



ARTICLE

Genomic and phenotypic characterization of 404 individuals with neurodevelopmental disorders caused by *CTNNB1* variants



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ABSTRACT

Purpose: Germline loss-of-function variants in *CTNNB1* cause neurodevelopmental disorder with spastic diplegia and visual defects (NEDSDV; OMIM 615075) and are the most frequent, recurrent monogenic cause of cerebral palsy (CP). We investigated the range of clinical phenotypes owing to disruptions of *CTNNB1* to determine the association between NEDSDV and CP. **Methods:** Genetic information from 404 individuals with collectively 392 pathogenic *CTNNB1* variants were ascertained for the study. From these, detailed phenotypes for 52 previously unpublished individuals were collected and combined with 68 previously published individuals with comparable clinical information. The functional effects of selected *CTNNB1* missense variants were assessed using TOPFlash assay.

Results: The phenotypes associated with pathogenic *CTNNB1* variants were similar. A diagnosis of CP was not significantly associated with any set of traits that defined a specific phenotypic subgroup, indicating that CP is not additional to NEDSDV. Two *CTNNB1* missense variants were dominant negative regulators of WNT signaling, highlighting the utility of the TOPFlash assay to functionally assess variants.

Conclusion: NEDSDV is a clinically homogeneous disorder irrespective of initial clinical diagnoses, including CP, or entry points for genetic testing.

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Introduction

Neurodevelopmental disorders (NDDs) are clinically diverse and predominantly genetic in origin. For NDDs, such as epilepsy, intellectual disability (ID), vision disorders, speech disorders, and movement disorders, early clinical genetic investigations have both financial and, more importantly, clinical benefits.¹ Until recently, cerebral palsy (CP), which is often comorbid with other NDDs, was underrepresented in clinical genomic research. Studies to date suggest at least one-quarter of CP is monogenic,²⁻⁴ however

consideration of individuals for clinical genomic investigation was (and may still be) overlooked owing to the pervasive view that CP is primarily a consequence of prenatal or perinatal brain injury. One example that shows this clinical ascertainment bias for genomic investigations is neurodevelopmental disorder with spastic diplegia and visual defects (NEDSDV; OMIM 615075) caused by heterozygous (typically de novo) loss-of-function variants of *CTNNB1*. In previous clinical reviews of NEDSDV, the most prominent contributors to the phenotype were (1) impairment in cognition, (2) impairment in speech, (3)

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impairment in movement owing to abnormal muscle tones or delays in acquiring motor skills, (4) impairment in morphology or physiology of the eye, (5) microcephaly, and (6) mild craniofacial dysmorphic features.^{5,6} Germline loss-of-function variants of *CTNGB1* have appeared in clinical sequencing cohorts in which the basis for ascertainment included ID, developmental delay (DD), and autism spectrum disorders.⁷⁻⁹ Notably, *CTNGB1* was also the most frequent recurrently affected gene (4% of all diagnoses) in a cohort of 1345 individuals analyzed retrospectively on the basis of a CP diagnosis.⁴ *CTNGB1* variants have been detected in other CP sequencing cohorts, occasionally being used as grounds for change of clinical diagnosis.^{10,11} This led us to examine the breadth of phenotypic variation owing to pathogenic and likely pathogenic (P/LP) germline *CTNGB1* variants.

CTNGB1 encodes β -catenin, a member of the highly conserved armadillo repeat protein family.¹² β -Catenin performs dual functions in cells: as a component of adherens junctions, it links transmembrane cadherins to the actin cytoskeleton through α -catenin and as an essential component of the WNT signaling pathway, it acts as a transcriptional coactivator in the nucleus.¹³ During brain development, the role of β -catenin in cell adhesions is essential for proper cell migration whereas the WNT signaling pathway regulates cell proliferation and cell fate determination.¹⁴⁻¹⁶

In this study, we aimed to assess the extent of clinical heterogeneity owing to P/LP variants of *CTNGB1* through analysis of clinical and genetic data from a previously unpublished cohort of 52 individuals combined with individuals who were previously described in the literature and clinical-genetic databases. We assessed the utility of the TOPFlash to show loss of β -catenin dependent transcriptional activity and thus, interpret the pathogenicity of missense variants. In combination, quantification of the frequencies of *CTNGB1*-related traits and improved interpretation of variant effects provide a framework for precise diagnosis and counseling.

Materials and Methods

Inclusion criteria and collection of clinical data

Previously unpublished individuals described in this study were ascertained using GeneMatcher¹⁷ and personal communications through the International Cerebral Palsy Genomics Consortium.¹⁸ Fields for clinical data in [Supplemental Tables 1 and 2](#) were selected on the basis of the range of traits previously associated with P/LP *CTNGB1* variants. For unpublished cases, the referring clinical team were required to specifically indicate the presence or absence of each trait when known and supply additional information, including parental ages, gestational ages, and the number and types of genetic tests performed up to and,

including the discovery of their *CTNGB1* variant, when available. When data were unavailable, it was treated as missing rather than absence of the trait and the corresponding individual was excluded from calculations of proportions of that particular trait in the disease population. Individuals previously reported in sequencing studies in the literature or public clinical databases with 4 or less of the 6 known *CTNGB1* traits described in the introduction were grouped with the unpublished individuals when new information was provided. Individuals previously published with more than 4 known *CTNGB1* traits were grouped with previously published, even when additional information was collected. For published individuals, at least 5 out of the 6 known *CTNGB1* traits were required for inclusion in the comparisons with the cohort of unpublished individuals, with the exception of individuals with P/LP variants in *CTNGB1* that cause nonsyndromic familial exudative vitreoretinopathy (FEVR) from the following publication,¹⁹ when FEVR diagnosis was the only requirement for inclusion. To quantify individual genetic diagnostic pathways, tests were grouped into 4 types: metabolic and biochemical screens; tests for chromosomal abnormalities using karyotyping, fluorescence in situ hybridization, and chromosomal microarray; tests for specific genetic diseases involving either a single or a few genes; and exome, genome, or large-scale disease gene panels, including *CTNGB1*. The strategy for identification of unpublished and previously published individuals with *CTNGB1* variants is summarized in [Supplemental Figure 1](#).

Identification of *CTNGB1* variants in literature and public clinical genetic databases

Published literature indexed in PubMed and supplementary data from large sequencing studies were reviewed to identify *CTNGB1* variants associated with neurodevelopmental phenotypes (see [Supplemental Table 3](#) for references). ClinVar²⁰ and DECIPHER (DatabasE of genomIc variation and Phenotype in Humans using Ensembl Resources)²¹ were queried using gene symbol to identify additional *CTNGB1* variants in NDDs (last accessed on April 30, 2022) and are identified in [Supplemental Table 2](#) by their respective accession numbers. All germline protein-truncating and canonical splice site variants in *CTNGB1* were included irrespective of the depth of phenotypic information except for 5 protein-truncating variants that were implicated in cancers ([Supplemental Table 4](#)). Missense, in-frame, and splice region variants were included only when NDD phenotypes were present. All somatic variants, associated with cancers were excluded. All available information was combined when an individual was counted from a publication and was also in ClinVar or DECIPHER to exclude duplication. Three variants reported to ClinVar that were likely reported in published literature by the same group but with no specific link to the corresponding articles were excluded from the list of published cases to avoid potential double-counting.

Excluded individuals are listed with accession numbers in [Supplemental Table 4](#). Variants that were likely double reported to ClinVar with a different submission identifier by a reporting laboratory and a testing laboratory were considered as 1 and both identifiers were noted in the Patient ID field in [Supplemental Table 2](#). Structural variants affecting *CTNNB1* only or *CTNNB1* and the adjacent predicted dosage insensitive and loss-of-function tolerant gene, *ULK4*, were also counted into the collection of published *CTNNB1* variants. For all single nucleotide variants (SNVs) and small indels within exons or splice sites of *CTNNB1* in this study, the complementary DNA annotations are from the same reference NM_001904.3. All genomic locations of both SNV and structural variants are annotated against NC_000003.11.

Identification of *CTNNB1* variants not associated with NDDs

Predicted benign variants in *CTNNB1* were obtained from Genome Aggregation Database (gnomAD) (v2.1.1).²²

Statistics

Statistical analyses were performed using R (version 4.0.4, The R Foundation, Vienna, Austria).

In silico prediction of pathogenicity of missense *CTNNB1* variants

Effects of *CTNNB1* missense variants were predicted using VEST3 (Variant Effect Scoring Tool), CADD (Combined Annotation Dependent Depletion), PROVEAN (Protein Variation Effect Analyzer), DANN (Deleterious Annotation of genetic variants using Neural Networks), Polyphen2 (Polymorphism Phenotyping v2), SIFT (Sorting Intolerant from Tolerant), Mutation Assessor, MetaSVM (Meta-analytic support Vector Machine), and FATHMM (Functional Analysis Through Hidden Markov Models) using ANNOVAR (Annotate Variation; hg19 dbNSFP version 3.5a).²³ Statistical significance of pathogenicity scores between different phenotypic groups was assessed for each predictive tool using 2-tailed *t* test assuming unequal variance.

Expression plasmids

A pcDNA 3.1 mammalian expression vector with the wild-type *CTNNB1* coding sequence with a C-terminal V5 tag was provided by Yoshitaka Sekido.²⁴ From this vector, we substituted the V5 tag for a Myc tag through polymerase chain reaction–based cloning. Using overlap polymerase chain reaction, we generated 4 *CTNNB1* missense variants identified in individuals with NDDs, c.1163T>C:p.Leu388Pro (rs1559474140), c.1723G>A:p.Gly575Arg (rs797044875), c.1271T>G:p.Leu424Arg (rs863224864), and c.2128C>T:p.Arg710Cys (rs748653573), and 2 predicted benign variants

from gnomAD, c.860A>G:p.Asn287Ser (rs35288908; allele frequency = 6.02E-04) and c.1188A>C:p.Glu396Asp (rs751375496; allele frequency = 4.38E-05). Cloning strategies for these variants are summarized in [Supplemental Table 5](#). Successful cloning of these variants were confirmed using Sanger sequencing. M50 Super 8x TOPFlash (Addgene plasmid catalog number 12456; <http://n2t.net/addgene:12456>; RRID:Addgene_12456) and M51 Super 8x FOPFlash (Addgene plasmid catalog number 12457; <http://n2t.net/addgene:12457>; RRID:Addgene_12457) were gifts from Randall Moon.²⁵ Renilla luciferase vector, pRL-TK plasmid, was obtained from Promega (catalog number E2241).

Cell culture and dual luciferase reporter assay

Culturing of HEK293T cells and dual luciferase reporter assay were performed as previously described.²⁶ *CTNNB1* constructs (200 ng; wild type, mutant, or 100 ng of both) were cotransfected with TOPFlash or FOPFlash plasmid (200 ng per well) and pRL-TK plasmid (5 ng per well) using lipofectamine 2000 (Invitrogen, catalog number 11668019). A pcDNA3.1 vector lacking *CTNNB1* coding sequence (empty vector) was used as a negative control.

Western blotting

Extraction of protein from HEK293T cells transfected with β -catenin expression constructs and luciferase reporter plasmids and western blotting were performed as previously described.²⁶ Primary antibodies used in this study were anti-Myc tag 9E10 antibody (1:2000; Invitrogen, catalog number MA1-980), anti-V5 tag antibody (1:2000; ThermoFisher Scientific, catalog number R960-25), anti- β -catenin antibody (1:1000; BD transduction laboratories, catalog number 610153), and anti- β -actin antibody (1:2000; Sigma, catalog number A2228).

Results

Ascertainment of individuals with germline P/LP *CTNNB1* variants

In total, 52 individuals, comprising 28 females and 24 males with P/LP *CTNNB1* variants were ascertained from the United States, Australia, and Europe ([Figure 1](#) and [Supplemental Table 1](#)). Of these, 3 individuals (individual 6, 8, and 50) were previously published with limited or no clinical information^{10,27,28} and 9 (individual 13, 15, 18, 22, 23, 45, 48, 49, and 52) were previously reported through ClinVar²⁰ or DECIPHER²¹ with no or limited clinical information, therefore, we considered their phenotypes as unpublished ([Supplemental Figure 1](#)). At the time of ascertainment, the remainder (40/52) had not been reported in either the literature or variant databases.

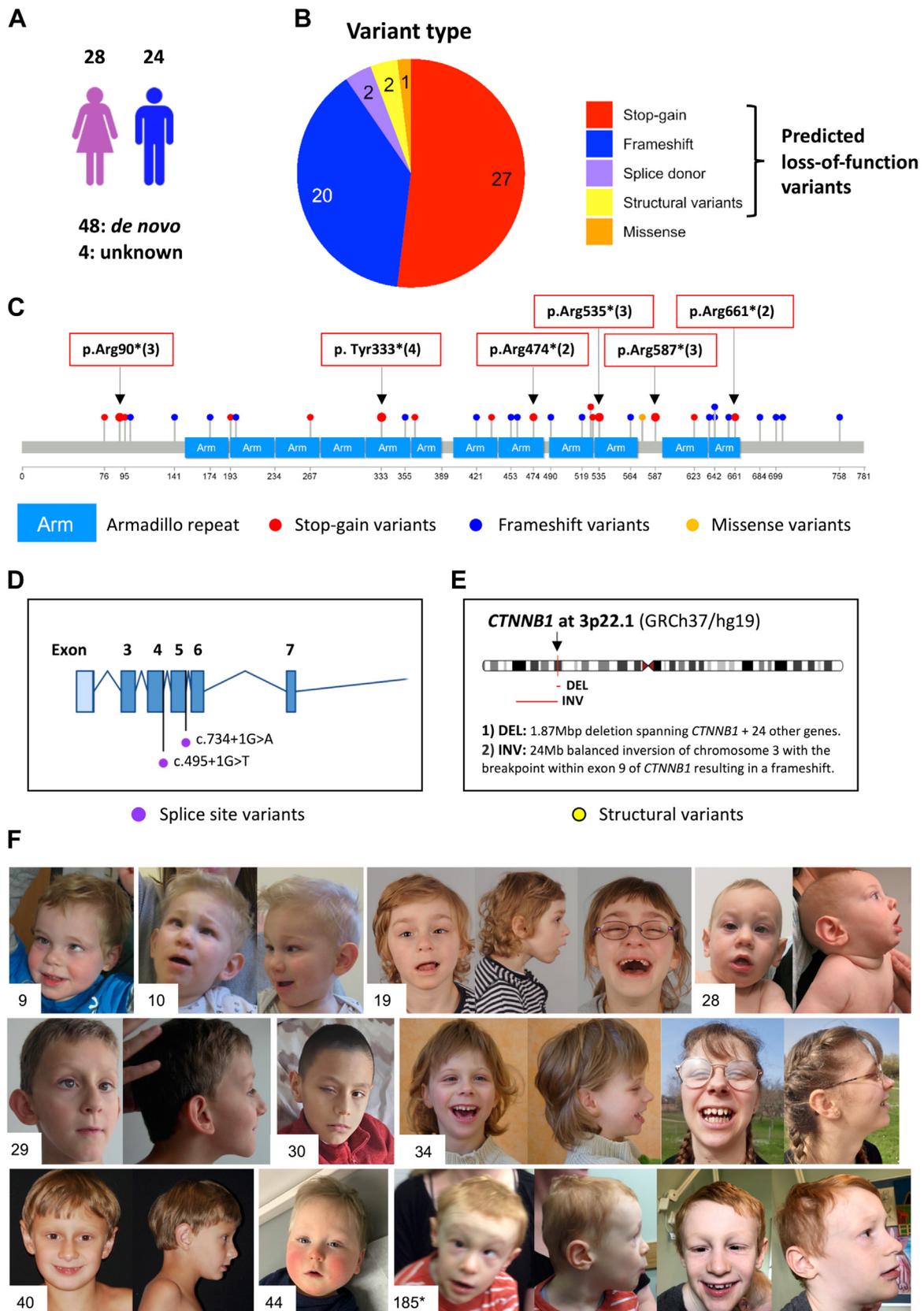


Figure 1 Graphical summary of 52 unpublished individuals with pathogenic variants in *CTNNB1*. A. The division of males and females in the cohort. B. The proportions of different types of variants. Colors represent different variant types: stop-gain variant (red), frameshift variant (blue), splice donor variant (purple), structural variant (yellow), and missense variant (orange). C. Lollipop plot shows β -catenin structure at the bottom and blue boxes represent Armadillo repeat domains in β -catenin. Each dot represents a *CTNNB1* variant identified in unpublished individuals. Recurrent variants identified in 2 or more unrelated individuals are labelled with the amino acid changes

CTNNB1 variants were confirmed as de novo in 48 affected individuals whereas inheritance of the other 4 variants was unknown because of lack of parental samples. In total, 50 individuals carried SNVs in *CTNNB1*, comprising 27 stop-gain, 20 frameshift, 2 splice donor variants, and 1 missense variant. This missense variant, NM_001904.3 (*CTNNB1*):p.Gly575Arg was recurrent,^{29,30} therefore, classified as pathogenic according to the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) guidelines.³¹ These SNVs were distributed throughout the *CTNNB1* gene with no apparent enrichment for variants in any domain (Figure 1C). All these SNVs were absent from the gnomAD (v2.1.1) and therefore considered to be rare events.²² One of the remaining 2 individuals had a de novo deletion (ISCN 2016): arr[hg19] 3p22.1(41227620_43101021)x1), whereas, the other had a de novo paracentric inversion of chromosome 3 NC_000003.11:g.16710965_41275270inv, with both of these structural variants affecting *CTNNB1* (Figure 1E). Similar structural variants spanning *CTNNB1* were absent in both gnomAD and the Database of Genomic Variants.^{22,32} Facial images of 10 individuals were provided with informed written consent. Thin upper lip vermilion was commonly identified in these individuals (Figure 1F). In line with previous reports of individuals with P/LP *CTNNB1* variants,^{5,6} predicted loss-of-function variants, including stop-gain, frameshift, canonical splice variants, and structural variants in *CTNNB1* were predominant.

We compared these 52 individuals with those previously published in clinical reports or reviews, as well as the large number of individuals that have been reported in the ClinVar²⁰ and DECIPHER²¹ clinical genetic databases without an associated publication. In total, we identified an additional 340 *CTNNB1* variants in 352 individuals that were likely involved in NDDs (Supplemental Table 2). We selected 68 individuals from this group of 352 on the basis that they had sufficient clinical information available to make meaningful comparisons to our cohort of 52 new individuals (Supplemental Figure 1 and Supplemental Table 6). The cohort of 68 previously published individuals included 9 individuals from 3 families with inherited *CTNNB1* variants: 2 families with nonsyndromic FEVR and 1 family with suspected parental germline mosaicism, otherwise, *CTNNB1* variants of published individuals were all de novo. This cohort comprised 35 females, 29 males, and 4 individuals of unreported sex.

Germline P/LP *CTNNB1* variants delineate a homogeneous syndrome

Comparison of frequencies of previously reported *CTNNB1*-related traits between unpublished ($n = 52$) and previously

published individuals ($n = 68$) identified significant differences in cognition (ID and/or DD), motor delay, and eye abnormalities (Fisher exact test, $P < .05$) (Supplemental Figure 2 and Supplemental Table 7). Exclusion of the 7 individuals with nonsyndromic FEVR who were clinically distinct from most individuals with *CTNNB1* pathogenic variants from the cohort of previously published individuals was sufficient to ablate any significant differences in traits between unpublished and previously published individuals suggesting minimal ascertainment biases in the new cohort (Fisher exact test, $P > .05$; Supplemental Table 7).

We combined data of 52 unpublished and 68 previously published individuals to delineate the common and rare traits associated with germline P/LP *CTNNB1* variants (Figure 2). Frequencies of the 9 most common neurological traits in a combined cohort of 120 individuals for which the information of each trait was available were as follows: 94.1% for ID/DD (111/118, 95% CI = 89.8%-98.3%), 93.7% for motor delay (104/111, CI = 89.2%-98.2%), 90.4% for delayed speech and language development (104/115, CI = 85.1%-95.8%), 87.5% for craniofacial dysmorphism (91/104, CI = 81.1%-93.9%), 86.0% for truncal hypotonia (80/93, CI = 79.0%-93.1%), 83.5% for mild to severe eye abnormalities (91/109, CI = 76.5%-90.5%), 79.8% for microcephaly (95/119, CI = 72.6%-87.0%), 77.9% for peripheral spasticity or hypertonia (81/104, CI = 69.9%-85.9%), and 74.2% for behavioral abnormalities (69/93, CI = 65.3%-83.1%). Neurological symptoms typically became apparent after age 2 months and by age 18 months at the latest. Onset of microcephaly was reported in 61 of 95 individuals with congenital onset (65.6%, $n = 40$) more frequent than postnatal onset (34.4%, $n = 21$). Available occipitofrontal circumference measurements of 73 of 120 individuals ranged from -8.18 SD to 0.50 SD (mean = -3.16 SD, median = -3.16 SD). Brain morphology was unremarkable for 74 of 96 (77.1%) of individuals when examined using magnetic resonance imaging, despite the high frequency of microcephaly in this cohort. The most frequently observed abnormalities were hypoplasia of the corpus callosum, delays in myelination, dilation of the lateral ventricles, and arachnoid cysts. The frequency of seizures was low with 11.4% (10/88, CI = 4.73%-18.0%). Of the 10 individuals, 7 had febrile seizures or a history of seizures that were likely self-limiting in early childhood, suggesting that P/LP *CTNNB1* variants rarely cause epilepsy.

Motor and neurological phenotypes are homogenous irrespective of a clinical diagnosis of CP

One-third (18/52) of unpublished individuals had a CP diagnosis, whereas, in previously published cases, CP was reported in only 7 of 68 individuals, but excluded in only 8

with the number of individuals in brackets. D. Variants affecting splice donor sites were identified in 2 individuals. E. Structural variants of a deletion and a balanced inversion in chromosome 3 were identified in 2 individuals. F. Facial images of 10 individuals with *CTNNB1* variants. Images were collected from unpublished individual 9, 10, 19 (at the age of 4 years and 9 years), 28, 29, 30, 34 (at the age of 6 years and 16 years and 9 months), 40, 44, and previously published individual 185.

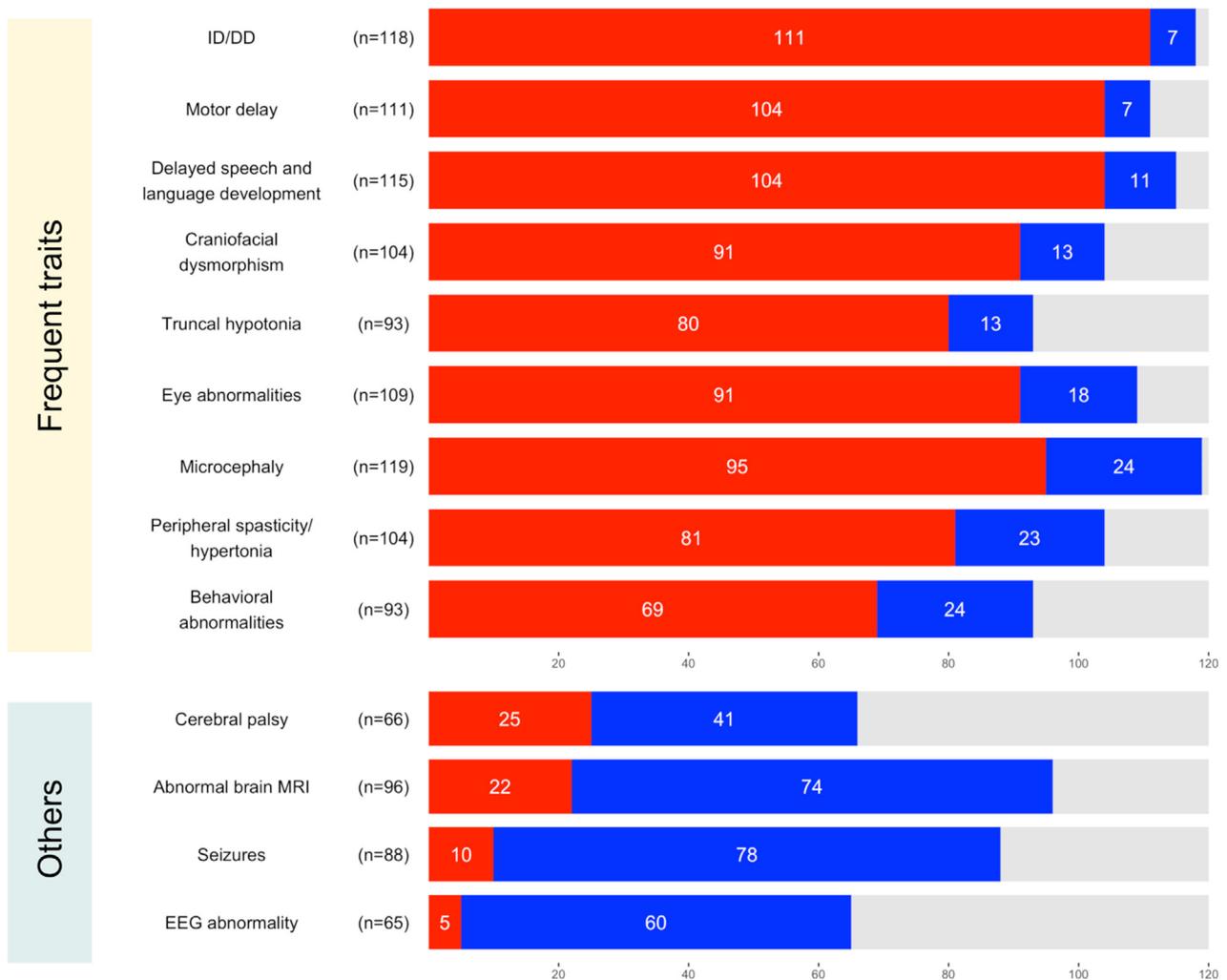


Figure 2 Neurological traits associated with pathogenic or likely pathogenic variants in *CTNNA1*. Traits that were frequently identified in a cohort of unpublished and previously published individuals are summarized at the top. Other relevant traits discussed in the present study are summarized at the bottom. Bar charts show the number of affected (red), unaffected (blue), and unknown (gray) individuals per trait. The number of individuals known for their affected status per trait is shown in brackets next to each trait. DD, developmental delay; EEG, electroencephalogram; ID, intellectual disability; MRI, magnetic resonance imaging.

of 68. Records of gestational ages available from 43 of 52 of the unpublished individuals indicated that most babies were born full term at an average of 39.3 (\pm 1.73) weeks (range 34 to 42 weeks), therefore, prematurity was not a factor associated with CP in this cohort. In previously published individuals with the records available (39/68), the average gestational age was 39.0 (\pm 2.94) weeks (range 24 to 42 weeks). Comparison of movement phenotypes between individuals diagnosed with CP ($n = 25$) and others ($n = 95$), which include those in whom the CP diagnosis had been explicitly excluded ($n = 41$, 33 unpublished and 8 previously published) and those who did not have specific mention of the diagnosis ($n = 54$, 1 unpublished and 53 previously published), found significantly increased frequency of peripheral spasticity/hypertonia in the group of individuals with CP (Fisher exact test, $P < .05$). However, the difference was not significant when we excluded 7

individuals with nonsyndromic FEVR who exhibited clinically distinct phenotypes from most individuals with P/LP *CTNNA1* variants from the analysis (Fisher exact test, $P > .1$). Regardless of inclusion or exclusion of individuals with nonsyndromic FEVR, no significant differences in frequencies of any other traits, motor delay, truncal hypotonia, ID and/or DD, delayed speech and language development, craniofacial dysmorphism, eye abnormalities, microcephaly, behavioral abnormalities, and seizures were found between individuals diagnosed with CP and others (Fisher exact test, $P > .05$, Supplemental Table 8). Movement impairments of individuals with P/LP *CTNNA1* variants were typically nonprogressive. Slowly progressive spasticity in lower limbs was only reported in 4 of 68 previously published individuals (Supplemental Table 6). In summary, individuals with P/LP *CTNNA1* variants were similarly affected irrespective of their CP diagnosis.

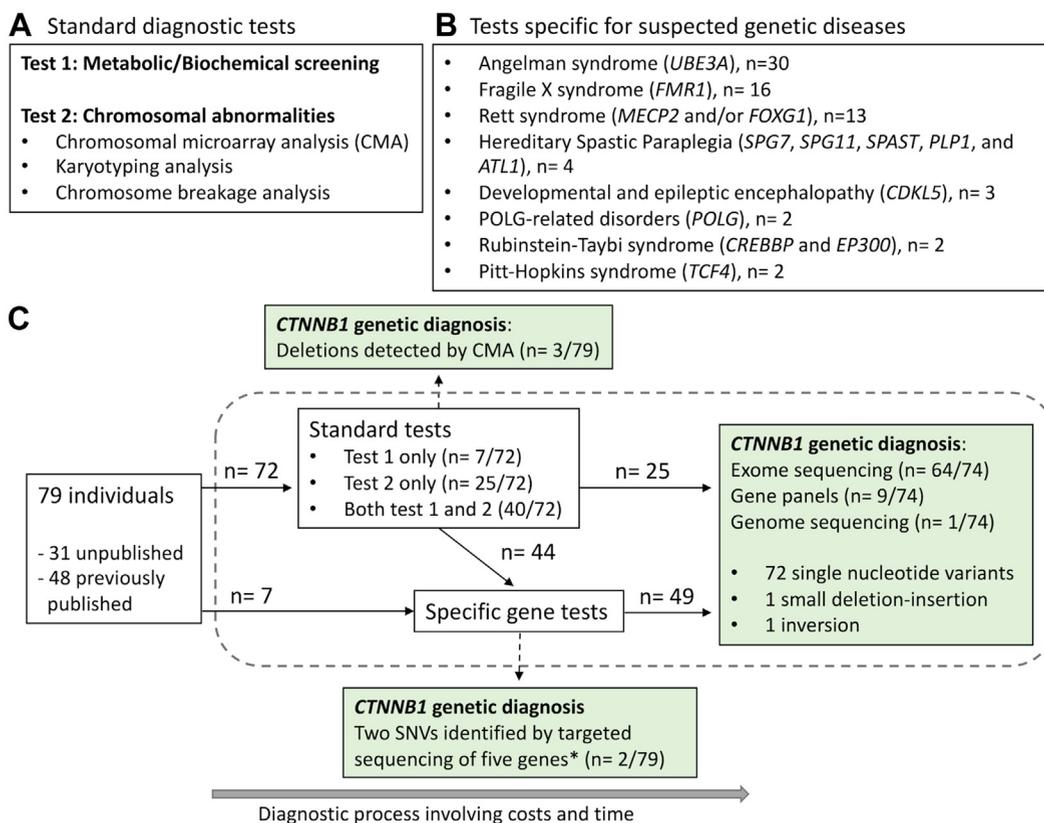


Figure 3 Diagnostic pathways of 79 individuals before their *CTNNB1* genetic diagnosis. A. Standard diagnostic tests performed during diagnostic process. B. A list of tests to assess suspected, specific genetic diseases that were performed in 2 or more individuals. C. A graphical summary of diagnostic pathways of 79 individuals before their *CTNNB1* genetic diagnosis. A total of 72 single nucleotide variants (SNVs) in *CTNNB1* were identified through exome sequencing, targeted next-generation sequencing panels, or genome sequencing. These variants included 31 frameshift, 29 stop-gain, 8 canonical splice site, and 4 missense variants. Two variants (*) were exceptionally identified through targeted sequencing of 5 intellectual disability genes, including *CTNNB1*.

Diagnostic pathways for discovery of *CTNNB1* genetic variants

We summarized diagnostic pathways that 79 individuals followed before the discovery of their P/LP *CTNNB1* variants (Figure 3). The information was newly collected from 31 unpublished and 2 previously published individuals and extracted from published information of 46 previously published individuals. Except for 3 deletions spanning *CTNNB1* that were identified through chromosomal microarray analysis and 2 published variants from a research cohort that were identified through targeted sequencing of five ID genes,⁸ P/LP *CTNNB1* variants were mostly identified through exome sequencing (86.5%, 64/74). Before their *CTNNB1* genetic diagnosis, all these individuals were assessed through standard diagnostic tests for abnormal metabolic/biochemical profiles and chromosomal abnormalities and/or tests specific for suspected genetic diseases (Figure 3 and Supplemental Table 9). In hindsight, early application of exome sequencing during testing process could have avoided

unnecessary testing to deliver faster diagnosis to most of these individuals.

Sex bias

Sex was specified for 225 individuals who we identified with neurological impairments likely because of *CTNNB1* variants, of whom, 121 were female and 104 were male, thus, the frequency of predicted P/LP *CTNNB1* variants does not appear to be biased toward a particular sex (Pearson's χ^2 test with Yates' continuity correction, $P = .48$). Limiting our analysis to the combined cohort of 120 individuals with detailed clinical data (comprising 63 females, 53 males and 4 of unreported sex), we compared frequencies of each *CTNNB1*-related neurological trait between males and females. Behavioral abnormalities were more frequently reported in females regardless of inclusion or exclusion of individuals with nonsyndromic FEVR (Fisher exact test, $P < .05$, Supplemental Table 10). Truncal hypotonia was significantly frequent in males only when individuals with

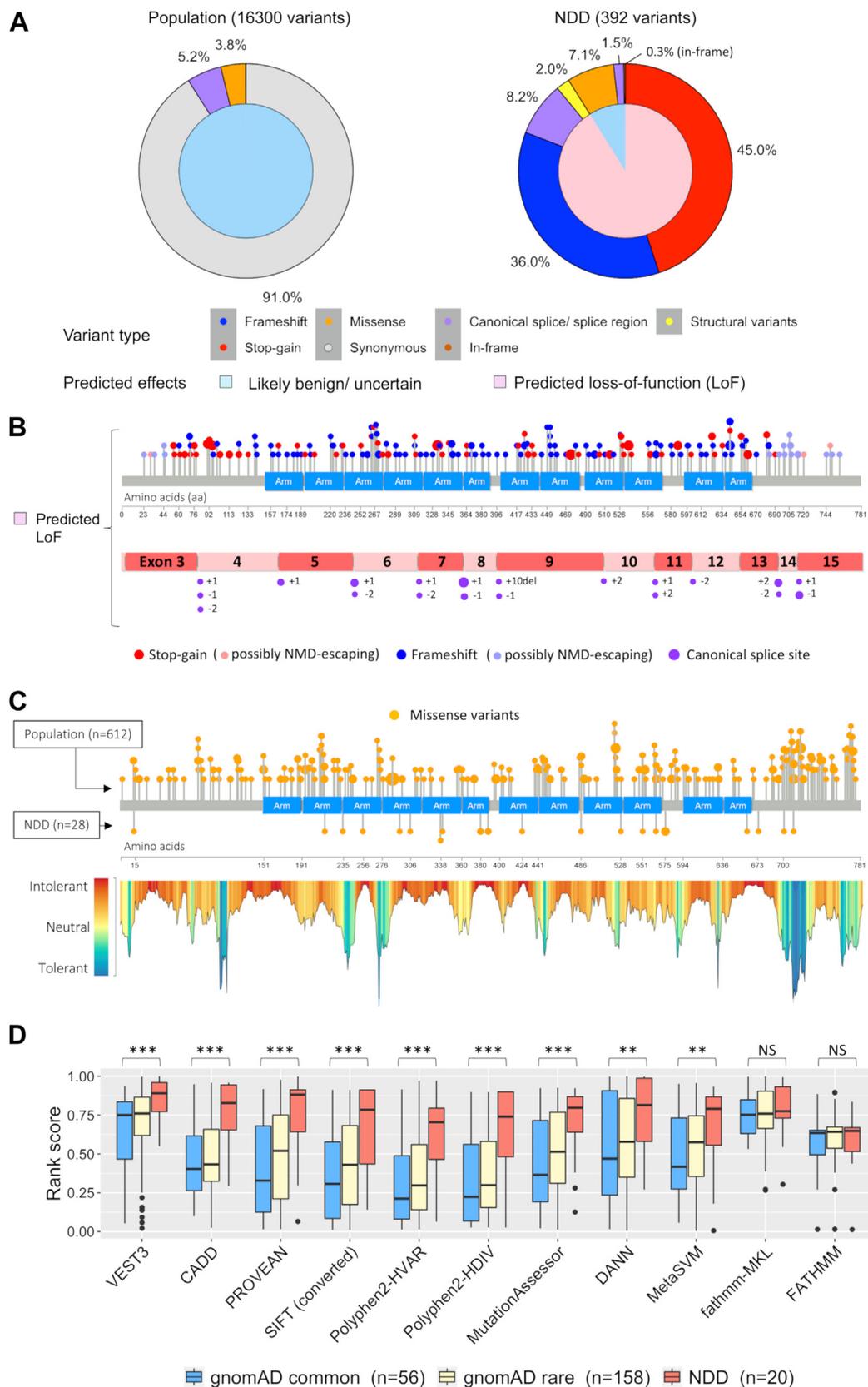


Figure 4 Analysis of phenotypic outcome by *CTNNB1* variants type. A. Distinct patterns of *CTNNB1* variants identified in the general population and NDDs with different predicted effects on *CTNNB1*. Inner pie charts show the ratio of variants with predicted effects of likely benign or uncertain (light blue) and loss of function (pink). Outer pie charts show the percentage of variants by type: synonymous (gray), missense (orange), frameshift (blue), stop-gain (red), splicing site (purple), in-frame insertions/deletions (brown), and structural variants

nonsyndromic FEVR were excluded from the analysis (Fisher exact test, $P < .05$, [Supplemental Table 10](#)).

Analysis of *CTNNB1* variants

CTNNB1 variants implicated in NDD were compared with predicted benign variants in gnomAD.²² Most *CTNNB1* variants in individuals with NDD phenotypes (91.1%, 357/392 variants) were predicted loss-of-function variants, predominantly stop-gain and frameshift variants that introduce premature termination codons in *CTNNB1* messenger RNA (mRNA) ([Figure 4A](#), [Supplemental Table 11](#)). These variants were expected to result in reduced expression of β -catenin because of nonsense-mediated mRNA decay except for 20 variants that were predicted to escape from nonsense-mediated mRNA decay because of their location in the last exon of *CTNNB1*, within 50 nucleotides upstream of the last exon-exon boundary, or proximal to the translation initiation codon³³ ([Figure 4B](#)). The vast majority of variants in gnomAD, a database in which P/LP *CTNNB1* variants causing NDDs were expected to be depleted, were synonymous changes (91.0%, 14826/16300) and only 2 predicted loss-of-function variants in *CTNNB1* were identified, each with an allele count of 1 ([Supplemental Table 11](#)). One of the 2 variants was a substitution at splice acceptor site of exon 14 (c.2077-2A>G), which likely alters normal splicing. The same variant was previously reported through ClinVar²⁰ (variant ID: VCV000985127.1) in a male with DD, delayed speech and language development, muscular hypotonia, and several craniofacial traits (submission ID: SCV001444047.1). The second variant, c.-48-2A>G, which was located at a splice acceptor site within the 5' untranslated region had a low confidence loss-of-function annotation and was of uncertain significance. Locations of *CTNNB1* canonical splice variants implicated in NDDs are shown in [Figure 4B](#).

Missense variants accounted for 7.1%, (28/392) of *CTNNB1* variants implicated in NDDs. Missense variants

identified through clinical sequencing are typically classified as variants of uncertain significance (VUS) according to ACMG/AMP guidelines³¹ because their effects on β -catenin functions are largely unknown. Missense variants reported in the gnomAD moderately clustered at the C terminus of β -catenin, as shown by areas tolerant to genetic variation identified using MetaDome³⁴ ([Figure 4C](#)). A VUS reported through ClinVar (p.Ile700Thr, rs2078481368, VCV001029547.1, SCV001522697.1) and a nonsyndromic FEVR variant (p.Arg710Cys, rs748653573) were located within this variation tolerant region at the C terminus. Most NDD-associated missense variants were located in regions intolerant to genetic variation, identified using MetaDome, supporting but not confirming the pathogenicity of these variants.

In silico analyses of *CTNNB1* missense variants

We investigated various in silico tools for predicting pathogenicity of missense variants in *CTNNB1*. Score distributions of each prediction for variants identified in NDDs were compared with those for common variants in gnomAD (56 variants with allele frequencies equal or greater than $1.0E-05$). NDD variants were scored significantly higher than common population variants by VEST3, CADD, PROVEAN, SIFT, Polyphen2, Mutation Assessor, DANN, and MetaSVM ([Figure 4D](#)). NDD variants were best distinguished from the common population variants by VEST3 (Student t test, $P = 7.72E-06$), followed by CADD (Student t test, $P = 1.46E-05$).

Functional investigation of *CTNNB1* missense variants

We tested the functional effect of missense *CTNNB1* variants identified in individuals with NDD phenotypes using TOP-Flash dual-luciferase reporter assay in HEK293T cells. Transfection of a mutant β -catenin expression vector along with a luciferase reporter that has TCF/LEF binding sites in

mainly with deletions (yellow). Percentage labels of variant types are shown when the values are greater than 0.1%. B. Variant plots showing distribution of stop-gain, frameshift, and canonical splice site variants of *CTNNB1* identified in individuals with NDD. Lollipop plot shows β -catenin structure at the bottom and blue box represents Armadillo repeat domains. Each dot represents an individual with stop-gain (red) or frameshift (blue) variant of *CTNNB1*. The larger size of a dot indicates multiple individuals with the same variant. Variants predicted to escape NMD are indicated with lighter blue (frameshift) and lighter red (stop-gain). *CTNNB1* messenger RNA structure shows exons with canonical splice site variants (purple) likely affecting normal splicing of *CTNNB1*. Locations of these splice site variants in intron regions were noted with the number of nucleotides from the last nucleotide of an exon (+) or the first nucleotide of an exon (-). C. Analysis of missense *CTNNB1* variants identified in individuals with NDD compared with likely benign variants identified in the general population. Lollipop plots show distribution of missense variants of *CTNNB1* (orange) identified in the general population (above) or individuals with NDD (below). Landscape of *CTNNB1* variant tolerance generated using MetaDome is shown under the lollipop plot. D. Summary of deleterious predictions of missense *CTNNB1* variants using 11 predictive tools. Box plots show first quartile (bottom) to third quartile (top) with each median value at the center. SIFT scores were calculated as 1-SIFT raw score. Student t test was applied to assess the difference of NDD variants against population variants with allele frequency equal or greater than $1.0E-05$ (gnomAD common). The significance marked with *** indicates $P = .001$, ** indicates $P = .01$, * indicates $P = .05$, or NS indicates not significant. CADD, Combined Annotation Dependent Depletion; DANN, Deleterious Annotation of genetic variants using Neural Networks; FATHMM, Functional Analysis Through Hidden Markov Models; HDIV, HumDIV; HVAR, HumVar; MKL, multiple kernel learning; NDD, neurodevelopmental disorder; NMD, nonsense-mediated mRNA decay; Polyphen, polymorphism phenotyping; PROVEAN, protein variation effect analyzer; SIFT, sorting intolerant from tolerant; VEST, Variant Effect Scoring Tool.

the promoter region specifically assesses the effect of the mutant β -catenin on regulation of the WNT signaling pathway. We cloned 4 P/LP variants: p.Leu388Pro, p.Leu424Arg, p.Gly575Arg, and a nonsyndromic FEVR variant, p.Arg710Cys, along with 2 predicted benign variants as controls, p.Asn287Ser and p.Glu396Asp, from the gnomAD. The variant, p.Leu388Pro was reported in a male exhibiting full *CTNNB1*-related neurological traits with an exception of abnormalities of the eye.⁵ The same variant reported in ClinVar²⁰ was classified as VUS (VCV000560986.1, SCV000807393.1). The variant, p.Leu424Arg was identified in a male with CP, DD, microcephaly, and dysmorphic traits.³⁵ The third variant, p.Gly575Arg was recurrently identified in 6 previously published individuals and 1 individual from this study (Supplemental Table 1 and 2). Neurological traits shared 2 or more among these 7 individuals were DD, motor delay, truncal hypotonia, microcephaly, craniofacial dysmorphism, and eye abnormalities, including FEVR, retinal detachment, and loss of vision.

The abundance of some transfected β -catenin variant proteins was variable in comparison to wild type (Figure 5A and 5B), but relatively similar at the mRNA level (Supplemental Figure 3), suggesting that some of these variants alter protein stability. We observed a significant difference in reporter activities with the addition of different epitope tags, Myc or V5, to the wild-type construct (Student *t* test, $P < .001$), therefore, we used Myc epitope tagged constructs for all comparisons between mutant and wild-type β -catenin (Figure 5C).

TOPFlash activity was absent for 2 NDD variants, p.Leu388Pro and p.Leu424Arg compared with the activity of wild-type β -catenin (Student *t* test, $P < .001$; Figure 5C). These 2 variants were dominant negative and significantly repressed TOPFlash activity when coexpressed with wild-type β -catenin (Figure 5C). In contrast, both of the predicted benign variants from gnomAD and the nonsyndromic FEVR variant significantly increased TOPFlash activity compared with the wild type (Student *t* test, $P < .05$). Increases in TOPFlash activity were also observed when each of these 3 variants were cotransfected an equal amount of the expression construct of the wild-type β -catenin; however, this was not statistically significant (Student *t* test, $P > .05$). Unexpectedly, TOPFlash activity was not altered by the recurrent p.Gly575Arg variant compared with wild-type β -catenin in this assay (Student *t* test, $P = .283$). None of the constructs tested in this assay had an effect on the negative control FOPFlash reporter, which had nonfunctional TCF/LEF binding motifs (Supplemental Figure 4). In summary, the TOPFlash assay facilitated functional assessment of *CTNNB1* missense variants. We were able to resolve p.Leu388Pro and p.Leu424Arg as likely dominant negative variants affecting the WNT signaling pathway, which was greater than the effect of haploinsufficiency caused by the known pathogenic loss-of-function variants. The functional effect of p.Gly575Arg was not evident using this assay; however, given that this variant is recurrent, there is already sufficient evidence to determine that it is pathogenic.

Discussion

Prompted by multiple prior observations of individuals with P/LP *CTNNB1* variants and clinical diagnosis of CP, we sought to identify whether this diagnosis defines a specific phenotypic subgroup. Combined phenotypes from 120 individuals however revealed that P/LP *CTNNB1* variants result in relatively consistent clinical traits in both males and females, suggesting that the CP diagnosis might reflect clinical ascertainment bias. These data support that genomic testing is beneficial for individuals with CP so that they have clear and fast genetic diagnosis irrespective of initial clinical diagnosis. Our results show overwhelming evidence for heterozygous loss-of-function of *CTNNB1* as the predominant disease mechanism. Analysis of missense variants however, showed that not all may affect WNT signaling, which may influence the design of future targeted therapies.

Inconsistent and incomplete clinical information for individuals with variants collected from the literature and public clinical databases is a limitation of this study. We did not exclude individuals from any population from these analyses; however, the 52 previously unpublished individuals in this study were sourced as a convenience cohort from countries where there is underlying bias in the represented races and ethnicities. We may have overestimated the overall number of reported variants when a single case was captured by multiple databases and we had no handle to determine such duplication.

Looking historically at the discovery of *CTNNB1* variants implicated in NDDs, cohorts were recruited for clinical sequencing studies of ID, autism, epilepsy, DD, FEVR and CP. Although several reviews pointed toward a consistent syndrome, it remained unclear that if some genotype–phenotype relationships exist or if these findings resulted from ascertainment biases. We assembled the majority of known individuals with *CTNNB1* variants identified to date and our data overwhelmingly support that P/LP *CTNNB1* variants result in a syndrome with consistent neurological traits, except in the case of nonsyndromic FEVR. A health care plan for a child genetically diagnosed with a P/LP *CTNNB1* variant should be prepared with oversight from their pediatrician, neurologist, and ophthalmologist and include access to physiotherapy, special education, speech, behavioral, and occupational therapy. Transitioning to adulthood, speech may not improve, complications owing to spasticity should be anticipated, and the risk of retinal detachment; however, it should be noted that less than 10 individuals older than 18 years have been described to date. At present, there are several international *CTNNB1*-specific support groups that families can be made aware of. In the case of CP, the prominent movement impairments in individuals with P/LP *CTNNB1* variants have been well characterized as truncal hypotonia and usually nonprogressive peripheral spasticity or hypertonia,^{5,6} which is consistent with Surveillance of Cerebral Palsy in Europe guidelines for diagnosing CP.^{36,37} In previous studies, a

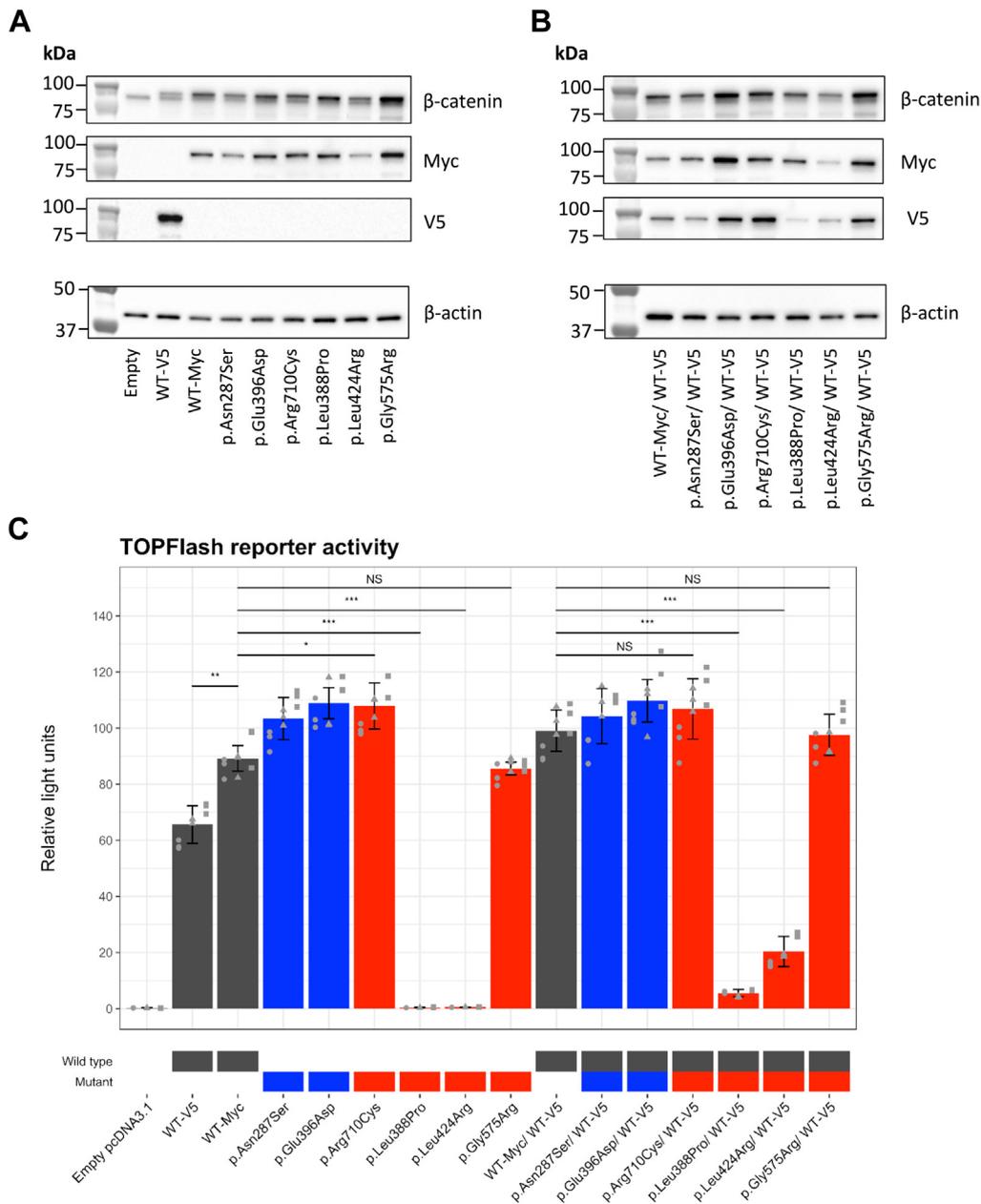


Figure 5 Functional assessment of missense *CTNNB1* variants using TOPFlash assay. A, B. Detection of Myc or V5-tagged WT β -catenin proteins and Myc-tagged mutant β -catenin proteins transfected into HEK293T cells by western blot. Expression constructs were transfected without cotransfection (A) and with cotransfection of a V5-tagged WT β -catenin (B). Molecular sizes of standard protein markers were indicated on the left of blots. Endogenous and exogenous β -catenin were detected using a β -catenin antibody (amino acid 571-781). Exogenous β -catenin was identified using a V5 antibody, and a Myc antibody. Endogenous levels of β -actin were detected to show equal loading in western blotting. Full blots are available in [Supplemental Figure 5](#). C. Effects of missense *CTNNB1* variants Wnt signaling as measured by the TOPFlash assay. Relative luciferase activity measured using the TOPFlash assay in HEK293T cells transfected with expression vectors for WT β -catenin or mutant β -catenin or an equal mixture using WT β -catenin tagged with V5. WT, Genome Aggregation Database variants, and pathogenic/likely pathogenic variants are highlighted in gray, blue, and red on the X-axis labels, respectively. Assay was performed in triplicate (shown with different shaped data points) with 3 technical replicate samples for each assay. Error bars indicate SDs between the 3 independent experiments. Student *t* test was applied to assess the difference of relative light unit of pathogenic/likely pathogenic variants against that of WT β -catenin. The significance marked with *** indicates $P = .001$, ** indicates $P = .01$, * indicates $P = .05$, or NS indicates not significant. WT, wildtype.

genetic diagnosis of *CTNNB1* variant was considered as grounds to remove a CP diagnosis;¹¹ however, based on our study, a clinical diagnosis of CP is appropriate and

recommended for individuals with P/LP *CTNNB1* variants associated with nonprogressive lower extremity hypertonia. Regardless of diagnostic clinical labels, it is most important

that these individuals have early and equitable access to genomic testing. More than half of individuals in whom it was possible to trace a diagnostic odyssey underwent a targeted single gene or gene-panel before receiving a diagnosis from exome or genome sequencing, which highlights the importance of genomic analysis for identifying P/LP *CTNNB1* variants (Figure 3, Supplemental Figure 6).

We assessed the functional effect of missense *CTNNB1* variants, including VUS, using TOPFlash dual-luciferase reporter assay. Using this established assay, effect of these variants on β -catenin mediated transactivation of WNT signaling pathway target genes can provide strong evidence for pathogenicity when loss-of-function or dominant negative effects are observed (PS3 in the ACMG/AMP guidelines). The TOPFlash assay does not account for all the functions of β -catenin, therefore, the result of the assay needs to be carefully interpreted. Negative results in the assay, as seen for the p.Gly575Arg variant assessed in this study, do not refute pathogenicity. The negative result of the p.Gly575Arg variant may be explained by cell-type specific effect of the variant on WNT signaling pathway. We note that delayed speech and language development, a trait frequently associated with P/LP *CTNNB1* variants, was specifically ruled out in 2 out of 7 individuals with p.Gly575Arg variant and not mentioned as a trait affecting the remaining 5 individuals. Therefore, this variant may only affect a subset of *CTNNB1* functions, manifesting as lack of speech delay.

CTNNB1 is currently associated with 2 neurological phenotypes: NEDSDV (OMIM 615075) and exudative vitreoretinopathy (EVR or FEVR; OMIM 617572), which is characterized by incomplete peripheral vascular development in the retina.³⁸ Whether the pathogenic mechanisms of NEDSDV and FEVR overlap is unknown. Detailed ophthalmologic examination was not available for most of our unpublished individuals, and reporting was variable in published individuals, therefore, we could not provide the exact frequency of FEVR in our cohort. Norrin induced Frizzled4/ β -catenin signaling, a particular derivative of WNT signaling pathway, likely attributes FEVR.³⁹ Mouse models with knockout mutations in FEVR genes (*Fzd4*, *Lrp5*, *Tspan12*, and *Ctnnb1*) developed defects in retinal vasculature, suggesting that reduced activity of WNT signaling pathway leads to FEVR.^{38,40-42} However, studies of non-syndromic FEVR variants in *CTNNB1* using the TOPFlash assay resulted in contradicting effects on the transcriptional activities.¹⁹ Further functional studies on these variants may be able to identify a specific cause of FEVR.

There are currently no established interventions or treatments for NEDSDV. Treatment with L-dopamine was used in 1 female with a stop-gain variant (p.Gln558*) of *CTNNB1* that resulted in improvements in her motor skills.⁴³ These encouraging results require confirmation in a larger case series with standardized outcomes and, if indicated, a full-scale randomized control trial to determine the benefits of L-dopamine treatment for individuals with P/LP *CTNNB1* variants. Clinical homogeneity of individuals with P/LP *CTNNB1* variants suggests that there is minimal effect

of individual-specific genetic or environmental factors on *CTNNB1*-related phenotypes, which would simplify modeling this disease for the purposes of identifying the potential interventions. The Batface (Bfc) mouse, which has a heterozygous missense variant, p.Thr653Lys, in *Ctnnb1* was proposed as a potential model for NEDSDV on the basis of the similar craniofacial features observed between the Bfc mouse and individuals with loss-of-function variants of *CTNNB1*.⁴⁴ Molecular characterization of the Bfc variant in mice identified reduced interaction between β -catenin and N-cadherin at cell adhesions in hippocampus⁴⁴ and surprisingly a gain of WNT signaling activity in embryos.⁴⁵ Thus, the Bfc mice do not model *CTNNB1* haploinsufficiency, which is the typical effect of variants in patients. The effect of the Bfc variant and the p.Gly575Arg variant in the TOPFlash assay may suggest that dysregulation of the role of *CTNNB1* in cell-cell adhesion may be the major contributor to phenotypes associated with NEDSDV. A robust assay that can reliably test the role of β -catenin missense variants in cell-cell adhesion remains to be developed, however, the coimmunoprecipitation approach used to quantify the interaction between the Bfc variant and N-cadherin could be applied to other missense variants.⁴⁴ Heterozygous *Ctnnb1* knockout mice also failed to recapitulate developmental abnormalities reported in individuals with P/LP *CTNNB1* variants,^{46,47} possibly indicating that differences in developmental process between human and mice are critical to model this NDD. In our accumulated 392 *CTNNB1* variants identified in NDDs, the most recurrent variant was p.Tyr333* (Supplemental Table 12). Recently, an induced pluripotent stem cell (iPSC) line, which was capable of differentiating into all 3 germ layers, was established from a male individual heterozygous for the p.Tyr333* variant.⁴⁸ This induced pluripotent stem cell line or equivalent human cell models are promising avenues elucidating the disease mechanism behind NEDSDV and potential identification of drugs capable of restoring normal development through stabilization of *CTNNB1*.

Data Availability

Where not otherwise indicated (by eg, ClinVar accession number), all source data for this article and supplemental information are available from the corresponding author on request. Requests for potentially identifiable data are subject to approval by the Women's and Children's Health Network Human Research Ethics Committee.

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Ethics Declaration

This study was approved by the Women's and Children's Health Network Human Research Ethics Committee number 2020/HRE01273. Written informed consent was obtained for all individuals for whom new data are presented in this study. Individual level data in this study are de-identified. Copies of explicit informed written consent for patients providing photographs (Figure 1F) are archived with the corresponding author.

Conflict of Interest

F.M. and M.M.M. are employees of GeneDX, Inc. All other authors declare no conflict of interest.

Additional Information

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