



CELF2-dependent RNA Regulation Supports Cortical Architecture and Synaptic Stability During Early Brain Development

Ishana Syed^{1,3} · Jing Jiang² · Su-Hyuk Ko^{1,3} · Shaoting Chen² · Zhao Zhang² · Zhijie Liu² · Lizhen Chen^{1,3}

Received: 22 February 2026 / Accepted: 13 May 2026
© The Author(s) 2026

Abstract

RNA regulation plays a central role in neurodevelopment by coordinating neuronal differentiation, migration, and circuit formation. The CUGBP Elav-like family member 2 (CELF2) is an RNA-binding protein with established roles in alternative splicing and mRNA regulation, yet its function in the developing brain remains poorly defined. Here, we investigated the role of CELF2 during neurodevelopment using a constitutive *Celf2* knockout (KO) mouse model. *Celf2* knockout pups exhibited neonatal lethality accompanied by impaired neuronal maturation and disrupted cortical organization. Bulk RNA sequencing revealed widespread transcriptional dysregulation, while splicing analyses identified reduced exon inclusion in multiple neurodevelopmental transcripts following CELF2 loss. Notably, *Camk2a* transcript and protein levels were markedly reduced in knockout brains, consistent with CELF2 binding to the *Camk2a* 3'UTR. Functional studies in *C. elegans* demonstrated that expression of human CAMK2A partially rescued synaptic puncta deficits in *unc-75* (CELF ortholog) mutants, supporting a conserved role for CELF-family proteins in synaptic maturation. Histological analyses revealed reductions in Nestin- and Doublecortin-positive immature neurons, thinning of upper cortical layers, and decreased CAMK2A expression. Single-nucleus RNA sequencing further revealed selective reductions in upper layer II/III excitatory neuron populations in the cortex. Cellular trajectory and pseudotime analyses revealed delayed maturation in certain cell types but accelerated progression in others in *Celf2* KO animals. Together, these findings establish CELF2 as a critical post-transcriptional regulator required for neuronal maturation and architectural stability during early brain development and highlight how disruption of RNA regulatory programs may contribute to neurodevelopmental disorders.

Keywords RNA-binding proteins · Single nucleus RNA sequencing · Neurodevelopment · Neuronal maturation · Synaptic development · Cortical organization

Introduction

Neurodevelopment depends on tightly coordinated gene regulatory programs that guide neural progenitor proliferation, neuronal differentiation, migration, synaptogenesis, and the assembly of cortical and hippocampal circuits [1–3]. Disruption of these processes contributes to a broad spectrum

of neurodevelopmental disorders (NDDs), including autism spectrum disorder, intellectual disability, and epilepsy [4, 5]. Large-scale sequencing studies have implicated hundreds of risk genes and pathways, yet the mechanisms by which these genes converge on early developmental events remain incompletely defined [6, 7]. In parallel with transcriptional control, post-transcriptional regulation, including alternative splicing, mRNA stability, localization, and translation, adds a critical layer of developmental gene regulation that is increasingly recognized as central to neuronal maturation and circuit formation [5, 8, 9].

RNA-binding proteins (RBPs) orchestrate these post-transcriptional programs by regulating splice site selection, transcript turnover, and translation in a cell type- and developmental stage-dependent manner [8, 9]. Several RBPs have been directly linked to neurodevelopmental phenotypes through their control of transcript networks required

✉ Lizhen Chen
ChenL7@uthscsa.edu

¹ Department of Cell Systems and Anatomy, University of Texas Health San Antonio, San Antonio, TX 78229, USA

² Department of Molecular Medicine, University of Texas Health San Antonio, San Antonio, TX 78229, USA

³ Barshop Institute for Longevity and Aging Studies, University of Texas Health San Antonio, San Antonio, TX 78229, USA

for neuronal identity, synaptic development, and circuit assembly [8–10]. The CUGBP Elav-like family (CELF) proteins comprise a conserved group of RNA-binding proteins that regulate multiple aspects of post-transcriptional gene expression. In mammals, the family consists of six members, CELF1–6, exhibiting both overlapping and tissue-specific expression patterns [11, 12]. In the brain, CELF proteins are dynamically regulated during development and display region- and cell type-specific expression patterns, consistent with roles in neuronal differentiation and maturation. Mechanistically, CELF proteins control alternative splicing, mRNA stability, polyadenylation, and translation, thereby shaping isoform diversity and fine-tuning protein output during critical developmental windows. Dysregulation of CELF-mediated RNA processing has been implicated in neuromuscular and neurological disorders, underscoring the importance of this family in maintaining tissue-specific gene expression programs [13–16].

Among these family members, CELF2 has emerged as a key regulator of alternative splicing and mRNA metabolism [13, 17]. While CELF2 has been extensively studied in immune contexts, including activity-dependent regulation of CELF2 expression and transcript stability, evidence increasingly suggests that CELF-family RBPs also support neuronal gene regulatory programs [12, 17]. In neurons, CELF proteins can influence axon-related RNA programs and post-transcriptional regulation of transcripts involved in neuronal structure and connectivity [12, 18, 19]. Notably, CELF2 has been implicated in neuronal biology through studies linking CELF2 to RNA regulatory events relevant to synaptic and developmental pathways, and through emerging *in vivo* work connecting *Celf2* disruption to neurodevelopmental phenotypes [19–22]. Recent studies have also begun to connect CELF2 biology to neurodevelopmental disease. *De novo* missense variants in CELF2, clustering within the nuclear localization signal of the RRM3 domain, have been identified in children presenting with simplified gyral patterns, global developmental delay, speech and language impairment, and epilepsy [21]. These variants cause aberrant cytoplasmic retention of CELF2, implicating disrupted nucleocytoplasmic shuttling as a pathogenic mechanism in human cortical malformation. Complementary mouse models have provided further mechanistic insight: acute shRNA-mediated knockdown in radial glial progenitors at E12.5–13.5 established CELF2 as a translational repressor of pro-differentiation mRNAs required for progenitor maintenance [21], while broad Nestin-Cre conditional deletion beginning at approximately E10.5 produced autism-relevant behavioral phenotypes, cortical thinning, and synaptic deficits, with transcriptomic changes enriched in synapse and dendrite pathways [20]. However, these models leave key questions unresolved. Neither approach defines which specific RNA targets and splicing programs are disrupted by CELF2 loss at a network

level, nor do they identify the downstream effectors through which CELF2 regulates neuronal maturation. Furthermore, the consequences of constitutive *Celf2* loss during the early postnatal period, when cortical lamination and excitatory circuit maturation undergo rapid refinement, have not been examined with cell-type resolution. Addressing these gaps is essential for understanding how CELF2-dependent RNA regulation contributes to neurodevelopmental outcomes and for situating CELF2 within the broader landscape of RBP-mediated risk for neurodevelopmental disorders.

Here, we used a constitutive *Celf2* knockout mouse model to define CELF2 function during early brain development by integrating bulk RNA sequencing, splicing analysis, immunohistochemistry, and single-nucleus RNA sequencing. We identify widespread transcriptomic disruption and reduced exon inclusion across multiple neurodevelopmentally relevant transcripts following CELF2 loss. Among affected genes, *Camk2a* emerged as a prominent CELF2-associated target, with reduced *Camk2a* transcript and protein levels in knockout brains. Furthermore, we demonstrate that *unc-75* (the *C. elegans* ortholog of CELF) mutants exhibit synaptic defects accompanied by reduced expression of *unc-43* (the CaMKII ortholog). Notably, transgenic expression of human CAMK2A partially rescues the synaptic phenotypes observed in *unc-75* mutants. Collectively, this study defines CELF2 as a key post-transcriptional regulator required for neuronal maturation and architectural stability during early brain development and provides a framework for understanding how disruption of RBP-mediated RNA regulation can contribute to neurodevelopmental.

Methods

Mouse Model and Tissue Collection

All animal care and experimental procedures were conducted in full accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of Texas Health Science Center at San Antonio.

Mouse Model and Breeding Strategy

The *Celf2* conditional knockout mouse line was generated by flanking exon 3 of the *Celf2* locus with loxP sites (*Celf2* fl/fl). To generate constitutive, germline knockout animals, *Celf2* fl/fl mice were crossed with Zp3-Cre transgenic mice (The Jackson Laboratory, RRID:IMSR_JAX:003651), in which Cre recombinase is expressed under the zona pellucida 3 promoter and mediates recombination in the oocyte, resulting in germline deletion of the floxed allele in all offspring. This cross produced *Celf2* heterozygous (*Celf2* +/-) founders, which were subsequently intercrossed (*Celf2*

+/- × Celf2 +/-) to maintain the colony and generate all three genotypes for experimental use. This intercross breeding strategy was necessitated by the fact that homozygous Celf2 knockout (Celf2^{-/-}) animals are neonatal lethal and therefore cannot contribute to colony propagation.

Genotyping and Genotype Distribution

Genotyping of the null allele was performed by PCR using the following primers: Forward: 5'-AACTGCACTGGA GGGATTTG-3'; Reverse: 5'-AAGGAAGGCGCACTAAAA CA-3', generating a PCR product of 570 bps. Genotyping of the WT allele was performed by PCR using the following primers: Forward: 5'-GAGGTGTCTGCCGA-3'; Reverse: 5'-CACTCAGTCCCTGTTTGTA-3', generating a PCR product of 370 bps. These primers distinguish wild-type, heterozygous, and knockout animals based on the presence or absence of the exon 3. Consistent with Mendelian expectations from an intercross of two heterozygous parents, litters exhibited a 1:2:1 ratio of wild-type (Celf2^{+/+}): heterozygous (Celf2^{+/-}): knockout (Celf2^{-/-}) offspring. Because the strain is maintained on a C57BL/6 background and typically produces relatively small litters (5–7 pups per litter), wild-type animals were sometimes limited. Therefore, heterozygous littermates were used as controls in certain experiments, as they were more readily available.

Phenotypic Characterization

Gross phenotypic assessment was performed at the time of collection on postnatal day 0 (P0). Wild-type and heterozygous pups were visually indistinguishable from one another and displayed no overt abnormalities. Knockout pups were identified not only by genotyping but also by the absence of a visible milk spot on the abdomen, indicating impaired nursing behavior, which is consistent with neonatal lethality. Body size and brain mass did not differ significantly across genotypes at P0, suggesting that gross developmental growth proceeds normally in the absence of CELF2 up to the time of birth, and that any observed molecular differences are not attributable to gross size differences between animals in early neonatal development.

Tissue Collection

P0 pups were euthanized and brains were rapidly dissected. Each brain was bisected along the sagittal plane: one hemisphere was snap-frozen on dry ice for downstream RNA and protein extraction and stored at -80 °C, and the contralateral hemisphere was immersion-fixed in 4% paraformaldehyde (PFA) for 24 h, cryoprotected in 30% sucrose, embedded in optimal cutting temperature (OCT) compound, and cryosectioned at 10 µm for immunohistochemical analysis.

Bulk RNA Sequencing and Analysis

Total RNA was extracted from $n=3$ frozen postnatal day 0 (P0) mouse hemibrains per genotype using TRIzol reagent (Invitrogen, cat no. 15596026) and further purified using a Qiagen RNA isolation kit (cat no. 74104). RNA integrity was assessed by the UT Health San Antonio sequencing core. Poly(A)+RNA libraries were prepared and sequenced on an Illumina platform. Sequencing reads were aligned to the mouse reference genome (mm9) using STAR. Differential gene expression analysis was performed using DESeq2, with significance defined as an adjusted p -value < 0.05. Alternative splicing analysis was conducted using rMATS with a false discovery rate (FDR) < 0.05. Genes that were both differentially expressed and alternatively spliced were identified by intersecting DESeq2 and rMATS outputs. Gene Ontology (GO) enrichment analysis was performed using ShinyGO, and additional DEG-focused analyses and visualizations were generated using the iDEP online platform. Genome browser visualizations were created using IGV from bigWig files aligned to the mouse mm9 reference genome. Previously published CELF2 CLIP-seq datasets generated by the Chen laboratory were re-analyzed and integrated with transcriptomic and splicing results to identify putative direct CELF2 RNA targets [18].

RT-PCR and qPCR

Total RNA ($n=3$ KO and Celf2^{+/-} mouse pups, $n=2$ WT mouse pups, $n=4$ replicate plates of *C.elegans* per strain) were reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, cat no. 18080085) with a mixture of oligo(dT) and random hexamer primers. For RT-PCR, gene-specific primers flanking the targeted exon were used to assess alternative isoform expression. RT-PCR reactions were performed using DreamTaq PCR Master Mix (2×, Thermo Scientific, cat no. K1081) in a final volume of 10 µL, and PCR products were resolved by agarose gel electrophoresis. For quantitative PCR (qPCR), reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad, cat no. 1725120) in a final reaction volume of 5 µL on a Bio-Rad real-time PCR system. All reactions were run in technical duplicate, and expression levels were normalized to *Gapdh* using the $\Delta\Delta C_t$ method. Primers were generated through Sigma-Aldrich. Primer sequences used in this study are listed as follows: mouse Dcl2 exon 5, F: 5'-ACTTTGTCCTGGATCATAGCG-3' and R: 5'-CTACTG GGAGTTCCATTCACTG-3'; mouse Camk2a exon 1–4, F: 5'-ACCCTCTACTTTCTCTCCTCC-3' and R: 5'-ACT TTGGTGTCTTCGTCTC-3'; mouse Camk2a exon 3–5, Forward: 5'-TTGAGGACGAAGACACCAAAG-3' and R: 5'-CTGGTTCAAAGGCTGTCATTC-3'; mouse Camk2a exon 5–6, F: GAATGACAGCCTTTGAACCAG-3' and R:

5'-GATGTGAGGGTTCAGGATGG-3'; mouse *Aplp2* exon 7, F: GAGAATCCAACCGAACCCAG-3' and R: 5'-TCC GCTGAGGTCTCAAAATAC; mouse *Gapdh* F: 5'-GTG AGGCCGGTGCTGAGTAT-3' and R: 5'-CTTTTGGCT CCACCTTCAA-3'; *C. elegans* *Unc-43*, F: 5'- GATGCC GCGTGTGTTGCTTAC-3' and R: 5'-CCCAACGGCCTT GTTTCTTTGAC-3'; *C. elegans* *Act-1*, F: 5'- CTCTTGCCC CATCAACCATG-3' and R: 5'-CTTGCTTGAGATCC ACATC-3'. Band intensities from RT-PCR gels were quantified using ImageJ. Images were converted to 8-bit grayscale, rectangular regions of interest were applied to each band, and mean pixel intensities were inverted ($255 - X$) and background-subtracted using adjacent non-band regions. Band intensities were normalized to GAPDH.

Western Blotting

Frozen postnatal day 0 (P0) hemibrains ($n = 2$ per genotype) were lysed in RIPA buffer supplemented with protease inhibitors and sonicated on ice. Lysates were centrifuged at $6000 \times g$ for 10 min at 4 °C, and supernatants were collected. Protein concentration was determined using a BCA assay. Equal amounts of protein (20 μ g) were mixed with $2 \times$ SDS sample buffer (Bio-Rad, cat no. 1610737), heated at 98 °C for 10 min, cooled on ice for 5 min, and briefly centrifuged at $16,000 \times g$ for 1 min. Samples were resolved on 10% SDS-PAGE gels run at 120 V for 90 min and transferred to PVDF membranes pre-activated in methanol using wet electrotransfer at 120 V for 90 min in transfer buffer containing methanol and an ice pack. Successful transfer was confirmed by Ponceau S staining. Membranes were washed in PBST and blocked in 5% non-fat milk in PBST for 30 min at room temperature. Membranes were incubated overnight at 4 °C with primary antibodies diluted 1:1000 or 1:2000, followed by HRP-conjugated secondary antibodies (1:2000) for 1 h at room temperature. Signal was detected using Clarity Western ECL substrate kit (Bio-Rad, cat no. 1705061). Band intensities were quantified by densitometry using ImageJ as described above and normalized to α -tubulin. The primary antibodies used are as follows: CELF2 (Abcam ab186430, rabbit); DCX (Abcam ab18723, rabbit); NeuN (Abcam ab177487, rabbit); CAMK2A (Abcam ab52476, rabbit); α -Tubulin (Abcam, ab7291, mouse, loading control).

Immunohistochemistry (IHC)

Cryosectioned brain tissue (10 μ m, $n = 6$ animals per genotype) was rehydrated in $1 \times$ PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Sections were washed three times in PBS (5 min each) and blocked for 30 min at room temperature in blocking solution containing 5% bovine serum albumin (BSA), 5% normal goat serum (NGS), and 0.05% Triton X-100 in PBS. Following

additional PBS washes, sections were incubated overnight at 4 °C with primary antibodies diluted 1:250 in blocking buffer containing 0.05% Triton X-100. The following day, sections were washed three times in PBS and incubated with Alexa Fluor-conjugated secondary antibodies (Jackson ImmunoResearch) diluted 1:750 in blocking buffer for 1 h at room temperature in the dark. Sections were counterstained with DAPI (1:1000 in PBS) for 5 min, washed twice in PBS, and mounted using Fluoromount-G. Imaging was performed using a Zeiss LSM 880 Airyscan confocal microscope. Identical acquisition settings were used across genotypes for each staining condition to allow quantitative comparison. Primary antibodies for IHC (1:250): CELF2: Santa Cruz, sc-47731 (mouse) and Abcam ab186430 (rabbit); Brn2: Santa Cruz, sc-393324 (mouse); Tbr1: Abcam, ab31940 (rabbit); DCX: Abcam, ab18723 (rabbit); Nestin: Millipore, MAB353 (mouse); NeuN: Abcam, ab177487 (rabbit); CAMK2A: Abcam, ab52476 (rabbit); Ki67: Invitrogen, 14-5698-82 (rat). Secondary antibodies (1:750): Goat anti-mouse Alexa Fluor 488 (Jackson 115-545-003); Goat anti-rabbit Alexa Fluor 568 (Jackson 111-575-144); Goat anti-rat Alexa Fluor 647 (Jackson 122-605-003).

Imaging and Quantification

Fluorescence imaging was performed using a Zeiss LSM 880 Airyscan confocal microscope at the UT Health San Antonio Glenn Biggs Institute. Images were acquired with a $20\times$ objective, with laser power, gain, and exposure settings held constant across animals within each antibody staining set. Image acquisition was performed blinded to genotype. For each animal and brain region ($n = 6$ animals per genotype), three non-overlapping z-stacks were collected per marker. Z-stacks were collapsed into a single image using mean intensity projection, and the three images per region were averaged to generate one biological data point per animal per brain region. Image analysis was performed using ImageJ (Fiji). Quantification metrics included percent area coverage (thresholded fluorescence-positive area normalized to ROI area), mean fluorescence intensity (normalized to ROI area or DAPI⁺ cell count, as indicated), and cell counts. Regions of interest (ROIs) and thresholding parameters were applied uniformly across genotypes using batch-processing macros, with identical ROI sizes used for corresponding brain regions to enable direct comparison.

C. elegans Transfection and Synaptic Rescue

C. elegans were maintained on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50 at 20 °C using standard methods. The N2 wild-type and CB950 [*unc-75(e950)*] strains were obtained from the Caenorhabditis Genetics Center (CGC). The synaptic reporter strain

NM2689 [jsIs821 (*Pmec-7::GFP::RAB-3*)] was kindly provided by the Erik Jorgensen laboratory. Transgenic strains expressing human *Camk2a* were generated by microinjecting plasmid DNA into the syncytial germline of young adult hermaphrodites. The *Camk2a* expression construct was injected at 10 ng/μL together with the co-injection marker *ttx-3::rfp* at 90 ng/μL. Microinjections were performed under differential interference contrast (DIC) microscopy using pulled borosilicate glass needles. Injected animals were recovered on seeded NGM plates and allowed to self-fertilize, and F1 progeny expressing the co-injection marker were selected to establish stable extrachromosomal lines. For quantitative RT-PCR, total RNA was isolated from mixed-stage worm populations ($n=4$ replicate plates per strain) using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA, and qRT-PCR was performed using a Bio-Rad CFX384 real-time PCR system. For synaptic imaging, day 1 adult worms ($n=10\text{--}18$ worms per strain) were immobilized with 30 mM muscimol on 4% agarose pads and imaged using a Zeiss LSM780 confocal microscope. Synaptic puncta areas were quantified using FIJI software.

Nuclei Isolation for snRNA-seq

Single nuclei were isolated from postnatal day 0 (P0) mouse brains using a detergent-based homogenization protocol optimized for RNA preservation. Each neonatal mouse brain was bisected sagittally, with one hemisphere allocated for histological analyses and the contralateral hemisphere flash-frozen for transcriptomic profiling. To ensure sufficient nuclei yield and obtain representative transcriptomic profiles, frozen hemispheres from two littermate pups with the same genotype were pooled per sample prior to nuclei isolation. This pooling strategy was used to ensure adequate input material and to reduce the impact of inter-individual variability on downstream analyses, resulting in $n=1$ per genotype (*Celf2* heterozygote control and *Celf2* KO). All steps were performed on ice with RNase-free reagents. Briefly, dissected brain tissue was minced and homogenized in ice-cold buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂) using a glass Dounce homogenizer (10 strokes with loose and tight pestles). Homogenates were centrifuged at $500\times g$ for 5 min at 4 °C, and pellets were resuspended in lysis buffer containing 0.0125% NP-40 and RNase inhibitor (0.2 U/μL) for 2 min on ice. Lysis was quenched with homogenization buffer, and nuclei were filtered through a 30 μm strainer. Filtered nuclei were washed twice in nuclei wash buffer (1×PBS, 1% BSA, 0.2 U/μL RNase inhibitor) and purified using a 25% OptiPrep density gradient by ultracentrifugation at 10,000 rpm for 30 min at 4 °C. Purified nuclei were collected, washed, and resuspended in wash buffer. Nuclear integrity was assessed by Trypan

Blue staining and viewing nuclei via a hemocytometer and brightfield microscope. Approximately 33,500 viable nuclei were counted in both the KO and control samples. Isolated nuclei were immediately processed for single-nucleus RNA sequencing library preparation using the 10× Genomics platform.

snRNA-seq Analysis

Data Processing and Filtering

Single-nucleus RNA sequencing data from pooled postnatal day 0 (P0) hemibrains (control and *Celf2* knockout) were processed using Seurat (v5.4.0) in R. Raw count matrices were imported and subjected to standard quality control filtering [23]. Nuclei with fewer than 200 detected genes, more than 10,000 detected genes, or greater than 10% mitochondrial transcript content were excluded. Genes expressed in only a minimal number of nuclei were removed from downstream analysis. After filtering, 67,559 nuclei and 33,696 genes were retained. Gene expression values were log-normalized using Seurat's standard normalization workflow, and 2000 highly variable genes were identified for dimensionality reduction. Principal component analysis (PCA) was performed on variable genes, and the top principal components were used to construct a shared nearest-neighbor graph.

Analysis and Data Visualization

Clustering was performed using the Louvain algorithm at a defined resolution. Two-dimensional visualization was generated using Uniform Manifold Approximation and Projection (UMAP) based on PCA space. Differential gene expression analysis was conducted using Seurat's FindMarkers function, applying the Wilcoxon rank-sum test to compare cells within each cluster to all remaining cells. P values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) correction. Differentially expressed genes were defined as those with adjusted $P<0.05$ and absolute log₂ fold change ≥ 1 . Cell type annotation was performed based on canonical marker gene expression and comparison to an external reference annotation table derived from a published single-cell dataset of autism [24]. For each reference-defined cell type, the top 20 most specific marker genes were used. Cluster identities were assigned based on overlap between cluster-enriched differentially expressed genes and reference marker sets. Trajectory inference was performed using Monocle 3 (v3.21) [25–27]. The Seurat object was converted to a Monocle cell_data_set using SeuratWrappers::as.cell_data_set. A specified early progenitor cluster was designated as the root population, and pseudotime values were computed using Monocle's graph-learning framework. UMAP



Fig. 1 Bulk RNA-sequencing identifies transcriptional expression- and splicing-level changes in *Celf2* KO. **(A)** Volcano plot of DEGs from bulk RNA sequencing, $n=3$ P0 hemibrain samples per group (KO vs Ctrl) were processed and analyzed for bulk sequencing. Y-axis corresponds to $-\log_{10}(\text{adjusted } p\text{-value})$ with the cutoff set to $\text{FDR}=0.05$. X-axis corresponds to $\text{Log}_2(\text{fold change})$, with the cutoff set to $\text{FC}=1.5$. *Camk2a* is among is significantly downregulated in the KO. **(B)** KEGG dot plot of DEGs from bulk RNA sequencing data. Dot size represents number of genes associated with a pathway; color scale represents confidence in pathway enrichment by $-\log_{10}(\text{FDR})$ where orange indicates stronger significance and purple indicates weaker significance. Top represented pathways include axon guidance, spliceosome, and Relaxin pathway. $\text{FDR}=0.05$. **(C)** KEGG dot plot of spliced transcripts identified via rMATs. Dot size represents number of genes associated with a pathway; color scale represents confidence in pathway enrichment by $-\log_{10}(\text{FDR})$ where orange indicates stronger significance and purple indicates weaker significance. Top represented pathways include long-term potentiation and circadian entrainment. $\text{FDR}=0.05$. **(D)** Venn diagram of significant DEGs counts vs significantly spliced transcript counts. 76 genes are both either significantly up- or down- regulated and alternatively spliced. Filtered by $p \leq 0.05$. **(E)** Heatmap plot of the 76 overlapping transcripts from DEG and rMATs analysis. Color scale indicates fold change

embeddings were used for trajectory visualization, with cells colored by pseudotime or annotated cell type. Cell type composition was calculated as the proportion of nuclei assigned to each annotated cell type per sample. Differences in cell type distribution were summarized descriptively. Data visualization was performed using ggplot2 and patchwork, with consistent scaling applied across UMAPs and composition plots for comparative interpretation. Key findings were validated by cross-referencing mRNA levels from bulk RNA sequencing.

Results

CEL2 Regulates Neurodevelopmental Gene Expression Programs

CEL2 is an RNA-binding protein implicated in post-transcriptional regulation, yet its physiological role during early mammalian brain development remains incompletely defined. We previously generated a conditional *Celf2* allele in which exon 3 is flanked by loxP sites [18]. Cre-mediated deletion of exon 3 disrupts splicing, producing *Celf2* mRNA that encodes non-functional CEL2 protein due to a frameshift and premature stop codon. To generate a constitutive knockout, *Celf2^{lox}* allele was crossed with the ZP3-Cre line (see “Materials and Methods”), yielding *Celf2* null allele. Survival analysis of pups from each genotype confirmed *Celf2* KO neonatal lethality as *Celf2* knockout (KO) pups died within 24 h of birth, whereas wild-type (WT) and heterozygous (Het) littermates survived, indicating an essential requirement for CEL2 during early postnatal life

(Fig. S1A). Genotype-dependent loss of CEL2 protein was confirmed by western blotting and immunohistochemistry (IHC) in the cortex and hippocampus revealed reduced CEL2 labeling in Het and near-complete loss in KO cortex (Fig. S1B-K). CEL2-positive area coverage, NeuN-positive neuronal area coverage, and the ratio of CEL2+/NeuN+ signal were significantly reduced in KO brains (Fig. S1E-K), suggesting impaired neuronal maturation in the cortex and hippocampus at birth.

To define the transcriptomic consequences of CEL2 loss, we performed bulk RNA sequencing on whole hemibrains from postnatal day 0 (P0) *Celf2* KO and control pups. Heterozygous animals were used as the reference group due to small litter size and limited availability of wild-type littermates. Importantly, Het brains retain substantial CEL2 expression and overall neurodevelopmental integrity relative to KO animals. Western blotting identified only a 20% reduction in CEL2 expression in *Celf2* heterozygotes, while IHC showed comparable CEL2 density in the cortex (Fig. S1B-G). Although slight reductions in CEL2 protein levels were observed in the hippocampus of Het brains, these changes were modest compared to the near-complete loss and severe cellular deficits observed in KO pups (Fig. S1H-I). Thus, comparison of KO to Het animals provides a conservative framework for identifying transcriptional programs that are most sensitive to CEL2 depletion. Differential expression analysis identified 1128 differentially expressed genes (DEGs) significantly altered between KO and Het brains (adjusted $p \leq 0.05$, Table S1). We generated a volcano plot to visualize DEGs and identified 20 down-regulated and 4 upregulated genes using a false discovery rate cutoff of $\text{FDR} < 0.05$ and an absolute fold change > 1.5 (Fig. 1A). Notably, several master transcriptional regulators critical for neurodevelopment, such as *Pax8*, *Nkx6-1* and *Hox* genes were among the 20 down-regulated genes, supporting a broad requirement for CEL2-dependent RNA regulation during this developmental window [5, 12, 13]. Gene ontology enrichment of differentially expressed genes revealed pathways related to axon guidance, focal adhesion, and MAPK signaling (Fig. 1B), processes critical for early neuronal development.

Given the role of CEL2 in regulating alternative splicing, we performed alternative splicing analysis using replicate Multivariate Analysis of Transcript Splicing (rMATs) and identified 995 transcripts exhibiting significant splicing alterations, based on adjusted $p\text{-value} \leq 0.05$ (Table S2). Gene ontology analysis of these transcripts demonstrated enrichment for long-term potentiation, circadian entrainment, and insulin secretion (Fig. 1C), suggesting that loss of CEL2 impacts pathways governing synaptic plasticity, neuronal activity-dependent transcription, and metabolic regulation. To comprehensively assess post-transcriptional regulation, we integrated DEGs with alternative splicing analysis.

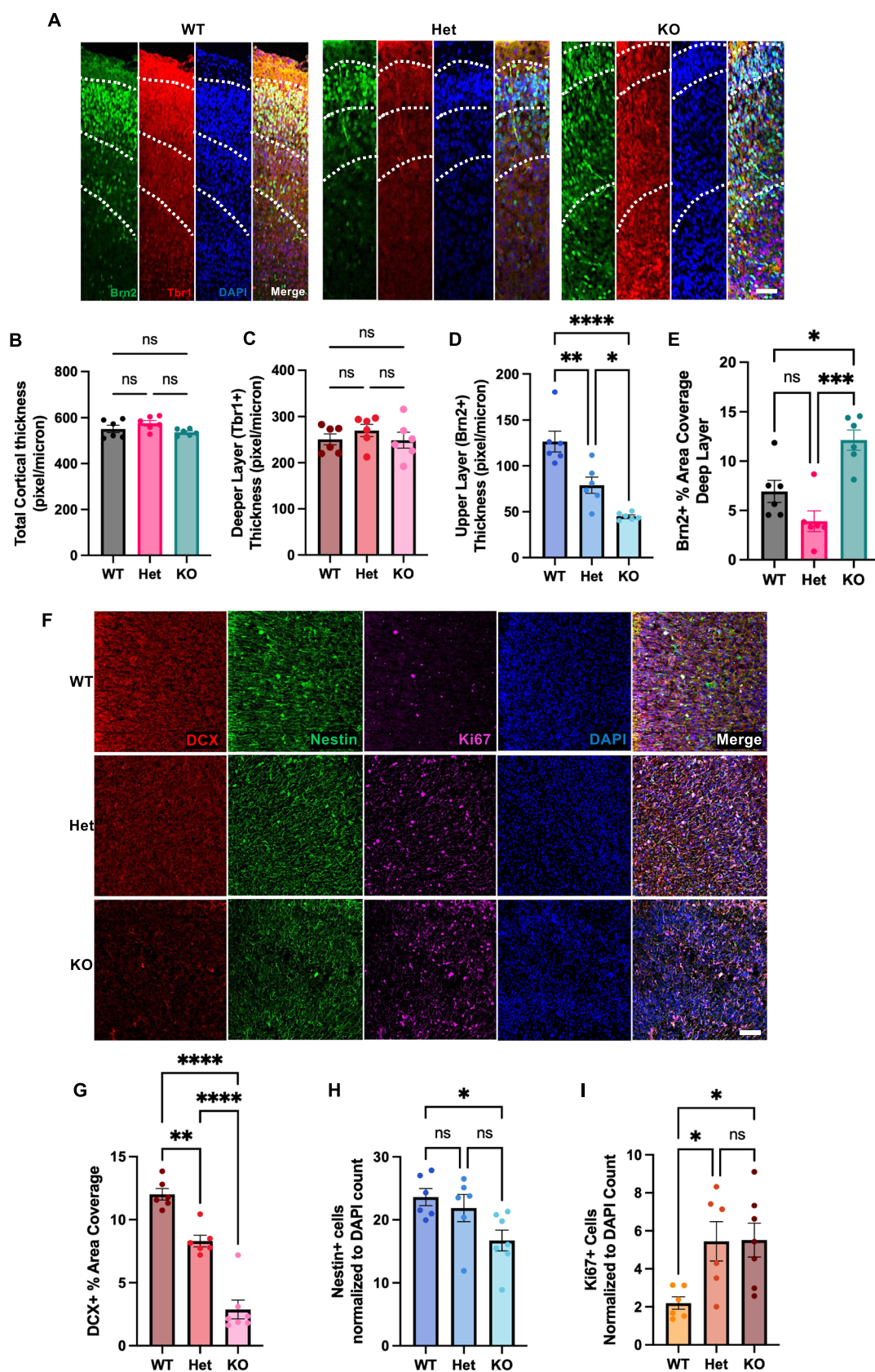


Fig. 2 Loss of CELF2 results in dysregulation of cortical organization and neurogenesis. IHC analyses were performed using $n=6$ animals per genotype, where each data point is an average of 3–6 slices from one animal, imaged via z-stacks that were collapsed by mean intensity. **(A)** IHC of cortical organization of neurons from P0 mice between genotype groups. Brn2 is a marker for upper layer cells, Tbr1 marks deeper cortical layer cells, DAPI is the counterstain. Traces of the upper, intermediate, and deeper layer overlay the images. Imaged at 20 $\times$ magnification, zoom 0.5 $\times$; scale bar=25 μ m. **(B)** Cortical thickness between genotype groups, measured by pixel/micron. No significant difference in total thickness of the cortex (sum of upper, intermediate, and deeper layer). Ordinary one-way ANOVA, $F=2.653$, $P=0.1032$. **(C)** Deeper layer (Tbr1+ dense area) thickness between genotype groups, measured by pixel/micron. No significant difference between genotype groups on actual thickness of deeper layer of cortex. Ordinary one-way ANOVA, $F=0.6636$, $P=0.5295$. **(D)** Upper layer thickness between genotype groups, measured by pixel/micron. Het mice show slight thinning of upper layer compared to WT controls. KO mice show severe upper layer thinning compared to WT and Het pups. Ordinary one-way ANOVA, $F=23.84$, **** $P<0.0001$. **(E)** Brn2+ percent coverage area within the Tbr1+ dense deeper layer of the cortex, normalized by ROI area. *Celf2* KO pups show increased Brn2+ coverage within the deeper layer of the cortex compared to WT and Het pups. Ordinary one-way ANOVA, $F=15.21$, *** $P=0.0002$. **(F)** IHC of proliferation (Ki67), migrations (Nestin), and neuronal differentiation (DCX) markers in the cortex of P0 brains between genotype groups. 20 $\times$ magnification, scale bar=30 μ m. **(G)** Differentiation index, calculated by percent coverage area of DCX+ staining normalized by image area. Reduced DCX+ coverage in the KO compared to WT and Het pups. Ordinary one-way ANOVA, $F=61.33$, **** $P<0.0001$. **(H)** Migration index, calculated by Nestin+ staining normalized to DAPI+ cell count. Reduced Nestin+ cells in KO pups, compared to WT. Ordinary one-way ANOVA, $F=4.393$, * $P=0.0301$. **(I)** Proliferation index, calculated by number of Ki67+ cells normalized to DAPI cell count. Increased Ki67+ cells in the Het and KO pups, compared to WT. Ordinary one-way ANOVA, $F=5.159$, * $P=0.0187$

This combined framework enabled detection of both changes in transcript abundance and differential exon usage. We identified 76 genes that were concurrently affected at both the expression and splicing levels (Fig. 1D). These dual-layer changes suggest coordinated regulation of transcript output and isoform composition, highlighting genes that may be particularly sensitive to perturbations in RNA processing mechanisms caused by CELF2 loss. Hierarchical clustering of these 76 overlapping genes clearly separated KO and control samples (Fig. 1E), with notable inclusion of neurodevelopmental genes such as *Camk2a*. Together, these findings demonstrate that CELF2 is essential for neonatal viability and regulates transcriptional and splicing programs that support early neurodevelopmental progression.

CELF2 is Required for Cortical Organization and Neuronal Maturation During Early Brain Development

Given the widespread dysregulation of neurodevelopmental gene expression and splicing programs observed following CELF2 loss, we next examined whether these molecular

changes were accompanied by defects in cortical organization and neuronal maturation at P0. Cortical architecture was evaluated by immunostaining for Brn2, a marker of upper-layer (II/III) excitatory neurons, and Tbr1, a marker of deep-layer (V/VI) neurons. These analyses revealed pronounced laminar disorganization in *Celf2* KO cortices (Fig. 2A–E, Fig. S2). KO brains displayed a reduction in densely packed Brn2+ neurons within the upper cortical layers and poorly defined boundaries between cortical layers. These findings are consistent with prior reports of cortical disorganization in neural-specific *Celf2* knockout models [17]. No significant difference in total cortical thickness was detected across genotypes (Fig. 2B), indicating that gross cortical expansion was preserved. While deep-layer thickness remained largely unchanged (Fig. 2C), upper-layer thickness was reduced in heterozygous animals and markedly diminished in KO cortices (Fig. 2D). Notably, Brn2+ cells were aberrantly distributed within deeper cortical layers in KO brains (Fig. 2E). As Brn2+ neurons are normally generated later in corticogenesis and migrate to superficial layers, this redistribution is consistent with impaired migration or maturation of post-mitotic neurons rather than altered progenitor output.

To determine whether these structural abnormalities reflected disrupted neurogenesis, we assessed markers of proliferation (Ki67), migration (Nestin), and neuronal differentiation (Doublecortin; DCX). Nestin+ migrating progenitors and DCX+ immature neurons were significantly reduced in KO cortices (Fig. 2F–H), indicating impaired neuronal maturation and radial migration. Interestingly, Ki67+ proliferative indices were increased in *Celf2* Het and KO pups (Fig. 2F, I). Consistent with these cortical findings, we observed a reduced percentage coverage of DCX+ immature neurons accompanied by a modest increase in Ki67+ proliferative indices in the hippocampus of *Celf2* KO pups (Fig. S3). The concomitant increase in Ki67+ proliferative cells and reduction in DCX+ immature neurons in the neonatal cortex suggests impaired cell-cycle exit and delayed neuronal differentiation following CELF2 loss. These data suggest that radial glial cells may be halted to remain in a proliferative state thereby reducing markers of migration and differentiation while notably increasing the population of proliferative cells. These cellular phenotypes are consistent with transcriptomic alterations affecting neurodevelopmental differentiation programs during a critical window of cortical and hippocampal circuit assembly [28–31].

Collectively, our data demonstrate that CELF2 is essential for proper cortical lamination and neuronal maturation during early brain development. Loss of CELF2 disrupts the balance between progenitor proliferation and differentiation, providing a cellular basis for the widespread transcriptional and post-transcriptional defects observed in *Celf2* KO brains.

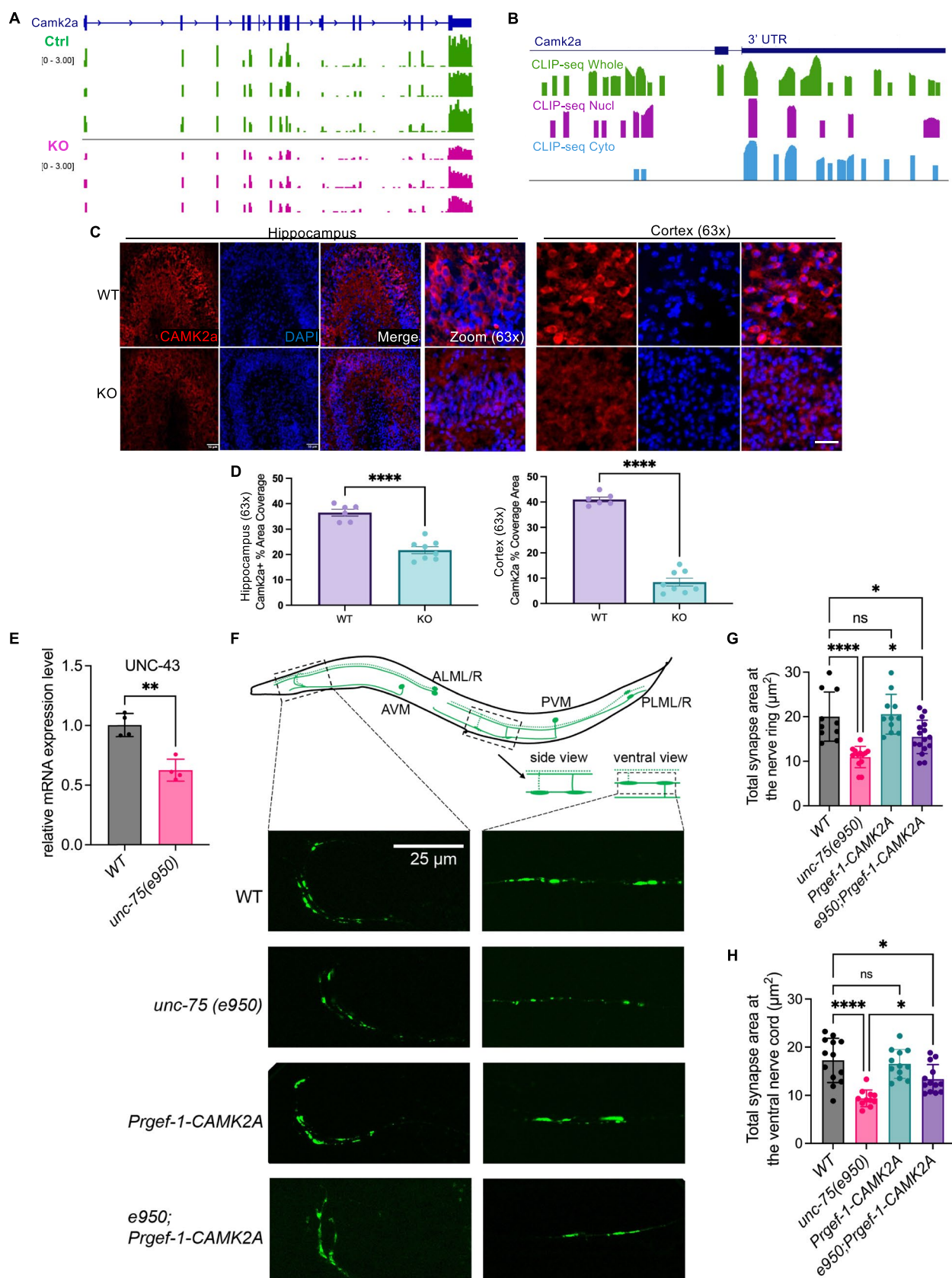


Fig. 3 Camk2a is a target of CELF2-mediated neurodevelopment. (A) IGV from bulk RNA sequencing of transcript levels expression for $n=3$ control samples and KO samples for *Camk2a*. *Camk2a* is downregulated in the KO. (B) CLIP-seq binding plot of CELF2 in N2A cells binding to *Camk2a* transcript. Celf2 primarily binds to *Camk2a* 3'UTR. (C) IHC of CAMK2a in the hippocampus and the cortex of P0 brains, between genotype groups, with DAPI counterstain. 70× magnification; scale bar=10 μ m. (D) Quantification of CAMK2a+percent coverage area in hippocampus and cortex at 63× magnification, normalized by area. IHC analyses were performed using $n=6$ animals per genotype, where each data point is an average of 3–6 slices from one animal, imaged via z-stacks that were collapsed by mean intensity. Comparison between *Celf2* WT and KO pups. Ordinary one-way ANOVA, $F=44.94$, **** $P<0.0001$. Increased percent coverage of CAMK2a cells in WT cortex, compared to KO. Ordinary one-way ANOVA, $F=160.4$, **** $P<0.0001$. (E) qRT-PCR analysis on UNC-43 expression. UNC-43 mRNA level in *unc-75(e950)* mutant is lower than that in wildtype. $N=4$ biological replicate plates per strain. Student's *t*-test, * $P<0.01$. (F) Schematic illustration and representative confocal images of touch neuron synapse labeled with *jsIs821(Pmec-7::GFP::RAB-3)* in the indicated genotypes. Touch neuron synapses were reduced in CELF/*unc-75* mutants, and the reduction was partially rescued by transgenic expression of human CamK2a under the pan-neuronal promoter *Prgef-1*. Scale bar=25 μ m. (G) Quantification of touch neuron synapse areas in the nerve ring. $N=10$ –18 worms per strain. One-way ANOVA, **** $P<0.0001$, * $P<0.05$. (H) Quantification of touch neuron synapse areas in the ventral nerve cord. $N=10$ –18 worms per strain. One-way ANOVA, **** $P<0.0001$, * $P<0.05$

CELF2 Regulates CAMK2A Expression and Coordinates Neurodevelopmental Splicing Programs

The marked reduction in mature neuronal populations, together with the identification of *Camk2a* as a significantly downregulated differentially expressed gene in *Celf2* KO brains, prompted us to investigate whether CELF2 directly regulates the *Camk2a* transcript, a key mediator of excitatory neuron development. Visualization of bulk RNA-seq data using the Integrated Genomics Viewer (IGV) demonstrated decreased *Camk2a* transcript abundance in KO samples compared with controls (Fig. 3A). Consistent with this observation, western blotting and qPCR targeting multiple regions of the *Camk2a* transcript confirmed significantly reduced mRNA and protein levels in KO brains (Fig. S4).

Integration of these findings with previously published CELF2 CLIP-seq datasets generated from Neuro2a cells revealed robust CELF2 binding across the *Camk2a* 3' untranslated region (UTR) (Fig. 3B), supporting a model in which CELF2 directly promotes *Camk2a* transcript stability and/or translational efficiency through post-transcriptional mechanisms. Immunostaining further demonstrated significantly reduced CAMK2A coverage in both the hippocampus and cortex of P0 *Celf2* KO pups compared with WT controls (Fig. 3C, D). This reduction is particularly notable given the well-established role of CAMK2A in excitatory synapse

maturation, activity-dependent plasticity, and neurodevelopmental disorders [32, 33].

To assess whether CAMK2A is a functional target of CELF2, we turned to *Caenorhabditis elegans*, in which UNC-75 is the CELF-family ortholog. RT-qPCR analysis revealed that *unc-75(e950)* mutants exhibit significantly reduced mRNA expression of *unc-43*, the *C. elegans* ortholog of *Camk2a*, compared with wild-type animals (Fig. 3E). To observe functional effects of mutant *unc-75* in *C. elegans*, we imaged and quantified synaptic area at the nerve ring and ventral nerve cord. Consistent with the deficit in *unc-43* expression, *unc-75* mutants displayed decreased synaptic puncta density in touch receptor neurons, visualized using the *jsIs821(Pmec-7::GFP::RAB-3)* synaptic reporter (Fig. 3F–H). Importantly, pan-neuronal transgenic expression of human CAMK2A under the *rgef-1* promoter partially restored synaptic puncta density in *unc-75* mutants (Fig. 3G–H). These findings demonstrate that CAMK2A functions downstream of CELF-family proteins to promote synaptic maturation across species. The partial rescue further suggests that, while CAMK2A represents a conserved and functionally significant effector, additional CELF2-regulated targets likely cooperate to ensure full synaptic development. Collectively, these data support a conserved CELF-CAMK2A regulatory axis governing neuronal maturation.

Beyond *Camk2a*, CELF2 loss was associated with widespread alterations in alternative splicing of neurodevelopmentally relevant transcripts. Integrated bulk RNA-seq and rMATS analyses identified significant reductions in exon inclusion for *Dclk2* and *Aplp2* in KO brains (Fig. S5). CELF2 CLIP-seq datasets demonstrated binding proximal to regulatory regions of both transcripts, consistent with direct RNA interactions. RT-PCR validation confirmed significant shifts toward exon exclusion for *Dclk2* exon 5 (~33%) and *Aplp2* exon 7 (~40%) in KO brains relative to WT and heterozygous controls. Although the precise functional consequences of these individual splicing events were not dissected here, both genes play established roles in neuronal differentiation, cytoskeletal organization, and synaptic architecture.

Together, these findings indicate that CELF2 exerts coordinated and multilayered control over neurodevelopmental RNA programs. By regulating both transcript abundance and alternative exon inclusion across key neuronal genes, CELF2 helps establish the molecular landscape required for excitatory neuron maturation, synaptic assembly, and proper cortical development during the early postnatal period.

Single-Nucleus RNA Sequencing Reveals Delayed Neuronal Maturation and Altered Cell State Stability Following CELF2 Loss

To explore cell type-specific consequences of CELF2 loss during early brain development, we performed

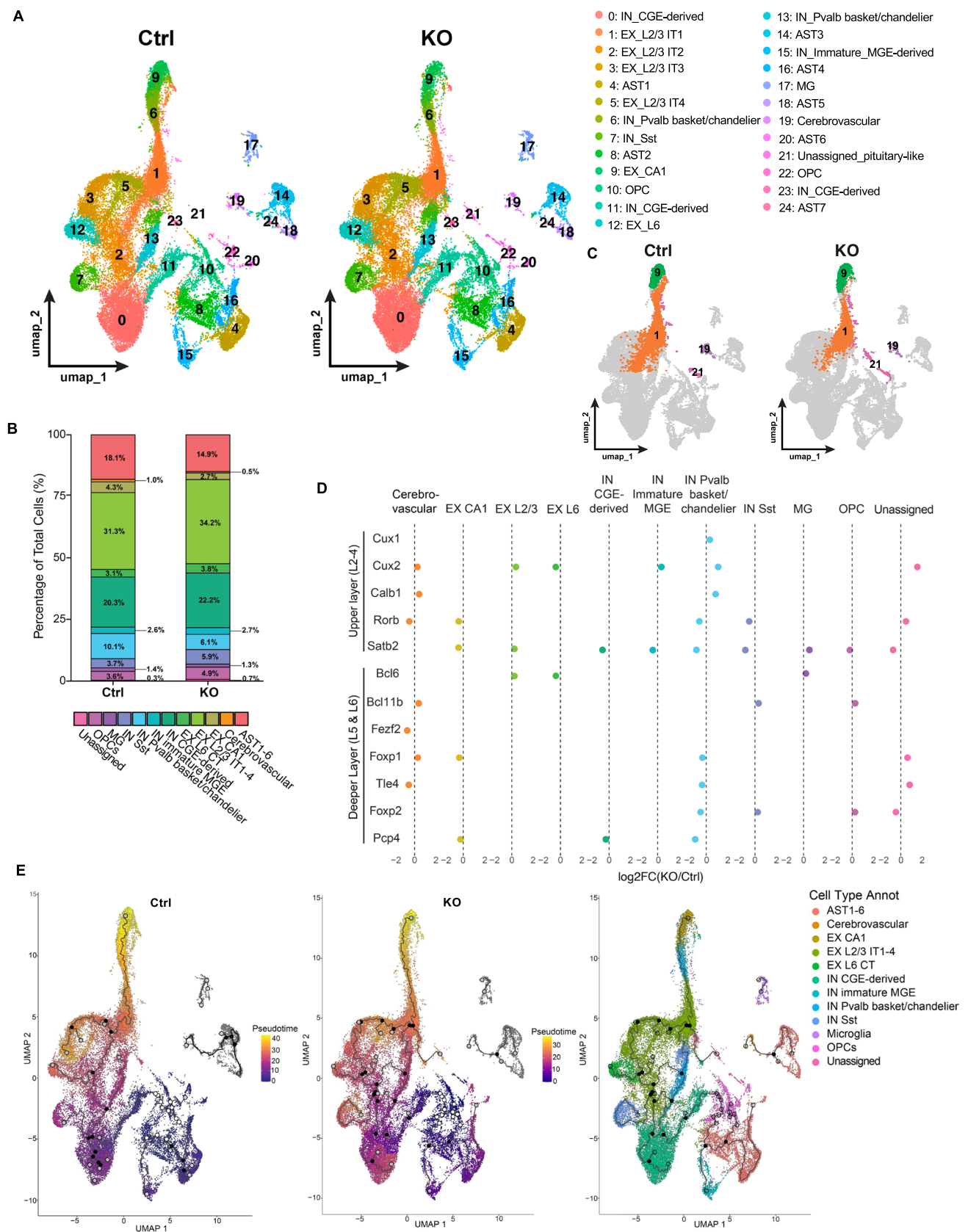


Fig. 4 Single-nucleus RNA sequencing reveals cellular and transcriptional alterations in newborn mouse brain following CELF2 knockout. Two hemibrains were pooled together for nuclei isolation per group, resulting in an $n=1$ per group to explore qualitative cell-type level differences. **(A)** Unsupervised clustering of snRNA-seq data from control (Ctrl) and CELF2 knockout (KO) newborn mouse brains, visualized by UMAP. Cell types were annotated based on the expression of established marker genes. **(B)** Stacked bar plot showing the proportional distribution of all annotated and pooled cell types in each sample. **(C)** UMAP visualization highlighting clusters 1, 9, 19 and 21 in each sample (colored as in panel A); all other cells are shown in grey. **(D)** Differential expression analysis of upper-layer and deep-layer cortex marker genes across pooled cell types; each dot represents a gene differentially expressed (KO versus Ctrl, adjusted $p < 0.05$), plotted by $\log_2$ fold change. **(E)** Monocle3-inferred trajectory analysis for Ctrl and KO samples, with the trajectory rooted in cluster 10. The two left panels are colored by pseudotime, and the right panel is colored by annotated cell type. Inhibitory clusters exhibited delayed progression relative to controls (Clusters 0 and 7 in panel A), and layer II/III and layer VI excitatory neurons (Clusters 3 and 12), despite preserved overall abundance, occupied earlier pseudotime states in KO samples. White dots indicate root nodes, black dots indicate branch nodes or cells populations that are differentiating, gray dots indicate leaf nodes or cells that have reach their final fate

single-nucleus RNA sequencing (snRNA-seq) on P0 brains from *Celf2* KO and het control mice. To ensure sufficient nuclei yield and obtain representative transcriptomic profiles, frozen hemispheres from two littermate pups of the same genotype were pooled per sample prior to nuclei isolation. This pooling strategy was used to provide adequate input material and to mitigate inter-individual variability. Each pooled sample was treated as a single biological replicate ($n=1$ per genotype). Therefore, the snRNA-seq data have been interpreted as exploratory and descriptive rather than hypothesis-confirming. Observed differences in cluster proportions and developmental trajectories are reported qualitatively, and key findings are contextualized by immunohistochemical and bulk RNA-sequencing data presented in prior figures, which provide orthogonal, statistically supported evidence for the major conclusions.

Unsupervised clustering and marker-based annotation identified major neural and non-neuronal cell types, including excitatory cortical and hippocampal neurons, inhibitory interneuron subtypes, astrocytes, oligodendrocyte precursor cells (OPCs), microglia, and a small cerebrovascular-associated population (Fig. 4A). In addition, we detected a small cluster uniquely represented in KO samples that lacked a clear lineage identity and exhibited features of pituitary-like cells (Cluster 21), suggesting an immature or transcriptionally unstable state. Comparison of cell type composition between genotypes revealed selective, rather than global, alterations (Fig. 4B). Excitatory hippocampal neurons were reduced in KO samples relative to controls, consistent with immunohistochemical evidence of diminished neuronal marker, NeuN, in the hippocampus as well as reduced

transcript levels of *Camk2a* (Fig. 3A–C). Conversely, OPCs showed a relative increase in the KO, consistent with the increased population of Ki67+ cells identified in Fig. 2H, suggestive of delayed lineage progression or incomplete transition toward mature neuronal fates. Astrocyte representation was modestly decreased in the KO, while cerebrovascular-associated cells that express endothelial, pericyte, and vascular smooth muscle markers were present at low abundance in both groups, further reduction was observed in the KO sample. In contrast, overall proportions of CGE-derived inhibitory neurons and immature MGE-derived interneuron mRNA were largely preserved. Subsequent trajectory analyses revealed altered developmental timing within these populations (see below). However, these findings remain observational and require future investigation.

To identify cell populations most impacted by CELF2 loss, we highlighted clusters exhibiting the largest genotype-associated differences (Fig. 4C). These included a subset of excitatory layer II/III neurons, excitatory hippocampal neurons, the cerebrovascular-associated cluster, and the transcriptionally unassigned population. Although total layer II/III excitatory neuron abundance was not uniformly reduced, one major subcluster was selectively diminished in the KO, suggesting selective vulnerability within specific maturation states rather than wholesale loss of this lineage. Consistent with the lack of cortical organization identified in Fig. 2, layer-specific marker analysis revealed coordinated transcriptional perturbations without complete loss of cell identity (Fig. 4D). Deep-layer markers, *Pcp4* and *Foxp1*, were broadly downregulated across neuronal populations in the KO. Conversely, snRNA-seq identified upper-layer markers such as *Cux2* and *Calb1* were aberrantly enriched in inhibitory Pvalb basket/chandelier interneurons. Differentiation-associated master regulator for callosal projection neurons, *Satb2*, observed reduced expression across cell types excluding excitatory cortical layer 2–3 neurons and microglia. The unassigned cluster displayed mixed expression of cortical layer-associated transcription factors, including *Cux2*, *Foxp1*, and *Tle4*, alongside reduced expression of *Satb2*. This hybrid transcriptional profile supports the interpretation of an unstable or incompletely differentiated state rather than a distinct lineage.

Developmental trajectory inference using pseudotime analysis further demonstrated an observational delay in neuronal maturation following CELF2 loss (Fig. 4E). In both genotypes, inferred trajectories progressed from proliferative OPC-like states through astrocytic and inhibitory intermediates, indicated by white root nodes, culminating in excitatory cortical and hippocampal neurons. However, more branch nodes were present in the KO, indicating a stall in the ability of cell populations to reach their final cell fate, indicated by gray leaf nodes, compared to controls. Despite this, inhibitory clusters in the KO exhibited accelerated progression

relative to the control sample though fewer leaf nodes were present, while layer II/III and layer VI excitatory neurons occupied delayed pseudotime states in KO samples, consistent with impaired maturation rather than altered specification. Microglia and cerebrovascular-associated cells were excluded from trajectory analysis due to distinct developmental programs.

Pseudobulk pathway analysis of pathway-associated transcripts reinforced these findings, revealing coordinated downregulation of gene programs associated with axon guidance, neurogenesis, neuronal migration, differentiation, and synapse development in KO samples (Fig. S6). Within hippocampal populations, differentiation-associated genes such as *Neurod1* were reduced, whereas progenitor-associated markers including *Prox1* were selectively enriched within the unassigned cluster, further supporting persistence of immature transcriptional states (Fig. S6). Notably, this unassigned population, although comprising a small fraction of total nuclei, was modestly expanded in the KO. Its mixed lineage markers, enrichment for progenitor-associated genes, and reduced differentiation signatures suggest accumulation of developmentally immature or aberrant cells. Given prior evidence that CELF2 can promote cell death pathways and limit survival of improperly specified cells in other systems [34], expansion of this population may reflect impaired elimination of cells that fail to execute appropriate maturation programs, rather than emergence of a novel lineage or technical artifact.

Collectively, these snRNA-seq data indicate that CELF2 KO changes cell type abundance and developmental trajectories. Loss of CELF2 disrupts developmental timing, selectively affects excitatory hippocampal and cortical populations, and promotes persistence of immature transcriptional states. These cellular-level alterations provide a mechanistic bridge linking CELF2-dependent RNA regulation to the gene expression, splicing, and histological defects observed in *Celf2* KO brains.

Discussion

This study identifies CELF2 as an essential post-transcriptional regulator of early postnatal brain maturation, coordinating RNA regulatory programs required for neuronal differentiation, synaptic stability, and cortical and hippocampal organization. Using integrated transcriptomic, splicing, histological, and single-nucleus RNA sequencing approaches, we show that constitutive loss of *Celf2* results in neonatal lethality accompanied by widespread molecular and cellular abnormalities in the developing brain. Together, these findings establish CELF2 as a nonredundant RNA-binding protein required during a narrow developmental window and

link CELF2-dependent RNA regulation to critical changes in early neural architecture.

Genetic variation in CELF2 and its related family members (CELF1–6) plays a critical role in human disease by disrupting post-transcriptional RNA processing, including alternative splicing and mRNA stability. Recent research identifies *de novo* heterozygous variants in *CELF2* as the cause of Developmental and Epileptic Encephalopathy-97 (DEE97) [35]. Unlike simple loss-of-function variants, pathogenic *CELF2* mutations typically disrupt activity-dependent nucleocytoplasmic shuttling, leading to protein mislocalization in the cytoplasm, neuronal hyperexcitability, and severe seizures. Beyond neurodevelopment, common genetic polymorphisms in *CELF2* have been identified as risk factors for late-onset Alzheimer's disease [37–39]. This disease association extends across the family; for example, CELF1 is a well-established modifier in Myotonic Dystrophy Type 1 (DM1) [40], while CELF4 deficiency is associated with autism-like behaviors and synaptic dysfunction [41]. However, additional studies will be required to establish causal relationships and to determine the precise molecular consequences of CELF variants.

Prior CELF-family CLIP-seq work supports direct RNA interaction across neuronal regulatory programs [18], and CELF2 has been shown to regulate RNA programs with relevance to neuronal structure and connectivity [36]. Building on this, two previous *Celf2* mouse models have provided important context for the present findings. MacPherson et al. established that cytoplasmic CELF2 translationally represses pro-differentiation mRNAs including *Tbr2*, *Neurog2*, and *Neurod1* to maintain progenitor identity during neurogenesis using shRNA-mediated knockdown of *Celf2* in embryonic radial glial progenitors [21]. Duan et al. subsequently generated a Nestin-Cre conditional knockout targeting neuroepithelial progenitors and their progeny broadly from approximately E10.5 onward, and found that *Celf2* haploinsufficiency produced autism-relevant behavioral deficits, cortical thinning, and reduced dendritic spine density, with transcriptomic changes enriched in synapse and dendrite pathways [20]. While these models established roles for CELF2 in embryonic progenitor maintenance and later neuronal morphological maturation, neither provided network-level resolution of the RNA targets and splicing programs disrupted by CELF2 loss, nor identified specific downstream effectors. Our data demonstrate that CELF2 loss disrupts coordinated gene expression and exon inclusion programs rather than isolated transcripts, consistent with CELF2 functioning as a network-level RNA regulator. Many affected genes participate in neuronal differentiation, cytoskeletal organization, and synaptic maturation, supporting a model in which CELF2 regulates convergent RNA programs required for early circuit formation. This mode of regulation is particularly relevant in the context of

neurodevelopmental disorders, where thousands of genetic risk loci have been identified, yet many converge on shared biological processes rather than single-gene mechanisms. CELF2-dependent RNA regulation may therefore represent one such convergent layer through which diverse upstream perturbations impair neuronal maturation, and may help explain the cortical malformations and broad neurodevelopmental phenotypes observed in children carrying pathogenic CELF2 variants [21].

Consistent with this idea, reduced exon inclusion and decreased transcript abundance occurred following CELF2 loss, supporting a role for CELF2 as a positive regulator of exon inclusion and transcript stability for a subset of neurodevelopmental targets. CELF2 is enriched in cortical and hippocampal neurons during embryonic and early postnatal development, and other CELF-family members do not compensate for its loss. Whereas prior conditional models entangle embryonic and postnatal contributions of CELF2 across broad neural lineages, the constitutive knockout examined here allows definition of the transcriptomic and splicing landscape disrupted by complete CELF2 loss during the early postnatal period, when cortical lamination and excitatory circuit maturation undergo rapid refinement. Our findings thereby extend prior work by providing molecular and cellular resolution into the mechanisms through which CELF2 supports early neuronal maturation and circuit stability, offering a developmental framework that may underlie the behavioral outcomes observed in conditional models.

Among the disrupted targets, *Camk2a* emerged as a prominent CELF2-dependent transcript. We observed marked reductions in *Camk2a* mRNA and CAMK2A protein levels in *Celf2* KO animals, consistent with prior CLIP-seq data demonstrating extensive CELF2 binding across the *Camk2a* 3'UTR. Functional studies in *C. elegans* provide direct in vivo evidence supporting conservation of this pathway: loss of the CELF ortholog *unc-75* reduced synaptic puncta density and transgenic expression of human CAMK2A partially rescued this phenotype. Use of *C. elegans* enabled rapid, circuit-level functional testing in an intact nervous system while avoiding confounds associated with in vitro models or mammalian circuit compensation. The partial nature of the rescue indicates that CAMK2A is a conserved but incomplete effector, reinforcing the conclusion that CELF2 regulates synaptic maturation through multiple converging RNA-dependent pathways.

In addition to *Camk2a*, the absence of CELF2 altered splicing of *Dclk2* and *Aplp2*, genes implicated in neuronal migration, cytoskeletal regulation, and synaptic organization. Rather than attributing direct causality to individual splice events, these findings point to broader disruption of RNA regulatory fidelity following CELF2 loss. Such widespread perturbation of RNA processing is a recurring theme

across neurodevelopmental disorders, where modest changes across many transcripts collectively destabilize neuronal maturation rather than producing single-gene phenotypes.

Histological analyses further revealed reduced Nestin- and DCX-positive populations alongside preserved overall cortical thickness in the KO, indicating intact early progenitor proliferation but impaired neuronal maturation and migration. Increased Ki67 signal and reduced NeuN expression support the interpretation that cells remain in immature or transitional states when CELF2-dependent RNA regulation is disrupted. Cortical laminar disorganization and thinning of upper layers are therefore more consistent with delayed maturation of late-born neurons than with altered fate determination, aligning with models of developmental delay rather than lineage misspecification observed in many neurodevelopmental conditions.

Our snRNA-seq data provided exploratory insight into cell-type-level changes accompanying CELF2 loss. Observed patterns included apparent reductions in excitatory hippocampal neurons and upper layer II/III excitatory neurons in knockout brains, delayed developmental progression across inhibitory and excitatory cortical populations in pseudotime analyses, and an expanded unassigned cluster enriched for stem-like transcriptional signatures. Importantly, these snRNA-seq datasets were generated from pooled hemibrains (two hemibrains per group) due to insufficient nuclei yield from individual samples, resulting in an effective $n = 1$ per group. As such, these data are not powered for FDR-corrected statistical comparisons between genotypes, and the observed differences in cluster proportions and pseudotemporal distributions cannot be interpreted as statistically supported claims. Rather, these findings should be understood as hypothesis-generating observations that are directionally consistent with, and contextualized by, the transcriptomic and histological evidence presented in prior figures. Where certain robust snRNA-seq patterns aligned with bulk RNA-seq and immunohistochemical findings, such as evidence of impaired neuronal maturation and reduced excitatory neuron populations, we interpret the convergence across orthogonal datasets as lending qualitative support to these conclusions. Patterns without such orthogonal support, including modest changes in astrocytic and cerebrovascular-associated populations, remain unvalidated and are noted only as observations warranting future investigation.

Several limitations should be acknowledged. Use of a constitutive global knockout model restricts temporal and cell-type-specific interpretation and likely conflates primary RNA regulatory effects with secondary developmental consequences. The constraints on snRNA-seq sample size reflect both the practical challenges of obtaining sufficient numbers of knockout pups within the required developmental window and the cost associated with running multiple samples per genotype, and represent a limitation that future

studies should address with appropriately powered experimental designs. Future work using conditional and temporally controlled models will also be essential to disentangle cell-autonomous effects from downstream network instability and to rigorously validate the cell-type-level observations reported here.

Conclusion

In summary, this study establishes CELF2 as a critical post-transcriptional regulator required for neuronal maturation, synaptic stabilization, and architectural organization of the developing brain. Loss of CELF2 disrupts coordinated gene expression and splicing programs, delays neuronal differentiation, destabilizes specific neuronal populations, and impairs early circuit formation, culminating in neonatal lethality. Our results suggest that CELF2 functions to stabilize developmental trajectories and ensure progression through maturation states. These findings provide mechanistic insight into how disruption of RNA regulatory networks can contribute to neurodevelopmental disorders, where diverse genetic risk factors converge on shared processes of neuronal maturation and synaptic function. Together, this work identifies CELF2-dependent RNA regulation as a convergent mechanism linking disrupted neuronal maturation to neurodevelopmental disease vulnerability.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12035-026-05933-6>.

Acknowledgements We would like to thank all the members of Liu and Chen labs for technical assistance and helpful discussion. We would also like to thank the Glenn Biggs Institute for Alzheimer's and Neurodegenerative diseases at the University of Texas Health Science Center in San Antonio, for use of the Zeiss Airyscan LSM 880 confocal microscope. Some worm strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). RNA-seq data was generated in the Genome Sequencing Facility, which is supported by UT Health San Antonio, NIH-NCI P30 CA054174 (Cancer Center at UT Health San Antonio) and NIH Shared Instrument grant S10OD030311 (S10 grant to NovaSeq 6000 System), and CPRIT Core Facility Award (RP220662).

Author Contributions Conceptualization, I.S., Z.L. and L.C.; methodology, I.S., S.K., S.C. and L.C.; formal analysis and investigation, I.S., J.J., Z.Z., and L.C.; resources L.C. and Z.L.; Figure preparation, I.S., J.J., and L.C.; writing- original draft preparation, I.S.; writing- review and editing I.S., J.J., and L.C.; supervision, Z.Z., Z.L. and L.C.; All authors have read and agreed to the published version of manuscript.

Funding This work was supported by the NIH Jointly Sponsored Predoctoral Training Program in the Neurosciences training grant (T32 NS082145) to I.S., the South Texas Alzheimer's and Neurodegeneration Disorders (STAND) training program (T32 AG082661) to I.S., NIA R01AG070214 to L.C., NIA R01AG071591 to L.C., NIA R01AG085545 to L.C., NIGMS R01GM137009 to Z. L., and NIGMS R35GM153228 to Z.L.

Data Availability The single nucleus RNA-sequencing data reported in this article have been deposited in the GEO and are accessible through the GEO accession number GSE319433. Other data generated or analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Consent for Publication Not applicable.

Competing interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

References

1. Taverna E, Götz M, Huttner WB (2014) The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. *Annu Rev Cell Dev Biol* 30:465–502
2. Sun T, Hevner RF (2014) Growth and folding of the mammalian cerebral cortex: from molecules to malformations. *Nat Rev Neurosci* 15:217–232
3. Rakic P (2009) Evolution of the neocortex: a perspective from developmental biology. *Nat Rev Neurosci* 10:724–735
4. Griffin A, Mahesh A, Tiwari VK (2022) Disruption of the gene regulatory programme in neurodevelopmental disorders. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1865:194860
5. Tao Y, Zhang Q, Wang H, Yang X, Mu H (2024) Alternative splicing and related RNA binding proteins in human health and disease. *Signal Transduct Target Ther* 9:26
6. Searles Quick VB, Wang B, State MW (2021) Leveraging large genomic datasets to illuminate the pathobiology of autism spectrum disorders. *Neuropsychopharmacology* 46:55–69
7. Nóbrega I, Silva A, Yokota-Moreno BY, Sertié AL, Nóbrega I, Silva A, Yokota-Moreno BY, Sertié AL (2024) The importance of large-scale genomic studies to unravel genetic risk factors for autism. *Int J Mol Sci*. <https://doi.org/10.3390/ijms25115816>
8. Schieweck R, Ninkovic J, Kiebler MA (2021) RNA-binding proteins balance brain function in health and disease. *Physiol Rev* 101:1309–1370
9. Parra AS, Johnston CA (2022) Emerging roles of RNA-binding proteins in neurodevelopment. *J Dev Biol* 10:23
10. Berto S, Usui N, Konopka G, Fogel BL (2016) ELAVL2-regulated transcriptional and splicing networks in human neurons link neurodevelopment and autism. *Hum Mol Genet* 25:2451–2464
11. Dasgupta T, Ladd AN (2012) The importance of CELF control: molecular and biological roles of the CUG-BP, Elav-like family of RNA-binding proteins. *Wiley Interdiscip Rev RNA* 3:104–121

12. Peng S, Cai X, Chen J, Sun J, Lai B, Chang M, Xing L (2024) The role of CELF family in neurodevelopment and neurodevelopmental disorders. *Neurobiol Dis* 197:106525
13. Ladd AN (2013) CUG-BP, Elav-like family (CELF)-mediated alternative splicing regulation in the brain during health and disease. *Mol Cell Neurosci* 56:456–464
14. Ladd AN, Charlet N, Cooper TA (2001) The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. *Mol Cell Biol* 21:1285–1296
15. Timchenko LT, Miller JW, Timchenko NA, DeVore DR, Datar KV, Lin L, Roberts R, Caskey CT et al (1996) Identification of a (CUG)_n triplet repeat RNA-binding protein and its expression in Myotonic Dystrophy. *Nucleic Acids Res* 24:4407–4414
16. Timchenko LT, Timchenko NA, Caskey CT, Roberts R (1996) Novel proteins with binding specificity for DNA CTG repeats and RNA CUG repeats: implications for Myotonic Dystrophy. *Hum Mol Genet* 5:115–121
17. Mallory MJ, Allon SJ, Qiu J, Gazzara MR, Tapescu I, Martinez NM, Fu X-D, Lynch KW (2015) Induced transcription and stability of CELF2 mRNA drives widespread alternative splicing during T-cell signaling. *Proc Natl Acad Sci U S A* 112:E2139–E2148
18. Chen L, Liu Z, Zhou B, Wei C, Zhou Y, Rosenfeld MG, Fu XD, Chisholm AD, Jin Y (2016) CELF RNA binding proteins promote axon regeneration in *C. elegans* and mammals through alternative splicing of Syntaxins. *eLife* 5:e16072. <https://doi.org/10.7554/eLife.16072>
19. Chatrikhi R, Mallory MJ, Gazzara MR, Agosto LM, Zhu WS, Litterman AJ, Ansel KM, Lynch KW (2019) RNA binding protein CELF2 regulates signal-induced alternative polyadenylation by competing with enhancers of the polyadenylation machinery. *Cell Rep* 28:2795–2806.e2793
20. Duan X, Peng X, Jia X, Tan S, Guo H, Tan J, Hu Z (2025) CELF2 deficiency demonstrates autism-like behaviors and interferes with late development of cortical neurons in mice. *Mol Neurobiol* 62:156–168
21. MacPherson MJ, Erickson SL, Kopp D, Wen P, Aghanoori MR, Kedia S, Burns KML, Vitobello A et al (2021) Nucleocytoplasmic transport of the RNA-binding protein CELF2 regulates neural stem cell fates. *Cell Rep* 35(10), 109226. <https://doi.org/10.1016/j.celrep.2021.109226>
22. Hua M, Aghanoori MR, MacPherson M, Ren Y, Yang Y, Or YY, Williams L, Gafuik CJ et al (2025) Genetic variants disrupting activity-dependent CELF2 shuttling cause neuronal hyperexcitability, learning deficits, and seizures. *medRxiv*. <https://doi.org/10.1101/2025.06.20.25329512>
23. Hao Y, Stuart T, Kowalski MH, Choudhary S, Hoffman P, Hartman A, Srivastava A, Molla G et al (2024) Dictionary learning for integrative, multimodal and scalable single-cell analysis. *Nat Biotechnol* 42:293–304
24. Velmeshev D, Schirmer L, Jung D, Haeussler M, Perez Y, Mayer S, Bhaduri A, Goyal N et al (2019) Single-cell genomics identifies cell type-specific molecular changes in autism. *Science* 364:685–689
25. Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ et al (2014) The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol* 32:381–386
26. Qiu X, Mao Q, Tang Y, Wang L, Chawla R, Pliner HA, Trapnell C (2017) Reversed graph embedding resolves complex single-cell trajectories. *Nat Methods* 14:979–982
27. Cao J, Spielmann M, Qiu X, Huang X, Ibrahim DM, Hill AJ, Zhang F, Mundlos S et al (2019) The single-cell transcriptional landscape of mammalian organogenesis. *Nature* 566:496–502
28. Zahr SK, Yang G, Kazan H, Borrett MJ, Yuzwa SA, Voronova A, Kaplan DR, Miller FD (2018) A translational repression complex in developing mammalian neural stem cells that regulates neuronal specification. *Neuron* 97:520–537.e526
29. Pachicano M, Mehta S, Hurtado A, Ard T, Stanis J, Brenningstall B, Bienkowski MS (2025) Laminar organization of pyramidal neuron cell types defines distinct CA1 hippocampal subregions. *Nat Commun* 16:10604
30. Gilmore EC, Walsh CA (2013) Genetic causes of microcephaly and lessons for neuronal development. *WIREs Dev Biol* 2:461–478
31. Cavalieri D, Angelova A, Islah A, Lopez C, Bocchio M, Bollmann Y, Baude A, Cossart R (2021) CA1 pyramidal cell diversity is rooted in the time of neurogenesis. *eLife* 10:e69270
32. Chia PH, Zhong FL, Niwa S, Bonnard C, Utami KH, Zeng R, Lee H, Eskin A et al (2018) A homozygous loss-of-function CAMK2A mutation causes growth delay, frequent seizures and severe intellectual disability. *eLife* 7:e32451
33. Rostas JAP, Skelding KA, Rostas JAP, Skelding KA (2023) Calcium/calmodulin-stimulated protein kinase II (CaMKII): different functional outcomes from activation, depending on the cellular microenvironment. *Cells*. 12, 3:401. <https://doi.org/10.3390/cells12030401>
34. Li J, Xian L, Zhu Z, Wang Y, Zhang W, Zheng R, Xue W, Li J (2023) Role of CELF2 in ferroptosis: potential targets for cancer therapy (review). *Int J Mol Med* 52(4). <https://doi.org/10.3892/ijmm.2023.5291>
35. Itai T, Hamanaka K, Sasaki K, Wagner M, Kotzaeridou U, Brosse I, Ries M, Kobayashi Y et al (2021) De novo variants in CELF2 that disrupt the nuclear localization signal cause developmental and epileptic encephalopathy. *Hum Mutat* 42:66–76
36. Dasgupta T, Ladd AN (2012) The importance of CELF control: molecular and biological roles of the CUG-BP, Elav-like family of RNA-binding proteins. *WIREs RNA* 3:104–121
37. Lee JH, Cheng R, Barral S, Reitz C, Medrano M, Lantigua R, Jimenez-Velazquez IZ, Rogaeva E, St George-Hyslop PH, Mayeux R (2011) Identification of novel loci for Alzheimer disease and replication of CLU, PICALM, and BIN1 in Caribbean Hispanic individuals. *Arch Neurol* 68:320–328
38. Yang P, Xu M, Liu Z, Tao Q, Lu S, Li H, Guo Q, Sun Y, Wu Z (2015) Genetic association of CUGBP2 and DNMBP with Alzheimer's disease in the Chinese Han population. *Curr Alzheimer Res* 12:228–232
39. Wijsman EM, Pankratz ND, Choi Y, Rothstein JH, Faber KM, Cheng R, Lee JH, Bird TD, Bennett DA, Diaz-Arrastia R, et al (2011) Genome-wide association of familial late-onset Alzheimer's disease replicates BIN1 and CLU and nominates CUGBP2 in interaction with APOE. *PLoS Genet* 7:e1001308
40. Chau A, Kalsotra A (2015) Developmental insights into the pathology of and therapeutic strategies for DM1: back to the basics. *Dev Dyn* 244:377–390
41. Barone R, Fichera M, De Grandi M, Battaglia M, Lo Faro V, Mattina T, Rizzo R (2017) Familial 18q12.2 deletion supports the role of RNA-binding protein CELF4 in autism spectrum disorders. *American Journal of Medical Genetics Part A* 173:1649–1655

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.