozygous state.

TABLE

Table 1

Study	Our study	Bisgaard et. al 2007	Fitzgibbon et al. 2009	Guo et. al 2019
Molecular anomalies⁵	c.362C>A p.(Ser121*) in <i>CSDE1</i> c.616C>T p.(Leu206Phe) in <i>GNB2L1</i>	del 1p13.1p21.1	del 1p13.1p13.2	7 frameshift, 6 stopgain, 3 splicing mutations in <i>CSDE1</i>
Gender, age	Male, from birth to 21 years old	Female, 13 years old	Female, 5 years old	11 males, 6 females 3 to 19 years old
Height	-2SD	<5th centile	n.d.	8/15: short (7), tall (1)
Weight	-2SD	Weight-for-height : 50th centile	n.d.	6/15: overweight (3), lean (3)
Head Circumference	0SD	<3rd centile	n.d.	6/14: macrocephaly
Neurodevelopmental features				
Intellectual disability	Severe	Severe	Severe	3/17: below average or learning disability, 14/17: mild to severe ID
Language impairment	Yes	Yes	Yes	17/17
Motor development delay	Walked at 8 years	Yes	Yes	15/17
Neurological features				
Epilepsy	Yes	Yes	n.d.	7/16
Brain MRI abnormalities	No	Delayed myelination	n.d.	7/14 (delayed myelination :1)
Facial features				
Telecanthus	Yes	n.d.	Mild	n.d.
Long eyebrows	Yes	Yes	Yes	n.d.
Epicanthic folds	Yes	No	No	n.d.
Short palpebral fissures	Yes	No	No	n.d.
Short/broad nose	Yes	Yes	Yes	n.d.
Macrostomia	Yes	Yes	No	n.d.
Philtrum	Short	Short	Long	n.d.
Low set ears	Yes	Yes	n.d.	n.d.
Musculoskeletal abnormalities				
Amyotrophy	Yes	n.d.	n.d.	n.d.
Hypotonia	Yes	Yes	Yes	6/13
Pectus excavatum	Yes	n.d.	n.d.	n.d.
Genu flexum	Yes	n.d.	n.d.	n.d.

Hand deformities	Mild camptodactyly	n.d.	n.d.	6/15: brachydactyly, polydactyly, and clinodactyly
Feet deformities	Brachydactyly	Syndactyly	n.d.	n.d.
Behavior characteristics				
Repetitive behavior	Yes	n.d.	n.d.	14/15
ADHD	No	n.d.	n.d.	9/13
Aggressive behavior	No	n.d.	n.d.	2/14
Self-injurious behavior	No	n.d.	No	3/12
Obsessive behavior	No	n.d.	n.d.	4/13
Anxiety	No	n.d.	n.d.	7/13
Other anomalies				
Eye abnormality	Divergent squint	Iris coloboma - Left alternating divergent squint	n.d.	7/13/ iris coloboma, hyperopia, and/or strabismus
Gastrointestinal problems	Constipation / diarrhea	Failure to thrive/gastrostomy at the age of 8	Constipation	2/15: intermittent constipation/diarrhea, constipation
Other	Lymphomatoid papulosis, testicular ectopia, scoliosis		Pain insensibility	

Figures

Figure 1

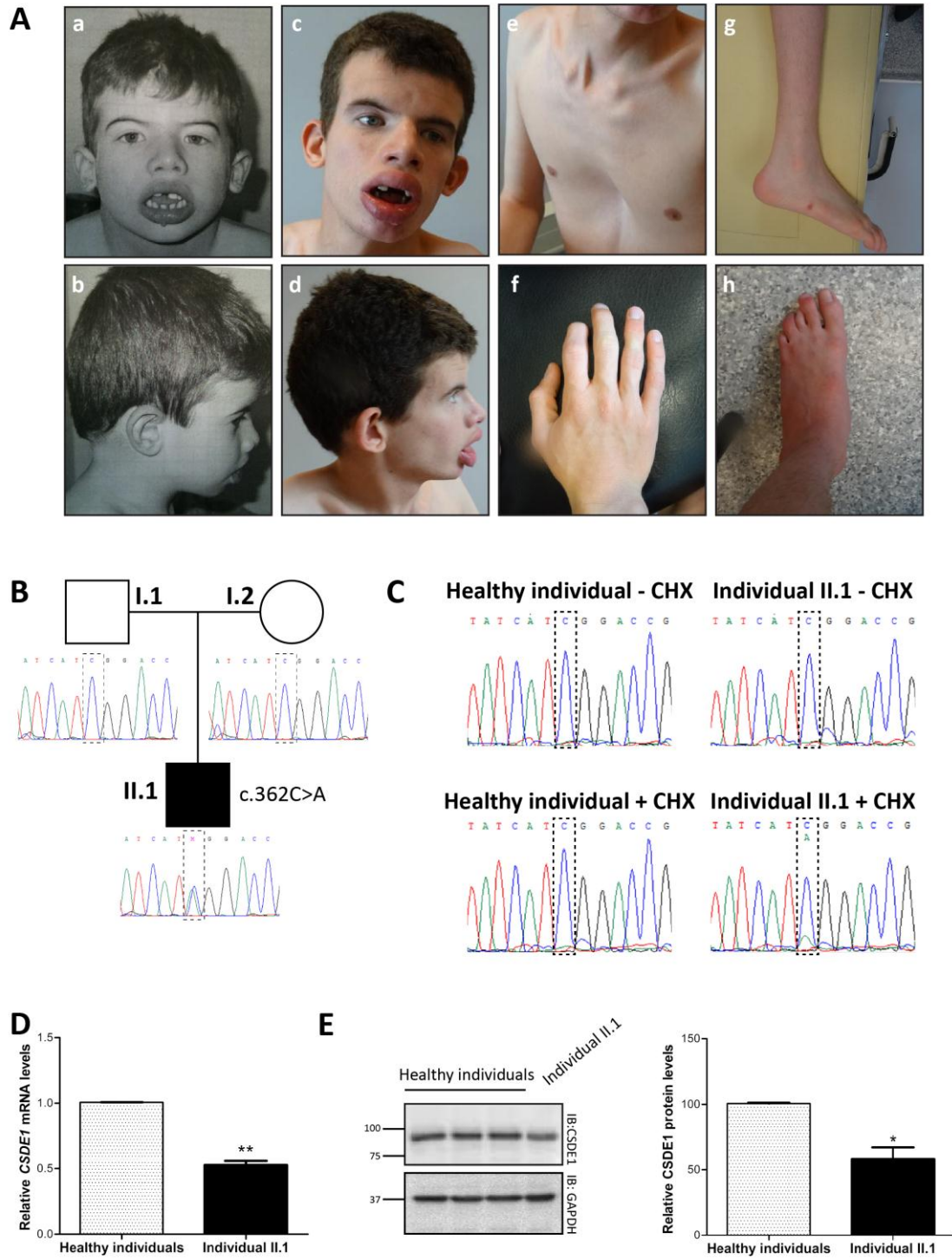


Figure 2

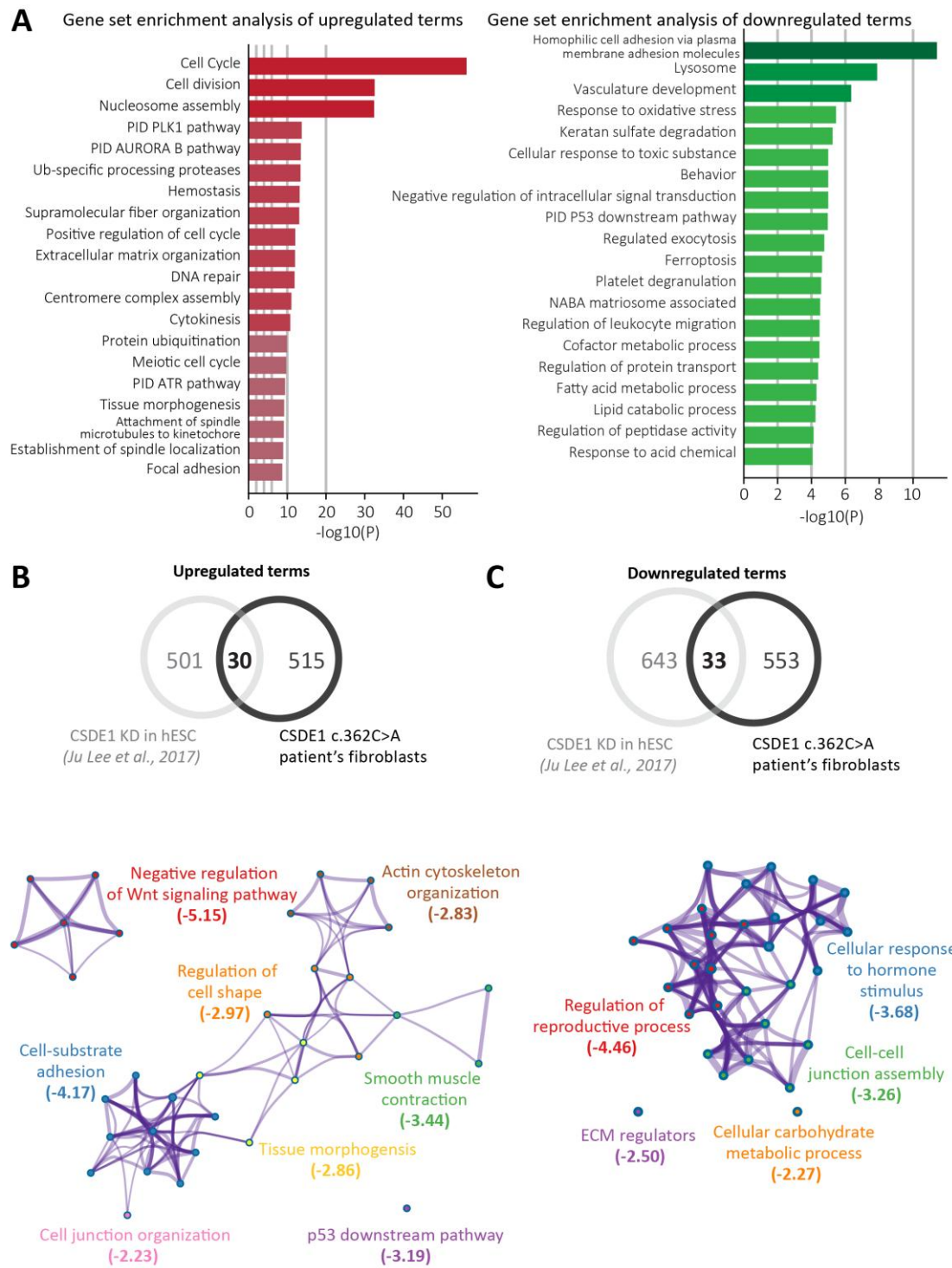


Figure 3

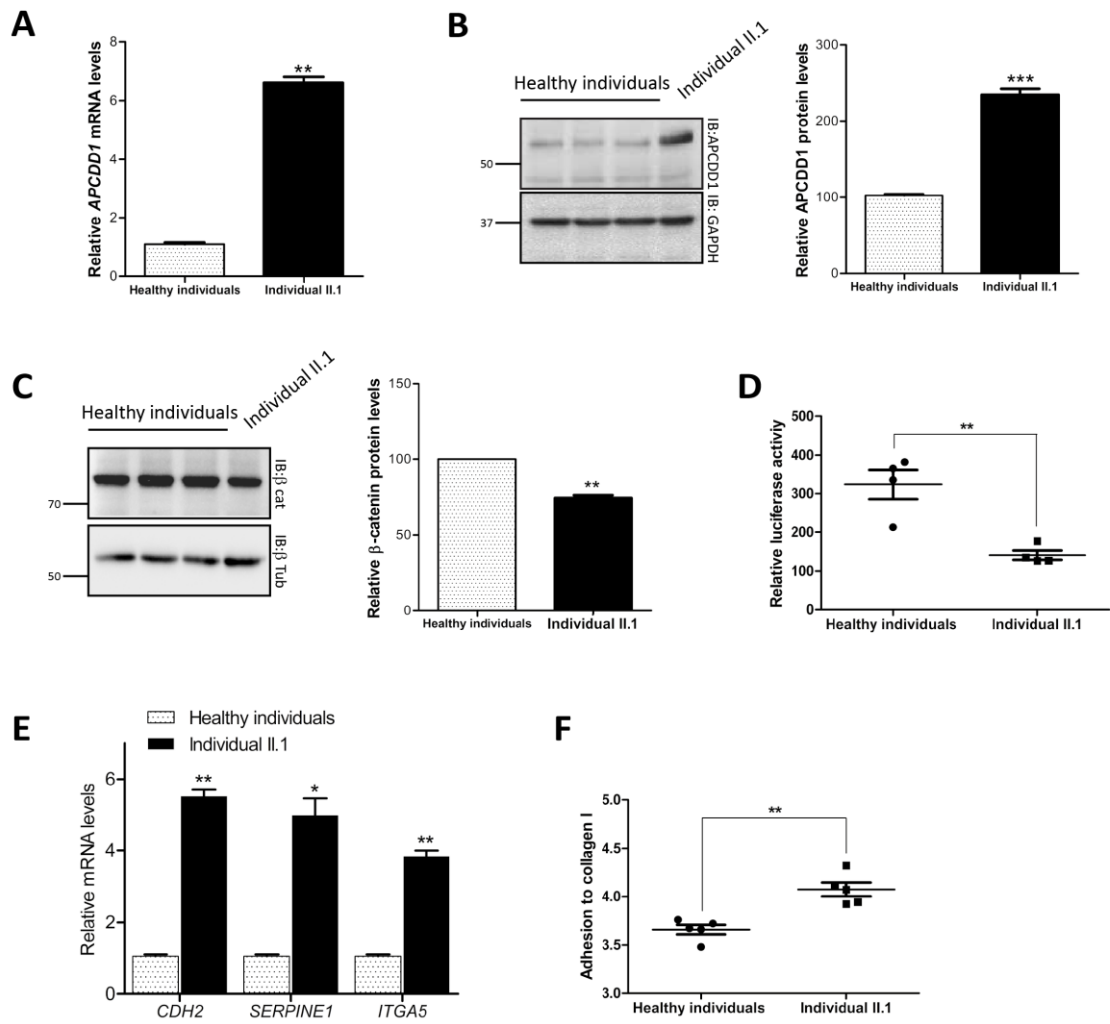
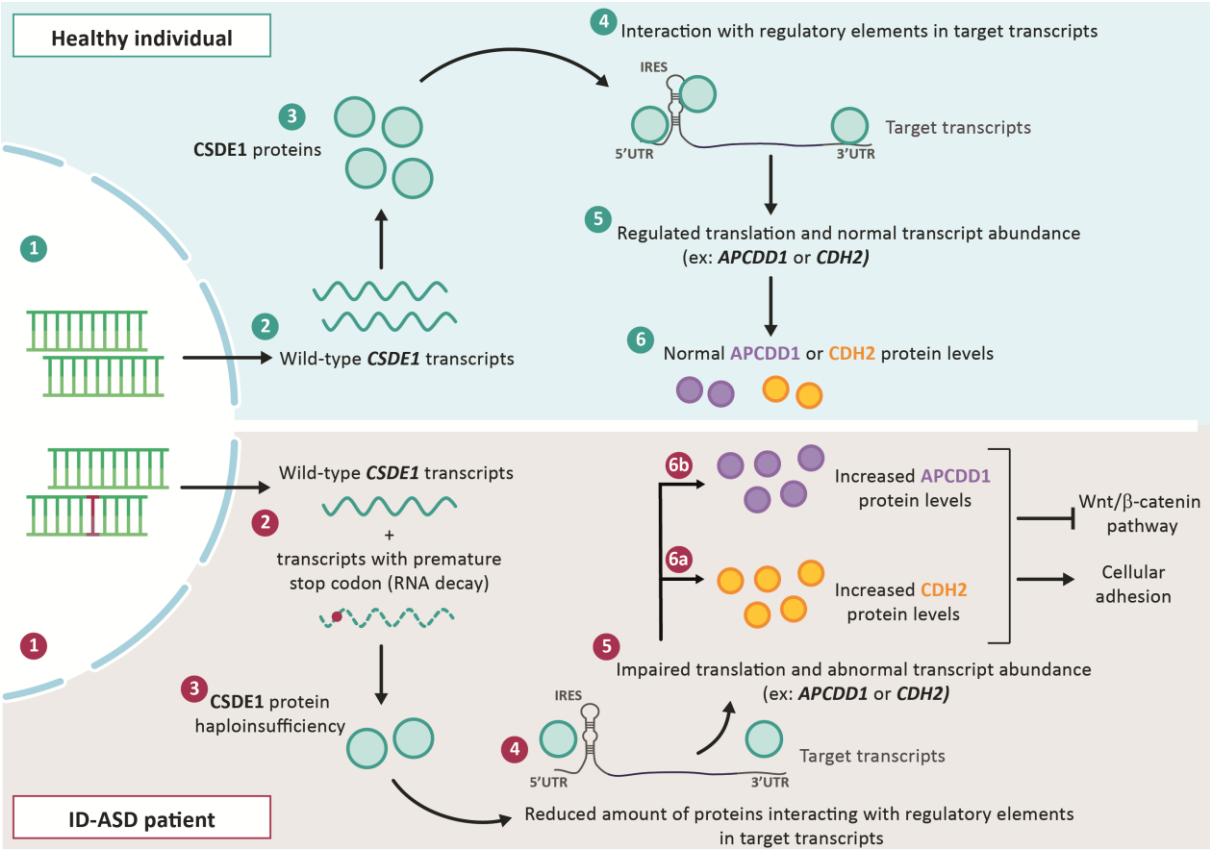
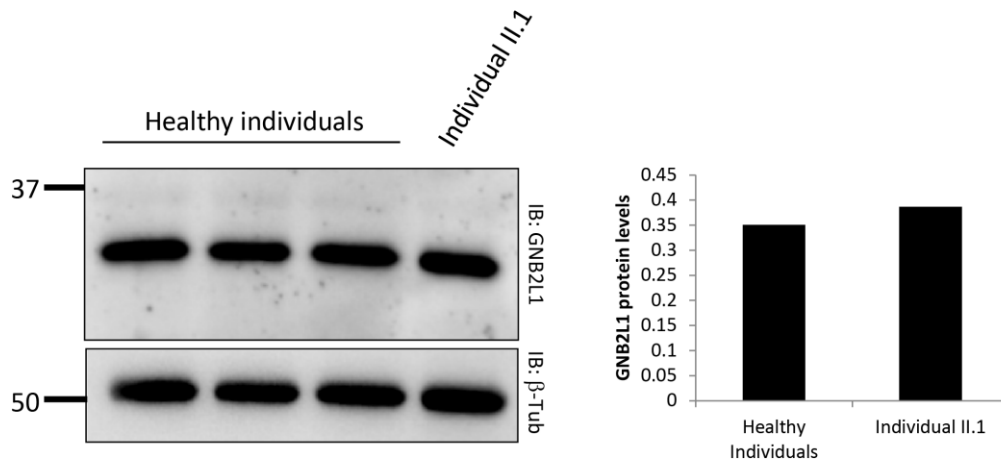


Figure 4



Supplementary Figure 1



GNB2L1 protein expression in fibroblasts of healthy individuals and individual II.1. Equal amounts of protein extracts from fibroblasts of healthy individuals and individual II.1 were subjected to SDS-PAGE and immunoblotted with GNB2L1 antibody. The GNB2L1 signal was quantified with ImageJ software and normalized to the amount of β -tubulin used as a loading control.