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RESEARCH ARTICLE

Id2 and *Id4* are not the major negative regulators of oligodendrocyte differentiation during early central nervous system development

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Abstract

Myelin sheathes ensure the rapid conduction of neural impulse and provide nutritional support for neurons. Myelin sheathes are formed by differentiated oligodendrocytes (OLs) in the central nervous system. During OL development, the differentiation of oligodendrocyte progenitor cells (OPCs) into mature OLs is controlled by both positive differentiation factors (drivers) and negative regulatory factors (brakes). Previous studies have suggested *Id2* and *Id4* as the key negative factors for OL differentiation. However, these conclusions were mainly based on in vitro studies and the reported OL phenotype in *Id4* mutants appear to be mild. In this study, we systematically investigated the in vivo function of *Id2* and *Id4* genes in OL differentiation in their genetic mutants and in embryonic chicken spinal cord. Our results showed that disruption of Id4 has no effect on OL differentiation and maturation, whereas *Id2* mutants and *Id2*/*Id4* compound mutants display a mild and transient precocity of OL differentiation. In agreement with these loss-of-function studies, *Id2*, but not *Id4*, is weakly expressed in OPCs. Despite their minor roles in OL differentiation, forced expression of *Id2* and *Id4* in embryonic chicken spinal cords strongly inhibit the differentiation of OPCs. Taken together, our detailed functional and expressional studies strongly suggest that *Id2* and *Id4* are not the major in vivo repressors of OPC differentiation during animal development, shedding new light on the molecular regulation of early OL development.

KEYWORDS
differentiation, *Id2*, *Id4*, oligodendrocytes, transcriptional factor

1 | INTRODUCTION

During development, neural progenitor cells sequentially generate neurons and glial progenitors of astrocytes or oligodendrocytes (OLs). Once oligodendrocyte progenitor cells (OPCs) are produced, they disperse into surrounding regions, proliferate rapidly to increase their number before they differentiate into mature myelinating OLs (Rowitch & Kriegstein, 2010). The differentiation and maturation of OLs is precisely controlled spatially and temporally, and coordinated by a large number of regulatory factors (Elbaz & Popko, 2019; Huang et al., 2013; Sock & Wegner, 2019). The differentiation factors can be generally classified into two categories: those that promote OL differentiation (positive factors or drivers) and those that repress OLs differentiation (negative factors or brakes). While the positive factors (e.g., *Sox10*, *Nkx2.2*, and *Myrf*) have been extensively investigated in details in their developmental expression and in vivo functions

Hao Huang and Huihui Wu contributed equally to this work.

(Aprato et al., 2020; Emery et al., 2009; Stolt et al., 2002; Zhu et al., 2014), many negative factors (e.g., *Id2*, *Id4*, and *Hes5*) in general are less well characterized in these aspects possibly due to their relatively less specific expression and mild phenotypes in their genetic mutants (Kondo & Raff, 2000; Liu et al., 2006; Marin-Husstege et al., 2006; Wang et al., 2001). For instance, although *Id2* (inhibitor of DNA binding 2) and *Id4* inhibitory factors are widely cited as the important negative regulators of OL differentiation, the in vivo functions of these two inhibitors in OL lineage have not been studied in the developing central nervous system (CNS) tissue.

ID2 and ID4 belong to the family of basic helix–loop-hex (bHLH) transcription factors, and function as downstream effector of bone morphogenetic protein (BMP) signaling (Ahuja et al., 2016; Kurooka et al., 2012; Yang et al., 2013). bHLH factors are known to regulate gene expression through the formation of a homodimer or heterodimer (Dennis et al., 2019). ID2 and ID4 are special members of the bHLH family as they do not contain the DNA-binding basic region, and thus function as dominant negative transcription factors (Roschger & Cabrele, 2017). Earlier studies have implicated *Id* genes in the regulation of the development of many tissues and cell types, including osteoblast cells, dendritic cells, natural killer cells and mammary gland (Dong et al., 2011; Hacker et al., 2003; Wang & Baker, 2015; Yokota et al., 1999), as well as neural cells (Bedford et al., 2005; Havrda et al., 2008; Park et al., 2013; Yun et al., 2004). In 2000, Kondo et al. reported that *Id4* is expressed in purified OPCs and down-regulated when these cells differentiate (Kondo & Raff, 2000). Meanwhile, they showed that overexpression of *Id4* inhibits the differentiation of OPCs in vitro, proposing that *Id4* controls the timing of OPC differentiation during development (Kondo & Raff, 2000). Soon after, another group demonstrated the similar expression pattern and function of *Id2* in vitro. Further, they found that OPCs purified from *Id2*-null mice brain are easier to differentiate than that from wild type mice in the presence of thyroid hormone (Wang et al., 2001). The same situation was also observed in OPC cultures from *Id4*-null mice (Marin-Husstege et al., 2006). After that, it was found that BMP4 strongly increases astroglial and decreases oligodendroglial lineage commitment both in vivo and in vitro (Gomes et al., 2003). Further, it was shown that *Id2/4* proteins function as the key downstream regulators of BMP4 and can directly repress OL lineage progression by interacting with *Olig1/2* (Samanta & Kessler, 2004). Of note, another two members of *Id* genes, *Id1* and *Id3*, were ruled out of OL development in these reports (Kondo & Raff, 2000; Samanta & Kessler, 2004; Wang et al., 2001). Based on these and other findings, it was proposed that *Id2* and *Id4* function as the potent negative inhibitors of OPC differentiation and maturation during mylinogenesis. However, although it was reported that disruption of *Id4* resulted in mild phenotypes in OL differentiation (Marin-Husstege et al., 2006), the in vivo function of *Id2/4* in OL lineage development and their expression in these cells have not been investigated systematically.

In this study, we investigated OL differentiation in *Id2* and *Id4* single and compound mutants, and re-examined the expression of *Id2/4* during the development of OL lineage. We found that disruption of *Id2* only cause a slight premature of OPC differentiation, whereas *Id4* mutation did not cause any detectable phenotypic changes in OL differentiation and myelin gene expression. The *Id2/Id4* compound mutants displayed a similar phenotype to that of *Id2* single mutants, indicating their lack of functional redundancy in the regulation of OL maturation. However, we still confirmed the negative regulation of OL differentiation by both *Id2* and *Id4* with *in ovo* electroporation assay. Furthermore, our data showed that *Id2*, but not *Id4*, is transiently expressed in OPCs. Together, these studies suggest that *Id4* is not a relevant negative regulator of OL differentiation during development, and *Id2* only plays a minor role in the regulation of OL differentiation.

2 | MATERIALS AND METHODS

2.1 | Mice

All procedures performed in studies involving animals were in accordance with the ethical standards of the Laboratory Animal Center, Hangzhou Normal University, and the protocol was approved by the Animal Ethics Committee of Hangzhou Normal University, China. There is no data that collected from human subjects in this study. Mouse lines for *Id2* and *Id4* conventional mutations were obtained from Jackson Labs (Stock No: 016224, 41,569) (Rawlins et al., 2009; Yun et al., 2004). Both mutant alleles have an EGFP reporter replacing most of the open reading frames. The genotyping methods of these mutant mice were performed according to the genotyping protocols of Jackson Labs.

2.2 | Immunostaining

Tissues were fixed in 4% PFA overnight at 4°C, and transferred to 20% sucrose in PBS overnight at 4°C. Tissues were then embedded in Frozen Section Medium (Thermo Scientific, #6502) and sectioned into 16 μm on a cryostat. Experimental procedures for immunofluorescence were described previously (Huang et al., 2018; Xu et al., 2020). Briefly, sections were first incubated in citrate antigen retrieval solution (Sangon Biotech, E673001) at 85°C for 30 min, and then blocked in 5% goat serum for 1 h. After that, sections were incubated with primary antibodies (diluted in PBS containing 5% goat serum and 0.1% Triton X-100) overnight at 4°C. On the following day, sections were washed in PBS for three times and incubated with secondary antibodies for 1 h at room temperature (diluted in 5% goat serum in PBS, 1:3000). Finally, the sections were counterstained with 0.1 μg/ml DAPI for 5 min before being mounted. The avidin-biotin complex (ABC) method was used for immunohistochemistry according to the manufacturer's instructions (Vector Laboratories, PK-4001). Antibodies were used as follows: rabbit-anti-ALDH1L1 (Oasis Biofarm, OB-RB001, 1:500), mouse-anti-OLIG2 (Millipore, MABN50, 1:500) for double labeling, rabbit-anti-OLIG2 (Oasis Biofarm, OB-RB009, 1:500), mouse-anti-MYC (DSHB, 9E 10, 1:100), rat-anti-MBP (Abcam,

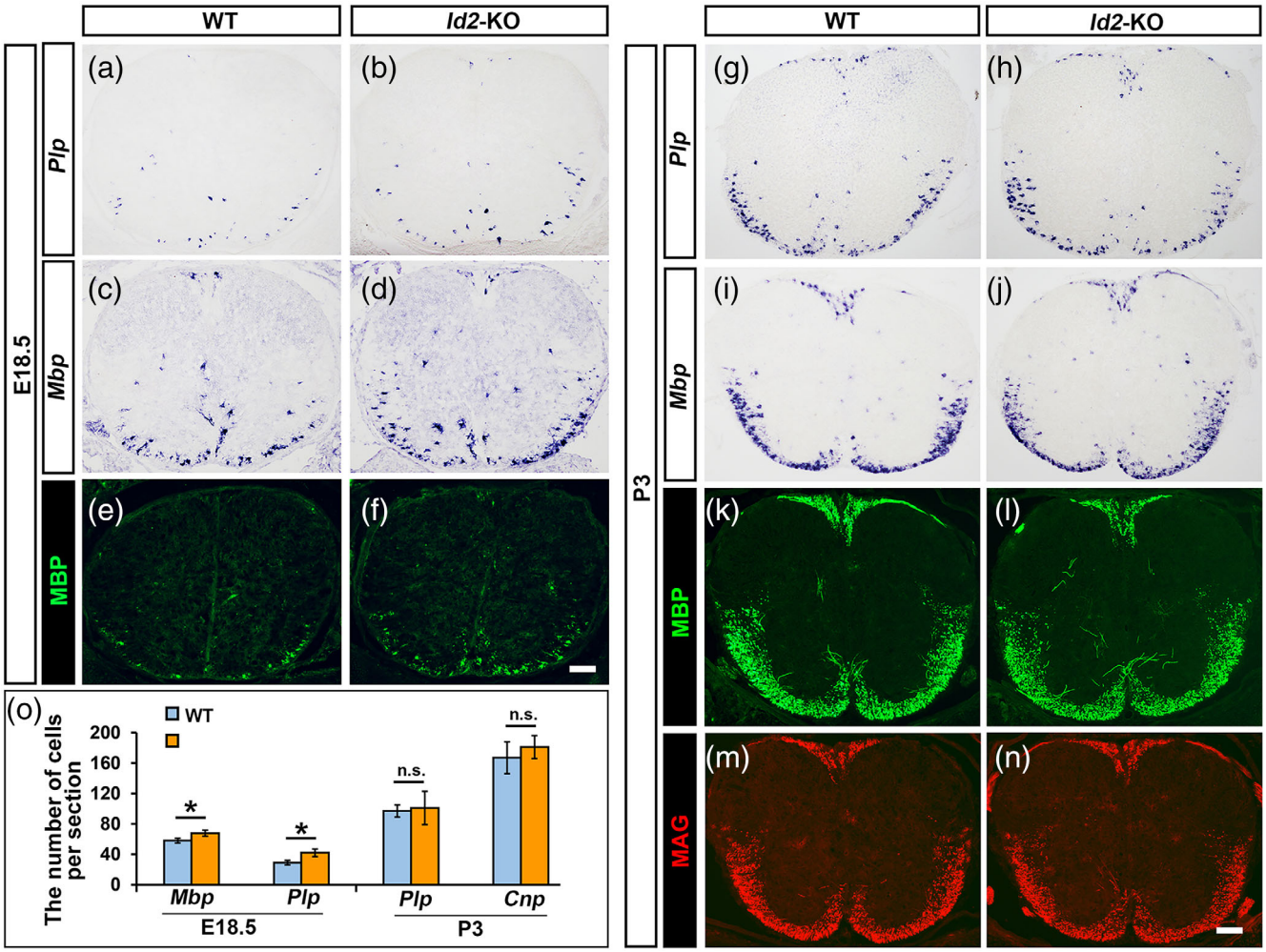

FIGURE 1 Ablation of *Id2* led to slight premature differentiation of OPCs in spinal cords. (a–f) Spinal cord sections from E18.5 wild type (WT) and *Id2*-KO mice were subjected with ISH for *Plp* and *Mbp*, or immunostaining for MBP. (g–n) Expression of myelin genes *Mbp*, *Plp* and MAG in P3 spinal cords from WT and *Id2*-KO mice. (o) Quantification of *Mbp*+, *Plp*+, and *Cnp* + cells per section in WT and Id2-KO mouse spinal cords at E18.5 and P3 stages (mean ± SD, $n = 3$). ISH, in situ hybridization; n.s., not significant; OPCs, oligodendrocyte progenitor cells. *$p < .05$. Scale bars: 100 μm

ab7349, 1:500), mouse-anti-MAG (Millipore, MAB1567, 1:500), rabbit-anti-Ki67 (Abcam, ab15580, 1:500), guinea pig-anti-SOX10 (Oasis Biofarm, OB-GP001, 1:500), rabbit-anti-EGFP (Oasis Biofarm, OB-RB014, 1:500) and rabbit-anti-MYRF (Oasis Biofarm, OB-RB007, 1:500). The Alexa Fluor 488 and 594 secondary antibodies were purchased from Thermo Scientific (A21125, A21121, A11012, A11034, A21135, A21131, A11076, and A11073).

2.3 | In situ hybridization

The procedure of RNA in situ hybridization (ISH) was described previously (Huang et al., 2017; Huang et al., 2018; Schaeren-Wiemers & Gerfin-Moser, 1993). To generate riboprobes for mouse tissues, the DNA fragments corresponding to *Mbp* (600 nt ~ 1600 nt of NM_001025251.2), *Plp* (400 nt ~ 1260 nt of NM_001359117.1), *Cnp* (450 nt ~ 1900 nt of NM_001146318.1), *Id1* (370 nt ~ 870 nt of NM_001355113), *Id2* (80 nt ~ 400 nt of NM_010496.3), *Id3* (10 nt ~ 800 nt of NM_008321.2), and *Id4* (70 nt ~ 570 nt of NM_031166.3) were cloned into pBluescript II KS(−) vectors. For generation of riboprobes used for chicken tissues, the DNA fragments corresponding to *PLP* (100 nt ~ 970 nt of NM_205277.1) were cloned into pBluescript II KS(−) vectors. The linearized plasmids were used as templates for in vitro transcription with T3 RNA polymerase (Promega, P4024) according to the manufacturer's instructions. The probes with a final concentration of 0.5 ng/μl were used for hybridization.

2.4 | *In ovo* electroporation

For chicken *in ovo* electroporation, the ORF of *Id2* fused with a *P2A-nlsGFP* cassette and ORF of *Id4* tagged with 3x Myc were cloned into *RCASBP(B)* vectors (Morgan & Fekete, 1996). P2A peptide mediates

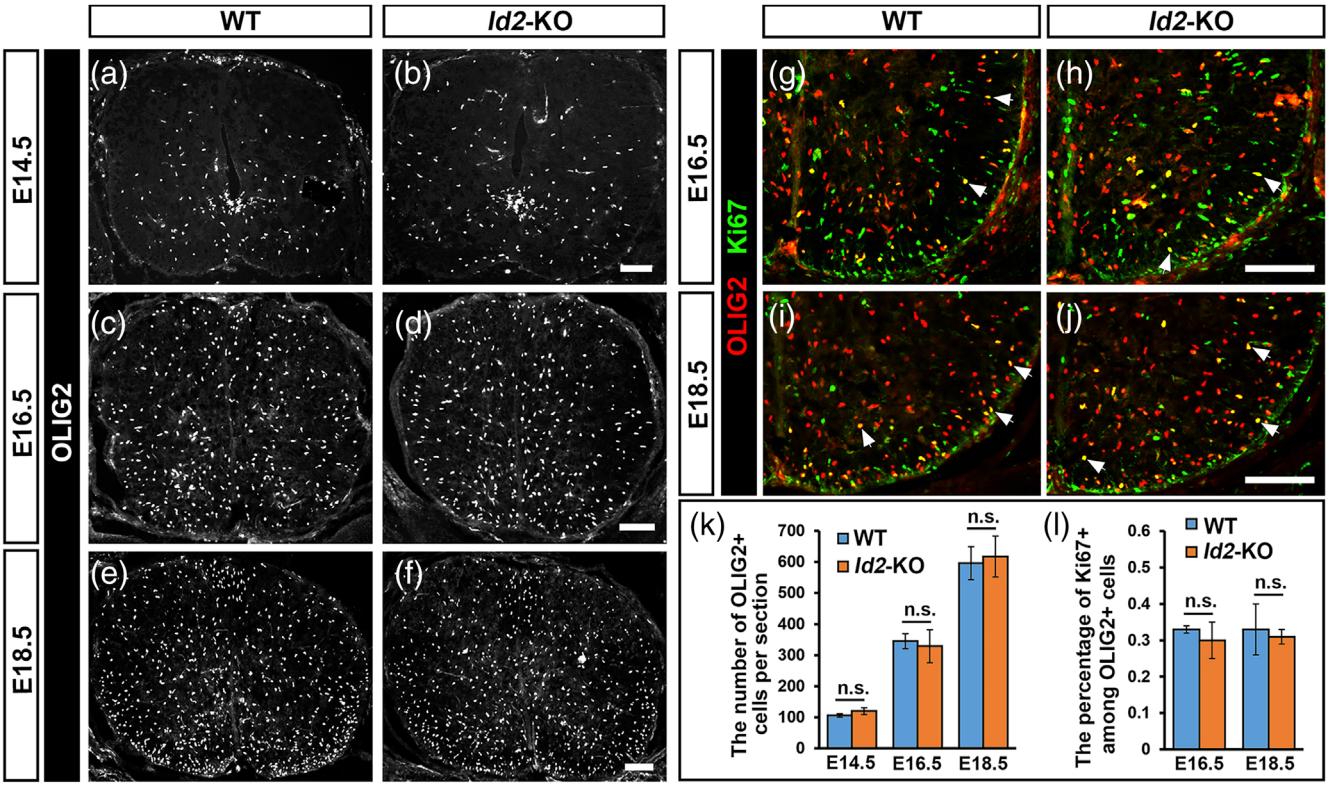

FIGURE 2 Ablation of *Id2* does not the early development of OPCs in the spinal cords. (a–f) OLIG2 immunostaining in E14.5 to E18.5 spinal tissues from WT and *Id2*-KO mice. (g–j) Double labeling of Ki67 and OLIG2 in E16.5 and E18.5 WT and *Id2*-KO mice. (k, l) quantification of OLIG2+ cells per section and the percentage of Ki67+ cells among OLIG2+ cells in WT and *Id2*-KO mouse spinal cords (mean ± SD, $n = 3$). n.s., not significant; OPCs, oligodendrocyte progenitor cells; WT, wild type. Scale bars: 100 μm

the self-cleavage between ID2 and nlsGFP, so that the expression of ID2 could be indicated by the detection of nuclear GFP. The forced expression of ID4 was confirmed by anti-Myc immunofluorescent staining.

The procedure of *in ovo* electroporation has been described previously (Huang et al., 2018). Briefly, the DNA solution containing 4 μg/μl RCASBP(B) plasmids and 1% Fast Green was injected into the central lumen of chick spinal cords at embryonic day 2 (cE2) (Momose et al., 1999). The injected embryos were then subjected to electrical shock (20 V, 50 ms for each pulse, 950 ms interval, 5 pulses) with a BTX-ECM 830 Electro Square Porator. Embryos were then allowed to develop to cE7 or cE9 as needed before analysis.

2.5 | Statistical analysis

Three mice were used in each experiment. All the cells labeled by specific markers on the spinal cord sections were counted. In the brain tissues, all *Mbp*+/*Cnp* + OLs of each section were calculated, while only OLIG2+ cells in the certain regions described in the text were calculated, and the results were presented as cell number per 0.1 mm^2 in the cortical area or per 0.25 mm^2 in the striatal region. Data were presented as mean ± SD. The two-tailed Student's t-test was performed to determine whether differences in cell numbers were statistically significant. Error bars represented the standard deviations. Statistical significance was considered to be at $^*p < .05$, $^{**}p < .01$, $n = 3$.

3 | RESULTS

3.1 | Slight precocity of OL differentiation in *Id2* mutants

Previous study demonstrated that inhibition of *Id2* expression in OPCs induced premature OL differentiation in vitro (Wang et al., 2001). To investigate whether it possesses a similar role in vivo, we examined the expression of several well-documented molecular markers for differentiated OLs in *Id2* mutants. The loss of *Id2* gene expression in *Id2* mutants was confirmed by ISH in P0 brain and spinal tissues (Figure S1g,l). During development, expression of mature OL markers *Plp* and *Mbp* in the spinal tissue first starts in the ventral white matter at around E18.5. In *Id2*-KO mutant tissue, the number of *Plp* + OLs was slightly increased at this stage (Figure 1a,b). However, a comparable number of *Plp* + OLs was observed in P3 spinal cord (Figure 1g,h). The similar number of mature OLs in P3 control and *Id2* mutant was also verified by ISH with *Mbp* and *Cnp*, and immunofluorescence against MBP and MAG (Figure 1c–f, i–n,o). Together, these

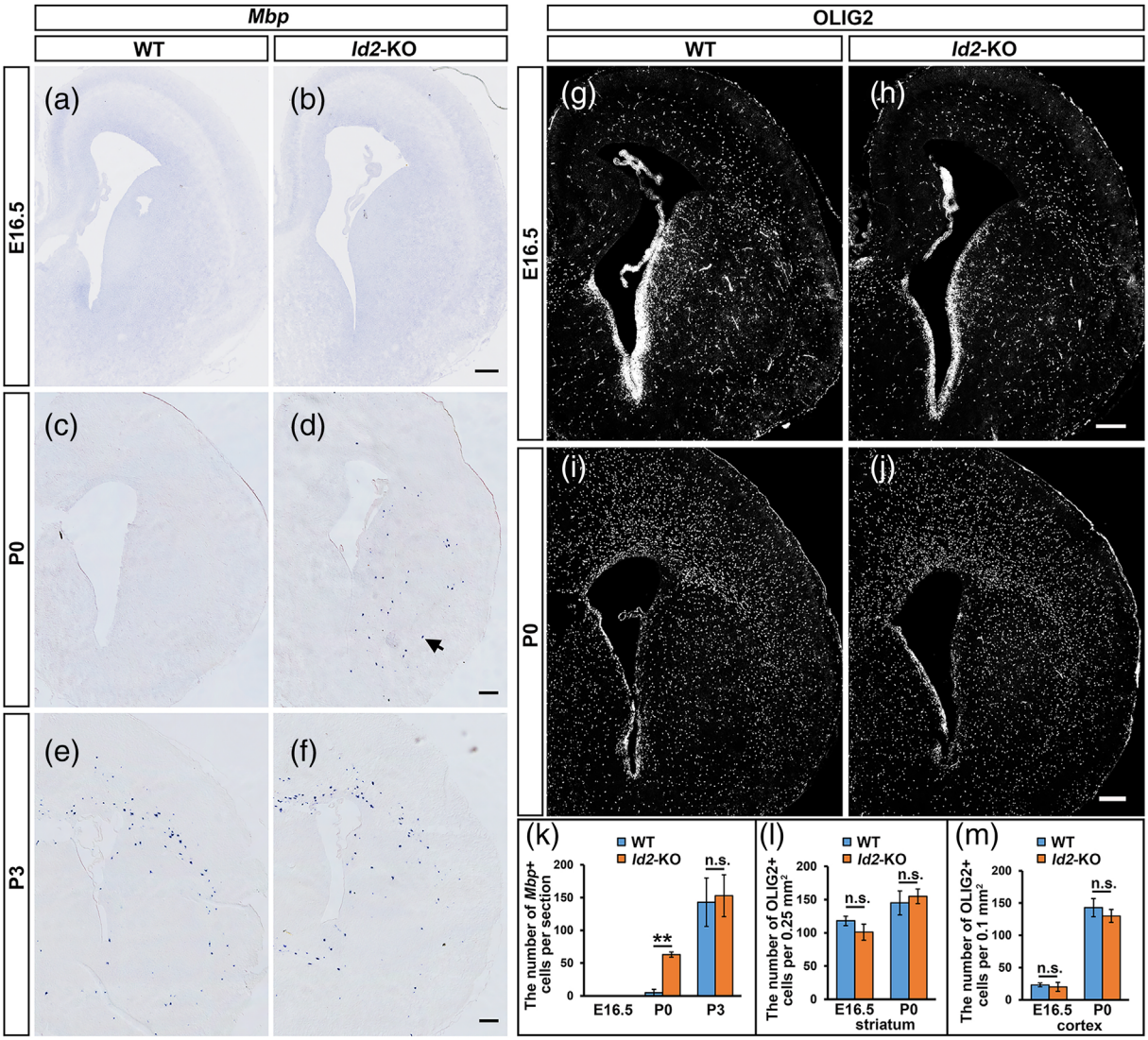

FIGURE 3 Ablation of *Id2* led to mildly premature differentiation of OPCs in brain. (a–f) Brain sections from WT and *Id2*-KO mice were subjected with ISH for *Mbp* from E16.5 to P3 stages. The black arrow indicates the premature of differentiating *Mbp* + OLs. (g–j) Immunostaining against OLIG2 in E16.5 and P0 forebrain of WT and *Id2*-KO mutants. (k–m) Quantification of *Mbp*+ and OLIG2+ cells in WT and *Id2*-KO mouse brain sections (mean ± SD, $n = 3$). ISH, in situ hybridization; n.s., not significant; OPCs, oligodendrocyte progenitor cells; WT, wild type. $^{**}p < .01$, . Scale bars: 200 μm

results indicated that *Id2* mutation only caused slight premature differentiation of OPCs during embryogenesis.

As premature differentiation of OPCs may also be caused by an excessive number of these cells, we next examined the expression of OLIG2, which selectively labels OPCs in late embryonic spinal cord. Immunostaining in E14.5 to E18.5 spinal tissues revealed the normal proliferation and distribution of OLIG2+ OPCs in *Id2* mutant (Figure 2a–f,k). Consistent with this observation, the percentage of Ki67+ proliferating cells in OLIG2+ OPCs was not affected in the mutants (Figure 2g–j,l).

Considering the possible regional difference in the molecular regulation of OL differentiation (Sperber et al., 2001; Zheng et al., 2018), we also analyzed the development of OLs in the brain tissue. At E16.5, *Mbp* + differentiated OLs were not observed in the forebrain in both wild-type and *Id2* mutants (Figure 3a,b). At P0, a few *Mbp* + OLs were detected in the striatum region of *Id2* mutant but not of the wild-type tissue (Figure 3c,d,k). However, at P3 stage, there was no significant difference in the number of *Mbp* + OLs in both genotypes (Figure 3e,f,k). Immunostaining study revealed a similar number and distribution of OLIG2+ cells in the striatal and cortical regions from E16.5 to P0 stages (Figure 3g–j,l,m). Together, these findings demonstrated that ablation of *Id2* caused a transient premature differentiation of OLs in both spinal and brain tissues without affecting the proliferation and migration of OPCs.

3.2 | Normal development of OLs in *Id4* mutants

In parallel, we also investigated the effects of *Id4* mutation on OL differentiation and maturation. The absence of *Id4* gene expression in

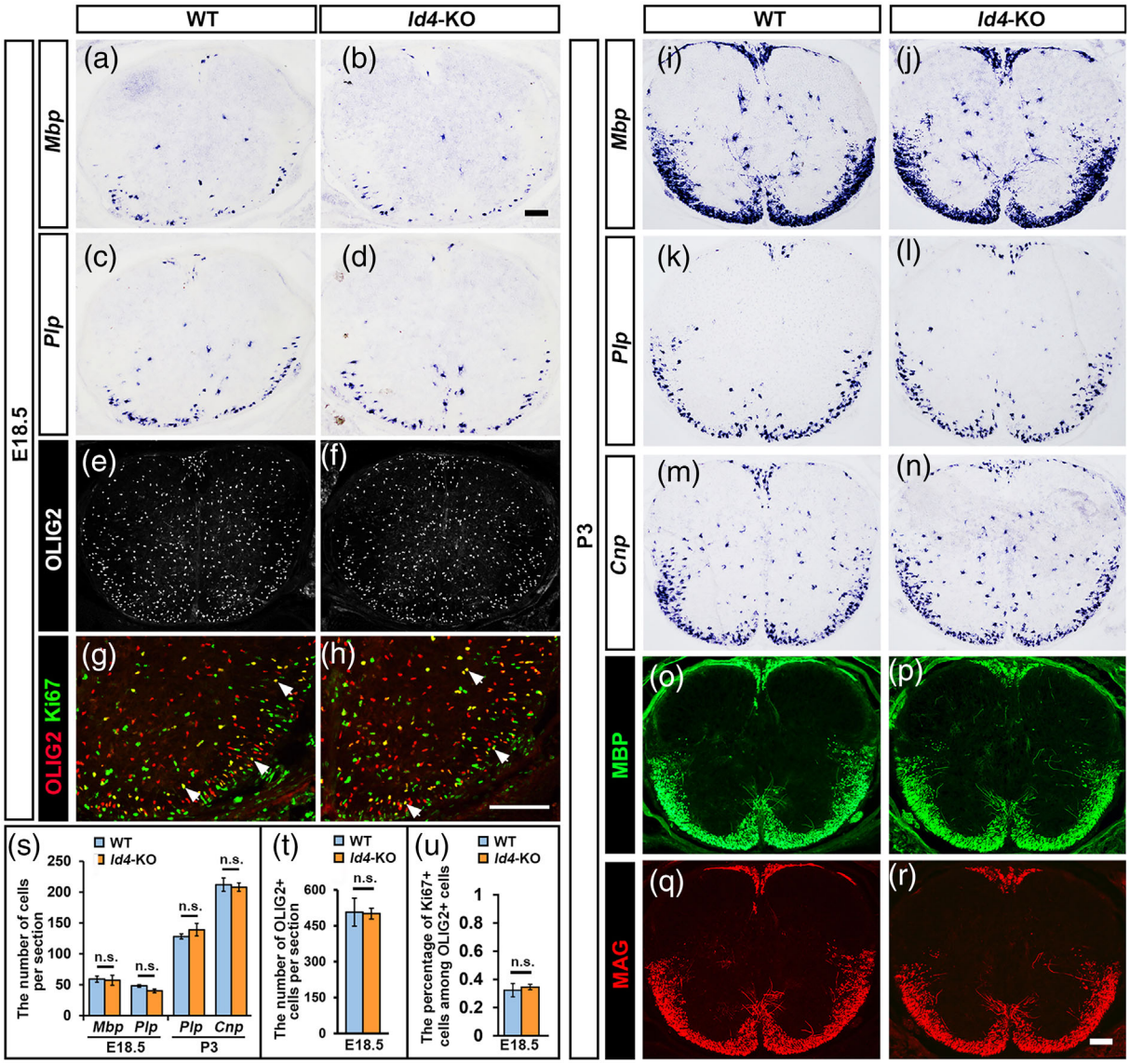

FIGURE 4 Normal development of oligodendrocytes in *Id4*-KO spinal cords. (a–f) Spinal cord sections from E18.5 wild type (WT) and *Id4*-KO mice were subjected with ISH for *Plp* and *Mbp*, or immunostaining for OLIG2. (g, h) double labeling of OLIG2 and Ki67 in E18.5 spinal cords of WT and *Id4*-KO mutants. (i–r) ISH and immunostaining against *Mbp*, *Plp*, *Cnp* and MAG in P3 spinal cord sections of WT and *Id4*-KO mice. (s–u) Quantification of cell numbers and the percentage of Ki67+ cells among OLIG2+ cells in WT and *Id4*-KO mouse spinal cords (mean ± SD, $n = 3$). ISH, in situ hybridization; n.s., not significant; WT, wild type. Scale bars: 100 μm

the mutant tissue was similarly verified by ISH in P0 mutant spinal cord and brain tissues (Figure S2g,l). Contradictory to the previous suggestion of *Id4* as an important negative regulator of OL differentiation in vitro (Kondo & Raff, 2000), *Id4* mutation had no effect on the expression of all OL markers examined including *Mbp*, *Plp*, *Cnp*, and MAG in E18.5 and P3 spinal cords (Figure 4a–d,i–r,s). Neither did it affect the number and localization of OLIG2+ OPCs, or their proliferation rate as determined by the percentage of OLIG2+ cells co-expressing Ki67 (Figure 4e–h,t,u).

In the brain tissues, a similar differentiation course was observed in the wild type and *Id4* mutants (Figure 5). *Mbp* + OLs were mainly detected in the corpus callosum regions at P3 stage, and there was no significant differece in their numbers between *Id4* mutants and their wild type littermates (Figure 5e,f,k). Unsurprisingly, the number and distribution of OLIG2+ OPCs were not affected in E16.5 and P0 forebrain (Figure 5g–j,l,m). Based on these observations, we conclude that ablation of *Id4* gene does not impair the development of OL lineage in the brain and spinal tissues.

3.3 | Precocious OL differentiation in *Id2*/ *Id4* double mutants as in *Id2* single mutants

The observations that *Id2* and *Id4* mutations cause mild or no effect on OL differentiation have raised the possibility of potential functional redundancy of these two genes. To test this possibility, we generated

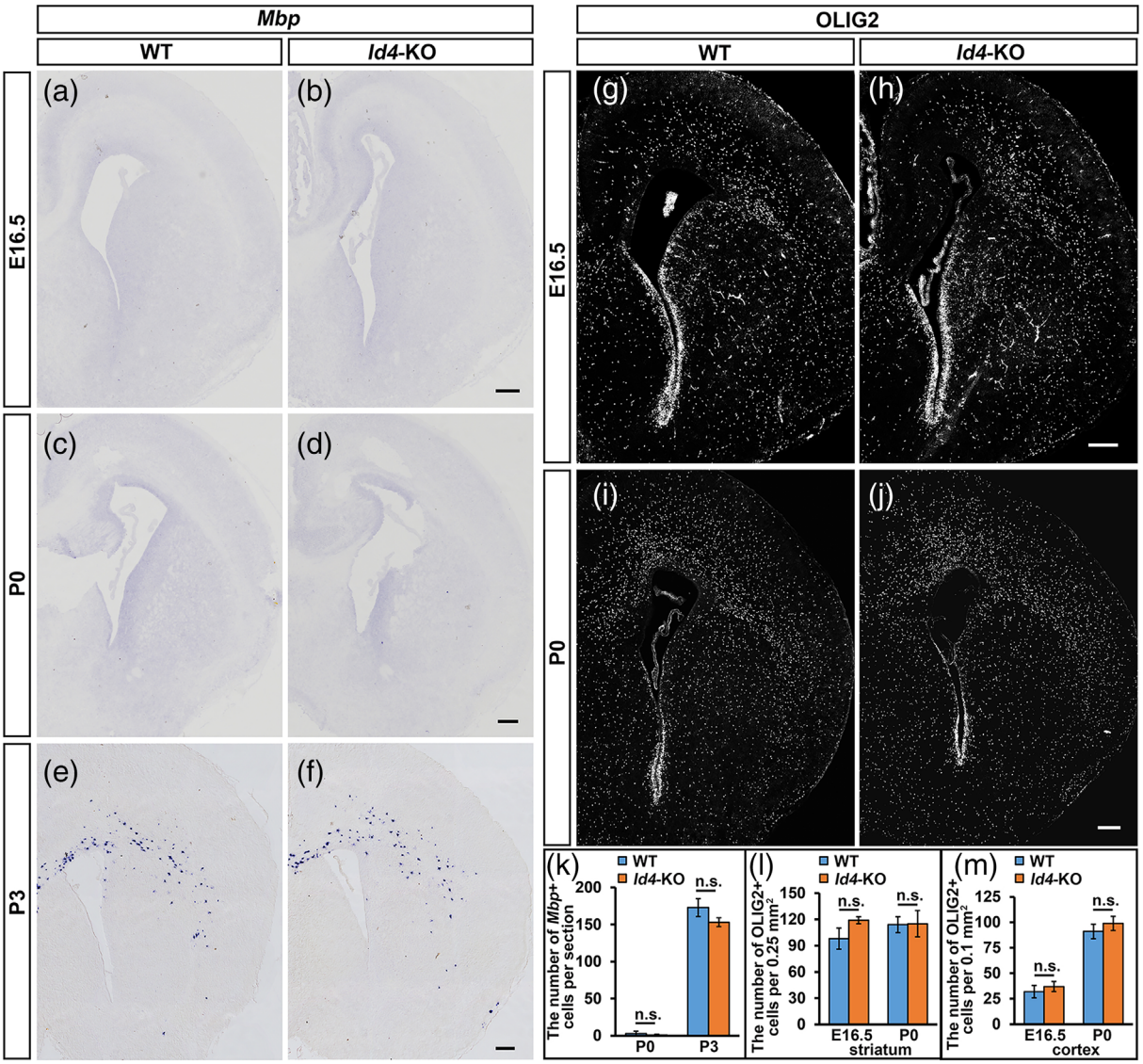

FIGURE 5 Normal development of oligodendrocytes in *Id4*-KO brain. (a–f) Brain sections from WT and *Id4*-KO mice were subjected with ISH for *Mbp* from E16.5 to P3 stages. (g–j) Immunostaining against OLIG2 in E16.5 and P0 forebrain of WT and *Id4*-KO mutants. (k–m) Quantification of *Mbp*+ and OLIG2+ cells in WT and *Id4*-KO mouse brain sections (mean ± SD, $n = 3$). ISH, in situ hybridization; n.s., not significant; WT, wild type. Scale bars: 200 μm

the *Id2*/*Id4* double mutants (*Id2*/*Id4*-dKO) by interbreeding. Examination of myelin gene expression in E18.5 spinal cords revealed the mild precocity of OL differentiation in *Id2*-KO and *Id2*/*Id4*-dKO as compared to the wild type and *Id4*-KO mutants, and no difference was observed between *Id2*-KO single mutants and *Id2*/*Id4*-dKO mutants (Figure 6a–h,q).

The effects of *Id2* and *Id4* genes on OL differentiation were also investigated in the developing forebrain. Since it was extremely difficult to obtain double mutant mice after birth, we only analyzed the differentiation of OLs in P0 forebrain tissues. Similar to the scenario in *Id2*-KO single mutants, a small number of *Mbp* + OLs were also observed in the striatum of *Id2*/*Id4*-dKO double mutants (Figure 6j,l,r), and no significant differences was detected between these two mutants. The same results were observed for the expression of *Cnp*, a specific marker for the differentiating OLs (Figure 6m–p). Thus, deletion of *Id2*, but not *Id4*, resulted in slight premature OL differentiation in both the spinal cord and brain tissues. The nearly identical phenotypes in *Id2*-KO and *Id2*/*Id4*-dKO mutants indicated the lack of functional redundance between these two *Id* genes in OL differentiation.

3.4 | Inhibition of OL differentiation by ID2 and ID4 in embryonic chicken spinal cord

Previous research demonstrated that overexpression of ID2 and ID4 proteins in purified OPCs can markedly inhibit OL differentiation and myelin gene expression (Kondo & Raff, 2000; Wang et al., 2001). To

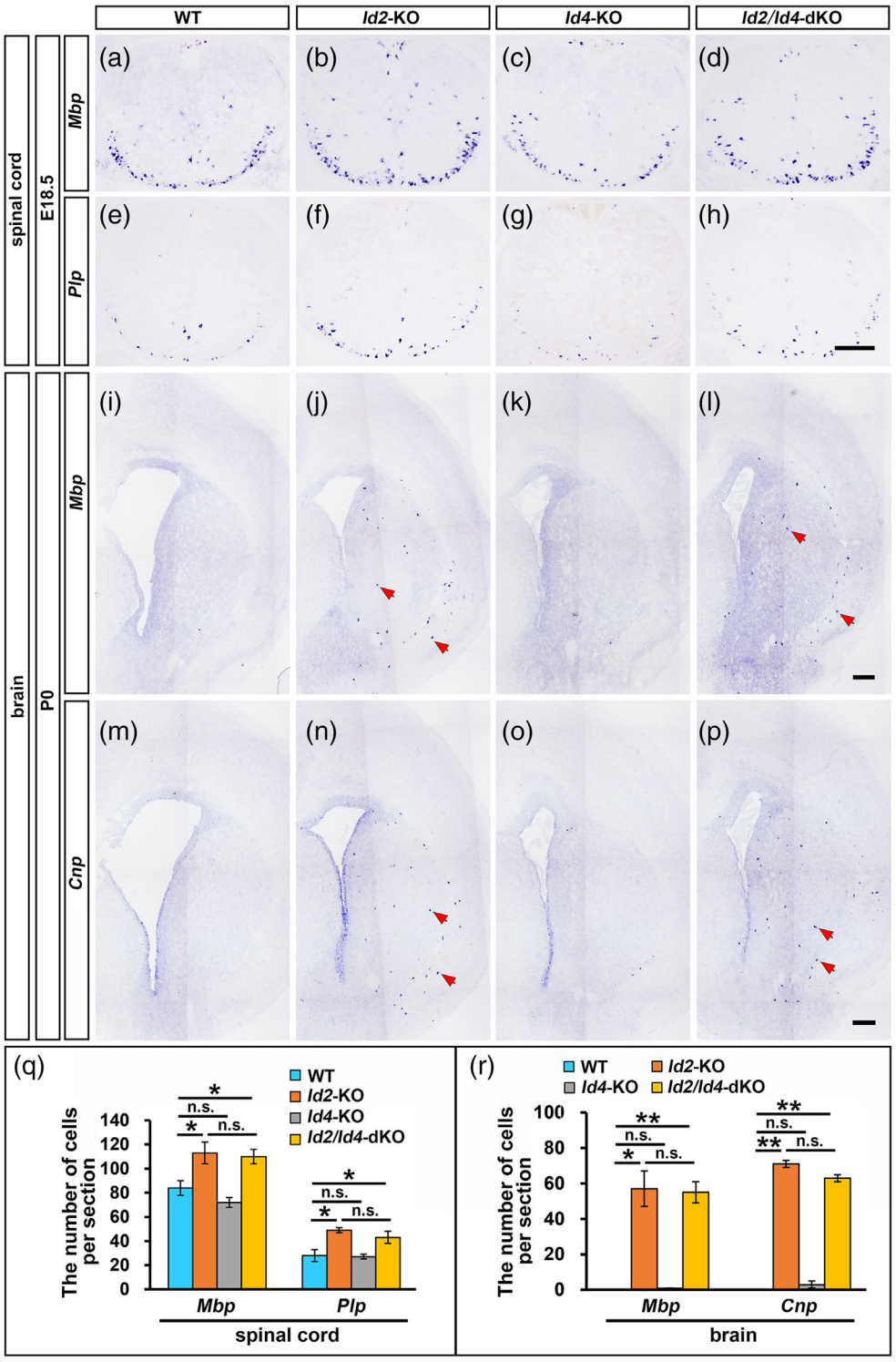

FIGURE 6 Lack of functional redundance between *Id2* and *Id4* in oligodendrocyte differentiation. (a–h) ISH for *Mbp* and *Plp* in E18.5 spinal cord sections from WT, *Id2*-KO, *Id4*-KO, and *Id2*/*Id4* double mutants (*Id2*/*4*-dKO). (i–p) ISH for *Mbp* and *Cnp* in P0 brain tissues of WT, *Id2*-KO, *Id4*-KO, and *Id2*/*4*-dKO mice. The premature of differentiating OLs are indicated by red arrows. (q, r) quantification of cell numbers per section (mean ± SD, $n = 3$). ISH, in situ hybridization; n.s., not significant; WT, wild type. $^{*}p < .05$, $^{**}p < .01$, . Scale bars: 200 μm

investigate whether expression of these molecules can possess similar functions in vivo, we overexpressed ID2 and ID4 proteins in embryonic spinal cords by *in ovo* electroporation. In this study, DNA constructs expressing ID2 or ID4 proteins were introduced into the ventricular neuroepithelial cells in day 2 (cE2) embryonic chicken spinal cord. Seven-days after electroporation at cE9, spinal tissues were processed to analyze the expression of myelin genes. We found that expression of *PLP* and MBP was almost completely suppressed in the ID2- and ID4-electroporated side (Figure 7a–f,k). In contrast, overexpression of either ID2 or ID4 had no effect on the expression of OLIG2 in the electroporated tissues (Figure 7g–j,l), indicating that ID expression did not alter the early development of OPCs from neuroepithelial cells. Taken together, these results demonstrated that forced expression of ID2 or ID4 proteins can potently inhibit OL differentiation and myelin gene expression in the developing neural tissue.

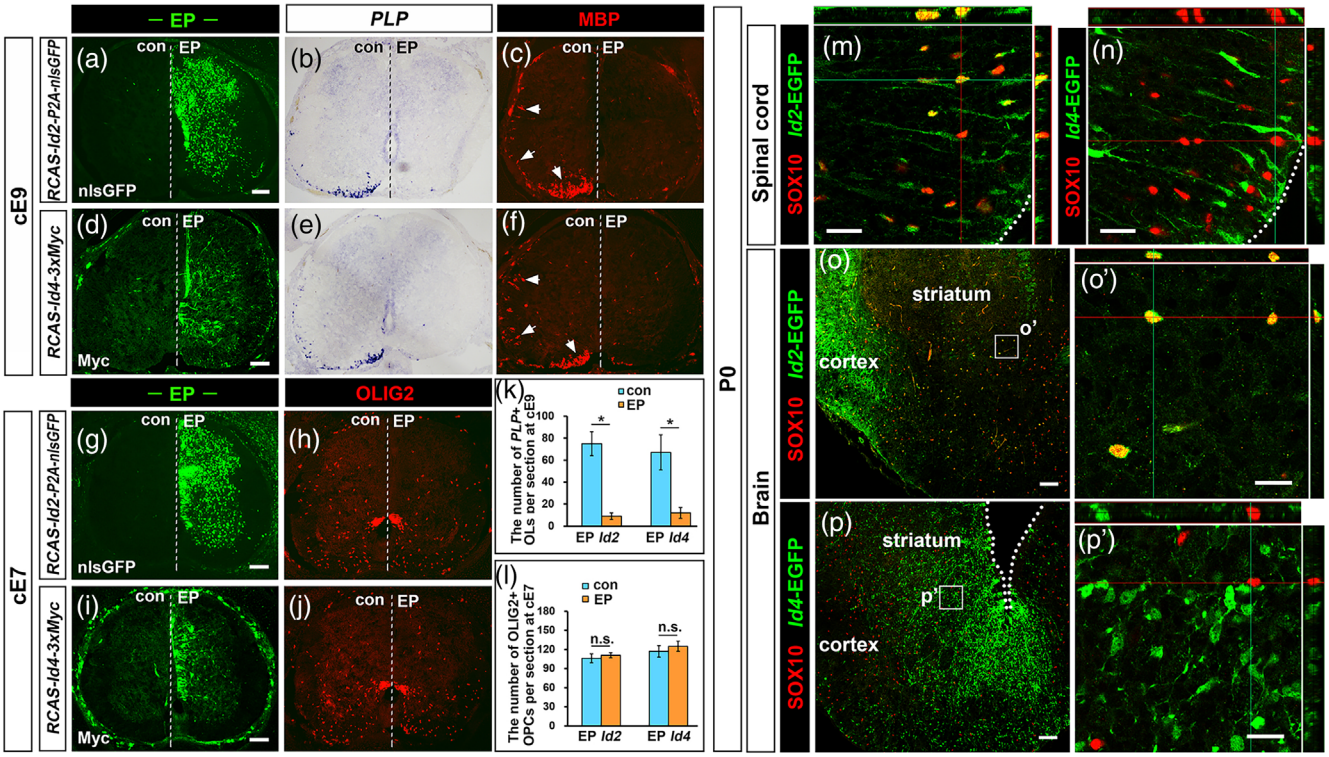

FIGURE 7 Forced expression of *Id2* and *Id4* inhibits myelin gene expression in embryonic chicken spinal cord. (a–f) cE9 chicken spinal cord sections were subjected to ISH for *PLP* and immunostaining against *MBP*. (g–j) Immunostaining against OLIG2 was performed on cE7 chicken sections. The electroporation of ID2 was referred by the nlsGFP, and ID4 expression was indicated by immunostaining against Myc tag. (k, l) quantity statistics of the number of *PLP* + and OLIG2+ cells of control (con) and electroplated (EP) sides at cE9 and cE7 stages (mean ± SD, $n = 3$). $^*p < .05$, n.s., not significant. (m–p, o', p') double immunostaining against SOX10 and EGFP in spinal cord and brain tissues of *Id2*-EGFP and *Id4*-EGFP heterozygous mice. Scale bars: 100 μm in (a–j, o, p) and 20 μm in (m, n, o', p'). ISH, in situ hybridization

3.5 | Developmental expression of *Id2* and *Id4* in the developing CNS

Considering the potent inhibitory effect *Id2* and *Id4* overexpression on OPC differentiation both in vitro and in vivo (Gokhan et al., 2005; Kondo & Raff, 2000; Wang et al., 2001) (Figure 7a-l), the fairly weak OL phenotypes in the mutants (Figures 1–6) imply that *Id2* and *Id4* genes may not be expressed in oligodendroglial lineage as strongly as previously thought in vivo. To investigate this possibility, we next examined the developmental expression of *Id2* and *Id4* in the developing CNS by RNA ISH due to the lack of well-verified antibodies.

In the spinal cord, prior to E16.5, *Id2/4* expression was mainly detected in the ventricular progenitor cells and their progenies in the gray matter of the spinal cord (Figures S1a and S2a). Starting from E16.5, weak expression of *Id2* and *Id4* emerged in the white matter region and persisted to about P15 before their eventual down-regulation at P35 (Figures S1b–f and S2b–f). These expression patterns suggest that *Id2* and *Id4* are gradually upregulated in white matter glial cells in the developing spinal cord. In brain tissues, *Id2* is predominantly expressed in the cortical neurons and subventricular zone at about E16.5 and thereafter (Figure S1h–l). Notably, a small number of cells with weak *Id2* expression were scattered around in the striatum at P0 stage (Figure S1k',m'). Interestingly, the expression pattern of *Id4* seems to be complementary to that of *Id2* in the forebrain. *Id4* is highly expressed in neural progenitor cells throughout the entire embryonic stages, especially in the cortical region, but in the striatal and septal regions as well (Figure S2h–l). Unlike *Id2*, *Id4* is only weakly expressed in postmitotic cortical neurons (Figure S2h–l). The expression patterns of *Id2* and *Id4* genes was confirmed by anti-EGFP immunofluorescence in *Id2*-EGFP and *Id4*-EGFP heterozygotes at P0 stages (Figures S1j,m and S2j,m).

To determine whether *Id2* and *Id4* are expressed in glial cells of OL lineage, we examined the expression of pan-OL marker SOX10 in EGFP+ cells in *Id2*-EGFP and *Id4*-EGFP heterozygous embryos, respectively. Double immunofluorescent staining in the spinal tissue revealed the SOX10 expression in a portion of *Id2*-EGFP+ cells, but in none of *Id4*-EGFP+ cells (Figure 7m,n), demonstrating that only *Id2* is expressed in OLs. In parallel, we also analyzed the co-expression of EGFP and SOX10 in the striatum region of brain tissues, given the expression of both *Id2* and *Id4* in this region (Figures S1k',m' and S2k,m). Again, despite the relatively low level of EGFP expression, many *Id2*-EGFP+ cells expressed SOX10 (Figure 7o,o'). By contrast, no *Id4*-EGFP and SOX10 double positive cells were found in P0 brain (Figure 7p,p'). Taken together, these expression studies clearly demonstrated that only *Id2*, but not *Id4*, is weakly expressed in OPCs in the developing CNS.

4 | DISCUSSION

Id2 and *Id4* genes have been thought as two important negative regulators that control the timing of OL differentiation both in vitro and in vivo (Kondo & Raff, 2000; Marin-Husstege et al., 2006; Wang et al., 2001). In this study, we showed that *Id2* mutation only caused transient and mild premature OL differentiation in E18.5 spinal cord and P0 striatum without apparent effect on the proliferation of OPCs (Figures 1 and 3). Unexpectedly, OL differentiation and maturation were not affected at all in *Id4* single mutant mice (Figures 4 and 5), contrary to the belief that *Id4* is one of the major negative regulators of OPC differentiation (Kondo & Raff, 2000; Marin-Husstege et al., 2006; Samanta & Kessler, 2004). Further, *Id2* and *Id4* double mutants displayed the same phenotype as the *Id2* single mutants, indicating the lack of potential functional redundancy between these two closely related members of the Id family. In keeping with these functional studies, our gene expression analyses demonstrated that only *Id2* gene is weakly expressed in OPCs at early postnatal stages, and down-regulated when OPCs differentiate (Figure S3j–l). By contrast, *Id4* is not expressed in cells of OL lineage in brain and spinal tissues at all stages examined. Based on these and other observations, we conclude that *Id2* is a minor negative regulator of OL differentiation during development, and *Id4* is not relevant to OL development under normal physiological conditions.

Since the expression of *Id1* and *Id3* was observed in cultured OPCs (Kondo & Raff, 2000; Wang et al., 2001), we also investigated their expression in P0 brain tissues. A weak expression of *Id1* was found outside the ventricular zone with a vessel-like pattern (Figure S3a,j). By contrast, *Id3* is highly expressed in neural tissues (Figure S3c,h). Double labeling experiments confirmed that all *Id3*+ cells outside the germinal zone are ALDH1L1+ astrocytes (Figure S3i), as reported by previous studies (Batiuk et al., 2020; Lamantia et al., 2014; Li et al., 2021). Collectively, these data argued against the expression of *Id1* and *Id3* in oligodendroglial cells during development. To examine the possible compensatory effects of other *Id* genes, we also analyzed the expression of *Id1*, *Id3*, and *Id4* in P0 brain tissues of *Id2*-null mutants by ISH, and no upregulation of these *Id* genes was detected in the mutant tissues. These data ruled out the involvement of other *Id* genes in OPC differentiation in *Id2*-KO mutants.

Other well-known negative regulators of OL differentiation include *Notch* signaling and its downstream effector *Hes5*, *Pdgfra* pathway and its upstream activators *Sox5/6*, and mutations of these inhibitory molecules in OPCs all lead to premature OL differentiation to different extents (Baroti et al., 2016; Calver et al., 1998; Ge et al., 2019; Liu et al., 2006; Stolt et al., 2006; Taylor et al., 2007; Zhang et al., 2009; Zhu et al., 2014). As compared to *Id2* single and *Id2*/*Id4* compound mutants, the number of precocious mature *Mbp*+ or *Plp1*+ OLs observed in these other mutants appears to be much larger and longer-lasting (Baroti et al., 2016; Calver et al., 1998; Ge et al., 2019; Stolt et al., 2006; Zhu et al., 2014), arguing against a prevailing role of *Id2* and *Id4* in repressing OPC differentiation in vivo. Consistent with these results, deletion of both *Bmpr1a* and *Bmpr1b* only slightly affected the differentiation of OLs in mouse spinal cord (See et al., 2007).

However, it is important to emphasize that forced expression of either *Id2* or *Id4* in OPCs significantly intercepts differentiation of OPCs both in vitro and in vivo (Kondo & Raff, 2000; Wang et al., 2001) (Figure 7), and OPCs purified from either *Id2* or *Id4* mutant mice are more likely to differentiate into GC+ OLs (Marin-Husstege et al., 2006; Wang et al., 2001). The inconsistent results between the in vitro and in vivo studies are probably due to the distinct extracellular environments. When OPCs are purified from CNS tissues, serum and some other components are introduced and thus activate the BMP pathway in these cells. As a result, *Id* genes in cultured OPCs from wild type mice are strongly activated in these cells by serum or BMP signaling in stark contrast to the meager expression of *Id* genes in OPCs in vivo. The induced ID2 and ID4 proteins can directly bind to OLIG2 protein and form heterodimers, repressing its function in OL fate specification and differentiation (Samanta & Kessler, 2004). However, the weak or absent expression of *Id2* and *Id4* in OPCs during development would also argue against a decisive role for them as a general inhibitor of differentiation/myelination.

Although *Id2* and *Id4* are not indispensable for the timely differentiation of OLs during development, they may be highly involved in injury-induced inhibition of OPC differentiation under pathological conditions (Petersen et al., 2017). It is known that injury can activate BMP signaling which strongly induces the expression of *Id* genes, which can potently inhibit OPC differentiation in culture (Kondo & Raff, 2000; Wang et al., 2001) and embryonic chicken spinal cord (Figure 7). Thus, it would be interesting and important to investigate whether these ID proteins are responsible for the inefficient differentiation of adult OPCs in pathological tissues with the *Id2* and *Id4* conditional ablation mouse models.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Hao Huang and Mengsheng Qiu conceived and designed the experiments; Huihui Wu and Hao Huang performed the majority of the experiments and collected the data, Fang Zhou, Wanjun He, Junqing Du, and Min Yi performed the experiments and statistically analyzed the data, Hao Huang and Mengsheng Qiu wrote the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Supplementary Fig. 1

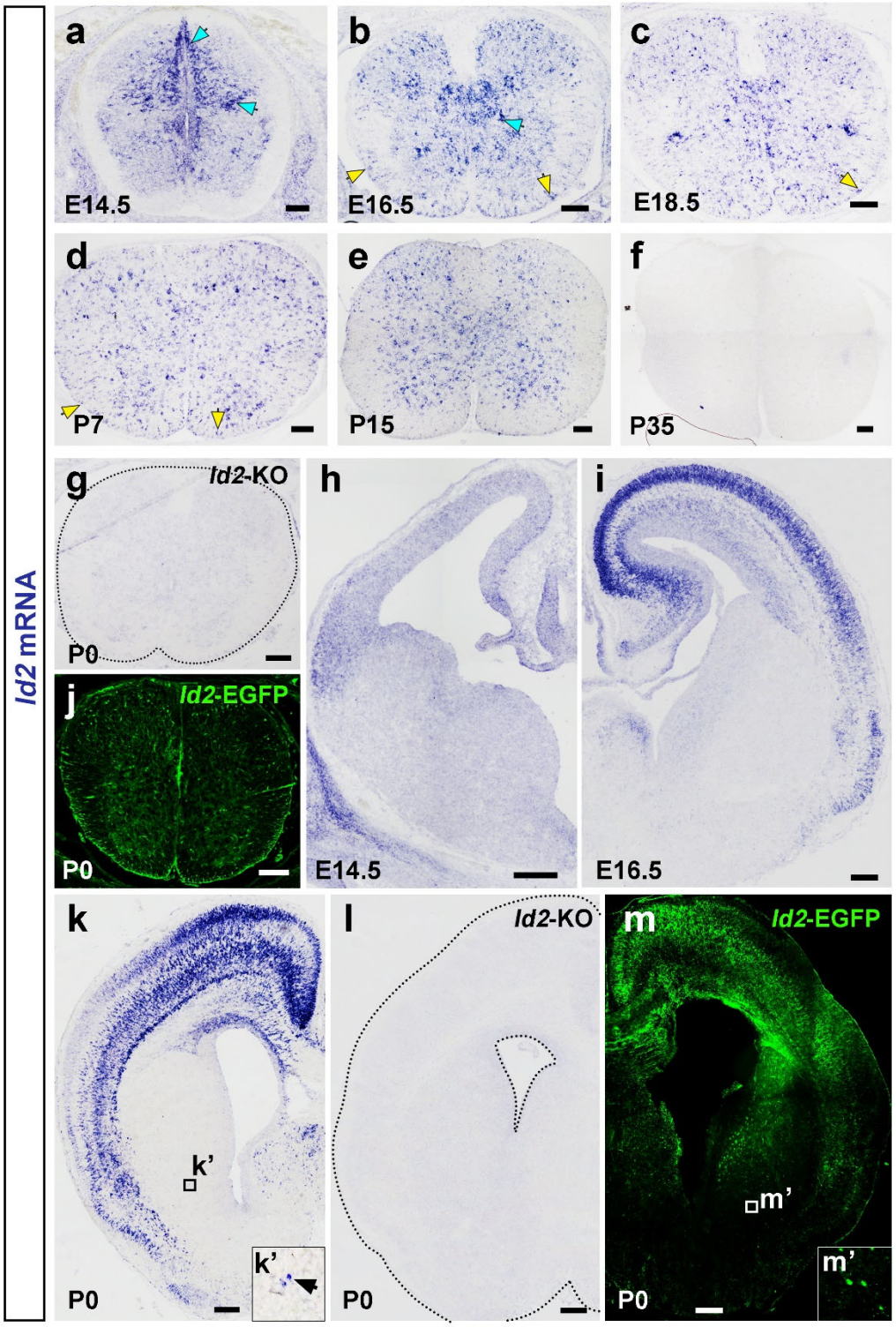

Fig. s1 Expression pattern of *Id2* in the developing mouse spinal cord and brain tissues. **a-f, h-k** ISH showed the expression of *Id2* mRNA in developing spinal cord (a-f) and forebrain (h-k). **g, l** *Id2* mRNA could not be detected in spinal cord and brain tissues of *Id2*-KO mice. **j, m** Immunostaining against EGFP in P0 spinal cord and forebrain of *Id2*-EGFP heterozygous mice. **k', m'** The expression of *Id2* mRNA and EGFP in the boxed areas (in k, m) in the striatum was shown in higher magnifications. Scale bars: 100 μm for spinal cord sections and 200 μm for brain sections.

Supplementary Fig. 2

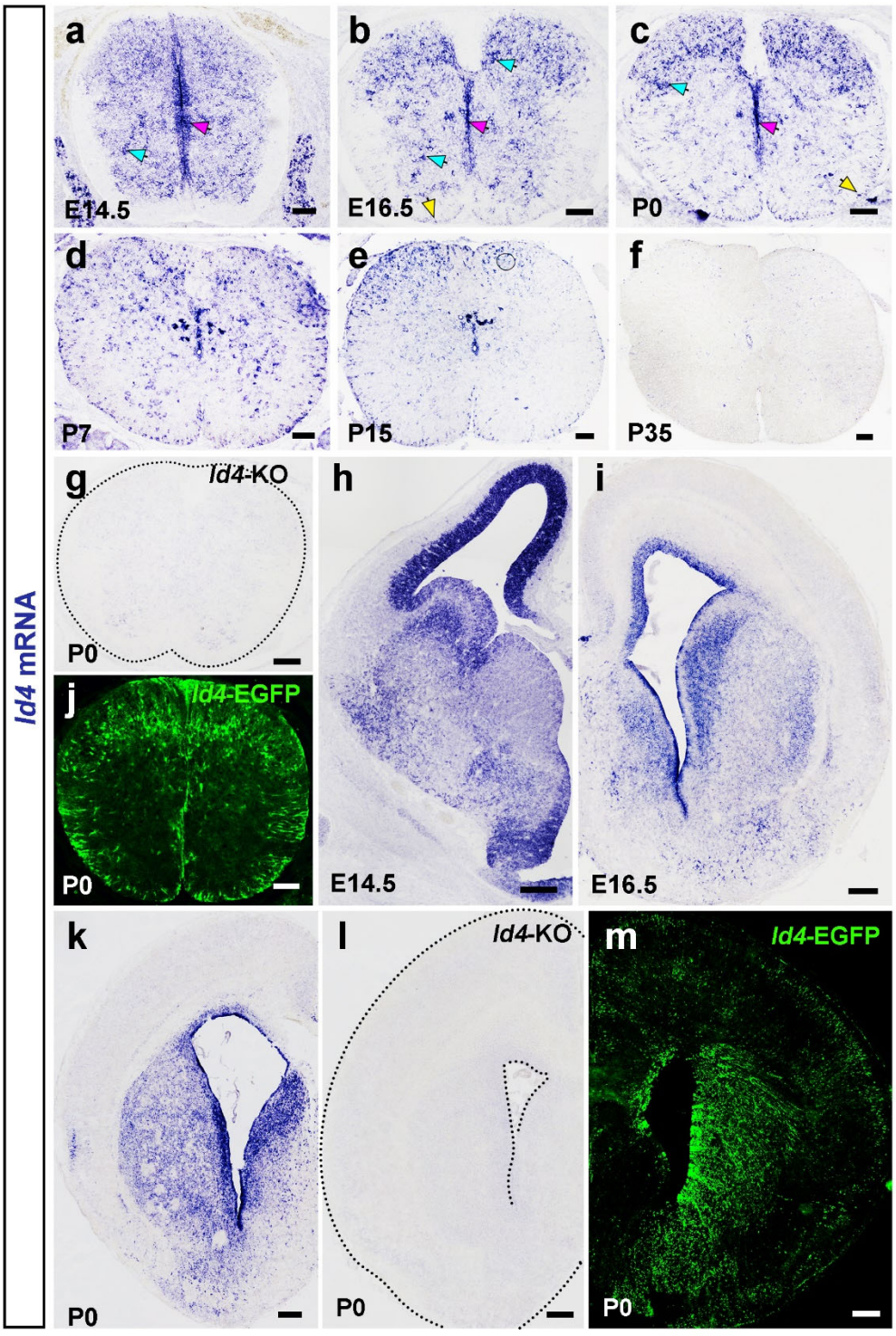

Fig. s2 Expression pattern of *Id4* in the developing mouse spinal cord and brain tissues. **a-f, h-k** ISH showed the expression of *Id4* mRNA in developing spinal cord (a-f) and forebrain (h-k). **g, l** *Id4* mRNA could not be detected in spinal cord and brain tissues of *Id4*-KO mice. **j, m** Immunostaining against EGFP in P0 spinal cord and forebrain of *Id4*-EGFP heterozygous mice. Scale bars: 100 μm for spinal cord sections and 200 μm for brain sections.

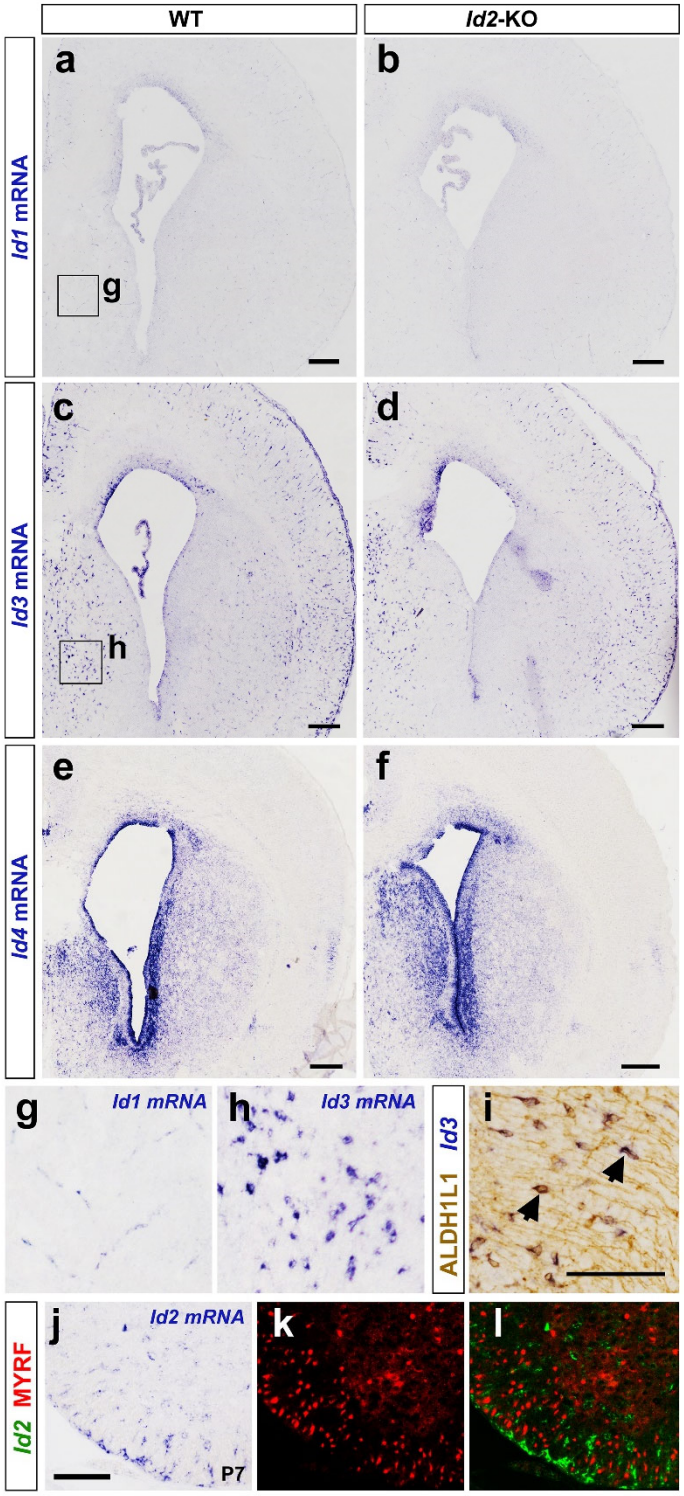

Fig. s3 Expression of other *Id* genes in P0 brain tissues from WT and *Id2*-KO mutants. **a-f** The expression of *Id1*, *Id3* and *Id4* mRNA revealed by ISH in wild type (WT) and mutant tissues. **g, h** Higher magnifications of *Id1* (g) and *Id3* (h) expression outlined in (a) and (c). **i** *Id3*+ cells were labeled by astrocyte marker ALDH1L1. **j-l** Immunostaining against MYRF after *Id2* ISH in P7 spinal cord. Scale bars: 200 μm in (a-f) and 100 μm in (i, j).