

TOP1MT rs2293925 is an enhancer-active regulatory SNP that shapes mitochondrial R-loop dynamics

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Mitochondrial topoisomerase 1 (TOP1MT) regulates mitochondrial DNA (mtDNA) topology during transcription and replication. Perturbed mtDNA maintenance and RNA metabolism have been implicated in neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS). Here we show that the common TOP1MT variant rs2293925 (R525W) has enhancer-like activity and is associated with increased mitochondrial R-loops (RNA : DNA hybrids). Tissue-dependent expression, quantitative trait locus analysis, chromatin-state annotation, reporter assays, and allele-specific DNA–protein binding assays support a transcriptional regulatory role for rs2293925. In isogenic cell models, rs2293925 increased TOP1MT mRNA and protein abundance, and this was accompanied by increased mitochondrial R-loop signal. TOP1MT trapping with lamellarin D supported increased TOP1MT-R525W occupancy at mitochondrial control region sites together with enhanced R-loops, consistent with altered TOP1MT–mtDNA interaction and/or increased TOP1MT abundance. Elevated mitochondrial R-loop signal was also detected in a pilot cohort of sporadic ALS samples carrying rs2293925 and in neural stem cells derived from C9orf72-positive ALS patients. These data support a dual-effect model in which rs2293925 increases TOP1MT expression and

Abbreviations

ALS, amyotrophic lateral sclerosis; CES, clinical exome sequencing; CLSM, confocal laser scanning microscopy; DRIP, DNA–RNA hybrid immunoprecipitation; dsDNA, double-stranded DNA; EMSA, Electrophoretic Mobility Shift Assay; eQTL, expression quantitative trait locus; FCS, fluorescent correlation spectroscopy; mtDNA, mitochondrial DNA; ssDNA, single-stranded DNA; TOP1MT, Mitochondrial topoisomerase 1.

is associated with altered mitochondrial R-loop dynamics, linking common genetic variation to mitochondrial nucleic acid stress in disease-relevant contexts.

Introduction

DNA topoisomerases are essential enzymes that maintain nucleic acid topology during DNA replication, transcription, recombination, and repair. They resolve torsional strain generated by moving RNA and DNA polymerases through transient cleavage and re-ligation reactions. Based on their catalytic mechanism, topoisomerases are classified into two major types. Type IA enzymes (e.g., TOP3A) introduce transient single-strand breaks and relax supercoils through strand-passage reactions, whereas type IB enzymes (e.g., TOP1 and TOP1MT) form a 3'-phosphotyrosyl intermediate and relax both positive and negative supercoils by controlled rotation of the cleaved strand. Type II enzymes (e.g., TOP2A and TOP2B) introduce transient double-strand breaks to untangle or decatenate DNA duplexes. Topoisomerases also differ in substrate preference, acting on double-stranded DNA, single-stranded DNA, or RNA-containing nucleic acid structures [1].

Unlike the nuclear genome, mitochondrial DNA is served by a minimal set of topoisomerases, reflecting the unique topological challenges posed by its circular, histone-free nature. Only two topoisomerases operate within human mitochondria: mitochondrial topoisomerase 1 (TOP1MT), a type IB enzyme, and topoisomerase 3 alpha (TOP3A), a type IA enzyme [2]. TOP1MT is encoded in the nucleus and imported into mitochondria, where it relieves transcription- and replication-associated supercoiling and thereby helps maintain mitochondrial DNA (mtDNA) topology [3]. TOP1MT has also been implicated in mitochondrial transcription, translation, and oxidative phosphorylation, consistent with a central role in mitochondrial genome function [4]. By contrast, TOP3A is thought to resolve hemicatenanes and recombination-like intermediates during mtDNA replication [2]. Notably, all other nuclear topoisomerases are excluded from mitochondria [1].

Expression data from the Human Protein Atlas [5] reveal unequal contributions of the two mitochondrial topoisomerases across tissues. TOP1MT is broadly expressed, with detectable protein levels across multiple tissues, including brain and peripheral blood/immune cell types, whereas TOP3A is generally

detected at lower protein levels in the same tissue panel. Transcript-level data follow a similar overall trend [5]. This pattern is consistent with TOP1MT serving as a major topological regulator of the mitochondrial genome in many cellular contexts, including tissues with high mitochondrial demand.

A key feature of mitochondrial DNA metabolism is the formation of R-loops, three-stranded structures composed of an RNA:DNA hybrid and a displaced single-stranded DNA strand [6,7]. In mitochondria, R-loops are not merely by-products of transcription [8]. They are essential at replication origins such as the heavy-strand origin (OH), where RNA can serve as a primer for mtDNA synthesis [9]. Their formation and stability are shaped by multiple factors, including RNA:DNA G-quadruplexes [10], RNase H1, mitochondrial RNA granules [11], RNA-binding proteins, and additional nucleic acid-processing enzymes [12–15] including helicases and endonucleases such as endonuclease G [16–18]. At the same time, dysregulated R-loops can trigger transcription-replication conflicts and genome instability, phenomena linked to aging, cancer, and neurodegeneration [19,20].

Despite growing interest in mitochondrial R-loops, the relationship between topological stress, topoisomerase function, and R-loop formation remains unresolved. In the nuclear genome, R-loops induce negative supercoiling behind RNA polymerase, which can recruit TOP1 to restore topological equilibrium [21]. An analogous relationship is likely to exist in mitochondria, where transcription occurs on a compact circular genome with limited topological buffering capacity. This raises the possibility that relatively subtle changes in TOP1MT activity or abundance could influence mitochondrial R-loop dynamics without necessarily causing overt defects in basal mitochondrial structure.

Common genetic variants provide one way to probe such mechanisms. Increasing evidence shows that non-coding and cis-regulatory SNPs can modulate gene dosage in a tissue-dependent manner by altering enhancer activity, chromatin accessibility, or transcription factor binding [22]. In some cases, coding SNPs may also reside within a broader regulatory region,

giving rise to alleles with both protein-level and expression-level consequences [23]. Such variants are especially relevant in genes involved in mitochondrial genome maintenance, where modest changes in enzyme abundance or behavior may produce context-dependent phenotypes rather than constitutive defects [24]. These considerations make TOP1MT an attractive candidate through which genetic variation could influence mitochondrial nucleic acid metabolism [25]. In particular, variants that alter TOP1MT dosage and/or enzyme behavior could be expected to affect mtDNA topology and, consequently, mitochondrial R-loop regulation. The TOP1MT linker domain may be especially relevant in this regard, as it has been implicated in DNA interaction dynamics and catalytic turnover [26].

These questions are particularly relevant to neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), where mitochondrial dysfunction and altered RNA metabolism are central pathological features [27–30]. ALS is a fatal neurodegenerative disorder characterized by progressive loss of motor neurons, leading to muscle weakness, atrophy, and death. Although ALS has traditionally been classified as familial or sporadic based on family history [31,32], this distinction has become less clear with broader genetic testing. A substantial fraction of patients (~20%) carry pathogenic variants irrespective of family history, while the majority remain genetically unresolved [33–35]. Mutations in *SOD1*, *TARDBP*, and *FUS* have been linked to familial forms, implicating oxidative stress and RNA metabolism in pathogenesis. Notably, pathological aggregation of TDP-43, encoded by *TARDBP*, is observed in nearly all ALS patients, regardless of genetic background. This underscores the central role of RNA-binding proteins and dysregulated RNA processing in ALS. The discovery of hexanucleotide repeat expansions in *C9orf72*—now the most common genetic cause of ALS—has further implicated toxic RNA species and impaired nucleocytoplasmic transport in disease [36]. Additional risk genes such as *NEK1*, *ANXA11*, and *ATXN2* reflect a polygenic architecture, encompassing pathways related to organelle dynamics, cytoskeletal transport, and autophagy [37].

In parallel, mitochondrial dysfunction has emerged as a key feature of ALS biology [38]. Evidence from ALS model systems and patient-derived cellular models, including primary fibroblasts and induced pluripotent stem cell (iPSC)-derived neural lineages, indicates fragmented mitochondria, impaired oxidative phosphorylation, increased reactive oxygen species, and defective mitophagy [39]. Mutations in

CHCHD10, involved in mitochondrial cristae stabilization, and *VAPB*, which regulates ER-mitochondria contact sites, also underscore the genetic links between mitochondrial integrity and ALS [40].

In this study, we investigated whether the common TOP1MT variant rs2293925, which encodes an arginine-to-tryptophan substitution at residue 525 (R525W), acts as a regulatory SNP that modulates TOP1MT expression and mitochondrial R-loop homeostasis. We combined epigenomic annotation, expression quantitative trait locus analysis, reporter assays, DNA–protein interaction studies, isogenic and neuronal-like cell models, and RNA:DNA hybrid profiling to test whether the rs2293925 allele alters TOP1MT expression, mtDNA association, and mitochondrial R-loop levels. We further examined whether these molecular phenotypes are detectable in ALS-relevant contexts, including sporadic ALS samples and neural stem cells derived from ALS patients.

Results

We investigated the common TOP1MT variant rs2293925, which encodes an arginine-to-tryptophan substitution at position 525 (R525W) within the TOP1MT linker domain [26]. Predictive tools (SIFTcat, PolyPhenCat, and CADD) indicate potential functional impact (CADD-Phred score: 20.2), and the variant occurs at high frequency in the general population (MAF = 0.43) [26,41].

To assess whether rs2293925 influences TOP1MT expression, we first performed GTEx expression QTL (eQTL) analysis [22] that identified rs2293925 as a tissue-dependent regulator of TOP1MT expression (Fig. 1A). The effect varied in both magnitude and direction across tissues, with the A allele associated with increased TOP1MT expression in central nervous system (CNS) tissues and reduced expression in subcutaneous adipose tissue (Fig. 1B). This pattern is consistent with context-dependent regulatory activity at the SNP.

Chromatin-state segmentation from the NIH Roadmap Epigenomics Project [42] (127 reference human epigenomes) further indicated that rs2293925 aligns with chromatin states annotated as active or genic enhancers across multiple tissues and cell types (Fig. 2A), consistent with a *cis*-regulatory module. To further evaluate the regulatory architecture of rs2293925 in a three-dimensional chromatin context, we analyzed public Hi-C data from CNS-relevant cell types using the 3DIV portal [43], centering the interaction view on rs2293925. In astrocytes, primary spinal cord cells, and an ectoderm-derived cell line, the

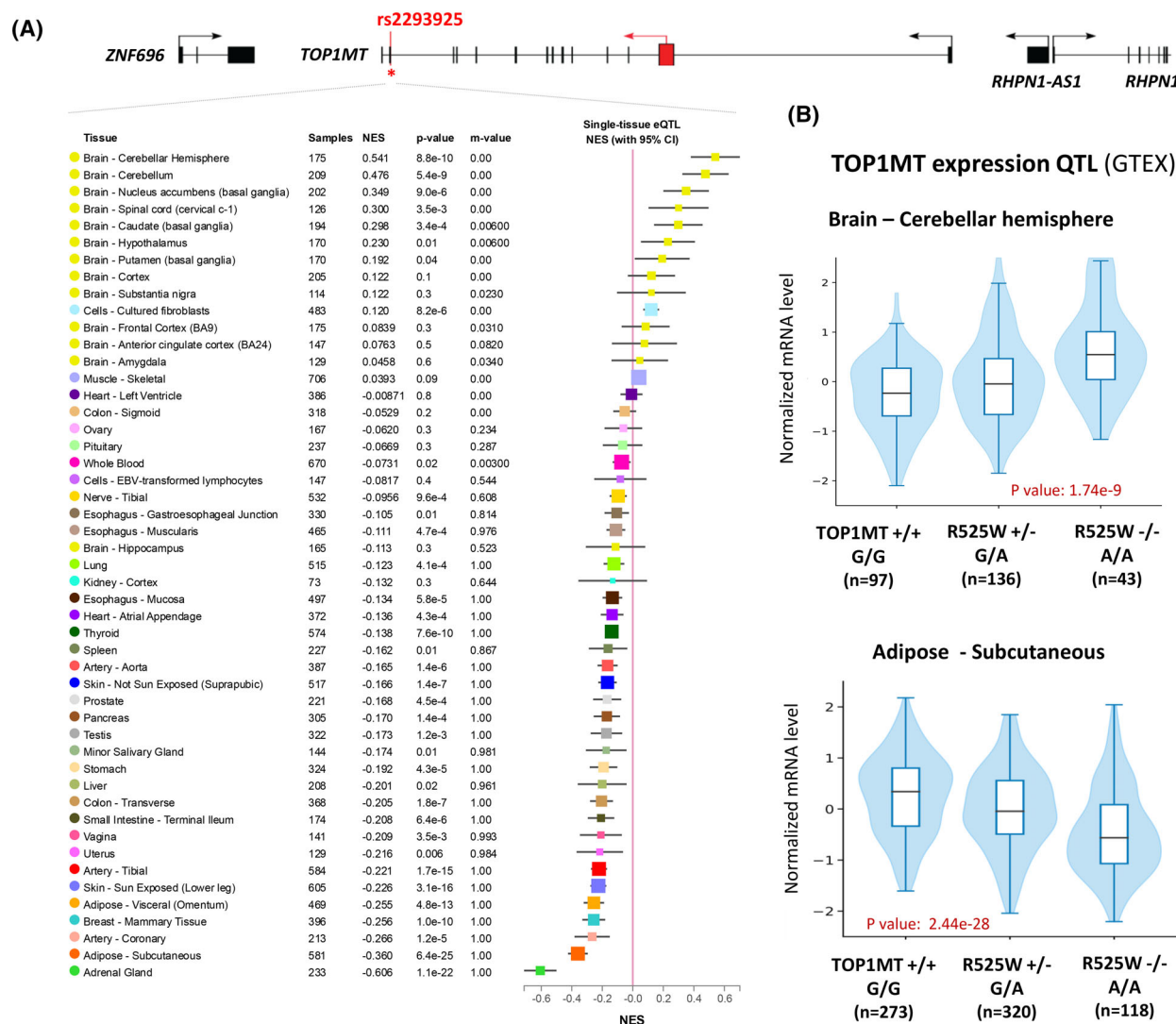


Fig. 1. GTEx eQTL analysis identifies rs2293925 as a tissue-dependent regulator of TOP1MT expression. (A) Top, genomic context of rs2293925 at the TOP1MT locus. GTEx multi-tissue eQTL summary showing the normalized effect size (NES) of rs2293925 on TOP1MT expression across tissues. NES represents the direction and magnitude of the association between rs2293925 and TOP1MT expression after GTEx normalization, with positive values indicating higher TOP1MT expression associated with the A allele relative to the G-allele, and negative values indicating lower expression associated with the A allele; points denote NES and horizontal bars indicate 95% confidence intervals. (B) Representative GTEx genotype-expression distributions (violin plots with embedded boxplots) for TOP1MT in selected tissues (top: brain cortex; bottom: subcutaneous adipose), stratified by rs2293925 genotype (G/G, G/A, A/A). In brain cortex, the A allele is associated with higher TOP1MT expression, whereas in subcutaneous adipose tissue the A allele is associated with lower TOP1MT expression (P -values are shown on the plots), illustrating bidirectional, context-dependent regulatory effects of rs2293925. Statistical significance (P -value) was determined via multiple linear regression analysis across n samples, as tracked by the GTEx consortium. Significant associations (red P -value) pass the tissue-specific False Discovery Rate (FDR) threshold of $\leq 5\%$.

variant mapped close to a topologically associating domain (TAD) boundary and displayed multiple local chromatin contacts within a 2 Mb interval (Fig. 2B). This organization places rs2293925 in a structurally dynamic regulatory interval separating adjacent interaction domains. The proximity of rs2293925 to a TAD boundary, together with the observed local interaction

structure, is consistent with the enhancer-like features of this SNP and supports a role in modulating TOP1MT expression in a context-dependent manner.

To test enhancer activity directly, we cloned a ~ 1 kb fragment spanning rs2293925 upstream of a minimal promoter and measured transcriptional output in luciferase assays. Constructs carrying the

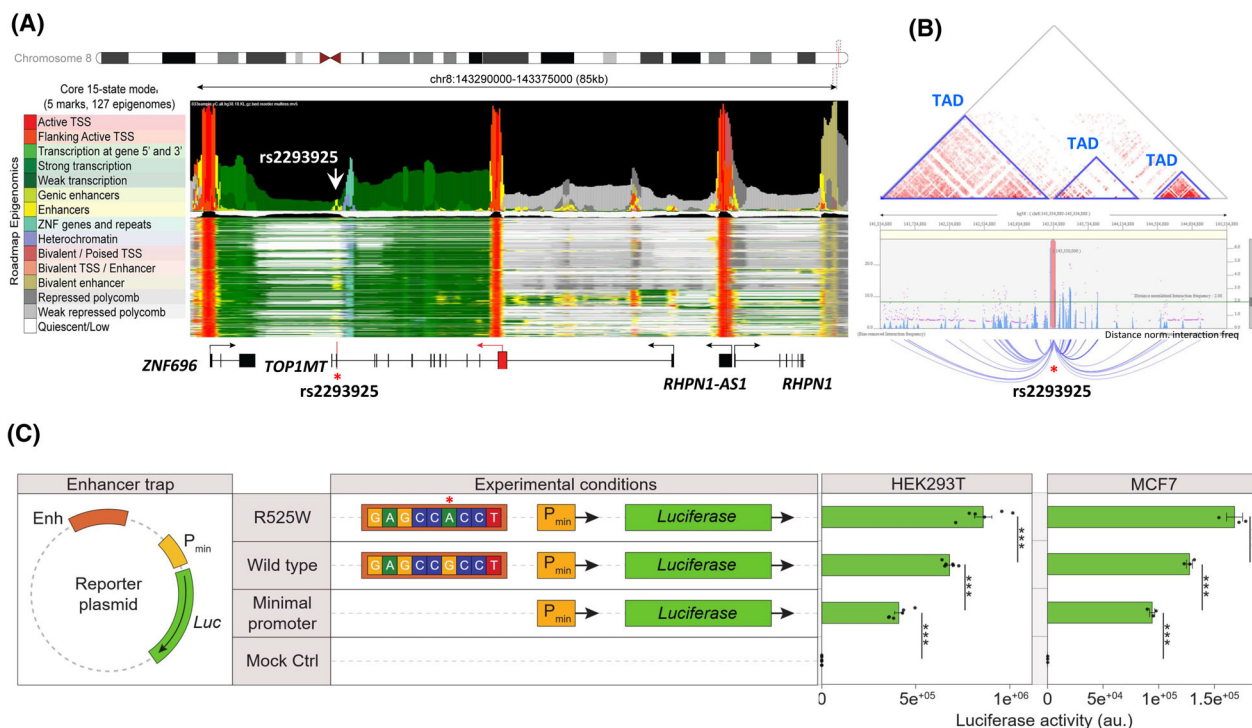


Fig. 2. TOP1MT rs2293925 is a transcriptional enhancer. (A) Chromatin state segmentation from the NIH Roadmap Epigenomics Project reveals that the genomic region encompassing rs2293925 maps to predicted active or genic enhancer states across multiple human tissues. Shown is the epigenomic landscape of the TOP1MT locus (chr8:143290000–143375000), with chromatin states for selected cell types based on enhancer-associated histone marks (H3K4me1 and H3K27ac). The white arrow marks the position of rs2293925, while the red box indicates the most transcriptionally active start site (TSS) of TOP1MT, supporting the presence of a cis-regulatory module at this locus. (B) rs2293925 localizes near a TAD boundary and shows multiple local 3D contacts within the surrounding interval. Public Hi-C data from the 3DIV portal were analyzed using the rs2293925 genomic position (hg38) as the bait. Shown is a 2 Mb interaction view in CNS-relevant cell types, including astrocytes, primary spinal cord cells, and an ectoderm-derived cell line, together with the local topologically associating domain (TAD) structure and distance-normalized interaction frequencies. (C) Luciferase reporter assay supports enhancer activity of the rs2293925 region in two cell types. A ~1 kb genomic fragment flanking rs2293925 was cloned upstream of a minimal promoter driving luciferase expression. Reporter constructs carrying the rs2293925 A allele (R525W-linked) produced higher luciferase activity than the G (reference) allele in both HEK293T and MCF7 cells. Mock control and minimal-promoter-only (empty vector) conditions are shown as negative controls. Data are presented as mean $\pm$ SEM from $n = 3$ –5 independent measurements (individual datapoints). Data were analyzed separately for each cell line by one-way ANOVA after confirming normality (Shapiro–Wilk test, $P = 0.608$) and homogeneity of variances (equal-variance test, $P = 0.650$). *Post hoc* multiple-comparison testing showed significant differences for all indicated pairwise comparisons (***) $P < 0.001$.

rs2293925 A allele produced significantly higher reporter activity than those carrying the reference G-allele in both HEK293T and MCF7 cells (Fig. 2C), consistent with the enhancer-like function of the variant-containing region.

We next asked whether rs2293925 alters transcription factor binding at the locus. Motif disruption analysis predicted allele-specific effects on several transcription factor motifs, including SP1, SP2, SMAD3, CTCFL, ZNF770, PATZ1, and SP4 (Fig. 3A). In electrophoretic mobility shift assays (EMSA) using HCT116 nuclear extracts, the rs2293925 A-allele probe showed altered formation of the dominant shifted DNA–protein

complex relative to the G-allele probe, with a significant difference detected at the 2.5 μ M nuclear extract concentration tested (Fig. 3B). An antibody-capture transcription factor DNA-binding assay using HEK293T nuclear extracts further showed increased binding of the rs2293925 A-allele for SP1 and CTCF, whereas SMAD3 did not reach statistical significance under these conditions (Fig. 3C). Together, these results support allele-dependent nuclear factor binding at rs2293925 and are consistent with a transcriptional regulatory role for the variant.

To determine whether this regulatory effect is reflected in living cells, we generated HCT116

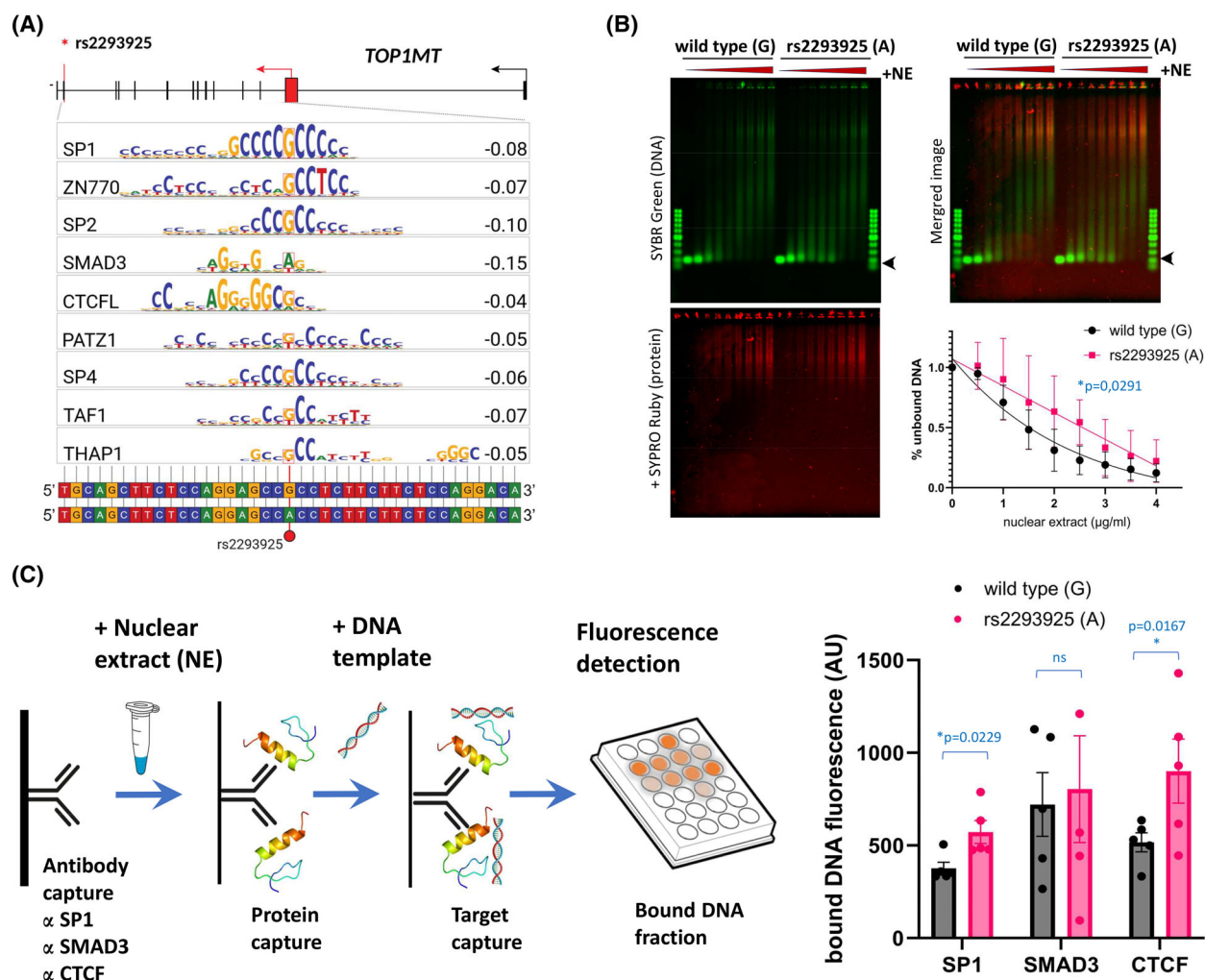


Fig. 3. Allele-specific transcription factor binding at rs2293925 predicts regulatory effects on TOP1MT expression. (A) *In silico* transcription factor motif disruption analysis was performed using motifbreakR with the HOCOMOCO v11 core human TF motif database. The variant rs2293925 (8:143310198:G>A; hg38) was predicted to significantly alter the binding affinity of multiple transcription factors. Strong disruptions were observed for motifs of SP1, SP2, ZNF770, and SMAD3, suggesting these TFs may play a direct role in mediating the variant's enhancer function. Additional moderate-to-weak disruptions were identified for CTCFL, PATZ1, SP4, TAF1, and THAP1, indicating a broader but subtler landscape of allele-specific TF interaction at this locus. (B) Electrophoretic mobility shift assays (EMSAs) were performed using a 200bp double-stranded DNA fragment spanning rs2293925 containing either the G (reference) or A (variant) allele (indicated by an arrowhead) and increasing amounts of nuclear extract from HCT116 cells. Native gel separation revealed allele-dependent differences in formation of the dominant shifted DNA–protein complex, consistent with variant-specific nuclear factor binding *in vitro*. Quantification of shifted-band integrated density is shown as a function of nuclear extract concentration; significant difference between alleles was observed at 2.5 μM nuclear extract ($P=0.0291$; two-tailed unpaired *t*-test, equal variances), indicated by *. Data are presented mean ± SD from $n=3–6$ independent measurements. (C) Fluorescence-based transcription factor DNA-binding assay (antibody-capture ELISA format) was used to quantify binding of rs2293925 G (reference) versus A (variant) allele DNA probes to immobilized transcription factors (SP1, SMAD3, and CTCF) captured from HEK293T nuclear extracts. The A allele showed increased binding for SP1 ($P=0.0229$) and CTCF ($P=0.0167$), whereas the difference for SMAD3 was not significant ($P=0.08$; two-tailed unpaired *t*-tests with variance assessed by *F*-test). Data are presented mean ± SEM from $n=3–5$ independent measurements. These results support allele-dependent TF binding consistent with the motif predictions and EMSA.

TOP1MT knockout cells [44] and reintroduced either wild-type or R525W TOP1MT alleles fused to tGFP. Confocal microscopy showed robust mitochondrial

localization of both proteins, and clear co-localization with MitoTracker (Fig. 4A). Fluorescence correlation spectroscopy (FCS) [45–47] revealed comparable

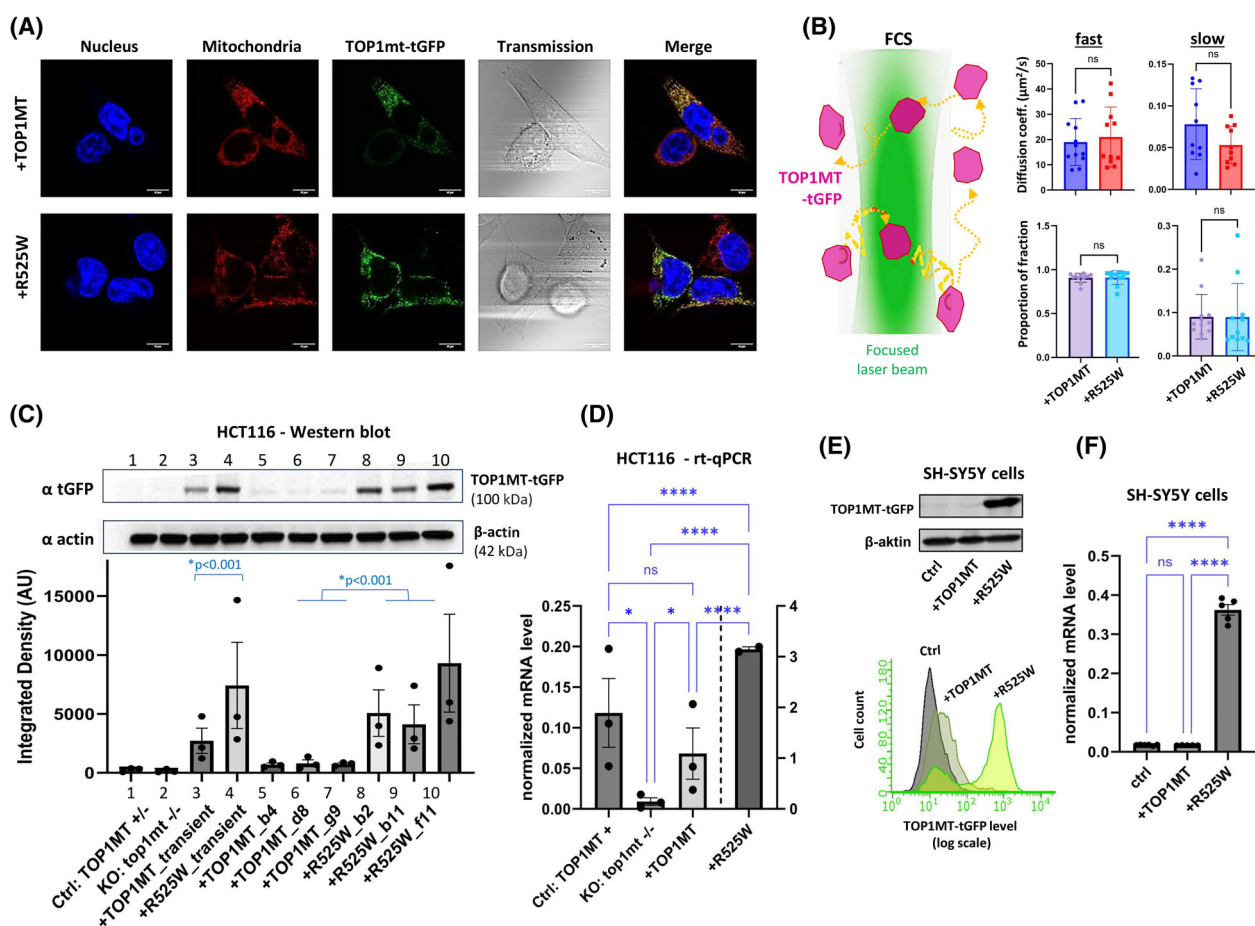


Fig. 4. TOP1MT R525W localizes to mitochondria and shows increased expression despite unchanged diffusion kinetics. (A) HCT116 cells with CRISPR/Cas9-mediated TOP1MT knockout were reconstituted with either wild-type (WT) or R525W TOP1MT alleles fused to tGFP. Confocal laser scanning microscopy (CLSM) revealed robust mitochondrial localization for both variants, confirmed by co-localization with MitoTracker Red. Scale bar: 10 μ m. (B) Fluorescence correlation spectroscopy (FCS) analysis of TOP1MT-WT-tGFP and TOP1MT-R525W-tGFP mobility in mitochondria. The bar plot shows one representative FCS measurement performed on 10 individual cells. Four independent FCS measurements were performed with similar results; however, because FCS measurements require fresh calibration for each experiment, individual measurements were not pooled. Statistical significance was determined using an unpaired, two-sided *t*-test (level of significance: $P \leq 0.05$; ns: non-significant). Data are presented mean $\pm$ SD from $n = 10$ cells per measurement. (C) Western blot analysis of TOP1MT expression in HCT116 isogenic lines shows higher TOP1MT protein abundance in TOP1MT-R525W addback clones compared with TOP1MT-WT addback clones (representative blot shown; loading control below). Quantification is shown beneath the blot; each point represents an independent biological replicate. Comparison of TOP1MT-R525W versus TOP1MT-WT is significant (two-tailed unpaired *t*-test, $P < 0.001$). Given the large number of groups shown in this panel, we do not annotate all possible pairwise comparisons; the underlying numerical source data are provided in Table S1. (D) RT-qPCR analysis of TOP1MT mRNA in the same panel of HCT116 lines shows increased TOP1MT transcript levels in TOP1MT-R525W addback clones relative to TOP1MT-WT. The TOP1MT-R525W versus TOP1MT-WT comparison was significant (one-way ANOVA, **** $P < 0.001$; * $P < 0.05$). Data are shown as mean with individual datapoints; error bars show SEM. The vertical dashed line visually separates the +R525W addback clone bars from the remaining samples and indicates that these bars are plotted against the right y-axis. This allows the lower-expression samples to remain visible and distinguishable from zero. The right y-axis represents the same relative TOP1MT mRNA expression parameter as the left y-axis but uses a higher numerical range. (E) TOP1MT-WT-tGFP and TOP1MT-R525W-tGFP expression in SH-SY5Y stable cell lines assessed by western blotting and flow cytometry. Upper panel, representative western blot showing TOP1MT-tGFP protein expression. Lower panel, representative flow cytometry analysis of tGFP fluorescence intensity. Western blotting and flow cytometry were each performed in three independent experiments, and one representative measurement is shown. For flow cytometry, 10 000 cells were collected per experiment. (F) RT-qPCR analysis of TOP1MT mRNA levels in the same SH-SY5Y cell populations shows higher TOP1MT transcript levels in TOP1MT-R525W cells than in TOP1MT-WT cells. Statistics: Data were analyzed by one-way ANOVA. *Post hoc* multiple-comparison testing showed significant differences for pairwise comparisons (**** $P < 0.0001$). Data are shown as mean with individual datapoints; error bars show SEM.

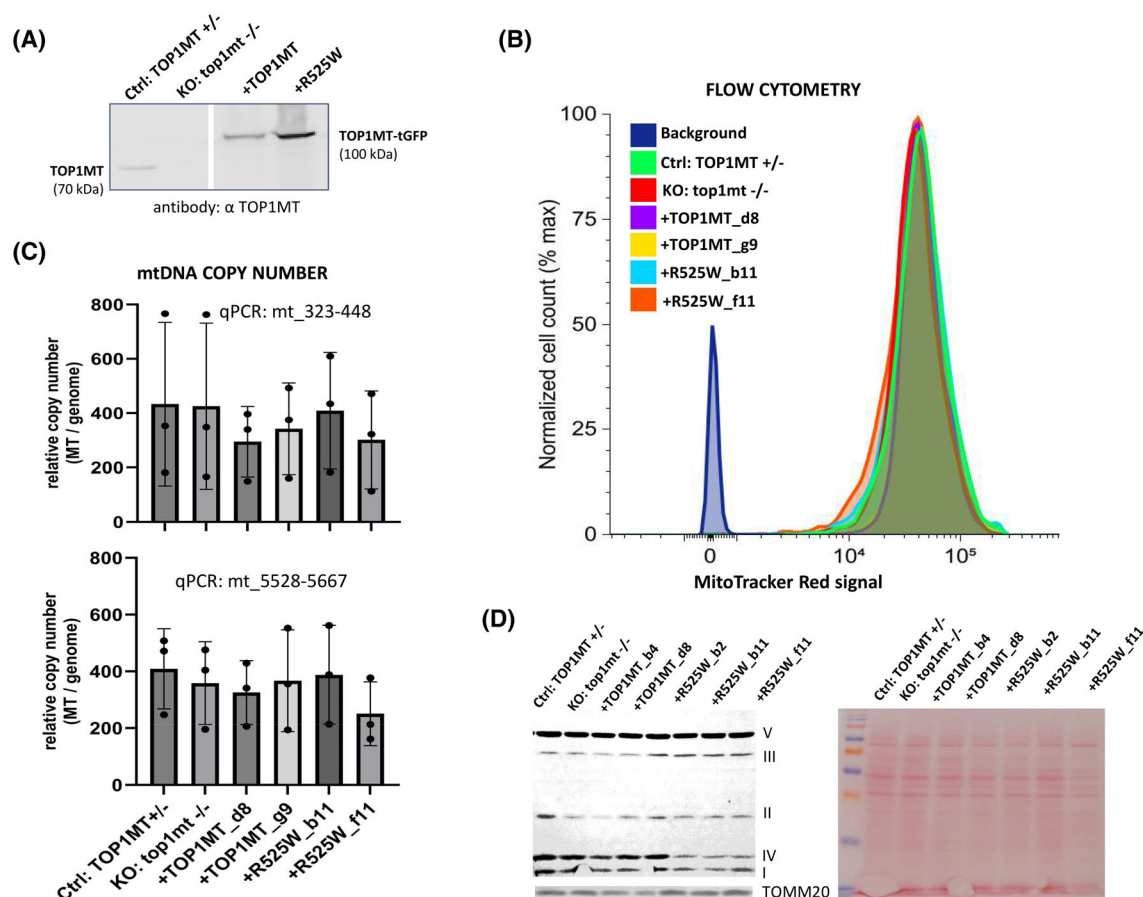


Fig. 5. TOP1MT R525W does not alter mitochondrial abundance or oxidative phosphorylation protein levels but increases TOP1MT expression. (A) Western blot analysis of transiently and stably transfected HCT116 cells confirmed consistently higher expression of TOP1MT R525W compared to WT, using anti-TOP1MT antibodies. Western blotting was performed in three independent experiments, and one representative blot is shown. (B) Quantification of mitochondrial content using MitoTracker staining in HCT116 cells reconstituted with WT or R525W TOP1MT showed no significant difference in mitochondrial abundance. (C) Relative mitochondrial DNA (mtDNA) copy number was quantified by qPCR using two independent mtDNA amplicons (mt:323–448 and mt:5528–5667) and normalized to a nuclear single-copy reference locus (ZNF554) measured in the same samples (MT/genomic ratio). Error bars represent standard deviation (SD). Comparable mtDNA content was measured between the cell lines tested. (D) Western blot analysis of oxidative phosphorylation (OXPHOS) complex proteins showed no detectable differences between the two genotypes, indicating preserved mitochondrial respiratory chain composition. OXPHOS western blotting was performed in three independent experiments, and one representative blot is shown.

diffusion kinetics for TOP1MT-tGFP (WT) and TOP1MT-R525W-tGFP (Fig. 4B), indicating similar intracellular mobility properties. In contrast, immunoblotting showed higher TOP1MT protein abundance in TOP1MT-R525W addback clones than in TOP1MT-WT addback clones (confirmed by anti-tGFP antibodies; Fig. 4C). RT-qPCR analysis similarly detected increased TOP1MT mRNA levels in TOP1MT-R525W addback clones relative to TOP1MT-WT (Fig. 4D), consistent with increased transcriptional output in the rs2293925 context. Enhanced TOP1MT protein expression in the TOP1MT-R525W addback clones was also confirmed by anti-TOP1MT antibodies (Fig. 5A).

We then asked whether this expression phenotype extends to a neuronal-like model that is more relevant to neurodegeneration. In SH-SY5Y cells stably expressing TOP1MT-tGFP (WT) or TOP1MT-R525W-tGFP, the R525W allele showed higher protein abundance by immunoblotting and flow cytometry (Fig. 4E), together with increased TOP1MT transcript levels by RT-qPCR (Fig. 4F). Thus, the expression upregulation observed in the isogenic HCT116 system was reproduced in an independent cell model.

Despite these expression changes, basal mitochondrial structural parameters were largely preserved. Mitochondrial abundance (Fig. 5B), mtDNA copy number (Fig. 5C), and oxidative phosphorylation

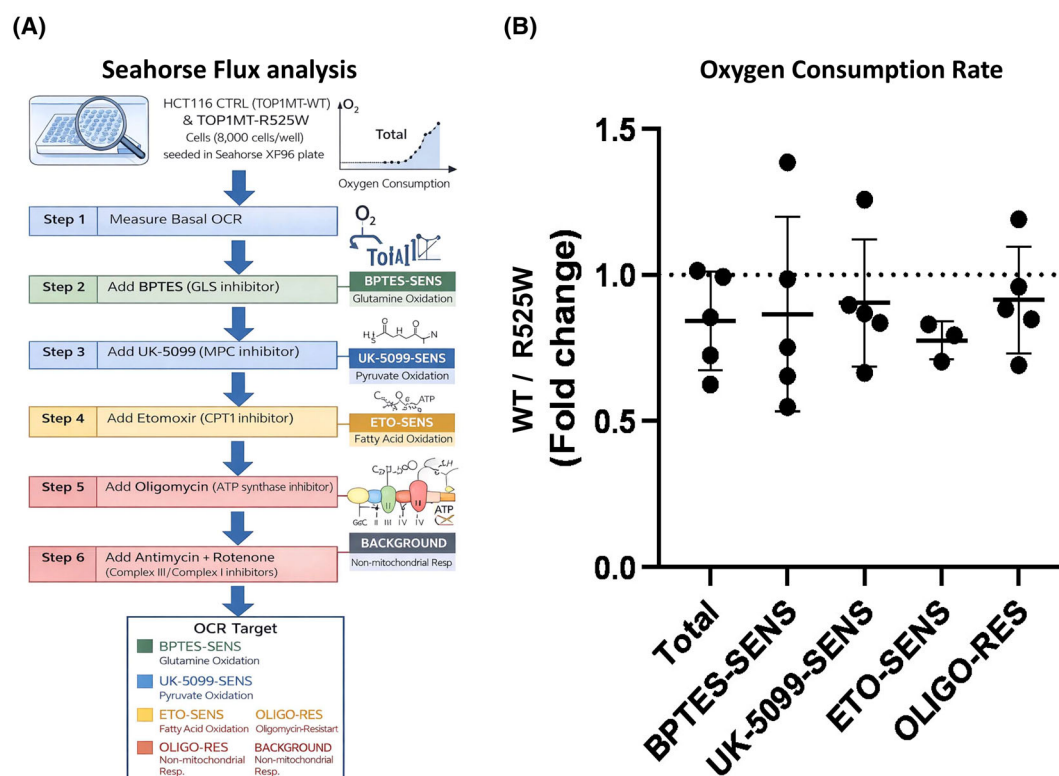


Fig. 6. Seahorse OCR analysis of substrate-pathway contributions in isogenic TOP1MT-WT and TOP1MT-R525W cells. (A) Flowchart of the measurement. HCT116 isogenic control (TOP1MT-WT) and TOP1MT-R525W cells (8000 cells per well) were plated in Seahorse XF96 microplates and oxygen consumption rate (OCR) was measured the next day. Basal OCR was recorded, followed by sequential addition of metabolic inhibitors to quantify pathway contributions: BPTES (10 μ M; glutaminase inhibitor), UK-5099 (10 μ M; mitochondrial pyruvate carrier inhibitor), etomoxir (50 μ M; CPT1 inhibitor), oligomycin (2 μ M; ATP synthase inhibitor), and finally antimycin A (10 μ M) plus rotenone (5 μ M) to determine non-mitochondrial background OCR. Background OCR was subtracted from each well and OCR values were normalized to cell number (sulforhodamine B assay). (B) Total OCR and inhibitor-defined components (BPTES-sensitive, UK-5099-sensitive, etomoxir-sensitive, and oligomycin-resistant respiration) were calculated and expressed as fold change (isogenic control/TOP1MT-R525W). 3–5 independent experiments were performed; 95% confidence intervals are shown. The etomoxir-sensitive (ETO-SENS) OCR component is reduced in TOP1MT-R525W cells by 20–30% compared with isogenic control. Abbreviations: CTL, isogenic control; SENS, inhibitor-sensitive component; RES, inhibitor-resistant component.

(OXPHOS) complex levels (Fig. 5D) did not differ detectably between WT and R525W cells, suggesting preserved basal organelle biogenesis and steady-state respiratory chain composition. Functional profiling by Seahorse extracellular flux analysis similarly showed that most respiratory parameters were comparable between genotypes (Fig. 6A,B). However, TOP1MT-R525W cells displayed a reduced etomoxir-sensitive oxygen consumption component, consistent with a subtle decrease in CPT1-dependent fatty acid oxidation under these assay conditions.

We next examined whether R525W is associated with altered TOP1MT occupancy on mtDNA and with changes in mitochondrial R-loop abundance. Target sites for ChIP-qPCR and DRIP-qPCR were selected within the mitochondrial Control Region (D-loop)

(Fig. 7A), guided by previous evidence for TOP1MT enrichment in this domain in mouse mtDNA mapping studies [48]. Because TOP1MT interactions with mtDNA are transient and highly variable under basal conditions (Fig. 7B), we performed TOP1MT ChIP-qPCR under trapping conditions using lamellarin D [49] (Fig. 7C). Lamellarin D dose-response profiling showed comparable sensitivity of TOP1MT-WT and TOP1MT-R525W HCT116 cells, supporting its use for short trapping treatments (Fig. 7C). In HCT116 cells, TOP1MT-R525W showed higher enrichment than TOP1MT-WT at two sites within the Control Region, while a nuclear locus served as a negative control (Fig. 7D). The same pattern was observed in SH-SY5Y cells expressing WT or R525W TOP1MT (Fig. 7E). DRIP-qPCR at the same Control Region

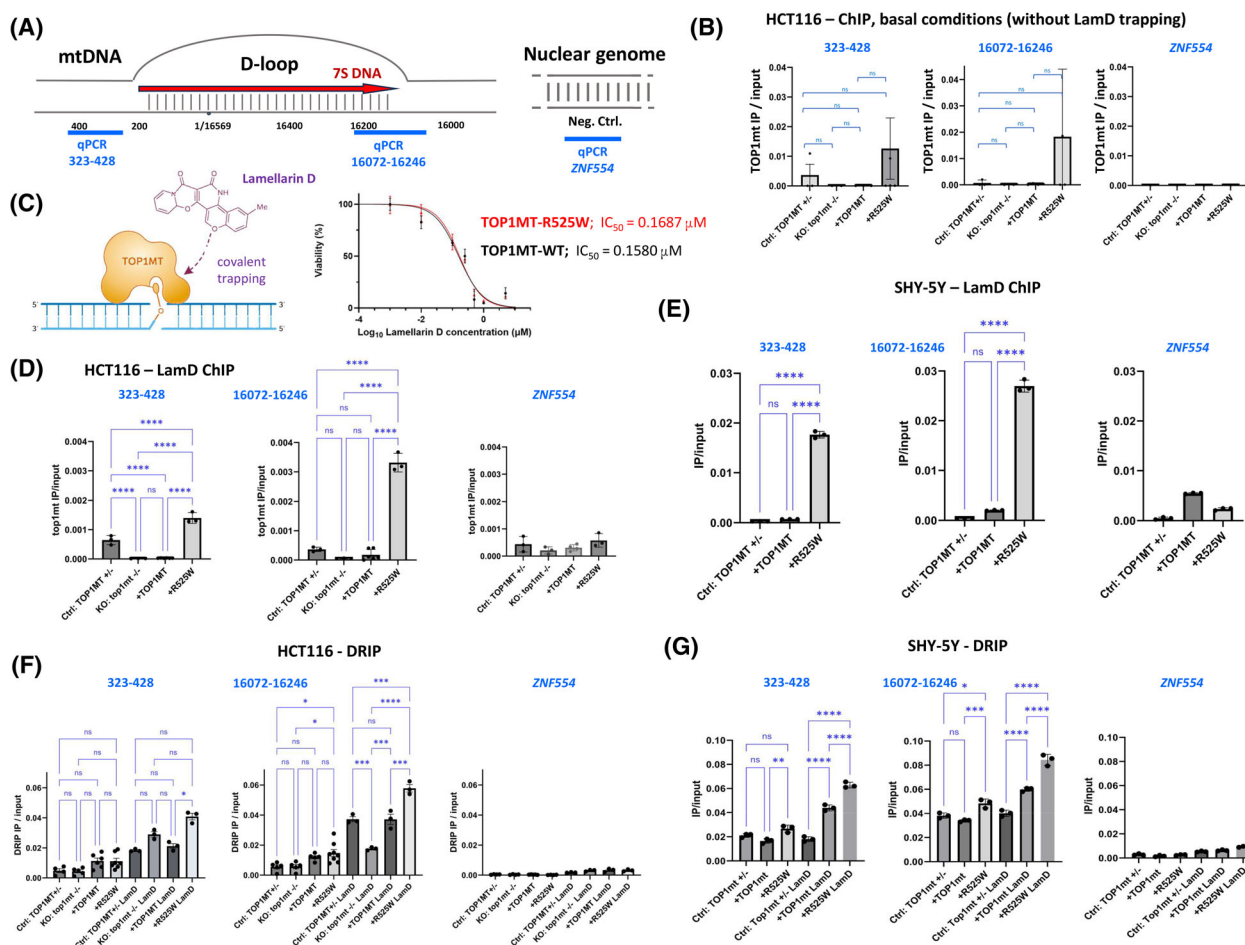


Fig. 7. rs2293925 increases TOP1MT association and R-loop abundance in the mitochondrial control region. (A) Schematic of the qPCR amplicons used for mitochondrial ChIP-qPCR and DRIP-qPCR. Two sites within the mitochondrial Control Region were assayed, and a nuclear genomic locus was included as a negative control for TOP1MT binding and R-loop formation. (B) Basal TOP1MT ChIP-qPCR without lamellarin D stabilization. TOP1MT-R525W shows a trend toward higher enrichment at the assayed mitochondrial Control Region sites relative to TOP1MT-WT. However, in the absence of lamellarin D, TOP1MT-mtDNA interactions were more variable, resulting in no statistically significant difference between genotypes under these conditions. Data are from 3 independent measurements and shown as mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA with *post hoc* multiple-comparison testing, as described in Methods. (C) Left: Lamellarin D stabilizes the transient TOP1MT-DNA cleavage complex by trapping TOP1MT on mtDNA. This increases recovery in ChIP-based assays and enables more robust detection of TOP1MT association with mtDNA. Right: Dose-response curves showing the effect of Lamellarin D on cell viability in HCT116 TOP1MT-WT and HCT116 TOP1MT-R525W cells after 48 h of treatment. Both cell lines exhibited a concentration-dependent decrease in viability, with nonlinear regression analysis yielding similar IC values (0.1580 μ M for WT and 0.1687 μ M for R525W). The curves display comparable slopes, indicating that the R525W variant does not markedly alter cellular sensitivity to Lamellarin D. Data are presented as mean $\pm$ SD from four technical replicates for each concentration. (D, E) TOP1MT ChIP-qPCR in HCT116 cells (panel D) and SH-SY5Y cells (panel E) overexpressing TOP1MT-tGFP (WT) or TOP1MT-R525W-tGFP performed after TOP1MT trapping with lamellarin D (1 μ M, 30 min) to stabilize TOP1MT cleavage complexes on mtDNA (see Methods). TOP1MT-R525W shows higher enrichment at Control Region sites compared with TOP1MT-WT. (F, G) DRIP-qPCR in HCT116 cells (panel F) and SH-SY5Y cells (panel G) performed with and without lamellarin D (1 μ M, 30 min) treatment. TOP1MT-R525W is associated with increased R-loop signal relative to TOP1MT-WT. Statistics: Data were analyzed by one-way ANOVA. *Post hoc* multiple-comparison testing showed significant differences for pairwise comparisons (**** P < 0.0001, * P < 0.05). Data are shown as mean with individual datapoints; error bars show SEM.

sites showed increased RNA:DNA hybrid signal in both HCT116 and SH-SY5Y cells expressing TOP1MT-R525W relative to TOP1MT-WT (Fig. 7F,

G). Together, these concordant data across two independent cell systems support increased TOP1MT occupancy at mitochondrial Control Region sites in the

R525W context, accompanied by elevated mitochondrial R-loop levels.

Next, we asked whether the above molecular changes associated with TOP1MT rs2293925 extend to an ALS-relevant setting. We first analyzed published ALS transcriptomic resources to assess allele-specific changes in TOP1MT expression in ALS. ALS Cell Atlas data [50] showed increased TOP1MT transcript levels in post-mortem human ALS spinal cord samples, as well as in SOD1^{G93A} and TARDBP^{Q331K/Q331K} mouse models of ALS (Fig. 8A). In parallel, analysis of patient-derived iPSC motor neurons from the Answer ALS dataset [51] showed that TOP1MT expression varied with rs2293925 genotype, with the highest mRNA levels observed in A/A homozygotes, intermediate levels in G/A heterozygotes, and the lowest levels in G/G homozygous wild-type samples (Fig. 8B). Genotype frequencies were similar between ALS and control groups in this dataset. Together, these observations are consistent with the increased TOP1MT-R525W expression detected in our experimental systems. We next examined our recent multi-omics dataset generated from a pilot cohort of sporadic ALS patient samples [52]. Although TOP1MT mRNA expression data were not available for this pilot cohort, DNA–RNA immunoprecipitation sequencing (DRIP-seq) revealed elevated RNA : DNA hybrid signal across the mitochondrial genome in ALS samples relative to healthy controls (Fig. 9A). Clinical exome sequencing did not identify rare coding variants in canonical R-loop regulators in this cohort [52], but detected TOP1MT rs2293925 in a subset of ALS patients (Fig. 9B, Table 1). Genotype-stratified analysis showed increased mitochondrial DRIP-seq signal in ALS cases carrying the rs2293925 A allele, with the strongest signal observed in A/A homozygotes relative to G/A heterozygotes and G/G reference cases (Fig. 9C). Prominent R-loop peaks mapped to regulatory regions of the mitochondrial genome, including the Control Region, and RNase H treatment markedly reduced the signal, confirming RNA : DNA hybrid specificity (Fig. 9D). Patient-resolved DRIP-seq analyses further indicated that the rs2293925 genotype association was not attributable solely to a single outlier, although inter-individual variability was evident and ALS62 displayed the highest signal within the A/A group (Fig. 9E). These data are best interpreted as consistent with an association between rs2293925 and elevated mitochondrial R-loop signal within the ALS group.

To test whether the elevated mitochondrial R-loop signal observed in the ALS pilot cohort is also present in an ALS-relevant neural lineage, we performed

mitochondrial DRIP-seq in neural stem cells derived from induced pluripotent stem cells generated from C9orf72-positive ALS patients and a healthy control (Fig. 10A). Neural stem cell identity was confirmed by Nestin immunostaining. ALS-derived lines showed higher mitochondrial DRIP-seq signal than the control line, and within the ALS lines, the rs2293925 A/G heterozygous line exhibited higher R-loop signal than the G/G homozygous reference line (Fig. 10B). RNase H treatment reduced the DRIP signal, supporting RNA : DNA hybrid specificity. These results extend the mitochondrial RNA : DNA hybrid phenotype to a neural lineage model and are consistent with the view that increased mitochondrial R-loop formation is associated with TOP1MT rs2293925 in pathological contexts relevant to ALS.

Discussion

Our data identify rs2293925 as a common TOP1MT variant with dual function that acts at the level of gene expression and mitochondrial R-loop homeostasis. This combination makes rs2293925 a candidate allele through which common genetic variation can influence mitochondrial genome dynamics in a context-dependent manner.

Several independent lines of evidence support a regulatory role for TOP1MT rs2293925. GTEx analysis indicates that rs2293925 behaves as a tissue-dependent expression quantitative trait locus (eQTL), while chromatin-state annotation places the variant within enhancer-associated chromatin across multiple tissues. Consistent with these observations, the rs2293925-containing region drives allele-dependent reporter activity, and *in vitro* DNA–protein binding assays support allele-specific transcription factor binding. Importantly, these regulatory changes are matched by expression data in living cells: in an isogenic HCT116 system, the rs2293925-linked A-allele is associated with increased TOP1MT transcript and protein abundance, and the same direction of change is reproduced in SH-SY5Y cells. Together, these findings support the view that rs2293925 can increase TOP1MT dosage in a cellular context-dependent manner.

In parallel with its gene expression effect, rs2293925 encodes the R525W substitution within the flexible TOP1MT linker domain, a region implicated in DNA interaction and catalytic turnover [26]. In targeted mitochondrial ChIP and DRIP assays, TOP1MT-R525W was associated with increased occupancy at Control Region sites and enhanced mitochondrial R-loop signal. These changes may reflect altered

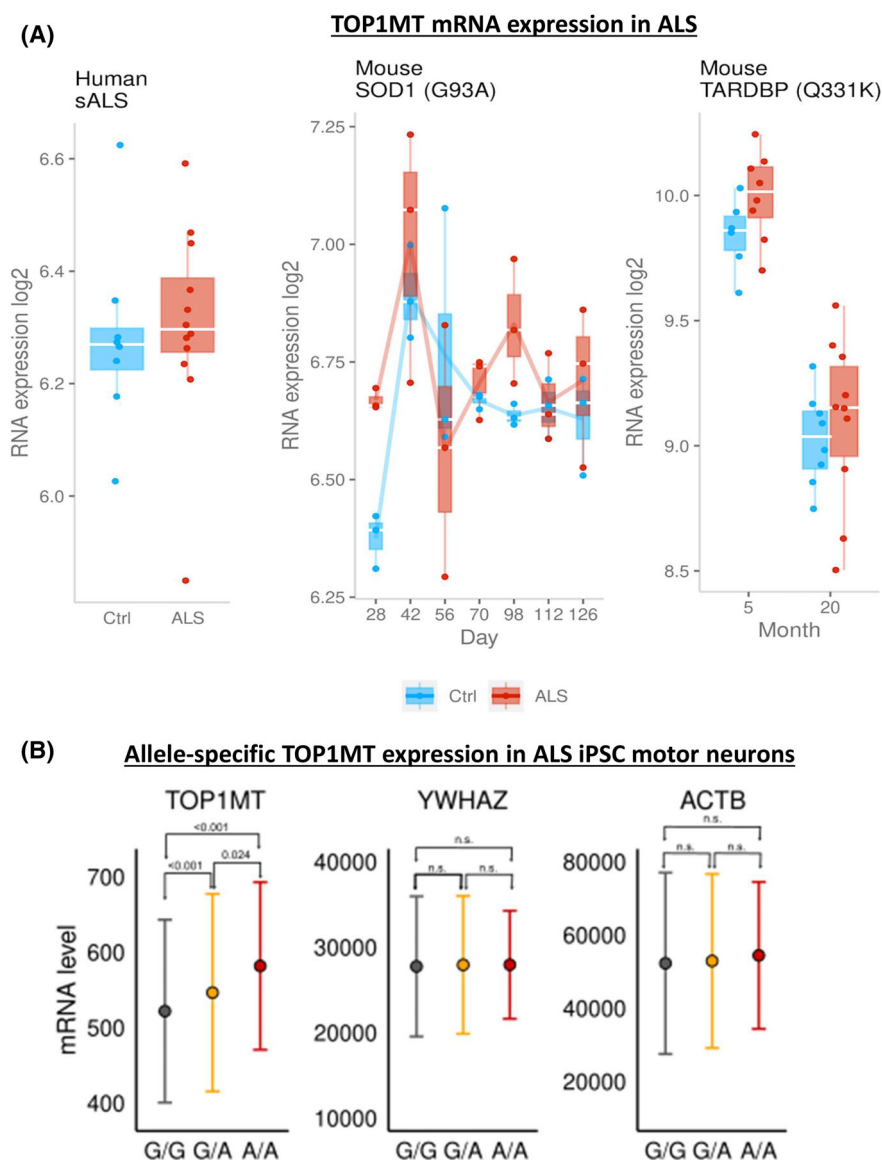


Fig. 8. Public ALS transcriptomic datasets showing TOP1MT expression in human and mouse ALS-related samples. (A) TOP1MT RNA transcript expression patterns from the ALS Cell Atlas in SOD1^{G93A} and TARDBP^{Q331K/Q331K} mouse models of ALS and in post-mortem human ALS spinal cord samples. The x-axis indicates age at sampling (days for SOD1^{G93A} mice, months for TARDBP^{Q331K/Q331K} mice, and post-mortem samples for human ALS cases). Data were retrieved from the ALS Cell Atlas; sample numbers varied across disease models and time points, with $n=3$ –11 biological samples per group. (B) TOP1MT mRNA expression in patient-derived iPSC motor neurons from the Answer ALS dataset stratified by rs2293925 genotype. TOP1MT expression was highest in A/A (R525W homozygous variant), intermediate in G/A heterozygotes, and lowest in G/G homozygous reference samples ($P < 0.001$). Sample numbers were $n=92$ (A/A), $n=424$ (G/A), and $n=350$ (G/G). Mean expression values were 581.85 ± 111.12 for A/A, 546.43 ± 131.02 for G/A, and 521.93 ± 121.25 for G/G. YWHAZ and ACTB are housekeeping genes serving as negative controls. The comparison of groups of variants was performed using a one-way ANOVA. A t -test was used for comparisons between subgroups of variants, and Bonferroni correction was applied for multiple comparisons.

interaction dynamics between TOP1MT–R525W and mtDNA, potentially increased recruitment and/or prolonged residence time, accompanied by elevated R-loop levels.

Despite these molecular changes, mitochondrial abundance, mtDNA copy number, and steady-state oxidative phosphorylation complex levels were not altered, arguing against a major defect in

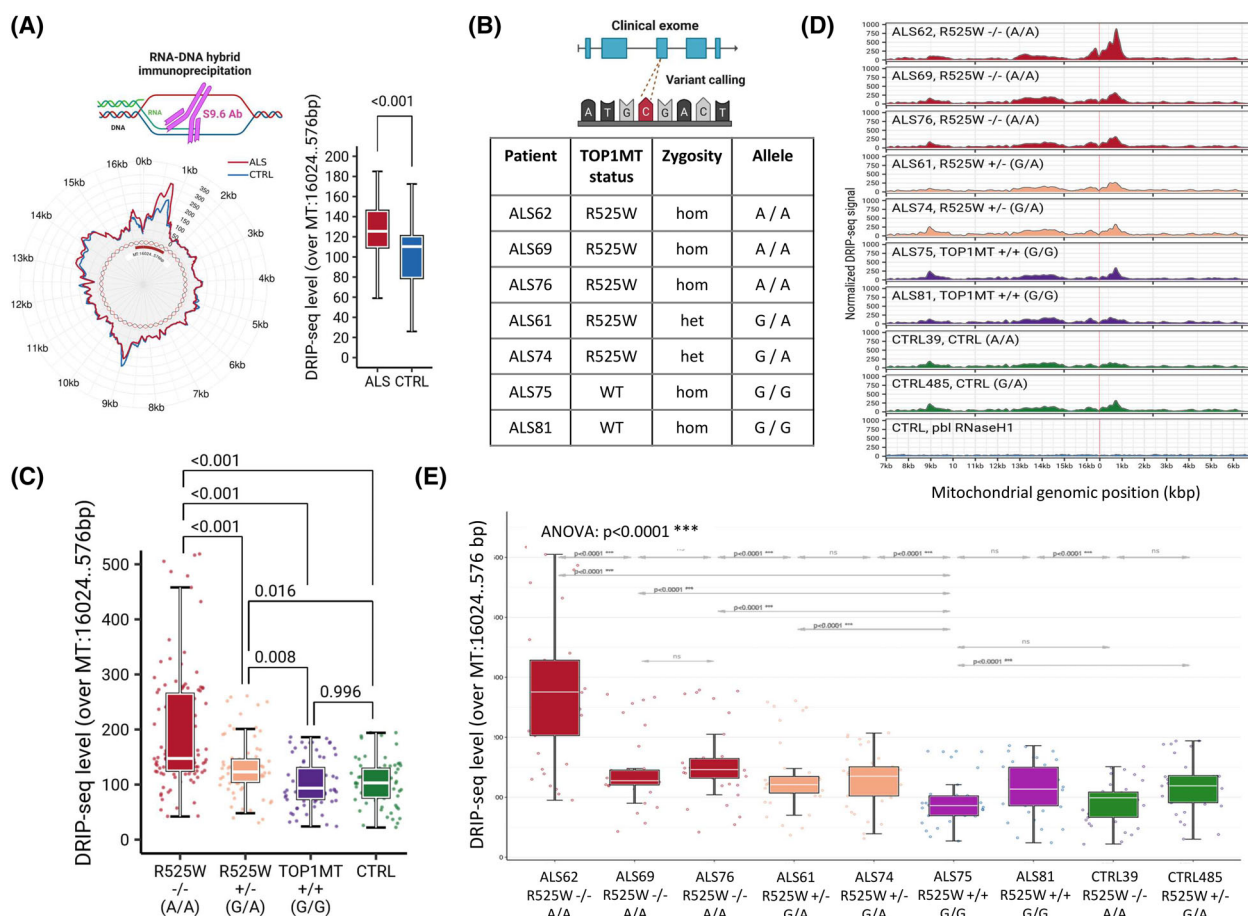


Fig. 9. DRIP-seq reveals elevated mitochondrial R-loop signal in sporadic ALS samples and an association with TOP1MT rs2293925 genotype. (A) Schematic of RNA:DNA hybrid immunoprecipitation (DRIP) using the S9.6 antibody and mitochondrial DRIP-seq readout. Left, circular representation of mean, normalized mitochondrial DRIP-seq signal across the mitochondrial genome for ALS (red) and healthy controls (blue). Right, boxplot quantification of mitochondrial DRIP-seq signal. Boxes indicate median and interquartile range; whiskers indicate the data range. A two-sided group comparison test was applied as described in Methods; the *P*-value is shown. (B) TOP1MT rs2293925 genotypes in the ALS cohort derived from clinical exome variant calling. Genotype groups are annotated as A/A (homozygous variant; R525W rs2293925), G/A (heterozygous), and G/G (homozygous reference; TOP1MT-WT). (C) Genotype-stratified distribution of mitochondrial DRIP-seq signal in ALS samples. Pairwise *P*-values for the comparisons indicated are shown on the plot (two-sided *t*-tests as specified in Methods). (D) Representative genome-browser tracks showing normalized mitochondrial DRIP-seq signal (IP/input) for individual ALS and control samples grouped by rs2293925 genotype (color scheme as indicated). An RNase H-treated sample (black) serves as a specificity control for S9.6 immunoprecipitation. (E) Sample-resolved distributions of mitochondrial DRIP-seq signal for each individual, displayed as boxplots. Overall differences were assessed by one-way ANOVA ($P < 0.0001$), followed by *post hoc* multiple-comparison testing; adjusted *P*-values for selected comparisons are annotated on the plot.

mitochondrial biogenesis or respiratory chain composition. Seahorse profiling likewise showed broadly preserved respiration, although the etomoxir-sensitive oxygen consumption was reduced in TOP1MT-R525W cells, consistent with decreased fatty acid oxidation. This effect raises the possibility that altered mitochondrial nucleic acid biochemistry may be coupled to modest metabolic rewiring rather than overt respiratory collapse. One plausible scenario is that persistent changes in mtDNA topology and RNA:DNA hybrid

abundance remodel mitochondrial signaling that lead to a change in substrate utilization.

These findings are consistent with prior studies on other TOP1MT variants, such as V256I and R111W [25], which showed partial mitochondrial phenotypes, perturbing nucleoid dynamics, relaxation capacity, and mitochondrial transcription. The R111W variant has been implicated in spinocerebellar ataxia and neurodevelopmental disorders, supporting the broader hypothesis that fine-tuned mitochondrial topoisomerase

Table 1. Patient data analyzed in this study.

Patient ID	Group	TOP1MT allele	Zygosity	Genotype	Sex	Age at sampling	Symptoms	Sites of symptoms
ALS62	ALS	R525W	Homozygous (variant)	A/A	Female	54	Classic (all 3 symptom groups), bulbar, lower and upper limb	Lower extremity weakness / right > left/ mainly lower MNs
ALS69	ALS	R525W	Homozygous (variant)	A/A	Male	51	classic (all 3 symptom groups), bulbar, lower and upper limb	bulbar: dysarthria, lower MNs > upper MNs
ALS76	ALS	R525W	Homozygous (variant)	A/A	Female	61	Lower and upper limb, bulbar not present	Slightly asymmetrical extremity weakness: mainly lower MNs
ALS61	ALS	R525W	Heterozygous	G/A	Female	52	Classic (all 3 symptom groups), bulbar, lower and upper limb, pseudobulbar	Bulbar: dysarthria, lower MNs > upper MNs
ALS74	ALS	R525W	Heterozygous	G/A	Female	45	Lower and upper limb, bulbar not present	Bilateral peroneal muscle weakness: difficulty in walking, lower and upper MNs
ALS75	ALS	Wild type	Homozygous (wild type)	G/G	Male	59	Lower and upper limb, bulbar not present	Lower extremity weakness and clumsiness: difficulty in waking, upper and lower MNs
ALS81	ALS	Wild type	Homozygous (wild type)	G/G	Female	69	Classic (all 3 symptom groups), bulbar, lower and upper limb	Left upper extremity weakness: lower MNs
K039	Ctrl	R525W	Homozygous (variant)	A/A	Female	52	N/A	N/A
K485	Ctrl	R525W	Homozygous (wild type)	G/A	Female	63	N/A	N/A

activity is essential for neuronal function [53,54]. More generally, these results highlight the importance of considering regulatory variants when interpreting mitochondrial genome maintenance, particularly when coding and *cis*-regulatory effects coexist within the same allele.

In our ALS pilot cohort, the R-loop signal was elevated in the mitochondria of rs2293925 carriers, especially among A/A homozygotes. Increased mitochondrial R-loop signal was also detected in neural stem cells derived from C9orf72-positive ALS patients. However, these associations do not establish rs2293925 as an ALS-specific driver. Instead, the current data are more consistent with a modifier/oligogenic model [55] in which the pathogenic effect of rs2293925 may only become apparent when combined with other genetic or epigenetic insults [56,57]. This interpretation fits with the broader view of ALS as a genetically heterogeneous disorder in which combinations of common and rare variants, together with environmental factors, shape disease susceptibility. In

such a framework, rs2293925 may not be sufficient to provoke pathology on its own, but could contribute to mitochondrial stress in cellular environments already sensitized by defects in RNA metabolism, proteostasis, or organelle quality control. Because mitochondrial R-loops influence replication, transcription, and genome stability, even modest increases in persistent RNA:DNA hybrids could become detrimental in neurons, which depend heavily on mitochondrial function and have limited capacity to buffer chronic mitochondrial stress.

Our study has limitations. First, the pilot ALS cohort was small and not powered for definitive genotype–phenotype inference at the population level. Second, neural validation was performed in neural stem cells and neuronal-like SH-SY5Y cells rather than mature motor neurons, and therefore does not capture the cellular state most relevant to ALS pathology. Future studies in iPSC-derived motor neurons and in larger, genotype-informed patient cohorts will be required to address these limitations.

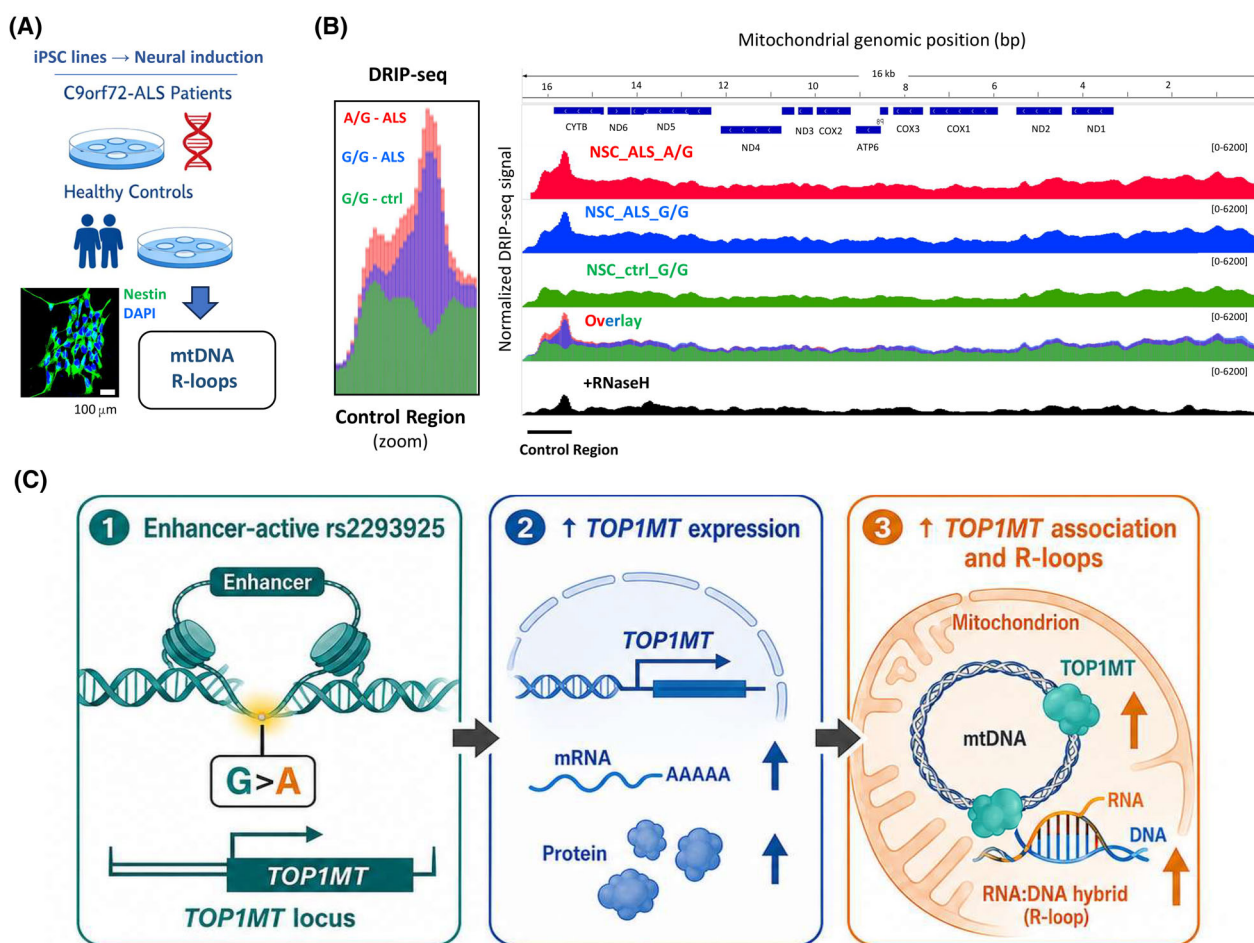


Fig. 10. Mitochondrial DRIP-seq analysis in neural stem cells (NSCs) derived from ALS patient iPSC lines. (A) Schematic workflow for generation of induced pluripotent stem cell (iPSC)-derived neural stem cells (NSCs) from healthy controls and C9orf72-ALS patients, including neural induction (7 days; PSC Neural Induction Medium), NSC expansion, and downstream characterization by Nestin staining and TOP1MT genotyping. Representative immunofluorescence image of iPSC-derived NSCs showing expression of the neural stem cell marker Nestin (green); nuclei are counterstained with DAPI (blue). Scale bar, 100 μ m. (B) Mitochondrial DRIP-seq analysis in neural stem cells (NSCs) derived from two ALS patient iPSC lines and one healthy control line. TOP1MT rs2293925 genotype is indicated for each line (A/G, heterozygous variant; G/G, homozygous wild type). Left, coverage profile over the mitochondrial Control Region shows higher DRIP-seq signal in the A/G ALS-derived NSC line relative to the G/G ALS-derived NSC line and the G/G control line. Right, genome-browser view of DRIP-seq read coverage across the mitochondrial genome for the three NSC lines. The RNase H-treated control (+RNase H) markedly reduces the signal, confirming RNA : DNA hybrid specificity. Together, these data extend the mitochondrial R-loop phenotype from the pilot ALS cohort to an ALS-relevant neural lineage context. (C) Model for rs2293925-mediated regulation of mitochondrial R-loops. The enhancer-active TOP1MT variant rs2293925 increases TOP1MT expression, leading to greater TOP1MT association with mtDNA and elevated mitochondrial R-loop formation. These molecular effects are supported by cellular models and ALS-relevant datasets.

Conclusions

TOP1MT rs2293925 is an enhancer-active common variant associated with increased TOP1MT expression and mitochondrial R-loop levels across isogenic and neuronal-like cell systems, with supportive evidence in ALS patient samples and iPSC-derived neural stem cells (Fig. 10C). These findings link common regulatory variants to mitochondrial nucleic acid topology and motivate future studies to establish the

pathological significance and therapeutic implications of this mechanism.

Materials and methods

Patient samples

This article contains experiments with human subjects published in [58]. See Table 1 for details. Written informed consent was obtained from all subjects and our experiments

were approved by the Medical Research Council (Ministry of Interior, Hungary) in accordance with all relevant ethical and legal requirements (approval numbers: 47066-3/2013/EKU(556/2013); 11920-2/2017/EÜIG; 12702-5/2018/EÜIG). We confirm that the ethics agreement allows for the open publication of anonymized genetic data and the participants understood all risks for the open publication of these data.

Cell lines and reagents

The HCT116 cell line ([RRID:CVCL_0291](#)), utilized in our experiments, was acquired from the NCI Development Therapeutics Program in 2014. Prior to employing CRISPR/Cas9 for genomic editing of TOP1MT, the cell line underwent authentication to ensure its integrity and identity. The HCT116 cells were maintained in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA; M8403), enriched with 10% fetal bovine serum (FBS), L-glutamine, and 1% penicillin/streptomycin, under controlled conditions of 37 °C and 5% CO₂. To maintain genetic stability and consistency in experimental outcomes, all experiments were conducted within 25 passages post-thaw. SH-SY5Y human neuroblastoma cells ([RRID:CVCL_0019](#)) were cultured in a 1 : 1 mixture of F12 medium (Sigma, N4888) and Minimum Essential Medium (MEM; Sigma-Aldrich, M2279), supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin, and maintained under standard culture conditions (37 °C, 5% CO₂). The SH-SY5Y cell line underwent authentication to ensure identity. Additionally, routine mycoplasma testing was performed to ascertain the absence of contamination, thereby ensuring the reliability of our experimental results.

Cell models

The following HCT116-derived cell lines were used in this study. HCT116 control (Ctrl) cells carry the endogenous TOP1MT/top1mt-R525W heterozygous genotype and therefore express both the wild-type and R525W alleles, providing the parental reference background. HCT116 top1mt^{-/-} (KO) cells lack TOP1MT expression and were used as the knockout model. HCT116 top1mt^{-/-} + TOP1MT (WT addback) cells are TOP1MT-knockout cells reconstituted with wild-type TOP1MT, such that the reintroduced wild-type allele serves as the sole source of TOP1MT activity. HCT116 top1mt^{-/-} + top1mt-R525W (R525W addback) cells are TOP1MT-knockout cells reconstituted with the R525W variant allele and were used to assess the functional consequences of the variant in an otherwise matched genetic background. The SH-SY5Y neuroblastoma cell line is likewise endogenous TOP1MT/top1mt-R525W heterozygous. In this background, we generated stable expression pools overexpressing either TOP1MT-

tGFP (WT) or TOP1MT-R525W-tGFP to evaluate whether the expression and mitochondrial nucleic acid phenotypes observed in the HCT116 isogenic system could be reproduced in a neuronal-like model.

Generation of neural stem cells (NSCs)

For the generation of NSCs, we started from four iPSC lines that we previously obtained by reprogramming of fibroblasts or peripheral blood mononucleate cells from two healthy control donors and two C9orf72-ALS patients after informed consent and approval from the local ethics committee (2015-03-31-07). The obtained iPSC lines were already described and characterized for the expression of stemness markers and for a normal karyotype [59]. Generation of NSCs was performed using the PSC Neural Induction Medium (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's indicated protocol. Briefly, the PSC Neural Induction Medium (Neurobasal plus 2% Induction supplement) was added to 15–25% confluent iPSCs colonies and maintained for 7 days. Thereafter, cells were harvested and expanded in complete Neural Expansion Medium (Neurobasal and advanced DMEM-F12 plus 2% Induction supplement) on Matrigel (Corning)-coated dishes for at least 6 passages as already described [60]. Characterization of the obtained NSCs was performed by immunocytochemistry using the specific marker Nestin (Anti-Nestin antibody, BD Biosciences, San Jose, CA, USA; 1 : 100). TOP1MT-R525W status of the generated NSC lines was determined by Sanger sequencing.

Transfection

HCT116 cells were cultured in 6-well plates and transfected at 70% confluence using polyethylenimine (PEI). A mixture of 12 µL of 1 µg·mL⁻¹ PEI and 1.5 µg of vector DNA was prepared, thoroughly mixed, and allowed to incubate for 30 min before application to the cells. We utilized a turboGFP (tGFP)-tagged human TOP1MT vector (tGFP-TOP1MT), acquired from OriGene Technologies, Inc., Rockville, MD, USA (Catalog Number RG234305, NM_001258446). The standard cell culture medium was substituted with Opti-MEM 1 h prior to transfection. Post 2–3 h of incubation with the PEI-vector blend, an additional 2 mL of Opti-MEM was added and the cells were incubated overnight. Subsequently, the medium was replaced with a selective medium the following day. To isolate transfected cells, the culture was maintained in a medium supplemented with 800 µg·mL⁻¹ G418. Following selection, tGFP-positive cells were isolated and monoclonal lines were established through single-cell plating. Three independent monoclonal lines were chosen for further analysis. These monoclonal lines were verified for the presence of top1mt-tGFP using Sanger sequencing and western blot analysis. SH-

SY5Y cells were transfected in 10 cm culture dishes using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions, employing the same TOP1MT-tGFP plasmids. Forty-eight hours post-transfection, selection was initiated using G418 at a final concentration of $0.5 \text{ mg} \cdot \text{mL}^{-1}$. Stable polyclonal cell populations were maintained under continuous selection for subsequent experiments.

Antibodies

Primary antibodies were obtained from the following sources: Mouse monoclonal turboGFP antibody (clone OTI2H8; TA150041, Origene), anti-TOP1MT antibody (CPTC-TOP1MT, Developmental Studies Hybridoma Bank), CTCF Rabbit polyclonal Antibody (TA375185, Origene), beta actin monoclonal antibody (8H10D10, ThermoFisher/Cell Signaling Technology, Danvers, MA, USA), anti-SP1 antibody (ab231778, Abcam, Cambridge, UK), anti-Smad3 antibody (ab208182, Abcam), S9.6 anti-RNA/DNA hybrid antibody (HB-8730, ATCC).

Lamellarin D treatment and cytotoxicity assay

Cells were seeded at 4000 cells per well in 96-well plates (day 0). On day 1, cells were treated with the TOP1MT-specific inhibitor Lamellarin D [49] (#T32545, TargetMol, Oberösterreich, Austria) over a concentration range of $0.001\text{--}10 \mu\text{M}$. Each concentration was tested in four technical replicates, and vehicle controls (DMSO; Sigma-Aldrich) were included in all experiments. After 48 h, cell viability was assessed by MTT assay. Briefly, cells were incubated with MTT (Thiazolyl Blue Tetrazolium Bromide; Sigma-Aldrich) at $0.5 \text{ mg} \cdot \text{mL}^{-1}$ for 3 h at 37°C . Formazan crystals were solubilized in 10% SDS (Sigma-Aldrich) and incubated overnight, and absorbance was measured at 560 nm using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific). Background absorbance was subtracted and values were normalized to the vehicle control (set to 100% viability). Dose–response curves were fitted by nonlinear regression (variable-slope model) in GRAPHPAD PRISM (v10), and IC₅₀ values were calculated for HCT116 TOP1MT-WT and HCT116 TOP1MT-R525W cell lines. For TOP1MT CHIP and mitochondrial DRIP experiments, Lamellarin D was applied at $1 \mu\text{M}$ for 30 min.

Western blot

For protein extraction, cells were washed twice with $1\times$ phosphate-buffered saline (PBS) and lysed in $50 \mu\text{L}$ of RIPA buffer containing 150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, and 50 mM Tris/HCl, supplemented with a protease inhibitor cocktail. Cells were collected using a cell scraper and sonicated at high intensity for 5 min (30 s ON/30 s OFF,

5 cycles). Lysates were then centrifuged at $15\,000g$ for 15 min at 4°C , and the resulting supernatants were collected. Protein concentrations were determined using a Qubit™ Protein and Protein Broad Range (BR) Assay Kit (Thermo Fisher) or BCA assay, and aliquots were stored at -70°C until further use. Prior to electrophoresis, protein samples were mixed with $6\times$ loading buffer (375 mM Tris/HCl, pH 6.8; 6% SDS; 4.8% glycerol; 9% 2-mercaptoethanol; 0.03% bromophenol blue) and denatured at 95°C for 10 min. Equal amounts of protein were separated on SDS/polyacrylamide gels. A stacking gel (4% acrylamide, 0.125 M Tris/HCl pH 6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED) was used, and separation was performed using a 10% running gel (10% acrylamide, 0.376 M Tris/HCl pH 8.8, 0.1% SDS, 0.1% APS, and 0.04% TEMED). Electrophoresis was carried out at 80 V for 20 min, followed by 120 V for 90 min in running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS). Proteins were transferred onto $0.2 \mu\text{m}$ nitrocellulose membranes (Amersham Protran, GE Healthcare, Cat# 10600001) or PVDF membrane (In the case of OXPHOS) using a blotting system at 90 V for 60 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), maintaining the temperature at 4°C . Membranes were blocked overnight at 4°C in 2% (w/v) bovine serum albumin (BSA) diluted in PBS-T (0.1% Tween-20 in PBS), followed by a 1-h incubation at room temperature. Primary antibodies were diluted in 2% BSA/PBS-T and incubated with membranes for 2 h at room temperature. The following primary antibodies were used: TOP1MT (DSHB Hybridoma Product CPTC-TOP1MT-3; dilution: 200 $\times$), tGFP (Origin, TA150041; dilution: 1000 $\times$), oxidative phosphorylation (OXPHOS Antibody Cocktail) complex proteins (Complexes II-III, V) and β -actin (Invitrogen, MA5-15739; dilution: 2000 $\times$). Membranes were washed five times with PBS-T (first wash for 1 min, second for 3 min, and three final washes for 5 min each). Secondary antibodies (HRP-conjugated secondary antibody or Alexa Fluor 647-conjugated anti-mouse IgG, Invitrogen A21236 or) were diluted 1:5000 in 2% BSA/PBS-T and applied for 1 h at room temperature, followed by the same washing steps. When we used HRP-conjugated secondary antibody, the protein detection was carried out using the Supersignal West Pico Plus Chemiluminescent Substrate, as per the manufacturer's instructions. When we used fluorescence secondary antibodies, the signal detection was performed using a Pharos FX Scanner (Bio-Rad Laboratories GmbH, Feldkirchen, Germany).

Mitochondrial labeling with MitoTracker red

Mitochondria were visualized in live cells using MitoTracker Red FM (Thermo Fisher Scientific). A 1 mM stock solution was prepared according to the manufacturer's instructions and diluted in Hank's Balanced Salt Solution (HBSS) to a final working concentration of 200 nM

immediately before use. Cells grown on Ibidi μ -Slide 8-well chamber slides were washed with 250 μ L HBSS and incubated with 200 μ L of 200 nM MitoTracker solution for 20 min at 37 °C. After incubation, the staining solution was removed, cells were washed once with 250 μ L HBSS, and fresh HBSS (250 μ L) was added prior to imaging or flow cytometric analysis.

Flow cytometry

Mitochondrial mass was quantified using MitoTracker Red FM (Thermo Fisher Scientific) and flow cytometry. HCT116 cells were harvested, washed once with pre-warmed phosphate-buffered saline (PBS), and stained in suspension with MitoTracker Red FM at a final concentration of 100 nM in HBSS. Staining was performed at 37 °C for 20 min in the dark. After incubation, cells were washed twice with cold PBS and resuspended in PBS for analysis. Flow cytometry was performed using a BD FACSCalibur instrument equipped with a 488 nm excitation laser. MitoTracker Red fluorescence was detected in the FL2 channel (585/42 nm). In addition, stable SH-SY5Y cell lines expressing TOP1MT-tGFP (WT) or TOP1MT-R525W-tGFP were measured on the same instrument using the green fluorescence channel (FL1; 530/30 nm) to assess relative TOP1MT protein expression based on tGFP intensity. A minimum of 10 000 events per sample were acquired, and data were analyzed using the Floreada software.

Confocal laser scanning microscopy (CLSM)

Confocal imaging of TOP1MT-tGFP and TOP1MT-R525W-tGFP fusion proteins was conducted to assess their subcellular localization relative to mitochondria stained with MitoTracker Red. After labeling, cells were maintained in phenol red-free HBSS and imaged live using a ZEISS LSM 880 microscope equipped with Airyscan and confocal scanning modules. Fluorophores were excited using 488 nm (turboGFP) and 561 nm (MitoTracker Red) laser lines. Emission signals were collected at 505–530 nm for turboGFP and 580–620 nm for MitoTracker Red. The pinhole was set to 1 Airy unit (34 μ m), resulting in an optical slice thickness of $\sim$ 1 μ m. Representative images were captured using standardized settings across conditions.

Fluorescence correlation spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) was performed to assess the diffusion kinetics of TOP1MT-tGFP fusion proteins in live cells. Measurements were carried out on transiently transfected cells using a ZEISS LSM 880 confocal microscope equipped with a Confocor 3 FCS/FCCS module, enabling high-resolution analysis of mobility within a defined 0.3 μ m³ observation volume. For calibrating the dimensions of the ellipsoidal detection volume, Alexa Fluor 488 dye was

used at 100 or 20 nM concentration in 10 mM Tris-EDTA buffer as described (Rehó *et al.*, <https://pubmed.ncbi.nlm.nih.gov/36639026/>). Excitation was achieved using a 488 nm argon ion laser, and emission was collected between 500 and 553 nm. Cells expressing either wild-type or R525W variant TOP1MT-tGFP were measured at 22 °C. Mitochondrial localization was confirmed prior to FCS via MitoTracker Red staining, and measurements were selectively performed within mitochondria displaying TOP1MT signals. Each FCS measurement involved 10 $\times$ 8 s runs, with 6–12 cells per variant group and one or more mitochondria analyzed per cell. Fluorescence intensity fluctuations were recorded, and autocorrelation curves were generated for each measurement point by the Zen data acquisition software. Autocorrelation functions $G(\tau)$ were fitted to a two-component free diffusion model also taking triplet state formation [61] into account:

$$G(\tau) = \frac{1-T + Te^{-\tau/\tau_{tr}}}{N(1-T)} \left[\rho_1 \left(1 + \frac{\tau}{\tau_1} \right)^{-1} \left(1 + \frac{\tau}{S^2\tau_1} \right)^{-1/2} + \rho_2 \left(1 + \frac{\tau}{\tau_2} \right)^{-1} \left(1 + \frac{\tau}{S^2\tau_2} \right)^{-1/2} \right]$$

where $G(\tau)$ is the autocorrelation function, N denotes the average number of diffusing fluorescent molecules present in the detection volume, τ is the lag time. T is the equilibrium molar fraction of fluorophores in triplet state, and τ_{tr} is the triplet correlation time, τ_1 and τ_2 are the diffusion times (average dwell times) of the fast (free) and slow (bound) molecular populations, and ρ_1 and ρ_2 are their respective fractions. S is the aspect ratio of the ellipsoidal detection volume, which together with the lateral beam waist radius (ω_{xy}) was determined from fitting the autocorrelation curve of the Alexa 488 solution [62]. The diffusion coefficients D_i ($i = 1, 2$) of the fast and slow components were calculated as:

$$D_i = \frac{\omega_{xy}^2}{4\tau_i}.$$

In total, three series of independent FCS experiments were performed, with consistent trends observed in diffusion profiles across replicates.

Seahorse flux analysis

Seahorse indirect oximetry was performed to determine cellular oxygen consumption (OCR) applying the considerations described in [63,64]. Cells were seeded into Seahorse XF96 Cell Culture Microplates at a seeding density of 8000 cells per well in 100 μ L growth media. Cells were incubated at 37 °C and 5% CO₂ overnight. On the following day, the growth media was changed to assay media (XF Assay Media Modified DMEM with 3 g·L⁻¹ glucose). Desired compounds were loaded into an appropriate port of the cartridge (20 μ L per port). Data integrations were

performed for 3 min for all measurement points. To assess the effects of ascites treatment on HCT-116 cells, 5 measurements were performed without any treatment to record the total oxygen consumption rate. Then, BPTES (10 μ M) was added to rows A and B, UK-5099 (10 μ M) was added to rows C and D, etomoxir (50 μ M) was added to rows E and F; finally, oligomycin (2 μ M) was added to rows G and H followed by 5 recordings. Finally, to measure background OCR, an antimycin (10 μ M) and rotenone (5 μ M) mixture was added to all wells, and OCR was recorded 5 times. Antimycin readings were subtracted from OCR reads for each well, and the OCR values were normalized for cell numbers. Total, oligomycin-resistant, BPTES resistant, UK-5099-resistant, and etomoxir-resistant respiration were calculated and these OCR values were expressed as fold change (mutant values/isogenic control values). Negative values and values outside 2 SDs were excluded. The ratios were assessed with the Rout method for outliers and one value was omitted on that ground. Calculations were performed using Microsoft Excel and GRAPHPAD PRISM 8.0.1 (GraphPad Software, Boston, MA, USA).

Sulforhodamine B assay

Sulforhodamine B assay to assess cell numbers was performed as described in [65,66].

Electrophoretic mobility shift assay (EMSA)

Allele-specific DNA–protein interactions at the rs2293925 locus were assessed using the LightShift® EMSA Optimization and Control Kit with SYBR™ Green & SYPRO™ Ruby stains (Thermo Fisher Scientific, Cat# E33075). A 200 bp double-stranded DNA fragment encompassing the rs2293925 region (either G or A allele) was generated by PCR from ALS and healthy control gDNA samples and purified by standard PCR clean up method. Nuclear extracts were prepared from HCT116 cells using the Nuclear Extraction Kit (Abcam, Cat# ab113474) according to the manufacturer's instructions. EMSA binding reactions were set up by incubating 20 fmol of the DNA probe with increasing amounts of nuclear extract (up to 4 μ g) in binding buffer (10 mM Tris pH7.5, 10 mM NaCl, 40 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 50 μ g·mL⁻¹ BSA) at room temperature for 20 min. Samples were separated on 0.8% agarose gels in 1× TBE buffer, run at 60 V for 2 h at 20 °C. Gels were sequentially stained with SYBR™ Green (DNA) and SYPRO™ Ruby (protein–DNA complexes). Fluorescent imaging was performed using the Molecular Imager PharoFX and PharoFX Plus Systems (Bio-Rad), ensuring high-resolution detection of DNA and DNA–protein complexes. Quantification of the unbound DNA fraction was carried out using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA). Differences in DNA–protein binding between alleles were assessed based

on DNA mobility shifts and the depletion of free DNA. Statistical analysis: for each nuclear extract concentration, band-intensity distributions obtained with the G-allele and A-allele probes were first assessed for equality of variance using an *F*-test; no significant differences in variance were detected. Accordingly, allele effects at each concentration were evaluated using two-tailed unpaired Student's *t*-tests assuming equal variances. Among the tested concentrations, a nominal difference between alleles was observed only at 2.5 μ M nuclear extract ($P = 0.0291$), whereas all other comparisons were not significant ($P > 0.05$). Statistical analyses were performed in GRAPHPAD PRISM (v10).

Luciferase transcriptional reporter assay

For cloning of reporter constructs, a 1 kbp genomic region encompassing the rs2293925 SNP was PCR-amplified from genomic DNA isolated from ALS patients and healthy controls. Primers contained flanking KpnI and HindIII restriction sites to facilitate directional cloning into the pNL3.1 [minP] NanoLuc® reporter vector (Promega). The forward primer sequence was 5'-GATGGTACCACACACACA TACAAAAGAAGT-3' and the reverse primer was 5'-GAAAAGCTTGCTGGGGTGGGGCTGAGCTT-3'. Amplified products were cloned into pNL3.1[minP] and transformed into *E. coli* DH5 α competent cells. Positive clones were screened by colony PCR using vector-specific primers (5'-GCAAAATAGGCTGTCCCC-3' and 5'-GACCATGGTGGCTTTACC-3'), followed by plasmid miniprep and diagnostic digestion with KpnI and HindIII to verify insert size. Sanger sequencing confirmed the identity of the insert and the rs2293925 allele. Endotoxin-free midiprep-grade plasmid DNA was prepared for transfection using standard purification kits. For luciferase measurements, HEK293T and MCF7 cells were seeded in 96-well plates (Corning) one day prior to transfection. Cells were transfected in technical triplicates using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions with 100 ng reporter plasmid per well. Luciferase activity was measured 24 h post-transfection using the Nano-Glo Luciferase Assay System (Promega Corporation, Madison, WI, USA; N1130) on a Hidex Sense microplate reader. Raw luminescence values were normalized to the minimal-promoter-only reporter (empty vector) and reported as relative luminescence units (RLUs). Statistical analysis. Luciferase activities were analyzed separately for each cell line. Data were first assessed for normality using the Shapiro–Wilk test and for homogeneity of variances using an equal-variance test; both assumptions were met (Shapiro–Wilk $P = 0.608$; equal variance $P = 0.650$). Differences among conditions (mock control, minimal promoter, rs2293925-G construct, rs2293925-A construct) were evaluated by one-way ANOVA, followed by *post hoc* multiple-comparison testing to assess the pre-specified pairwise contrasts (mock vs. minimal promoter;

minimal promoter vs. rs2293925-G; rs2293925-G vs. rs2293925-A). Most pairwise comparisons were significant at $P < 0.001$, whereas the rs2293925-G vs. rs2293925-A comparison was significant at $P < 0.05$. Statistical analyses were performed in GRAPHPAD PRISM (v10).

Transcription factor motif disruption analysis

We evaluated the potential effect of the SNP rs2293925 on transcription factor binding by utilizing the MOTIFBREAKR package (v1.22.0) in R (v4.3.0) [67]. This tool determines whether a DNA variant alters the binding potential of transcription factors by comparing the sequence context of each allele against known position weight matrices (PWMs). Genomic coordinates for rs2293925 were obtained using the *snps.from.rsid* function and matched to the GRCh38/hg38 reference genome via the *BSgenome.Hsapiens.UCSC.hg38* and *SNPlocs.Hsapiens.dbSNP144.GRCh38* resources [68]. Motif predictions were performed using PWMs from the HOCOMOCOv11 core collections (A, B, and C) retrieved from the MotifDb database [69]. We selected the “information content” scoring method and used equal background nucleotide frequencies ($A = 0.25$, $C = 0.25$, $G = 0.25$, $T = 0.25$). Predictions were filtered using a match score threshold of 0.001 to retain high-confidence hits. Additional filtering was applied to keep motifs with at least 80% sequence similarity for one of the alleles ($P_{ct} > 0.8$). The results were sorted by predicted functional impact and difference in allele binding strength. To improve accuracy, P -values were refined using the *calculatePvalue* function with a resolution of $1e-6$. Visualizations of the predicted disruptions were generated using *plotMB* function to highlight strong, weak, and total effects.

Fluorescent DNA–protein binding assay

Allele-specific transcription factor binding to the rs2293925 locus was assessed using the DNA–Protein Binding Assay Kit (Fluorometric) (Abcam, ab117140) following a modified manufacturer’s protocol. Wells were coated with antibodies against the indicated transcription factors (SP1: Abcam ab231778; SMAD3: Abcam ab208182; CTCF: OriGene TA375185) and incubated to allow immobilization. Nuclear extracts from HEK293T cells were then added to capture endogenous transcription factors. After washing, 200 bp double-stranded DNA probes spanning rs2293925 and containing either the G (reference) or A (variant) allele were added and incubated to enable binding to the immobilized proteins. Following washes to remove unbound DNA, a DNA-specific fluorescent dye was added according to the kit instructions and fluorescence was measured on a Hidex Sense microplate reader (E_x/E_m 485/535 nm). Background signal from wells lacking primary antibody was subtracted, and fluorescence intensity was used as a proxy for probe DNA retained through transcription factor

binding. Statistical analysis. For each transcription factor, binding of the G- and A-allele probes was compared using a two-tailed unpaired Student’s t -test. Equality of variance between groups was evaluated by an F -test prior to t -testing. The rs2293925 A allele showed higher binding for SP1 ($P = 0.0229$) and CTCF ($P = 0.0167$), whereas the difference for SMAD3 did not reach statistical significance ($P = 0.08$). Statistical analyses were performed in GRAPHPAD PRISM (v10).

Reverse transcription quantitative real-time PCR (rt-qPCR)

Total RNA was isolated from cells with TRIzol according to a standard protocol and quantified using a Qubit™ RNA BR Assay Kit (Invitrogen, Q10210). For each reaction, 1000 ng of RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland; Cat. No. 04896866001) according to the manufacturer’s protocol, employing random hexamer primers in a single-tube 20 μ L reaction, and the cDNA was diluted fivefold before qPCR. Quantitative PCR was performed on a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc) using SYBR Green chemistry in 10 μ L reaction volumes containing: 5.0 μ L 2 $\times$ SYBR Green Master Mix, 0.375 μ L each of forward and reverse primers (10 μ M; final concentration 375 nM), 0.05 μ L ROX passive reference dye, and 0.20 μ L nuclease-free water. Thermal cycling consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 20 s and 72 °C for 30 s, with fluorescence acquisition at the extension step. Melt-curve analysis (65–95 °C) verified amplicon specificity. Data were analyzed using the QuantStudio software with automatic threshold settings. The threshold cycle (C_t) was used to calculate relative quantity (RQ) values, which were normalized to the geometric mean of the average of YWHAZ, ACTB and 18S rRNA house-keeping genes. Normalized expression values were used for data visualization by GraphPad. Primer design: Gene-specific primers were designed using Primer-BLAST (NCBI) to span exon–exon junctions, generating 100–200 bp amplicons. The “TOP1MT 13–14. exon” primer pair targeted the rs2293925 position. Primer sequences can be found in the Supplementary material.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed to assess the DNA-binding profile of TOP1MT in human cells. We conducted the ChIP assay using a variety of cell lines: TOP1MT/top1mt-R525W heterozygote, TOP1MT knockout, and two knockout cell lines each complemented with either the wild-type TOP1MT allele or the top1mt-R525W allele, serving as the sole source of Top1mt

activity. A total of 2×10^7 HCT116 cells were fixed in T175 flasks using 1% formaldehyde (20 mL) for 10 min at room temperature and crosslinking was quenched with 416 mM glycine for 5 min. After three washes with ice-cold PBS containing 0.1% Tween-20 (PBS/T), cells were scraped, pelleted at 1000 *g* for 5 min at 4 °C, and stored at –80 °C until use. Cell pellets were resuspended in 1.5 mL ChIP Lysis Buffer (50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA, pH 8.0; 1% Triton X-100; 0.1% sodium deoxycholate; 1% SDS; protease inhibitors) and lysed using Bioruptor (Diagenode, Seraing (Ougrée), Belgium) (2 cycles: 6 m·s^{–1} for 40 s, with 120 s pause). Chromatin was sonicated in Diagenode Bioruptor Plus using 1.5 mL LoBind tubes (600 µL sample per tube) with 2 × 5 cycles of 30 s ON/OFF at low power. Debris was removed by centrifugation at 16 000 *g* for 20 min at 4 °C. Fragmentation was verified by reverse crosslinking (65 °C, 6 h), followed by DNA extraction using the Zymo Chip DNA Clean & Concentrator kit (Zymo Research), and 1% agarose gel electrophoresis. For immunoprecipitation, 1% of the sonicated chromatin was saved as input. The remaining chromatin was diluted 1 : 10 in IP Dilution Buffer (1% Triton X-100; 2 mM EDTA, pH 8.0; 20 mM Tris/HCl, pH 8.0; 150 mM NaCl; protease inhibitors), and incubated overnight at 4 °C with rotation with 100 µL Dynabeads™ Protein G pre-coated with 8 µg of anti-TOP1MT antibody (CPTC-TOP1MT, Developmental Studies Hybridoma Bank). Beads were sequentially washed at 4 °C for 1 min in the following buffers: low salt wash buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris/HCl; 150 mM NaCl), high salt wash buffer (same as above with 500 mM NaCl), LiCl wash buffer (0.25 M LiCl; 1% NP-40; 1% sodium deoxycholate; 1 mM EDTA; 10 mM Tris/HCl), and TE buffer (10 mM Tris/HCl, 1 mM EDTA). Immunoprecipitated DNA and input were eluted in 100 µL of elution buffer (0.1 M NaHCO₃, 1% SDS) at 30 °C for 15 min with intermittent vortexing. DNA was purified and stored at –80 °C until analysis. Quantitative PCR (qPCR) was performed using the LightCycler® 480 SYBR Green I Master mix (Roche) on a QuantStudio12KFlex instrument (Applied Biosystems). Enrichment was calculated as percent of input and corrected for dilution. Primer sequences can be found in the Supplementary material.

DNA–RNA immunoprecipitation (DRIP)

DRIP experiments were performed using the same HCT116-derived cell lines as in the ChIP assays, and mitochondrial DRIP-seq was additionally carried out on ALS-derived neural stem cells (NSCs) and control NSCs. For DRIP, 5–7 × 10⁶ cells were harvested per sample and stored at –70 °C until processing. Genomic and mitochondrial DNA were isolated using the Macherey–Nagel NucleoSpin Tissue Kit according to the manufacturer's protocol, with the following modification: the lysis step was performed at 55 °C

for 4 h to ensure efficient recovery of nucleic acids for RNA : DNA hybrid analysis [70]. Post-RNase A digestion (37 °C, 18 min), DNA concentration was measured. For each sample type, 6 µg of genomic DNA (gDNA) was sonicated for 5 cycles at high intensity (1 cycle: 30 s ON, 30 s OFF) to achieve fragment sizes of 400–600 bp, verified by agarose gel electrophoresis. We reserved 2% of each sample as INPUT. As a negative control, 6 µg of DNA underwent RNase H digestion in a thermoshaker at 37 °C for 2 h (RNase H selectively cleaves RNA–DNA hybrids). RNase H-digested samples were then purified using the Kapa Pure bead system according to the manufacturer's instructions. For immunoprecipitation, 100 µL of protein G beads were washed twice with 0.5% BSA in PBS/EDTA, then incubated with 30 µg of S9.6 antibody (R-loop specific) in 0.5% BSA PBS/EDTA for 4 h at 4 °C on a rotator. The S9.6-bead complexes were washed twice, then immunoprecipitation was performed in IP buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% Triton-X, 0.1% Na-deoxycholate) containing Protease Inhibitor. The samples were added to the bead-S9.6 complex and incubated overnight at 4 °C on a rotator. The next day, bead-S9.6-R-loop complexes were washed thrice with IP buffer and thrice with TE buffer, then resuspended in elution buffer (50 mM Tris/HCl pH 8, 10 mM EDTA, 1% SDS, proteinase K), and incubated at 55 °C for 2 h at 1000 rpm in a thermos shaker. The supernatant was then transferred to a new tube and purified using a 3× bead-to-sample ratio with Kapa Pure beads. Quantitative PCR (Q-PCR) was performed on the IP, RNase H IP, and INPUT samples to measure R-loop levels. Primer sequences can be found in Table S1.

NGS and bioinformatics analysis

NGS library preparation and sequencing were performed as described [58] using the Illumina TruSeq ChIP Sample Preparation protocol. Amplified libraries were sequenced using an Illumina HiSeq 2500 instrument (2 × 125 paired-end sequencing). FASTQ reads were aligned to the Homo sapiens GRCh38.p12 reference genome using Bowtie2 v2.5.2 [71]. The SAM-format alignments were sorted, indexed, and converted to BAM format with SAMtools v1.19.2 [72]. BAM files were subjected to deduplication using Picard.jar 3.4.0 [73]. From the alignment files, BigWig coverage files were generated after RPKM normalization using deepTools v3.5.4 [74]. A figure of coverage over the mitochondrial genome was produced using IGV v2.15.2 [75].

Statistical analysis

Statistical analyses were performed in GRAPHPAD PRISM (v10) unless stated otherwise. Data are presented as mean with individual datapoints where applicable, and error bars are defined in the corresponding figure legends. Normality and homogeneity of variance were assessed where required

using the Shapiro–Wilk test and an equal-variance test, respectively. For comparisons between two groups, two-tailed unpaired Student's *t*-tests were used; equality of variances was evaluated by F-test when applicable (e.g., EMSA densitometry and transcription factor binding assays). For comparisons involving more than two conditions, one-way ANOVA was performed followed by *post hoc* multiple-comparison testing for pre-specified contrasts (e.g., luciferase reporter assays). For multi-group analyses of mitochondrial DRIP-seq signal stratified by genotype, overall differences were assessed by one-way ANOVA with *post hoc* multiple comparisons; adjusted *P*-values are shown where indicated. For Seahorse OCR analyses, inhibitor-defined OCR components were calculated after background subtraction and normalization to cell number, expressed as fold change (isogenic control/TOP1MT-R525W), and summarized across independent experiments with 95% confidence intervals. For all tests, $P < 0.05$ was considered statistically significant and significance levels are indicated in the figures/legends. Unless stated otherwise, data were derived from 2 to 3 independent biological replicates (as indicated in the relevant Methods subsections).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

DV, ZsR, ÉN, BBO, GyV: performed all experiments otherwise stated; ET and PBa performed Seahorse flux measurements; SS, PBo, AR created and characterized iPSC and NSC cell lines; ZsK, LH, BLB: performed bioinformatic analyses, data curation, and visualization; LSz, YP, VG: Conceptualization, draft editing; BLB, YP, VG, ZsR: review and editing. LSz: writing—review and editing, supervision, funding acquisition, visualization, formal analysis.

Peer review

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/febs.70649>.

Data availability statement

NGS datasets (clinical exome sequencing (CES), DNA–RNA immunoprecipitation sequencing (DRIP-seq)) were deposited in Gene Expression Omnibus (GEO) under the accession numbers [GSE242475](#) and [GSE319153](#). Source data accompanying this study can be found in Table S1.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of PCR oligos and source data accompanying this study.