SARA regulates neuronal migration during neocortical development through L1 trafficking

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Supplementary Materials and Methods

Antibodies

The following antibodies were used in this study: α-tubulin mouse antibody (1:10,000, Sigma #T9026), β1 integrin rat antibody (1:100, BD Pharmingen #552828), γ-tubulin mouse antibody (1:500, Sigma #T6557), BrdU rat antibody (1:500, Abcam #ab6326), cleaved PARP rabbit antibody (1:600, Cell Signaling #9541), Ctip2 rat antibody (1:600, Abcam #ab18465), Cux1 rabbit antibody (1:300, Novus #NBP2-13883), DCX goat antibody (1:400, Santa Cruz #sc-8066), GFP chicken antibody (1:1,000, Abcam #ab13970), Ki67 rabbit antibody (1:1000, Abcam #ab833), L1CAM mouse antibody (1:400, Abcam #ab24345), nestin mouse antibody (1:400, DB #556309), Pax6 rabbit antibody (1:600, Covance #PRB-278P), PH3 rat (1:600, Abcam #ab10543), Rab5 rabbit antibody (1:300, Santa Cruz #sc-309), RFP rabbit antibody (1:1000, Rockland #600-401-379), SARA rabbit antibody (1:400, Hu et al., 2002), SARA rabbit antibody (1:400, Santa Cruz #sc-9135), Tbr2 rabbit antibody (1:400, Abcam #ab23345), Tuj1 mouse antibody (1:1000, Sigma #T8660). Various Alexa-dye conjugated secondary antibodies (1:1000, Invitrogen) and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) were also used.

DNA constructs and cell line transfection

All expression constructs were directed by CAG promoter and all sh constructs were directed by U6 promoter. The knockdown (KD) effect of SARAsh and SARAsh/SARA* have been previously validated (Chuang et al., 2007). SARAsh-IRES-GFP plasmid was generated by replacing HcRed of SARAsh-IRES-HcRed plasmid with GFP. The pCAG-L1-YFP was generated by inserting L1-YFP (a kind gift from Hiroyuki Kamiguchi [Nishimura et al., 2003]) into a pCAG vector using standard cloning method. The targeting construct of L1sh

was 5’ GGGTCTCTGATCTTGAGTAACGCCATGG CGTTACTCAAGATCAGAGACCC 3’; the DNA fragment containing pU6-L1sh was inserted into pCAG-mCherry vector to generate L1sh-IRES-mCherry.

HEK cells were transfected using the polyethylenimine method (Li et al., 2011). Protein expression levels were estimated by immunoblotting and quantified by Odyssey Infrared scanner (LI-COR).

Imaging and quantitative analysis

All immunolabelled sections were acquired with Leica TCS SP2, Olympus FV1000 or Zeiss LSM 780 confocal microscopes, as previously described (Li et al., 2011).

To quantify the distribution of endogenous SARA or Rab5 endosomes during mitosis at the ventricle border, only dividing cell pairs were taken into account whose nuclei presented characteristic condensed segregating chromatin observed in anapahase/telophase. GFP signal was used to delimit the cells borders and normalized SARA or Rab5 fluorescence intensity was obtained for each cell with the Measure tool in ImageJ. The cleavage plane angle between the dividing pair was also scored. Finally, a ratio was calculated between the highest intensity of the two cells by the lowest.

Morphology assessment was made by considering the maximal projections images from individualized neurons to reconstruct their whole extent. In all cases, similar areas in the transfected neocortices were selected for quantification analysis.

Neurons form the upper IZ and CP were considered for orientation angle analysis. Quantification of the migration scope was carried out by dividing the cortex into its main areas: VZ, SVZ, IZ, CP and MZ; or into ten-equally sized bins; and counting the number of cells in each of these subdivisions. Measurements were normalized to cell number per slice by percentage.

Quantifications of neuronal processes mean fluorescence intensity, lengths and orientation angles were scored with the morphometric tools in ImageJ. Average background fluorescent intensity from three different empty areas in the image was subtracted to fluorescence intensity measurements over neuronal processes or cell bodies. Only individually traceable neuronal processes were considered for length quantitation. Linearity index corresponds to the average ratio of total LP's length divided by the linear length from the base to the tip of the same LP. For analyzing the interaction between neurons and progenitors, the overlapping lengths of the longest neurites either with other DCX+ cells or nestin+ cells were measured.

All images were processed using Adobe Photoshop (CS3) for presentation. Statistics was performed with Statistica software (StatSoft, v8.0). For all quantification assays, over 300 cells were counted from at least 3 brains for each condition. Image capture and analysis were done at separate times in a double-blind fashion. A t-test was designed for the comparison of two groups. One-way analysis of variance (ANOVA) was applied when more than two groups were being analyzed. A post hoc Tukey test was used for comparison between different groups. Statistical significance was defined as $P< 0.05$*, 0.01** or 0.001***.

For Movie 1, a full slice tissue 3D reconstruction and volume rendering were carried out using 94 confocal 0.4 µm-thick optical sections, with the 3D Viewer plugin in ImageJ. To highlight the contact sites in Fig. 5F, surfaces of 31 confocal sectioning planes of several individual traceable neurons and their processes were rendered; the signals from the neighboring somata and cell processes were excluded for simplicity.

Supplementary References

Nishimura, K., Yoshihara, F., Tojima, T., Ooashi, N., Yoon, W., Mikoshiba, K., Bennett, V. and Kamiguchi, H. (2003). L1-dependent neuritogenesis involves Ankyrin B that mediates L1-CAM coupling with retrograde actin flow. J. Cell Biol. **163**, 1077-1088.

Supplementary Figures and Movies.

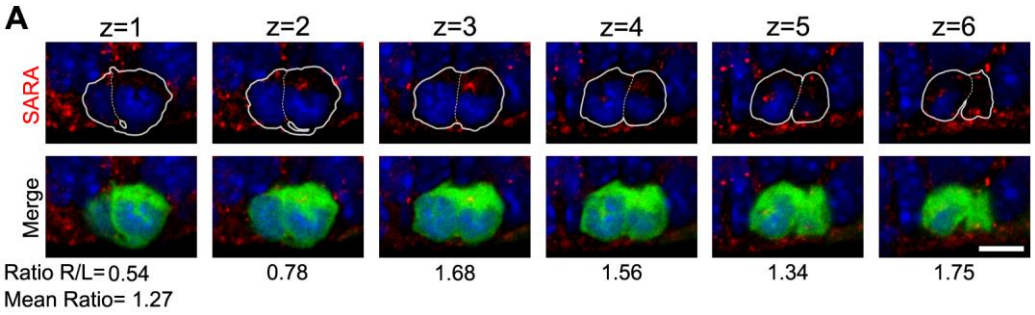


Figure S1. Related to Figure 1.

(A) Six different focal planes of a confocal z-stack showing a dividing pair of cells at the ventricle border. GFP signal delimits the cells borders. DAPI in blue shows condensed chromatin. Endogenous SARA is shown in red. Normalized SARA fluorescence intensity for each cell and plane was measured. A ratio between the fluorescence intensity of the cell to the right divided the fluorescence intensity of the left one (Ratio R/L) is provided for each z-plane. A mean ratio considering all the z-planes is also shown. Scale bar= 5 µm.

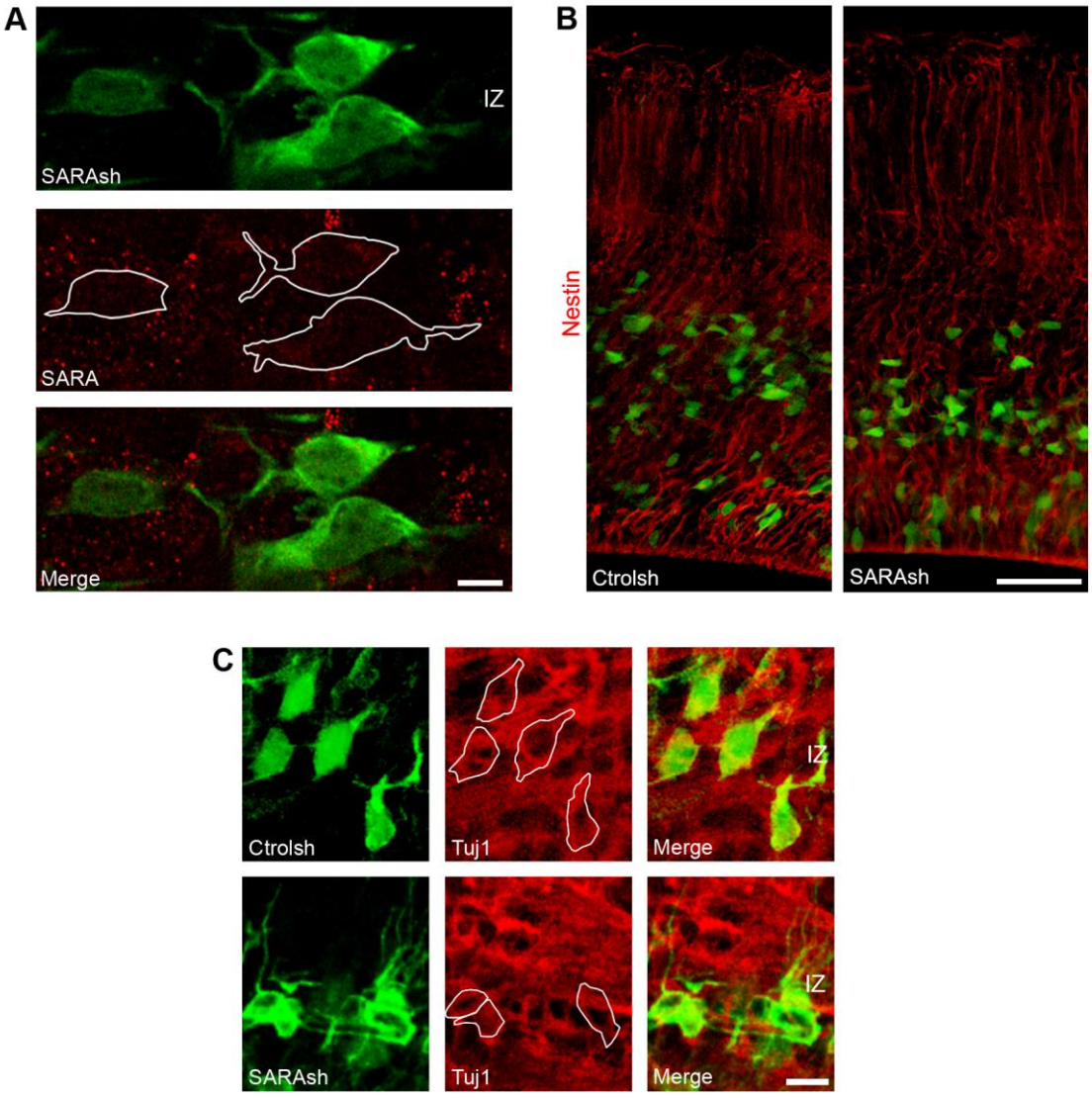


Figure S2. Related to Figure 2.

(A) Section of a mouse brain electroporated with SARAsh at E13.5, harvested 40h later and counterstained for endogenous SARA (red). Transfected cells have a decreased SARA expression compared to surrounding untransfected cells. (B) Sections of mouse brains transfected with the indicated plasmids, harvested 40h later and counterstained for the RG marker nestin. Under both conditions progenitors' processes extend radially and exhibit their apical and basal end-feet attached to the ventricle and pial borders, respectively. (C) High power images at the IZ region as in Fig. 2H. SARAsh transfected cells also expressed Tuj1, like Ctrolsh transfected cells. Scale bars A= 5 µm, B= 50 µm, C= 10 µm.

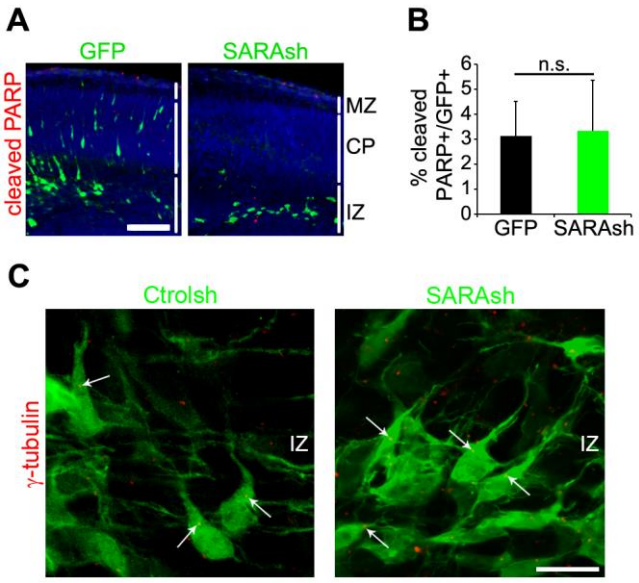


Figure S3. Related to Figure 3.

(A) Confocal images of cleaved PARP immunolabeling (red) of mouse cortical slices electroporated at E13.5 with either GFP or SARAsh for 3 days. (B) Quantification of transfected cells with the indicated plasmid positive for cleaved PARP. Data are mean ± s.e.m.; n= 4 brains for each condition. P=0.95, t-test. (C) High power images of brains sections transfected with the indicated plasmids at E13.5 and processed for counterstaining against centrosome marker γ-tubulin (arrows) 3 days later. Scale bar A= 100µm, B= 10 µm.

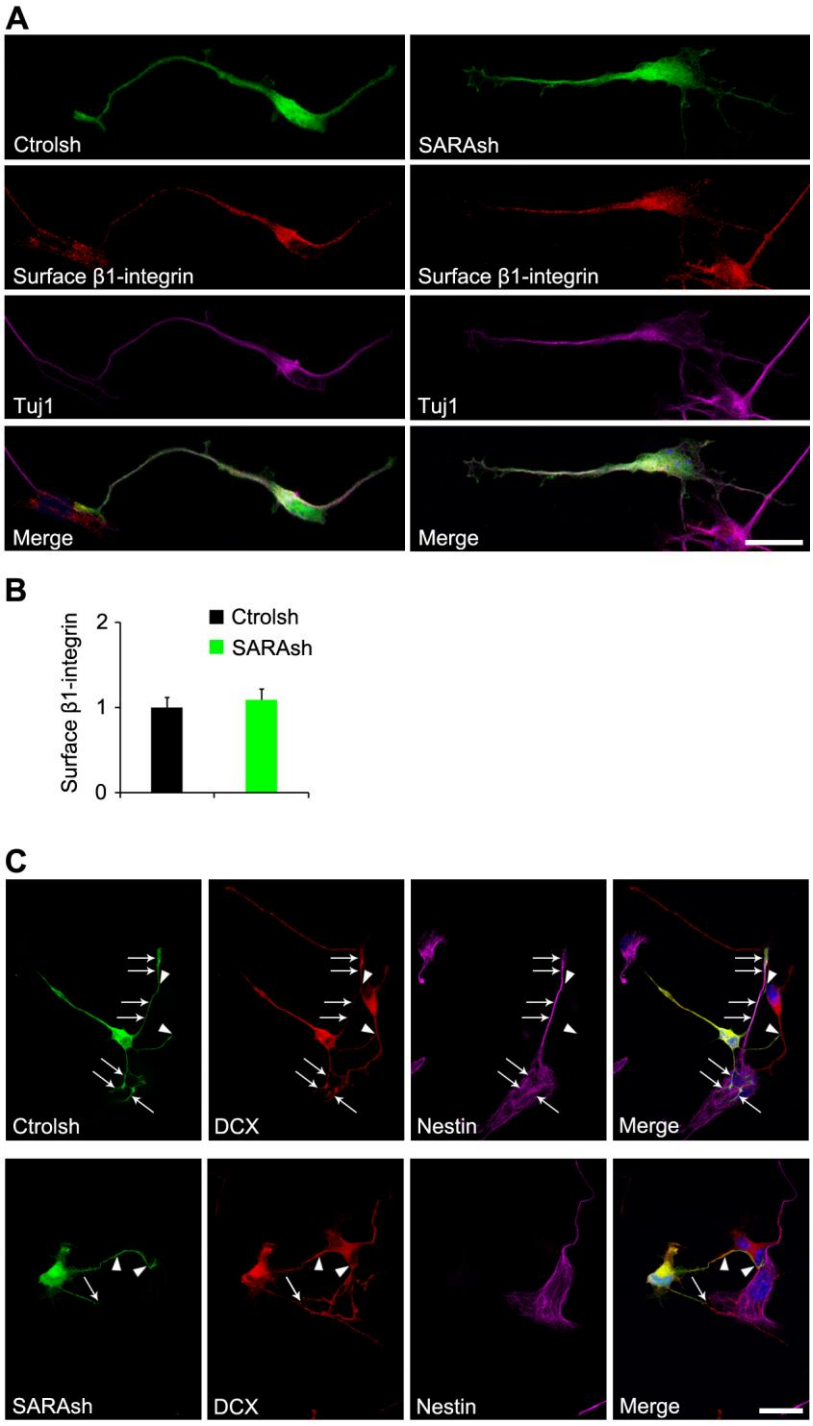


Figure S4. Related to Figure 4.
(A) Cortical neurons were isolated from brains transfected with Ctrolsh or SARAsh and cultured for 2 DIV. Cells were immunolabelled for surface β1-integrin (red) under non-permeabilized conditions. Tuj1 (magenta) staining required permeabilizing the membranes and was conducted only after incubation of primary and secondary antibodies directed to detect β1-integrin. (B) Mean fluorescence intensity of surface β1-

integrin on the longest neurite. Data represent mean intensity ± s.e.m., P= 0.61, t-test. At least 15 neurons were scored from three cultures for each condition. (C) Split channels of the images shown in Fig. 4E for better visualization of cell-cell contacts. Note that unlike SARA-KD neurons, Ctrolsh expressing and untransfected neurons (DCX+) preferentially extend and branch their processes over nestin+ progenitor cells. Arrows point to processes of transfected neurons growing over nestin+ progenitor cells. Arrowheads point to neuronal processes that contact other DCX+ neurons.
Scale bars A= 20 µm, C= 10 µm.

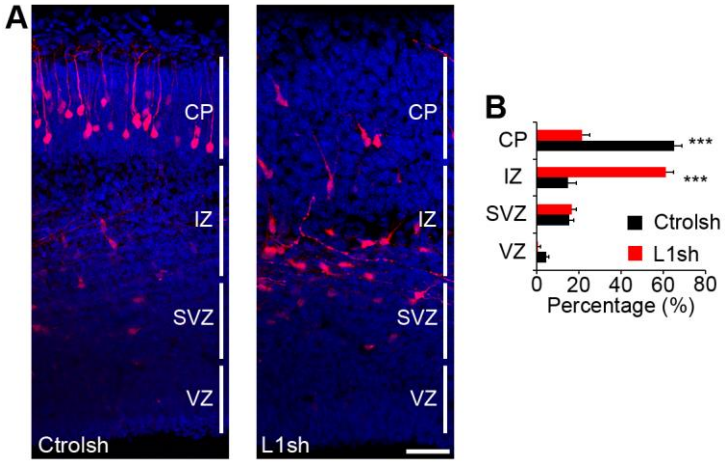


Figure S5. Related to Figure 5.
(A) Cortical slices transfected with Ctrolsh and L1sh at E13.5 and harvested 3 days later. (B) Quantification shows the percentage of transfected cells in different cortical regions with the indicated plasmids. Data are means ± s.e.m.; *** P<0.0001, one-way ANOVA. Three brains for each condition. Scale bar 50 µm.

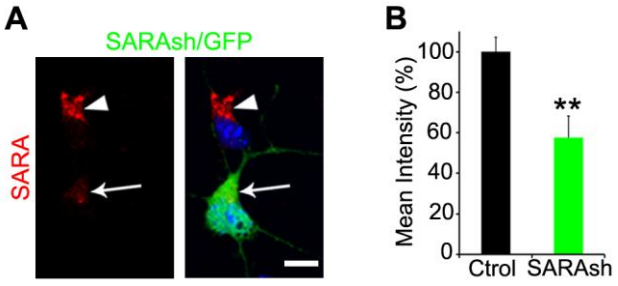
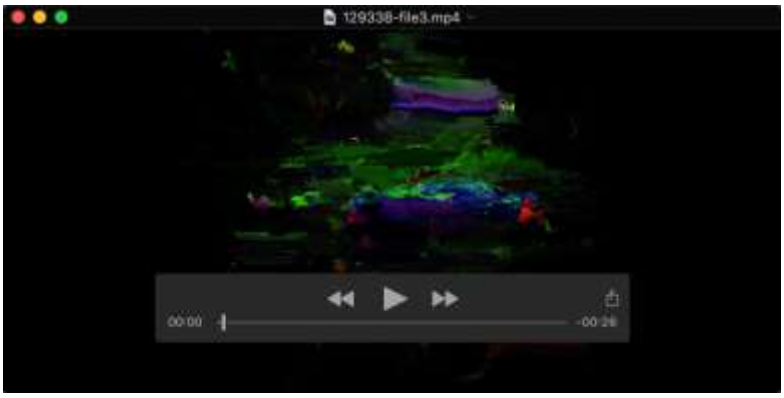
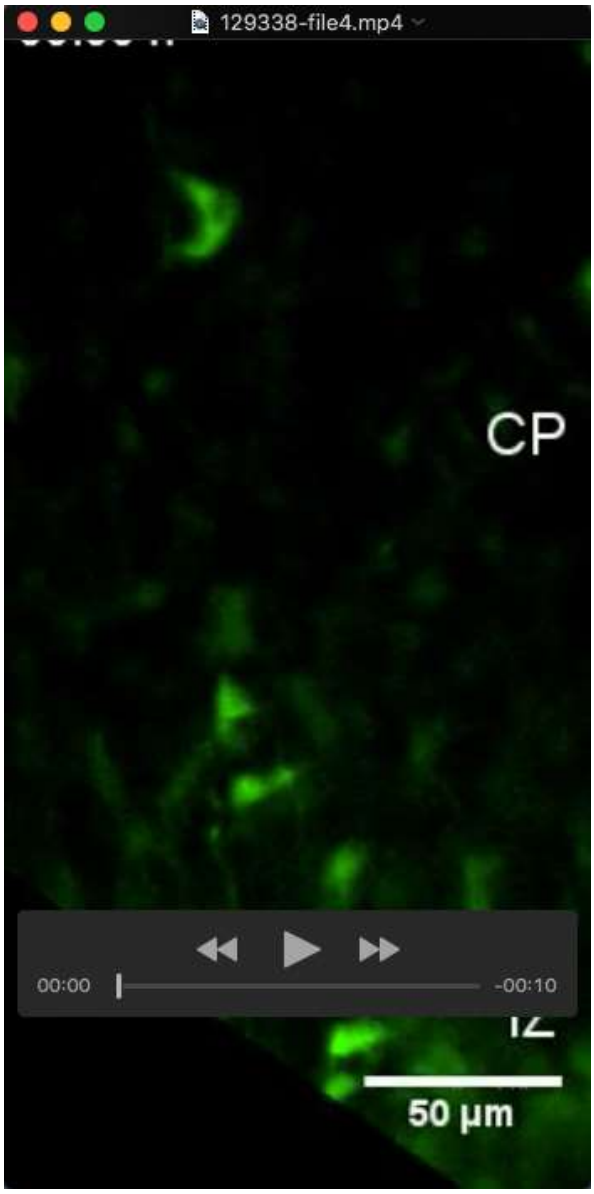


Figure S6. Related to Figure 7.

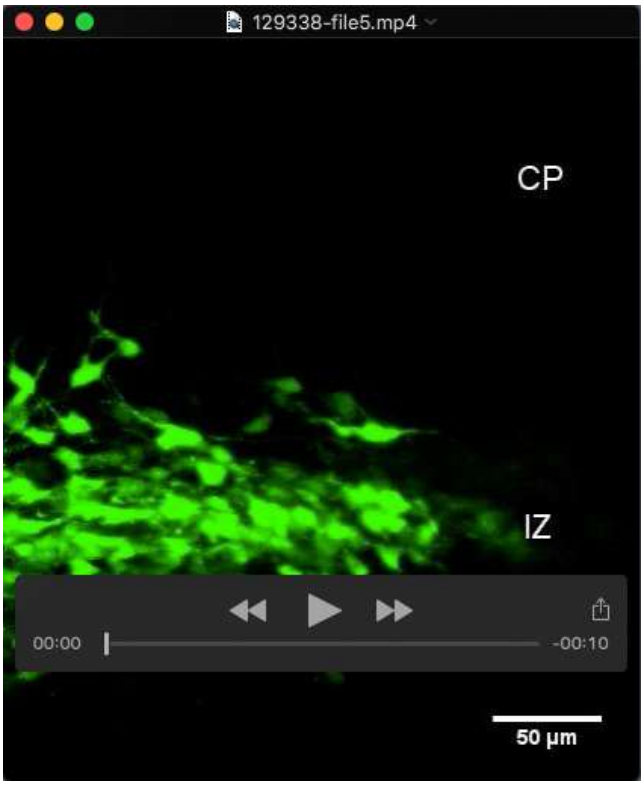
(A) Immunofluorescence of cells isolated from E13.5 brains electroporated with SARAsh and cultured in vitro for 5 days. Blue=DAPI. An arrow and arrowhead point to a transfected and an untransfected cell, respectively. Scale bar= 5 µm. (B) Quantification of immunolabeled endogenous SARA expression. Data represent mean intensity ± s.e.m.; ** P=0.0025, t-test. n= 3 independent cultures.



Movie 1. Full slice tissue 3D reconstruction. Related to Figure 4. Slice of a brain cotransfected with HcRed and L1-YFP. Note that several L1+ neuronal processes tangentially distributed along the IZ, coming from neurons outside the image, tangle with transfected neurons at this cortex area.



Movie 2. Related to Figure 6. Organotypic slice culture of a brain electroporated with Ctrolsh at E13.5 and processed for time-lapse imaging at E15.5 for 14 hours. Note that transfected cells exit the IZ with a vertical orientation and migrate radially towards the CP.



Movie 3. Related to Figure 6. Organotypic slice culture of a brain electroporated with SARAsh at E13.5 and processed for time-lapse imaging at E15.5 for 14 hours. Note that transfected cells display a tilted orientation around the IZ and are unable to migrate radially towards the CP.