Abnormal Behaviors and Developmental Disorder of Hippocampus in Zinc Finger Protein 521 (ZFP521) Mutant Mice

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Abstract

Zinc finger protein 521 (ZFP521) regulates a number of cellular processes in a wide range of tissues, such as osteoblast formation and adiopose commitment and differentiation. In the field of neurobiology, it is reported to be an essential factor for transition of epiblast stem cells into neural progenitors in vitro. However, the role of ZFP521 in the brain in vivo still remains elusive. To elucidate the role of ZFP521 in the mouse brain, we generated mice lacking exon 4 of the ZFP521 gene. The birth ratio of our ZFP521<sup>+/+</sup> mice was consistent with Mendel’s laws. Although ZFP521<sup>−/−</sup> pups had no apparent defect in the body and were indistinguishable from ZFP521<sup>+/+</sup> and ZFP521<sup>+/−</sup> littermates at the time of birth, ZFP521<sup>−/−</sup> mice displayed significant weight reduction as they grew, and most of them died before 10 weeks of age. They displayed abnormal behavior, such as hyper-locomotion, lower anxiety and impaired learning, which correspond to the symptoms of schizophrenia. The border of the granular cell layer of the dentate gyrus in the hippocampus of the mice was indistinct and granular neurons were reduced in number. Furthermore, Sox1-positive neural progenitor cells in the dentate gyrus and cerebellum were significantly reduced in number. Taken together, these findings indicate that ZFP521 directly or indirectly affects the formation of the neuronal cell layers of the dentate gyrus in the hippocampus, and thus ZFP521<sup>−/−</sup> mice displayed schizophrenia-relevant symptoms. ZFP521<sup>−/−</sup> mice may be a useful research tool as an animal model of schizophrenia.

Introduction

ZFP521/Evi-3 protein in the mouse, also known as ZNF521/EHZF in humans, is a 1311 amino-acid-long nuclear factor that contains a putative nuclear localization signal, a nuclear remodeling and histone deacetylation complex (NuRD), and 30 zinc-finger motifs (ZF). The 6152 nucleotide-long mRNA comprises eight exons. Among them, exon 4 is the longest, consisting of 3353 nucleotides (approximately 85%), and encodes the motifs from ZF2 to ZF30 [1].

ZFP521 regulates the differentiation of several kinds of stem cells [2–4]. In CD34<sup>+</sup> human hematopoietic progenitor cells, it suppresses their erythroid differentiation by inhibiting the transcriptional activity of hematopoietic transcriptional factor GATA-1 [5]. In mesenchymal stem cells, it suppresses their adipogenic determination and differentiation by binding to early B cell factor 1 (Ebf1) and inhibiting the expression of zinc finger protein 423 (ZFP423) [6], while it induces their osteogenic differentiation by inhibiting Runx related protein 2 (Runx2) [7–9].

In the field of neurobiology, Kamiya, et al. reported that ZFP521 is an essential factor for transition of epiblast stem cells into neural progenitors in vitro. They demonstrated that forced expression of ZFP521 leads to the neural conversion of embryonic stem (ES) cells even in the presence of bone morphogenetic protein 4 (BMP4); conversely, deprivation of ZFP521 by short hairpin RNA (shRNA) tends to halt the cell at the epiblast stage. The first eight zinc-finger motifs, from ZF1 to ZF8, were found to be essential for the neuralizing activity of ZFP521, because it triggers the neural differentiation of ES cells by associating with transcriptional co-activator p300 through these motifs. Also, ZF9 to ZF30 were found to be essential for this activity, because deletion of the motifs resulted in loss of activity [10]. ZF26 to ZF30 were found to be essential for this activity, because deletion of the motifs resulted in loss of activity [10]. ZF26 to ZF30 were found to be essential for this activity, because deletion of the motifs resulted in loss of activity [10].

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To elucidate the role of ZFP521 in the mouse brain, we generated mice lacking exon 4 of the ZFP521 gene, and analyzed them in detail.

Materials and Methods

Generation of ZFP521 Mutant Mice

A genomic fragment containing exon 4 of mouse \( \text{ZFP521} \) from C57BL/6J mouse was used to construct the targeting vector. For positive selection, the fragment containing most of exon 4 was replaced with a loxP/PGK-Neo-pA/loxP cassette (Figure 1A). The targeting vector was electroporated into ES cells. The clones resistant to G418 were screened for homologous recombination by PCR and confirmed by Southern blot analysis. Chimeric mice were generated by injection of targeted ES cells into blastocysts, and they were mated with C57BL/6J mice to obtain heterozygous mutant mice. The mice were backcrossed onto C57BL/6J for more than 10 generations, and after that intercrossed to obtain homozygous ZFP521 mutant mice (Acc. No. CDB0952K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html).

Animals

All mice were housed in an SPF facility, with a twelve-hour light/dark cycle, and fed water and a pellet chow diet \textit{ad libitum}. The protocol for \( \text{ZFP521} \) mutant mice studies was approved by the Institutional Review Board of Ehime University Graduate School of Medicine (Permit Number:05-SO-38-16). All animal studies were carried out in accordance with the guidelines of the...
Table 1. Genotype analysis of 142 offspring derived from intercrossing of ZFP521+/− mice.

|       | +/+ | +/Δ | Δ/Δ |
|-------|-----|-----|-----|
| Male  | 18  | 35  | 18  |
| Female| 18  | 37  | 16  |
| Total | 36  | 72  | 34  |

From 15 ZFP521+/− breeding pairs, 142 pups were produced. Note that the birth ratio of each genotype is consistent with Mendel’s laws. doi:10.1371/journal.pone.0092848.t001

Ehime University School of Medicine Committee on Animals. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Humane endpoints were employed during all animal experiments. Any of these animals were monitored daily, weighed weekly and euthanized at the humane endpoint after dramatic weight loss indicative of severe illness that would progress to death.

Southern Blot Analysis
Genomic DNA was prepared from the tail using a DNeasy kit (QIAGEN, Tokyo, Japan), digested with EcoRI restriction enzyme, electrophoresed through a 1.0% agarose gel and transferred to nylon membrane Hybond-N+ (GE Healthcare, New York, USA). Then, a 0.7-kb DNA probe (probe A in Figure 1A) was made by PCR, purified with a Gel Extraction Kit (QIAGEN) and 32P-labeled with a Random Primer DNA Labeling Kit Ver.2 (TaKaRa, Otsu, Japan). Hybridization was carried out according to the standard method [13].

Northern Blot Analysis
Total RNA was extracted from mouse whole brain using Isogen II (Nippon-Gene, Tokyo, Japan) as described in the manufacturer’s protocol. RNAs (40 µg/lane) were dissolved in sample buffer (50% formamide, 2.2 M formaldehyde, 10 mM EDTA, 1x MOPS solution) and heated to 98°C for denaturing. Denatured samples were electrophoresed through 1% agarose-MOPS gel and transferred to nylon membrane (GE Healthcare). The blotted membrane was hybridized with a 32P-labeled probe corresponding to the DNA sequence of ZFP521 (probe B in Figure 1A) or β-actin [14,15].

Genotyping
Mice were genotyped by polymerase chain reaction (PCR) using genomic DNA extracted from the tail, using the primers ZFP521-a: 5’-CAGCAGCTGTAATGATATGTCCCTA-3’ and ZFP521-b: 5’-GGTGTTAGTCTTATATGGATCTTG-3’ for the wild-type allele, and ZFP521-a and ZFP521-c: 5’-AGAAAGCGAAGGAGCAAGCCTG-3’ for the targeted allele [16].

Body and Organ Weights
The living mice were weighed once a week. All data were derived from the healthy mice. If necessary, mice were euthanized at the predetermined humane endpoint. Five-week-old mice were anesthetized, perfused with phosphate-buffered saline (PBS) and dissected. Each organ was removed from the mice and weighed. Data were normalized by body weight.

Growth Plate Histological Examination and Safranin O Staining
Femurs from five-week-old mice were processed for paraffin embedding with decalcification as described by Correa et al., and cut into 10-µm sections [7]. The sections were deparaffinized, stained with safranin-O and analyzed under a BZ-9000 microscope (Keyence, Osaka, Japan). The thickness of the femoral growth plate was measured using BZ-H1C software (Keyence).
Behavioral Assays

An open field test was used to estimate locomotor activity [17,10]. All the mice were individually placed in a corner of an open field apparatus (L60 cm × W60 cm × H30 cm) just after the start of the dark cycle. The field was evenly illuminated at 20 lux. The open field was divided into a 30 cm × 30 cm central zone with a surrounding 15 cm wide border zone. The mouse was allowed to explore freely in the open field for one hour, and the movement of the central part of its body was monitored with a CCD camera connected to a computer. The distance of movement of the central point of its body was monitored with Ethovision XT software (Brain Science Idea, Osaka, Japan). This test was repeated every 24 hours for three days.

To estimate the level of anxiety and spatial learning, an elevated plus maze test was employed [19,20]. The maze consisted of two open arms (30 cm × 5 cm, no wall) and two closed arms (30 cm × 5 cm, surrounded by 15 cm high walls), which emerged from a central platform (5 cm × 5 cm) and was aligned perpendicularly. The apparatus was elevated 45 cm above the floor. All the mice were individually placed on the central area of the maze during the dark phase of the light/dark cycle, and allowed to move freely for 10 min. The movement of the mouse was monitored with a CCD camera connected to a computer. Time spent in the open arms, closed arms, and the central area were scored with Ethovision XT software.

Behavioral assays were evaluated as described previously [21,22]. Briefly, tests were initiated by placing an animal onto a round platform (an inverted 20 cm high ×13.5 cm diameter glass cylinder). The time from an initial placement on the platform to falling down was recorded. If the animal remained to be on the platform after 7 min test, the jumping time was estimated as 7 min. Cumulative jumping events (%) were calculated according to the formula: (the number of falling animals/the number of total testing animal) ×100.

The cliff-avoidance test and jumping events were evaluated as described previously [17,18]. All the mice were individually placed into a 20 cm high ×13.5 cm diameter glass cylinder filled to 12 cm of depth with water (23°C). Immobility floating time was measured for 3 min.

The prepulse inhibition (PPI) test was carried out as described previously [24,25]. Each mouse was placed individually in a Plexiglas tube mounted in a sound-attenuated chamber (Panlab, Barcelona, Spain). The startle responses of the mice were detected by a piezoelectric accelerometer. A computer was used to control the timing and presentation of acoustic stimuli and record the corresponding startle responses. The animals were placed into the chamber and allowed to habituate for 10 min under a presence of 65 dB white noise background. After habituation, the animals received 60 different trials (10 startle trials, 10 no-stimulus trials and 40 PPI trials) pseudo-randomly. The startle trial consisted of a single 120 dB white noise burst lasting 40 msec. The PPI trials consisted of a prepulse (20 msec burst of white noise with intensities of 69, 73, 77 or 81 dB) 100 msec later, followed by the startle stimulus (120 dB, 40 msec white noise). Each of prepulse trials was repeated 10 times. As to the no-stimulus trial, no stimulus was presented but the movement of the animal was scored. The result of the animal moving was measured during 100 msec after startle stimulus onset. PPI score for each prepulse level is expressed as % prepulse inhibition, calculated according to the formula: (1-(mean startle amplitude in prepulse trial/mean startle amplitude in control trials)) ×100.

Brain Histological Examination and Hematoxylin & Eosin (HE) Staining

Anesthetized five-week-old mice were perfused with PBS followed by 4% paraformaldehyde. The whole brain was removed from the skull and fixed for 16 h in 4% paraformaldehyde at 4°C and then washed in PBS, dehydrated, and embedded in paraffin for sectioning. Sections (10 μm thick) were stained with HE to examine the histological appearance of the brain under light microscopy [20]. To quantify the total cell number, hematoxylin positive cells were counted in 0.01-mm² area of each brain section.

Thionin Staining

For frozen sections, fixed brains were incubated in PBS containing 10% sucrose (pH 7.4). The buffer was changed three times over the next 24 hours. Then 8-μm sections of frozen brain were cut and mounted directly on AP-C-coated slides (Matsumani, Kishiwada, Japan). After drying, the brain sections were stained with 0.25% thionin solution (0.25% thionin, 0.55 μM lithium carbonate) [26].

Table 2. Organ weights of 5-week-old ZFP521Δ/Δ mice.

|                  | Δ/Δ          | Mean ± S.D.  |
|------------------|--------------|--------------|
|                  | Weight (mg)  | %            |
| Body             | 11400±6700   | 19900±1270   |
| Brain            | 237±55.3     | 356±83.5     |
| Thymus           | 260±7.98     | 54.5±12.1    |
| Lung             | 96.6±6.33    | 149±12.9     |
| Heart            | 91.7±10.6    | 123±7.60     |
| Liver            | 722±111      | 1040±73.7    |
| Kidney           | 117±5.72     | 161±9.35     |
| Spleen           | 371±23.6     | 630±7.44     |
| Adrenal gland    | 2.05±0.165   | 3.50±0.458   |
| Pituitary gland  | 1.17±0.176   | 2.13±0.0882  |

Values are presented as mean ± S.D. n = 6.

Note that all organs of ZFP521Δ/Δ mice were significantly lighter than those of ZFP521+/+ mice. doi:10.1371/journal.pone.0092848.t002
Figure 3. Behavioral analysis. Five-week-old ZFP521Δ/Δ mice and ZFP521+/+ mice were used for all the analyses. (A–D) Open field test. (A) Representative locomotor tracks of ZFP521Δ/Δ mouse (right panel) and ZFP521+/+ mouse (left panel) during 60 minutes. Green dashed box, central area of open field. (B) Total distance of ambulation of ZFP521Δ/Δ mice (right bar) and ZFP521+/+ (left bar) mice during 60 min. Error bars indicate SD. n = 6. *, P < 0.01, compared to ZFP521+/+ mice. (C) Time spent in central area. ZFP521Δ/Δ mice spent more time in the central area of the open field than did ZFP521+/+ mice during 60 minutes. Error bars indicate SD. n = 6. *, P < 0.01, compared to ZFP521+/+ mice. (D) Open field test was repeated three times every 24 hours.▲, ZFP521Δ/Δ; ■, ZFP521+/+. Data represent mean ± SD. n = 6. *, P < 0.01, compared to ZFP521+/+ mice. Note that the total distance of ambulation in the second test was significantly shorter than that in the first test for ZFP521Δ/Δ mice. (E–F) Elevated plus maze test. (E) Representative locomotor tracks of ZFP521+/+ mouse (left panel) and ZFP521Δ/Δ mouse (right panel) during 10 minutes. C, closed arm; O, open arm. (F) Duration time spent in closed arms, open arms, and central area. Error bars indicate SD. n = 6. *, P < 0.05, compared to ZFP521+/+ mice. Note that ZFP521Δ/Δ mice spent more time in the open arms than did ZFP521+/+, and less time in the central area. (G) Cliff-avoidance test. The cumulative frequency of jumping was determined. n = 5. *, P < 0.01, compared to ZFP521+/+ mice. Note that the cliff avoidance of ZFP521Δ/Δ mice was impaired. (H) Forced swim test. Immobility time was measured during the forced swim for 3 min. Data represent mean ± SD. n = 5 *, P < 0.05, compared to
The density of each band was quantified by densitometric analysis as described previously [16]. The anti-ZFP521 antibody was raised against a polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting experiments were performed as described previously [16]. Neuronal and GFAP-positive cells were counted in a 0.01-mm² area of each brain section.

### Western Blotting and Densitometric Quantification

The brains of five-week-old mice were quickly removed, dissected and frozen in liquid nitrogen. Before the analyses, each whole brain was homogenized at 4°C in lysis buffer (0.1 M MES, pH 6.8, 0.5 mM MgSO4, 1 mM EGTA, 2 mM dithiothreitol, 0.75 M NaCl, 2 mM PMSF, 20 mM NaF, 0.5 mM sodium orthovanadate, 1 mM benzamide, 25 mM β-glyceroophosphate, 10 mM p-nitrophenylphosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μM okadaic acid). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed as described previously [16]. The anti-GFP, anti-neuron-specific enolase (NSE), and anti-β-actin antibodies were used in this study. The density of each band was quantified using ImageJ software, and normalized by β-actin.

### Statistical Analysis

Data are presented as mean ± S.D. Statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA). Differences were determined by one-way analysis of variance (ANOVA) in the sniff-avoidance test and by the Log-rank test in the Kaplan-Meier survival curve. Means of variables in the other tests were compared by Student’s t test.

### Results

#### Generation and Confirmation of ZFP521 Mutant Mice

The mouse ZFP521 gene was disrupted in ES cells using a targeting vector as shown in Fig. 1A. In this targeting vector, a 3.3-kb fragment containing most of exon 4 in the ZFP521 genomic DNA was replaced with neomycin resistance gene (neo). Simultaneously, an in-frame stop codon was newly inserted downstream of the remainder of exon 4. This deletion caused a loss of ZF2 to ZF30 motifs from ZFP521 protein, among which the ZF9 to ZF30 motifs are essential for ZFP521's neuroprotective activity [10].

From the G418-resistant ES cell clones, correctly targeted clones were screened by PCR and confirmed by Southern blot analysis with the probe shown in Fig. 1A. Chimeric mice were generated by injection of targeted ES cells into blastocysts, and they were mated with C57BL/6j mice to obtain heterozygous mice. The ZFP521 allele was disrupted in ES cells using a targeting vector as shown in Fig. 1A. In this targeting vector, a 3.3-kb fragment containing most of exon 4 in the ZFP521 genomic DNA was replaced with neomycin resistance gene (neo). Simultaneously, an in-frame stop codon was newly inserted downstream of the remainder of exon 4. This deletion caused a loss of ZF2 to ZF30 motifs from ZFP521 protein, among which the ZF9 to ZF30 motifs are essential for ZFP521's neuroprotective activity [10].

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Figure 4. Histopathological analysis of the brain. (A) Photographs of the whole brains of two-week-old (a) and twenty-week-old (b) mice. The brain of ZFP521+/Δ mice (right brain) was smaller than that of ZFP521+/Δ littermate (left brain). (B) HE-stained sections of cerebellum and cerebral cortex of five-week-old mice. Bars = 100 μm. a and b, cerebellum; c and d, cerebral cortex; a and c, ZFP521+/+ mice, b and d, ZFP521+/Δ mice. Note that the cerebellum and cerebral cortex of ZFP521+/Δ mice appeared histologically normal. (C) Quantification of the number of cells in cerebellum and cerebral cortex of five-week-old mice. We counted hematoxylin positive cells, and statistically analyzed. Data represent mean ± SD. n = 5. Note that there was no significant difference in the number of cells between ZFP521+/+ mice and ZFP521+/Δ mice. (D) Cortical cell layers in five-week-old mice. Sagittal sections of cerebral cortex from ZFP521+/+ mouse (a) and ZFP521+/Δ mouse (b) were stained with thionin. Bars = 100 μm. Note that the formation of six neural cell layers in the cerebral cortex of ZFP521+/Δ mice appeared normal. (E) Immunohistochemical detection of neurons in five-week-old mice. Cerebral cortex (a and b) and cerebellum (c and d) of ZFP521+/+ mouse (a and c) and ZFP521+/Δ mouse (b and d) were immunohistochemically stained with anti-NeuN antibody. Bars = 100 μm. Note that no significant abnormality, including the number, size and positioning of differentiated neurons, was found. (F) Immunohistochemical detection of astroglial cells in five-week-old mice. Hippocampus (a and b)
and cerebellum (c and d) of ZFP521<sup>+/−</sup> mouse (a and c) and ZFP521<sup>Δ/Δ</sup> mouse (b and d) were immunohistochemically stained with anti-GFAP antibody. Bars = 100 μm. Note that no abnormality of astroglial cells was found. (G) Quantification of the number of anti-NeuN and anti-GFAP positive cells in cerebellum, dentate gyrus and cerebral cortex of five-week-old mice. Data represent mean ± SD. n = 6. Note that there was no significant difference in the number of cells between ZFP521<sup>+/−</sup> mice and ZFP521<sup>Δ/Δ</sup> mice. (H) Immunoblot detection. Immunoblots of lysates from the cerebellum of five-week-old ZFP521<sup>+/−</sup> and ZFP521<sup>Δ/Δ</sup> were probed with anti-GFAP (upper panel) and anti-NeuN antibody (middle panel). Anti-β-actin antibody (bottom panel) was used as control for protein loading. The experiment was repeated at least three times, and all results were similar. A representative result is shown. (I) Densitometric quantification of GFAP protein in the cerebellum of five-week-old mice. The density of the GFAP band shown above was quantified using Image-J software, and normalized by β-actin. The vertical axis indicates the quantified density of GFAP protein relative to that in ZFP521<sup>+/−</sup> mice. Error bar indicates SD. n = 6. Note that there was no significant difference between ZFP521<sup>+/−</sup> mice and ZFP521<sup>Δ/Δ</sup> mice.

### Histopathological Analysis of Brain

The whole brains of two-week-old (a) and twenty-week-old (b) ZFP521<sup>Δ/Δ</sup> mice were smaller than those of ZFP521<sup>+/−</sup> mice (Fig. 4A). The shapes of the cerebellum and cerebral cortex of ZFP521<sup>Δ/Δ</sup> mice were morphologically normal, and HE-stained sections of both brain regions also showed no abnormality (Fig. 4B). The numbers of hematoxylin-positive cells in the granular layer of the cerebellum and layer II of the cerebral cortex were counted (Fig. 4C). There was no significant difference in cell density between ZFP521<sup>+/−</sup> and ZFP521<sup>Δ/Δ</sup> mice, in both parts of the cerebral cortex. In addition to HE-staining, we also employed thionin-staining to clarify the neuronal cell layer, in order to confirm that the six neuronal cell layers in the cerebral cortex of ZFP521<sup>Δ/Δ</sup> mice had formed normally (Fig. 4D).

Next, we immunohistochemically stained the cerebral cortex and cerebellum of ZFP521<sup>Δ/Δ</sup> mice with an antibody against NeuN, which is a specific marker of differentiated neurons [28,29], but no significant abnormality in the number, size or positioning of differentiated neurons was found (Fig. 4E and 4G). Nor could we find any abnormality of astroglial cells in the hippocampus (Fig. 4F, a and b) and cerebellum (Figure 4F, c and d) of ZFP521<sup>Δ/Δ</sup> mice, using an antibody against GFAP, which is a specific marker of astrocytes [30,31]. In addition to immunohistochemical studies, immunoblot analyses with anti-GFAP and anti-NeuN antibodies were performed to quantify the levels of proteins in the cerebellum of five-week-old mice (Fig. 4H-J). This revealed no significant differences in the expression levels of both GFAP and NeuN between ZFP521<sup>+/−</sup> and ZFP521<sup>Δ/Δ</sup> mice.

The dentate gyrus in the hippocampus of five-week-old ZFP521<sup>Δ/Δ</sup> mice was also morphologically normal, but granular neurons were clearly reduced in number and the border of the granular cell layer was indistinguishable compared with that in control ZFP521<sup>+/−</sup> mice (Fig. 5A and 5D). In the dentate gyrus of five-day-old ZFP521<sup>Δ/Δ</sup> mice, the number of granular neurons was smaller than that in ZFP521<sup>+/−</sup> mice (Fig. 5B and 5D). We further examined the brain of E19.5 mice (Fig. 5C). At this stage, the hippocampus of the ZFP521<sup>Δ/Δ</sup> brain was poorly organized, whereas it was well organized in ZFP521<sup>+/−</sup> mice. These results suggest that formation of the neuronal cell layers in the hippocampus and dentate gyrus of E19.5 ZFP521<sup>Δ/Δ</sup> embryos was affected, and that the neural cells may still have been in the process of migrating to the appropriate region.

Taken together, the results of histopathological analysis suggest that ZFP521 is concerned with formation of the neuronal cell layers of the dentate gyrus in the hippocampus.

### Detection of Undifferentiated Neural Precursor Cells in ZFP521<sup>Δ/Δ</sup> Brain

ZFP521 is reported to be essential for driving the intrinsic neural differentiation of mouse ES cells in vitro [10]. If so, the behavioral abnormalities of ZFP521<sup>Δ/Δ</sup> mice might be caused not
only by the morphological abnormality of the dentate gyrus and hippocampus, but also by suppression of transition of ES cells to neuronal cells.

We examined the presence of neural progenitor cells in the dentate gyrus and cerebellum of five-week-old ZFP521A/A mice, using a Sox1-specific antibody. This revealed fewer Sox1-positive cells in the dentate gyrus and cerebellum of ZFP521A/A mice than those in ZFP521+/+ mice (Fig. 6A). Similarly, immunoblotting with the antibody demonstrated that Sox1 expression was significantly decreased in the cerebellum of five-week-old ZFP521A/A mice (Fig. 6B and 6C). These results suggest that ZFP521 is involved in the differentiation of ES cells to neural progenitor cells, also in vivo.

**Discussion**

We generated mice lacking exon 4 of the ZFP521 gene, and having an in-frame stop codon inserted downstream of the remainder of exon 4. As a result, ZF2-30 were deleted from ZFP521 protein in ZFP521A/A mice.

Seriwatanachai, et al. originally generated chondrocyte-specific ZFP521-deficient mice, and using them, they reported that ZFP521 is required downstream of PTHR1 signaling to act on chondrocyte proliferation and differentiation [7,32,33]. In our ZFP521A/A mice, the thickness of the femoral growth plate was decreased by 54%, compared with that in ZFP521+/+ (Fig. 7). Our result is in agreement with that of Seriwatanachai, et al. and confirms that our mutant mice were successfully generated.

Kamiya, et al. reported that ZFP521 is essential and sufficient for driving the intrinsic neural differentiation of mouse ES cells. Using cultured ES cells and shRNA-mediated gene knockdown techniques, they clearly demonstrated that ZFP521-depleted ES cells do not undergo neural conversion but tend to halt at the epiblast stage in vitro. They also demonstrated that deletion of ZF9-30 from ZFP521 protein resulted in loss of its neuralizing activity [10]. Based on these findings, we expected that ZFP521A/A mice would be embryonically lethal. However, contrary to our expectation, the mice were viable, and their birth ratio was consistent with Mendel’s laws. This might have been due to the difference in the experimental system employed; Kamiya, et al. performed their experiments in vitro, whereas ours were done in vivo. The existence and induction of a compensatory gene for ZFP521 in vivo is suspected. On the other hand, our results are partly consistent with those of Kamiya, et al. in that the number of Sox1-positive neural progenitor cells was lower in the brain of our ZFP521A/A mice.

Kang, et al. have also generated and analyzed ZFP521-deficient mice, and reported recently that Zfp521 acts as a key regulator of adipose commitment and differentiation in vitro and in vivo [6]. As their homozygous ZFP521-deficient mice died shortly after birth, they used E18.5 embryos for their analysis. On the other hand, our ZFP521A/A mice lived longer, but most of them died before 10 weeks after birth. It seems unlikely that an eating disorder would have been the cause, because the tendency for growth retardation remained constant during and after weaning. The cause of death in our ZFP521A/A mice remains unknown, and is a matter for further investigation.

The results of the open field test suggested that ZFP521A/A mice have a hyper-locomotive phenotype (Fig. 3A and 3B). Hyper-locomotion is a classical feature of rodent models of schizophrenia and corresponds to the psychomotor agitation evident in schizophrenic patients [34,35]. The results of the open field test (Fig. 3C), elevated plus maze test (Fig. 3E and 3F), cliff-avoidance test (Fig. 3G) and forced swim test (Fig. 3H) suggested that ZFP521A/A mice have a significantly lower anxiety level and a higher impulsivity level. The prepulse inhibition of startle response

### Figure 5. HE-staining of dentate gyrus at each developmental stage. (A) HE-staining of dentate gyrus in five-week-old mice. Bars = 50 μm. a, ZFP521+/+ mouse; b, ZFP521A/A mouse. Note that the density of neuronal cells was obviously lower and the neuronal cell layer was more obscure in ZFP521A/A mouse. (B) HE-staining of dentate gyrus in five-day-old mice. Bars = 100 μm. a and b, ZFP521+/+ mouse; c and d, ZFP521A/A mouse. Note that the number of neuronal cells appear smaller in five-day-old ZFP521A/A mouse than ZFP521+/+ mouse. (C) HE-staining of dentate gyrus and hippocampus in E19.5 embryos. Bars = 100 μm. a, ZFP521+/+ embryo; b, ZFP521A/A embryo. Note that the dentate gyrus and hippocampus of the ZFP521A/A embryo were poorly organized, and the number of cells in the neuronal cell layer was smaller in the ZFP521A/A embryo than in the ZFP521+/+ embryo. (D) Quantification of the number of cells in dentate gyrus. Hematoxylin positive cells were counted. Error bar indicates SD. n = 6, **, P<0.05, compared to ZFP521+/+ mice. Note that the number of cells in dentate gyrus of both five-week-old (Sw.o) and five-day-old (P5) mice were significantly decreased compared with ZFP521+/+ littermates. Error bar indicates SD. n = 6.

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One of the major tests for schizophrenic behavior. Deficits in prepulse inhibition are observed in patients suffering from schizophrenia, and other psychiatric disorders [36,37] and can be induced in animals by administration of various schizophrenomimetics [38,39,40]. Our ZFP521/D mice displayed significantly reduced inhibition at 69 dB and 73 dB prepulse (Fig. 3I). These results strongly suggest that ZFP521/D mice have schizophrenia-relevant symptoms.

The hippocampus is likely to be an impacted region in schizophrenic patients [41,42]. In patients with schizophrenia, as...
well as model mice, histopathological alteration in the dentate gyrus of the hippocampus has been reported. There, neural stem cell proliferation and adult hippocampal neurogenesis in the dentate gyrus were decreased [43,44]. The results of histopathological studies of our \textit{ZFP521} mutant mice were consistent with these reports. In our \textit{ZFP521}\textsubscript{D/D} mice, Sox1-positive neural stem cells in the dentate gyrus were significantly reduced in number (Fig. 6A). Furthermore, in the dentate gyrus of five-day-old and five-week-old \textit{ZFP521}\textsubscript{D/D} mice, the border of the granular cell layer was indistinct and granular neurons were clearly reduced in number compared with those in control \textit{ZFP521}\textsubscript{+/+} mice (Fig. 5A, 5B and 5D). At the E19.5 stage, the hippocampus of \textit{ZFP521}\textsubscript{D/D} embryos was poorly organized, whereas it was well organized in \textit{ZFP521}\textsubscript{+/+} embryos (Fig. 5C). Therefore, the abnormal behavior of \textit{ZFP521}\textsubscript{D/D} mice may be due to such histopathological abnormalities in the dentate gyrus.

Many neurons of the adult brain are generated prenatally, but in the hippocampus, cerebellum, and olfactory bulb, neurons are also formed in postnatal life. This occurs through the proliferation and differentiation of adult neural stem cells, which exist at two locations under normal conditions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Newborn neurons in the SGZ migrate a short distance into the granular cell layer of the dentate gyrus, and integrate into the existing neural circuitry [45]. Lack of \textit{ZFP521} does not prevent, but may attenuate, the transition of

![Figure 6. Histological analysis of the femoral growth plate.](image)

(A) Femoral growth plate from five-week-old mice, stained with safranin O solution. The growth plate layer is strongly stained. Bars = 100 \(\mu\)m (a and b) and 50 \(\mu\)m (c and d), a and c, \textit{ZFP521}\textsubscript{+/+} mouse; b and d, \textit{ZFP521}\textsubscript{D/D} mouse. Note that the growth plate of \textit{ZFP521}\textsubscript{D/D} femur appears thinner than that of \textit{ZFP521}\textsubscript{+/+}. (B) Histomorphometric analysis of thickness of femoral growth plate. Error bars indicate SD. n = 22. * , \(P<0.001\). Note that the thickness of the growth plate of \textit{ZFP521}\textsubscript{D/D} femur is decreased by 54%, compared with that of \textit{ZFP521}\textsubscript{+/+} femur.

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epiblast stem cells into Sox1-positive neural stem cells, or otherwise suppress the proliferation of slow-dividing stem cells within the SGZ, which results in a reduced number of granular neurons and an indistinct border of the granular cell layer of the dentate gyrus.

Mice lacking exons 2 and 3 of the disrupted-in-schizophrenia (DISC1) gene displayed schizophrenia-relevant behaviors, similarly to our ZFP521 mutant mice [46]. DISC1 is a promising candidate gene for susceptibility to schizophrenia, and is involved in newborn neuron migration in the SGZ [47,48]. Although the candidate gene for susceptibility to schizophrenia, and is involved in hippocampal formation and beyond. Stem Cell Res Ther 2: 20.

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Author Contributions

Conceived and designed the experiments: NO EM RA JY. Performed the experiments: NO EM RA JY. Analyzed the data: NO EM RA JY. Contributed reagents/materials/analysis tools: TA HK. Wrote the paper: NO MA NY.
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