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

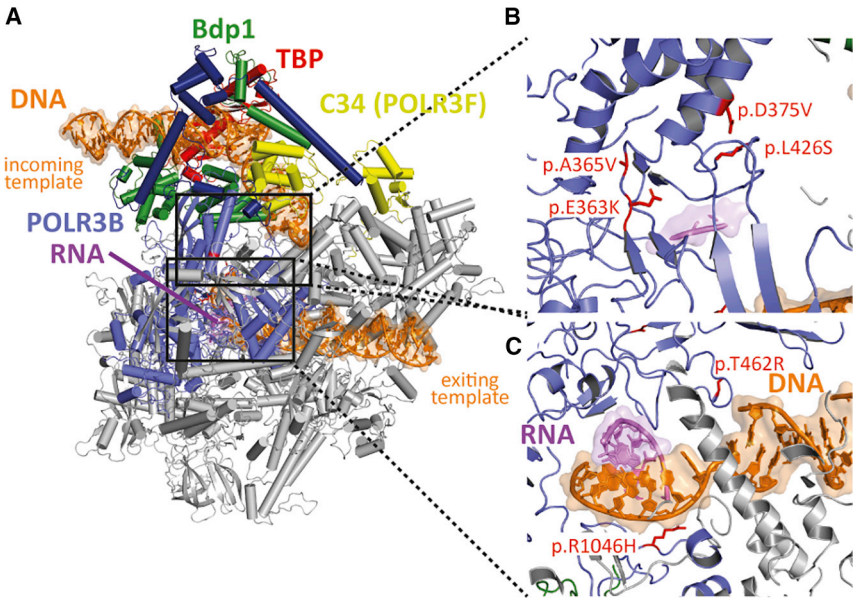

Figure 1. Structural protein modeling
(A) In order to localize the *POLR3B* variants found in the patients on the yeast POLR3B structure, we used the model of POLR3 in transcription initiation state.[13]
(B) Of the six variants identified, four of them cluster in a region of POLR3B where transcribed 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

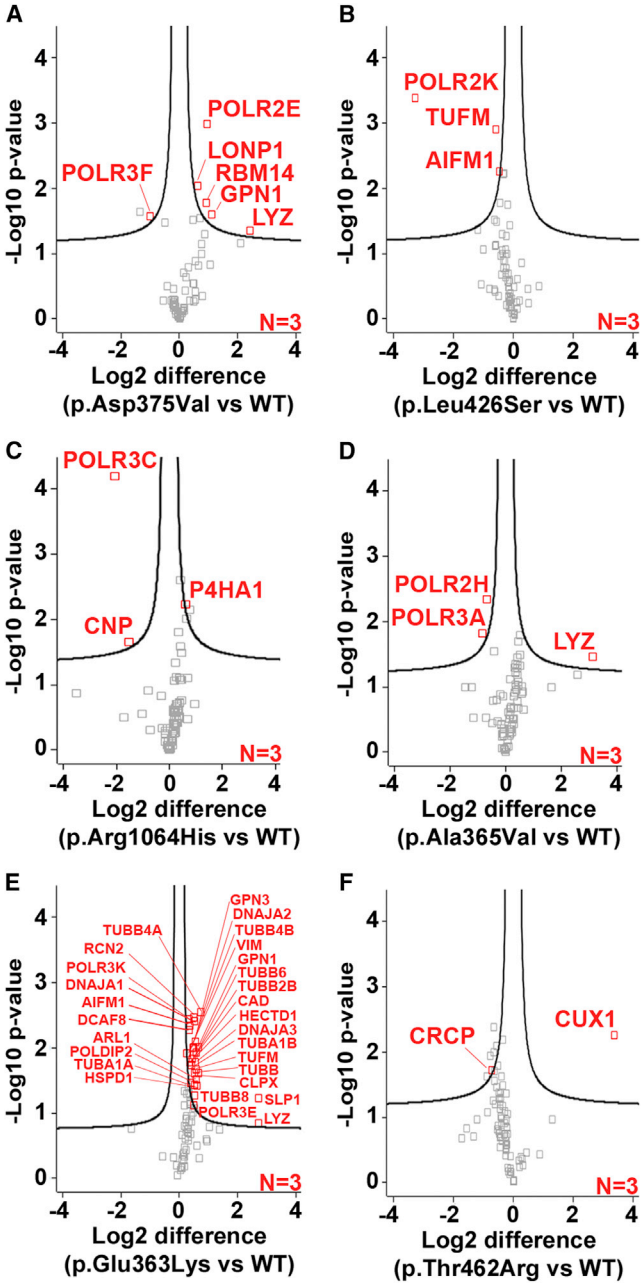

Figure 2. Different POLR3B *de novo* variants distinctively affect the assembly of specific RNA polymerase III subunits

(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.

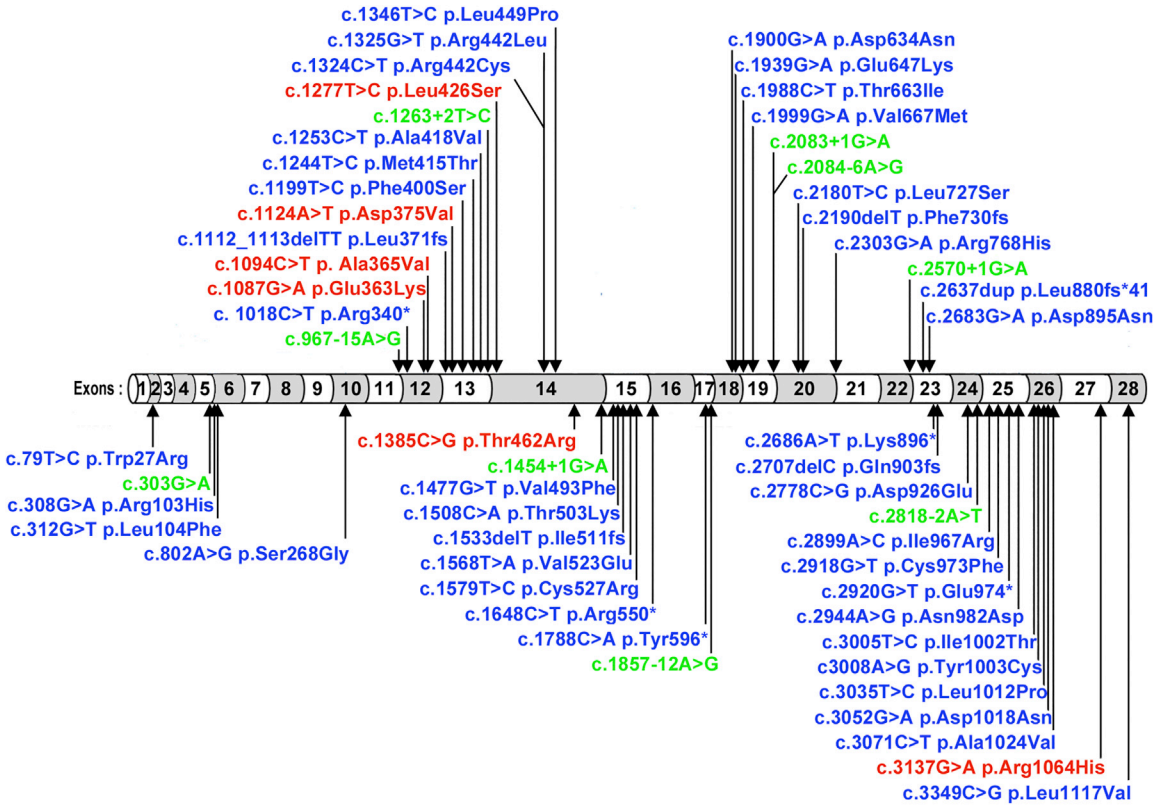

Figure 3. *De novo POLR3B* variants differ from known recessive *POLR3B* pathogenic variants
Recessive variants in *POLR3B* exons (blue) or introns (green) include missense, frameshift, premature truncation, and splicing variants.[18,19] *De novo POLR3B* pathogenic missense variants reported in this 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.

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Declaration of interests

N.I.W. served as advisor for Orchard and PassageBio and participates in a clinical multicenter trial led by Shire/Takeda. G.B. has no relevant conflict of interest. She has received compensation for advisory boards from Ionis (2019), Shire (2013), Actelion Pharmaceuticals (2011), and Santhera Pharmaceutical (2011) and speaker honoraria from Genzyme (2013) and Actelion Pharmaceutical (2012). She received research grants from Shire/Takeda and Bluebird Bio. B.B. is an employee of GeneDx, Inc.

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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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Supplemental Data

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

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Supplemental Data

Supplemental Case Histories

Subject 1

The proband is a 16 year old female born to non-consanguineous parents of East Indian descent at 36 weeks gestational age. Birth weight was 2130g and delivery was uncomplicated. She had global developmental delay and achieved walking at 3.5 years and first words at 5 years. Early infant feeding difficulties and axial hypotonia in infancy later resolved. Formal cognitive testing revealed intellectual delay with nonverbal cognitive abilities, verbal reasoning and adaptive skills less than first percentile for age. She remains dysarthric and mainly speaks in single word phrases. She can print her name but requires assistance to feed and dress. She is ambulatory but with an unsteady, spastic gait. She is unable to perform tandem gait. She has had progressive spasticity and intermittent dystonic posturing. Additional pyramidal signs include 4+ deep tendon reflexes, sustained ankle clonus, positive Hoffman's sign, and extensor plantar responses. She has full power throughout on MRC grading, apart from 4/5 power in her tibialis anterior muscle and extensor hallucis longus. Cerebellar testing revealed mirror movements on rapid alternating movement testing, an intention tremor and mild dysmetria. She has left esotropia but a normal retinal exam, and normal dentition apart from multiple caries. She has oligomenorrhea but normal LH, FSH and estrogen levels. Metabolic testing and microarray were unremarkable. Her brain MRI at age 10 years shows mild cerebellar atrophy and T2 FLAIR hyper intensities of the periventricular white matter, which are nonspecific. She has had normal VEPs, delayed upper brainstem conduction bilaterally on BAEPs and abnormal SSEPs with delayed cortical responses bilaterally. EMG/NCS at 13 and 15 years of age showed a length dependent axonal neuropathy with secondary demyelination (markedly decreased velocities in lower limb motor fibres).

Subject 2

The proband is a 4-year-old boy of Turkish heritage and unremarkable pregnancy and perinatal histories. He was born at 37 weeks with a birth weight of 1928g. He initially demonstrated food refusal and restriction, but this resolved over time. He presented at 18 months old with developmental delay affecting speech and gross motor skills. Generalized epilepsy was diagnosed at 3 years of age, and seizures were characterized by drop attacks and tonic-clonic events that were initially well controlled on Levetiracetam but later required addition of Valproic acid with good control achieved. EEG at 3.5 years of age revealed normal background, with bilateral spike and slow wave complexes, poly spike and wave, associated with bilateral arm drop and/or head drops; with treatment by 4 years the EEG had normalized. He was also diagnosed with intellectual disability and attention deficit hyperactivity disorder. His history is negative for regression. On examination he speaks in 2-3 word phrases and speech is dysarthric. He is ambulatory but walks with an awkward in-toed gait.

Subject 3

The proband was born at term following an uneventful pregnancy and delivery to non-consanguineous parents of mixed Northern European descent. He was born at 40 weeks with a birth weight of 3175g. The patient walked at around 16 months. Other early developmental milestones and growth parameters were reported to be within normal limits. The parents noted difficulties with running coordination and frequent trips/falls starting around age 7 years. The patient had some progression in these symptoms, so he was evaluated by a pediatric neurologist at age 9 years. Physical examination by pediatric neurologist revealed patient had normal muscle tone, bulk and strength but impaired sensation to pinprick, joint position, and vibration in all extremities along with foot drop and a wide-based and high-stepping gait. He had ataxia of limbs

with eyes closed, and he was diagnosed with a sensory ataxia and a peripheral neuropathy was suspected. Nerve conduction study performed at age 10 years revealed a severe demyelinating sensorimotor polyneuropathy. MRI of brain/spine revealed a Chiari type I malformation. He underwent a trial of IVIG at age 11 years that provided no apparent clinical benefit. The patient currently attends a regular school and is reported to perform grade-level academic work. He has difficulty with some fine motor skills such as writing neatly.

Subject 4

The proband is a 22 year-old male of mixed Irish and French heritage with non-contributory family history, pregnancy and perinatal histories. He was born at 40 weeks with a birth weight of 3940g. He presented at six months of age with myoclonic seizures. Over the years, he developed other generalized seizures: atonic and absence seizures. He never had generalized tonic-clonic seizures. His seizures are intractable with more than 20 anti-epileptic medications tried and two trials of ketogenic diet. He underwent a vagal nerve implantation at 5 years of age and an anterior callosotomy at age 10 years. At the age of 19 years, his seizures were noted to be better controlled. EEG at 2 years of age revealed frequent generalized bursts of spike and wave activity during sleep. Repeat study at 3 years revealed clinical myoclonic seizures with multifocal independent and generalized epileptiform discharges, and at 3.5 years showed generalized slowing, bursts of polyspike and wave discharges, with interval improvement. He was diagnosed with global developmental delay around 12 months of age. He is also known for moderate intellectual disability, ADHD with impulsivity. As an adolescent, he had significant behavior difficulties. He is also known to have sensorimotor polyneuropathy, chronic back pain attributed to degenerative disc disease and L5-S1 spondylolisthesis. He also had oromotor dyspraxia, and progressive dysphagia. His examination at 22 years was significant for mild dysarthria, mild

dystonia, spasticity, more so in the lower than the upper extremities, brisk reflexes, except at the ankle where they were reduced, and bilateral Babinski sign, hypersensitivity of the feet, gait ataxia and a positive Romberg sign. Current medications include Valproic acid, Oxcarbazepine, Gabapentin, Clonidine, Depo- Provera, Pantoprazole, and Ranitidine.

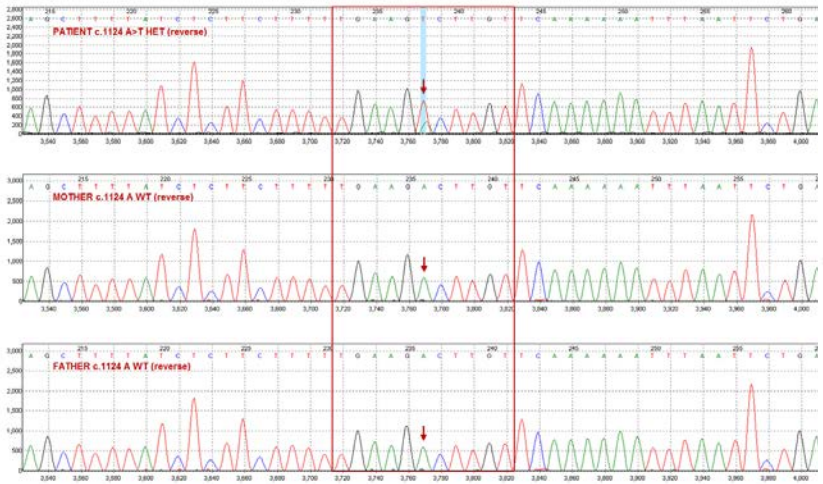
Subject 5

This 8-year-old female proband was born to unrelated parents of Western European origin after an uneventful pregnancy. Delivery was term and uncomplicated, and birth weight was 3350g. She had plagiocephaly treated with helmet therapy and global developmental delays. She learnt to walk without support at age 2.5 years and her IQ was 55 at age 7 years. Her speech is slightly dysarthric, and she uses simple sentences. She was found to have partial growth hormone deficiency (age 2.5 y), which was not treated as her growth was stable. From age 12 months, she had epilepsy with mainly atonic seizures (loss of tone of neck muscles), from age 5 years she also developed atypical absences. Numerous EEGs from 12 months of age consistently revealed spikes and spike waves, which increased during night-time to up to 30% of the recording. Treatment with several antiepileptic drugs (valproic acid, levetiracetam, clobazam, topiramate), methylprednisolone and ketogenic diet did not consistently improve seizures until topiramate was reintroduced at age 8 years with good effect. Neurological examination at age 7 years was remarkable for saccadic pursuit, mild intention tremor and dysmetria, pyramidal signs (legs) and inability to stand on her heels. She had mild scoliosis. Brain MRI at age 6 years was normal. Nerve conduction velocities at age 7 years showed moderately severe motor and sensory polyneuropathy with both de/hypomyelinating and axonal characteristics.

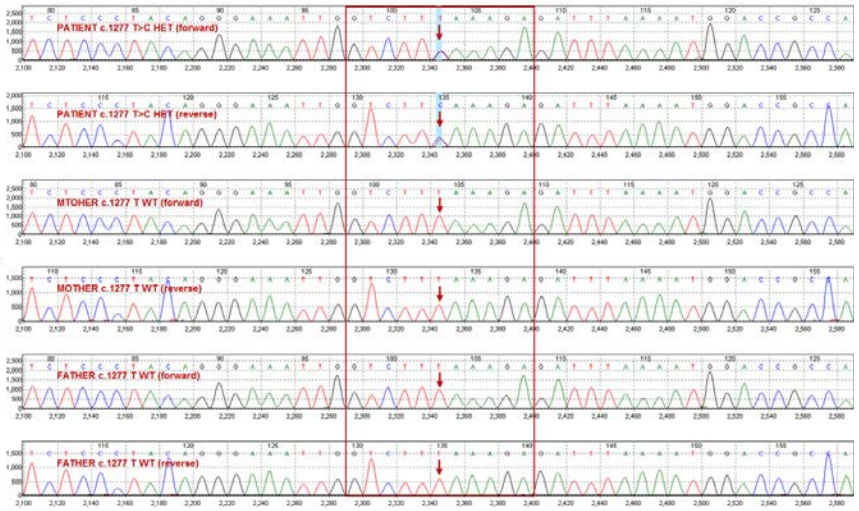
Subject 6

The proband is a 14 year old female born at term to non-consanguineous parents of Italian, Scottish and English descent. Birth weight was 2948g. Delivery was uncomplicated. She is microcephalic. She had global developmental delay and achieved walking at 15 months and speaking in phrases at 3 years. Early infant feeding difficulties later resolved. She is currently reading and writing at a senior kindergarten level. She remains difficult to understand and speaks in short phrases with limited vocabulary. She is ambulatory and now can run following an early gross motor delay, however is unable to perform tandem gait. She has spasticity which is more pronounced in the lower limbs. Additional pyramidal signs include 3+ deep tendon reflexes throughout and extensor plantar responses. She has full power throughout on MRC grading. Metabolic testing and microarray were unremarkable. Her brain MRI at age 14 years is normal. She has had abnormal SEPs, showing absent peripheral and cortical response of posterior tibial nerves. EMG/NCS at 6 and 8 years of age showed a predominantly demyelinating polyneuropathy with slowed conduction velocities, more in the lower limbs.

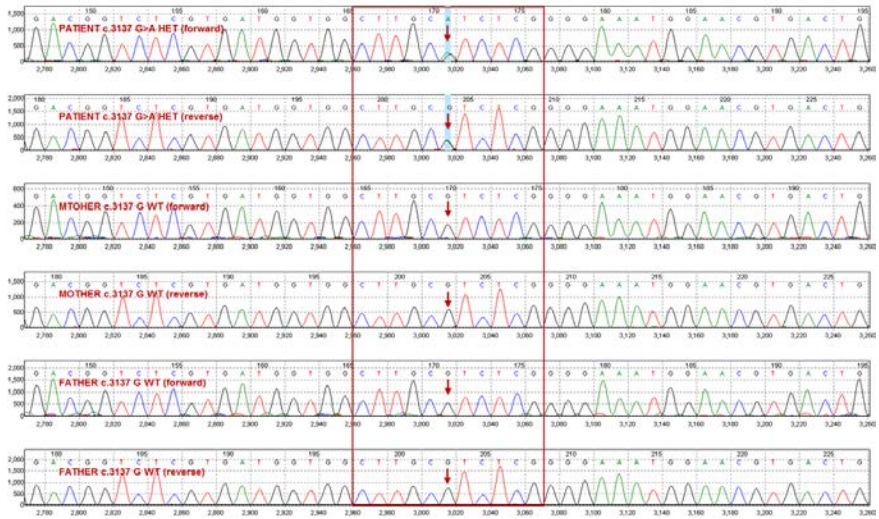
Subject 1: c.1124A>T p.Asp375Val



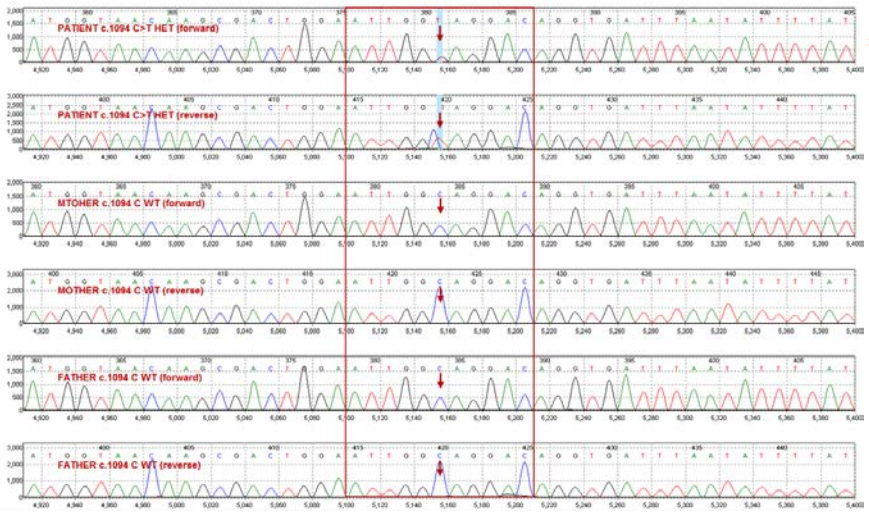
Subject 2: c.1277T>C p.Leu426Ser



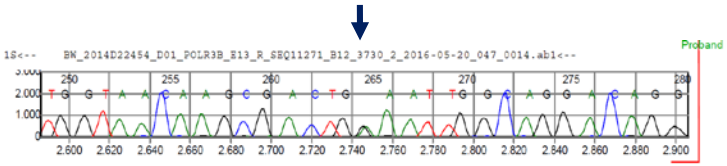
Subject 3: c.3137G>A p.Arg1046His



Subject 4: c.1094C>T p.Ala365Val



Subject 5: c.1087G>A p.Glu363Lys



Subject 6: 1385C>G p.Thr462Arg

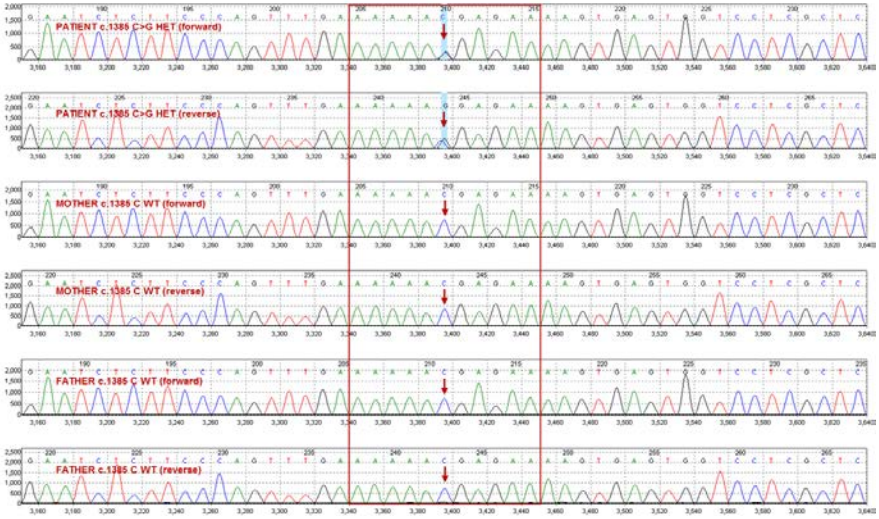


Figure S1. Sanger electropherograms of all probands and parents. Normal parental sequences for Subject 5 are not shown, but all other families clearly demonstrate *de novo* occurrence of the *POLR3B* variants.

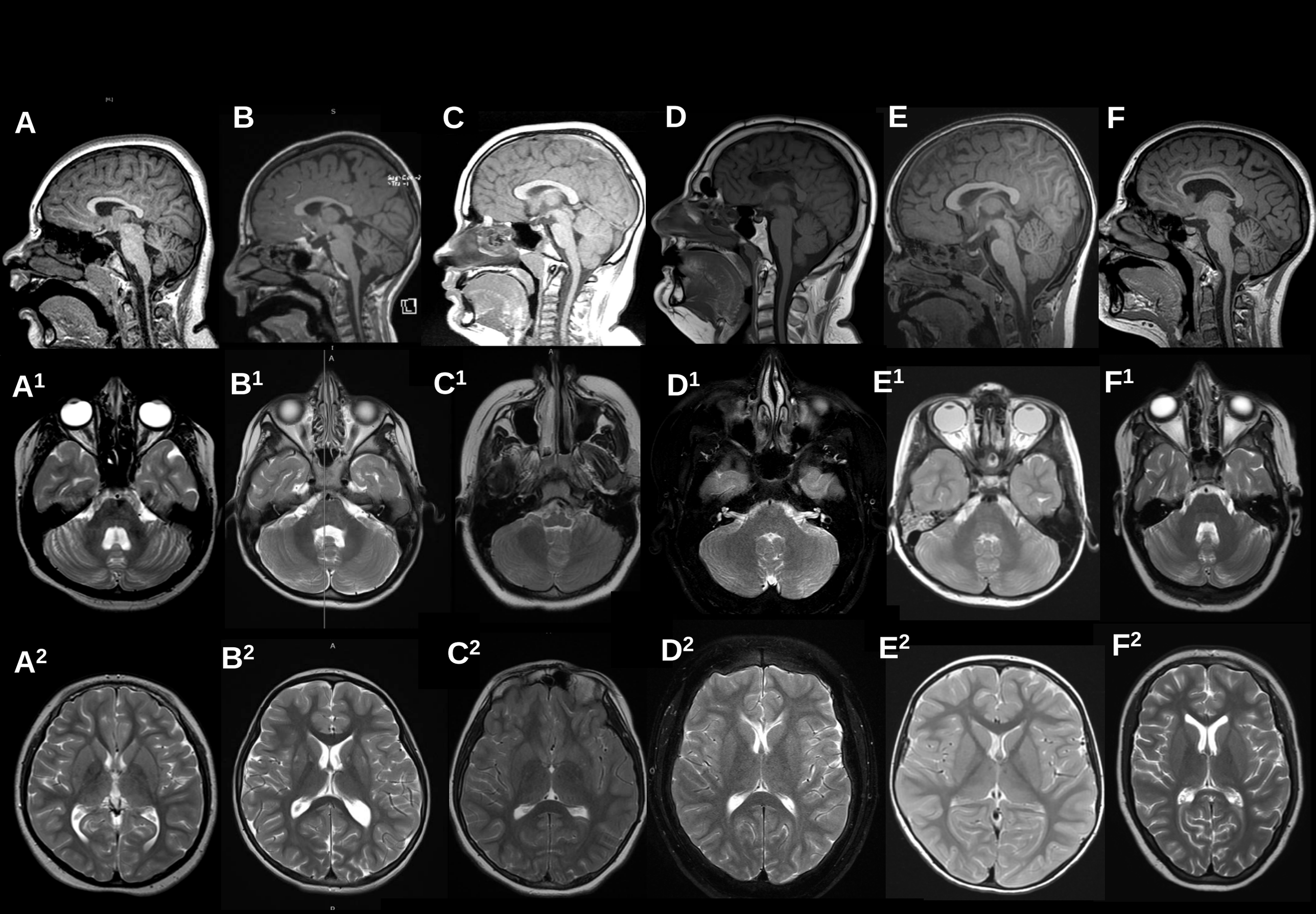

A
B
C
D
E
F
A1
B1
C1
D1
E1
F1
A2
B2
C2
D2
E2
F2

Figure S2. Brain imaging of all subjects 1-6 (A-F). MRI images of sagittal T1 (A-F), axial T2 (at middle cerebellar peduncles, A^1-F^1) and axial T2 (at lateral ventricles, A^2-F^2). There is evidence of mild cerebellar atrophy (A) and T2 hyper intensities (A^2) in the brain MRI of Subject 1, and Subject 4 (D^2) but this is otherwise not present in the other subjects. All 6 subjects had normal brain myelination, with no evidence of hypomyelination or leukodystrophy.

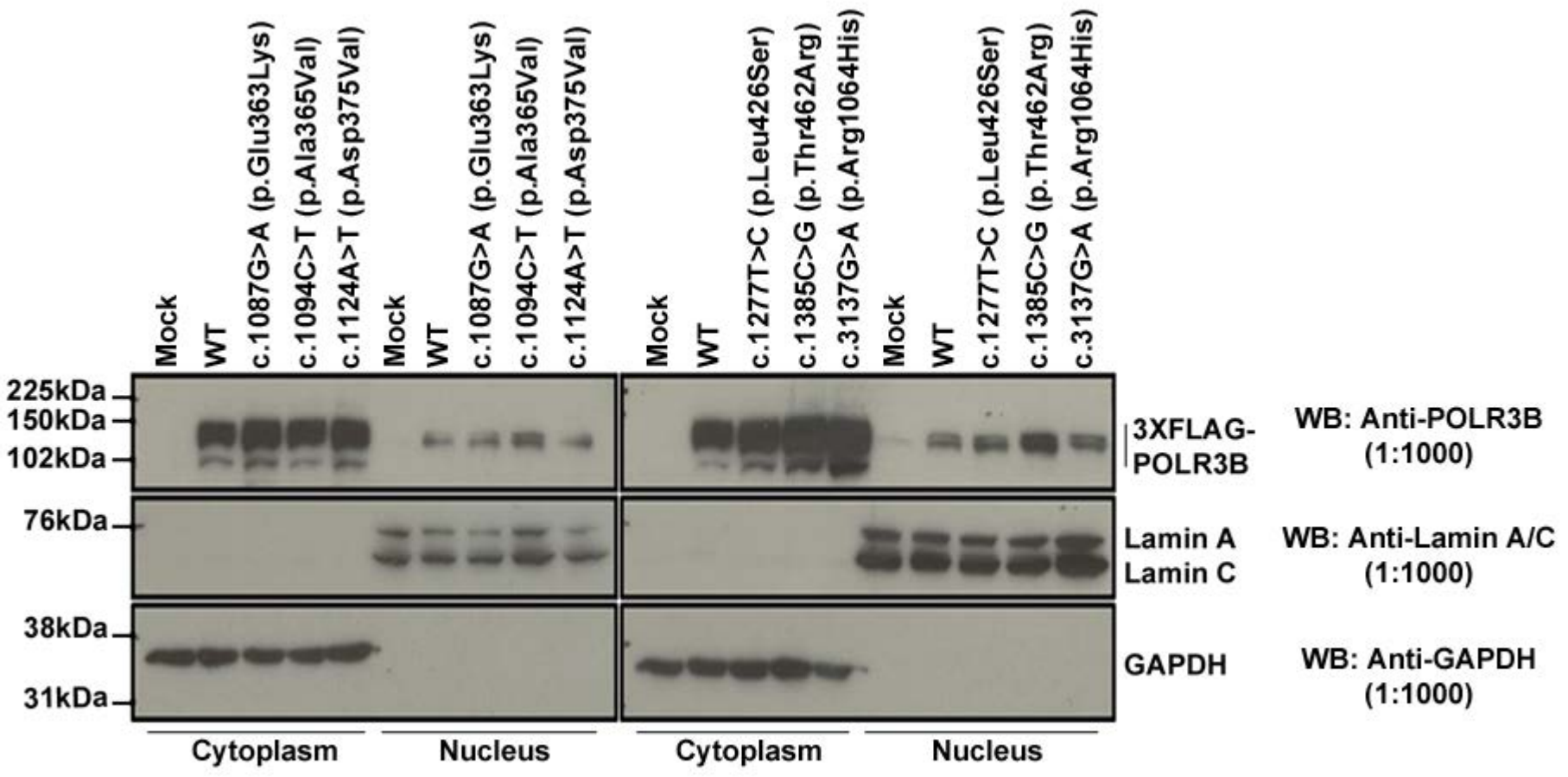

Mock
WT
c.1087G>A (p.Glu363Lys)
c.1094C>T (p.Ala365Val)
c.1124A>T (p.Asp375Val)
Mock
WT
c.1087G>A (p.Glu363Lys)
c.1094C>T (p.Ala365Val)
c.1124A>T (p.Asp375Val)
Mock
WT
c.1277T>C (p.Leu426Ser)
c.1385C>G (p.Thr462Arg)
c.3137G>A (p.Arg1064His)
Mock
WT
c.1277T>C (p.Leu426Ser)
c.1385C>G (p.Thr462Arg)
c.3137G>A (p.Arg1064His)
225kDa
150kDa
102kDa
76kDa
38kDa
31kDa
3XFLAG-POLR3B
Lamin A
Lamin C
GAPDH
WB: Anti-POLR3B (1:1000)
WB: Anti-Lamin A/C (1:1000)
WB: Anti-GAPDH (1:1000)
Cytoplasm
Nucleus
Cytoplasm
Nucleus

Figure S3. *POLR3B de novo* mutations do not appear to affect protein localization.

Subcellular fractions were obtained from HEK 293 cells transiently expressing either POLR3B Wt or the various mutants as indicated. Results of a representative immunoblot are shown. HEK293 cells treated with transfection reagent were used as a negative control (Mock). POLR3B-3XFlag localization was assessed by western blot with an anti-FLAG primary antibody (1:1000). GAPDH (1:1000) and Lamin A/C (1:1000) were used as loading controls and markers of cytoplasmic or nucleus (nucleoplasm and chromatin) extracts respectively.

Table S1. Equivalent *POLR3B* variants in humans and yeast.

Subject	***POLR3B* variant**	**Amino acid Change**	**Yeast Equivalent**
1	c.1121AA>T	p.Asp375Val	Asp390
2	c.1277T>C	p.Leu426Ser	Leu444
3	c.3137G>A	p.Arg1046His	Arg1061
4	c.1094C>T	p.Ala365Val	Ala380
5	c.1087C>G	p.Glu363Lys	Glu378
6	c.1385C>G	p.Thr462Arg	Ser480

Table S2. Detailed electrophysiological results of the nerve conduction studies. Underlined values are interpreted as being abnormal. NA:Not Available, NR:Non-Recordable, L:Left, R: Right

Subject	Motor nerve conduction	Distal latency (ms) (normal ≤ms)[1,2]	Amplitude (mV) (normal ≥mV)[1,2]	Velocity (m/s) (normal ≥ m/s)[1,2]
1 - age 15	L Median L Peroneal L Tibial	3.7 (4.0) 4.5 (6.5) 5.8 (5.0)	13.4 (4.0) 0.6 (2.0) 0.2 (4.0)	47.3 (50) 30.5 (45) 35.9 (40)
2 - age 5	R Tibial	3.1 (4.5)	8.8 (4.0)	40 (40)
3 - age 10	L Ulnar L Median L Peroneal L Tibial	+ prolonged (3.3) ++ prolonged (4.0) NR (6.5) NR (5.0)	Normal (6.0) Normal (4.0) NR (2.0) NR (4.0)	+++ slowing (50) +++ slowing (50) NR (45) NR (40)
4 - age 12	R Ulnar R Median R Peroneal R Tibial	NA (3.3) NA (4.0) Normal (6.5) NA (5.0)	NA (6.0) Normal (4.0) Normal (2.0) <1.0 (4.0)	49.4 (50) 46.3 (50) 32.1 (45) 34.7 (40)
5 - age 7	R Median L Peroneal	3.54 (4.0) 6.3 (6.5)	4.3 (4.0) 1.5 (2.0)	44.2 (50) 28.4 (45)
6 - age 8	L Median L Peroneal L Tibial	4.1 (4.0) 6.2 (6.5) 6.5 (5.0)	9.2 (4.0) 1.2 (2.0) 3.4 (4.0)	41 (50) 36.3 (45) 33.7 (40)

Subject	Sensory nerve conduction	Distal latency (ms) (normal ≤ms)[1,2]	Amplitude (mV) (normal ≥mV)[1,2]	Velocity (m/s) (normal ≥m/s)[1,2]
1 - age 15	L Median L Peroneal	1.4 (3.1) NR (4.4)	33 (20) NR (6)	50.7 (50) NR (40)
2 - age 5	R Sural	1.6 (4.4)	18.8 (6)	50 (40)
3 - age 10	L Median L Sural L Ulnar	NR (3.1) NR (4.4) NR (3.1)	NR (20) NR (6) NR (17)	NR (50) NR (40) NR (50)
4 - age 12	R Median R Sural R Ulnar	NA (3.1) NR (4.4) NA (3.1)	8.2 (20) NR (6) 4.6 (17)	NA (50) NR (40) NA (50)
5 - age 7	R Median L Peroneal	2.34 (3.1) 3.89 (4.4)	9.7 (20) 6.4 (6)	38.4 (50) NA (40)
6 - age 8	L Median	2.1 (3.1)	3.1 (20)	31 (50)

Table S3. List of significantly different protein interactions between p.Glu363Lys variant and WT POLR3B. Q-values were obtained by adjusting p-values for multiple hypothesis testing using a permutation-based test by considering a False Discovery Rate (FDR) of 5% adjusted using an s0 correction factor of 0.1 with 10 000 iterations in Perseus (Version 1.6.10.43).

Gene names	Log2 Difference (p.Glu363Lys-WT)	-Log10 p-value (p.Glu363Lys-WT)	q-value (p.Glu363Lys-WT)
AIFM1	0.373	2.342	0.009
ARL1	0.565	1.514	0.019
CAD	0.431	1.836	0.014
CLPX	0.658	1.607	0.010
DCAF8	0.322	2.274	0.018
DNAJA1	0.516	2.416	0.000
DNAJA2	0.501	2.015	0.000
DNAJA3	0.561	1.775	0.011
GPN1	0.666	2.008	0.000
GPN3	0.558	2.102	0.000
HECTD1	0.370	1.774	0.019
HSPD1	0.515	1.435	0.020
LYZ	2.715	0.842	0.030
POLDIP2	0.379	1.468	0.027
POLR3E	0.506	1.125	0.044
POLR3K	0.331	2.438	0.015
RCN2	0.492	2.470	0.000
SLPI	2.727	1.227	0.014
TUBA1A;TUBA3E	0.592	1.417	0.016
TUBA1B;TUBA4A	0.424	1.736	0.017
TUBB	0.516	1.613	0.013
TUBB2B	0.491	1.883	0.009
TUBB4A	0.750	2.548	0.000
TUBB4B	0.480	2.003	0.012
TUBB6	0.578	1.928	0.000
TUBB8	0.519	1.272	0.019
TUFM	0.564	1.671	0.016
VIM	0.267	1.918	0.037

Supplemental Methods

Affinity purification coupled with mass spectrometry (AP-MS)

Human embryonic kidney cell line 293 (HEK293) cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 2mM glutamine and transfected with FLAG-tagged WT or mutant POLR3B (p.Asp375Val, p.Leu426Ser, p.Arg1064His, p.Ala365Val, p.Glu363Lys, or p.Thr462Arg) expressing plasmids by using Jet Prime transfection reagent (PolyPlus). Transfected cells were incubated at 37°C for 24h, washed with sterile PBS, pelleted at 3500RPM and snap frozen. Affinity purifications were performed in three independent replicate experiments for each mutant and WT. The speedvac protein extracts were re-solubilized in 10µL of a 6M urea buffer, reduced (45mM DTT, 100mM ammonium bicarbonate) for 30min at 37°C, and alkylated (100mM iodoacetamide, 100mM ammonium bicarbonate) for 20min at 24°C. Proteins were digested in 10µL of trypsin solution (5ng/µL of trypsin, Promega, 50mM ammonium bicarbonate) at 37°C for 18 h. The digests were acidified with trifluoroacetic acid and cleaned by MCX (Waters Oasis MCX 96-well Elution Plate). Peptides were identified by LC-MS/MS using HPLC coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific) through a Nanospray Flex Ion Source. MS/MS raw data were searched against the human SwissProt database (updated on April 24th 2019), and a reverse decoy, using MaxQuant (version 1.6.10.43)[3]. Label-free quantification (LFQ)-intensities were further analyzed with Perseus (Version 1.6.10.43)[4]. A log2 transformation was applied to LFQ intensities. LFQ intensities reported as 0 by MaxQuant were replaced by randomly generated intensities normally distributed with a width of 0.3 times and a downshift of 1.8 times the standard deviation of non-zero intensities. For simplification, proteins that did not show enrichment compared to the empty vector control (p-value <0.05 and intensity difference higher or equal to 2) were excluded.

Statistical differences between protein intensities from WT and mutant experiments were determined using a two-tailed T-test adjusted for multiple hypothesis testing using a permutation-based test with a False discovery rate (FDR) of 5% adjusted using a s0 correction factor of 0.1 with 10 000 iterations[5]. The level of differential interaction with POLR3B was considered statistically significant when the adjusted P value was <0.05.

Fibroblast culture

MCH 073 fibroblasts were isolated from a healthy control female subject. MCH073 and WM253.0 (c.1124A>T p.Asp375Val mutant) fibroblasts were maintained in culture in DMEM media supplemented with 10% fetal bovine serum and 2 mM glutamine.

Whole cell extract and Subcellular fractionation

Fibroblast whole cell extracts were obtained followed by incubation with total lysing buffer (25mM HEPES pH8.0, 50 mM KCl, 1 mM EDTA, 0,05% NP40, 5% glycerol, 0.5 mM DTT, protease inhibitor cocktail (Roche), 1 mM PMSF, 10 mM NaF and 1 mM Na_3VO_4) for 30 min at 4°C. 10 units of benzonase was added and each sample was sonicated three times for 10 s at an amplification with a Sonic Dismembrator model 100 (Fisher Scientific) and centrifuged at 16000xg for 30 min at 4°C. Protein concentrations were determined by Bradford assay. HEK293 cells were grown in 6 wells plates and transfected with Jet Prime reagent (PolyPLUS) with either p3XFLAG-CMV14-POLR3B WT, E363K, A365V, D375V, L426S, T462R and R1064H or with the transfection reagent alone (Mock) according to the manufacturer's recommendations for 24 h. The cytoplasmic fractions were obtained by incubation with hypotonic lysis buffer (10 mM

HEPES, 10% Glycerol, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 340 mM Sucrose, protease inhibitor cocktail (Roche), 1 mM PMSF, 10 mM NaF and 1 mM Na_3VO_4) for 10 min on ice. 0.1% of triton X100 was added, mixed for 10 seconds and centrifuged at 1200xg at 4°C to pellet the nuclei. The nucleus pellets were washed with hypotonic lysis buffer. Nuclear fractions were obtained by incubation with nuclear lysis buffer (10 mM HEPE, 420 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail) complemented with 10 units of benzonase (EMD millipore) for 30 min at 4°C with agitation. Each sample was sonicated three times for 10 s at an amplification setting of 30% with a Sonic Dismembrator model 100 (Fisher Scientific) and centrifuged at 16000xg for 30 min at 4° C. Protein concentrations were determined by Bradford assay.

Western blot

40 µg of whole cell extract from MCH073 and WM253.0 was loaded on a 10% polyacrylamide gel, transferred on a PVDF membrane (EMD millipore) and the membrane was blocked with PBS + 0.5% Tween20 + 5% milk. The membrane was washed six times with PBS + 0.5% Tween20 after antibody incubation and western blotting was performed with rabbit anti-POLR3B (EPR8719, AbCam) and mouse anti-GAPDH (0411, Santa Cruz). For subcellular fractions, 23.5 µg of cytoplasmic extract and 15 µg of nuclear extract (each value corresponding roughly to 1/20 of each fraction) were loaded on an 10% polyacrylamide gel, transferred on a PVDF membrane (EMD millipore) and the membrane blocked with PBS + 0.5 % Tween20 + 5% milk. The membrane was washed three times with PBS + 0.5% Tween20 after antibody

incubation and western blotting was performed with mouse anti-FLAG (M2, Sigma), mouse anti-GAPDH (0411, Santa Cruz), and rabbit anti-Lamin A/C (EPR4068, AbCam) antibodies.

Subcellular fractionation

HEK293 cells were grown in 6 wells plates and transfected with Jet Prime reagent (PolyPLUS) with either p3XFLAG-CMV14-POLR3B WT, E363K, A365V, D375V, L426S, T462R and R1064H or with the transfection reagent alone (Mock) according to manufacturer's recommendations for 24 h. The cytoplasmic fractions were obtained by incubation with hypotonic lysis buffer (10 mM HEPES, 10% Glycerol, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 340 mM Sucrose, protease inhibitor cocktail (Roche), 1 mM PMSF, 10 mM NaF and 1 mM NaVO4) for 10 min on ice. 0.1% of triton X100 was added, delicately mixed for 10 seconds and centrifuged at 1200xg at 4°C to pellet the nuclei. The nucleus pellets were washed with hypotonic lysis buffer. Nuclear fractions were obtained by incubation with nuclear lysis buffer (10 mM HEPE, 420 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail) complemented with 10 units of benzonase (EMD millipore) for 30 min at 4°C with agitation. Each sample was sonicated three times for 10 s at an amplification setting of 30% with a Sonic Dismembrator model 100 (Fisher Scientific) and centrifuged at 16000xg for 30 min at 4°C. Protein concentrations were determined by Bradford assay and 23.5 µg of cytoplasmic extract and 15 µg of nuclear extract (each value corresponding roughly to 1/20 of each fraction) were loaded on an 10% polyacrylamide gel. Western blotting was performed with mouse anti-FLAG (M2, Sigma), mouse anti-GAPDH (0411, Santa Cruz), and rabbit anti-Lamin A/C (EPR4068, AbCam) antibodies.

Supplemental References

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