Smaller Body Size, Early Postnatal Lethality, and Cortical Extracellular Matrix-Related Gene Expression Changes of Cyfip2-Null Embryonic Mice

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Cytoplasmic FMR1-interacting protein 2 (CYFIP2) is a key component of the WAVE regulatory complex (WRC) which regulates actin polymerization and branching in diverse cellular compartments. Recent whole exome sequencing studies identified de novo hotspot variants in CYFIP2 from patients with early-onset epileptic encephalopathy and microcephaly, suggesting that CYFIP2 may have some functions in embryonic brain development. Although perinatal lethality of Cyfip2-null (Cyfip2−/−) mice was reported, the exact developmental time point and cause of lethality, and whether Cyfip2−/− embryonic mice have brain abnormalities remain unknown. We found that endogenous Cyfip2 is mainly expressed in the brain, spinal cord, and thymus of mice at late embryonic stages. Cyfip2−/− embryos did not show lethality at embryonic day 18.5 (E18.5), but their body size was smaller than that of wild-type (WT) or Cyfip2+/− littermates. Meanwhile, at postnatal day 0, all identified Cyfip2−/− mice were found dead, suggesting early postnatal lethality of the mice. Nevertheless, the brain size and cortical cytoarchitecture were comparable among WT, Cyfip2+/−, and Cyfip2−/− mice at E18.5. Using RNA-sequencing analyses, we identified 98 and 72 differentially expressed genes (DEGs) from the E18.5 cortex of Cyfip2+/− and Cyfip2−/− mice, respectively. Further bioinformatic analyses suggested that extracellular matrix (ECM)-related gene expression changes in Cyfip2−/− embryonic cortex. Together, our results suggest that CYFIP2 is critical for embryonic body growth and for early postnatal survival, and that loss of its expression leads to ECM-related gene expression changes in the embryonic cortex without severe gross morphological defects.

Keywords: Cyfip2-null mice, Embryo, body size, postnatal lethality, extracellular matrix
INTRODUCTION

The Cytoplasmic FMR1-interacting protein (CYFIP1 and CYFIP2) family is a critical component of the heteropentameric WAVE regulatory complex (WRC) which regulates actin polymerization and branching in diverse cellular compartments (Abekhoukh and Bardoni, 2014; Lee et al., 2017). Despite their high sequence homology at the protein level (88% identity and 95% similarity) (Schenck et al., 2001), several lines of evidence indicate that CYFIP1 and CYFIP2 have distinct and non-complementable functions in vivo (Cioni et al., 2018). For example, both Cyfip1- and Cyfip2-null mice are lethal at different developmental stages. Specifically, Cyfip1-null (Cyfip1−/−) embryos die before embryonic day 9.5 (E9.5) (Chung et al., 2015). In the case of Cyfip2−/− mice, perinatal lethality was reported (Kumar et al., 2013; Han et al., 2015), but the exact developmental time point and cause of lethality, and whether Cyfip2−/− embryos have molecular or morphological brain abnormality remain unknown.

Clinically, variants of CYFIP1 have been associated with neurodevelopmental and neuropsychiatric disorders, including autism spectrum disorders, intellectual disability, and schizophrenia (Abekhoukh and Bardoni, 2014). Although the genetic associations between CYFIP2 and brain disorders are relatively unknown, two recent whole exome sequencing studies identified de novo hotspot variants of CYFIP2 (at the Arg87 residue) in patients diagnosed with West syndrome (Nakashima et al., 2018; Peng et al., 2018). The variants may disrupt the inhibitory interaction between CYFIP2 and WAVE in the WRC, leading to aberrant activation of the WRC and downstream actin polymerization (i.e., gain-of-function effects on the WRC) (Nakashima et al., 2018). The West syndrome is characterized by early-onset epileptic encephalopathy and developmental delay, and the symptoms typically start between three and twelve months of age (D’alongo et al., 2018). The patients with CYFIP2 variants showed signs of microcephaly and began experiencing seizures around three to six months of age (Nakashima et al., 2018). Therefore, it is conceivable that CYFIP2 may have some roles in embryonic brain development.

To examine the function of CYFIP2, we characterized expression patterns of endogenous Cyfip2 in embryonic mice, we performed in situ hybridization analysis with two independent probes against Cyfip2 transcripts. In E16.5 and E18.5 WT mice, Cyfip2 transcripts were detected throughout the central nervous system, and relatively strong signals were observed in the cortex (Figure 1A). Intriguingly, strong Cyfip2 expression was also detected in the thymus but not in other organs. Consistent with the in situ hybridization analysis, qRT-PCR analysis showed that Cyfip2 and Wasf1 (encoding WAVE1) transcripts were more abundant in the brain compared with the liver and intestine of E18.5 mice (Figure 1B). Meanwhile, Cyfip1 transcripts in the intestine were as abundant as those in the brain. At the protein level, CYFIP1, CYFIP2, and WAVE1 were expressed in the E18.5 brain at levels higher than those in the liver and intestine (Figure 1C). We observed similar results from tissue samples of postnatal day 0 (P0) mice (Figure S1).

By using two primer sets (one for the targeting cassette of Cyfip2-mutant mice, and the other for intron 7 of Cyfip2), we could identify WT, Cyfip2+/−, and Cyfip2−/− mice at E18.5 (Figure 1D). We could confirm that Cyfip2 mRNAs and proteins in the cortex and cerebellum were reduced by approximately 50% in Cyfip2−/− mice compared with WT mice, and not detected in Cyfip2+/− mice at E18.5 (Figures 1E,F). There was no change in Cyfip1 and Wasf1 mRNA levels in either Cyfip2+/− or Cyfip2−/− embryonic mice compared with WT littermates (Figure 1E). However, at the protein level, WAVE1, but not CYFIP1, was reduced in Cyfip2−/− cortex at E18.5 (Figure 1F), which is consistent with previous reports showing decreased WAVE1 protein stability without CYFIP (Zhao et al., 2013; Han et al., 2015).

More information about materials and methods is provided in Supplementary Materials.
Cyfip2−/− mice at P0. During the counting, we often observed dead pups in the cages. Indeed, after genotyping PCR, we found that all identified Cyfip2−/− mice were dead at P0 (Figure 1I).

The smaller body size and early postnatal lethality of Cyfip2−/− embryos prompted us to investigate brain abnormalities of the mice. However, brain size, as measured by length and width of the cortical region, was comparable among WT, Cyfip2−/−, and Cyfip2+/− mice at E18.5 (Figure 2A). Moreover, DAPI staining of the brain sections showed generally normal morphology of Cyfip2−/− brains at E18.5 (Figure 2B).

To further analyze the details of the cortical cytoarchitecture of the embryos, we performed fluorescent immunohistochemistry on the cortical area using antibodies against Brain-2 (Brn2, marker for layers 2/3, and S) and COUP-TF-interacting protein 2 (Ctip2, marker for layer 5/6) (Figure 2C). We found that total width of the cortex (from layer 2/3 to intermediate zone), and relative width of each layer to total width (percentage of total width) were similar among WT, Cyfip2+/−, and Cyfip2−/− mice at E18.5 (Figure 2D). We further confirmed these results with additional antibodies against Cut like homeobox 1 (Cux1, marker for layer 2/3) and T-box brain protein 1 (Tbr1, marker for layer 6) (Figure S3). However, the relative width of layers 2-4 to the total width was significantly larger in Cyfip2−/− embryos than in the Cyfip2+/− littermates. We also examined F-actin levels in the cortex, but they were not different among WT, Cyfip2+/−, and Cyfip2−/− embryos. Furthermore, the total neurite lengths of WT and Cyfip2−/− cultured cortical neurons (at days in vitro 4) were comparable, but those between Cyfip2+/− and Cyfip2−/− neurons were slightly, but significantly different (Figure S4).

Next, we performed transcriptomic analyses (RNA-sequencing [RNA-seq]) of cortical tissue from E18.5 WT, Cyfip2+/−, and Cyfip2−/− mice, to identify any molecular changes (Table S1). After applying adjusted P values to the analyses, we identified 98 and 72 differentially expressed genes (DEGs) in the Cyfip2+/− and Cyfip2−/− cortex, respectively, compared with the WT cortex (Figure 2E and Tables S2, S3). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of the 98 DEGs in the Cyfip2+/− cortex showed no significant term in any category, possibly because of heterogeneity of the DEGs. However, same
analyses on the 72 DEGs in the Cyfip2−/− cortex revealed extracellular matrix (ECM)-related terms to be significant (Figure 2F and Table S4). Furthermore, Gene Set Enrichment Analysis (GSEA), which is used to identify molecular signatures based on the broader expression changes in the transcriptome, also suggested “ECM receptor interaction” as a significantly enriched term especially from the up-regulated genes in the Cyfip2−/− cortex (Figure 2G and Table S5).

Taken together, our results suggest that CYFIP2 is critical for embryonic body growth and for early postnatal survival. CYFIP2 is not essential for overall embryonic brain development, in terms of gross morphology, as assessed by brain size and cortical cytoarchitecture of Cyfip2−/− mice. However, at the molecular level, ECM-related genes are significantly altered in the cortex of Cyfip2−/− embryonic mice. ECM affects many aspects of brain development, ranging from neuronal migration to synapse formation (Barros et al., 2011). Therefore, further detailed analyses about the cortical ECM in Cyfip2−/− mice may potentially provide better insight toward understanding the functions of CYFIP2 in embryonic mice. However, our results may not be directly implicated in the pathophysiology that underlies early-onset epileptic
encephalopathy and developmental delay associated with West syndrome, because the CYFIP2 variants found in patients have gain-of-function effects on the WRC and do not affect CYFIP2 stability (Nakashima et al., 2018).

**AUTHOR CONTRIBUTIONS**

YZ, YL, YK, BL, JK, CJ, SK and KH designed and performed the experiments. HKa, HKi, and KH analyzed and interpreted the data. HKa and KH wrote the paper. All authors read and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00482/full#supplementary-material

**REFERENCES**

Abekhoukh, S., and Bardoni, B. (2014). CYFIP family proteins between autism and intellectual disability: links with Fragile X syndrome. *Front. Cell. Neurosci.* 8:81. doi: 10.3389/fncel.2014.00081

Barros, C. S., Franco, S. J., and Muller, U. (2011). Extracellular matrix: functions in the nervous system. *Cold Spring Harb. Perspect. Biol.* 3:a005108. doi: 10.1101/cshperspect.a005108

Chung, L., Wang, X., Zhu, L., Towers, A. J., Cao, X., Kim, I. H., et al. (2015). Parental origin impairment of synaptic functions and behaviors in cytoplasmic FMRP interacting protein 1 (Cyfip1) deficient mice. *Brain Res.* 1629, 340–350. doi: 10.1016/j.brainres.2015.10.015

Cioni, J. M., Wong, H. H., Bressan, D., Kodama, L., Harris, W. A., and Holt, C. E. (2018). Axon-Axon interactions regulate topographic optic tract sorting via CYFIP2-dependent WAVE complex function. *Neuron* 97, 1078–1093 e1076. doi: 10.1016/j.neuron.2018.01.027

D’alonzo, R., Rigante, D., Mencaroni, E., and Esposito, S. (2018). West syndrome: a review and guide for paediatricians. *Clin. Drug Investig.* 38, 113–124. doi: 10.1007/s40261-017-0595-z

Han, K., Chen, H., Gennarino, V. A., Richman, R., Lu, H. C., and Zoghbi, H. Y. (2015). Fragile X-like behaviors and abnormal cortical dendritic spines in Cytoplasmic FMR1-interacting protein 2-mutant mice. *Hum. Mol. Genet.* 24, 1813–1823. doi: 10.1093/hmg/ddu595

Kumar, V., Kim, K., Joseph, C., Kourrich, S., Yoo, S. H., Huang, H. C., et al. (2013). C57BL/6N mutation in cytoplasmic FMRP interacting protein 2 regulates cocaine response. *Science* 342, 1508–1512. doi: 10.1126/science.1245563

Lee, Y., Kim, D., Ryu, J. R., Zhang, Y., Kim, S., Kim, Y., et al. (2017). Phosphorylation of CYFIP2, a component of the WAVE-regulatory complex, regulates dendritic spine density and neurite outgrowth in cultured hippocampal neurons potentially by affecting the complex assembly. *Neuroreport* 28, 749–754. doi: 10.1097/WNR.0000000000000838

Nakashima, M., Kato, M., Aoto, K., Shiina, M., Belal, H., Mukaida, S., et al. (2018). De Novo hotspot variants in CYFIP2 cause early-onset epileptic encephalopathy. *Ann. Neurol.* 83, 794–806. doi: 10.1002/ana.25208

Peng, J., Wang, Y., He, F., Chen, C., Wu, L. W., Yang, L. F., et al. (2018). Novel West syndrome candidate genes in a Chinese cohort. *CNS Neuropsi. Ther.* 24, 1196–1206. doi: 10.1111/cns.12860

Schenc, A., Bardoni, B., Moro, A., Baggi, C., and Mandel, J. L. (2001). A highly conserved protein family interacting with the fragile X mental retardation protein (FMRP) and displaying selective interactions with FMRP-related proteins FXR1P and FXR2P. *Proc. Natl. Acad. Sci. U.S.A.* 98, 8844–8849. doi: 10.1073/pnas.15121598

Zhao, L., Wang, D., Wang, Q., Rodal, A. A., and Zhang, Y. Q. (2013). *Drosophila* cyfip regulates synaptic development and endocytosis by suppressing filamentous actin assembly. *PLoS Genet.* 9:e1003450. doi: 10.1371/journal.pgen.1003450

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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