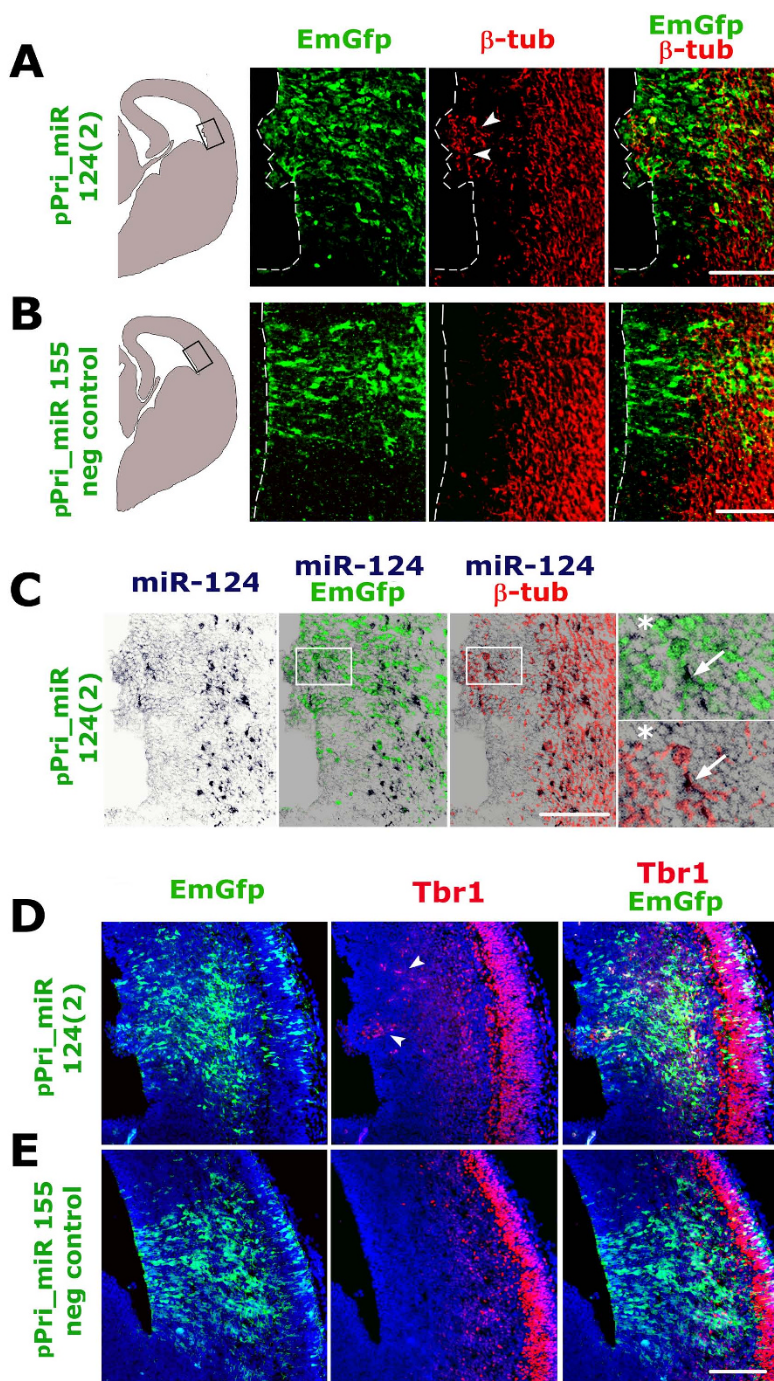


**Figure 5**

**Immunoprofiling of cortical periventricular layers after *in utero* electroporation of plasmids pPri-miR-124(2) and pPri-miR-155/neg_control; part II**. **(A, B, D, E)** Distribution of β-tubulin, Tbr1 and pCMV-driven EmGFP on E14.5 mid-frontal sections from brains electroporated *in utero* at E12.5 with plasmids pPri-miR-124(2) and pPri-miR-155/neg_control. In (A, D), arrowheads point to periventricular cells expressing β-tubulin and Tbr1, respectively. **(C)** Comparison of pCMV-driven EmGFP and neuron-specific β-tubulin with miR-124 expression in the electroporated area shown in (A, B, D, E). Magnifications of boxed insets to the right show an EmGFP$^+$ electroporated cell co-expressing huge amounts of miR-124 and β-tubulin (arrow), as well as another EmGFP$^+$/miR-124$^+$ cell negative for β-tubulin (asterisk). Electroporated zones shown throughout Figure 5 correspond to the boxed areas in the schematics of (A, B). Scale bars = 100 μm.

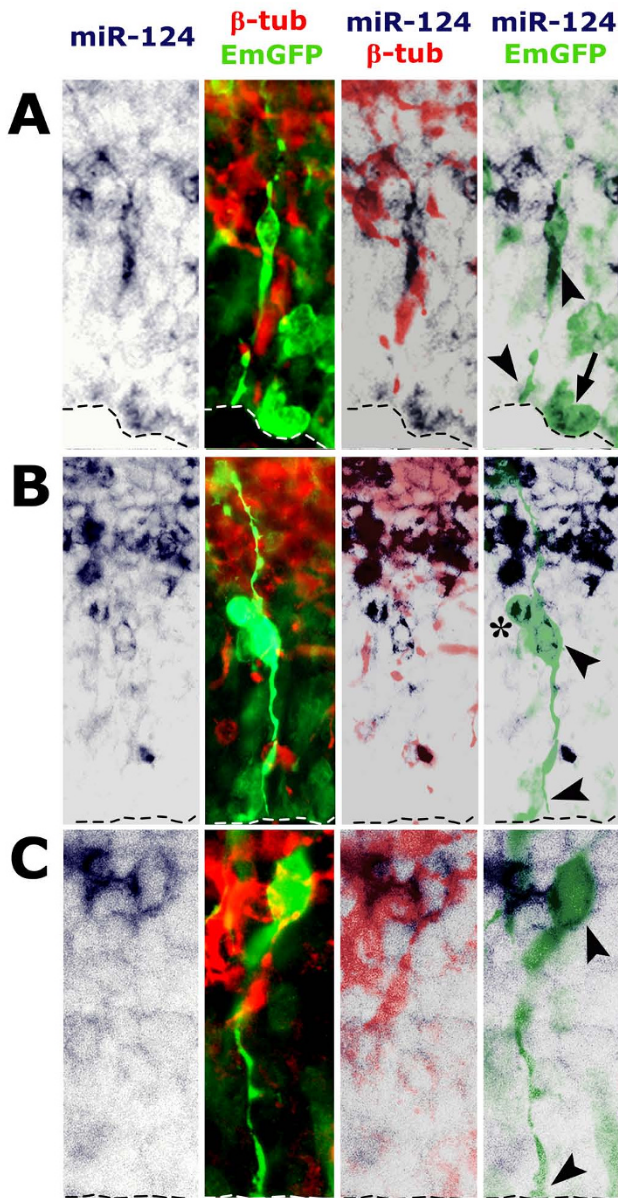


**Figure 6**
**Specific miR-124 overexpression in pPri-miR-124(2) electroporated periventricular neural precursors of the E14.5 cortex**. Distribution of miR-124, pCMV-driven EmGFP and neuron-specific β-tubulin in mid-frontal sections from brains electroporated *in utero* at E12.5 with pPri-miR-124(2). miR-124 may be specifically detected in: apical progenitors still connected to the ventricle (arrowheads in (A, B)) or undergoing mitosis (arrow in (A)); basal progenitors (asterisk in (B)); and nascent neurons still connected to the ventricle (arrowheads in (C)).

processing of this chimeric transcript to mature miRNA. This hypothesis is consistent with the discrepancy between the expression profiles of miR-124, which is mainly restricted to abventricular layers (Figures 1 and 2), and its precursors, which are conversely detectable at E14.5 at similar levels throughout the cortical wall [58]. This may also account for the progressive lowering of DsRed2 fluorescence we found in *in vitro* differentiating neurons harboring a DsRed2/Pri-miR-124(2) transgene. The idea that substantial modulation of miRNA levels may occur after transcription is not novel. In addition to transcriptional regulation [39,62], it has been suggested and experimentally proven that biogenesis of many miRNAs may be regulated at a variety of levels, including Drosha-dependent conversion of Pri-miR to Pre-miR [63], translocation of Pre-miR from the nucleus to the cytoplasm [64], Dicer-dependent conversion of Pre-miR to miR [55], and incorporation of miRNAs into RISC [65]. Modulation of Pri-miR processing is especially relevant to the proper regulation of neuro-specific and neuro-enriched miRNAs, including *let-7* family members, *miR-128* and *miR-138*, whose post-transcriptional maturation may dramatically increase with the transition from stem cells to post-mitotic differentiated elements [53-55]. Preferential confinement of the maturation of many miRNA precursors to late histogenesis is consistent with the integrity of stem cells within the cortical VZ of *Dicer* conditional-null mutants [38,43], as well as with the impaired differentiation abilities of *Dicer*$^{-/-}$ embryonic stem cells [66]. Post-transcriptional regulation of miR-124 has already been addressed in the developing *Drosophila* nervous system, where dFMR1 is required for its proper biogenesis [67]. Further studies are required to clarify modulation of miR-124 expression in vertebrates.

By electroporating a Pri-miR-124(2) precursor into the developing mouse cortex, we were able to promote cortical neuronogenesis. We forced a fraction of ventricular precursors to leave the apical compartment and move to the basal compartment (Figure 4). We occasionally anticipated β-tubulin activation in pin-like cells (Figure 6C) and elicited an ectopic burst of neuronogenesis from apical progenitors within the VZ (Figure 5; Additional file 5). We replicated the last result *in vitro* by over-expressing Pri-miR-124(2) in dissociated cortical neuroblasts, but only when these precursors were kept under differentiating medium (Figure 3F-I). Inhibition of BrdU uptake and stimulation of direct neuronogenesis has been reported already in the chicken embryonic spinal cord, specifically upon electroporation of mature miR-124 [40,42]. A reduction in the number of dividing cells also takes place *in vivo* in the adult mouse SVZ upon Pri-miR-124(3) over-

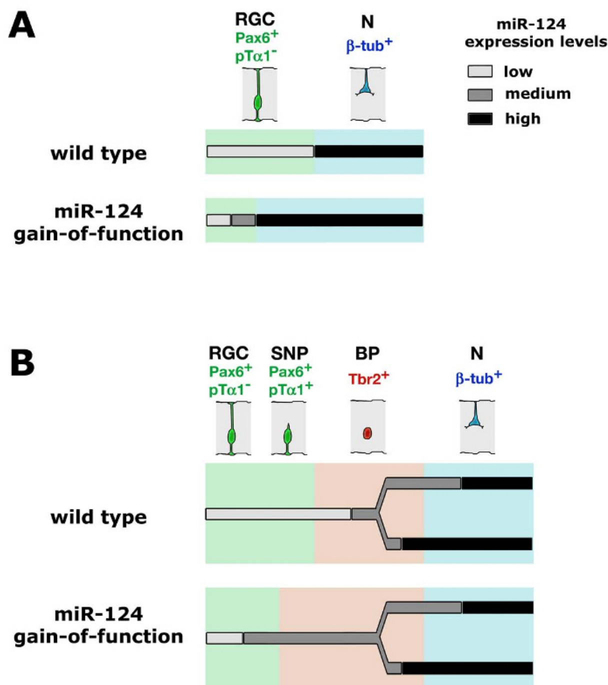


**Figure 7**

**Schematic of the miR-124 expression profile along neuronogenic lineages and the phenotype of miR-124 gain-of-function electroporated cortices. (A, B)** One-step and two-step changes in miR-124 expression levels during direct (A) and indirect (B) neuronogenesis. Stimulation of direct neuronogenesis and expansion of the basal compartment occurs at the expense of the apical one upon miR-124 over-expression. RGC, radial glial cell; SNP, short neural precursor; BP, basal progenitor; N, neuron.

expression. Consistently, administration of antisense miR-124 to *in vitro* cultures of SVZ elements increases BrdU uptake by C-type transit amplifying cells and A-type neuroblasts, slowing down transition from the former to the latter [36]. Remarkably, we also found that miR-124 facilitates neuronogenesis in a permissive molecular environment, but is not able to initiate such a process *per se*, similar to what was previously described [37,38,68]. Finally, we did not find any increase in cell death upon Pri-miR-124(2) electroporation (Additional file 6), in contrast to what was previously reported for the chicken embryo [42]. This may be due to a variety of reasons, including differences between animal models, different CNS tracts studied, and different constructs used and electroporation protocols.

Lastly, by analyzing electroporated brains, we noticed a previously undescribed technical artifact. We detected a pronounced displacement of apical Pax6+ and basal Tbr2+ progenitors, just beneath the cortical plate, in both pPri-miR-124(2) and pPri-miR-155_neg_control electroporated brains (Figure 4A, B, arrowheads). This phenomenon was replicated upon electroporation of pEGFP-C1 (Additional file 4, arrowheads), which shares the pCMV-EGFP module with the above two plasmids but does not harbor the Pri-miR stem-loop moiety, indicating that miR-124 or stem-loop specificity are not involved in it. Displacement of apical and basal precursors took place only on the electroporated side, was mainly restricted to the middle of the electroporated zone, being undetectable in its surroundings, and was not cell-autonomous (Additional file 4). Despite the locality of the displacement, the electric field we applied was uniform throughout the E12.5 telencephalon, thanks to the 7 mm tweezer electrodes we used. This implies that this effect was not due to the electrical stress *per se*. Reasonably, it might originate from heavy metabolic loads weighing on electroporated precursors, possibly impairing the correct scaffold structure of the cortical wall. The mechanical damage induced by the injection needle might contribute to the priming of such an effect. Nevertheless, displacement of Pax6+ and Tbr2+ progenitors was equally present in controls and Pri-miR-124(2) electroporated embryos, and so does not affect the results of the miR-124 gain-of-function analysis.

## Conclusion

Our study makes two main observations. First, miR-124 is expressed in the developing embryonic cortex according to a complex pattern. It is upregulated sharply in apical precursors undergoing direct neuronogenesis and, via an intermediate expression level, in late basal progenitors during indirect neuronogenesis (Figure 7). Differential post-transcriptional processing seems to contribute to this pattern. Second, miR-124 overexpression stimulates direct neuronogenesis and promotes transition of neural precursors from the apical to the basal compartment (Figure 7).

These findings shed light on the role of miR-124 during early cortical development in mammals. Understanding the role of miRNAs during neurogenesis may be fundamental to uncovering the mechanisms that regulate the sizes of the different cell compartments in the CNS primordium.

## Materials and methods

### *Animals and bromodeoxyuridine injection*

Mice (*Mus musculus* strain CD1, purchased from Harlan-Italy Srl (San Pietro al Natisone, UD, Italy)) were maintained at the SISSA-CBM mouse facility and were staged by timed breeding and vaginal plug inspection. Animal handling and subsequent procedures were in accordance with European laws (European Communities Council Directive of November 24, 1986 (86/609/EEC)) and with National Institutes of Health guidelines. Embryos (E10.5

to E18.5) were harvested from pregnant dames killed by cervical dislocation. When required, BrdU was injected intraperitoneally into previously electroporated pregnant dams 45 minutes before the sacrifice at 150 μg/g bodyweight. Electroporated embryos were harvested immediately afterwards.

### *Pri-miRNA and cDNA expression constructs*

The pPri-miR-124(2) construct contains the 285-bp mouse Pri-miR-124(2) genomic fragment (chr3 (+):17695562-17695846) cloned into the BLOCK-iT™ expression vector (Invitrogen - Life Technologies Corporation, Carlsbad, CA, U.S.A.) in-between the pCMV-EmGFP and TK_pA modules using *Sal*I and *Xba*I enzyme restriction sites. pPri-miR-155neg_control contains the Pri-miR155 sequence in-between the pCMV-EmGFP and TK_pA modules (BLOCK-iT™, Invitrogen). The plasmid pmiR-124-sensor contains the 477-bp 3' untranslated region fragment of mouse *Lhx2* (chr2 (+):38224759-38225235) cloned into the pDsRed2-N1 plasmid (Clontech Laboratories Inc., Mountain View, CA, U.S.A) in-between the pCMV-DsRed2 and SV40pA modules using *Not*I and *Eco*RV enzyme restriction sites. The pTα1-EGFP plasmid (a kind gift of E Ruthazer) harbors the GFP coding sequence under the control of the α-tubulin 1 promoter (pTα1). pLV_Pri-miR-124(2) and pLV_Pri-miR-155neg_control, encoding lentiviral RNA genomes, were generated as follows. Briefly, the Pri-miR-124(2) and Pri-miR-155neg_control *Dra*I/*Bgl*II fragments were transferred from pPri-miR-124(2) and pPri-miR-155neg_control, respectively, into the pDsRed2-N1 *Not*I-blunted/*Bgl*II-cut plasmid downstream of the DsRed2 module. Subsequently, the DsRed2-Pri-miR-124(2) and the Dsred2-Pri-miR-155neg_control *Age*I/*Sma*Ifragments were transferred from the resulting plasmids into the pCCLsin.PPT.prom.EGFP.Wpre [51]*Age*I/*Sal*I-blunted cut vector. pEGFP-C1 (Clontech) was used as control for *in utero* electroporation.

### *Production and titration of lentiviral vectors*

Plasmids pLV_Pri-miR-124(2) and pLV_Pri-miR-155neg_control were used to produce lentiviral vectors LV_Pri-miR-124(2) and LV_Pri-miR-155neg_control as previously described [51]. Titration of lentiviral vectors was performed by real-time PCR, as previously reported [69].

### *miR-activity assay*

HeLa cells grown in 10% FCS and Dulbecco's modified Eagle's medium with Glutamax (DMEM/Glutamax; Invitrogen) were co-transfected with either pPri-miR-124(2) or pPri-miR-155neg_control, each pre-mixed with pmiR-124-sensor plasmid at a molar ratio of 30:1, using Lipofectamine (Invitrogen) and according to the manufacturer's instructions. Forty-eight hours after transfection, photos of ten different randomly chosen fields of each plate were taken, using a Nikon Eclipse 80 i fluorescent microscope (20× lens) and a DS-2 MBWC digital microscope camera. Pictures were processed using Photoshop CS3 software and specific attenuation of DsRed2 signal was evaluated by comparing the numbers of single- and double-labeled cells. All cell counting was performed on coded samples, so that the experimenter was blind to the condition. The experiment was repeated three times and data analyzed using Excel 2008 and SigmaPlot.

### *Lentiviral gene transduction on differentiating primary cortical precursor cells*

To evaluate the fraction of β-tubulin$^+$ cells among differentiating primary cortical precursor cells, cerebral cortices of E12.5 embryonic brains were dissected as previously described [70]. Cells ($3 \times 10^6$) were plated onto each well of a 12-multiwell Falcon plate (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of $10^3$ cells/μl and cultured in DMEM/F12/Glutamax medium (Invitrogen) with N2 supplement (Invitrogen), 0.6% w/v glucose, 2 μg/ml heparin, 10 pg/ml fungizone and with or without 2.5% FCS. Cortical precursors were transduced with lentiviral vectors at a multiplicity of infection of 40. Medium was replaced 36 h post-transduction. For the experiments on neurite outgrowth, $5 \times 10^5$ cells were plated onto each well of a polylysined 12-multiwell plate (Falcon) at a density of 200 cells/μl and cultured as above. This lower density culture was necessary to allow for subsequent NeuriteTracer® analysis of differentiating cells.

### *Evaluation of neuronal frequencies* in vitro

Seventy-two hours after lentiviral infection, *in vitro* transduced cells were dissociated with trypsin-EDTA for 5 minutes, left to attach on poly-L-lysine coated glass coverslips for 30 minutes and finally fixed in 4% paraformaldehyde. Staining was performed as previously described [71] with primary mouse monoclonal antibody anti-β-tubulin (1:300; clone Tuj1, Covance, Princeton, NJ, U.S.A.) and anti-mouse secondary antibody Alexa fluor 594 conjugates (1:500; Invitrogen). DAPI (4',6'-diamidino-2-phenylindole) was used as nuclear counterstaining. For each experiment 5 subject and 5 control fields were captured using a fluorescent Nikon Eclipse 80 i microscope (20× lens) and a DS-2 MBWC digital microscope camera. For each experiment, at least 300 subject and 300 control cells were counted. The experiment was repeated three times and data were analyzed as follows. Frequencies of β-tubulin$^+$ cells within each field were calculated. They were averaged for each experiment and each lentivirus; results (± standard error of the mean) are therefore plotted against experiment number. Finally, the 3 subject and the 3 control average frequencies obtained were analyzed by *t*-test (one-way, paired) and the *P*-value reported on the graph.

#### *Evaluation of* in vitro *neurite outgrowth*

Seventy-two hours after lentiviral infection, *in vitro* transduced cells were fixed for 15 minutes in 4% paraformaldehyde. β-Tubulin/DAPI staining was performed as described in the Evaluation of neuronal frequencies *in vitro* section above, replacing the Alexa fluor 594 antibody with Alexa fluor 488. For each experiment 30 subject and 30 control fields were captured, using a fluorescent Nikon Eclipse 80 i microscope (40× lens) and a DS-2 MBWC digital microscope camera. For each experiment, at least 150 subject and 150 control β-tubulin$^+$ cells were sampled. Electronic files were imported into ImageJ and processed using the NeuriteTracer® plugin according to the authors' instructions [52], and NeuriteTracer® outputs - that is, average neurite lengths per neuron calculated per each field - were collected. The experiment was repeated three times and data were analyzed as follows. Average neurite lengths per neuron calculated for each field were averaged for each experiment and each lentivirus. The results (± standard error of the mean) were plotted against experiment number. Finally, the 3 subject and the 3 control average frequencies obtained were analyzed by *t*-test (one-tail, paired) and the *P*-value reported on the graph.

#### In utero *electroporation*

Electroporation was carried out to transfect VZ cells *in utero* with mammalian expression vectors as described previously [4,72,73]. Briefly, uterine horns of E12.5 pregnant dams were exposed by midline laparotomy after anesthetization with ketamine (200 μg/g bodyweight) and xylazine (40 μg/g bodyweight). Then, 1.5 μl of a solution containing 3 μg of DNA plasmid mixed with 0.02% fast-green dye in phosphate buffered saline (PBS) was injected in the telencephalic vesiscle using a sharp pulled micropipette (hole external diameter about 30 μm) through the uterine wall and the amniotic sac. Platinum tweezer-style electrodes (7 mm diameter) were placed outside the uterus over the telencephalon and four pulses of 40 mV were applied (each 50 ms long; interval between consecutive pulses 950 ms) using a BTX ECM830 square wave pulse generator (Genetronics, San Diego, CA, U.S.A.). Electroporation was performed in about half of the embryos found in each uterine horn to avoid prolonged surgery time. The uterus was then replaced within the abdomen, the cavity was filled with warm sterile PBS, and the abdominal muscle and skin incisions were closed with silk sutures. Animals were left to recover in a warm clean cage. Harvesting of electroporated embryos was performed 2 days later, as described above.

#### In situ *microRNA hybridization*

Brains from E10.5 to E18.5 embryos and 4-day-old CD1 mice (Harlan lab), as well as brain from E14.5 embryos electroporated 2 days earlier, were perfused with 4% paraformaldehyde overnight. Afterwards, brains were immersed in 30% sucrose (w/v) and embedded in OCT mounting medium. *In situ* hybridization was carried out on 10 μm coronal brain slices using miRCURY 5' DIG labeled detection probes (LNA) for mmu-miR-124, mmu-miR-425 and mmu-miR-207 according to the manufacturer's instructions (Exiqon, Vedbaek, Denmark), as previously described [44].

#### *Tissue immunofluorescence*

Immunofluorescence analyses were performed as previously described [72]. Briefly, frozen sections were boiled in 10 mM sodium citrate, pH 6.0, and blocked in 10% fetal bovine serum and 0.1% Triton X-100 for 1 h at room temperature. Incubation with primary antibodies was performed at 4°C overnight. In the case of BrdU detection, epitopes were made accessible by HCl treatment, as previously described [75]. Secondary antibodies were applied to sections for 2 h at room temperature. The following primary antibodies were used: anti-β-tubulin mouse monoclonal (1:300; clone Tuj1, Covance, Princeton, NJ, U.S.A.), anti-Egfp chicken polyclonal (1:600; AbCam, Cambridge, MA, U.S.A.), anti phosphohistone-H3 rabbit polyclonal (1:400; Chemicon-Millipore, Billerica, MA, U.S.A.), anti-Tbr1 rabbit polyclonal (1:2,000; a gift from R Hevner, Seattle, USA), anti-Tbr2 rabbit polyclonal (1:600; AbCam), anti-active_caspase3 rabbit polyclonal (1:300; BD Biosciences Pharmingen, San Diego, CA, U.S.A.), anti-Pax6 rabbit polyclonal (1:500; AbCam), anti-β1-integrin rat monoclonal (1:500; clone VLA, Chemicon-Millipore, Billerica, MA, U.S.A.), and anti-BrdU mouse monoclonal (1:50; clone B44, BD Biosciences Pharmingen, San Diego, CA, U.S.A.). Secondary antibodies were conjugates of Alexa Fluor 488 and Alexa Fluor 594 (1:500; Invitrogen). DAPI was used as nuclear counterstaining. Finally, slices were washed and mounted in Vectashield Fluorescent Mounting Medium (Vector Labs, Burlingame, CA, U.S.A.). Immunofluorescence analyses on *in situ* hybridized coronal brain slices was performed as described above, after washing the LNA-hybridized sections for 1 h in PBS.

#### *Acquisition, processing and statistical analysis of* in vivo *immunoprofiling data*

*In situ* hybridized sections with or without immunofluorescence analysis were imaged using a fluorescent Nikon Eclipse 80 i microscope and a DS-2 MBWC digital microscope camera. Such images were processed using Adobe Photoshop CS3 software.

For each marker under analysis, cell counting was performed on at least three different electroporated embryos for both pPri-miR-124(2) and pPri-miR-155 constructs (N ≥ 3+3); three sections from each electroporated embryo spaced 100 μm apart along the rostro-caual axis were inspected. In total, at least 400 EmGFP$^+$ cells per embryo were scored for double labeling, paying special attention to compare embryonic tissue electroporated at

similar rostro-caudal and medio-lateral levels. Sections were photographed using a TCS SP2 Leica confocal microscope, generally collected as 5.0-μm-thick Z-stacks of 1,024 × 1,024 pixel images. Images were then imported into Photoshop CS3, where all cell countings were performed on coded samples, so that the experimenter was blind to the condition. Results were imported into Excel 2008, percentages of labeled cells were calculated for each brain and data relative to all brains electroporated with the same construct were averaged. Results are expressed as mean value ± standard error of the mean and were tested for statistical significance by one-way-ANOVA. Results shown are normalized against controls.

## Abbreviations

BrdU: bromodeoxyuridine; CNS: central nervous system; DMEM: Dulbecco's modified Eagle's medium; E: embryonic day; EGFP: enhanced green fluorescent protein; EmGFP: emerald green fluorescent protein; FCS: fetal calf serum; LNA: locked nucleic acid; miRNA: microRNA; PBS: phosphate buffered saline; SVZ: subventricular zone; VZ: ventricular zone.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

NAM and AM designed the study, NAM performed the experiments, and NAM and AM analyzed the data and wrote the manuscript. Both authors read and approved the final manuscript.

## Additional material

### Additional file 1

***Specific faint expression of miR-124 in the E14.5 VZ. (A-C)*** In situ *hybridization of miR-124 (A), miR-425 (B) and miR-207 (C) probes on mid-frontal E14.5 telencephalic sections. miR-425 and miR-207 are two miRNAs not expressed in the developing CNS [45,46]. Magnifications of boxed areas illustrate the faint staining detectable in the VZ (black arrowheads) but not in mesenchymal tissue (black harrow) upon miR-124 hybridization, as well as the absence of any signal in samples hybridized with miR-425 or miR-207 (asterisks). Scale bar = 100 μm. Abbreviations: cp, cortical plate; mes, mesenchymal tissue; svz, subventricular zone; vz, ventricular zone.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1749-8104-4-40-S1.tiff]

### Additional file 2

***Levels of miR-124 expression in the E14.5 VZ after*** **in vivo** ***E12.5 pPri-miR-124 electroporation***. *Arrowheads in boxed inset magnifications denote mid-to-high miR-124 expression levels, which are specifically restricted to heavily electroporated elements. Scale bar = 100 μm.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1749-8104-4-40-S2.tiff]

### Additional file 3

***Time-course DsRed2 fluorescence in primary cortical precursor cultures infected with Pri-miR expressing lentiviruses***. *Divergent temporal progression of DsRed2 fluorescence in E12.5 neuroblasts infected by LV_Pri-miR-124(2) or LV_Pri-miR-155/neg_control and allowed to differentiate in FCS. PI, post-infection.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1749-8104-4-40-S3.tiff]

### Additional file 4

***Displacement of apical, Pax6+, and basal, Tbr2+, precursors within the cortical wall of E14.5 brains electroporated 2 days earlier with pEGFP-C1***. *Arrowheads point to abventricularly displaced Pax6+ and Tbr2+ elements, both positive and negative for electroporated EGFP. Such displaced cells were not detectable in the controlateral, non-electroporated side of same embryos (N = 3). Scale bar = 100 μm.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1749-8104-4-40-S4.tiff]

### Additional file 5

***VZ neuronal differentiation in E14.5 cerebral cortex electroporated 2 days earlier with pPri-miR-124(2)***. *Arrowheads show outgrowing neurites within the VZ. Scale bar = 10 μm.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1749-8104-4-40-S5.tiff]

### Additional file 6

***Distribution of activated-Caspase3+ apoptotic cells within the cortical wall of E14.5 brains electroporated 2 days earlier with pPri-miR-124(2) or pPri-miR-155/neg_control***. *N = 4+4. Scale bar = 100 μm.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1749-8104-4-40-S6.tiff]

### Additional file 7

***Distribution of β1-integrin within the cortical wall of E14.5 brains electroporated 2 days earlier with pPri-miR-124(2) or pPri-miR-155/neg_control***. *The arrowhead in (B) points to the pPri-miR-124(2)-electroporated region, which does not display any overt reduction of β1-integrin immunoreactivity. Arrows in (A-C) denote the cortical plate, where β1-integrin is down-regulated and restricted to radial glial fibers. Scale bar = 100 μm.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1749-8104-4-40-S7.tiff]

## Acknowledgements

We thank Stefano Gustincich for critical reading of the manuscript. We also thank Marco Brancaccio for help in lentivirus production and Micaela Grandolfo for technical assistance in confocal microscopy. This work was entirely supported by intramural SISSA funding.

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