Smad anchor for receptor activation nuclear localization during development identifies Layers V and VI of the neocortex

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Abstract
Smad anchor for receptor activation (SARA, zfyve9) has been classically observed in early endosomes of different cell types where it regulates vesicular transport of proteins and membrane components. Very few other members of the zinc finger FYVE-domain-containing family (zfyve) have different functions other than controlling membrane trafficking. By analyzing SARA localization throughout mouse embryonic brain development, we detected that besides the endosomal localization it also targets neuronal nuclei, specifically of the cortical layers V/VI. These findings were confirmed in human brain organoids. When evaluating neuronal cell lines, we found that SARA accumulates in nuclei of PC-12 cells, but not Neuro-2a, highlighting its specificity. SARA functions as a specific marker of the deep cortical layers until the first postnatal week. This temporal regulation corresponds with the final phases of neuron differentiation, such as soma ventral translocation and axonal targeting. In sum, here we report that SARA localization during brain development is temporarily regulated, and layer specific. This defined pattern helps in the identification of early born cortical neurons. We further show that other zfyve family members (FYCO1, WDFY3, Hrs) also distribute to nuclei of different cells in the brain cortex, which raises the possibility that this might be an extended feature within the protein family.

KEYWORDS
development, endosome, neocortex, nucleus, SARA, zfyve

1 | INTRODUCTION

The brain cortex is composed of six different layers. Each of them consists of diverse neuronal subtypes with particular morphological, anatomical projection, and electrophysiological properties. Various genes with layer and neuronal subpopulation specificity have been identified; and their role in brain development and cortical layering is continuously studied. In this sense, the unique identity of each cell layer is accomplished during development by means of the expression of a differential subset of genes (Molyneaux, Arlotta, Menezes, & Macklis, 2007). Understanding the gene expression programs during brain formation will further help to elucidate neuron subtypes specification.

Originally, Smad anchor for receptor activation (SARA) has been discovered in the TGF-β pathway (Tsukazaki, Chiang, Davison, Attisano, & Wrana, 1998), and it is closely related to the endosomal machinery (Hu, Chuang, Xu, McGraw, & Sung, 2002). Here, we report SARA (zfyve9) as a new specific molecular marker of layers V and VI during embryonic and early postnatal development.

SARA belongs to the zinc finger FYVE (Fab1 [1-phosphatidylinositol 3-phosphate 5-kinase], YOTB [hypothetical Caenorhabditis elegans protein ZK632.12], Vac1b [Vesicle transport protein 1b], and EEA1 [Early Endosome Antigen 1]) domain-containing protein family. In the human, the zfyve family currently has 31 members. Differently to other zinc fingers that bind nucleic acids or proteins (Cassandri et al., 2017), the zinc
finger FYVE domain binds phosphatidylinositol 3-phosphate (PI3P) with high specificity (Chuang, Zhao, & Sung, 2007; Stenmark & Aasland, 1999). PI3P recruits zfyve members to endosomes and other membrane organelles such as the Golgi apparatus, lysosomes/autophagosomes, and the endoplasmic reticulum. This specific membrane affinity has led to classically associate the zfyve family to membrane transport and protein sorting functions (Kutateladze, 2006).

In cell lines and cultured hippocampal neurons, SARA presents a vesicular labeling partially colocalizing with the early endosome (EE) marker Rab5 (Arias, Siri, & Conde, 2015; Hu et al., 2002). Accordingly, we have previously reported a punctate vesicle-like distribution of endogenous SARA in the mice embryonic neocortex (Mestres, Chuang, Calegari, Conde, & Sung, 2016). We have also addressed its role in controlling membrane and membrane proteins trafficking and how this function is important for normal development of the nervous system (Mestres & Sung, 2017). Particularly, during neocortical development, SARA fine-tunes the amount of the cell adhesion protein L1 that distributes to the cell surface of migrating neurons (Mestres et al., 2016). Also, in the eye, SARA tethers vesicles with the outer segment of rod photoreceptors, thereby participating in photoreceptor renewal (Chuang et al., 2007).

Now, we inform that SARA might have an additional role regarding its novel localization into deep-layer nuclei of the brain cortex. Importantly, we also show that the nuclear localization aspect is shared with other members of the zfyve family. Although in a different spatiotemporal pattern, FYCO1 (zfyve7), Hrs (zfyve8), and WDFY3 (zfyve25), also localize to nuclei of different cell types in the cortex during development.

2 Methods

2.1 Animals

Mouse (C57BL/6; Janvier Labs, Saint Berthevin, France) brains from E13 to P2 were collected after decapitation, and immediately fixed in 4% PFA in 0.1 M phosphate buffer (pH 7.4), overnight at 4°C. For postnatal brains (P5–P30), isoflurane anesthetized animals were transcardially perfused through the ascending aorta with 10 ml of heparin saline (1,000 units/ml), followed by 20 ml of 4% PFA in 0.1 M phosphate buffer. Later, brains were dissected out of the skull and postfixed for 1 hr in the same fixative. Fixed brains were embedded in low melting point agarose, and sectioned by vibratome (40 μm). For all ages, at least three brains were evaluated. Mice of both sexes were used, which were kept in standard cages with 12 hr dark/light cycle, and with access to food and water ad libitum. All animal procedures were approved by local authorities (TVV 39/2015).

2.2 Brain organoids

Slices of human brain organoids were a generous gift from M. Heide and W. Huttner (MPI-CBG, Dresden, Germany), and were generated as described elsewhere (Lancaster et al., 2013; Mora-Bermudez et al., 2016).

2.3 Cell culture

Primary neurons were obtained from E15 mouse brains. Briefly, the brains were removed from the embryo, the cortex dissected out and the meninges removed. The tissue was incubated with trypsin at 37°C for 15 min, and later rinsed in PBS. Mechanical dissociation was performed by pipetting several times in culture medium containing Neurobasal, Penicillin/Streptomycin, B27 and Glutamine (Gibco). Neuro-2a cells were grown in DMEM supplemented with 10% fetal bovine serum, and 1% Penicillin/Streptomycin. Rat pheochromocytoma (PC-12) cells grown in RPMI supplemented with 10% horse serum, 5% fetal bovine serum, and 1% Penicillin/Streptomycin. For all cases, 40,000 cells were seeded per well, of a 24 multiwell, in poly-D-lysine and laminin (neurons and Neuro-2a cells) or collagen (PC-12 cells) coated coverslips. After 1 day in vitro, cells were fixed in 4% PFA for 15 min. FGF2 (Peprotech) was incubated at 10 ng/ml in serum-free culture medium for the indicated timepoints.

2.4 Immunohistochemistry

Immunolabeling of brain sections was carried out using free-floating methods. Briefly, brains sections (40 μm thick) were incubated in 5% donkey serum with PBS 0.1% triton (PBST) for 1 hr at room temperature. Primary and secondary antibodies were incubated in 1% donkey serum in PBST, followed by three washes in PBST for 15 min each. Primary antibodies were incubated for three overnights, while secondary antibodies were incubated one overnight, rotating at 4°C. Cryosections of human brain organoids (14 μm thick) were obtained mounted onto glass slides and processed for immunolabeling similarly as for the mouse brain slices, except primary antibodies were incubated one overnight.

A detailed list of the primary antibodies used in this study can be found in Table 1. Various Alexa-dye conjugated secondary antibodies (1:500; Molecular Probes) were also used. Phalloidin coupled to Alexa dye (633) was used to detect the actin cytoskeleton, and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was employed to stain the nuclei.

2.5 Specificity of the antibodies

Most of the antibodies used here were validated by the commercial providers (Table 1). As indicated in their supplier’s data sheet, all antibodies showed a band of the respective molecular weight of the protein detected as revealed by Western blot. The only exception is the SARA (C137) antibody which was validated by Dr Sung (Hu et al., 2002). Also, our Western blots show a single band at ~160 kDa when using the C137 antibody (or H300 antibody); which corresponds to SARA’s molecular weight.

Additionally, in some control tissue sections, we omitted either the primary or the secondary antibodies. No labeling was observed in these sections.
2.6 | In situ hybridization

Vibratome sections (40 μm thick) of E15 or P0 mouse brains were used. Primers to generate the probes were: forward 5'-CTTTGTACAGCTTTACCGGGAC-3', reverse 5'-GACTTGGCAAGGTATCCTGAAG-3'. Reverse transcription-PCR using SP6 or T7 RNA polymerases generated digoxigenin (DIG)-labeled cRNA antisense or sense probes, which cover a 663-bp region of the three SARA isoforms (between Exons 8 and 13). Hybridization signals were detected with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche; RRID: AB_2734716), followed by color development with BM purple (Roche).

2.7 | Subcellular fractioning and Western blot

Cytoplasmic and nuclear protein extractions of PC-12 cells were done using the NE-PER Extraction Reagents (Thermo Fisher, #78833), supplemented with protease inhibitor mix (Diagenode, #C12010012), following manufacturer's instructions with minor modifications. Before extracting the nuclear fraction, the pellet was washed extensively in CER I and CER II. From either fraction, 30 μg were run per lane in 4–12% Bis-Tris gels (Thermo Fisher, #NP0335BOX), transferred to PVDF membranes, and blotted with SARA antibodies (C137 or H300), actin antibody or LaminB1 antibody. Immunodetections were carried out with the ECL method (Thermo Fisher, #34577).

2.8 | Confocal imaging and analysis

Immunolabeled sections were examined with a confocal Zeiss LSM 780 microscope, and processed in ImageJ and Photoshop. Low power tile images were acquired in the confocal microscope or in a Zeiss Axio Scan with a 20x objective, and were stitched together in Zen. Nuclear SARA intensity signal was measured in ImageJ (NIH). The area within the nucleus was selected for each cell in the DAPI channel. This area was used to determine the mean intensity gray values for the SARA signal. Background signal was negligible (<1 a.u.). At least 20 cells, from three independent cultures were measured.

2.9 | Statistical analysis

Data are presented as mean ± SEM. Statistical significance was evaluated by one-way analysis of variance (ANOVA) and a Tukey post hoc
Smad anchor for receptor activation (SARA) endogenous expression during embryonic development. (a–c) Coronal brain sections at E13. (a) Low power image shows lateral to medial SARA expression. Dashed lines delimit the upper and deep borders of the CP. (b) Higher power image of the inset in (a) depicts SARA (green) colocalizing with Ctip2 (red) at the CP. The VZ is labeled by Sox2 (magenta). (c) Single plane of a high-power image showing SARA (green) distributes into the nucleoplasm, except heterochromatin, in cells localizing in the Tuj1+ (red) CP. Empty arrows point to cell nuclei in the MZ that lack SARA immunoreactivity. 4′,6-Diamidino-2-phenylindole (DAPI) is in blue. (d,e) E15 sagittal brain section shows the anteroposterior distribution of SARA. Dashed line delimits the ventricular border. (e) Higher power image of the inset in (d) depicts SARA nuclear localization is restricted to the CP. (f) In situ hybridization for SARA mRNA in E15 brain slices. SARA mRNA expression is higher in the CP, delimited by dashed lines. (g,h) Rab5 distribution in the brain cortex. Low (g) and high (h) power images of a brain slice labeled for Rab5 (red) shows early endosomes (EEs) distribute to the basal side of the neuronal soma toward the pia (empty arrows). Nuclei (DAPI, in blue) are negative for Rab5 antibody immunoreactivity (arrows). CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; MZ, marginal zone; ob, olfactory bulb; Str, striatum; SVZ, subventricular zone; VZ, ventricular zone. Scale bar a = 200 μm; b, f = 20 μm; c, e = 100 μm; d = 500 μm; g = 50 μm; and h = 10 μm [Color figure can be viewed at wileyonlinelibrary.com]
test in Prism6. For each treatment, three independent cultures were analyzed.

2.10 | Nuclear localization signal analysis

FASTA amino acid sequence for the different zfyve members were analyzed in NLS Mapper (Kosugi et al., 2009) to determine the sequence of putative nuclear localization signals. Peptide sequences with Scores 1 or 2 localize to the cytoplasm; that with Scores 3, 4, or 5 distribute both to the cytoplasm and the nucleus; that with Scores 6 or 7 partially localize to the nucleus; while Scores 8, 9, or 10 distribute mainly to the nucleus.

2.11 | Gene ontology analysis

Two publicly available curated databases (BioGrid and IntAct) confirmed 43 binding partners for SARA (Chatr-Aryamontri et al., 2017; Orchard et al., 2014). The list was processed for gene functional
annotation clustering in DAVID (Huang, Sherman, & Lempicki, 2009). A GO term, enrichment score and \( p \)-value were obtained for each cluster.

### 3 \| RESULTS

#### 3.1 \| Endogenous SARA localizes to nuclei of early born neurons

To first assess endogenous SARA distribution, we immunolabeled coronal brain slices of mouse embryo as early as embryonic Day (E) 13. We found that it distributed into a punctate pattern throughout the cortex (Figure 1a,b). Interestingly, SARA highly localized to nuclei in the developing cortical plate (CP), following the lateral-to-medial axis of neurogenesis (Takahashi, Goto, Miyama, Nowakowski, & Caviness Jr, 1999). The nuclear distribution of SARA overlapped with that of the deep-layer marker Ctip2, but the cells in the proliferative area (Sox2+) or the transitional intermediate zone (IZ) were negative for SARA nuclear immunostaining (Figure 1b). This suggests that nuclear SARA is not involved in early differentiation of neuron progenitors. Single-plane high-power images at the CP revealed that besides the well-known endosomal localization, SARA distributed to Tuji+ neuron nucleoplasm except the heterochromatin (Figure 1c). Importantly, the cells nuclei within the marginal zone (MZ) were not immunoreactive for SARA. Analysis of sagittal brain sections at E15 showed that SARA distributed to nuclei in the CP without constraints in the rostrocaudal axis (Figure 1d). As described previously, the vesicular localization of SARA is enriched at the ventricle border (Figure 1e) (Mestres et al., 2016). As for the earlier timepoint, SARA nuclear distribution was restricted to nuclei of postmitotic neurons residing in the CP, once they finished migrating throughout the IZ. To confirm our results, we evaluated the expression of SARA mRNA. In situ hybridization experiments in E15 brain sections revealed that it is predominantly expressed in the CP of the developing cortex (Figure 1f). At the same timepoint (E15), we evaluated the distribution of the EE marker Rab5. In postmitotic neurons that reached the CP, Rab5 localized into vesicular structures at the basal side of the soma. Importantly, Rab5 was not found in any nucleus of the cortical wall (Figure 1g,h). This indicates that SARA nuclear localization is independent of the Rab5 pathway, and of the endosomal machinery.

To test whether SARA might also localize to nuclei of later born neurons, we immunolabeled neonatal (P0–P2) coronal brain sections. We found that within the cortex SARA distributed to all layers of the CP within EEs (Figure 2a,b). Interestingly, only neurons of layers V/VI were positive for SARA also in their nucleus, together with the classical early born neuron marker Ctip2 (Figure 2a–c). Of note, SARA and Ctip2 immunolabeling in the deep layers were complementary. That is, while Ctip2 was expressed at higher levels in Layer V than VI, SARA expression was higher in Layer VI compared to Layer V (Figure 2c). Outside the cerebral cortex, we found that SARA also localized to neuronal nuclei in the thalamus (Figure 2d). To determine SARA nuclear localization along the rostrocaudal axis, we analyzed sagittal P0 brain sections immunolabeled with the upper layers (II–IV) marker Satb2. The examination of posterior and anterior regions confirmed the specific distribution of SARA in Layers V and VI along the brain cortex (Figure 2e–g). Consistent with our previous results, independently of the SARA protein subcellular localization either within EEs and/or in the nucleus, SARA mRNA expression was highest at the CP within the neonatal brain cortex (Figure 2h–j).

Our findings were confirmed by two different SARA antibodies (Figure 3a). Both rabbit antibodies recognize separate regions of SARA, and their results were comparable. A strong nuclear SARA localization in deep-layers neurons was detected using either one of the antibodies (Figure 3b).
Additionally, we performed cell fractioning to obtain proteins from the cytosolic or nuclear compartments. Immunoblots of either fraction confirmed the presence of SARA (Figure 3c).

Altogether, our results suggest that the SARA nuclear localization might be important for postmitotic neurons residing exclusively in layers V/VI of the CP, but neither for their specification nor migration.

3.2 | SARA distributes to neuronal nuclei of all layers upon cortical maturation

Next, we wondered about the localization of SARA at later stages. To this end, we stained coronal brain sections at different timepoints postnatally.

At P5, SARA distributed strongly to deep layer nuclei, while the nuclei of upper layers were also positive for SARA localization but to a lesser extent (Figure 4a–d). Even though at this age (P5), SARA localized to neuronal nuclei of all layers, the higher distribution in deep layers nuclei (compared to the upper layers) still allows the identification of Layers V/VI. This was no longer the case as development proceeded (see below). Single-plane confocal images of neurons in deep layers (V/VI) showed a strong SARA nuclear localization in 50–70 speckles (Figure 4c,d). In upper layers (II/IV), SARA distributed to neuronal nuclei also in a dotted pattern but to a milder degree (10–15 speckles) (Figure 4b). Orthogonal views in the YZ- and XZ-axis of the same cells confirmed SARA subcellular distribution. In addition, SARA was visible in perinuclear vesicular structures consistent with its endosomal localization (Figure 4b–d).

Later, at P15, SARA distributed indistinguishably across all layers of the CP (Figure 5a–d). Single-plane confocal images revealed that at the subcellular level, it presented a dotted localization in both the neuronal nucleus (Ctip2 and/or NeuN+) and soma (Figure 5b–d). A comparable staining pattern was found when analyzing P30 brain sections (not shown).

3.3 | Human deep-layer neurons are also positive for SARA

Next, we evaluated whether the human SARA homologue might also target a specific cell population similarly as in the mouse cortex. To this end, we analyzed cryosections of human brain organoids. At developmental Day 54, SARA localized into vesicular structures which were enriched at the ventricular border; comparable with our observations in E15 mouse brain sections. Also in the human cells, SARA colabeled the early generated neurons along with Ctip2; while the proliferative Sox2+ area was negative for SARA nuclear immunolabeling (Figure 6a,b).

These results point that SARA functions as a novel cortical deep-layer marker in both, mouse and human tissue.
SARA identifies early born cortical neurons grown in culture

Subsequently, we tested SARA distribution in neuronal cells grown in culture. We examined primary mouse cortical neurons, rat pheochromocytoma (PC-12) cells, and Neuro-2a cells. Primary neurons were obtained from E15 mouse brains. At this timepoint, most of the Tuj1 positive primary neurons were also immunoreactive for Ctip2 (not shown). In these cells, SARA localized to vesicle-like structures; namely, EEs. Consistent with our brain slices immunolabeling, the early born neurons also exhibited an SARA nuclear distribution (Figure 7a). Notably, the nucleolus and heterochromatin were predominantly devoid of SARA staining.

Similar to the cultured neurons, in undifferentiated PC-12 cells SARA showed a punctate distribution throughout the cytoplasm; and it was also highly enriched in the nucleus of the cells (Figure 7b). Contrarily, in Neuro-2a cells SARA distributed mainly into endosomes, while it was not evidently accumulated in the cell nucleus (Figure 7c). These results point that comparably to the different cells of the cortex, the nuclear distribution of SARA might be differentially regulated between cell types.

Functional aspects of nuclear SARA distribution

We next evaluated whether SARA nuclear localization might be dependent on PI3P levels. To assess this, we incubated Neuro-2a cells with FGF2 to induce PI3-Kinase activation; the enzyme that synthesizes PI3P. Already 10 min after induction, SARA exhibited nuclear distribution in these cells; besides to its endosomal localization (Figure 8a,d). After 120 min incubation with FGF2, Neuro-2a cells showed larger SARA+ EEs and an increased SARA nucleoplasm localization (Figure 8b–d). Altogether, this suggests that cell type specificity of SARA nuclear localization might rely on PI3P concentration.

Finally, we wondered about the role of SARA in the nuclear localization described here. To this end, we compile the 43 known SARA binding partners. Gene ontology analysis for functional clustering provided with different annotation terms associated with this set of proteins. The top five highest enriched terms are shown in Table 2. GO terms such as circadian rhythm and phosphatase complex is representative of the known functions of SARA. SARA has been shown to interact with Rhodopsin in the light-sensing organelle of the eye (Chuang et al., 2007), and to be part of a complex together with Smad2/3 and the phosphatase PP1c (Bennett & Alphay, 2002). Also, recent evidence suggests a major role of SARA in embryonic development; particularly of the nervous system (Kressmann, Campos, Castanon, Fürthauer, & González-Gaitán, 2015; Mestres et al., 2016; this article).

Interestingly, the most enriched GO term identified was nucleus; in agreement with the evidence presented here. In this sense, transcription regulation was another term associated with SARA binding...
partners. In sum, this points that nuclear SARA might have a hitherto unforeseeable function in gene transcriptional control; possibly in cooperation with its binding partners.

3.6 | Other zfyve members also localize to cell nuclei in the cortex

To determine whether neocortical nuclear distribution of SARA was exclusive within the zfyve family, we tested other members: FYCO1 (zfyve7), Hrs (zfyve8), RUFY1 (zfyve12), and WDFY3 (zfyve25).

At E15, FYCO1 exhibited a nuclear localization in all areas of the cortex. In addition to neurons that finished migrating and reached the CP, FYCO1 distributed to nuclei of neurons undergoing locomotion through the IZ, and it also colocalized with the neural stem cell marker Pax6 in the ventricular zone (Figure 9a). Differently to SARA which at this stage identified specifically neurons of the developing CP, FYCO1 labeled several cells types, undergoing distinct developmental programs. Later, at P0, FYCO1 signal was restricted to the neonatal VZ, and the neurons of all cortical layers (Figure 9b). Even more, it also localized to the hippocampus, together with Ctip2. The white matter, which derives developmentally from the IZ, mainly lacked FYCO1 signal.

Both, WDFY3 and Hrs localized only to a subset of clustered cells lining the ventricle border in E15 brain slices (Figure 9c,h). These group of cells were positive for the nuclear stem cell marker Sox2, but negative for the intermediate progenitor marker, Tbr2 (Figure 9d,i). At this timepoint, postmitotic neurons either migrating through the IZ or residing in the CP did not display significant amounts of WDFY3 or Hrs. However, later in development at E18, when both deep- and upper cortical layers have been established, WDFY3 and Hrs exhibited a nuclear localization in postmitotic neurons throughout all layers (Figure 9e–g,j–l). In these brain slices, the clustered pattern of cells at the VZ was not longer observed. In sum, during cortical development these two zfyve members (WDFY3 and Hrs), exhibited a shift in their cell-type affinity, which might reflect different functions at separate times.

We also performed immunofluorescence with a RUFY1 antibody (sc-398740). In our hands, it did not show a clear and consistent nuclear distribution within the cortex (not shown).
Importantly, analysis of nuclear localizing signals among the zfyve members studied here identified the amino acid sequences with high score of putative nuclear localization (Table 3).

**DISCUSSION**

Although highly controversial, SARA was initially identified in the TGF-β signaling pathway (Bakkebø et al., 2012; Runyan, Liu, & Schnaper, 2012). Upon ligand binding, the downstream effector Smad2 is recruited to the TGF-β receptor. After internalization into EEs, Smad2 dissociates from the TGF-β receptor-SARA complex which allows Smad2 nuclear localization and gene transcription regulation (Runyan, Schnaper, & Poncelet, 2005). Despite extensive research in different model organisms and cell types, SARA itself was never reported to distribute to the nucleus.

It has been shown before that a pool of nuclear phosphoinositides, including PI3P, exist independently of the cytosolic pool (Gonzales & Anderson, 2006). Also, electron microscopy revealed that PI3P distributes within the nucleus (Gillooly et al., 2000). Importantly, the probe used for these experiments derived from the FYVE domains of two zfyve members: Hrs and EEA1 (zfyve2). However, the current view supports the idea that the nucleus does not attract zfyve proteins perhaps due to its neutral or mildly alkaline pH compared to the lower pH found in endosomes, phagosomes, and Golgi apparatus (Kutateladze, 2006; Lee et al., 2005). Nevertheless, the findings presented here add evidence that challenges that notion.

A seminal work identified the first zfyve member to localize in the cell nucleus. RUFY2 (zfyve13), although not in the brain, exhibits a nuclear localization in the mouse primordial cartilage and otic capsule at very specific ages, from E12 to E14 (Dunkelberg & Gutierrez-Hartmann, 2001). Also, unlike most other zfyve members that localize to endosomes, WDFY3 mainly localizes to the nuclear envelope in HeLa cells at steady state, but shifts to the autophagosome upon starvation (Simonsen et al., 2004).

Our work reveals that SARA also has the ability to localize to the nucleus in a very specific time- and cell-type dependent manner. During embryonic corticogenesis until P5, SARA functions as a reliable and consistent novel deep-layer marker. Two independently generated antibodies exhibited this feature; both in vivo, and in vitro. Moreover, SARA immunolabeling provided neuronal specificity in rodent as well as human samples. Arguing for cell type specificity, among the different neuronal cell lines tested some exhibited SARA nuclear accumulation (PC-12), while others did not (Neuro-2a). Nevertheless, Neuro-2a cells also exhibit nucleoplasm SARA distribution provided PI3K stimulation with FGF2.

| Term                  | Enrichment score | p-Value  |
|-----------------------|------------------|----------|
| Nucleus               | 4.24             | 3.2E-5   |
| Circadian rhythm      | 4.09             | 5.4E-6   |
| Phosphatase complex   | 2.93             | 1.0E-4   |
| Embryonic development | 2.79             | 2.0E-4   |
| Transcription regulation | 2.69         | 2.5E-4   |

| Term                  | Enrichment score | p-Value  |
|-----------------------|------------------|----------|

Abbreviation: SARA, Smad anchor for receptor activation.
The lack of SARA in the neuronal stem cells nuclei indicates that deep-layer neurons are not "primed" by SARA in neuron progenitors to specify these neuronal subtypes. Accordingly, SARA does not localize to the cell nucleus until neurons finished migrating and reached the CP.

Although the function of SARA in the nucleus remains to be investigated, it is possible to speculate that given the spatiotemporal pattern it might control neuronal final positioning within deep layers (e.g., somal translocation), or their postmitotic development; namely, dendritic arborization and axonal pathfinding. Once the pyramidal neurons reside in the CP, their soma translocate ventrally as later-born neurons surpass their predecessors; following an inside-out lamination gradient (Barnes & Polleux, 2009). The somatic ventral translocation occurs until the first postnatal week. Also, after migration the neuronal apical...
TABLE 3  Nuclear localization signal scores. The zfyve members studied here, their putative sequences responsible for nuclear localization and corresponding score, as identified by NLS mapper

| UniProtKD | Gene     | Position | Sequence                        | NLS score |
|----------|----------|----------|---------------------------------|-----------|
| A2A8R0   | SARA (zfyve9) | 683      | RFTFTKRRHHCRCAGKVFASCSCSLKCLLYMD | 6.1       |
| Q8VDC1   | FYCO1 (zfyve7) | 1392     | RGQLKVPQILYYLIDNFTSFRISSKLYH | 5.7       |
| Q99L18   | Hrs (zfyve8) | 178      | RKIHCRACQAIFCGKCSSKSTIPKFGIE   | 4.0       |
| G3UYW1   | WDFY3 (zfyve25) | 2483     | VKPPLKRSRSA                    | 9.0       |

Abbreviation: SARA, Smad anchor for receptor activation.

dendrite begins to branch, and their axonal track elongates to its targets; which in deep-layer projection neurons last up to P7 (Lewis Jr, Courchet, & Polleux, 2013). Interestingly, SARA is able to identify Layers V/VI of the cortex at least until P5; that is, when these morphological changes finish. In favor of this view, loss of WDFY3 leads to axonal midline crossing defects (Dragich et al., 2016).

While the classical deep-layer marker Ctip2 is also found in neuronal nuclei of the striatum, the hippocampus, and the olfactory bulb (Arliotta et al., 2005), SARA was not evident in the nuclei of these extracortical cells. Nevertheless, neuronal nuclei in the thalamus were also immunoreactive for SARA. Interestingly, neurons from Layer VI and some of the Layer V conforms the corticofugal neurons that project to the thalamus (Tau & Peterson, 2010). This observation raises the possibility that SARA might be relevant for the development of corticothalamic circuitry.

In line with the evidence presented here, analysis of the GO terms associated with SARA binding partners identified “nucleus” and “transcription regulation” as enriched annotated terms. These findings suggest a novel role for SARA in the nucleus, additionally to the long-known function in membrane transport. A possible contribution of SARA to the transcriptional regulation, and the identity of putative genes regulated by SARA remains to be investigated.

As a proof-of-concept, we analyzed four other zfyve members and evaluated whether they also localized to the cell nucleus. Three of which; FYCO1, WDFY3, and Hrs, show a nuclear distribution within different cells of the developing cortex. Except for WDFY3, whose nuclear localization was known in cultured HeLa cells (Simonsen et al., 2004); this work shows for the first time that FYCO1 and Hrs can distribute to subcellular structures other than their previously known localization into late endosomes and lysosomes (Pons et al., 2008; Raiborg et al., 2015). Altogether, these results strongly point that the nuclear localization might be a feature extended among the zfyve family.

Notably, some zfyve members have been related to neurodevelopmental diseases such as autism; including WDFY3, FYCO1, and FGD1 (zfyve3) (Orosco et al., 2014; Orrico et al., 2004; Voineagu et al., 2011). For example, a mutation found to generate autism-like features; including forebrain overgrowth and enlarged ventricles, introduces a stop site right before the WD40 and FYVE domains at the end of the WDFY3 gene. Despite the important function of WDFY3 in selective macroautophagy, mutant embryos did not show a disruption in the autophagy pathway (Orosco et al., 2014). Whether the developmental defects observed after loss of WDFY3 in mutant mice are due to its nuclear localization in the cortical wall as shown here, constitutes a hypothesis to be further tested. Also, it will be interesting to evaluate if other zfyve members are involved in brain developmental disorders as a result of their role in the cell nucleus.

Structurally, it was demonstrated that the isolated FYVE domain of several zfyve members, mainly localize to nucleus and the cytoplasm, but upon dimerization stimulation the FYVE domains shift to the endosomes (Hayasaka et al., 2004; Hayasaka, Hayes, Leonard, Lambright, & Corvera, 2007). This implies that oligomerization favors endosomal localization. It remains to be elucidated whether the opposite holds true; that is, do FYVE domains shift to the nucleus upon oligomerization inhibition?

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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