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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 due 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 From these, detailed phenotypes for 52 previously unpublished individuals were collected and combined with 68 previously published individuals with comparable clinical information available. The functional effects of selected *CTNNB1* missense variants were assessed by 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 signalling, highlighting the utility of the TOPFlash assay to functionally assess variants.

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

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Conflicts of Interest

Two members of our authorship Dr Michelle Morrow and Dr Francisca Millan declare their employment with GeneDx as a conflict of interest in this study and there are no other conflicts to declare.

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.

Introduction

Neurodevelopmental disorders (NDDs) are clinically diverse and predominantly genetic in origin. For NDDs such as epilepsy, intellectual disability (ID), vision, speech and movement disorders, early clinical genetic investigations have both financial and more importantly, clinical benefit.[1] Until recently, cerebral palsy (CP) which is often comorbid with other NDDs, was under represented 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 due to the pervasive view that CP is primarily a consequence of prenatal or perinatal 'brain injury.' One example that demonstrates this clinical ascertainment bias for genomic investigations is Neurodevelopmental Disorder with Spastic Diplegia and Visual Defects (NEDSDV; OMIM: 615075) is 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 impairments in (i) cognition, (ii) speech, (iii) movement due to abnormal muscle tones or delays in acquiring motor skills, (iv) morphology or physiology of the eye, (v) microcephaly, and (vi) mild craniofacial dysmorphic features.[5,6] Germline loss-of-function variants of *CTNNB1* have appeared in clinical sequencing cohorts where the basis for ascertainment included ID, developmental delay (DD) and autism spectrum disorders.[7–9] Notably, *CTNNB1* was also the most frequent recurrently affected gene (4% of all diagnoses) in a cohort of 1,345 individuals analysed retrospectively on the basis of a CP diagnosis.[4] *CTNNB1* 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 due to pathogenic and likely pathogenic (P/LP) germline *CTNNB1* variants.

CTNNB1 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 signalling pathway, it acts as a transcriptional co-activator in the nucleus.[13] During brain development, the role of β-catenin in cell adhesions is essential for proper cell migration while the WNT signalling pathway regulates cell proliferation and cell fate determination.[14–16]

Here, we present phenotypes of 52 previously unpublished individuals with NEDSDV due to P/LP variants in *CTNNB1* and compare them to 68 previously described individuals. We show that there is a common phenotype among all individuals with NEDSDV except for eye-related pathologies. A clinical diagnosis of CP was not significantly associated with any other set of traits that defined a specific phenotypic subgroup, indicating that CP is not due to an additional environmental or secondary genetic cause.

Materials and Methods

Inclusion criteria and collection of clinical data

Fields for clinical data in Supplementary Table 1 were selected based on the range of traits previously associated with P/LP *CTNNB1* variants. For unpublished cases, the referring

clinical team were required to specifically indicate presence or absence of a trait when known. Where 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 four or less of the six known *CTNNB1* traits described in the introduction were grouped with the unpublished individuals when new information was provided. Individuals previously published with more than four known *CTNNB1* traits, were grouped with previously published even when additional information was collected. For published individuals, at least five out of the six known *CTNNB1* traits were required for inclusion in the comparisons with the cohort of 52 unpublished individuals. Identification of unpublished and previously published individuals with *CTNNB1* variants is summarized in Supplementary Figure 1.

Identification of 340 CTNNB1 variants in literature and public clinical genetic databases

Published literature indexed in PubMed and supplementary data from large sequencing studies were reviewed to identify *CTNNB1* variants associated with neurodevelopmental phenotypes (see Supplementary Table 2 for references). ClinVar[17] and DECIPHER[18] were queried to identify additional *CTNNB1* variants in NDDs (last accessed on April 30th, 2022) and are identified in Supplementary Table 3 by their respective accession numbers. All germline protein-truncating and canonical splice site variants in *CTNNB1* were included irrespective of the depth of phenotypic information except for five protein-truncating variants that were implicated in cancers (Supplementary 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. Genomic and phenotypic 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 Supplementary Table 4. Variants which were likely double reported to ClinVar with a different submission identifier by a reporting laboratory and a testing laboratory were considered as one and both identifiers were noted in the Patient ID field in Supplementary Table 3. Structural variants impacting *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.

Identification of CTNNB1 variants not associated with NDDs

Predicted benign variants in *CTNNB1* were obtained from gnomAD (v2.1.1)[19].

Statistics

Statistical analysis was performed using R (version 4.0.4).

In-silico prediction of pathogenicity of missense CTNNB1 variants

Effects of *CTNNB1* missense variants were predicted by VEST3, CADD, PROVEAN, DANN, Polyphen2, SIFT, Mutation Assessor, MetaSVM, and FATHMM using ANNOVAR (hg19 dbNSFP version 3.5a).[20] Statistical significance of pathogenicity scores between different phenotypic groups was assessed for each predictive tool using two-tailed t-test assuming unequal variance.

Expression plasmids

A pcDNA 3.1 mammalian expression vector carrying wild-type *CTNNB1* coding sequence with a C-terminal V5 tag was provided by Dr. Yoshitaka Sekido.[21] From this vector, we substituted the V5 tag for a Myc tag by PCR-based cloning. Using overlap PCR, we generated four *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), c.2128C>T:p.Arg710Cys (rs748653573), and two predicted benign variants from gnomAD database, c.860A>G:p.Asn287Ser (rs35288908; Allele frequency [AF] 6.02E-04) and c.1188A>C:p.Glu396Asp (rs751375496; AF 1.77E-05). Cloning strategies of these variants are summarized in Supplementary Table 5. Successful cloning of these variants was confirmed by Sanger sequencing. M50 Super 8x TOPFlash (Addgene plasmid # 12456; http://n2t.net/addgene:12456; RRID:Addgene_12456) and M51 Super 8x FOPFlash (Addgene plasmid # 12457; http://n2t.net/addgene:12457; RRID:Addgene_12457) were a gift from Randall Moon.[22] Renilla luciferase vector, pRL-TK plasmid was obtained from Promega (Cat #E2241).

Cell culture and dual luciferase reporter assay

Culturing of HEK293T cells and dual luciferase reporter assay were performed as previously described.[23] CTNNB1 constructs (200ng; wild type, mutant or 100ng of both) were co-transfected with TOPFlash or FOPFlash plasmid (200 ng per well) and pRL-TK plasmid (5ng per well) using lipofectamine 2000 (Invitrogen, Cat #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.[23] Primary antibodies used in this study were anti-Myc tag 9E10 antibody (1:2000), anti-V5 tag antibody (1:2000, ThermoFisher Scientific, Cat #R960-25), anti-β-catenin antibody (1:1000, BD transduction laboratories, Cat#610153), and anti-β-actin antibody (1:2000, Sigma, Cat #A2228).

Results

Ascertainment of individuals with germline P/LP CTNNB1 variants

Fifty two individuals, comprising 28 females and 24 males with P/LP *CTNNB1* variants were ascertained from the United States, Australia, and Europe using GeneMatcher[24] and personal communications through the International Cerebral Palsy Genomics Consortium[25]

(Figure 1 and Supplementary Table 1). Three of these (Individual 6, 8, and 50) were previously published with limited or no clinical information[10,26,27] and nine (Individual 13, 15, 18, 22, 23, 45, 48, 49, and 52) were previously reported through ClinVar[17] or DECIPHER[18] with no or limited clinical information, therefore we considered their phenotypes as unpublished (Supplementary Figure 1). At the time of ascertainment, the remainder (40/52) had not been reported in either the literature or variant databases.

CTNNB1 variants were confirmed as *de novo* in 48 affected individuals while inheritance of the other four variants was unknown due to lack of parental samples. Fifty individuals carried single nucleotide variants (SNVs) in *CTNNB1* comprising 27 stop-gain, 20 frameshift, two splice donor variants, and one missense variant. This missense variant, NM_001904.3(*CTNNB1*):p.Gly575Arg was recurrent,[28,29] therefore, classified as pathogenic according to the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) guidelines.[30] 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 database (v2.1.1) and therefore considered to be rare events.[19] One of the remaining two individuals had a *de novo* deletion (ISCN 2016): arr[hg19] 3p22.1(41227620_43101021)x1), while the other had a *de novo* paracentric inversion of chromosome 3 NC_000003.11:g.16710965_41275270inv, with both of these structural variants impacting *CTNNB1* (Figure 1E). Similar structural variants spanning *CTNNB1* were absent in both gnomAD and the Database of Genomic Variants.[19,31] Facial images of 10 individuals were provided with informed written consent. Thin upper lip vermillion 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 ClinVar[17] and DECIPHER[18] clinical genetic databases without an associated publication. In total, we identified an additional 340 *CTNNB1* variants in 352 individuals which were likely involved in NDDs (Supplementary Table 3). 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 (Supplementary Figure 1 and Supplementary Table 6). The cohort of 68 previously published individuals included nine individuals from three families with inherited *CTNNB1* variants: two families with non-syndromic familial exudative vitreoretinopathy (FEVR) and one family with suspected parental germline mosaicism, otherwise *CTNNB1* variants of published individuals were all *de novo*. This cohort comprised 35 females, 29 males, and four 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’s exact

test, p< 0.05) (Supplementary Figure 2, Supplementary Table 7). Exclusion of the seven individuals with non-syndromic FEVR who were clinically distinct from the majority of individuals carrying *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's exact test, p> 0.05; Supplementary 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. Frequencies of the nine most common neurological traits in a combined cohort of 120 individuals where the information of each trait was available, were as follows: 94.1% with ID/DD (111/118, 95% confidence interval (CI)= 89.8%–98.3%), 93.7% with motor delay (104/111, CI=89.2%–98.2%), 90.4% with delayed speech and language development (104/115, CI= 85.1%–95.8%), 87.5% with craniofacial dysmorphism (91/104, CI= 81.1%–93.9%), 86.0% with truncal hypotonia (80/93, CI= 79.0%–93.1%), 83.5% with mild to severe eye abnormalities (91/109, CI = 76.5%–90.5%), 79.8% with microcephaly (95/119, CI = 72.6%–87.0%), 77.9% with peripheral spasticity or hypertonia (81/104, CI = 69.9%–85.9%), and 74.2% with behavioral abnormalities (69/93, CI = 65.3%–83.1%). Neurological symptoms typically became apparent after two months of age and by 18 months at the latest. Onset of microcephaly was reported in 61/95 individuals with congenital onset (65.6%, n=40) more frequent than postnatal onset (34.4%, n=21). Available occipitofrontal circumference measurements of 73/120 individuals ranged from −8.18SD (standard deviations) to 0.50SD (mean= −3.16SD, median= −3.16SD). Brain morphology was unremarkable for 74/96 (77.1%) of individuals when examined by magnetic resonance imaging (MRI), despite the high frequency of microcephaly in this cohort. The frequency of seizures was low 11.4% (10/88, CI= 4.73%–18.0%). Seven out of the 10 individuals had febrile seizures or a history of seizures that were likely self-limiting in early childhood, suggesting 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, while in previously published cases, CP was reported in only 7/68 individuals but excluded in only 8/63. Records of gestational ages available from 43/52 of the unpublished individuals indicated most babies were born full term at an average of 39.3 (± 1.73) weeks; range 34– 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 (± 2.94) weeks; range 24– 42 weeks. Comparison of movement phenotypes between individuals diagnosed with CP (n= 25) and others (n= 95) which include those where 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's exact test, p< 0.05). However, the difference was not significant when we excluded seven individuals with non-syndromic FEVR who exhibited clinically distinct phenotypes from the majority

of individuals with P/LP *CTNNB1* variants from the analysis (Fisher's exact test, p> 0.1). Regardless of inclusion or exclusion of individuals with non-syndromic FEVR, no significant difference 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 was found between individuals diagnosed with CP and others (Fisher's exact test, p> 0.05, Supplementary Table 8). Movement impairments of individuals with P/LP *CTNNB1* variants were typically non-progressive. Slowly progressive spasticity in lower limbs was only reported in 4/68 previously published individuals (Supplementary Table 6). In summary, individuals with P/LP *CTNNB1* variants were similarly affected irrespective of their CP diagnosis.

Diagnostic pathways for discovery of CTNNB1 genetic variants

We summarized diagnostic pathways that 79 individuals followed prior to 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 three deletions spanning *CTNNB1* that were identified by chromosomal microarray analysis (CMA) and two 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). Prior to their *CTNNB1* genetic diagnosis, all these individuals were assessed by standard diagnostic tests for abnormal metabolic / biochemical profiles and chromosomal abnormalities and / or tests specific for suspected genetic diseases (Figure 3 and Supplementary Table 9). In hindsight, early application of exome sequencing during testing process could have avoided unessential testing to deliver faster diagnosis to the majority of these individuals.

Sex bias

Sex was specified for 225 individuals that we identified with neurological impairments likely due to *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 towards a particular sex (Pearson's Chi-squared test with Yates' continuity correction, p= 0.48). Limiting our analysis to the combined cohort of 120 individuals with detailed clinical data (comprising 63 females, 53 males and four 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 non-syndromic FEVR (Fisher's exact test, p<0.05, Supplementary Table 10). Truncal hypotonia was significantly frequent in males only when individuals with non-syndromic FEVR were excluded from the analysis (Fisher's exact test, p<0.05, Supplementary Table 10).

Analysis of CTNNB1 variants

CTNNB1 variants implicated in NDD were compared to predicted benign variants in gnomAD.[19] The majority of *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* mRNA (Figure

4A, Supplementary Table 11). These variants were expected to result in reduced expression of β-catenin due to nonsense-mediated mRNA decay (NMD) except for 20 variants which were predicted to escape from NMD due to their location in the last exon of *CTNNB1*, within 50 nucleotides upstream of the last exon-exon boundary, or proximal to the translation initiation codon[32] (Figure 4B). The vast majority of variants in gnomAD, where P/LP *CTNNB1* variants causing NDDs were expected to be depleted, were synonymous changes (91.0%, 14826/16300) and only two predicted loss-of-function variants in *CTNNB1* were identified, each with an allele count of one (Supplementary Table 11). One of the two 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 ClinVar[17] (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 donor 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 by clinical sequencing are typically classified as variants of uncertain significance (VUS) according to ACMG/AMP guidelines[30] because their effects on β-catenin functions are largely unknown. Missense variants reported in the gnomAD database moderately clustered at the C terminus of β-catenin, as demonstrated by areas tolerant to genetic variation identified by MetaDome[33] (Figure 4C). A VUS reported through ClinVar (p.Ile700Thr, rs2078481368, VCV001029547.1, SCV001522697.1) and a non-syndromic FEVR variant (p.Arg710Cys, rs748653573) were located within this variation tolerant region at the C terminus. The majority of NDD-associated missense variants were located in regions intolerant to genetic variation identified by 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 to 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's t-test, p= 7.72E-06), followed by CADD (Student's t-test, p= 1.46E-05).

Functional investigation of CTNNB1 missense variants

We tested the functional impact of missense *CTNNB1* variants identified in individuals with NDD phenotypes by TOPFlash dual-luciferase reporter assay in HEK293T cells. Transfection of a mutant β-catenin expression vector along with a luciferase reporter carrying TCF/LEF binding sites in the promoter region specifically assesses the impact of the mutant β-catenin on regulation of the WNT signalling pathway. We cloned four P/LP

variants: p.Leu388Pro, p.Leu424Arg, p.Gly575Arg, and a non-syndromic FEVR variant, p.Arg710Cys, along with two predicted benign variants as controls, p.Asn287Ser and p.Glu396Asp from the gnomAD database. 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 ClinVar[17] was classified as VUS (VCV000560986.1, SCV000807393.1). The variant, p.Leu424Arg was identified in a male with CP, DD, microcephaly, and dysmorphic traits.[34] The third variant, p.Gly575Arg was recurrently identified in six previously published individuals and one individual from this study (Supplementary Table 1 and 3). Neurological traits shared two or more among these seven 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 (Supplementary Figure 3) suggesting 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's t-test, $p<0.001$), therefore, we used Myc epitope tagged constructs for all comparisons between mutant and wild type β-catenin (Figure 5C).

TOPFlash activity was absent for two NDD variants, p.Leu388Pro and p.Leu424Arg compared to the activity of wild type β-catenin (Student's t-test, $p<0.001$; Figure 5C). These two variants were dominant-negative and significantly repressed TOPFlash activity when co-expressed with wild type β-catenin (Figure 5C). In contrast, both of the predicted benign variants from gnomAD and the non-syndromic FEVR variant significantly increased TOPFlash activity compared to the wild type (Student's t-test, $p<0.05$). Increases in TOPFlash activity were also observed when each of these three variants were co-transfected an equal amount of the expression construct of the wild type β-catenin; however, this was not statistically significant (Student's t-test, $p>0.05$). Unexpectedly, TOPFlash activity was not altered by the recurrent p.Gly575Arg variant compared to wild type β-catenin in this assay (Student's t-test, $p=0.283$). None of the constructs tested in this assay had an effect on, the negative control FOPFlash reporter which has non-functional TCF/LEF binding motifs (Supplementary 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 signalling pathway which is greater than the effect of haploinsufficiency caused by the known pathogenic loss-of-function variants. The functional impact 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 if 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 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 signalling which may influence the design of future targeted therapies.

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. Though several reviews pointed towards a consistent syndrome it remained unclear 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 supports that P/LP *CTNNB1* variants result in a syndrome with consistent neurological traits, except in the case of non-syndromic FEVR. 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 non-progressive peripheral spasticity or hypertonia[5,6] which is consistent with Surveillance of Cerebral Palsy in Europe guidelines for diagnosing CP.[35,36] In previous studies, a genetic diagnosis of *CTNNB1* variant was considered as grounds to remove a CP diagnosis[11]; however, our study now suggests that clinical CP diagnosis might also be appropriate for some individuals with P/LP *CTNNB1* variants. Regardless of diagnostic clinical labels, it is most important that these individuals have early and equitable access to genomic testing. Over half of individuals in whom it possible to trace a diagnostic odyssey underwent a targeted single gene or gene-panel prior to a receiving a diagnosis from exome or genome sequencing, which highlights the importance of genomic analysis for identifying P/LP *CTNNB1* variants (Figure 3).

We assessed the functional impact of missense *CTNNB1* variants, including VUS, using TOPFlash dual-luciferase reporter assay. With this established assay, impact of these variants on μ-catenin mediated transactivation of WNT signalling 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 in one of the NDD variants; p.Gly575Arg assessed in this study, do not refute pathogenicity. The negative result of the p.Gly575Arg variant may be explained by cell-type specific impact of the variant on WNT signalling pathway. We note that delayed speech and language development, a trait frequently associated with P/LP *CTNNB1* variants, was specifically ruled out in two out of seven individuals carrying the p.Gly575Arg variant and not mentioned as a trait affecting the remaining five individuals. Therefore, this variant may only affect a subset of *CTNNB1* functions, manifesting as lack of speech delay.

CTNNB1 is currently associated with two neurological phenotypes: NEDSDV (MIM: 615075) and exudative vitreoretinopathy (EVR or FEVR, MIM: 617572) that is characterized by incomplete peripheral vascular development in the retina.[37] Whether the pathogenic mechanisms of NEDSDV and FEVR overlap is unknown. Detailed ophthalmologic examination was not available for the majority 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 signalling, a particular derivative of WNT signalling pathway likely attributes FEVR.[38] Mouse models with knockout mutations in FEVR genes (*Fzd4*, *Lrp5*, *Tspan12,* and *Ctnnb1*) developed defects in retinal vasculature, suggesting that reduced activity of WNT signalling pathway leads to FEVR.[37,39–41] However, studies of non-syndromic FEVR variants in *CTNNB1* using the TOPFlash assay resulted in contradicting effects on the transcriptional activities.[42] 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 one female with a stop-gain variant (p.Gln558*) of *CTNNB1.*[43] The outcome was positive with improvements in her motor skills; however, a full scale randomised control trial is required 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 there is minimal impact of individual-specific genetic or environmental factors on *CTNNB1*-related phenotypes, which would simplify modelling 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 based on the similar craniofacial features observed between the Bfc mouse and individuals carrying 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 hippocampus[44] and surprisingly a gain of WNT signalling activity in embryos.[45] Thus the Bfc mouse does 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. 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* (Supplementary table 13). Recently, an induced pluripotent stem cell (iPSC) line, which was capable of differentiating into all three germ layers, was established from a male individual heterozygous for the p.Tyr333* variant.[48] This iPSC 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 stabilisation of *CTNNB1.*

Supplementary Material

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Data Availability Statement

Where not otherwise indicated (by e.g. ClinVar accession number) all source data for this paper and supplementary information is available from the corresponding author on reasonable 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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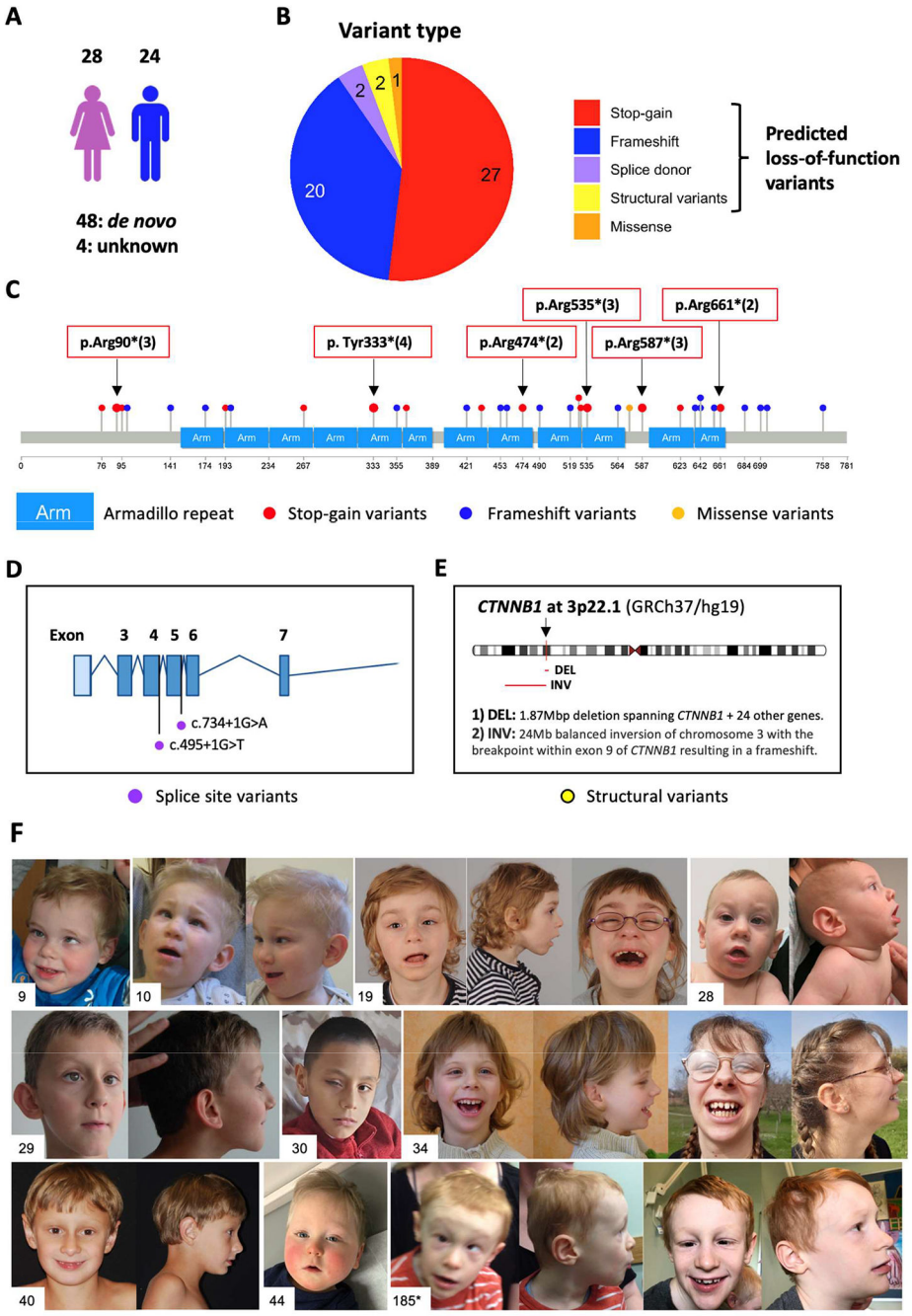

Figure 1.

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 two or more unrelated individuals

are labelled with the amino acid changes with the number of individuals in brackets. (D) Variants affecting splice donor sites were identified in two individuals. (E) Structural variants of a deletion and a balanced inversion in chromosome 3 were identified in two individuals. (F) Facial images of 10 individuals with *CTNNB1* variants. Images were collected from unpublished individual 9, 10, 19 (at the age of four years old and nine years old), 28, 29, 30, 34 (at the age of six years old and 16 years and 9 months old), 40, 44, and previously published individual 180.

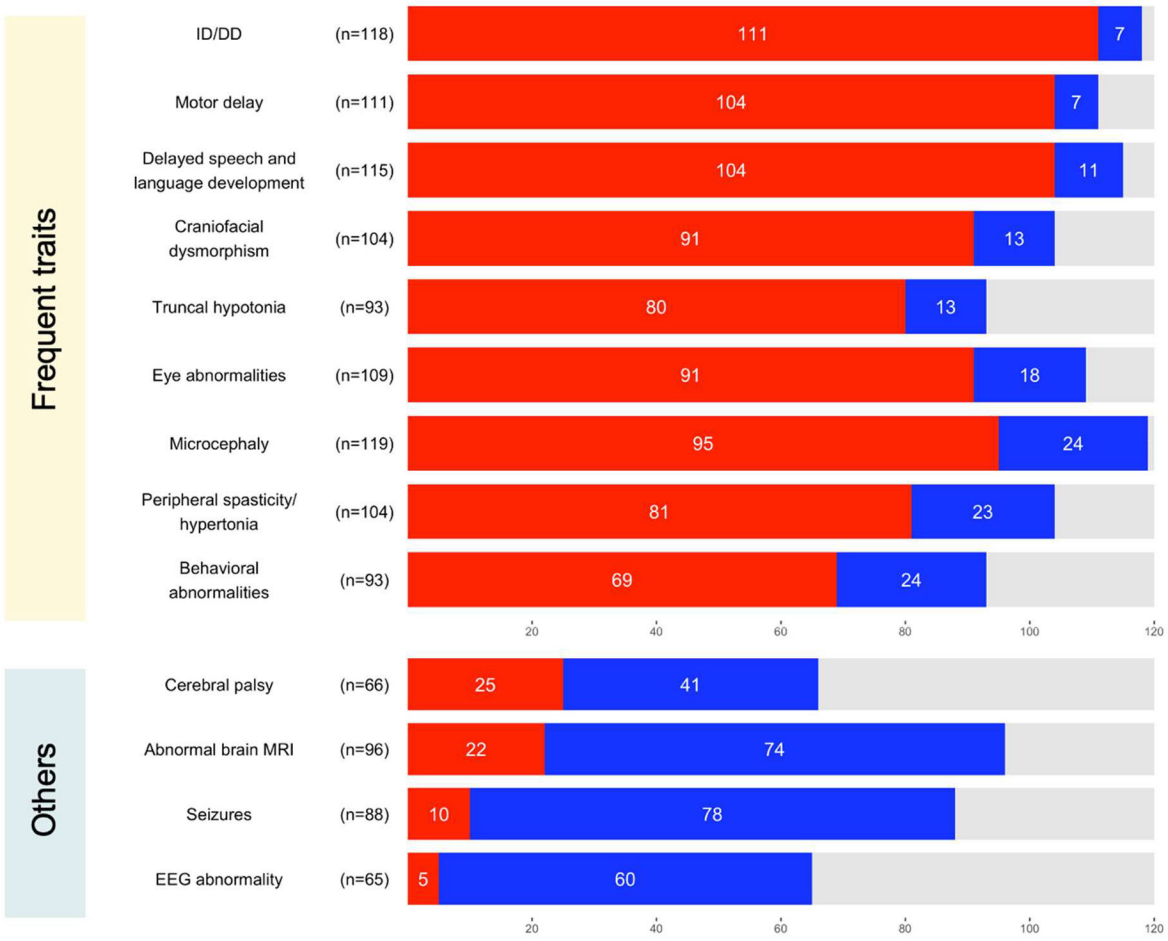

Figure 2.

Neurological traits associated with pathogenic or likely pathogenic variants in *CTNNB1.* 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 (grey) individuals per trait. The number of individuals known for their affected status per trait is shown in brackets next to each trait. ID, intellectual disability; DD, developmental delay.

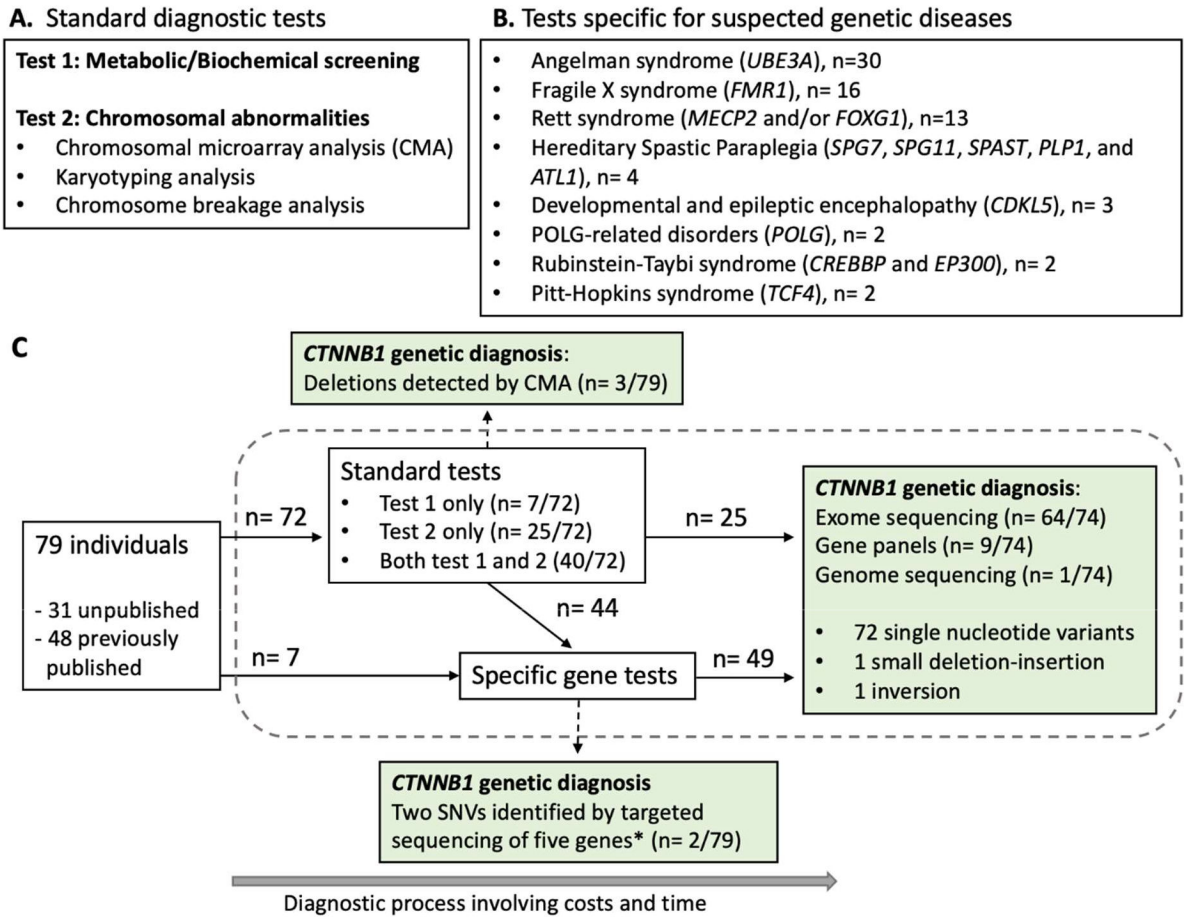

Figure 3.

Diagnostic pathways of 79 individuals prior to 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 two or more individuals. (C) A graphical summary of diagnostic pathways of 79 individuals prior to their *CTNNB1* genetic diagnosis. Seventy-four 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, eight canonical splice site, and four missense variants. Two variants (*) were exceptionally identified by targeted sequencing of five intellectual disability genes including *CTNNB1.*

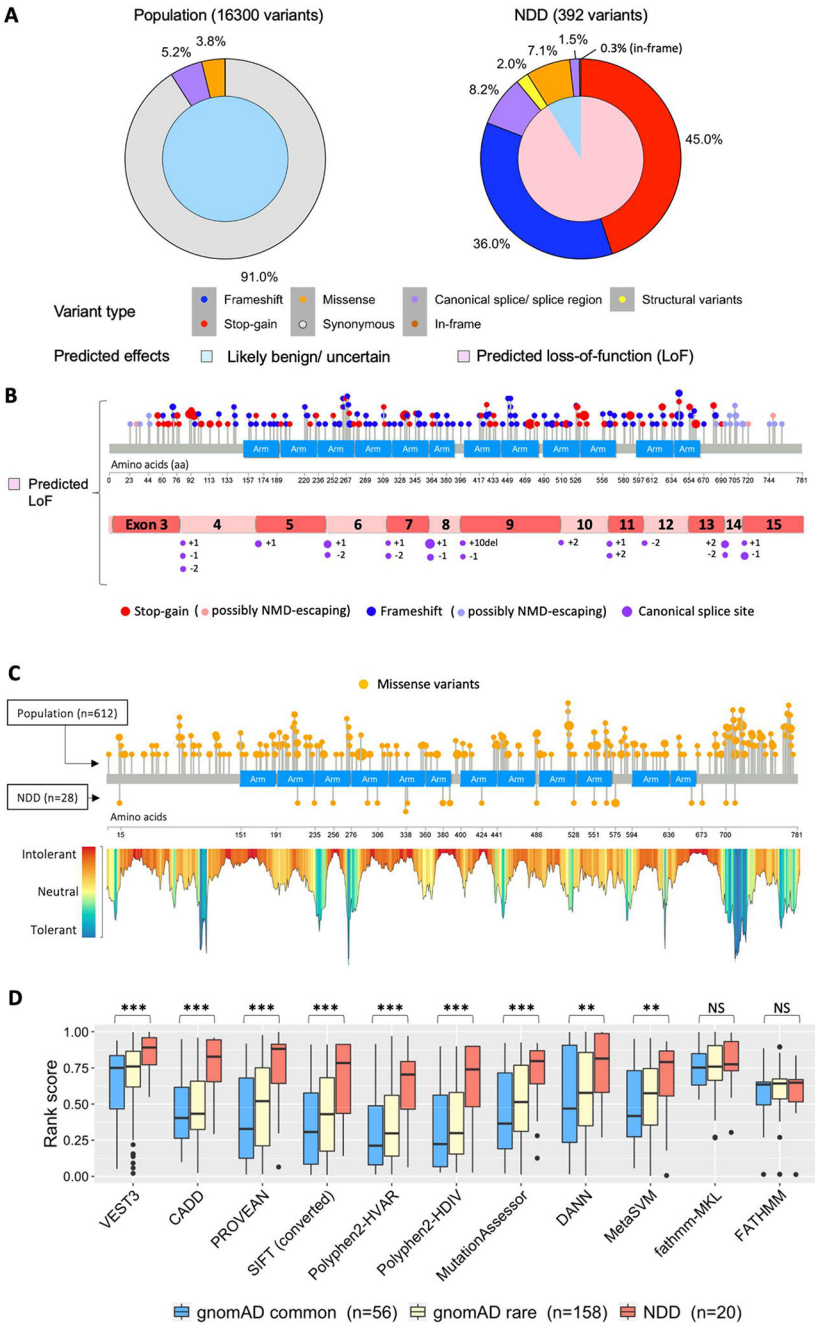

Figure 4.

Analysis of phenotypic outcome by *CTNNB1* variants type. (A) Distinct patterns of *CTNNB1* variants identified in the general population and neurodevelopmental disorders (NDD) 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 (grey), missense (orange), frameshift (blue), stop-gain (red), splicing site (purple), in-frame insertions / deletions (brown), and structural variants mainly with deletions (yellow).

Percentage labels of variant types are shown when the values are larger 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 μ-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 nonsense-mediated mRNA decay (NMD) are indicated with lighter blue (frameshift) and lighter red (stop-gain). *CTNNB1* mRNA 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 to 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 1st quartile (bottom) to 3rd quartile (top) with each median value at the center. SIFT scores were calculated as 1-SIFT raw score. Student's t-test was applied to assess the difference of NDD variants against population variants with allele frequency equal or greater than 1.0 × 10E-05 (gnomAD common). The significance marked with "***"=0.001,"**"=0.01,"*"=0.05, or "NS"=Not significant.

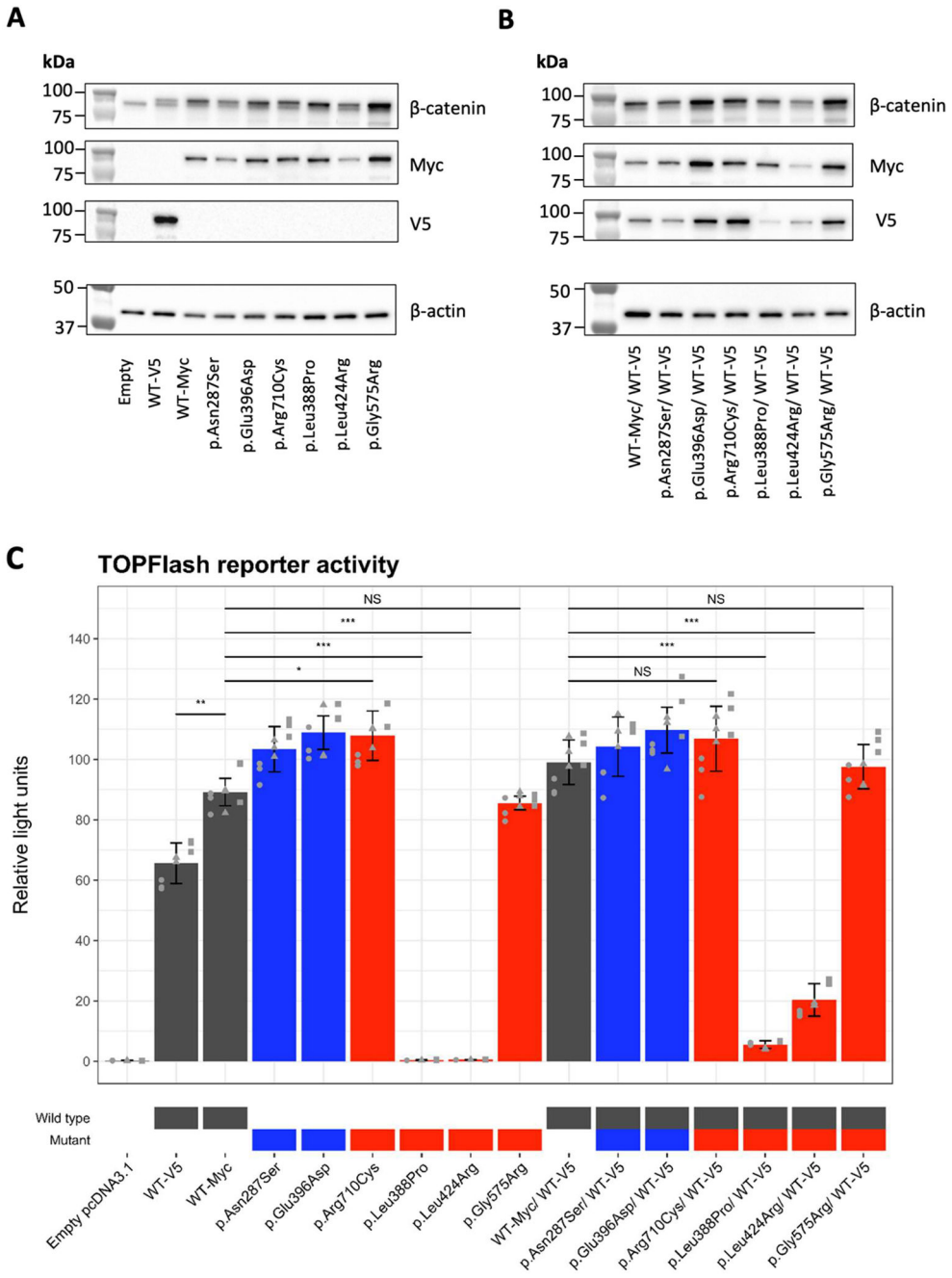

Figure 5.

Functional assessment of missense *CTNNB1* variants using TOPFlash assay. (A-B) Detection of Myc or V5-tagged wild-type (WT) β-catenin proteins and Myc-tagged mutant β-catenin proteins transfected into HEK293T cells by western blot. Expression constructs were transfected without co-transfection (A) and with co-transfection of a V5-tagged wild-type β-catenin (B). Molecular sizes of standard protein markers were indicated on the left of blots. Endogenous and exogenous β-catenin were detected with a β-catenin antibody (amino acid 571–781). Exogenous β-catenin was identified with a V5 antibody, and a Myc antibody.

Endogenous levels of β-actin were detected to show equal loading by western blot. Full blots are available in Supplementary 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 wild-type β-catenin or mutant β-catenin or an equal mix with wildtype β-catenin tagged with V5. Wild type, gnomAD variants, and pathogenic/likely pathogenic variants are highlighted in grey, blue, and red on the X-axis labels, respectively. Assay was performed in triplicate (shown with different shaped data points) with three technical replicate samples for each assay. Error bars indicate standard deviations between the three independent experiments. Student's t-test was applied to assess the difference of relative light unit of pathogenic/likely pathogenic variants against that of wild-type β-catenin. The significance marked with "***"=0.001, "**"=0.01,"*"=0.05, or "NS"=Not significant.