



# AATYK is a Novel Regulator of Oligodendrocyte Differentiation and Myelination

Chunxia Jiang<sup>1</sup> · Wanqing Yang<sup>1</sup> · Zhihong Fan<sup>1</sup> · Peng Teng<sup>3</sup> · Ruyi Mei<sup>3</sup> · Junlin Yang<sup>1</sup> · Aifen Yang<sup>1</sup> · Mengsheng Qiu<sup>1,2</sup> · Xiaofeng Zhao<sup>1</sup>

Received: 24 October 2017 / Accepted: 21 November 2017 / Published online: 19 March 2018  
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**Abstract** Oligodendrocytes (OLs) are myelinating glial cells that form myelin sheaths around axons to ensure rapid and focal conduction of action potentials. Here, we found that an axonal outgrowth regulatory molecule, AATYK (apoptosis-associated tyrosine kinase), was up-regulated with OL differentiation and remyelination. We therefore studied its role in OL differentiation. The results showed that AATYK knockdown inhibited OL differentiation and the expression of myelin genes *in vitro*. Moreover, AATYK-deficiency maintained the proliferation status of OLs but did not affect their survival. Thus, AATYK is essential for the differentiation of OLs.

**Keywords** AATYK · Differentiation · Oligodendrocytes

## Introduction

Myelin sheaths elaborated by oligodendroglia (OLs) ensure rapid conduction and the integrity of axons in the central nervous system (CNS) [1, 2], which are crucial for the

function of neural circuitry mediated by extensive neuron-glia interactions. Loss of OL function and myelin sheaths causes neurological diseases represented by multiple sclerosis [1, 3, 4]. In addition, emerging evidence has revealed OL and myelin deficits in psychiatric disorders such as schizophrenia [5]. It is now clear that OLs function in neuron survival, as OL impairment has been detected in neurodegenerative diseases including multiple sclerosis, Alzheimer's disease, and Parkinson's disease [6, 7]. To develop novel strategies to enhance OL differentiation and myelination in neurological disorders, it is essential to understand the mechanisms that underlie the control of OL differentiation and myelination.

OL progenitor cells (OPCs) are generated from pluripotent neural stem cells, and ultimately develop into mature and myelinating OLs after proliferation and differentiation. To identify candidate genes involved in OL myelinogenesis, we compared the mRNA array data from *Nkx2.2*-knockout mice with those from transgenic mice overexpressing *Nkx2.2*, two mouse models in which the differentiation of OLs is promoted or delayed, respectively, and many genes responded to altered OL differentiation (unpublished data). Interestingly, the axonal outgrowth regulatory molecule AATYK (apoptosis-associated tyrosine kinase) was up-regulated with OL differentiation. We therefore hypothesized that AATYK participates in OL differentiation and myelination.

Here, we further explored the role of AATYK in OL differentiation and myelinogenesis to test this hypothesis.

✉ Mengsheng Qiu  
m0qiu001@yahoo.com

✉ Xiaofeng Zhao  
xiaofengzhao@yahoo.com

<sup>1</sup> 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

<sup>2</sup> Department of Anatomical Sciences and Neurobiology, School of Medicine, University of Louisville, Louisville, KY 40202, USA

<sup>3</sup> College of Life Sciences, Zhejiang University, Hangzhou 310036, China

## Materials and Methods

### Animals

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

### Immunohistochemistry and *In Situ* Hybridization (ISH)

Mice were deeply anesthetized with 2% tribromoethanol (Avertin) at the dose of 20  $\mu\text{L/g}$  *via* intraperitoneal injection and perfused with 4% paraformaldehyde (PFA), and tissues were isolated and postfixed in 4% PFA overnight at 4°C, then kept in 20% sucrose in phosphate-buffered saline overnight at 4°C. The tissues were then embedded in OCT and cut into 16- $\mu\text{m}$  sections. Immunohistochemistry and ISH were performed according to the description by Chen *et al.* [8]. The riboprobe sequence for *AATYK* was F: 5'-GCACAAAGGAATCACTCCCTAC-3'; R: 5'-GCTTACAGACCCCATAGACCC-3'. The dilution of anti-Olig2 antibody (Abcam, Cambridge, MA) was 1:1000.

### Immunofluorescence Staining

Double-immunofluorescence procedures were as described by Chen *et al.* [8]. The dilutions of antibodies were as follows: anti-MBP (myelin basic protein) (Abcam, 1:500), anti-MAG (myelin-associated glycoprotein) (Millipore, Burlington, MA; 1:500), and anti-Ki67 (Thermo, Grand Island, NE, 1:500).

### Cuprizone Treatment

Wild-type mice (8–10 weeks old) were fed a diet containing 0.2% cuprizone (*w/w*) (biscyclohexane oxaldihydrazone; Sigma-Aldrich) for 6 weeks to induce demyelination. Remyelination was assayed after returning them to normal chow for 2 weeks. Control mice were maintained on a normal diet.

### Western Blotting

Cells were lysed in lysis buffer (Sigma) with a protease inhibitor cocktail (Sigma). Western blotting was performed as previously described [8]. SDS-PAGE gels were

subsequently detected with anti-MBP (Abcam, 1:2 000), anti-caspase3 (Millipore, 1:1 000), and anti-GAPDH (Sigma, 1:5 000) antibodies.

### Rat OPC Culture, Lentivirus Construction, and OPC Infection

Cerebral cortices from newborn Sprague-Dawley rats were used for OPC primary culture, according to protocols described by Chen *et al.* [8]. For RNAi-mediated depletion of *AATYK*, a lentivirus was used to express validated shRNA specifically targeting *AATYK* mRNA with the following sequence: 5'-GGC AAG AAG CAA GUG UCU TAC-3' (GenePharma, Shanghai, China). A negative control shRNA lentivirus expressing a scrambled sequence without homology to any known mammalian mRNA was used in parallel. Rat OPCs were transfected with lentiviral *AATYK*-shRNA 72 h before experiments. The infection efficiency was analyzed on day 2 post-infection using GFP expression, and revealed to be > 90%. Six days after infection, OPCs were collected for experiments.

### Statistical Analysis

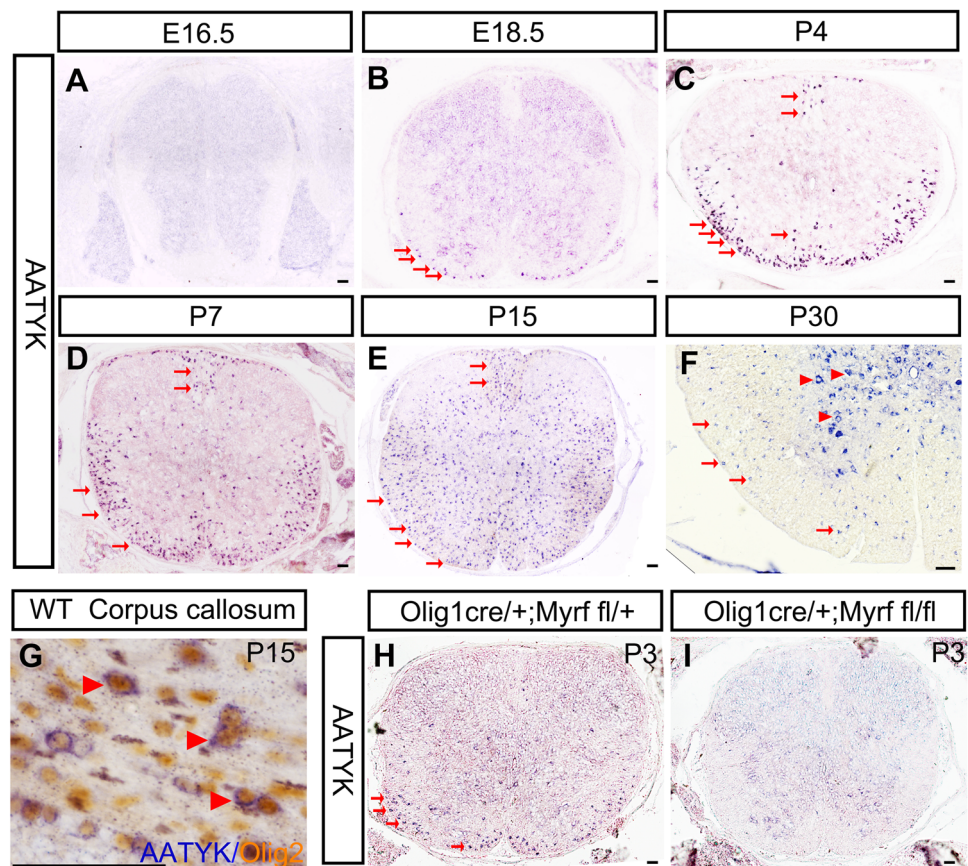
Data are presented as mean  $\pm$  SEM and were analyzed by Student's *t*-test for two-group comparison.  $P < 0.05$  was considered to be statistically significant.

## Results and Discussion

### *AATYK* is Selectively Expressed in Differentiating OLs

As our microarray results suggested that *AATYK* is involved in OL differentiation (unpublished data), we further tested this possibility by studying the *AATYK* expression pattern in the developing spinal cord using RNA ISH. The results showed that *AATYK* started to be expressed around embryonic day 18.5 (E18.5) in the spinal white matter, showing no specific expression at E16.5 (Fig. 1A, B). Its expression progressively increased thereafter (Fig. 1C, D), and at postnatal day 15 (P15), its expression was detected throughout the spinal cord (Fig. 1E). By P30, its expression was down-regulated in the white matter (Fig. 1F). These results suggested that *AATYK* is expressed in OLs. To test this possibility, we performed double-staining in the white matter of the brain. As expected, all *AATYK*<sup>+</sup> cells in the P15 corpus callosum were co-labeled with the OL-specific marker Olig2 [9] (Fig. 1G). In addition, we examined *AATYK* expression in *Olig1* mutant and *Myrf* conditional knock-out (CKO) mice, in which OL differentiation is significantly delayed and

**Fig. 1** *AATYK* is up-regulated in differentiating OLs. **A–F** Sections of spinal cord from E16.5, E18.5, P4, P7, P15, and P30 mice subjected to ISH with *AATYK* riboprobe. Expression of *AATYK* started at ~ E18.5 in the spinal white matter. Its expression progressively increased between E18.5 and P7, and then was scattered throughout the cord at P15. By P30, *AATYK* expression had gradually decreased in the white matter and was detectable in the gray matter. Arrows indicate white matter cells; arrowheads indicate gray matter cells. **G** *AATYK*-expressing cells immunoreactive to Olig2 in the corpus callosum at P15. **H–I** The numbers of *AATYK*<sup>+</sup> cells were reduced drastically in *Myrf* CKO mice at P3. Arrows indicate white matter cells; arrowheads indicate *AATYK*<sup>+</sup> Olig2<sup>+</sup> double-positive cells. Scale bars, 50  $\mu$ m.



impaired [10–12]. We found that the *AATYK*<sup>+</sup> cells in the white matter were completely lost in *Olig1* mutant and *Myrf*-CKO mice (Fig. 1H, I). Together, these findings demonstrated that *AATYK* is expressed in differentiating OLs in the CNS. *AATYK* is known to be highly expressed in neurons, and is involved in axonal outgrowth [13]. Here we presented the first evidence that *AATYK* is also expressed in oligodendrocytes (Fig. 1A–G), although it was also detectable in neurons in the spinal gray matter (Fig. 1F). Moreover, *AATYK* was highly expressed by differentiating OLs, suggesting that *AATYK* helps to promote and stabilize OL differentiation.

#### Up-regulation of *AATYK* During Remyelination in a Cuprizone-Induced Demyelination Model

The overlap of the spatiotemporal pattern of *AATYK* expression with the myelination process (Fig. 1) indicated that *AATYK* is involved in myelinogenesis in the CNS. Therefore we evaluated the expression of *AATYK* in the remyelination model induced by cuprizone [14]. Wild-type mice were fed cuprizone for 6 weeks, which induced massive demyelination in the corpus callosum, followed by spontaneous myelin repair within 2 weeks after cuprizone removal (Fig. 2A–I). We performed ISH with MBP and myelin proteolipid protein

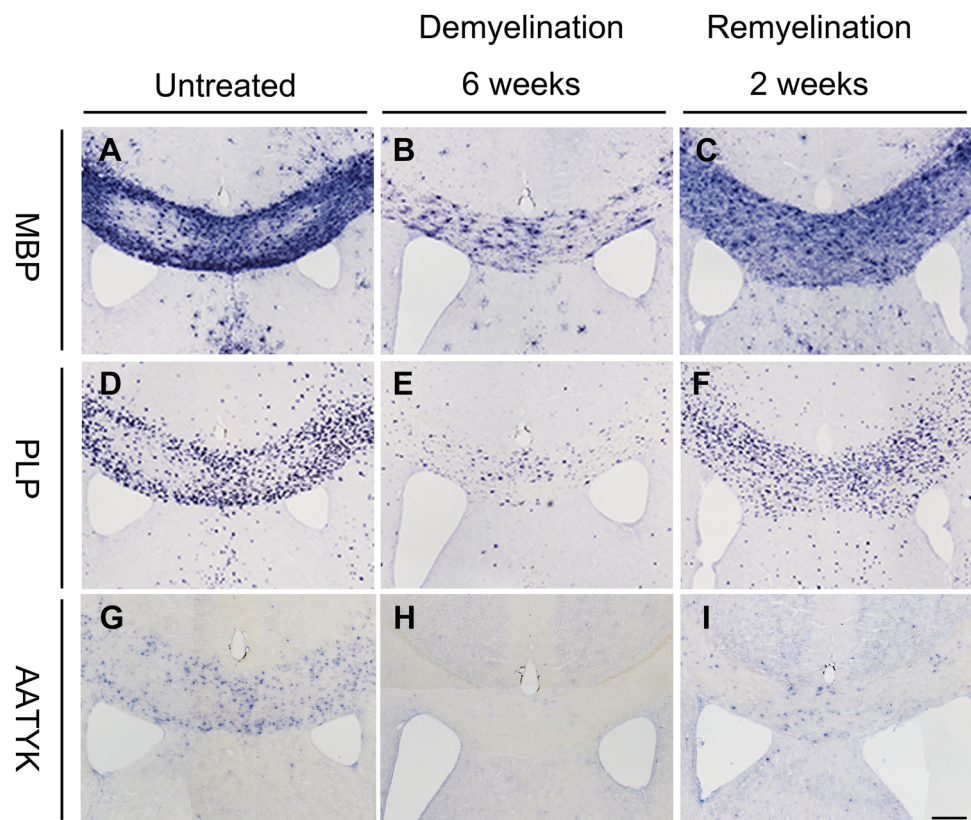
(PLP) probes to assess the remyelination status. The results showed that after 6 weeks of cuprizone treatment, severe demyelination of the corpus callosum occurred (Fig. 2B, E, H). Two weeks of recovery resulted in more *MBP*<sup>+</sup> and *PLP*<sup>+</sup> cells in the corpus callosum (Fig. 2C, F), similar to the *AATYK*<sup>+</sup> cells (Fig. 2I), supporting the hypothesis that *AATYK* is involved in OL myelinogenesis.

#### Absence of *AATYK* Inhibits Oligodendrocyte Differentiation

Since *AATYK* is expressed in differentiating OLs and up-regulated during myelinogenesis, its deletion may affect the differentiation of OLs. To test this, primary cultures of OPCs were infected with lentivirus co-expressing green fluorescent protein (GFP) and short hairpin (sh) RNA of *AATYK* 3 days prior to differentiation assays. As expected, the qPCR results showed that *AATYK* expression in OLs was significantly inhibited by shRNA interference (Fig. 3Q). Meanwhile, the numbers of mature *MAG*<sup>+</sup> and *MBP*<sup>+</sup> OLs were remarkably decreased in the RNAi-*AATYK* cells compared to controls (Fig. 3C, G, K, O). Quantitative analysis also showed that the percentages of *MBP*<sup>+</sup> and *MAG*<sup>+</sup> OLs in GFP<sup>+</sup> RNAi-*AATYK*-infected cells were significantly lower than in controls (Fig. 3R).



**Fig. 2** *AATYK* is up-regulated during remyelination. *In situ* hybridization with *MBP* (A, B) or *PLP* probes (D, E) showed severe demyelination of the corpus callosum in wild-type mice after 6 weeks of cuprizone treatment as compared to untreated animals. Compared with the recovered expression of *MBP* and *PLP* (C, F), *AATYK* was observed in the corpus callosum of mice after recovery for 2 weeks (G, H, I). Scale bar, 100  $\mu$ m.



Moreover, OLs from shRNA-*AATYK* cells displayed typical immature processes (Fig. 3F, N). Therefore, these results demonstrated that *AATYK* positively regulates the differentiation of OLs, and its absence inhibits OL differentiation. These findings raise the possibility that *AATYK* is essential for the differentiation of OLs and consequential OL myelination. This expression pattern (Fig. 1) and the function of *AATYK* in OL development are similar to myelin regulatory factor (MRYP), which is also expressed in differentiating OLs, and is required for CNS myelination and its maintenance [11, 15].

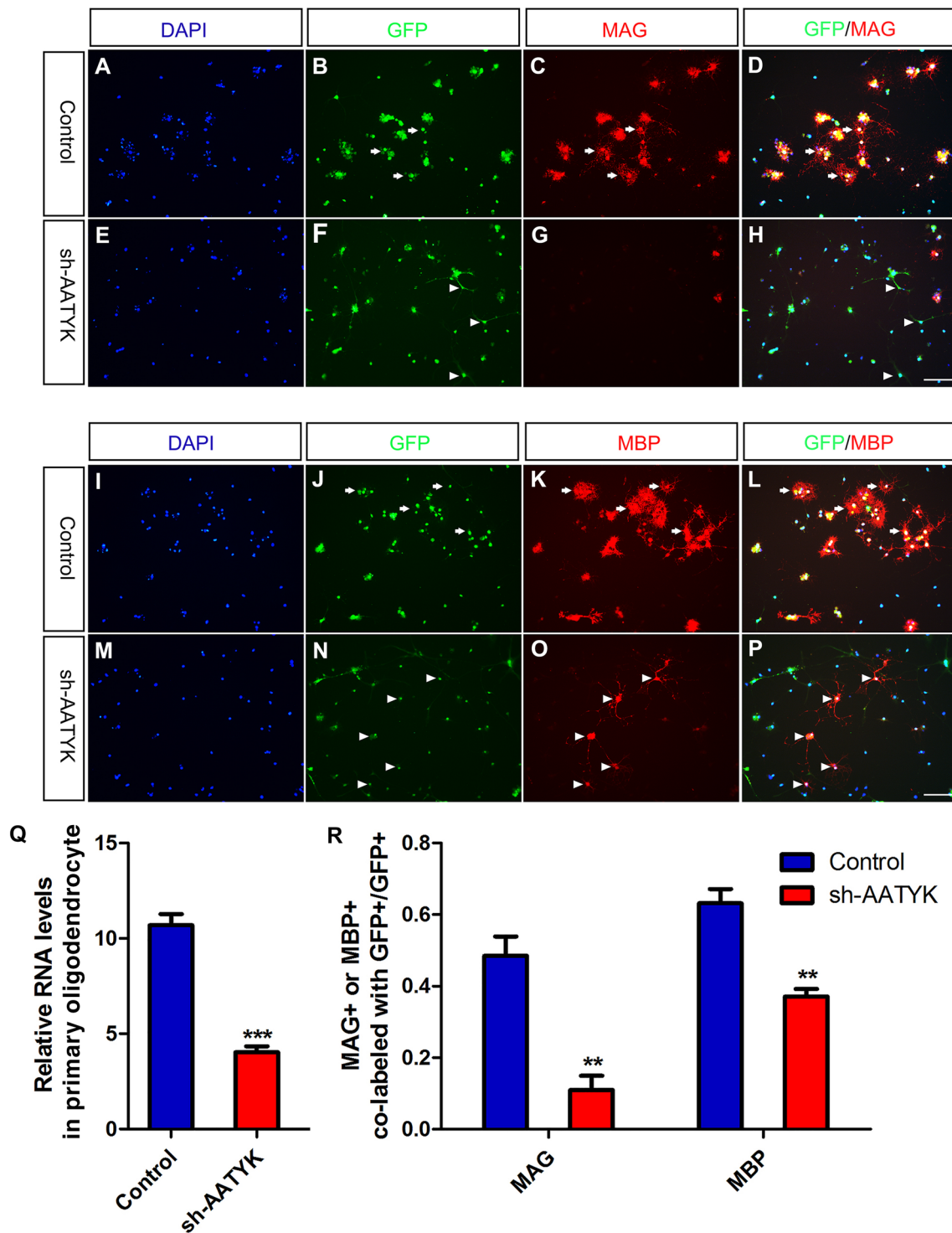
#### Ablation of *AATYK* Affects the Proliferation of OPCs but not Their Survival

To address the possibility that inhibition of OPC differentiation by *AATYK* ablation is due to the induction of cell death or proliferation, western blot with anti-MBP and anti-caspase3 antibodies and Ki67 immunostaining were performed. Consistent with the above results, the western immunoblotting revealed a similar decrease of MBP expression in *AATYK*-knockdown cells. However, there was no significant difference in caspase3 expression between control and RNAi-*AATYK* OPCs, indicating that cell death and apoptosis were not affected by *AATYK*-deficiency (Fig. 4C, D). Interestingly, we found that there

were more Ki67<sup>+</sup> and GFP<sup>+</sup> co-labeled cells among the Sh-*AATYK*-treated OPCs than in controls (Fig. 4A), and statistical analysis showed significantly higher percentages of Ki67<sup>+</sup> and GFP<sup>+</sup> co-labeled cells among GFP<sup>+</sup> cells treated with sh-*AATYK* than those with the control vector (Fig. 4B). These results suggest that *AATYK* knockdown does maintain the proliferation status of OLs, in other words, *AATYK* is essential for the differentiation of OLs.

In the CNS, both extracellular signals and intracellular pathways control OPC differentiation and myelination during development [16]. Recently, a number of researchers have demonstrated that endocytosis plays a key role in regulating signal transduction during cell differentiation. For example, after activation by Wnt-ligands, Frizzled is internalized *via* a clathrin-mediated pathway and subsequently degraded, which can induce aberrant embryonic development [17–20]; GPR17, which plays a crucial role in OPC differentiation [21], is partially degraded or recycled to the plasma membrane through recycling endosomes after endocytosis [22]. Interestingly, *AATYK* regulates the trafficking of recycling endosomes in CHO-K1 cells and is involved in axonal outgrowth [23]. It is conceivable that *AATYK*-deficiency affects the formation of recycling endosomes, and thus upsets the balance between degradation and recycling for modulation of receptor levels on the cell surface such as GPR17, which is highly expressed in





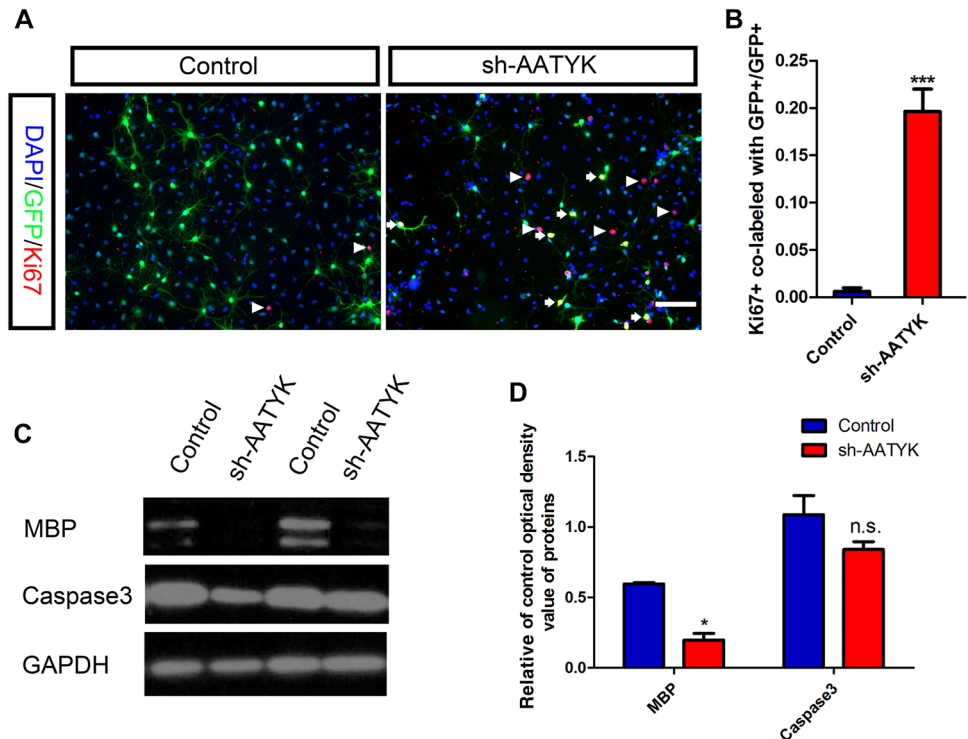
**Fig. 3** Ablation of *AATYK* inhibits OL differentiation. Primary cultured OPCs infected with GFP-tagged ShRNA-*AATYK* or control lentivirus (A, B, E, F, I, J, M, and N). *AATYK* was significantly down-regulated by shRNA in OPCs by qPCR (Q). Compared to control cells, the numbers of  $MAG^+$  and  $MBP^+$  cells were remarkably decreased in the RNAi-*AATYK* treated cells (C, G, D,

H, K, O, L, and P). The percentages of co-labeled  $MAG^+$  or  $MBP^+$  with  $GFP^+$  OLs in  $GFP^+$  cells among RNAi-*AATYK* cells were significantly lower than that in the control (R). Arrows indicate  $GFP^+$  and  $MAG^+/MBP^+$  double-positive cells; arrowheads indicate immature cells. Scale bars, 100  $\mu m$ .

OPCs but down-regulated in mature OLs to ensure full myelination [24–26], and thereby, affect OL differentiation

and myelination. However, the underlying mechanisms are currently unknown, and future studies with the conditional

**Fig. 4** *AATYK* knockdown does not affect the survival but maintains proliferation of OPCs. **A** Ki67 immunostaining showed there were more Ki67<sup>+</sup> and GFP<sup>+</sup> co-labeled cells in the sh-*AATYK*-treated OPCs than in the control. **B** Percentages of Ki67<sup>+</sup> and GFP<sup>+</sup> co-labelled OLs in GFP<sup>+</sup> cells among RNAi-*AATYK* cells were significantly higher than in the control. **C–D** Compared to control cells, the expression of MBP protein was significantly decreased in shRNA-*AATYK*-treated cells and there was no difference in caspase3 between the two groups. Scale bar, 100  $\mu$ m.



knockout approach are needed to elucidate the functions of *AATYK* and related signaling pathways in OL differentiation and myelination.

**Acknowledgements** This work was supported by the National Natural Sciences Foundation of China (31471955) and the Natural Science Foundation of Zhejiang Province, China (LY17C090006; Q16C090017; LY18H090014).

#### Compliance with Ethical Standards

**Conflict of interest** All authors claim that there are no conflicts of interest.

#### References

- Miron VE, Kuhlmann T, Antel JP. Cells of the oligodendroglial lineage, myelination, and remyelination. *Biochim Biophys Acta* 2010, 1812: 184–193.
- Nave KA. Myelination and support of axonal integrity by glia. *Nature* 2011, 468: 244–252.
- Bruce CC, Zhao C, Franklin RJ. Remyelination - An effective means of neuroprotection. *Horm Behav* 2009, 57: 56–62.
- Steinman L. Multiple Sclerosis: a coordinated immunological attack against myelin in central nervous system. *Cell* 1998, 85: 299–302.
- Feng Y. Convergence and divergence in the etiology of myelin impairment in psychiatric disorders and drug addiction. *Neurochem Res* 2008, 33: 1940–1949.
- Bartzokis G. Age-related myelin breakdown: a developmental model of cognitive decline and Alzheimer's disease. *Neurobiol Aging* 2004, 25: 5–18; author reply 49–62.
- Sokolov BP. Oligodendroglial abnormalities in schizophrenia, mood disorders and substance abuse. Comorbidity, shared traits, or molecular phenocopies? *Int J Neuropsychopharmacol* 2007: 1–9.
- Chen YD, Mei RY, Teng P, Yang AF, Hu XM, Zhang ZY, *et al.* TAPP1 inhibits the differentiation of oligodendrocyte precursor cells via suppressing the Mek/Erk pathway. *Neurosci Bull* 2015: 1–10.
- Redwine JM, Evans CF. Markers of central nervous system glia and neurons *in vivo* during normal and pathological conditions. *Curr Top Microbiol Immunol* 2002, 265: 119–140.
- Zhu Q, Zhao XF, Zheng K, Li H, Huang H, Zhang Z, *et al.* Genetic evidence that Nkx2.2 and Pdgfra are major determinants of the timing of oligodendrocyte differentiation in the developing CNS. *Development* 2014, 141: 548–555.
- Emery B, Agalliu D, Cahoy JD, Watkins TA, Dugas JC, Mulinyawe SB, *et al.* Myelin gene regulatory factor is a critical transcriptional regulator required for CNS myelination. *Cell* 2009, 138: 172–185.
- Koenning M, Jackson S, Hay CM, Faux C, Kilpatrick TJ, Willingham M, *et al.* Myelin gene regulatory factor is required for maintenance of myelin and mature oligodendrocyte identity in the adult CNS. *J Neurosci* 2012, 32: 12528–12542.
- Takano T, Tomomura T, Yoshioka N, Tsutsumi K, Terasawa Y, Saito T, *et al.* LMTK1/AATYK1 is a novel regulator of axonal outgrowth that acts via Rab11 in a Cdk5-dependent manner. *J Neurosci* 2012, 39: 6587–6599.
- Chang A, Tourtellotte WW, Rudick R, Trapp BD. Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. *N Engl J Med* 2002, 346: 165–173.
- Koenning M, Jackson S, Hay CM, Faux C, Kilpatrick TJ, Willingham M, *et al.* Myelin gene regulatory factor is required for maintenance of myelin and mature oligodendrocyte identity in the adult CNS. *J Neurosci* 2012, 32: 12528–12542.
- Fu P, Tang R, Yu Z, Li C, Chen X, Xie M, *et al.* Rho-associated kinase inhibitors promote microglial uptake via the ERK signaling pathway. *Neurosci Bull* 2016, 32: 83–91.

17. Piddini E, Marshall F, Dubois L, Hirst E, Vincent JP. Arrow (LRP6) and Frizzled2 cooperate to degrade Wingless in *Drosophila* imaginal discs. *Development* 2005, 132: 5479–5489.
18. Rives AF, Rochlin KM, Wehrli M, Schwartz SL, Di Nardo S. Endocytic trafficking of Wingless and its receptors, Arrow and DFrizzled-2, in the *Drosophila* wing. *Dev Biol* 2006, 293: 268–683.
19. Seto ES, Bellen HJ. Internalization is required for proper Wingless signaling in *Drosophila melanogaster*. *J Cell Biol* 2006, 173: 95–106.
20. Yu A, Rual JF, Tamai K, Harada Y, Vidal M, He X, *et al.* Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling. *Dev Cell* 2007, 12: 129–141.
21. Chen Y, Wu H, Wang H, Koito H, Li J, Ye F, *et al.* The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination. *Nat Neurosci* 2009, 12: 1398–1406.
22. Fratangeli A, Parmigiani E, Fumagalli M, Lecca D, Benfante R, Passafaro M, *et al.* The regulated expression, intracellular trafficking and membrane recycling of the P2Y-like receptor GPR17 in Oli-neu oligodendroglial cells. *J Biol Chem* 2013, 288: 5241–5256.
23. Takano T, Tsutsumi K, Saito T, Asada A, Tomomura M, Fukuda M, *et al.* AATYK1A phosphorylation by Cdk5 regulates the recycling endosome pathway. *Genes Cells* 2010, 15: 783–797.
24. Boda E, Viganò F, Rosa P, Fumagalli M, Labat-Gest V, Tempia F, *et al.* The GPR17 receptor in NG2 expressing cells: focus on in vivo cell maturation and participation in acute trauma and chronic damage. *Glia* 2011, 59: 1958–1973.
25. Chen Y, Wu H, Wang S, Koito H, Li J, Ye F, *et al.* The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination. *Nat Neurosci* 2009, 12: 1398–1406.
26. Fumagalli M, Daniele S, Lecca D, Lee PR, Parravicini C, Fields RD, *et al.* Phenotypic changes, signaling pathway, and functional correlates of GPR17-expressing neural precursor cells during oligodendrocyte differentiation. *J Biol Chem* 2011, 286: 10593–10604.