

TAPP1 inhibits the differentiation of oligodendrocyte precursor cells *via* suppressing the Mek/Erk pathway

Yidan Chen¹, Ruyi Mei¹, Peng Teng¹, Aifen Yang¹, Xuemei Hu², Zunyi Zhang¹, Mengsheng Qiu^{1,2}, Xiaofeng Zhao¹

¹*Institute of Developmental and Regenerative Biology, Zhejiang Key Laboratory of Organ Development and Regeneration, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China*

²*Department of Anatomical Sciences and Neurobiology, School of Medicine, University of Louisville, Louisville, KY 40202, USA*

Corresponding authors: Xiaofeng Zhao and Mengsheng Qiu. E-mail: xiaofengzhao@yahoo.com; m0qiu001@yahoo.com

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ABSTRACT

Oligodendrocytes (OLs) are glial cells that form myelin sheaths around axons in the central nervous system (CNS). Loss of the myelin sheath in demyelinating and neurodegenerative diseases can lead to severe impairment of movement. Understanding the extracellular signals and intracellular factors that regulate OL differentiation and myelination during development can help to develop novel strategies for enhancing myelin repair in neurological disorders. Here, we report that *TAPP1* was selectively expressed in differentiating OL precursor cells (OPCs). *TAPP1* knockdown promoted OL differentiation and myelin gene expression in culture. Conversely, over-expression of *TAPP1* in immature OPCs suppressed their differentiation. Moreover, *TAPP1* inhibition in OPCs altered the expression of Erk1/2 but not AKT. Taken together, our results identify *TAPP1* as an important negative regulator of OPC differentiation through the Mek/Erk signaling pathway.

Keywords: TAPP1; differentiation; oligodendrocyte; spinal cord

INTRODUCTION

In the central nervous system (CNS), myelin sheaths

elaborated by oligodendrocytes (OLs) govern axonal conductance and integrity^[1,2]. OLs are developmentally generated through the differentiation and maturation of OL precursor cells (OPCs)^[3,4]. Impairment of OL function and CNS myelin can cause devastating neurological disorders such as multiple sclerosis^[1,5,6], and psychiatric disorders such as schizophrenia and depression^[7]. In addition, OL impairment has also been found in neurodegenerative diseases including multiple system atrophy, Alzheimer's disease, and Parkinson's disease^[8,9]. Elucidating the molecular mechanisms underlying OL differentiation is a crucial prerequisite for developing novel strategies for enhancing myelin repair in neurological disorders.

Recently, we demonstrated that Nkx2.2 directly binds to the promoter of platelet-derived growth factor receptor alpha (Pdgfra) and inhibits its gene expression to turn off Pdgfra signaling in OPCs and initiate the intrinsic program for OL differentiation^[10]. To identify the downstream targets of the Nkx2.2-Pdgfra pathway, we compared the gene expression array data in spinal cord tissues from Nkx2.2 conditional-knockout and Nkx2.2 over-expressing transgenic mice and found that many regulatory molecules respond to altered Nkx2.2 expression. One of the regulatory molecules is TAPP1, a pleckstrin homology (PH) domain-containing adapter protein that specifically binds to the lipid phosphatidylinositol 3, 4-bisphosphate [PI(3,4)P₂] via its C-terminal PH domain^[11]. TAPP1 is recruited to the plasma membrane of cells stimulated with platelet-derived

growth factor (PDGF), a mitogen that produces PI(3,4)P2^[12]. Since PDGF signaling is a key pathway for regulating OPC proliferation and differentiation, it is possible that TAPP1 is involved in OL maturation and myelination in CNS development.

We therefore set out to determine the effect of *TAPP1* on OPCs differentiation and the signaling involved in this development process.

MATERIALS AND METHODS

Animals

The mice used in this study were handled according to the protocols approved by Institutional Animal Care and Use Committee (IACUC), University of Louisville (IACUC: 12034). C57BL/6N mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Statistics of cell numbers and relative expression levels of target proteins were calculated for each genotype ($n = 3$).

In Situ RNA Hybridization and Immunofluorescence Staining

In situ hybridization (ISH) was performed according to Schaeren-Wiemers and Gerfin-Moser^[13] with minor modifications. Mice were deeply anesthetized and perfused with 4% paraformaldehyde (PFA), and spinal cord and brain tissues were isolated and postfixed in 4% PFA at 4°C overnight. Fixed spinal cord tissues were embedded in OCT medium and sectioned on a cryostat (Microm HM525, German). Frozen sections (16 µm thick) were subjected to ISH with digoxigenin-labeled riboprobe. The riboprobe sequence for *TAPP1* was F: 5'-GGA ATT CAT CAT CAC GCC GAC GCA GAA AGA -3'; R: 5'-CGG GAT CCA GGC TGG GGA ATG TGA GGC GGT-3'. Double immunofluorescence procedures were carried out as previously described^[14]. The dilution ratios of antibodies were as follows: anti-TAPP1 (Sigma, St. Louis, MO, 1:50), anti-Olig2 (Millipore, Billerica, MA, 1:1 000), anti-MBP (Abcam, Cambridge, MA, 1:500), anti-MAG (Millipore, 1:500), anti-Ki67 (Thermo, Grand Island, NE, 1:500), and anti-caspase3 (Millipore, 1:1 000).

Western Blotting

Spinal cord tissues were lysed in lysis buffer (Sigma) with protease inhibitor cocktail (Sigma). Proteins from control

and mutant tissues (30 µg each) were loaded for SDS-PAGE electrophoresis and subsequently detected with anti-TAPP1 (Sigma, 1:250), anti-MBP (Abcam, 1:2 000), anti-CNPase (Cell Signaling, Boston, MA, 1:1 000), anti-p44/42 MAPK (Erk1/2) (Cell Signaling, 1:2 000), anti-phospho-p44/42 MAPK-T202/Y204 (pErk1/2) (Cell Signaling, 1:2 000), and mouse anti-GAPDH (Sigma, 1:5 000) antibodies according to the standard protocol. The optical density of blots was assessed with Quantity One software (ChemiDoc XRS; Bio-Rad, USA) and the relative densitometric values were used for statistical analyses of the expression levels of target proteins.

Rat OPC Culture

Cerebral cortices from P0 SD rats were dissected out, minced and digested in 0.125% trypsin at 37°C. The digestion was stopped by the addition of Dulbecco's modified Eagle's medium (DMEM)/F12 containing 20% fetal bovine serum (FBS). The dissociated cells were plated in 75-cm² tissue-culture flasks coated with 100 µg/mL poly-L-lysine, and the medium was changed the next day. After 7 days in culture, the cells were rinsed three times with medium and shaken for 1 h at 200 r/min to remove microglia. Then the flasks were sealed and shaken at 250 r/min at 37°C for 15–18 h to collect OPCs. The medium with the detached cells was collected and first plated on tissue-culture dishes (not coated with poly-D-lysine) for 30–60 min at 37°C to allow firm attachment of microglia and astrocytes, while OPCs were loosely attached and were collected by gently shaking the dishes. The non-adherent OPCs were collected, centrifuged and re-plated in DMEM containing 20% FBS in 4 hours, then replaced by OPCs medium. To expand the OPCs and keep them undifferentiated, the medium was supplemented with PDGF-AA (10 nmol/L, PeproTech, Rocky Hill, NJ). For differentiation assay, OPCs were allowed to grow in differentiation medium containing 40 ng/mL T3 for 3 days. OPCs collected in the medium were then re-plated at 5 000 to 50 000 cells/cm² onto poly-D-lysine-coated plates, dishes, or coverslips for differentiation cultures.

Lentivirus Construction and OPC Infection

For RNAi-mediated depletion of *TAPP1*, lentivirus encoding small hairpin RNA (shRNA) for *TAPP1* was prepared by GenePharma (Shanghai, China). The shRNA sequence for

TAPP1 was 5'-GGC AAG AAG CAA GUG UCU TAC-3'. To construct the lentiviral vector expressing *TAPP1*-shRNA, the shRNA was amplified by PCR and subcloned into pWP vector. To produce lentivirus containing *TAPP1*-shRNA, HEK-293T cells were co-transfected with pWP-TAPP1 plasmid and ViraPower Packaging Mix using Lipofectamine 2000 according to the manufacturer's guidelines. OPCs were transfected with lentiviral *TAPP1*-shRNA 72 h before experiments.

For *TAPP1* overexpression *in vitro*, rat *TAPP1* cDNA was cloned into the pCDH-MCS-EF1-CopGFP vector by primers as follows: forward 5'-GGA ATT CGC CAC CAT GCC TTA TGT GGA TCG ACA GAA T-3; reverse 5'-CGG GAT CCT CAA GCG TAA TCT GGA ACA TCG TAT GGG TAG CTG TAA GGA GGA GCG AGT CCA GG-3'. Lentiviruses were prepared by co-transfecting pCDH-MCS-EF1-CopGFP or pCDH-rTAPP1-EF1-CopGFP with pMD2.G and psPAX2 packaging vectors (Addgene, Cambridge, MA) into 293T cells. The harvested viral supernatants were used to infect OPCs. Infection efficiency was analyzed on day 2 post-infection using GFP expression, and efficiencies >95% were obtained. Six days after infection, OPCs were collected for the next experiments.

Real-Time PCR

mRNA levels were assessed by real-time PCR using a Bio-Rad QX100 Droplet Digital PCR system. cDNA was synthesized by reverse transcription using oligo (dT) as the primer and proceeded to real-time PCR with gene-specific primers in the presence of SYBR Premix Ex Taq (DRR041A, Takara Biotechnology, Dalian, China). Quantification was performed by the comparative cycle threshold method, using β -actin as the internal control. The following forward (F) and reverse (R) primers were used for amplification: *TAPP1*-F: 5'-ACA CAG AAA GAA GAA GTA AAC G-3', *TAPP1*-R: 5'-ACA GGT TGT CCC TCA TCA TAA T-3', β -actin-F: 5'-CGC ACC ACT GGC ATT GTC AT-3', β -actin-R: 5'-TTC TCC TTG ATG TCA CGC AC-3'.

Statistical Analysis

Statistical differences were determined by Student's *t*-test for two-group comparison or by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. $P < 0.05$ was considered to be statistically significant. Data are presented as mean $\pm$ SEM.

RESULTS

TAPP1 Is Selectively Expressed in Differentiating Oligodendrocytes

Our microarray analysis has identified *TAPP1* as one of the Nkx2.2 downstream target genes that may be involved in OL development (unpublished data). To investigate this possibility, we first examined its expression pattern in the developing CNS. RNA ISH revealed that *TAPP1* started to be expressed in the white matter of the spinal cord at around embryonic day 18.5 (E18.5); no specific expression was detected at earlier stages (Fig. 1A, B). Its expression progressively increased between E18.5 and P7 (Fig. 1B–E) and then gradually decreased thereafter (Fig. 1F–H). By P30, *TAPP1* expression was restricted to the gray matter (Fig. 1H). Consistent with the ISH results, western blotting revealed a similar increase of *TAPP1* protein expression in the spinal cord from E18.5 to P10, but a decrease after P15 (Fig. 1I, J). The spatiotemporal pattern of *TAPP1* expression suggested that it is likely to be expressed in OLs. To test this possibility, we performed immunofluorescence staining with anti-TAPP1 and anti-Olig2 on P3 and P15 spinal cord sections and P21 brain sections. The results showed that most *TAPP1*⁺ cells co-localized with Olig2⁺ cells in both the white matter of the spinal cord and the corpus callosum (Fig. 2A–C).

The spatiotemporal pattern of *TAPP1* expression suggested that it is likely to be expressed in differentiating OLs but not in mature myelinated OLs. To further confirm this, we examined the expression of this gene in Olig1 mutant and Nkx2.2 mutant spinal cords using *TAPP1* ISH. It was previously shown that the differentiation of OLs is delayed and/or reduced in the spinal cords of these mutants^[10, 15]. Consistently, *TAPP1* was expressed in differentiating OLs, as expression of this gene was dramatically reduced in P3 Olig1 and Nkx2.2 mutant spinal cords (Fig. 2D–G).

TAPP1 Knockdown Promotes Oligodendrocyte Differentiation

To investigate the function of *TAPP1* deficiency in OL lineage development, OPC cultures were infected with lentivirus co-expressing GFP and shRNA of *TAPP1* three days prior to differentiation assays. The infection efficiency was defined by GFP fluorescence (Fig. 3B, F, J, N). As expected, the *TAPP1* expression in OPCs was significantly inhibited by shRNA treatment (Fig. 3Q). Meanwhile, down-

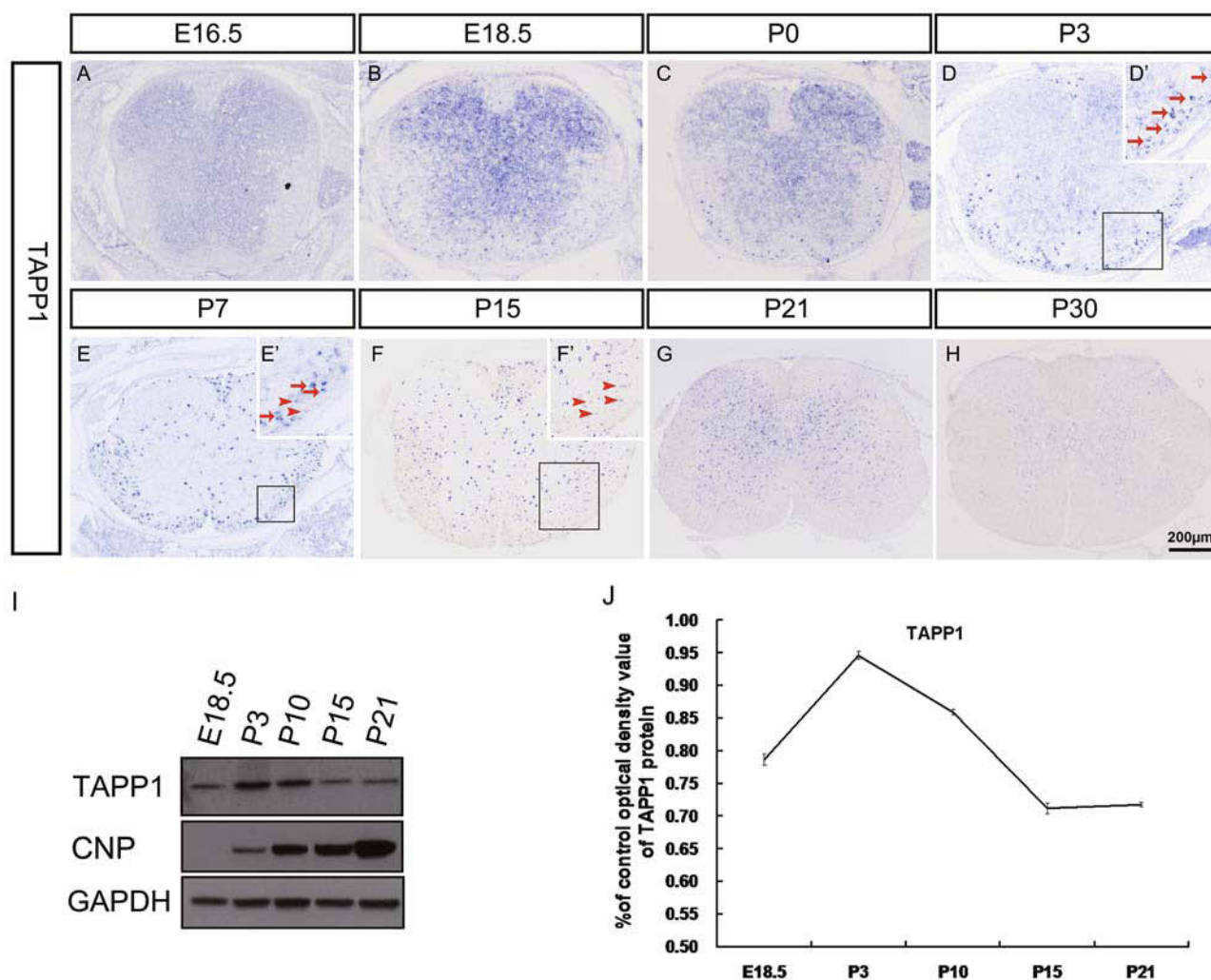


Fig. 1. Dynamic expression of *TAPP1* in spinal cord. A–H: Expression of *TAPP1* started at around embryonic day 18.5 (E18.5) in the white matter of the spinal cord. Its expression progressively increased between E18.5 and P7, and then gradually decreased thereafter. By P30, *TAPP1* expression was restricted to the gray matter. D'–F': Higher magnification of boxes in D–F. Arrows indicate positive signals. Scale bar, 200 μm. I–J: Consistently, Western blotting revealed a similar pattern of *TAPP1* protein expression in spinal cords from E18.5 to P21.

regulation of *TAPP1* by shRNA remarkably increased the number of mature myelin-associated glycoprotein-positive (MAG⁺) and myelin basic protein-positive (MBP⁺) OLs compared to control vectors (Fig. 3C, G, K, O). Quantitative analysis showed that the percentage of GFP⁺ with MAG⁺ or MBP⁺ OLs in GFP⁺ RNAi-*TAPP1*-infected cells was significantly higher than that in control cells (Fig. 3R). Moreover, RNAi-*TAPP1* cells displayed multipolar processes instead of the typical bipolar processes of OPCs (Fig. 3D, H, L, P). Together, these data indicated

that *TAPP1* negatively regulates OPC differentiation, and inhibition of *TAPP1* expression promotes OL differentiation.

Over-expression of *TAPP1* Inhibits OPC Differentiation

We then investigated whether *TAPP1* overexpression was capable of inhibiting OPC differentiation. Cultured OPCs were infected with a constitutively active *TAPP1* plasmid, pCDH-rTAPP1-EF1-CopGFP, followed by T3 treatment for 3 days. Real-time PCR confirmed that *TAPP1* expression was markedly enhanced after pCDH-rTAPP1-

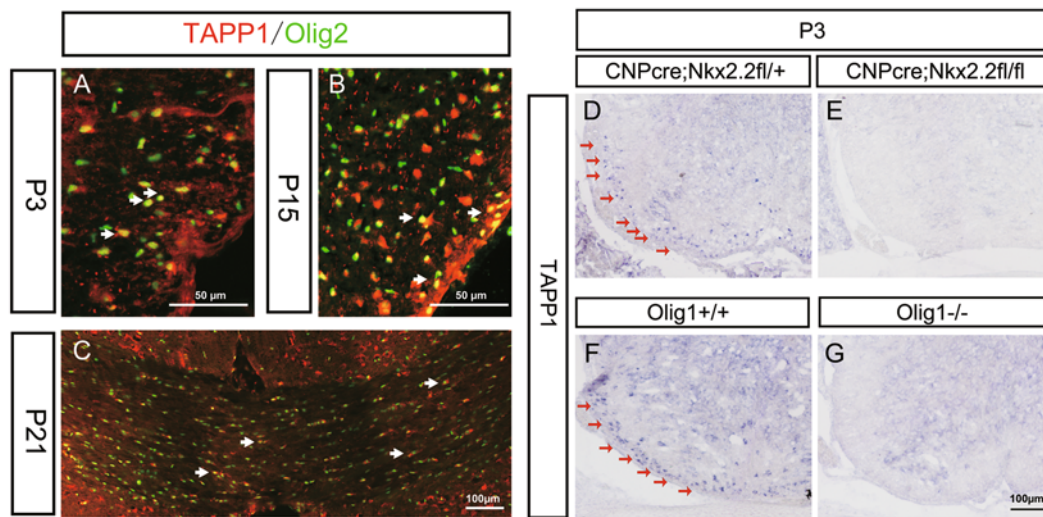


Fig. 2. TAPP1 is selectively expressed in differentiating oligodendrocytes. A–B: Transverse sections from wild-type mouse spinal white matter at P3 and P15 double-immunostained with anti-TAPP1 and anti-Olig2. Double-positive cells are indicated by arrows. Scale bars, 50 μm. C: Transverse section of wild-type mouse corpus callosum at P21 double-immunostained with anti-TAPP1 and anti-Olig2. Double-positive cells are indicated by arrows. Scale bar, 100 μm. D–G: TAPP1 was significantly reduced in P3 Nkx2.2 conditional mutant spinal cords and absent from Olig1 mutants. Arrows indicate positive signals. Scale bar, 100 μm.

EF1-CopGFP infection (Fig. 4Q). Concomitantly, MAG and MBP expression was also significantly decreased (Fig. 4C, G, K, O). Analysis showed significantly lower percentages of GFP⁺ with MAG⁺ or MBP⁺ OLs in GFP⁺ cells over-expressing TAPP1 than that in the control vector (Fig. 4R). Moreover, OPCs over-expressing TAPP1 displayed typical bipolar rather than multipolar processes (Fig. 4D, H, L, P), contrary to the morphology of TAPP1-knockdown OPCs. These data suggested that activation of TAPP1 inhibits OPC differentiation.

Over-expression of TAPP1 Does Not Affect the Survival and Proliferation of OPCs

It may be argued that the inhibition of OPC differentiation by TAPP1 over-expression is due to its induction of cell death or inhibition of cell proliferation. To address this question, caspase3 and Ki67 immunostaining were performed. But there was no significant difference between control and TAPP1 over-expressing OPCs, indicating that cell death and apoptosis were not affected by TAPP1 over-expression (Fig. 5C, G, Q). In addition, there was also no difference in the Ki67 score (Ki67⁺/GFP⁺) between OPCs treated with pCDH-rTAPP1-EF1-CopGFP for 72 h and control OPCs (Fig. 5K, O, Q), suggesting that TAPP1 over-expression

does not influence the proliferation of OPCs.

TAPP1 Inhibition Alters Erk1/2 Expression

TAPP1 is a PH domain-containing adapter protein which is recruited to the plasma membrane of cells in response to phosphoinositol 3-kinase (PI3K) activation^[11]. Several studies have implicated the PI3K/Akt pathway in the proliferation, migration, or survival of OPCs^[16–22], so we examined the effects of TAPP1 inhibition on the expression of Akt during OPC differentiation. Surprisingly, TAPP1 inhibition had no significant effects on the expression levels of total and phospho-Akt proteins (Fig. 6).

Recently, it has been reported that Erk1/2 MAPK and PI3K/Akt/mTOR signaling sequentially regulates distinct stages of OL differentiation *in vitro*^[23], so we next asked whether Erk1/2 signaling is affected by TAPP1 inhibition during OPC differentiation. Our studies showed that both total and phospho-Erk(1/2) were induced in TAPP1-knockdown cells. Consistent with the immunostaining results, MBP and CNPase expression was also induced in cells treated with shRNA-TAPP1 (Fig. 6). Together, these findings suggested that TAPP1 ablation induces Erk1/2 expression, which in turn causes the precocious differentiation of OLs.

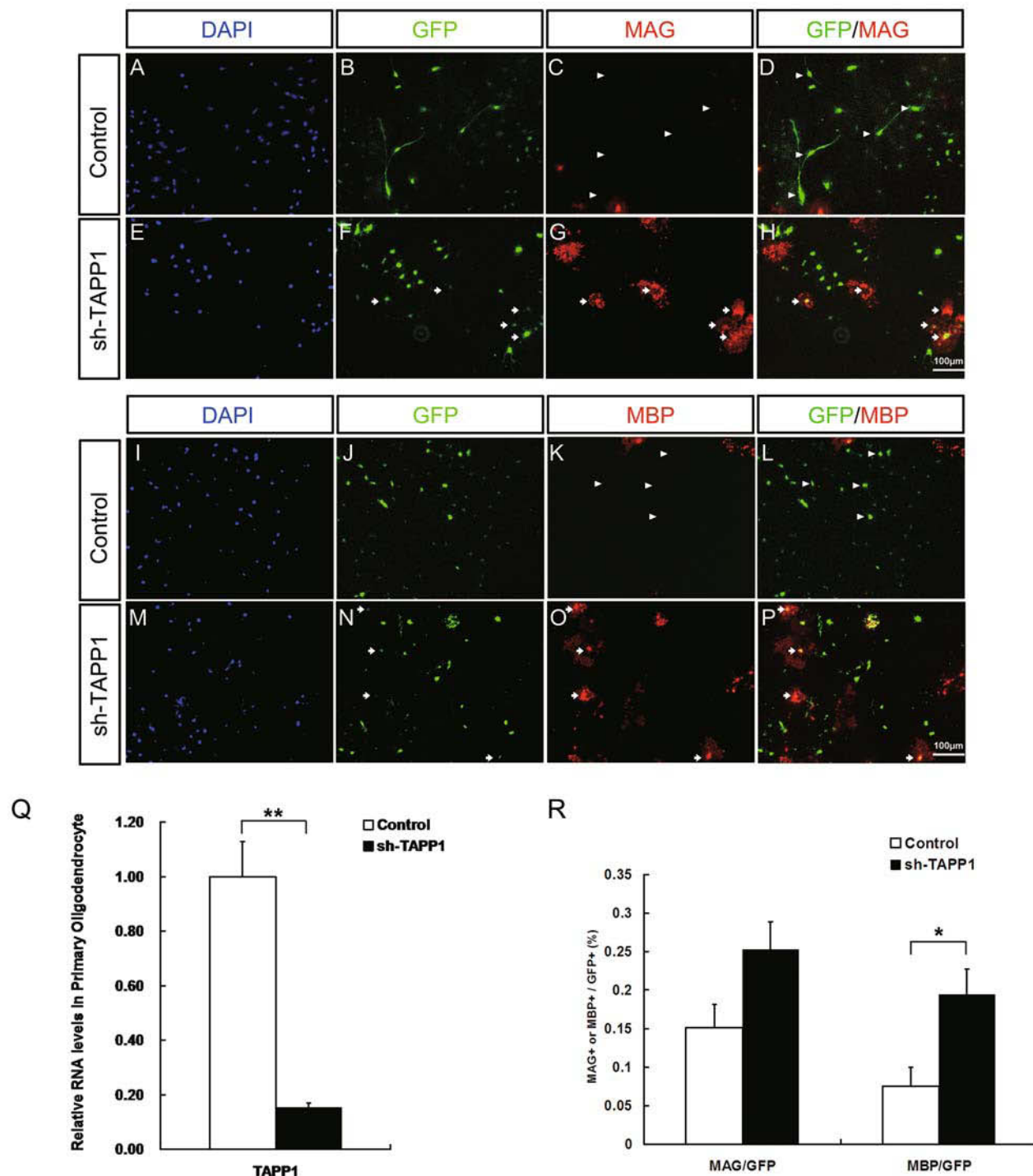


Fig. 3. *TAPP1* knockdown promotes OL differentiation. A–R: Cultured OPCs infected with GFP-tagged ShRNA-*TAPP1* or control lentivirus (A, B, E, F, I, J, M, and N). *TAPP1* expression in OPCs was significantly inhibited by shRNA (Q). Compared to control cells, *TAPP1* down-regulation by shRNA remarkably increased the number of MAG⁺ and MBP⁺ cells (C, G, D, H, K, O, L, and P). Analysis showed that the percentages of GFP⁺ with MAG⁺ or MBP⁺ OLs in GFP⁺ cells among RNAi-*TAPP1* cells were significantly higher than that in the control (R). Arrows indicate GFP⁺ and MAG⁺/MBP⁺ double-positive cells; arrowheads indicate GFP⁺ single-positive cells. Statistical analyses with Student's *t*-test. Error bar, standard deviation ($n = 3$, * $P < 0.05$, ** $P < 0.01$). Scale bars, 100 μm .

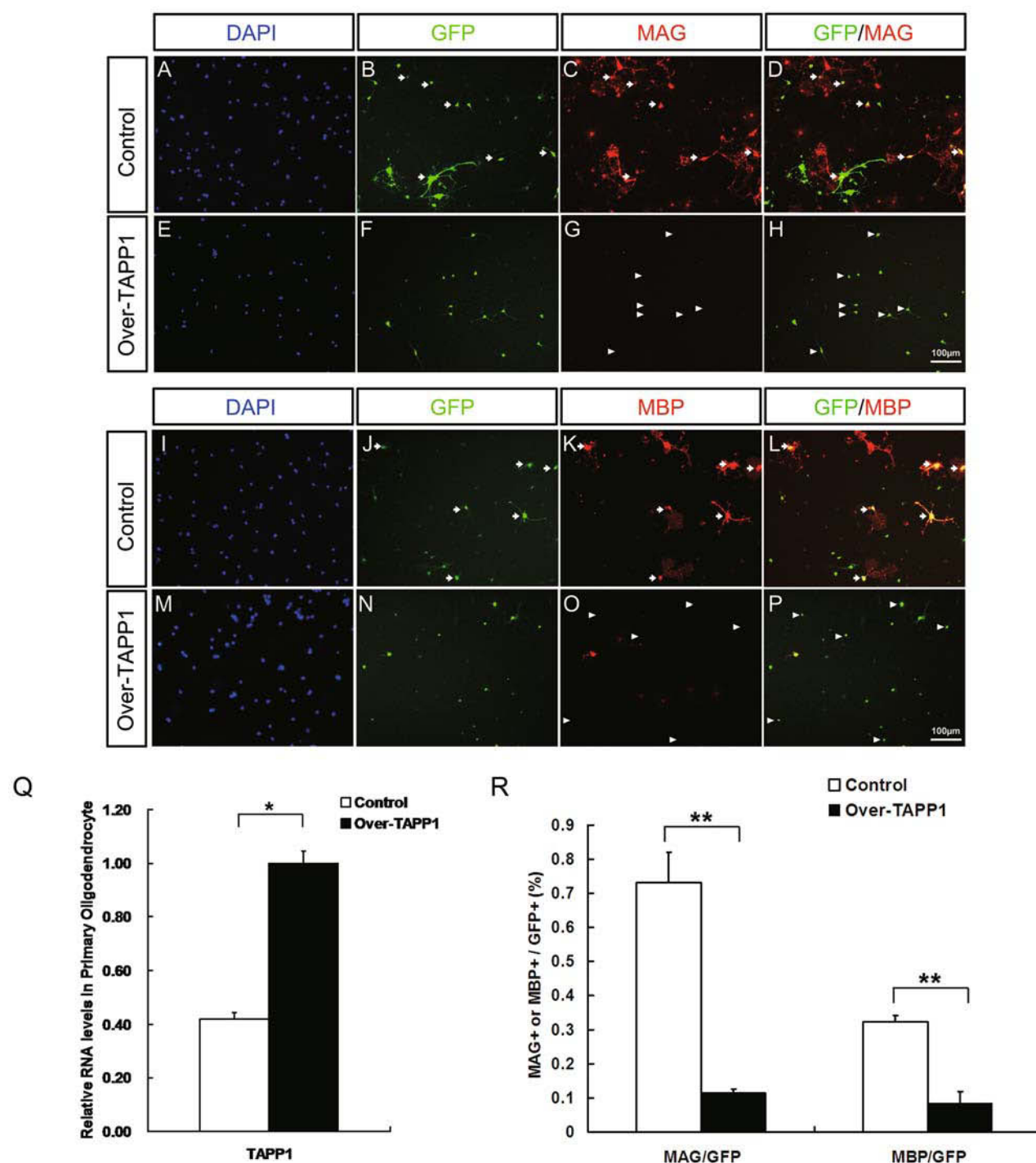


Fig. 4. Over-expression of TAPP1 inhibits OPC differentiation. A–R: Cultured OPCs infected with control and GFP-tagged pCDH-rTAPP1 viruses (A, B, E, F, I, J, M, and N). Expression of TAPP1 was significantly enhanced after pCDH-rTAPP1-EF1-CopGFP transfection in OPCs (Q). Meanwhile, MAG and MBP expression was significantly decreased (C, G, D, H, K, O, L and P). Analysis demonstrated significantly lower percentages of GFP⁺ with MAG⁺ or MBP⁺ OLs in GFP⁺ cells over-expressing TAPP1 than that in the control (R). Arrows indicate GFP⁺ and MAG⁺/MBP⁺ double-positive cells; arrowheads indicate GFP⁺ single-positive cells. Statistical analyses with Student's *t*-test. Error bar, standard deviation ($n = 3$, * $P < 0.05$, ** $P < 0.01$). Scale bars, 100 μ m.

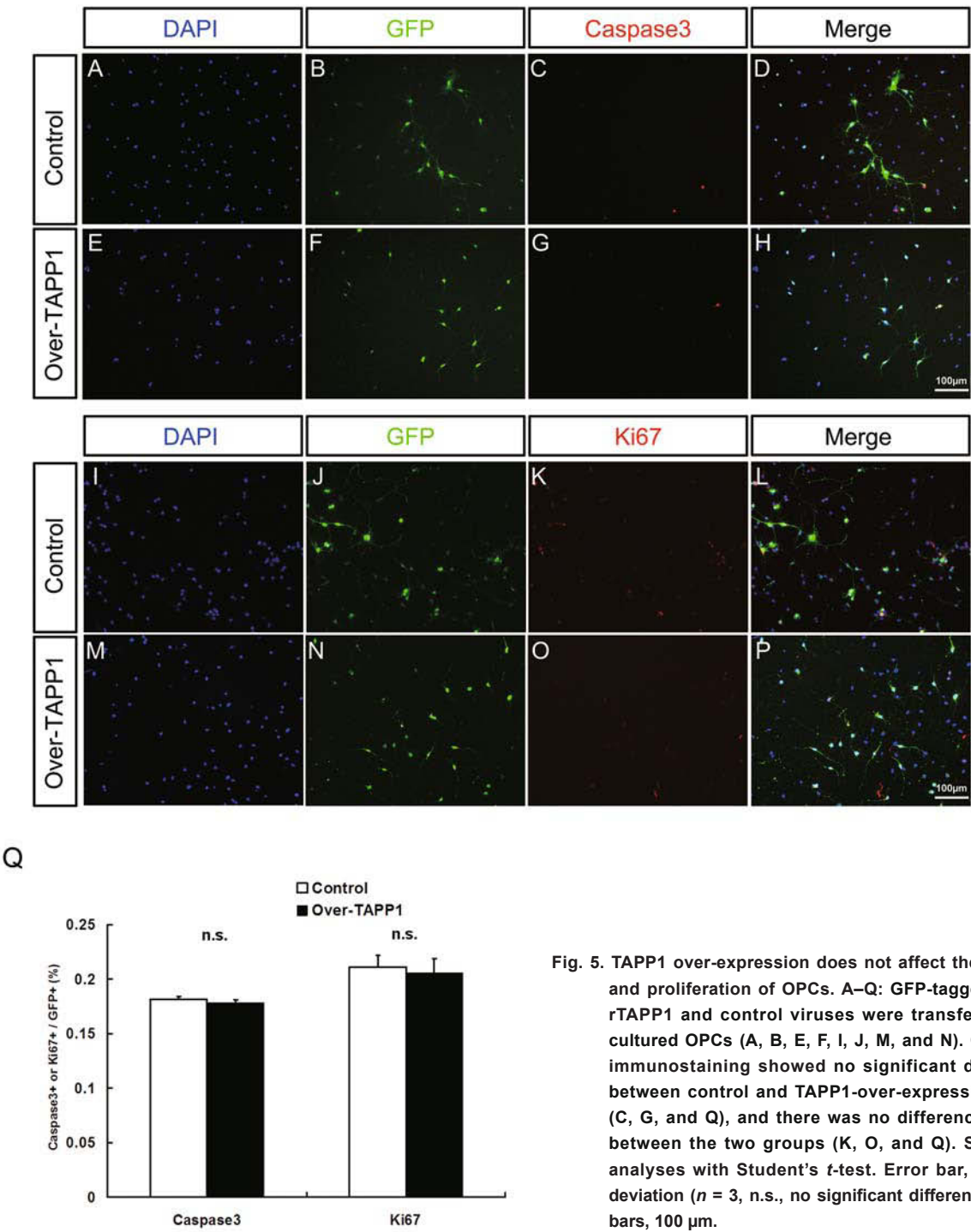


Fig. 5. TAPP1 over-expression does not affect the survival and proliferation of OPCs. A–Q: GFP-tagged pCDH-rTAPP1 and control viruses were transfected into cultured OPCs (A, B, E, F, I, J, M, and N). Caspase3 immunostaining showed no significant difference between control and TAPP1-over-expressing OPCs (C, G, and Q), and there was no difference in Ki67 between the two groups (K, O, and Q). Statistical analyses with Student's *t*-test. Error bar, standard deviation (*n* = 3, n.s., no significant difference). Scale bars, 100 μ m.

DISCUSSION

In this study, we present the first evidence that the TAPP1 adaptor protein is selectively expressed by differentiating

OPCs (Fig. 1). *TAPP1* was initially expressed in the white-matter OLs in the perinatal spinal cord, and was expressed in both the postnatal spinal cord and corpus callosum (Fig.

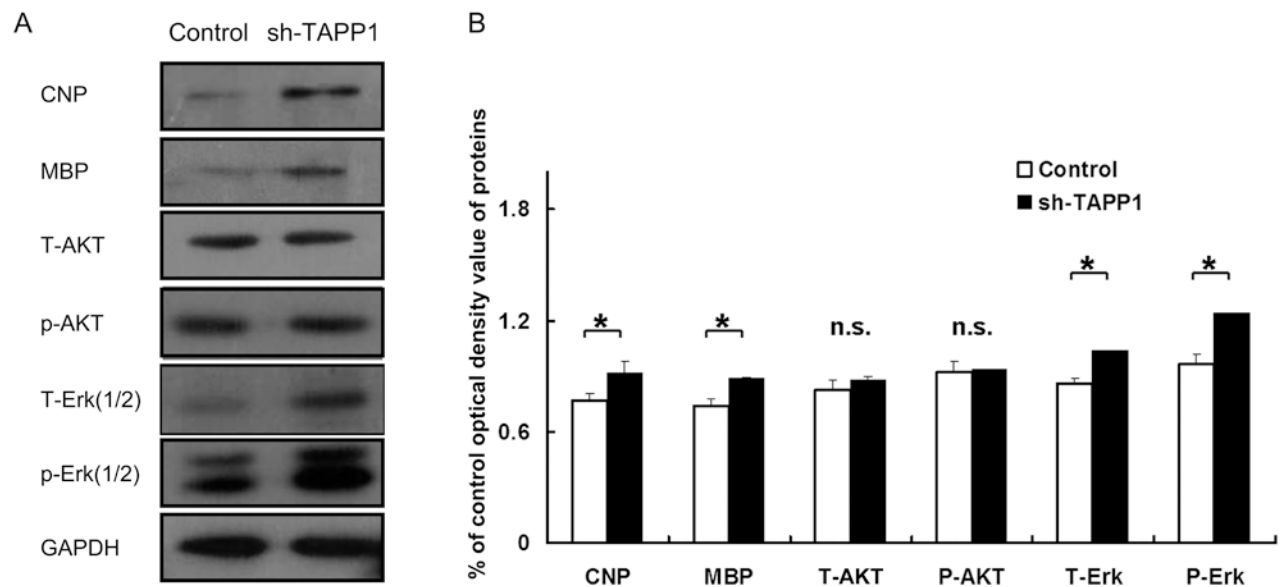


Fig. 6. TAPP1 inhibition alters the expression of Erk1/2 but not Akt. **A:** Compared to control cells, the expression of MBP and CNPase proteins in shRNA-TAPP1-treated cells significantly increased. Conversely, TAPP1 knockdown induced the expression of both total and phospho-Erk(1/2), but not that of total and phospho-Akt. **B:** Statistics of the relative expression levels of CNP, MBP, Akt, and Erk in control and shRNA-TAPP1-treated cells (Student's *t*-test; error bar, standard deviation; *n* = 3; **P* < 0.05, n.s., no significant difference).

2A–C). In the spinal cord, its expression level increased between E18.5 and P10, but gradually decreased at later stages when OPCs were fully differentiated (Fig. 1A–H, I, J). Moreover, *TAPP1* expression was markedly reduced in P3 Olig1 and Nkx2.2 mutant spinal cords in which the differentiation of OLs is inhibited or delayed (Fig. 2D–G).

Functional analyses revealed that knockdown of *TAPP1* expression in early OPCs by shRNA led to precocious OL differentiation and myelin protein production *in vitro* (Fig. 3). Conversely, prolonged *TAPP1* expression in OLs *in vitro* inhibited OL differentiation and myelin gene expression, but did not affect the survival and proliferation of OPCs (Figs. 4, 5). These findings raised the possibility that TAPP1 may function to negatively regulate OL differentiation. Thus, we propose that *TAPP1* is up-regulated in differentiating OLs to maintain the undifferentiated state at early stages and therefore prevents the premature differentiation of OPCs. At later stages, TAPP1 acts as a suppressor of myelin gene expression in differentiated OLs, and down-regulation of TAPP1 may be necessary for the rapid synthesis of myelin proteins in mature OLs that undergo myelin sheath formation. This expression and function are similar to Gpr17, which is

also upregulated in differentiating OPCs, but functions to inhibit OPC differentiation^[24]. It is conceivable that TAPP1 functions downstream of Gpr17 to prevent the premature differentiation of OPCs during development.

TAPP1 has been identified as an adaptor molecule that binds to PI(3,4)P2^[11]. PI(3,4)P2 is generated by the inositol 5'-phosphatase SHIP from PI(3,4,5)P3, which in turn is produced through the phosphorylation of PI(4,5)P2 by PI3K. The PI(3,4,5)P3-dependent recruitment and activation of the Akt/PKB, PDK1, and Btk kinases promote cell survival and proliferation^[24]. However, neither the expression levels of total and phospho-Akt proteins nor cell survival was affected by changing *TAPP1* expression in cell culture (Fig. 6). Interestingly, we found that the Erk1/2 MAPK signaling pathway was significantly induced when OL differentiation was induced by knockdown of *TAPP1* *in vitro* (Fig. 6). It is plausible that the Erk1/2 but not the PI3K/Akt/mTOR signaling pathway regulates the transition of early progenitors to the immature OL stage^[23]. However, the underlying mechanisms are currently unknown, and future studies with genetic approaches are necessary to elucidate the functions of TAPP1 in OL development and axonal myelination.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Sciences Foundation of China (31471955 and 31372150) and the National Basic Research Development Program (973 Program) of China (2013CB531300).

Received date: 2015-01-05; Accepted date: 2015-05-19

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