Title:

Brain malformations and seizures by impaired chaperonin function of TRiC

Florian Kraft[1]†, Piere Rodriguez-Aliaga[2]†, Weimin Yuan[3]†, Lena Franken[1], Kamil Zajt[4], Dimah Hasan[5], Ting-Ting Lee[2], Elisabetta Flex[6], Andreas Hentschel[7], A. Micheil Innes[8], Bixia Zheng[9], Dong Sun Julia Suh[1], Cordula Knopp[1], Eva Lausberg[1], Jeremias Krause[1], Xiaomeng Zhang[4], Pamela Trapane[10], Riley Carroll[10], Martin McClatchey[11], Andrew E. Fry[12,13], Lisa Wang[4], Sebastian Giesselmann[1], Hieu Hoang[3], Dustin Baldridge[3], Gary A. Silverman[3], Francesca Clementina Radio[14], Enrico Bertini[15], Andrea Ciolfi[14], Katherine A Blood[16], Jean-Madeleine de Sainte Agathe[17,18], Perrine Charles[17], Gaber Bergant[19], Goran Čuturilo[20], Borut Peterlin[19], Karin Diderich[21], Haley Streff[22], Laurie Robak[22], Renske Oegema[23], Ellen van Binsbergen[23], John Herriges[24], Carol J. Saunders[25,26,27], Andrea Maier[28,29], Stefan Wolking[30], Yvonne Weber[30], Hanns Lochmüller[31], Stefanie Meyer[31], Alberto Aleman[31], Kiran Polavarapu[31,32], Gael Nicolas[33,34], Alice Goldenberg[33], Lucie Guyant[33], Kathleen Pope[35,36], Katherine N. Hehmeyer[36], Kristin G Monaghan[37], Anne Quade[38], Thomas Smol[39], Roseline Caumes[39], Sarah Duerinckx[40], Chantal Depondt[41], Wim Van Paesschen[42,43], Claudine Rieubland[44], Claudia Poloni[44], Michel Guipponi[45], Severine Arcioni[46], Marije Meuwissen[47], Anna C. Jansen[48], Jessica Rosenblum[47], Tobias B. Haack[49], Miriam Bertrand[49], Lea Gerstner[49], Janine Magg[50], Olaf Riess[49], Jörg B. Schulz[28,29], Norbert Wagner[51], Martin Wiesmann[5], Joachim Weis[4], Thomas Eggermann[1], Matthias Begemann[1], Andreas Roos[31,52,53], Martin Häusler[29,38], Tim Schedl[54], Marco Tartaglia[14], Juliane Bremer[4], Stephen C. Pak[3]*, Judith Frydman[2]*, Miriam Elbracht[1,29]‡*, Ingo Kurth[1,29]‡*

Affiliations:

1 Institute for Human Genetics and Genomic Medicine, Medical Faculty, RWTH Aachen University, Aachen, 52074, Germany.
2 Department of Biology, Stanford University, Stanford, 94305 California, United States.
3 Department of Pediatrics, Washington University in St Louis School of Medicine, St Louis, Missouri, 63110 United States.
4 Institute of Neuropathology, RWTH Aachen University Hospital, Aachen, 52074, Germany.
5 Department for Diagnostic and Interventional Neuroradiology, RWTH Aachen University Hospital, Aachen, 52074, Germany.
6 Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, 00161, Italy.
7 Leibniz- Institut für Analytische Wissenschaften -ISAS- e.V., Dortmund, 44139, Germany.
8 Department of Medical Genetics and Alberta Children's Hospital Research Institute, University of Calgary, Calgary, T2N 1N4, Canada.
9 Nanjing Key Laboratory of Pediatrics, Children's Hospital of Nanjing Medical University, Nanjing, 210008, China.
10 Division of Pediatric Genetics, Department of Pediatrics, University of Florida College of Medicine-Jacksonville, Jacksonville, Florida, 32209, United States.
11 Institute of Medical Genetics, University Hospital of Wales, Cardiff, CF14 4XW, United Kingdom.
12 All Wales Medical Genomics Service, University Hospital of Wales, Heath Park, Cardiff CF14 4XW, United Kingdom.

13 Division of Cancer and Genetics, Cardiff University, Heath Park, Cardiff CF14 4XN, United Kingdom.
14 Molecular Genetics and Functional Genomics, Ospedale Pediatrico Bambino Gesù IRCCS, Rome, 00165 Italy.
15 Neuromuscular Disorders, Ospedale Pediatrico Bambino Gesù IRCCS, Rome, 00146, Italy.

16 Department of Medical Genetics, University of British Columbia, Vancouver, BC, V6T 2A1,Canada.
17 Department of Medical Genetics, Pitié-Salpêtrière Hospital, AP-HP.Sorbonne University, Paris, 75005, France.
18 Laboratoire de Médecine Génomique Sorbonne Université, LBM SeqOIA, Paris, 75014, France.
19 Clinical Institute of Genomic Medicine, University Medical Centre Ljubljana, Ljubljana, 1000, Slovenia.
20 Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia AND University Children's Hospital, 11000 Belgrade, Serbia.
21 Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, 3015 GD, Netherlands.
22 Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, 77030, United States.
23 Department of Genetics, University Medical Centre Utrecht, Utrecht University, Utrecht, 3584 CX, Netherlands.
24 Department of Pathology and Laboratory Medicine, Children's Mercy-Kansas City, Kansas City, 64108 USA.
25 Department of Pathology and Laboratory Medicine, Children's Mercy Hospital, Kansas City, Missouri, 64108, USA
26 School of Medicine, University of Missouri Kansas City, Kansas City, Missouri, 64108, USA
27 Genomic Medicine Center, Children's Mercy Research Institute, Kansas City, Missouri, 64108 USA
28 Department of Neurology, University Hospital, RWTH Aachen University, Aachen, 52074, Germany.
29 Center for Rare Diseases Aachen (ZSEA), RWTH Aachen University Hospital, Aachen, 52074, Germany.
30 Department of Epileptology and Neurology, Medical Faculty, RWTH Aachen University, Aachen, 52074, Germany.
31 Children's Hospital of Eastern Ontario Research Institute; Division of Neurology, Department of Medicine, The Ottawa Hospital, and Brain and Mind Research Institute, University of Ottawa, Ottawa, K1H 8L1, Canada.
32 Department of Neurology, National Institute of Mental Health and Neuro Sciences, Bangalore, 560030, India.
33 Univ Rouen Normandie, Normandie Univ, Inserm U1245 and CHU Rouen, Department of Genetics and Reference Center for Neurogenetics Diorders, F-76000 Rouen, France.
34 Laboratoire multi-sites SeqOIA, Paris, 75014, France.
35 University of South Florida, College of Public Health, Tampa, Florida, 33612, United States
36 Nemours Children's Health, Dept of Pediatrics, Division of Genetics, Orlando, Florida, 32827, United States
37 GeneDx, Gaithersburg, Maryland, 20877, USA.
38 Division of Pediatric Neurology and Social Pediatrics, Department of Pediatrics, University Hospital RWTH Aachen, Aachen, 52074, Germany.
39 Department of Clinical Genetics, Lille University Hospital, CHU Lille, Lille, 59000, France.
40 Department of Pediatric Neurology, Hôpital Universitaire de Bruxelles, Hôpital Erasme, Université Libre de Bruxelles, Brussels, 1070, Belgium.
41 Department of Neurology, Hôpital Universitaire de Bruxelles, Hôpital Erasme, Université Libre de Bruxelles, Brussels, 1070, Belgium.
42 Laboratory for Epilepsy Research, KU Leuven, Leuven, 3000, Belgium.
43 Department of Neurology, University Hospitals Leuven, Leuven, 3000, Belgium.
44 Department of Medical Genetics, Central Institute of the Hospitals, Hospital of the Valais, Sion, 1951, Switzerland.
45 Department of Genetic Medicine, University Hospitals of Geneva and University of Geneva Medical Faculty, Geneva, 1205, Switzerland.
46 Division of Medical Genetics, Central Institute of Hospitals, Valais Hospital, Sion, 1951, Switzerland.
47 Center of Medical Genetics, Antwerp University Hospital/ University of Antwerp, Edegem, 2650, Belgium.
48 Department of Pediatrics, Division of Child Neurology, Antwerp University Hospital, University of Antwerp, Edegem, 2650, Belgium.
49 Institute of Medical Genetics and Applied Genomics, University of Tübingen, Tübingen, 72074, Germany.
50 Department of Neuropediatrics, Developmental Neurology, Social Pediatrics, University Children's Hospital, University of Tübingen, Tübingen, 72076, Germany.
51 Department of Pediatrics, University Hospital RWTH Aachen, Aachen, 52074, Germany.
52 Department for Pediatric Neurology, University Medicine Essen, Duisburg-Essen University, 45147, Germany.
53 Institute of Neurology, University Hospital Düsseldorf, Heinrich Heine University Düsseldorf, 40225, Germany.
54 Department of Genetics, Washington University in St Louis School of Medicine, St Louis, Missouri, 63110, United States.

† Joint first author

‡ Joint last authors

* Corresponding: S.P., J.F., M.E., I.K.: stephen.pak@wustl.edu, jfrydman@stanford.edu , mielbracht@ukaachen.de , ikurth@ukaachen.de

Abstract: 111 words

Malformations of the brain are common and vary in severity, from negligible to potentially fatal. Their causes have not been fully elucidated. Here, we report pathogenic variants in the core protein folding machinery TRiC/CCT in individuals with brain malformations, intellectual disability, and seizures. The chaperonin TRiC is an obligate hetero-oligomer and we identify variants in seven of its eight subunits, all of which impair function or assembly through different mechanisms. Transcriptome and proteome analyses of patient-derived fibroblasts demonstrate the various consequences of TRiC impairment. The results reveal an unexpected and potentially widespread role for protein-folding in the development of the central nervous system and define a unique disease spectrum of "TRiCopathies".

Main Text:

Anatomical alterations in the brain structure are present in approximately 20% of healthy individuals, with 4% of these incidental findings having clinical consequences (*1*). Genetic factors substantially contribute to brain malformations and impact different steps of cortical development. Abnormal cell proliferation and apoptosis affect the size of brain structures (hypoplasia), impaired neuronal migration results in abnormally positioned cells (heterotopia), and postmigration cortical organization can result in excessive folding of the cortex and laminar alterations (polymicrogyria) (*2, 3*). Together, these widely overlapping disorders represent a major cause of intellectual disability, autism, epilepsy, and cerebral palsy (*4*). Although the determinants of brain malformations are not yet fully understood, defects in microtubules, collagens, ion channels and transporters, as well as mTOR signaling proteins have been implicated in disease (*5*). Regardless of which protein is involved in the development of the disorder, they all have one thing in common: they must be folded correctly in order to function. Chaperones are key factors supporting the necessary protein folding processes and contributing to proteostasis (*6*). The eukaryotic chaperonin TRiC is a large barrel-shaped one megadalton 16-mer oligomeric complex that plays an essential role in protein-folding. TRiC forms two rings, each consisting of eight different but related proteins encoded by *CCT1-CCT8* (*7*). These form a central chamber in which unfolded polypeptide substrates bind and fold in an ATP-driven reaction, shielded from the external environment (**Fig. 1, A and fig. S1**) (*8*). TRiC facilitates the co- and post-translational folding of approx. 5-10% of the entire cellular proteome (*8-10*). Its substrates are characterized by aggregation-prone and topologically complex domains, including many essential proteins such as actins, tubulins, F-box proteins, DNA and RNA replication factors, cell cycle proteins as well as key regulators including p53, subunits of mTORC, and protein kinases (*11-14*). Here we investigated the mechanisms mediating brain malformations and demonstrated a role for the protein folding machinery and TRiC.

Results:

De novo variants in *CCT3* cause human disease

We analyzed brain magnetic resonance imaging (MRI), muscle biopsy, and transcriptomic data from an individual (CCT3 #9) with severe global developmental disorder, muscle hypotonia, ataxia, microcephaly, and intellectual disability (**Fig. 1**). Muscle biopsy revealed moderate widening of the muscle fiber caliber spectrum and peculiar accumulations of globular and granular material in intermyofibrillar membrane-bound vacuoles, suggestive of aggregation and altered proteostasis (**Fig. 1, C-J, fig. S2**). Similarly, several axons showed signs of altered proteostasis. Trio genome sequencing revealed a *de novo* frameshift deletion in the *chaperonin containing TCP1 subunit 3* (*CCT3*) which has not yet been shown to cause human disease and which is highly intolerant to loss-of-function variants (gnomAD pLI=1) (**table S3,5**). Subsequent investigation of individuals with similar phenotypes identified additional cases with *de novo* variants in different domains of CCT3, including two loss-of-function variants (CCT3 #10, #12) and an individual with a *de novo* missense variant (CCT3 #11) (**Fig. 1, B**). Phenotypic overlap included developmental delay, microcephaly, and visual impairment. MRI data showed hypomyelination of the white matter and atrophy of the optic tracts (**table 1**). These findings establish CCT3 as a disease-relevant chaperonin subunit.

CCT3 disruption is deleterious in different taxa

Consistent with its essential function in mediating folding of newly translated proteins, TRiC is highly conserved in sequence and structure among different taxa (**Fig. 2, A, table S2**) (*7, 15*). We next performed functional studies in yeast, worm and fish to determine if the identified *CCT3* variants affected actin and tubulin folding, and brain development. First, we used a plasmid-shuffling approach in yeast to examine whether the *cct3* variants impair TRiC function (**fig. S8**). Briefly, yeast strains in which the essential *cct3* gene was deleted but which contained a modified wild-type plasmid for survival were transformed with either cct3-WT or the respective orthologous variants cct3-Q12R, cct3-A401L*fs**27 or cct3-R522* (**Fig. 2, B, fig. S11-18**). When co-expressed with cct3-WT, the variants did not show typical TRiC/CCT-impairment phenotypes, such as sensitivity to a microtubule destabilizing drug (benomyl) or to actin-stressing hypertonic media (600mM NaCl). However, when the WT survival plasmid was removed by 5-fluoroorotic acid (5-FOA) treatment, the cct3 variants were lethal under all conditions tested, except for wildtype cct3 and cct3-Q12R. These experiments indicate that the disease-linked variants leading to protein truncations in CCT3 cause loss-of-function in yeast TRiC (*16*).

For *C. elegans* studies, we generated the orthologous *cct-3* missense variant Q14R (individual #11) by CRISPR/Cas9 editing in three lines together with two control lines containing the corresponding synonymous changes (Q14Q). We also generated a deletion of the entire coding region of *cct-3* (*cct-3(del)*) as a model of *CCT3* loss-of-function variants (**fig. S3**). Homozygous *cct-3(del)* mutant worms (*cct-3 -/-*) arrest at the second to third larval (L2-L3) stages, whereas heterozygous mutants were viable, but showed a reduced crawling speed suggesting haploinsufficiency for this phenotype (**Fig. 2, C, fig. S4**). Homozygous Q14R lines were less severely affected: although viable and fertile, the animals showed reduced motility and a reduced number of progeny consistent with the missense variant being deleterious in worms (**Fig. 2, C, fig. S5**). Heterozygous Q14R animals had a substantial crawling speed defect indicating that the variant is a weak loss of function allele (**fig. S5**). The function in folding of actin, an obligatory TRiC substrate, was investigated using fluorescently labelled intestinal actin-5 (mCherry::ACT-5) (*17, 18*). In wildtype *cct-3* worms, labelled actin localized to the apical surface of the intestine, as expected, and showed no aggregates (**Fig. 2, D**). In contrast,

cct-3 -/- mutants showed numerous bright aggregate-like mCherry::ACT-5 punctate structures throughout the intestinal cell cytoplasm, consistent with a general requirement of *cct-3* for actin folding and localization (**Fig. 2, D**). Homozygous Q14R worms also contained aggregate-like actin puncta, albeit smaller than the *cct-3 -/-* mutants, indicating a hypomorphic, but nevertheless deleterious allele (**Fig. 2, E, fig. S6**). To test for a role of *cct-3* in the function of beta tubulin, also an obligate TRiC substrate, we tested for a genetic interaction between *gfp::tbb-2* and *cct-3* Q14R following heat shock. We found that a knock-in *gfp::tbb-2* strain (*19*) was hypersensitive to heat shock. In the *gfp::tbb-2* background, Q14R was more sensitive than the Q14Q control to heat shock, suggesting that *cct-3* promotes beta-tubulin function in *C. elegans*, consistent with its role in tubulin folding (**Fig. 2, F, fig. S7**). These observations corroborate that the disease-associated *cct-3* alleles are deleterious and impair TRiC function, including the ability to properly fold obligate substrates.

To address the impact of TRiC impairment on altered brain development in vertebrates, we used a CRISPR/Cas9 approach to generate loss-of-function alleles of *cct3* in zebrafish. At four days post fertilization (dpf), WT zebrafish were larger in size compared to cct3 mutants and showed a normally developing cerebellum with regular granule and Purkinje cell layers. In contrast, the cerebellar granule cell layer in aged-matched homozygous *cct3* mutants was almost entirely absent and Purkinje cells were strongly reduced in number compared to WT and unevenly distributed in few small cell groups (**Fig. 2, G and H**). Moreover, developing hindbrains of zebrafish at 3 dpf showed focal irregularities when labeled with tetramethylrhodamine (TRITC)-conjugated phalloidin, suggesting F-actin disorganization and focal accumulations in neural cells (**Fig. 2, I**). These observations are in agreement with the cerebellar hypoplasia observed in humans. Overall, the results confirm a conserved developmental role for CCT3 among different taxa.

TRiC variants in other CCT subunits are associated with brain pathologies

Given that TRiC is an obligate hetero-oligomer of the eight ATP-binding subunits CCT1-CCT8, we hypothesized that variants in other TRiC subunits may lead to similar clinical phenotypes. Analysis of over 5,000 *in-house* exomes and genomes with a focus on intellectual disability ~~(ID)~~ revealed a nonsense variant in *TCP1 (CCT1)* in an individual with intellectual disability, multifocal epilepsy, and heterotopia (CCT1 #1). Additionally, another individual with intellectual disability, epilepsy and polymicrogyria harbored a *de novo* frameshift variant in *CCT8* (CCT8 #21). Subsequently, international collaborations, matchmaking platforms and RD-Connect GPAP (*20-22*) identified additional TRiC variants in individuals from different ethnicities. A total of 22 individuals with an overlapping disease spectrum were identified (**table1, table S1**). Brain MRIs showed early differentiation and proliferation disorders and/or white matter disorders as well as early and late neuronal migration disorders (**Fig. 3, A-J**). Mapping the variants on the structure of the TRiC/CCT complex showed that disease-linked alleles were widely dispersed along the three domains of every TRiC subunit and distributed across seven out of the eight subunits, as follows: TCP1 (CCT1) (n=8), CCT3 (n=4), CCT4 (n=1), CCT5 (n=1), CCT6A (CCT6) (n=5), CCT7 (n=1), CCT8 (n=2) (**Fig. 3, K, L and M, fig. S26,27, table S4**). The spectrum of variants included missense- (n=6), frameshift- (n=8), nonsense- (n=6) and splice-site-variants (n=1), as well as a two-exon deletion (n=1). *De novo* occurrence was confirmed in all cases for which parental samples were available (17/22).

TRiC missense variants differently impact yeast viability and actin folding and localization in *C. elegans*

The identification of a wide range of CCT variants with similar clinical manifestations strongly suggests these alleles lead to impaired TRiC function. It was reasonable to assume that alleles that will result in truncated or grossly altered CCT protein subunits such as frameshift, nonsense and splice site variants as well as the two-exon deletion, should lead to a loss-of-function of the affected subunits and thus reduced TRiC levels or activity. Testing these variants in yeast using the plasmid shuffling approach supported this idea. Additionally, the yeast experiments revealed these variants caused haploinsufficiency in several conditions (**fig. S11-18**), consistent with previous observations that CCT deletions are haploinsuficient in diploid yeast (*23*). We next examined if the patient-derived *missense* variants also impair TRiC function (**fig. S28**). Since all missense variants affect highly conserved amino acids, we investigated the pathogenicity of the orthologous missense variants in yeast using the plasmid-shuffling approach described above (**Fig. 3, O**). The cct1-K167R, cct5-K191R and cct6-R318C variants induced yeast lethality in 5-FOA media at all temperatures, indicating they lead to a severe loss-of-function. Two disease-linked variants, cct1-K167R (K159R in humans), and cct5-K191R (K176R in humans) affect the same amino acid in the highly conserved nucleotide-sensing loop (NSL) of these subunits. (**Fig. 3, L, N, P, fig. S29)**. The NSL is a conserved element in all CCT subunits that senses the hydrolysis status of the bound nucleotide, ATP or ADP, and participates in the allosteric regulation controlling the timing of ATP-driven cycling between the open and closed states (*24*). We carried out structural modelling analysis of these seemingly conservative NSL variants, using mutagenesis in Chimera, and Alphafold2 (see Methods). Both approaches showed that the K to R variant in both CCT1 and CCT5 induces the formation of a pseudo-bond with a nearby aspartate in Helix 12, likely inducing the formation of an electrostatic bond that is not present in wildtype. Therefore, the sensing capability of these NSLs may be impaired to properly detect the nucleotide state of the corresponding subunit because it interacts more with the negatively charged aspartate rather than with the gamma phosphate of ATP. Upon co-expression with a corresponding wildtype subunit, both loss-of-function NSL variants showed dominant negative effects on TRiC function, including microtubule and actin defects. In addition, cct1-K167R, but not cct5-K191R, leads to restricted growth with glycerol as the carbon source, indicative of defective mitochondrial function (**fig. S14-16,18**). Since each TRiC/CCT complex contains two copies of each subunit, the dominant negative effect of these variants can be rationalized by the formation of allosterically dysfunctional TRiC complexes containing a wildtype and a mutant allele. For cct1-P46L and cct7-E383K no detectable phenotypes in yeast were observed (**Fig. 3, O**). However, when tested in worms, the orthologous *cct-1*-P42L and *cct-7*-E377K exhibited actin aggregates, similar to *cct-3*-Q14R, and in line with a suspected pathogenicity (**Fig. 3, Q and R**). In addition, *cct-1* and *cct-7* null heterozygous worms displayed crawling defects providing additional support for haploinsufficiency of *cct* genes (**fig. S3, fig. S8**). The distinct effect of some missense mutants in yeast and worms likely arises from an increased burden or demand on TRiC function in more complex multicellular organisms. These experiments combined show that all *cct* variants tested were deleterious *in vivo* and that missense variants in different subunits can affect the function of TRiC in distinct ways and to varying degrees, likely reflecting the complex interplay between TRiC's hetero-oligomeric nature and its cellular functions.

TRiC dysfunction impairs different pathways in patient-derived cells

To better understand shared molecular pathways affected in TRiCopathies we investigated patient-derived fibroblasts carrying variants in four different TRiC subunits. Fibroblasts from

CCT3 #9, CCT6 #15, and CCT8 #21 all harbor frameshift variants, and CCT5 #14 contains a missense variant of the highly conserved NSL (**Fig. 4, A**). As expected, the three frameshift variants resulted in reduced mRNA and protein amounts of the affected subunit (RNA vs protein: CCT3 #9: 0.48 vs 0.68, CCT6A #15: 0.24 vs 0.46, CCT8 #21: 0.44 vs 0.59) (**Fig. 4, D**). Consistent with the obligate hetero-oligomeric nature of TRiC, the frameshift variants of these different subunits reduced the protein expression for all TRiC subunits in patient-derived cells. In contrast, the missense variant in the NSL loop in CCT5 did not impact expression of any TRiC subunit at the RNA or protein level, indicating this variant directly impairs TRiC function rather than TRiC expression (**Fig. 4, D**). In each patient-derived cell line a reduction in actin and tubulin isoforms was observed, in line with an impact on these major obligate TRiC substrates (**Fig. 4, E**). The results from the proteomics analyses were confirmed by direct biochemical analyses of assembled TRiC complexes by native PAGE (**fig. S24)** and native assembled TRiC was reduced relative to healthy controls (HC) in the patient-derived cells carrying frameshift mutations, but not in those with the NSL variant. Additionally, immunoblot confirmed reduced α-tubulin amounts in patient-derived cells, particularly observed for CCT6 (**fig. S24**). An ATP-AlFx closure assay (*25, 26*) revealed all mutant TRiC complexes could reach the ATP-induced closed state, even those with an NSL mutation (**fig. S25)**. As these are lysate-based endpoint assays, these experiments cannot capture potential kinetic defects in ATP cycling by the NSL variant.

We also examined shared RNA and protein changes affecting all four CCT genotypes. We observed an interesting common link to focal adhesion (**Fig. 4, B and C, fig. S19,20**). Three of the four proteins consistently upregulated (FC ≥ 1.5) in all four genotypes, FMN2, NTM, and ITGBL1, affected focal adhesions (**Fig. 4, C**).

Network analyses of proteome alterations in the CCT-deficient patient-derived cells also identified shared changes upregulating or downregulating key cellular pathways (**Fig. 4, F and G**). We observed downregulation of lysosomal, ubiquitination and metabolism pathways, all of which are linked to TRiC substrates (*9, 27*). Another major downregulated pathway corresponded to mitochondrial proteins, suggesting a role for TRiC in mitochondrial biogenesis and function (**Fig. 4, F and G)**. Immunofluorescence analyses in patient-derived fibroblasts, however, did not reveal gross alteration in tubulin or actin staining (**fig. S21**) nor in mitochondrial morphology **(fig. S22,23**). By contrast, we observed higher expression of proteasome and stress-induced chaperones in patient-derived cells, which likely compensate for the increased misfolded-protein load due to TRiC impairment and thus alleviate some of the cellular deficits caused by lower TRiC expression or function.

Discussion:

We show here that *de novo* genetic variants in virtually every subunit of the TRiC chaperonin cause a broad spectrum of brain malformations. Heterozygous variants in 22 independent individuals are linked to impaired brain development with clinical phenotypes ranging from mild to severe epilepsy, intellectual disability, ataxia, and other features of cerebral malfunction. For variants in *CCT1* and *CCT8*, polymicrogyria so far emerges as a leading pattern of malformation, whereas variants in *CCT3, CCT5* and *CCT6A* are rather associated with volume reduction of various cortical structures. Since this huge multi-subunit complex has numerous points of vulnerability, we suspect a broader clinical spectrum will emerge with the description of further cases. The variants suggest impaired TRiC function as a major disease mechanism, which is consistent with population data where seven of the eight TRiC subunits are strongly loss-of-function intolerant (pLI=1, gnomAD, v4). Multiple lines of experimental evidence suggest that the defect of one copy of a CCT gene is sufficient to cause disease, in line with previous observations that TRiC forms an obligate hetero-oligomer (*7, 16*). Consistent with this, we found that TRiC frameshift variants in one subunit, which lead to production of non-functional subunits, not only affect the expression of the variant-containing subunit but also reduce the levels of the entire oligomeric TRiC, which may explain overlapping clinical phenotypes regardless of which subunit is primarily affected.

The different TRiC subunits as well as different domains within a single subunit play distinct roles in TRiC mechanism and function (*8*). The variants are widely distributed along the structure of each subunit. Therefore, it is likely that these variants induce TRiC's impairment through different mechanisms and with variable clinical outcomes. Possible mechanisms include impairment of ATPase activity, inhibiting substrate binding or release, or hindering the assembly of functional complexes. The C-terminally located frameshift variants may escape nonsense-mediated mRNA decay and could possibly have a dominant-negative effect by becoming incorporated into TRiC complexes.

Protein encapsulation and folding *via* TRiC is ATP-driven and binding and subsequent hydrolysis of ATP promotes lid closure in both octameric rings. Of central importance is an ultra-conserved nucleotide sensing loop (NSL) adjacent to the ATP binding site which directly impacts the rate of nucleotide hydrolysis and thus the timing of the protein folding reaction (*24*). Our data show that the same NSL core residue is affected in two individuals, CCT1-K167R (#7) and CCT5-K191R (#14). In contrast to the frameshift variants, patient-derived cells with the NSL variant did not lead to reduced levels in TRiC subunits, indicating different avenues of TRiC impairment leading to disease. Structural modeling of the NSL variants suggest they will affect nucleotide status sensing. Given the role of the NSL loop in controlling the kinetics of ATP hydrolysis (*24*), it is likely that incorporation of a subunit carrying an NSL variant will change the TRiC "folding timer" even in complexes containing a wildtype and a mutated copy of the affected subunit. This may explain the dominant negative phenotype of these loss-of-function missense variants in yeast. The phenotype of the individual with the CCT5-NSL variant is particularly severe, likely reflecting the central role of ATP binding and hydrolysis of CCT5 in the hierarchical allosteric regulation of TRiC ATP-driven cycling (*28*).

The CCT3-Q12R (#11) variant observed in a mildly affected individual had a less severe actin disorganization/aggregate formation phenotype in worms. This variant affects the structurally disordered N-terminus of CCT3, which projects towards the central folding chamber and has been suggested to mediate substrate specific roles in TRiC function (*15, 25*). Another missense variant, CCT6-R314C (#16) changes the charge properties of the closed central chamber (*25*), and our yeast data indicate that this variant mainly affects folding of TRiC substrates other than tubulin or actin. The MRI of the latter individual was structurally normal, with an otherwise severe clinical phenotype. Both the CCT1-P38L (#4) and CCT7-E379K (#20) missense-

variants had no pathogenic effect in yeast, but showed actin aggregates in *C. elegans*. Together with their respective *de novo* occurrence, evolutionary conservation, and clinical phenotype this indicates disease causality. In summary, the data suggest that heterozygous missense variants are associated with TRiC impairment and support a model in which different TRiC-linked disease mechanisms operate depending on the location of the variant and subunit affected.
Variants in *CCT2* and *CCT5* have previously been reported as the only subunits involved in monogenic disorders, but in contrast to the pathology described here, with recessive inheritance. A single family with retinal dystrophy and compound heterozygous missense variants in *CCT2* has been identified (*29*) and homozygous missense-variants in *CCT5* have been reported in two families with different types of complicated neuropathies (*30, 31*). The recessive inheritance indicates that the individual heterozygous missense variants have a comparatively minor effect on TRiC function and that one affected allele is not sufficient for disease expression in contrast to the individuals reported here. Conversely, it is to be expected that mutation-specific inheritance patterns and phenotypes with variable penetrance can occur for TRiC/CCT disorders. It is also plausible that certain changes in *CCT2* will lead to brain malformations.
A central question is why heterozygous variants in this ubiquitously expressed and constantly active folding complex predominantly lead to a neurodevelopmental phenotype. A likely explanation is the uniqueness of early brain development: division of neuronal precursor cells during embryogenesis places highest demands on the pace of generating new cells, remodeling the cytoskeleton, and folding proteins. This may create a spatio-temporal bottleneck in *de novo* protein folding to which the developing brain is particularly sensitive. According to estimates, several million neurons are generated per hour during the neurogenic period of human central nervous system development (*32*). It is conceivable that an optimal TRiC operation is most critically required at this stage of cell formation and proliferation, but also in the phase of neuronal migration, and is therefore particularly susceptible to haploinsufficiency (*33*). Indeed, TRiC is highly expressed in neuronal stem cells, and is required for their resistance to proteostatic stress (*34*). The developmental steps described here critically depend on the efficient folding of actin and tubulin, which are controlled largely by TRiC (*2, 35*). Our proteomics data indeed show that actin and tubulin isoforms are less abundant in the TRiC-deficient patient-derived cells. Since TRiC is essential for neuronal tubulin folding, this might additionally explain the primary neuronal phenotypes of the individuals (*36-38*). In line with this, the disruption of many tubulin (*39-44*) and actin genes (*45*) is known to lead to similar malformations of the brain. Furthermore, cct3 knockout in zebrafish supports a model in which F-actin disorganization and focal accumulations are associated with impaired brain development. Consistent with the extensive contribution of TRiC to cellular folding, the consequences of CCT variants will extend beyond cytoskeletal defects (*46-49*).

Altered folding capacities with impaired neuronal proteostasis is typically associated with neurodegenerative rather than neurodevelopmental phenotypes (*50*). Our study establishes a direct and unanticipated link between a central proteostasis factor, TRiC/CCT, and brain development. The analysis of the muscle biopsy of individual #9 (CCT3 #9) also revealed aggregates in the cell bodies, which supports further defects in protein folding and degradation. One major downregulated protein group are mitochondrial proteins. TRiC does interact co-translationally with many nuclear-encoded mitochondrial precursors in yeast (*11*) and is strongly upregulated during mitochondrial stress (*51*). This suggests that TRiC may play an unanticipated role in mitochondrial function, in line with mitochondrial alterations observed in the muscle biopsy. The association of abnormal autophagosomes with mitochondria in intramuscular axons likewise suggests abnormal mitophagy.

Our observations support a scenario where TRiC variants may yield a wide spectrum of clinical manifestations, leading us to propose that mild variants in TRiC should contribute to less

recognizable, but more common and clinically relevant brain developmental disorders, consistent with some of our individuals being mildly affected and managing their daily lives without help. Based on publicly available genomic databases we anticipate there will be more individuals with TRiC-related disorders. Besides clear loss-of-function variants, more frequent TRiC variants could have mild effects on the function of the complex and thus on the balance of protein folding and proteostasis. It is quite possible that such factors play a substantial role in neurodevelopmental disorders currently being classified as “idiopathic”. Such correlations need to be investigated in larger studies.

In summary, the data show the role of the individual subunits of the TRiC chaperonin folding machinery for corticogenesis and demonstrate how an essential protein folding machinery is required for proper brain development. We propose that the TRiC-related brain disorders are designated as "TRiCopathies".

Acknowledgments:

We thank all patients and families who contributed to the study. We thank Ken Sato (Gunma University, Japan) for providing the mCherry::ACT-5 strain and Asako Sugimoto (Tohoku University, Japan) for providing the GFP::TBB-2 strain. Research reported in this manuscript was supported by National Institutes of Health, National Institute of Child Health and Human Development, R01 HD110556 (S.C.P. and colleagues Solnica-Krezel, Johnson, Kroll, Ornitz). Some *C. elegans* strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). This work was supported by the Flow Cytometry Facility, a core facility of the Interdisciplinary Center for Clinical Research (IZKF) Aachen within the Faculty of Medicine at RWTH Aachen University. This research was made possible through access to data in the National Genomic Research Library, which is managed by Genomics England Limited (a wholly owned company of the Department of Health and Social Care). The National Genomic Research Library holds data provided by patients and collected by the NHS as part of their care and data collected as part of their participation in research. The National Genomic Research Library is funded by the National Institute for Health Research and NHS England. The Wellcome Trust, Cancer Research UK and the Medical Research Council have also funded research infrastructure.

Funding:
Funding was also provided by the Children's Discovery Institute, St Louis Children's Hospital Foundation (G.A.S. and S.C.P.). Funding to M.T.: Italian Ministry of Health (RCR-2022-23682289, PNRR-MR1-2022-12376811, 5 per 1000 and Current Research funds). H.L. receives support from the Canadian Institutes of Health Research (CIHR) for Foundation Grant FDN-167281 (Precision Health for Neuromuscular Diseases), Transnational Team Grant ERT-174211 (ProDGNE) and Network Grant OR2-189333 (NMD4C), from the Canada Foundation for Innovation (CFI-JELF 38412), the Canada Research Chairs program (Canada Research Chair in Neuromuscular Genomics and Health, 950-232279), the European Commission (Grant # 101080249) and the Canada Research Coordinating Committee New Frontiers in Research Fund (NFRFG-2022-00033) for SIMPATHIC, and from the Government of Canada Canada First Research Excellence Fund (CFREF) for the Brain-Heart Interconnectome (CFREF-2022-00007). K.P. is a recipient of a CIHR Postdoctoral fellowship. S.W. is funded by the German Research Foundation (WO 2385/2-1). J.W. is supported by the Deutsche Forschungsgemeinschaft (DFG) (WE 1406/16-1 and WE 1406/17-1). T.B.H. was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 418081722, 433158657. Proteomics (A.R., A.H.): AH acknowledges the support by the "Ministerium für Kultur und Wissenschaft des Landes Nordrhein-Westfalen" and "Der Regierende Bürgermeister von Berlin, Senatskanzlei Wissenschaft und Forschung". A.R. also acknowledges the support by the "Ministerium für Kultur und Wissenschaft des Landes Nordrhein-Westfalen" for MODS [grant no. PROFILNRW-2020–107-A]. P.R.-A. received a postdoctoral fellowship from The Hereditary Disease Foundation (2019-2023). The German Research Foundation (DFG) provided funding to J.B. and I.K. for a spinning disc confocal microscope in the major research instrumentation program (project number 499059538; INST 222/1458-1 FUGG). J.F. is supported by NIH grants GM74074 and GM56433. I.K., T.E., M.E. receive funding for sequencing by Illumina. F.K., I.K., T.B.H. receive funding for sequencing by Oxford Nanopore Technologies (ONT) within the lonGER consortium. I.K. is supported by the Deutsche Forschungsgemeinschaft (DFG) (KU 1587/6-1, KU 1587/9-1, KU 1587/10-1, KU 1587/11-1). I.K. receives funding from the European Union's Horizon 2020 research and innovation programme under the EJP RD COFUND-EJP N° 825575.

Author contributions:
Study design, experiments, data evaluation: Florian Kraft, Miriam Elbracht, Ingo Kurth, Piere Rodriguez-Aliaga, Judith Frydman, Weimin Yuan, Stephen Pak
Writing: Florian Kraft, Miriam Elbracht, Ingo Kurth, Piere Rodriguez-Aliaga, Judith Frydman, Stephen Pak with input from all co-authors
Cell experiments, genomics, transcriptomics: Lena Franken, Sebastian Gießelmann, Matthias Begemann, Thomas Eggermann, Jeremias Krause, Tobias B. Haack, Elisabetta Flex, Enrico Bertini, Andrea Ciolfi, Marco Tartaglia, Ting-Ting Lee, Piere Rodriguez-Aliaga, Florian Kraft, Miriam Elbracht, Ingo Kurth
Yeast experiments: Piere Rodriguez-Aliaga, Judith Frydman
Zebrafish & Neuropathology: Kamil Zajt, Xiaomeng Zhang, Lisa Wang, Joachim Weis, Juliane Bremer
Neuroradiology: Dimah Hasan, Martin Wiesmann
C.elegans: Weimin Yuan, Hieu Hoang, Dustin Baldridge, Gary A. Silverman, Tim Schedl, Stephen C. Pak
Proteomics: Andreas Hentschel, Andreas Roos
In silico Mutagenesis: Piere Rodriguez-Aliaga, Judith Frydman
Clinical data, sampling, genetics: Miriam Elbracht, Florian Kraft, Ingo Kurth, A. Micheil Innes, Bixia Zheng, Dong Sun Julia Suh, Cordula Knopp, Eva Lausberg, Pamela Trapane, Riley Carroll, Martin McClatchey, Andrew Fry, Francesca Clementina Radio, Katherine A Blood, Jean-Madeleine de Sainte Agathe, Perrine Charles, Gaber Bergant, Goran Čuturilo, Borut Peterlin, Karin Diderich, Haley Streff, Laurie Robak, Renske Oegema, Ellen van Binsbergen, John Herriges, Carol Saunders, Andrea Maier, Stefan Wolking, Yvonne Weber, Hanns Lochmüller, Stefanie Meyer, Alberto Aleman, Kiran Polavarapu, Gael Nicolas, Alice Goldenberg, Lucie Guyant, Kathleen Pope, Katherine N. Hehmeyer, Michelle Morrow, Anne Quade, Thomas Smol, Roseline Caumes, Sarah Duerinckx, Chantal Depondt, Wim Van Paesschen, Claudine Rieubland, Claudia Poloni, Michel Guipponi, Severine Arcioni, Marije Meuwissen, Anna C. Jansen, Jessica Rosenblum, Miriam Bertrand, Lea Gerstner, Janine Magg, Olaf Riess, Jörg B. Schulz, Norbert Wagner, Martin Häusler

Competing interests: The authors declare no competing interests.

Data and materials availability: Details of genetic analyses, yeast studies, zebrafish and worm studies, patient-derived cell experiments, RNA-seq, transcriptomics, modelling, and statistical analyses are provided in the **Supplementary Appendix and via the Dryad (https://datadryad.org/stash) repository** DOI: 10.5061/dryad.sj3tx96d3. Written informed consent was provided by the patients or their legal representatives at the respective institutions. All experiments in *D. rerio* were performed with the approval of the institutional review board in Aachen, Germany. All human genetic variants will be submitted to ClinVar by the time of publication.

References and Notes:

1. Y. Li *et al.*, Rates of Incidental Findings in Brain Magnetic Resonance Imaging in Children. *JAMA Neurol* **78**, 578-587 (2021).
2. E. Klingler, F. Francis, D. Jabaudon, S. Cappello, Mapping the molecular and cellular complexity of cortical malformations. *Science* **371**, (2021).
3. M. Severino *et al.*, Definitions and classification of malformations of cortical development: practical guidelines. *Brain* **143**, 2874-2894 (2020).
4. R. Oegema *et al.*, International consensus recommendations on the diagnostic work-up for malformations of cortical development. *Nat Rev Neurol* **16**, 618-635 (2020).
5. S. K. Akula *et al.*, Exome Sequencing and the Identification of New Genes and Shared Mechanisms in Polymicrogyria. *JAMA Neurol* **80**, 980-988 (2023).
6. F. U. Hartl, A. Bracher, M. Hayer-Hartl, Molecular chaperones in protein folding and proteostasis. *Nature* **475**, 324-332 (2011).
7. A. Leitner *et al.*, The molecular architecture of the eukaryotic chaperonin TRiC/CCT. *Structure* **20**, 814-825 (2012).
8. D. Gestaut, A. Limatola, L. Joachimiak, J. Frydman, The ATP-powered gymnastics of TRiC/CCT: an asymmetric protein folding machine with a symmetric origin story. *Curr Opin Struct Biol* **55**, 50-58 (2019).
9. A. Y. Yam *et al.*, Defining the TRiC/CCT interactome links chaperonin function to stabilization of newly made proteins with complex topologies. *Nat Struct Mol Biol* **15**, 1255-1262 (2008).
10. V. Thulasiraman, C. F. Yang, J. Frydman, In vivo newly translated polypeptides are sequestered in a protected folding environment. *EMBO J* **18**, 85-95 (1999).
11. K. C. Stein, A. Kriel, J. Frydman, Nascent Polypeptide Domain Topology and Elongation Rate Direct the Cotranslational Hierarchy of Hsp70 and TRiC/CCT. *Mol Cell* **75**, 1117-1130 e1115 (2019).
12. A. Freund *et al.*, Proteostatic control of telomerase function through TRiC-mediated folding of TCAB1. *Cell* **159**, 1389-1403 (2014).
13. A. Y. Dunn, M. W. Melville, J. Frydman, Review: cellular substrates of the eukaryotic chaperonin TRiC/CCT. *J Struct Biol* **135**, 176-184 (2001).
14. C. Spiess, A. S. Meyer, S. Reissmann, J. Frydman, Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets. *Trends Cell Biol* **14**, 598-604 (2004).
15. M. Jin, C. Liu, W. Han, Y. Cong, TRiC/CCT Chaperonin: Structure and Function. *Subcell Biochem* **93**, 625-654 (2019).
16. K. Betancourt Moreira *et al.*, A hierarchical assembly pathway directs the unique subunit arrangement of TRiC/CCT. *Mol Cell* **83**, 3123-3139 e3128 (2023).
17. K. Saegusa *et al.*, Caenorhabditis elegans chaperonin CCT/TRiC is required for actin and tubulin biogenesis and microvillus formation in intestinal epithelial cells. *Mol Biol Cell* **25**, 3095-3104 (2014).
18. C. R. Huang *et al.*, Host CDK-1 and formin mediate microvillar effacement induced by enterohemorrhagic Escherichia coli. *Nat Commun* **12**, 90 (2021).
19. Y. Honda, K. Tsuchiya, E. Sumiyoshi, N. Haruta, A. Sugimoto, Tubulin isotype substitution revealed that isotype combination modulates microtubule dynamics in C. elegans embryos. *J Cell Sci* **130**, 1652-1661 (2017).

20. N. Sobreira, F. Schiettecatte, D. Valle, A. Hamosh, GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. *Hum Mutat* **36**, 928-930 (2015).
21. A. A. Philippakis *et al.*, The Matchmaker Exchange: a platform for rare disease gene discovery. *Hum Mutat* **36**, 915-921 (2015).
22. S. Laurie *et al.*, The RD-Connect Genome-Phenome Analysis Platform: Accelerating diagnosis, research, and gene discovery for rare diseases. *Hum Mutat* **43**, 717-733 (2022).
23. A. M. Deutschbauer *et al.*, Mechanisms of haploinsufficiency revealed by genome-wide profiling in yeast. *Genetics* **169**, 1915-1925 (2005).
24. J. H. Pereira *et al.*, Mechanism of nucleotide sensing in group II chaperonins. *EMBO J* **31**, 731-740 (2012).
25. D. Gestaut *et al.*, Structural visualization of the tubulin folding pathway directed by human chaperonin TRiC/CCT. *Cell* **185**, 4770-4787 e4720 (2022).
26. A. S. Meyer *et al.*, Closing the folding chamber of the eukaryotic chaperonin requires the transition state of ATP hydrolysis. *Cell* **113**, 369-381 (2003).
27. M. Y. Hein *et al.*, A human interactome in three quantitative dimensions organized by stoichiometries and abundances. *Cell* **163**, 712-723 (2015).
28. S. Reissmann *et al.*, A gradient of ATP affinities generates an asymmetric power stroke driving the chaperonin TRIC/CCT folding cycle. *Cell Rep* **2**, 866-877 (2012).
29. Y. Minegishi *et al.*, CCT2 Mutations Evoke Leber Congenital Amaurosis due to Chaperone Complex Instability. *Sci Rep* **6**, 33742 (2016).
30. A. Bouhouche, A. Benomar, N. Bouslam, T. Chkili, M. Yahyaoui, Mutation in the epsilon subunit of the cytosolic chaperonin-containing t-complex peptide-1 (Cct5) gene causes autosomal recessive mutilating sensory neuropathy with spastic paraplegia. *J Med Genet* **43**, 441-443 (2006).
31. V. Antona *et al.*, A Novel CCT5 Missense Variant Associated with Early Onset Motor Neuropathy. *Int J Mol Sci* **21**, (2020).
32. J. C. Silbereis, S. Pochareddy, Y. Zhu, M. Li, N. Sestan, The Cellular and Molecular Landscapes of the Developing Human Central Nervous System. *Neuron* **89**, 248-268 (2016).
33. G. G. Jayaraj, M. S. Hipp, F. U. Hartl, Functional Modules of the Proteostasis Network. *Cold Spring Harb Perspect Biol* **12**, (2020).
34. W. I. M. Vonk *et al.*, Differentiation Drives Widespread Rewiring of the Neural Stem Cell Chaperone Network. *Mol Cell* **78**, 329-345 e329 (2020).
35. R. Ayala, T. Shu, L. H. Tsai, Trekking across the brain: the journey of neuronal migration. *Cell* **128**, 29-43 (2007).
36. J. J. Kelly *et al.*, Snapshots of actin and tubulin folding inside the TRiC chaperonin. *Nat Struct Mol Biol* **29**, 420-429 (2022).
37. C. Liu *et al.*, Pathway and mechanism of tubulin folding mediated by TRiC/CCT along its ATPase cycle revealed using cryo-EM. *Commun Biol* **6**, 531 (2023).
38. L. Fei *et al.*, Systematic identification of cell-fate regulatory programs using a single-cell atlas of mouse development. *Nat Genet* **54**, 1051-1061 (2022).
39. D. A. Keays *et al.*, Mutations in alpha-tubulin cause abnormal neuronal migration in mice and lissencephaly in humans. *Cell* **128**, 45-57 (2007).
40. M. Breuss *et al.*, Mutations in the beta-tubulin gene TUBB5 cause microcephaly with structural brain abnormalities. *Cell Rep* **2**, 1554-1562 (2012).

41. T. D. Cushion *et al.*, De novo mutations in the beta-tubulin gene TUBB2A cause simplified gyral patterning and infantile-onset epilepsy. *Am J Hum Genet* **94**, 634-641 (2014).
42. X. H. Jaglin *et al.*, Mutations in the beta-tubulin gene TUBB2B result in asymmetrical polymicrogyria. *Nat Genet* **41**, 746-752 (2009).
43. K. Poirier *et al.*, Mutations in the neuronal ss-tubulin subunit TUBB3 result in malformation of cortical development and neuronal migration defects. *Hum Mol Genet* **19**, 4462-4473 (2010).
44. K. Poirier *et al.*, Mutations in TUBG1, DYNC1H1, KIF5C and KIF2A cause malformations of cortical development and microcephaly. *Nat Genet* **45**, 639-647 (2013).
45. J. B. Riviere *et al.*, De novo mutations in the actin genes ACTB and ACTG1 cause Baraitser-Winter syndrome. *Nat Genet* **44**, 440-444, S441-442 (2012).
46. J. Cuellar *et al.*, Structural and functional analysis of the role of the chaperonin CCT in mTOR complex assembly. *Nat Commun* **10**, 2865 (2019).
47. H. Weng, X. Feng, Y. Lan, Z. Zheng, TCP1 regulates PI3K/AKT/mTOR signaling pathway to promote proliferation of ovarian cancer cells. *J Ovarian Res* **14**, 82 (2021).
48. Z. Ying *et al.*, CCT6A suppresses SMAD2 and promotes prometastatic TGF-beta signaling. *J Clin Invest* **127**, 1725-1740 (2017).
49. N. Tang, X. Cai, L. Peng, H. Liu, Y. Chen, TCP1 regulates Wnt7b/beta-catenin pathway through P53 to influence the proliferation and migration of hepatocellular carcinoma cells. *Signal Transduct Target Ther* **5**, 169 (2020).
50. C. Soto, S. Pritzkow, Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases. *Nat Neurosci* **21**, 1332-1340 (2018).
51. F. Boos *et al.*, Mitochondrial protein-induced stress triggers a global adaptive transcriptional programme. *Nat Cell Biol* **21**, 442-451 (2019).
52. K. W. Nolte, A. R. Janecke, M. Vorgerd, J. Weis, J. M. Schroder, Congenital type IV glycogenosis: the spectrum of pleomorphic polyglucosan bodies in muscle, nerve, and spinal cord with two novel mutations in the GBE1 gene. *Acta Neuropathol* **116**, 491-506 (2008).
53. D. Mair *et al.*, Differential diagnosis of vacuolar myopathies in the NGS era. *Brain Pathol* **30**, 877-896 (2020).
54. D. Torre, A. Lachmann, A. Ma'ayan, BioJupies: Automated Generation of Interactive Notebooks for RNA-Seq Data Analysis in the Cloud. *Cell Syst* **7**, 556-561 e553 (2018).
55. M. E. Ritchie *et al.*, limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**, e47 (2015).
56. A. Hentschel *et al.*, Protein signature of human skin fibroblasts allows the study of the molecular etiology of rare neurological diseases. *Orphanet J Rare Dis* **16**, 73 (2021).
57. K. A. Mullan *et al.*, ggVolcanoR: A Shiny app for customizable visualization of differential expression datasets. *Comput Struct Biotechnol J* **19**, 5735-5740 (2021).
58. S. Babicki *et al.*, Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res* **44**, W147-153 (2016).
59. M. V. Kuleshov *et al.*, Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res* **44**, W90-97 (2016).
60. S. Brenner, The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94 (1974).
61. T. Boulin *et al.*, Functional analysis of a de novo variant in the neurodevelopment and generalized epilepsy disease gene NBEA. *Mol Genet Metab* **134**, 195-202 (2021).
62. H. Huang *et al.*, A dominant negative variant of RAB5B disrupts maturation of surfactant protein B and surfactant protein C. *Proc Natl Acad Sci U S A* **119**, (2022).

63. M. C. Mullins, M. Hammerschmidt, P. Haffter, C. Nusslein-Volhard, Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr Biol* **4**, 189-202 (1994).
64. J. A. Gagnon *et al.*, Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLoS One* **9**, e98186 (2014).
65. W. Y. Hwang *et al.*, Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* **31**, 227-229 (2013).
66. Y. K. Bae *et al.*, Anatomy of zebrafish cerebellum and screen for mutations affecting its development. *Dev Biol* **330**, 406-426 (2009).
67. S. Tam, R. Geller, C. Spiess, J. Frydman, The chaperonin TRiC controls polyglutamine aggregation and toxicity through subunit-specific interactions. *Nat Cell Biol* **8**, 1155-1162 (2006).
68. D. Gestaut *et al.*, The Chaperonin TRiC/CCT Associates with Prefoldin through a Conserved Electrostatic Interface Essential for Cellular Proteostasis. *Cell* **177**, 751-765 e715 (2019).
69. J. Grantham, The Molecular Chaperone CCT/TRiC: An Essential Component of Proteostasis and a Potential Modulator of Protein Aggregation. *Front Genet* **11**, 172 (2020).
70. M. R. Leroux, E. P. Candido, Characterization of four new tcp-1-related cct genes from the nematode Caenorhabditis elegans. *DNA Cell Biol* **14**, 951-960 (1995).
71. M. R. Leroux, E. P. Candido, Molecular analysis of Caenorhabditis elegans tcp-1, a gene encoding a chaperonin protein. *Gene* **156**, 241-246 (1995).
72. M. R. Leroux, E. P. Candido, Subunit characterization of the Caenorhabditis elegans chaperonin containing TCP-1 and expression pattern of the gene encoding CCT-1. *Biochem Biophys Res Commun* **241**, 687-692 (1997).
73. K. Nishida *et al.*, Expression Patterns and Levels of All Tubulin Isotypes Analyzed in GFP Knock-In C. elegans Strains. *Cell Struct Funct* **46**, 51-64 (2021).
74. M. Roy, R. C. Fleisher, A. I. Alexandrov, A. Horovitz, Reduced ADP off-rate by the yeast CCT2 double mutation T394P/R510H which causes Leber congenital amaurosis in humans. *Commun Biol* **6**, 888 (2023).
75. A. Suga, Y. Minegishi, M. Yamamoto, K. Ueda, T. Iwata, Compound heterozygous mutations in a mouse model of Leber congenital amaurosis reveal the role of CCT2 in photoreceptor maintenance. *Commun Biol* **7**, 676 (2024).
76. F. Scalia *et al.*, Structural and Dynamic Disturbances Revealed by Molecular Dynamics Simulations Predict the Impact on Function of CCT5 Chaperonin Mutations Associated with Rare Severe Distal Neuropathies. *Int J Mol Sci* **24**, (2023).
77. W. Min *et al.*, A human CCT5 gene mutation causing distal neuropathy impairs hexadecamer assembly in an archaeal model. *Sci Rep* **4**, 6688 (2014).
78. J. H. Pereira *et al.*, Structure of the human TRiC/CCT Subunit 5 associated with hereditary sensory neuropathy. *Sci Rep* **7**, 3673 (2017).
79. O. A. Sergeeva, M. T. Tran, C. Haase-Pettingell, J. A. King, Biochemical characterization of mutants in chaperonin proteins CCT4 and CCT5 associated with hereditary sensory neuropathy. *J Biol Chem* **289**, 27470-27480 (2014).
80. Z. Lin *et al.*, Evolutionary-scale prediction of atomic-level protein structure with a language model. *Science* **379**, 1123-1130 (2023).
81. K. Tunyasuvunakool *et al.*, Highly accurate protein structure prediction for the human proteome. *Nature* **596**, 590-596 (2021).

Figure and Table legends:

Fig. 1. *CCT3* variants in human developmental disorder. (**A**) MRI of individual #9 at age four years and five months showing hypoplastic cerebellar vermis (arrowhead) and hypomyelination of the white matter. (**B**) The heterozygous variants of the individuals (#9-#12) in CCT3 are indicated and locate across the different operational domains of the subunit. The apical domain of CCT3 (green) at the tip of the ring has a substrate recognition site and the lid-forming loop, the intermediate domain (red) controls ATP hydrolysis and thus the movement of the apical domain, and the equatorial domain (blue) includes the ATP-binding site (light green). Protein substrates of the complex are encapsulated in a folding chamber formed by TRiC to assist ATP-dependent folding of proteins. CCT3 is highlighted in the upper octameric ring of TRiC. The entire 16-meric complex is formed by two octameric rings. (**C-J**) Muscle biopsy findings. (**C**) Light microscopy of toluidine-blue stained semithin section showing the spectrum of muscle fiber calibers. Arrowhead: intramuscular nerve fascicle containing myelinated nerve fibers. Scale bar = 40 µm. (**D**) intramuscular nerve fiber showing intraaxonal accumulation of abnormal autophagic material (arrowhead). EM; scale bar = 2 µm. (**E**) In the intramuscular nerve fiber, the abnormal intraaxonal autophagic material (arrowheads) is associated with mitochondria. EM; scale bar = 1 µm. (**F**) Motor end plate displaying a paucity of synaptic folds and swelling of mitochondria in axon endings (arrowheads). EM; scale bar = 1 µm. (**G** and **H**) Two membrane-bound intermyofibrillar vacuoles containing globular and granular material of variable osmophilia. EM; scale bars = 600 nm. (**I**) intermyofibrillar accumulation of degenerating mitochondria and of vesicular material (dashed circle). EM; scale bar = 500 nm. (**J**) Representation of Z-band material. EM; scale bar = 1 µm.

Fig. 2. Functional consequences of *CCT3* alterations in different species. (**A**) Conservation of the missense variant (individual #11) and position of the C-terminal loss-of-function variants (individual #9, #10 and #12) in different species. (**B**) Functional studies of the *cct3* variants by yeast plasmid-shuffling assays. (**C**) Representative brightfield images of *C. elegans* phenotype 48-hour post egg-lay. Scale bar, 100 µm. (**D**) Actin staining in the intestine of *cct-3* -/- and *cct-3* Q14R/Q14R animals. Maximum intensity images of mCherry::ACT-5 in the intestine of wildtype (*cct-3* +/+), heterozygous (*cct-3* +/-) and homozygous (*cct-3* -/-) L2 stage animals. Scale bar, 50 µm. (**E**) Quantification of cytoplasmic mCherry::ACT-5 puncta in adult worms. (**F**) Lethality in the presence of a GFP-β-tubulin fusion protein following heat shock. Shown are relative survival rates following heat shock at 37 ºC for 2 hours. Five independent biological replicates were combined for each genotype. Differences between groups were determined using ordinary 1-way ANOVA followed by a Tukey multiple-comparisons test. ns, not significant; * $p< 0.05$; ** $p< 0.01$. (**G**) *Danio rerio cct3* knockout animals. Overview images of *cct3* wildtype ($cct3^{+/+}$) and homozygous knockout ($cct3^{-/-}$) zebrafish at 4 days post fertilization (4 dpf) are shown. (**H**) Cerebellum of $cct3^{+/+}$ and $cct3^{-/-}$ animals at 4 days dpf. Whole mount immunohistochemistry of vglut1 as a marker of cerebellar granule cells (magenta) and pvalb7 as a marker of Purkinje cells (green). Scale bars are 40 µm. (**I**) F-actin was labeled using TRITC-

conjugated phalloidin. Images of the hindbrains of WT zebrafish at 3 dpf (n=5) and *cct3* mutants (n=4). Representative single planes are shown. Scale bar is 10 µm. The examined hindbrain area is marked with a red box in the diagram of the zebrafish larva (right).

Fig. 3. Brain phenotype and cellular consequences of TRiC dysfunction. (**A-J**) Magnetic resonance images from affected individuals with CCT gene variants. (**A**) Individual age 29 years with heterotopia at the posterior horn of right lateral ventricle (arrowheads). (**B**) Child age 12 years with bilateral perisylvian polymicrogyria with frontal and parietal extension. (**C**) Child age 9 years with asymmetrical bilateral polymicrogyria, most severe over the right frontoparietal hemisphere (arrowheads left, right panels). Mild findings perisylvian in the left hemisphere (arrowheads, middle). (**D**) Child age 14 years with bilateral symmetrical polymicrogyria, frontoparietal and temporal. (**E**) Child age 4 years with cerebellar atrophy and hypoplastic cerebellar vermis (arrowheads, right), hypomyelination of white matter, especially periventricular and temporal (middle). (**F**) Child age 2 years with reduced supratentorial white matter volume with abnormal signal, and thinning of the corpus callosum (middle). Reduced volume of the thalami bilaterally, with mild associated abnormal signal. Mildly small optic chiasm and optic nerves (left). (**G**) Individual with bilateral temporoparietal polymicrogyria (middle) and suspected dysplasia of posterior insular cortex (left) as well as hypogenesis of the corpus callosum (right), hypoplasia of both cerebellar hemispheres and pons (middle). (**H**) Child age 2 years with cerebellar atrophy (arrows). (**I**) Child age 3 years with bilateral polymicrogyria. Extensive findings over the left hemisphere frontoparietal and temporal (arrows left and right panels) and over the posterior end of sylvian fissure in the right hemisphere (arrow, middle panel). (**J**) Individual with extensive right hemispheric polymicrogyria, frontoparietal and temporal. (**K**) 16-mer TRiC complex with position of the CCT variants indicated in pink. (**L**) ATP-dependent protein folding via two octameric double rings which build the folding-chamber. (**M**) Pathogenic variants of all subunits projected onto a single prototype CCT primary structure ("panCCT") with the three protein domains highlighted. (**N**) Structural modelling of the NSL variants. (**O**) Functional studies of missense mutations in CCT subunits in yeast. (**P**) High conservation of the nucleotide sensing-loop (NSL). (**Q**) Maximum intensity projection of actin aggregates in *C. elegans* harboring the orthologous CCT1-P38L human variant (*cct-1* P42L) and CCT7-E379K human variant (*cct-7* E377K), respectively. Scale bar, 50 µm (**R**) Quantification of actin aggregates.

Fig. 4. Downstream effects of human TRiC dysfunction. (**A-C**) Transcriptome and proteome analysis in patient-derived fibroblast cell lines. Differences between groups were determined using t-test and a p-value ≤ 0.05. Volcano plots show the differentially expressed genes (B) and proteins (C) in the patient-derived cell lines (CCT3 #9, CCT5 #14, CCT6 #15, CCT8 #21) compared to healthy controls. Dashed lines indicate the thresholds for log2 fold change (≥ 0.585) and significance level (p-value ≤0.05). Upregulated genes are shown in yellow, whereas downregulated genes are colored in blue. Genes with expression changes common to all four genotypes (≥1.5-fold and with a p-value ≤0.05) are labeled by gene/protein name. (**D** and **E**) Expression changes of all TRiC subunits as well as the genes/proteins of the actin and tubulin family are shown per genotype. (**F** and **G**) Pathway analysis of combined differentially regulated proteins from the patient-derived cells reveals upregulated (F) and downregulated (G) pathways.

Table 1. Clinical and genetic spectrum of TRiCopathies. Abbreviations: y = year; n.k. = not known; n.s. = not specified; ID = intellectual disability; CC = corpus callosum; DD = developmental delay; § = age at last follow up. Head circumference: * = at last appointment; P = percentile; z = z-score.