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Original Article

# CRISPR/Cas9 based gene editing of Frizzled class receptor 6 (*FZD6*) reveals its role in depressive symptoms through disrupting Wnt/β-catenin signaling pathway

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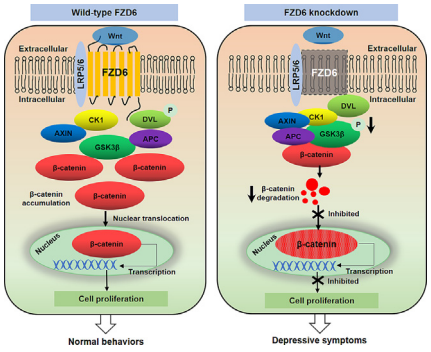
## HIGHLIGHTS

- FZD6 expression was significantly reduced in MDD patients.
- *Fzd6* knockdown mice presented significant changes in depressive-like behaviors.
- The cell proliferation was decreased in the hippocampus of *Fzd6* knockdown mice.
- By applying both *in vitro* and *in vivo* models, we demonstrated that the significant role of FZD6 in depression is mainly mediated by Wnt/β-catenin signaling pathway.

## GRAPHICAL ABSTRACT

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## ABSTRACT

*Introduction:* As one of the common psychiatric diseases, depression poses serious threats to human health. Although many genes have been nominated for depression, few of them were investigated in details at the molecular level.
*Objectives:* To demonstrate Frizzled class receptor 6 (FZD6) functions in depression through disrupting Wnt/β-catenin signal pathway.
*Methods:* The *FZD6* edited cell line and mouse model were generated by using CRISPR/Cas9 technique. The expression of key genes and proteins in Wnt/β-catenin pathway was determined by qRT-PCR and Western blotting, respectively. Animal behavioral tests, including open field test (OFT), elevated plus maze test (EPM), forced swimming test (FST), tail suspension test (TST), and sucrose preference test (SPT), were employed to determine anxiety- and depressive-like behaviors. Immunofluorescent staining was used to assess cell proliferation in the hippocampus of mouse brain.
*Results:* Among patients with depression, FZD6, one of the receptors of Wnt ligand, was significantly decreased. In CRISPR/Cas9-based *FZD6* knockdown cells, we showed that *FZD6* plays a significant role in regulating expression of genes involved in Wnt/β-catenin pathway. Subsequently behavioral studies on *Fzd6* knockdown mice (with a 5-nucleotide deletion; *Fzd6*-Δ5) revealed significant changes in

*Abbreviations:* MDD, Major depressive disorder; FZD6, Frizzled class receptor 6; OFT, Open field test; FST, Forced swimming test; SPT, Sucrose preference test; TST, Tail suspension test; KD, Knockdown; EPM, Elevated plus maze; PCNA, Proliferating Cell Nuclear Antigen.

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depressive symptoms, including increased immobility duration in FST, less preference of sucrose in SPT, reduction of distance traveled in OFT, and decreased time spent in open arms in EPM. Immunofluorescent staining showed decreased cell proliferation in the hippocampus of *Fzd6*-Δ5 mice with reduced number of $Ki67^+$ and $PCNA^+$ cells. Moreover, decreased *Gsk3β* mRNA expression, phosphorylated GSK3β, and cytoplasmic β-catenin in the hippocampus of *Fzd6*-Δ5 mice provided further evidence supporting the role of Fzd6 in depression.

*Conclusion:* Together, above findings proved the significant role of FZD6 in depression through its effect on hippocampal cell proliferation and its ability to regulate canonical Wnt/β-catenin pathway.



## Introduction

>300 million person suffer from major depressive disorder (MDD) worldwide, which is one of the most commonly diagnosed psychiatric disorders [1]. It contributes significantly to the global burden of diseases and is still increasing annually [2,3]. By 2030, the World Health Organization predicted that MDD will rank as the first of global burden of disease (GBD) [4]. Considering depression is a complex disease, numerous studies have been conducted so far with the goal of discovering susceptibility genes for the disease and their underlying mechanisms.

It has been recently reported that G protein-coupled receptors (GPCRs) are key regulators for depressive-like behaviors [5]. Frizzled (FZD) proteins belong to GPCRs, which are seven-pass transmembrane cell surface receptors for secreted Wnt proteins, and they are encoded by ten *FZD* genes in vertebrates [6,7]. Both Fzd4 and Fzd6 expressed more abundantly in the adult mouse primary brain endothelial cells than any of other eight Fzd genes [8], indicating their essential roles in brain.

There are accumulating evidences showing that the Wnt/β-catenin pathway plays a significant role in the pathophysiology of depression and to be a potential plausible target for antidepressants [9–12]. FZD6 was reported to participate in Wnt/β-catenin pathway, which was documented in the process of many diseases, such as cancers [13], neural tube defects [14,15] and brain morphogenesis defect [16]. However, whether and how FZD6 is involved in depression is largely unknown. Voleti *et al.* showed that virus-mediated inhibition of *Fzd6* in the brain resulted in depressive-like behaviors in a rodent model [17]. Very recently, we found a missense single nucleotide polymorphism (SNP) located in *FZD6* associated significantly with depressive symptoms [18], but the underlying mechanism of such an association was not addressed.

Considering decreased expression of *FZD6* in MDD patients and significantly association of genetic variants in the gene with MDD, our primary purpose was to determine how this gene participates in the pathogenesis of depression at molecular level by using a combination of cellular analysis, animal behavioral tests, morphological analysis, and molecular analysis with *in vitro* and *in vivo* models.

## Materials and Methods

### *Expression analysis of FZD6 in patients with depression*

To characterize the correlation between depression and expression of *FZD6* in human beings, we downloaded two datasets (GSE54562 and GSE92538) from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/). After extracting *FZD6* mRNA expression values from each sample, we examined the expression difference of *FZD6* between MDD patients and healthy control subjects by applying unpaired *t*-test.

### *Cell culture and transfections*

The HEK293T cells and SH-SY5Y neuronal cells were obtained from ATCC (Manassas, VA, USA), and cultured in DMEM medium (Hyclone, Logan, UT, USA) supplied with 10% FBS (GIBCO, Grand Island, NY, USA) and 1% penicillin/streptomycin (GIBCO) at 37 °C with 5% $CO_2$ atmosphere.

CRISPR/Cas9 plasmid with a 2A-Puro co-expression vector *pSp*-Cas9(BB)-2A-Puro (PX459) was purchased from Addgene (https://www.addgene.org/). We applied the CRISPR online tool (https://tools.genome-engineering.org/) to design single guide-RNA (sgRNA) aiming exon 4 of *FZD6*, and the designed sgRNA (i.e., 5′-GAACAAGTCCAAAGAGACAT-3′) was then cloned into PX459 (namely *FZD6*-Cas9-Puro) as previously described [19–21]. The *FZD6*-specific targeting plasmid was transfected into HEK293T or SH-SY5Y cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Cells were selected with puromycin (3 μg/ml) for 96 h one day after transfection, and then subjected to validate the guided target site by Sanger sequencing (Sango Biotech, Shanghai, China).

### *Animals*

All mice (GemPharmatech Co. Ltd, Nanjing, China) were housed under standard conditions (12-hour light–dark cycle) and were provided with free food and water at the Core Animal Facility at Zhejiang University. Mice were deeply anesthetized before sacrifice.

### *Ethics statement*

The Animal Care and Use Committee of the First Affiliated Hospital of Zhejiang University approved the experimental procedures in this study (Approval No. 1093–1).

### *Creation of Fzd6 knockdown (Fzd6-KD) mouse model with CRISPR/Cas9 technique*

Based on C57BL/6J background mice, we created *Fzd6*-KD mice by CRISPR/Cas9 editing system [22] at GemPharmatech (Nanjing, China). Briefly, the Cas9 mRNA and sgRNA were co-injected into the zygotes of mouse. The sgRNA was designed following above website with the sequence of 5′-CACCAAAATCCAATGTCTCT-3′, which was targeted to exon 4 of *Fzd6*. Through homologous recombination, the broke double-strand was repaired, leading to generation of *Fzd6* knockdown heterozygote mice. The pups were genotyped by PCR followed by Sanger sequencing (Sango Biotech, Shanghai, China). The primers for genotyping and sequencing were forward: 5′-TCTGTGAATGCAGCAAAGTCATGG-3′, reverse: 5′-GTCT CTCTGGGTATCTGAATCGTC-3′. By crossbreeding the heterozygote mice, we obtained both homozygous *Fzd6*-KD mice and wild-

type (WT) mice. Since only five nucleotides were deleted in the *Fzd6*-KD mice, we named them as *Fzd6*-Δ5 mice thereafter.

*Behavioral tests*

The male and female homozygous WT and *Fzd6*-Δ5 mice (2 ∼ 3-month old) were used for the following tests. Before each behavioral test, mice were adapted to testing room for about 1 h. After finishing behavioral tests, the hippocampus from each mouse brain was collected and stored at −80 °C refrigerator.

*Forced swim test (FST)*

FST was conducted as reported before [23]. Briefly, each mouse was put into a clear 12 cm diameter cylinder filled with 23–25 °C water (∼16 cm depth) for 6 min. We monitored the behaviors and counted the total immobility during the last 4 min. The animals with no any movement except breathing was defined as immobility.

*Tail suspension test (TST)*

TST was performed as previously reported [24]. Briefly, the tail of each mouse was stuck with adhesive tape and suspended 50 cm above the floor. The behaviors were recorded for 6 min by a camera from side and counted the total immobility during the last 4 min.

*Sucrose preference test (SPT)*

The SPT was performed following previously reported protocol [24–26]. Animal was singly housed and adapted to two bottles filled with water. After 48-hour habituation, all mice were subjected to two bottles filling with either 2% water or sucrose for 48 h. To avoid side preference, we switched the positions of two bottles every 6 h. The amount of consumed sucrose relative to the total amount of consumed fluid during testing phase was used to calculate the sucrose preference.

*Open field test (OFT)*

Every mouse was put individually into the OFT apparatus (44 × 44 × 44 $cm^3$) facing the wall. The locomotor activity was recorded 10 min with a camera above the apparatus under dim light. The anxiety-like behavior was measured with the total distance travelled, center entries, and time spent in the central zone. After each test, we cleaned the apparatus with 75% ethanol. We used Any-maze software (Stoelting Co., Wood Dale, IL, USA) to analyze the collected data.

*Elevated plus maze (EPM)*

The EPM apparatus comprised of two closed arms (30 × 5 × 20 $cm^3$) and two open arms (30 × 5 $cm^2$). The maze was placed above the floor at a height of 50 cm. Being faced to one of the open arms, we put every mouse into the middle of the apparatus and allowed to explore the apparatus for 6 min. The track of each animal was recorded with a camera above the apparatus. Total time spent and total distance travelled in each arm were counted. We used Any-maze software (Stoelting) to analyze the collected data.

*Serum corticosterone detection*

The blood was collected from each animal and centrifuged at 3000 rpm at 4 °C for 15 min. The supernatants were collected and stored at − 80 °C for later use. Serum corticosterone level was measured by ELISA kit (Abcam, ab108821).

*RNA extraction and quantitative real-time PCR (qRT-PCR)*

The total RNA from cells or hippocampus was isolated with TRIzol reagent (Life Technologies, Grand Island, NY, USA). RNA quantity was determined by NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Primers for each gene of interest were designed and synthesized at Sangon Biotech Co., Ltd (Shanghai, China) and are listed in **Supplementary Table S1**. One μg RNA was used to synthesize the cDNAs with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The qRT-PCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems). A total of 10 μl mixture containing 5 μl Master Mix, 2.5 μl sense and antisense primers (the final concentration is 1 μM), as well as 2.5 μl diluted cDNA in a 384-well plate. The PCR was conducted with 7900 HT Fast Real Time PCR system (Applied Biosystems). The relative gene expression was normalized to *β-actin* for *in vivo* study and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) for *in vitro* study using $2^{-\Delta\Delta Ct}$ method [27].

*Western blotting*

The cells or brain tissues were homogenized by ultrasonic disruptor in RIPA solution (Beyotime Biotechnology, Shanghai, China) containing Phenylmethanesulfonylfluoride (PMSF) protease inhibitors. All samples were centrifuged at 12,000 rpm for 15 min after being incubated on ice for 30 min, and collected the liquid supernatant. Cytoplasmic protein was extracted following nuclear and cytoplasmic extraction instruction (Pierce, Rockford, IL, USA). The protein concentration was measured with the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology). A mixture of protein loading buffer and equal amount of protein was electrophoresed on an 8% SDS-PAGE gel, and they were then electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 5% BSA in TBST for 1 h at room temperature, the membrane was incubated overnight at 4 °C with the following primary antibodies: FZD6, GSK3β, phospho-GSK3β (Ser9) and phospho-β-catenin (Ser675) (1:1000 for all of them, Cell Signaling Technology, Beverly, MA, USA), β-catenin (1:5000, Proteintech, Wuhan, China). Following three times of washing with TBST, immunoblots were incubated with a 1:4000 dilution of HRP-conjugated anti-rabbit secondary antibody (1:5000, Proteintech) for 1 h, followed by detection with Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA). The total protein expression was normalized to β-actin (1:10000, Abclonal, Wuhan, China). The β-tublin (1:1000, Proteintech) was used as the cytoplasmic loading control, and Lamin B1 (1:2000, Proteintech) was used as the nuclear loading control. We applied ImageJ software (NIH) to quantify the immunoblot results.

*Immunofluorescence staining*

Mice were deeply anesthetized and subjected to transcardial perfusion with cold saline followed by 4% paraformaldehyde (PFA). Whole brain was collected and post-fixed in 4% PFA for 24 h. Then, samples were given graded dehydration and embedded in paraffin, and immunostaining was conducted with 5 μm sections. Next, sections were deparaffinized, followed by rehydrated and subjected to block with blocking solution containing 0.1% Triton X-100 for permeabilization, and then incubated at 4 °C overnight with anti-Ki67 (1:200, Servicebio, Wuhan, China), anti-PCNA (1:500, Abcam, Cambridge, MA, USA), or anti-NeuN (1:200, Servicebio). After being washed three times in PBS for 5 min, each section was incubated with secondary antibodies conjugated to

Alexa Fluor 488 and/or Cy3 (1:400, Servicebio) at room temperature for 1 h. Finally, sections were mounted with antifade mounting media containing DAPI after washing in PBS. Images were scanned by Pannoramic 250FLASH slice scanner (3DHISTECH, Budapest, Hungary). Quantitative analysis was conducted using ImageJ software as a measure of positive cells in the DG region of hippocampus.

### *Statistical analysis*

All data were analyzed with GraphPad Prism 8.0 (San Diego, CA, USA) and are presented as Mean ± SEM. Two-tailed unpaired *t*-test was applied to perform statistical analysis. *p*-value < 0.05 was defined as statistically significant difference.

## Results

### *Characterization of FZD6 in depression*

To determine whether there existed any expression difference of *FZD6* between MDD patients and healthy controls, we compared the expression level of *FZD6* between healthy controls and MDD patients in two human expression datasets (GSE54562 and GSE92538). The results showed that the mRNA expression level of *FZD6* significantly reduced in MDD patients ($p < 0.05$; **Supplementary** Fig. S1A a**nd S1B**), indicating FZD6 may play significant roles in depression.

### *Demonstration of the effects of FZD6 knockdown on Wnt/β-catenin pathway in HEK293T and SH-SY5Y cells*

To determine FZD6 roles in depression, we constructed two CRISPR/Cas9-based *FZD6*-knockdown cell lines (Fig. 1**A**). As FZD6 is one of the receptors for Wnt proteins involving in Wnt/β-catenin signaling pathway, several key genes within this pathway were measured. In SH-SY5Y neuronal cells, qRT-PCR analysis showed a 61% reduction of *FZD6* mRNA expression in *FZD6*-KD cells compared with controls ($p < 0.001$; Fig. 1**B**). Consistent with the mRNA results, we also detected a downregulation at the FZD6 protein level ($p < 0.05$; Fig. 1**F**). We then examined the effect of decreased *FZD6* expression on the downstream genes in the Wnt/β-catenin pathway. We found that knockdown of *FZD6* resulted in reduced *GSK3β* mRNA expression ($p < 0.05$; Fig. 1**C**) and decreased phosphorylation of GSK3β protein expression normalized to the total GSK3β ($p < 0.01$; Fig. 1**G**). The *β-catenin* mRNA expression was also downregulated significantly in *FZD6*-KD cells ($p < 0.001$; Fig. 1**D**), a targeted downstream gene regulated by GSK3β. In addition, most of the other gene expression levels in the Wnt/β-catenin pathway, such as Casein kinase 1 (*CK1*), Adenomatous polyposis coli (*APC*), and Dishevelled 3 (*DVL3*) also showed significant decrease in *FZD6*-KD cells ($p < 0.01$) (Supplementary Fig. S2A).

Consistently, we also constructed *FZD6* knockdown HEK293T cells with CRISPR/Cas9 system. Both the FZD6 mRNA and protein expression were markedly decreased in *FZD6*-KD HEK293T cells (Supplementary Fig. S3A and **S3E**). Similarly, as shown in **Supplementary** Fig. S2B**, S3B, and S3C**, *GSK3β*, *β-catenin*, *APC*, *DVL3*, *AXIN1*, and *AXIN2* mRNA expression were markedly decreased in the *FZD6*-KD cells ($p < 0.05$). The GSK3β protein phosphorylation ($p < 0.05$; **Supplementary** Fig. S3F) was also significantly reduced, but not for the total GSK3β between the WT and *FZD6*-KD cells ($p > 0.05$).

## Generation of Fzd6-KD mice

To further investigate FZD6 roles in depression, the *Fzd6*-KD mouse model was created with the CRISPR/Cas9 system targeting the fourth exon of *Fzd6* (Fig. 2**A**). After crossbreeding between

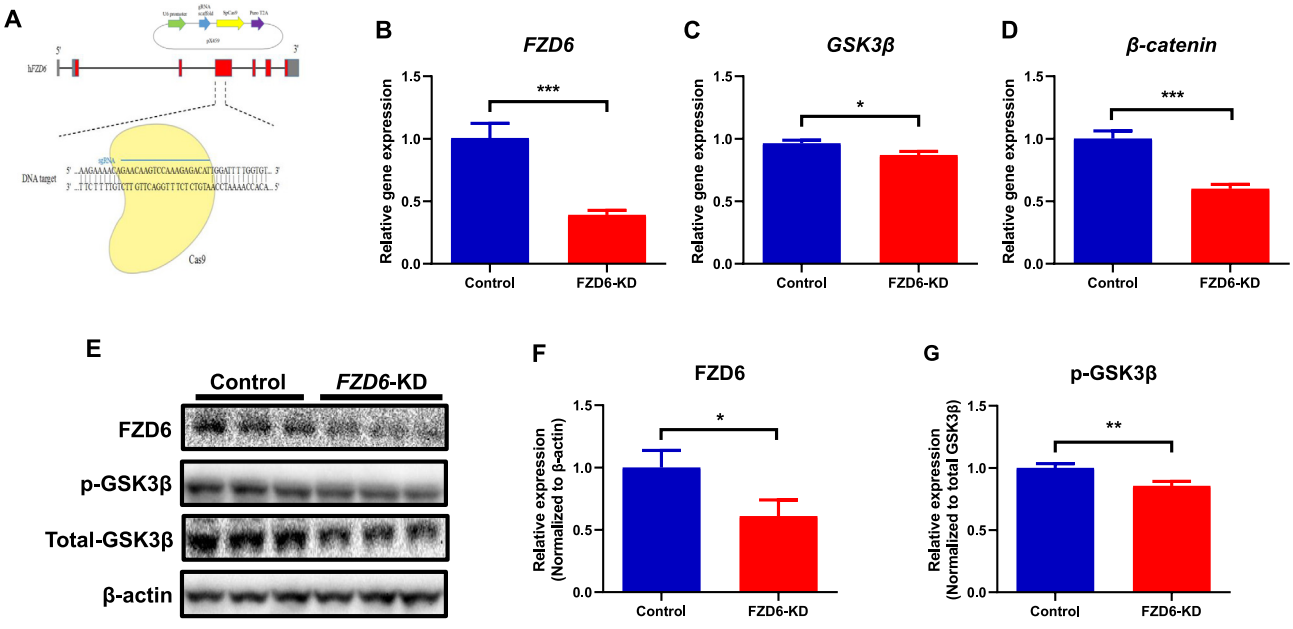


**Fig. 1.** Regulatory effects of *FZD6* knockdown (*FZD6*-KD) on Wnt/β-catenin signaling pathway in SH-SY5Y cells. (A) Schematic of *FZD6*-KD created by CRISPR/Cas9 technology. The sgRNA (blue) was cloned into a Cas9 nuclease (yellow) expression plasmid (PX459), then the Cas9 nuclease was targeted to exon 4 of *FZD6* by a 20-nt guide sequence and induced a double-strand break. **(B-D)** *FZD6*-KD significantly decreased mRNA expression of **(B)** *FZD6*, **(C)** *GSK3β*, and **(D)** *β-catenin* in Wnt/β-catenin pathway, which were assessed by qRT-PCR in SH-SY5Y cells (n = 3). **(E)** Representative Western blotting images of FZD6, total GSK3β, and phosphorylation of GSK3β (p-GSK3β) at Ser9 with β-actin as loading control in SH-SY5Y cells. **(F, G)** *FZD6*-KD remarkably reduced expression of **(F)** FZD6 protein and **(G)** phosphorylation of GSK3β (p-GSK3β) at Ser9 in SH-SY5Y cells (n = 3). Optical densities were normalized to loading control (β-actin), and p-GSK3β was expressed as ratio of p-GSK3β over total-GSK3β. All data are shown as mean ± SEM. Unpaired *t*-test was used to conduct the statistical analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the heterozygote mice, we obtained *Fzd6*-Δ5 homozygote mice with a five-nucleotide deletion (Fig. 2**B**), which led to generation of an early stop codon in the amino acid of Fzd6 (Fig. 2**C**). Subsequent qRT-PCR and Western blotting analyses presented that the level of *Fzd6* mRNA reduced by 50% ($p < 0.001$), and the level of Fzd6 protein decreased by 40% ($p < 0.05$) in *Fzd6*-Δ5 mice, respectively (Fig. 2**D**). These results demonstrated that we have successfully generated a *Fzd6*-KD mice, which could be used for further functional studies.

### *Alterations of depressive-like and anxiety-like behaviors in Fzd6-Δ5 mice*

To determine *Fzd6* knockdown on depressive-like behaviors in animals, we applied FST, TST and SPT to evaluate despair or anhedonia of mice. Compared with WT mice, *Fzd6*-Δ5 mice showed increased immobile durations in FST (Fig. 3**A**; $p < 0.05$) and a similar trend but at a less extent in TST (Fig. 3**B**; $p = 0.0834$). In the SPT, *Fzd6*-Δ5 mice showed less preference for sucrose (Fig. 3**C**) compared with WT mice ($p < 0.05$), suggesting *Fzd6* knockdown induced the mice presenting depressive-like behaviors.

To further measure the anxiety along with depression after *Fzd6* knockdown, we used OFT (Fig. 4**A**) and EPM tests (Fig. 4**D**) to determine anxiety-like behaviors. In the OFT, compared with the WT mice, we found that *Fzd6*-Δ5 mice took less time in the center area ($p < 0.05$; Fig. 4**B**), but there are no markedly changes for the distance travelled were detected between the WT and *Fzd6*-Δ5 mice ($p > 0.05$; Fig. 4**C**). In EPM, compared with the WT mice, the *Fzd6*-Δ5 mice travelled less distance (Fig. 4**E**) and took less time (Fig. 4**F**) in the open arms ($p < 0.05$). Together, above results indicated that the *Fzd6*-Δ5 mice exhibited anxiety-like behaviors.

We also found that the serum corticosterone, a major hallmark of stress hormones for depressive disorder [28], was significantly elevated in *Fzd6*-Δ5 mice compared with the WT mice ($p < 0.05$; Fig. 4**G**). This suggests that the *Fzd6*-Δ5 mice not only presented changed depressive-like and anxiety-like behaviors, but also showed altered hormones level, which is related to depressive disorder.

## Alteration of cell proliferation in the hippocampus of Fzd6-Δ5 mice

To measure the cell proliferation in the hippocampus, an important region implicated in depression [29], we used Ki67 and PCNA (Proliferating Cell Nuclear Antigen) as markers to label the proliferating cells in the dentate gyrus (DG) region of hippocampus. As shown in Fig. 5**A and 5B**, compared to the WT mice, the number of $Ki67^+$ cells and $PCNA^+$ cells were markedly decreased by 40% ($p < 0.05$; Fig. 5**C**) and 54% ($p < 0.001$; Fig. 5**D**) in the DG of *Fzd6*-Δ5 mice, respectively, suggesting a significant decrease of proliferating cells in *Fzd6*-Δ5 mice. However, no significant difference of $NeuN^+$ (mature neuron marker) cells in the DG region was detected between the WT and *Fzd6*-Δ5 mice ($p > 0.05$; Fig. 5**E**). This indicated that *Fzd6* knockdown reduced cell proliferation in the hippocampus but had no significant effect on the mature neuron.

### *Regulation of Wnt/β-catenin pathway in hippocampus of Fzd6-Δ5 mice*

To further study the molecular mechanisms of *FZD6* in depression, the mRNA and protein expressions of GSK3β and β-catenin in Wnt/β-catenin pathway in hippocampus were examined. We found the relative mRNA expression of *Gsk3β* reduced significantly

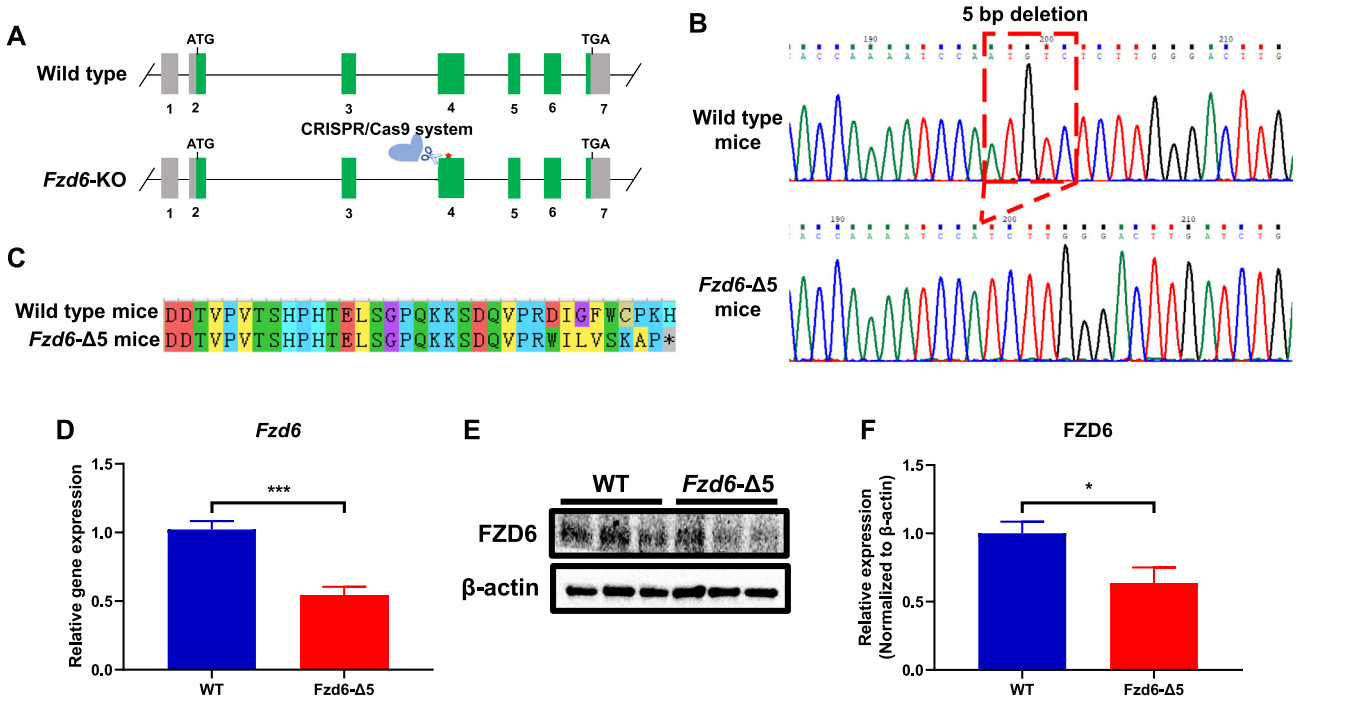


**Fig. 2.** Generation of *Fzd6* knockdown (*Fzd6*-KD) mice by CRISPR/Cas9 system. (A) Schematic of strategy for construction of *Fzd6*-KD mice via the CRISPR/Cas9 system. The system targeted to the fourth exon of *Fzd6*. **(B)** Sequencing chromatogram to identify wild-type and *Fzd6* knockdown mice. Dashed red line indicated 5-bp deletion in *Fzd6*-KD mice, named as *Fzd6*-Δ5 thereafter. **(C)** BLAST of amino acid from wild-type (WT) and *Fzd6*-5 bp deletion (*Fzd6*-Δ5) mice. The asterisk indicates a stop codon in *Fzd6*-Δ5 mice. **(D)** Comparison of *Fzd6* mRNA expression in WT and *Fzd6*-Δ5 mice as detected by qRT-PCR. **(E)** Representative Western blotting images of FZD6 with β-actin as loading control in WT and *Fzd6*-Δ5 mice. **(F)** Quantitative analysis of FZD6 expression in WT and *Fzd6*-Δ5 mice. All data are shown as mean ± SEM. Unpaired *t*-test was used to conduct the statistical analysis. $^*p < 0.05$, $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

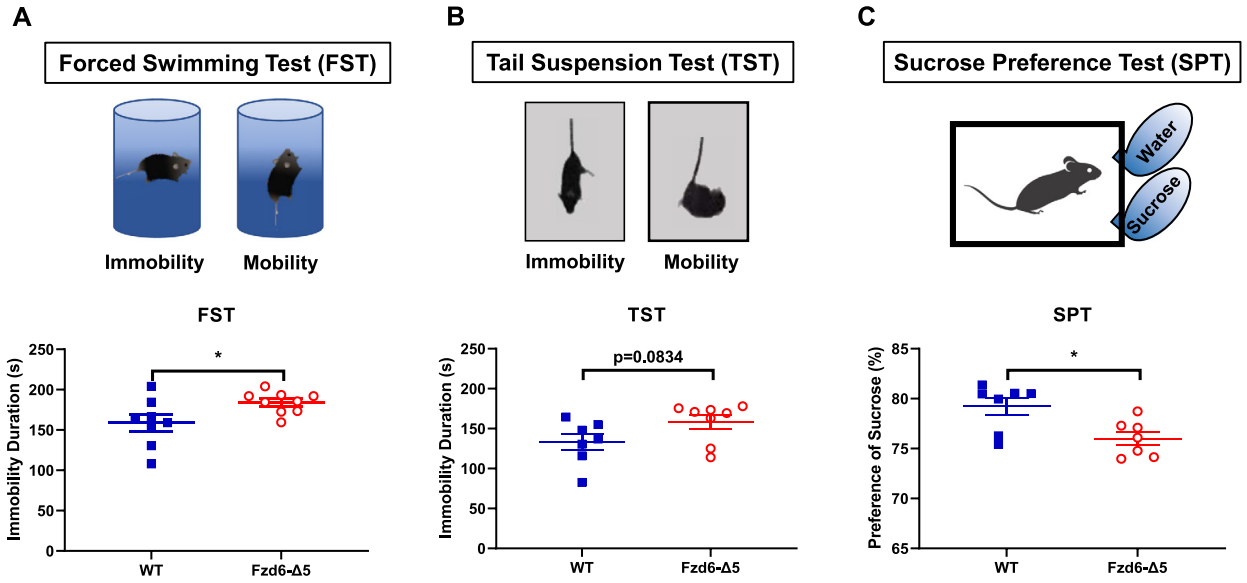


**Fig. 3.** Effects of *FZD6* on depressive-like behaviors in *Fzd6*-Δ5 mice. The forced swimming test (FST), tail suspension test (TST), and sucrose preference test (SPT) were used to measure depressive-like behaviors in WT and *Fzd6*-Δ5 mice. **(A)** The immobility seconds in FST. **(B)** The immobility seconds in TST. **(C)** The percentage of sucrose consumption over total fluid consumption in SPT. All behavioral tests were evaluated with number of mice from 7 to 9 per genotype. Data were analyzed using unpaired *t*-test and are shown as mean ± SEM. $^{*}p < 0.05$.

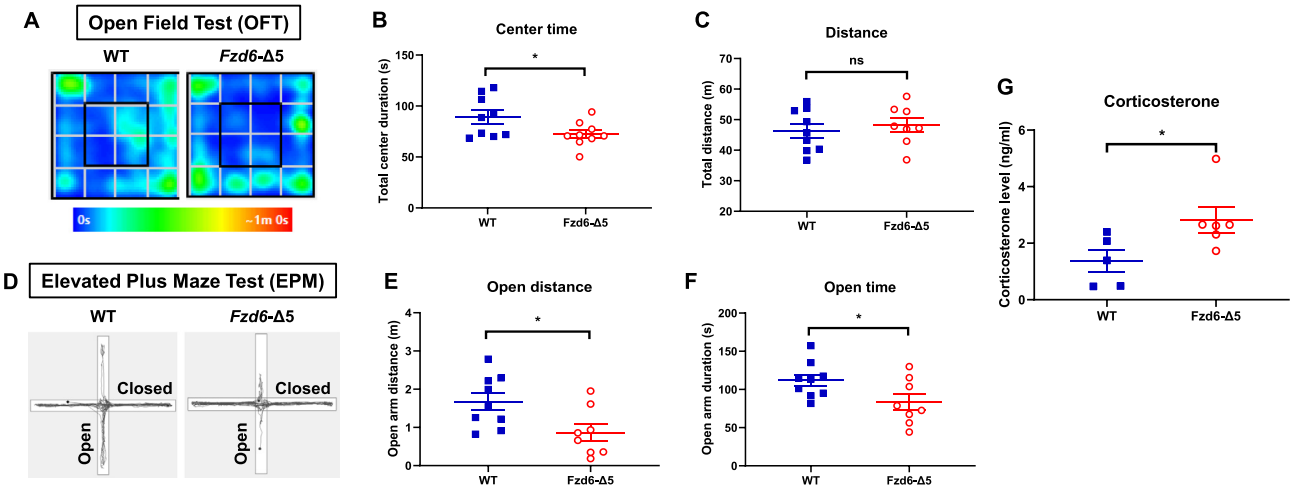


**Fig. 4.** Effects of *FZD6* on anxiety-like behaviors in *Fzd6*-Δ5 mice**.** The open field test (OFT) and elevated plus maze (EPM) test were applied to measure anxiety-like behaviors in WT and *Fzd6*-Δ5 mice. **(A)** The representative tracking plot in OFT. Graphs show **(B)** the time spent in center area and (**C**) the total distance travelled in OFT. **(D)** Representative tracking plot in EPM test. Graphs present **(E)** distance travelled and **(F)** time spent in open arms in EPM. All behavioral tests were evaluated with number of mice from 7 to 9 per genotype. Data were analyzed using unpaired *t*-test and are presented as mean ± SEM. $^{*}p < 0.05$, ns = not significant.

($p < 0.05$; Fig. 6**A**) in *Fzd6*-Δ5 mice, but no markedly difference was found in *β-catenin* mRNA level (Fig. 6**B**). Further, we found that, compared with the WT mice, phosphorylation of GSK3β was significantly decreased in *Fzd6*-Δ5 mice normalized to the total GSK3β ($p < 0.05$; Fig. 6**D**), but only a decrease trend was detected for phosphorylation of β-catenin in *Fzd6*-Δ5 mice normalized to the total β-catenin ($p = 0.07$; Fig. 6**E**). Given that β-catenin translocate from cytoplasm to nucleus when the Wnt/β-catenin pathway was activated [30], we next determined both the cytoplasmic and nuclear β-catenin levels. As shown in Fig. 6**G**, we observed lower protein level of β-catenin in the cytoplasmic fraction in *Fzd6*-Δ5 mice ($p < 0.05$), but β-catenin protein level in the hippocampal nuclear fraction only showed a decrease trend ($p > 0.05$; Fig. 6**I**). All above results clearly demonstrated the role of Fzd6 in depression mainly functions in hippocampus through disrupting the Wnt/β-catenin signaling pathway, especially GSK3β and β-catenin.

## Discussion

Here, we first found a significantly decreased expression of *FZD6* in MDD patients compared with healthy controls. By generating a gene knockdown animal model, we found that *Fzd6* knockdown mice presented depressive-like and anxiety-like behaviors, a direct evidence of the gene function in depression. Moreover, decreased *GSK3β* and *β-catenin* mRNA levels, reduced GSK3β phosphorylation, and the imbalance of β-catenin level in cytoplasm and nucleus of hippocampus in *Fzd6* knockdown mice provided further evidence for FZD6 roles in depression at mechanistic level.

So far, several pathways have been documented in the pathogenesis of depression. For example, proinflammatory cytokines were increased in the patients suffering from depression, in which nod-like receptor pyrin containing 3 (NLRP3) and toll-like receptor (TLR) are two known signaling pathways participated in neuroin-

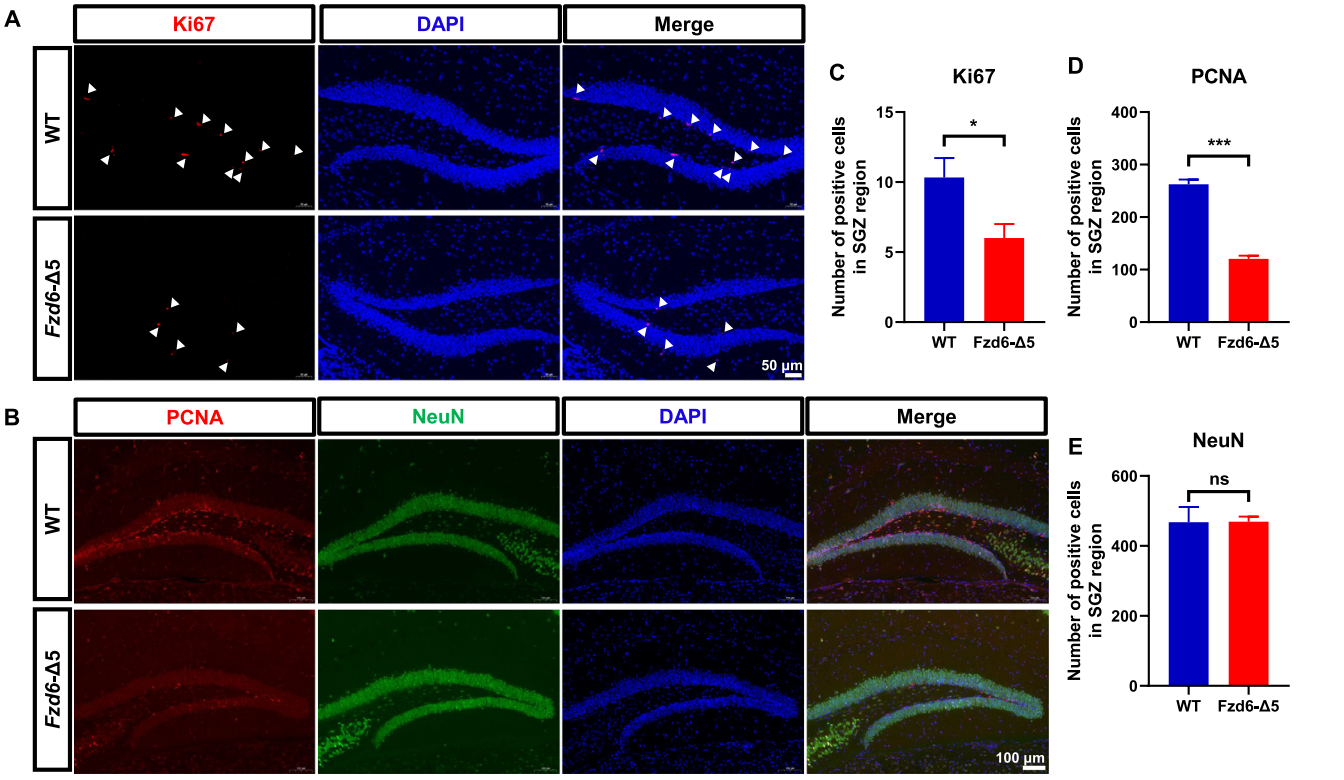


**Fig. 5.** Effects of *FZD6* on cell proliferation in the dentate gyrus (DG) region of hippocampus of *Fzd6*-Δ5 mice. (A) The representative immunofluorescent staining images of hippocampus staining with Ki67 (red) in WT and *Fzd6*-Δ5 mice. Nuclei were counterstained with DAPI (blue). The arrowheads show Ki67 positive cells. Scale bar = 50 μm. **(B)** The representative immunofluorescent staining images of NeuN (green) and PCNA (red) staining in the hippocampus of WT and *Fzd6*-Δ5 mice. Nuclei were counterstained with DAPI (blue). Scale bar = 100 μm. Quantification analysis of Ki67 **(C)**, PCNA **(D)**, and NeuN **(E)** positive cells in the DG region of hippocampus in WT and *Fzd6*-Δ5 mice. Data were analyzed using unpaired *t*-test and are presented as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, ns = not significant, n = 3 animals per group. PCNA, proliferating cell nuclear antigen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flammation involving depression [31–33]. Further, the levels of quinolinic acid (QUIN) and kynurenic acid (KYNA), two key metabolites in kynurenine (KYN) pathway, were reported to be abnormal, an indication of the contribution of aberrant metabolic pathway to the pathophysiology of depression [34]. The deficiency of trophic factors is another neurobiological mechanism involved in depression, in which brain derived neurotrophic factor (BDNF) is the main factor related to hippocampal neurogenesis [35]. In addition, the Wnt/β-catenin pathway, nuclear factor kappa-B (NF-κB) pathway, as well as mammalian target of rapamycin (mTOR) pathway were all reported involving in the process of neurogenesis and antidepressant [9,35,36]. However, the regulatory role of many abovementioned pathways in depression, especially the Wnt/β-catenin pathway, is largely unknown.

Frizzled receptor family was widely reported to mediate Wnt/β-catenin pathway, which is involving in several diseases including neural tube defects brain morphogenesis defects [37]. Recently, accumulating reports indicated that the FZD family is related to depression. For instance, Calabro and colleagues [38] found that SNP rs352428 in *FZD3* was strongly liked to depression, where the person who carries G allele having greater risk of experiencing depressive episode. Li and colleagues [5] reported FZD7 to be a new GPCR regulators involving depression. FZD6 was found to be related to depression according to an observation of altered depressive-like behaviors after *Fzd6* knockdown in rats [17]. All these studies strongly demonstrated a significant role of this family in depression [17]. Very recently, we found a SNP rs61753730 located in *FZD6* was linked to depression and allele specifically affected the expression of FZD6 [18].

It is documented that *Fzd6*-KD in the hippocampus region of rat brain can induce depressive-like behavior (i.e., decreased sucrose preference) and anxiety-like behaviors (i.e., repressed feeding and presentation in the EPM) [17]. Mice with knockdown expression of the FZD6 ligand Wnt2/Wnt3 also showed decreased immobility duration in TST and FST, as well as reduced sucrose consumption, an indication of involvement of Wnt2/Wnt3 in depressive-like behaviors [39]. To study the role of FZD6 involving depression, here, we generated *Fzd6*-Δ5 mice with *Fzd6* knockdown by employing CRISPR/Cas9 technique and found that the *Fzd6*-Δ5 mice showed significant depressive-like behaviors, such as increased immobility in FST and less prefer for sucrose. These behavioral alterations further demonstrated that Fzd6 plays a regulation role in depression-related behaviors. Because the hypothalamic pituitary adrenal (HPA) axis and elevated level of corticosterone in serum and different brain regions have been found as major hallmark of depressive disorder [28], we also measured corticosterone level in serum which was significantly elevated in *Fzd6*-Δ5 mice. MDD patients not only presented the changes of hormones, but also showed disturbances in neurotransmitters in the periphery and brain, including dopamine, glutamate, γ-aminobutyric acid (GABA), and serotonin (5-HT) [40,41]. Although we did not measure neurotransmitters level in the brain of *Fzd6*-Δ5 mice by ourselves in this study, a recent study reported that the neurotransmitters level (including BDNF, 5-HT, and NE) were all significantly decreased in *Fzd6* mutant mice [42]. These results demonstrated that Fzd6 plays an important role in depression.

FZD6 is the receptor for Wnt involving in Wnt/β-catenin pathway. This pathway is not only important for neuron development for instance neuron proliferation, neuron survival, migration, and differentiation in the brain, but also has been implicated in synaptic plasticity modulation, which is correlated with learning and

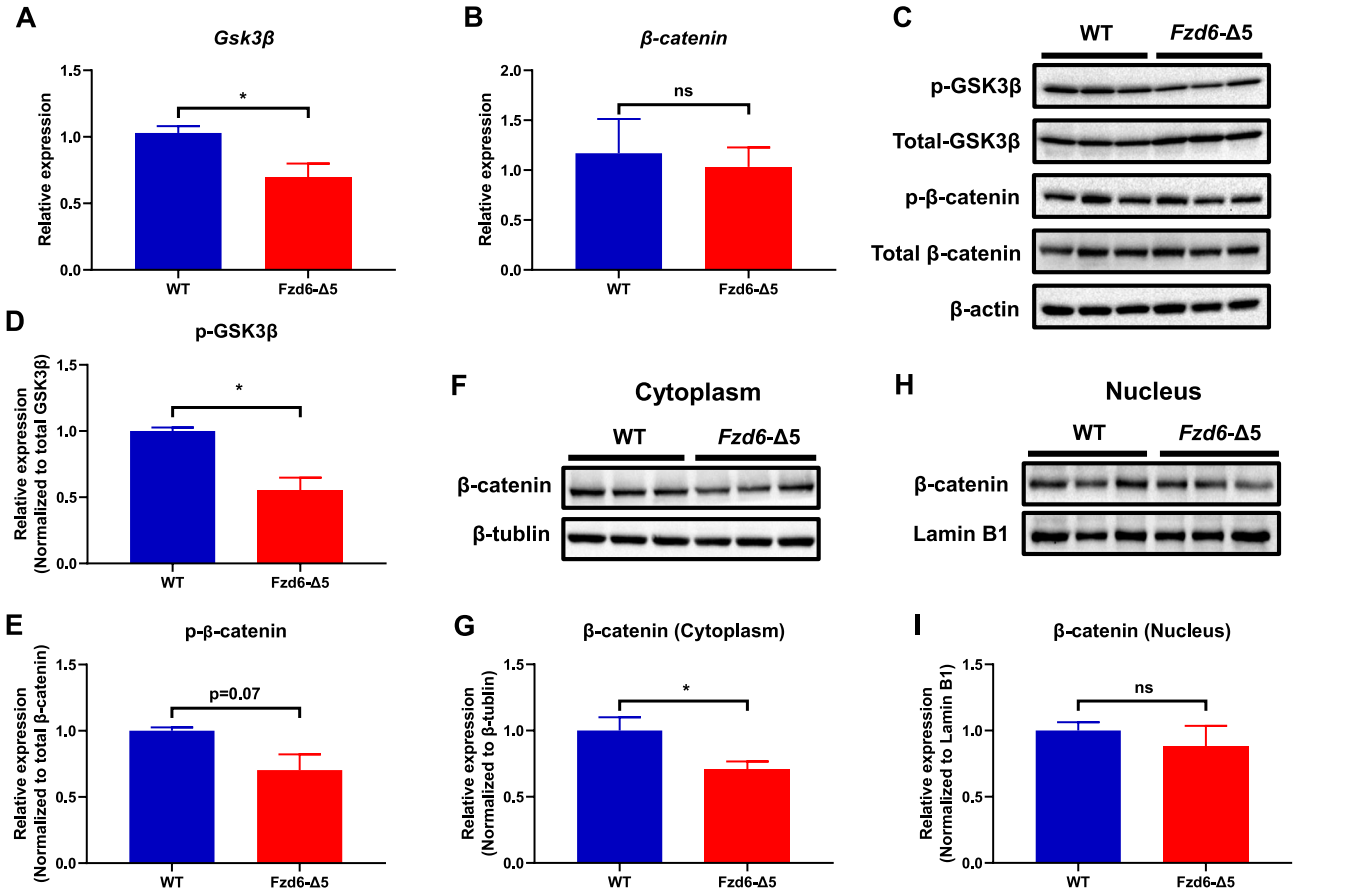


**Fig. 6.** Effects of *FZD6* on Wnt/β-catenin pathway in hippocampus of *Fzd6*-Δ5 mice. The mRNA relative expression of (A) *Gsk3β* and (B) *β-catenin* in hippocampus of WT and *Fzd6*-Δ5 mice. **(C)** Representative immunoblot images of total GSK3β and β-catenin, phospho-GSK3β (p-GSK3β) at Ser9, and phospho-β-catenin (p-β-catenin) at Ser675 in hippocampus of *Fzd6*-Δ5 mice. β-actin was applied as the loading control. Quantitative analysis of **(D)** p-GSK3β and **(E)** p-β-catenin expression levels in hippocampus, which were normalized to total GSK3β and total β-catenin, respectively. Representative immunoblot images of β-catenin in **(F)** hippocampal cytoplasmic fraction and **(H)** nuclear fraction of WT and *Fzd6*-Δ5 mice. Quantitative analysis of β-catenin expression in **(G)** cytoplasm and **(I)** nucleus of hippocampus normalized to loading control β-tubulin and Lamin B1, respectively. Data were analyzed using unpaired *t*-test and are presented as mean ± SEM. $^{*}p < 0.05$, ns = not significant.

memory, and emotional disorders including depression [43,44]. Here, we demonstrated that *Fzd6* knockdown significantly altered the cell proliferation with decreased Ki67 and PCNA positive cells. It is well known that GSK3β phosphorylation at Ser9 negatively regulates GSK3β activity and further increasing cytosolic β-catenin level, but GSK3β phosphorylation at Tyr216 positively regulates GSK3β activity further promoting β-catenin degradation [45,46]. The chronic stress induces depressive-like behaviors, decreases phosphorylation of GSK3β at Ser9 but increases phosphorylation of GSK3β at Tyr216, reduces β-catenin in the hippocampus of rodents [47,48]. These alterations also were found in the postmortem brains of depressed persons [49]. In the present study, we detected a decreased mRNA expression of *GSK3β* and *β-catenin*, as well as decreased phosphorylation of GSK3β in CRISPR/Cas9-based *FZD6*-KD HEK293T and SH-SY5Y cells. Similarly, in the hippocampus of *Fzd6*-Δ5 mice, we also detected a decreased mRNA expression of *Gsk3β* and a reduction of GSK3β phosphorylation. Although no significant change was shown in the *β-catenin* mRNA expression, we did detect decreased β-catenin level in the cytoplasm of the hippocampus. The reduced β-catenin expression in protein but not in mRNA level may due to the posttranslational modifications such as ubiquitination [50].

To gain a clear picture on roles of FZD6 in depression, we proposed a working model for its function (see Fig. 7**, left**). Normally, Wnt protein binds to its ligand FZD6 and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) to activate the canonical Wnt/β-catenin signaling pathway, then recruits the scaffold protein DVL to form a ligand–receptor complex. The activated DVL suppresses the kinase activity of GSK3β and destabilizes a "β-catenin destruction complex" consisted of CK1, AXIN, APC, and GSK3β. Then, β-catenin accumulates in the cytoplasm, which further translocate into the nucleus to promote Wnt target genes transcription and neurons proliferation [30,51]. There is an increasing evidences indicating that disruption of canonical Wnt/FZD signaling is related to multiple neurodevelopmental and neuropsychiatric disorders [10,52]. For instance, blocking *Dvl2* in the nucleus accumbens of mice makes them more vulnerable to depression [53]. Meanwhile, studies on the antidepressants target CREB led to the discovery of CREB and Wnt/FZD pathway connections [54]. Chronic ECS resulted in enhanced CREB binding to the *Fzd6* promoter and greater hippocampal *Fzd6* expression as determined by chromatin immunoprecipitation followed by microarray analysis (ChIP-chip) [17]. As a result of *FZD6* knockdown (see Fig. 7**, right**), the binding of Wnt to the receptors is blocked, or at least attenuated, which results in the aberrant inactivation of the Wnt/β-catenin pathway. GSK3β in the β-catenin destruction complex was dephosphorylated at Ser 9 and further phosphorylated in β-catenin, which resulted in a low concentration of β-catenin in cytoplasm. Attributing to the ubiquitination and proteasomal degradation, β-catenin reduction further results in inhibition of β-catenin nuclear translocation and suppression of the cell proliferation, ultimately, it may present depressive symptoms.

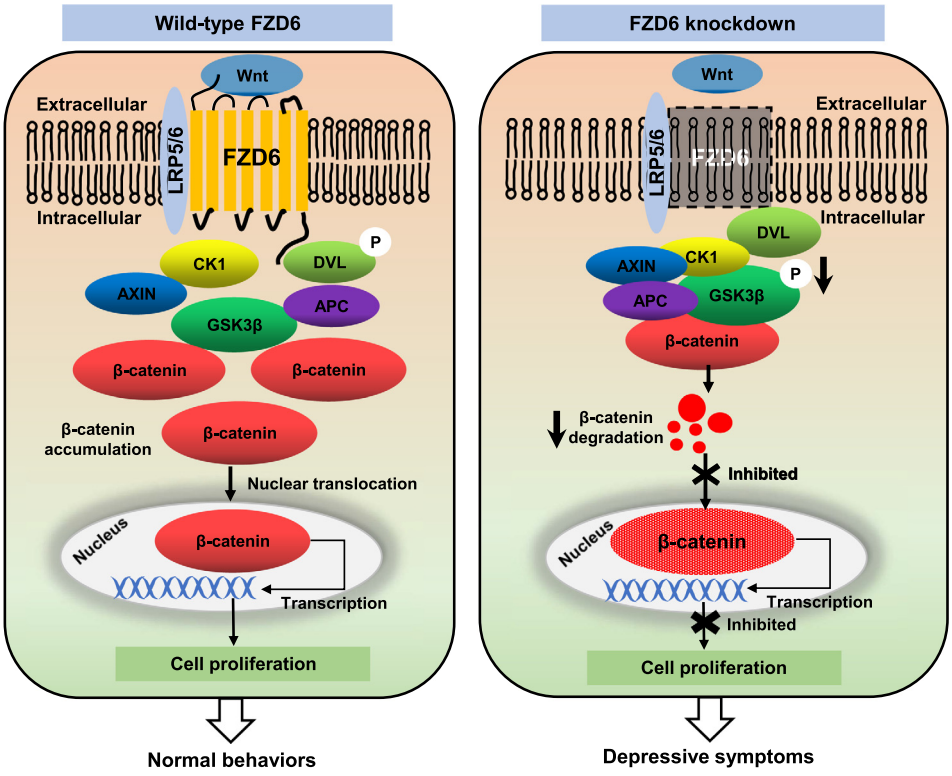


**Fig. 7.** Proposed model describing role of *FZD6* knockdown on disrupting Wnt/β-catenin signaling pathway in depression. Left schema shows physiological process of Wnt signaling in wild-type subjects. Wnt glycoproteins bind to FZD6 and a low-density lipoprotein (LRP)5/6 co-receptor, resulting in activation of Disheveled (DVL). This leads to phosphorylation of GSK3β, resulting in disruption of protein complex consisting of AXIN, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and GSK3β. This causes stabilization of β-catenin and its subsequent translocation to nucleus, which will activate transcription of target genes. Schema on right shows the role of FZD6 in this signaling pathway. Knockdown of *FZD6* expression might affect formation of ligand-receptor complex and increase stability of destruction complex. Dephosphorylation of GSK3β leads to degradation of β-catenin accompanied by decreased expression. The process of β-catenin into nucleus is inhibited, expression of downstream genes is restrained, and cell proliferation is also inhibited, this eventually results in depressive symptoms.

## Conclusions

In summary, based on our recent study where we found a missense SNP in *FZD6* markedly associated with depressive symptom and allele specifically affected the structure and expression of FZD6. In this study, to further investigate the mechanism and functional role of FZD6 in depression, firstly, we found decreased expression of *FZD6* in MDD patients. Then, by using various animal behavioral tests on *Fzd6* knockdown mice, we demonstrated that *Fzd6* was highly related to depressive symptoms. Finally, by applying both *in vitro* and *in vivo* approaches, we proved the above effect was caused, at least partly, by altering the expression and phosphorylation of β-catenin and GSK3β in hippocampus of the Wnt/β-catenin signaling pathway, and further affecting cell proliferation of DG region, which contributes to depression occurrence. Taken together, our current findings clearly demonstrate that *FZD6* is an important gene for depression. More studies are necessary to conduct to investigate the mechanisms of how *FZD6* in the Wnt/β-catenin pathway affects neuronal development such as neurogenesis and contributes to depression.

Compliance with Ethics Requirements

*The Animal Care and Use Committee of the First Affiliated Hospital of Zhejiang University approved the experimental procedures in this study (Approval No. 1093-1).*

## CRediT authorship contribution statement

**Haijun Han:** Data curation, Visualization, Investigation, Writing – original draft, Funding acquisition. **Mengxiang Xu:** Data curation, Investigation, Validation, Writing – original draft. **Ju Wang:** Methodology, Software, Investigation, Writing – original draft. **Ming D. Li:** Conceptualization, Supervision, Funding acquisition, Investigation, Project administration, Resources, Writing - original draft, Writing - review & editing. **Zhongli Yang:** Conceptualization, Supervision, Funding acquisition, Investigation, Project administration, Resources, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

*The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.*

## Acknowledgments

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2023.06.001.

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