

Regulatory Factor X Transcription Factors Control Musashi1 Transcription in Mouse Neural Stem/Progenitor Cells

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The transcriptional regulation of neural stem/progenitor cells (NS/PCs) is of great interest in neural development and stem cell biology. The RNA-binding protein Musashi1 (Msi1), which is often employed as a marker for NS/PCs, regulates Notch signaling to maintain NS/PCs in undifferentiated states by the translational repression of Numb expression. Considering these critical roles of Msi1 in the maintenance of NS/PCs, it is extremely important to elucidate the regulatory mechanisms by which Msi1 is selectively expressed in these cells. However, the mechanism regulating Msi1 transcription is unclear. We previously reported that the transcriptional regulatory region of *Msi1* is located in the sixth intron of the *Msi1* locus in NS/PCs, based on in vitro experiments. In the present study, we generated reporter transgenic mice for the sixth intronic *Msi1* enhancer (*Msi1*-6IE), which show the reporter expression corresponding with endogenous Msi1-positive cells in developing and adult NS/PCs. We found that the core element responsible for this reporter gene activity includes palindromic Regulatory factor X (Rfx) binding sites and that *Msi1*-6IE was activated by Rfx. Rfx4, which was highly expressed in NS/PCs positive for the *Msi1*-6IE reporter, bound to this region, and both of the palindromic Rfx binding sites were required for the transactivation of *Msi1*-6IE. Furthermore, ectopic Rfx4 expression in the developing mouse cerebral cortex transactivates *Msi1* expression in the intermediate zone. This study suggests that ciliogenic Rfx transcription factors regulate *Msi1* expression through *Msi1*-6IE in NS/PCs.

Introduction

NEURAL STEM CELLS (NSCs) are somatic stem cells that can generate the three major types of cells that constitute the central nervous system (CNS), that is, neurons, astrocytes and oligodendrocytes, and maintain themselves in an undifferentiated state during neural development. These cells are identified in the ventricular zone (VZ) of developing neural tube by selective markers, including the RNA-binding protein Musashi1 (Msi1) and the intermediate-filament protein Nestin [1].

The Musashi family is an evolutionarily conserved group of RNA-binding proteins that were isolated as mammalian homologues of the *Drosophila* Musashi [2–5]. In mammals, two Musashi proteins, Msi1 and Msi2, have been identified by characterization of two RNA recognition motifs (RRMs) [3,6]. From our previous studies using knock-out mice, Msi1 and Msi2 are known to contribute to the maintenance of stemness of neural stem/progenitor cells (NS/PCs) by repressing the translation of target mRNAs [4,7]. We have identified a consensus RNA sequence that binds to the RRM1s in both mammals [8] and *Drosophila* [9]. Msi1 binds to the 3'-untranslated region of the Notch signaling inhibitors *m-numb* [8] and *p21*^{WAF} [10] through RRM1s and is

thought to control stemness and the cell cycle through that interaction.

The transcriptional regulation of stem-cell markers has been intensively investigated, and these studies have made crucial contributions to the elucidation of the regulatory mechanisms of stem-cell fate and developmental biology. Key roles for transcription factors in rewiring transcriptional networks have been revealed by the investigation of genomic regions. The NSC-specific regulatory region of the *Nestin* gene was found in its second intron and contains binding sites for SOX (Sry-like HMG box protein) and POU (Pit-Oct-Unc) factors [11,12]. Oct4, a member of the POU family, directly binds with Sox2, and it forms a core transcriptional network with Nanog to activate or repress downstream genes for maintaining embryonic stem cells (ESCs) [13]. During the transition from ESCs to NSCs, Sox2 changes its partner from the POU family member to Brn2, and they cooperatively regulate the second intron enhancer of *Nestin* [14,15]. Indeed, Brn2 is important for the induction of NSCs [15]. Many transcription factors, including Tcf3, Smad1, Stat3, Esrrb, Zfx and Klf4, have been shown to play important roles in the control of ESC state; remarkably, the transcription factors associated with the LIF, Wnt, and BMP4 signaling pathways (Stat3, Tcf3, and

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Smad1) tend to co-occupy enhancers bound by Oct4, Sox2, and Nanog [13]. Compared with the transcriptional network in ESCs, the mechanisms in NSCs are not well understood. Thus, further investigation into the transcriptional network in NSCs is required.

We have previously demonstrated that the regulatory region of *Msi1* in NS/PCs is located in the sixth intron of the *Msi1* genomic locus (*Msi1*-6IE): The activity of a 184 kb *Msi1*-BAC reporter is dependent on the *Msi1* locus (exons and introns), including 595 bp of *Msi1*-6IE [16]. *Msi1*-6IE is highly conserved in mammals and is an active enhancer that is H3K4me1-positive and includes the p300-binding region in NS/PCs. The reporter gene containing *Msi1*-6IE shows activity specifically in *Msi1*-positive NS/PCs during the process of neural differentiation of ESCs to NS/PCs in vitro, and it does not show activity in differentiated neurons and astrocytes.

In the present study, we demonstrate that the *Msi1*-6IE reporter gene is specifically expressed in *Msi1*-positive NS/PCs in the embryonic and adult mouse brain and show that *Msi1* is regulated by a master regulatory factor for ciliogenesis, the regulatory factor X (Rfx) transcription factor, through *Msi1*-6IE.

Materials and Methods

Generation of the transgenic mice

The *Msi1*-P1-ffLuc (*Msi1*-P1) and *Msi1*-6IE-ffLuc plasmids (*Msi1*-6IE) have been previously generated and described [16]. DNA was linearized at a site within the vector with *Bsi*WI and *Apa*LI, and the plasmids were injected into the pronuclei of fertilized BDF1 background mouse eggs in RIKEN Brain Science Institute (BRC05764). Five lines of P1 and four lines of 6IE transgenic (tg) mice were obtained, and one line was chosen for the studies. All animal care and treatment procedures were performed in accordance with institutional guidelines approved by the Experimental Animal Care Committee of the Keio University School of Medicine (Approval number: 09091-10). Genotypes were determined by polymerase chain reaction (PCR) of genomic DNA prepared from tail biopsies. The primer sequences for the genotyping were as follows: 5'-CTACCTGAGCTACCA GTCCGCCCTG-3' and 5'-CAGCTAACGACATCTACAA CGAGCGCGAG-3'.

Histological analysis

Frozen sections (12 μm) of embryonic brains fixed with 4% (w/v) paraformaldehyde were prepared using a cryostat (CM3050S-IV; Leica). Adult brains were perfused with 4% paraformaldehyde, and the brains were dissected, postfixed with 4% paraformaldehyde at 4°C overnight, and sliced into 40-μm sections using a vibratome (VT1200S; Leica). To detect Mash1-positive cells, tissue sections were pretreated with ice-cold acetone for 30 s. The sections were preblocked with TNB blocking buffer (PerkinElmer) for 1 h at room temperature and incubated with primary antibodies at 4°C overnight, followed by incubation with Alexa Fluor-biotin-conjugated secondary antibodies for 1 h at room temperature. The Vectastain Elite ABC kit (Vector Laboratories) and Tyramide Signal Amplification-Red (PerkinElmer) were used for visualization. To detect BrdU incorporation, tissues were pretreated with 1 N HCl at 37°C for 30 min. The

following antibodies were used: anti-*Msi1* [rat immunoglobulin G (IgG); [43]; 1:400], anti-brain lipid binding protein (Blbp) (rabbit IgG; Chemicon AB9558; 1:200), anti-GFAP (rat IgG; Invitrogen 13-0300; 1:200), anti-Nestin (mouse IgG; BD 556309; 1:250), anti-Sox2 (rabbit IgG; Millipore mab377; 1:100), anti-Mash1 (mouse IgG; BD 556604; 1:200), anti-DCX (goat IgG; Santa Cruz Biotechnology sc-8066; 1:500), anti-βIII tubulin (mouse IgG; Sigma-Aldrich T8660; 1:500) anti-BrdU (rat IgG; Abcam ab6326; 1:100), and anti-GFP (chick IgY; Aves GFP-1010; 1:500). The images were captured by confocal laser-scanning microscopy (LSM700; Carl Zeiss).

Cell culture

Neurosphere culture. Cortical cells were dissected from E14.5 *Msi1*-6IE mice, pipetted 20 times and cultured in previously described media hormone mix (MHM) medium supplemented with 20 ng/mL fibroblast growth factor (FGF)-2 and 20 ng/mL epidermal growth factor (EGF) for 6 days [16,17].

Neuronal and glial differentiation of NS/PCs. The neurospheres were dissociated with TrypLE Select (Life Technologies), plated on poly-L-ornithine/fibronectin-coated 10-mm cover glass at a cell density of 1.5×10^5 cells/0.75 cm² in a 48-well culture plate and allowed to differentiate for 4 days in MHM with 1% fetal bovine serum.

Neural induction of ESCs. ESCs (EB3 tg14 line) were cultured in suspension conditions for 2 days and induced to neural lineage by the addition of all-trans retinoic acid (Sigma R2625) to a final concentration of 10^{-8} M for 4 days, as previously described [16,17].

Flow cytometer

Neurospheres were dissociated with TrypLE Select (Life Technologies), resuspended in MHM containing 1 mg/mL propidium iodide, and filtered (30 μm). Cell sorting and analyses were performed using a MoFlo (Beckman Coulter) flow cytometer/cell sorter equipped with CELLQuest software (Becton-Dickinson).

Plasmids

The genomic regions used to find the core enhancer in 6IE were generated with the following primers: 6IE 316 (5'-gtcgacGATCTGGGTCCAAGACGCAG-3' and 5'-atgcatCTCGCTCCCTGGCAGCCTATTC-3'), 6IE 161 (5'-gtcgacGATCTGGGTCCAAGACGCAG-3' and 5'-gtcgacCCTCCAA TCACAGCTGCTGGATCAG-3'), 6IE 178 (5'-gtcgacGATCCAGCAGCTGTGATTGGAGG-3' and 5'-gtcgacCCTCC AATCACAGCTGCTGGATCAG-3'), 6IE 279 (5'-gtcgacG GGGACAGCCGGGAATGCCAAG-3' and 5'-gtcgacCTCC TGAGGCTGGCTGAG-3'), 6IE 181 (5'-gtcgacCCGCCAT GGCTGCCACTCAG-3' and 5'-gtcgacCTCCTGAGGCTG GCTGAG-3').

Mutagenesis. The mutations of transcription-factor binding sites in 6IE were generated with the following primers: C/EBP (5'-AGCAGCTGTGtgcacAGGCCGCTG-3'), Rfx#1 (5'-CCGGGGCCGGCacgtaaGTGGCAACCGG-3'), Rfx#2 (5'-GGCGTTGCCGTacgcgtCGGGCCCCAGGA-3'), Rfx#2 for Rfx double mutation (5'-GGCACGTAAGTacgcgtCGGGC CCCAGGA-3'), Ap2 (5'-TGGCAACCGGttaCCAttAGAAG CCGTCA-3'), Gfi1 (5'-TTCGGGCCTAacgctGGCGTTGG-CA-3'), Nfi (5'-CCTAAATCGGCGTtggcatGTGAAACCGG

CC-3'). The underlined letters in the sequences represent the mutated base sequences. The primers were phosphorylated by T4-PNK, and the substituted sequences of *Msi1*-6IE plasmids were amplified by PCR and selected with *DpnI* treatments following with ampicillin selection of transformed *Escherichia coli*.

All Rfx expression vectors and Nfl expression vectors for the luciferase assay were constructed in an entry vector (pENTR-3xHA) and recombined into CIIEF-RfA-IRES2-Venus.

Rfx expression vectors for chromatin immunoprecipitation (ChIP)-PCR and in utero electroporation were constructed on pENTR-3xHA and recombined into pCAGGS2-RfA.

Luciferase assays

Neurospheres derived from mouse E14.5 telencephalons were dissociated, and 1×10^5 cells were cultured on 24-well plates in MHM (+20 ng/mL FGF-2, +20 ng/mL EGF) for 2 days, after which they were transfected with 400 ng reporter plasmids and pRL-SV40 as an internal control, using Gene Juice (Novagen). After 2 days, the cells were washed with phosphate-buffered saline and then lysed with 100 μ L lysis buffer (Toyo Ink) for 20 min at room temperature. For each sample, 25 μ L luciferase substrate solution (Toyo Ink) was added to 5 μ L lysate, and the luminescence was measured with a Berthold Centro LB960 luminometer. The assays were internally calibrated with a standardized *Renilla* luciferase solution (Toyo Ink) to ensure uniformity between experiments. For the reporter activity with transcription factors, 10 ng of the Rfx or Nfl expression vectors was co-transfected into NIH3T3 cells, which were cultured for 2 days and measured for luminescence as described above.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from cells with the RNeasy Mini Kit (Qiagen). Synthesis of cDNA was performed with Superscript II RNase H reverse transcriptase (Invitrogen) at 42°C for 50 min according to the manufacturer's instructions. Quantitative PCR was performed with SYBR Premix Ex Taq II (Tli RNaseH Plus) (RR820A; TaKaRa) in MX3000P Real-Time PCR Machine (Stratagene). The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay was conducted with amplification conditions at 95°C 10 min for enzyme activation, 50 cycles of denaturation at 95°C for 30 s, 60°C for 1 min for annealing, 72°C for 30 s for extension. Serial dilutions ranging from 10^8 , 10^6 , 10^4 , to 10^2 copies per 1 μ L of DNA solution were prepared as standard samples by subcloning each PCR product into the Zero Blunt TOPO PCR Cloning Kit (K2800; Invitrogen). To analyze the relative expression in different samples, the amount of cDNA was normalized to the level of ubiquitously expressed β -actin mRNA. The primer sets used were as follows: GFP (5'-TGAACCGCATCGAGCTGAAGGG-3' and 5'-TCCAGCAGGACCATGTGATCGC-3'), *Msi1* (5'-GGGATGGACGCCTCATGCTG-3' and 5'-TGGCTTGAACCCTGGGTAAC-3'), Rfx1 (5'-TAGTCCAGGCCACTCCACAGA-3' and 5'-TACTGCACCTGGCTGGAATACAC-3'), Rfx2 (5'-CCAGTTCCTGCTCAAGTGGTC-3' and 5'-ACACGGTGCTCCACAGGTA-3'), Rfx3 (5'-CAAGCGCTGAAGCCCTATGAA-3'

and 5'-CTAGCAGCACTGCGCAAGGTTA-3'), Rfx4 (5'-TTGCCTCATGACACAGCCATC-3' and 5'-TGCTCATCAAA CAAGGTTCCATC-3'), Rfx5 (5'-TACAGAAATTCTCGGA CAACGACAA-3' and 5'-TGC GGATCCACCTATAGGCAT AC-3'), Rfx6 (5'-CCAAGGGTCAACATTGCCTTC-3' and 5'-GTTCCACCATCGATCCAAGCCTA-3'), Rfx7 (5'-CCATT AAGTGCTGCTGACTTTGG-3' and 5'-TGGGCAGTGTG GCATATGAA-3'), β -actin (5'-GGTGGGCCGCCCTAGGC ACCA-3' and 5'-TTGGCCTTAGGGTTCAGGGG-3').

Chromatin immunoprecipitation-polymerase chain reaction

For ChIP-PCR, 3×10^6 NS/PCs derived from E14.5 cortex were electroporated with 30 μ g of CAGGS2-3xHA or pCAGGS2-Rfx4-3xHA using NEPA21 Type2 electroporator (NEPA GENE), and these cells were cultured on poly-L-ornithine/fibronectin-coated 10-cm dishes. After 3 days in culture, cells were cross-linked by incubation in 1% formaldehyde for 10 min, and chromatin fractions were sheared into 200-1,000-bp fragments by sonication, after which immunoprecipitation was conducted for 15 h at 4°C using the rat monoclonal anti-HA antibody (Roche 3F10). The resultant immune complexes were collected using protein G-beads, after which the DNA was purified by phenol/chloroform extraction, precipitated with ethanol and resuspended in water.

The primer sequences for the PCR reaction were as follows: 6IE core 1 (5'-GTGTCTTTGTGTCTGAGCACC-3' and 5'-CTGGCAGCCTATTCTCCAGCTC-3'), 6IE core 2 (5'-CACTAATCTGATCCAGCAGCTG-3' and 5'-GGTTT CACATGCCAACG-3'), Upstream (5'-CTTCTGTAGCCA GTTCAATCC-3' and 5'-CTGAGAGCTAGTGACCTCTC AG-3'). Downstream (5'-CTATTAACAGCTGAAGCAGC TG-3' and 5'-GATTCCACAGTCTGTAAATGG-3').

In utero electroporation

A plasmid expressing GFP driven by the CAG promoter was used with either an empty pCAGGS2-3xHA vector or pCAGGS2-Rfx4-3xHA. The introduction of DNA by in utero electroporation was performed essentially as described previously [18]. Briefly, ICR pregnant mice were anesthetized with isoflurane and nitrous oxide, and 1 μ L of DNA solution was injected into the lateral ventricle at E13.5. The solution contained 0.3 μ g GFP plasmid and 3 μ g of either the Rfx4 plasmid or the empty vector. After injection, electroporation was performed using CUY-21 (NEPA GENE) to deliver eight cycles of square pulse (50-ms pulses of 37 V with 950-ms intervals) per embryo. Embryos were reimplanted in utero, and embryonic brains were harvested 3 days later.

Results

Msi1-6IE reporter gene is expressed in *Msi1*-positive NS/PCs in vivo

To evaluate *Msi1*-6IE reporter expression in vivo, we generated *Msi1*-6IE reporter tg mice. We used *Msi1*-6IE constructs containing 595 bp of 6IE aligned downstream of ffluc, as previously reported ([16], 6IE is described as D5E2 in this paper) (Fig. 1A). The ffluc reporter gene is a fused gene of Venus and firefly luciferase sequences, and it allows both visualization of the expressing cells by

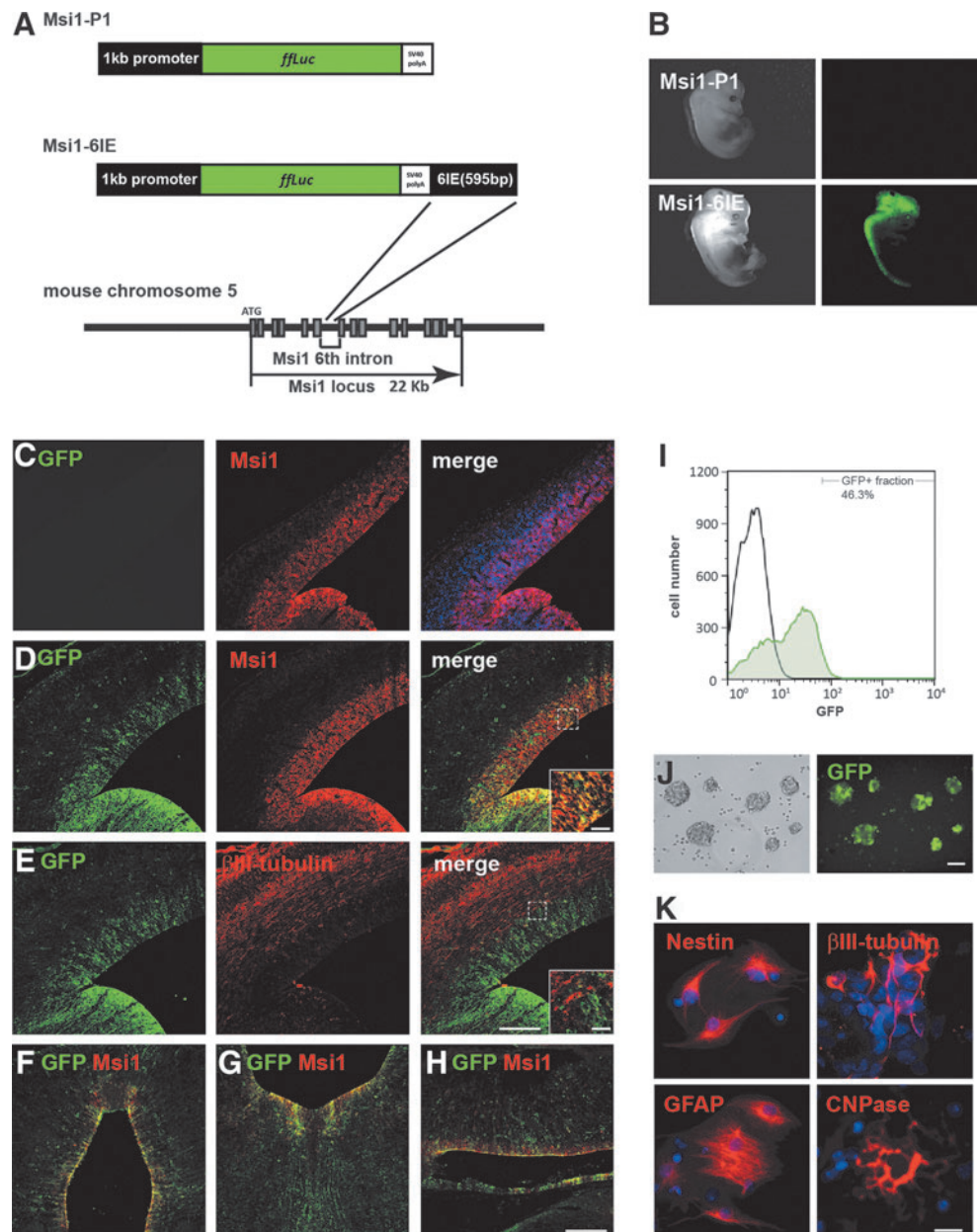


FIG. 1. Expression of the *Msi1*-6IE reporter corresponds to endogenous *Msi1* expression in NS/PCs in vivo. **(A)** Schematic *Msi1*-P1, *Msi1*-6IE and *Msi1*-BAC reporter constructs are shown. A 1-kb *Msi1* promoter was allocated at the 5' end of the *ffLuc* reporter gene in *Msi1*-P1 and *Msi1*-6IE constructs. 6IE was aligned at the 3' end of the SV40 polyA, which mimics its intronic enhancer position in *Msi1*-BAC and genomes. **(B)** Stereomicroscopic activity of the reporter genes in vivo. *ffLuc* activity was observed in the nervous systems of all *Msi1*-6IE Tg mice (four of four), and no expression was observed in *Msi1*-P1 (five of five). **(C–E)** Immunohistochemical analysis of the reporter expression in embryonic Tg mouse cortex. *Msi1* expression colocalized with Venus expression in undifferentiated cells in the ventricular zone of E14.5 *Msi1*-6IE **(D)** but not with β -III tubulin-positive neurons **(E)**. Venus expression in *Msi1*-P1 cortex could not be detected in any embryonic stage (E12.5 cortex is shown). The *dashed boxes* are magnified and shown in the *right-hand panels* (Scale bar: 10 μ m). **(F–H)** Reporter expression in E14.5 midbrain **(F)** and hindbrain **(G, H)**. Scale bar: 100 μ m. **(I)** Dissected *Msi1*-6IE mice cortex were dissociated and assessed by flow cytometry, and the Venus-positive fraction was selected based on fluorescent intensity. Tg-mice were used as a negative control. The ratio of Venus-positive cells to the total Tg+ cells is indicated. **(J)** Neurospheres derived from Venus-positive cells cultured for 4 days. *Left panel*: brightfield, *right panel*: fluorescence. Scale bar: 200 μ m. **(K)** Cells derived from Venus-positive neurospheres differentiated into β III-tubulin-positive neurons (*upper right*), GFAP-positive astrocytes (*lower left*) and CNPase-positive oligodendrocytes (*lower right*) after culture for 4 days without growth factors, or remained undifferentiated as Nestin-positive cells when cultured in the growth condition (*upper left*). Scale bar: 20 μ m. NS/PCs, neural stem/progenitor cells; Tg, transgenic.

fluorescence and quantification of the transcriptional activity by bioluminescence [19]. A 1-kb *Msi1* promoter upstream of the *Msi1* transcription start site was placed in the upstream portion of ffLuc. Similar to the Venus expression in 180kb *Msi1*-BAC tg mice [16], GFP fluorescence was observed in the CNS from forebrain to spinal cord of the E12.5 *Msi1*-6IE tg mice (four of four lines) (Fig. 1B), and this fluorescence was not detected with the 1-kb *Msi1* promoter reporter tg without 6IE (*Msi1*-P1, 0 of 5). Next, we examined the immunohistochemical characterization of Venus (which can be detected with anti-GFP antibody) and endogenous *Msi1* expression using embryos from P1 and 6IE tg mice (Fig. 1C, D). Venus expression was observed in the VZ and subventricular zone (SVZ) in *Msi1*-6IE tg mice, where NS/PCs are present, and colocalized with *Msi1* in E14.5 cortex (Fig. 1D), midbrain (Fig. 1F) and hindbrain (Fig. 1G, H). Venus-expressing cells were not stained with the neuronal marker β III tubulin (Fig. 1E). We could not detect the Venus expression in *Msi1*-P1 tg mice brain by immunostaining with GFP antibody (Fig. 1C). These results support our previous in vitro data showing that 6IE regulates *Msi1* expression in the neural lineage and that the 1-kb promoter region is not sufficient for transactivation of the reporter gene.

Msi1-6IE-positive cells are self-renewing and multipotent in vitro

To reveal the properties of Venus-expressing NS/PCs, we dissected and dissociated the cortex from E14.5 *Msi1*-6IE tg mice, and Venus-positive cells were sorted by flow cytometry. The Venus-positive cells proliferated in culture medium containing FGF-2 and EGF, which maintains Venus expression, and formed neurospheres in floating culture (Fig. 1I, J). The neurospheres were dissociated and cultured in differentiation medium without growth factors to test the multipotency of *Msi1*-6IE cells by immunocytochemistry. Under the growth conditions, *Msi1*-6IE cells expressed the NS/PC marker Nestin (Fig. 1K). Under the differentiation conditions, the purified NS/PCs produced β -III-tubulin-positive neurons, glial fibrillary acidic protein (GFAP)-positive astrocytes and 2',3'-cyclic nucleotide 3' phosphodiesterase-positive oligodendrocytes (Fig. 1K).

Msi1-6IE reporter recapitulates the spatio-temporal regulation of endogenous *Msi1*

To gain insight into the temporal regulation of 6IE, we immunostained adult *Msi1*-6IE tg mouse brain and analyzed the distribution of Venus-positive cells in the hippocampus and cortex, where NS/PCs are present. In the subgranular zone (SGZ) of hippocampus, radial-glia-like stem cell (Type 1 cell) gives rise to three consecutive stages of transiently amplifying PCs (Type 2a, Type 2b and Type 3 cells). These stages lead to a differentiation of transient postmitotic granule cells (GCs) following to terminally differentiated mature GCs [20]. Endogenous *Msi1* is mostly expressed in the SGZ, colocalized with cells expressing *Blbp*, a marker of Type 1 and Type 2a cells, and it is not expressed in NeuN-positive mature neuronal cells (Supplementary Fig. S1A, C; Supplementary Data are available online at www.liebertpub.com/scd). A very small population of *Msi1*-positive cells expressed doublecortin (*Dcx*), which is expressed during neuronal differentiation

process from Type 2b to immature GCs (Supplementary Fig. S1B). Thus, endogenous *Msi1* is specifically expressed in NS/PCs in adult hippocampus (Supplementary Fig. S1X). *Msi1*-6IE reporter expression was observed in two morphologically distinct cell types in the SGZ. One population displayed radial-glia-like cell morphology, with a long process, and the other population lacked the radial process (Supplementary Fig. S1D). The *Msi1*-6IE-Venus cells strongly colocalized with *Msi1* (Supplementary Fig. S1E), *Blbp* (Supplementary Fig. S1F) and *Sox2* (Type 1 and Type 2a marker) (Supplementary Fig. S1J) ($93.6 \pm 2.3\%$, $95.7 \pm 0.9\%$, $87.3 \pm 3.1\%$, respectively, double-positive cells/total Venus-positive cells, mean \pm standard error of the mean, total cell number: 544, 714 and 558, respectively), and colocalized with 17.9% of the *Dcx*-positive cell population (total cell number: 546) (Supplementary Fig. S1G) and never stained with NeuN (Supplementary Fig. S1N). These *Dcx* or Venus double-positive cells did not have radial processes. Although both Type 1 cells and *Dcx*-positive GCs extend their processes to the granular layer, the radial processes in Venus-positive cells were stained with Type 1 markers *Blbp* (Supplementary Fig. S1I) and GFAP (Supplementary Fig. S1L) but were negative for *Dcx* (Supplementary Fig. S1K), and non-radial Venus-positive cells were Mash1-positive Type 2a precursors (Supplementary Fig. S1M) or *Tbr2*-positive Type 2b cells (data not shown for *Tbr2* immunostaining). The 2-week BrdU pulse-labeling experiments showed that a small fraction of Venus-positive cells were proliferating (Supplementary Fig. S1O). Based on these observations, most of the Venus-expressing cells were *Msi1*-positive Type 1 and Type 2a cells, and some were Type 2b or Type 3 cells.

In the cortex, Venus-positive cells were observed in subependymal zone along the lateral ventricle. These cells showed good co-localization with *Msi1* (Supplementary Fig. S1T). The majority of endogenous *Msi1*-positive cells were observed in *Sox2*-positive and Nestin-positive Type B radial glia-like cells or Type C precursors (Supplementary Fig. S1P, Q), and some PSA-NCAM-positive and *Dcx*-positive Type A neuroblasts were *Msi1* positive cells during neuronal differentiation process (Supplementary Fig. S1R, S). The *Msi1*-6IE reporter was able to mimic the expression in these cells (*Mash1* was used for labeling Type C cells. See Supplementary Fig. S1U–W). Based on these observations, we concluded that the transactivation of the sixth intronic enhancer can reflect the authentic spatio-temporal expression of *Msi1*.

The 178-bp core enhancer site is responsible for the transactivation of *Msi1*-6IE

Msi1-6IE includes many transcription-factor binding sites, and several candidate binding sites have been observed from the correlation of the localization with *Msi1* and/or the functional aspects with *Msi1*, as we previously reported [16]. For example, *Sox* and *Ap-2* are expressed in NS/PCs in a subtype-specific fashion and control the balance between proliferation and differentiation [21–24]. To elucidate which transcription factors regulate *Msi1*, we performed a deletion study of the reporter gene to find the regulatory region responsible for the transactivation of 6IE. We first generated two *Msi1*-6IE reporter constructs that were gradually shortened from the 3' end of 6IE (6IE-316

and 6IE-161) (Fig. 2A). The luciferase activity of fLuc in 6IE-316-transfected cells was significantly higher than with the 1-kb promoter alone (P1) at a level similar to the full length of the 6IE reporter gene in NS/PCs derived from E14.5 cortex. However, the activity of 6IE-161 was markedly decreased, to almost the same level as P1 (Fig. 2B). These results indicated that the critical regulatory region was included in the region from 161 to 316 bp of 6IE. To confirm this result, we generated reporter genes including the common sequences between 6IE-161 and 6IE-316 (6IE-178), the region between full-length 6IE and 6IE-316 (6IE-279), and the 3' 181 bp of 6IE (6IE-181). 6IE-178 had transcriptional activity at the same level as 6IE-316, whereas 6IE-279 and 6IE-181 showed low activity, consistent with their relationships with 6IE-316. These data show that 6IE-178 is required for the transactivation in NS/PCs as a cis-element, suggesting the presence of important transcription factor(s)-binding sites regulating *Msi1* transcription in this 178-bp genomic region.

Palindromic Rfx transcription-factor binding sites determine the efficiency of transactivation in the 6IE region

To identify the transcription factor(s) regulating *Msi1*, we searched for potential transcription-factor binding sites in 6IE-178 by in silico analysis, and we observed that there were highly homologous motifs with consensus sequences for NF-Y (or C/EBP), Rfx, Ap-2, Gfi1 and Nfi (Fig. 2C). Two palindromic Rfx-binding sites (5'-GTTGCC-3') were present across two bases of 5'-GT-3' (Rfx#1 and Rfx#2). We compared the genomic sequences of 6IE-178 among 60 species including 40 mammals and 20 other species including fishes, reptiles, birds and amphibians (UCSC genome browser conservation track). Each binding site was identical among the most of mammals including mouse, rat, human and dog, and Rfx, Ap-2 and Nfi sites were completely conserved even in lizard (*Anolis carolinensis*). To test whether these binding sites were required for activation of 6IE, we disrupted them by introducing mutations (see the "Materials and Methods" section for their mutation sequences). Unexpectedly, when either of the mutations in the Rfx-binding sites was introduced into 595 bp of *Msi1*-6IE reporter gene, enhancer activity was obviously attenuated in NS/PCs (Fig. 3A, B). The mutation in the Nfi-binding site also showed mildly but significantly decreased activity against wild type (no mut.), but the mutations in other binding sites were not significantly different. Noteworthy, simultaneous mutations of both Rfx#1 and Rfx#2 sites did not exhibit any additive effect compared to single mutants. These results suggest that the Rfx binding sites and the Nfi binding site are involved in the regulation of *Msi1*-6IE.

Genes encoding seven Rfx proteins have been identified in vertebrates, based on a highly conserved DNA binding domain [25–27]. The conserved dimerization domains, which mediate homo- and heterodimerization, are present in Rfx1 through 4 but are lacking in Rfx5 and Rfx7. Rfx proteins recognize a highly conserved regulatory element called the X-box, which is frequently observed as a palindromic sequence (5'-GTNRCC (0-3N) RGYAAC-3') [28].

To establish the contribution of Rfx transcription factors to the regulation of the *Msi1*-6IE, we exogenously expressed

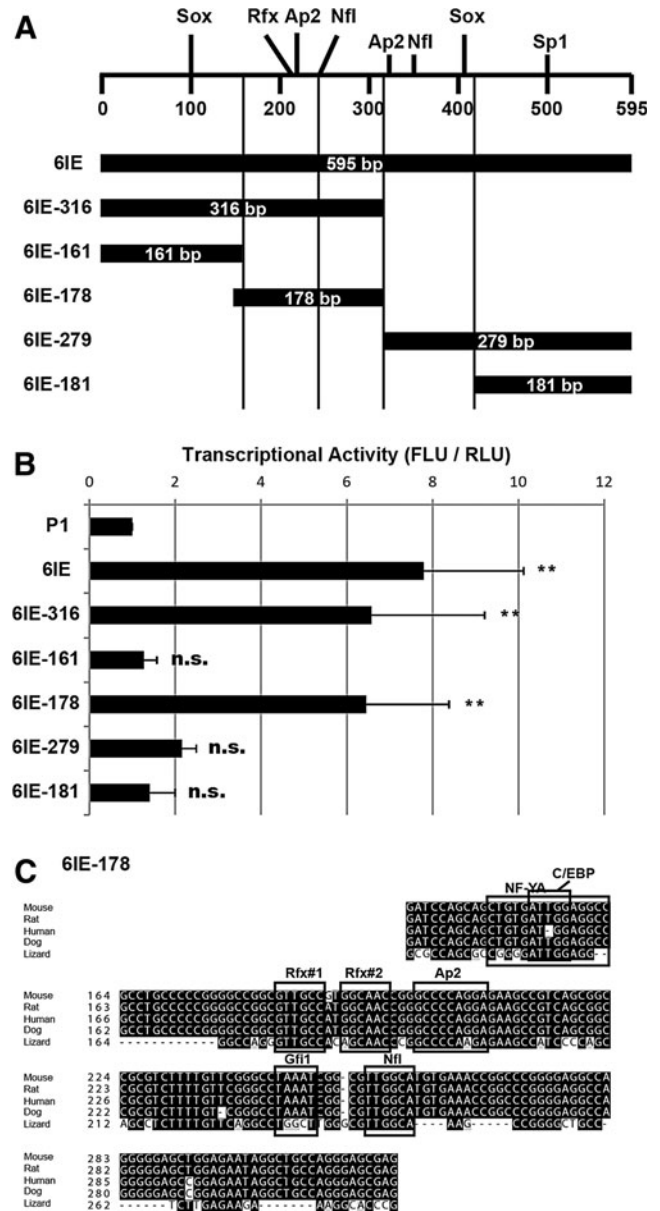


FIG. 2. Identification of core enhancer site in 6IE. (A) Map of *Msi1* sixth intron fragments assayed for finding the genomic region responsible for *Msi1* transcription in NS/PCs. (B) Relative luciferase activity of six *Msi1*-6IE reporters and *Msi1*-P1 (P1, P1=1). The data represent the mean \pm SEM of three independent experiments. The data were subjected to non-repeated-measures ANOVA, and *P*-values were calculated with Bonferroni multiple comparison tests. ***P* < 0.01; n.s., not significant. FLU/RLU: firefly luciferase light unit/renilla luciferase light unit. (C) Nucleotide sequence of 178 bp-genomic region in mouse 6IE which was responsible for *Msi1*-6IE reporter activity. The sequence is aligned with the corresponding sequences of rat, human, dog and lizard. Black shading indicates conserved nucleotide sequences. Transcription-factor binding sites shown in the boxes were identified from the JASPAR CORE database. SEM, standard error of the mean; ANOVA, analysis of variance.

Rfx1-4 in NIH3T3 cells (Fig. 3C). When each Rfx was expressed, the cotransfected *Msi1*-6IE reporter was activated (no mut. in the Rfx expression) compared to reporter alone (no gene). We also tested whether functionally disrupted Rfx4 expression (Rfx4^{L298P}) could activate wild-type 6IE. Rfx4^{L298P} has a point mutation in the dimerization domain and shows impaired transcriptional activation ability due to its failure of nuclear localization, as previously reported [29]. As expected, the Rfx4^{L298P} could not activate the *Msi1*-6IE reporter compared to wild-type Rfx4 expression (Fig. 3C).

To confirm that the reporter activities induced by Rfx transcription factor were dependent on Rfx binding sites,

Msi1-6IE reporters carrying either Rfx#1 or Rfx#2 mutations or Rfx#1 and Rfx#2 double mutation were used for the luciferase assay (Fig. 3C). The overactivation produced by the expression of each Rfx was significantly attenuated when mutations in #1, #2 or both (#1 and #2) were introduced into the 6IE sequence. Each single mutation repressed the activity to the same degree as the double mutations. The similar results were obtained by the reporter assay in NS/PCs (Fig. 3A). Thus, the palindromic Rfx binding sites are necessary for efficient 6IE activation, suggesting the dimer of Rfx transcription factors may regulate 6IE.

Rfx expression in embryonic forebrain and NS/PCs

Rfx members belong to the winged-helix subfamily of helix-turn-helix transcription factors, which have been identified in various eukaryotic organisms from yeast to human [30]. The expression levels of Rfx family transcription factors vary among mouse tissues [25]. Rfx1, Rfx5 and Rfx7 are ubiquitously expressed in essentially all types of tissues, whereas Rfx6 shows highly tissue-specific expression in liver, heart and pancreas. Despite these many differences, all Rfx transcription factors except for Rfx6 are highly expressed in brain. The loss of Rfx4 function results in pleiotropic brain defects, and ablation of Rfx3 produced hydrocephalus and abnormal differentiation of ependymal cells [28,29,31]. Knockdown of Rfx7 results in failure of neural tube closure in *Xenopus* embryos [32]. However, the precise localization of Rfx genes in brain is poorly understood. To gain a quantitative understanding of the expression of Rfx in NS/PCs, we quantified the mRNA levels of *Rfx* genes in neurospheres and embryonic forebrains by qRT-PCR. All of the *Rfx* genes except for Rfx6 were detected in the forebrain from E12.5 to E18.5 (Fig. 4A). *Rfx1*, *Rfx3*, *Rfx4* and *Rfx7* were also strongly expressed in neurospheres dissociated from the E12.5 to E18.5 cerebral cortices. Noteworthy, at any stage examined, the expression levels of *Rfx4* were much higher in neurospheres than in the corresponding forebrains (Fig. 4B). These results suggest that *Rfx4* is highly expressed in cortical

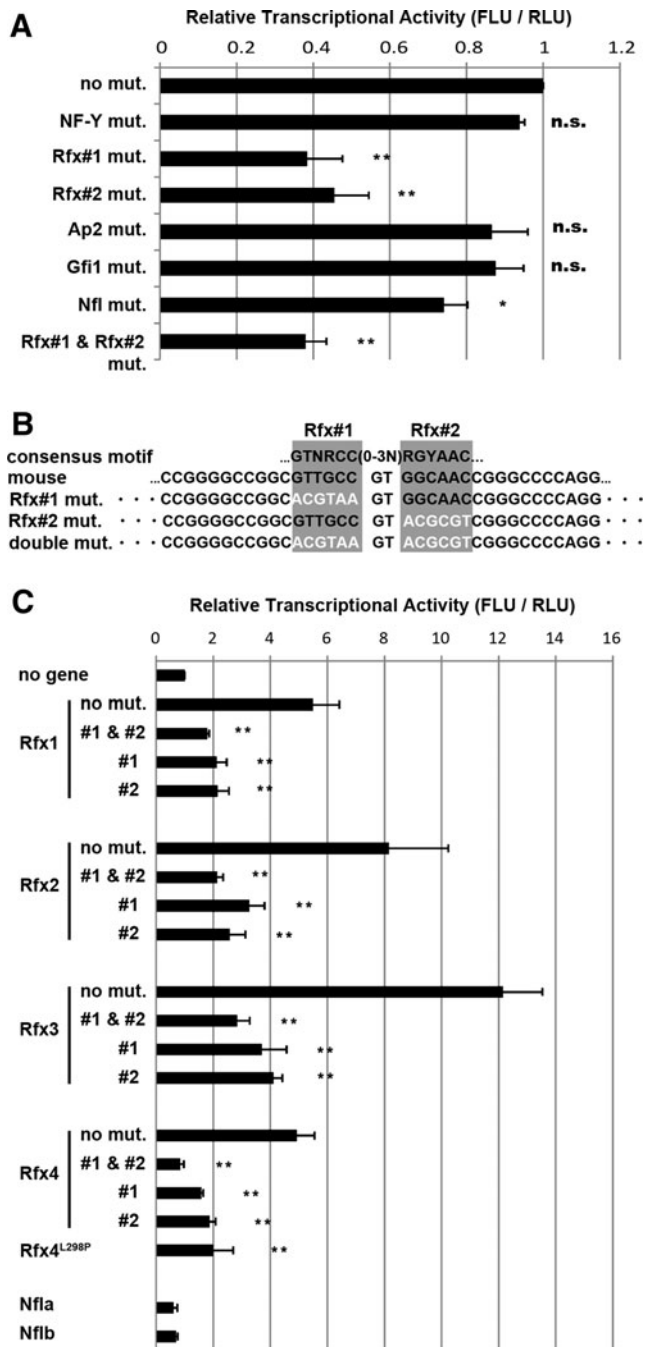


FIG. 3. Rfx transcription factor binding sites regulate 6IE. (A) Activity of *Msi1*-6IE and the mutant forms of its transcription factor binding sites in NS/PCs derived from E14.5 cortex. The activity of wild-type *Msi1*-6IE (no mut.) = 1. (B) Nucleotide sequences of palindromic Rfx binding sites aligned in mouse 6IE (Rfx#1 and Rfx#2) completely match with consensus motif. Mutated sequence of each Rfx binding site (Rfx#1 mut. or Rfx#2 mut.) and both Rfx binding sites (double mut.) used in this figure are shown in white. (C) Activation of *Msi1*-6IE and mutant forms of *Msi1*-6IE by Rfx expression. Each *Msi1*-6IE construct is cotransfected with an Rfx expression vector (Rfx1, Rfx2, Rfx3 and Rfx4, respectively) in NIH3T3 cells. Single Rfx binding site mutants (#1 or #2) or the double-mutant (#1 and #2) was used to assess the ability of Rfx to transactivate the 6IE region. No gene indicates *Msi1*-6IE activity without exogenous transcription factor expression (no gene = 1). The data were compared within each Rfx expression experiment (“no mut.” vs. “#1,” “#2” or “#1 and #2”) and subjected to non-repeated-measures ANOVA, and *P*-values were calculated with Bonferroni multiple comparison tests. ***P* < 0.01, n.s., not significant. Rfx, regulatory factor X.

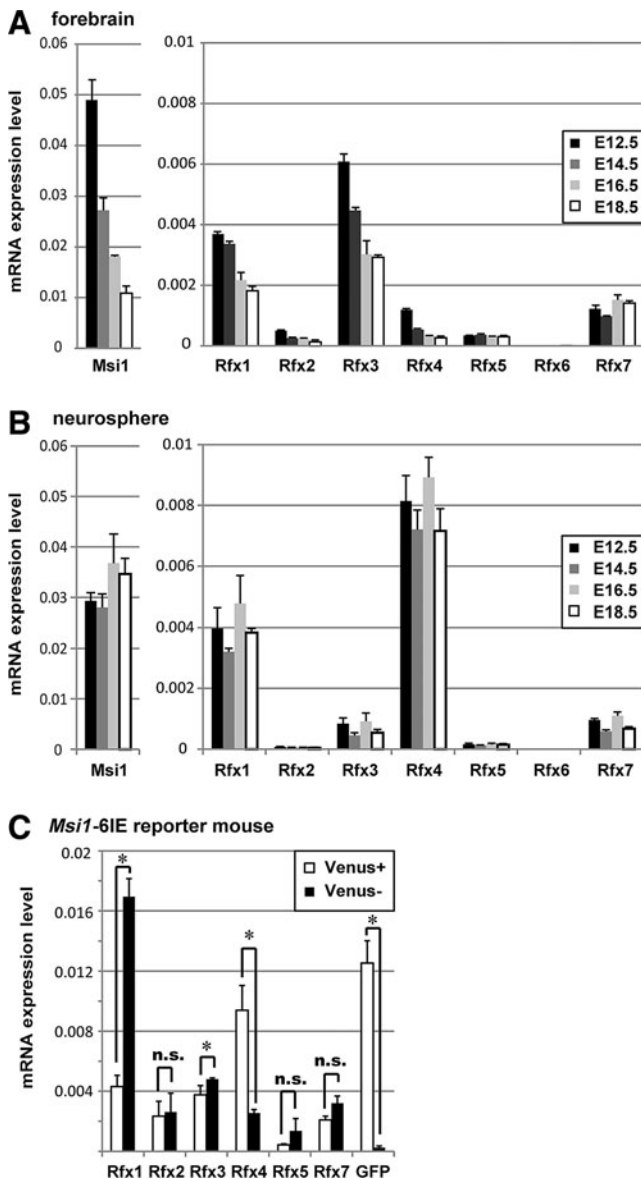


FIG. 4. Quantification of *Rfx* expression in NS/PCs. (A, B) Quantitative analysis of *Msi1* and *Rfx* mRNA expression by qRT-PCR in E12.5-E18.5 forebrain (A) and neurospheres dissociated from cortex and cultured for 6 days (B) ($n=3$, mean \pm SEM of three independent experiments). *Rfx4* is more highly expressed in neurospheres than in forebrain. (C) *Rfx* expression levels in *Msi1*-6IE-positive and -negative cells. Day 6 neurospheres derived from E14.5 *Msi1*-6IE Tg mice cortex were dissociated and divided into Venus-positive and Venus-negative cells by flow cytometry, and mRNA expression levels were quantified by qRT-PCR. Venus expression was quantified with GFP primers to confirm the selection of Venus-expressing or non-expressing cells. The data were compared between *Msi1*-6IE-positive cells and -negative cells in each *Rfx* primers and subjected to Mann-Whitney's U test ($n=3$, mean \pm SEM of three independent experiments, * $P < 0.05$, n.s., not significant). qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

NS/PCs. To address the regulation of *Msi1*-6IE by *Rfx*, NS/PCs dissociated from neurospheres derived from the cortices of E14.5 *Msi1*-6IE tg mice were divided into Venus-positive and Venus-negative cells by flow cytometry, and the expression of *Rfx* genes was quantified in each population (Fig. 4C). Consistent with the higher expression in neurosphere culture, the expression of *Rfx4* was significantly highly enriched in Venus-positive NS/PCs compared with Venus-negative cells. In contrast, *Rfx1*, *Rfx3*, and *Rfx7* did not exhibit such an enrichment in Venus-positive cells. We also analyzed the expression of *Rfx* genes in Nestin-second intronic enhancer reporter mice (*Nestin*-d4-Venus) in embryonic forebrain to confirm this finding in NS/PCs (Supplementary Fig. S2A) [33]. Consistent with the expression of *Rfx4* in VZ of forebrain revealed by in situ hybridization [28], significantly higher amounts of *Rfx4* mRNA were found in *Nestin*-Venus positive NS/PCs than in Venus-negative cells. *Rfx3* had a similar expression pattern to *Rfx4*, probably because these samples included the ventral cortex area. *Rfx3* was detected in the ganglionic eminence by immunohistochemistry, but it was very weakly expressed in dorsal cortex (data not shown). Further analysis in embryoid bodies derived from ESCs was conducted to compare *Rfx* expression to *Msi1* expression during the process of differentiation to NS/PCs (Supplementary Fig. S2B). *Msi1* expression was increased along with neural differentiation, and it was relatively high in neural induced cells (+RA) and low in -RA cells. *Rfx4* expression was increased in a similar way as the *Msi1*, and it was specifically expressed by neural induction, whereas non-neural *Rfx6* showed opposite results. All of these expression profiles suggested that *Rfx4* was the most likely candidate gene involved in the transcriptional regulation of *Msi1* transcription among the seven *Rfx* genes, although there may be redundant regulation by *Rfx1*, *Rfx3* and *Rfx7* in NS/PCs.

Binding of *Rfx4* to the *Msi1*-6IE in NS/PCs

To test whether *Rfx4* binds to the 6IE in NS/PCs, ChIP followed by PCR (ChIP-PCR) was performed (Fig. 5). The NS/PCs dissociated from the neurospheres derived from E12.5 cortex were electroporated with control plasmid or HA-tagged *Rfx4* expression plasmid. The *Rfx4*-binding genomic regions were immunoprecipitated with anti-HA antibody. Only the 6IE region precipitated by *Rfx4* was amplified, suggesting that *Rfx4* binds to 6IE but not to each region upstream or downstream of 6IE.

Overexpression of *Rfx4* shows ectopic *Msi1* expression in vivo

Because *Rfx4* transactivated the regulatory region of *Msi1* in NS/PCs, we examined whether overexpression of *Rfx4* could ectopically promote the endogenous *Msi1* expression in the cerebral cortex. HA-*Rfx4*, along with GFP, was overexpressed in the developing dorsal cortex by in utero electroporation at E13.5. At E15.5, the control GFP-expressing cells were mainly observed in the intermediate zone (IZ), and those cells did not express *Msi1* (Fig. 6). In contrast, HA-*Rfx4*-expressing cells in the IZ ectopically and highly expressed *Msi1*. Taken together, these results demonstrate that *Rfx4* induces endogenous *Msi1* expression through *Msi1*-6IE in the developing nervous system.

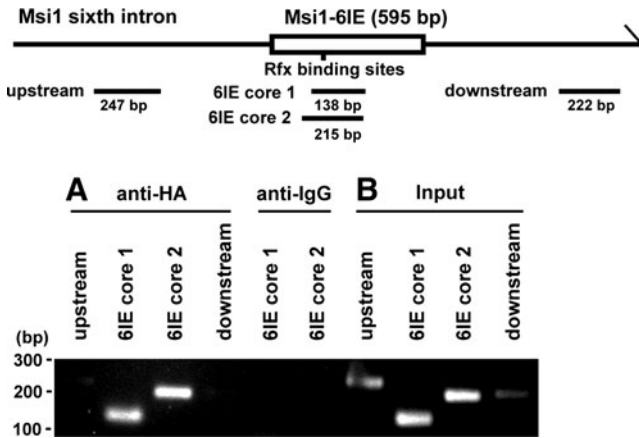


FIG. 5. Rfx4 binds to the *Msi1*-6IE region. chromatin immunoprecipitation-PCR analysis of Rfx4 binding to *Msi1*-6IE in NS/PCs. (A) Immunoprecipitation with anti-HA for Rfx4 binding or anti-immunoglobulin G for the negative control. The various regions in *Msi1* sixth intron indicated on the top were amplified by PCR. The upstream region or the downstream region of *Msi1*-6IE were about 500-bp or 700-bp distant from Rfx binding sites respectively, and two of the 6IE core regions contained Rfx binding sites. (B) Input DNA samples were amplified with the same primer sets.

Discussion

To better understand the regulation of NS/PCs, we focused on the transcriptional regulation of the *Msi1* gene, which marks these cells. Our study took advantage of the properties of *Msi1* based on the previous observation that *Msi1* is expressed preferentially in NS/PCs in embryonic brain and functions to maintain the NS/PCs in an undifferentiated state. In this study, we show that *Msi1* expression is regulated by Rfx transcription factors through the sixth intronic enhancer of the *Msi1* gene in NS/PCs.

We previously identified the NS/PC-specific enhancer of the *Msi1* gene in the sixth intron [16]. The stable lines of

ESCs containing the *Msi1*-6IE reporter gene showed the increased activity by the induction to neural lineage in embryoid bodies, and they continued the expression of reporter gene in tertiary neurosphere culture in vitro. Therefore, 6IE was thought to be involved in *Msi1* expression from developmental stages to adult brain in vivo. In the present study, we confirmed this temporal expression by using the *Msi1*-6IE tg mouse in both embryonic and adult brain. Further, the *Msi1*-6IE reporter expression was well colocalized with endogenous *Msi1*-positive NS/PCs, and these cells were negative for neuron markers. Our present data indicate that 6IE is the authentic regulatory region for *Msi1* transcription, and it is useful for understanding the transcriptional regulation of *Msi1*. While endogenous *Msi1* is highly expressed in NS/PCs, it is also weakly expressed in GFAP-positive astrocytes in adult brain [34]. The reporter expression in *Msi1*-BAC tg mouse was confirmed in GFAP-positive astrocytes [16]. In contrast to these expression patterns, *Msi1*-6IE reporter expression was not detected in GFAP-positive astrocytes in the cerebral cortical layer and in the area outside the SGZ by immunostaining with anti-GFP antibody (Supplementary Fig. S1E). This result suggests that *Msi1*-6IE is an active genomic region specifically in NS/PCs, and the potential regulatory regions for *Msi1* transcription in astrocytes are present in other regulatory regions included in the 180-kb *Msi1*-BAC reporter.

We identified a 178-bp core element of 6IE containing NF-YA (or C/EBP), palindromic Rfx, Ap-2, Gfi1 and Nfl binding sites. It has been reported that the NF-Y transcription factor regulates Sox3 promoter activity: Sox3 is expressed in NSCs from the earliest stages of development [35,36]. It is also known that NF-Y consists of three subunits, NF-YA, NF-YB, and NF-YC, and binds to the CCAAT sequence of the Y box in the major histocompatibility complex class II (MHC-II) promoter [37]. Rfxank forms a complex with the Rfx5 dimer and Rfxap, and this complex associates with NF-Y to transactivate the MHC-II gene [38,39]. From these reports, we hypothesized that NF-Y and Rfx form a complex for *Msi1* transcription in the CNS. Therefore, we disrupted each binding site in *Msi1*-6IE.

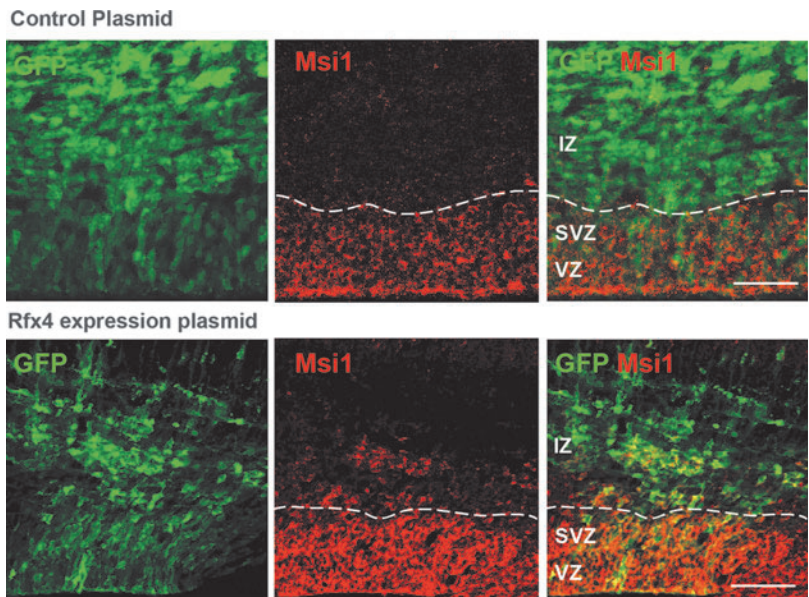


FIG. 6. Rfx4 expression shows ectopic *Msi1* expression in IZ. Immunostaining of cortical periventricular layers at E15.5 after in utero electroporation of control plasmid (upper) and Rfx4 (lower) at E13.5. *Msi1* expression in IZ was observed only in Rfx4-electroporated slices with colocalization with GFP. IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: 50 μ m.

The reporter activity was significantly repressed by the mutation in tandem Rfx binding sites, but it was not affected by mutation of the NF-Y binding site in NS/PCs. This result indicates that Rfx regulates 6IE, but NF-Y is not required for the transactivation. The mutation in the Nfi binding site in the *Msi1*-6IE reporter gene also reduced transcriptional activity. Nfla, Nflb, and Nflx are expressed in the developing mouse brain by E15 and E16, and Nfla and Nflb are expressed in the VZ and SVZ [40]. Nfi targets the GFAP promoter prior to the induction of astrocyte differentiation in NS/PCs [41,42]. Because endogenous *Msi1* is also expressed in both NS/PCs and in the astrocyte [43], we tested whether the *Msi1*-6IE reporter was activated by Nfla or Nflb expression in NIH3T3 cells. However, these Nfi proteins could not transactivate the reporter. This result is consistent with the *Msi1*-6IE reporter expression in *Msi1*-6IE tg mice, that is, the activity was specific to NS/PCs and was not detected in astrocytes in the cerebral cortex or hippocampus. Thus, it was conceivable that other transcription factors regulate this Nfi-binding site. AP2 and Gfi1 were other candidates for 6IE regulatory factors. The AP2 binding sequence in 6IE-178 (GCCCCAGG) was homologous to the consensus AP2 γ binding sequence (GCC^CT_G^G/C^GA_CGG^G/C) [44]. AP2 γ is expressed in Pax6-positive NSCs, where *Msi1* is expressed, and a subset of apical VZ progenitors, and it specifies basal progenitor fate in the occipital cortex [45]. The Gfi1 proto-oncogene encodes a zinc-finger protein that binds to the sequence AATC, and the mRNA of Gfi1 is expressed in the developing brain and in several specialized sensory cells of the peripheral nervous system [46]. The binding sites for AP2 and Gfi1 are conserved in mammals; thus, we disrupted the AP2 and Gfi1 binding sequences, but significant differences of reporter activities were not observed with mutant constructs compared to the wild-type sequence of the 6IE reporter.

In addition to the repression of the *Msi1*-6IE reporter activity by the introduction of mutations in the Rfx binding sites, the expression of all four Rfx transcription factors could significantly activate the *Msi1*-6IE reporter gene. ChIP-PCR experiment revealed that Rfx4 binds to the 6IE region of the genome in NS/PCs (Fig. 5). Further supporting ChIP-sequence data was reported in Creighton et al. [53] that clearly showed that Rfx1 also binds to 6IE with an active enhancer signature (H3K27ac) in NS/PCs (Supplementary Fig. S3) [15]. Moreover, the exogenous Rfx4 expression in the developing cortex could transactivate the endogenous *Msi1* expression ectopically in the IZ. Rfx transcription factors bind to tandem binding sites, called the “X-box,” in the regulatory regions of several genes, including IL-5Ra, HLA-DRA, and c-myc, and disruption of one of the two binding sites inhibits the DNA binding of Rfx [30]. Our luciferase assays for *Msi1*-6IE activity agreed well with these observations. The transcriptional activities of single mutations in each Rfx binding site were sufficient to reduce the activity to the same level as the mutations of both binding sites. Taken together, we concluded that Rfx transcription factors regulate *Msi1* expression through *Msi1*-6IE.

Rfx is known as a master transcription factor for the development of both primary cilia and motile cilia. The loss of a Rfx transcription factor known as Daf-19 in *Caenorhabditis elegans* causes the absence of cilia [47], and Rfx2 and Rfx3 are broadly required for the proper devel-

opment of cilia in mammals [48,49]. Rfx4 has been reported to be a key regulator of primary cilia development that modulates sonic hedgehog signaling in the neural tube [29,50]. The lack of *Rfx4* transcripts or the functional disruption of *Rfx4* (*Rfx4*^{L298P}) lead to dorsal midline defects in the forebrain, hydrocephalus, and patterning defects in the telencephalon [28,29]. Because the *Msi1* knockout mouse also shows hydrocephalus, there might be a relationship with the phenotype of the *Rfx4*-disrupted mouse. Although the functional role of Rfx transcription factors in NS/PCs has not been reported, primary cilia are required for the expansion and establishment of postnatal hippocampal progenitors [50–52]. Thus, it is speculated that Rfx transcription factors contribute to the regulation of NS/PCs regarding their maintenance or differentiation. Interestingly, Rfx binding site was enriched at H3K4 me1 positive enhancers and Sox2-Brn2 co-occupied regions in NS/PCs [15,53]. We quantified the mRNA expression of *Rfx* in NS/PCs; *Rfx1*, 3, 4 and 7 were strongly expressed in NS/PCs. Rfx1 is ubiquitously expressed in brain, however, it transcriptionally represses neuronal marker MAP1A gene in non-neuronal cells but not in neuronal cells, and Rfx3 also shows repressive regulation of MAP1A in non-neuronal cells [54]. This indicates Rfx transcription factors function redundantly and in a cell type specific manner. Furthermore, both Rfx1 and Rfx3 were recently suggested to regulate the transcription of *GPR56* in NS/PCs, and the deletion of Rfx binding sites in *GPR56* E1m promoter correlated with pathogenesis of polymicrogyria in entire cortex through putative decreased proliferation of NS/PCs and reduced neuronal migration [55]. Therefore, Rfx transcription factors are good candidates for the regulation of NS/PCs. Our present study revealed that Rfx transcription factors were involved in NS/PC marker expression, and this insight will be applied to the functional analysis of these genes in NS/PCs.

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Author Disclosure Statement

H.O. is a scientific consultant of San Bio, Inc. and Daiichi Sankyo Co., Ltd. The remaining authors have no conflicts of interest to declare.

References

- Okano H. (2002). Stem cell biology of the central nervous system. *J Neurosci Res* 69:698–707.
- Nakamura M, H Okano, JA Blendy and C Montell. (1994). Musashi, a neural RNA-binding protein required for *Drosophila* adult external sensory organ development. *Neuron* 13:67–81.
- Sakakibara S, T Imai, K Hamaguchi, M Okabe, J Aruga, K Nakajima, D Yasutomi, T Nagata, Y Kurihara, et al. (1996). Mouse-Musashi-1, a neural RNA-binding protein

- highly enriched in the mammalian CNS stem cell. *Dev Biol* 176:230–242.
4. Okano H, T Imai and M Okabe. (2002). Musashi: a translational regulator of cell fates. *J Cell Sci* 115:1355–1359.
 5. Okano H, H Kawahara, M Toriya, K Nakao, S Shibata and I Takao. (2005). Function of RNA binding protein Musashi-1 in stem cells. *Exp Cell Res* 306:349–356.
 6. Sakakibara S, Y Nakamura, H Satoh and H Okano. (2001). RNA-binding protein Musashi2: developmentally regulated expression in neural precursor cells and subpopulations of neurons in mammalian CNS. *J Neurosci* 21:8097–8107.
 7. Sakakibara S, Y Nakamura, T Yoshida, S Shibata, M Koike, H Takano, S Ueda, Y Uchiyama, T Noda and H Okano. (2002). RNA-binding protein Musashi family: roles for CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation. *Proc Natl Acad Sci U S A* 99:15194–15199.
 8. Imai T, A Tokunaga, T Yoshida, M Hashimoto, K Mikoshiba, G Weinmaster, M Nakafuku and H Okano. (2001). The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. *Mol Cell Biol* 21:3888–3900.
 9. Okabe M, T Imai, M Kurusu, Y Hiromi and H Okano. (2001). Translational repression determines a neuronal potential in *Drosophila* asymmetric cell division. *Nature* 411:94–97.
 10. Battelli C, GN Nikopoulos, JG Mitchell and JM Verdi. (2006). The RNA binding protein Musashi-1 regulates neural development through the translational repression of p21WAF-1. *Mol Cell Neurosci* 31:85–96.
 11. Latchman DS. (1999). POU family transcription factors in the nervous system. *J Cell Physiol* 179:126–133.
 12. Catena R, C Tiveron, A Ronchi, S Porta, A Ferri, L Tangelo, M Cavallaro, R Favaro, S Ottolenghi, et al. (2004). Conserved POU binding DNA sites in the Sox2 upstream enhancer regulate gene expression in embryonic and neural stem cells. *J Biol Chem* 279:41846–41857.
 13. Young RA. (2011). Control of the embryonic stem cell state. *Cell* 144:940–954.
 14. Tanaka S, Y Kamachi, A Tanouchi, H Hamada, N Jing and H Kondoh. (2004). Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. *Mol Cell Biol* 24:8834–8846.
 15. Lodato MA, CW Ng, JA Wamstad, AW Cheng, KK Thai, E Fraenkel, R Jaenisch and LA Boyer. (2013). SOX2 co-occupies distal enhancer elements with distinct POU factors in ESCs and NPCs to specify cell state. *PLoS Genet* 9:e1003288.
 16. Kawase S, T Imai, C Miyauchi-Hara, K Yaguchi, Y Nishimoto, S Fukami, Y Matsuzaki, A Miyawaki, S Itoharu and H Okano. (2011). Identification of a novel intronic enhancer responsible for the transcriptional regulation of musashi1 in neural stem/progenitor cells. *Mol Brain* 4:14.
 17. Okada Y, T Shimazaki, G Sobue and H Okano. (2004). Retinoic-acid-concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells. *Dev Biol* 275:124–142.
 18. Tabata H and K Nakajima. (2001). Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. *Neuroscience* 103:865–872.
 19. Hara-Miyauchi C, O Tsuji, A Hanyu, S Okada, A Yasuda, T Fukano, C Akazawa, M Nakamura, T Imamura, et al. (2012). Bioluminescent system for dynamic imaging of cell and animal behaviour. *Biochem Biophys Res Commun* 419:188–193.
 20. Kempermann G, S Jessberger, B Steiner and G Kronenberg. (2004). Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* 27:447–452.
 21. Pfisterer P, J Ehlermann, M Hegen and H Schorle. (2002). A subtractive gene expression screen suggests a role of transcription factor AP-2 alpha in control of proliferation and differentiation. *J Biol Chem* 277:6637–6644.
 22. Eckert D, S Buhl, S Weber, R Jäger and H Schorle. (2005). The AP-2 family of transcription factors. *Genome Biol* 6:246.
 23. Kamachi Y, M Uchikawa and H Kondoh. (2000). Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet* 16:182–187.
 24. Sarkar A and K Hochedlinger. (2013). The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell* 12:15–30.
 25. Aftab S, L Semenech, JS Chu and N Chen. (2008). Identification and characterization of novel human tissue-specific RFX transcription factors. *BMC Evol Biol* 8:226.
 26. Gajiwala KS, H Chen, F Cornille, BP Roques, W Reith, B Mach and SK Burley. (2000). Structure of the winged-helix protein hRFX1 reveals a new mode of DNA binding. *Nature* 403:916–921.
 27. Emery P, B Durand, B Mach and W Reith. (1996). RFX proteins, a novel family of DNA binding proteins conserved in the eukaryotic kingdom. *Nucleic Acids Res* 24:803–807.
 28. Blackshear PJ, JP Graves, DJ Stumpo, I Cobos, JL Rubenstein and DC Zeldin. (2003). Graded phenotypic response to partial and complete deficiency of a brain-specific transcript variant of the winged helix transcription factor RFX4. *Development* 130:4539–4552.
 29. Ashique AM, Y Choe, M Karlen, SR May, K Phamluong, MJ Solloway, J Ericson and AS Peterson. (2009). The Rfx4 transcription factor modulates Shh signaling by regional control of ciliogenesis. *Sci Signal* 2:ra70.
 30. Morotomi-Yano K, K Yano, H Saito, Z Sun, A Iwama and Y Miki. (2002). Human regulatory factorX4 (RFX4) is a testis-specific dimeric DNA-binding protein that cooperates with other human RFX members. *J Biol Chem* 277:836–842.
 31. Baas D, A Meiniehl, C Benadiba, E Bonnafé, O Meiniehl, W Reith and B Durand. (2006). A deficiency in RFX3 causes hydrocephalus associated with abnormal differentiation of ependymal cells. *Eur J Neurosci* 24:1020–1030.
 32. Manojlovic Z, R Earwood, A Kato, B Stefanovic and Y Kato. (2014). RFX7 is required for the formation of cilia in the neural tube. *Mech Dev* 132:28–37.
 33. Sunabori T, A Tokunaga, T Nagai, K Sawamoto, M Okabe, A Miyawaki, Y Matsuzaki, T Miyata and H Okano. (2008). Cell-cycle-specific nestin expression coordinates with morphological changes in embryonic cortical neural progenitors. *J Cell Sci* 121(Pt 8):1204–1212.
 34. Sakakibara S and H Okano. (1997). Expression of neural RNA-binding proteins in the postnatal CNS: implications of their roles in neuronal and glial cell development. *J Neurosci* 17:8300–8312.
 35. Kovacevic Grujicic N, M Mojsin, A Krstic and M Stevanovic. (2005). Functional characterization of the human SOX3 promoter: identification of transcription factors implicated in basal promoter activity. *Gene* 344:287–297.
 36. Krstic A, M Mojsin and M Stevanovic. (2007). Regulation of SOX3 gene expression is driven by multiple NF-Y binding elements. *Arch Biochem Biophys* 467:163–173.

37. Celada A, SR McKercher and RA Maki. (1996). Identification of the transcription factors NF-YA and NF-YB as factors A and B that bound to the promoter of the major histocompatibility complex class II gene I-A beta. *Biochem J* 317(Pt 3):771–777.
38. Jabrane-Ferrat N, N Nekrep, G Tosi, LJ Esserman and BM Peterlin. (2002). Major histocompatibility complex class II transcriptional platform: assembly of nuclear factor Y and regulatory factor X (RFX) on DNA requires RFX5 dimers. *Mol Cell Biol* 22:5616–5625.
39. Zhu XS, MW Linhoff, G Li, KC Chin, SN Maity and JP Ting. (2000). Transcriptional scaffold: CIITA interacts with NF-Y, RFX, and CREB to cause stereospecific regulation of the class II major histocompatibility complex promoter. *Mol Cell Biol* 20:6051–6061.
40. Mason S, M Piper, RM Gronostajski and LJ Richards. (2009). Nuclear factor one transcription factors in CNS development. *Mol Neurobiol* 39:10–23.
41. Miura M, T Tamura and K Mikoshiba. (1990). Cell-specific expression of the mouse glial fibrillary acidic protein gene: identification of the cis- and trans-acting promoter elements for astrocyte-specific expression. *J Neurochem* 55:1180–1188.
42. Cebolla B and M Vallejo. (2006). Nuclear factor-I regulates glial fibrillary acidic protein gene expression in astrocytes differentiated from cortical precursor cells. *J Neurochem* 97:1057–1070.
43. Kaneko Y, S Sakakibara, T Imai, A Suzuki, Y Nakamura, K Sawamoto, Y Ogawa, Y Toyama, T Miyata and H Okano. (2000). Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev Neurosci* 22:139–153.
44. McPherson LA and RJ Weigel. (1999). AP2alpha and AP2gamma: a comparison of binding site specificity and trans-activation of the estrogen receptor promoter and single site promoter constructs. *Nucleic Acids Res* 27:4040–4049.
45. Pinto L, D Drechsel, MT Schmid, J Ninkovic, M Irmeler, MS Brill, L Restani, L Gianfranceschi, C Cerri, et al. (2009). AP2gamma regulates basal progenitor fate in a region- and layer-specific manner in the developing cortex. *Nat Neurosci* 12:1229–1237.
46. Wallis D, M Hamblen, Y Zhou, KJ Venken, A Schumacher, HL Grimes, HY Zoghbi, SH Orkin and HJ Bellen. (2003). The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. *Development* 130:221–232.
47. Swoboda P, HT Adler and JH Thomas. (2000). The RFX-type transcription factor DAF-19 regulates sensory neuron cilium formation in *C. elegans*. *Mol Cell* 5:411–421.
48. Bonnafé E, M Touka, A AitLounis, D Baas, E Barras, C Ucla, A Moreau, F Flamant, R Dubruille, et al. (2004). The transcription factor RFX3 directs nodal cilium development and left-right asymmetry specification. *Mol Cell Biol* 24:4417–4427.
49. Chung MI, SM Peyrot, S LeBoeuf, TJ Park, KL McGary, EM Marcotte and JB Wallingford. (2012). RFX2 is broadly required for ciliogenesis during vertebrate development. *Dev Biol* 363:155–165.
50. Gerdes JM, EE Davis and N Katsanis. (2009). The vertebrate primary cilium in development, homeostasis, and disease. *Cell* 137:32–45.
51. Breunig JJ, MR Sarkisian, JI Arellano, YM Morozov, AE Ayoub, S Sojitra, B Wang, RA Flavell, P Rakic and T Town. (2008). Primary cilia regulate hippocampal neurogenesis by mediating sonic hedgehog signaling. *Proc Natl Acad Sci U S A* 105:13127–13132.
52. Han YG and A Alvarez-Buylla. (2010). Role of primary cilia in brain development and cancer. *Curr Opin Neurobiol* 20:58–67.
53. Creighton MP, AW Cheng, GG Welstead, T Kooistra, BW Carey, EJ Steine, J Hanna, MA Lodato, GM Frampton, et al. (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci U S A* 107:21931–21936.
54. Nakayama A, H Murakami, N Maeyama, N Yamashiro, A Sakakibara, N Mori and M Takahashi. (2003). Role for RFX transcription factors in non-neuronal cell-specific inactivation of the microtubule-associated protein MAP1A promoter. *J Biol Chem* 278:233–240.
55. Bae BI, I Tietjen, KD Atabay, GD Evrony, MB Johnson, E Asare, PP Wang, AY Murayama, K Im, et al. (2014). Evolutionarily dynamic alternative splicing of GPR56 regulates regional cerebral cortical patterning. *Science* 343:764–768.

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