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Short communication

SFPQ associates to LSD1 and regulates the migration of newborn pyramidal neurons in the developing cerebral cortex

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ABSTRACT

The development of the cerebral cortex requires the coordination of multiple processes ranging from the proliferation of progenitors to the migration and establishment of connectivity of the newborn neurons. Epigenetic regulation carried out by the COREST/LSD1 complex has been identified as a mechanism that regulates the development of pyramidal neurons of the cerebral cortex. We now identify the association of the multifunctional RNA-binding protein SFPQ to LSD1 during the development of the cerebral cortex. *In vivo* reduction of SFPQ dosage by *in utero* electroporation of a shRNA results in impaired radial migration of newborn pyramidal neurons, in a similar way to that observed when COREST or LSD1 expressions are decreased. Diminished SFPQ expression also associates to decreased proliferation of progenitor cells, while it does not affect the acquisition of neuronal fate. These results are compatible with the idea that SFPQ, plays an important role regulating proliferation and migration during the development of the cerebral cortex.

1. Introduction

The generation of pyramidal glutamatergic neurons during the development of the cerebral cortex proceeds through a sequence of steps that involves the exit from cell cycle of neural progenitors, the suppression of progenitor cell identity, the initiation of neuronal differentiation and radial migration towards the cortical plate of newborn neurons (Takahashi et al., 1999; Noctor et al., 2004; Kriegstein et al., 2006). The adequate execution of the process requires a fine regulation of gene expression that results from the coordinated action of both transcriptional and post-transcriptional mechanisms (Molyneaux et al., 2007; Darnell, 2013). Epigenetic regulation plays an important role in the control of spatial and temporal patterns of gene expression during neural development (Riccio, 2010). Epigenetic modifications of chromatin are carried out by multicomponent coactivator or corepressor complexes which are selectively recruited towards specific regulatory elements in the DNA by transcription factors, resulting in covalent modifications of the chromatin surrounding the transcription factor binding site.

Changes of the acetylation and methylation status of histones are important mechanisms of epigenetic regulation. COREST (also known as RCOR1) and the histone demethylase LSD1 (also known as KDM1A) are key regulators of these covalent modifications of the chromatin and have been shown to play a critical role during cerebral cortex development (You et al., 2001; Shi et al., 2004; Fuentes et al., 2012; Lopez et al., 2016). The unveiling of how these proteins assemble in complexes, how their function is targeted to particular domains of chromatin and which genes they regulate is important to understand the coordination of processes that underlie the complex genesis of the multilayered mammalian cerebral cortex. COREST is a multidomain transcriptional corepressor that regulates gene expression through its ability to recruit and form complexes with chromatin-modifying proteins including class I histone deacetylases (HDAC1/2) (Humphrey et al., 2001; You et al., 2001; Hakimi et al., 2002) and LSD1 (Shi et al., 2005; Foster et al.,

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2010). COREST, first identified as a corepressor of REST (also known as NRSF) (Andrés et al., 1999), interacts with LDS1 and enables its histone H3 demethylase activity (Forneris et al., 2007; Yang et al., 2006). In addition, COREST inhibits LSD1 proteosomal degradation (Lee et al., 2005; Shi et al., 2005). Conversely, conditional deletion of LSD1 from embryonic stem cells leads to a reduction of COREST protein (Foster et al., 2010), suggesting a close functional relationship between these proteins. shRNA mediated knock-down of COREST or LSD1 during cortex development leads to a transitory migratory delay of newborn pyramidal neurons that is accompanied by an increase in the number of progenitor cells (Fuentes et al., 2012; Lopez et al., 2016). The function of COREST/LSD1 complex during cortical development appears to be independent of the function of REST, as REST depleted neurons migrate normally to the cortical plate (Fuentes et al., 2012). These observations are in agreement with previous studies showing that COREST may operate independently of REST in the regulation of specific gene networks responsible for inducing and maintaining neuronal identity (Ballas et al., 2005; Abrajano et al., 2009). Thus, it is reasonable to propose that the function of the complex COREST/LSD1 during development may be associated to other unidentified transcriptional regulators.

We conducted a study aimed to identify proteins associated to COREST/LSD1 which could contribute to direct and complement the function of this complex in the context of cerebral cortex development. In this article we communicate that the multi-functional protein SFPQ (splicing factor proline-glutamine rich) is associated to LSD1 in the developing cerebral cortex. Decreasing the levels of SFPQ in the developing mouse cerebral cortex *in vivo* leads to marked defects in the radial migration of pyramidal neurons without affecting neuronal differentiation, as well as to changes in the dynamics of progenitor population, in a way similar to the one observed with the depletion of LSD1 (Fuentes et al., 2012; Lopez et al., 2016).

2. Materials and methods

2.1. Animals

Animal studies described here were carried out in the CF-1 strain mice and followed NIH (USA) and CONICYT (Chile) guidelines. The institutional Bioethics Committee (Universidad de Chile) approved all animal protocols (CBA, 0474).

2.2. Plasmids

pCAGIG-COREST-HA and pCAGIG-LSD1-HA were previously engineered in our laboratory (Fuentes et al., 2012; Lopez et al., 2016). pMYC-PSF-WT (Rosonina et al., 2005) was obtained from Addgene (ID# 35183). pLV-hU6-mPKG-green pre designed shRNA kit (cat# RNAi-B06-NM_023603.3) was purchased by Biosettia (San Diego, CA). It includes a non-sense ("shControl") and four SFPQ shRNAs expressing plasmids (sh-NM-023603-1095; sh-NM-023603-1332; sh-NM-023603-1433; sh-NM-023603-1542). The sequences of the shRNAs used were:

"shControl":

AAAAGCTACACTATCGAGCAATTTTGGATCCAAAATTGCTC GATAGTGTAGC

sh-NM-023603-1095 (sh1SFPQ):

AAAAGGGTTCATTAAGCTTGAATTTGGATCCAAATTCAAG CTTAATGAACCC

sh-NM-023603-1332 (sh2SFPQ):

AAAAGGCATTGTTGAGTTTGCTTTTGGATCCAAAAGCAAAC TCAACAATGCC

sh-NM-023603-1433 (sh3SFPQ):

AAAAGGAACCACTTGAACAGTTATTGGATCCAATAACTGTTC AAGTGGTTCC

sh-NM-023603-1542 (sh4SFPQ):

AAAAGGCACATTTGAGTATGAATTTGGATCCAAATTCATACTC AAATGTGCC

2.3. Isolation of LSD1 interacting proteins

Mouse E14.5 embryonic brain cortices were homogenized by sonication in RIPA buffer (50 mM Tris, pH 7,5; 150 mM NaCl; 1 mM EDTA; 0,25% Deoxicolate; 1% Nonidet P-40) supplemented with a protease inhibitor cocktail (Roche, cat# 04693116001). Homogenates were centrifuged at 17,000*g* for 20 min at 4 °C to remove debris. Precleared embryonic cortex protein extract (3 mg in 1 ml) was incubated with rabbit polyclonal anti LSD1 (Abcam, cat# ab17721) or normal rabbit IgG (Santa Cruz, cat# sc-2027) cross-linked to protein G-agarose (KPL, cat# 223-51-00) overnight at 4 °C. Protein G-agarose cross-link was performed with dimethyl pimelimidate (Thermo Scientific, cat# 21666) according to the manufacturer's instructions. Eluates were run in polyacrilamide gels on PROTEAN® II xi Cell system and silver stained (Shevchenko et al., 1996). Protein enriched bands in anti-LSD1 immunoprecipitates were selected for mass spectrometry.

2.4. Mass spectrometry

Samples were proteolyzed with trypsin, separated by high-pressure chromatography (EASY-nLC II, Proxeon, Thermo Scientific) and detected by mass spectrometry (LTQ Velos, Thermos Scientific). Data were processed with MS Cleaner 2.0 (http://mendel.bii-sg.org/mass-spectrometry/MSCleaner-2.0/) and analyzed with MASCOT Server 2.1.03 version (Matrix Science, UK) and local server PEAKS Studio 6.0, using the SwissProt (2016) database, considering *Mus musculus* taxonomy, trypsin proteolysis, one missed cleavages, methionine oxidation, monoisotopc mass, mass tolerance 0.8 Da, fragmentation tolerance 0.8 Da, peptide charge +1, +2 and +3. We used a decoy database to eliminate false positives. Only statistically significant proteins obtained from both servers were taken for further analysis. Identified proteins were analyzed and classified according their biological functions with DAVID (http://david.abcc.ncifcrf.gov).

2.5. Cell culture and transfection

The Neuro-2a (N2a) cell line was obtained from ATCC and grown under standard conditions in DMEM (Invitrogen) medium supplemented with fetal bovine serum (Hyclone) to a final concentration of 10%. For transfection experiments, 10^5 cells were grown in 35 mm dishes and transfected using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's instructions. After 72 h of transfection, cell cultures were lysed to evaluate SFPQ expression by Western blot or to perform co-immunoprecipitation assays.

2.6. Co-immunoprecipitation assays

N2a cell cultures were non-transfected (control) or transfected with: pCAGIG-LSD1-HA; pCAGIG-COREST-HA or pMYC-PSF-WT (Rosonina et al., 2005). Whole cell lysates were prepared by sonication in RIPA buffer and incubated with 2 μg rabbit polyclonal anti HA (Abcam, cat# ab9110) or mouse monoclonal anti MYC (Sigma, cat# M4439) overnight at 4 °C. Co-immunoprecipitation was performed with Pierce Classic Magnetic IP/Co-IP kit (cat# 88804) according to the manufacturer's instructions. Cell lysates and immunoprecipitates were analyzed by Western blot. To analyze the interaction between LSD1 and SFPQ in the developing

cortex, nuclear extracts from embryonic E17.5 cortices were prepared by using NE-PER® nuclear extraction kit (Thermo Scientific, cat# 78833), according to the manufacturer's instructions. Extracts were incubated with 2 μg of rabbit polyclonal anti LSD1 or mouse monoclonal anti SFPQ (Abcam, cat# ab11825) overnight at 4 °C and purified with protein A-agarose (Pierce, cat# 20333). Inputs and eluates were analyzed by Western blot to determine the interaction.

2.7. Western blot analysis

Transfected N2a cells or fresh cortical tissue were lysed in 10 mM TRIS-base, 0.1% SDS supplemented with complete protease inhibitor cocktail (Roche, #cat 04693116001). Cortical tissue was homogenized using a Dunce grinder while cell culture lysates were passed several times through a 27-gauge syringe. Lysates were cleared by centrifugation at 10,000 × *g* for 10 min. Protein samples were subjected to electrophoresis on 8–10% SDS-PAGE minigels and then transferred to nitrocellulose membrane (Bio-Rad, cat# 1620112) for Western blotting. Membranes were blocked with 5% skim milk 2 h at RT and incubated with mouse anti SFPQ (Abcam, cat# ab11825; used 1:3000; Santa Cruz, cat# sc-101137; used 1:1000) overnight at 4 °C. Anti TFII-β (Santa Cruz, cat# sc-225, used 1:1000) or HRP-conjugated anti β-tubulin (AbCam, cat# ab21058; used 1:5000) were used as loading control. Membranes were incubated with a donkey anti rabbit HRP-conjugated secondary antibody (Jackson Immunoresearch; used 1:10000). For co-immunoprecipitation, immunoblots membranes were incubated with rabbit polyclonal anti HA (used 1:5000) or mouse monoclonal anti MYC (used 1:1000) for the cell culture eluates; or anti LSD1 (used 1:1000) or anti SFPQ (used 1:1000) for cortex eluates and True Blot horseradish peroxidase conjugated polyclonal anti rabbit (Rockland cat# 18-8816-21, used 1:5000) or anti mouse (Rockland cat# 18-8817-31, used 1:5000).

2.8. In utero *electroporation*

In utero electroporation procedure and subsequent tissue processing was performed as described previously (Saito and Nakatsuji, 2001; Cánovas et al., 2015). Briefly, timed pregnant female mice (E14.5) were deeply anesthetized with isofluorane and the uterine horns were exposed through a midline incision. A volume of 1–1.5 μl of DNA solution (4 μg/μl) mixed with Fast Green (0.05%; Fisher Scientific, #cat F99-10) was injected into the brain lateral ventricle of the embryos of either gender using a microcapillary, followed by five square pulses (35 V each, 50 ms duration at 1 s intervals) delivered across the embryo's head using 0.5 cm diameter electrodes connected to an electroporator (GenePulse Xcell, Bio-Rad). At the selected developmental stage, the animals were fixed to process the brains for direct visualization of GFP or for immunohistochemistry analysis. After fixation, each brain was visually inspected for GFP intensity and electroporation area. The brains that showed either poor GFP expression or misplaced electroporation (*i.e.* outside the sensory motor area) were not included in subsequent analyses.

2.9. Analysis of neuronal migration

Neuronal migration was evaluated at E17.5 in coronal brain slices containing the sensory motor cortex. After the acquisition of high-resolution microscopy images of electroporated brains, 100 μm wide fields perpendicular to the lateral ventricle were selected for quantitative analysis of GFP-expressing cells located in the ventricular/subventricular zone (VZ/SVZ), intermediate zone (IZ) and cortical plate (CP). The boundaries between these structures were defined according to histological differences (*i.e.* nuclear density) detected by Hoechst staining (Schambra, 2008). The GFP positive cells contained in each of the mentioned regions were manually counted using the ImageJ Cell Counter tool. In average, we counted 150 GFP positive cells per brain slice and expressed the results as the percentage of cells located in the VZ/SVZ, IZ and CP.

2.10. Immunohistochemistry

Embryonic or P0 mice of either gender were transcardially perfused with chilled PBS 1X, followed by fresh 4% paraformaldehyde (PFA) in PBS 1X. The brains were dissected and postfixed in 4% PFA overnight. Whole brains were embedded into low melting point agarose (2% in PBS 1X) and coronally-sectioned (70 μm thick) using a vibratome (VT1000S, Leica). After blocking with 3% donkey serum/0.25% Triton X-100 in PBS 1X during 2 h at room temperature, sections were incubated overnight at 4 °C with primary antibodies. Sections were washed 3 times with PBS 1X, followed by incubation during 1.5 h with secondary antibodies at room temperature. Sections were rinsed three times with PBS 1X, stained with Hoëchst (Invitrogen, cat# H1399) and mounted on microscope slides in Vectashield mounting medium (Vector Laboratories, cat# H1000). For SFPQ immunohistochemistry, brain slices were subjected to antigen retrieval procedure in citrate buffer pH 8.5 (30 min at 78–80 °C) before blocking step. We used the following primary antibodies: anti SFPQ (cat# sc-101137; used 1:100), anti CUX1 (cat# sc-13024; used 1:300) and anti doublecortin (cat# sc-8066; used 1:250) were purchased from Santa Cruz Biotechnology; anti SOX2 (cat# AB5603; used 1:400) was purchased from Millipore Bioscience Research Reagents; anti Ki67 (cat# ab9260; used 1:400) and anti TBR2 (cat# ab23345; used 1:200) were purchased from Abcam. All fluorophore-conjugated secondary antibodies were purchased from Jackson Immunoresearch and were used at 1:250 dilutions.

2.11. Microscopy, images, and statistics

Images were acquired using an Olympus disk-spinning unit microscope (BX61WIDSU) and an Olympus FluoView FV1000 confocal microscopy. Processing and quantization of images was performed using the ImageJ software suite (NIH). The montage of figures was performed in GIMP (GNU Image Manipulation Program). Results are presented as mean ± SEM. For comparison between two groups unpaired Student's *t*-test was used to determine statistical significance. We performed F test to determine variance homogeneity and Shapiro Wilk test to corroborate that data present normal distributions. The level of significance was defined as probability values (p) < 0.05.

3. Results

In order to identify interacting partners of the COREST/LSD1 complex we performed co-immunoprecipitation (Co-IP) experiments followed by mass spectrometry analysis. A fundamental requirement for successful immunopurification of multiprotein complexes is the use of primary antibodies that efficiently bind and immunoprecipitate some of the proteins present in the target complex. Thus, we first screened the effectiveness of commercial antibodies against COREST and LSD1 to co-immunoprecipitate COREST/LSD1 complex from embryonic day 14.5 (E14.5) whole cerebral cortex lysates. We tested two anti COREST antibodies and one anti LSD1 antibody. We observed that both anti COREST antibodies tested barely co-immunoprecipitated LSD1, whereas the anti LSD1 antibody efficiently co-immunoprecipitated COREST, even more abundantly than the amount recovered by the anti COREST antibodies tested (data not shown) thus, we used the anti LSD1 antibody to carry out a preparative immunoprecipitation from an

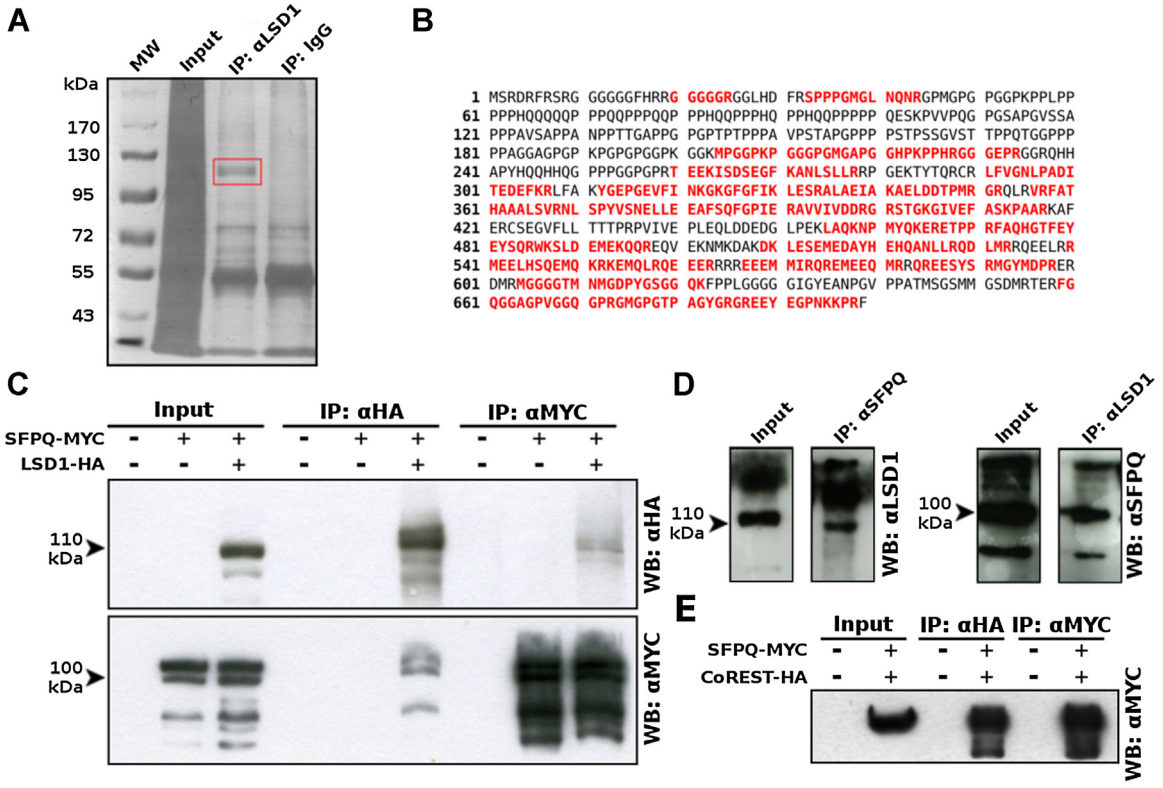

Fig. 1. Identification of proteins associated to LSD1 and COREST. (**A**) Anti-LSD1 and control immunoprecipitates (using a rabbit IgG) were resolved in a SDS-PAGE gel and then silver stained. A 100 kDa band (red rectangle) present in the anti-LDS1 immunoprecipitation but not in the control immunoprecipitation was isolated and subsequently analyzed by mass spectroscopy (100 kDa band in Table S1). (**B**) Aminoacidic sequence of SFPQ. Letters in red denote the segments of the protein that were identified in the mass spectrometric sequencing of the band shown in A. (**C**) Whole-cell extracts from N2a cells transfected with pLSD1-HA and/or pSFPQ-MYC plasmids were immunoprecipitated (IP) with the indicated antibodies. Precipitated immunocomplexes were resolved by SDS-PAGE and revealed by Western blot (WB) with anti-HA or anti-MYC antibodies. LSD1-HA and SFPQ-MYC proteins migrated according to their predicted molecular masses (110 kDa and 100 kDa, respectively). (**D**) Nuclear extracts obtained from E17.5 cortices were immunoprecipitated with anti-LSD1 or anti-SFPQ antibodies. Immunoprecipitates were resolved by SDS-PAGE and endogenous LSD1 and SFPQ proteins were detected by Western blot. **(E)** Interaction between COREST and SFPQ. Whole-cell extracts from N2a cells transfected (Transf) with pCOREST-HA and/or pSFPQ-MYC plasmids were immunoprecipitated (IP) with anti-HA or anti-MYC antibodies. Precipitated immunocomplexes were resolved by SDS-PAGE and revealed by Western blot (WB) with anti-MYC antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

E14.5 cerebral cortex lysate which was analyzed by SDS-PAGE and visualized by silver staining. Six bands with molecular masses of around 100, 73, 70, 68, 66 and 55 kDa, that were consistently found in anti LSD1 immunoprecipitates but that were absent in control immunoprecipitations (using a normal rabbit IgG) were excised and analyzed by mass spectrometry (Figs. 1 **A** and S1). As expected, the proteomic analysis of these chosen bands evidenced the presence of LSD1 (KDM1, 100 kDa band), COREST1 (RCOR1, 55 kDa band) and COREST2 (RCOR2, 70 kDa band) among the immunoprecipitated proteins (Table S1), with a considerable protein sequence coverage (45.4, 40.9 and 58.1%, respectively). The classification of LSD1-associated proteins according to their biological function revealed a wide variety of process in which LSD1 is potentially involved, including RNA processing, transcription, intracellular trafficking and cellular differentiation (Table S2). Interestingly, the analysis of the 100 kDa (Fig. 1**A**) showed that the SFPQ protein is abundantly represented in that band as the 56.8% of its polypeptidic sequence was detected in the mass spectrometry analysis (Fig. 1**B** and Table S1). Our analysis also detected NONO (75.3% protein sequence coverage, 55 kDa band), a nuclear factor highly homologous to the C-terminal portion of SFPQ, which is known to form nucleic acid-binding heterodimers with SFPQ (Shav-Tal and Ziporu, 2002) (Table S1). Thus, the proteomic analysis strongly indicates the association of SFPQ with LSD1 in the developing cerebral cortex.

SFPQ is a unique multidomain protein that possesses dual RNA/DNA-binding capacities and the ability to interact with a number of proteins within the nucleus (Shav-Tal and Zipori 2002; Yarosh et al., 2015). Thus, SFPQ has been recognized as a multifaceted protein involved in a wide range of nuclear processes including pre-mRNA splicing, retention of defective RNAs within the nucleus, DNA repair and transcriptional activation and repression of multiple genes (Patton et al., 1993; Straub et al., 1998; Akhmedov and Lopez, 2000; Urban et al., 2000; Mathur et al., 2001; Zhang and Carmichael, 2001; Urban et al., 2002; Morozumi et al., 2009; Salton et al., 2010; Rajesh et al., 2011). It has been reported that SFPQ is expressed during cortex development (Chanas-Sacré et al., 1999), but its functional role in this context is not understood.

To confirm the interaction between SFPQ and LSD1, we performed Co-IP assays using whole-cell lysates from double-transfected N2a cells with expression vectors for full length tagged forms of LSD1 (pLSD1-HA) and SFPQ (pSFPQ-MYC). Western-blot analysis revealed the presence of the MYC epitope in anti-HA immunoprecipitates; conversely, the HA epitope was detected in anti-MYC immunoprecipitates corroborating the physical association between LSD1 and SFPQ (Fig. 1**C**). As expected, these interactions were not observed in cultures transfected solely with pLSD1-HA or pSFPQ-MYC plasmid (Fig. 1**C**). In complementary experiments cerebral cortices were dissected out from E17.5 brains, and nuclear extracts were immunoprecipitated using an anti SFPQ antibody or an anti LSD1 antibody. Western blotting showed the presence of both LSD1 and SFPQ in the anti SFPQ and the anti LSD1-immunoprecipitates respectively, thus confirming the interaction between these proteins *in vivo* during cerebral cortex development (Fig. 1**D**). Interestingly, a previous study detected SFPQ protein in a

COREST-containing complex in mouse embryonic stem cells (Ding et al., 2009); therefore, additional Co-IP experiments were made using whole-cell extracts obtained from N2a cells transfected with pCOREST-HA and pSFPQ-MYC that were immunoprecipitated with anti MYC or anti HA antibodies and then blotted with anti MYC antibody. This assay revealed physical interaction between COREST and SFPQ (Fig. 1**E**), suggesting that COREST is also part of the complex formed by LSD1 and SFPQ.

In agreement with a previous report showing the developmental expression of *Sfpq* mRNA in cerebral cortex homogenates (Chanas-Sacré et al., 1999), we observed that the SFPQ protein is highly expressed in the cerebral cortex during embryonic stages and then declines after birth (Fig. 2**A** and **B**), indicating that SFPQ is developmentally regulated and that its higher expression level spans the time period of cortical neurogenesis and neuronal differentiation. To gain insight into the function that SFPQ plays during cortex development, we examined the cell populations that express SFPQ in the embryonic cortex using immunofluorescence analysis. At E16.5, we observed that SFPQ is differentially expressed through different cortical domains. Thus, SFPQ immunoreactivity is faintly detected in progenitor cells located in the ventricular and subventricular zones (VZ and SVZ, respectively) identified by the expression of SOX2 and TBR2, respectively (Fig. 2**C** and **D**); however SFPQ expression becomes enriched toward the intermediate zone (IZ) and cortical plate (CP), indicating that post-mitotic neurons progressively express higher levels of SFPQ as they migrate and differentiate (Fig. 2**C** and **D**). Consistently, SFPQ is weakly detected in proliferating cells identified by the expression of Ki67 but it is highly expressed by post-mitotic cells identified by the expression of DCX, and in particular in cells located at the cortical plate at E17.5 (Fig. 2**E**). Previous studies also reported elevated expression levels of SFPQ in differentiating neurons in the mouse and zebrafish developing nervous system suggesting that SFPQ may have a role during neuronal differentiation (Chanas-Sacré et al., 1999; Lowery et al., 2007). In contrast to SFPQ, COREST and LSD1 are ubiquitously expressed across the developing cortex exhibiting high expression levels in proliferative zones, IZ and CP (Fuentes et al., 2012; Zhang et al., 2014).

To explore the function of SFPQ during cortical development we carried out short hairpin RNA (shRNA)-mediated knock-down experiments *in vivo*. We screened a set of four plasmids, each expressing GFP plus a candidate shRNA targeting the mouse *Sfpq* transcript (Fig. 3**A**). We selected the construct expressing sh4SFPQ (hereafter called pshSFPQ) as it reduced endogenous SFPQ expression in transfected N2a cells by 65 ± 9% relative to control cultures transfected with a plasmid expressing a control shRNA (pshControl) ($n = 3$, $p < 0.001$) (Fig. 3**A** and **B**). To test the ability of pshSFPQ to inhibit the expression of SFPQ *in vivo*, we performed *in utero* electroporation of this construct into cortices at E14.5 and analyzed electroporated cells and their progeny at E17.5. As opposed to pshControl electroporated cells (*i.e.* GFP-positive cells), we observed a robust decrease in SFPQ immunoreactivity in cells electroporated with pshSFPQ (Fig. 3**C**). Interestingly, we observed that SFPQ depletion led to marked defects in cell migration in brains electroporated at E14.5 and analyzed at E17.5 (Fig. 4**A** and **B**). Thereby, in brains electroporated with pshControl, 38 ± 2% of cells were found in the VZ/SVZ and 27 ± 2% had reached the CP. In contrast, 45 ± 1.7% of the pshSFPQ electroporated cells remained in the VZ/SVZ and only 19 ± 2% reached the CP ($n = 18$ brains pshControl and $n = 16$ brains pshSFPQ, $p = 0.0162$ for VZ/SVZ; $p = 0.0067$ for CP)(Fig. 4**A** and **B**). Previous reports have shown that small reductions in SFPQ expression trigger apoptosis in human cultured cells (Heyd and Lynch, 2011) and that SFPQ loss-of-function associates to widespread cell death in the developing zebrafish embryo (Lowery et al., 2007), suggesting a general suppressive effect of SFPQ on apoptosis. Thus, we examined if the decrease in the number of cells that reach the CP in pshSFPQ electroporated brains is due to an increase in cell death. We detected very few apoptotic cells (identified as activated caspase-3 expressing cell) in the cortex of electroporated brains at E17.5 and no differences were observed in the number of GFP-positive/caspase-3 positive cells between pshControl and pshSFPQ electroporation conditions (pshControl: 0.7 ± 0.2%; pshSFPQ: 0.6 ± 0.5%, $n = 4$ brains per condition, $p = 0.7154$) suggesting that, in our experimental conditions, SFPQ silencing does not affect neuronal survival in a significant manner (data not shown).

The *in vivo* SFPQ function has been linked to the differentiation of specific neuronal classes during zebrafish nervous system development (Lowery et al., 2007). Thus, it is possible that the defects in radial migration observed in SFPQ electroporated brains are a secondary consequence of defective neuronal differentiation. Considering the strong SFPQ expression observed in post-mitotic neurons at the cortical plate we explored the ability of SFPQ-depleted cells to acquire neuronal fate. We found that all SFPQ-depleted (GFP-positive) cells located in the IZ/CP boundary at E17.5 expressed TUJ1 (β3-tubulin) (Fig. 4**C**) and DCX (Fig. 4**D**), which are widely considered indicators of neuronal identity; this indicates that SFPQ does not influence the execution of the broad features of the differentiation program of cortical pyramidal neurons. The subtypes of pyramidal neurons that populate the different layers of the cerebral cortex are generated in a timely stereotyped manner from neural progenitors. Thus, most neurons born at around E14.5 become layer IV neurons which receive inputs from the thalamus and mainly project to layers II–III (Molyneaux et al., 2007); among other features, they can be distinguished by its enriched expression of the transcription factor CUX1 (Nieto et al., 2004). Consistently, in brains electroporated with pshControl at E14.5 and analyzed at P0 most of the GFP-expressing neurons were located at layer IV and 43.65% (SEM 8.31%) expressed CUX1 (Fig. 4**E**). Similarly, in pshSFPQ electroporated brains 57.34% (SEM 3,6%; $p = 0.1$) of the cells that reached the layer IV expressed CUX1. However, we found an abnormal cellular distribution in pshSFPQ electroporated brains, as many GFP-positive cells were located in deep layers and in the white matter. Noticeably, 56.27% (SEM 2.3%) of misplaced SFPQ depleted neurons expressed CUX1 regardless of their position in the cortex (Fig. 4**E**).

Since COREST/LSD1 function influences neural progenitor dynamics in the cerebral cortex (Fuentes et al., 2012; Lopez et al., 2016), we next examined the effect of the decrease of SFPQ levels on progenitor proliferation. We analyzed the number of GFP-positive cells that expressed Ki67 in the VZ/SVZ of brains electroporated at E14.5 with pshControl or pshSFPQ and fixed at E17.5. In brains electroporated with pshControl, 24.2 ± 1.8% of GFP-positive cells were Ki67-positive, whereas in brains electroporated with pshSFPQ, the percentage of Ki67-positive cells was significantly decreased to 16.7 ± 1.5% ($n = 10$ brains per condition; $p = 0.0051$) (Fig. 5**A** and **B**). This result suggests that SFPQ depletion modifies the proliferative behavior of progenitor cells in the developing cerebral cortex.

Overall, these results are consistent with the idea that SFPQ function is required for the regulation of progenitor proliferation and the correct positioning of cortical pyramidal neurons within the cortex but not for the specification of their fate.

4. Discussion

During the development of the cerebral cortex, neural progenitors at the dorsal pallium generate pyramidal neurons that migrate out of proliferative zones and initiate their differentiation program, including their “layer identity”. The mechanisms that integrate these sequential developmental processes into a coherent and ordered schedule are not well understood. Previously we demonstrated that the epigenetic regulators COREST and LSD1 function

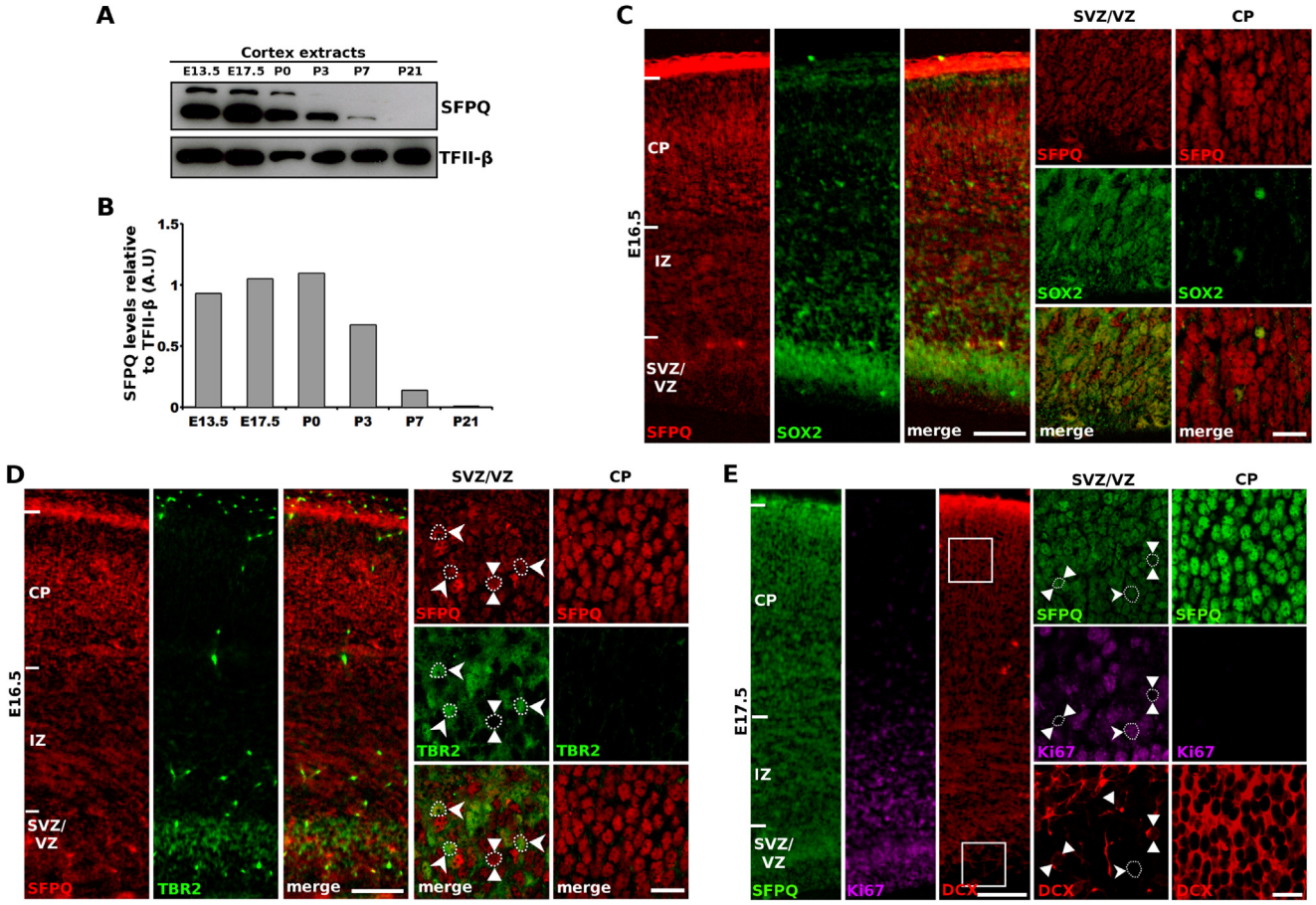

Fig. 2. SFPQ expression in the developing cortex. (**A**) Western blot analysis of SFPQ abundance in the cerebral cortex at different stages of development. (**B**) Densitometric analysis of SFPQ abundance relative to TFII-β in cortical extracts at different developmental stages. (**C**) Coronal section of an E16.5 brain showing the expression of SFPQ in the cortex. SFPQ expression is contrasted with the pattern of SOX2, which is abundantly expressed in progenitor cells at the VZ. Right, high-magnification views of the VZ/SVZ and the CP. Note the difference in SFPQ intensity between VZ/SVZ and the CP. Most cells in the VZ co-express SOX2 and SFPQ. (**D**) Coronal section of an E16.5 brain contrasting SFPQ and TBR2 expression, which is abundantly expressed in progenitor cells at the SVZ. SFPQ is expressed in most TBR2-positive cells at the SVZ. Right, high-magnification views of the VZ/SVZ and the CP. Note the difference in SFPQ intensity between VZ/SVZ and the CP. Notched arrowheads and arrowheads indicate SFPQ positive cells expressing TBR2 and not expressing TBR2, respectively. Scale bars: Scale bars: low-magnification images: 100 μm; high-magnification images: 20 μm. (**E**) Coronal section of an E17.5 cortex displaying the expression of SFPQ compared with the expression of Doublecortin (DCX) and Ki67. Right, magnified views showing the VZ/SVZ and the CP. SFPQ is highly expressed in DCX-positive cells at the CP, but exhibits a lower expression in both, proliferating cells (Ki67-positive) and post-mitotic cell (DCX-positive) located at the VZ/SVZ. Arrowheads show cells expressing SFPQ and DCX. Notched arrowhead showa a cell expressing SFPQ and Ki67. Scale bars: low-magnification images in C and D: 100 μm; High-magnification images in C and D: 20 μm.

together in the proper execution of the developmental program of the glutamatergic neurons of the cerebral cortex (Fuentes et al., 2012), and particularly in the process of radial migration. Since these functions do not appear to be related to the function of REST in recruiting the COREST/LSD1 complex we used an unbiased proteomic approach to identify nucleic acid binding protein that could be associated to LSD1 and thus participate in directing their action to particular nuclear locations. Using LSD1 immunoprecipitation we isolated a number of potential interactors, among which it called our attention the presence of SFPQ.

SFPQ is a multidomain protein highly conserved in vertebrates that has been shown to participate directly in a wide array of nuclear activities. It has been suggested that the functional versatility of SFPQ relies on its complex domain structure, providing the ability to bind nucleic acids (DNA and RNA) and form complexes with several protein counterparts in a context-dependent fashion (Shav-tal and Zipori 2002; Yarosh et al., 2015; Knott et al., 2016). Most reports concerning SFPQ protein are focused on its molecular and biochemical characterization using *in vitro* systems or cell culture models, however studies addressing the *in vivo* SFPQ function are limited.

The idea that SFPQ forms physical complexes with LSD1 is supported by the direct identification by mass spectrometry of a large portion of the SFPQ aminoacidic sequence in the material immunoprecipitated with an LSD1 antibody, and also by the immunorecognition of SFPQ in the anti-LSD1 immunoprecipitate obtained from developing cerebral cortices; moreover, we demonstrated that recombinant LSD1 tagged with HA epitope (and also COREST tagged with HA), are able to associate with SFPQ tagged with MYC, when they are coexpressed in a cell line. As an additional argument, we detected in our mass spectrometry analysis the presence of NONO, a protein that forms functional dimers with SFPQ. Although these data strongly indicate that LSD1 associates with SFPQ during cerebral cortex development, we cannot dismiss the possibility that nucleic acids may mediate or stabilize the interaction between these proteins due to the ability of SFPQ or LSD1-containing complexes to interact with DNA or RNA (Tsai et al., 2010). Altogether, these results allow us to predict a functional interaction of SFPQ with LSD1 and COREST.

The identification of the mechanisms through which SFPQ or the SFPQ and LSD1/COREST-containing complexes function in the development of the cerebral cortex poses significant challenges, taking into account their pleomorphic biochemical features as well

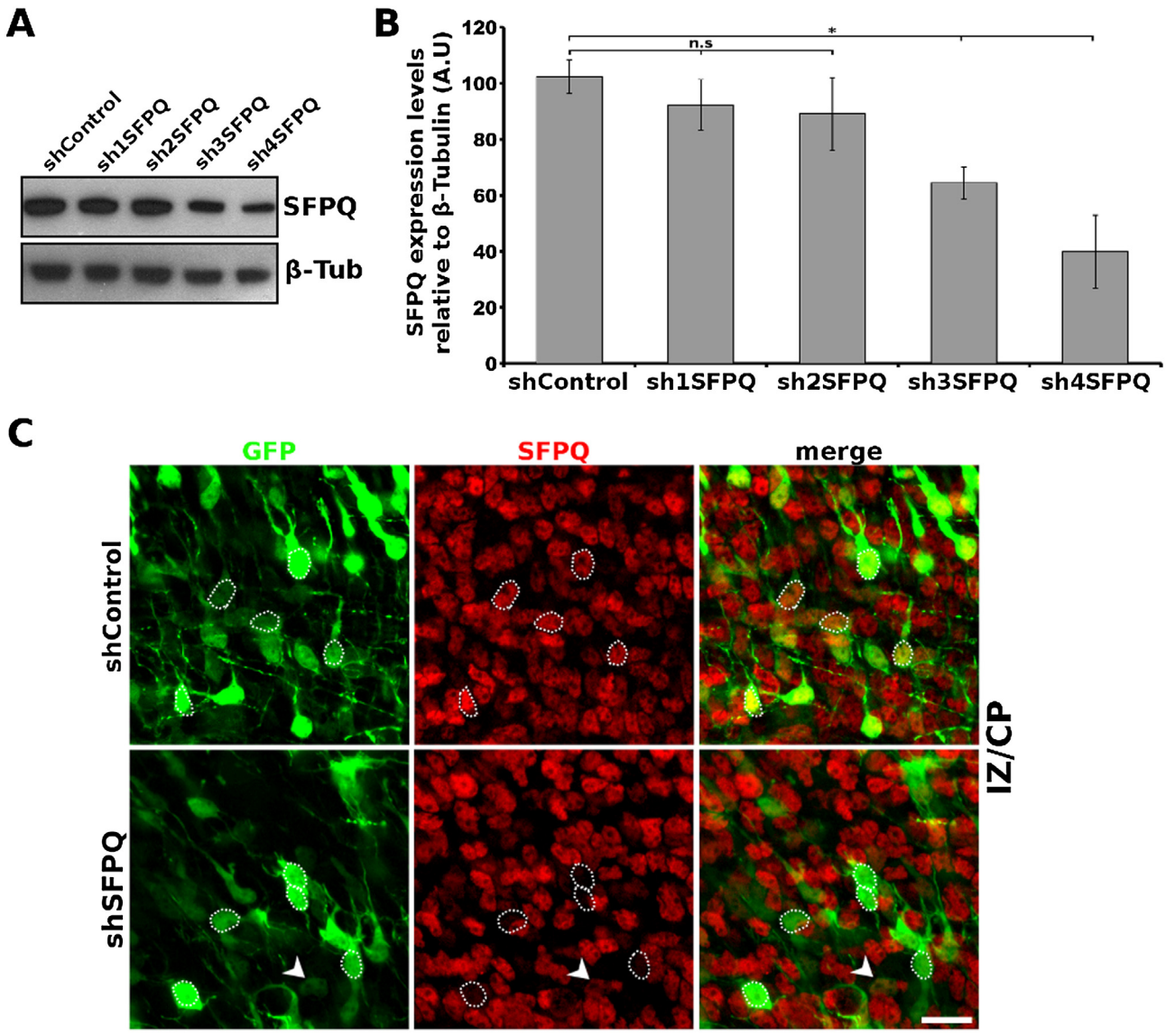

Fig. 3. shRNA-mediated SFPQ knock-down *in vitro* and *in vivo*. (**A**) Western-blot analysis of SFPQ expression in whole-cell extract obtained from N2a cells 72 h post-transfection with plasmids encoding either a shControl or different shRNA's targeting SFPQ (shSFPQ). (**B**) Densitometric analysis of SFPQ expression relative to β-tubulin in transfected N2a cells. Error bars represent ± SEM. Student's *t*-test $^{*}p < 0.05$, n = 3. (**C**) SFPQ immunodetection in coronal sections of brains electroporated *in utero* with pshControl or pshSFPQ at E14.5 and analyzed at E17.5. High magnification confocal images of electroporated neurons (GFP-positive) located at the intermediate zone/cortical plate boundary (IZ/CP) are shown. The notched arrowhead indicates an electroporated cell in which SFPQ was not efficiently inhibited. Scale bar 20 μm.

as the reports showing that alterations in SFPQ expression or function associate to various diseases ranging from cancers such as leukemia, renal and prostate cancer to degenerative and neurological disorders as Alzheimer's disease and autism (Clark et al., 1997; Duhoux et al., 2011; Dolnik et al., 2012; Ke et al., 2012; Jiang et al., 2013; Stamova et al., 2013), which illustrates a critical role of SFPQ for normal cell biology in different contexts. Besides this, very little is known about the functions of SFPQ in the development of the nervous system and neuronal biology. It has been shown that SFPQ displays an enriched expression in the developing central nervous system of zebrafish and that mutant fishes for *sfpq* exhibit an abnormal development of midbrain and hindbrain, alterations in the differentiation of specific neuronal classes and a marked increase in cell death throughout the embryo (Lowery et al., 2007). More recently, it was reported that SFPQ is required to maintain axon viability in neuronal cultures by means of its ability to bind several mRNAs and coordinate their subcellular localization (Cosker et al., 2016).

Our results show that SFPQ is also relevant for the development of the cerebral cortex, participating in the control of radial migration of pyramidal neurons and also affecting the dynamics of their progenitors. The multi-functional nature of SFPQ represents a difficult challenge for the understanding of the specific contribution of this protein to cortical development from a mechanistic perspective. Taking into consideration that SFPQ interacts with LSD1 and COREST during cortical development and the similarity of the migratory phenotypes observed upon LSD1, COREST and SFPQ knock-down in cortical neurons (Fuentes et al., 2012; Lopez et al., 2016), it may be proposed that SFPQ serves a role in targeting the components with enzymatic activity to particular nucleic acid domains. Nevertheless, it must be acknowledged that our experimental approach does not allow distinguishing whether the migratory defects associated to SFPQ depletion are related to alterations in the function of COREST/LSD1 complex or whether originates from impairments of some of the other SFPQ functions independent of this complex.

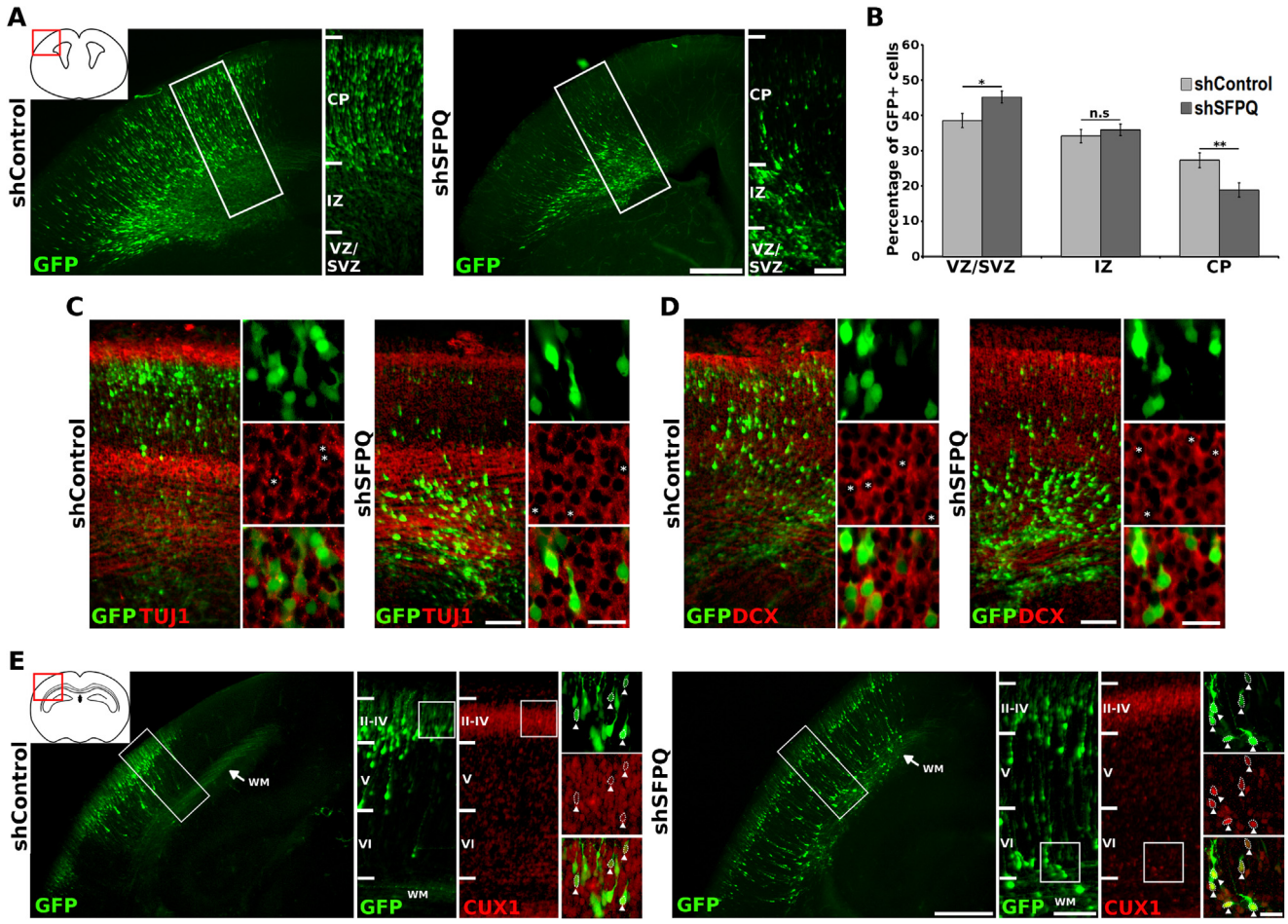

Fig. 4. SFPQ knock-down impairs radial migration but not neuronal differentiation. (**A**) Representative coronal sections of brains electroporated *in utero* with pshControl or pshSFPQ at E14.5 and processed at E17.5. Right, magnified views of the areas within the white rectangles. A schematic representation of the brain area analyzed is shown (Upper left). VZ/SVZ, ventricular zone/subventricular zone; IZ, intermediate zone; CP, cortical plate. (**B**) Quantification of cell position in the cortex of experiments showed in A. The results are expressed as the percentage of GFP-positive cells distributed in the indicated cortical regions. Error bars represent ± SEM. Student's *t*-test, $^{*}p < 0.05$; $^{**}p < 0.01$; pshControl, n = 18 brains; pshSFPQ, n = 16 brains. (**C**) Cortical sections of brains electroporated *in utero* with the indicated plasmids at E14.5 and processed at E17.5. Sections were stained with the pan-neuronal marker TUJ1 (β-III tubulin). For each electroporation condition, high-magnification images of electroporated cells at the IZ/CP boundary are shown. Note that all pshControl and pshSFPQ electroporated cells express Tuj1 (see cells labeled with asterisks). Scale bars: low-magnification images: 100 μm; High-magnification images: 20 μm. (**D**) Cortical sections of brains electroporated *in utero* with the indicated plasmids at E14.5 and fixed at E17.5. Sections were stained with the post-mitotic marker Doublecortin (DCX). Right, high-magnification images of electroporated cells at the IZ/CP boundary are shown. Note that all pshControl and pshSFPQ electroporated cells express DCX (see cells labeled with asterisks). (**E**) Coronal sections of brains electroporated *in utero* with pshControl or pshSFPQ at E14.5 and processed at P0. A schematic representation of the brain area analyzed is shown (Upper left). Sections were immunostained with anti-CUX1 antibody, a marker of the cortical layers II to IV. Middle panels show magnified views of the areas within the white rectangles. Right, high-magnification images of the areas enclosed in the white squares are shown for each electroporation condition. Arrows indicate axons from electroporated cells extending medially across the white matter (WM). Arrowheads point out shControl or shSFPQ electroporated cells that display a high CUX1 expression. Note that misplaced pshSFPQ electroporated cells express high levels of CUX1. Scale bars: low-magnification images in A and D: 500 μm; medium-magnification images in A and D: 100 μm; high-magnification images in C and D: 20 μm.

Interestingly and despite of the wide variety of nuclear processes that have been reported to be regulated by SFPQ and which could, when perturbed, dramatically alter the viability or differentiation program of cortical neurons, the defects caused by the decrease of SFPQ expression appear to be restricted to discrete aspects of cortical development (*i.e.* migration and proliferation of neural precursors), suggesting that SFPQ is involved in the control of specific steps of corticogenesis instead of more general processes determining the cellular viability. It must be taken into account that our approach involves the decrease of the protein dose rather than the complete suppression of its expression, which may unveil subtler functions than those affected by its complete ablation.

We found that SFPQ expression in progenitor cells at the VZ/SVZ is low, and that it is progressively increased in maturing neurons at the IZ and CP. Interestingly and regardless the low SFPQ expression levels observed in the germinal regions of the cortex, we showed that the decrease of SFPQ protein associates to a decrease in the proportion of proliferating progenitor cells. From our results it is not possible to ascertain whether the effect of the decrease of SFPQ was primarily to induce a premature cell cycle exit of neural progenitors or to trigger their neuronal differentiation. Interestingly, SFPQ was first characterized as regulator of splicing and as a physical interactor of the polypyrimidine tract-binding protein 1 (PTBP1) (Patton et al., 1993), a RNA-binding protein described as a repressor of neuron-specific splicing that is expressed in non-neuronal cells and neural progenitors (Ashiya and Grabowski, 1997; Chan and Black, 1997; Valcarcel and Gebauer, 1997; Makeyev et al., 2007). It has been shown that PTB is downregulated in differentiating and mature neuronal cells by the microRNA 124 (*Mir-124-1*), allowing the switch from general to neuron-specific alternative splicing and neuronal differentiation (Makeyev et al., 2007). Importantly, the inhibition of PTB proved to be sufficient to trigger the activation of neuron-specific splicing, supporting an inhibitory role of PTB over neuronal differentiation (Makeyev et al., 2007). It is not known if

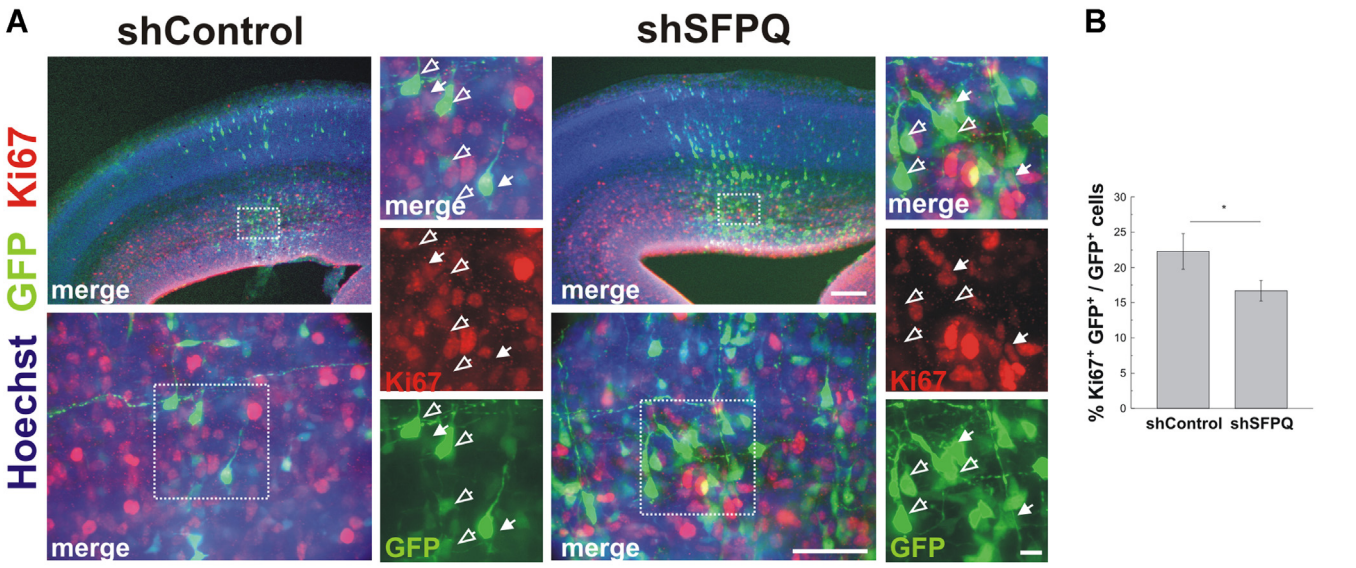

Fig. 5. SFPQ knock-down alters the proportion of proliferating cells in the VZ/SVZ. (**A**) Coronal sections of brains electroporated *in utero* with pshControl or pshSFPQ at E14.5 and processed at E17.5. Sections were stained with Ki67 and Hoechst. Bottom left, magnified views of the areas within the white rectangles drew in the low-magnification images are shown for each condition. Right, high-magnification images of the areas enclosed in the white squares are shown for each condition. Filled arrows indicate electroporated cells that express Ki67, whereas empty arrows indicate electroporated cells that do not express Ki67. Scale bars: low-magnification images: 500 μm; medium-magnification images: 50 μm; high-magnification images: 10 μm. (**B**) Quantitation of experiments shown in A. The results are expressed as the percentage of GFP-positive cells that express Ki67 at the VZ/SVZ. Error bars represent ± SEM. Student's *t*-test, *p < 0.05; pshControl, n = 10 brains; pshSFPQ, n = 10 brains.

SFPQ contributes to PTB's inhibitory role over neuronal differentiation, however our data indicating that SFPQ decrease of function inhibits the proliferation of progenitor cells could be in agreement with this idea. It would be interesting to analyze the mRNA splicing events that are sensitive to SFPQ knock-down in neural progenitors as a way to explore its participation in the regulation of transcriptome at post-transcriptional level.

The most noticeable defect produced by SFPQ depletion is the inability of cortical neurons to migrate to their final destination in the cortex. Nevertheless, disruption of SFPQ expression throughout the stages from neural progenitor cells to post-mitotic neurons does not appear to impair the acquisition neuronal identity *in vivo*, suggesting that migration defects of SFPQ-depleted cells may not be attributed to misspecification of the neuronal fate. Several lines of evidence suggest that neuronal migration and differentiation are temporally related, but independently controlled processes as specific mouse and human gene mutations exist that affect only one of the two events (Ge et al., 2006). For instance, in patients with mutations in Filamin-1 gene (*Flna*), some neurons fail to migrate but still retain the ability of differentiate normally (Nagano et al., 2004). Moreover, studies using Neurogenins 1 and 2 mutants showed that distinct neurogenic basic helix-loop-helix (bHLH) transcription factors control neuronal migration independently of neuronal differentiation, suggesting that these cellular processes are regulated in parallel by neurogenic bHLH transcription factors (Ge et al., 2006). The data shown here are consistent with the notion that SFPQ controls cell migration independently of the neuronal differentiation program.

Mutations in human genes that control different aspects of neuronal migration associate to severe brain malformations, such as lissencephaly, and periventricular and cortical heterotopias (Guerrini et al., 2008). Additionally, genetic studies have identified several candidate genes involved in neuronal migration as susceptibility loci for neurological and cognitive disorders, underscoring the importance of the migration process. The *Sfpq* gene is located in a chromosomal region that has been related to speech sound disorder, language impairment, and dyslexia (Rabin et al., 1993). Interestingly, SFPQ has been shown to form a complex with TFII-I and PARP1 that positively regulate the expression of *Dyx1c1* gene (Tapia-Páez et al., 2008), the first candidate dyslexia gene and an important regulator of neuronal migration during cortex development (Taipale et al., 2003; Wang et al., 2006). Preliminary results from our laboratory using chromatin immunoprecipitation assays confirmed that SFPQ is enriched in regulatory regions of the *Dyx1c1* gene in the E17.5 cortex. Additionally, we found that LSD1 is also bound to the same region tested for SFPQ, suggesting that both proteins might act together in the regulation of *Dyx1c1* gene during cortex development (K.S. and M.K; unpublished results).

5. Conclusion

This report defines SFPQ as a component of LSD1 containing complexes expressed in the developing cerebral cortex and establishes its role as regulator of the process of radial migration of pyramidal neurons. This role is consistent with the previously demonstrated requirement of COREST/LSD1 for its adequate execution and opens a new and complex avenue to the study of cerebral corticogenesis.

Declaration of interests

The authors declare no competing financial interest or other conflict of interest regarding the word described in this article.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijdevneu.2016.12.006.

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