REPORT

Bi-allelic variants in *CHKA* cause a neurodevelopmental disorder with epilepsy and microcephaly

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Abstract

The Kennedy pathways catalyze the *de novo* synthesis of phosphatidylcholine and phosphatidylethanolamine, the most abundant components of eukaryotic cell membranes. In recent years, these pathways have moved into clinical focus since four out of ten genes involved have been associated with a range of autosomal recessive rare diseases such as a neurodevelopmental disorder with muscular dystrophy (*CHKB*), bone abnormalities and cone-rod dystrophy (*PCYT1A*), and spastic paraplegia (*PCYT2*, *SELENOI*).

We identified six individuals from five families with bi-allelic variants in *CHKA* presenting with severe global developmental delay, epilepsy, movement disorders, and microcephaly. Using structural molecular modeling and functional testing of the variants in a in a cell-based *S. cerevisiae* model, we determined that these variants reduce the enzymatic activity of *CHKA* and confer a significant impairment of the first enzymatic step of the Kennedy pathway.

In summary, we present *CHKA* as a novel autosomal recessive gene for a neurodevelopmental disorder with epilepsy and microcephaly.

Keywords: neurodevelopmental disorder; epilepsy; Kennedy pathway; exome sequencing; choline kinase alpha

Abbreviations: ACMG = American College of Medical Genetics; ADP = Adenosine diphosphate; cMRI = cranial MRI; COX = cytochrome c oxidase; DD/ID = developmental delay/intellectual disability; ES = exome sequencing; H&E = hematoxylin and eosin; NMD = nonsense-mediated mRNA decay; OFC = occipitofrontal circumference; ORF = open reading frame; PC = phosphatidylcholine; PE = phosphatidylethanolamine; RPE = retinal pigment epithelium, SDH = succinate dehydrogenase

Introduction

Eukaryotic membranes are dependent on the precise compositions of glycerophospholipids, the most abundant being PC and PE. PC and PE account for more than half of the phospholipid species in eukaryotic membranes and are synthesized *de novo* by the Kennedy pathways.[1]

CHKA encodes for choline kinase alpha, an enzyme that catalyzes the first step of phospholipid synthesis in the Kennedy pathway. Together with its paralog *CHKB*, it phosphorylates either choline or ethanolamine using ATP resulting in phosphocholine or phosphoethanolamine and ADP.[2,3] Bi-allelic variants in *CHKB* are associated with a neurodevelopmental disorder with muscular dystrophy characterized by intellectual disability, microcephaly, hypotonia and structural mitochondrial abnormalities (MIM 602541).[4–6] In recent years, variants in further genes involved in the Kennedy pathway have been described to cause recessive hereditary disorders, ranging from bone abnormalities with cone rod dystrophy (*PCYT1A*, MIM 608940)[7] to neurodevelopmental disorders such as complex spastic paraplegia (*PCYT2*, MIM 618770; *SELENOI*, MIM 618768).[8,9] Similar lipid metabolic pathways have also been associated with hereditary motor neuron degenerative diseases.[10]

In this study, we describe six individuals from five families with homozygous and compound-heterozygous pathogenic variants in *CHKA*. They present with a severe neurodevelopmental disorder characterized by DD/ID, epilepsy, and microcephaly. We also verified altered protein function using structural *in silico* modeling and functional testing of variants in a cell-based model.

Materials and methods

Standard protocol approvals

The study was approved by the ethics committee of the University of Leipzig, Germany (402/16-ek). All families provided informed consent for clinical testing and publication.

Research cohort and identification of variants

All individuals were ascertained in the context of local diagnostic protocols. As no causative variants were identified in known disease genes, research evaluation of the sequencing data was performed identified potentially damaging rare variants in *CHKA*. By using matchmaking platforms and international collaborations, six individuals from five families harboring rare homozygous and compound-heterozygous variants in *CHKA* were identified.[11] Phenotypic and genotypic information were obtained from the referring collaborators using a standardized questionnaire. Causality of both truncating and missense variants were assessed according to the guidelines of the ACMG (Table S1).[12]

For individuals 1.1 and 1.2, quattro ES for the parents and the two affected children were performed. Variants identified by ES were validated using Sanger sequencing. For individuals 2, 3 and 5 Singleton ES was performed. Validation of all variants identified by ES and bi-allelic segregation analysis were done by Sanger sequencing. For individual 4, trio ES was performed, and Sanger sequencing validated the identified variants and segregation analysis (for further details see Supplemental Methods 1).

Subcloning of human *CHKA* allelic variants

The DNA for a wild type ORF of human *CHKA* C-terminally tagged with Myc and FLAG epitopes was amplified by PCR from Origene plasmid RC219747 using HiFi Platinum *Taq* polymerase and subcloned into the yeast expression vector p416-GPD. Variants were generated by site directed mutagenesis on the p416-GPD-CHKA (missing the GC rich region) using the QuikChange mutagenesis kit (Agilent) following manufacturer's instructions. DNA sequencing was used to confirm the ORF for each plasmid (for further details see Supplemental Methods 2).

Yeast transformation and culture

Wild type BY4742 and otherwise isogenic *cki1*Δ::KanMX6 strains were transformed with plasmid DNA following standard yeast protocols and selected on media for plasmid maintenance. This strain is part of the yeast gene knockout collection and is known to be devoid of endogenous choline kinase activity. The strain is viable as yeast contain a second pathway, the phosphatidylethanolamine methylation pathway, for the synthesis of PC. Transformed cells were grown to logarithmic phase at 30°C in liquid medium enabling plasmid selection and retention.

Protein extraction and western blot analysis

Logarithmic grown yeast cells were harvested, washed, and taken up in lysis buffer (50 mM Tris-HCl, 0.3 M sucrose, 1 X Complete protease inhibitor cocktail (Roche), 2 mg/ml pepstatin A, 1 mM PMSF) at 30 OD units/ml. Cells were broken by glass bead beating and supernatants of a 500 *g* x 5 min centrifugation were collected. Protein amount was determined by the Bradford method and equal amounts of protein were subjected to SDS-PAGE analysis followed by

western blotting. Monoclonal antibodies against Myc were used to determine CHKA expression with yeast Pgk1 used as a loading control.

Choline kinase activity

Choline kinase activity was estimated by the synthesis of phosphocholine from radiolabeled choline using yeast cytosolic fractions as sources of enzyme, followed by TLC to separate substrate from product (for further details see Supplemental Methods 2).

Skeletal muscle biopsy

Details on muscle biopsy and staining are available in the Supplemental Methods 3.

Structural modeling

The structural effect of the variants was investigated based on the crystal structures of CHKA in complex with ADP (PDB:3G15[13]) or phosphocholine (PDB: 2CKQ[14]). Variants were modelled with SwissModel[15] and RasMol[16] was used for structure analysis and visualization.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

Results

Clinical description

All five individuals aged between 2 and 11 years were affected by a neurodevelopmental disorder. The initial clinical presentation was in the first year of life with severe DD/ID, seizures, and microcephaly. Further signs include movement disorders, and abnormal muscle tone. An overview of the clinical symptoms is presented in Table 1. Further clinical data is presented in Supplemental case reports including MRI images and MRI structure analysis.

Individuals 1.1 and 1.2

Individuals 1.1 and 1.2 [homozygous p.(Arg141Trp)] are siblings born to healthy consanguineous Iranian parents. First signs were noted in the first months of life and included severe DD/ID, cerebral palsy, and seizures (epileptic spasms, focal and generalized seizures). While individual 1.1 did not acquire free walking, developmental delay for individual 1.2 was noted to be less severe as she was able to walk on her own. Further signs for both siblings include absent speech, microcephaly, hyperreflexia, and nystagmus.

Individual 2

Individual 2 [homozygous p.(Arg141Trp)] is the fourth child of consanguineous Indian parents. First signs were noted in the first year of life. He showed severe DD/ID, hyperreflexia, microcephaly, and muscular hypotonia. The first epileptic spasms occurred at the age of three years. All three older siblings succumbed between the ages of 18 months to 17 years to a similar

disorder comprising DD/ID, seizures, microcephaly and muscular hypotonia. Genetic test results are not available for these siblings.

Individual 3

Individual 3 [homozygous p.(Pro194Ser)] is a two-year-old boy and the first child of healthy consanguineous Egyptian parents. First clinical signs were noted after the age of seven months when he showed regression of developmental milestones. Tonic seizures with cyanosis and myoclonic seizures occurred at the age of one year. He has severe DD/ID with no speech as well as autistic symptoms with repetitive head movements and secondary microcephaly. He did not achieve walking. Further neurological signs include muscular hypotonia, hyperreflexia and excessive abnormal movements.

Individual 4

Individual 4 [compound heterozygous p.(Cys6Leufs*19), p.(Phe341Leu)] is an eleven-year-old boy and the first child of healthy non-consanguineous German parents. After birth, he presented with muscular hypertonia and reduced mobility of the left side. The first epileptic spasms occurred at the age of 11 months leading to the diagnosis of West syndrome. He showed severe DD/ID with absent speech and inability to walk, frequent uncoordinated movements, and secondary microcephaly. cMRI imaging at the age of 10 months revealed delayed myelination but subsequent neuroimaging at age six years showed normal age-appropriate findings. A muscular biopsy performed at the age of one year showed slightly enlarged and dense mitochondria without impairment of mitochondrial function (Fig. 1).

Individual 5

Individual 5 [homozygous p.(Met1?)] is a six-year-old girl and the second child of healthy consanguineous Bangladeshi parents. She showed severe to profound DD/ID with absent speech and inability to walk, microcephaly, muscular hypotonia, continuous discrete movement of the limbs and first seizure at age 6 months.

Genotypic spectrum and structural modeling

Three different missense variants, one start-loss variant and one truncating variant have been observed in this cohort.

Individuals 1.1, 1.2 and 2 carry the homozygous variant p.(Arg141Trp). Individuals 1.1 and 1.2 are siblings. The CHKA structure indicates that Arg141 is in the vicinity of the ADP binding site and stabilizes the structure by forming hydrogen bonds to Pro130 and Thr133 [Fig. 2B(i)]. These interactions cannot be formed by the uncharged aromatic Trp141 sidechain in the variant [Fig. 2B(ii)] thereby causing destabilization close to the ADP binding site.

In case of the variant p.(Pro194Ser) identified in individual 3, a similar destabilization is assumed. The variant is also located near the ADP binding site what may lead to steric clashes resulting from the altered sidechain geometry of Ser194 in the protein [Fig. 2B(iii and iv)].

Individual 4 carries two compound-heterozygous variants p.(Phe341Leu) and p.(Cys6Leufs*19). The variant p.(Cys6Leufs*19) likely leads to a complete loss of the allele, likely through NMD[17]. The residue Phe341 affected by the missense variant p.(Phe341Leu) is part of a hydrophobic cluster that forms the choline binding site [Fig. 2B(v)]. A change to Leu results in a loss of hydrophobic interactions [Fig. 2B(vi)], which are expected to destabilize the structure and the interaction with choline.

Individual 5 carries the homozygous start-loss variant p.(Met1?). *CHKA* has no known alternative start codons in other transcripts. The second next possible start codon occurs at amino acid position 123, potentially removing around 26% percent of the protein, and may therefore significantly impair gene expression and protein function.[18]

The variants p.(Arg141Trp) and p.(Pro194Ser) have each been observed once in a heterozygous state in the gnomAD database.[19] For individual 4, both variants p.(Phe341Leu) and p.(Cys6Leufs*19) are absent in the gnomAD database and also no variants affecting the initiation codon have been observed (last accessed September 2021). All missense variants affect highly conserved amino acid residues and multiple *in silico* tools predict a pathogenic effect (Supplemental Table S2 and Figure S1).

Expression of human *CHKA* in yeast

To investigate the functional significance of these variants, we assessed the effect of the individual-derived variants on *CHKA* catalytic activity. To do so we expressed the *CHKA* ORF, and the patient-derived alleles encoding these variants, from a constitutive promoter in a *S. cerevisiae* strain devoid of endogenous choline kinase activity.

Western blots showed that *CHKA* and each patient-derived variant was expressed in yeast cells at their projected molecular weight of 46 kDa and at comparable levels (Fig. 2C). Choline kinase activity for variants p.(Arg141Trp) and p.(Pro194Ser) was 20 % and 15 %, respectively, of the activity for wild type *CHKA* (Fig. 2C). These two variants were identified in homozygosity (individuals 2-4). For variant p.(Phe341Leu) catalytic activity was reduced by half. This variant is present in compound heterozygosity with a frameshift variant in the *CHKA* gene on the alternate allele (individual 4), implying a total CHKA activity of ~25% in this individual.

Discussion

We present six individuals with bi-allelic variants in *CHKA* and establish a novel neurodevelopmental disorder of the Kennedy pathway. All affected individuals presented with a consistent phenotype of a neurodevelopmental disorder characterized by severe DD/ID, seizures starting in the first years of life, and microcephaly.

Individuals 1.1, 1.2 and 2 carry the same homozygous missense variant p.(Arg141Trp). While the phenotype was similar in almost all aspects, such as severe ID and occurrence of epileptic spasms in early infancy, individual 1.2 achieved independent walking at age three years as the only one in the cohort. What caused this difference in developmental course in this individual is unclear.

Functional testing of the variants in an *S. cerevisiae* model revealed a marked reduction of enzymatic activity ranging between 15-20% of wild-type activity for the missense variants p.(Arg141Trp) and p.(Pro194Ser). The missense variant p.(Phe341Leu) showed a reduction by half and is in a compound-heterozygous state with the frameshift variant p.(Cys6Leufs*19) that likely leads to nonsense-mediated decay mRNA transcribed from this allele. Therefore net enzyme activity is assumed to be around 25 % in this individual, which is on a comparable level to the homozygous missense variants. The functional consequence of p.(Met1?) affecting the initiation codon of *CHKA* cannot be as readily assessed.[18] But (1) considering the consistent phenotype of individual 5 compared to the rest of the cohort, (2) the absence of an alternative start codons in other transcripts of *CHKA* and (3) the next possible methionine start codon AUG occurring at amino acid position 123, a loss-of-function mechanism and disease causality for this variant is highly likely.

Our structural *in silico* modeling of missense variants supports the assumption of reduced enzymatic activity. The variants are located near the binding sites of ATP/ADP [p.(Arg141Trp), p.(Pro194Ser)] and choline [p.(Phe341Leu)] and are therefore suggested to impair enzymatic function through structural changes or destabilization of these regions.

Compared to the other disorders described for genes of the Kennedy pathway, DD/ID and seizures seem particularly prominent in individuals of the *CHKA* cohort. When compared with its paralog *CHKB*, affected individuals also show a neurodevelopmental disorder, but seizures have rarely been reported. Severity of DD/ID ranges between mild to severe and recently, pathogenic variants in *CHKB* have been associated with autism spectrum disorder and atypical Rett syndrome.[5,20] The abnormalities on muscle biopsy include muscular dystrophy as well as mitochondrial enlargement and placement in the periphery of muscle fibers. The muscular biopsy of individual 4 in this study also showed mitochondrial abnormalities with dense matrix and regular cristae, but mitochondria were evenly distributed throughout the cell. Even though CHKA and CHKB share a similar molecular structure and catalyze the same reaction in PC/PE biosynthesis, the phenotypic differences might be explained by different expression patterns throughout different tissues in the body.[21] Nevertheless, no tissue specificity could be observed concerning mRNA expression or presence of protein in the cytosol.[22] Most pathogenic variants known for *CHKB* are truncating variants leading to a complete loss of enzymatic function. In animal models, homozygous *Chkb(-/-)* mice presented with progressive muscular weakness similarly observed for the human phenotype. In comparison, *Chka(-/-)* is embryonically lethal in mice implying complete loss of CHKA activity is not compatible with vertebrate life.[23] *Chka(+/-)* mice showed a reduction of choline kinase activity of approximately 30% and appeared to be without obvious behavioral abnormalities although this has not been explored in detail.

Interestingly, a screen of 1,566 mouse lines identified 198 genes whose disruption yielded neuroanatomical phenotypes, with *Chka(+/-)* mice among these 198 mouse lines.[24] These observations in mice reinforce the assumption that decreased *CHKA* function through bi-allelic recessive inheritance can lead to neurological phenotypes in humans.

The phenotypic spectrum of the *CHKA* cohort also resembles, in part, to the other disorders of the Kennedy pathway. The overlap between the phenotypes associated with *CHKA*, *SELENOI*, and *PCYT2* comprises DD/ID, microcephaly, short stature, visual impairment, seizures, hyperreflexia, abnormalities of muscle tone, and movement disorder (Supplemental Table 3).[8,9,25–27] Particularly noteworthy are the eye abnormalities observed in individual 3: nystagmus, diffuse retinal pigmentary epithelium, severe conduction dysfunction and moderate retinal dysfunction in both eyes. A retinal phenotype of cone-rod dystrophy and macular pigmentary changes was described for *PCYT1A* and *SELENOI*.[7,8]

Taken together, our findings establish bi-allelic variants in *CHKA* as a novel cause of a neurodevelopmental disorder with epilepsy and microcephaly adding to the description of genetic disorders associated with the Kennedy pathway.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

References

1. Gibellini F, Smith TK. The Kennedy pathway-De novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life*. Published online 2010:n/a-n/a. doi:10.1002/iub.337

2. Aoyama C, Yamazaki N, Terada H, Ishidate K. Structure and characterization of the genes for murine choline/ethanolamine kinase isozymes alpha and beta. *J Lipid Res*. 2000;41(3):452-464.

3. Liao H, Aoyama C, Ishidate K, Teraoka H. Deletion and alanine mutation analyses for the formation of active homo- or hetero-dimer complexes of mouse choline kinase-alpha and -beta. *Biochim Biophys Acta*. 2006;1761(1):111-120. doi:10.1016/j.bbalip.2006.01.005

4. Mitsuhashi S, Ohkuma A, Talim B, et al. A congenital muscular dystrophy with mitochondrial structural abnormalities caused by defective de novo phosphatidylcholine biosynthesis. *Am J Hum Genet*. 2011;88(6):845-851. doi:10.1016/j.ajhg.2011.05.010

5. Bardhan M, Polavarapu K, Bevinahalli NN, et al. Megaconial congenital muscular dystrophy secondary to novel CHKB mutations resemble atypical Rett syndrome. *J Hum Genet*. Published online March 12, 2021. doi:10.1038/s10038-021-00913-1

6. Sher RB, Aoyama C, Huebsch KA, et al. A rostrocaudal muscular dystrophy caused by a defect in choline kinase beta, the first enzyme in phosphatidylcholine biosynthesis. *J Biol Chem*. 2006;281(8):4938-4948. doi:10.1074/jbc.M512578200

7. Hoover-Fong J, Sobreira N, Jurgens J, et al. Mutations in PCYT1A, encoding a key regulator of phosphatidylcholine metabolism, cause spondylometaphyseal dysplasia with cone-rod dystrophy. *Am J Hum Genet*. 2014;94(1):105-112. doi:10.1016/j.ajhg.2013.11.018

8. Ahmed MY, Al-Khayat A, Al-Murshedi F, et al. A mutation of EPT1 (SELENOI) underlies a new disorder of Kennedy pathway phospholipid biosynthesis. *Brain*. 2017;140(3):547-554. doi:10.1093/brain/aww318

9. Vaz FM, McDermott JH, Alders M, et al. Mutations in PCYT2 disrupt etherlipid biosynthesis and cause a complex hereditary spastic paraplegia. *Brain*. 2019;142(11):3382-3397. doi:10.1093/brain/awz291

10. Rickman OJ, Baple EL, Crosby AH. Lipid metabolic pathways converge in motor neuron degenerative diseases. *Brain*. 2020;143(4):1073-1087. doi:10.1093/brain/awz382

11. Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. *Hum Mutat*. 2015;36(10):928-930. doi:10.1002/humu.22844

12. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405-424. doi:10.1038/gim.2015.30

13. Hong BS, Allali-Hassani A, Tempel W, et al. Crystal structures of human choline kinase isoforms in complex with hemicholinium-3: single amino acid near the active site influences inhibitor sensitivity. *J Biol Chem*. 2010;285(21):16330-16340. doi:10.1074/jbc.M109.039024

14. Malito E, Sekulic N, Too WCS, Konrad M, Lavie A. Elucidation of human choline kinase crystal structures in complex with the products ADP or phosphocholine. *J Mol Biol*. 2006;364(2):136-151. doi:10.1016/j.jmb.2006.08.084

15. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*. 1997;18(15):2714-2723. doi:10.1002/elps.1150181505

16. Sayle RA, Milner-White EJ. RASMOL: biomolecular graphics for all. *Trends Biochem Sci*. 1995;20(9):374. doi:10.1016/s0968-0004(00)89080-5

17. Popp MWL, Maquat LE. Organizing principles of mammalian nonsense-mediated mRNA decay. *Annu Rev Genet*. 2013;47:139-165. doi:10.1146/annurev-genet-111212-133424

18. Abou Tayoun AN, Pesaran T, DiStefano MT, et al. Recommendations for interpreting the loss of function PVS1 ACMG/AMP variant criterion. *Hum Mutat*. 2018;39(11):1517-1524. doi:10.1002/humu.23626

19. Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*. 2020;581(7809):434-443. doi:10.1038/s41586-020-2308-7

20. Kutluk G, Kadem N, Bektas O, Eroglu HN. A Rare Cause of Autism Spectrum Disorder: Megaconial Muscular Dystrophy. *Ann Indian Acad Neurol*. 2020;23(5):694-696. doi:10.4103/aian.AIAN_98_19

21. Tavasoli M, Lahire S, Reid T, Brodovsky M, McMaster CR. Genetic Diseases of the Kennedy Pathway for Phospholipid Synthesis. *J Biol Chem*. Published online 22 2020. doi:10.1074/jbc.REV120.013529

22. Uhlén M, Fagerberg L, Hallström BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. 2015;347(6220):1260419. doi:10.1126/science.1260419

23. Wu G, Aoyama C, Young SG, Vance DE. Early embryonic lethality caused by disruption of the gene for choline kinase alpha, the first enzyme in phosphatidylcholine biosynthesis. *J Biol Chem*. 2008;283(3):1456-1462. doi:10.1074/jbc.M708766200

24. Collins SC, Mikhaleva A, Vrcelj K, et al. Large-scale neuroanatomical study uncovers 198 gene associations in mouse brain morphogenesis. *Nat Commun*. 2019;10(1):3465. doi:10.1038/s41467-019-11431-2

25. Horibata Y, Elpeleg O, Eran A, et al. EPT1 (selenoprotein I) is critical for the neural development and maintenance of plasmalogen in humans. *J Lipid Res*. 2018;59(6):1015-1026. doi:10.1194/jlr.P081620

26. Kaiyrzhanov R, Wortmann S, Reid T, et al. Defective phosphatidylethanolamine biosynthesis leads to a broad ataxia-spasticity spectrum. *Brain*. 2021;144(3):e30. doi:10.1093/brain/awaa442

27. Vélez-Santamaría V, Verdura E, Macmurdo C, et al. Expanding the clinical and genetic spectrum of PCYT2-related disorders. *Brain*. 2020;143(9):e76. doi:10.1093/brain/awaa229

Figure legends

Figure 1 Muscle biopsy of individual 4 and an aged-matched control. The histochemical analysis revealed a prominent mitochondrial patterning. The scale bar represents 20 μm.: (A) H&E staining, in the sarcoplasm basophilic dots indicate dense and enlarged mitochondria in the affected individual. (B) cytochrome c oxidase staining, the mitochondria are slightly increased in size compared to the control (C) succinate dehydrogenase staining, mitochondria are evenly distributed., (D) Electron microscope (without aged-matched control), the scale bar represents 300 nm. Mitochondria show dense matrix and regular cristae. Due to the high electron density, the mitochondria appear prominent**.** The arrows indicate the broadened cristae by material of higher density.

Figure 2 Overview on location of variants, structural modeling, and functional testing in an *S. cerevisiae* model. (**A**) Location of variants in *CHKA* with respect to reported domain structure.[14] (**B**) Structural effect of *CHKA* sequence variants. (**i**) Arg141 forms stabilizing hydrogen bonds (black dotted lines) with Pro130 and Thr133 in the vicinity of the ADP binding site. Interacting residues and ADP are shown in stick presentation (atom-type coloring) and are labelled. The CSKH protein backbone is depicted as cyan ribbon. (**ii**) Trp141 cannot form the stabilizing hydrogen bonds to Pro130/Thr133 and adopts a different sidechain orientation. Color coding as in (**i**). (**iii**) Pro194 (grey) is located in a turn close to the ADP binding site. (**iv**) The Ser194 sidechain causes steric problems (indicated as red arrow) with the adjacent Ile209 thereby destabilizing the structure. (**v**) Phe341 (grey) is part of a hydrophobic cluster close to the binding site of phosphocholine (PC; shown as sticks). Residues of the hydrophobic cluster are shown in space-filled presentation. (**vi**) The presence of the nonaromatic Leu341 results in a loss

of hydrophobic interactions (denoted as red circle). (**C**) Western blot of human CHKA expressed in yeast demonstrates that each allele was expressed at a similar level. Pgk1 is the loading control. Choline kinase activity of each allele, normalized to the level of CHKA expressed, was determined. It was determined that each patient-derived allele possessed reduced choline kinase activity.

Table 1 Clinical information on individuals with bi-allelic variants in *CHKA*

Individual ID	**1.1**	**1.2**	**2**	**3**	**4**	**5**
Genomic position (NC_000011.9)	g.67864527G>A	g.67864527G>A	g.67864527G>A	g.67842234G>A	g.67888631dup, g.67833357A>G	g.67888643A>G
cDNA (NM_001277.2)	c.421C>T	c.421C>T	c.421C>T	c.580C>T	c.14dup, c.1021T>C	c.2T>C
Protein alteration (NP_001268.2)	p.(Arg141Trp)	p.(Arg141Trp)	p.(Arg141Trp)	p.(Pro194Ser)	p.(Cys6Leufs*19), p.(Phe341Leu)	p.(Met1?)
Zygosity	Homozygous	Homozygous	Homozygous	Homozygous	Compound-heterozygous	Homozygous
Consanguinity	Yes	Yes	Yes	Yes	No	Yes
Age at last assessment	9y	9y	3y 3m	2y 1m	11y 5m	6y
Sex	Male	Female	Male	Male	Male	Female
Microcephaly	Yes (42 cm, − 7 SD)	Yes (45 cm, − 4 SD)	Yes (44 cm, − 6 SD)	Yes (41,8 cm, − 5 SD)	Yes (49,6 cm, − 3.3 SD)	Yes (44,5 cm, − 6 SD)
Short stature	Yes	Yes	Yes (82 cm, − 4 SD)	No	No	Yes (106 cm, − 2.3 SD)
Global developmental delay / Intellectual Disability	Severe	Severe	Severe	Severe/profound	Severe	Severe/profound
Gross motor delay	No walking achieved	Assisted walking since age 3 years	No walking achieved	No walking achieved	No walking achieved	No walking achieved
Speech and language	No speech	No speech	No speech	No speech	No speech	No speech
Seizures						
Epileptic encephalopathy	Yes	Yes	Yes	Yes	Yes	Yes
Age at seizure onset	Infancy	Infancy	3y 2m	1y	<1y	0y 6m
Seizure type at onset	Epileptic spasms	Epileptic spasms	Epileptic spasms	Generalized seizures	Epileptic spasms	Generalized seizures
Further seizure type	Focal and generalized seizures	Focal and generalized seizures		Myoclonic seizures	Tonic-clonic seizures	Tonic seizures
Brain MRI	Normal	Normal	Normal	Deep white matter hypomyelination, thin corpus callosum, faint increased signal intensity in lentiform nucleus at T-FLAIR	Hypomyelination of occipital white matter, initially pronounced (age 15 months), normal MRI at follow-up (age 6 years)	Not done
Movement Disorder	None	None	None	Dyskinesia, rigidity	Dyskinesia	choreoathetotic movements
Hyperreflexia	Yes	Yes	Yes	Yes	Yes	Unknown
Muscle tone	Hypertonia	Hypertonia	Hypotonia	Hypotonia	Hypertonia	Hypotonia
Additional Symptoms	Scoliosis, nystagmus, aggressive behavior	Scoliosis, nystagmus, aggressive behavior	Hyperactivity, self injurious behaviour	Autistic behavior, nystagmus, high arched palate, poor visual acuity, cortical visual loss, diffuse retinal pigment epithelium, moderate retinal dysfunction, dysmorphic facial features	Scoliosis, myopia, recurrent kidney stones	Aggressive behavior, poor sleep, feeding problems

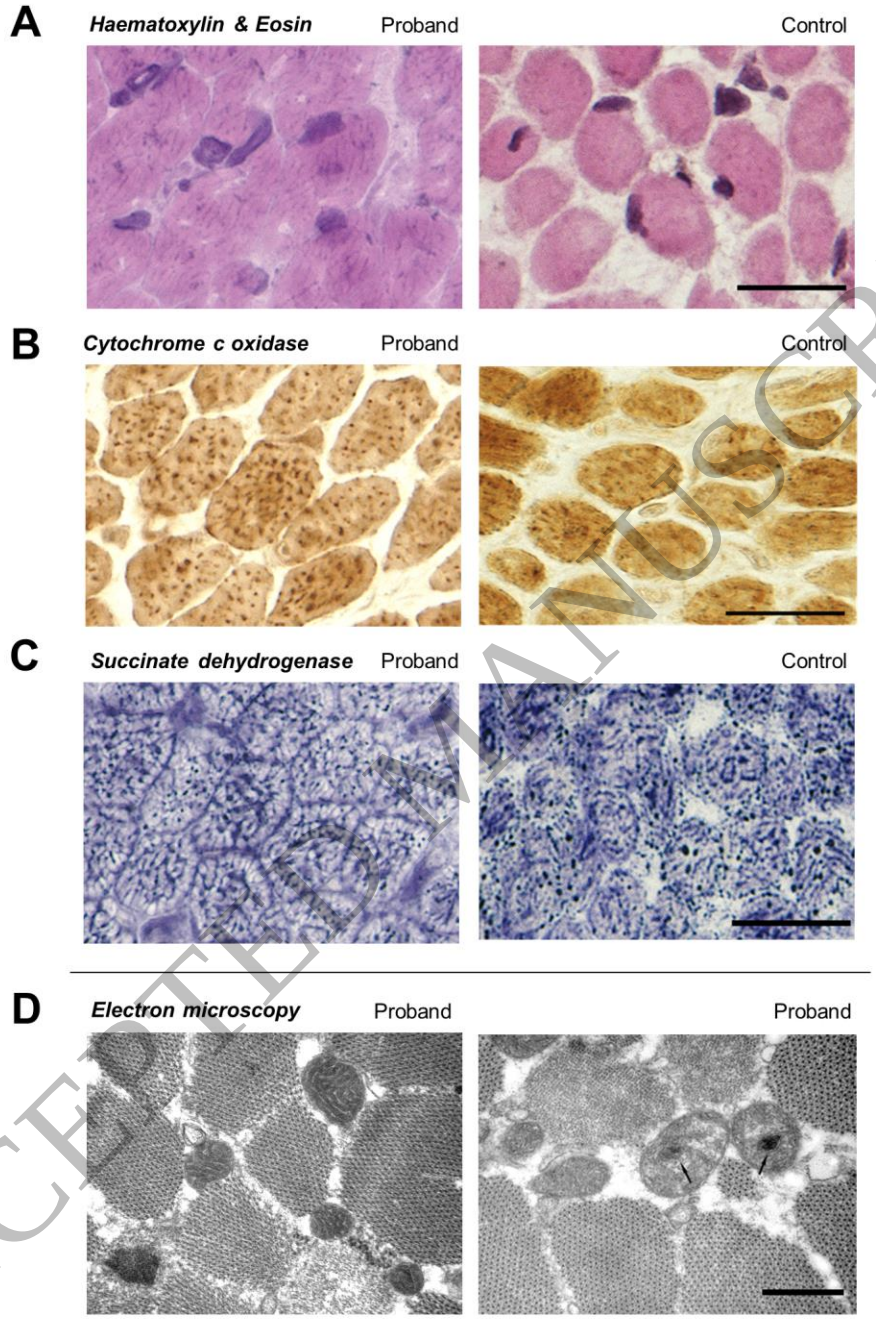

Figure 1
150x200 mm (0.5 x DPI)

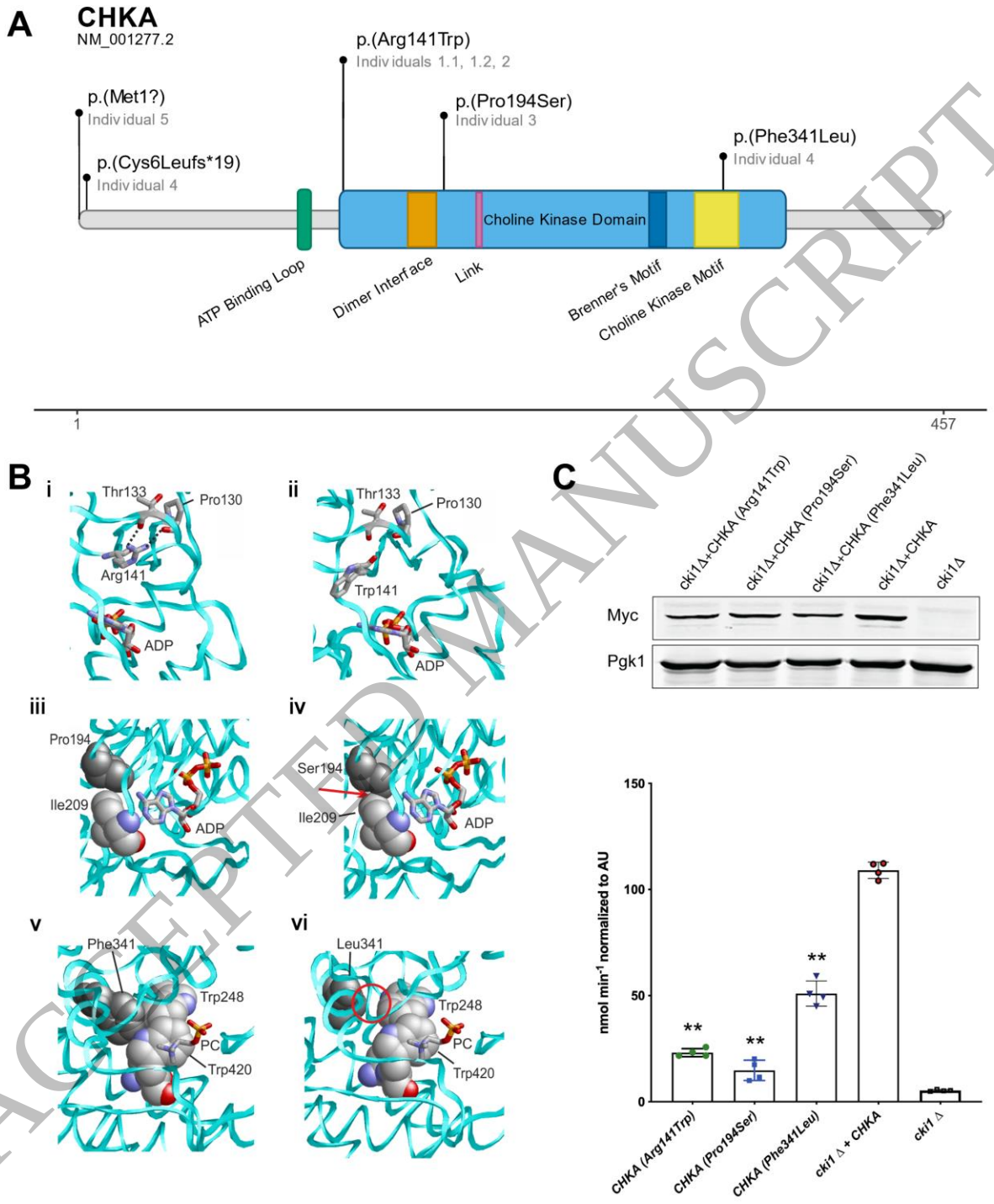

Figure 2
165x206 mm (0.5 x DPI)