

Supplementary Information:

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

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Methods and Materials:

Reverse transcription polymerase chain reaction (RT-PCR)

Relative amounts of endogenous and exogenous *CTNNB1* mRNA were quantified by RT-PCR. HEK293T cells transfected with β -catenin expression constructs and luciferase reporter plasmids were homogenized in TRIzol reagent (Invitrogen, Cat# 15596026) and RNA was isolated using RNeasy kit (QIAGEN, Cat#74104). Complementary DNA (cDNA) was synthesized using random hexamers and the SuperScript IV First-Strand Synthesis System (Invitrogen, Cat# 18091050). Total endogenous and exogenous *CTNNB1* mRNA was detected by primers targeting N-terminal *CTNNB1* cDNA sequence. Exogenous *CTNNB1* mRNA was detected by primers with sequences of a Myc tag or a V5 tag. Equal abundance of total cDNA among samples was confirmed by assessing expression of the housekeeping gene esterase D. Sequences of primers used in RT-PCR can be found in a table below.

Primer sequences designed for RT-PCR.

Target	Primer orientation	Sequence (5' -> 3')
<i>ESD</i> (housekeeping)	Forward	GGAGCTTCCCCAACTCATAAATGCC
	Reverse	GCATGATGTCTGATGTGGTCAGTAA
Total <i>CTNNB1</i> mRNA	Forward	CAGAGTGCTGAAGGTGCTATC
	Reverse	ATTCCTGAGAGTCCAAAGACAG
<i>CTNNB1</i> -Myc mRNA	Forward	CAATCAGCTGGCCTGGTTTG
	Reverse	GATGATGACCGGTACGCAGA
<i>CTNNB1</i> -V5 mRNA	Forward	CAATCAGCTGGCCTGGTTTG
	Reverse	TCGAGACCGAGGAGAGGGTTA

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Table_S3: List of references to previously published studies from which we collected *CTNNB1* variants and clinical data for the present study.

Table_S4: Summaries of exclusion criteria applied to *CTNNB1* variants identified in clinical genetic databases.

Table_S5: Summary of cloning strategies of *CTNNB1* variants tested in TOPFlash dual-luciferase reporter assay.

Table_S6: Summaries of a cohort of 120 individuals from 52 unpublished and 68 selected, previously published individuals with sufficient clinical information.

Table_S7: Comparison of the frequency of traits between unpublished and previously published individuals.

Table_S8: Comparison of the frequency of traits between individuals with or without a diagnosis of cerebral palsy.

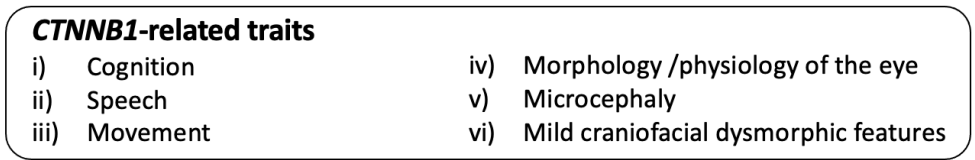
Table_S9: Summary of diagnostic tests performed prior to discovery of pathogenic *CTNNB1* variants.

Table_S10: Comparison of the frequency of traits between different sex.

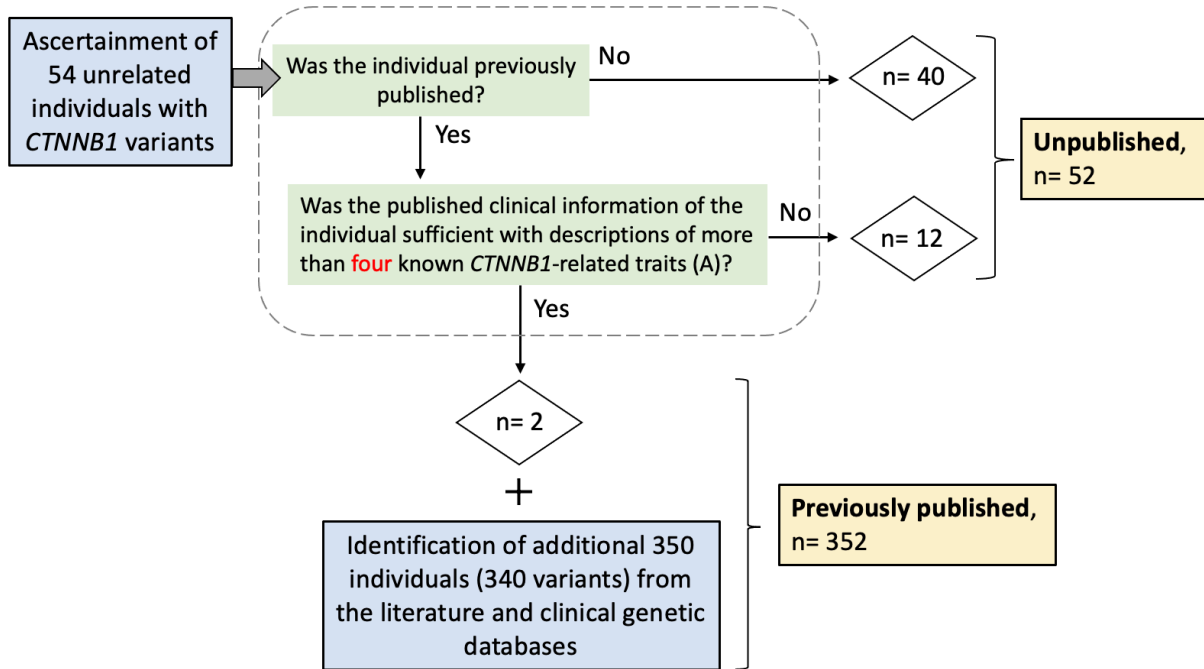
Table_S11: Ratio of different types of *CTNNB1* variants identified in population and neurodevelopmental disorders (NDD).

Table_S12: List of *CTNNB1* variants identified in neurodevelopmental disorders with the number of observations in unrelated individuals.

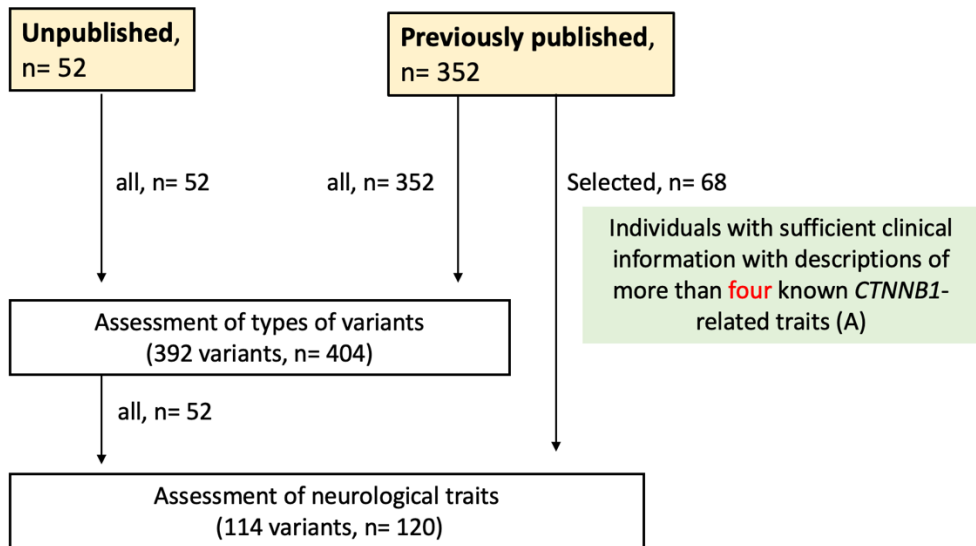
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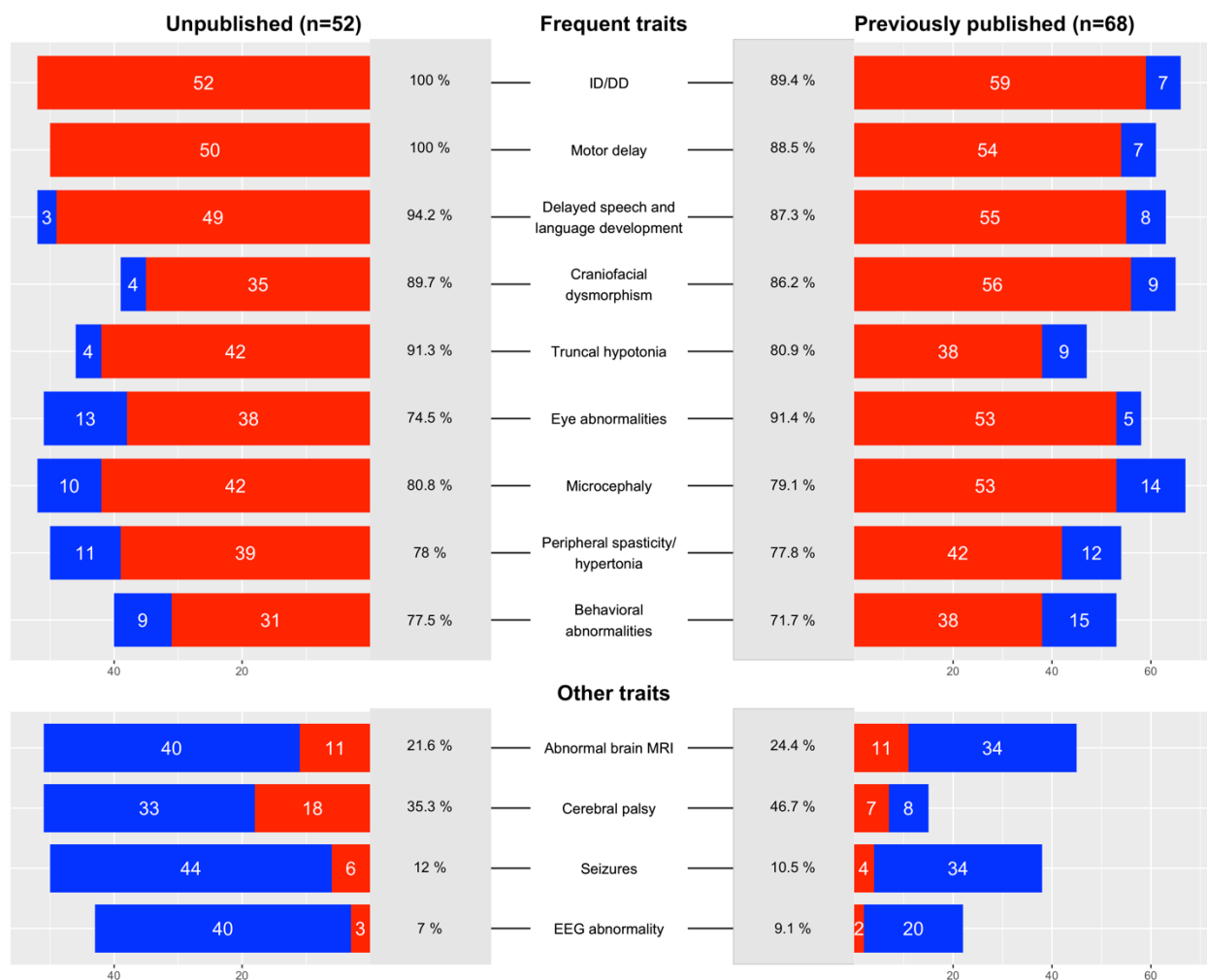
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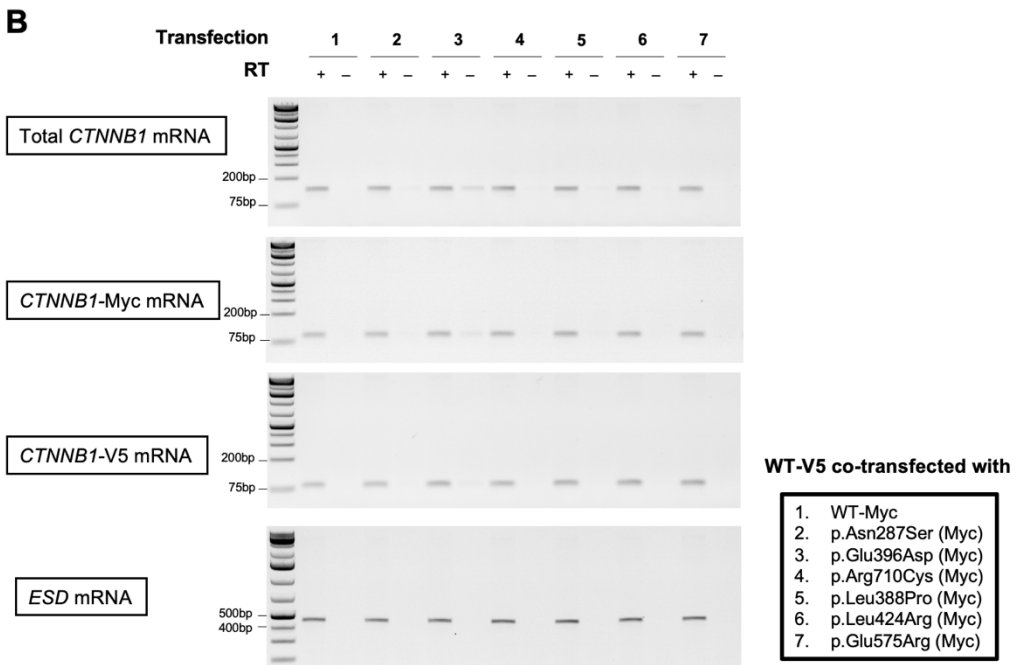
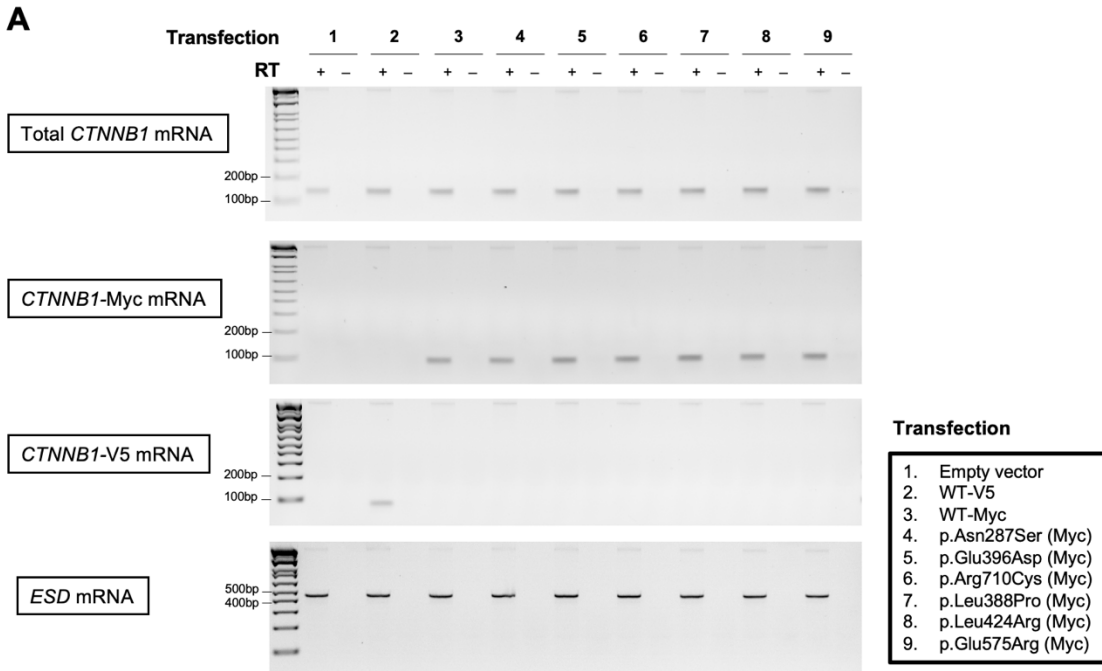
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Supplementary Figure 1. Identification of 52 unpublished and 352 previously published individuals carrying *CTNNB1* deleterious variants and study design. (A) A summary of six *CTNNB1*-related traits that were defined from previous clinical reviews of individuals carrying pathogenic *CTNNB1* variants. (B) A flow chart shows how we identified 52 unpublished and 352 previously published individuals with *CTNNB1* deleterious variants. (C) A flow chart summarizes design of this study.

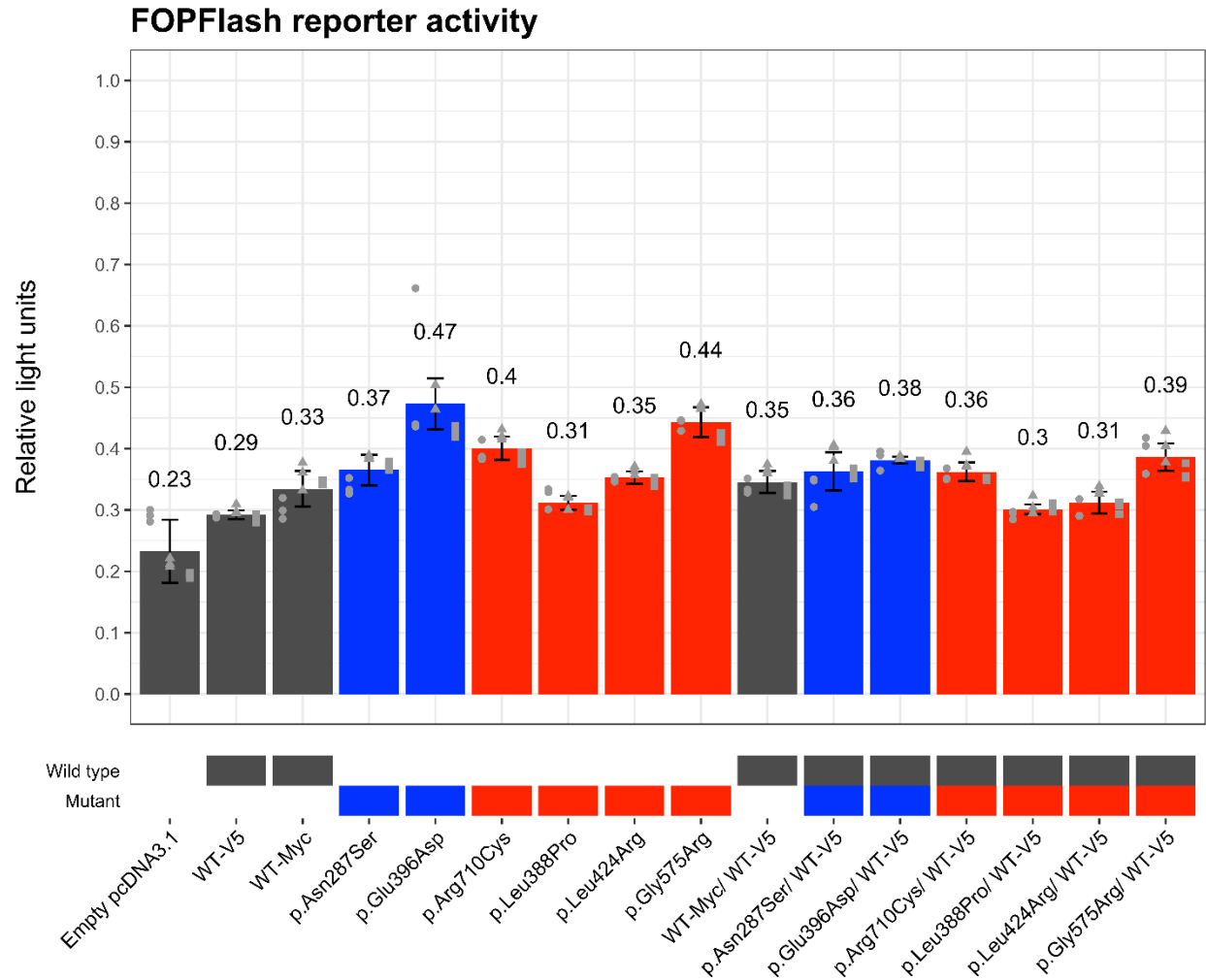


Supplementary Figure 2. Comparison of the frequency of traits between unpublished and previously published individuals. Neurological traits which were frequently identified in unpublished (left) and previously published (right) individuals are summarized at the top. Other relevant traits discussed in the present study are summarized at the bottom. Bar charts show the number of affected (red) and unaffected (blue) individuals per trait. The length of bars reflects the number of individuals with available information per trait. The percentages of affected individuals to total reported cases are shown in the grey boxes.

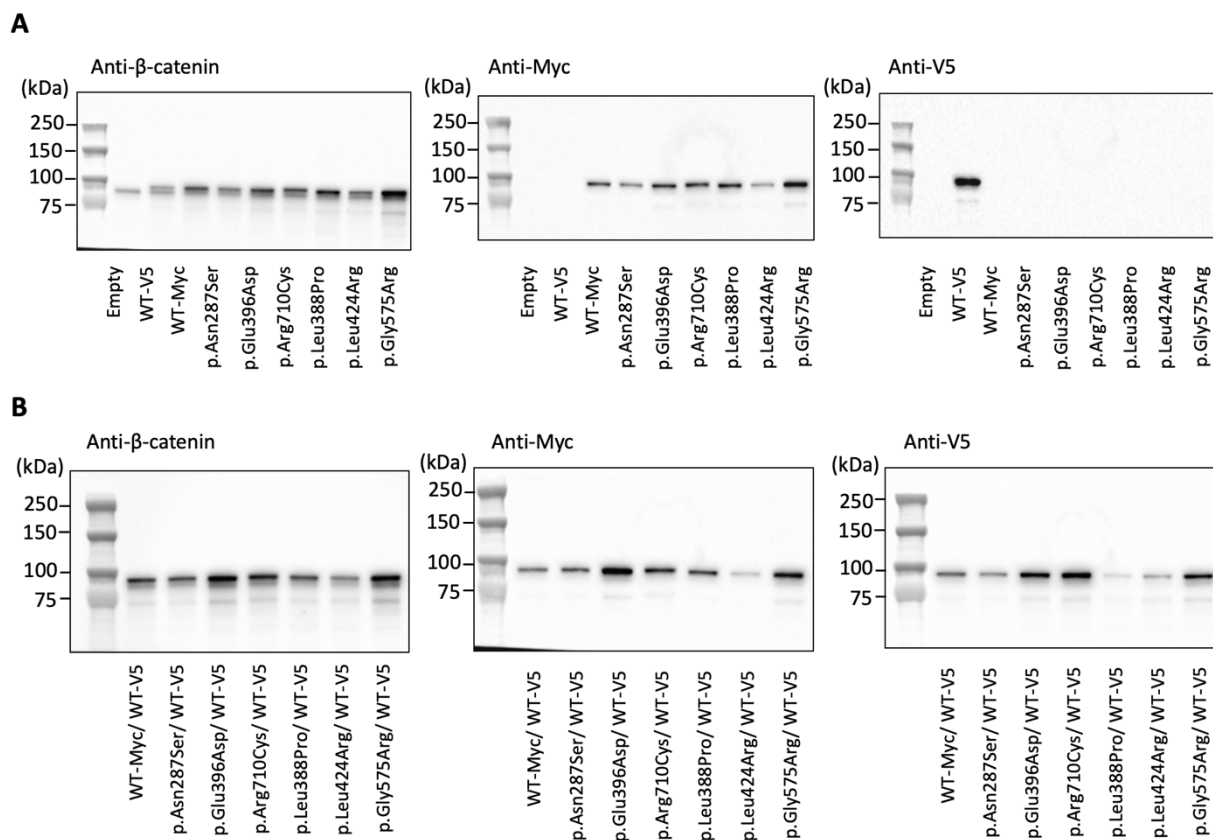


Supplementary Figure 3. Expression of Myc or V5-tagged wildtype β -catenin proteins and Myc-tagged mutant β -catenin proteins transfected into HEK293T cells detected by reverse transcription polymerase chain reaction (RT-PCR). Reverse transcription polymerase chain reaction (RT-PCR) was performed to detect mRNA expression of Myc or V5-tagged wildtype

(WT) β -catenin transcripts and Myc-tagged mutant β -catenin transcripts. Expression constructs were transfected without co-transfection (A) and with co-transfection of a V5-tagged wildtype β -catenin (B). Synthesis of complementary DNA (cDNA) from RNA was performed in the presence (+) or absence (-) of reverse transcriptase (RT). Total *CTNNB1* mRNA was detected with primers which amplify *CTNNB1* coding region (proximal to the N terminus). Exogenous *CTNNB1* mRNA (*CTNNB1*-Myc or *CTNNB1*-V5) were selectively amplified using a primer containing a Myc tag or a V5 tag sequence. Amplifications with primers specific to esterase D (*ESD*) cDNA were performed to confirm success of cDNA synthesis in samples treated with RT.

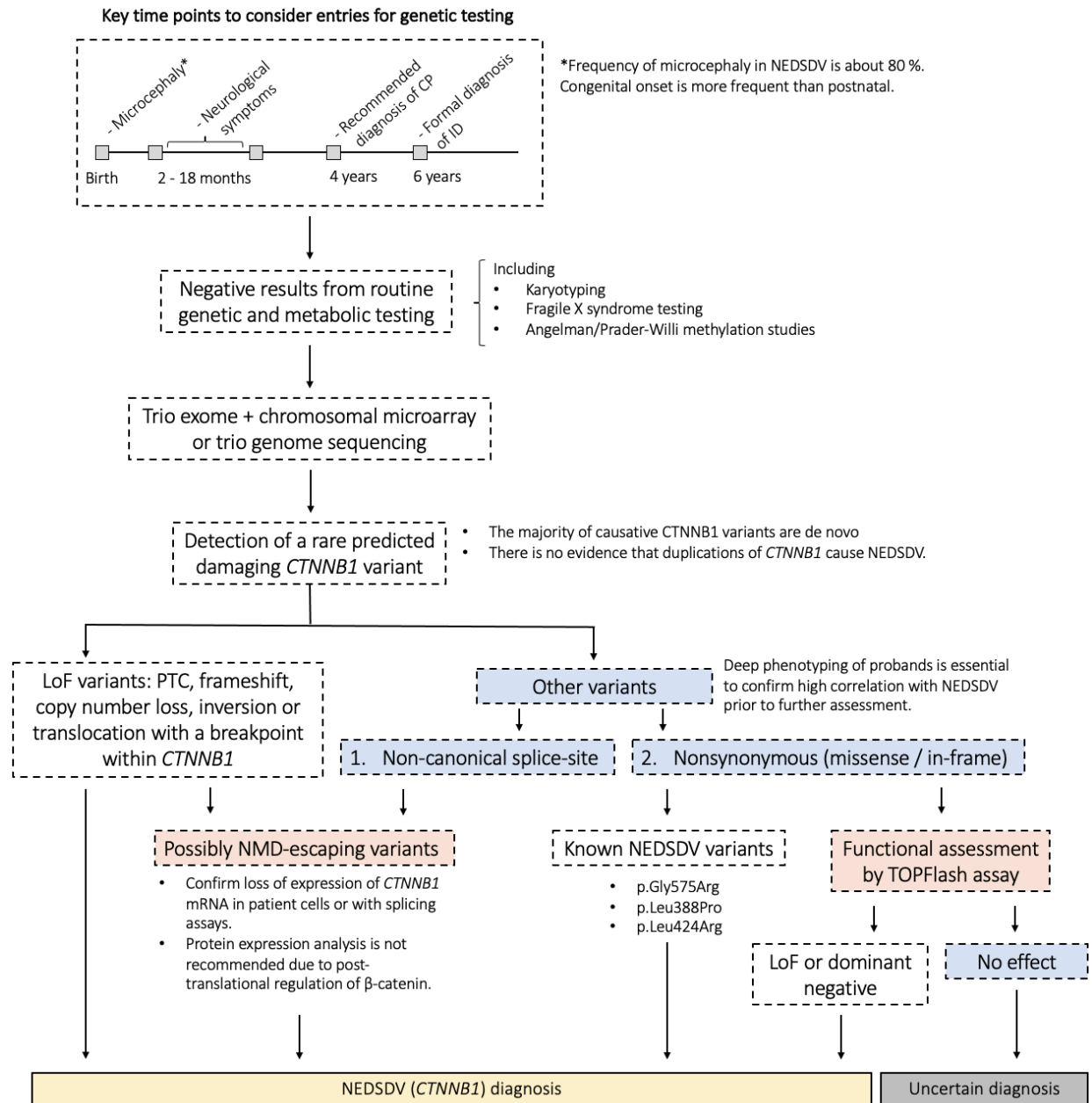


Supplementary Figure 4. CTNNB1 constructs had minimal effects on luciferase reporter gene expression in the absence of wild type LEF/TCF responsive elements. Relative luciferase activity measured from the FOPFlash reporter construct in HEK293T cells co-transfected with expression vectors for wildtype 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. Results indicate a functional LEF/TCF binding site is required for transactivation.



Supplementary Figure 5. Full blots of western blots shown in Figure 5A and 5B in the main text.

(A) Detection of wild-type (WT) and mutant β -catenin proteins transfected as solo into HEK293T cells by western blot, shown in Figure 5A in the main text. (B) Detection of wildtype and mutant β -catenin proteins co-transfected with a V5-tagged wildtype β -catenin into HEK293T cells by western blot, shown in Figure 5B in the main text. Endogenous and exogenous β -catenin were detected with a β -catenin antibody (Anti- β -catenin, left). Myc-tagged wild-type and mutant β -catenin were detected with an anti-Myc antibody (Anti-Myc, middle). A V5-tagged wildtype β -catenin was detected with an anti-V5 antibody (Anti-V5, right).



Supplementary Figure 6. Recommendations for molecular and clinical diagnosis of NEDSDV.

Flow diagram indicates neurological signs consistent with NEDSDV appear between 2 - 18 months of age, typically microcephaly is observable at birth. The recommended testing pathway includes available newborn screening and if these tests are negative, trio (parents and affected child) exome sequencing or genome sequencing with structural variant analyses by chromosomal microarray and/or from the sequencing data. Variant interpretation should follow American

College of Medical Genetics and Genomics and Association for Molecular Pathology guidelines or an equivalent National standard. Heterozygous loss-of-function (LoF) is a known disease mechanism for *CTNNB1* therefore these variants constitute very strong evidence for pathogenicity. Other types of variants (blue boxes) may require further investigation (red boxes) either by gene expression analysis for non-sense mRNA decay (NMD) escaping for intronic or splice variants or the TOPFlash assay for nonsynonymous variants, with the exception of recurrent nonsynonymous variants. Abbreviations; CP: cerebral palsy, ID: intellectual disability, PTC: premature termination codon.

Supplementary Table 5: Summary of cloning strategies of *CTNNB1* variants tested in TOPFlash dual-luciferase reporter assay.

Variant / purpose	Primer	Sequences (5' -> 3')	Orientation	Primer pair for PCR	Annealing temperature (°C)	Cloning strategy
Overlap PCR	T7	TAATACGACTCACTA TAGGG	Forward			
Overlap PCR	CTNNB1_c598 (F1)	CGTACCATGCAGAA TACAAATG	Forward			
Overlap PCR	CTNNB1_c1123 (F2)	CAACGTCTTGTTTCAG AACTG	Forward			
Overlap PCR	CTNNB1_c1973 (R1)	ACAGCAGCTGCATA TGTCG	Reverse			
Overlap PCR	pcDNA_vector (R2)	CCATTGAGTTTAAAC CCGCTG	Reverse			
Substitution of a C-terminal tag	CTNNB1_myc_R (sequence of a Myc tag highlighted in red.)	TGGTGATGATGACC GGTACGCAGATCCTC TTCTGAGATGAGTTT TTGTTCTTCGAAGGG CCCTCT	Reverse	F2	62.0	PCR product was purified and digested with AgeI (NEB# R3552S) and EcoRI (NEB# R3101S) prior to ligation into a vector digested with the same set of restriction enzymes.
NM_001904.3: c.860A>G: p.Asn287Ser (rs35288908)	Fragment 1	ATGGTTGCCTTGCTC AGCAAAACAAATGT TA	Forward	R1	72.0	Equal amount of fragment 1 and 2 were mixed in PCR reaction with outer primers (T7 x R1). Amplified product and a vector were digested with Bsu36I (NEB# R0524S) and EcoRI.
	Fragment 2	AACATTTGTTTTGCT GAGCAAGGCAACCA TT	Reverse	T7	62.0	
NM_001904.3: c.1188A>C: p.Glu396Asp (rs751375496)	Fragment 1	CTGCAACTAAACAG GACGGGATGGAAGG TC	Forward	R1	72.0	
	Fragment 2	GAGACCTTCCATCCC GTCCTGTTTAGTTGC	Reverse	T7	62.0	
NM_001904.3:	Fragment 1	TGGACTCTCAGGAAT CCTTCAGATGCTGCA AC	Forward	R1	72.0	Equal amount of fragment 1 and 2 were mixed in PCR

Variant / purpose	Primer	Sequences (5' -> 3')	Orientation	Primer pair for PCR	Annealing temperature (°C)	Cloning strategy
c.1163T>C: p.Leu388Pro (rs1559474140)	Fragment 2	TAGTTGCAGCATCTG AAGGATTCCTGAGA GTC	Reverse	T7	66.7	reaction with outer primers (T7 x R1). Amplified product was digested with BamHI (R3136S) and EcoRI and cloned into a pcDNA3.1-CTNNB1-V5 vector digested with the same set of enzymes. For this variant only, the V5 tag was replaced with the Myc tag after insertion of <i>CTNNB1</i> variant of interest.
NM_001904.3: c.1271T>G: p.Leu424Arg (rs863224864)	Fragment 1	CTGTGCAGCTGGAAT TCGTTCTAACCTCAC TTG	Forward	R2	72.0	Equal amount of fragment 1 and 2 were mixed in PCR reaction with outer primers (F1 x R2). Amplified product and a vector were digested with AgeI and EcoRI.
	Fragment 2	AGTGAGGTTAGAAC GAATTCCAGCTGCAC AGGT	Reverse	F1	62.0	
NM_001904.3: c.1723G>A: p.Gly575Arg (rs797044875)	Fragment 1	GTTGAAGGTTGTACC AGAGCCCTTCACATC	Forward	R2	62.0	Equal amount of fragment 1 and 2 were mixed in PCR reaction with outer primers (F2 x R2). Amplified product and a vector were digested with AgeI and EcoRI.
	Fragment 2	TGTGAAGGGCTCTG GTACAACCTTCAACT A	Reverse	F2	62.0	
NM_001904.3: c.2128C>T: p.Arg710Cys (rs748653573)	Fragment 1	AACCCCTTGGATATT GCCAGGATGATCC	Forward	R2	72.0	
	Fragment 2	TAGGATCATCCTGGC AATATCCAAGGGGT TC	Reverse	F2	62.0	

“c” in name of primers refers to a position at *CTNNB1* cDNA coordinate (NM_001904.3).

Supplementary Table 11: Ratio of different types of *CTNNB1* variants identified in population and neurodevelopmental disorders (NDD).

Variant type	Population (gnomAD)		NDD	
	Count	Ratio (%)	Count	Ratio (%)
Synonymous	14826	90.96	0	0.00
Missense	612	3.75	28	7.14
Stopgain	0	0.00	176	44.90
Frameshift	0	0.00	141	35.97
In-frame	5	0.03	1	0.26
Splicing	2	0.01	32	8.16
Splice site	854	5.24	6	1.53
Structural variants	0	0.00	8	2.04
Start lost	1	0.01	0	0.00
Total (n)	16300		392	