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Prmt4-mediated methylation of NF-κB is critical for neural differentiation of embryonic stem cells

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ABSTRACT

Neural differentiation is a complex process regulated by multiple signaling at different regulatory levels. Though great progresses have been made in understanding the mechanisms of neural differentiation, post-translational regulation of neural differentiation remains largely unknown. In this study, we found Prmt4, one of the methyltransferases catalyzing protein arginine methylation, is highly expressed in neural stem cells (NSCs) and associated with neural differentiation. Knockout of Prmt4 in mESCs blocked neural differentiation by inhibiting NF-κB activation. Mechanistically, Prmt4 interacts with NF-κB component p65 to promote its methylation, resulting in increased activation of NF-κB signaling during neural differentiation. Our study not only identified Prmt4 as novel regulator of neural differentiation, but also highlighted the importance of protein arginine methylation in cell fate transition.

1. Introduction

Neural differentiation is a complex process that involves dramatic remodeling in cell morphology, metabolism and epigenetics, *etc* [1–3]. The mechanisms controlling neural differentiation have been revealed at diverse levels. Although transcriptional and translational control of neural differentiation have been extensively studied, post-translational regulation of neural differentiation remains unclear [1,4].

Post-translational modifications (PTMs) play crucial roles in various biological processes and are involved in almost all signaling cascades. Arginine methylation, one of the prevalent PTM of proteins, is catalyzed by protein arginine methyltransferases (Prmts) and involved in many cellular functions [5,6]. Prmt family contains nine members, which can be divided into three subclasses according to their catalytic activity: type I (Prmt1, Prmt2, Prmt3, Prmt4, Prmt6 and Prmt8), type II (Prmt5 and Prmt9) and type III (Prmt7) [5]. It has been reported that Prmt4, Prmt5, Prmt6, Prmt7 and Prmt8 are highly expressed in human or mouse embryonic stem cells (hESCs or mESCs) and play roles in pluripotency maintenance [7–12]. However, little is known about roles of Prmts in differentiation, such as neural differentiation.

In our study, we found Prmt4 is highly expressed in neural stem cells (NSCs) and associated with neural differentiation. Prmt4 deficiency in mESCs results in failure of neural differentiation by inhibiting NF-κB activation. Mechanistically, we elucidated that Prmt4 interacts with NF-κB component p65 to enhance its methylation, leading to increased activation of NF-κB signaling during neural differentiation. Our study not only identified Prmt4 as crucial regulator of neural differentiation, but also highlighted the importance of protein arginine methylation in cell fate transition.

2. Materials and methods

2.1. Cell culture and transfection

mESCs were cultured on gelatin-coated plate in Dulbecco's modified Eagle medium (DMEM) high-glucose medium supplemented with GlutaMax (Gibco), non-essential amino acids (Gibco), sodium pyruvate (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), N2 (Gibco), B27 (Gibco), 1000U/ml leukemia inhibitory factor, 3 μM CHIR99021 and 1 μM PD0325901, incubated at 37 °C in a humidified incubator in an atmosphere of 5% CO2. Transfection was performed with lipofectamine 2000 reagent according to the manufacturer's instructions. Prmt4 was cloned into pMX vector.

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2.2. Neural differentiation

To induce neural differentiation, mESCs were dissociated and plated at a density of 1×10^4 cells/cm^2 on gelatin coated plates in N2B27 medium supplemented with GlutaMax (Gibco), non-essential amino acids (Gibco), sodium pyruvate (Gibco), incubated at 37 °C in a humidified incubator in an atmosphere of 5% CO_2.

2.3. Generation of Prmt4 knockout mESCs

To generating Prmt4 knockout mESCs, donors were constructed by inserting a loxP-flanked PGK-puromycin cassette or loxP-flanked PGK-neomycin cassette into left and right homology arms, which were amplified from genomic DNA. Guide RNA (gRNA) was design on the website (crispr.mit.edu). For targeting, 1×10^6 mESCs were electroporated with 3 μg of donor and 6 μg of pX330 plasmid containing gRNA. Positive clones were selected using 2 μg/ml puromycin and 5 μg/ml neomycin for 3 days, and then picked and analyzed.

2.4. Reverse transcription-quantitative polymerase reaction (RT-qPCR)

Total RNA was extracted using TRIzol® reagent (Invitrogen) and reverse-transcribed into cDNA using Premix Ex Taq (Takara). cDNA was analyzed with CFX96 Real-Time System (Bio-Rad). All the primers using were from the Primer Bank database (pga.mgh.harvard.edu). Primers for *Prmt4*: forward TGACATCAGTATTGTGGCA and reverse TGAGGAGCCTAAGGGAATCA; Primers for *Pax6*: forward TACCAGTGTCTACCAGCCAAT and reverse TGCACGAGTATGAGGAGGTCT; Primers for *Actin*: forward GGCTGTATTCCCCTCCATCG and reverse CCAGTTGGTAACAATGCCATGT.

2.5. Western blot

Cells are lysed in RIPA buffer (Beyotime) supplemented with protease inhibitors (Selleck). The proteins were analyzed by SDS-PAGE and transferred to 0.45 μm PVDF membrane. The membrane was exposed to X film after incubation with indicated antibodies. Actin were used as control. Antibodies used were as follows: Actin (Cell Signaling Technology, 3700), Prmt4 (Cell Signaling Technology, 12495), p65 (Cell Signaling Technology, 8242), phos-p65 (Cell Signaling Technology, 3033), IKKα (Cell Signaling Technology, 11930), IKKβ (Cell Signaling Technology, 8943), mono-methylarginine (Cell Signaling Technology, 8015).

2.6. Co-immunoprecipitation

Cells were lysed in ice-cold cell lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP40, protease inhibitors) for 30 min. Lysates were incubated with the indicated antibodies for 3 h, followed by incubating with protein-A beads (Millipore) for 3 h at 4 °C. After immunoprecipitation, resin was washed with lysis buffer five times and was boiled in SDS buffer for 5 min, and analyzed by Western blot as mentioned above.

2.7. Cell immunofluorescence

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 containing 10% FBS (GIBCO) in PBS at room temperature for 15 min. Samples were then incubated with primary antibodies overnight at 4 °C, and stained with 0.1 μg/ml DAPI (Sigma). The antibody used for cell immunofluorescence were Pax6 (Cell Signaling Technology, 60433).

2.8. NF-κB reporter assay

NF-κB activity in cells treated with AdOx or AMI-1 were measured using a NF-κB reporter kit (DBS Bioscience, 60614) according to the manual with minor revisions. The NF-κB responsive elements and luciferase gene from the kit were cloned into pRLenti vector and used to produce lentivirus. After transfection, Cells were induced for neural differentiation and lysed for further analysis.

2.9. Statistical analysis

Data are presented as mean ± SD. Statistical comparisons were performed using the unpaired two-tailed student's t tests or a one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Prmt4 is highly expressed in neural stem cells and associated with neural differentiation

To identify which of the Prmt family members are important for neural differentiation of mouse embryonic stem cells (mESCs), we first analyzed the mRNA levels of nine members of Prmt gene family in mESCs and neural stem cells (NSCs) by qRT-PCR. The results showed that *Prmt5*, *Prmt6*, *Prmt7* and *Prmt8* were overexpressed in mESCs, while only *Prmt4* was highly expressed in NSCs (Fig. 1A). The high expression of Prmt4 was further confirmed at the protein levels (Fig. 1B), indicating possible roles for *Prmt4* in NSCs. Therefore, we focused on the role of Prmt4 during neural differentiation of mESCs. We induced neural differentiation of mESCs by using N2B27 medium [13] and confirmed the decrease of pluripotency gene *Oct4* and increase of neural marker gene *Pax6* (Fig. 1C). Next, we measured the expression of Prmt4 in this system and found both mRNA and protein levels of Prmt4 were increased during neural differentiation (Fig. 1C and D). Taken together, these results demonstrate Prmt4 is highly expressed in NSCs and is associated with neural differentiation.

3.2. Knockout of Prmt4 blocks neural differentiation of mESCs

To determine the roles of Prmt4 in mESCs and neural differentiation, we knocked out *Prmt4* in mESCs using CRISPR/Cas9-mediated homologous recombination [14]. We designed single guide RNA (sgRNA) targeting the first exon of *Prmt4* and co-transfected sgRNA/Cas9 with a homologous donor vector containing puromycin or neomycin resistant cassette. The mESC clones were subsequently selected by puromycin and neomycin, picked and cultured for further analysis (Fig. 2A). The knockout of *Prmt4* in mESC was verified by qRT-PCR, Western blot and genomic PCR (Fig. 2B). Because Prmt4 has been shown to be involved in pluripotency regulation [7], we analyzed the mRNA levels of several pluripotency genes *Oct4*, *Nanog* and Rex1 in WT and Prmt4 knockout (Prmt4 KO) mESCs. However, no significant difference in the expression of pluripotency genes were observed between WT and Prmt4 KO mESCs (Fig. 2C). As Prmt4 is highly expressed in NSCs and is up-regulated during neural differentiation, we ask whether Prmt4 play a role in neural differentiation of mESCs. To this end, we induced WT and Prmt4 KO mESCs to differentiate towards a neural fate and found Prmt4 KO mESCs failed to differentiate into NSCs, evidenced by decreased expression of *Pax6* and formation of neural rosettes (Fig. 2D). Overall, these results suggest that knockout of Prmt4 does not impact pluripotency maintenance, but is required for neural differentiation of mESCs.

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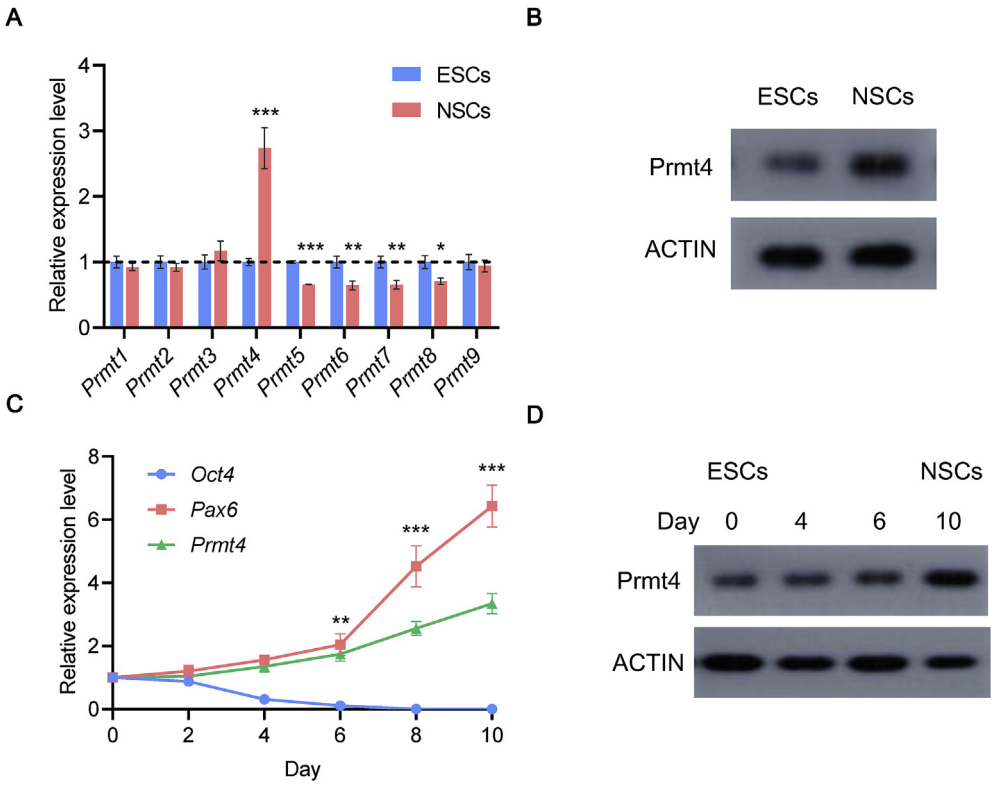

Fig. 1. Prmt4 is highly expressed in neural stem cells and associated with neural differentiation.
(A) qRT-PCR analysis of Prmt gene family expression is mouse embryonic stem cells (mESCs) and neural stem cells (NSCs). Data are presented as mean ± SD, n = 3, *P < 0.05, **P < 0.01, ***P < 0.001.(B) Western blot analysis of Prmt4 expression in mESCs and NSCs. (C) qRT-PCR analysis of *Prmt4* expression during neural differentiation of mESCs. Data are presented as mean ± SD, n = 3, **P < 0.01, ***P < 0.001. (D)Western blot analysis of Prmt4 expression during neural differentiation of mESCs.

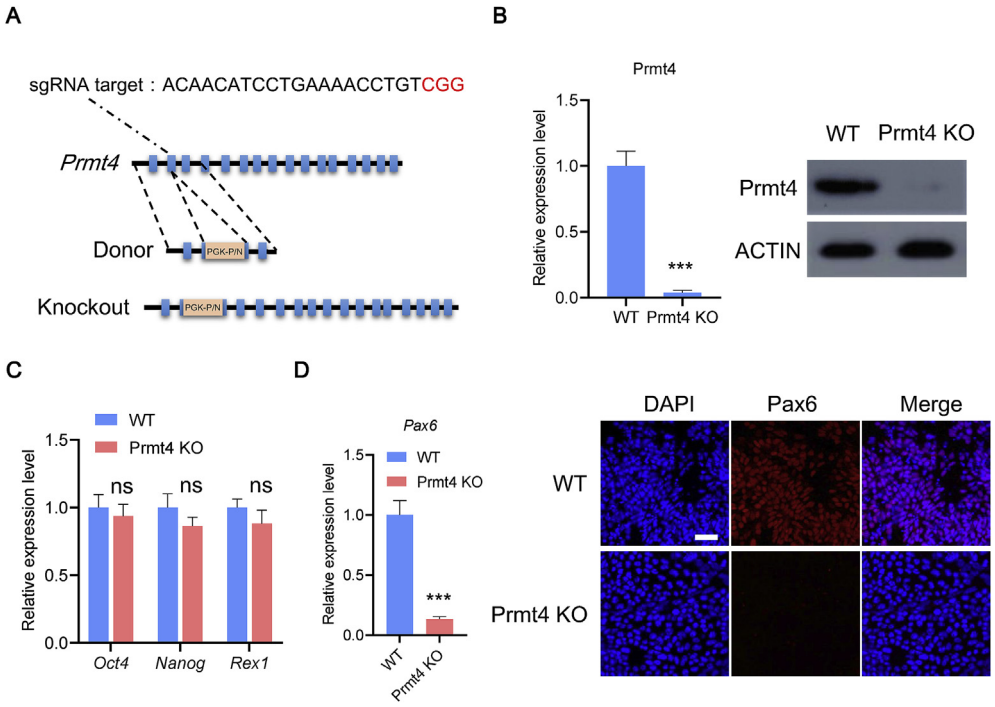

Fig. 2. Knockout of Prmt4 blocks neural differentiation of mESCs.
(A) Schematic overview showing the targeting strategy for the *Prmt4* locus using CRISPR/Cas9. PGK-P/N: PGK promoter-Puromycin/Neomycin. (B) qRT-PCR and Western blot analysis of Prmt4 expression in wild type (WT) and Prmt4 knockout (Prmt4 KO) mESCs. Data are presented as mean ± SD, n = 3, ***P < 0.001. (C) qRT-PCR analysis of pluripotency gene expression (Oct4, Nanog, Rex1) and in WT and Prmt4 KO mESCs. Data are presented as mean ± SD, n = 3, ns: not significant. (D) qRT-PCR and immunofluorescence analysis of PAX6 expression on day 10 during neural differentiation of WT and Prmt4 KO mESCs. Scale bar represents 100 μm, Data are presented as mean ± SD, n = 3, ***P < 0.001.

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3.3. Loss of Prmt4 inhibits NF-κB signaling during neural differentiation

To gain deeper insights into the role of Prmt4 in neural differentiation, we performed genome-wide transcriptome profiling during neural differentiation of WT and Prmt4 KO mESCs by RNA-sequencing. KEGG analysis showed that genes changed significantly upon Prmt4 knockout (fold change>2 and p value < 0.05) were associated with NF-κB pathway, metabolic pathway, ECM pathway, mTOR signaling and STAT signaling (Fig. 3A). We also performed GO analysis and found Prmt4 deficiency was involved in transcription, inflammatory response, macromolecule biosynthesis, cell differentiation and adhesion (Fig. 3B). We noted that NF-κB signaling might be the most prominent pathway regulated by Prmt4 in the KEGG pathway analysis, and was also play important roles in most of the GO terms showed above [15–17](Fig. 3A and B). Therefore, we focused on NF-κB signaling and analyzed the activation of NF-κB by measuring the phosphorylation level of NF-κB component p65 (Fig. 3C). The results showed that knockout of Prmt4 significantly inhibited phosphorylation of p65, without affecting total p65 protein level (Fig. 3C). Next, we sought to determine whether NF-κB signaling is downstream of Prmt4 during neural differentiation. We employed NF-κB activator betulinic acid (BA) or PMA, which successfully increases phosphorylation of p65 during neural differentiation of Prmt4 KO mESCs (Fig. 3D), and tried to rescue deficiency in neural differentiation. The results showed that addition of either BA or PMA could restore neural differentiation of Prmt4 KO mESCs, evidenced by increased *Pax6* expression (Fig. 3E). Together, these results suggest that Prmt4 is critical for the activation of NF-κB signaling during neural differentiation.

3.4. Prmt4-mediated methylation of NF-κB is required for neural differentiation

Prmt family members, Prmt2 and Prmt5, were shown to regulate NF-κB through interacting with different components in the NF-κB signaling pathway, such as p50/p65 and Ikkα/β [18,19]. These led us to ask whether Prmt4 interacts with NF-κB components and upstream kinases. Specially, we examined the interaction between Prmt4 and p65, Ikkα or Ikkβ and found only p65 interacted with Prmt4 (Fig. 4A). As Prmt family members often function through catalyzing protein arginine methylation, we performed Western blot to measure arginine methylation of NF-κB p65. NF-κB p65 was immunoprecipitated and blotted using antibody against monomethylarginine (me1) (Fig. 4B). Notable, knockout of Prmt4 resulted in a markedly decrease in arginine methylation of p65, while overexpression of Prmt4 restored the methylation level (Fig. 4B), suggesting Prmt4 interacted with and methylated NF-κB p65 to increase NF-κB activity. As NF-κB p65 signaling is activated and required for neural differentiation [20], we ask whether arginine methylation of NF-κB p65 is involved in the activation of NF-κB during neural differentiation of mESCs. To this end, we employed methyltransferase inhibitors AdOx and AMI-1 [21], and found both of them inhibited arginine methylation of NF-κB p65 during neural differentiation (Fig. 4C). To test whether the phosphorylation and methylation of p65 occur together for NF-kB activation, we analyzed the phosphorylation of p65 upon treatment with AdOx or AMI-1. The results showed that AdOx or AMI-1 reduced phosphorylation of p65 as well as methylation (Fig. 4D). Further, we analyzed NF-κB activity in cells undergoing neural differentiation using a NF-κB reporter assay and found NF-κB activity was markedly reduced by AdOx or AMI-1 (Fig. 4E). In addition, methyltransferase inhibitors AdOx or AMI-1 decreased neural

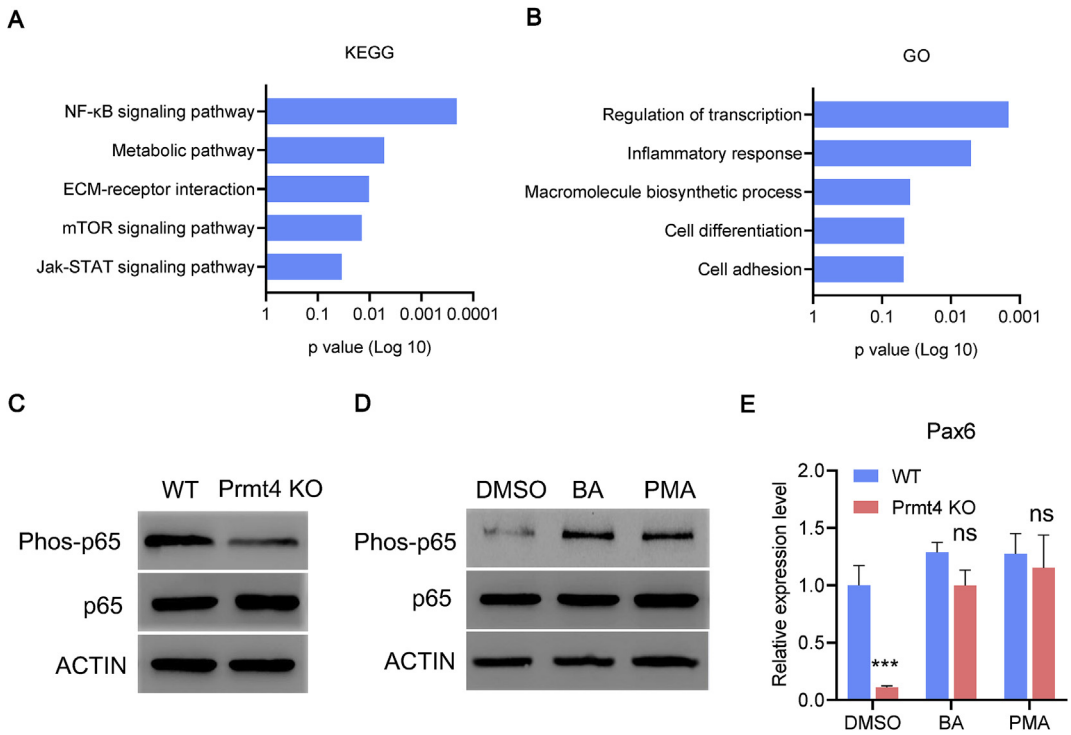

Fig. 3. Loss of Prmt4 inhibits NF-κB signaling during neural differentiation
(A) KEGG analysis of RNA-seq data from neural differentiation of WT and Prmt4 KO mESCs (day 10). (B) Gene ontology (GO) analysis of RNA-seq data from neural differentiation of WT and Prmt4 KO mESCs (day 10). (C) Western blot analysis of NF-κB activation during neural differentiation of WT and Prmt4 KO mESCs (day 10). (D) Western blot analysis of NF-κB activation in Prmt4 KO mESCs treated with NF-κB activators Betulinic acid (BA) or PMA. (E) qRT-PCR analysis of *Pax6* expression during neural differentiation of WT and Prmt4 KO mESCs upon treatment of NF-κB activators BA or PMA. Data are presented as mean ± SD, n = 3, ***P < 0.001, ns: not significant.

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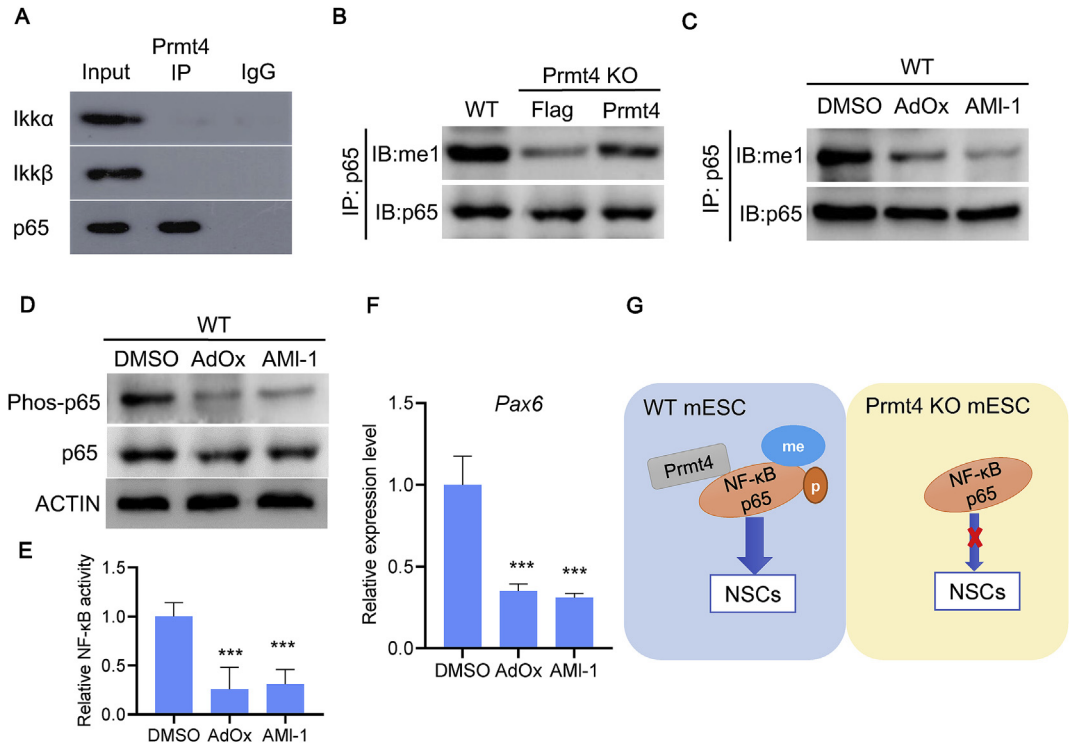

Fig. 4. Prmt4-mediated methylation of NF-κB is required for neural differentiation

(A) Co-Immunoprecipitation analysis of Prmt4 and NF-κB p65 and upstream kinases Ikkα and Ikkβ. (B) Western blot analysis of arginine methylation of NF-κB p65 during neural differentiation of WT and Prmt4 KO mESCs with or without Prmt4 expression. (C) Western blot analysis of arginine methylation of NF-κB p65 during neural differentiation of WT mESCs treated with methyltransferase inhibitors AdOx or AMI-1. Immunoprecipitated NF-κB p65 was blotted with an antibody against mono-methylarginine (me1), p65 was used as loading control.(D) Western blot analysis of phosphorylation of NF-κB p65 during neural differentiation of WT mESCs treated with methyltransferase inhibitors AdOx or AMI-1. (E) Relative NF-κB activity during neural differentiation of WT mESCs upon treatment of methyltransferase inhibitors AdOx or AMI-1. Data are presented as mean ± SD, n = 3, ***P < 0.001.(F) qRT-PCR analysis of PAX6 expression during neural differentiation of WT mESCs upon treatment of methyltransferase inhibitors AdOx or AMI-1. Data are presented as mean ± SD, n = 3, ***P < 0.001.(G) The proposed model of Prmt4 functions in NSCs.

differentiation, evidenced by decreased *Pax6* expression (Fig. 4F). Taken together, our results indicate that Prmt4-mediated methylation of NF-κB p65 is required for neural differentiation (Fig. 4G).

4. Discussion

Protein arginine methylation, a prevalent post-translational modification, has recently drawn increasing attention as crucial regulators in a wide range of cellular processes, including transcription regulation, metabolism, carcinogenesis, development and differentiation [5,6]. Prmts are methyltransferases which are responsible for catalyzing the formation of arginine methylation. The mammalian Prmt family contains nine members with distinct cellular functions. Prmt4, Prmt5, Prmt6, Prmt7 and Prmt8 have been reported to be highly expressed in embryonic stem cells and associated with stemness [7–11]. However, little is known about the roles and mechanisms of Prmts in differentiation of mESC to a specific cell type. Here, we found that Prmt4 is highly expressed in NSCs and is associated with neural differentiation of mESCs. Knockout of Prmt4 blocked neural differentiation by inhibiting NF-κB signaling. Mechanistically, Prmt4 interacts with NF-κB component p65, leading to increased arginine methylation of p65, which is required for neural differentiation (Fig. 4E). Our results identified Prmt4 as crucial regulator of neural differentiation and elucidated underlying mechanism of Prmt4.

NF-κB is a ubiquitously expressed transcription factor that plays critical roles in various biological processes, including inflammation, tumor progression, proliferation and differentiation [16,22]. Posttranslational modifications of core components of NF-κB signaling pathway is crucial for regulation of NF-κB activity [23,24]. Although great progress has been made in understanding the roles of phosphorylation and ubiquitination in NF-κB regulation, little is known about methylation [25]. Our study identified Prmt4 as previously unknown regulator of NF-κB methylation, and showed that Prmt4-mediated methylation of NF-κB is critical for neural differentiation. In addition, as coordinated regulation of NF-κB at different levels is crucial for cell fate transitions, including oncogenesis, pluripotency regulation and development [17,20,26–29], our results linking arginine methylation to NF-κB should provide insights into understanding the arginine methylation regulation of cell fate transitions.

In all, our finding demonstrated that Prmt4 and NF-κB activation is required for neural differentiation and identified Prmt4 as critical regulator of NF-κB methylation and activation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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