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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.

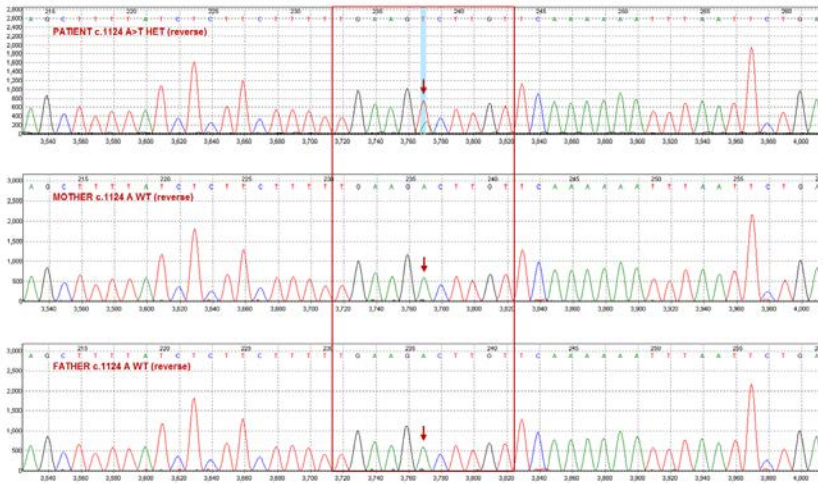
Subject 5

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.

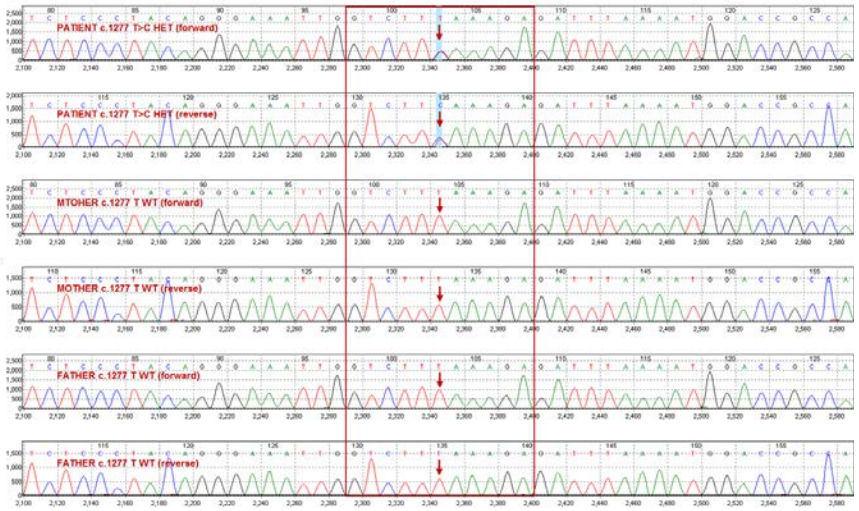
Subject 6

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.

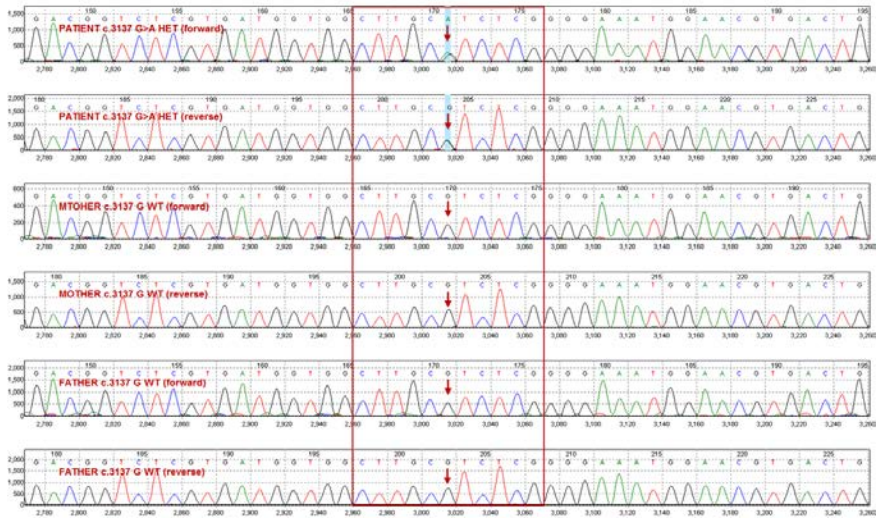
Subject 1: c.1124A>T p.Asp375Val



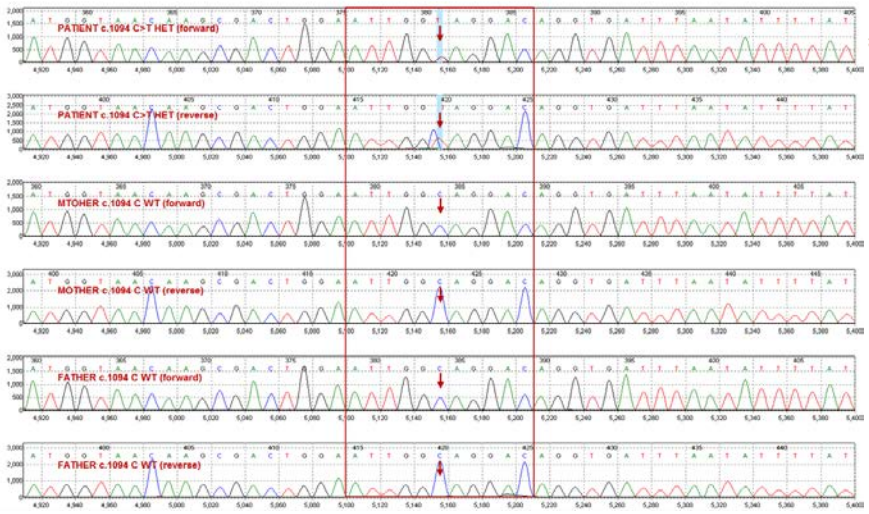
Subject 2: c.1277T>C p.Leu426Ser



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



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



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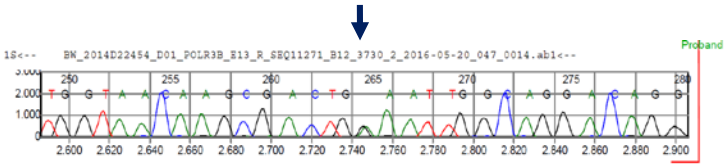

Subject 6: 1385C>G p.Thr462Arg

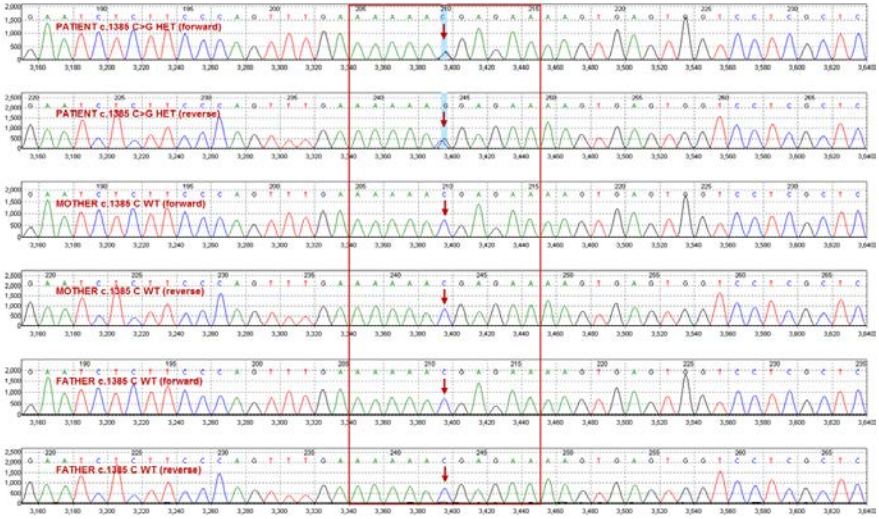

Figure S1. Sanger electropherograms of all probands and parents. Normal parental sequences for Subject 5 are not shown, but all other families clearly demonstrate *de novo* occurrence of the *POLR3B* variants.

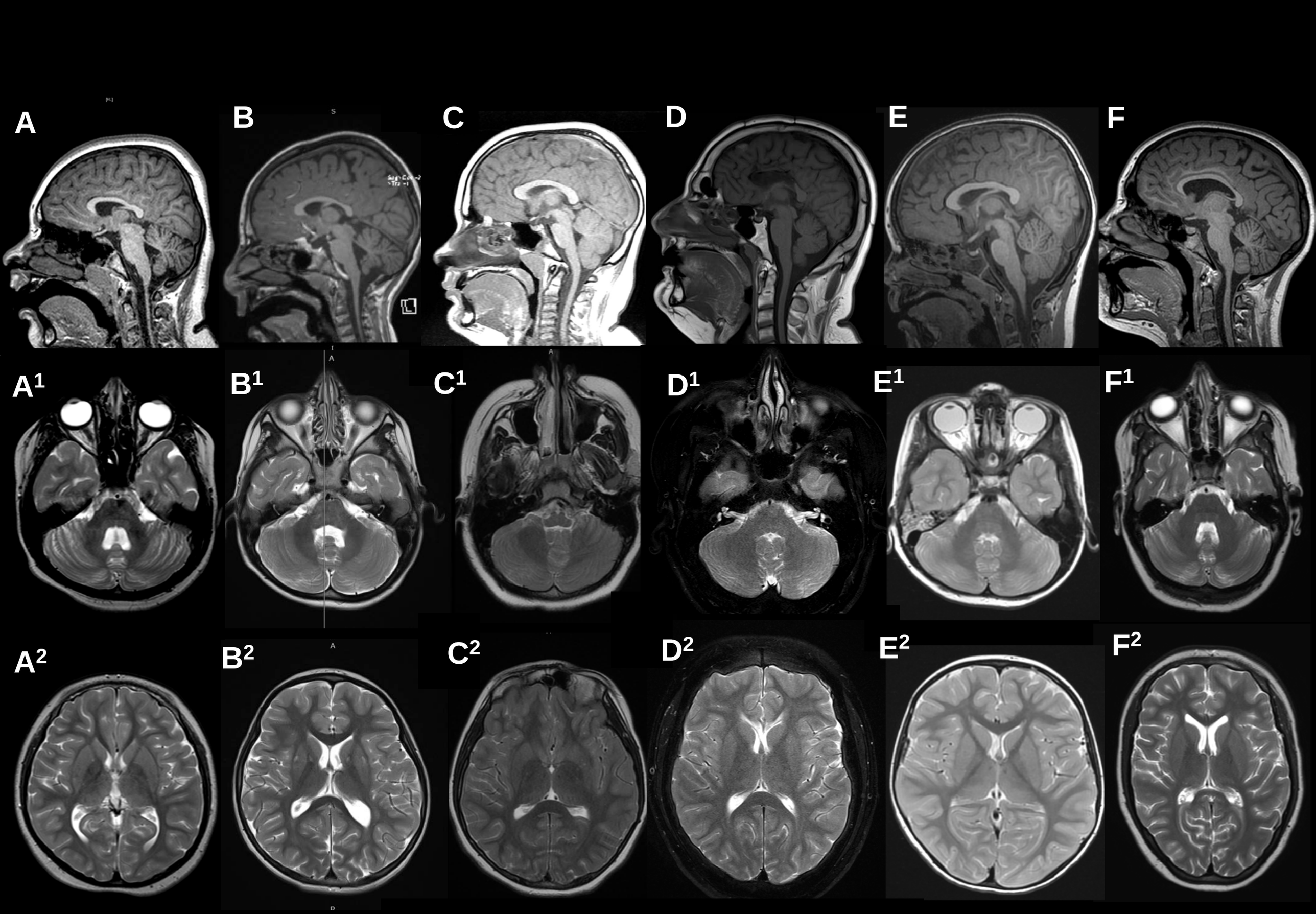
A
B
C
D
E
F
A1
B1
C1
D1
E1
F1
A2
B2
C2
D2
E2
F2

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.

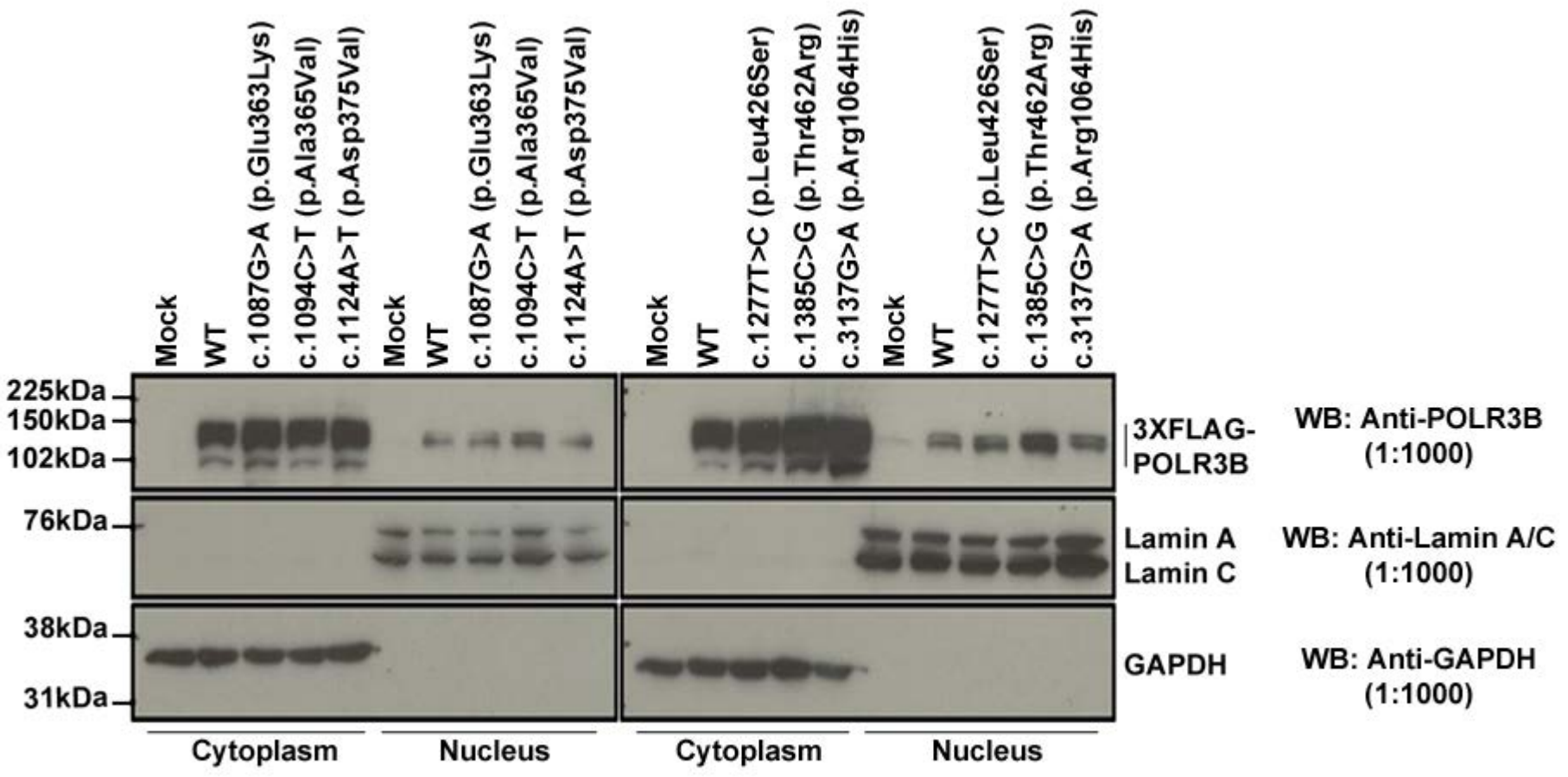
Mock
WT
c.1087G>A (p.Glu363Lys)
c.1094C>T (p.Ala365Val)
c.1124A>T (p.Asp375Val)
Mock
WT
c.1087G>A (p.Glu363Lys)
c.1094C>T (p.Ala365Val)
c.1124A>T (p.Asp375Val)
Mock
WT
c.1277T>C (p.Leu426Ser)
c.1385C>G (p.Thr462Arg)
c.3137G>A (p.Arg1064His)
Mock
WT
c.1277T>C (p.Leu426Ser)
c.1385C>G (p.Thr462Arg)
c.3137G>A (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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