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Advances

Genotypic, functional, and phenotypic characterization in CTNNB1 neurodevelopmental syndrome

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Summary

CTNNB1 neurodevelopmental syndrome is a rare disorder caused by *de novo* heterozygous variants in the *CTNNB1* gene encoding β-catenin. This study aimed to characterize genetic variants in individuals with CTNNB1 neurodevelopmental syndrome, systematically assess the spectrum of clinical phenotypes using standardized measures, and explore potential genotype-phenotype correlations. In this cross-sectional cohort study, individuals diagnosed with CTNNB1 neurodevelopmental syndrome underwent structured interviews using standardized scales to evaluate motor skills, speech, communication, feeding abilities, visual function, neurodevelopment, and psychopathology. Genetic variants were analyzed, and, in a subset of cases, the impact of β-catenin variants on the Wnt/β-catenin signaling pathway was assessed. Across the 127 included participants (mean age, 70 months; range, 7–242 months) from 20 countries, we identified 88 different variants of the *CTNNB1* gene, 87 of which were predicted to lead to loss of *CTNNB1* function. Functional assays demonstrated reduced Wnt signaling activity, including 11 variants that also exhibited a dominant-negative effect. One missense variant demonstrated a gain-of-function effect. Dominant-negative variants were not clearly associated with a distinct phenotype; however, those with missense variants presented a milder phenotype, including earlier achievement of independent walking, fewer motor impairments, better conceptual and social skills, improved communication, and fewer feeding difficulties. This study describes the genetic, functional, and phenotypic characteristics in individuals with CTNNB1 neurodevelopmental syndrome. Further investigation into the genotypic and phenotypic characteristics of this syndrome and their interrelationships is essential to deepen our understanding of the disorder and inform the development of targeted therapies.

Introduction

β-catenin is an evolutionarily conserved protein that exerts a crucial role in a multitude of developmental and homeostatic processes in animals.[1,2] During embryonic development, Wnt-regulated β-catenin plays a critical role in establishing the body axis and the orchestration of tissue and organ development. In adult organs, Wnt signaling continues to play indispensable roles in tissue homeostasis, cell renewal, and regeneration.[1] Wnt signaling includes a canonical pathway, often referred to as the Wnt/β-catenin pathway, that involves the nuclear translocation of β-catenin and activation of target genes primarily to control cell proliferation and a non-canonical pathway that regulates cell polarity and migration.

Wnt signaling is highly regulated, including mutual regulation of the canonical and non-canonical pathways,[3] and dysregulation is associated with serious outcomes, including cancer and neurodevelopmental disorders.[4,5]

CTNNB1 neurodevelopmental syndrome (MIM: 615075) is a rare disorder caused by germline *de novo* heterozygous variants in the *CTNNB1* gene encoding β-catenin (i.e., autosomal dominant). Approximately 400 individuals have been diagnosed with this syndrome worldwide,[6] with an estimated incidence of about 2.6–3.2 in 100,000 live births.[7] Due to the syndrome's rarity, insufficient phenotypic characterization, and limited medical awareness, CTNNB1 neurodevelopmental syndrome is likely underdiagnosed. Research indicates that at least one-quarter of individuals diagnosed with cerebral palsy (CP) have an

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underlying monogenic etiology,[8] with *CTNNB1* variants accounting for up to 4% of these cases.[9,10] While data from more than 400 individuals have been reported to date, only small cohorts have undergone systematic phenotypic evaluation.[11–15] Recently, baseline data from an in-person natural history study were published, involving 32 persons living with CTNNB1 neurodevelopmental syndrome.[11] However, this study mainly involved English-speaking participants, and no genotype-phenotype correlations were assessed.

The aim of this study was to systematically characterize genetic variants in a cohort of individuals diagnosed with the CTNNB1 neurodevelopmental syndrome, evaluate the spectrum of clinical phenotypes using standardized measures, investigate potential genotype-phenotype correlations, and integrate functional genomic data to guide potential therapeutic strategies.[12]

Subjects and methods

This is a cross-sectional cohort study, performed between May 2021 and November 2022. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (0120-80/2021/4) and preregistered on ClinicalTrials.gov (NCT04812119).

Recruitment
A call for participation was broadcast through various social media support groups for individuals diagnosed with a CTNNB1 neurodevelopmental disorder. The inclusion criteria were a genetically confirmed pathogenic or likely pathogenic variant in the *CTNNB1* gene and a signed informed consent form by the participant or a parent or caregiver of the participant if the affected participant was unable to provide it. There were no age, nationality, language, or phenotypic restrictions for recruitment. Upon signing the consent form, respondents were asked to provide (1) a copy of the genetic report, (2) a copy of their head and/or spine magnetic resonance imaging (MRI) report (if available), and (3) a copy of their electroencephalogram (EEG) report (if available). Respondents were then invited to an online interview, for which a translator was provided if needed.

Data collection and management
Study data were collected and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted at the University Medical Center Ljubljana's secure institutional server.[13]

Genetic data
Bulk tissue gene expression data from 176 *postmortem* human brain samples from the dorsolateral prefrontal cortex[14] were assessed at nucleotide resolution across development by calculating the median read count per base (Figure S1). These were represented as a track in the UCSC genome browser[15] to facilitate comparison across genomic regions. Gene expression for exon subdivisions (Table S1) based on GRCh38/GENCODEv39 were assessed using DEX-seq and plotted across developmental time (Figure S2A). The ratio of expression for pairs of exon subdivisions was assessed across development using a linear model

(Figure S2B). More details on these methods for per exon gene expression have been described previously.[16]

Genomic data were extracted from clinical genetics reports collected. Genomic coordinates were treated as the gold standard; where not present, the cDNA and protein description of the variant were used to derive the genomic coordinates. Where necessary, coordinates were lifted over from GRCh37 to GRCh38 and all variants were annotated to the 3,661-bp MANE Select ENST00000349496.11, NM_001904.4 transcript from GENCODEv39 of the ENSG00000168036.18, *CTNNB1* gene. Mutability for each variant was estimated based on trinucleotide genomic sequence.[17] Protein structure and domain information were derived from the UniProt Feature viewer.[18]

Cell constructs used for the analysis of β-catenin protein levels in *CTNNB1* variants
Plasmids
FLAG-tagged wildtype (WT) and mutant *pcDNA3.1(+)-CTNNB1* constructs were synthesized by Genewiz (Suzhou, China). The 42 *CTNNB1* variants included in this study are p.Ser23 LysfsTer27, p.Ser47GlufsTer3, p.Arg90Ter, p.Arg95Ter, p.Pro100 ArgfsTer5, p.Ile140SerfsTer3, Gln193Ter, p.Ile231LeufsTer2, p. Leu294Ter, p.Ala317ValfsTer8, p.Gln322ProfsTer31, p.Val325-GlufsTer24, p.Met328GlufsTer24, p.Thr330AspfsTer23, p.Tyr 333Ter, p. Ser352fsTer, p.Pro355GlnfsTer2, p.Gly367Ter, p. Leu385GlnfsTer9, p.Arg386GlnfsTer9, p.Val406PhefsTer9, p. Glu462SerfsTer10, p.Arg474Ter, p.Gln482Ter, p.Gly490Alaf-sTer33, p.Leu498PhefsTer32, p.Arg535Ter, p.Val564GlyfsTer7, p.Gly575Arg, p.Leu577Arg, p.Arg587Ter, p.Ile607LeufsTer7, p. Cys619LeufsTer2, p.Cys619Ter, p.Glu634Ter, p.Glu642Argfs Ter6, p.Tyr654Ter, p.Ser663ArgfsTer15, p.Tyr670Ter, p.Glu 692Asp, p.Ala694LeufsTer41, and p.Ile700AsnfsTer14. The TOPFlash-luciferase and FOPFlash-luciferase were kind gifts from Professor Randall Moon (Addgene plasmids # 12456 and 12457). The TOPFlash-luciferase construct harbors seven consecutive WT β-catenin binding sites, while all β-catenin binding sites in the FOPFlash-luciferase construct are mutated.
Cell culture and transfection
The human neuroblastoma SK-N-MC cells (HTB-10TM, American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (11995065, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (F7524, Sigma-Aldrich, St Louis, MO, USA) and 1% penicillin–streptomycin (15140122, Thermo Fisher Scientific). The SK-N-MC cells were transfected with plasmids using Lipofectamine 2000 (11668019, Thermo Fisher Scientific). Transfection was carried out according to the manufacturer's instructions.
Dual-luciferase reporter assay
The dual-luciferase reporter assay (E1910, Promega, Madison, WI, USA) was carried out as described previously.[1] The *pTK-RL Renilla* luciferase vector (E2241, Promega) was used as an internal control reporter construct for the normalization of transfection efficiency. Both firefly and *Renilla* luminescence were recorded on a Spark multimode microplate reader (Tecan, Morrisville, NC, USA). The relative luciferase activity was calculated by dividing the firefly luminescence reading by the *Renilla* luminescence reading.
Cellular thermal shift assay
SK-N-MC cells were seeded in a six-well plate at a cell density of 5×10^5 cells/well and grown until 70% confluency. Cells were transfected with 3.0 µg of plasmids using Lipofectamine 2000. At 18 h after transfection, cells were harvested, collected by
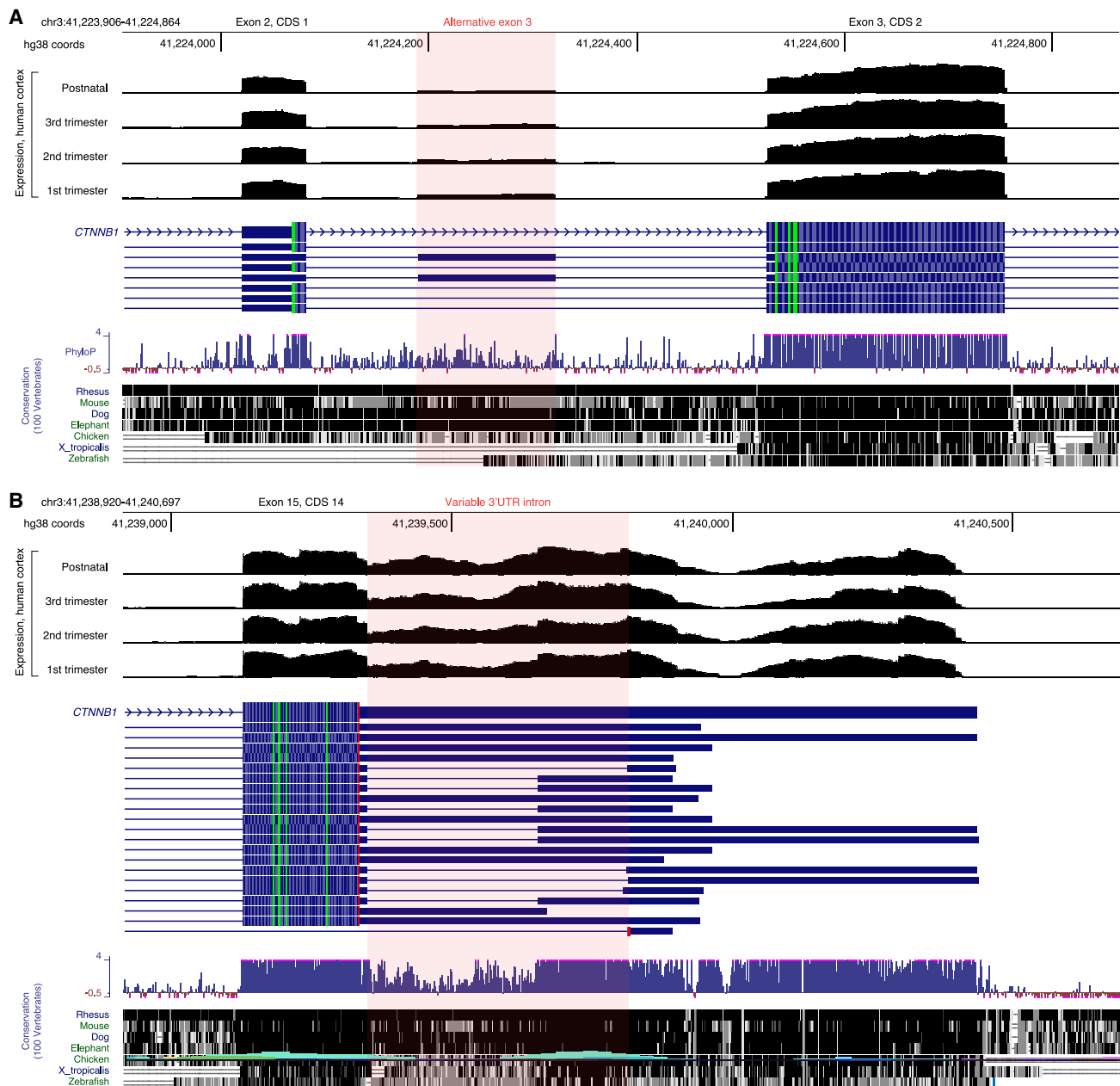
Figure 1. Alternative splicing in *CTNNB1*

(A) Genomic co-ordinates GRCh38/hg38 are shown at the top for the region around exons 2 and 3. Nucleotide resolution of the median read counts of RNA sequencing gene expression data from 176 *postmortem* human prefrontal cortices are shown organized by developmental stage; data are auto-scaled and include zero. A representative subset of protein-coding transcripts from GENCODEv46 are shown, the alternative splicing event of an additional 132 bp exon (chr3:41,224,190-41,224,321, hg38) is highlighted in light red. Species conservation across 100 vertebrates is represented at the bottom, including PhyloP scores (top) and homology across species (bottom).

(B) Corresponding data for an intron with variable inclusion in the 3′ UTR. Images were generated from UCSC genome browser.

centrifugation (200×*g*, 3 min) and washed with PBS twice. The whole cell pellet was resuspended in 800 μL of PBS and divided into 14 equal aliquots. The cell suspensions were incubated at a temperature range of 40.0°C, 42.3°C, 44.7°C, 47.0°C, 48.7°C, 50.7°C, 53.3°C, 55.2°C, 57.0°C, 60.0°C, 64.0°C, and 68.0°C for 6 min using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), followed by cooling to room temperature for 6 min. One aliquot was kept at room temperature and one aliquot was kept on ice as controls. The samples were then snap frozen in liquid nitrogen for 1 min, with a total of three freeze-and-thaw cycles. Subsequently, the samples were briefly vortexed and centrifuged at 16,100×*g* for 25 min at 4°C to pellet cell debris together with precipitated and aggregated proteins. The soluble supernatant fraction was collected for western blot analysis. The protein signal was detected using the anti-FLAG antibody (1:1,000; F3165, Sigma-Aldrich). The band intensities were quantified using ImageJ software and normalized to the band intensity of the 40°C sample. The melting curves were fit
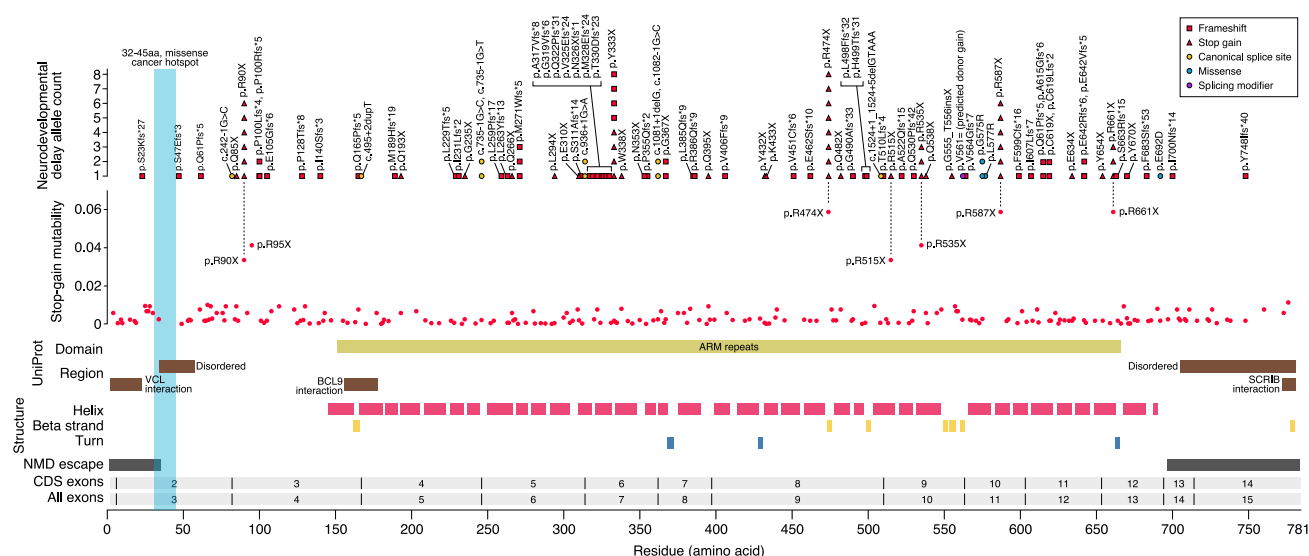
Figure 2. Neurodevelopmental disorder variants in *CTNNB1*
Variants reported by clinical genetics reports are annotated against the MANE Select transcript (ENST00000349496.11), the 125 variants are listed in Table S2. Predicted functional impact is indicated by shape and color (see legend). A blue bar indicates the region (p.32–45) frequently associated with somatic GoF mutations in cancer. For stop-gain single nucleotide variants, the predicted mutability based on trinucleotide genomic sequence is shown under the variants; predicted highly mutable stop gain sites are labeled. Below, protein domains, regions, and structure from UniProt and AlphaMissense are shown. At the bottom, the exon number, CDS exon number, and regions predicted to escape nonsense-mediated decay (NMD) are shown with protein residue number.
Nine variants disrupt the canonical splice site, and all of these are predicted to impact splicing (SpliceAI score ≥0.8). Two additional variants had high SpliceAI scores, suggesting they act as cryptic splice sites: the missense variant p.E692D (SpliceAI donor loss score 0.94, donor gain score 0.78) and the synonymous variant p.V561= (SpliceAI donor loss score 0.41, donor gain score 0.76).

by non-linear regression using the Boltzmann sigmoidal equation in GraphPad Prism 7 to determine the apparent melting (unfolding) temperature (Tm).

Immunoblotting

Protein samples were harvested from SK-N-MC cells using the SDS sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 40% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue). Samples were heated at 99°C for 10 min before being subjected to the immunoblotting analysis. The protein samples were then transferred to a PVDF membrane (IPVH00010, pore size 0.45 μm, Merck Millipore, Burlington, MA, USA). The membrane was blocked using 5% non-fat milk at 25°C for 1 h, followed by incubating primary antibodies at 4°C for 16 h. Primary antibodies used in this study were rabbit anti-FLAG (1:1,000; F3165, Sigma-Aldrich) and anti-β-tubulin (1:2,000, ab6046) from Abcam (Cambridge, UK).

The membrane was washed three times with 1× TBST each for 10 min before being subjected to the incubation of secondary antibodies at 25°C for 1 h. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G (H + L) (11-035-045, 1:5,000) and HRP-conjugated goat anti-mouse IgG (H + L) (115-035-062, 1:10,000) from Jackson ImmunoResearch (West Grove, PA, USA). The membrane was washed three times with 1× TBST each for 10 min, followed by chemiluminescent signal detection. The signal was developed using Immobilon Forte Western HRP substrate (WBLUF0100, Merck Millipore), and the images were captured and processed using ChemiDoc Touch Imaging System. β-tubulin was used as the loading control. Only representative blots are shown.

Structured medical interview

All structured interviews were performed by a single physician-investigator (N.Ž.). Participants and/or their parents or caregivers

were guided through a detailed medical questionnaire, containing more than 500 questions regarding medical history, functional status, and general demographic characteristics, including (1) family history of cancer, (2) detailed genetic information, (3) physical appearance data to ascertain potential dysmorphic features, (4) pregnancy and delivery data, (5) information on reaching developmental milestones and potential developmental delay, (6) first symptoms observed, (7) data on previous intellectual disability testing (if available), (8) current weight, height, and head circumference, (9) previous and current gait, muscle tone, and feeding status data, (10) data on potential neurological and cardiac symptoms, (11) potential presence of scoliosis, (12) head and/or spine MRI results (if available), (13) EEG results (if available) and potential abnormalities, (14) current list of medications, and (15) an array of standardized questionnaires on participant's behavioral, emotional, and functional status, as discussed further below.

Clinical instruments

An array of standardized tests was used to determine participant's behavioral, emotional and functional status. The tests were used in age groups pertaining to their respective standardization and validity.

(1) The Modified Checklist for Autism in Toddlers, Revised with Follow-Up: a parent-reported screening tool for autistic traits, standardized for children between 16 and 30 months of age.[19]
(2) The Autism Spectrum Quotient (AQ)-Children's Version: a parent-reported screening tool for autistic traits, standardized for children between 4 and 11 years of age.[20]
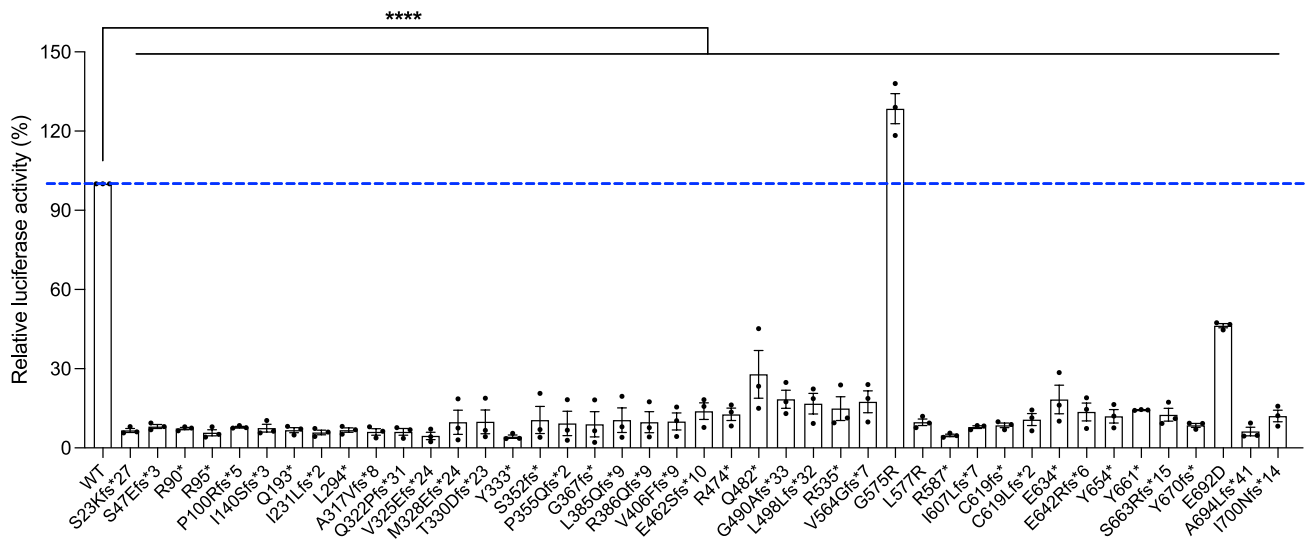
Figure 3. TOP/FOP Flash luciferase assay-based functional assessment of mutant β-catenin on Wnt transcriptional activity
Dual luciferase activity assay results from SK-N-MC cells transfected with *CTNNB1^{wt}* or *CTNNB1^{mutant}*. Each bar indicates the relative luciferase activity for each construct following normalization to *CTNNB1^{wt}* group. Results are from three independent experiments. Error bars denote SEM. ****$p < 0.0001$.

(3) The AQ-Adolescent Version: a self- or parent-reported screening tool for autistic traits, standardized for children above the age of 11 years.[21]

(4) Viking Speech Scale (VSS): a scale that classifies the speech performance of children with CP aged 4 years and older, incorporating the presence of a motor speech disorder and the severity of limitations in speech performance in everyday life.[22]

(5) Functional Communication Classification System: a scale that classifies how children with CP aged 4 years and older communicate with familiar and unfamiliar communication partners.[23]

(6) Brief Sleeping Questionnaire: a parent-reported screening test used to assess sleep patterns, parent perception, and sleep-related behaviors in children younger than 3 years.[24] Since sleep problems have previously been reported in children with CTNNB1 neurodevelopmental syndrome and the questionnaire is quite general, we decided to use it for all the children in our study.

(7) Pediatric Sleep Questionnaire (PSQ): a parent-reported screening test for sleep problems in children aged 2 years and older.[25]

(8) Eating and Drinking Ability Classification System (EDACS): a scale that classifies the ability to eat and drink safely and efficiently in children with CP aged 3 years and older.[26]

(9) Mini Manual Ability Classification System: a scale that classifies self-initiated ability to handle objects and the need for assistance or adaptation in daily activities in children with CP between 1 and 4 years of age.[27]

(10) Manual Ability Classification System: a scale that classifies self-initiated ability to handle objects and the need for assistance or adaptation in daily activities in children with CP between 4 and 18 years of age.[28]

(11) Gross Motor Function Classification System (GMFCS): a 5-level scale describing the gross motor function of children and youth with CP based on their self-initiated movement with emphasis on sitting, walking, and wheeled mobility.[29]

(12) Visual Function Classification System (VFCS): a five-level scale that describes how toddlers and youth (1–19 years) with CP use visual abilities in daily life.[30]

(13) Adaptive Behavior Assessment System (ABAS-3): a scale that assesses adaptive behavior and skills for people from birth through age 89.[31]

(14) The Achenbach System of Empirically Based Assessment (ASEBA): a scale for rating behavioral/emotional/social problems and adaptive characteristics for ages 1½ to more than 90 years.[32]

Please see the supplementary text for more precise description of the levels of the following scales: VSS, FCSS, GMFCS, VFCS, and EDACS.

Statistical analysis

Statistical analysis conducted in the R language for statistical computing[33] included the calculation of basic descriptive statistics for the demographic and phenotypical characteristics of participants. More specifically, for each phenotypical characteristic we analyzed the proportion of occurrence in our cohort with appropriate statistical confidence intervals (CIs) and/or calculated measures of central tendency and variability for continuous phenotypic measures (e.g., birthweight). We also calculated the proportions for each response category on measures of functioning in specific domains (VSS, FCSS, GMFCS, VFCS, and EDACS).

Finally, we used Bayesian regression implemented in the R *brms* package[34] to examine the phenotype-genotype correlation, i.e., the impact of *CTNNB1* variant type and *CTNNB1*-specific germline *de novo* variant on neurodevelopment. We examined the impact of the functional status of the *CTNNB1* gene (a) loss-of-function (LoF) dominant negative vs. LoF not dominant negative and (b) LoF dominant negative vs. LoF not dominant negative,
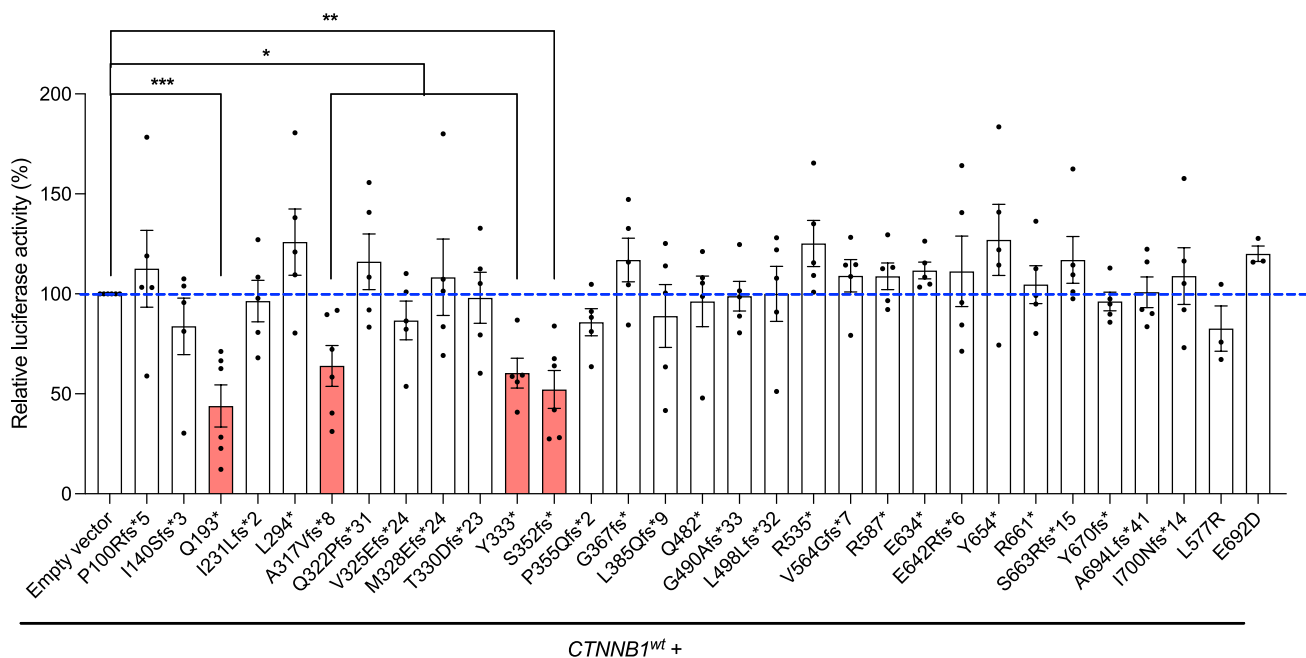
Figure 4. Study of dominant-negative effect of β-catenin variants on Wnt transcriptional activity using TOP/FOP Flash luciferase assay

Dual luciferase activity assay results from SK-N-MC cells co-transfected with *CTNNB1^wt* and empty vector (*pcDNA3.1(+)*) or *CTNNB1^mutant*. Each bar indicates the relative luciferase activity for each construct following normalization to the *CTNNB1^wt* (WT) + empty vector group. Variants marked in red are those for which a significant dominant-negative effect was observed. Results are from three to six independent experiments. Error bars denote SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

LoF, and LoF presumed, as well as the variant type (missense vs. predicted haploinsufficiency, including frameshift, nonsense/stop gain, canonical splice site, and whole gene deletion) on the age at which individuals were able to walk independently using Bayesian survival models. We then examined the impact of variant type and variant functional impact on individuals' daily functioning as measured by the VSS, FCSS, GMFCS, VFCS, and EDACS by using Bayesian ordinal regression. To analyze differences between genotypes in their age of independent walking acquisition, we used Bayesian hurdle regression models. In the models, we included age at independent walking as the dependent variable and achievement of these milestones as the censoring variable. In addition to using genotype as a predictor, we also used the age of the child at entry into the study as a covariate in the hurdle part of the regression model. We examined the association of genotype with the presence of different neurological symptoms, adaptive skills, and symptoms of psychopathology using Bayesian regression with continuous response variables (ABAS-3, ASEBA, and age at independent walking) being modeled as skewed Gaussian distributions. For ABAS-3 adaptive skills scales, we used a regression analysis with a truncated response distribution, truncated at the lowest possible value of scale scores. Finally, binary response variables were modeled using Bayesian logistic regression. Each of the regression models was estimated using four separate Markov chain Monte Carlo (MCMC) chains and run for 10,000 iterations, with the last one-half being used for parameter estimation. We ensured that convergence was achieved for each model by checking the MCMC plots, *Rhat* statistic, and the effective sample size. We report the results of Bayesian analysis using both Bayes factors, regions of practical equivalence, as well as Bayesian equivalents of classical probability measures (*p* values).

We calculated Kaplan-Meier curves for the probability of achieving independent walking by using the R "survival" package.[35]

Results

Participants

Our study included 127 participants with a genetically confirmed pathogenic or likely pathogenic variant in the *CTNNB1* gene. Two additional participants whose genetic report indicated a variant in the *CTNNB1* gene, but without mentioning the specific variant, were not included. At the time of enrollment, the mean age of participants was 70 ± 50 months (range, 7–242 months) and 55 (43.3%) were female. The reporting individuals were mostly mothers (86.6%). Participants in the study were residents of 20 different countries: United States ($N = 28$), People Republic of China ($N = 26$), Spain ($N = 15$), Germany ($N = 12$), France ($N = 8$), Italy ($N = 7$), UK ($N = 6$), Poland ($N = 4$), Belgium ($N = 4$), Australia ($N = 3$), Mexico ($N = 2$), Portugal ($N = 2$), Russian Federation ($N = 2$), Switzerland ($N = 2$), Canada ($N = 1$), Ecuador ($N = 1$), Peru ($N = 1$), Slovenia ($N = 1$), Taiwan ($N = 1$), and Turkey (N = 1) (Figure S3). It is likely that several of these individuals were previously published in case reports or small cohorts.

Analysis of *CTNNB1* transcripts in the developing human brain

The MANE Select project has identified a canonical transcript for each gene based on multiple annotation features
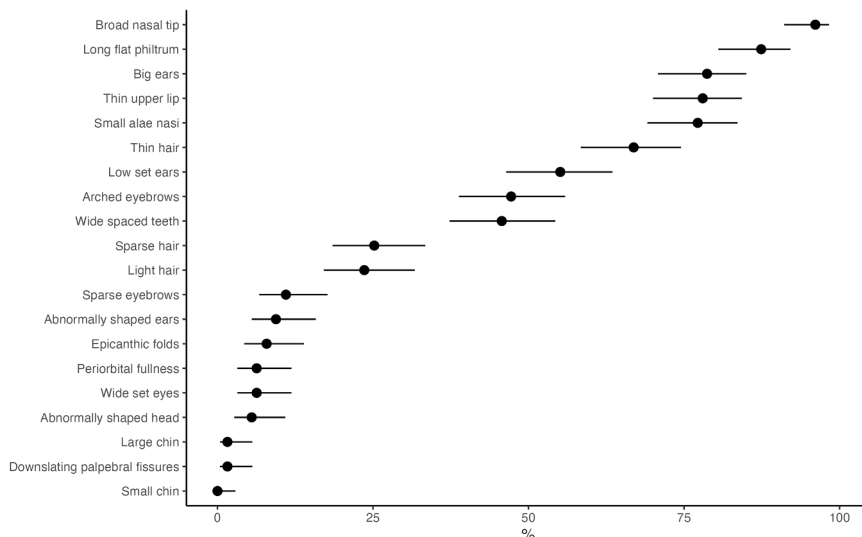
Figure 5. Prevalence of dysmorphic features in participants with CTNNB1 neurodevelopmental syndrome
Percentage with 95% CIs.

aligned between Ensembl/GENCODE and RefSeq.[36] Using bulk tissue gene expression data from 176 *postmortem* human prefrontal cortex samples across development, we assessed whether the MANE Select transcript was appropriate for a disorder with predominantly neurodevelopmental symptoms. These human cortex expression data support the use of the MANE Select transcript ENST00000349496.11, NM_001904.4 for this gene (Figure S1). We also assessed whether there were substantial differences in splicing patterns across human brain development; in contrast with other genes,[16] splicing remained mostly constant. The most developmental variability was observed in weakly expressed non-canonical transcripts (exon subdivisions 085 and 093 in Table S1; Figure S2).

We noticed three transcriptional features of interest (Figure S1). First, several alternative transcripts identified a transcription start site (TSS) upstream of the canonical TSS. We did not see evidence of this being expressed in the human prefrontal cortex but note that the genetic sequence at this locus is very highly conserved across species. This feature may represent a non-coding regulatory sequence related to *CTNNB1*. Second, multiple transcripts describe an additional 132-bp exon (alternative exon 3) between exons 2 and 3 in the MANE transcript that is predicted to lead to a β-catenin protein missing the first (N-terminus) five amino acids. We see evidence of this additional exon being expressed at moderate levels across the developing human cortex and the region is somewhat conserved across species (Figure 1A).[14] It is unclear if this additional exon impacts *CTNNB1* expression or β-catenin levels and function. Third, we see the variable inclusion of an intron in the 3′UTR of *CTNNB1* (Figure 1B, exon 16),[15] and this is supported by long-read RNA sequencing of the human cortex.[37] This event has previously been reported as being very frequently dysregulated in cancer,[38] furthermore, preventing inclusion of the intron reduced *CTNNB1* levels. This implies that promoting inclusion of this intron might lead to higher β-catenin levels, a potential therapeutic strategy in a haploinsufficient disorder.

Analysis of *CTNNB1* variants in neurodevelopmental disorders

We collected and curated 125 variants of the *CTNNB1* gene from the participant's clinical genetic reports (Table S2, including predicted protein impact and SpliceAI score). Of these, 91 (73%) were reported as pathogenic and 34 (27%) were reported as likely pathogenic. In 60 participants (48%), both parents also provided blood samples for genetic analysis; of these, all proband variants were found to be germline *de novo* mutations. Variants were annotated against the MANE Select transcript (Figure 2). The predominant pattern is of premature termination codons (PTCs), caused by frameshift variants, stop gain/nonsense, or canonical splice site variants. All three PTC mechanisms would be predicted to lead to nonsense-mediated decay and a haplo-insufficient (heterozygous LoF) mechanism of action, distinct from the gain-of-function (GoF) variants at the p.32–45 hotspot observed in cancer (Figure 2). Three PTCs (p.S23Kfs*27, p.I700Nfs*14, and p.Y748Ifs*40) were in regions predicted to escape nonsense-mediated decay (Figure 2).[18,39]

Only four missense variants were reported, giving a PTC: missense ratio of 29.5 (118:4). Similar ratios are observed in neurodevelopmental-associated genes in which PTCs cluster in the terminal exon (e.g., *PPM1D*, *ASXL3*) suggesting a toxic truncated protein (e.g., dominant negative)[40,41]; however, such clustering is not observed in *CTNNB1*. Among neurodevelopmental-associated genes with high PTC:missense ratios without PTC clustering (e.g., *ADNP*, *WAC*), the PTC enrichment in *CTNNB1* seems especially prominent. It is unclear whether this PTC enrichment represents patient ascertainment (e.g., missense variants lead to milder symptoms and less genetic testing or are not being reported as pathogenic) or mechanism (e.g., many missense variants cause more severe symptoms that are not compatible with life).

While the missense variant NM_001904.4:c.2076G>C is predicted to be benign at the protein level (p.(Glu692Asp)), *in silico* analysis using SpliceAI yielded a high score of 0.94, strongly suggesting a potential impact on pre-mRNA splicing. This raises the possibility that the variant could create or disrupt a splice site, leading to an altered transcript. Such an alteration might result in a frameshift and the introduction of a PTC, which would likely trigger
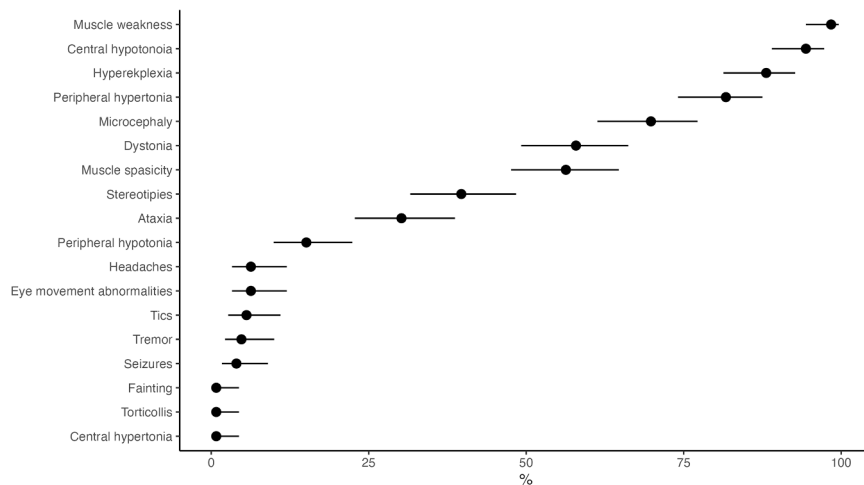
Figure 6. Neurological status of CTNNB1 participants as reported by parents
Percentage with 95% CIs.

nonsense-mediated mRNA decay and significantly reduce functional protein levels. Although experimental validation of this splicing effect was beyond the scope of the current study, the strong *in silico* evidence warrants the consideration of a potential LoF mechanism contributing to the observed phenotype, despite the benign missense prediction.

A recent analysis has raised the possibility that LoF variants in *CTNNB1* lead to a selective advantage in the male germline, as observed in achondroplasia. This mechanism would predict an older paternal age and a bias toward mutations on the paternal allele. Comparing the age of fathers at birth of the proband in this cohort (median, 33 years median; Table S2) with the Simons Simplex Collection (median, 32 years),[42] we do not see a difference ($p = 0.85$, Wilcoxon test, two-tailed). Estimation of parent of origin would require access to DNA samples for both parents and the child, which are not currently available.

Recurrent variants
We noted several PTC variants observed in multiple individuals (Figure 2). To understand these recurrent PTC variants, we assessed patterns of stop-gain mutability based on genomic sequence, driven by the 10-fold higher mutation rate of CpG variants.[17,43] We identified seven PTCs predicted to have higher mutation rates, and five of these aligned with observed recurrent PTCs: p.R474X ($n = 8$), p.R90X ($n = 6$), p.R587X ($n = 6$), p.R535X ($n = 3$), and p.R661X ($n = 3$), while p.R515X was observed only once and p.R95X was not observed at all. Surprisingly, eight individuals had a PTC predicted to lead to a stop gain at residue 333 (p.Y333X); however, the actual variant differed: c.998dupA ($n = 4$), c.999C>A ($n = 3$), and c.999C>G ($n = 1$). This recurrent variant cannot be explained by CpG mutability, homopolymers (recurrent nucleotides that enrich for indels), repetitive sequence, predicted splice sites, or predicted polyA sites.[44] This raises the possibility that the recurrence is driven by patient ascertain-

ment, for example, a truncated protein with dominant negative effects leading to more severe symptoms. However, it is not clear why p. Y333X, but not other PTCs in *CTNNB1*, might act in such a manner. There is a high density of frameshift variants upstream of p. Y333X (e.g., residues 317–330) that might reflect a similar mutational or functional mechanism. Two individuals were reported to have the same missense variant (p.G575R), and this can also be explained by higher mutability driven by CpG (Table S3).

CTNNB1 variants can both perturb and stimulate the Wnt/β-catenin pathway
We used a luciferase-based reporter system (TOP/FOP Flash) to examine the molecular mechanism of the disease activity of the Wnt/β-catenin pathway in SK-N-MC cells overexpressing 43 mutant β-catenin constructs observed in persons living with the CTNNB1 syndrome. The TOPFlash- and FOPFlash-luciferase constructs are commonly used to evaluate β-catenin-dependent signaling, which drives the expression of TCF. When compared with the WT *CTNNB1*-transfected cells, cells expressing all *CTNNB1* variants, except for one, showed reduced levels of TOPFlash-luciferase activity. This strongly suggests a LoF mechanism for these *CTNNB1* variants, in keeping with nonsense-mediated decay predictions and functional loss of an allele. Intriguingly, cells transfected with the *CTNNB1*[G575R] missense variant showed a significantly higher TOPFlash-luciferase activity. This finding indicates that, unlike the other Wnt/β-catenin LoF variants, the *CTNNB1*[G575R] variant might act via stimulating the Wnt/β-catenin pathway (Figure 3); however, more studies are needed in other cell types.

Missense variants can lead to normal protein levels, as was observed for the variant p.G575R, or reduced protein levels, e.g., by inducing protein instability, as was observed for the variant p.L577R. In contrast, all PTC variants are predicted to induce nonsense-mediated decay and minimal protein levels, unless they occur 50 bp downstream of the last exon splice junction (Figure 2) or in the first 100 bp from the first codon. Of the 29 PTC variants assessed, 19 follow this prediction (Table S4; Figure S4). Fifteen PTC variants (p.L294*, p.T330Dfs*23, p.P355Qfs*2, p.R386Qfs*9, p.Q482*, p.G490Afs*33, p.L498Lfs*32, p.R535*, p.V564Gfs*7, p.R587*, p.I607Lfs*7, p.C619fs*, p.E634*, p.S663Rfs*15, and p.A694Lfs*41) showed reduced β-catenin levels and a further five PTC

Table 1. Levels of functioning across domains of speech and language (VSS, FCCS), feeding (EADCS), motor function (GMFCS), and visual functioning (VFCS)

	Level 1 (%)	Level 2 (%)	Level 3 (%)	Level 4 (%)	Level 5 (%)
VSS	2.8	30.6	38.9	27.8	N/A
FCCS	8.3	31.9	22.2	33.3	4.2
GMFCS	17.3	38.6	29.1	11.8	3.1
VFCS	29.1	12.6	54.3	2.4	1.6
EDACS	42.4	34.8	17.4	4.3	1.1

FCCS, Functional Communication Classification System; N/A, not applicable. Note: Higher levels represent greater levels of impairment across all scales. For further details please see subsection clinical instruments.

variants (p.R90*, p.R95*, p.V406Ffs*9, p.E462Sfs*10, and C619fs*) led to undetectable protein levels. However, 10 PTC variants (p.I140Sfs*3, p.Q193*, p.I231Lfs*2, p.V325 Efs*24, p.M328Efs*24, p.Y333*, p.G367fs*, p.E642Rfs*6, p.Y654*, and p.Y670fs*) showed a similar protein level as the WT control.

CTNNB1 variants that exhibit a dominant-negative effect on the Wnt/β-catenin pathway

The presence of a truncated protein has the potential to lead to additional damaging effects, including a dominant negative mechanism. We therefore assessed the impact of 31 variants on TOPFlash-luciferase activity when co-transfected with WT CTNNB1. Most variants had similar activity to the co-transfection of WT CTNNB1 and empty vector, consistent with a simple LoF mechanism. However, four variants (p.Y333*, p.Q193*, p.A317Vfs8*, and p.S352fs*) showed reduced levels of TOPFlash-luciferase activity suggesting a dominant negative mechanism (Figure 4).

Arginine substitution variants between ARM repeats 10 and 11 affect β-catenin protein stability in opposite directions

We identified two variants in the proximity (p.G575R and p.L577R) at the junction of ARM repeats 10 and 11. Both the glycine and leucine residues were mutated to arginine in individuals. Interestingly, these two neighboring arginine substitution variants result in a totally opposite effect on TOPFlash-luciferase activity (Figure 3). The p.G575R caused increased Wnt activity while the p.L577R resulted in reduced TOPFlash-luciferase activity.

We attempted to understand the opposite TOPFlash-luciferase pattern by looking into the biophysical stability of these mutant proteins. With a single point variant, proteins may undergo conformational changes or different interactions with cellular partners, which might subsequently affect their function. A cellular thermal shift assay (CETSA) was conducted to investigate whether the p. G575R and p.L577R variants have different thermal stability of the mutant proteins. CETSA is a well-established method used to determine cellular protein stability, since proteins typically unfold and aggregate when exposed to

elevated temperatures. Proteins with greater stability are more resistant to temperature changes and have a higher T_m.[2,3] The T_m of the β-catenin WT protein was determined as 51.11 ± 0.80°C, meaning that 50% of the β-catenin WT protein remained soluble at this temperature. For p.G575R, the T_m was shifted to 55.32 ± 0.51°C, indicating that the p.G575R mutant protein is more stable than the WT protein and might be degraded less efficiently by the degradation complex. In contrast, the T_m of p.L577R was 46.02 ± 0.84°C, suggesting that the p.L577R mutant protein was less stable, which may be responsible for its loss of function (Figure S5).

Clinical phenotypes
Prenatal and perinatal characteristics
The mean gestational age at birth was 39 ± 1.8 weeks, with the youngest participant being born at 33 gestational weeks. The mean birthweight was 2,955 ± 497 g (range, 1,500–4,139 g). Several prenatal (Figure S6A) and perinatal (Figure S6B) characteristics were identified in our cohort: intrauterine growth restriction, microcephaly at birth, and feeding issues after birth. Perinatal birth complications were rare, as evidenced by the high Apgar scores at 1 and 5 min (median, 9 [interquartile range (IQR), 2] and 10 [IQR, 1], respectively).

Anthropomorphic measures
Compared with their peers, at the time of enrollment the participants tended to be of smaller stature, being 0.70 SD shorter (95% CI, 0.30–1.00), and weight, 0.80 SD lighter (95% CI, 0.50–1.00), according to World Health Organization z-scores for weight and height in children.[45]

Dysmorphic features
The most common dysmorphic features of the CTNNB1 phenotype in our cohort were a broad nasal tip (96.1%; 95% CI, 91.1%–98.3%), a long flat philtrum (87.4%; 95% CI, 80.5%–92.1%), small alae nasi (77.2%; 95% CI, 69.1%–83.6%), a thin upper lip (78.0%; 95% CI, 70.0%–84.3%), large ears (78.7%; 95% CI, 70.8%–85.0%), and thin hair (66.9%; 95% CI, 58.4%–74.5%) (Figure 5), consistent with previous reports.[46]

Neurological signs and symptoms
By far the most prevalent neurological symptoms in participants were muscle weakness (98.4%; 95% CI, 94.4%–99.6%), central hypotonia (94.5%; 95% CI, 89.1%–97.3%), syndromic atypical hyperekplexia (88.2%; 95% CI, 81.4%–92.7%), and peripheral hypertonia (81.9%; 95% CI, 74.3%–87.6%) (Figure 6). Many parents also reported dystonia (57.5%; 95% CI, 48.8%–65.7%), spasticity (56.7%; 95% CI, 48.0%–65.0%), and peripheral hypotonia (15.0%; 95% CI, 9.8%–22.2%). Stereotypical movements were also relatively common, occurring in 40.2% of participants (95% CI, 32.0%–48.9%). Among other conditions diagnosed at the time of the participants' inclusion in the study were microcephaly (70.1%; 95% CI, 61.6%–77.4%; defined as −2 SD), headaches (6.3%; 95% CI, 3.2%–11.9%), and seizures (3.9%; 95% CI, 1.7%–8.9%).
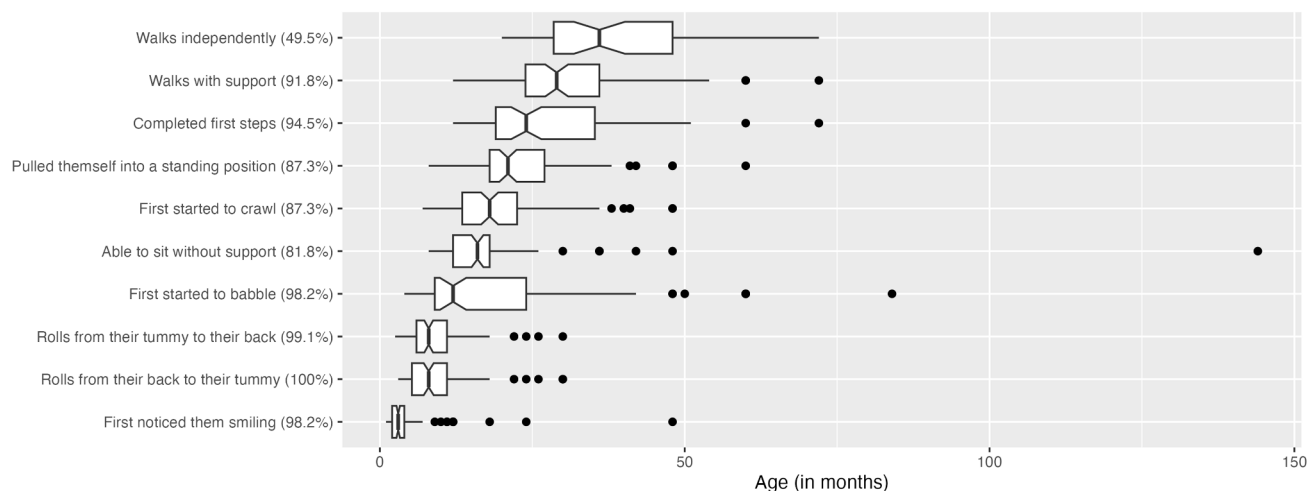
Figure 7. Developmental milestones of CTNNB1 neurodevelopmental syndrome participants aged 24 months or older (*N* = 111)
Percentages represent the proportion of participants achieving a particular milestone. Boxplots represent the median and IQR. Dots represent outliers.

Neurophysiological studies

EEG was performed in 81 of the 127 participants (63.8%). Most participants had a normal EEG, although seven (8.6%; 95% CI, 4.2%–16.8%) participants had an abnormal EEG. In two participants, an abnormal slowing of the background activity was reported, while in five participants, epileptiform discharges were reported. Five participants reported taking anti-seizure medications (valproic acid, phenobarbital, levetiracetam, oxcarbazepine, or clobazam, with some more taking than one).

MRI studies

MRI was performed in 121 of the 127 participants (95.3%). Most participants had a normal MRI, while 34 (28.1%; 95% CI, 20.9%–36.7%) had abnormal MRI findings. White matter abnormalities were reported in 26 of these 34 participants (76.5%). Among these, 15 participants were reported to have white matter volume loss, 11 participants had abnormal signal intensity, and 8 had delayed myelinization. Of these 26 participants, 5 also had an abnormal corpus callosum (CC) morphology, accompanied with a smaller CC volume in 4 participants. Gray matter abnormalities were specifically reported in three participants, while seven participants presented with wider cortical cerebrospinal fluid spaces.

Functional assessment

Assessment of participants' speech, language, feeding, motor and visual function using standardized scales is presented in Table 1.

Visual function

Another major area of reported symptomology were ophthalmologic issues (Figure S7). Most of the participants had strabismus (81.1%; 95% CI, 73.4%–87.0%) and 47.2% had farsightedness (95% CI, 38.7%–55.9%). Less common problems were near-sightedness (16.5%; 95% CI, 11.1%–24.0%), familial exudative vitreoretinopathy (FEVR; 7.1%; 95% CI, 3.8%–12.9%), and gaze palsy (3.1%; 95% CI, 1.2%–7.8%).

Motor development

The age at which CTNNB1 participants achieved their developmental milestones in the motor domain was significantly delayed (Figure 7). Considering participants older than 24 months, 91.9% were (95% CI, 85.2%–95.6%) able to walk with support with the average age of attaining this milestone being 30 months. Far fewer were able to walk independently (49.5%; 95% CI, 40.4%–58.7%; walked without aid for at least 10 m), with those that did achieve this milestone doing so at 39 months of age on average. Most individuals used a wheelchair in their daily life (72.7%; 95% CI, 59.8%–82.7%).

Looking more specifically at walking in our cohort of persons living with the CTNNB1 syndrome, up to 28.6% are not able to walk independently in any age group (Table 2). The probability of independent walking increases with advancing age (Figure 8).

Neurodevelopment

On the ABAS-3 (Figure S8), the majority of participants' scores were in the range of children and adolescents with mild (50.8%; 95% CI, 41.8%–59.9%) or moderate to severe intellectual disability (26.3%; 95% CI, 19.0%–35.1%). A minority achieved scores observed in neurotypical participants (3.5%; 95% CI, 1.3%–8.7%) or participants with borderline intellectual abilities (19.2%; 95% CI, 13.1%–27.5%). Among those CTNNB1 individuals who had already been psychologically assessed before participating in the study (40.9%), more than three-quarters had been given a diagnosis of intellectual disability (78.8%; 95% CI, 66.0%–97.8%).

Among the adaptive subdomains in which the participants had the greatest difficulties were subdomains included in the practical composite score (self-care, health and safety, and home living), as well as the communication subdomain. In concordance with the latter, we found that 38.9% (95% CI, 28.5%–50.4%) had unclear speech not usually understandable to unfamiliar listeners and

Table 2. Number and percentage of persons living with the CTNNB1 syndrome walking independently across age groups

Age, mo	No.		%	
	No	Yes	No	Yes
≤24	16	0	100.0	0.0
25–60	36	21	63.2	36.8
61–120	12	17	41.4	58.6
121–180	6	15	28.6	71.4
≥181	2	1	66.7	33.3

27.8% (95% CI, 18.8%–39.0%) had no understandable speech (Table S5).

On the ABAS-3, participants also displayed substantial impairments in pre-academic and academic skills. We found that, among the children who attended school or kindergarten (62.2%; 95% CI, 53.3%%–70.2%), most required additional support in school (92.7%; 95% CI, 80.6%%–97.5%) and one-half attended a special education kindergarten or school (48.1%; 95% CI, 37.4%–58.9%).

Before entering the study, most participants had not had a formal psychological assessment. However, among those who did, approximately one-half had been diagnosed with autism spectrum disorder (ASD; 48.3%; 95% CI, 39.6%–57.2%). This group of individuals also scored beyond the critical value for ASD on the autism spectrum scale of the Child Behavior Checklist 1½ 1–5 (mean, 72.7 ± 8.3) and higher than the remaining participants (mean, 65.3 ± 10.4). Among those diagnosed with ASD, 57.9% also had a diagnosis of intellectual disability (95% CI, 36.3%–76.9%), while among those without the diagnosis, 36.4% had a diagnosis of intellectual disability (95% CI, 19.7%–57.0%)

In our cohort, other psychopathological symptoms were also more common than in the general population. On the ASEBA scales, respondents reported increased rates of withdrawn behavior, symptoms of anxiety and depression, attention problems, and thought problems. Symptoms of ASD, as assessed with the ASEBA preschool scales, were also more prevalent, with about half of the participants scoring above the clinical cut-off of a T-score of 70.

Developmental regression
A substantial proportion (35.1%; 95% CI, 26.9%–44.4%) of respondents reported signs of developmental regression in participant's ability to talk or babble (19.8%; 95% CI, 13.5%–28.2%), and walk independently (5.4%; 95% CI, 2.5%–11.3%). When present, most respondents noticed the regression at 31 ± 40 months of age (range, 3–180 months).

Parents also reported that 56.3% of children living with the CTNNB1 syndrome (95% CI, 47.6%–64.7%) had sleeping problems. The average score on the PSQ was 7.1 ± 2.6 points (range, 2–15), with the established cut-off value suspected of obstructive sleep apnea being 7.3 points.[25]

Genotype-phenotype correlations
Genotype-phenotype correlations were examined by comparing motor development, neurodevelopment, and the functional status of individuals with (1) LoF dominant negative ($N = 11$) vs. LoF not dominant negative variant ($N = 38$), (2) LoF dominant negative variant ($N = 11$) vs. LoF not dominant negative ($N = 38$), LoF ($N = 24$), and LoF presumed variant ($N = 48$); and (3) different variant types (missense vs. predicted haploinsufficiency including frameshift, nonsense/stop gain, canonical splice site, and whole gene deletion).

LoF dominant-negative variants vs. LoF not-dominant-negative variants
We did not see evidence of differences between individuals with LoF dominant negative and individuals with LoF non-dominant variants in terms of age at which they were able to walk independently (LoF dominant negative, 36.0 months; LoF not dominant negative, 33.7 months; Bayes factor [*BF*], 1.65; Table S6), nor in terms of their adaptive functioning (*BF* values <2.1, *pd* values <0.678; Table S7), psychopathology (*BF* values <9.5, *pd* values <0.904; Table S8), or results of functional assessment (*BF* values <6.9, *pd* values <0.873; Table S9). The largest *BF* was from autism spectrum, with more symptoms reported in children with LoF not dominant negative ($BF = 9.46$, $pd = 0.904$).

LoF dominant-negative variants vs. LoF not-dominant-negative, LoF, and LoF presumed variants
Results were similar (Tables S10–S13) with the addition of 72 samples with variants predicted to be LoF and not known to have dominant negative effects (LoF nDN+). Of note, parents of individuals with the LoF nDN+ variants reported that these individuals had more symptoms of ASD compared with individuals with LoF dominant negative variants ($BF = 26.9$, $pd = 0.964$).

Variant type
Clearer genotype-phenotype correlations emerged when looking at different variant types. We found plausible evidence that individuals with a missense variant ($n = 4$) started to walk earlier (median estimate, 27.7 months) compared with individuals with a nonsense (median estimate, 41.0 months) or frameshift variant (median estimate, 39.2 months) or a whole gene deletion (median estimate, 45.7 months; *BF* values >11.0, *pd* values >0.917; Table S14). Individuals with a missense variant were also rated as having better conceptual and social skills compared with individuals with other variant types (*BF* values >39.7, *pd* values >0.975; Table S15). In terms of psychopathology, individuals with a missense variant did not differ from other variant types, although their parents tended to report fewer symptoms of anxiety and depression compared with individuals with a frameshift variant ($BF = 16.11$, $pd = 0.924$) or splice variant ($BF = 16.59$, $pd = 0.943$); however, parents also tended to report more
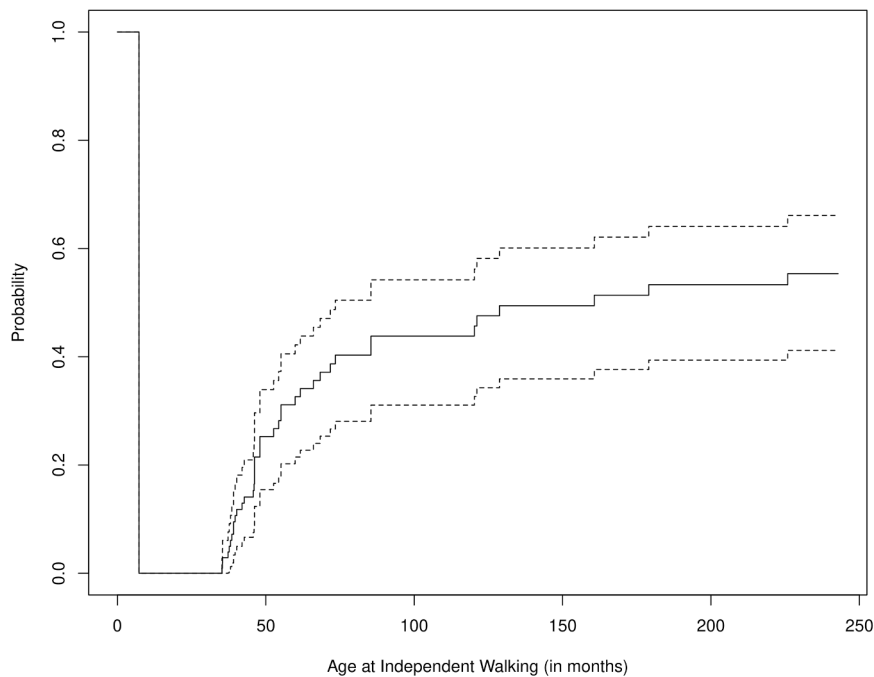
Figure 8. Kaplan-Meier curves showing the probability of independent walking by age with 95% CIs

Discussion

We present the phenotypic spectrum of a large cohort of individuals living with the CTNNB1 neurodevelopmental syndrome and report possible genotype-phenotype correlations between missense and PTC variants. Rare diseases, like CTNNB1 neurodevelopmental syndrome, are often insufficiently characterized due to underdiagnosis, the rarity of identified individuals, and the lack of comprehensive disease registries that facilitate research. Additionally, research initiatives in the field of rare diseases are commonly limited. However, gaining a deeper understanding of the genetics, pathophysiology, and phenotypes of a specific rare disease is crucial for improving symptom management, developing treatment guidelines, and advancing the potential for disease-modifying therapies.[12]

somatic problems compared with individuals with a nonsense or frameshift variant or a whole gene deletion (*BF* values <0.13, *pd* values <0.117). In terms of psychopathology, the greatest symptom burden, compared with individuals with a missense variant, was present in individuals with a canonical splice variant. Their parents reported more pronounced symptoms of anxiety and depression (*BF* = 16.59, *pd* = 0.943), ASD (*BF* = 11.11, *pd* = 0.917), and aggressive behavior (*BF* = 10.37, *pd* = 0.912; Table S16).

On the functional assessment scales, individuals with a missense variant were rated as having better communication skills on the VSS compared with individuals with a nonsense variant, frameshift variant, or a whole gene deletion (*BF* values >12.6, *pd* values >0.927). They also showed the least difficulties in terms of feeding as rated by the EDACS (*BF* values >56.3, *pd* values >0.983). Finally, the analysis of GMFCS scores indicated better motor abilities than individuals with other variant types (*BF* values >1,000.0, *pd* values >0.999; Table S17; Figure 9).

Gain and loss of function

Given that only two individuals had a confirmed GoF variant in CTNNB1, we could only examine the genotype-phenotype correlation using descriptive means. When examining the age of onset for independent walking, as well as the median scores on measures of adaptive functioning and psychopathology, we found no evidence of greater impairment in individuals with a GoF variant. On the contrary, they showed better adaptive skills compared with the remaining individuals (Figure 10).

Genetic characterization and Wnt transcriptional activity of *CTNNB1* variants

In our cohort of 125 participants with a genetically confirmed CTNNB1 neurodevelopmental syndrome, the predominant genetic pattern found was PTC, caused by frameshift variants, stop-gain, or canonical splice site variants. These variants were predicted to lead to nonsense-mediated decay and a haploinsufficient mechanism of action. Only four missense variants were reported (3.2% [4/125 variants]). Similarly, in the study of Kayumi et al.,[6] most *CTNNB1* variants (91.1% [357/392 variants]) were predicted to be LoF variants, predominantly stop gain/nonsense and frameshift variants, while missense variants accounted for only 7.1% of variants (28/392). This high PTC:missense ratio could represent that missense variants lead to milder symptoms postponing genetic testing in these participants or that missense variants cause more severe, life-limiting symptoms. Our data from four individuals with a missense variant supports the former explanation, with a milder phenotype compared with participants with other variants.

Our functional characterization of 43 variants supports the notion that haploinsufficiency, along with reduced Wnt activity, are the predominant pathological mechanisms. This suggests that strategies to increase gene expression, such as CRISPR activation or antisense oligonucleotides, could potentially be suitable therapies. The variable inclusion of an intron in the 3′ UTR of *CTNNB1*[38]
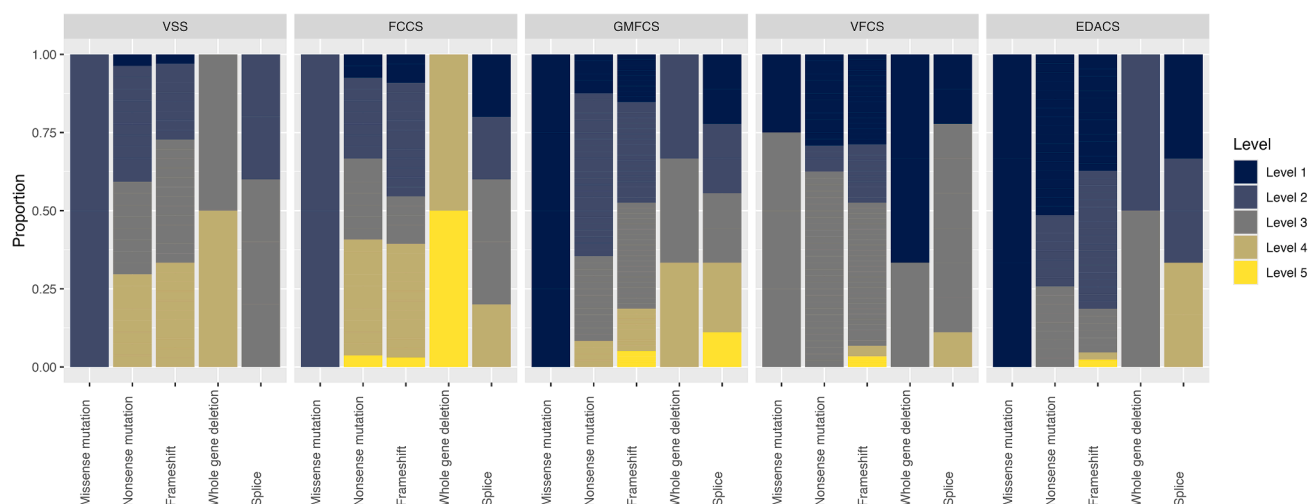
Figure 9. Scores on functional assessment scales by individuals in relation to their variant type (missense variants, nonsense variants, frameshift variants, whole gene deletions and (canonical) splice variants)
Level 1, milder phenotype; level 5, more severe phenotype.

(Figure 1B, exon 16), supported by long-read RNA sequencing of the human cortex,[37] presents one such therapeutic opportunity for increasing expression by promoting intron inclusion.

Surprisingly, the p.Y333* variant was observed in eight participants (four single nucleotide variations and four insertion/deletions), despite no there being evidence of genomic factors to account for this recurrence via mutational mechanisms. Participant ascertainment, driven by particularly severe symptoms in one or more domains, might explain this. Analysis of the cell construct showed that the variant did not lead to nonsense-mediated decay, as predicted, but rather a truncated protein. Co-expression with a WT construct suggested a dominant negative mechanism. The p.Q193*, p.A317Vfs8*, and p.S352fs* variants were also found to result in a truncated protein and exert a dominant negative effect, although these variants were only observed in single participants. As a subgroup, we did not find evidence that variants with a dominant negative effect led to clear genotype-phenotype correlations that would phenotypically distinguish them from other LoF variants.

We also identified one missense variant that increased Wnt activity and was found to encode a more thermally stable protein, p.G575R (two participants in our cohort). This suggests a GoF effect. Although our cohort consisted of only two participants with this variant, descriptive observation suggests these participants were not different regarding the onset of independent walking, adaptive functioning, and psychopathology, but showed better adaptive skills compared with the remaining individuals.

These putative dominant-negative and GoF effects warrant further examination, since a therapy that would upregulate both *CTNNB1* alleles might have unintended consequences in individuals harboring these variants. Somatic missense variants of the *CTNNB1* gene in cancer patients also merit further investigation, as somatic GoF variants are functionally different from therapeutic overexpression, such as gene replacement.[47] In conclusion, genetic characterization of larger cohorts, combined with functional assessment of mutant β-catenin, would enable more precise genotype-phenotype analysis. This could better inform the development of targeted therapies and guide selection of treatment candidates once such treatments become available.

Typical phenotypic presentation

A significant proportion of participants experienced intrauterine growth restriction, with a low birth weight (<2,500 g), small head circumference, and postnatal feeding difficulties. At enrollment, many had reduced height and weight, microcephaly, and dysmorphic features (e.g., broad nasal tip, small alae nasi, long philtrum, thin upper lip, and large ears), observed in at least 75% of the cohort. Recognition of these features may help to decrease diagnostic delays in CTNNB1 syndrome.

Neurological findings were prominent, with more than 50% showing muscle weakness, hypotonia (central and peripheral), spasticity, dystonia, and atypical hyperekplexia—consistent with previous reports.[48] Motor disorders were often accompanied by developmental delay: 91.9% of children older than 24 months could walk with support, but only 49.5% achieved independent walking, often delayed. Wheelchair use was common (72.7%), and developmental regression was reported in 35.1%.

Participants frequently had speech and language impairments, feeding difficulties, visual issues (strabismus in 81.1%, while FEVR [7.1%] and gaze palsy [3.1%] were reported less commonly), and behavioral problems. Mild intellectual disability was found in more than 50%, while 26.3% had moderate to severe disability. ASD was reported in 48.3%, along with academic challenges and increased rates of anxiety, depression, attention deficits, and cognitive issues.
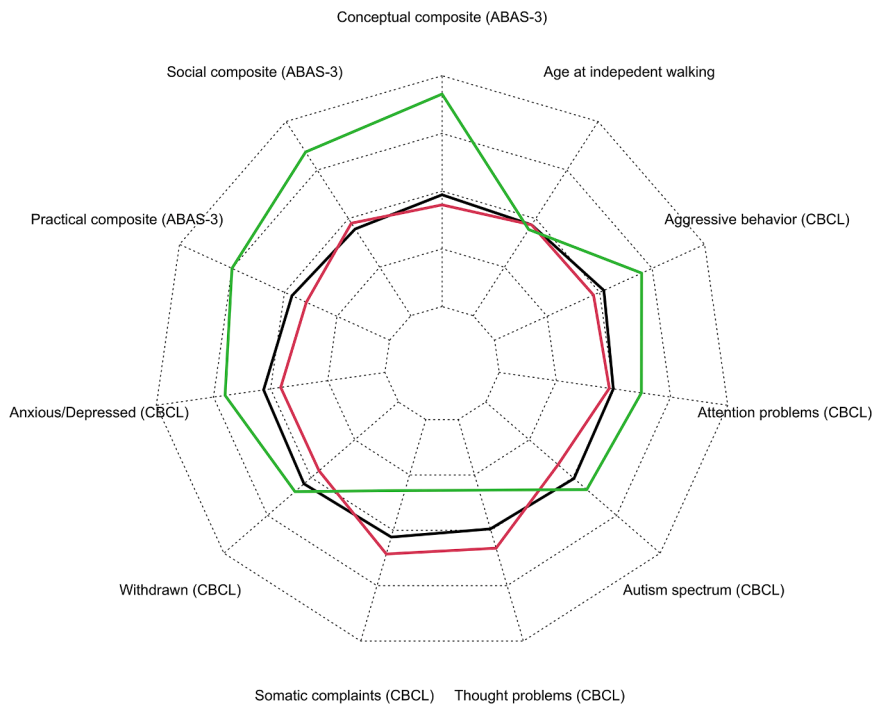
MRI abnormalities were seen in 28.1% of participants, primarily nonspecific white matter changes (76.5% of those with findings). EEG abnormalities were rare, observed in only 8.6% of cases.

Genotype-phenotype correlations

As mentioned above, we were not able to find clear evidence that the 11 participants with dominant-negative LoF variants have specific phenotypes that would distinguish them from other participants. However, the four participants with a missense variant in the *CTNNB1* gene were more likely to start walking earlier compared with participants with other variants. Participants with a missense variant were also rated as having better conceptual and social skills compared with participants with other variants. On the functional assessment scales, participants with a missense variant were rated as having better communication skills and showed fewer difficulties in terms of feeding as rated by the EDACS. Finally, the analysis of GMFCS scores indicated that their motor abilities were clearly superior to those of participants with other types of variants. In terms of psychopathology, the greatest symptom burden was found in participants with a splice variant (n = 9), as their parents reported more pronounced symptoms of anxiety and depression, ASD and aggressive behavior.

Limitations

Although this is the largest cohort of CTNNB1 participants studied systematically with standardized tests, only a proportion of known individuals living with the CTNNB1 neurodevelopmental syndrome were enrolled. The number of participants was low for statistical

purposes; therefore, more or different genotype-phenotype correlations could possibly be identified in larger cohorts of participants. The voluntary enrollment process through social media could also lead to selection bias, which could significantly skew the landscape of genetic variants and phenotypes described. Evaluating participants using standardized questionnaires and scales through online interviews—sometimes with a translator—provides an overview of their symptoms and enables an inclusive approach, allowing participation from individuals with diverse backgrounds and those living far from investigation centers. However, it cannot replace in-person examinations, such as psychological, ophthalmological, and neurological evaluations. Finally, there may be some overlap between the present cohorts and previously published cases. Due to data confidentiality, it was not possible to systematically assess overlap with previous publications, although we note that many of the individuals were based in Europe.

Suggestions for future studies

A detailed natural history study with in-person and longitudinal examinations using standardized measures would overcome many of the limitations mentioned above and would be essential for better understanding the CTNNB1 neurodevelopmental syndrome. Examinations such as MRI and wearable devices for movement analysis[49] could provide crucial additional data in understanding the syndrome. Identifying reliable blood biomarker(s) of the disease severity would be a significant aid in objectively assessing participants. Furthermore, such data would also provide important information for the design and development of potential disease modifying treatments.[12]

Conclusions

Although the CTNNB1 neurodevelopmental syndrome is a rare and understudied syndrome, rapidly expanding knowledge related to it is stimulating scientific and clinical inquiry. In the cohort of 125 participants described in this study, we have found that most carry a variant

leading to a loss of *CTNNB1* gene function and a reduction in Wnt signaling activity. However, we have identified a minority of variants with associated dominant negative or a GoF effect, warranting further studies of the genetic landscape of the CTNNB1 neurodevelopmental syndrome. This finding could be potentially important in the context of the development of potential disease modifying treatments.

Data and code availability

The majority of the data are provided in the manuscript and its supplementary materials. A reasonable request for R code should be addressed to the corresponding author.

Declaration of interests

The authors declare no conflict of interest.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.xhgg.2025.100483.

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