



TTC14 dysfunction contributing to microcephaly and lissencephaly spectrum features through protein mislocalization and impaired RNA processing

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Abstract

Congenital microcephaly and lissencephaly spectrum disorders are characterized by disrupted neuronal proliferation and migration, often driven by underlying genetic variants. Here, we identified and characterized of a novel gene (Tetratricopeptide Repeat Domain 14, *TTC14*) and its homozygous missense variant (c.89 A>G; p.His30Arg (p.H30R)) in a female proband presenting with microcephaly, epileptic spasms, global developmental delay, and neuroimaging features of simplified gyral pattern, focal pachygyria, and corpus callosum thinning. Trio-based whole exome sequencing revealed this variant to be rare and autosomal recessively inherited from both asymptomatic parents. Structural modeling demonstrated that H30 lies at the start of an α -helical region and is evolutionarily conserved. The p.His30Arg substitution caused significant steric clashes and disrupted local folding. Interaction network analysis and molecular dynamics simulations confirmed structural destabilization, increased conformational flexibility, and loss of stability in the mutant protein. While mRNA and protein levels remained unaltered, the *TTC14*-p.His30Arg protein mislocalized from the nucleus to the cytosol and formed aggregates. Functionally, proband fibroblasts exhibited increased cell death and altered cell cycle progression. *TTC14* interacts with RNA splicing and mRNA processing proteins, and gene ontology analysis implicated it in RNA binding and spliceosomal complex assembly in key brain regions including the cerebral cortex, hippocampus, and white matter. These findings collectively suggest that *TTC14* plays a crucial role in RNA metabolism during neurodevelopment, and that the p.His30Arg variant impairs its function, possibly leading to a neurodevelopmental disorder within the lissencephaly spectrum. This study identifies *TTC14* as a likely pathogenic candidate gene in cortical malformation syndromes.

Introduction

Lissencephaly, a severe malformation of cortical development, belongs to a broader group of conditions referred to as the lissencephaly spectrum. These disorders are

characterized by the smoothening of the cerebral cortex due to abnormal neuronal migration during early fetal development, typically between the 12th and 24th weeks of gestation (Guerrini and Dobyns 2014). The classical presentation of lissencephaly includes absent or diminished gyri and sulci, resulting in a smooth brain surface that is often accompanied by thickened cortical layers, ventriculomegaly, and corpus callosum hypoplasia. Clinically, individuals with lissencephaly spectrum disorders present with a range of neurodevelopmental phenotypes, including severe global developmental delay, epileptic seizures, hypotonia, microcephaly, and often early childhood mortality (Wheway et al. 2019; Donato et al. 2017). The severity and distribution of cortical malformations can vary, leading to subclassifications such as classical lissencephaly, subcortical band heterotopia, and pachygyria.

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The genetic basis of the lissencephaly spectrum is highly heterogeneous. Several causative genes have been identified, most of which encode proteins critical for neuronal migration, microtubule dynamics, and cytoskeletal remodeling. Variants in *LIS1* (*PAFAH1B1*) and *DCX* are the most frequently implicated in classical lissencephaly, particularly in posterior and anterior-predominant forms, respectively (Dobyns 2010; Portes et al. 1998). Other genes such as *TUBA1A*, *ARX*, *RELN*, and *DYNC1H1* have also been associated with variant lissencephaly phenotypes, reinforcing the role of cytoskeletal and intracellular transport mechanisms in normal brain development (Kumar et al. 2010; Poirier et al. 2013).

Among the families of proteins involved in intracellular transport and scaffolding, tetratricopeptide repeat (TPR)-containing proteins have gained increasing importance due to their versatile roles in protein-protein interactions, organelle positioning, and signal transduction (Blatch and Lässle 1999). TPR domains mediate the assembly of multiprotein complexes, a function that is particularly important in the developing brain, where dynamic interactions coordinate neuronal proliferation, migration, and differentiation. Several TPR-containing proteins have been directly implicated in human neurodevelopmental disorders. For example, Pericentrin (PCNT), a large centrosomal protein with multiple TPR motifs, is mutated in microcephalic osteodysplastic primordial dwarfism type II, a disorder characterized by profound microcephaly and cortical malformations (Rauch et al. 2008). Similarly, O-GlcNAc transferase (OGT), which contains multiple N-terminal TPR repeats, is associated with X-linked intellectual disability (MIM number: 300997), highlighting the critical involvement of TPR-mediated scaffolding in chromatin regulation and transcriptional control in neurons (Pravata et al. 2020). Another TPR protein, TTC19, is linked to early-onset neurodegeneration and cerebellar ataxia due to defective mitochondrial complex III function (MIM number: 615157) (Koch et al. 2015). Additionally, TTC21B, a ciliary TPR protein, has been associated with ciliopathies that can involve neurodevelopmental features (MIM numbers: 613820 and 613819). These examples underscore the essential roles of TPR-domain proteins in neurodevelopment and provide strong conceptual support for the hypothesis that TTC14 dysfunction, another TPR-containing protein, may contribute to cortical malformations and microcephaly.

TTC14, which encodes the tetratricopeptide repeat domain-containing protein 14, is an evolutionarily conserved gene that remains poorly characterized. Preliminary functional studies suggest that TTC14 localizes to the nucleus, implicating it in processes such as mRNA homeostasis (Fagerberg et al. 2014; Wheway et al. 2019). Nuclear localization strongly suggests a role in mRNA homeostasis

because the nucleus is the central site for RNA splicing, maturation, quality control, and ribonucleoprotein (RNP) assembly. Proteins enriched in the nucleus frequently interact with spliceosomal components or RNA-binding proteins, and many TPR-containing proteins function as scaffolds that organize these complexes (Carrocci and Neugebauer 2019). Thus, TTC14's nuclear presence implies that it may support the assembly or stabilization of RNA-processing machinery, a function critical for maintaining neuronal gene expression programs. Given the established role of TPR proteins in organizing intranuclear architecture and genome maintenance, variants in TTC14 could plausibly disrupt neuronal function and survival, ultimately contributing to malformations in brain development such as those observed in the lissencephaly spectrum.

In this study, we report the identification of a novel variant in the *TTC14* gene in a proband presenting with radiological and clinical features consistent with the lissencephaly spectrum. Through genetic, radiological, and bioinformatic analyses, we explore the potential pathogenic mechanisms by which TTC14 dysfunction likely contributes to this neurodevelopmental phenotype, and provide evidence for its role in the molecular landscape of lissencephaly.

Results

Clinical case description

The proband is a second-born female child born to non-consanguineous, healthy Saudi Arabian parents (Fig. 1A) following an uneventful pregnancy and delivery. She was delivered at term via spontaneous vaginal delivery, with a birth weight of 3.04 kg. No perinatal complications were reported. She first presented for clinical evaluation at the age of 9 months due to concerns regarding developmental delay. At 1 year of age, her anthropometric measurements revealed a weight of 8.9 kg (50th percentile, z-score of 0.0 SD), height of 61 cm (1st percentile, z-score of -4.5 SD), and a significantly reduced head circumference of 38 cm, indicating microcephaly. Detailed neurological and developmental assessments revealed global developmental delay, including delayed attainment of major motor milestones such as independent sitting and walking. The child also showed cognitive impairment (reflected by poor social engagement, limited eye contact, reduced responsiveness to parents and other people, and absence of age-appropriate interactive behaviors such as reciprocal smiling or object exploration), poor language acquisition (no sign of age-expected babbling sounds, showed no attempt to imitate vocalizations, did not respond to simple verbal cues), and feeding difficulties. Additionally, the child exhibited mild

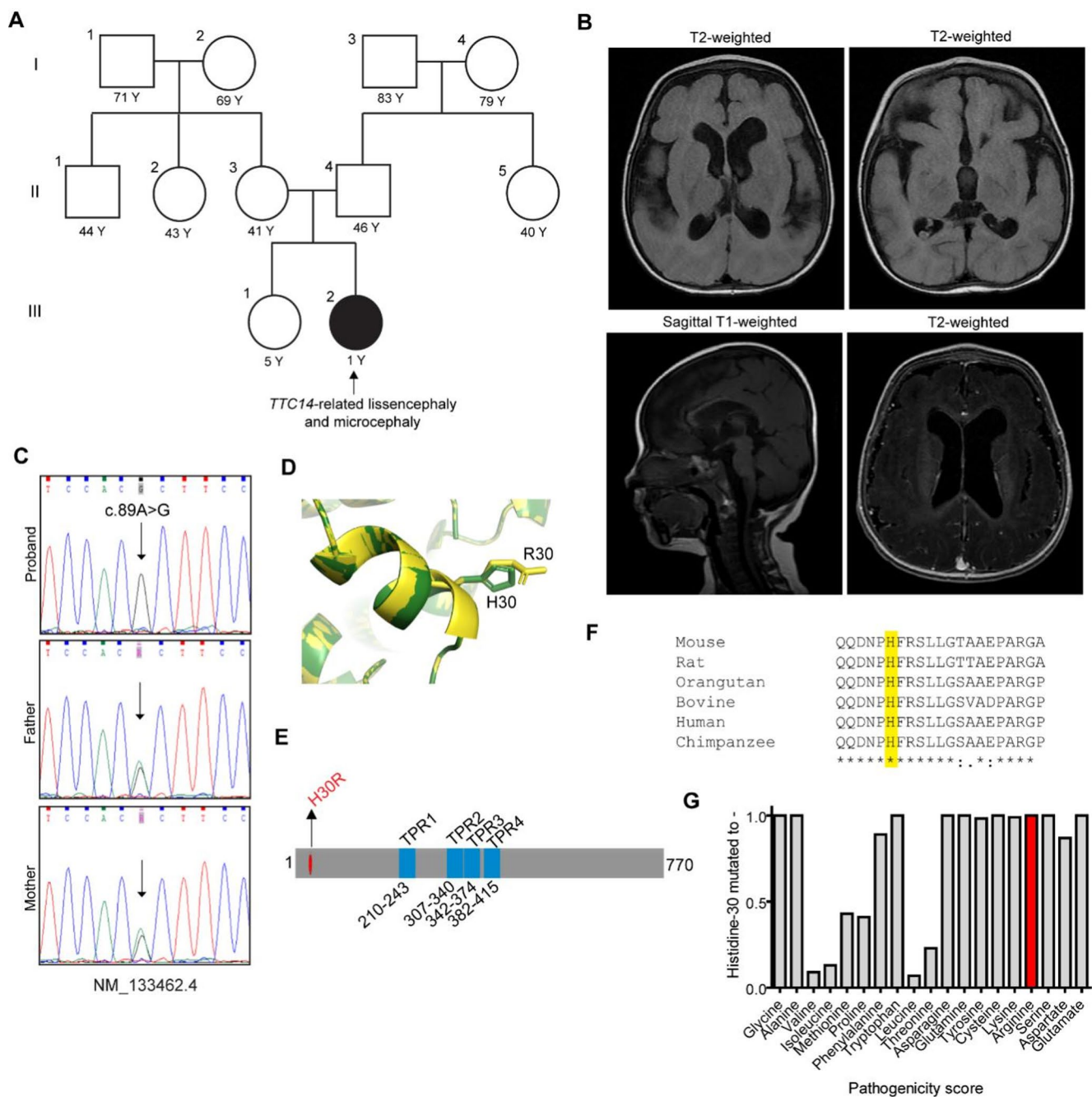


Fig. 1 Clinical, genetic, and structural analysis of the *TTC14*-p.His30Arg variant in the proband with microcephaly and lissencephaly spectrum disorder. **(A)** Pedigree of the affected family showing autosomal recessive inheritance of the *TTC14* variant. The proband (filled circle) presented with *TTC14*-associated lissencephaly and microcephaly. Both parents are heterozygous carriers, while the affected child is homozygous for the variant. **(B)** Brain MRI of the proband shows multiple structural abnormalities. Axial T2-weighted images reveal diffuse volume loss involving both cerebral hemispheres, thinning of the corpus callosum, widened ventricles, and prominent subarachnoid spaces over the frontoparietal convexities. The smoothed appearance of bilateral parietal and temporal lobes and simplified cortical pattern are suggestive of focal pachygyria, indicating a neuronal migration defect. The sagittal T1-weighted image further confirms thinning of

the corpus callosum and reduced brain volume. **(C)** Sanger sequencing chromatograms showing the *TTC14* c.89 A>G (p.His30Arg) variant in the proband (homozygous) and both parents (heterozygous), confirming recessive inheritance. **(D)** Structural overlay of the *TTC14* wild-type (green) and mutant (yellow) proteins showing the substitution of histidine (H30) with arginine (R30) in a helical region. **(E)** Schematic representation of the *TTC14* protein showing the location of the p.His30Arg variant (red) outside the tetratricopeptide repeat (TPR) domains (blue). **(F)** Multiple sequence alignment of *TTC14* orthologs in different species shows strong evolutionary conservation of the histidine residue at position 30 (highlighted in yellow). **(G)** Pathogenicity scores for 19 possible substitutions at position H30, as predicted by computational modeling. The p.His30Arg variant (highlighted in red) shows a high pathogenicity score, consistent with a damaging effect

generalized hypotonia and had developed epileptic spasms, which were confirmed through clinical observation and electroencephalogram (EEG) findings. There was no family history of neurological disorders, and both parents and the proband's sibling were clinically asymptomatic, with no prior history of neurodevelopmental abnormalities or epilepsy. Genetic analysis of the proband's sibling was not performed, as the parents of the proband were not inclined to the genetic analysis of the unaffected child.

Magnetic Resonance Imaging (MRI) of the brain in the proband revealed significant structural neurodevelopmental abnormalities. There was generalized volume loss involving both cerebral hemispheres, manifested as white matter paucity, widened cerebral sulci, and ventricular enlargement (Fig. 1B). Despite these atrophic changes, the cortical grey matter thickness remained preserved at approximately 3 mm. A simplified gyral pattern was noted, consistent with microcephaly with a simplified gyral pattern (Fig. 1B), indicating disrupted cortical organization. Additionally, there was a marked paucity of sulcations in the bilateral parietal and temporal lobes, giving a smoothed cortical appearance suggestive of focal pachygyria (Fig. 1B). The corpus callosum appeared thinned (Fig. 1B), indicating compromised interhemispheric connectivity. The subarachnoid spaces, particularly over the bilateral frontoparietal convexities, were prominently widened (Fig. 1B), likely secondary to underlying cerebral atrophy. Bilateral lateral ventricles (more prominent on the left) and the third ventricle were enlarged, suggesting ex vacuo dilatation due to adjacent white matter loss. Myelination was found to be appropriate for the child's age, and the signal intensities of the remaining cerebral hemispheres, cerebellum, brainstem, hippocampi, and parahippocampal regions were normal. No intra- or extra-axial lesions or abnormal leptomeningeal or pachymeningeal enhancements were observed. These findings collectively support a diagnosis of a congenital brain malformation and neuronal migration disorder, likely genetic in origin, associated with disrupted neuronal proliferation and migration, correlating well with lissencephaly spectrum and microcephaly.

To further investigate the etiology of the proband's neurodevelopmental disorder, trio whole exome sequencing was performed, which identified a homozygous missense variant in the *TTC14* gene, specifically c.89 A>G in transcript NM_133462.4 (genomic coordinate: chr3 g.180602352 A>G), resulting in a p.His30Arg amino acid substitution (Fig. 1C). This variant was absent from major population variant databases, including gnomAD, suggesting that it is ultra-rare and potentially novel. No other variants of clinical relevance or potential pathogenic significance were identified from the trio exome sequencing analysis that could explain the proband's phenotype. Both

parents were found to be asymptomatic heterozygous carriers of the same variant, indicating an autosomal recessive mode of inheritance in this case.

Detailed analysis of the identified *TTC14* missense variant (c.89 A>G; p.His30Arg) revealed that the substituted residue histidine at position 30 (H30) is located at the beginning of a predicted α -helical region (Fig. 1D) that lies outside of the four annotated tetratricopeptide repeat (TPR) domains of the TTC14 protein (Fig. 1E). Importantly, H30 is evolutionarily conserved (GERP score is 4.97, showing high evolutionary conservation) across a broad range of eukaryotic animal species (Fig. 1F). To assess the potential impact of the variant on protein function, multiple pathogenicity prediction tools were employed. PolyPhen-2.0 classified the p.His30Arg substitution as 'possibly damaging,' with a high confidence score (score of 0.808, with sensitivity: 0.84 and specificity: 0.93) (Fig. 1G). Similarly, CADD (Combined Annotation Dependent Depletion) scored (23.5) the variant as deleterious. MutationTaster predicted the variant to be 'polymorphism', and ClipPred classified the p.His30Arg variant as 'pathogenic'.

p.His30Arg variant disrupts structural stability and folding dynamics of the protein

Structural and dynamic analyses of the TTC14-p.His30Arg mutant shows that the p.His30Arg substitution is predicted to have a destabilizing impact on TTC14's folding and function. Structural modeling showed that the wild-type histidine at position 30 is located at the start of an N-terminal α -helix and interacts stably with Met1 (M1) without any steric hindrance (Fig. 2A). In contrast, substitution with arginine introduces a larger and more flexible side chain that causes significant steric clash with M1 (Fig. 2A), as illustrated by overlapping van der Waals volumes. This local interference suggests conformational strain at the N-terminal region of TTC14-p.His30Arg, potentially compromising helix stability and initiating misfolding.

To further probe the impact of the variant, residue interaction network (RIN) analysis was performed for both wild-type and p.His30Arg mutant proteins. In the wild-type protein, H30 and M1 reside within a well-connected interaction cluster that maintains structural integrity at the N-terminus (Fig. 2B). However, in the mutant, R30 disrupts this topology by forming altered interactions with surrounding residues including Q26, D27, N28, and G36 (Fig. 2B), indicating a substantial reorganization of local contacts. This network remodeling is indicative of structural destabilization, potentially altering TTC14's folding pathway or its interaction with binding partners.

The energetic consequences of H30 substitutions were further examined through computational mutagenesis. Free

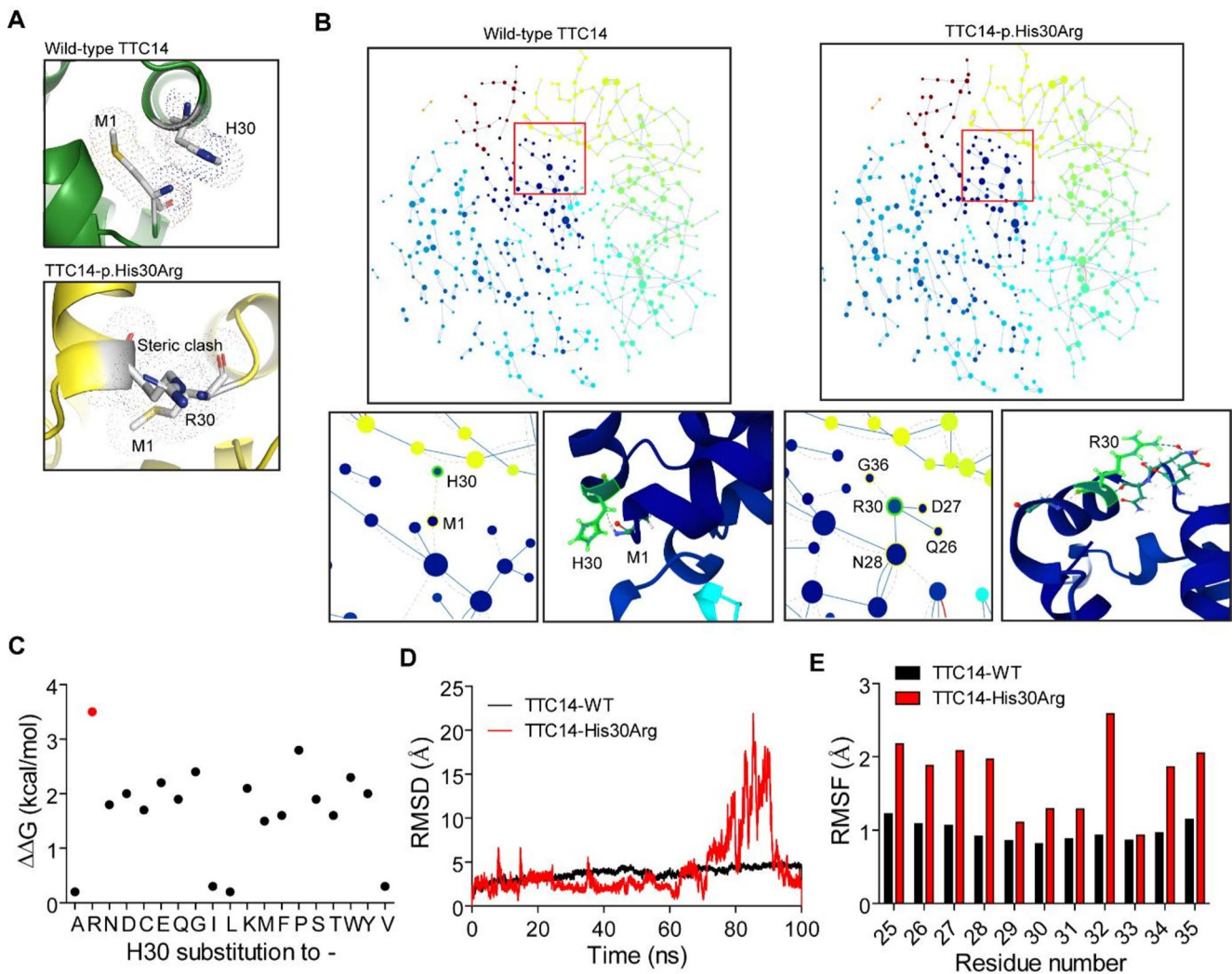


Fig. 2 Structural and dynamic consequences of the TTC14-p.His30Arg variant. **(A)** Structural modeling of wild-type TTC14 (top) shows the side chain of H30 positioned without steric interference, maintaining proper interaction with M1. TTC14-p.His30Arg mutant (bottom) introduces a bulky arginine residue at position 30, causing a steric clash with M1. **(B)** Residue interaction network (RIN) analysis reveals altered interaction topology in the mutant protein. The upper panels show global inter-residue interaction networks of wild-type (left) and TTC14-p.His30Arg (right). Zoomed-in panels below show: wild-type

interaction between H30 and M1 (left); mutant interaction network around R30 (middle) involving new contacts with Q26, D27, N28, and G36. **(C)** $\Delta\Delta G$ values upon substitution of H30 with each of the other 19 amino acids. The p.His30Arg variant (red dot) exhibits the highest destabilizing effect (+3.5 kcal/mol). **(D)** Root Mean Square Deviation (RMSD) plots from molecular dynamics simulations of wild-type and TTC14-p.His30Arg over 100 ns. **(E)** Root Mean Square Fluctuation (RMSF) plots for residues 25–35 surrounding the variant site

energy calculations showed that most substitutions at position 30 result in positive $\Delta\Delta G$ values, suggesting a general destabilizing effect (Fig. 2C). Notably, the p.His30Arg substitution yielded the highest destabilizing energy change (Fig. 2C), strongly indicating that this variant significantly impairs the thermodynamic stability of the protein. This finding supports the notion that H30 is a structurally and functionally constrained residue, with arginine substitution being particularly deleterious.

To assess conformational stability over time, molecular dynamics simulations (MDS) were performed for both wild-type and TTC14-p.His30Arg proteins over a 100 ns

timescale. The root mean square deviation (RMSD) profile for wild-type TTC14 remained low and stable throughout the simulation, consistent with a folded and dynamically stable protein (Fig. 2D). In contrast, the TTC14-p.His30Arg mutant exhibited a sharp increase in RMSD after approximately 60 ns, ultimately reaching over 20 Å (Fig. 2D). This drastic shift reflects large-scale conformational fluctuations, suggesting that the mutant protein undergoes progressive destabilization or structural loosening over time.

Additionally, root mean square fluctuation (RMSF) analysis focused on residues near the 30th position revealed that the p.His30Arg variant induced substantial local flexibility.

In the wild-type protein, this region showed relatively low fluctuations (Fig. 2F), reflecting its stable α -helical conformation. However, in the p.His30Arg mutant, residue R30 and its immediate neighbors displayed significantly higher fluctuations (Fig. 2F), indicative of increased local disorder. These dynamic perturbations imply that the p.His30Arg variant compromises both local secondary structure and global stability of TTC14.

Taken together, these multi-layered analyses demonstrate that the TTC14-p.His30Arg variant introduces steric clashes, disrupts native residue interaction networks, destabilizes folding energy, and increases both local and global flexibility. These structural defects likely impair TTC14's function in human cells, including neurons.

TTC14-pHis30Arg variant leads to cytosolic mislocalization, aggregation, and correlates with increased cell death in proband fibroblasts

To investigate the functional consequence of the TTC14-p.His30Arg variant at the cellular level, transcript and protein expression, subcellular localization, and cell cycle profiles were assessed in proband-derived and control fibroblasts. The relative transcript levels of *TTC14* were comparable between wild-type and c.89 A>G mutant expressing control and proband fibroblasts (Fig. 3A), indicating that the c.89 A>G variant does not affect mRNA stability or expression. Similarly, the total protein levels of wild type TTC14 and TTC14-p.His30Arg remained similar in control and proband fibroblasts (Fig. 3B), suggesting that the variant does not impact translational efficiency or protein stability under steady-state conditions. However, we observed a striking difference in subcellular localization between wild-type and

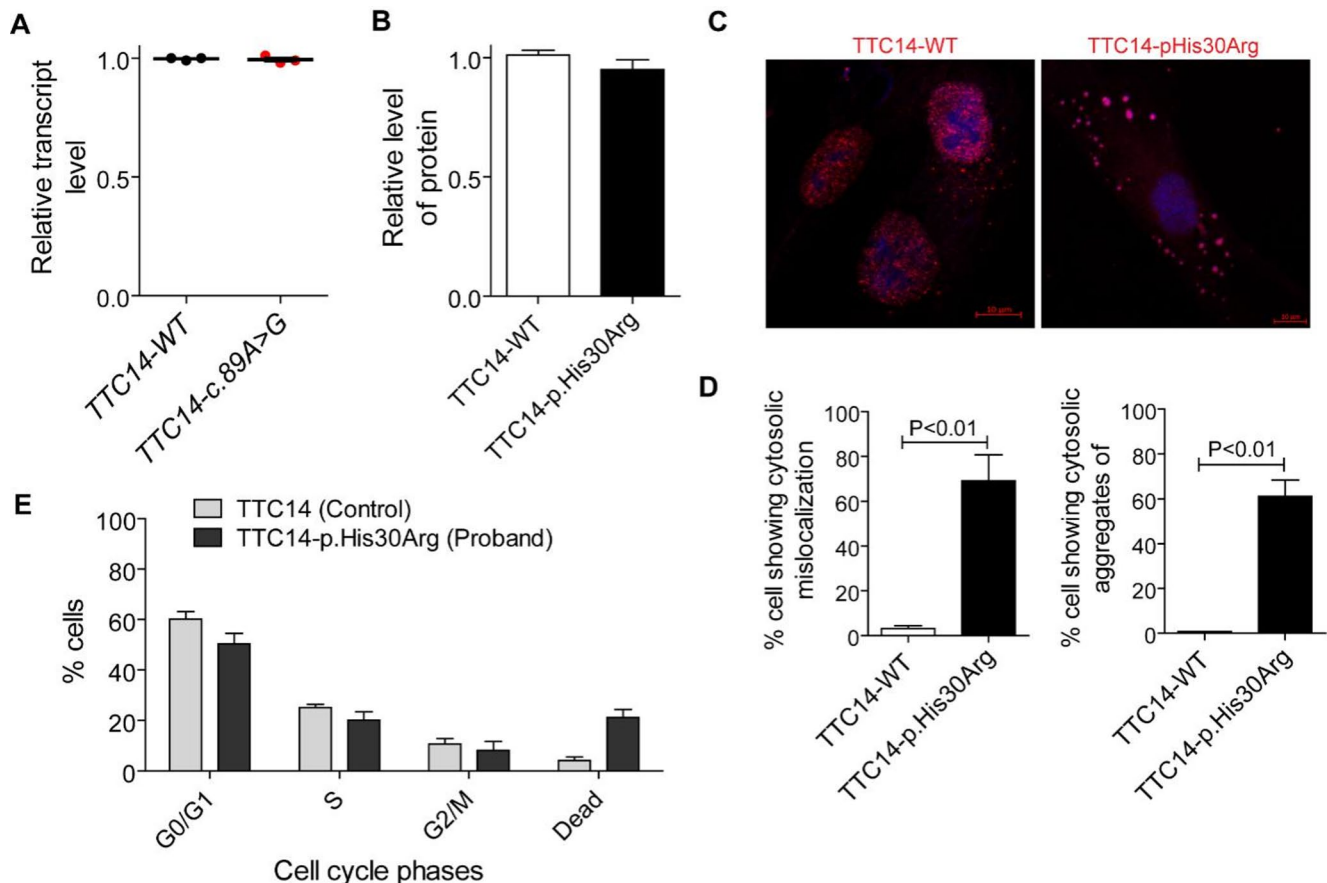


Fig. 3 The TTC14-p.His30Arg variant does not alter transcript or protein levels but leads to mislocalization, aggregation, and cell death. **(A)** Quantitative PCR analysis shows no significant difference in *TTC14* mRNA expression between wild-type and mutant fibroblasts. Data normalized to GAPDH ($n=3$). **(B)** ELISA quantification of TTC14 protein levels reveals comparable expression between TTC14-WT and TTC14-p.His30Arg in proband and control fibroblasts ($n=3$). **(C)** Immunocytochemistry showing subcellular localization of TTC14.

TTC14 is shown in red; nuclei stained with DAPI (blue). Scale bars: 10 μ m. **(D)** Quantification of immunofluorescence data showing a significant increase ($p<0.01$) in the percentage of cells with cytosolic mislocalization (left) and cytosolic aggregation (right) in TTC14-p.His30Arg mutant fibroblasts compared to TTC14-WT ($n=50$ cell in each group). **(E)** Flow cytometry analysis of cell cycle phases in fibroblasts ($n=25,000$ cell in each group). All experiments were done in three biological and technical replicates

mutant TTC14 proteins. In control fibroblasts expressing wild-type TTC14, the protein was predominantly localized to the nucleus (Fig. 3C and D). In contrast, the TTC14-p.His30Arg mutant protein displayed aberrant cytosolic localization (Fig. 3C and D), and notably, formed visible cytosolic aggregates (Fig. 3C and D), indicating potential misfolding and impaired cytosol-to-nuclear trafficking.

Furthermore, flow cytometry-based cell cycle analysis revealed functional consequences of the TTC14-p.His30Arg variant on cellular viability and proliferation. While both control and proband fibroblasts had similar distributions in the G0/G1, S, and G2/M phases, the proband cells showed a markedly higher percentage of dead cells (Fig. 3E), indicating increased susceptibility to cell death. This increase in cell death is likely linked to the cytosolic mislocalization and aggregation of TTC14-p.His30Arg.

Overall, these findings demonstrate that although the TTC14-p.His30Arg variant does not affect gene expression or total protein levels, it causes mislocalization, aggregation, and cytotoxicity, providing further evidence of its likely pathogenic role in neurodevelopmental dysfunction.

TTC14 interactome and functional landscape suggest a role in RNA processing and neurodevelopmental regulation

To gain mechanistic insights into the cellular role of TTC14 and how the p.His30Arg variant may contribute to disease pathogenesis, we investigated the TTC14 protein interaction network, its associated gene ontology functions and biological processes, and its expression profile across brain regions.

The curated protein-protein interaction (PPI) network for TTC14 identified a diverse array of direct interactors (Fig. 4A). These include proteins involved in RNA metabolism (e.g., DDX23, DHX8, DHX40, SNW1, SRSF1), splicing and mRNA processing (e.g., SRRM2, HNRNPL, RBM39, CDC5L, SF3B2), and signaling/regulatory proteins such as MYC, CDK9, and KRAS (Fig. 4A). Several interacting partners, like SNIP1, DDX23, and CHERP, are also associated with spliceosomal complex formation (Fig. 4A), further indicating that TTC14 may function in RNA processing and post-transcriptional gene regulation. Notably, proteins such as DGCR14 and PRNP are implicated in neurodevelopmental and neurodegenerative disorders, providing a potential link between TTC14 dysfunction and the observed neurological phenotype.

Gene ontology (GO) analysis of TTC14 and its interacting proteins revealed significant enrichment for key molecular functions. The top-enriched categories include RNA binding, mRNA binding, RNA helicase activity, and ATP-dependent RNA metabolism, all of which are essential for

proper regulation of gene expression in neurons (Fig. 4B). Additional enrichment for cyclin-dependent protein serine/threonine kinase activity suggests potential crosstalk with cell cycle regulatory pathways (Fig. 4B), which could explain the increased cell death observed in TTC14-p.His30Arg mutant fibroblasts. The GO enrichment for biological processes, also highlights TTC14's involvement in RNA splicing via the spliceosome, spliceosomal complex assembly, mRNA processing, and regulation of mRNA metabolic processes (Fig. 4C). These functions are particularly critical in neurons, where alternative splicing and fine-tuned transcript regulation are essential for development, synaptic function, and response to environmental signals. The substitution of histidine with arginine at position 30, which disrupts TTC14's folding and localization, could therefore interfere with its ability to properly interact with these partners, potentially abrogating its RNA-related molecular functions in neurons, and leading to widespread transcriptomic dysregulation. Analysis using the eLIM database revealed that His30 is embedded within a predicted short linear interaction motif (sLIM), suggesting that this region may contribute to TTC14's protein-protein interaction interface. This sLIM is predicted to associate with interaction modules such as Cyclin-binding, ARC, PTB, and GSK3 motifs, indicating that the H30R mutation may disrupt a potential interaction hotspot rather than solely destabilizing local structure. The mutant TTC14 is therefore likely to impair neuron-specific splicing events, contributing to abnormal neuronal differentiation or connectivity and aligning with the structural brain abnormalities seen on MRI in the proband.

TTC14's expression profile across various regions of the human brain demonstrates that it is widely expressed, with notably high transcript levels in the cerebral cortex, hippocampus, thalamus, white matter, and choroid plexus (Fig. 4D). These regions are central to higher-order cognitive functions, memory processing, motor control, and cerebrospinal fluid regulation - all of which are commonly affected in neurodevelopmental disorders. The high expression of TTC14 in these areas supports its essential role in normal brain physiology. Mislocalization or aggregation of TTC14 - as observed in the p.His30Arg mutant - may disturb its native function in RNA processing in these regions, potentially explaining the global developmental delay, seizures, microcephaly, and structural malformations.

Together, these findings highlight TTC14 as a critical component of neuronal RNA processing machinery and establish a possible mechanistic link between its dysfunction due to the p.His30Arg variant and the neurodevelopmental disorder phenotype observed in the proband.

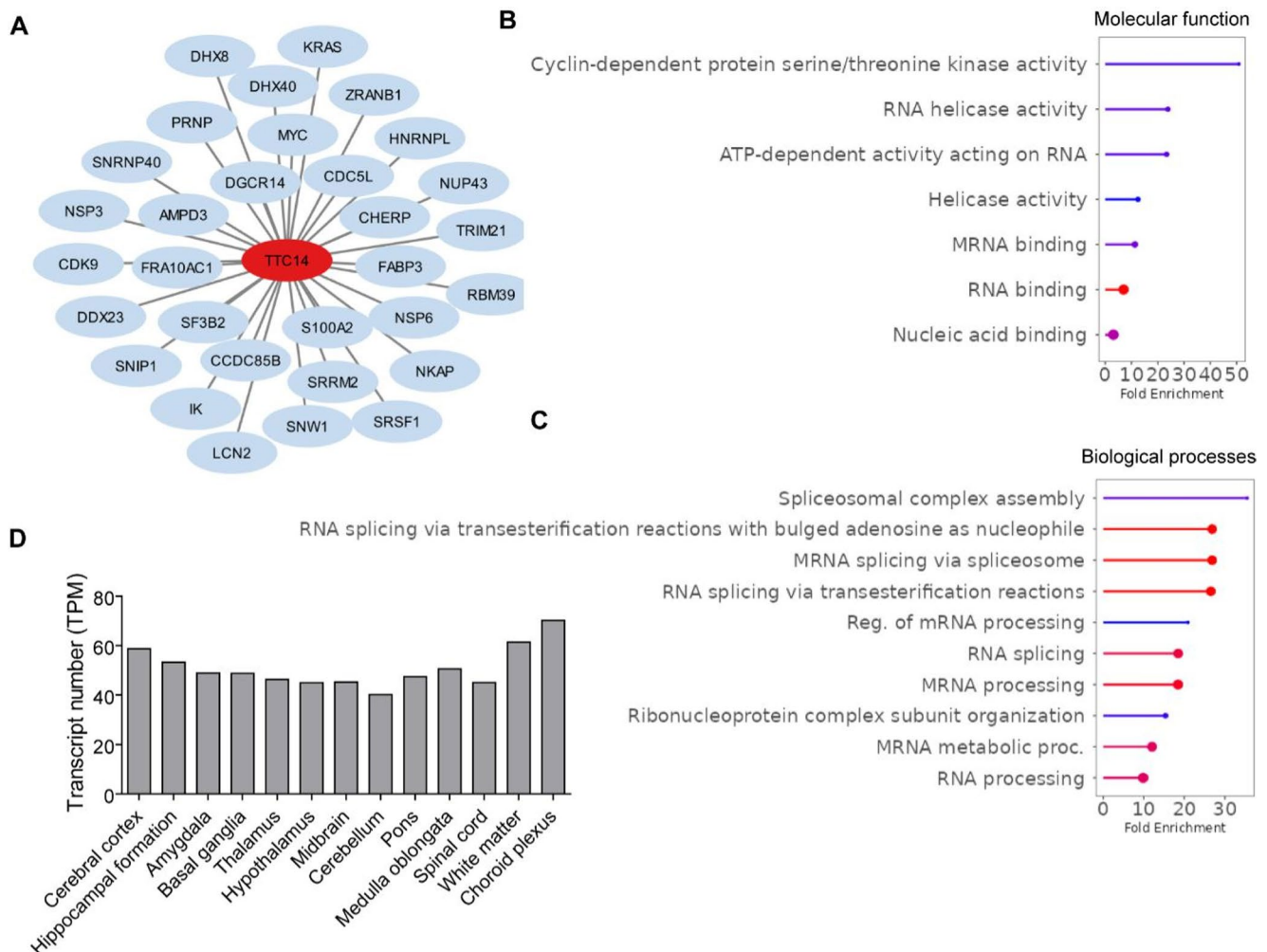


Fig. 4 TTC14 interacts with RNA-binding proteins and is enriched in brain regions associated with RNA processing and splicing. **(A)** Protein-protein interaction network of TTC14 constructed using data curated from STRING and BioGRID databases. TTC14 (red node) interacts with multiple proteins involved in RNA metabolism, including splicing factors (e.g., SRSF1, SRRM2, DDX23), helicases (e.g., DHX8, DHX40), and regulatory proteins (e.g., MYC, PRNP), suggesting a role in RNA-binding and post-transcriptional regulation. **(B)**

Gene ontology analysis for molecular function of TTC14 and its interactors shows strong enrichment for RNA binding, nucleic acid binding, helicase activity, and mRNA binding. **(C)** GO analysis for biological processes reveals that TTC14 and its associated proteins are enriched in RNA splicing via transesterification, spliceosomal complex assembly, mRNA processing, and ribonucleoprotein complex organization. **(D)** Brain-region-specific transcript levels of TTC14

Discussion

Lissencephaly spectrum disorders are a group of neurodevelopmental malformations caused by defective neuronal proliferation, migration, and cortical organization during fetal brain development. They are classically characterized by smooth or simplified cerebral surfaces, thickened cortical plate, reduced or absent sulci and gyri, and often associated with microcephaly, seizures, and global developmental delay. Several genes have been implicated in the pathogenesis of lissencephaly and its variants, including *LIS1*, *DCX*, *TUBA1A*, *ARX*, and *RELN*, which are primarily involved in cytoskeletal regulation, neuronal migration, and intracellular trafficking. In the current study, we identify a

novel homozygous missense variant in *TTC14* (c.89 A>G; p.His30Arg) in a proband presenting with clinical and radiological features that fall within the lissencephaly spectrum, including microcephaly, developmental delay, epileptic spasms, and simplified gyral pattern with focal pachygyria. MRI further revealed thinning of the corpus callosum and prominence of ventricles and subarachnoid spaces, consistent with cortical malformation due to neuronal migration defects.

TTC14 encodes a poorly characterized protein containing four tetratricopeptide repeat (TPR) motifs - structural elements known to mediate protein-protein interactions and contribute to scaffolding and molecular chaperone functions (Blatch and Lässle 1999). TPR-containing proteins

play pivotal roles in neurodevelopment, including regulation of centrosomal functions, cytoskeletal integrity, and RNA metabolism (D'Andrea and Regan 2003). Although TTC14 lacks prior disease association, its interaction profile strongly implicates it in RNA splicing and processing, as it interacts with proteins such as DDX23, HNRNPL, SRRM2, and SRSF1 - key components of the spliceosomal machinery. TTC14 was also found to localize predominantly to the nucleus in wild-type cells, suggesting its role in nuclear RNA metabolic processes.

The His30 residue is highly conserved across eukaryotic species, located at the N-terminal helix of TTC14, distinct from its TPR domains. Structural modeling showed that substitution of histidine with arginine at this position leads to significant steric clashes with Met1, disrupting the local helical structure. Computational energy calculations indicated that the p.His30Arg substitution yields the highest increase in unfavorable folding free energy changes among all possible substitutions at this position, indicating strong destabilization. This structural perturbation was confirmed through molecular dynamics simulations showing elevated RMSD and RMSF in the p.His30Arg mutant, suggesting loss of global stability and local rigidity. Additionally, inter-residue contact network analysis revealed altered interaction patterns, supporting a loss of structural integrity and folding fidelity. The destabilization likely renders the mutant protein prone to misfolding and aggregation, as evidenced by cytoplasmic retention and aggregation of TTC14-p.His30Arg in proband-derived fibroblasts. This misfolding could impair interaction with nuclear partners and lead to protein quality control stress.

The wild-type TTC14 protein showed nuclear localization, supporting its role in RNA splicing and nuclear events. In contrast, the p.His30Arg mutant mislocalized to the cytosol and formed distinct aggregates. This mislocalization likely results from impaired nuclear translocation due to folding defects or loss of recognition by nuclear import machinery. Nuclear mislocalization of RNA-binding proteins has been associated with severe cellular dysfunction, as these proteins often regulate pre-mRNA splicing, mRNA export, and transcript stability (Kim and Taylor 2017). Disruption of these processes in neurons, which rely heavily on tightly regulated transcriptomic programs, could lead to transcript misprocessing and subsequent neuronal dysfunction or death.

Furthermore, cell cycle analysis revealed increased cell death in proband fibroblasts expressing the TTC14-p.His30Arg mutant. Misfolded proteins that aggregate in the cytoplasm can lead to proteostasis stress and activation of cell death pathways, including senescence. In neurons, which are highly sensitive to proteotoxic stress, the accumulation of cytosolic aggregates and loss of critical

RNA regulatory functions could exacerbate neurodegeneration, contributing to the cortical atrophy and microcephaly observed in the proband. These findings align with similar pathogenic mechanisms seen in TDP-43 and FUS proteinopathies in ALS and frontotemporal dementia (Ling et al. 2013).

TTC14 is also highly expressed in brain regions critical for cognitive and motor function, including the cerebral cortex, hippocampus, thalamus, and white matter. These regions correspond to the areas most severely affected in the proband's MRI scans, suggesting that loss of TTC14 function in these areas disrupts developmental gene expression programs and contributes to the observed phenotype. An additional aspect relevant to interpreting the pathogenicity of the TTC14-p.His30Arg variant is the presence of multiple TTC14 isoforms in humans. According to RefSeq, *TTC14* encodes at least three protein isoforms, isoform a (770 aa; NM_133462.4), isoform b (439 aa; NM_001042601.3), and isoform c (653 aa; NM_001288582.2), generated through alternative splicing at the 3' end of the transcript. Although these isoforms differ substantially in their C-terminal regions, all share an identical N-terminal segment that includes the conserved His30 residue mutated in our proband. Importantly, all three isoforms preserve the ribosomal S1-like RNA-binding domain and the first four TPR motifs, indicating that core functions of TTC14, including protein interactions and RNA-associated roles, are common across isoforms. Expression datasets (GTEx, Ensembl, HPA) show that isoforms a and b are expressed at comparable levels in brain tissues, whereas isoform c is moderately abundant, suggesting that multiple TTC14 isoforms may be functionally relevant in neurodevelopment. Since the p.His30Arg substitution lies upstream of the first structured domain and is shared by all isoforms, the variant is predicted to disrupt the folding, stability, and localization of every TTC14 protein variant. This broad impact across isoforms may help explain the severity of the neurodevelopmental phenotype observed in the proband. However, because *TTC14* remains poorly characterized experimentally, definitive functional differences between isoforms cannot yet be ascertained, and future studies will be required to clarify whether specific isoforms have distinct or overlapping roles in neural development and RNA processing.

In the context of population genetics, it is notable that *TTC14* harbors several rare variants in population databases such as gnomAD, including missense, frameshift, and predicted deregulation-of-function alleles. Inspection of the gnomAD v4 dataset for TTC14 (ENSG00000163728) shows that these variants are collectively extremely rare, with most alleles observed only once or a few times in large cohorts and no homozygous deregulation-of-function individuals reported, consistent with a gene under functional constraint.

Several frameshift or nonsense variants pass quality filters in gnomAD, yet these occur exclusively in heterozygous state, supporting the interpretation that *TTC14*-related disease follows an autosomal recessive mode of inheritance, as demonstrated in our proband. Additional support for the functional importance of His30 comes from population genetic data. Examination of the gnomAD database revealed several ultra-rare variants affecting the same codon, including the missense substitutions p.His30Tyr, p.His30Asp, and p.His30Gln, each observed only once among more than 1.6 million alleles (allele frequency $\approx 6.2 \times 10^{-7}$), together with a rare synonymous variant (p.His30His; allele frequency $\approx 1.86 \times 10^{-5}$). Notably, all of these variants have been identified exclusively in the heterozygous state, with no homozygous carriers reported. Although these variants have not been functionally characterized or classified as pathogenic, their extreme rarity and the absence of homozygous individuals suggest that the His30 residue is under strong evolutionary and functional constraint. Importantly, none of the population variants affect the highly conserved N-terminal region containing His30, and no variants have been annotated as pathogenic, which further strengthens the relevance of the homozygous p.His30Arg substitution identified in this study. While these uncharacterized variants may represent carrier alleles with incomplete penetrance or benign consequences due to isoform-specific effects, their presence does not contradict our findings. Instead, the rarity and heterozygous nature of these variants underscore the likelihood that biallelic damaging variants, such as the homozygous p.His30Arg identified here, are necessary to produce the severe neurodevelopmental phenotype. Given the limited functional annotation of *TTC14*, further research will be essential to determine the consequences of other naturally occurring variants. However, the available population data align well with the recessive pathogenic model supported by our clinical, molecular, and cellular findings.

Although our multidisciplinary analyses strongly support the involvement of *TTC14* in the proband's neurodevelopmental disorder, it is important to acknowledge the limitations inherent in single-patient studies. While the homozygous p.His30Arg variant is absent in population databases, occurs at a highly conserved residue, and demonstrates clear structural destabilization, mislocalization, aggregation, and downstream cellular dysfunction in patient fibroblasts, these findings alone cannot provide definitive proof of causality. At present, no additional unrelated patients with pathogenic *TTC14* variants and comparable phenotypes have been reported, and thus the possibility of an incidental association cannot be fully excluded. Our results therefore support a likely pathogenic effect of the *TTC14*-p.His30Arg variant rather than establishing absolute causation. We emphasize that our cellular, structural, and

computational data provide strong mechanistic evidence that *TTC14* dysfunction disrupts RNA-processing pathways and proteostasis, biological processes highly relevant to cortical development, but further validation in additional patients, as well as in vivo or neuronal model systems, will be required to conclusively define *TTC14* as a monogenic cause of lissencephaly and microcephaly. Future studies identifying additional *TTC14* variants in individuals with similar cortical malformations will be essential to confirm the gene-disease relationship and to refine the phenotypic spectrum associated with *TTC14* deficiency.

In summary, this study provides compelling genetic, structural, and cellular evidence implicating *TTC14* as a potentially novel contributor to neurodevelopmental disorders within the lissencephaly spectrum. The *TTC14*-p.His30Arg variant leads to protein misfolding, mislocalization, and functional impairment in RNA processing, thereby possibly resulting in neuronal dysfunction and brain malformation. These findings expand our understanding of TPR-containing proteins in neurodevelopment and identify *TTC14* as a potentially novel candidate gene in congenital cortical malformation syndromes.

Methods and materials

Clinical diagnosis and genetic counselling

The proband, a female infant born to non-consanguineous Saudi Arabian parents, was referred to the Department of Public Health at King Khalid University, at the age of 9 months due to concerns regarding global developmental delay, hypotonia, and episodes of epileptic spasms. A comprehensive clinical assessment was carried out by a pediatric neurologist, including detailed medical history, perinatal records, developmental milestones, and seizure characteristics. Anthropometric parameters were recorded at 1 year of age. Neurological and developmental evaluations were performed using standardized developmental screening tools. MRI of the brain was performed to assess structural abnormalities and interpreted by a board-certified neuroradiologist. Following the identification of neurological and structural abnormalities consistent with a congenital neurodevelopmental disorder, the proband and her parents were referred for genetic counseling. A trained clinical geneticist conducted pre-test counseling sessions to explain the nature and scope of genetic testing, potential outcomes, implications of findings, and the possibility of uncovering incidental or secondary findings. The family was also informed about the confidentiality and long-term significance of genetic data. Written informed consent was obtained from both parents prior to genetic testing, in accordance with the

ethical standards of the institutional review board and the Declaration of Helsinki. The consent included approval for genetic analysis, data sharing for research purposes, and publication of anonymized clinical findings. This study and all associated procedures involving human participants were reviewed and approved by the Institutional Ethics Committee of Hiralal Mazumdar Memorial College for Women, West Bengal State University.

Exome sequencing and Sanger sequencing

To identify the genetic basis of the proband's neurodevelopmental disorder, trio-whole exome sequencing (WES) was performed, involving the proband and both parents according to standard procedure (Udupa and Ghosh 2024). Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Germany), following the manufacturer's protocol. DNA quality and quantity were assessed using NanoDrop spectrophotometry and Qubit fluorometric quantification. WES libraries were prepared using the SureSelect Human All Exon V6 kit (Agilent Technologies), which captures approximately 60 Mb of exonic content. Library preparation included DNA fragmentation, end repair, adapter ligation, and hybridization-based target enrichment. Paired-end sequencing (2×150 bp) was carried out on the Illumina NovaSeq 6000 platform to achieve a mean coverage depth of >100×, ensuring >95% of targeted exons were covered at ≥20× read depth.

Raw paired-end sequencing reads generated from trio whole-exome sequencing were subjected to quality assessment to remove low-quality reads and adapter contamination before downstream analysis. High-quality reads were aligned to the human reference genome (GRCh38/hg38) using the Burrows–Wheeler Aligner (BWA-MEM). PCR duplicates were identified and removed, followed by base quality score recalibration and local realignment around insertion/deletion regions using the Genome Analysis Toolkit (GATK) Best Practices workflow. Single nucleotide variants (SNVs) and small insertions/deletions (indels) were called using GATK HaplotypeCaller, and variant quality filtering was performed to retain high-confidence variants for subsequent analysis. Variant annotation was carried out using ANNOVAR, incorporating information from population frequency databases including gnomAD and dbSNP, as well as disease databases such as ClinVar. Variants were filtered based on minor allele frequency (<0.01), predicted functional consequence, inheritance pattern, and clinical relevance. Priority was given to protein-altering variants, including missense, nonsense, frameshift, splice-site, and coding insertion/deletion variants. Given the unaffected parents and affected child, variants consistent with an autosomal

recessive mode of inheritance were prioritized, with particular emphasis on homozygous variants inherited from heterozygous carrier parents. Candidate variants were further evaluated using multiple in silico pathogenicity prediction tools, including PolyPhen-2, MutationTaster2, CADD, and ClinPred, together with evolutionary conservation analysis across vertebrate orthologues. Variants were interpreted according to the recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines.

Sanger sequencing was performed using PCR amplification of the region encompassing exon 1 of *TTC14* was done using gene-specific primers. The amplified products were purified and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), followed by capillary electrophoresis on an ABI 3730xl DNA Analyzer.

Bioinformatics analysis

The three-dimensional structure of the human TTC14 protein was curated from the AlphaFold Protein Structure Database (identifier: AF-Q96N46-F1).

In silico site-directed mutagenesis of the histidine at position 30 to arginine (p.His30Arg) was performed using PyMOL (Schrödinger, LLC), which was also used for structural visualization, residue interaction interpretation, and steric clash evaluation.

Pathogenicity prediction of the TTC14-p.His30Arg variant was performed using PolyPhen-2.0 (Adzhubei et al. 2010), CADD (Combined Annotation Dependent Deletion) (Rentzsch et al. 2019), MutationTaster (Schwarz et al. 2014) and ClinPred (Alirezaie et al. 2018).

Multiple sequence alignment (MSA) of TTC14 orthologs was performed. Protein sequences were retrieved from UniProtKB, and MSA was carried out using Clustal Omega (Sievers et al. 2011).

Protein inter-residue interaction networks were analyzed using RING 2.0 (Piovesan et al. 2016).

To predict the change in folding free energy ($\Delta\Delta G$) upon variant, FoldX algorithm was used (Schymkowitz et al. 2005).

Prediction of short linear interaction motifs (sLIMs) within TTC14 was performed using the eLIM database, which identifies evolutionarily conserved linear motifs involved in protein–protein interactions. The full-length human TTC14 protein sequence was submitted to the server, and predicted interaction motifs were analyzed to determine whether the H30 residue resided within a putative protein-binding interface. The identified sLIMs were further examined for their predicted interacting partners and functional relevance to TTC14-mediated protein interactions.

Molecular dynamics simulations: molecular dynamics (MD) simulations were conducted to evaluate the structural and dynamic impact of the TTC14 p.His30Arg variant using GROMACS version 2021.3, following the process as described previously (Mandal et al. 2025, Saha et al. 2024). Structures were energy-minimized using FoldX's Repair-PDB function to remove steric clashes. The simulations were carried out using the AMBER99SB-ILDN force field, with each protein placed in a triclinic box solvated with explicit TIP3P water molecules. Sodium and chloride ions were added to neutralize the system and mimic physiological ionic strength (0.15 M NaCl). Energy minimization was followed by two-step equilibration: a 100 ps NVT ensemble to heat the system to 310 K and a 100 ps NPT ensemble to stabilize pressure at 1 bar, employing the V-rescale thermostat and Parrinello-Rahman barostat. Position restraints were applied to protein heavy atoms during equilibration. Production MD simulations were run for 100 ns with periodic boundary conditions and a 2 fs time step, saving snapshots every 10 ps. Post-simulation analyses included RMSD to assess global structural deviation, and RMSF for residue-level flexibility, to evaluate stability changes.

To analyze TTC14's protein-protein interaction (PPI) network, interaction data were curated from BioGRID (Oughtred et al. 2019) and STRING v11.5 (Szklarczyk et al. 2019). The interaction network was constructed and visualized using Cytoscape v3.9.1 (Shannon et al. 2003).

Gene ontology (GO) analysis of TTC14 and its interactors was performed using ShinyGO v0.76.3 (Ge et al. 2020), with enrichment for molecular functions and biological processes assessed using FDR-adjusted p-values.

To investigate the regional transcript distribution of TTC14 in the human brain, expression data were retrieved from The Human Protein Atlas (Uhlén et al. 2015), including normalized transcript levels across cerebral cortex, hippocampus, thalamus, white matter, and other brain regions.

Cell culture

Primary fibroblasts were cultured from dermal punch biopsies obtained from the proband and an age-matched healthy control (unrelated, age- and sex-matched individual with no known neurological or genetic disorder, and was not a parent or sibling of the proband) under standard sterile conditions (Udupa et al. 2024). Skin biopsy samples were collected in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Dermal tissues were mechanically minced into small fragments under aseptic conditions. The tissue fragments were placed into 6-well culture plates and allowed to adhere for 10–15 min before being covered with growth medium comprising high-glucose

DMEM supplemented with 20% (v/v) fetal bovine serum (FBS; Gibco), 100 U/mL penicillin-100 µg/mL streptomycin (Gibco), and 2mM L-glutamine (Gibco). Cultures were maintained at 37 °C in a humidified incubator with 5% CO₂. Confluent cells were harvested using 0.25% trypsin-EDTA and expanded into larger flasks. Cells were periodically checked for mycoplasma contamination.

Quantitative PCR analysis of wild-type and mutant TTC14 transcripts

Total RNA was extracted from cultured fibroblasts derived from the proband and the healthy control using the RNeasy Mini Kit (Qiagen, Germany), following the manufacturer's protocol. RNA purity and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA integrity was confirmed by agarose gel electrophoresis. 1 µg of total RNA was reverse transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad, USA) in a 20 µL reaction, according to the manufacturer's instructions. The resulting cDNA was diluted 1:5 with nuclease-free water and used as a template for quantitative real-time PCR. Quantitative PCR was performed using SYBR Green-based detection in a CFX96 Real-Time PCR System (Bio-Rad) using specific primers designed against a conserved region of the *TTC14* transcript (NM_133462.4), spanning exons flanking the variant site to avoid amplification of genomic DNA. GAPDH was used as a housekeeping gene control. Each reaction was run in technical triplicates, and experiments were repeated independently in three biological replicates. Relative expression levels of *TTC14* were calculated using the $2^{-\Delta\Delta C_t}$ method, normalizing to GAPDH and comparing mutant samples to the control.

Quantitative estimation of wild-type and mutant TTC14 protein levels by ELISA

The expression levels of TTC14 protein in fibroblasts derived from the proband and a healthy control were quantified using the Human Tetratricopeptide Repeat Protein 14 (TTC14) ELISA Kit (Catalogue No: abx551690; Abnova, UK), following the manufacturer's instructions. Cells were washed with cold PBS and lysed using the RIPA lysis buffer (Thermo Scientific), supplemented with protease inhibitor cocktail (Sigma-Aldrich). Lysates were incubated on ice for 30 min and clarified by centrifugation at 14,000 × g for 15 min at 4 °C. The supernatant containing total soluble protein was collected, and protein concentration was measured using the BCA Protein Assay Kit (Pierce, Thermo Scientific). For the ELISA, 100 µL of appropriately diluted cell lysates (normalized to equal protein concentrations) were

added to each well of the 96-well microplate pre-coated with anti-TTC14 antibody. Samples were incubated at 37 °C for 90 min. After discarding the unbound material and washing the wells with the provided wash buffer, 100 µL of biotinylated detection antibody was added and incubated for 1 h at 37 °C. Following additional washes, avidin-HRP conjugate was added and incubated for 30 min. TMB substrate solution (3,3',5,5'-Tetramethylbenzidine) was then added to initiate color development, and the reaction was stopped after 15 min using the stop solution provided in the kit. The absorbance was measured at 450 nm using a microplate reader (BioTek Synergy HT). A standard curve was generated using serial dilutions of the TTC14 standard provided in the kit, and TTC14 concentrations in the samples were calculated accordingly. All assays were performed in biological triplicates, and each sample was analyzed in technical duplicates.

Immunocytochemistry

Immunocytochemistry was done according to established standard procedures (Saha et al. 2025, Udupa et al. 2023). Cells were cultured on sterile 12 mm glass coverslips placed in 24-well plates and grown to ~70% confluency. Cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. After fixation, cells were washed and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Non-specific binding was blocked by incubating the cells with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Primary antibody incubation was performed overnight at 4 °C using rabbit polyclonal anti-TTC14 antibody (Thermo Fisher Scientific, Catalog No. PA5-52663), diluted 1:200 in PBS containing 1% BSA. Subsequently, coverslips were washed three times with PBS and incubated for 1 h at room temperature in the dark with the secondary antibody: Goat anti-Rabbit IgG (H+L), Alexa Fluor™ 555-conjugated, cross-adsorbed secondary antibody (Thermo Fisher Scientific, Cat No. A-21428) diluted 1:500 in PBS with 1% BSA. After secondary incubation, coverslips were washed thoroughly and counterstained with DAPI for 5 min. Coverslips were mounted on glass slides using ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific) and imaged using a Zeiss LSM 900 confocal microscope with appropriate filters for DAPI and Alexa Fluor 555. Fluorescence images were processed and analyzed using ImageJ/Fiji software.

Cell cycle analysis by flow cytometry

Cell cycle profiling was performed on fibroblasts derived from the proband and a healthy control using propidium

iodide (PI)-based DNA staining and flow cytometry (Kumar et al. 2025). For each sample, 1×10^6 cells were trypsinized, collected by centrifugation at $300 \times g$ for 5 min, and washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4). The cell pellet was gently resuspended in 0.5 mL PBS and fixed by adding ice-cold 70% ethanol dropwise while vortexing to prevent cell clumping. Fixed cells were stored at -20 °C for at least 2 h to allow proper DNA fixation. Prior to analysis, cells were washed with PBS to remove ethanol and resuspended in 500 µL of PI/RNase staining solution containing 50 µg/mL propidium iodide (Sigma-Aldrich) and 100 µg/mL RNase A (Thermo Scientific). Samples were incubated in the dark at 37 °C for 30 min. Stained cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences), acquiring a minimum of 25,000 events per sample. Data were collected using the FL2 (585/42 nm) channel and analyzed using FlowJo v10.8 (BD Biosciences). Each sample was analyzed in triplicate, and the results were averaged across three independent biological replicates.

Statistical analysis

All quantitative data obtained from experimental assays were analyzed using GraphPad Prism version 9.5.1 (GraphPad Software, San Diego, USA). Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. For comparisons between two groups, unpaired two-tailed Student's t-tests were performed for normally distributed data. In experiments involving multiple comparisons, one-way ANOVA followed by Tukey's post hoc test was applied for normally distributed datasets. A p-value of less than 0.05 was considered statistically significant.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare that they have no competing interests [financial, personal, or professional] that could have influ-

enced the outcome or interpretation of this work.

Ethical approval The study was approved by the Institutional Ethics Committee of Hiralal Mazumdar Memorial College for Women (Approval No. 2024/Oct/RARE/12-C/SRA). Written informed consent was obtained from the proband's parents prior to participation.

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