REPORT

De novo variants in POLR3B cause ataxia, spasticity, and demyelinating neuropathy

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Summary

POLR3B encodes the second-largest catalytic subunit of RNA polymerase III, an enzyme involved in transcription. Bi-allelic pathogenic variants in POLR3B are a well-established cause of hypomyelinating leukodystrophy. We describe six unrelated individuals with de novo missense variants in POLR3B and a clinical presentation substantially different from POLR3-related leukodystrophy. These individuals had afferent ataxia, spasticity, variable intellectual disability and epilepsy, and predominantly demyelinating sensory motor peripheral neuropathy. Protein modeling and proteomic analysis revealed a distinct mechanism of pathogenicity; the de novo POLR3B variants caused aberrant association of individual enzyme subunits rather than affecting overall enzyme assembly or stability. We expand the spectrum of disorders associated with pathogenic variants in POLR3B to include a de novo heterozygous POLR3B-related disorder.

RNA polymerase III (pol III) is a 17-subunit enzyme involved in the transcription of small non-coding RNAs, which regulate transcription in eukaryotes.[1] POLR3B (MIM: 614366) encodes the second-largest catalytic subunit of pol III, POLR3B. Bi-allelic mutations in POLR3B and genes encoding other POLR3 subunits cause hypomyelinating leukodystrophy type 8 (MIM: 614381) with a wide clinical spectrum of associated symptoms. These include endocrine dysfunction (hypogonadism, reproductive failure, and delayed puberty), ocular abnormalities, and abnormal dentition (hypodontia and oligodontia).[1,2]

The original descriptions of POLR3-related leukodystrophy were of individuals with hypodontia, ataxia, and hypomyelination,[3,4] followed by individuals with additional endocrine abnormalities of hypogonadotropic hypogonadism.[2] The initial identification of the genetic basis of POLR3-related leukodystrophy enabled definitive association of this constellation of clinical features with recessive mutations in POLR3A (MIM: 614258), POLR3B, POLR1C (MIM: 610060), and POLR3K (MIM: 606007).[1,5–9] In total, five previously distinct clinical phenotypes were described prior to the identification of their shared molecular basis. These include hypomyelination, hypodontia, hypogonadotropic hypogonadism (4H syndrome); ataxia, delayed dentition, and hypomyelination (ADDH); tremor-ataxia with central hypomyelination (TACH); leukodystrophy with oligodontia (LO); and hypomyelination with cerebellar atrophy and hypoplasia of the corpus callosum (HCAHC). These previously distinct clinical phenotypes were subsequently re-classified as 4H leukodystrophy after the discovery of the shared phenotypic spectrum and genetics[10] and are now collectively referred to as POLR3-related leukodystrophy.

The classic neurological features of individuals with POLR3B-related leukodystrophy consist primarily of central nervous system involvement, and abnormal electromyogram and nerve conduction studies (EMG/NCSs) have not been reported in these individuals.[1,2] To our knowledge, no prior publications have reported sensory or peripheral motor abnormalities of clinical significance in individuals with bi-allelic POLR3B mutations.

Six unrelated individuals were enrolled in the study, which was approved by the research ethics board of the Hospital for Sick Children (REB #1000009004) and the McGill University Health Center (11-105-PED and 2019-4972). Five individuals (subjects 1–4 and 6) were found to have pathogenic variants in POLR3B from exome sequencing completed at GeneDx (Gaithersburg, MD). One additional individual (subject 5) was diagnosed at the University Medical Center Utrecht. All parents/legal guardians provided written informed consent for their

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Table 1. Clinical and genetic characteristics of individuals with *de novo* POLR3B variants

Subject	1	2	3	4	5	6
POLR3B variant (NM_018082.5)	c.1124A>T (*de novo*)	c.1277T>C (*de novo*)	c.3137G>A (*de novo*)	c.1094C>T (*de novo*)	c.1087G>A (*de novo*)	c. 1385C>G (*de novo*)
Predicted effect on protein	p.Asp375Val	p. Leu426Ser	p. Arg1046His	p.Ala365Val	p.Glu363Lys	p.Thr462Arg
Age at last review	16 years	4 years	11 years	22 years	8 years	14 years
Gender	female	male	male	male	female	female
Current height (centile)	166 cm (50%)	96 cm (1%)	152 cm (95%)	165 cm (5%)	116 cm (10%)	157 cm (30%)
Current weight (centile)	74.3 kg (85%)	13.5 kg (1%)	51.9 kg (95%)	95.45 kg (95%)	19.9 kg (20%)	55.4 kg (63%)
Current OFC (centile)	51 cm (<3%)	48 cm (5%)	55 cm (85%)	58 cm (~98%)	47 cm (~3%)	51 cm (<2%)
Age achieved walking	3.5 years	22 months	16 months	18 months	2.5 years	16 months
Global developmental delay/intellectual disability	severe; on formal testing verbal reasoning and adaptive skills <1% (ABAS-II, Leiter-R, PPVT-4)	language DQ 51%, visual motor DQ 51%	normal intellectual functioning; grade-level academic performance	IQ at age 21 years: FSIQ 46 (moderate ID)	IQ at age 7 years: »55 (mild to moderate ID)	severe; reading, writing, and adaptive function at kindergarten level at age 14
Communication/ speech	first words at 5 years, sentences at 7 years, mainly uses single words and short phrases at 16 years, dysarthria	first words at 2 years, 2–3-word phrases, dysarthria	normal speech	first word at 13 months, put 2 words together at 2.5 years, dysarthria	language development initially normal, can use simple sentences, dysarthria	significant early delay, phrases at 3 years but limited vocabulary, now short phrases, dysarthria
Gross motor	ambulatory, spastic gait, ataxia, occasional dystonic posturing	ambulatory, in-toeing L > R	mild gross motor delay as an infant	ambulatory, ataxia, falls monthly, spasticity and increasing unsteadiness	ambulatory, spastic gait	gross motor delay, ambulatory, spastic gait, mild ataxia
Fine motor	needs assistance to feed herself, dress, and bathe, can print her name	feeds self with spoon and fork	normal	can use utensils but prefers finger feeding, does not color within lines, needs assistance with most ADLs	needs assistance with all activities of daily living	cuts own food but needs help with buttons and zippers, feeds self, needs assistance to bathe and toilet
Appendicular hypertonia/spasticity	severe, progressive	none	none	severe, progressive, LEs>>UEs	clearly present, progressive	moderate, all 4 limbs, LE > UE

(Continued on next page)

Table 1. *Continued*

Subject	1	2	3	4	5	6
Deep tendon reflexes	4+ with sustained ankle clonus, +Hoffman sign	normal and symmetric	1+ in biceps, triceps, absent in patella and ankles bilaterally	3+ throughout, except 1+ at the ankles	arms, 3+; legs, 4+ (crossed adductor reflex, sustained ankle clonus)	3+ bilaterally
Extensor plantar responses	yes	no	no	yes	yes	yes
Muscle weakness	MRC grade 5/5 UEs, tibialis anterior and extensor hallucis longus 4/5	normal strength	prominent distal leg weakness	not tested formally but grossly normal, no asymmetry	not tested formally, no obvious weakness	MRC grade 5/5 throughout
Sensory examination	not able to cooperate with formal exam	grossly normal	decreased vibration and proprioception in distal limbs	uncooperative for detailed exam, hypersensitivity in the feet bilaterally	detailed sensory exam not possible, light touch preserved	unable to cooperate with formal exam
Cerebellar testing	mirror movements, intention tremor, mild dysmetria, gait ataxia, unable to perform tandem gait	normal for age	slightly wide-based gait	slow finger to nose movements, clearly ataxic gait with bilateral circumduction, unable to perform tandem gait or stance	mild intention tremor and dysmetria, unable to balance on one foot or perform tandem gait	no overt appendicular ataxia but mild gait ataxia and unable to perform tandem gait
Seizures	no	onset 3 years, head and bilateral arm drop attacks, also generalized seizures	no	onset 6 months, refractory generalized epilepsy, myoclonic, rarely atonic and absence	onset 12 months, head drop attacks, refractory to medications and ketogenic diet	no
Brain MRI	mild cerebellar atrophy, non-specific T2 and FLAIR hyperintensities	normal	normal (except for Chiari I malformation)	non-specific white matter signal abnormalities, stable over time	normal (age 5 years)	initially delayed myelination that normalized by 14 years
EMG/NCS (age when performed)	length-dependent primary axonal neuropathy with secondary demyelination, markedly decreased velocities in motor fibers of tibial and deep peroneal nerves (13 years)	normal (4 years)	severe demyelinating sensory motor polyneuropathy, normal amplitudes, mild-moderate prolonged distal latencies, significant slowing of conduction velocities (10 years)	predominantly demyelinating sensory motor neuropathy, slow motor conduction velocities, markedly decreased amplitudes in tibial nerve, absent sural responses (12 years)	combined axonal and demyelinating polyneuropathy of both motor and sensory nerves (7 years)	predominantly demyelinating polyneuropathy with slow conduction velocities (6 and 8 years)

Abbreviations are as follows: OFC, occipitofrontal circumference; DQ, development quotient; ID, intellectual disability; L, left; R, right; ADLs, activities of daily living; LE, lower extremity; UE, upper extremity; MRC, Medical Research Council
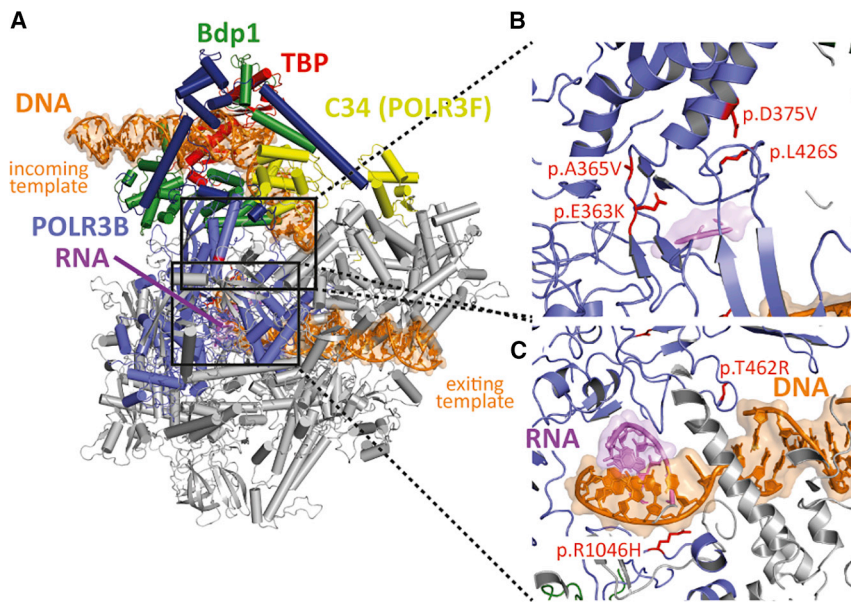
Figure 1. Structural protein modeling

(A) In order to localize the *POLR3B* variants found in the patients on the yeast POLR3B structure, we used the model of POLR3 in transcription initiation state.[13]

(B) Of the six variants identified, four of them cluster in a region of POLR3B where transcribed DNA is melted.

(C) No direct contacts with other POLR3 subunits were identified, but the mutated region is at the transcription bubble and may have an impact on transcription itself. Similarly, the two remaining variants are located in the exiting DNA tunnel and have direct contact with the duplex DNA. These variants may affect transcription activity. Of note, none of the identified variants are at the interface with transcription factors or transcription repressors (e.g., Maf1). POLR3B-altered residues found in the six patients and their equivalent yeast positions are as follows: Asp375 = Asp390, Leu426 = Leu444, Arg1046 = Arg1061, Ala365 = Ala380, Glu363 = Glu378, and Thr462 = Ser480.

children to participate in the study and for publication of clinical information.

We identified six unrelated individuals, from non-consanguineous families, who harbor *de novo* heterozygous variants in *POLR3B* (Figure S1) with consistent clinical features that differ from those associated with previously reported bi-allelic mutations[1,2] (Table 1).

The six participants each had some degree of gait dysfunction ranging from mild instability to more severe gait ataxia. Five participants (subjects 1, 3, 4, 5, and 6) had truncal and/or appendicular ataxia with wide-based ataxic gait, inability to perform tandem gait, or gait instability (Table 1, Supplemental notes). Three participants had hyperreflexia, while one had normal deep tendon reflexes, one had diminished reflexes, and one had a mixed picture of proximal hyperreflexia with distal hyporeflexia.

The majority of individuals also had some degree of delay in other developmental domains; intellectual disability ranging from mild to moderate severity was diagnosed in 5/6 participants. Only one individual (subject 3) had normal academic performance and development apart from early mild motor delay. This individual also had normal language, while the other 5/6 participants had dysarthria and/or varying degrees of delayed early speech development ranging from normal to markedly delayed (the most severely affected, subject 1, achieved first words at 5 years of age). Four individuals (subjects 1, 4, 5, and 6) required assistance with basic activities of daily living, suggestive of significant degrees of intellectual disability and/or coordination and movement difficulties, however no subject had developmental regression. Motor delay also varied from mild to severe; independent ambulation was achieved by all participants between 16 months and 3.5 years of age.

Other clinical features were less consistently present. Seizures appeared to be variably present: 3/6 participants (subjects 2, 4, and 5) had seizures of diverse semiologies. Two are refractory to medical management with seizure onset at 6–12 months of age, while subject 2 responded well to anti-epileptic medications and had seizure onset later at 3 years of age. There did not appear to be an association between presence of seizures and other features, including brain MRI abnormalities, severity of clinical phenotype, or developmental history. Although there was no clear pattern of bulbar involvement, subject 4 had dysphagia and oromotor dyspraxia. Feeding was otherwise normal; only one other subject had early food aversion, which resolved.

Although various additional clinical features were noted (Table 1, Supplemental notes), there were no consistent abnormalities found with respect to growth, endocrinopathies, dentition, vision, or cardiovascular or skeletal systems. All participants were born at term, had normal birth weights, and were born to non-consanguineous parents of varied ethnic origins.

EMG/NCSs for the majority of individuals (5/6) revealed predominantly demyelinating sensory and motor neuropathy (Table 1, Table S2). Subject 4 had a muscle biopsy that showed neuropathic changes. Subject 2 had NCSs at a young age (4 years) that was reported as normal. Brain MRI did not reveal specific abnormalities; three participants (subjects 2, 3, and 5) had normal brain imaging, and two participants (subjects 1 and 4) had non-specific white matter signal abnormalities, one of whom (subject 1) also had mild cerebellar atrophy (Figure S2). Other routine clinical investigations (microarray and metabolic testing) were noncontributory (Supplemental notes).

Using genomic DNA from subjects 1, 2, 3, 4, and 6 and their respective parents, we captured the exonic regions and flanking splice junctions of the genome by using the IDT xGen Exome Research Panel v.1.0. Massively parallel next-generation sequencing (NGS) was performed on an
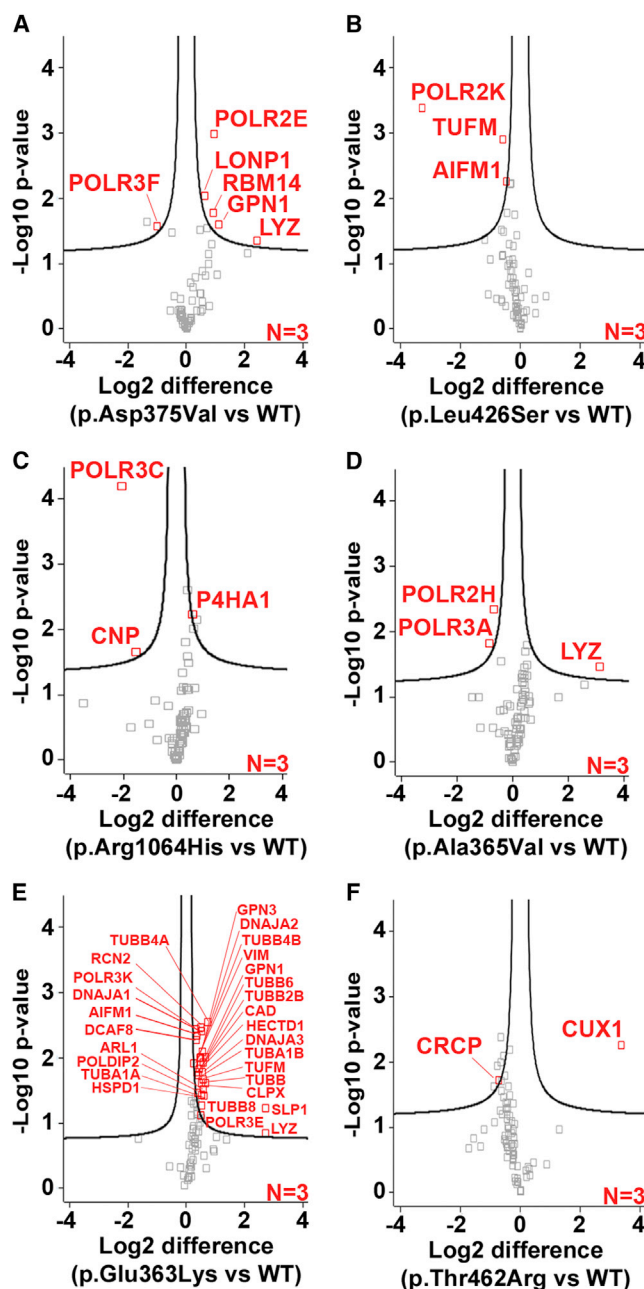
Figure 2. Different POLR3B *de novo* variants distinctively affect the assembly of specific RNA polymerase III subunits

(A–F) FLAG-tagged POLR3B wild type (WT), p.Asp375Val (A), p.Leu426Ser (B), p.Arg1064His (C), p.Ala365Val (D), p.Glu363Lys (E), and p.Thr462Arg (F) were expressed in HEK293 cells for 24 h, purified with an anti-FLAG antibody, and digested with trypsin. The co-purified proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The label-free quantification (LFQ) intensity of each peptide was computed via MaxQuant and Perseus. Volcano plots illustrate the $\log_2$-transformed average LFQ-intensity difference between mutant and WT (x axis), and the $-\log_{10}$ p value obtained via a two-tailed t test adjusted with a permutation-based multiple hypothesis testing with 10,000 iterations and an s0 correction factor of 0.1 (y axis). Proteins marked in red are considered significantly different between the conditions.

Illumina system with 150 bp paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19 and analyzed for sequence variants with a custom-developed analysis tool. Additional sequencing technology and variant interpretation protocols have been previously described.[11] The general assertion criteria for variant classification are publicly available on the GeneDx ClinVar submission page. For subject 5, trio whole-exome sequencing was performed via the SureSelect XT Human All Exon V5 kit (Agilent) at a mean target depth of 100×. Reads were aligned to Hg19 via BWA (BWA-MEM v.0.7.5a) and variants were called with the GATK haplotype caller (v.2.7-2). Detected variants were annotated, filtered, and prioritized via the Bench lab NGS v.3.1.2 platform (Cartagenia, Leuven, Belgium) and confirmed by Sanger sequencing.

POLR3B variants identified in the participants were localized and correlated on the yeast POLR3 structure. A sequence alignment of the POLR3B subunit was generated and the equivalent positions (Table S1) were then displayed on the yeast model of POLR3 in the transcription initiation state (Figure 1A).[12,13] Four of the six *de novo* variants cluster in a region of POLR3B where transcribed DNA is melted (Figure 1B). No direct contacts with other POLR3 subunits were identified, but the affected region is at the transcription bubble, which may have an impact on transcription itself. The two remaining variants are located in the exiting DNA tunnel and have direct contact with the duplex DNA (Figure 1C) and may thereby affect transcription activity.

To further define the impact of the *de novo* variants on POLR3B, we carried out affinity purification coupled with mass spectrometry (AP-MS) to evaluate the assembly of specific RNA pol III subunits in human embryonic kidney cell line 293 (HEK293) cells (Supplemental notes). AP-MS demonstrated that five of the six variants in *POLR3B* (GenBank: NM_018082.5) caused impairment of the association of at least one individual subunit, either POLR3F (c.1124A>T [p.Asp375Val]), POLR2K (c.1277T>C [p.Leu426Ser]), POLR3C (c.3137G>A [p.Arg1064His]), POLR3A and POLR2H (c.1094C>T [p.Ala365Val]), and CRCP (c. 1385C>G [p.Thr462Arg]), with the enzyme (Figure 2). Although this remains to be directly confirmed, the loss of a pol III subunit is expected to render the enzyme inactive, as has been shown for RNA polymerase II.[14–16] The c.1087G>A (p.Glu363Lys) variant was associated with abnormal POLR3B interactions with multiple proteins (Figure 2, Table S3), but their exact functional role in this context remains to be determined. Expression of POLR3B in fibroblasts from subject 1 was similar to that of control fibroblasts (data not shown), suggesting that the c.1124A>T (p.Asp375Val) variant does not have a direct effect on expression of the protein. Notably, all six *de novo POLR3B* variants localized in the nucleus as measured by immunoblots of cytoplasmic and nuclear fractions (Figure S3), suggesting that a defect in nucleocytoplasmic pol III shuttling is unlikely.
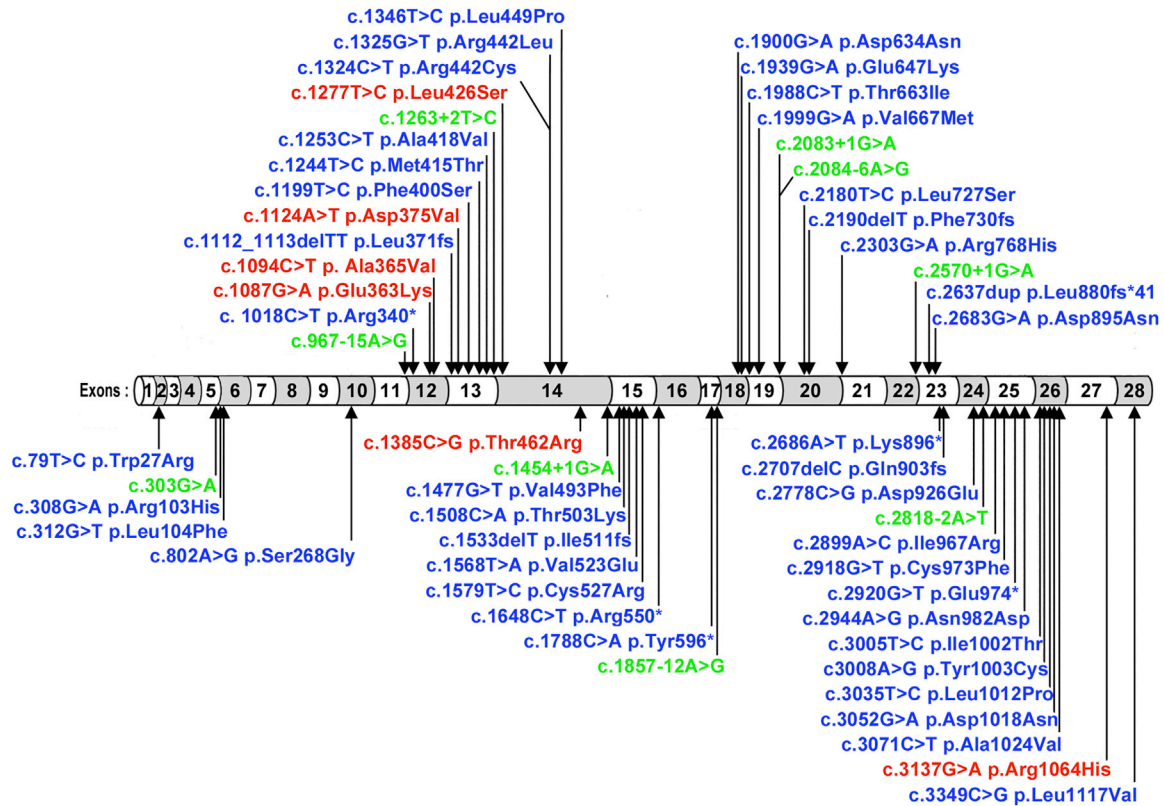
Figure 3. *De novo POLR3B* variants differ from known recessive *POLR3B* pathogenic variants
Recessive variants in *POLR3B* exons (blue) or introns (green) include missense, frameshift, premature truncation, and splicing variants.[18,19] *De novo POLR3B* pathogenic missense variants reported in this study are marked in red and do not overlap with the recessive variants.

Recessive mutations in *POLR3A, POLR3B, POLR1C*, and *POLR3K* are a well-established cause of hypomyelinating leukodystrophy.[1,5–7,9] The individuals with *de novo* heterozygous missense variants in *POLR3B* reported in this study manifest a different set of clinical features, further expanding the phenotypes of POLR3B-related disease to include this distinct disorder. Importantly, brain imaging did not reveal evidence of hypomyelination or leukodystrophy. Participants had pyramidal signs and gait dysfunction of varying severity, as well as some degree of intellectual disability ranging from mild to moderate severity. Most notably, EMG/NCSs for the majority of individuals (5/6) revealed predominantly demyelinating sensory and motor neuropathy. The remaining subject had NCSs at a young age and may not yet have developed this symptom. The shared clinical phenotype of these six individuals provides strong evidence for the pathogenicity of these variants.

In previous studies, we have shown that the recessive mutations in *POLR3B*, which generate variant proteins (p.Arg103His and p.Val523Glu) that do not associate with multiple subunits of the 17-subunit RNA pol III and which clearly show impaired ability to assemble into the active enzyme, cause hypomyelinating leukodystrophies.[17] In contrast, our studies of these *de novo POLR3B* variants suggest they cause disruption in the association of one or two enzyme subunits rather than impairment of full enzyme assembly or nuclear transport of the complex. These results

are suggestive of a distinct mechanism of pathogenicity for these six *de novo* heterozygous mutations, most likely a dominant-negative effect. Of note, recessive variants in *POLR3B* include missense, frameshift, premature truncation, and splicing variants, whereas the *de novo POLR3B* pathogenic variants we report in this study are missense and do not overlap with the recessive variants (Figure 3). Protein structural modeling suggests that these variants may exert their pathogenicity either via alterations in the local environment or by disruption of the assembly of a functionally active pol III pre-initiation complex,[12] however definitive proof will require further studies.

Our results contribute to understanding how mutations targeting several different amino acids of the same pol III subunit (i.e., POLR3B) can exhibit distinct pathological effects in human beings. Our study provides a description of the clinical phenotype and functional evidence for a *de novo* heterozygous POLR3B-related disorder characterized by spasticity, ataxia, and neuropathy. We expand the spectrum of disorders associated with pathogenic variants in *POLR3B*.

Data and code availability

This study did not generate or analyze any new datasets or code.

Supplemental Data

Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2020.12.002.

Web resources

Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), https://evs.gs.washington.edu/EVS/
GeneDx ClinVar submission page, https://www.ncbi.nlm.nih.gov/clinvar/submitters/26957/
gnomAD, https://gnomad.broadinstitute.org/
OMIM, https://omim.org/

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