MCPIP1 is a recently identified immune regulator that plays critical roles in preventing immune disorders, and is also present in the brain. Currently an unresolved question remains as to how MCPIP1 performs its non-immune functions in normal brain development. Here, we report that MCPIP1 is abundant in neural progenitor cells (NPCs) and newborn neurons during the early stages of neurogenesis. The suppression of MCPIP1 expression impairs normal neuronal differentiation, cell-cycle exit, and concomitant NPC proliferation. MCPIP1 is important for maintenance of the NPC pool. Notably, we demonstrate that MCPIP1 reduces TET (TET1/TET2/TET3) levels and then decreases 5-hydroxymethylcytosine levels. Furthermore, the MCPIP1 interaction with TETs is involved in neurogenesis and in establishing the proper number of NPCs in vivo. Collectively, our findings not only demonstrate that MCPIP1 plays an important role in early cortical neurogenesis but also reveal an unexpected link between neocortical development, immune regulators, and epigenetic modification.

INTRODUCTION

The cerebral cortex is the center of the mammalian brain and provides the structural basis for complex perceptual and cognitive functions. The formation of the cortex relies on the expansion of neural progenitor cells (NPCs) and the subsequent generation of postmitotic neurons. Recent studies have shed light on neurogenesis, the process that underlies expansion of the neocortex whereby NPCs generate neurons. It has been reported that numerous immune proteins are expressed in neural stem cells, suggesting that immune signaling could be involved in the process of neurogenesis (Carpentier and Palmer, 2009). For a better understanding of this new role of immune proteins in brain development and function, it is first necessary to have a basic understanding of their known functions.

Due to the existence of the blood-brain barrier and the immunosuppressive microenvironment, the CNS has been traditionally considered an immune-privileged organ (Sallusto et al., 2012). It has been reported that immune proteins classically thought to have specific immune function such as cytokines, major histocompatibility complex class I molecules, and T cell receptor subunits, are also expressed in the regions of the CNS (Boulanger, 2009; Komal et al., 2014; Syken and Shatz, 2003). Immune molecules play essential roles in various aspects throughout neural development of the CNS (Bauer et al., 2007; Boulanger, 2009). However, the expression, function, and mechanisms of action for the large majority of immune molecules in normal brain development have not yet been studied. Monocyte chemoattractant protein (MCP)-1-induced protein 1 (MCPIP1) is a recently identified protein harboring a CCCH-type zinc-finger domain (Liang et al., 2008; Xu et al., 2012). It is encoded by the ZC3H12A (zinc-finger CCCH-type containing 12A) gene, which is expressed in interleukin-1β (IL-1β)-induced human monocyte-derived macrophages and MCP-1-stimulated human peripheral blood monocytes (Skalniak et al., 2009; Zhou et al., 2006). MCPIP1 is necessary to inhibit unwanted immune reactions mediated by T cells through destabilizing a set of mRNAs (Uehata et al., 2013). Its deficiency leads to a complex phenotype involving severe anemia, severe inflammatory response, autoimmune response, and premature death (Liang et al., 2010; Matsushita et al., 2009). Structural studies of MCPIP1 reveal that the N-terminal conserved domain shows a PilT N-terminus-like RNase structure, providing further evidence that MCPIP1 has RNase activity. Recently, several studies have focused on the RNase activity of MCPIP1, which targets the mRNAs for IL-6, IL-1β (Matsushita et al., 2009; Mizgalska et al., 2009), and pre-microRNAs (Suzuki et al., 2011). The functional diversity and the RNase structure of MCPIP1 make it an attractive candidate as an immune regulator that mediates normal brain development.
mammals and influences a variety of biological processes, including transcriptional regulation, imprinting, and the maintenance of genomic stability. Hydroxymethylcytosine is emerging as the active demethylation modification that targets a specific 5-methyl group on cytosine for net removal by a complex base excision repair mechanism (Guo et al., 2011a, 2011b). Consistent with the idea that hydroxymethylcytosine is involved as a specific mechanism for active cytosine demethylation, recent studies identified the ten-eleven translocation (TET) family of proteins in active DNA demethylation (Ito et al., 2010; Tahiliani et al., 2009). The three mammalian TET proteins, TET1, TET2, and TET3, have changed our understanding of the process of DNA demethylation as they can oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (He et al., 2011; Ito et al., 2010; Tahiliani et al., 2009). Recent studies have shown that TET-mediated DNA demethylation can play vital roles in various biological processes, not only in development but also in disease. Despite these advances, the functions of TET proteins and their regulation in brain development need further investigation.

Here, we report the unique roles of MCPIP1 during early neocortical development. We found a dramatic expression pattern of MCPIP1 during early cortical neurogenesis. MCPIP1 regulates various aspects of neurogenesis. Notably, we observed that MCPIP1 directly targets Tets, and represses TET and 5hmC expression levels. Importantly, the interaction of MCPIP1 and TETs is involved in neurogenesis and NPC pool maintenance. Our current data demonstrate a direct and important molecular link between the immune regulatory molecule, TETs, and epigenetic regulation.

RESULTS

Expression of MCPIP1 in the Developing Neocortex

To elucidate the role of MCPIP1 in early neocortical development, we identified the expression of MCPIP1 in the embryonic cortex. We performed immunostaining experiments in vivo and in vitro to confirm the expression of MCPIP1. The results showed that MCPIP1 was expressed at high levels in the cortical plate (CP), subventricular zone (SVZ), and ventricular zone (VZ) in the developing neocortex, but expressed at low levels in the intermediate zone (Figure 1A). Notably, we found that the expression of MCPIP1 decreased significantly in the VZ but increased significantly in CP from embryonic day 12 (E12) to E18, the main phase of cortical neurogenesis in the mouse developing brain (Figure 1A). These data suggested that MCPIP1 may be expressed in NPCs and neurons. To determine this possibility, we immunostained E12 brain sections and found that MCPIP1 was preferentially expressed in SOX2- and PAX6-expressing NPCs, whereas it was also expressed in TBR2-positive progenitors (Figures 1B–1D). We further determined that MCPIP1 was also strongly expressed in newborn neurons of the CP, as identified by CTIP2 and β-III-tubulin (TUJ1) staining (Figures 1E and 1F). To further confirm these results, we isolated NPCs from E12 mouse brains and analyzed MCPIP1 expression in vitro. The double immunostaining results revealed that MCPIP1 was expressed at high levels in cultured NPCs and newborn neurons (Figures S1A–S1E). Taken together, these data indicate that MCPIP1 is abundantly expressed in NPCs and newborn neurons during the early stages of neurogenesis.

NPCs are multipotent cells characterized by their capability to self-renew and differentiate into multiple cells. Our study showed that at the beginning of neurogenesis MCPIP1 was largely expressed in the expanding cell population of the VZ, which maintains the appropriate pool of neural progenitors and subsequently establishes a functional neocortex. Therefore, we focused our studies on the potential roles of MCPIP1 in early cortical neurogenesis.

MCPIP1 Regulates Neurogenesis in the Developing Neocortex

To determine the potential roles of MCPIP1 in NPCs, we performed gain- and loss-of-function studies to elucidate its function in vivo and in vitro. Two different small hairpin RNAs (shRNAs) to silence Mcpip1 expression and a DNA plasmid encoding Mcpip1 were constructed and used for in utero electroporation. All constructs expressed GFP as an indicator. Western blotting results demonstrated that the two shRNAs were capable of reducing MCPIP1 protein levels (Figures S2A–S2D), and the Mcpip1 expression plasmid could effectively increase MCPIP1 protein expression (Figures S2E and S2F).

Next, we examined the effects of Mcpip1 knockdown or overexpression in the VZ/SVZ of the mouse cortex using in utero electroporation (Lv et al., 2014a). We electroporated E13 mouse embryonic brains with Mcpip1 shRNAs or Mcpip1 expression plasmid, and harvested the electroporated brains 3 days later at E16. We observed a significantly altered distribution of GFP-positive cells in the developing neocortex following Mcpip1 expression changes. Mcpip1 knockdown led to an obvious increase in the proportion of GFP-positive cells in the VZ and SVZ with a concomitant decrease in the proportion of GFP-positive cells in the CP. In contrast, Mcpip1 overexpression resulted in a distribution of GFP-positive cells in the three cortex zones (Figures 2A and 2B). The phenotypes resulting from treatment with the two different shRNAs were identical (Figures S2G and S2H). Together, these results demonstrate that MCPIP1
regulates cell distribution during early neocortical development. To exclude apoptosis as a potential cause for the observed phenotypes, we performed TUNEL staining at E15 and E16 brain sections, but did not observe significant differences following *Mcpip1* alteration (Figures S3A–S3D).

Meanwhile, we performed immunostaining experiments using cleaved caspase-3 antibody in vitro. The results also showed no obvious differences after *Mcpip1* expression alteration (Figure S3E).

The fact that different proportions of GFP-positive cells remained in the CP following *Mcpip1* expression alteration suggested possible changes in neuronal differentiation. To determine whether MCPIP1 had an effect on neuronal differentiation, we next examined the overlap of the GFP-positive cell population with TUJ1 and MAP2, markers of differentiated neurons, on E16 electroporated brain sections. We found that *Mcip1* knockdown significantly decreased the levels of TUJ1 and MAP2 compared with controls (Figures 2C–2F). To further confirm the effects of MCPIP1 on neuronal differentiation, we performed in vitro experiments. Primary NPCs isolated from E12 mouse embryonic brains were infected with control,
Figure 2. MCPIP1 Regulates Neurogenesis in the Developing Neocortex

(A and B) Mcpip1 expression variation resulted in altered cell distribution in the cortex. (A) Control, Mcpip1 shRNA, or Mcpip1 expression plasmid was electroporated into embryonic brains at E13. The brains were then harvested at E16. Scale bar, 50 μm. (B) The percentage of GFP cells in each region was quantified. Values are presented as mean ± SD; n = 3 independent experiments. **p < 0.01, one-way ANOVA with Tukey’s test for post hoc multiple comparisons; NS, not significant.

(C–H) MCPIP1 regulates cortical neuronal differentiation. (C and E) The electroporated E16 brain sections were immunostained with anti-TUJ1 or anti-MAP2 antibody. Inset shows zoom view of colocalization cells in brain sections with Mcpip1 knockdown or overexpression. (legend continued on next page)
**Mcpip1** knockdown, or **Mcpip1** overexpression lentivirus. After culture in differentiation medium for 3 days, cells were harvested and fluorescence-activated cell sorting (FACS) analysis was performed to ensure the extent of **Mcpip1** overexpression or knockdown (Figures S2A and S2J). Then **TuJ1** and **Map2** mRNA levels were examined by real-time qRT-PCR. The results further confirmed that **Mcpip1** expression variation resulted in significant changes of **TuJ1** and **Map2** expression (Figures 2G and 2H). In addition, the infected NPCs were also examined by immunostaining (Figures S4A and S4B) and western blot (Figures S4C–S4E) with **TuJ1** antibody after culture in differentiation medium for 3 days. These results in vitro are consistent with those in vivo. Collectively, these results in vivo and in vitro indicate that **MCPIP1** promotes premature neuronal differentiation. Premature neuronal differentiation is closely associated with early cell-cycle exit. To test this possibility, we analyzed the cell-cycle exit index. The embryonic brains were electroporated at E13 and harvested at E16. Bromodeoxyuridine (BrDU) (100 mg/kg) was injected into mice 48 hr after electroporation. The results demonstrated that **Mcpip1** knockdown obviously decreased the cell-cycle exit index (Figures 2I and 2J).

The generation of the proper number of neurons in the developing neocortex depends on a carefully regulated spatial and temporal balance between differentiation and proliferation. Because **Mcpip1** knockdown resulted in defects in neuronal differentiation, we asked whether these defects coincided with changes in NPC proliferation. To investigate this possibility, we performed the NPC culture experiments in vitro. The results showed increased cell proliferation activity following **Mcpip1** inhibition, which were identified by ethynyldeoxyuridine (a proliferation marker also labeling S-phase dividing cells) and Ki67 (a proliferation marker that labels cells in all active phases of the cell cycle) (Figures S4F–S4I). Furthermore, the embryonic brains were electroporated at E13 and BrDU was injected to label S-phase dividing cells 2 hr before euthanasia at E16. The immunostaining results showed that BrDU labeling in the GFP-positive population significantly increased after **Mcpip1** knockdown (Figures S4J and S4L), indicating an overall increase in cell proliferation. Meanwhile, the E16 electroporated brains were also stained with the mitotic marker phosphohistone H3 (pH3), and the results demonstrated a significant increase in mitotic activity (Figures S4K and S4M). Conversely, **Mcpip1** overexpression resulted in a significant decrease in cell proliferation and mitotic index. Taken together, these findings demonstrate that **Mcpip1** knockdown disrupts normal neuronal differentiation, cell-cycle exit, and concomitant NPC proliferation.

**MCPIP1 Regulates the Composition of the NPC Pool and Transition of NPCs**

Ordery cortical development requires appropriate numbers and types of cells to be derived from the progenitor pools. Two main types of neurogenic progenitor cells are present in the developing cerebral cortex, apical progenitors, and basal progenitors. Apical progenitors, which are capable of self-renewal, are in charge of the maintenance of the progenitor pool during neurogenesis. Basal progenitors, also known as intermediate neural progenitors, are capable of generating the majority of neurons for all layers. The changed neuronal differentiation, cell-cycle exit index, and cell proliferation due to **Mcpip1** expression variation suggested that **MCPIP1** may play an important role in regulating the makeup of the neural progenitor pool. To investigate this possibility, we analyzed the electroporated brains at E16 to examine the makeup of the progenitor pool by immunohistochemistry for **Pax6**, the apical progenitor marker, and **Tbr2**, the basal progenitor marker. These results indicated that **MCPIP1** decreased the number of apical progenitors and increased the number of basal progenitors (Figures 3A–3D). Meanwhile, in vitro FACS and subsequent qRT-PCR analysis further confirmed that **Mcpip1** expression variation caused correlated changes of **Pax6** and **Tbr2** mRNA expression levels (Figures 3E and 3F). Taken together, these findings reveal that **MCPIP1** is critical for the maintenance of the NPC pool.

Comparatively, apical progenitors are often considered as the predominant progenitor due to their ability to...
Figure 3. MCPIP1 Regulates the Composition of the NPC Pool and Transition of NPCs

(A–F) MCPIP1 regulates the makeup of the NPC pool. The electroporated E16 brain sections were immunostained with anti-PAX6 or anti-TBR2 antibody. Confocal images of the immunostained brain section (A and B; scale bars, 20 μm) and the quantification results within SVZ/VZ (C–F) are shown. MCPIP1 was overexpressed or knocked down in E12 mouse primary NPCs, and cells were harvested for FACS and qRT-PCR analysis to examine Pax6 and Tbr2 mRNA levels after culture for 3 days. Arrows indicate GFP+ PAX6+ or GFP+ TBR2+ cells. Values are presented as mean ± SD; n = 3 independent experiments. *p < 0.05, **p < 0.01, one-way ANOVA with Tukey’s test for post hoc multiple comparisons.

(legend continued on next page)
maintain the NPC pool and generate a second, transient neural progenitor population of basal progenitors, which are exclusively neurogenic and have limited self-renewal capacity. We speculated that the increased number of apical progenitors was due to enhanced apical progenitor generation, and the reduction in the number of basal progenitors may be caused by an abnormal transition from apical progenitors to basal progenitors. We explored this possibility by electroporating E13 brains with Mcpip1 shRNA or Mcpip1 expression plasmid followed by BrdU injection 2 hr before being euthanized at E16. The electroporated brains were stained with PAX6, BrdU, and TBR2 antibodies. The results showed that Mcpip1-inhibited brains exhibited markedly increased the proportion of PAX6-BrdU double-positive apical progenitors and reduced the proportion of PAX6-TBR2 double-positive progenitors, whereas Mcpip1-overexpressing brains displayed the opposite proportions (Figures 3G–3J). Taken together, these findings strongly suggest that Mcpip1 inhibits apical progenitor production and enhances basal progenitor transition.

MCPIP1 Binds Tet Transcripts Analyzed by PAR-CLIP

Next, we investigated the molecular mechanism by which MCPIP1 regulates neurogenesis during early cortical development. MCPIP1 is a recently identified immune regulator harboring a CCCH-type zinc-finger domain (Liang et al., 2008; Xu et al., 2012). Most of the characterized CCCH-type zinc-finger proteins bind to RNA and regulate mRNA processing, including mRNA maturation, export, modification, and turnover (Brown, 2005; Hall, 2005). We hypothesized that MCPIP1 as an immune regulator may also regulate neurogenesis by regulating processing of RNAs. To identify the targets of MCPIP1, we performed a photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) assay (Zhao et al., 2014) in mouse neural N2a cells (Figure 4A). In brief, the experiment was performed by overexpression of FLAG-tagged Mcpip1 in N2a cells. Two days after transfection, 4-thiouridine (4-SU), a photoreactive ribonucleoside analog, was added to the medium. After 16 hr of incubation, cells were collected after UV crosslinking and subjected to immunoprecipitation with anti-FLAG M2 magnetic beads. Immuno blotting was performed to detect the expression (Figure 4B) and immunoprecipitation efficiency of FLAG-Mcpip1 (Figure 4C). The RNA fragments bound by MCPIP1 were labeled and detected using a biotin labeling kit (Figure 4C). Next, the target protein-RNA complexes were cut out and subsequently treated to separate RNAs. RNAs were sent for cDNA library construction and deep sequence. After basic analysis, the resulting sequence reads were mapped to the mouse genome (mm10), and analyzed using the PARalyzer software. We identified 9,999 MCPIP1 binding sites located in gene regions. Among them, 3,620 sites (36.2%) bound to MCPIP1 via their intronic regions, 2,981 sites (29.8%) bound to MCPIP1 via 3’ UTR, and 1,427 sites (14.3%) were in coding sequence (CDS) (Figure 4D and Table S1). We then used the CREME algorithm (Bailey, 2011) to analyze all utilized sequence reads of binding sites and define the sequence logo of the MCPIP1 recognition motif (Figure 4E).

The molecular basis of DNA demethylation during mammalian development has been prompted by the recent discovery and characterization of the TET family of dioxygenases (Tahiliani et al., 2009). Although some essential roles of TET enzymes in brain development have been reported (Hahn et al., 2013; Lv et al., 2014b; Zhang et al., 2013), the function of TET proteins and their regulation are still poorly elucidated. Based on these previous studies, we focused our work on the TET family among the MCPIP1-binding RNAs. From PAR-CLIP results, we found that both Tet2 and Tet3 were included in MCPIP1-binding RNAs, and their binding sites were located in the coding sequence (Table S1). We analyzed all the binding genes, and Tet2 and Tet3 had higher mode scores among these genes (Figure 4F). In particular, Tet3 has two binding sites for MCPIP1 (Figure 4G). These results demonstrate that MCPIP1 directly binds to Tet2 and Tet3 and that their interaction is strong. Collectively, these data suggest that the TET family is one of the major targets for MCPIP1. To confirm the direct binding of MCPIP1 to Tet2 and Tet3 mRNAs, we performed in vitro experiments with electromobility shift assay (EMSA). The results showed that MCPIP1 protein bound directly to Tet2 and Tet3 mRNA (Figure S5A). However, the interaction between MCPIP1 and Tet2 is not as strong as the interaction between MCPIP1 and Tet3. These results are consistent with those in PAR-CLIP experiments, which show that Tet3 has two binding sites for MCPIP1.

Considering that the mammalian TET family contains three members (TET1, TET2, and TET3) and the PAR-CLIP results, we wondered whether Tet1 was also an MCPIP1-binding RNA but not detected. Therefore, we further examined the endogenous expression of Tet1, Tet2, and Tet3 in N2a cells using absolute qRT-PCR. The results showed

(G–J) MCPIP1 regulates the generation and transition of apical progenitors. (G and H) Embryonic brains were electroporated at E13 and collected at E16. BrdU (100 mg/kg) was injected into mice 2 hr before being euthanized. The electroporated brains were immunostained with anti-PAX6 and anti-BrdU or anti-TBR2 antibody. Arrows indicate GFP+ PAX6+ BrdU+ or GFP+ PAX6+ TBR2+ cells. Scale bars, 20 μm. (I and J) Quantification of results within SVZ/VZ. Values are mean ± SD; n = 3 independent experiments. *p < 0.05, **p < 0.01, one-way ANOVA with Tukey’s test for post hoc multiple comparisons.
Figure 4. MCPIP1 Binds Tet Transcripts Analyzed by PAR-CLIP

(A) Illustration of PAR-CLIP analysis. N2a cells were transfected with FLAG or Flag-Mcipip1 plasmids. After culture for 2 days, 200 μM 4-SU was supplemented to the culture medium for 16 hr, and cells were irradiated with 0.4 J/cm², 365 nm UV light. After a series of subsequent treatments, MCPIP1-binding RNAs were separated and deep sequenced.

(B) Immunoblot analysis of N2a cells transfected with FLAG or Flag-Mcipip1 plasmids. The expression levels of MCPIP1 protein in whole-cell extract (WCE) was detected by western blotting with FLAG antibody. Asterisk indicates the position of MCPIP1 protein. Ctrl, FLAG empty vector. β-Actin was used as loading control.

(C) Immunoprecipitation efficiency of MCPIP1 and biotin-labeled MCPIP1 protein-RNA complex. MCPIP1 protein-RNA complex was pulled down with FLAG M2 magnetic beads, and then RNA was labeled and detected according to instructions of biotin labeling kit. Asterisk indicates the FLAG-MCPIP1-RNA complex.

(D) Percentage of MCPIP1 PAR-CLIP tags in mature mRNAs (5’ UTR, CDS, and 3’ UTR), Introns, and non-coding RNAs.

(E) Sequence logo of the MCPIP1 recognition motif generated by DREME analysis of all utilized sequence reads of binding sites.

(F) The ranks of Tet2 and Tet3 binding sites on ModeScore among all MCPIP1 binding sites. Asterisk indicates the ModeScore of Tet2 and Tet3 binding sites.
that Tet1 mRNA levels were significantly lower than Tet2 and Tet3 mRNA levels (Figure 4H), which is similar to the results in mouse embryonic neocortex (Hahn et al., 2013). Low Tet1 expression levels provide a possible explanation for the reason that Tet1 was not detected in PAR-CLIP experiments. Therefore, in the following experiments we analyzed the regulation of MCPIP1 on TET2 and TET3, as well as TET1.

**MCPIP1 Regulates Tet mRNA and 5hmC Levels**

The potential roles of TET proteins in the immune system have been implied (Suarez-Alvarez et al., 2012; Tsagaratou and Rao, 2013). Combined with the PAR-CLIP results, we originally hypothesized that MCPIP1 as an immune regulator may also regulate the expression of Tet genes. To determine the regulation of MCPIP1 on TETs, we performed in vitro NPC culture experiments. Primary NPCs were infected with lentivirus to knock down or overexpress Mcpip1 and cultured under differentiation conditions. Cells were harvested 3 days later for FACS and qRT-PCR analysis. Consistent with our prediction, Tet mRNA levels were increased by Mcpip1 inhibition, whereas Tet mRNA levels were decreased by Mcpip1 overexpression (Figures 5A and S5B). Further western blot results confirmed that TET protein levels were decreased by Mcpip1 overexpression (Figures S5C–S5H). These results demonstrate that MCPIP1 regulates the expression of TETs. Due to the ability of TET proteins to convert 5mC to 5hmC (Ito et al., 2010; Tahiliani et al., 2009), we investigated whether MCPIP1 affected ShmC levels by dot-blot and immunostaining analysis. In the dot-blot experiment, NPCs were infected with Mcpip1 shRNA or Mcpip1 expression lentivirus and cultured in differentiation medium for 3 days, and the cells harvested for dot-blot analysis. Consistent with the effects of MCPIP1 on the expression of TETs, Mcpip1 inhibition markedly enhanced the levels of ShmC, whereas Mcpip1 overexpression reduced the levels of ShmC (Figures S5B, S5C, S5I, and S5J). However, global ShmC levels showed no obvious changes following Mcpip1 expression variation (Figures S5B, S5C, S5I, and S5J), consistent with previous reports (Fu et al., 2013; Lv et al., 2014b). This is not difficult to understand, considering that the level of ShmC in cortical DNA only accounts for about 1% of all cytosines or 20%–25% of total ShmC (jin et al., 2011), so the change of ShmC would not dramatically affect the global ShmC levels. Our study established a correlation between the expression levels of MCPIP1 and TETs, as well as ShmC. Collectively, these data demonstrate that MCPIP1 can regulate TET expression and ShmC levels.

Based on these results, we performed the following experiments to confirm the regulation of MCPIP1 on Tet mRNA. Mouse N2a cells were transfected with Mcpip1 overexpression or vector plasmids, and actinomycin D was added to stop transcription after 48 hr. The cells were harvested to examine the residual level of Tet mRNA at different time points. The results showed that MCPIP1 caused a faster decay rate of Tet mRNA (Figure 5D). These results indicate that MCPIP1 is directly involved in the regulation of Tet mRNA levels.

Our PAR-CLIP analysis showed that MCPIP1 binds to Tet mRNA via the coding sequence. To confirm whether MCPIP1 regulates Tet mRNA levels through the coding sequence, we constructed Tet expression plasmids containing coding sequence but without 3′ UTRs. Mcpip1 contains a PiII N-terminal (PIN) domain, which is responsible for its enzymatic activity (Matsushita et al., 2009; Mizgalaska et al., 2009). Aspartic acid at position 141, which forms the enzymatic pocket of PIN domain, is critical for RNase activity (Matsushita et al., 2009). To confirm that RNase activity of MCPIP1 is required for its function, we also constructed Mcpip1 mutant plasmids with a substitution of aspartic acid for asparagine at position 141 (D141N) (Figure S5K). Additionally the CCCH zinc-finger domain of MCPIP1, located within amino acid residues 305–325, is essential for RNA-binding capacity. Mcpip1-D305–325 (lacking amino acids 305–325) has been used to demonstrate the importance of this CCCH domain in mRNA and pre-microRNA degradation (Lin et al., 2013; Matsushita et al., 2009; Suzuki et al., 2011). To confirm whether the CCCH domain of MCPIP1 bound Tet mRNA and was necessary for its RNase activity, we constructed the Mcpip1-D305–325 mutant plasmid (Figure S5K). Tet expression plasmid was cotransfected with vector plasmid, wild-type Mcpip1, Mcpip1-D141N, or Mcpip1-D305–325 plasmid into human embryonic kidney cells (HEK 293T). The qRT-PCR analysis was performed after culture for 3 days, and the results showed that wild-type Mcpip1 obviously reduced the expression levels of Tet mRNA, whereas the point mutation of D141N or the Mcpip1-D305–325 mutant significantly abolished this effect of Mcpip1 (Figure 5E). These

(G) Sequence alignments of MCPIP1 PAR-CLIP cDNA sequence reads to the corresponding regions of Tet2 and Tet3 transcripts. The number of sequence reads (# reads) and mismatches (errors) are indicated. Red bars indicate the 4-nt MCPIP1 recognition sequence and nucleotides marked in red indicate T-to-C or T-to-G mutations.

(H) Absolute copy numbers of Tet1, Tet2, and Tet3 mRNA transcripts in N2a cells were detected by absolute qRT-PCR. N2a cells were collected to extract total RNAs, and qRT-PCR analysis was performed using Tet1, Tet2, and Tet3 primers, respectively. Values are mean ± SD; n = 3 independent experiments. **p < 0.01, one-way ANOVA with Tukey’s test for post hoc multiple comparisons. See also Figure S5 and Table S1.
Figure 5. MCPIP1 Regulates Tet mRNA Expression and 5hmC Levels

(A) MCPIP1 regulates Tet mRNA levels. Mouse NPCs isolated from E12 embryonic brains were infected with Mcpip1 knockdown or overexpression lentivirus, and collected for FACS and qRT-PCR analysis after culture in differentiation medium for 3 days. Relative Tet mRNA levels were normalized to the expression of β-Actin. Values are mean ± SD; n = 3 independent experiments. *p < 0.05, **p < 0.01, one-way ANOVA with Tukey's test for post hoc multiple comparisons.

(B and C) Dot blot (B) shows that the relative 5hmC not 5mC levels significantly change after Mcpip1 expression alteration. Mouse NPCs isolated from E12 embryonic brains were infected with Mcpip1 knockdown or overexpression lentivirus, and collected for DNA extraction after culture for 3 days in differentiation medium. DNA (500 ng) for each sample was used for dot-blot analysis with anti-5hmC or anti-5mC antibody. Graphs (C) show the quantification results. Values are mean ± SD; n = 3 independent experiments. **p < 0.01, one-way ANOVA with Tukey's test for post hoc multiple comparisons.

(D) Effects of MCPIP1 on the decay rate of Tet mRNA. Mcpip1 or vector plasmids were transfected into N2a cells. Actinomycin D (5 μg/mL) was added to stop transcription after 48 hr. The cells were harvested for RNA extraction at the indicated times, and the Tet mRNA levels were determined by qRT-PCR and normalized by β-Actin. The quantification shows the relative Tet mRNA at each time point, compared with the level of Tet mRNA at time zero, taken as 1. Meanwhile, the half-life of Tet mRNA is calculated and shown as T value. Values are mean ± SD; n = 3 independent experiments. *p < 0.05, **p < 0.01, two-tailed Student's t test.

(E) The RNase activity of MCPIP1 and the CCCH zinc-finger domain of MCPIP1 are required for the regulation of Tets. Tet (Tet1, Tet2, or Tet3) plasmids were cotransfected with Mcpip1 (WT), Mcpip1-D141N mutant, the Mcpip1-Δ305–325 mutant or vector plasmids into HEK293T (legend continued on next page)
results suggest that MCPIP1 regulates *Tet* mRNA levels by its RNase activity and that the CCCH domain of MCPIP1 is essential for its RNase activity. However, our results showed that MCPIP1 could regulate *Tet* mRNA by targeting its coding sequence but not 3′ UTR as inflammation-related mRNAs as in previous reports (Lin et al., 2013; Matsushita et al., 2009; Uehata et al., 2013). Taken together, these results suggest diversity of MCPIP1 action mode in regulating gene expression as an RNA-binding protein with RNase activity.

To demonstrate how TETs or DNA demethylations affect early cortical neurogenesis, we constructed *Tet* knockdown plasmids that efficiently silenced *Tet* expression (Figures S6A–S6C), and tested the expression change of several important genes required for early cortical neurogenesis after *Tet* expression was reduced. Primary NPCs infected with *Tet* (Tet1, Tet2, or Tet3) knockdown lentivirus were collected for FACS and qRT-PCR analysis after culturing in differentiation medium for 3 days. The results showed that *Tet* knockdown resulted in markedly decreased expression of the transcription factor SRY (sex-determining region) box 2 (Sox2), yes-associated protein 1 (Yap1), and REST silencing transcription factor (REST) in cortical NPCs under differentiation conditions, whereas β-Catenin expression was only significantly affected by Tet1 knockdown, and Cyclin D1 showed no significant change after *Tet* expression inhibition (Figure S5L). These results demonstrated that TETs affect early cortical neurogenesis by regulating downstream genes required for neurogenesis.

**MCPIP1/TETs Functionally Regulate Neurogenesis and Maintain the NPC Pool**

Our findings raised the possibility that MCPIP1 interaction with TETs may be involved in co-regulation of early cortical neurogenesis and maintenance of the NPC pool. To further examine the relationship between MCPIP1 and TETs during early neocortical development, we determined whether silencing *Tet* expression could rescue the in vivo cellular phenotypes due to reduced *Mcpip1* expression. In utero electroporation was performed at E13, followed by BrdU injection 2 hr before euthanasia at E16. Notably, we observed that the cell distribution change caused by *Mcpip1* knockdown in the cortex was completely rescued by TETs (Figures 6A and 6B). Co-expression of *Tet* shRNA with *Mcpip1* shRNA completely rescued the defects of neurogenesis caused by *Mcpip1* knockdown. We observed that *Tet* knockdown led to a significant increase in MAP2-positive (Figures 6C and 6D) and TUJ1-positive cells (Figures S6D and S6E), as well as an obvious reduction in BrdU and pH3 incorporation (Figures S6F–S6I) compared with *Mcpip1* knockdown. Meanwhile, the unbalanced proportion of apical and basal progenitors in the NPC pool caused by *Mcpip1* knockdown was reversed (Figures 6F–6I). To further confirm these results, we performed in vitro FACS and qRT-PCR analysis and obtained consistent results (Figures 6J and 6K). In addition, the increased 5hmC level caused by *Mcpip1* knockdown could be rescued by knockdown of Tets, which demonstrate TETs as mediators of the effects on 5hmC levels (Figures S6J and S6K). Collectively, these data strongly supported the functions of MCPIP1 in regulating early cortical neurogenesis and maintenance of the NPC pool through mediating *Tet* expression.

**DISCUSSION**

Although the brain has traditionally been regarded as immune-privileged, many studies suggest that there is extensive communication between the immune system and the nervous system in both healthy and diseased conditions. We sought here to learn about the basic roles of specific and crucial immune molecules in the nervous system. We observed that MCPIP1 was abundant in early neural progenitors and that its expression decreased over time during early mouse neocortical development. In the murine cortex, neurogenesis begins at approximately E12, reaches a peak at approximately E15, and terminates at approximately E18 (Qian et al., 2000). Therefore, MCPIP1 expression in the developing neocortex suggested precise temporal and spatial roles for MCPIP1 during this period. We identified these roles by demonstrating that altered *Mcpip1* expression resulted in changes of various aspects of neurogenesis. Notably, the composition of the neural progenitor pool was altered following *Mcpip1* expression variation, resulting in a change in the relative proportion of apical progenitors to basal progenitors. A potentially interesting possibility was explored, showing that MCPIP1 inhibited apical progenitor generation but promoted its transition.

Next, we investigated the underlying mechanism for MCPIP1 in early cortical neurogenesis. It is known that active DNA demethylation is prevalent in mammals (Tan and Shi, 2012). TET proteins were recently identified as enzymes that promote DNA demethylation (Ito et al., 2010; Tahiliani et al., 2009). Direct regulation of *Tet* expression
Figure 6. MCPIP1/TETs Functionally Regulate Neurogenesis and Maintain the NPC Pool

(A and B) The changed cell distribution caused by Mcpip1 inhibition is rescued by Tet knockdown (shTet1, shTet2, and shTet3). (A) Embryonic brains were electroporated at E13 and collected at E16. Scale bar, 50 μm. (B) The percentage of GFP cells in each zone was quantified. Values are presented as mean ± SD; n = 3 independent experiments. **p < 0.01, one-way ANOVA with Tukey’s test for post hoc multiple comparisons; NS, not significant.

(C–E) Decreased MAP2-positive cells caused by Mcpip1 knockdown is rescued by Tet knockdown. (C) The electroporated E16 brain section was immunostained with anti-MAP2 antibody. Scale bar, 50 μm. Graph (D) shows the quantification of MAP2-GFP double-positive cells. Map2 mRNA level downregulation caused by Mcpip1 knockdown in E12 mouse primary NPCs is rescued by Tet knockdown, determined by FACS and qRT-PCR analysis (E). Values are presented as mean ± SD; n = 3 independent experiments. *p < 0.05, **p < 0.01, one-way ANOVA with Tukey’s test for post hoc multiple comparisons; NS, not significant.

(F–K) The unbalanced number of apical progenitor and basal progenitor in the NPC pool is rescued by TETs. The electroporated E16 brain sections were immunostained with anti-PAX6 (F) or anti-TBR2 antibody (G). Arrows indicate GFP+ PAX6+ or GFP+ TBR2+ cells. Scale bars, 20 μm. The percentage of PAX6-GFP (H) or TBR2-GFP (I) double-positive cells within SVZ/VZ is quantified. (J and K) Pax6 and Tbr2 mRNA levels in E12 mouse primary NPCs were analyzed (legend continued on next page)
should be a rather straightforward means of modulating the level of DNA modification. Although regulation of Tet expression at the transcriptional level is well documented (Fu et al., 2013; Song et al., 2013), insights into the direct regulation of Tet mRNA by an RNA-binding protein are reported herein.

Recent studies have demonstrated that the post-transcriptional control of gene expression at the mRNA level is as important as transcriptional control (Anderson, 2008; Hao and Baltimore, 2009). MCPIP1 is an RNase and is responsible for degrading a set of inflammatory transcripts as well as pre-microRNAs (Matsushita et al., 2009; Mizgalska et al., 2009; Suzuki et al., 2011). PAR-CLIP analysis has been extensively used in target identification for RNA-binding proteins (Hafner et al., 2010; Yoon et al., 2014). Therefore, we performed PAR-CLIP analysis to identify genes bound by MCPIP1 in mouse neural N2a cells, but not human HEK293 cells, which have been widely used in CLIP analysis. This is because the N2a cell is a mouse neural cell, which more closely resembles mouse NPCs than human HEK293 cells. However, the mouse genome is less annotated, and the results from two cell lines may be different. A large number of genes binding to MCPIP1 were identified by PAR-CLIP analysis. Among them, we found that Tets have a strong interaction with MCPIP1. Low levels of Tet1 expression may provide an explanation for its non-detection. An in vitro EMSA experiment further confirmed the direct interaction between MCPIP1 protein and Tet mRNA. These results indicate that TETs are one of the major targets for MCPIP1.

There are many post-transcriptional regulation modes of RNA-binding proteins for their target genes. Binding sites located in coding sequence contribute most to translational repression while binding sites located in 3’ UTR mainly affect transcript stabilization (Brummer et al., 2013). Our findings showed that MCPIP1 bound to the coding sequence of Tets and regulated Tet mRNA levels. Further studies showed that its RNase activity was necessary for MCPIP1 regulation on Tet mRNA. However, the mechanism of MCPIP1 regulation on Tet mRNA by its coding sequence needs further work. Our current findings indicate the diversity of MCPIP1 action mode in regulating gene expression as an RNA-binding protein with RNase activity. These results will be essential to further our understanding of direct regulation of Tet expression.

In summary, this study clearly demonstrated an essential contribution for MCPIP1 in early neocortical development, which provides a basis for understanding the functions of MCPIP1 in the developing brain. Thus, these results suggested that MCPIP1 may be a potential therapeutic target for the treatment of neural developmental disorders by direct manipulation of immune or non-immune signaling within the brain.

**EXPERIMENTAL PROCEDURES**

**Animals**
Pregnant ICR mice were purchased from Vital River Laboratories. All animal studies were performed in accordance with experimental protocols and approved by Animal Care and Use Committees at the Institute of Zoology, Chinese Academy of Sciences.

**Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation Analysis**
The PAR-CLIP analysis was carried out as in previous work (Zhao et al., 2014), and performed in N2a cells by overexpression of FLAG-tagged Mcpip1. Detailed information of PAR-CLIP can be found in Supplemental Experimental Procedures.

**In Utero Electroporation**
In utero electroporation was performed as in previous work (Lv et al., 2014a), and described in detail in Supplemental Experimental Procedures.

**Statistical Analysis**
Statistical analyses, including two-tailed Student’s t tests and one-way ANOVA followed by a Tukey’s test for post hoc multiple comparisons, were performed using SPSS 16.0 for Windows. Differences were considered statistically significant at *p < 0.05 and **p < 0.01. Data are presented as mean ± SD.

Additional methods are provided in Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at [http://dx.doi.org/10.1016/j.stemcr.2016.07.011](http://dx.doi.org/10.1016/j.stemcr.2016.07.011).

**AUTHOR CONTRIBUTIONS**
H.J. and X.L. designed parts of the study, performed the research, analyzed the data, and wrote the manuscript; X.L., Y.Y., and X.Y. performed the research; J.J. designed parts of the study, supported the finance, and approved the final manuscript.

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level variation caused by Mcpip1 knockdown could be rescued by Tet knockdown, determined by FACS and qRT-PCR analysis. Values are mean ± SD; n = 3 independent experiments. *p < 0.05, **p < 0.01, one-way ANOVA with Tukey’s test for post hoc multiple comparisons; NS, not significant.

See also Figure S6.
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