

Mutations in Citron Kinase Cause Recessive Microlissencephaly with Multinucleated Neurons

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1 Mutations in Citron-Kinase a cause of autosomal recessive micro-lissencephaly with

- 2 multinucleated neurons.
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26 Abstract:

27 Primary microcephaly is a neurodevelopmental disorder caused by a reduction in brain size 28 attributed to defects in early neurogenesis. Mutations in numerous genes encoding proteins that 29 localize to the mitotic spindle and centrosome are implicated in the pathogenicity of primary 30 microcephaly. In contrast, involvement of the contractile ring and midbody required for cleavage 31 furrow constriction and abscission have not previously been implicated in primary microcephaly. 32 Citron Kinase (CIT), a component of the mitotic contractile ring, is a multi-domain protein that 33 localizes to the cleavage furrow and midbody of mitotic cells, where it is required for the 34 completion of cytokinesis. Rodent models of *Cit* deficiency highlighted the role of this gene in 35 neurogenesis and microcephaly over a decade ago. Here we identify recessively inherited 36 pathogenic variants in CIT as the genetic basis of severe microcephaly and neonatal death. We 37 present postmortem data showing that CIT is critical to build a normally sized human brain. 38 Consistent with cytokinesis defects attributed to CIT, multinucleated neurons are observed 39 throughout the cerebral cortex and cerebellum of an affected proband, expanding our 40 understanding of mechanisms attributed to primary microcephaly.

41 **Main**:

Primary 42 microcephaly is a genetically heterogeneous neurodevelopmental disorder characterized by a severe reduction in brain growth¹. This decreased brain volume often stems 43 44 from a primary defect in neurogenesis. The cerebral cortex is composed of neurons born from 45 neural progenitor cells (NPCs) that reside adjacent to the lateral ventricle during early 46 neurodevelopment². The mitotic machinery of NPCs is critical not only for rapid expansion of 47 the progenitor pool required for normal brain growth, but also for maintaining the balance 48 between proliferation and differentiation. Human genetics has identified many primary 49 microcephaly genes which impact the fidelity of mitotic spindle placement and centrosome stability required to build a human brain of normal size ³⁻⁸. Surprisingly, other steps in mitosis 50 51 have not been implicated by neurogenetics in the pathophysiology of microcephaly, despite a 52 series of rodent models showing that cleavage furrow placement, constriction of the contractile ring and abscission by the midbody are critical for neurogenesis ⁹⁻¹¹. 53

54

55 CIT is a multi-domain protein that localizes to the cleavage furrow and midbody where it functions in cytokinesis and abscission, the final steps of mitosis (Fig. 1a) ^{12,13}. CIT facilitates 56 57 protein-protein interactions between contractile ring components (with anillin, actin, myosin and 58 RhoA) and has a N-terminal kinase domain, both of which are critical for abscission ^{12,14,15}. 59 Studies in drosophila and mammals demonstrate the evolutionarily conserved function of CIT in 60 cytokinesis, with mutations that disrupt abscission resulting in binucleate cells. The impact of 61 cytokinesis defects on brain development and size are evident in the Cit knockout mouse and 62 the Flathead rat with a spontaneous nonsense mutation in Cit exon 1. In rodents Cit is critical 63 for proliferation of NPCs and male germ cell precursors. Cit null animals are characterized by testicular hypoplasia, microcephaly, ataxia, growth deficiencies and lethal epilepsy ^{10,11}. These 64 65 phenotypes have been linked to cytokinesis defects and the presence of multinucleated cells 66 throughout the cortex and cerebellum. Premature differentiation of early NPCs and widespread

apoptosis are compounded across development, resulting in a cerebral cortex and cerebellum
less than 50% the size of normal brain. Cortical hypoplasia and layer disorganization are
predicted to account for the recurrent spontaneous seizures and premature death in the mice ¹⁶.
Roles for *CIT* in human brain development and primary microcephaly have been predicted but
not yet reported.

72

73 Here we describe three independent families, each with multiple affected children who 74 presented with severe micro-lissencephaly associated with neonatal mortality (Fig. 1b). The 75 clinical features of the probands are provided in Table 1. Egyptian Family A is consanguineous 76 with two microcephalic male siblings. The first affected child died in the neonatal period (Fig. 77 1b). Proband A was delivered at term and weighed 2600 grams (-2SD) consistent with 5th 78 percentile for growth. Head circumference, not measured until 3.5 months of age, was 27cm (-79 11 to -12 SD below the age/sex mean). His length at 3.5 months was 53 cm (-4 to -5 SD), and 80 he continued to experience failure to thrive thereafter. Dysmorphisms were noted including 81 hypotelorism, sloping forehead and large ears (Fig. 2a). He exhibited axial hypotonia, upper and 82 lower extremity hypertonia, increased deep tendon reflexes and lack of head support. 83 Microcephaly was confirmed by MRI, which also revealed lissencephaly, enlarged ventricles, 84 agenesis of the corpus callosum, cerebellar hypoplasia and brainstem hypoplasia (Fig. 2b). T2 85 hyperintensity was also noted throughout the white matter consistent with spastic tetraplegia.

86

Family B has two affected children born to first cousin parents from United Arab Emirates. Male Proband B was noted by ultrasound at 30 gestational weeks (GWs) to have microcephaly, intrauterine growth retardation, and oligohydramnios. Echocardiogram indicated cardiomegaly, biventricular dilatation and tricuspid regurgitation. He was delivered by spontaneous vaginal delivery at 39 GWs with a head circumference of 24cm, 8 standard deviations below the age/sex mean, and a body length measurement of 27.2cm crown-rump (2.5 SD below the mean) consistent with 36 GWs. Despite Apgar scores of 8 at one minute, and 9 at 5 minutes, he
died the following day.

95

96 At birth, the brain weight of Proband B was 40g (10% the average newborn brain weight of 97 390q). The two plate-like brain hemispheres were lissencephalic, separated by shallow Sylvian 98 fissures. Corpus callosum agenesis, large ventricles and small well-delineated basal ganglia 99 and thalami were noted (Fig. 2c-e). These structural defects were accompanied by brain stem 100 and cerebellum hypoplasia with small cerebellar folia (Fig. 3a). Dysmorphic facial features were 101 noted including prominent occiput, absent fontanelle, large ears, wide nasal bridge, prominent 102 upper lip, highly arched palate, cloudy corneas, right-sided single palmar crease and 103 hypoplastic nails. There was renal aplasia with few primitive glomerular and tubular structures 104 embedded in connective tissue and absence of ureters and bladder. These features were 105 consistent with those observed during the pregnancy and birth of a previously affected female 106 infant from the same pedigree who presented with microcephaly and renal agenesis, and 107 survived only 4 hours following birth (Fig. 1b).

108

109 Family C is French and had no reported consanguinity. Proband C was born at full term with 110 weight 2900g (-1 SD), length 46cm (- 2 SD), and a head circumference of 30cm (-3.5 SD) 111 consistent with a diagnosis of microcephaly. Proband C has a sloping forehead, prominent 112 nose, and relatively large ears. His neurological examination was remarkable for upper and 113 lower limb hypertonia and brisk tendon reflexes in the lower limbs. He walked independently at 114 18 months of age. He has developmental delay with moderate to severe ID and mild autistic 115 features. No metabolic, ERG or EEG abnormalities were detected. By age 10 years, his head 116 circumference was 6.5SD below the age/sex mean and an MRI performed at 10 years of age 117 showed a simplified gyral pattern and hypoplastic cerebellum (Fig. 2f-g). A second child in this

family (II:2) was also confirmed to have microcephaly by fetal MRI at 29 GWs (Fig. 2h-i). The
biparietal diameter measured from the fetal MRI at that time was consistent with 23 GWs.

120

121 Whole exome sequencing (WES) and candidate gene screening were employed to identify a 122 molecular diagnosis for these probands. Following WES for Family A (mother, father, proband 123 and unaffected sibling), bioinformatics filtering removed common variants (minor allele 124 frequency (MAF) >0.005). Annotated rare variants analyzed according to a consanguineous 125 recessive mode of inheritance identified a homozygous G>A transition (c.1111+1 G>A; g. chr12: 126 120,260,623, hg19) disrupting the splice donor site of exon 9 in CIT (NM_007174) (Fig. 1c). 127 Sanger sequencing confirmed the presence of this mutation and its appropriate segregation in 128 the family, with both parents being heterozygous carriers. In silico analysis of the exonic and 129 intronic sequence flanking the c.1111+1 G>A nucleotide variant was evaluated with Human 130 Splicing Finder (HSF) and identified cryptic splice donor sites 54 bp 5' of and 2 bp 3' of the 131 abolished WT site (Supp. Fig 1a). A minigene reporter assay was used to assess alternative 132 splicing. CIT WT and c.1111+1 G>A variant exon 9 were cloned into the pCAS2 reporter 133 plasmid between pseudoexons A and B. Plasmids were transfected into HeLa cells to test cryptic splice site usage ¹⁷. Sequencing the RT-PCR products confirmed that both cryptic splice 134 135 donors are alternatives, with the splice site 2 bp 3' of the WT site most frequently used (Supp. 136 Fig 1b,c). This major splice product results in a premature stop mutation in exon 10 that is 137 predicted to undergo nonsense mediated decay. The spliced CIT transcript generated from the 138 54 bp 5' splice donor is a minor product and generates an 18 amino acid in-frame deletion in the kinase domain of CIT (CIT^{-18aa}). The stability of mutant CIT^{-18aa} has not been evaluated, but 139 140 could disrupt kinase activity.

141

142 Resequencing of *CIT* coding exons and splice junctions in 35 probands with primary 143 microcephaly identified two additional patients, Probands B and C, with recessive pathogenic 144 variants. A homozygous pathogenic mutation consistent with consanguinity was identified in 145 Proband B. This 10bp deletion in exon 2 of CIT, creates a premature stop codon after 15 146 codons (NM 007174; c.29 38delATCCTTTGGA; g.chr12:120,313,935-120,313,944 hg19; 147 p.Asn10Metfs*15) and is predicted to function as a null allele (Fig. 1a,d). Proband C and Subject 148 II:2 of Family C were found to carry compound heterozygous CIT variants. Both variants are 149 located in the kinase domain; one generates a nonsense variant in exon 4 (c.412 C>T; g. 150 chr.12: 120,295,329 hg19; p. Gln138*) and one generates a missense variant in exon 5 (c.473 151 C>G; g. chr12: 120,288,021 hg19; p. Pro158Arg) (Fig. 1a,f). The p. Pro158Arg may express a 152 partially functional protein that ameliorates the severity in the patient, compared with the 153 homozygous null phenotypes in Probands A and B.

154

155 The CIT mutations presented here were absent from online genomic databases, including the 156 1000 Genomes Project, National Heart, Lung and Blood Institute (NHLBI) ESP6500, dbSNP 141, and the Exome Aggregation Consortium (ExAC)¹⁸⁻²⁰. Bioinformatic variant annotation 157 158 using SeattleSeq Variation Annotation revealed that the point mutations in our probands affect 159 highly conserved bases with high GERP and CADD scores that are predicted to be pathogenic 160 (Table 1) ^{21,22}. Further analysis of the genetic variation in *CIT* showed that loss-of-function 161 alleles are infrequent. Fifteen heterozygous and no homozygous nonsense or frameshift alleles 162 were present in the ExAC browser, and each had a frequency of <0.001. This suggests high 163 evolutionarily pressure against these alleles and supports the deleteriousness of the CIT 164 variants. These findings correspond to a Residual Variation Intolerance Score (RVIS) of -2.35 165 indicating CIT is in the top 1.14% of genes in the genome intolerant to common functional 166 genetic variation ²³.

167

168 Given the well established role for CIT in cytokinesis, our three cases implicate a novel 169 pathogenic mechanism for microcephaly, a disorder previously linked primarily to gene products 170 that localize to the centrosome or mitotic spindle ²⁴. Severe microcephaly with widespread 171 multinucleated neurons is characteristic of the structural and cellular pathology observed in the 172 *Cit* knockout mouse and the *Flathead* rat (Supplemental table 1)^{10,11}. In accordance with 173 institutional research board and ethics committee approval, post-mortem analysis of Proband B 174 allowed the human cell and molecular neuropathology associated with pathogenic variants in 175 CIT to be analyzed. All non-neural tissue examined had normal cytology, but multinucleated 176 neurons were observed throughout the neuraxis of Proband B, a hallmark of cytokinesis defects 177 (Supplemental table 2). Microscopically, the profoundly microcephalic cerebral cortex showed 178 both cytological and organizational abnormalities in many areas. The neocortex was excessively 179 thick, with the six cortical layers replaced by a molecular layer and two broad layers comprised 180 of loosely and irregularly scattered neurons (Fig. 2j,k). The underlying white matter was 181 unmyelinated and contained scattered ectopic neurons. The overlying leptomeninges were 182 greatly thickened with reticulin and collagen fibers, prominent vascularity, and heterotopic 183 astrocytes and neurons, some of which were multinucleated (Fig. 2I). The hippocampi were 184 dysplastic, small and under-rotated, with hypoplastic dentate gyri (Fig. 2m-o). Cortical 185 hypoplasia and layer disorganization account for the recurrent spontaneous seizures associated with premature death in rodent models ¹⁶. Extrapolating from these models, the cytoarchitectural 186 187 abnormalities observed in Proband B are consistent with cytokinesis defects that contribute to 188 reduced neuronal precursor proliferation, multinucleation of neurons and increased cell death. 189 Despite the phenotypic similarities, pathogenic *CIT* variants have not been detected in epilepsy 190 cohorts, including the Epi4K exome sequence collection of 264 epileptic encephalopathy trios ²⁵. 191

The cerebellar cortex of Proband B was hypoplastic and dysplastic (Fig. 3). Laminar disorganization was more evident in the hemispheres than the vermis, where folia were fused and Purkinje cells were observed in multilayered islands interspersed by abnormal granule cell domains (Fig. 3b-h). At birth, the external granule cell layer (EGL) is usually compact, with 4-6 rows of bipolar cells abutting the molecular layer (ML) (Fig. 3b,d,g). In Proband B the EGL was wider, the ML narrower, and were merged with the Purkinje cell layer (Fig. 3g-h). In this case the EGL was comprised of 2 or 3 compact rows of cells overlying a looser band of horizontally oriented, thin, elongated cell bodies, some clearly binucleate (Fig. 3e,f,h). Purkinje cells had short simplified dendritic arborization compared to controls and many were multinucleated (Fig. 3g-i). Likewise, the internal granular layer (IGL) was severely hypo-cellular (Fig. 3g-h).

202

203 In summary, we report the identification of pathogenic variants in CIT as a genetic basis for 204 primary microcephaly and cerebellar hypoplasia, with phenotypic features remarkably similar to 205 Cit^{-/-} rodent models. These findings highlight the evolutionarily conserved function of CIT in 206 neurodevelopment and the disproportionate sensitivity of the neuroaxis to pathogenic variants in 207 this gene. Multinucleated neurons are rarely observed in the nervous system, apart from their 208 occurrence in ganglion cell tumors. These genetic findings implicate novel mechanisms in the 209 pathogenesis of primary microcephaly and allow us to describe the human presentation of this 210 very interesting multinucleated neuronal phenotype.

211

212 Materials and Methods

Growth Standards The Child Growth Standards published by World Health Organization were applied for weight, height and head circumference assessment. These standards can be found at http://www.who.int/childgrowth/standards/en/.

216

DNA Extraction DNA was extracted from peripheral blood lymphocytes for Family A and C using Qiagen Blood DNA extraction kit according to manufacturer's instructions. Proband B DNA was extracted from formalin-fixed, paraffin-embedded brain tissue using Qiagen QIAamp DNA FFPE Tissue Kit (Catalog Number 56404) per the manufacturer's instructions. Three independently extracted Proband B DNA samples were analyzed. Extracted DNA was quantified with the use of Life Technologies Qubit dsDNA BR Assay Kit (Catalog NumberQ32850) and a Qubit 2.0 Fluorometer.

224

Whole exome sequencing Quad family WES was performed for family A, including Proband A, 225 226 father, mother and unaffected sibling. The exome was captured with in-solution Agilent 227 SureSelect All Exon XT2 50 Mb Kit and sequenced with an Illumina HiSeg 2000 instrument at 228 Beijing Genomics Institute, resulting in 97X coverage of more than 99% of the targeted exons. 229 SNPs and indels were mapped and called with the SOAPAligner/SOAP2-SOAPSnp-BWA-230 GATK BGI analysis pipeline ^{26,27}. Reads were mapped against hg19, with an average of 65457 231 SNPs and 4455 Indels called per individual. Annotation, ranking, and stringent filtering of 232 genetic variants was performed to exclude non-pathogenic variants with a MAF > 0.005 in 233 dbSNP, 1000 Genomes, EVS or an in-house database. Nonsynonymous rare variants were 234 filtered against reference databases and as potential candidates. Databases used for annotation 235 include: CCDS (https://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi), RefSeq 236 (http://www.ncbi.nlm.nih.gov/refseg/), Ensembl (www.ensembl.org), Encode and 237 (www.encodeproject.org). Variants were analyzed according to a recessive mode of inheritance. 238

239 Candidate gene analysis PCR primers for coding region amplification were designed using 240 ExonPrimer from the UCSC Genome Bioinformatics Genome Browser 241 (http://genome.ucsc.edu/cgi-bin/hgGateway) for assembly hg19. For Proband B, PCR reactions 242 were prepared with Promega GoTag Green Master Mix (Catalog #M712B), and products were 243 cleaned up with Affymetrix ExoSAP-IT (Product Number 78201). The 46 coding exons of CIT on 244 chromosome 12 were screened by dideoxy sequence analysis on an ABI 3730 sequencer 245 (Applied Biosystems, Life Technologies), and sequence data was analyzed with Sequencher 246 5.1 (Gene Codes Corporation). Microcephaly gene panel testing for Proband C was paired with 247 RainDance microdroplet PCR and 2X150bp sequencing with Illumina MiSeq. Deep sequencing

(142X) reads were mapped to hg19 with MiSeq analysis software and BWA-GATK with 99%
coverage. Variants were filtered against MAF (>0.5%), dbSNP, 1000 Genomes, EVS and an inhouse dataset. Rare variants were annotated for functional features of coding nucleotides with
publically available databases outlined for WES.

252

253 Online Databases CIT variants identified were absent from 1000 Genomes Project 254 (http://browser.1000genomes.org), National Heart, Lung and Blood Institute (NHLBI) ESP6500, 255 NHLBI Sequencing (ESP) Exome Exome Project Variant Server 256 (http://evs.gs.washington.edu/EVS/), dbSNP 141 (http://www.ncbi.nlm.nih.gov/projects/SNP/) 257 and ExAC Browser (http://exac.broadinstitute.org/). SeattleSeg Variation Annotation 138 258 (http://snp.gs.washington.edu/SeattleSeqAnnotation138/) was used to generate GERP 259 (Genomic Evolutionary Rate Profiling) conservation scores and CADD (Combined Annotation 260 Dependent Depletion) scores. Alternative splicing was investigated with Human Splicing Finder 261 version 3.0 (http://www.umd.be/HSF3/HSF.html). Residual Variation Intolerance Score was 262 generated using http://genic-intolerance.org

263

264 Mini-gene Construction and Splicing Assay

265 Functional analysis of splicing abnormalities caused by c.1111+1 G>A was performed using an 266 *in vitro* minigene splicing assay according to previously published guidelines ¹⁷. *CIT* exon 9 and 267 approximately 150 bp of flanking intronic sequences were amplified from a control DNA sample 268 and then cloned in the pCAS2 vector. The c.1111+1 G>A substitution was subsequently 269 introduced using site-directed mutagenesis and analyzed for specific splicing abnormalities 270 following transfection in HeLa cells. Forty-eight hours after transfection, transcriptional analysis 271 was performed and the effect on splicing identified using RT-PCR and further confirmed using 272 direct Sanger sequencing and agarose gel electrophoresis.

Immunohistochemistry Brain sections were examined with hematoxylin-eosin. Selected
sections were stained with Kluver-Barrera, and immunostained for GFAP, 1:400 (Dako,
Carpenteria, CA, #M0761), phosphorylated neurofilament NF-P, 1:10 (clone TA51, gift of Dr. J.
Trojanowski, Hospital of University of Pennsylvania, PA), synaptophysin 1:100 (Dako,
monoclonal #M7315) and calbindin 1:100 (monoclonal, Novocasta).

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Table 1. Genetic and major clinical features

	Proband A	Proband B	Proband C	Subject II:2 of Family C
	Chr12: 120,260,623	Chr12: 120,313,935 - 120,313,944	Chr12: 120,295,329 and Chr12: 120,288,021	Chr12: 120,295,329 and Chr12: 120,288,021
<i>CIT</i> variant (hg19, NM_007174)	c.1111+1 G>A	c.29_38delATCCTTTGGA	c.412 C>T and c.473 C>G	c.412 C>T and c.473 C>G
		p. Asn10Metfs*15	p. Gln138* and p. Pro158Arg	p. Gln138* and p. Pro158Arg
GERP Score	5.900	N/A	4.660 and 5.280	4.660 and 5.280
CADD Score	31.000	N/A	38.000 and 27.800	38.000 and 27.800
Gender	Male	Male	Male	Male
Gestational Length	Full Term	Full Term	Full Term	Pregnancy terminated at GW 29+2
Birth Weight	2.6 kg (-2 SD)	1.730 kg (-4 SD)	2.92 kg (-1 SD)	N/A
Birth Length	Unknown (home birth)	27.2 cm (crown-rump -2.5 SD)	46 cm (-2 SD)	N/A
Birth Head Circumference (HC)	Unknown (home birth)	24 cm (-8 SD)	30 cm (-3.5 SD)	N/A
HC at Most Recent Evaluation	27 cm (-11 to -12 SD) measured at 3.5 months	N/A	43 cm (-6.5 SD) measured at 10.5 years	N/A
Brain Abnormalities	MRI revealed micro- lissencephaly, agenesis of corpus callosum, cerebellar and brainstem hypoplasia, and T2 hyperintensity of the whole white matter	Autopsy revealed micro- lissencephaly, absent corpus callosum, hindbrain and cerebellum hypotrophy, cerebral cortex hypotrophy, and comparatively large ventricles. Detailed neuropathology included in the main text.	Microcephaly (MRI not performed)	Fetal MRI revealed a microcephaly, biparietal diameter equivalent to a fetus of 23 WG, and gyration equivalent to a fetus of 26 WG
Neurological Findings	Upper and lower extremity hypertonia, axial hypotonia, increased DTRs, and spastic tetraplegia	N/A	Upper and lower extremity hypertonia, and brisk DTRs for the lower extremities	N/A
Seizures	No	N/A	No	N/A
Intellectual Disability	Not assessed	N/A	Moderate to severe ID with mild autistic features	N/A
Development	Delayed	N/A	Delayed	N/A
Dysmorphisms	Hypotelorism, sloping forehead, and relatively large ears	Prominent occiput, absent fontanelle, large ears, wide nasal bridge, prominent upper lip, high arched palate, cloudy corneas, a right-sided sinlge palmar crease and small nails	Sloping forehead, prominent nose, and relatively large ears	N/A
Status	Unknown	Deceased; one day after birth	Alive	Terminated pregnancy



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357 Figure 1 Autosomal recessive CIT variants in micro-lissencephaly. (a) Protein domain 358 organization of CIT showing pathogenic coding variants relative to protein domains. (b) 359 Pedigrees of the families investigated with probands noted. (c) Chromatographs and schematic 360 of the WT reference CIT exon 9/intron boundary, homozygous Proband A splice donor variant 361 (c.1111+1 G>A) and cryptic splice donor site two bases downstream (boxed). Parents and 362 unaffected sibling are heterozygous carriers. (d) Chromatograms defining the homozygous 363 10bp (c.29_38delATCCTTTGGA) deletion in *CIT* exon 2 (chr12:120,313,935-120,313,944) 364 amplified from Proband B that creates a stop after 15 codons (not shown). (e) Chromatographs 365 and schematic of reference CIT exon 4 and exon 5 and the corresponding nonsense (c.412 366 C>T; p. Gln138*) and missense (c.473 C>G; p. Pro158Arg) variants identified in Proband C.



Figure 2. Structural and cellular neocortical phenotypes. (**a**) Proband A displays a microcephalic cranium, sloping forehead, wide nasal bridge and hypotelorism. (**b**) T2-weighted axial magnetic resonance imaging (MRI) of Proband A at 3.5 months. Cerebral cortical size is markedly reduced with simplification of gyral folding and enlarged ventricles (red asterisks). (**c**)

372 Coronal section of newborn control brain. Width is in centimeters (cm) and lateral ventricles 373 marked by red asterisks. (d) Lateral view of lissencephalic newborn brain from Proband B (scale 374 in cm), (e) Coronal section through mid-thalamus of Proband B brain showing enlarged lateral 375 ventricles (red asterisks). (f) Coronal brain MRI of Proband C at 10 years of age showing 376 microcephaly, simplified gyral pattern and enlarged ventricles (red asterisks). (g) Mid-sagittal 377 Proband C MRI showing sloping forehead, hypoplastic brainstem and cerebellum (red arrow) 378 (h) Coronal and (i) axial fetal brain MRI of affected subject II:2 of Family C at 29 GWs. Reduced 379 brain volume, gyrification and enlarged ventricles (red asterisks) were noted. (i) Control and (k) 380 Proband B cerebral cortex stained with hematoxylin and eosin (H&E). Compared to control (j) 381 leptomeninges (LM; blue arrow) are excessively thick and contiguous with molecular layer (ML; 382 pink arrow) in proband (k). Cortex (CX; dark purple arrow) is thickened and cortical layering 383 obscure when compared with control (j). (I) High magnification of Proband B cortex stained with 384 Kluver-Barrera. Disorganized parenchyma includes many multinucleated neurons: inserts show 385 detail of multinucleated (red double arrowheads) cells labeled 1,2,3. (m-n) Kluver-Barrera 386 stained sections of control (m) and Proband B (n) temporal lobe. The proband hippocampus (n) 387 is very small with hypoplastic pyramidal layer (red asterisk) and greatly reduced dentate gyrus 388 (arrowheads). Image of normal control hippocampus (m) imaged at half the magnification to 389 allow for comparison of overall structure of the hippocampus. (o) Detail of Proband B dentate 390 gyrus (red box in H), showing binucleated granule cells (double arrowheads).



392 Figure 3 Structural and cellular cerebellar CIT phenotypes. (a) Section though Proband B 393 midbrain with external view of hypoplastic cerebellum. (b-f) histologic sections of cerebellum, 394 stained H&E. Cerebellar folia are poorly developed and the cortex disorganized in Proband B 395 (c,e & h). Cerebellar lamination in Proband B is disrupted with clustered Purkinje cells (PC) 396 interspersed with granule cells (GC) within fused folia (c, e) as compared to control cerebellum 397 (b, d). (f) Many GC appear binucleated (double blue arrowheads). (g-j) Cerebellum 398 immunostained for calbindin shows abnormally thick EGL, reduced ML (blue line) and 399 multilayered collections of small PCs (arrows) with stubby irregular dendritic processes in

- 400 Proband B (h), as compared with control (g). (i-j) Dashed boxes are enlarged to show
- 401 binucleated PC (blue arrow heads) within a small soma in Proband B tissue (j) as compared to
- 402 controls (i). EGL=external granule layer, IGL=internal granule layer, ML=molecular layer

403 Supplementary Data

а

chr.12: 120,260,608-120,260,684 - CIT Exon 9 Intron boundary





Supplemental Figure 1. Alternative splicing between *CIT* exon 9 and pseudo-exon B (exon B) in minigene assay. Minigene splicing assay was performed according to previously published guidelines ¹⁷. (a) Schematic of *CIT* sequence across the exon-intron border of *CIT* exon 9. WT (pink nucleotides) and cryptic splice sites (blue, orange and green nucleotides and asterisks) 410 were delineated and scored with Human Splice Finder (HSF). Exonic sequence is boxed and 411 upper case. Intronic sequence is lower case. Proband A c.1111+1 G>A substitution (red 412 arrowhead) breaks the WT splice donor site. (b) CIT exon 9 and approximately 150 bp of 413 flanking intronic sequence was amplified from genomic DNA and cloned into the pCAS2 vector 414 between pseudo- exon A and exon B. The c.1111+1 G>A substitution was introduced using 415 site-directed mutagenesis. Forty-eight hours after transfection in HeLa cells, alternative spliced 416 mRNA transcripts from empty (V), WT CIT exon 9 (WT), and c.1111+1 G>A CIT exon 9 (Mut) 417 pCAS2 vectors were amplified by RT-PCR. (c) Differentially spliced products were detected by 418 size separation on an agarose gel. Each product is labeled with a corresponding composition of 419 the various splice products. Transcript composition was confirmed by Sanger sequencing. With 420 loss of the WT splice donor, alternative splice transcripts were generated from nearby cryptic 421 splice sites with the highest HSF scores. The major product included exon 9 along with an 422 additional four base pairs of intron 9 (green asterisk and exon). The minor product includes only 423 a portion of exon 9 with 54 base pairs excluded (blue asterisk and exon).

424 425 Supplemental Table 1. Mammalian models of null mutations in CIT.

			1
Species	Proband B	<i>Cit^{/-}</i> Knockout Mouse (Di Cunto et al. 2000)	<i>Flathead fh/fh</i> Mutant Rat (Sarkisian et al. 2002) (Ackman et al. 2007)
Citron Kinase Mutation	10 bp deletion in exon 2	Conditional excision exon 2 by <i>Cre</i> mediated homologous recombination	Spontaneous G/C bp deletion in exon 1
Predicted Mutational Impact	Frameshift causing a premature stop codon 25 amino acids from start site	Premature stop codon	Frameshift causing a premature stop 27 amino acids from start site
CIT Transcript	Predicted NMD	NMD	NMD
Brain Size and Weight	Microcephalic and ~1/10 of the brain weight of an average newborn	Microcephalic 50% brain weight reduction	50% brain size reduction
Cerebral Cortex Abnormalities	Cortex shows low cellularity and disorganized three-layer arrangement. Presence of multinucleated neurons.	40% reduction in cerebral cortex thickness. Disorganized lamination of 6- layer cortex. Presence of binucleated cells.	Neocortex displays a reduced number of neurons but normal layering. Presence of binucleated neurons in neocortex.
Cerebellar Abnormalities	Global hypotrophy and disrupted laminar architecture. Crowded layer of Purkinje cells with simple dendrite projections. Binucleated Purkinje cells and granule cells.	70% cerebellum size reduction. Crowded layer of Purkinje cells with simple dendrite projections. Presence of binucleated cells.	Cerebellum displays a reduced number of neurons. Presence of binucleated neurons.
Hippocampal Abnormalities	Small and disorganized Ammon's horns. Only a small remnant of dentate fascia. Binucleated granule cells.	Normal Ammon's horn cell density and lamination. Essentially absent dentate gyrus.	Dentate gyrus displays a reduced number of neurons. Presence of binucleated neurons.
Additional Locations of Multinucleated Cells	Thalamus, striatum, brain stem and spinal cord	Thalamus	Striatum, thalamus, midbrain, hindbrain, and spinal cord
Other Phenotypoic Abnormalities	Kidney and heart defects, facial dysmorphisms, and rotated lower limbs	Ataxia, seizures, and failure to thrive	Seizures and disrupted development of the retina

426 427

NMD=Nonsense mediated decay

428 **Supplemental Table 2.** Presence of binucleated neurons by anatomic area

Presence of binucleated neurons by anatomic area				
neocortex				
hippocampus (pyramidal and granule cells)				
thalamus				
striatum (large and small cells)				
pallidum				
cerebellum	cerebellar cortex (Purkinje and granule cells)			
	dentate nucleus			
brain stem	midbrain tectum			
	oculomotor nuclei			
	pontine reticular formation			
	nuclei pontis			
	abducens nucleus			
	inferior olive			
	red nucleus			
spinal cord & PNS	anterior horn			
	autonomic ganglion			

429 PNS- Peripheral nervous system



- 430
- 431 **Supplementary Figure 2:** Multinucleated neurons throughout the neuraxis of the Proband B.
- 432 (A) Thalamus, (B) Spinal cord anterior horn. Cells indicated by arrowheads are enlarged in the
- 433 inserts and multinucleated.