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Supplemental Data

Mutations in *CRADD* Result in Reduced Caspase-2-Mediated Neuronal Apoptosis and Cause Megalencephaly with a Rare Lissencephaly Variant

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SUPPLEMENTAL NOTE: CASE REPORTS

LR00-150. This 51-year-old woman was born in the United States to parents of Finnish ancestry. She had a normal birth history and early motor milestones, but her language development was moderately delayed. She eventually learned to talk in complete sentences, and attended self-contained special education classes throughout childhood. Spells of marked aggressive behavior (screaming, hitting, throwing objects) began by 2 years that lasted a few minutes up to about a half hour as recalled by her younger sister, who is now her legal guardian. These were eventually diagnosed as seizures and seizure medications began, although the nature of the spells was never certain.

As an adult, her health has been generally good except for type 2 diabetes and hypothyroidism. She has lived in a series of nursing homes, largely because of her episodic very aggressive behavior. In between these spells, her sister describes her as friendly and caring. She speaks in complete sentences, with language skills estimated as similar to a 9- or 10-year-old child, and can follow most commands. She does not read or follow local or national events. Her legal and financial concerns were managed by her parents and more recently by her sister. Her spells have been somewhat less frequent as an adult. A diagnosis of schizophrenia has been suggested, again with unclear basis. A recent occipitofrontal circumference (OFC) was 60.9 cm (+3.6 standard deviations, SD). She was enrolled in our research study at 36 years, at which time review of brain MRI demonstrated the “thin” variant of lissencephaly (TLIS) (**Figures S2 A–C**).

LR02-006. This 18-year-old male was born at 37 weeks gestation following a pregnancy complicated by pre-eclampsia at ~34 weeks gestation. His birth weight was 2.8 kg (+0.1 SD) and length 48.6 cm (-0.3 SD). His head circumference at 2 weeks was 36 cm (+1.21 SD). Inguinal hernias were repaired at 6 weeks bilaterally. When evaluated at 1-2 years, he had global developmental delay, hypotonia, and significant behavior problems consisting of short attention span, distractibility, tactile sensitivity, and echolalia. Brain MRI first performed at 5 years (**Figures S2 D–F**) demonstrated TLIS.

He had a generalized convulsive seizure at 11 years. Repeat brain MRI demonstrated a tumor in the left lateral ventricle. Following resection, pathological examination was consistent with an atypical choroid plexus papilloma. He had no further seizures. At 18 years he still lives with his parents and is enrolled in school, where he attends mainstream classes without grades or exams. Testing at school was consistent with mild intellectual disability. He has no external anomalies, and has been in good general health. Chromosome microarray using the Illumina Human660W-Quad BeadChip detected a 3.07 Mb deletion in 12q21.33q22 (chr12:92443579-95515465; hg38) that included 12 defined genes (*DLEU2_6*, *BTG1* [MIM: 109580], *CLLU1* [MIM: 616988], *PLEKHG7*, *EEA1* [MIM: 605070], *NUDT4* [MIM: 609229], *UBE2N* [MIM: 603679], *MRPL42* [MIM: 611847], *SOCS2* [MIM: 605117], *CRADD*, *PLXNC1* [MIM: 604259], *CEP83* [MIM: 615847]) and numerous other transcripts.[1] Several are oncogenes such as *BTG1*, *CLLU1* and *SOCS2*, but none have been linked to choroid plexus papilloma (MIM: 260500).[2-4] Duplications of this region, but not deletions, have been associated with choroid plexus papilloma.[5; 6]

LR04-101a1. The eldest of two sisters (see also LR04-101a2 below), this individual was born to non-consanguineous Mennonite parents. Pregnancy, labor and delivery were uneventful and she was recalled to have normal birth measurements. Her development was delayed with walking at 16 months. Although she spoke her first word at 10-12 months, her language development plateaued after seizure onset at 12 months. EEG showed slow posterior rhythm (4-5 Hz) and frequent bursts of polyspike, spike and slow wave discharges at a frequency of about 3 Hz lasting from 3 to 5 seconds associated with eyelid fluttering. She was diagnosed with generalized non-convulsive seizures. At 17 years she had mild intellectual disability with more severely impaired speech, as well as self-abusive and aggressive behavior. Her behavior anomalies required frequent psychiatric care.

LR04-101a2. The younger sister also had normal pregnancy, labor and delivery histories. Her development was better than her older sister. She walked at 13 months and used about ten words by 2 years. Her seizures began at approximately 18 months, and were associated with an EEG pattern similar to her sister's (LR04-101a1), so she was also diagnosed with generalized non-convulsive epilepsy. She had no loss of developmental skills with onset of seizures. Her behavior was normal. She had mild intellectual disability. At age 13 years she attended a special school and could manage her everyday tasks with minimal help.

LR05-279a1. This boy and his affected sister (see LR05-279a2 below) were born to Turkish parents who were first cousins. The older boy was born at 38 weeks gestation after a normal pregnancy with birth weight 3.76 kg (+1.03SD) and length 51 cm (-0.04SD). OFC at 10 weeks was 42.8 cm (+2.4SD).

He had early global developmental delay, but improved and is able to speak in complete sentences. He has had no seizures and his intellectual disability was described as mild. Brain MRI performed at 12 years of age demonstrated TLIS (**Figures S2 G–I**). He was enrolled in a special education program and at 23 years was still living with his parents. He was employed in a sheltered workshop, and required a legal guardian but minimal help with everyday life.

LR05-279a2. His younger sister was born at 40 weeks gestation following a normal pregnancy with normal birth measurements and OFC at 90th centile. OFC at 8 weeks was 42.6 cm, (+3.4 SD). She was more severely affected than her brother. In addition to TLIS, she had a Chiari malformation type I (MIM: 118420) that led to hydrocephalus and required an emergency shunt at 13 years. She spoke in short sentences using simple words. She also had scoliosis that worsened significantly during puberty and required bracing, bilateral hallux valgus, and myopia (-2 diopters). She received special education and did not graduate from high school. At 21 years, she worked in a sheltered workshop and required extra help with even the simplest of everyday tasks (meals, shower, etc.). Both siblings had overlapping but nonspecific minor anomalies including broad forehead, thick and horizontal eyebrows, and wide nasal bridge. None of the other individuals with TLIS *CRADD* mutations shared the same facial gestalt.

LR15-293a1-a7. All affected members of this previously reported family[7] underwent detailed neuropsychological evaluation and showed mild-borderline (IQ 70-80) to mild (IQ 55-70) intellectual disability with highly non-homogenous ability profiles. An example of brain MRI from this family, demonstrating mild TLIS, is presented in **Figures S2 J–L** (Subject LR15-293a1;

male at 19 years). A detailed clinical report of this family (LR15-293) will be published elsewhere.

SUPPLEMENTAL FIGURES

Figure S1A

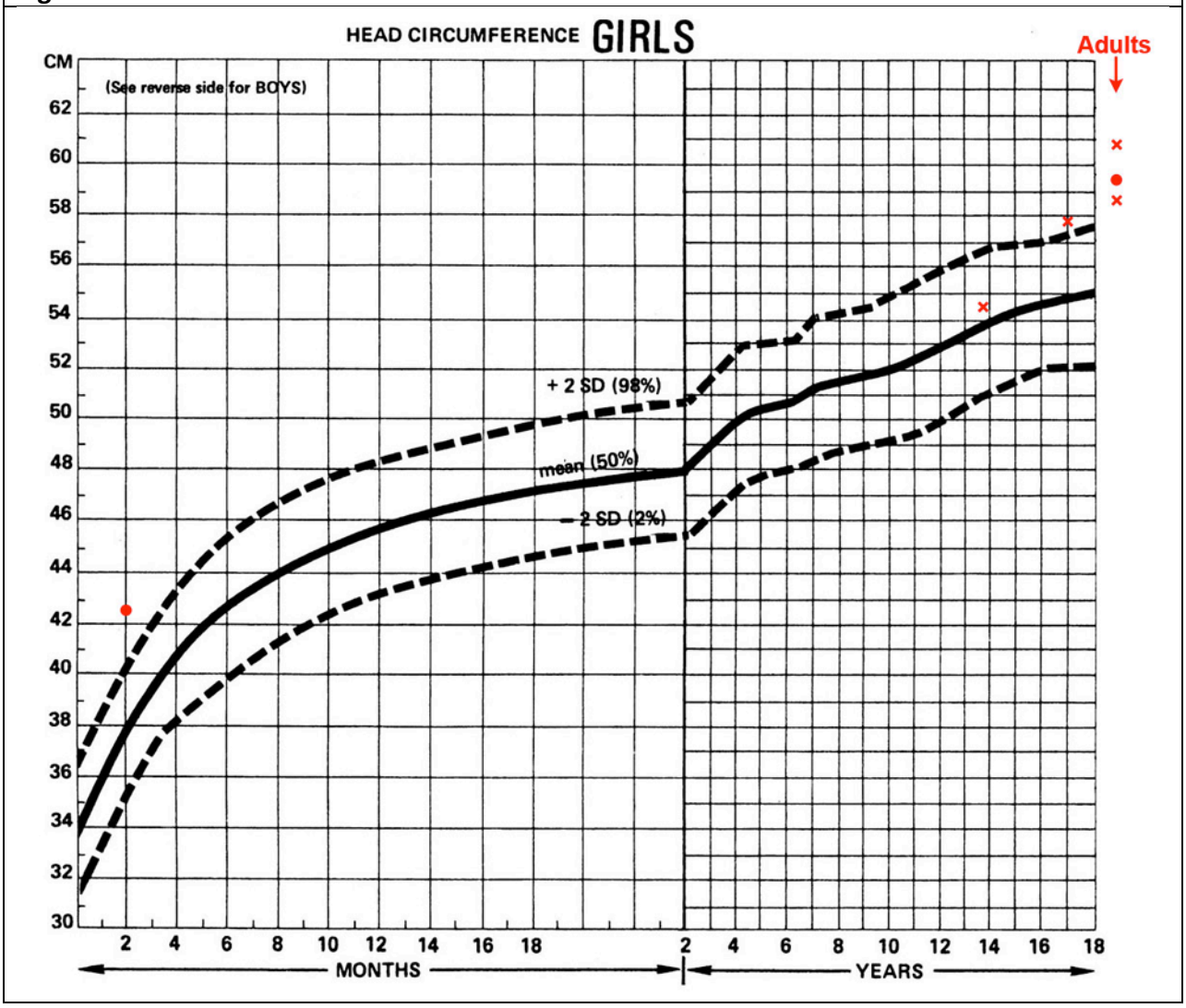


Figure S1B

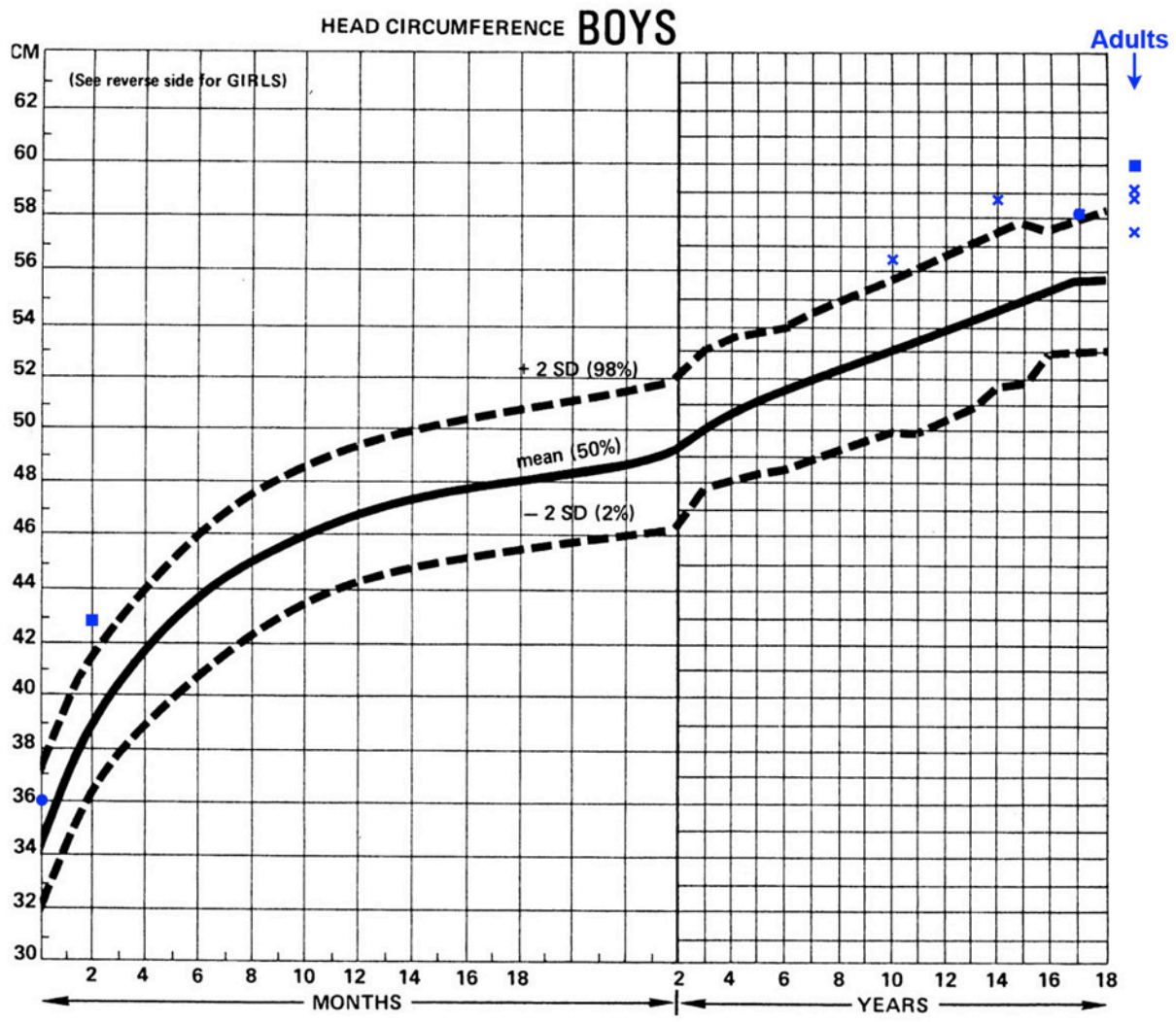

HEAD CIRCUMFERENCE BOYS
(See reverse side for GIRLS)
CM
62
60
58
56
54
52
50
48
46
44
42
40
38
36
34
32
30
+ 2 SD (98%)
mean (50%)
– 2 SD (2%)
Adults
2 4 6 8 10 12 14 16 18
MONTHS
2 4 6 8 10 12 14 16 18
YEARS

Figure S1. Occipitofrontal Circumference (OFC) Measurements in Five Females (A) and Seven Males (B) with TLIS and *CRADD* Mutations.

Four of five females and seven of eight males have mild or true megalencephaly (see **Table S1**). One female with OFC near the mean at 13 years has relative megalencephaly when corrected for height, which is 2 SD below the mean. Standard deviations (SD) were calculated based on Nellhaus charts.[8] True megalencephaly was defined as OFC >3 SD above the mean, mild megalencephaly as OFC 2-3 SD above the mean, and relative megalencephaly as OFC 2 SD or more greater than height SD.[9] Serial measurements in three subjects showed either stable (blue squares for LR05-279a1, and red circles for LR05-279a2) or progressive (blue circles for LR02-006) megalencephaly.

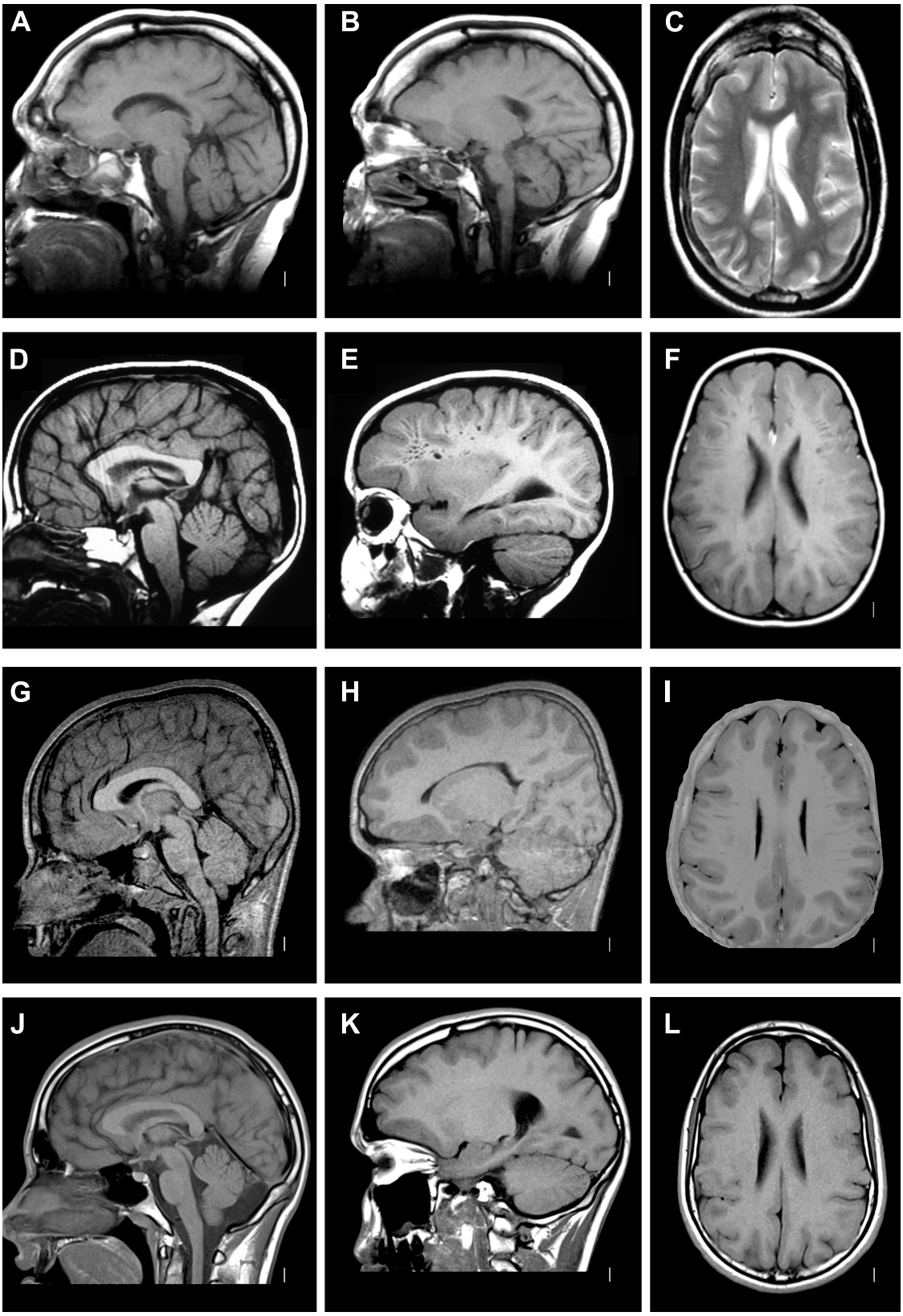


Figure S2

Figure S2. Additional Brain Images of TLIS Individuals with *CRADD* Mutations

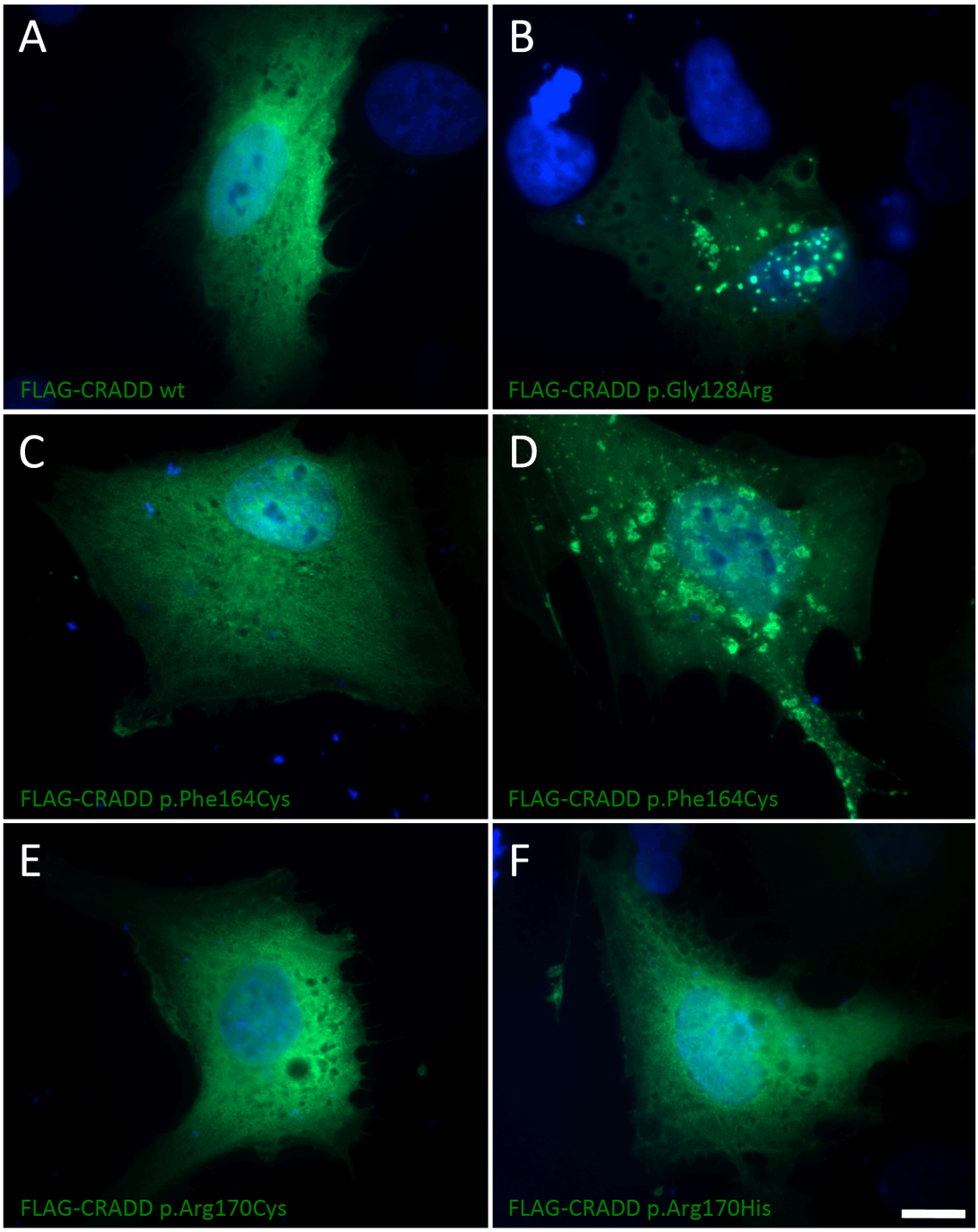
(A-C) TLIS subject LR00-150 at 38 years. Note normal brainstem and cerebellum (A), frontal pachygyria with reduced number of gyri, shallow sulci and mildly thickened cortex (B, C).

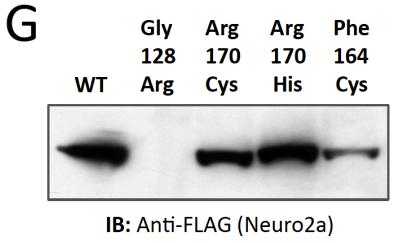
(D-F) TLIS subject LR02-006 at 5 years showing normal brainstem and cerebellum with mildly thickened corpus callosum (D), pachygyria pattern identical to LR00-150 (E, F). Note enlarged perivascular spaces (E).

(G-I) TLIS subject LR05-279a1 at 12 years demonstrating pachygyria and thickened cortex in a pattern overlapping with the TLIS subjects described above (A-F). Note relatively thickened corpus callosum (G).

(J-L) TLIS subject LR15-293a1 at 19 years showing normal midsagittal scan (J), significantly simplified gyration with frontal predominance (K and L) and mildly thickened cortex (mild thin pachygyria). The overall appearance is similar but mild relative to other TLIS subjects.

MRI image section planes: A-B, D-E, G-H, J-K, sagittal T1-weighted images; C, axial T2-weighted image; F, I, L, axial T1-weighted images.


A
FLAG-CRADD wt
B
FLAG-CRADD p.Gly128Arg
C
FLAG-CRADD p.Phe164Cys
D
FLAG-CRADD p.Phe164Cys
E
FLAG-CRADD p.Arg170Cys
F
FLAG-CRADD p.Arg170His


G
Gly 128 Arg
Arg 170 Cys
Arg 170 His
Phe 164 Cys
WT
IB: Anti-FLAG (Neuro2a)

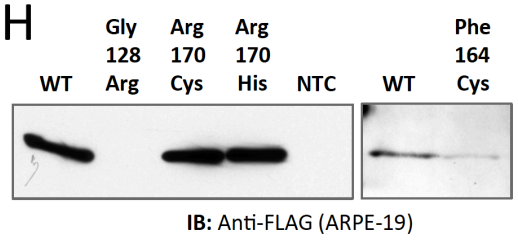

H
Gly 128 Arg
Arg 170 Cys
Arg 170 His
Phe 164 Cys
WT
NTC
WT
IB: Anti-FLAG (ARPE-19)

Figure S3

Figure S3. Overexpression of TLIS CRADD Variants *in vitro*

(A–F) Overexpression of FLAG-CRADD WT and TLIS variants in human ARPE-19 cells *in vitro*. FLAG-CRADD WT (A), p.Phe164Cys (C), p.Arg170Cys (E), and p.Arg170His (F) (each in green) localized relatively uniformly to the cytosol and nucleus (DAPI – blue). FLAG-CRADD p.Gly128Arg (B) characteristically formed distinct clusters in the cytosol and nucleus. Similar clusters were occasionally observed in cells overexpressing FLAG-CRADD p.Phe164Cys (D). Scale bar in F = 10 μm in A–F. (N=3).

(G–H) Immunoblots (IB) of recombinant FLAG-CRADD WT and TLIS variants overexpressed in (G) Neuro2a cells (N=3) and in (H) ARPE-19 cells (N=3). NTC – non-transfected control.

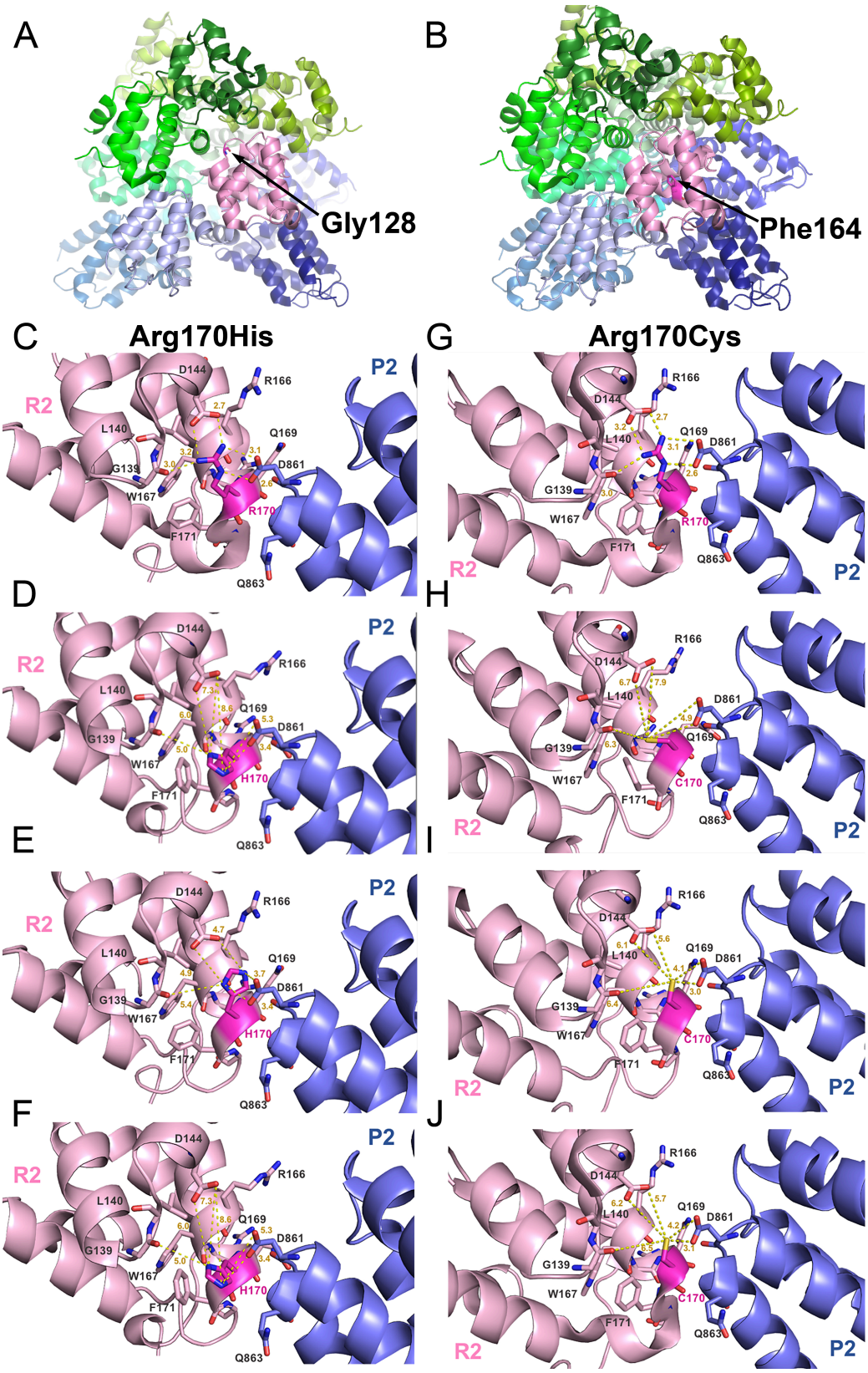

A
Gly128
B
Phe164
C
Arg170His
G
Arg170Cys
D
H
E
I
F
J
R2
P2
D144
R166
L140
Q169
G139
D861
W167
R170
H170
C170
F171
Q863

Figure S4

Figure S4. CRADD-DD and PIDD-DD Interaction in Protein Models of the PIDDosome Incorporating TLIS CRADD Amino Acid Substitutions

(A) Gly128 within the CRADD Death Domain (DD) has no direct contact with any residues of the PIDD-DD; CRADD-DD with Gly128 in sticks (highlighted with a circle) is shown in pink, the other CRADD-DDs are depicted in green and PIDD-DDs in blue.

(B) Phe164 is located internal to the CRADD-DD and has no direct contact with other CRADD or PIDD residues; CRADD-DD with Phe164 in sticks shown in pink, other CRADD-DDs in green and PIDD-DDs in blue.

(C–F) CRADD:PIDD interaction in the presence of the p.Arg170His substitution; CRADD-DD (pink) and PIDD (blue) are displayed as cartoons. The wild-type Arg170 (magenta) and all residues within 4Å are shown as sticks. (C) Arg170 forms several polar contacts (yellow dashes) with CRADD and PIDD residues. (D–F) Three protein models of the amino acid substitution p.Arg170His generated by SWISS-MODEL (D), IRECS (E), and PyMOL mutagenesis wizard (F). In each model, the mutated His170 residue is located too far from neighboring CRADD and PIDD residues to form polar contacts like those predicted for Arg170 in WT CRADD.

(G–J) CRADD:PIDD interaction in the presence of the p.Arg170Cys substitution. (G) is similar to (C) but shows a different aspect of the PIDD residues interacting with Arg170 in WT CRADD. (H–J) Three protein models of the amino acid substitution p.Arg170His generated by SWISS-MODEL (H), IRECS (I) and PyMOL mutagenesis wizard (J). Cys170 is predicted to fail to form a salt bridge with Asp861 of PIDD and to be too distant from the other residues to form polar contacts like those predicted for Arg170 in WT CRADD.

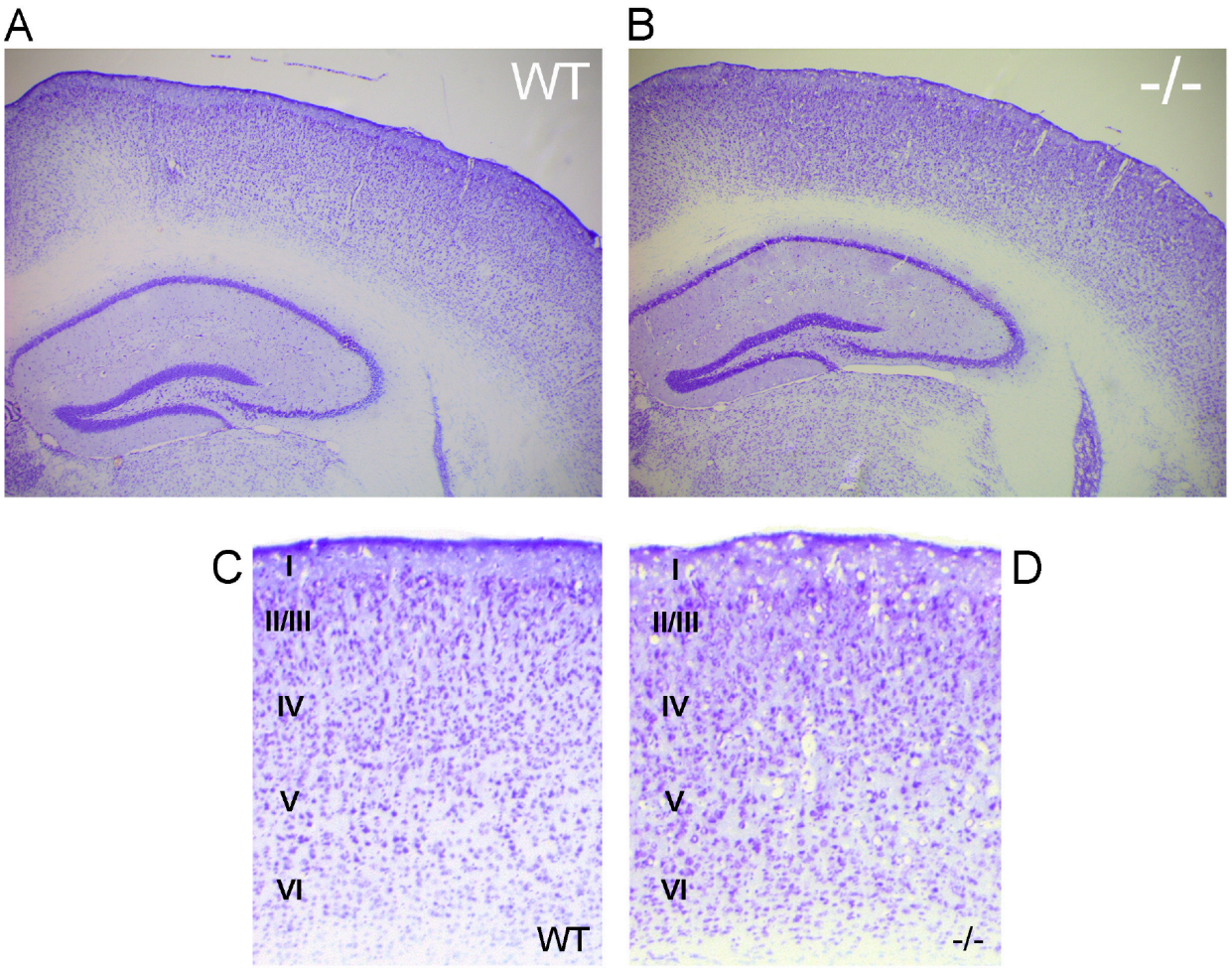


Figure S5. *Cradd* Knockout Mouse Cerebral Cortex

(A–B) Nissl-stained coronal sections of representative wild-type (A) and *Cradd*$^{-/-}$ knockout (B) mouse brains displaying similar gross morphology.

(C–D) *Cradd*$^{-/-}$ brains display normal cortical morphology demonstrated by cortical thickness and lamination that is indistinguishable from wild-type (WT). Higher magnification micrographs of coronal sections through the cerebral cortex of wild-type (C) and *Cradd*$^{-/-}$ (D) mice with each of the six cortical layers labeled and aligned demonstrating little difference in cellular organization.

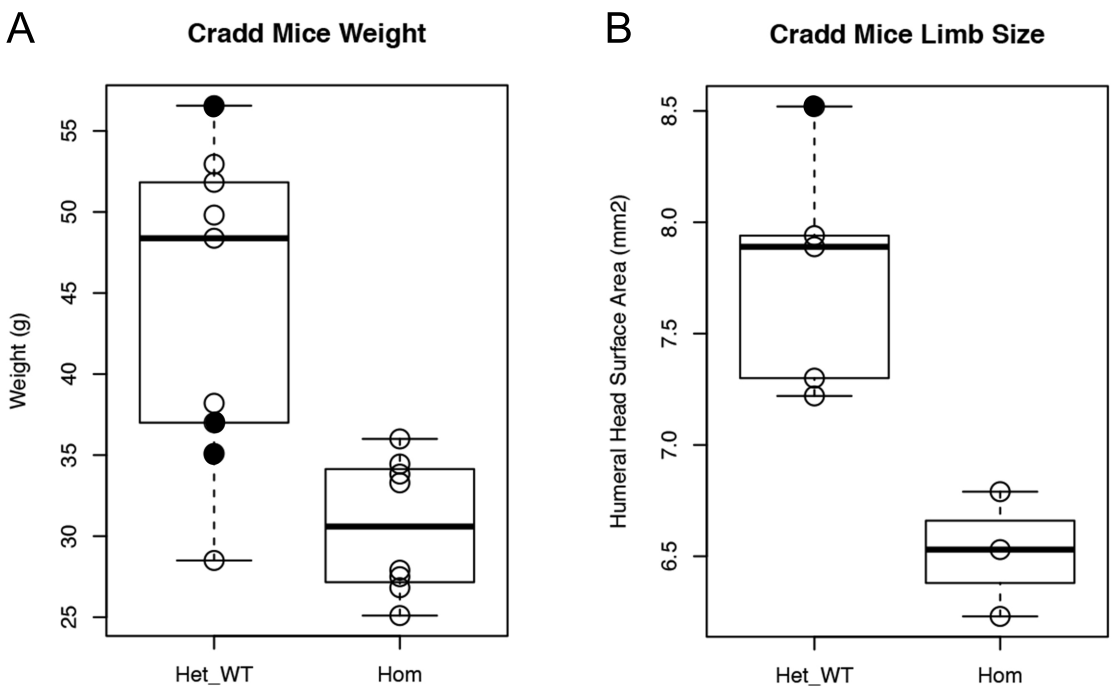


Figure S6. Body Measurements In *Cradd* Knockout Mice

Box plots show (A) body weight and (B) humeral surface area in *Cradd* control (heterozygous and wild-type, Het-WT) and *Cradd*$^{-/-}$ knockout mice (Hom). Knockout and control groups differ significantly in both parameters. Wild-type and heterozygous mice are shown with closed and open circles, respectively.

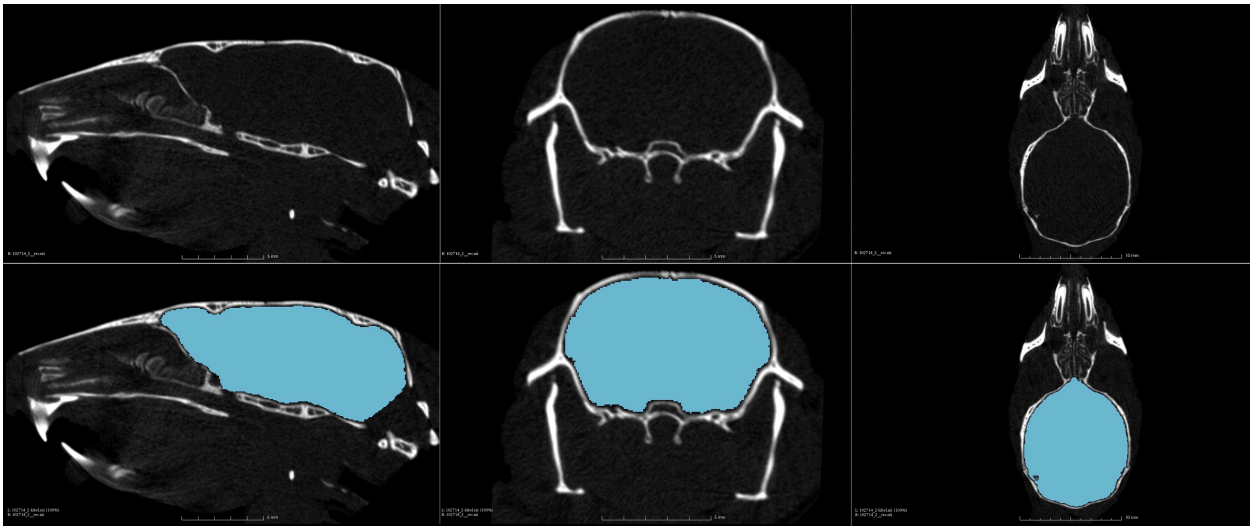


Figure S7. MicroCT Imaging of *Cradd* Knockout Mouse Endocasts.

Endocast segmentation of a representative *Cradd*$^{-/-}$ mouse. First row shows sagittal, coronal, and axial images of one of the *Cradd*$^{-/-}$ knockout mice (102714_2) used for the volumetric analysis. Second row demonstrates the same images with the label overlay used for the endocast segmentation.

SUPPLEMENTAL TABLES

Table S1. Clinical Presentation in Individuals with *CRADD*-associated Lissencephaly

Subject ID	Sex	Age last seen	Family history	OFC[a]	Body size	MEG[c]	ID	Seizures	Other anomalies
LR00-150	F	51y	Unremarkable, healthy female sibling	**60.9 cm (+3.6 SD)**	Not determined	True	Mild	Well controlled	None
LR02-006	M	18y	Unremarkable, two healthy siblings	**59.7 cm (+2 SD)**	176.5 cm (-0.3 SD) 67 kg (-0.6 SD)	Mild	Mild	2 generalized tonic-clonic seizures at time of papilloma diagnosis, no recurrence and no treatment after surgery	Atypical choroid plexus papilloma, WHO grade 2 (onset 8 years)
LR04-101a1	F	17y	Affected sister	**57.8 cm (+2 SD)**	157.5 cm (-1.51 SD) 60.4kg (+0.24SD)	Mild	Mild	Generalized nonconvulsive seizures, onset 12 mos., well controlled	None
LR04-101a2	F	13y	Affected sister	54.5 cm (+0.1 SD)	146.7 cm (-2 SD) 40.1 kg (-1.55 SD)	Relative	Mild-moderate	Generalized nonconvulsive seizures, onset 18 mos., well controlled	None
LR05-279a1	M	23y	Affected sister; parents first cousins	**60 cm (+2.7 SD)**	178 cm (-0.4 SD) 79.7 kg (+0.85 SD)	Mild	Mild	None	None
LR05-279a2	F	21y	Affected brother; parents first cousins	**59.5 cm (+3.5 SD)**	163.5 cm (-0.7 SD) 71.1 kg (+1.3 SD)	True	Mild-moderate	None	Chiari malformation, hydrocephalus with shunt
LR15-293a1	M	19y	Affected x7	57.5 cm (+0.4 SD)	177,8 cm (-0.19 SD) 75 kg (-0.19 SD)	None	Borderline-mild	None	None
LR15-293a2	M	14y	Affected x7	**58.8 cm (+2.1 SD)**	162.6 cm (-1.1 SD) 62 kg (+0.27 SD)	Mild	Mild	None	None
LR15-293a3	M	47y	Affected x7	**58.9 cm (+2.1 SD)**	170.2 cm (-1.53 SD) 81.1 (+0.98 SD)	Mild	Mild	None	None
LR15-293a4[b]	M	17y	Affected x7	**58.3 cm (+2 SD)**	174.6 cm (-0.61 SD) 78 kg (+0.59 SD)	Mild	Mild	None	None
LR15-293a5[b]	M	10y	Affected x7	**56.5 cm (+2 SD)**	143.5 cm (-0.16 SD) 52.6 kg (+1.45 SD)	Mild	Mild	None	None
LR15-293a6[b]	M	38y	Affected x7	**59.2 cm (+2.2 SD)**	173.4 cm (-0.83 SD) 82.6 kg (+0.85 SD)	Mild	Mild	None	None
LR15-293a7[b]	F	33y	Affected x7	**58.5 cm (+2.4 SD)**	160.7 cm (-0.79 SD) 70.1 kg (+0.89 SD)	Mild	Mild-moderate	None	None

Family LR15-293 previously reported.[7]
[a]Standard deviations (SD) calculated based on Nellhaus charts.[8]
[b]Brain MRI not done.
[c]We define true megalencephaly (MEG) as occipitofrontal circumference (OFC) >3 SD above the mean, mild megalencephaly as OFC 2-3 SD above the mean, and relative megalencephaly OFC >2 SD greater than height.[9]
ID, intellectual disability.

Table S2. Mouse MicroCT Endocast Data.

Animal Number	Sex	Age (weeks)	Genotype (PCR)	Parents	Weight (g)	Endocast (mm3)	Humerus SA (mm2)	isoData threshold	Length (mm)	Width (mm)	Nasal (mm)	Frontal (mm)	Rest (mm)	EQ (head/weight)	EQ (head/humerus)
102714_1	F	25	−/−	+/−; +/−	27.50	466.03	n.a.	53	22.858	12.439	8.017	7.192	8.224	16.95	n.a.
110614_7	F	25	−/−	+/−; +/−	25.10	426.20	n.a.	54	19.771	12.066	6.373	6.334	7.625	16.98	n.a.
100514_1	F	26.1	−/−	−/−; −/−	34.45	489.33	n.a.	53	22.951	12.484	7.964	7.74	7.926	14.20	n.a.
102714_2	F	26.1	−/−	−/−; −/−	36.00	491.83	n.a.	52	23.011	12.51	8.076	7.508	8.025	13.66	n.a.
110614_6	F	29.3	−/−	−/−; −/−	27.90	424.36	n.a.	52	19.99	12.117	6.671	6.259	7.494	15.21	n.a.
32	M	72.4	−/−	−/−; −/−	33.83	436.53	6,53	42	22.239	13.093	7.993	7.217	7.551	12.90	2.97
34	M	72.4	−/−	−/−; −/−	33.29	423.44	6,79	43	22.009	12.946	7.864	7.49	7.31	12.72	2.88
36	F	72.4	−/−	−/−; −/−	26.82	398.35	6,23	43	20.956	12.506	7.388	7.395	6.892	14.85	2.95
110614_5	F	25	+/−	+/−; +/−	28.50	432.79	n.a.	53	22.208	11.998	7.606	7.525	7.756	15.19	n.a.
102314_1	F	26.7	+/−	+/−; +/−	37.00	443.54	n.a.	56	22.098	12.105	7.825	7.067	7.829	11.99	n.a.
100314_1	M	43.7	+/−	+/+; +/−	52.95	429.26	7,22	44	22.596	12.556	7.982	7.381	7.844	8.11	2.81
100314_3	F	43.7	+/−	+/+; +/−	49.81	427.71	7,94	47	21.948	12.128	7.617	8.01	7.028	8.59	2.67
100314_4	F	43.7	+/−	+/+; +/−	51.83	449.60	7,89	46	21.789	12.237	7.587	7.569	7.167	8.67	2.73
100314_5	F	43.7	+/−	+/+; +/−	48.38	440.25	7,3	47	22.219	12.329	7.603	7.59	7.649	9.10	2.82
102314_2	F	26.7	+/+	+/−; +/−	38.20	462.51	n.a.	56	22.327	12.292	7.943	7.076	7.975	12.11	n.a.
102314_3	F	26.7	+/+	+/−; +/−	35.10	458.93	n.a.	55	22.243	12.148	7.729	7.276	8.008	13.08	n.a.
100314_2	M	43.7	+/+	+/+; +/−	56.56	440.25	8,52	44	22.243	12.593	8.127	7.517	7.243	7.78	2.61

EQ - encephalization quotient

EQ (head/weight) - ratio endocast (mm3) to weight (g)

EQ (head/humerus) - ratio cubic root of endocast volume to square root of humerus surface area (SA)

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