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ORIGINAL PAPER

A critical role of noggin in developing folate-nonresponsive NTD in *Fkbp8*$^{-/-}$ embryos

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Abstract
Purpose Maternal folate intake has reduced the incidence of human neural tube defects by 60–70 %. However, 30–40 % of cases remain nonresponsive to folate intake. The main purpose of this study was to understand the molecular mechanism of folate nonresponsiveness in a mouse model of neural tube defect.
Methods We used a folate-nonresponsive *Fkbp8* knockout mouse model to elucidate the molecular mechanism(s) of folate nonresponsiveness. Neurospheres were grown from neural stem cells isolated from the lumbar neural tube of E9.5 *Fkbp8*$^{-/-}$ and *wild-type* embryos. Immunostaining was used to determine the protein levels of oligodendrocyte transcription factor 2 (Olig2), Nkx6.1, class III beta-tubulin (TuJ1), O4, glial fibrillary acidic protein (GFAP), histone H3 Lys27 trimethylation (H3K27me3), ubiquitously transcribed tetratricopeptide repeat (UTX), and Msx2, and quantitative real-time (RT)-PCR was used to determine the message levels of *Olig2*, *Nkx6*.1, *Msx2*, and *noggin* in neural stem cells differentiated in the presence and absence of folic acid.
Results Fkbp8$^{-/-}$-derived neural stem cells showed (i) increased noggin expression; (ii) decreased Msx2 expression; (iii) premature differentiation—neurogenesis, oligodendrogenesis (Olig2 expression), and gliogenesis (GFAP expression); and (iv) increased UTX expression and decreased H3K27me3 polycomb modification. Exogenous folic acid did not reverse these markers.
Conclusions Folate nonresponsiveness could be attributed in part to increased noggin expression in *Fkbp8*$^{-/-}$ embryos, resulting in decreased Msx2 expression. Folate treatment further increases Olig2 and noggin expression, thereby exacerbating ventralization.

Keywords Neural stem cell · H3K27me3 · Noggin · Msx2 · Olig2 · UTX

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Introduction

Maternal folic acid (FA) intake has reduced the prevalence of neural tube defects (NTDs) in the human population by 60–70 %. These NTDs are termed as folate-responsive; however, the remaining 30–40 % of NTDs which do not respond to FA are termed as folate-nonresponsive [1–6]. FA alters the methylation status of key genes involved in stem cell proliferation and neurogenesis during neural tube (NT) formation [7–9]. In previous studies, our laboratory elucidated the molecular mechanisms of FA responsiveness using folate-responsive *Splotch* (*Pax3*$^{-/-}$) mutant mice [8–11]. Pax3 regulates aspects of dorsal sensory neurogenesis, as well as melanogenesis and musculogenesis [8, 9]. Maternal intake of FA rescued NTDs in *Splotch* (*Pax3*$^{-/-}$) homozygous embryos, in part, by decreasing levels of select microRNAs (miR-138, 148a, 185, 339-5p) which target KDM6B, a histone H3K27 demethylase. The FA rescued *Pax3*$^{-/-}$ embryos showed an increase in *Hes1* expression which rescued the proliferation potential of progenitor neural crest stem cell populations [8]. Recently, our laboratory has also shown that FA acts through FRα (Folr1), which can function as a transcription factor regulating Pax3 downstream targets such as *Hes1* and *Fgfr4* by binding to AANTT sequences within the *cis*-regulatory elements on their promoters [12]. Thus, folate responsiveness in *Splotch* (*Pax3*$^{-/-}$) embryos involves (i) regulation of miRNA levels, (ii) chromatin remodeling (H3K27 methylation) at Pax3 downstream target promoters, and (iii) regulation of some Pax3 downstream targets genes via FRα binding to their *cis*-regulatory elements.

Using a *curly tail* mouse model that exhibits folic acid-resistant neural tube defects, Leung et al. [13] tested the effect of specific combinations of pyrimidine and purine nucleotide precursors and observed a significant protective effect. In the present study, another FA-nonresponsive mutant mouse model *fkbp8* (*Gt* (*neo*)) [14] (*Fkbp8*$^{-/-}$) was used to investigate the mechanism of FA nonresponsiveness. Fkbp8 is a 38-kD protein with extensive amino acid homology to the FK-506 rapamycin-binding PPIase domain of Fkbp1a [15]; however, Fkbp8 does not bind FK506 or rapamycin, nor does it possess PPIase activity [15, 16]. Fkbp8 contains a three-unit tetratricopeptide repeat and a leucine zipper repeat, suggesting that it may form multimeric complexes with other proteins. It is ubiquitously expressed in vertebrate cells and plays a role in various processes such as cell death and viral replication [17]. It is highly expressed in the brain, spinal cord, kidney, liver, and testis and moderately expressed in the lung, spleen, heart, and ovary [15, 16]. During development, Fkbp8 controls neural cell fate through antagonism of sonic hedgehog (Shh), critical for proper neural tube ventralization [18–21]. The absence of Shh is associated with the midline defect holoprosencephaly, whereas increased Shh signaling is associated with exencephaly and spina bifida [22]. *Fkbp8*$^{-/-}$ embryos show abnormal neural tube development, skeletal defects including scoliosis, rib deformities, club foot, and curled tail [14]. In these embryos, the neuroepithelium is disorganized, and the formation of dorsal root ganglia is defective [21].

To examine the mechanism of FA nonresponsiveness in *Fkbp8*$^{-/-}$ embryos, neurospheres (NS) were grown from neural stem cells (NSCs) isolated from the lumbar neural tube of E9.5 *Fkbp8*$^{-/-}$ and *wild-type* (*WT*) embryos. The mutant NSCs exhibited increased noggin expression. Noggin is an antagonist of BMP4 signaling [23] and acts in concert with Shh to establish the dorsoventral axis during development [24]. FA treatment further increased noggin expression and decreased Msx2 levels. Mutant NSCs exhibited greater proliferation and premature neurogenesis compared to *WT*, with the observed changes unaffected by FA. In contrast, decreased proliferation and premature neurogenesis seen in NS from *Splotch* (*Pax3*$^{-/-}$) embryos are both reversed by FA [8]. Differentiated NSCs from *Fkbp8*$^{-/-}$ embryos displayed an increase in the ventral neural tube marker, oligodendrocyte transcription factor 2 (Olig2) which was further increased by FA treatment. FA treatment further decreased the polycomb modification histone H3 Lys27 trimethylation (H3K27me3). These results suggest that FA-mediated increase in noggin levels and decreased levels of H3K27me3 may play a crucial role in the development of folate-nonresponsive NTDs.

Material and methods

Ethics statement

All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH) (NIH Publication 8523, revised 1985). The Institutional Animal Care and Use Committee (IACUC) at Lurie Children's Hospital of Chicago Research Center reviewed and approved all animal protocols (the approval ID: IACUC 2009-13).

Animal care

The ES cell clone OST287985 containing a gene trap insertion in the *Fkbp8* gene was identified from the Omnibank database from Lexicon Pharmaceuticals Inc. (The Woodlands, TX, USA) and microinjected into C57BL/6 blastocysts to generate germline chimera. Chimeric males were bred to C57BL/6 females for germline transmission [14]. *Fkbp8* heterozygous males and females were mated to produce homozygous *Fkbp8*$^{-/-}$ embryos. PCR was performed on tails from dissected embryos, to confirm the *Fkbp8*$^{-/-}$ genotype. PCR primers were as described [14].

Preparation of neurospheres

To ascertain the level of ventral neural tube markers, these studies were conducted on neurosphere cultures obtained from the lumbar region of the closed neural tubes of *WT* and open neural tube region of *Fkbp8*$^{-/-}$ embryos (E9.5), and the reason for doing was embryo lethality and mortality before E9.5. Primary NS were prepared from the lumbar regions of E9.5 *Fkbp8*$^{-/-}$ and *WT* embryos and confirmed to originate from a NSC lineage, as previously described [9]. Secondary NS were cultured using 5×10^4 cells/mL.

NS proliferation colony-forming assay and EdU staining

Proliferation potential of *Fkbp8*$^{-/-}$ and *WT* NS was determined in the presence and absence of FA (2 μg/mL) (Sigma F8758). Triturated single cells dissected from E9.5 embryos were grown on PolyHema (2-hydroxyethyl methacrylate; Sigma P3932)-coated 24-well plates. NS were grown in NeuroBasal (Gibco-Invitrogen) supplemented with B-27 (Gibco-Invitrogen), N-2 (Gibco-Invitrogen), L-glutamate (2 mM) (Invitrogen), heparin sodium (J.T. Baker), and penicillin-streptomycin (Gibco-Invitrogen) together with epidermal growth factor (EGF) (20 ng/mL; Sigma) and basic fibroblast growth factor (bFGF) (20 ng/mL; R&D systems). On day 5, NS with diameters greater than 50 μm were counted and considered to be colony-forming units (CFUs). After counting, the Click-iT® EdU Imaging Kit (Invitrogen), which detects and quantifies newly synthesized DNA, was used to further examine proliferation. EdU was detected as green fluorescence, using a Leica DMIRB Inverted Microscope. Differentiation of these neurospheres was initiated by growing them in Neurobasal media devoid of growth factors for 1 week.

Immunostaining

The NS from *WT* and *Fkbp8*$^{-/-}$ embryos (E9.5) were incubated for 2 weeks on laminin (Sigma L2020)-coated 24-well plates in the absence of growth factors. They were then incubated with primary antibodies against the following: Nkx6.1 (Santa Cruz 1:100, sc15027) and Olig2 (Santa Cruz 1:100, sc19967) as NT ventral markers, TuJ1 (Covance 1:100; 435P) for neuron-specific class III b-tubulin; ubiquitously transcribed tetratricopeptide repeat (UTX) (KDM6A) rabbit polyclonal antibody (Abcam; ab-36938) and H3K27me3 rabbit polyclonal antibody (Upstate; 07-449); and Msx2 (M-70) (Santa Cruz 1:100, sc-15396). Secondary antibodies (from Jackson ImmunoResearch) included the following: CyTM2-conjugated donkey anti-Mouse and anti-rabbit IgG (H+L) (Jackson Immuno Research); CyTM3-conjugated donkey anti-rabbit IgG (H+L) (Jackson Immuno Research) donkey anti-goat IgG H&L (Cy3®) (Abcam; ab6949); and Alexa Fluor 488-labeled donkey anti-mouse IgG (A21206) (Invitrogen).

Differentiated NS were fixed for 15 min with 4 % paraformaldehyde at RT and washed three times with PBS. Cells were permeabilized, blocked with 10 % normal donkey serum with 0.1 % Triton X-100 for 1 h, and incubated with primary antibody overnight at 4 °C. NS were then incubated with a secondary antibody for 1 h at RT. Nuclei was counterstained with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich). NS were photographed using a Leica DMIRB inverted fluorescence microscope. A minimum of five fields were selected for counting cells (×20). Cells expressing ventral, dorsal, and differentiation markers were counted. Values of positive cell numbers were expressed as % DAPI ratios.

RNA extraction and PCR

Total RNA was extracted from cell lysates of tertiary *fkbp8*$^{-/-}$ NS using the E.Z.N.A.TM MicroElute™ Total RNA Kit (OMEGA). RNA concentration was measured by NanoDrop (Fischer Scientific). cDNA was synthesized using 500 ng of total RNA and reverse transcription reagents, qScript cDNA SuperMix (95048-025, Quanta Biosciences). Primers (Table 1) were designed using Primer Express software (PerkinElmer Life Sciences) and synthesized by Eurofins MWG Operon. Relative amount of messenger RNA (mRNA) was compared to *β-actin*. Quantitative real-time PCR (qRT-PCR) was done as previously described [10].

Table 1 The list of murine forward and reverse primers used in this study for qRT-PCR

Primer name	Sequence 5′-3′
β-actin forward	ACGGCCAGGTCATCACTATTG
β-actin reverse	TGGATGCCACAGGATTCCA
Nkx6.1 forward	AAACACACCAGACCCACGTTCTCT
Nkx6.1 reverse	TTCTGGAACCAGACCTTGACCTGA
Noggin forward	GATCTGAACGAGACGCTGCT
Noggin reverse	CCTTTGATCTCGCTCGGCAT
Olig2 forward	TATTACAGACCGAGCCAACACC
Olig2 reverse	GGGCAGAAAAAGATCATCGGG
Msx2 forward	CCTGAGGAAACACAAGACCA
Msx2 reverse	AGTTGATAGGGAAGGGCAGA
Fkbp8 forward	GCTGGATCCTTTAGCAGAGAAGGT GGCCAGAGTC
Fkbp8 WT reverse	CCAACAAGCACAGAAAGACAAAGCCCAGCC
Fkbp8 MUT reverse	AGCTGGAACCTTCCAACAAGACGA

Statistical analysis

Statistical differences were determined using two-sided Student's *t* test. Values are expressed as means±standard error of the mean; $p<0.05$ was considered significant (StatView J ver.5.0).

Results

Noggin expression is upregulated in *Fkbp8*$^{-/-}$ embryo-derived NSCs, and FA exacerbates it

Fkbp8 is a negative regulator of Shh signaling. In the absence of Fkbp8 expression, Shh downstream targets are expected to be upregulated. Since noggin is a Shh-dependent gene [25], its expression is expected to be increased in *Fkbp8*$^{-/-}$ embryos, and FA treatment will exacerbate it. To test this hypothesis, we performed qRT-PCR using the undifferentiated neurospheres obtained from E9.5 *WT* and *Fkbp8*$^{-/-}$ embryos in the presence or absence of FA. *Noggin* expression is significantly (**$p<0.001$) upregulated both in *WT* and *Fkbp8*$^{-/-}$ embryo-derived neurospheres by FA (Fig. 1). These observations suggest that noggin is upregulated in *Fkbp8*$^{-/-}$ embryos, and FA treatment upregulates it even further.

Msx2 expression is downregulated in *Fkbp8*$^{-/-}$ embryo-derived NSCs, and FA treatment downregulates its expression even further

Noggin is a secreted polypeptide important for neural tube fusion. It diffuses through extracellular matrices more efficiently than other members of the transforming growth factor-beta (TGF-beta) superfamily thereby creating morphogenic gradients. Noggin binds and inactivates members of the TGF-beta superfamily signaling proteins, such as bone morphogenetic protein-4 (BMP4) [26]. With increased noggin expression as observed above, the BMP4 signaling is expected to decrease. It has been reported that Msx2 expression is induced by BMP4 signaling [27]. Therefore, we hypothesized that Msx2 expression will be decreased in *Fkbp8*$^{-/-}$ embryos, and FA treatment will decrease it even further. Immunocytochemistry data (Fig. 2a) shows that Msx2 expression is significantly downregulated in *Fkbp8*$^{-/-}$ embryo-derived differentiated neural stem cells. FA treatment to differentiated neural stem cells (Fig. 2a) caused a further significant decrease in Msx2-positive immunostaining. Quantitative RT-PCR data (Fig. 2b) showed that *Msx2* message level was significantly reduced in *Fkbp8*$^{-/-}$ NSCs ($p<0.001$), and FA treatment caused a further significant decrease in its levels ($p<0.0001$). These immunostaining and qRT-PCR data clearly demonstrated that *Msx2* expression is downregulated in *Fkbp8*$^{-/-}$ embryo-derived NSCs, and FA treatment downregulated its expression even further.

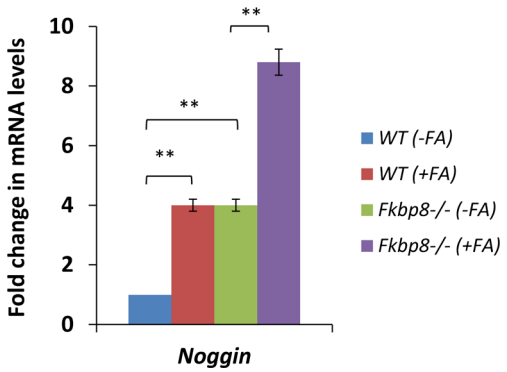


Fig. 1 Effect of FA on *Noggin* expression in *Fkbp8*$^{-/-}$ embryo lumbar neural tube-derived NSCs. E9.5 *Fkbp8*$^{-/-}$ embryo lumbar neural tube-derived undifferentiated NSCs showed significantly higher levels (**$p<0.001$) of *noggin* mRNA expression as compared with *WT*. FA treatment caused a significant increase in expression (**$p<0.001$) compared to non-FA-treated NS from *WT* and *Fkbp8*$^{-/-}$ undifferentiated NSCs. Each experiment was performed six times, with each data point in triplicate. Data is presented as fold changes in gene expression of non-FA-treated NS. *β-actin* was used as a control. Student's *t* test was used for statistical analysis; data is mean±SEM

Proliferation in NSCs derived from *Fkbp8*$^{-/-}$ embryos is unchanged by FA treatment

Msx2 is required for the survival of the subpopulations of neural crest cells, and *Msx2* mutant embryos are reported to show increased apoptosis and unchanged proliferation [28]. It is therefore expected that in *Fkbp8*$^{-/-}$ embryos, which shows a decreased level of Msx2, the proliferation of NSCs will be affected. Earlier we reported that in *Splotch* embryos [9], FA increased the proliferation of NSCs from NS (also referred to as CFUs). To examine the effects of FA on the proliferation of NSCs from *Fkbp8*$^{-/-}$ embryonic NS, primary NS were cultured from E9.5 *Fkbp8*$^{-/-}$ and *WT* NTs, in the presence and absence of FA. NS derived from *Fkbp8*$^{-/-}$ embryos showed insignificantly increased CFUs compared to *WT* in the absence of FA as evident from EdU staining (Fig. 3a, b). Although FA treatment increased the proliferation levels of *WT* NS (*$p<0.05$), there was no significant change in CFU in NS derived from *Fkbp8*$^{-/-}$ embryos, suggesting that the proliferation of NSCs from *Fkbp8*$^{-/-}$ embryos was unchanged by FA treatment, which is in sharp contrast with the FA-induced proliferation of NSCs from *Splotch* (*Pax3*$^{-/-}$) embryo neural tubes [8, 9].

FA treatment further decreases the H3K27me3 in differentiated NSCs derived from *Fkbp8*$^{-/-}$ embryos

Earlier we [8] reported that in FA-responsive *Splotch* (*Pax3*$^{-/-}$) embryos, the repressive H3K27me3 polycomb modification was downregulated by FA treatment. Noggin, a BMP

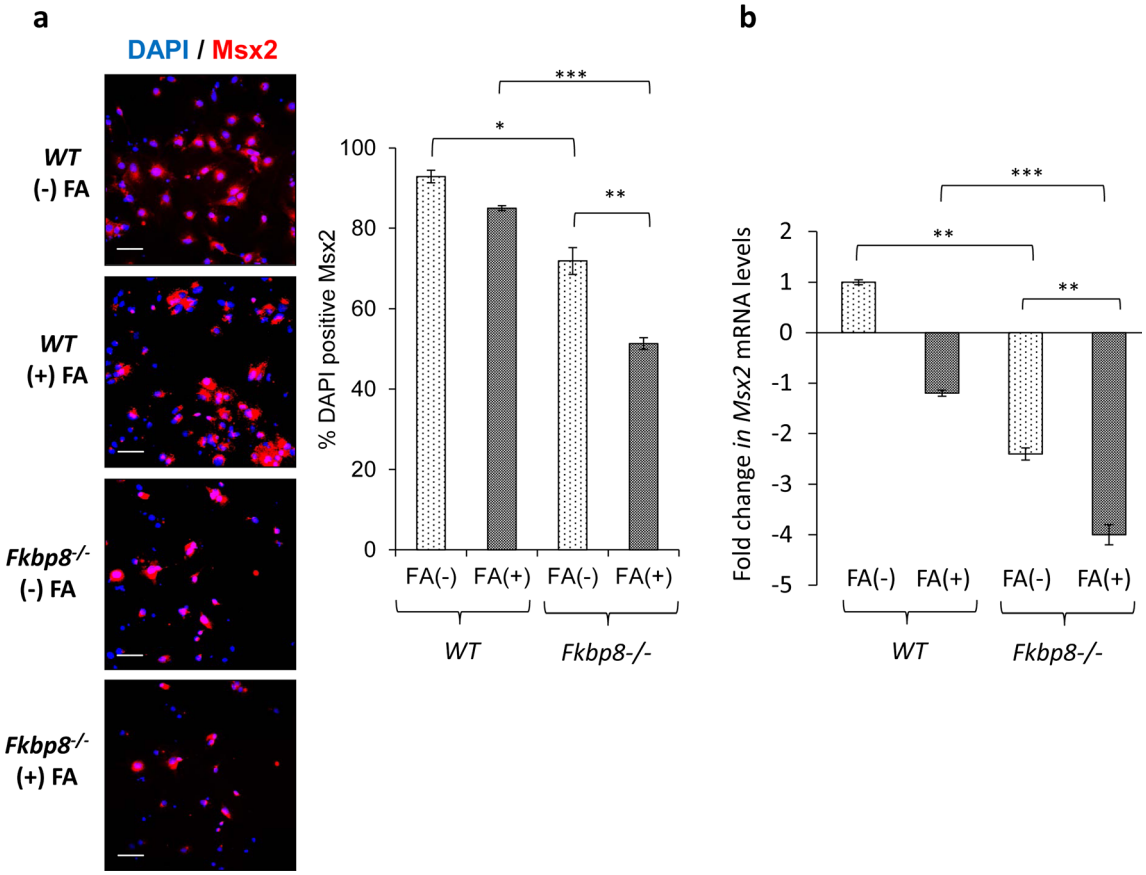


Fig. 2 Effect of FA on Msx2 expression in *Fkbp8*$^{-/-}$ embryo lumbar neural tube-derived NSCs. **a** NSCs from *WT* and *Fkbp8*$^{-/-}$ embryos' lumbar neural tubes differentiated in the presence or absence of FA were immunostained by Msx2 antibody and counterstained by DAPI. % DAPI positive Msx2 immuno-positive cells were counted. Student's *t* test was used for statistical analysis; data is mean±SEM: *$p<0.05$; **$p<0.001$; ***$p<0.0001$. **b**. qRT-PCR depicting fold changes in *Msx2* mRNA levels in NSCs obtained from *WT* and *Fkbp8*$^{-/-}$ embryo neural tubes differentiated in the presence or absence of FA. Each experiment was performed six times, with each data point in triplicate. Data is presented as fold changes in gene expression of non-FA-treated NS. *β-actin* was used as a control. Student's *t* test was used for statistical analysis; data is mean± SEM: *$p<0.05$; **$p<0.001$; ***$p<0.0001$

antagonist, is repressed by increased H3K27me3 [29]. We therefore hypothesized that in *Fkbp8*$^{-/-}$ embryos where we observe increased *noggin* expression, FA treatment would cause a decrease in H3K27me3 polycomb modification. To determine whether FA treatment decreases H3K27me3 modification in *Fkbp8*$^{-/-}$, differentiated NSCs derived from E9.5 *Fkbp8*$^{-/-}$ and *WT* embryos were immunostained for this histone mark in the presence and absence of FA. The data show that *Fkbp8*$^{-/-}$-derived NSCs had an overall lower (**$p<0.001$) H3K27me3 expression compared to *WT* (Fig. 4a). FA treatment further decreased H3K27me3 polycomb modification, both in *WT*-derived (**$p<0.001$) and *FKbp8*$^{-/-}$-derived (*$p<0.05$) neurospheres. UTX (KDM6A), a H3K27 demethylase, levels were also examined, with the hypothesis that its levels would be inversely related to H3K27me3 levels. UTX showed higher (*$p<0.05$) levels in *Fkbp8*$^{-/-}$ NS relative to *WT*. FA treatment increased UTX expression in *Fkbp8*$^{-/-}$ and *WT* NS even further (Fig. 4b), although the increase was not statistically significant. The results show that FA further decreases the levels of H3K27me3 histone modification and further increases the levels of UTX in *Fkbp8*$^{-/-}$ embryos.

Fkbp8$^{-/-}$ embryo-derived NSCs show premature differentiation—neurogenesis, oligodendrogenesis, and gliogenesis—and FA treatment does not exacerbate it

In FA-responsive *Splotch* (*Pax3*$^{-/-}$) embryos, we observed premature sensory neurogenesis [10]. Since Fkbp8 is a negative regulator of Shh [23], we hypothesized that *Fkbp8*$^{-/-}$ embryo-derived NCSs when differentiated would show premature motor neuron differentiation and increased expression of ventral motor neuron makers such as Olig2 (oligodendrocytes lineage transcription factor) and O4 (oligodendrocytes surface marker) and Nkx6.1 (ventral neural tube progenitor marker). To test this hypothesis, NSCs taken from E9.5 embryo lumbar neural tubes were subjected to differentiation (Neurobasal medium devoid of bFGF and EGF) in the absence and presence of FA and immunostained with Olig2 and

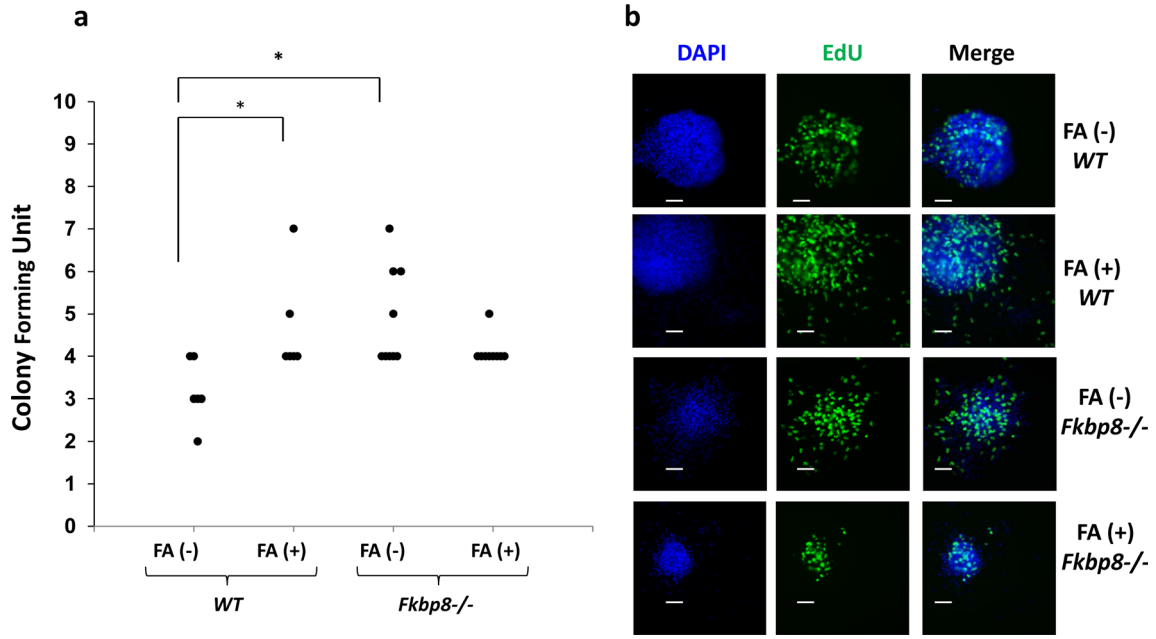


Fig. 3 Proliferation in NSCs derived from *Fkbp8*$^{-/-}$ embryos' lumbar neural tube is unchanged by FA treatment. **a** EdU incorporation and CFUs were higher in E9.5 *fkbp8*$^{-/-}$ embryo NS compared with those in *WT*. FA did not increase proliferation (CFU) of NS derived from *fkbp8*$^{-/-}$ embryos, whereas it did increase the proliferation of *WT* NS. Magnification, ×20. *Scale bars*: 50 μm for bright images and 50 μm for fluorescent images. Each experiment was performed six times with each data point in triplicate. Student's *t* test was used for statistical analysis; *$p<0.05$

Nkx6.1 antibodies. The results (Fig. 5a) show a modest increase ($p<0.05$) in the expression of ventral neural tube marker Olig2 in E9.5 *Fkbp8*$^{-/-}$ embryos, compared to that in *WT*. Nkx6.1 expression showed a slight insignificant increase in E9.5 *Fkbp8*$^{-/-}$ embryo-derived NSCs, compared to that in *WT*. FA treatment increased the expression of Olig2, but not Nkx6.1 both in *WT* and in *Fkbp8*$^{-/-}$ embryo-derived NSCs.

To confirm the results at the mRNA level, neurospheres from NT tissue from E9.5 *Fkbp8*$^{-/-}$ and *WT* embryos were subjected to quantitative RT-PCR (Fig. 5b). In the NSCs obtained from E9.5 *Fkbp8*$^{-/-}$ embryos, the ventral NT markers, *Olig2*, showed a 2-fold and *Nkx6.1* showed a 2.5-fold increase in mRNA levels relative to *WT* controls. FA treatment increased the expression of *Olig2*, but not *Nkx6*.1 both in *WT* and in *Fkbp8*$^{-/-}$ embryo-derived NSCs.

Since the immunostaining and the qRT-PCR data did not coincide exactly, we counted the percentage of class III beta-tubulin (TuJ1)-positive neurons that also show Olig2- and

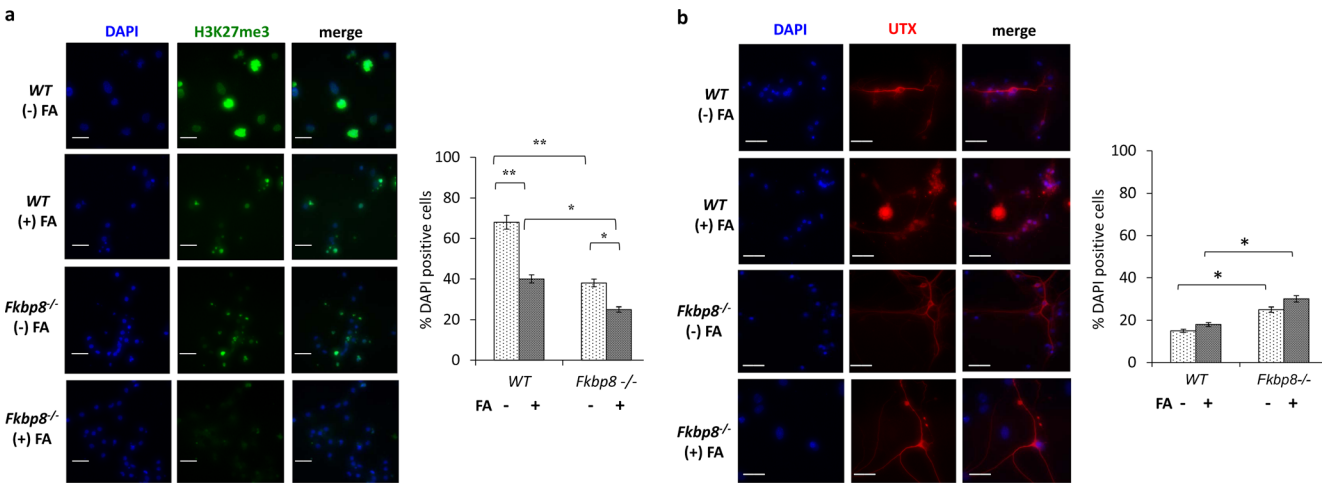


Fig. 4 FA treatment decreases the repressive H3K27me3 polycomb modification in *Fkbp8*$^{-/-}$ embryos. NSCs from E9.5 *WT* and *fkbp8*$^{-/-}$ lumbar neural tubes, treated or not treated with FA, were immunostained using H3K27me3 antibody (*green*) (**a**) or UTX antibody (*red*) (**b**) and counterstained with DAPI. Each experiment was performed three times, with each data point in triplicate. Student's *t* test was used for statistical analysis. *$p<0.05$; **$p<0.001$. Data is mean±SEM. Magnification, ×20. *Scale bars*, 50 μm

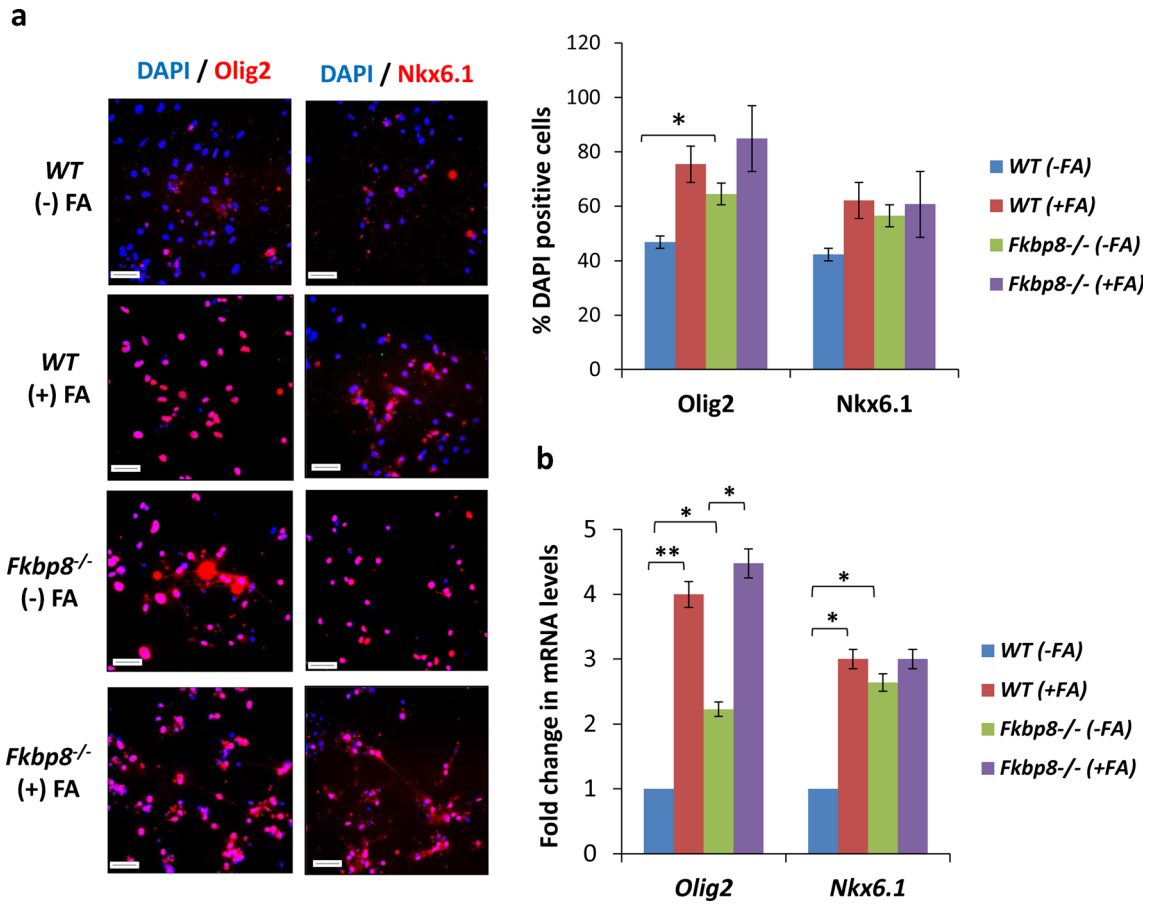


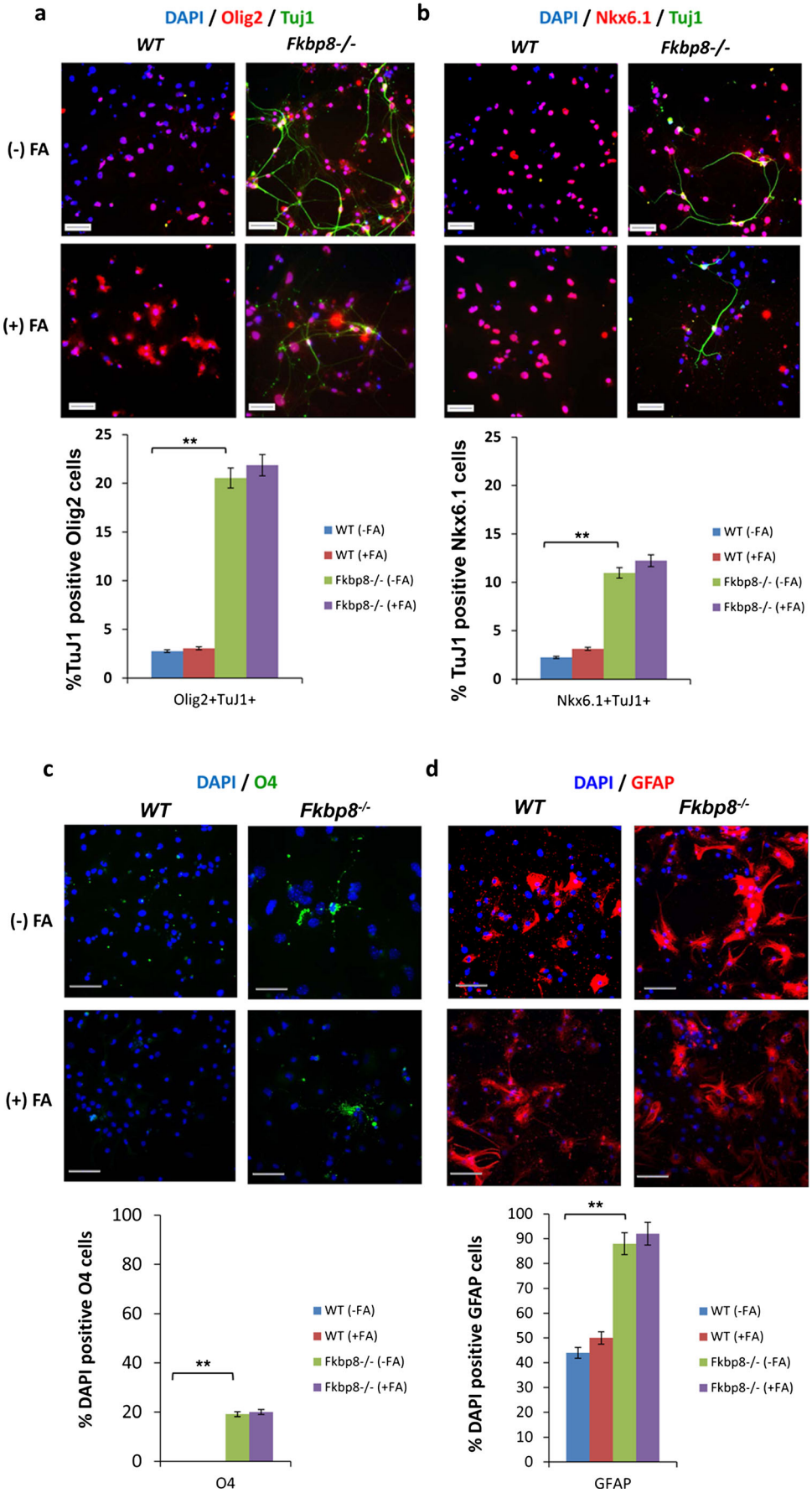
Fig. 5 Effect of FA on Olig2 and Nkx6.1 expression in *Fkbp8*$^{-/-}$ embryo lumbar neural tube-derived NSCs. **a** NSCs from *WT* and *Fkbp8*$^{-/-}$ embryos' lumbar neural tubes differentiated in the presence or absence of FA were immunostained by Olig2 and Nkx6.1 antibody and counterstained by DAPI. % DAPI positive Olig2 and Nkx6.1 immuno-positive cells were counted. Student's *t* test was used for statistical analysis; data is mean±SEM; *$p<0.05$. **b** qRT-PCR depicting fold changes in *Olig2* and *Nkx6.1* mRNA levels in NSCs obtained from *WT* and *Fkbp8*$^{-/-}$ embryo neural tubes differentiated in the presence or absence of FA. Each experiment was performed six times, with each data point in triplicate. Data is presented as fold changes in gene expression of non-FA-treated NS. *β-actin* was used as a control. Student's *t* test was used for statistical analysis; data is mean±SEM; *$p<0.05$; **$p<0.001$

Nkx6.1-positive immunostaining. It is worth noting that although DAPI+Olig2+ and DAPI+Nkx6.1+ (in Fig. 5a) showed almost similar immunostaining patterns, the percentage of TuJ1+Olig2+ and TuJ1+Nkx6.1+ cells were more in *Fkbp8*$^{-/-}$-derived differentiated neurospheres (Fig. 6a). The Olig2 immunostaining was significantly higher (**$p<0.001$) in *Fkbp8*$^{-/-}$ as compared to that in the *WT* controls. Similarly, Nkx6.1 immunostaining was higher (*$p<0.005$) in *Fkbp8*$^{-/-}$ embryo NSCs than that in age-matched *WT* controls (Fig. 6b). This data clearly suggest that *Fkbp8*$^{-/-}$ embryos may show premature motor neurogenesis. FA treatment had no effect on the expression of Olig2+TuJ1+ and Nkx6.1+TuJ1+ expression, in *WT* as well as *Fkbp8*$^{-/-}$-derived differentiated NSCs.

To determine if premature differentiation—oligodendrogenesis and gliogenesis—are also associated with ventral motor neurogenesis, the differentiated NSCs from E9.5 *Fkbp8*$^{-/-}$ embryos were immunostained with another oligodendrocyte surface marker, O4 (Fig. 6c), and a glial cell marker, glial fibrillary acidic protein (GFAP) (Fig. 6d). Levels of these differentiation markers were significantly higher (**$p<0.001$) in cells from E9.5 *Fkbp8*$^{-/-}$ embryos relative to age-matched *WT* controls. Taken together, the E9.5 *Fkbp8*$^{-/-}$ embryos appear to exhibit premature neurogenesis (TuJ1+), oligodendrogenesis (O4+), and gliogenesis (GFAP+). FA treatment does not seem to have any effect on the level of premature neurogenesis in *Fkbp8*$^{-/-}$ embryos.

Discussion

Maternal FA supplementation has reduced the prevalence of NTDs in the human population. Nonetheless, significant morbidity remains due to those NTDs that are FA-nonresponsive [30]. Understanding the mechanisms associated with FA nonresponsiveness is critical to developing newer and more effective intervention strategies [5]. Such possibilities certainly exist, as recently demonstrated in mice with nucleotide precursors [13]. Mouse models for NTDs [31, 32] serve to


a
DAPI / Olig2 / Tuj1
WT
Fkbp8-/-
(-) FA
(+) FA
%TuJ1 positive Olig2 cells
Olig2+TuJ1+
b
DAPI / Nkx6.1 / Tuj1
WT
Fkbp8-/-
% TuJ1 positive Nkx6.1 cells
Nkx6.1+TuJ1+
WT (-FA)
WT (+FA)
Fkbp8-/- (-FA)
Fkbp8-/- (+FA)
c
DAPI / O4
WT
Fkbp8-/-
(-) FA
(+) FA
% DAPI positive O4 cells
O4
d
DAPI / GFAP
WT
Fkbp8-/-
% DAPI positive GFAP cells
GFAP

◀ **Fig. 6** Neurosphere cultures from the lumbar neural tube of E9.5 *Fkbp8*$^{-/-}$ embryos show premature differentiation. Differentiated *Fkbp8*$^{-/-}$ NSCs show premature neurogenesis as evident by **a** TuJ1+ Olig2+ and **b** TuJ1+Nkx6.1+ staining (**$p<0.05$). Differentiation in the presence or absence of FA shows no difference in premature neurogenesis. *Fkbp8*$^{-/-}$ NS showed premature oligodendrogenesis (**c**) as evident by O4 (a surface marker for oligodendrocytes) as compared with *WT* (**$p<0.001$). *Fkbp8*$^{-/-}$ NS showed premature gliogenesis (**d**) as compared to *WT* (**$p<0.001$). Each experiment was performed four times, and each data point was done in triplicate. Student's *t* test was used for statistical analysis; data is mean±SEM. Magnification, ×20. *Scale bars*, 50 μm

help elucidate some of these potential underlying mechanisms. Previously, our lab used a folate-responsive mouse model *Splotch*, to examine the mechanism of folate responsiveness [8–12]. As a counterpoint to this initial work, here we have used the folate-nonresponsive *Fkbp8* knockout mouse, to elucidate mechanisms of folate-nonresponsive NTD. This model was chosen because Fkbp8 is a negative regulator of Shh; therefore, *Fkbp8*$^{-/-}$ embryos have higher Shh levels [18–21]. Increased Shh levels and subsequent signaling is in turn associated with anencephaly [31–35], exencephaly, and spina bifida [22]. The present results show that (i) Noggin expression is upregulated in *Fkbp8*$^{-/-}$ embryo-derived NSCs, and FA exacerbates it; (ii) Msx2 expression is downregulated in *Fkbp8*$^{-/-}$ embryo-derived NSCs, and FA treatment downregulate its expression even further; (iii) FA treatment further decreases the H3K27me3 in differentiated NSCs derived from *Fkbp8*$^{-/-}$ embryos; and (iv) NSCs from *Fkbp8*$^{-/-}$ embryos exhibit premature differentiation —neurogenesis, oligodendrogenesis, and gliogenesis.

Fkbp8 is a negative regulator of Shh signaling. In the absence of *Fkbp8* expression, Shh downstream targets are expected to be upregulated. In animal studies, the absence of Shh-negative regulators such as Fkbp8 [18], rab23 [33], tectonic [34], and tulp3 [35], consistently induces spina bifida and exencephaly. Excess Shh may inhibit bending of posterior NT at the dorsolateral hinge point (DLHP) [36], or may affect bone formation in the thoracicolumbar spine [37–39] and dysgenesis of mesenchyme surrounding the NT [40–44], thus preventing proper NT closure. Constitutively, active Shh signaling in *Fkbp8*$^{-/-}$ embryos leads to expansion of ventral fates in the NT [20, 22]; this may be the reason for the abnormal shape and alignment of spine and regional dysgenesis of mesenchyme in *Fkbp8*$^{-/-}$ embryos. Since *Hes1*, a stem cell maintenance gene is a direct transcriptional target of Shh signaling [41], it suggests that Shh plays a role in stem cell maintenance, which may in part account for the characteristic morphology of the NT in *Fkbp8*$^{-/-}$ embryos, including the observed overgrowth of the dorsolateral NT at the lumbar level [14].

Shh signaling mediates cell specification through the simultaneous repression of class I homeodomain transcription factors (i.e., Pax6, Pax7, Irx3, and Dbx1/2) and the induction of class II homeodomain transcription factors (i.e., Nkx2.2, Nkx6.1) in ventral spinal cord progenitors [42–44]. Previous findings showed that in *Fkbp8*$^{-/-}$ embryos, Olig2 and Nkx6.1 expressions were increased [14] and subsequently generate oligodendrocytes [45].

Epigenetic modifications, such as changes in the levels of the repressive H3K27me3 mark, have been shown to be important for the animal posterior neural tube development [46]. In *Splotch* homozygous embryos with open neural tube defects, we observed an increased H3K27me3 levels. In *Fkbp8*$^{-/-}$ embryos, H3K27me3 levels are decreased relative to *WT*, while folate treatment further reduced H3K27me3. Decreased H3K27me3 has been linked to increased *noggin* expression [29]. This might play an important role in folate nonresponsiveness in *Fkbp8*$^{-/-}$ embryos.

Increased Shh may also play a role in folate nonresponsiveness. High levels of Shh may cause excessive stem cell proliferation; premature differentiation which includes neurogenesis, oligodendrogenesis, and gliogenesis; and NT ventralization, as there is a cross talk between the Shh signaling cascade with WNT, RTK, Notch, and BMP/TGF-β signaling cascades to regulate the balance of stem cells, progenitor cells, and terminally differentiated cells during embryogenesis [26]. Interestingly, *noggin* is a Shh-dependent gene [25], and hence increased Shh signaling may also increase noggin expression. FA treatment further increases *noggin* expression and exacerbates NT ventralization. Our data is supported by the observation that *Ptch1*$^{-/-}$ mutants with constitutive Shh pathway activity show premature differentiation of motor neurons at the expense of ventral neural progenitors [26]. This study provides an impetus for investigations into the mechanisms of folate nonresponsiveness to prevent human neural tube defects in individuals that do not respond to folate.

Conclusions

Folate enhances neural tube over-ventralization in *Fkbp8*$^{-/-}$ embryos, through overexpression of noggin, and downregulating BMP4 signaling and thereby increasing Msx2 expression. This in turn may cause over-ventralization of developing neural tube. Folate may exacerbate neural tube defects in some folate-nonresponsive individuals.

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Conflict of interest We declare no conflict of interests.

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