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Recessive mutations in *POLR1C* cause a leukodystrophy by impairing biogenesis of RNA polymerase III

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A small proportion of 4H (Hypomyelination, Hypodontia and Hypogonadotropic Hypogonadism) or RNA polymerase III (POLR3)-related leukodystrophy cases are negative for mutations in the previously identified causative genes *POLR3A* and *POLR3B*. Here we report eight of these cases carrying recessive mutations in *POLR1C*, a gene encoding a shared POLR1 and POLR3 subunit, also mutated in some Treacher Collins syndrome (TCS) cases. Using shotgun proteomics and ChIP sequencing, we demonstrate that leukodystrophy-causative mutations, but not TCS mutations, in *POLR1C* impair assembly and nuclear import of POLR3, but not POLR1, leading to decreased binding to POLR3 target genes. This study is the first to show that distinct mutations in a gene coding for a shared subunit of two RNA polymerases lead to selective modification of the enzymes' availability leading to two different clinical conditions and to shed some light on the pathophysiological mechanism of one of the most common hypomyelinating leukodystrophies, POLR3-related leukodystrophy.

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to confirm segregation. In total, 13 *POLR1C* mutations were detected in eight cases (Supplementary Table 2a, Fig. 1). Similar to what is observed in cases with the disease caused by mutations in *POLR3A* or *POLR3B*, clinical and radiological characteristics of these eight cases were compatible with POLR3-related leukodystrophy; however, patients did not necessarily have all clinical (that is, neurological, dental, ophthalmological and endocrine abnormalities) and MRI features^{1,3,12} (Table 1, Fig. 2, Supplementary Tables 4 and 5) of the disease.

Impact of *POLR1C* recessive mutations on POLR1 and POLR3.

To investigate the potential pathogenic role of these mutations, we evaluated the impact of the two homozygous mutations (Table 1) on the function of nuclear POLR1 and POLR3. FLAG-tagged versions of the wild-type (WT) form of *POLR1C* and its variants having the p.Asn32Ile (N32I) or p.Asn74Ser (N74S) substitution were expressed in HeLa cells. Anti-FLAG affinity purification was performed on cell extracts and the purified proteins were analysed using shotgun proteomics. These experiments were performed in triplicate. The expression level of the various forms of *POLR1C* (that is, WT and mutants) were equivalent and comparable (see Supplementary Fig. 2), and protein expression levels were normalized by the expression level of the bait in each purification (Supplementary Table 6). The tagged WT *POLR1C* pulled down all subunits of both POLR1 and POLR3 (Fig. 3a), a finding that was expected since *POLR1C* is a shared subunit of both polymerases (see Fig. 3c for a schematic representation). Both tagged mutated *POLR1C* (N32I and N74S) pulled down amounts of POLR1-specific (POLR1A, 1B, 1E, CD3EAP, TWISTNB and ZNRD1) and POLR1/POLR3-shared subunits (POLR2E, 2F, 2H, 2K, 2L, 1C and 1D) that were not significantly different from those pulled down by the WT (this is especially true for the POLR1-specific subunits). However, both mutated subunits pulled down lower amounts of POLR3 (most specific subunits) relative to WT. This finding suggests that the mutations lead to a selective defect in POLR3 assembly, and not in POLR1. Indeed, assembly of nuclear RNA polymerases has previously been shown to occur in the cell's cytoplasm and defects in RNA polymerase assembly caused by functional disruption of the RNA Polymerase-Associated proteins was previously shown to lead to cytoplasmic accumulation of polymerase subunits^{15–21}. Notably, the position of mutated residues in the proposed structure of POLR3 is compatible with defects in enzyme

assembly and/or folding (see Supplementary Fig. 1). To further confirm that mutated *POLR1C* variants (N32I and N74S) are impaired in supporting enzyme assembly and nuclear import, immunofluorescence studies were performed using anti-FLAG antibodies. The results reveal an accumulation of both mutated *POLR1C* subunits, but not the WT subunit, in the cytoplasm (Fig. 3d). We then performed chromatin immunoprecipitation (ChIP) of FLAG-tagged *POLR1C* followed by high-throughput sequencing (ChIP-Seq), as a proxy of gene transcription activity^{22–24}, to investigate the impact of *POLR1C* mutations on gene occupancy by POLR1 and POLR3. After alignment of the reads to the human reference genome (hg19), we compared occupancy of WT and mutated *POLR1C* variants over 659 *POLR3*-transcribed genes, including all transfer RNA (tRNA) and 5S ribosomal RNA genes (Supplementary Table 7). As expected, mutated *POLR1C* variants displayed reduced binding to *POLR3*-transcribed genes compared with WT *POLR1C* for all three classes of *POLR3*-transcribed genes (classified according to their regulatory elements; see legend to Fig. 4a). In contrast, there were no differences in WT and mutated *POLR1C* occupancy over the ribosomal RNA gene transcribed by POLR1 (Fig. 4c). Together, these results indicate that the N32I and N74S substitutions in the POLR1/POLR3-shared subunit *POLR1C* specifically interfere with assembly, nuclear import and chromatin association of POLR3. To compare the roles of leukodystrophy versus TCS-causing mutations in the biogenesis of POLR1 and POLR3, we expressed FLAG-tagged *POLR1C* with the p.Arg279Gln (R279Q) mutation in HeLa cells, affinity-purified the tagged subunit and identified the purified interactors using mass spectrometry. Contrary to *POLR1C* (N32I) and *POLR1C* (N74S), none of the subunits of POLR1 and POLR3 were pulled down by tagged *POLR1C* (R279Q) in amounts that were statistically significantly different from the WT (Fig. 5a,b, Supplementary Table 6), suggesting that this TCS-causing mutation does not affect the assembly of these polymerases. Notably, however, immunofluorescence results indicate that *POLR1C* (R279Q) targeting to the nucleolus is impaired as compared with the WT subunit (Fig. 5c) and the N32I- and N74S-mutated subunits (see Fig. 3d).

Discussion

With the advent of exome sequencing, it is becoming increasingly apparent that allelic heterogeneity in genes encoding essential

Table 1 | Major clinical and MRI findings in index patients with mutations in *POLR1C*.

Patient numbers	Family nos	Gene	Mutation 1	Mutation 2	Main clinical characteristics			Main MRI characteristics			
					Neurological abnormalities	Myopia	Dental abnormality	Hypogonadotropic hypogonadism	Hypo-myelination	Thin corpus callosum	Cerebellar atrophy
1	I	<i>POLR1C</i>	c.95A>T; p.Asn32Ile	c.95A>T; p.Asn32Ile	+	–	+	Too young	+	+	–
2	II	<i>POLR1C</i>	c.221A>G; p.Asn74Ser	c.221A>G; p.Asn74Ser	+	–	–	Too young	+	+	–
3	III	<i>POLR1C</i>	c.436T>C; p.Cys146Arg	c.883_885delAAG; p.Lys295del	+	–	–	Too young	+	+	–
4	IV	<i>POLR1C</i>	c.77C>T; p.Thr26Ile	c.326G>A; p.Arg109His	+	–	+	Too young	+	+	+
5	V	<i>POLR1C</i>	c.193A>G; p.Met65Val	c.572G>A; p.Arg191Gln	+	+	–	Too young	+	+	+
6	VI	<i>POLR1C</i>	c.326G>A; p.Arg109His	c.970G>A; p.Glu324Lys	+	+	–	–	+	+	+
7	VII	<i>POLR1C</i>	c.395G>A; p.Gly132Asp	c.461_462delAA; p.Lys154Argfs*4	+	+	–	–	+	+	+
8	VIII	<i>POLR1C</i>	c.281T>C; p.Val94Ala	c.785T>C; p.Ile262Thr	+	–	+	Too young	+	+	+

–, absent; +, present; MRI, magnetic resonance imaging.

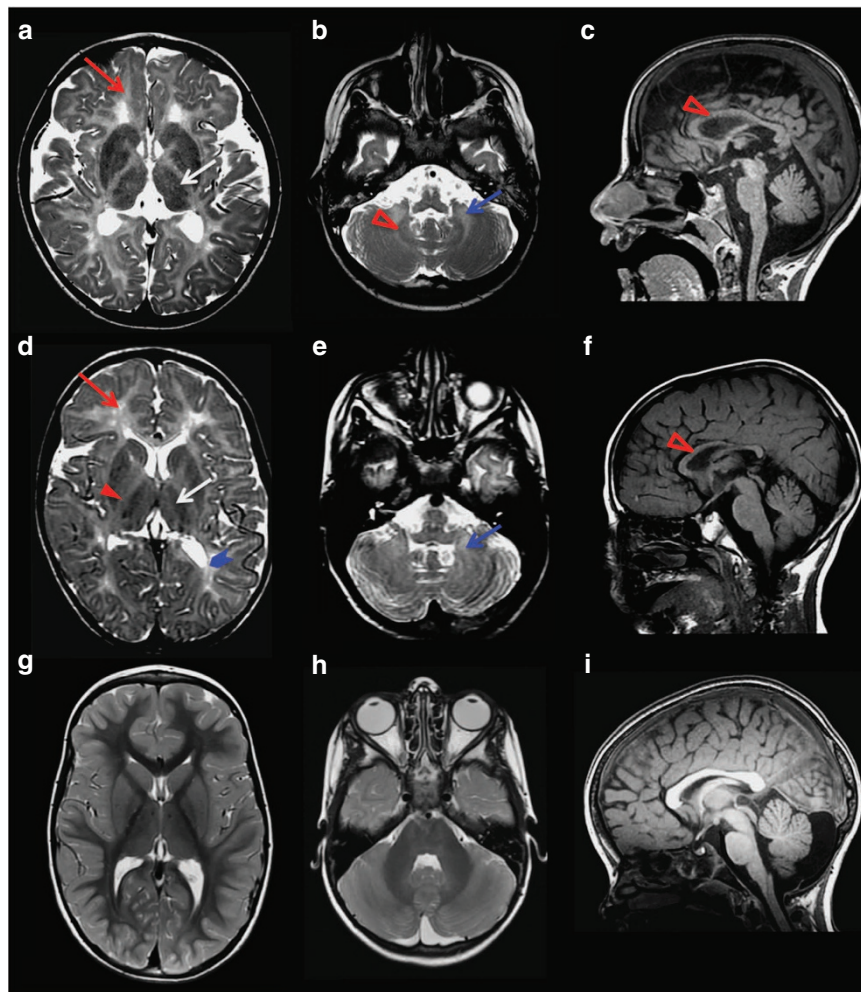


Figure 2 | MRI characteristics of POLR3-related leukodystrophy caused by *POLR1C* mutations. Axial T2-weighted (**a,b,d,e,g,h**) and sagittal T1-weighted (**c,f,i**) images of case 1 aged 6 years (**a-c**) and case 2 aged 4.5 years (**d-f**) compared with a healthy control aged 4 years (**g-i**). Diffuse hyperintense signal of the supratentorial (red arrow, **a,d**) and cerebellar (blue arrow, **b,e**) white matter is visible on the T2-weighted images, indicating hypomyelination. There is no cerebellar atrophy. As typical for POLR3-related leukodystrophy, the ventrolateral thalamus (white arrow, **a,d**), the optic radiation (thick arrowhead blue, **d**) and the dentate nucleus (open red arrowhead, **b**) show a relative hypointense signal on the T2-weighted images resulting in an easily visible dentate nucleus (**b**) as compared with the control (**h**) as well as a small dot in the posterior limb of the internal capsule (red arrowhead, **d**). The corpus callosum is slightly thinned in case 1 and thinned in case 2 (Open red arrowhead, **c,f**).

proteins, such as those involved in transcription, results in highly variable phenotypes. A previous report of mutations in *POLR1C* (ref. 14) highlighted the discovery of the first cases of TCS with an autosomal recessive mode of inheritance. TCS (MIM 154500, 613717, 248390) is characterized by an abnormal craniofacial development and is caused, from most to least frequent, by mutations in *TCOF1* (dominant), *POLR1D* (dominant) or *POLR1C* (recessive)¹⁴. TCS caused by mutations in *POLR1D* or *POLR1C* has been proposed to arise as a consequence of a decreased quantity of functional ribosomes in the neuroepithelium and the neuronal crest cells during critical points of embryogenesis^{14,25}. We assessed the role of a TCS-causing mutation (R279Q) in the biogenesis of POLR1 and POLR3. Our results (see Fig. 5, Supplementary Table 6) indicate that this mutation does not impair polymerase assembly, as opposed to leukodystrophy-causing mutations (see Fig. 3), but affect targeting to the nucleolus, the site for Pol I transcription.

Our findings suggest that improper assembly and nuclear import of POLR3 resulting from leukodystrophy-causative mutations lead to decreased availability of the complex at the chromatin. As POLR3 binding is well correlated to tRNA

expression^{22–24}, this decreased POLR3 occupancy is likely to cause reduced transcription of tRNAs and other essential small non-coding RNAs. One hypothesis is that mutations in *POLR3A*, *POLR3B* or *POLR1C* lead to decreased levels of certain tRNAs crucial for the synthesis of proteins essential for central nervous system myelin development. tRNA function has also been suggested to be impaired in other white matter disorders^{26–30} caused by mutations in tRNA-aminoacyl synthetases, including hypomyelinating leukodystrophies such as *RARS*-associated hypomyelination³⁰ and Hypomyelination with Brain Stem and spinal cord involvement and Leg spasticity²⁹. Of note, tRNA synthetases have not been found in our POLR3 purifications, suggesting that tRNA aminoacylation is not coupled with POLR3 transcription. An alternative hypothesis would involve changes in the expression of other essential small non-coding RNAs synthesized by POLR3.

In conclusion, our sequencing study of 18 cases with compatible clinical and/or radiological features with 4H or POLR3-related leukodystrophy identified 13 different *POLR1C* mutations in eight cases. *POLR1C* joins an emerging group of genes with dual roles in pathogenesis of human diseases³¹,

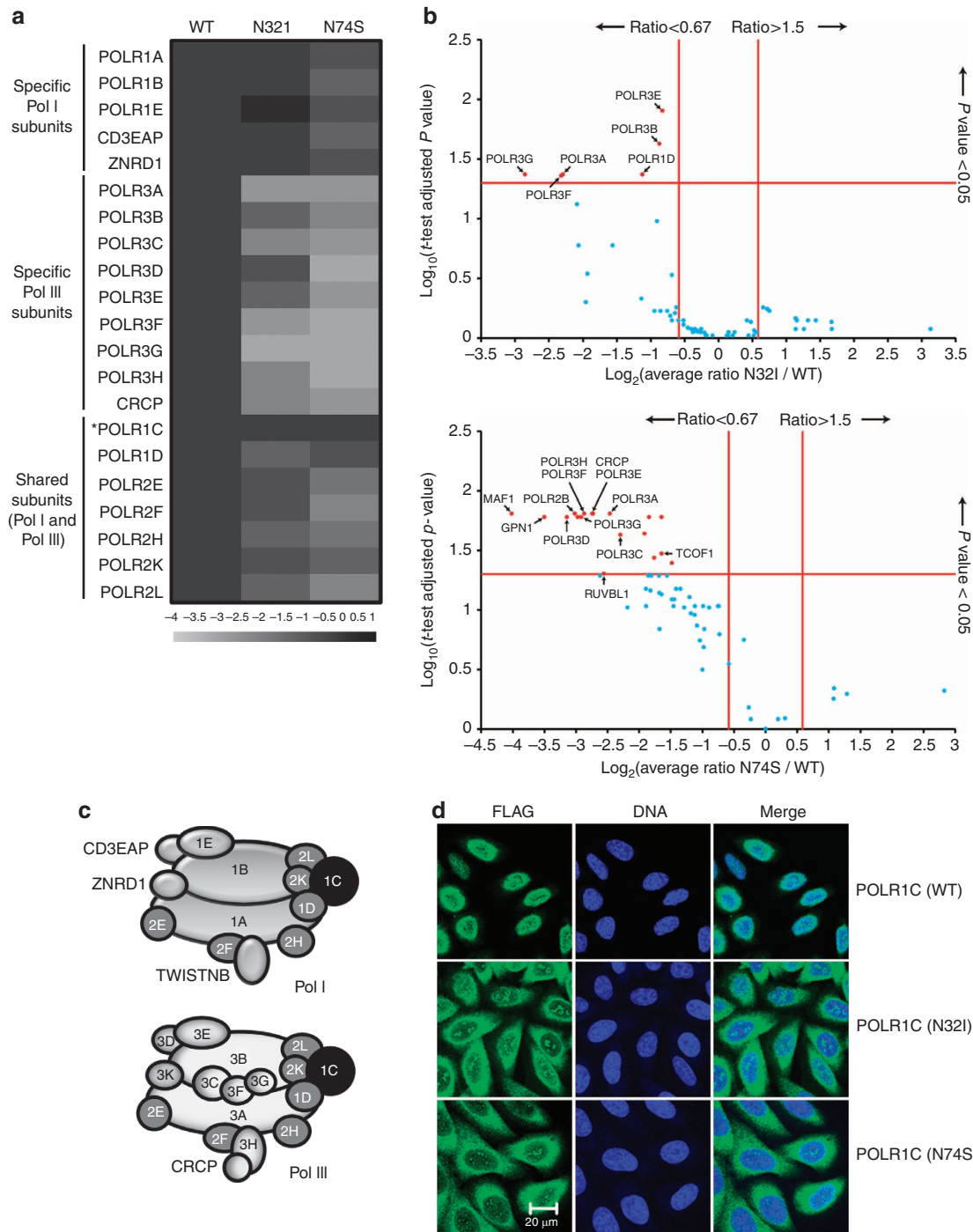


Figure 3 | Impact of *POLR1C* mutations on polymerase assembly and nuclear import. (a) FLAG-tagged *POLR1C* variants, either the wild-type (1C) polypeptide or mutated versions having a N321 or a N74S substitution, were expressed in HeLa cells and purified using anti-FLAG affinity chromatography. The co-purified proteins were identified using LC-MS/MS mass spectrometry. The heatmap contains the \log_2 -transformed average spectral count ratios N321 or N74S/WT across all three replicates. Spectral counts were computed with Mascot (see Supplementary Table 6 for the complete data set). Specific and shared *POLR1* (Pol I) and *POLR3* (Pol III) subunits are identified on the left. *POLR1C* (the bait) is identified by an asterisk. (b) Volcano plots of the \log_2 -transformed average spectral count ratios N321 or N74S/WT (x axis) and the $-\log_{10}$ -transformed *P* values (adjusted with the Benjamini-Hochberg procedure) resulting from the two-tailed one-sample *t*-tests of the high-confidence interactors of *POLR1C*. Red proteins show a level of differential interaction with *POLR1C* that is statistically significant, while blue proteins do not. (c) Schematic representation of the subunit composition of *POLR1* (Pol I) and *POLR3* (Pol III; see refs 53,54 for details). Shared subunits are in grey and *POLR1C* in black. (d) Immunofluorescence experiments showing the cellular localization of tagged *POLR1C* variants. Nuclei are stained using TO-PRO-3 iodine. Scale bar, 20 μm .

expanding the clinical phenotype associated with constitutional mutations in this gene and opening new aspects in the annotation and assessment of pathogenicity of sequence variants.

Furthermore, our functional analyses on the mutational impact of *POLR1C* bring the first insights into the pathophysiology of *POLR3*-related disorders.

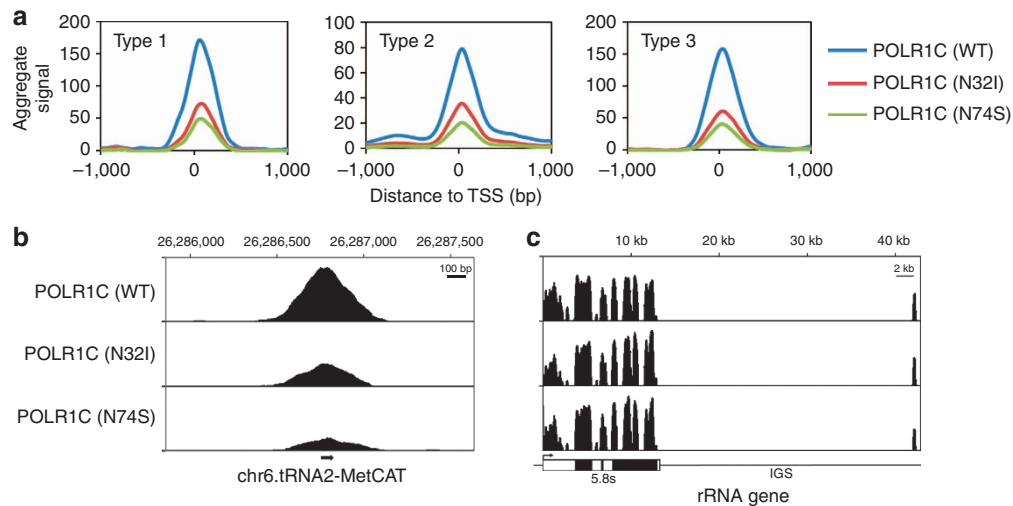


Figure 4 | Impact of *POLR1C* mutations on polymerase association with chromatin. (a) ChIP-Seq experiments of FLAG-tagged *POLR1C* variants (wild type, N32I or N74S). Aggregate profile produced with the annotation mode of the Versatile Aggregate Profiler shows ChIP-Seq data sets over the three classes of *POLR3*-transcribed genes, as defined by the promoter structure. Type 1 genes have an internal promoter composed of A and C boxes (5S rRNA genes). Type 2 genes have an internal promoter composed of A and B boxes (tRNA genes, for example), while the promoter of type 3 genes (U6, 7SK, RNase P and others) is located upstream of the transcription start site (TSS)⁵⁵. The TSS was used as the reference point. (b) IGView of a tRNA-Met gene transcribed by *POLR3*. *POLR1C* binding is decreased in mutated variants compared with wild type. IGS, intergenic spacer. (c) IGView of one ribosomal DNA (rDNA) repeat. The rDNA gene encodes a 45S pre-rRNA precursor that will generate the 5.8S, 18S and 28S rRNAs. There are ~400 copies of the rDNA gene arranged in tandem repeats in the human genome. rDNA repeats are not present in the reference genome assemblies; therefore, unique reads were aligned directly to the human rDNA reference sequence (NCBI accession number: HSU13369)⁵⁶. No differences were observed in *POLR1C* occupancy between wild-type and mutant variants. A schematic of a rDNA repeat is included below the graph.

Methods

Patients and exome sequencing. Informed consent was obtained from all participants. The project was approved by the research ethics committee of the Montreal Children Hospital (11-105-PED), the institutional review board of the VU University Medical Center, Neuroscience Campus, Amsterdam, the Netherlands, the Children's National Health System as part of the Myelin Disorders Bioregistry Project in Washington, DC and the University of Queensland, Australia. Genomic DNA was extracted from peripheral blood leukocytes of patients and family members using the Qiagen Genom Puregen Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Exome sequencing was performed in two cases by PerkinElmer (Branford, Connecticut, USA) and in a third case by the Institute for Molecular Biology at the University of Queensland. Cases one and two were sequenced using PerkinElmer's sequencing service using the Agilent Sure Select Human All Exon Capture V4 Kit and exome sequencing for these two cases was performed (two paired-end 100-bp reads) with the Illumina HiSeq 2000 system. Reads were aligned to the reference human genome (UCSC Genome Browser hg19) with the Genome Analysis Toolkit (GATK)^{32,33}, SAMtools^{32,34}, Picard (see web resource) and CASAVA v1.8 (ref. 35) and annotated using the snpEff software tool (<http://snpeff.sourceforge.net/>), as well as visualization tools from PerkinElmer. Subsequent analyses were performed using the Ingenuity software package (Qiagen, Redwood City, USA). Exome enrichment for case three was performed using the Illumina Nextera Rapid Capture kit and sequenced on an Illumina HiSeq 2000 (2 × 100 bp paired-end). Reads were aligned to the reference human genome (GRCh37) and pedigree-informed variant calling was performed using the Real Time Genomics (RTG) integrated analysis tool rtg Family v3.2 (ref. 36). Variants were annotated using SnpEff v3.4 (ref. 37). In all cases, filtering queries were created as specific presets that allowed *in silico* reduction of variant lists down to candidates with correlation to phenotype, transmission mode of inheritance and alteration classification of pathogenicity. Sanger sequencing and co-segregation analysis were performed on genomic DNA using primer pairs designed with the primer3 software package and the genomic sequence of *POLR1C* (NM_203290; GRCh37/hg19). PCR products were forward- and reverse-sequenced at the McGill University and Genome Quebec Innovation Centre using an ABI 3730xl DNA Analyzer (ABI; Applied Biosystems, Foster City, CA, USA). Sequences were analysed using SeqMan 4.03 (DNASar, Wisconsin, USA) and Chromas 1.62 (Technelysium Pty, Ltd, Australia).

Immunofluorescence and western blot analyses. Transfection experiments for generating stable HeLa cell lines expressing FLAG-tagged versions of *POLR1C* variants used lipofectamine, as described by the supplier (Invitrogen, Carlsbad, CA, USA)¹⁸. The antibodies used in this study were obtained from the following sources: anti-FLAG monoclonal primary antibody (Sigma-Aldrich, St Louis,

Missouri, USA), Alexa Fluor 488 fluorescence secondary antibody (Invitrogen) and anti-GAPDH (FL-335; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunofluorescence studies used an anti-FLAG (dilution 1/300) antibody and the secondary antibody Alexa Fluor 488 (1/200) to localize exogenously expressed FLAG-*POLR1C* variants in HeLa cells. For western blot analysis, anti-FLAG (dilution 1/3,000) was used to detect the FLAG-*POLR1C* variants and anti-GAPDH (dilution 1/2,000) for loading control¹⁸.

Protein affinity purification coupled to mass spectrometry. Generation of cell lines stably expressing FLAG-tagged *POLR1C* subunits (WT and mutated) and affinity purification from the soluble fraction were performed using standard procedures^{38,39}. The eluates were digested with trypsin and the resulting tryptic peptides were purified and identified with tandem mass spectrometry (LC-MS/MS) using a microcapillary reversed-phase high-pressure liquid chromatography-coupled LTQ-Orbitrap (ThermoElectron) quadrupole ion trap mass spectrometer with a nanospray interface, as recently described⁴⁰. Protein database searching and protein spectral count quantification were performed with Mascot (version 2.3.02)⁴¹. The NCBI_Human protein sequence database was downloaded on 20 February 2014. Known protein contaminants such as keratins, which are not expressed in HeLa cells, were excluded from the data set. Undistinguishable protein isoforms were considered as a single protein. For each LC-MS/MS analysis, protein spectral counts were normalized by the spectral count of the FLAG-*POLR1C* in order to allow the comparison of different purifications. To simulate the background noise of the LC-MS/MS analysis of a given sample, spectral counts reported as 0 by Mascot were replaced by randomly generated spectral count values that are normally distributed with a mean and s.d. equal to those of the lowest 20% spectral count values from the LC-MS/MS analysis. Each replicate LC-MS/MS analysis of the affinity purifications of the FLAG-*POLR1C* mutants (MUT; N32I, N74S and R279Q) was paired with a LC-MS/MS analysis of the WT FLAG-*POLR1C* that was performed at the same time. The set of high-confidence interactors of *POLR1C* for a given mutant analysis was identified by comparing the spectral counts of the interactors obtained from the purifications of the paired WT *POLR1C* and the MUT *POLR1C* to those of the proteins purified with an empty vector (EV) of the FLAG tag (nonspecific interaction). A protein is labelled as a high-confidence interactor if it was identified and quantified in all three replicates of WT *POLR1C* or MUT *POLR1C* and that the ratio of the average spectral counts across the three replicates (WT/EV or MUT/EV) was greater than 5. These stringent criteria allow us to eliminate the vast majority of nonspecific interactors of *POLR1C* for the analysis of the interactions of each mutant. For each high-confidence interacting protein, a two-tailed one-sample *t*-test was performed on the spectral count ratios (MUT/WT). The resulting *P* values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg procedure⁴². To maximize the

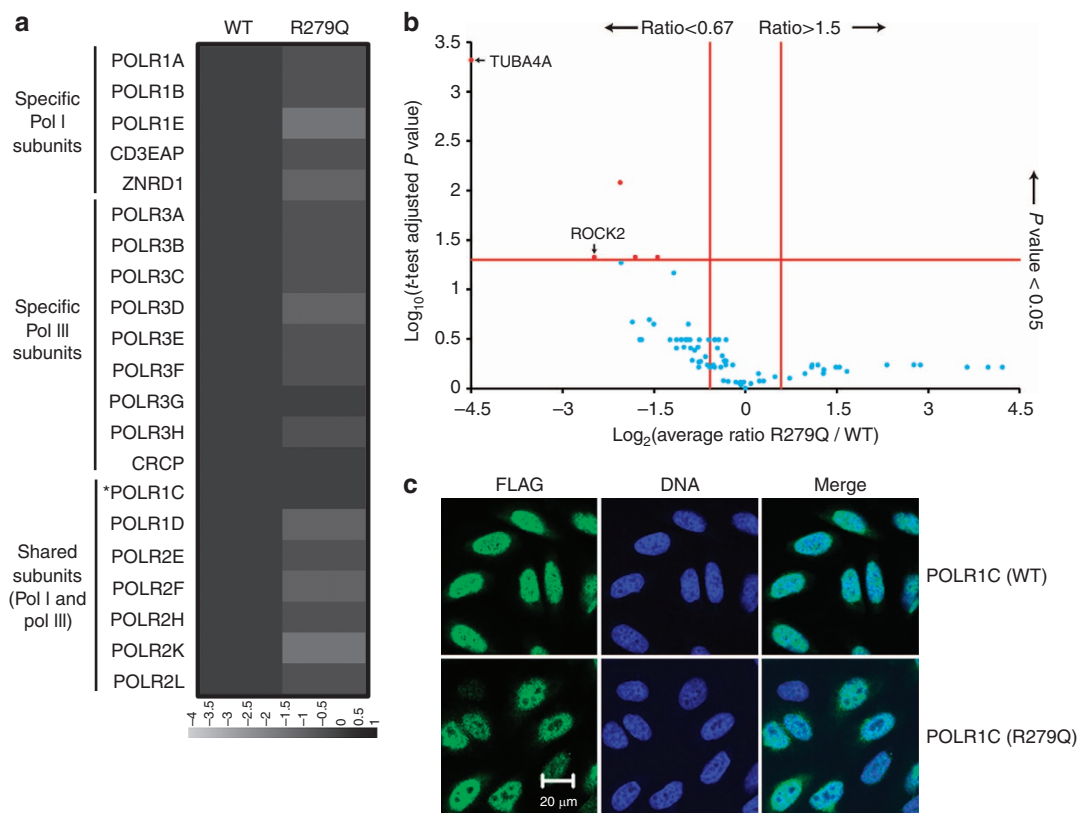


Figure 5 | Impact of a TCS-causative mutation in *POLR1C* on polymerase assembly and cellular localization. FLAG-tagged *POLR1C* variants, either the wild type (1C) or a mutated version with the R279Q substitution, were expressed, affinity-purified and used in anti-FLAG immunofluorescence experiments as in Fig. 3. **(a)** Affinity purification coupled to mass spectrometry data is represented in the form of a heatmap that contains the \log_2 -transformed average spectral count ratios R279Q/WT across all three replicates. Spectral counts were computed with Mascot (see legend to Fig. 3 for details). **(b)** Volcano plot of the \log_2 -transformed average spectral count ratios R279Q/WT (x axis) and the $-\log_{10}$ -transformed *P*-values (adjusted with the Benjamini-Hochberg procedure) resulting from the two-tailed one-sample *t*-tests of the high-confidence interactors of *POLR1C*. Red proteins show a level of differential interaction with *POLR1C* that is statistically significant, while blue proteins do not. Proteins with a \log_2 -transformed average spectral count ratio < -4.5 were capped to -4.5 for display purposes. **(c)** Immunofluorescence data showing the cellular localization of tagged *POLR1C* variants are shown. Scale bar, 20 μm .

specificity of our approach, a protein is deemed to show a level of differential interaction with *POLR1C* that is statistically significant when its adjusted *P* value is < 0.05 and that its average spectral count fold-change (MUT/WT) is < 0.6 or > 1.5.

ChIP-sequencing and data analysis. Stable HeLa cell lines expressing FLAG-tagged *POLR1C* (WT or mutated) were cultured to 80% confluence and cross-linked with 1% formaldehyde directly in the cell medium for 5 min followed by a 5-min quenching in 125 mM glycine. For ChIP experiments⁴³, nuclei from 3×10^6 cells were lysed and re-suspended in sonication buffer (10 mM Tris-HCl pH 8, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.5% Triton, 0.5% SDS and protease inhibitors). Chromatin was prepared by sonicating the nuclei in a Covaris E-Series E22 for 6 min at Duty 2. This generated chromatin fragments of 500 bp on average. Sonicated chromatin was immunoprecipitated using 25 μl of anti-FLAG M2 Magnetic beads (Sigma) for 4 h. The beads were then washed and eluted, followed by phenol:chloroform-isoamyl (25:24:1 pH 8, Invitrogen) extraction and ethanol precipitation. Sequencing libraries were prepared from input and ChIP eluates (WT and mutated) using the TruSeq DNA library preparation kit (Illumina). Libraries were sequenced on an Illumina HiSeq 2000 (2×50 cycles paired-end). Quality control of the sequencing data was performed with FastQC and low-quality bases and adapter sequences were trimmed with Trimmomatic⁴⁴. Unique reads were aligned to the reference human genome (build hg19) or to the human rDNA reference sequence (rDNA; NCBI accession number: HSU13369) with Bowtie version 2.1.0 (ref. 45). Input DNA signal was subtracted from ChIP signal. *POLR1C* peaks were called using MACS version 2.0.10 (ref. 46). Peaks were annotated with HOMER version 4.5.0 (ref. 47) using GENCODE Genes V19 annotations⁴⁸ and the tRNA Genes track of the UCSC Genome Browser^{49,50}. Data visualization was performed with the Integrative Genomics Viewer⁵¹. To compare chromatin binding between WT and mutated *POLR1C* variants over all *POLR3*-transcribed genes divided into three classes according to their regulatory elements

(see legend to Fig. 4), we used the annotation mode of the Versatile Aggregate Profiler with the transcription start site as the only reference point and 100 windows of 10 base pairs each on both sides of the reference point⁵².

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Additional information

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