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Copb2 is essential for embryogenesis and hypomorphic mutations cause human microcephaly

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Abstract

Primary microcephaly is a congenital brain malformation characterized by a head circumference less than three standard deviations below the mean for age and sex and results in moderate to severe mental deficiencies and decreased lifespan. We recently studied two children with primary microcephaly in an otherwise unaffected family. Exome sequencing identified an autosomal recessive mutation leading to an amino acid substitution in a WD40 domain of the highly conserved *Coatomer Protein Complex, Subunit Beta 2 (COPB2)*. To study the role of *Copb2* in neural development, we utilized genome editing technology to generate an allelic series in the mouse. Two independent null alleles revealed that *Copb2* is essential for early stages of embryogenesis. Mice homozygous for the patient variant (*Copb2*$^{R254C/R254C}$) appear to have a grossly normal phenotype, likely due to differences in corticogenesis between the two species. Strikingly, mice heterozygous for the patient mutation and a null allele (*Copb2*$^{R254C/Znf}$) show a severe perinatal phenotype including low neonatal weight, significantly increased apoptosis in the brain, and death within the first week of life. Immunostaining of the *Copb2*$^{R254C/Znf}$ brain revealed a reduction in late-born (CTIP2^{+}) neurons, while the overall cell density of the cortex is unchanged. Moreover, knockdown of *Copb2* in mouse neurospheres resulted in reduced proliferation. These results identify a general requirement for *COPB2* in embryogenesis and a specific role in corticogenesis. We further demonstrate the utility of CRISPR-Cas9 generated mouse models in the study of potential pathogenicity of variants of potential clinical interest.

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INTRODUCTION

Autosomal recessive primary microcephaly is a rare congenital brain defect resulting in a reduction of occiptofrontal head circumference by at least 3 standard deviations. Patients with microcephaly are typically afflicted with varying degrees of intellectual disability and low intelligence quotient mental retardation and can either be part of a syndromic condition or an isolated malformation (1, 2). To date, mutations in at least twelve genes have been identified in patients with primary microcephaly. The roles of these genes include proper orientation of the mitotic spindle, the duplication, formation or function of the centrosome, DNA-damage response, transcriptional regulation and vesicle trafficking. Most of these mutated genes ultimately play roles in neural progenitor proliferation during the intricate development and organization of the cerebral cortex (3-11).

While there has been significant progress identifying genetic causes of primary microcephaly, the genetics of neurodevelopmental disorders remains incompletely understood (12). In these cases, exome sequencing has become an efficient tool for the identification of rare disease variants. We performed exome sequencing in a pedigree with two affected siblings with primary microcephaly and identified a homozygous recessive variant in the coding region of *Coatomer Protein Complex Subunit Beta 2 (COPB2).*

COPB2 encodes β'-COP, a subunit of the Golgi Coatomer complex (COPI), which is necessary for retrograde trafficking from the Golgi to the Endoplasmic Reticulum (ER) (13-16). In addition to its structural role in the COPI complex, β'-COP has been shown to directly interact with cargo (17, 18) and with regulators of intracellular trafficking. In order to test our hypothesis that the microcephaly and other congenital malformations seen in the affected patients are due to the missense mutation in the coding region of *COPB2*, we used genome editing technology to generate an allelic series in the mouse. Our results indicate that complete loss of *Copb2* is incompatible with embryonic survival to

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organogenesis stage of embryonic development, and that compound heterozygotes for a null allele and the missense allele of *Copb2* show both brain and growth defects in mice. Thus, we have identified a novel causal gene for primary microcephaly and a requirement for this gene in early mammalian development.

RESULTS

Homozygous recessive mutation in COPB2 identified in two patients with primary microcephaly

Two affected children presented in our hospital with abnormal head size, severe developmental delay, failure to thrive, cortical blindness and spasticity. Both parents and a sibling were healthy with no features of microcephaly (Fig 1A). Magnetic resonance imaging confirmed microcephaly with a simplified gyral pattern, thin corpus callosum, slight dilation of the lateral, third and fourth ventricles, enlarged extra-axial space and delayed myelination (Fig 1B-E). Occipital head circumference was well below the 3rd percentile at all ages for both affected children (Table 1). The low body weight and proportional reduction in head size became more severe in each child as they aged (Fig 1 F-I). To further define the microcephaly phenotype based on clinical classifications (2) and to determine whether the clinical phenotype could differentiate between a role for proliferation and/or atrophy in the underlying mechanism, we performed a detailed analysis of the brain imaging studies. We measured the extra-axial fluid spaces in the area of the cisterna magna and sylvian fissures and sizes for the atrium of the lateral, third and fourth ventricles over development of the affected children. This analysis showed a progressive increase in brain ventricle size and minimal change in extra-axial space dimensions (Table 2) suggesting progressive central brain volume loss.

To determine the genetic origin of this syndrome, an initial series of microcephaly genes (*ASPM, CK5RAP2, CENP, MCPH1, STIL*) was sequenced in the proband with negative results. The affected siblings had a SNP microarray analysis performed using the Illumina Infinium Assay to assess copy number

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variation (CNV) and regions of homozygosity (ROH). Subsequent analysis did not identify any pathogenic CNV's but did find a single large region of homozygosity (ROH) in both the proband and her affected brother, despite parental report of non-consanguinity (Fig 1J). The 16.8 Mb ROH was identified at 3q22.1q24 and contained approximately 90 Refseq genes.

Whole exome sequencing (Illumina Hi Seq 2000) was performed for the two affected children and their parents after obtaining informed consent according to our Cincinnati Children's Hospital Medical Center (CCHMC) IRB-approved protocol. Alignment and variant detection was performed using the Broad Institute's web-based Genome Analysis Tookit (GATK; Genome Reference Consortium Build 37) (19). Quality control and data filtering were performed on VCF files in Golden Helix's SNP and Variation Suite. Non-synonymous coding variants were compared to three control databases, including NHLBI's ESP6500 exome data (20), the 1000 genomes project (21), and an internal CCHMC control cohort (22). The initial bioinformatics analysis identified 126,752 variants (Table 3). After filtering for quality control using filters previously described (22), coding variants, non-synonymous variants, and minor allele frequencies, we identified one single mutation which followed a homozygous recessive inheritance pattern. The identified variant was compared to known disease genes in the OMIM and Human Gene Mutation (HGMD) (23) databases, and to reported variants in dbSNP (24) and the Exome Aggregation Consortium (ExAC; (25). There is one reported heterozygous variant in this position in the Exac database coding for a different missense variant (p.R253H). The variant was also analyzed using Interactive Biosoftware's Alamut v2.2 to determine location of mutation within a protein domain, the conservation of the amino acid, the Grantham score (26) and the designation of the mutation by three existing in-silico software tools; including a SIFT classification of "damaging" (27), and a Mutation Taster designation as "disease-causing" (28).

The identified variant is a missense mutation in the gene *Coatomer Protein Complex, Subunit Beta 2* (*COPB2*). Consistent with the microarray data, this mutation was within the previously identified

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region of homozygosity on 3q22.1q24. We confirmed this exome result by Sanger Sequencing in all participants sequenced and extended the Sanger analysis to the unaffected sibling, who was heterozygous for the *COBP2* mutation (Fig 1K). The missense mutation in exon 8 of *COPB2* (n.C760T; p.R254C) occurs at a highly conserved residue in a conserved region of the protein (Fig 1L). The affected amino acid is within a predicted WD40 protein-binding domain (Fig 2A). *Copb2* is highly expressed in the mouse ventricular zone where neuroprogenitor cell division occurs in both mouse and human (29). This expression pattern is consistent with a model wherein reduction or loss of *COPB2* function could cause primary microcephaly.

A Copb2 allelic series in mouse

In order to validate our results in a mammalian system and to further explore the role of *COPB2* in development, we generated several alleles in the mouse. The first allele, $Copb2^{em1Rstot}$ ($Copb2^{Zfn}$), is a Zinc-Finger nuclease mediated 5bp deletion within exon 12 which codes a premature stop codon in ten amino acids (Fig 2A-D). PCR amplification of a 79bp region surrounding the deletion allowed us to distinguish the two species upon gel electrophoresis (Fig 2E). We then used CRISPR-Cas9 technology to generate a "humanized" allele, $Copb2^{em2Rstot}$ ($Copb2^{R254C}$), with the missense variant seen in the probands (Fig 2A,F-I). In the process of creating the $Copb2^{R254C}$ allele, animals were also created with a one base pair insertion downstream of desired missense mutation site, creating another premature stop, $Copb2^{em3Rstot}$, ($Copb2^{null}$;Fig 2A,F,H,I).

Copb2 is indispensable for embryogenesis

Given the integral role *Copb2* plays in cellular trafficking as a subunit of the Golgi Coatomer Complex, we hypothesized that loss of *Copb2* would severely impair embryogenesis. Results from both of our presumed null alleles ($Copb2^{zfn}$ and $Copb2^{null}$) supported this. Of 35 embryos from E11.5-E18.5, and 11

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from E7.5-E10.5 recovered from *Copb2*$^{Zfn/wt}$ intercrosses, no homozygous *Copb2*$^{Zfn/Zfn}$ embryos were recovered. Similarly, no *Copb2*$^{null/null}$ embryos were recovered at E12.5. In many cases, there was evidence of early embryonic resorption, consistent with early lethality.

Copb2$^{R254C/R254C}$ *mice do not display any developmental abnormalities*

Mice homozygous for the *R254C* allele are born in Mendelian ratios (Table 4) and are not physically distinct from littermate controls. Gross brain morphology and histology using Nissl staining revealed no discernable abnormalities in the brains of *R254C* homozygous or heterozygous animals compared to controls (Fig 3A-I). Body mass at both P21 and P60 was similar across all genotypes (Fig 3J). At 2 months of age, brain mass, forebrain area, and cell density appeared similar across all three genotypes (Fig 4K-M). Immunoblots for COPB2 show no significant decrease in protein levels in *Copb2*$^{R254C/wt}$ heterozygous or *Copb2*$^{R254C/R254C}$ homozygous animals (Fig 4N).

Copb2$^{R254C/Zfn}$ *mice are perinatal lethal with cortical malformations.*

As *Copb2*$^{R254C/R254C}$ mice appeared phenotypically normal, we hypothesized that the mouse brain is less sensitive to reductions in *Copb2* function. In order to test this hypothesis and further assess pathogenicity of the *R254C* allele, we generated *Copb2*$^{R254C/Zfn}$ animals. These pups were not born in Mendelian ratios (Table 4) and could be easily identified among littermate controls due to their small and sickly appearance, usually lacking a milk spot. Body mass was decreased in both P0 and P1 *Copb2*$^{R254C/Zfn}$ mice as compared to controls (Fig 4A). (With the exception of one litter in which all 4 *Copb2*$^{R254C/Zfn}$ survived the perinatal period but were morbid at P19, all *Copb2*$^{R254C/Zfn}$ animals were euthanized by P3 due to the excessive morbidity.) Additionally, both the mass and the cortical area of the *Copb2*$^{R254C/Zfn}$ brains were reduced compared to controls (Fig 4B,C). Western blots revealed a significant reduction in COPB2 protein levels in *R254C/Zfn* mice (approximately 50%; Fig 4D). Histological

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analysis of *Copb2*$^{R254C/Zfn}$ brains showed morphologically normal but reduced cortical tissues as compared to wild type controls (Fig 4E-J).

Knockdown of Copb2 results in reduced proliferation in mouse neurospheres

Based on the decreased patient occipital head circumference and reduced brain size in the *Copb2*$^{R254C/Zfn}$ mice, we hypothesized that loss or reduction of COPB2 is associated with neural cell proliferation defects. To determine whether loss of *COPB2* affects neural cell proliferation, mouse neurospheres containing neural stem/progenitor cell populations (NPCs) were treated with virally introduced *Copb2* RNAi plasmids and proliferation was analyzed. Neurospheres were dissected from mouse embryos on embryonic days (E) 12.5-13.5 and cultured in DMEM-F12 medium with B27 and growths factor as described previously (30). We then used RNAi to deplete *COPB2* from NPCs and measured their proliferation. Over a three-day time course, control-infected NPCs increased in cell number over three-fold (Fig 4K). *Copb2* knockdown NPCs only proliferated approximately 1.6-fold (Fig 4K). To further confirm specific functional effects of our RNAi constructs we created clonal lines via lentiviral transfection and assayed for abortive autophagy, a known *Copb2* knockdown phenotype. We transfected nontargeted or *Copb2*-silenced intramedullary collecting duct (IMCD) cells with an LC3-RFP plasmid to measure autophagic flux. We used RFP-positive puncta in LC3-RFP transfected cells as a marker of autophagosome production, and GFP-RNAi constructs to identify cells with *Copb2* knockdown. In control IMCD cells, very few cells showed intense accumulation of LC3-RFP indicative of autophagic processing (Fig S1A). As a positive control, treatment with Brefeldin-A caused a significant accumulation of autophagosomes (Fig S1B). Cells treated with brefeldin-A (Fig S1B) or stably expressing the *Copb2* siRNA (as indicated by GFP, data not shown) showed significant increases in LC3-RFP puncta, indicative of increased numbers of autophagosomes (Fig S1C). We further labeled the cells with LysoTracker to mark the lysosomes. We first noted a significant increase in the number of lysosomes in *Copb2*

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knockdown cells as compared to controls (Fig. S1H). We also noted a significant amount of co-localization of the Lysotracker and the LC3-RFP, indicating fusion of the lysosomes and autophagosomes (Fig. S1I), consistent with previous results (31, 32). Taken together and in combination with previously published reports on the function of COPB2, these data show that reduction of COPB2 function leads to, 1) an increase in formation of autophagosomes which are not able to fully mature, 2) abortive autophagy and, 3) lowered cellular proliferation in mouse neurospheres. We were unable to generate clonal cell lines with either RNAi or CRISPR/CAS9 editing that lacked COPB2 entirely, consistent with the failure to recover homozygous null mouse embryos and previous literature (31).

To further determine a potential mechanism for the reduction in cortical size, we began a molecular analysis of the *Copb2*$^{R254C/Zfn}$ phenotype. Immunohistochemistry for post-mitotic neurons using TuJ1 showed a robust population in both *Copb2*$^{R254C/Zfn}$ brains and controls (Fig. 5A,B). Layer marker analysis was performed and we saw no reduction in TBR1-positive (layer VI) neurons in the *Copb2*$^{R254C/Zfn}$ brains compared to control (Fig. 5 C,D). In contrast, we saw a reduction in the later-born CTIP2-positive neurons (layer V) with a 32% reduction in *Copb2*$^{R254C/Zfn}$ perinatal brains ($P<.05$, Fig. 5F,G). Staining for apoptosis using Cleaved-Caspase 3 (CC3) at P0 showed significant increases in the *Copb2*$^{R254C/Zfn}$ animals within the cortex (2.68 fold-increase relative to wild-type controls; Fig. 5I,J). Increased cell death was also observed in the hippocampus, midbrain, cerebellum, and hindbrain of *R254C/Zfn* animals compared to wild-type at P0-P3 (data not shown). *In vitro* experiments had indicated an increase in abortive autophagy following RNAi inhibition of *Copb2*, but we did not detect any change in the ratio of LC3-I to LC3-II levels in *R254C/Zfn* brains (Fig S2A).

DISCUSSION

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Here, we have identified a novel variant in the coding region of *COPB2* in two patients with primary microcephaly, seizures, and failure to thrive. To potentially demonstrate a role for *COPB2* in neural development and pathogenicity for this variant, we generated an allelic series in the mouse. We show that *Copb2* is required for survival to organogenesis stages. Strikingly, *Copb2*$^{R254C/R254C}$ mice appear phenotypically indistinct from littermates and are viable and fertile. However, reducing the proportion of functional *Copb2* further by generating *Copb2*$^{R254C/Zfn}$ mice leads to perinatal death and cortical malformations. In contrast to previous reports studying depletion of COPI components, the *Copb2*$^{R254C/Zfn}$ phenotype does not appear to involve an increase in abortive autophagy or ER stress. This study represents the first discovery of a disease-causing variant in *COPB2* as well as the first known link between primary microcephaly and a subunit of the COPI complex.

Like most genes linked to microcephaly, *COPB2* apparently acts as part of a highly conserved pathway that is essential for life in a wide variety of cellular lineages. This conclusion is supported by our discovery that *Copb2*$^{null/null}$ and *Copb2*$^{Zfn/Zfn}$ embryos do not survive to organogenesis (Table 4), and that our attempts to create COPB2-null cell lines were unsuccessful (data not shown). These results are also supported by the literature, where it has been demonstrated that siRNA-mediated reduction of *COPB2* in breast cancer cells leads to a drastic reduction in replating efficiency (31). Notably, an early lethality phenotype has also been observed in mice null for other known microcephaly-associated genes, including *MCPH7* (*STIL*), which die *in utero* after E10.5 (33), and *MCPH10 (ZNF335)*, which die as early as E7.5 (34). Thus, it appears human malformations are more likely due to hypomorphic mutations in these genes where neural development is especially susceptible, likely due to the extremely high rates of neurogenesis in primate cortical development.

The early lethality of *Copb2*$^{null/null}$ embryos strongly suggests that the patient mutation is hypomorphic, as an effectively null allele would presumably arrest embryogenesis at a much earlier stage. This assumption proved true in our mouse models: immunoblots of perinatal *Copb2*$^{R254C/R254C}$

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brains indicate no significant decrease in COPB2 levels, whereas *Copb2*$^{R254C/ZnF}$ brains have approximately half the normal amount of protein. The *Copb2*^{R254C} mutation is located within the N-terminal WD40 repeat (β-propeller) domain of β'-COP, which has been shown to bind COPI cargo (35). Given the hypomorphic nature of the mutation, one could hypothesize that it interferes with the interaction between β'-COP and this cargo, causing an incremental reduction in COPI trafficking efficiency which is sufficient to cause defects during neurogenesis. Interestingly, the α-COP subunit of COPI shares significant N-terminal homology with β'-COP and has also been shown to bind cargo using its own WD40 repeat domain (36). *COPA* mutations in this domain result in Copa Syndrome, an immune dysregulatory syndrome in which the mutated α-COP protein has a reduced ability to bind di-lysine tagged cargo (37). It remains to be seen why *COPA* mutations result in such a syndrome while *COPB2* mutations in the homologous domain result in neurogenesis defects. Presumably, this can be attributed either to the fact that COPB2 binds a unique set of COPI cargo, or to the fact that the *COPB2*^{R254C} mutation affects protein function to a different degree than known *COPA* mutations do.

A major difference between the patients discussed here and the mouse models we created was the ability of the mice to tolerate the *Copb2*$^{R254C/R254C}$ genotype without an observable phenotype, only exhibiting major defects when overall *Copb2* expression was reduced by a *Copb2*$^{R254C/Zfn}$ genotype. This issue is not unique to our study, as microcephaly has been historically difficult to model in the mouse, perhaps because mouse corticogenesis only generates around 14 million neurons while the human cortex has around 16 billion (38). Additionally, neurogenesis in gyrencephalic species is much more reliant on basal progenitor proliferation (39), suggesting that phenotypes involving this cell type may not be easily recapitulated in lissencephalic models such as the mouse. In some mouse models of microcephaly, such as *MCPH1* (40), and *MCPH2* (*Wdr62*) (41), reductions in cortical size are accompanied by comparable reductions in other brain structures and in overall body size, as was observed in *Copb2*$^{R254C/Zfn}$ mice. Additionally, we did find a reduction in layer V CTIP2-positive neurons in

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the *COPB2*$^{R254C/Zfn}$ mice, which is a reported feature in mouse models of microcephaly (42). It is also worth noting that the probands themselves were significantly underweight in addition to being microcephalic (Fig 1).

To date, *Copb2* variants have not been connected to any human disease, although mutations in *Copb2* as well as *Copa* (α-COP) and *Copb* (β-COP) have been linked to notochord defects in Zebrafish (43). Notably, a protein associated with neurodegeneration, SCYL1, was shown to interact with COPI subunits and to regulate retrograde Golgi to ER traffic (44). Also of note, COPI function (specifically, the δ-COP subunit) was implicated in the trafficking and metabolism of amyloid precursor protein, suggesting a role in Alzheimer's progression (45).

MATERIALS AND METHODS

Subjects. Informed consent was obtained according to Cincinnati Children's Hospital Medical Center (CCHMC) institutional review board protocol # 2012-0203. Following consent, whole blood was collected on the parents, residual DNA on the affected individuals, and spit on the unaffected sibling.

Microarray. Affected siblings had microarray analysis performed using the Illumina Infinium Assay (San Diego, CA) with the Illumina HD Human OMNI-quad BeadChip platform. The chip contains approximately 1,140,419 probes to assess copy number variation and regions of homozygosity.

Genotyping. Library generation, exome enrichment, sequencing, alignment and variant detection were performed in the CCHMC Genetic Variation and Gene Discovery Core Facility (Cincinnati, OH). Briefly, sheared genomic DNA was enriched with NimbleGen EZ Exome V2 kit (Madison, WI). The exome library was sequenced using Illumina's Hi Seq 2000 (San Diego, CA). Alignment and variant detection was

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performed using the Broad Institute's web-based Genome Analysis Tookit (GATK) (19). All analyses were performed using Genome Reference Consortium Build 37.

Variant Filtering and Pathogenicity Assessment. Quality control and data filtering were performed on VCF files in Golden Helix's SNP and Variation Suite (Bozeman, MT). Non-synonymous coding variants were compared to three control databases, including NHLBI's ESP6500 exome data (20), the 1000 genomes project (21), EXAC Browser (25)and an internal CCHMC control cohort (22). Remaining variants were subject to autosomal recessive analysis with emphasis on homozygous recessive variants found in the region of homozygosity identified by SNP microarray. The identified variant was compared to known disease genes in the OMIM (REF) and Human Gene Mutation (HGMD) (23) databases, and to reported variants in dbSNP (24) and the Exome Variant Server. The variant was also analyzed using Interactive Biosoftware's Alamut v2.2 (San Diego, CA) to determine location of mutation within a protein domain, the conservation of the amino acid, the Grantham score (26) and the designation of the mutation by three existing in-silico software tools, SIFT (27), Polyphen (46) and Mutation Taster (28). *COPB2* mutations were validated by Sanger Sequencing (see Table 5).

COPB2 RNAi and cell proliferation RNAi constructs were purchased from the Thermo-Fisher pGIPZ collection. Lentivirus was made using standard transfection protocols of HEK293t cells and used to infect mIMCD cells. Cells were selected for knockdown by both puromycin resistance and GFP expression. For all hairpins, clonal lines were also created by limiting dilution. Cell proliferation assays were performed using the Cell Titer-Blue Cell Viability Assay (Promega). 10,000 IMCD cells were plated for each clone and cell titer assays were performed in triplicate 6 and 48 hours after plating. NPC proliferation assays were performed as previously described (47). Statistical significance was calculated with Student's t-test. All antibodies were purchased from Sigma (St. Louis, MO).

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Autophagy Assays. IMCD cells which had undergone clonal selection for pGIPZ-RNAi constructs were further transfected with pmRFP-LC3 (Addgene: (48)). Three days after transfection cells were fixed and examined. As a positive control, Brefeldin-A was added at a final concentration of 10ug/mL for 1 hour before fixation. LysoTracker Deep Red (Molecular Probes) was used to visualize lysosomes by addition of a 50nM stock 2 hours before fixation.

Allele Generation.

Zinc finger nuclease guides were purchased from Sigma (St. Louis, MO) and submitted to the CCHMC Transgenic Core for blastocyst injection. Founder animals were screened with PCR primers (Table 5). CRISPR guides targeting the region of interest within exon 8 (ENMUSE00000230688) of m*Copb2* were designed using the MIT CRISPR design tool (crispr.mit.edu). Three potential guide RNA (gRNA) sequences were selected and ordered as complementary oligonucleotide pairs with BbsI overhangs (Table 5). These were ligated into the pSpCas9(BB)-2A-Puro (px459) vector and transfected into 3T3 using the Lipofectamine® 3000 transfection reagent (Thermo Fisher Scientific, Massachusetts). 48 hours after transfection, the cells were harvested and genomic DNA was purified before being subjected to the Surveyor® mutation detection kit in order to test cutting efficiency (Integrated DNA Technologies, Coralville, IA). As a control, cutting efficiencies of potential m*Copb2* guides were compared with that of a previously-published m*Tet2* gRNA (Integrated DNA Technologies, Iowa). A donor sequence was designed to introduce the desired missense mutation *Copb2*^{R254C} modeling the patients' mutation (Table 5). The donor and the plasmid encoding the most efficient m*Copb2* gRNA (Guide 3, Table 5) were sent to the CCHMC Transgenic Core for blastocyst injections. Potential founders were validated with Sanger Sequencing. pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988). All transgenic injections were done into animals from a mixed C57BL/6;DBA/2 background. (C57BL/6 x

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DBA/2, 2nd generation). *Copb2*^{Zfn} mice were maintained on a C57BL/6J background and all CRISPR/CAS9 alleles were maintained on a CD-1 background.

Animal husbandry

All animals were housed under and approved protocol and standard conditions. All euthanasia and subsequent embryo/organ harvests were preceded by Isoflurane sedation. Euthanasia was accomplished via dislocation of the cervical vertebrae. For embryo collections, noon of the day of vaginal plug detection was designated as E0.5.

Sample measurements (body mass, brain mass). Sacrificed animals were weighed immediately following euthanasia. Brains were removed, weighed, and imaged using a Zeiss SteREO Discovery.V8 stereomicroscope. Relative area was measured using Axiovision 40 software (v.4.8.2.0). Magnification scale was the same for samples of the same age. Box plots were generated with BoxPlotR (http://shiny.chemgrid.org/boxplotr/).

Western immunoblotting. Following euthanasia, P0-P3 brains were dissected, flash-frozen, and weighed. These samples were then homogenized in RIPA buffer and stored at -80°C. Homogenized samples were diluted in 50:1 Laemelli Buffer: 2-Mercaptoethanol and heated to 90°C for 5 minutes. Samples were then loaded into Novex™ WedgeWell 10-16% Tris-Glycine gels and electroporated at 130V for 100-130 minutes in Tris-Buffered Saline. Transfers were performed on ice at 35V for 90 minutes in Transfer Buffer (25 mM Tris, 190 mM Glycine, 20% Methanol). The membranes were then washed in TBS and blocking was performed at room temperature in 1:1 Odyssey® Buffer: PBS. Primary antibody incubation was performed at 4°C overnight in 1:1 Odyssey® Buffer: PBS. Membranes were then washed three times in TBS. Membranes were then incubated in IRDye® 680RD and 800CW at room

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temperature for 1 hour and visualized using an Odyssey® CLx Imaging System. Analysis was performed using Image Studio Lite V5.2. Relative band intensity was compared to α-Tubulin loading controls in order to quantify results.

Histological and immunohistochemical analysis. For adult histology and immunohistochemistry (IHC) adult (2 months of age) littermate animals underwent transcardial perfusion using cold heparinized phosphate buffered saline (PBS) and 4% paraformalydehyde (PFA) solution. Brains were dissected and fixed for 72 hours in 4% PFA at room temperature followed by immersion in 70% ethanol (for histology). Samples were then paraffin embedded, sectioned at 5um, and processed through hematoxylin and eosin (H&E) staining. Sections were sealed using Cytoseal Mounting Medium (Thermo-Scientific).

For postnatal IHC analysis, P0-P3 pups were euthanized and brains dissected and fixed overnight in 4% paraformaldehyde. Following this, samples were immersed in 30% sucrose solution for 24-48 hours until sufficiently dehydrated. Brains were embedded in OCT compound (Tissue-Tek) and sectioned at a thickness of 10uM. For IHC sections were immersed in PBS followed by blocking in 5% normal goal serum in PBST for one hour and overnight incubation in the following primary antibodies: TuJ1 (1:500, Sigma), Tbr1 (1:500, Abcam), Ctip2 (1:200, Abcam), CC3 (1:300, Cell Signaling), and GFAP (1:500, Abcam). Sections were washed three times in PBS and incubated at room temperature in AlexaFluor 488 goat anti-rabbit or goat anti-mouse secondary (1:500, Life Technologies) for 1 hour followed by incubation in DAPI (1:1000) for 15 minutes. Sections were then rinsed in PBS and sealed with ProLong Gold Antifade Reagent (Life Technologies). For postnatal histology sections were rinsed in PBS followed by processing through H&E and Nissl staining. Sections were sealed using Cytoseal Mounting Medium (Thermo-Scientific). All IHC sections were imaged on Nikon C2 Confocal microscope. All paired images were taken at the same magnification.

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Quantification of cortical cell number. Images were imported into Image J (Schneider et al., 2012) and areas were established around the region of interest in the cortex. Thresholding was used to detect specific populations in each channel (DAPI and GFP) and quantified using the Analyze Particles function. All cell measurements are normalized to the areas recorded in each respective sample.

FIGURE LEGENDS

Figure 1. Microcephaly in two patients with a single region of homozygosity on chromosome 3. (A) Pedigree of family with autosomal recessive primary microcephaly in two affected children with unaffected parents and sibling. (Proband is indicated with arrow) (B, C) Computed tomography (CT) scan of the proband (B) and the affected sibling (C) revealed decreased sulci and enlarged extra axial fluid spaces and ventricles. Sagittal T1 weighted MR image of the proband at age 4.5 (D) depicts microcephaly with a simplified gyral pattern, dilation of the lateral ventricle, and enlarged extra axial space. Axial T2 weighted image of the proband (E) again demonstrates simplified gyral pattern and enlarged lateral ventricles and extra axial spaces with increased signal in the white matter (arrows) consistent with delayed myelination. (F-I) The proband (F,H) and affected sibling (G,I) are consistently below 5th percentile in weight and below the 3rd percentile for head circumference. (J) SNP microarray analysis identified a single large region of homozygosity (ROH, red box) was identified in both affected siblings at 3q22.1q24. Parents denied consanguinity and all other ROH were less than 2.6 Mb in size. (K) Sanger Sequencing confirms exome results that affected children are homozygous for the n.760C>T sequence variant while both parents and the unaffected sibling are heterozygous. (L) The mutation results in a p.R254C coding change in a highly conserved region of the protein (mutated residue in red, human amino acid positions 250,255,260 indicated).

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Figure 2. Mouse alleles of COPB2. (A) Schematic of the mouse COPB2 protein domains. Asterisks denote sites of mutations. (B-E) The *Copb2*^{Zfn} allele is a 5bp deletion in exon 12 (B,D), which results in a frameshift and creates a premature stop codon (C). (D) Sanger sequencing of the PCR products from a heterozygous animal. Sanger peaks that differ from wild-type are indicated by red arrows and this analysis identifies the precise nature of the deletion. (E) Genotyping was performed by PCR amplification of a 79bp region surrounding the deletion and gel electrophoresis (4% metaphor agarose). (F-H) The *Copb2*^{R254C} (F,G) and *Copb2*^{null} (F,H) alleles were created using CRISPR-Cas9 technology targeting exon 8. Red letters indicate nucleotide changes affecting the amino acid sequence while blue letters indicate silent mutations. (I) *Copb2*^{R254C} creates an amino acid change orthologous to the patient mutation at amino acid position 254, while *Copb2*^{null} creates a frameshift resulting in a premature stop codon (*) at position 264.

Figure 3. *COPB2*$^{R254C/R254C}$ mice are viable and do not have cortical malformations. (A-C) Gross images of *Copb2*$^{wt/wt}$(A), *Copb2*$^{R254C/wt}$(B) *Copb2*$^{R254C/R254C}$(C) brains at 2 months of age. (D-I) Histological Nissl stain shows similar brain structures across all three genotypes (J,K) Overall body and brain mass were similar between genotypes and forebrain area was not significantly different across genotypes (L). Additionally, high mag images of the cortex of all three genotypes (G-I) were quantified and showed no significant difference in cell density (M). For plots in J-M, center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; data points are plotted as open circles. Measurement of COPB2 protein showed similar levels across all three genotypes (N).

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Figure 4. *COPB2*$^{R254C/Zfn}$ mice are perinatal lethal with reduced brain size and mass. (A-B) Body and brain mass were significantly lower in *COPB2*$^{R254C/Zfn}$ mice compared to wild-type controls at both P0 and P1. (C) Cortical area was measured from gross images and showed a significant reduction in *COPB2*$^{R254C/Zfn}$ mice compared to control at both P0 and P1. For plots in A-C, center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; data points are plotted as open circles. Significance between groups was determined by a student's t-test. (*:$p<0.05$; **:$p<0.01$). (D) COPB2 protein is reduced in *COPB2*$^{wt/R254C}$ and *COPB2*$^{R254C/Zfn}$ mice relative to wild-type controls. (E-J) Gross images of wild-type control (E) and *COPB2*$^{R254C/Zfn}$ brains (H) at P0. H&E stained sagittal sections of wild-type (F) and *COPB2*$^{R254C/Zfn}$mice (I) mice show similar brain structure and similar cortical organization (I,L). (K) Overall cell density was similar between the cortices of control and *COPB2*$^{R254C/Zfn}$ mice. (L) Knockdown of COPB2 in mouse neurospheres resulted in reduced proliferation after 3 days in vitro (DIV). All paired images taken at same magnification.

Figure 5. Reduced layer V neuron production and increased cell death in *COPB2*$^{R254C/Zfn}$ brains. (A-B) IHC for TuJ1-positive post-mitotic neurons highlights a robust population of cells in control (A) and *COPB2*$^{R254C/Zfn}$ mice (B). TBR1 IHC showed no significant reduction between control (C) and *COPB2*$^{R254C/Zfn}$ mice (D). CTIP2 IHC revealed a reduction in layer V neurons in *COPB2*$^{R254C/Zfn}$ mice (G) relative to control (F). Additionally, levels of CC3 to mark apoptotic cells were increased in the cortex of *COPB2*$^{R254C/Zfn}$ mice (J) compared to control (I). (E,H,K) Quantification of TBR1,CTIP2 and CC3-immunoreactive cells was performed with an $n \geq 3$ for each genotype. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; data points are plotted as open circles. Significance between groups was determined by a student's t-test. (*:$p<0.05$; **:$p<0.01$).

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Table 1. Occipital head circumference measurement (cm) and standard deviation from mean WHO head circumference over time.

Age (months)	Median (female)	3rd percentile (female)	Patient 1/ Proband	Standard Deviation*
0	33.9	31.7	29.2	-4.0
4	39.5	37.2	35.5	-4.0
35	48.4	45.8	40.5	-5.6
45	49.2	46.5	42.0	-5.0
57	49.8	47.1	42.0	-5.5
	Median (male)	**3rd percentile (male)**	**Patient 2**	**Standard Deviation#**
0	34.5	32.1	30.5	-3.1
2	39.1	36.9	31.1	-6.9
5	42.6	40.3	33.7	-7.3
7	44.0	41.7	36.8	-5.8
11	45.8	43.4	37.5	-6.5
23	48.1	45.6	37.0	-8.2

* Based on World Health Organization Head Circumference for age z-scores in girls.
# Based on World Health Organization Head Circumference for age z-scores in boys.

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Table 2. Measurement of extra axial space (mm) and brain ventricles (mm) demonstrate progressive enlargement of ventricles over time with minimal progressive change in the extra axial space.

Age (months)	Extra Axial CM	SF-L	SF-R	Left Lateral Ventricle (T) *	Right Lateral Ventricle (T)*	Third Ventricle (T)*	Forth Ventricle AP	T
0	11.9	13.7	13.9	19.8	18.8	6.7	7.3	7.9
13	11.6	14.0	14.9	22.7	23.5	9.4	9.4	10.7
50	11.6	15.0	16.4	28.6	25.4	10.6	12.0	15.6
Age (months)	**Extra Axial CM**	**SF-L**	**SF-R**	**Left Lateral Ventricle**	**Right Lateral Ventricle**	**Third Ventricle**	**Forth Ventricle AP**	**T**
0	8.2	15.0	14.6	13.8	7.4	4.3	5.2	8.2
7	7.8	17.3	13.4	15.6	7.4	7.4	6.3	12.1
39	9.4	17.2	16.7	15.2	9.2	8.6	7.0	15.6

* In a large study of cerebral ventricle size in children (49), the greatest transverse length of the lateral ventricle was 10 mm and the third ventricle was 5 mm.

CM= Cisterna Magna
SF-L= Left Sylvian Fissure
SF-R= Right Sylvian Fissure
AP= anterior-posterior
T= transverse

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Table 3. Exome Variant Analysis

Familial Variant Analysis	# of Variants
Total Variants	126,752
Quality Control	103,431
Coding variants	28,875
Non-synonomous variants	14,312
European American variants with MAF<0.01 (NHLBI ESP6500 exome data (20))	2,943
Variants with MAF<0.01 (1000 genomes project (21))	1,801
Variants with MAF<0.01 (CCHMC AA altering Variants (22))	769
Recessive analysis	1

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Table 4 – Genotypes recovered from crosses of *Copb2* alleles.

Copb2^{Zfn}			
	Copb2$^{wt/wt}$	*Copb2$^{Zfn/wt}$*	*Copb2$^{Zfn/Zfn}$* **
E11.5 - E18.5	13	22	0 *(8.75)*
E7.5 - E10.5	3	8	0 *(2.75)*
Copb2^{null}			
	Copb2$^{wt/wt}$	*Copb2$^{null/wt}$*	*Copb2$^{null/null}$* *
E12.5	7	11	0 *(4.5)*
Copb2^{R254C}			
	Copb2$^{wt/wt}$	*Copb2$^{R254C/wt}$*	*Copb2$^{R254C/R254C}$*
weaning	41	57	34 *(33)*
Copb2$^{R254C/Zfn}$			
	control	*Copb2$^{R254C/Zfn}$* *	
P0	70	15 *(21.25)*	
P1	60	11 *(18)*	

Copb2^{Zfn}, *Copb2^{null}*, *Copb2^{R254C}* data are from matings of heterozygous mice. *Copb2$^{R254C/Zfn}$* data are from matings of *Copb2^{Zfn}* and *Copb2^{R254C}* mice. Expected numbers of mutant animals are indicated by parentheses. Statistical significance of the reductions in mutant numbers was determined with a chi-squared analysis. (**:$p \leq 0.01$; *:$p \leq 0.05$).

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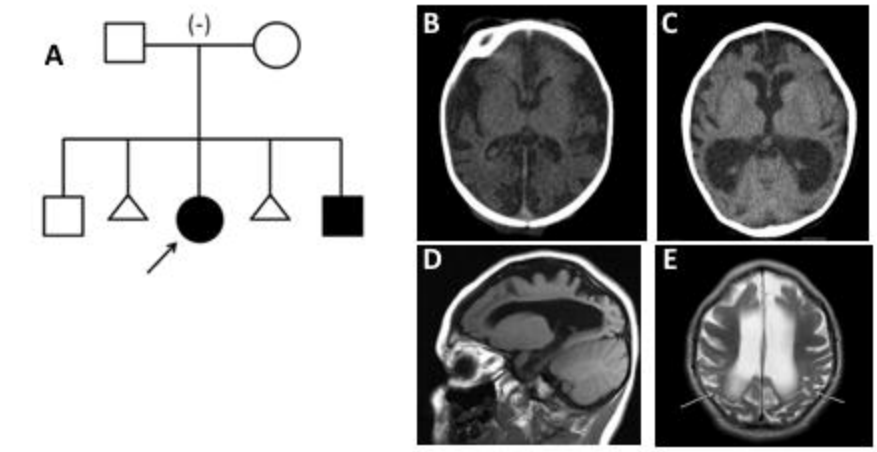
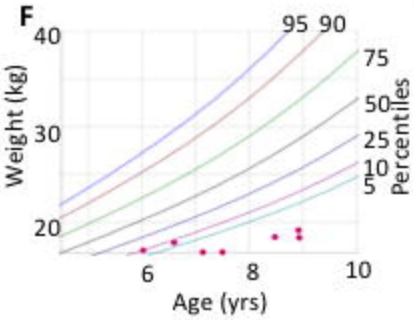
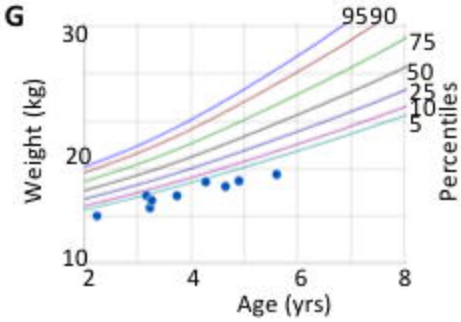
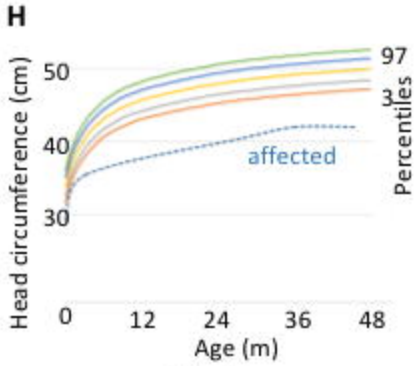
Table 5. Primers used in this study.

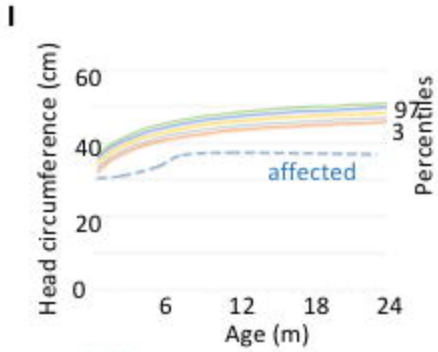
h*COPB2*F	tgtaaaacgacggccagtggtcatta
h*COPB2*R	tcacacaggaaacagctatgacgctc
Copb2 Zfn guides	
ZFN Primer F	taaacccgattttggagctg
ZFN Primer R	acagtgttcagcacagtggg
Copb2 R254C CRISPR guides	
Guide 1	gaagatggaacagtacgtatt
Guide 2	gtttggcattcaagcacctac
Guide 3 (used)	gtctggcattctagcacctac
Copb2 CRISPR donor oligonucleotide	Tttattttggatgttcatatccagagggaatgtttacattgttgatttttag GAACCGTATGTATATGGCACTCAAGTACCTAT CGACTTGAGAGCACGCTGAATTACGGAATGG AGAGGGTG
Genotyping *Copb2*^{Zfn} allele	
m*Copb2 Zfn F*	atctatgggggcttcttgct
m*Copb2 Zfn R*	tgacacgagggactcagaca
Genotyping *Copb2 CRISPR* alleles	
m*Copb2* CRISPR flank F	cggcttccagtagctcattc
m*Copb2* CRISPR flank R	atctttccattggcatccat

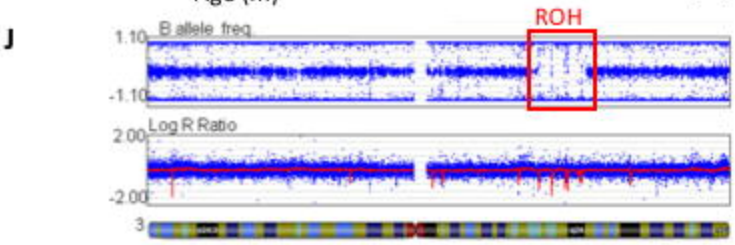


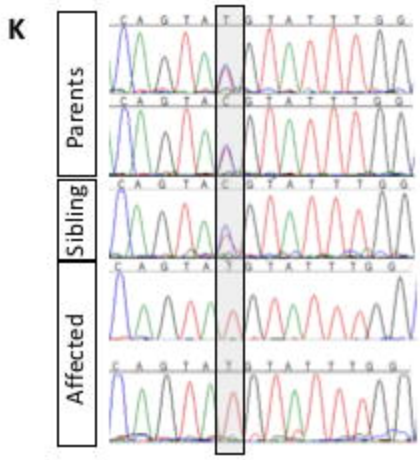








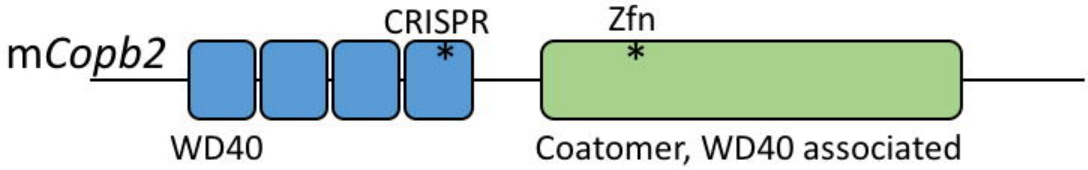




L

	250 255 260
H. Sapiens	GSEDGTVRIWHSSTYRL
M. mulatta	GSEDGTVRIWHSSTYRL
M. musculus	GSEDGTVRIWHSSTYRL
X. tropicalis	GSEDGTVRIWHSSTYRL
D. rerio	GSEDGTVRIWHSSTYRL
D. melanogaster	GSEDGTVRIWHSGTYRL
C. elegans	GSEDSTVRLWHANTYRL

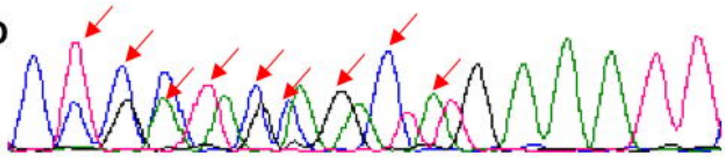
A



B

Copb2^{wt} CCGCAGAATTGAAATT
Copb2^{Zfn} CCGCAGA-----AATT

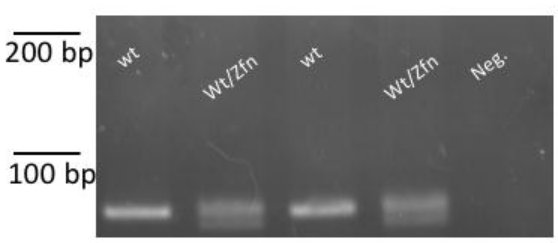
D



C

Copb2^{wt} LIRRIEIQPKHIFW
Copb2^{Zfn} LIRRNSAQAHLLV*

E



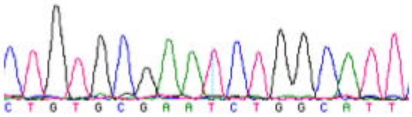
F

Exon 8

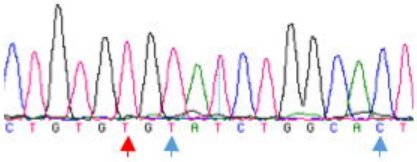
Copb2^{wt} ttgatttttagGAACTGTGCGAATCTGGCATTCTAGCACCT-ACCG
Copb2^{R254C} ttgatttttagGAACTGTGTGTATCTGGCACTCTAGTACTT-ATCG
Copb2^{null} ttgatttttagGAACTGTGCGAATCTGGCATTCTAGCACCTTACCG

G

Copb2^{wt}

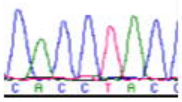


Copb2^{R254C}

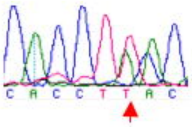


H

Copb2^{wt}

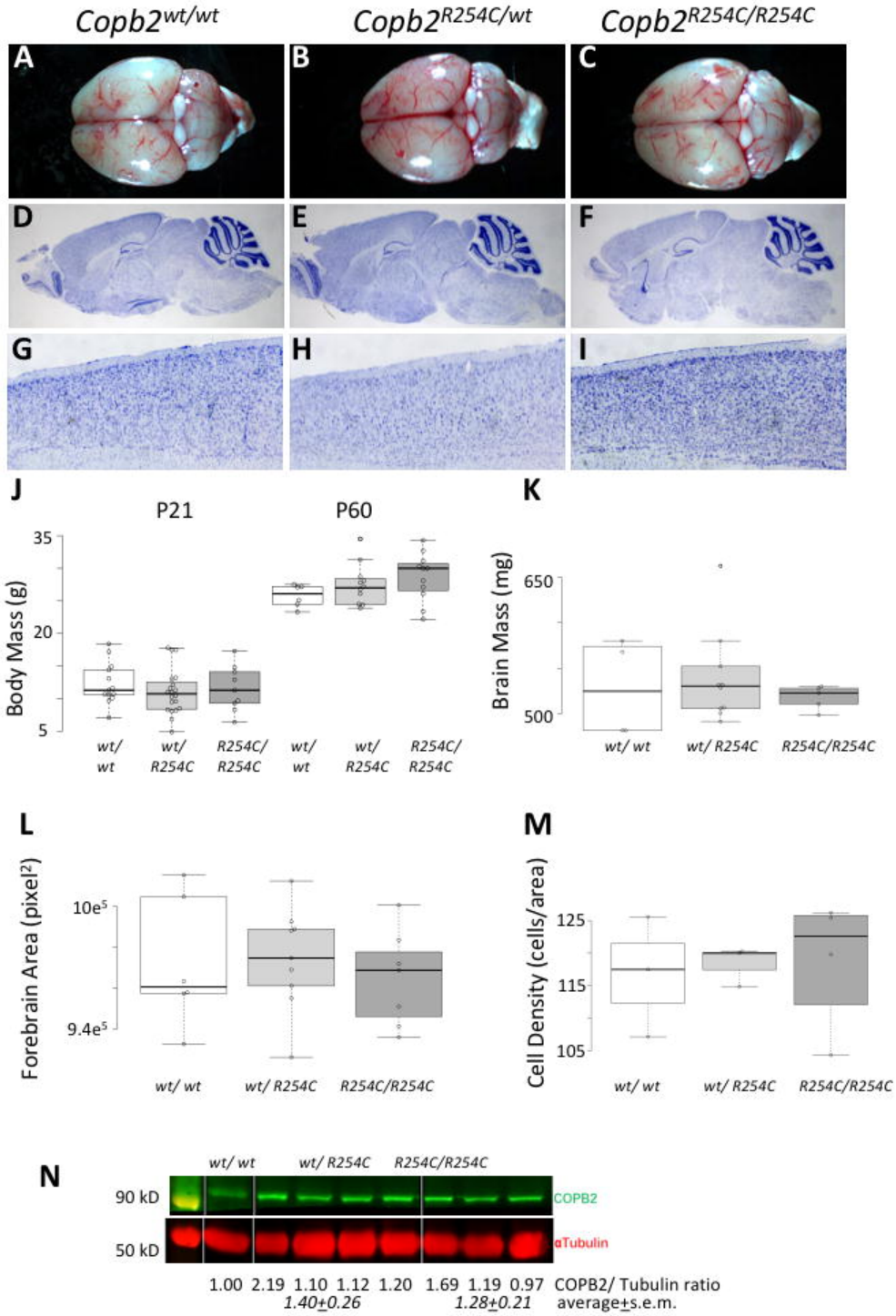


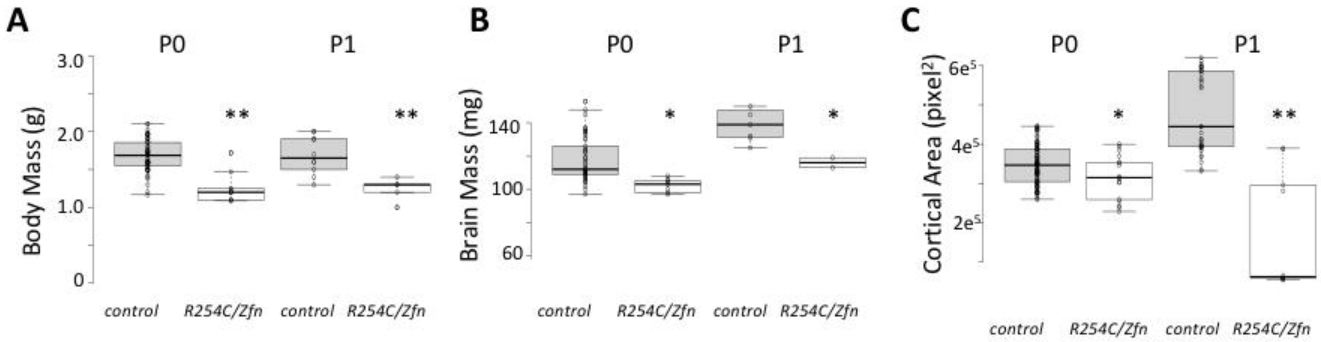
Copb2^{null}

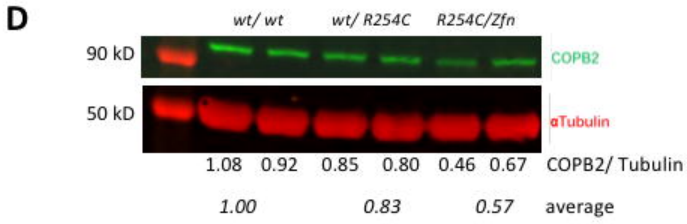


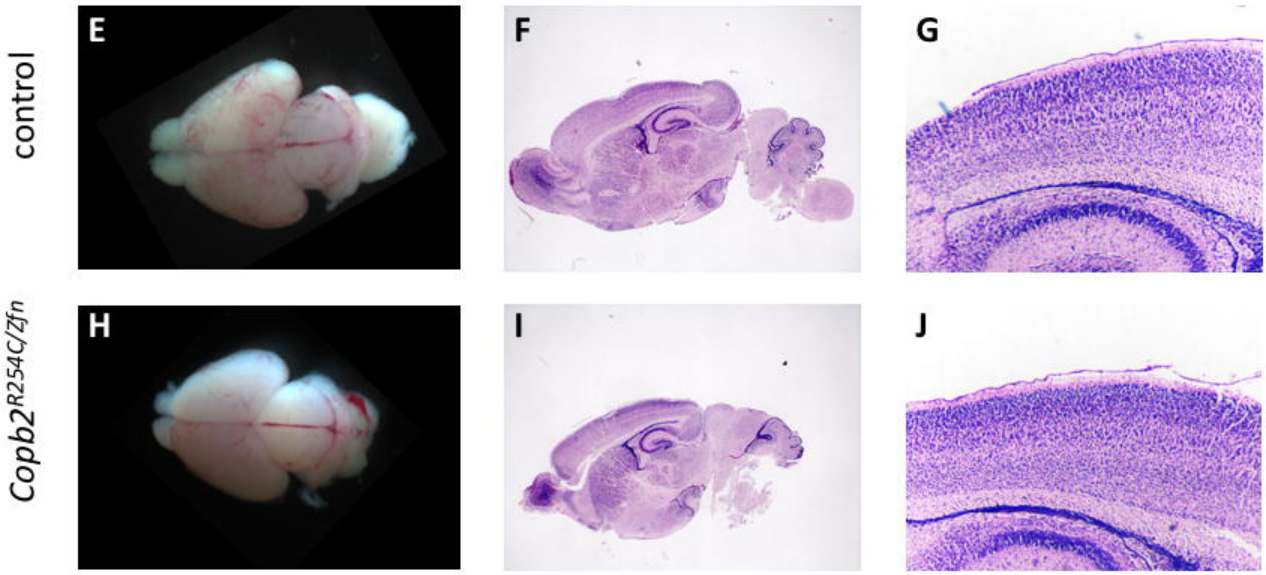
I

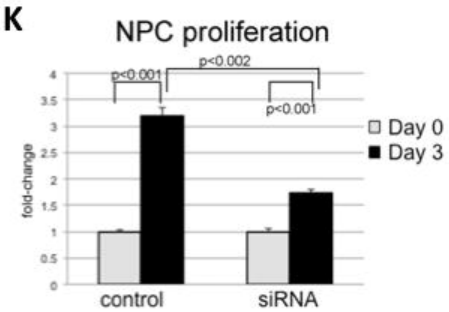
Copb2^{wt} GTVRIWHSSTYRLE
Copb2^{R254C} GTVCIWHSSTYRLE
Copb2^{null} GTVRIWHSSTLPA*

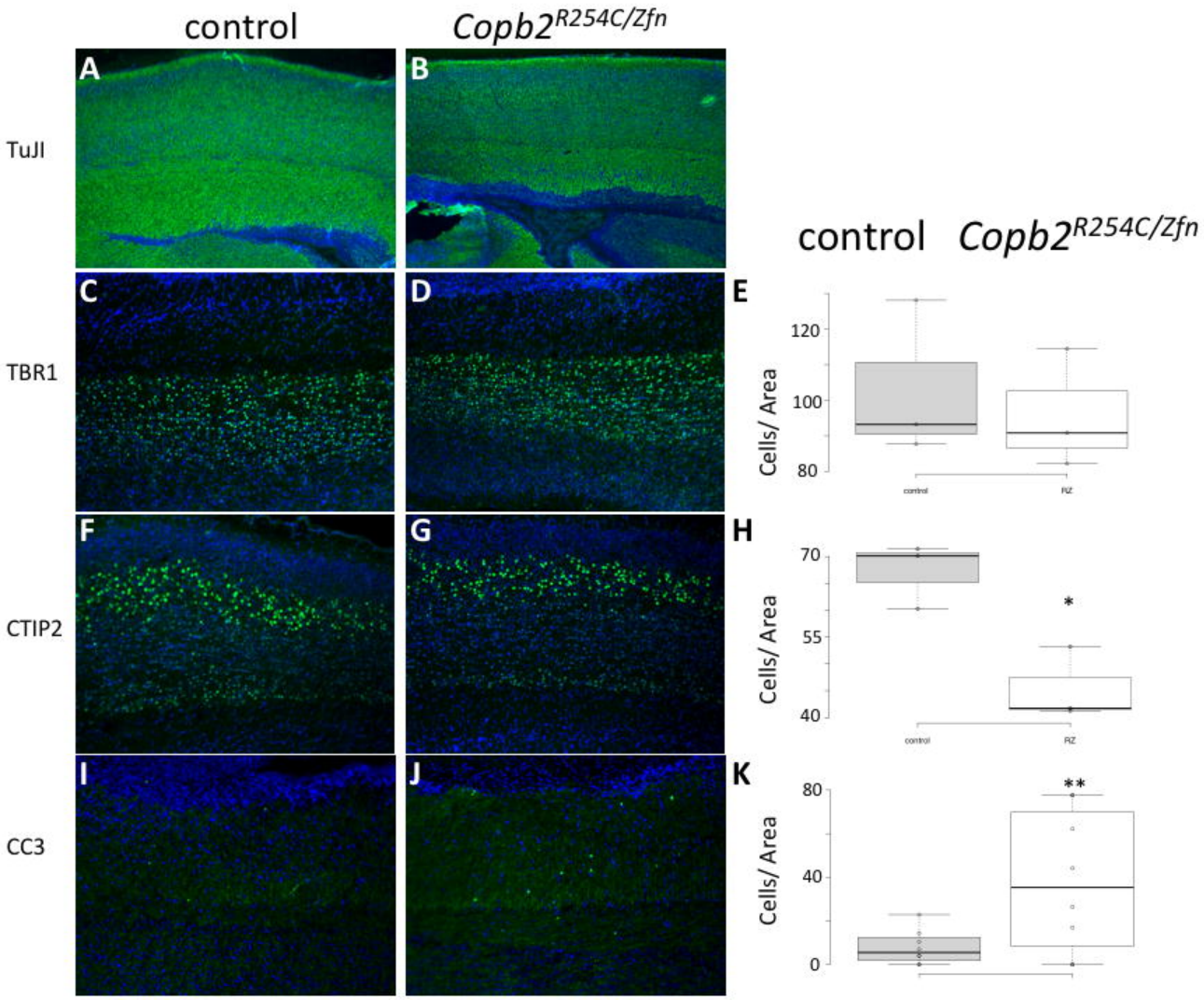

Copb2wt/wt
Copb2R254C/wt
Copb2R254C/R254C
A
B
C
D
E
F
G
H
I
J
P21
P60
Body Mass (g)
35
20
5
wt/ wt
wt/ R254C
R254C/ R254C
K
Brain Mass (mg)
650
500
wt/ wt
wt/ R254C
R254C/R254C
L
Forebrain Area (pixel2)
10e5
9.4e5
M
Cell Density (cells/area)
125
115
105
N
90 kD
50 kD
COPB2
αTubulin
1.00 2.19 1.10 1.12 1.20 1.69 1.19 0.97 COPB2/ Tubulin ratio
1.40±0.26
1.28±0.21
average±s.e.m.


A
P0
P1
Body Mass (g)
3.0
2.0
1.0
0
**
**
control
R254C/Zfn
control
R254C/Zfn
B
P0
P1
Brain Mass (mg)
140
100
60
*
*
control
R254C/Zfn
control
R254C/Zfn
C
P0
P1
Cortical Area (pixel²)
6e⁵
4e⁵
2e⁵
*
**
control
R254C/Zfn
control
R254C/Zfn


D
wt/ wt
wt/ R254C
R254C/Zfn
90 kD
COPB2
50 kD
αTubulin
1.08 0.92 0.85 0.80 0.46 0.67 COPB2/ Tubulin
1.00 0.83 0.57 average


control
Copb2R254C/Zfn
E
F
G
H
I
J


K
NPC proliferation
p<0.001
p<0.002
p<0.001
fold-change
4
3.5
3
2.5
2
1.5
1
0.5
0
Day 0
Day 3
control
siRNA


control
Copb2R254C/Zfn
A
B
TuJI
C
D
TBR1
F
G
CTIP2
I
J
CC3
control
Copb2R254C/Zfn
E
Cells/ Area
120
100
80
control
RZ
H
Cells/ Area
70
55
40
*
control
RZ
K
Cells/ Area
80
40
0
**