Regulation of cell-type-specific transcriptomes by microRNA networks during human brain development

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MicroRNAs (miRNAs) regulate many cellular events during brain development by interacting with hundreds of mRNA transcripts. However, miRNAs operate nonuniformly upon the transcriptional profile with an as-yet unknown logic. Shortcomings in defining miRNA–mRNA networks include limited knowledge of in vivo miRNA targets and their abundance in single cells. By combining multiple complementary approaches, high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation with an antibody to AGO2 (AGO2-HITS-CLIP), single-cell profiling and computational analyses using bipartite and coexpression networks, we show that miRNA–mRNA interactions operate as functional modules that often correspond to cell-type identities and undergo dynamic transitions during brain development. These networks are highly dynamic during development and over the course of evolution. One such interaction is between radial-glia-enriched ORC4 and miR-2115, a great-ape-specific miRNA, which appears to control radial glia proliferation rates during human brain development.
primary samples of the developing human brain from stages corresponding to peak neurogenesis (gestational week 15 (GW15) and GW16.5, early stage) and early gliogenesis (GW19–20.5, late stage); nine samples in total were harvested from prefrontal cortex, motor cortex area 1, visual cortex area 1 and other regions (Supplementary Table 1). AGO2-bound miRNAs and mRNAs were identified after sequencing (Fig. 1a and Supplementary Tables 1 and 2; see Methods for details). In total, 921 human miRNAs were identified after sequencing (Fig. 1a and Supplementary Tables 1 and 2). To our surprise, hundreds of the in vivo miRNA targets we detected were also identified by other high-throughput sequencing techniques (Supplementary Fig. 1). We validated a subset of the canonical AGO site interactions using luciferase reporter assays in human cells in vitro (Supplementary Fig. 1 and Supplementary Table 3). Among the detected interactions, we considered only sites identified in CDS and 3′ UTRs, reflecting canonical miRNA–mRNA interactions. We identified 3,693 and 2,705 genes at early and late stages of development, respectively, that were actively targeted by miRNAs through CDS or 3′ UTR parts of the transcript (Supplementary Fig. 1). We validated a subset of the canonical AGO site interactions using luciferase reporter assays in human cells in vitro (Supplementary Fig. 1). Among the detected interactions, we considered only sites identified in CDS and 3′ UTRs, reflecting canonical miRNA–mRNA interactions. We identified 3,693 and 2,705 genes at early and late stages of development, respectively, that were actively targeted by miRNAs through CDS or 3′ UTR parts of the transcript (Supplementary Fig. 1).
mRNAs (Fig. 1b). Using a bipartite community detection algorithm, we revealed modules of miRNA–mRNA interactions (Fig. 1b, Supplementary Figs. 2 and 3, and Supplementary Table 5; see Methods for details). Notably, the average abundance of a bound miRNA was negatively correlated with the total number of different miRNAs bound in each module (Supplementary Fig. 4). This suggests that miRNA targeting uses two strategies: (1) targeting with one or very few abundant miRNAs and (2) targeting with multiple low abundance miRNAs. To contextualize these interactions in the framework of cell diversity we projected bipartite graph modules onto cell-type-specific information calculated from published scRNA-seq data sets (Fig. 1c,d and Supplementary Table 6). Our analysis showed marked enrichment of cell-type-specific transcripts among bipartite graph modules, suggesting that miRNAs acquire targets according to the cognate transcriptional landscape of individual cell types.

**Cell-type enrichment of miRNAs**. To further investigate how miRNA–mRNA interactions relate to the emerging diversity of cell types of the developing brain, we used an innovative protocol for combined detection of miRNAs and mRNAs in the same single cells (Fig. 2) using an automated microfluidic platform to perform automated cell capture, reverse transcription and targeted preamplification of miRNA and mRNA (Fig. 2a–c and Supplementary Tables 7 and 8). In addition to long-established markers of distinct cell types in the developing cortex, we selected miRNA targets according to the specificity of their expression in distinct cell types (Fig. 2b), as determined in Pollen et al. We profiled single cells isolated from human cortex samples at GW14 (deep layer neurogenesis) and GW17 (upper layer neurogenesis) and GW18 (upper layer neurogenesis). To enrich for progenitor cells and newborn neurons, we microdissected samples of the cortical germinal zone, and to capture maturing neuron populations and interneurons, we microdissected cortical plate regions. In total, we retained data from 312 cells with more than ten genes detected. Clustering analysis performed based on marker gene abundance revealed 11 clusters (Supplementary Table 7). We inferred the identities of individual cell clusters as radial glia, intermediate progenitors, upper and deep cortical layer neurons, and interneurons (Fig. 2b,c). Furthermore, spatial microdissections supported further refinement of our interpretations with respect to neuronal maturation state (newborn neurons captured from the cortical germinal zone and maturing neurons captured from the cortical plate regions) (Fig. 2c). Next, for every miRNA profiled, we quantified the abundance in every cell and calculated an expression enrichment score for every cell type (Fig. 2d). To our surprise, the vast majority of miRNAs we profiled showed significant enrichment in at least one cell type, suggesting robust variation in miRNA abundance across closely related cells of the developing brain. For example, miR-221–miR-222 and miR-92a were enriched in cortical intermediate progenitor cells, in agreement with recent reports and consistent with their proposed roles in controlling proliferation, whereas miR-124 was enriched in postmitotic neurons, consistent with its neuronal role in development. Furthermore, we grouped miRNAs according to the shared pattern of abundance across single cells using weighted gene coexpression network analysis (WGCNA; Supplementary Table 8). Some of these specific miRNA coexpression modules operate within specific cell types, whereas others are broadly distributed across multiple cell types (Fig. 2e). Our analysis revealed dynamic changes in miRNA abundance in concordance with neuronal differentiation and maturation, a critical axis of transcriptional variation in the developing brain.

**Dynamic changes in miRNA–mRNA network during development**. Next, we explored the temporal axis of miRNA–mRNA interactions. We compared the abundance of miRNAs at two stages of development, GW15–16.5 and GW19–20.5 (Fig. 3), and found 69 differentially expressed miRNAs between these two stages including recently evolved miRNAs (Fig. 3a,b, Supplementary Figs. 5–8 and Supplementary Table 9). Two miRNAs, miR-449a and miR-449b-5p, which control mitotic spindle orientation during mammalian brain development, showed the highest overall fold change in expression level between GW15–16.5 and GW19–20.5. We confirmed the expression of miR-2115, miR-449c, miR-455 and miR-362 by in situ hybridization (Fig. 3b and Supplementary Figs. 5–7). We also found that miRNAs miR-1286, miR-142, and miR-548aa were enriched in the occipital lobe compared to the frontal lobe (Supplementary Figs. 8–10 and Supplementary Table 2), suggesting, in agreement with recent studies, that miRNAs may regulate regionally divergent transcriptional states in the developing human cortex.

By independently performing bipartite network analyses for samples at each of the two stages studied, we found marked preservation of most co-regulatory modules, as well as a set of distinct interactions predominantly present at one stage (Fig. 3c, Supplementary Fig. 11a and Supplementary Table 10). Notably, many of these modules
were also highly preserved as compared to adult human brain interactions previously surveyed using the same experimental strategy. Together, our findings suggest that miRNA-mediated regulation forms a developmentally dynamic network of interactions related to cell type, developmental stage and cortical area specificity.

Recent studies suggest that perturbations in miRNA expression may underlie human developmental neuropsychiatric disorders, but the specific molecular consequences remain poorly understood. Notably, genes implicated in autism spectrum disorders (ASD) were enriched in the magenta module (Supplementary Fig. 11b). Together, our findings suggest that miRNA-mediated regulation forms a developmentally dynamic network of interactions related to cell type, developmental stage and cortical area specificity.

we found that the expression of several miRNAs recently implicated in ASD was biased toward expression in excitatory neurons in developing mid-gestational human samples (Supplementary Fig. 12), suggesting that in vivo target interactions of these miRNAs may underlie human developmental neuropsychiatric disorders.

The heat map of miRNA target genes used to interpret cell identities (Fig. 2a) was based on values obtained from two biologically independent specimens. Although their expression patterns may change over the course of brain development, genes targeted by miR-137 in the developing brain differ greatly from targets identified in adult human brain tissue, suggesting that in vivo target interactions of these miRNAs may underlie human developmental neuropsychiatric disorders.
Fig. 3 | Dynamic changes in miRNA regulatory networks during development. a, Differential expression analysis identifies miRNAs differentially expressed between GW15–16 and GW19–20 developing human cortex. Red dots indicate primate-specific miRNAs. Heatmap displays expression z-score of each miRNA in that sample. Each column represents a specimen, and specimens are arranged according to age. b, Validation of differentially expressed miRNAs by in situ hybridization in developing human neocortex sections. Images show staining in outer subventricular zone. For every specimen, the experiment was repeated three times in different sections with similar results. c, Module preservation analysis for networks generated across GW15–16 and across GW19–20 samples as well as a set of distinct interactions present predominantly at one stage (for example, association of turquoise and lightyellow with GW19–20 and modules brown and blue with GW15–16). GW15–16 module names are used to compare GW15–16 modules with their homologs in GW19–20. CPM, count per million. d, Stage-specific changes in miRNA targets according to their specificity to distinct cell types of developing brain identified using single-cell RNA-seq27. Heatmap shows enrichment P value of miRNAs in distinct cell types in each stage of development.
miRNAs contribute to cell-type-specific function. a, HITS-CLIP data tracks showing reads spanning a predicted miR-2115 response element in the CDS of ORC4 mRNA. b, Luciferase reporter assay demonstrating functionality of miR-2115 interaction through CDS site identified in a (**P < 0.0001, unpaired two-sided t test), n = 3 biologically independent experiments. Center value represents mean and error bars represent s.e.m. c, d, miRNA-2115 influences radial glia development. (c) Primary radial glia were transfected with miR-2115 expression plasmid and cultures were immunostained for markers of radial glia (SOX2) and intermediate progenitors (EOMES). (d) Quantification of immunopositive cells (n = 3 biological replicates). miR-1, GFP-miR1 small RNA overexpression control construct; miR-2115, GFP-miR2115 overexpression construct; anti-miR-2115, miR-2115 inhibitor co-transfected with GFP expression construct. All constructs and reagents are described in Methods. *P < 0.05, two-sided Student’s t test. Center values represent mean and error bars represent s.e.m. e, Experimental design of cumulative BrdU labeling in human cells in vitro performed to assess impact of miR-2115 on progenitor proliferation. f, Immunostaining of human cultured cells. Arrowheads indicate GFP+ SOX2+ cells. Scale bar, 25 μm. g, h, Quantification of BrdU labeling of SOX2+ cells (g) and estimates of S-phase length (Ts) and cell cycle length (Tc) (h). Center values are relative to control conditions (n = 3 specimens). *P < 0.05, two-sided Student’s t test.
miR-2115 regulates cell cycle in human radial glia. The developmental transition between GW15–16.5 and GW19–20.5 coincides with changes in proliferation rates of radial glia and depletion of proliferative capacity in the human ventricular zone46. Among the top five miRNAs differentially expressed between these stages, a great-ape-specific miRNA, miR-2115, was prominently upregulated at GW19–20 in the germinal zones (Fig. 3a,b and Supplementary Figs. 5 and 7). Among miR-2115 targets, ORC4, a known regulator of DNA replication46, was enriched in radial glia at early stages of development47, and is a member of the turquoise module, which is enriched for GW19–20.5 HITS-CLIP interactions within a segment corresponding to a putative miR-2115 response element (Fig. 3c and Supplementary Table 11). Mutations in ORC4 are linked to Meier–Gorlin syndrome, which is frequently associated with microcephaly, suggesting that this gene may have an important role in normal brain development46. We hypothesized that miR-2115 acts through a radial-glia-enriched gene regulatory network involving ORC4 to regulate cell cycle dynamics and thereby influences cortical progenitor cell function. To test this hypothesis, we first confirmed the binding of the ORC4 miRNA response element and miR-2115 using a reporter assay and inhibitor studies (Fig. 4a–d and Supplementary Fig. 1) as well as a nearly zero P value using the stringent target prediction algorithm PACCMIT-CDS48. Next, we overexpressed a synthetic miRNA, mmu-miR-2115 (see Methods), in developing mouse cortex and found a greater proportion of radial glia, but a lower proportion of radial glia in mitosis, among the cells electroporated with mmu-miR-2115as compared to control electroporation (Supplementary Fig. 13). Similarly, manipulation of miR-2115 expression influenced the development of human primary radial glia cells in vitro. Both overexpression and inhibition of miR-2115 changed the proportion of cells expressing SOX2, indicating a possible role for this miRNA in proliferation or differentiation (Fig. 4c,d and Supplementary Fig. 13b). The phenotype was rescued by the addition of wild-type ORC4 reporter construct expressed together with miR-2115 (Fig. 4d).

To more specifically test for a possible cell cycle phenotype, we performed a cumulative bromodeoxyuridine (BrdU) incorporation assay (Fig. 4e–g) in human radial glia using GFP–miR2115 construct. The change in cell cycle rate caused by miR-2115 overexpression was rescued by addition of ORC4 protein expression construct (full-length) (Fig. 4h). This showed that miR-2115 expression regulates normal cell cycle duration in human radial glia by controlling ORC4 protein levels. Together, these findings suggest that miR-2115 emerged recently in evolution and integrated into post-transcriptional regulatory networks controlling cell cycle dynamics during human cortical development.

Discussion

Our study reveals several distinct mechanisms by which miRNA regulatory pathways contribute to human brain development. We developed a new single-cell-profiling approach for combined mRNA and miRNA profiling in the same cell. Using this approach, we examined cell-type-specific patterns of miRNA abundance, which revealed highly dynamic changes in miRNA expression, even among the closely related cells of the developing brain. Our findings support the emerging view that many miRNAs are expressed in cell-type-specific patterns42,43.

To gain insight into miRNA–miRNA interactome remodeling during cell-type transitions, we combined high-throughput profiling of miRNA–mRNA interactions with cell-type-specific gene expression profiles. Many miRNAs, including those expressed in multiple cell types, regulate the expression of cell-type-specific genes. Dynamic changes in cellular transcriptomes occurring during developmental lineage progression are probably controlled through a variety of regulatory networks involving transcription factors, signaling pathways, and post-transcriptional, epigenetic and epitranscriptomic mechanisms. Our analysis further emphasizes the contribution of miRNAs to the vast majority of such networks, including genes regulating excitatory neuron laminar and projection fates, such as regulators of callosal projection neurons (BHLHE22 and SATB2; ref. 38), corticothalamic neurons (TBR1; ref. 49) and interneurons (LHX6 and DLX5; ref. 38). Understanding the implications of these interactions for neuronal subtype specification will require highly multiplexed approaches for functional validation.

Furthermore, by projecting cell-type-specific miRNA and mRNA expression patterns against the modular framework of the bipartite network of miRNA–mRNA interactions, our study reveals dynamic developmental remodeling of miRNA–miRNA interaction networks involving conserved and recently evolved miRNAs, as well as cell-type-specific miRNA regulatory networks in the developing human brain (Supplementary Table 12). Comprehensive understanding of cell-type-specific miRNA–mRNA interactions may reveal previously unappreciated patterns of the selective vulnerability of cell types in neurodevelopmental disorders, including ASDs.

This multimodal approach to cell-type identity reveals an additional regulatory element introduced into radial glial cells. The expression of a great-ape-specific miRNA (miR-2115) targets ORC4, a gene involved in cell division. Because the target site is located in the conserved CDS, it does not have the same need for coevolution as the presumably less-constrained 3′ UTR, indicating that this regulatory interaction would be more likely to evolve. Functional sites have been extensively reported in the CDS47–50 and photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) experiments in humans show that the numbers of CDS and 3′ UTRs are similar48, a finding supported by predictive algorithms49. The functional data here on the control of cell cycle duration in radial glia are consistent with the microcephalic primordial dwarfism phenotype of Meier–Gorlin syndrome due to mutations in ORC4. These individuals have lissencephaly and hypoplastic frontal lobes among other structural abnormalities. In addition to specific miRNA coexpression modules associated with specific cell types (Fig. 2e), many miRNAs are expressed in many cell types, and they are likely regulated by different trans-factors,
depending on cell-type-specific transcriptomes. In these latter cases, some miRNA modules can be broadly distributed across multiple cell types (Fig. 2e). These latter modules may represent shared functionalities across different cell types or poorly resolved intermediate states. Setting boundaries for cell clusters is a statistical matter that can accrue additional support with more miRNA expression data. miRNA abundance is not easily measured with commonly used single-cell RNA-sequencing platforms. With more highly resolved single-cell quantification, novel cell identities may become apparent or cell type boundaries may emerge as less bistable and instead depend on an analog model.

The developing primate cerebral cortex forms a layered structure that contains an astonishing diversity of cell types. These diverse cell identities emerge during early development from highly dynamic progenitor cells, which undergo sequential changes in cellular transcriptome while simultaneously generating daughter cells that differentiate into one of dozens of terminal cell identities. Mitosis is a pivotal event in which both daughter cells have to decide to reenter the cell cycle or differentiate. Upon differentiation, fate specification and refinement are controlled by a suite of transcription factors. Post-transcriptional regulation of their expression by miRNAs acts a network-level control mechanism that can serve as a critical tuner of precise and robust identities. We found that groups of miRNAs can assume cell-type-specific patterns of expression and can regulate hundreds of cell-type-specific transcripts. Postmitotic cells undergo sequential changes in gene expression as they migrate to their architectonic destinations, and they often alter their states in response to environmental cues before establishing terminal identities. Notably, the dynamic process of cell-type maturation must use miRNAs differently than stably differentiated cells, in which the prominent role of miRNAs is homeostatic to buffer change. This strategic difference in the role of miRNAs—to foster change rather than stability—suggests further realms of cellular systems control over cell identity.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41593-018-0265-3.

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Author contributions
K.S.K., N.R., T.J.N., H.R.Z., L.R.P. and A.R.K. designed and supervised the study. J.A.W., A.L. and B.A. designed and optimized single-cell miRNA and mRNA PCR protocol. B.A., N.R., T.J.N., A.A.P. and B.A. performed experiments. H.R.Z., M.G., K.H., B.A., N.R. and T.J.N. performed data analysis. T.J.N., N.R. and K.S.K. wrote the paper with contribution from all authors.

Competing interests
The authors declare no competing interests.

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Methods

dissociation cell culture. Cortical cells were dissociated using papanin (Worthington Biochemical) and cultured on tissue culture plates coated with matrigel (BD Biosciences). Cells were plated at about 100,000–200,000 cells per well of a 12-well plate. Culture media used in this experiment consisted of DMEM (Invitrogen, 11965) supplemented with N2 (Invitrogen, 12587-010), B27 (17502-048), penicillin and streptomycin, but without serum. At the time of plating, culture medium was spiked with recombinant human fibroblast growth factor (FGF) (10 ng/mL, Peprotech, AF-100-188). About 24–48 h after plating, cells were transfected with plasmids using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. BrdU (Sigma) was diluted in the culture medium for dissociated cells (DMEM, supplemented with B27 and N2, with penicillin and streptomycin) at 50 μg mL⁻¹.

Sample collection. All mice in this study were obtained from Simonsen Laboratories and maintained according to protocols approved by the Institutional Animal Care and Use Committee at University of California, San Francisco (UCSF). De-identified human tissue samples were collected with subject consent in strict observance of the legal and institutional ethical regulations for elective pregnancy termination. Protocols were approved by the Human Gamete, Embryo and Stem Cell Research Committee (UCSF Institutional Review Board). Sample processing for dissociation, fixation, cryosectioning and long-term storage was performed as described21.

In utero electroporation. Survival in utero surgery was performed in strict observance of protocols and recommendations approved by the Institutional Animal Care and Use Committee at UCSF. Plasmids were injected at ~1.5μL/mL as described22,23. Although the ORC4 protein sequence is highly conserved, the miR-2115-5p miRNA recognition element (MRE) sequence is not fully conserved in mouse. We generated a mutant miR-2115-5p hairpin sequence (mumu-miR-2115) whose seed would be complementary to the mouse ORC4 mRNA coding sequence at the site homologous to the human miR-2115-5p MRE.

Plasmid constructs. gBlocks gene fragments for the respective target sites or miRNAs (Supplementary Table 3) with restriction site(s) at their ends were purchased from IDT. Restriction site cloning was performed using the standard method. Full-length ORC4 expression plasmid was generated using gene synthesis using a full-length human ORC4 and cloned into CAG-IRES-GFP vector using the GeneArt service (Thermo Fisher).

Luciferase activity assay. Luciferase activity assay was performed as described24 in HEK293 cells. The target sites were cloned in psiCHECK2 (Promega) plasmid (Supplementary Table 3), and miR-2115 and miR-9 were cloned in pCAG-GFP (Addgene 11150) plasmid (Supplementary Table 2). miRNA mimics (Life Technologies) were used for other miRNA assays. For ORC4, wild-type MRE reporter was named WT-ORC4, and a reporter lacking the miR2115 MRE was called MT-ORC4.

Fluorescence reporter expression measurement. Fluorescence reporters were generated by cloning the respective target site, as indicated above, into the Noti site of the GFP expression vector (Addgene, plasmid 11153), and validated by Sanger sequencing. For each culture condition, 100 ng of plasmid was transfected into primary dissociated human cells in culture, alone or together with LNA anti-miRNA inhibitor (Qiagen) (50 pmol). Mutant reporters were generated as gBlocks from IDT lacking the miRNA response element being tested. After 72 h in culture, cells were fixed and imaged using fluorescent microscopy. Using SPS confocal microscope at constant laser power and detector conditions. About ten fields of view were considered at random, and all cells in every field of view were used to quantify the average intensity of fluorescence signal (per pixel) across four conditions for every miRNA-mRNA interaction examined: control reporter with wild-type MRE, control reporter with wild-type MRE together with anti-miRNA inhibitor, reporter lacking an MRE, and reporter lacking an MRE together with anti-miRNA inhibitor. For wild-type and mutant reporter, we calculated the ratio of average fluorescence per pixel with and without anti-miRNA inhibitor. The experiment was repeated for cells derived from three independent biological specimens (n = 3, at GW14, GW18 and GW20), except for miR-2115-ORC4 CDS interactions, which were tested in three additional biological specimens (GW15, GW17 and GW19). For experiments presented in Supplementary Fig. 1, loading control CAG-Driven expression plasmid (100 ng) was co-transfected with the reporter to increase the accuracy of our quantification efforts. DsRed immunofluorescence was to normalize the GFP fluorescence signal in every cell analyzed.

In situ hybridization. In situ hybridization in primary tissue sections was performed as described25, except that we did not perform a probe linearization step. Digoxigenin-conjugated probes for miRNA detection were purchased from Roche or Qiagen. Immunofluorescence. Thin 20-μm cryosections were collected on superfrost slides (VWR) using Leica CM3050S cryostat. Immunohistochemistry-based detection of specific antigens was performed according to standard protocols. In short, heat-mediated antigen retrieval was performed in 10 mM sodium citrate for 15 min. Cells were permeabilized in PBS (pH 7.4) supplemented with 2% Triton X-100. Blocking buffer consisted of PBS supplemented with 10% donkey serum, 0.2% gelatin and 2% Triton X-100. The antibodies used in this study included chicken anti-GFP (1:1,000, Aves Labs GFP-1020), rabbit anti-PAX6 (1:300, Covance prb-278p), and mouse anti-pH3 (1:100, Abcam ab1791). These antibodies have been used previously in human tissues26,27. Secondary antibodies were obtained from Life Technologies. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma).

After cell fixation, BrdU epitope was unmasked using 2 N hydrochloric acid, neutralized using 0.1 M boric acid, and stained using a rat antibody to BrdU (BU1/75S, ICRI; 1:50, Abcam ab6526). This antibody has been used previously in human tissues28,29. Coverslips were mounted with Aqua-mount (Lerner Laboratories). Anti-pH3 and dsRed immunofluorescence cortical staining was quantified as described23. Quantification results for every biological replicate (embryo) represent an average of quantification across three nonadjacent sections. Replication shown from at least two independent litters. Quantification of BrdU incorporation into Sox2+ primary cells in culture was performed by imaging randomly selected fields in the well using tile-scanning feature. GFP+ were first evaluated for expression of Sox2, and after that BrdU immunoreactivity was assessed. Some 100–200 SOX2+ GFP+ cells were evaluated per well. Quantification of cell-cycle phenotypes was performed by fitting linear regression, and cell cycle parameters were calculated as described23.

AGO2-HITS-CLIP. We chose AGO2 as a target for HITS-CLIP experiments as most other studies did for miRNA target identification because among the Argonaute family of proteins, AGO2 has slicer activity, which is not the case for AGO1, AGO3 or AGO4 in mammals30,31. This mechanism of mRNA destruction by miRNA binding is both prevalent and implements a more rapid and readily detectable effect on downstream pathways. Moreover, we chose AGO2 because the data would be suitable for comparison with the previously generated HITS-CLIP data in the adult brain32, which we analyzed to gain insights into the preservation of the miRNA–mRNA interaction modules in Supplementary Fig. 1b. The experiments were performed as described32 except for a few modifications. Monoclonal antibody to Ag02 (Sigma, 11A9 clone, SAB4200085) was used to perform immunoprecipitation of protein on protein G dynabeads (Invitrogen, 100-05D). For the negative control, goat anti-rat IgG antibody (Sigma, A9037) was used. RNase dilution of 1:50,000 was used after optimization. Primary tissue samples at stages corresponding to peak neurogenesis (GW13–15.5) upper layer neurogenesis and early gliogenesis were analyzed. Sample quality and data processing metrics were comparable to published results for adult human brain HITS-CLIP study33. Some 3,258 genes were actively targeted by miRNAs through protein coding (CDS) or 3′ UTRs of the transcript. Notably, ~80% of gene targets were detected in at least two samples, suggesting that we recovered the majority of in vivo mRNA targets.

Library preparation for sequencing RNA tags on Ion Torrent. For library preparation, all steps were performed as described34, except that the primers with adapter sequences were modified according to the Ion Torrent sequencing platform.

Primers with adapter sequences were 5′-CCATCTCATTCCCCGTTGCTTCGGCTACGAGGAGGAGATGCCGCGG-3′ and 5′-CCCTCTCTATGGGGCAGTCGTGAGCAGCCTGGAATGTGACGAC-3′. The bands corresponding to AGO2-miRNA-target complexes (130 kDa) were cut. miRNA libraries and target mRNA libraries were made separately.

Single-cell quantitative PCR analysis. Capture of single cells was done using the C1 Single-Cell Auto Prep Integrated Fluidic Circuit (Fluidigm Corporation), which uses a microfluidic chip to capture the cells and perform lysis, reverse transcription and cDNA amplification in nanoliter reaction volumes of miRNA and mRNA species at the same time. The details of the cell capture protocol and primer set described in protocol of GISP2 (http://www.lifeblog.com/) were followed. During the reverse transcription step, miRNAs are reverse transcribed to cDNA using stem loop reverse transcription (RT) primers from the Megaplex RT primer pool (Life Technologies) that are specific for mature miRNA species and reagents from the Single-Cell-to-CT kit (Life Technologies). mRNA species were reverse transcribed at the time of LNA capture during this processing using primers present in the Single-Cell VILO RT mix. Megaplex primers and mRNA primers were added at the recommended concentrations. The choice of sc-qPCR miRNA targets was constrained by the pool of miRNA pre-enrichment primers from Life Technologies, and by the cost of Taqman primers. Therefore, the decision to include a primer for particular miRNA was driven by a combination of previous analysis and literature search. In particular, we leveraged published expression profiling data from developing human or nonhuman primate brain tissues35.
During the PCR step, products were uniformly amplified from cDNA templates using Megaplex PreAmp Primers (Life Technologies), a pool of DELTAgene primers and Single-Cell Preamp mix from the Ambion Single-Cell-to-CT kit (Life Technologies).

Cycling conditions were as followed: for reverse transcription, primers were annealed at 16 °C for 2 min, followed by extension at 42 °C for 1 min and extension at 50 °C for 1 min. This cycle was repeated 40 times, followed by a hold step at 85 °C for 5 min. For preamplification, polymerase was activated at 95 °C for 10 min, followed by incubation at 55 °C for 2 min and 72 °C for 2 min. Preamplification cycling steps were performed by denaturing step at 95 °C for 15 min followed by extension at 60 °C for 4 min. At the end, the reaction was incubated at 99.9 °C for 10 min, and the reaction was cooled to 4 °C.

After preamplification PCR, the amplicons were diluted at 1:4 with 1:1 DNA Dilution reagent (Fluidigm 100-3007) and stored in −20 °C until needed. qPCR was carried out using the 96.96 dynamic array (Fluidigm) according to the manufacturer’s protocol (100-3909 and 100-9792). Gene expression analysis was done using Fluidigm Real-Time PCR Analysis software (version 3.0.2). Cycle threshold (Ct) values were obtained and then square root normalized to stabilize the variance.

Clustering of single cells was performed using a recently developed method combining Louvain clustering of single-cell sample coordinates with Lasscard distance metric. Principal components analysis (PCA) dimensionality reduction and clustering was performed in the space of the following genes meaningful for cell type classification: COL1A1, COL1A2, FGF12, HCN2, NTRK2, NRG1, NRG3, ORMDL3, SLC4A1, SLC29A3, TBR1, TPR, TUBB3, TUBB8, TUBG1, TUBG1D, URI1, VIM, WDR73, ZIC1, ZIC3.

Preprocessing and mapping of AGO2-HITS-CLIP tags.

Barcodes were identified and reads were separated into each sample. Adapter sequences at both ends of reads were removed using Cutadapt. Trimmed reads were mapped to the human genome (hg19) with novoalign (http://www.novocraft.com/). Identical alignments were collapsed in each sample to remove PCR replicates. Strand-specific read coverage was then calculated using the alignments from each sample.

miRNA profiling and differential expression analysis. Adapter-trimmed AGO2 reads from miRNA libraries were mapped to human miRNA precursors from miRbase version 21 using miRDeep2 (ref. 64). DESeq2 was used to identify differentially expressed miRNA profiling and differential expression analysis. (Supplementary Fig. 11a,b, Supplementary Table 2). An edge exists between only one node in mode I and one node in mode II if there is the total number of miRNA and target genes combined. Each entry in the matrix

| Gene expression analysis. To formally demonstrate that cell-type-specific genes are regulated by miRNAs, we used published scRNA-seq data to calculate cell-type-specificity scores using the gene-set correlation approach for every miRNA target identified by HITS-CLIP (Supplementary Table 7). We defined a gene to be a signature of a cell type if its Pearson correlation was 3e higher than the mean Pearson correlation score of all genes in all cell types (histogram in Fig. 1c)23,24.

To calculate the overlap with ASD genes, we used genes associated with ASD annotated by SFAIRI Gene. A module was defined to be enriched in ASD if the FDR corrected P value of hypergeometric test was <0.05 (the total number of expressed genes in prenatal brain tissues was also used as the background).

Bipartite community detection analysis. An unweighted (binary) bipartite network was constructed such that there exists an edge between each miRNA–mRNA pair if and only if such interaction is detected by AGO2-HITS-CLIP. First, this network was shown to be scale-free (P <0.001), and then by generating random networks while constraining the number of edges and nodes to the original miRNA–mRNA network and calculating Barber’s modularity score (null distribution), the miRNA–mRNA network was shown to be significantly modular (P < 2 × 10−16 for permutation test). Label propagation followed by the bipartite recursively induced modularity algorithm (LP-BRIM) was used for community detection in this bipartite network. Due to the stochasticity of this method, we obtained robust communities by repeating LP-BRIM 2,500 times and determining the overlap among all the iterations.

Bipartite network construction. The bipartite networks were generated using all detected target genes (3,463 nodes in mode I) and all miRNA (514 nodes in mode II). An edge exists between only one node in mode I and one node in mode II if such interaction is present in the HITS-CLIP data set (Supplementary Table 2) (no edge is allowed between nodes in mode I or mode II). Hence in this study, the bipartite network was constructed using GW15–16, GW19–20, and the combined GW15–16 and GW19–20 HITS-CLIP data set with 31,859, 20,734, and 36,176 total edges, respectively.

Bipartite network modularity statistics. First, the R bipartite package was used to show that the constructed network was scale-free. For consistency with the literature definition of scale-free networks, we showed that this network significantly obeyed power law, truncated power law and exponential distribution (Supplementary Fig. 11a,b, P < 0.001). Next, to demonstrate that these networks were significantly modular, we first generated a null distribution by randomly shuffling the edges between the nodes and calculating Barber’s modularity score. Finally, we showed that the network was significantly modular using a permutation test (P < 2 × 10−16).

Bipartite community detection. We used R lparim package to detect communities in the described networks. Due to stochasticity of the method, we ran the community detection algorithm 25 times and obtained the best solution among all 25 runs (with maximum Barber’s modularity score). We repeated this procedure 100 times and found consensus clustering as follows.

We first defined the overlap rate matrix (ORM) as a symmetric n by n matrix where n is the total number of miRNA and target genes combined. Each entry ORM, shows the probability (or rate) of which target gene (or miRNA) lies within the same cluster as target gene (or miRNA) upon 100 runs.

The obtained ORM was further clustered using hierarchical clustering (Supplementary Fig. 11c). Each obtained diagonal block after hierarchical clustering represents one community (containing both target genes and miRNAs that have significant intramodular interactions compared to their intermodular interactions with target genes (or miRNAs) in other communities).
Finally, dynamic branch cutting implemented in the dynamicTreeCut R package was used for tree cutting and assigning cluster identification numbers to each node (Supplementary Fig. 11c,d). We repeated this procedure to show that the obtained clusters are robust and reproducible (Supplementary Fig. 11c,d).

**Bipartite module preservation analysis.** To determine whether each identified module in the GW15–16 network was statistically preserved in GW19–20, we first found the closest module in GW19–20 to GW15–16 in terms of the number of shared nodes in that module. Then, using the hypergeometric test, module preservation statistics were obtained and corrected using the Benjamini–Hochberg method for multiple comparison. As shown in Fig. 3f, two homolog modules in GW15–16 and GW19–20 had similar interaction levels, suggesting preserved topology of modules as well. Names of GW15–16 module colors were used to label Fig. 3f.

**Inference of evolutionary history of miR-2115.** To infer the evolutionary history of miR2115, we used genome sequences obtained from the University of California Santa Cruz (UCSC) Genome Browser (using the most recent genome assemblies). Alignments were done using the Geneious bioinformatics platform (version 9.1.8). Human SPINK8 gene sequence was annotated with introns, exons, CDS and UTR regions and miR-2115 location according to National Center for Biotechnology Information RefSeq track on the UCSC Genome Browser. SPINK8 gene orthologs were located in chimpanzee, gorilla, orangutan and gibbon genomes using the other RefSeq track on UCSC Genome Browser. We excluded Bonobo because of the poor quality of genome assembly at the area of interest. Primate SPINK8 sequences were aligned with human SPINK8 sequences individually using the MUSCLE Alignment algorithm. Primate SPINK8 sequences were annotated with introns, exons, CDS and UTR regions and miR-2115 location according to alignment with the annotated human SPINK8 sequence. Originally primate SPINK8 genes were annotated according to the other RefSeq track on UCSC Genome Browser but this yielded varied and unreliable results. The presence or absence of miR-2115 was determined based on alignment of human miR-2115 to the orthologous primate sequence (with mature transcript and seed region taken into consideration). Annotated human, chimpanzee, gorilla, orangutan and gibbon SPINK8 intron 3/4 sequences, where intron miR-2115 is located, were aligned together to visualize changes in intron sequences between species. Boundaries of insertions and deletions in SPINK8 intron 3/4 occurring between species were defined based on evolutionarily chronological alignments of SPINK8 intron 3/4 sequences (for example, gibbon and orangutan alignment, orangutan and gorilla alignment, and so on). Evolution of the SPINK8 intron 3/4 was predicted using the fewest mutations that would give rise to observed insertions and deletions.

**Statistics.** Statistical tests, sample sizes and assumptions are indicated in each corresponding figure legend, except for bipartite network analysis, which is described above. Data distribution was assumed to be normal but this was not formally tested. Across all experiments and quantification, each tissue specimen was considered a biological replicate. Although we did not use formal methods of randomization, quantification of immunostaining was always performed blind, such that identity of sample and condition was not known to the person performing the quantification. Across all experiments, specimens with low viability of cells after plating, specimens contaminated with bacteria, or specimens for which we could not perform the entire experiment were not included in the analysis and not reported. For mouse embryos, we selected embryos with highest quality of electroretropion based on visual assessment under a stereotaxic fluorescence microscope. For animal and human embryonic tissue experiments, we did not examine the embryos for sex. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported earlier. Across all experiments, data distribution was assumed to be normal but this was not formally tested.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Code availability.** Scripts used in data analysis for this manuscript can be found at GitHub: https://github.com/mgolkaram/Nature-Neuroscience-2018-miRNA-miRNA-paper.

**Data availability** The data used in this study are available as part of the publicly available Gene Expression Omnibus database under accession number GSE107468.

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Reporting Summary

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement
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- For null hypothesis testing, the test statistic (e.g. $F$, $t$, $r$) with confidence intervals, effect sizes, degrees of freedom and $P$ value noted. Give $P$ values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s $d$, Pearson’s $r$), indicating how they were calculated
- Clearly defined error bars

State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

- Data collection: https://github.com/mgolkaram/Nature-Neuroscience-2018-miRNA-mRNA-paper , protocol 100-6667 at http://www.fluidigm.com/
- Data analysis: https://github.com/mgolkaram/Nature-Neuroscience-2018-miRNA-mRNA-paper , Geneious® bioinformatics platform (version 9.1.8), http://www.novocraft.com/). Fluidigm Real-Time PCR Analysis Software (v.3.0.2), mirdeep2, DESeq2, R version 3.3.1 (2016-06-21)

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data used in this study are available as part of the publicly available Gene Expression Omnibus database under the accession number GSE107468.
### Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | We generated data based on the availability of primary tissue samples. In total we processed 9 primary tissue samples for AGO2-clip, and used saturation analysis to determine that this number was effective in recovering the majority of targeted genes. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | For single cell qPCR we excluded any assay that did not give positive value after 30 cycles of amplification in at last two single cells. We removed cells with positive signal from at least 5 assays. |
| Replication | We used strict measures to highlight high confidence AGO2 binding clusters in the mRNA. Firstly, we used multiple biological replicates for each age-range of specimens and curated the dataset to the list of target sites detected in at least two biological specimens. For all other experiments that were carried out, all attempts at replication were successful. |
| Randomization | Samples were randomized due to the random collection of primary tissue specimens and were processed in random order. Sc-qPCR data was generated and processed in random order. |
| Blinding | Sample collection was performed by researchers at UCSF, the samples were processed for AGO2-CLIP at UCSB without cross-referencing with sample metadata. For single cell qPCR,m data was processed in parallel and only analyzed together following a melting curve analysis.miR2115 overexpression analysis and brdU analysis experiment was performed by blinding the experimental conditions for brdu/sox2 quantification. |

### Reporting for specific materials, systems and methods

#### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑️ | Unique biological materials |
| ☑️ | Antibodies |
| ☑️ | Eukaryotic cell lines |
| ☑️ | Palaeontology |
| ☑️ | Animals and other organisms |
| ☑️ | Human research participants |

#### Eukaryotic cell lines

Policy information about [cell lines](#)

| Cell line source(s) | HEK293 cells were obtained from a commercial source. |
|---------------------|----------------------------------------------------|
| Authentication      | This cell line was never authenticated |
| Mycoplasma contamination | We have not tested this line for mycoplasma contamination |
| Commonly misidentified lines (See ECILAC register) | We used the HEK293 cell line and there was no particular reason for choosing it, other than that it is commonly used in the lab for luciferase experiments. |

#### Animals and other organisms

Policy information about [studies involving animals](#): ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | We have pregnant dams Swiss-Webster in this experiment, but only analyzed embryonic animals. We never test embryos for sex, therefore we expect approximately even representation of males and females. We used three pregnant dams in this |
experiments. Because Simonsen does not inform us about the age of these animals, we do not know what ages they are. The embryos used in the analysis were at E15.5 stage of development. However, we did not confirm the age by Theiler criteria, and the age is only based on the plug check information provided by Simonsen.

| Wild animals                        | this study did not involve wild animals |
|-------------------------------------|----------------------------------------|
| Field-collected samples             | this study did not involve field-collected animals |