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Zfp462 deficiency causes anxiety-like behaviors with excessive self-grooming in mice

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Zfp462 is a newly identified vertebrate-specific zinc finger protein that contains nearly 2500 amino acids and 23 putative C2H2-type zinc finger domains. So far, the functions of Zfp462 remain unclear. In our study, we showed that Zfp462 is expressed predominantly in the developing brain, especially in the cerebral cortex and hippocampus regions from embryonic day 7.5 to early postnatal stage. By using a piggyBac transposon-generated Zfp462 knockout (KO) mouse model, we found that Zfp462 KO mice exhibited prenatal lethality with normal neural tube patterning, whereas heterozygous (Het) Zfp462 KO (Zfp462$^{+/-}$) mice showed developmental delay with low body weight and brain weight. Behavioral studies showed that Zfp462$^{+/-}$ mice presented anxiety-like behaviors with excessive self-grooming and hair loss, which were similar to the pathological grooming behaviors in Hoxb8 KO mice. Further analysis of grooming microstructure showed the impairment of grooming patterning in Zfp462$^{+/-}$ mice. In addition, the mRNA levels of *Pbx1* (pre-B-cell leukemia homeobox 1, an interacting protein of Zfp462) and *Hoxb8* decreased in the brains of Zfp462$^{+/-}$ mice, which may be the cause of anxiety-like behaviors. Finally, imipramine, a widely used and effective anti-anxiety medicine, rescued anxiety-like behaviors and excessive self-grooming in Zfp462$^{+/-}$ mice. In conclusion, Zfp462 deficiency causes anxiety-like behaviors with excessive self-grooming in mice. This provides a novel genetic mouse model for anxiety disorders and a useful tool to determine potential therapeutic targets for anxiety disorders and screen anti-anxiety drugs.

Keywords: Anxiety disorders, behavior, Hoxb8, Pbx1, Zfp462

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Zfp462 is a newly identified vertebrate-specific zinc finger protein that contains nearly 2500 amino acids and 23 putative C2H2-type zinc finger domains (Iuchi 2001). Although the exact functions of Zfp462 are still unknown, current evidences show its critical roles in early embryonic development and neuronal differentiation (Laurent *et al.* 2009a,2009b). Zfp462 can be detected at mouse embryonic day 6.5 (E6.5) and reach the peak level at E18.5 (Chang *et al.* 2007). In the central nervous system (CNS), Zfp462 is expressed in a graded pattern in the cerebral cortex (Chang *et al.* 2007). How Zfp462 regulates the development of CNS remains unclear, but it was thought that Zfp462 might be implicated as a molecular determinant in the process of cortical development through similar mechanisms as Emx2 and Pax6 that Emx2 and Pax6 mutants result in abnormal neocortical development (Bishop *et al.* 2000, 2002; Chang *et al.* 2007).

As the regulative role of Zfp462 in the CNS is not well understood, its functions may be examined by its interacting proteins. A recent study reported that Zfp462 interacts with Pbx1 (pre-B-cell leukemia homeobox 1) in a two-hybrid system, as was further confirmed by GST (Glutathione-S-transferase) pull-down experiments *in vitro* (Laurent *et al.* 2007). Pbx1 is a three-amino acid loop extension class homeodomain protein and functions as a cofactor for Hox class homeodomain proteins. Pbx1 acts together with multiple Hox proteins in the development of mice (Manley *et al.* 2004). In human studies, single nucleotide polymorphisms such as rs4387163 and rs2066331 close to *PBX1* gene were reported to be highly associated with obsessive-compulsive disorders (OCDs) and 'anxiety' facet of neuroticism (Nestadt *et al.* 2012; van den Oord *et al.* 2008). These studies suggested that Pbx1 could be involved in the pathogenesis of anxiety or OCDs. Pbx1 is a transcription factor that critically regulates numerous embryonic processes including morphologic patterning, organogenesis and

hematopoiesis (DiMartino *et al.* 2001; Ficara *et al.* 2008; Kim *et al.* 2002; Manley *et al.* 2004; Schnabel *et al.* 2003). Pbx1 knockout (KO) mice showed delayed or absence of caudal pharyngeal pouch formation, leading to abnormal development (Manley *et al.* 2004). In addition, Pbx1 KO embryos had marked defects in exocrine and pancreatic hypoplasia and endocrine cell differentiation prior to death at E15 or E16 (DiMartino *et al.* 2001; Kim *et al.* 2002). Interestingly, Pbx1 forms a complex with Hoxb8 protein through a hexameric sequence appointed to the Pbx1-interaction motif (Knoepfler *et al.* 2001; Neuteboom *et al.* 1995).

Previous studies indicated that Pbx1 was a cofactor of Hoxb8 protein (Knoepfler *et al.* 2001; Neuteboom *et al.* 1995). Hoxb8 is a member of the mammalian Hox (homeobox-containing) complex, a group of transcription factors for providing positional information along the anteroposterior axis during early development (Capecchi 1997; Greer & Capecchi 2002). Interestingly, Hoxb8 is highly expressed in brain regions including the striatum, the orbitofrontal cortex and the anterior cingulate cortex (so-called OCD circuit), which are lined to grooming behaviors (Greer & Capecchi 2002). Furthermore, Hoxb8 KO mice exhibit excessive grooming behaviors compared with control littermates, resulting in hair loss and self-inflicted open skin lesions on the lateral and ventral body walls; more surprisingly, those Hoxb8 KO mice also excessively groomed their wild-type (WT) littermates (Chen *et al.* 2010; Greer & Capecchi 2002). This suggests that the complex of Pbx1 and Hoxb8 may be involved in the regulation of OCD or anxiety-like behaviors. It is not clear whether Zfp462 KO mice have similar phenotypes through the interaction with the complex of Pbx1 and Hoxb8.

As a partner protein of Pbx1 and Hoxb8 proteins, which are highly associated with anxiety/OCD in human studies and animal studies, the functional change or KO of Zfp462 protein may alter the stability of Pbx1/Hoxb8 or the affinity to their downstream targets. Therefore, we hypothesized that Zfp462 KO mice have anxiety-like behaviors or OCD-like behaviors by affecting Pbx1/Hoxb8 expression or functions.

To examine the role of Zfp462 protein on the development of the CNS and determine the behavioral phenotypes of Zfp462 deficiency mice, we generated a Zfp462 KO mouse model by the piggyBac (PB) transposon system. By using this mouse model, we performed a series of behavioral tests including anxiety-related tests (open field test, elevated plus maze, dark–light test and marble-burying test), putative OCD test (self-grooming test) and depression-related tests (forced swimming test and tail suspension test); also, memory tests and a motor test were administered as a part of a behavioral test panel. Finally, we found that Zfp462 deficiency leads to the CNS developmental abnormalities and anxiety-like behaviors with hair loss and excessive self-grooming.

Materials and methods

Animals

Zfp462 mutant mice were isolated from a large-scale insertional mutagenesis project on the FVB/NJ background. In this project, mice carrying a single PB transposon PB[Act-RFP] in the genome was mated with the transposase line Act-PBase (Ding *et al.* 2005), which were generated by conventional pronuclei injection of linear plasmids. Double-positive male progeny were then crossed with WT mice to generate transposase negative mutants carrying remobilized PB insertions. The Zfp462 mutation was one of the mutagenesis lines generated in the offsprings. The mutation was mapped by inverse polymerase chain reaction (PCR) as described (Ding *et al.* 2014). Zfp462 mutant carries the PB insertion in the seventh intron of the *Zfp462* gene (Chr4; 55 036 508 bp, as Ensemble Version 85 indicated). Presence of the mutation was confirmed by genotyping PCR with the primers provided in Table S1 (Supporting information). More detailed information showed on the PB mice database (http://idm.fudan.edu.cn/PBmice). Mice were maintained in specific pathogen-free animal housing at Soochow University Experimental Animal Center. All mice were housed with a maximum of five mice per cage in a temperature-controlled room (22 ± 2°C), with a 12-h light (0700–1900 h)/dark (1900–0700 h) cycle. Behavioral tests were carried out in the light phase of the cycle with an illumination background of 120 lx except the light–dark box test. The separate cohorts of mice were used for most behavioral tests. Mice used in the behavioral tests were males aged 2–6 months. The WT mice were the littermates of $Zfp462^{+/-}$ mice and Zfp462 KO mice. All procedures and protocols were approved by the Institutional Animal Care and Use Committee and followed the Guidelines for the Care and Use of Laboratory Animals.

Western blot analysis

After mice were sacrificed, fresh tissues from the heart, liver, lung, kidney, muscles, whole brain, brain stem, cortex, amygdala, hippocampus, striatum and hypothalamus were collected. In brief, 20–40 μg of total proteins were used for western blotting following the procedure described previously (Ren *et al.* 2014; Xu *et al.* 2010). Primary antibodies were goat polyclonal antibody against Zfp462 (Santa Cruz Biotechnology, Delaware, CA, USA; 1:500), rabbit polyclonal antibody against Pbx1 (Cell Signaling Technology Inc., Danvers, MA, USA; 1:1000), mouse monoclonal antibody against actin and mouse monoclonal antibody against tubulin (Sigma, St Louis, MO, USA; 1:20 000).

Immunoprecipitation

Brain samples were homogenized in lysis buffer and homogenates were centrifuged at 160 000 ***g*** for 30 min. After supernatants were collected, protein concentrations were then determined. Samples containing 500 μg protein in 500 μl were pre-absorbed with protein A agarose beads (50 μl, Santa Cruz Biotechnology) for 1 h at 4°C with gentle shaking. After gentle spinning, supernatants were incubated with 5 μl of Zfp462 antibody or control goat IgG at 4°C overnight. On the second day, protein A beads (50 μl) were added and incubated for 2 h at room temperature. Samples were centrifuged for 5 min at 2000 ***g*** at 4°C. Beads were further washed with lysis buffer three times. The bound proteins were eluted with sample buffer containing 100 mM Tris–HCl (pH 6.8), 200 mM dithiothreitol, 4% sodium dodecyl sulfate, 0.2% bromochlorophenol blue and 20% glycerol by heating at 96°C for 10 min. Elutes were collected for western blot analysis.

Fluorescent staining

For immunofluorescent staining, mouse brains were fixed with 4% paraformaldehyde through heart perfusion. After the incubation with 15% and 30% sucrose, brain tissues were cut to sections (8–10 μm thickness) with a cryostat at −20°C. Frozen brain sections were mounted on glass slides, washed by phosphate-buffered saline (PBS) and then blocked with buffer that contains 0.3% Triton-X-100/5% bovine serum albumin/PBS for 1 h at room temperature. Sections were then incubated with primary antibodies overnight at 4°C. After washing with PBS, brain sections were incubated with secondary antibodies and 4′,6-Diamidino-2-Phenylindole (DAPI) for 1 h at 4°C. Images were digitized by a camera accompanying with a fluorescence microscope (Axio ScopeA1, Carl Zeiss, Oberkochen, Germany). Mouse embryos were collected, fixed and embedded at E10.5. Primary antibodies were sued in this study as following: Pbx1 (Cell Signaling Technology Inc.; 1:1000), Shh, Pax3, Isl1/2 (Developmental

Studies Hybridoma Bank, IA, USA; 1:10), Olig2 (Millipore, Billerica, MA, USA; 1:200) and Arl13b (Proteintech, Chicago, IL, USA; 1:500).

Reverse transcription and quantitative PCR analysis

Total RNA was extracted from brain tissues using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the instructions. Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) as described previously (Niu *et al.* 2012). Real-time PCR was performed using 10 μl of 2XSYBR Green PCR Master Mix (Roche), 1 μl primers (10 μM) and 50 ng cDNA in a final volume of 20 μl by a 7500 Real-Time PCR machine (Applied Biosystems, Foster city, CA, USA). Data were normalized against GAPDH as internal controls and calibrated with control cDNAs. The relative expression ratio was calculated with a $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed in Table S1.

Body weight and brain weight evaluation

Body weight was measured by an electronic balance. When the mice were sacrificed, the whole brains were isolated and weighted.

Elevated plus maze test

The elevated plus maze was about 70 cm above the floor. The maze consisted of two opposite closed arms with 14-cm high opaque walls and two opposite open arms ($30 \times 5\ cm^2$). A single 10-min testing session was carried out. To begin a trial, the 2–3-month-old male mice were placed in the center of the plus maze facing an open arm and their behaviors were recorded for 10 min. After each trial, the maze was cleaned with 50% ethanol solution in order to eliminate possible odor left by the previous subjects. The time spent in open arms and closed arms was recorded as previously described (Shmelkov *et al.* 2010).

Open field test

The open field apparatus contained a white plastics inner wall ($40 \times 40 \times 40\ cm^3$) and a circular white outer wall (30 cm away from the arena wall) to prevent the testing room from being visible in the trial. Male mice were placed into the center of the open field area; their movements in the field were recorded over a 6-min session. The total time spent in the inside area ($20 \times 20\ cm^2$) and center entries was calculated.

Light–dark box test

This test is based on the conflict of the innate aversion to illuminated areas and the spontaneous exploratory activity (Crawley & Goodwin 1980). The apparatus consisted of a polypropylene cage with a dimension of $45 \times 27 \times 30\ cm^3$. The cage was separated into two compartments by a rectangular opening ($7 \times 7\ cm^2$) at floor level. The bigger compartment ($27 \times 27\ cm^2$) was open-topped, transparent and brightly lit (900 lx). The smaller one ($18 \times 27\ cm^2$) had black painted walls and was covered with black Plexiglass at the top. For each test, mice were placed in the center of the dark compartment. The time spent in the light compartment and the transitions between two compartments were recorded over a period of 5 min.

Marble-burying test

The procedure for marble burying was slightly modified from the method as described previously by Deacon (2006). Briefly, mice were individually placed in a standard cage ($26.5 \times 16 \times 14\ cm^3$) without a lid individually. Twenty opaque (black or blue) glass marbles were evenly placed on a 5-cm thickness layer of sawdust in cages. Mice were then placed in cages for 30 min. After mice were removed from the cage, total marbles that were buried over two-thirds of their height was counted.

Self-grooming test

Mice (aged 2–6 months) were placed individually in clean empty cages without bedding to observe spontaneous grooming behaviors. After a 10-min habituation period in empty cages, mouse behaviors were then recorded in another 10 min. The grooming patterning in rodents usually proceeds in a cephalo-caudal direction and involves several distinct phases: phase 1, paw and nose grooming; phase 2, face grooming; phase 3, head grooming; phase 4, body grooming (Kalueff *et al.* 2016). The cumulative time spent in grooming, the number of grooming bouts, the average time per bout and the number of interrupted grooming bouts (non-chain bouts) were evaluated by using a stopwatch as described recently (Kalueff *et al.* 2016). The percentage of interrupted grooming bouts was calculated.

Drug administration

Imipramine (IM; Sigma-Aldrich Corp., St Louis, MO, USA) was dissolved in normal saline (NS) and freshly prepared before treatment. Mice were intraperitoneally (20 mg/kg) treated with IM or NS for 3 weeks.

Statistical analysis

All data were expressed as mean ± SEM. Difference between two groups was determined with a Student's *t*-test (factor: genotype). The differences among groups were determined with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (factors: variables tissue or time course) or two-way ANOVA followed by Bonferroni *post hoc* tests (the body weight curve and the escape latency in Water Maze test, factors: mouse group and time; behavioral tests after IM treatment, factors: mouse genotype and treatment). A *P*-value of <0.05 was considered statistically significant.

Results

Zfp462 protein expression was characterized

To characterize the expression of Zfp462 protein, we examined the distribution of Zfp462 protein in different organs as well as different brain regions. Our data indicated that Zfp462 protein was expressed in the heart, liver, lung, kidney, muscle and the whole brain. Among these tissues, the whole brain had the highest level of Zfp462 expression (Fig. 1a,b). Zfp462 expression was more abundant in the cortex and the hippocampus than other brain regions (Fig. 1c,d). Owing to the role of Zfp462 in the brain development, we observed Zfp462 expression in different developmental stages. Consistent with previous study, the brain had the highest level of Zfp462 expression from E7.5 to EP7. After this period, Zfp462 expression in the brain began to decline and maintained in a low level since 1 month old (Fig. 2).

Zfp462 KO resulted in prenatal death in mice

As Zfp462 protein was abundant in the brain, we generated Zfp462 KO ($Zfp462^{-/-}$) mice to study the functions of Zfp462 in the brain. We collected the brains from E12.5 and extracted the proteins and total RNA. Our results showed that no Zfp462 protein is expressed in brain samples of $Zfp462^{-/-}$ mice (Fig. 3a). Consistent with this, Zfp462 mRNA was not present in the brains of $Zfp462^{-/-}$ mice (Fig. 3b), indicating that the *Zfp462* gene had been successfully disrupted. Surprisingly, there were no $Zfp462^{-/-}$ mice found in the survival postnatal mice, suggesting that the lack of Zfp462 caused prenatal death in mice. The living $Zfp462^{-/-}$ embryos were found at E18.5 and dying KO embryos showed smaller size with abnormal eyes (Fig. 3c). Because most prenatal death resulted from neural tube defects, we then examined

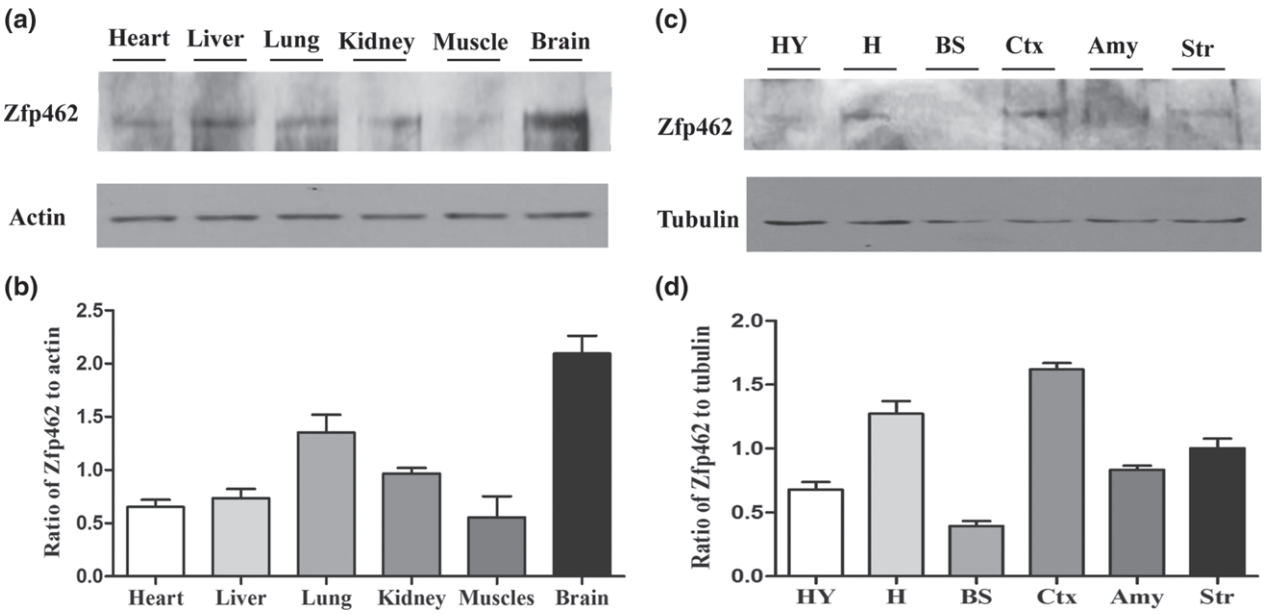

Figure 1: Zfp462 expressed in different tissues and brain regions. After WT mice were scarified, tissues from different organs including the heart, liver, lung, kidney, muscle and the whole brain were collected. Zfp462 protein expression was then examined by western blot analysis (a). Quantitative analysis for Zfp462 protein in different tissues was performed (b). Zfp462 protein expression was examined in different brain tissues including the hypothalamus (HY), hippocampus (H), brain stem (BS), cortex (Ctx), amygdala (Amy) and the striatum (Str) (c). Quantitative analysis for Zfp462 protein expression in different brain regions was performed (d). $N = 3–4$.

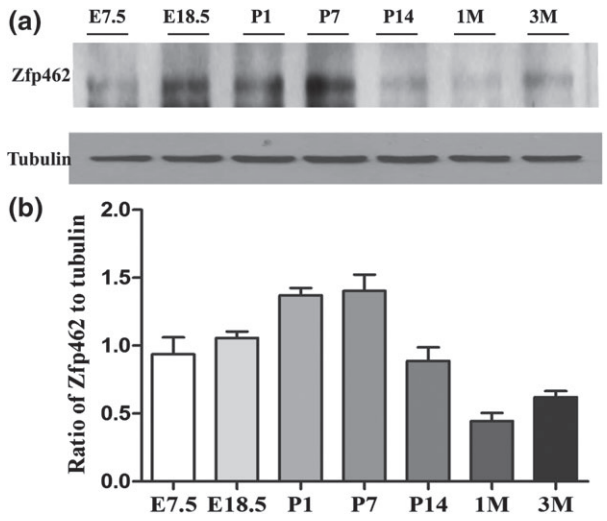

Figure 2: Time-course of Zfp462 expression in brains. Whole mouse brains at different ages (from E7.5 to 3 months) were collected and Zfp462 protein expression was examined by western blot analysis (a). Quantitative analysis for Zfp462 protein expression was performed (b). $N = 3–4$.

neural tube patterning in prenatal embryos. Neural tube patterning-related genes including *Pax3*, *Arl13b*, *Isl1/2*, *Olig2* and *Shh* were found to have normal distributions in Zfp462 KO mice (Figs. 3d,S1).

Zfp462$^{+/-}$ mice showed reduced Zfp462 expression with delayed development in postnatal mice

As homozygous Zfp462$^{-/-}$ mice died prenatally, we further examined the expression of Zfp462 in postnatal heterozygous Zfp462 KO mice (Zfp462$^{+/-}$) and WT mice. We found that Zfp462 protein significantly decreased in the orbitofrontal cortex ($t(5) = 5.984$, $P < 0.05$; Fig. 4a) and amygdala ($t(5) = 4.492$, $P < 0.05$; Fig. S5), but not in the hippocampus ($t(4) = 1.237$, $P > 0.05$; Fig. 4a) of Zfp462$^{+/-}$ mice. However, Zfp462 mRNA expression decreased significantly both in the orbitofrontal cortex ($t(5) = 4.211$, $P < 0.05$) and the hippocampus ($t(5) = 4.490$, $P < 0.05$) of Zfp462$^{+/-}$ mice (Fig. 4b). Therefore, the reduction of Zfp462 expression in Zfp462$^{+/-}$ mice provided a chance to examine the functions of Zfp462 and their phenotypes in adult mice. Our results showed delayed growth in Zfp462$^{+/-}$ mice from postnatal day 1 to 2 months ($F_{1,49} = 10.02$, $P < 0.05$; Fig. 4c). At postnatal day 1, body weight in Zfp462$^{+/-}$ mice was less than that in WT mice ($t(51) = 3.437$, $P < 0.05$; Fig. 4d). At 2 months, Zfp462$^{+/-}$ mice were smaller and weighed less than WT mice (Fig. 4e). In addition, brain weight also significantly reduced in Zfp462$^{+/-}$ mice compared with WT mice ($t(8) = 7.020$, $P < 0.05$; Fig. S2).

Pbx1 and Hoxb8 protein expressions decreased in Zfp462$^{+/-}$ mice

Previous study reported that Zfp462 interacts with Pbx1 in an *in vitro* experiment (Laurent *et al.* 2007); therefore, we further examined *Pbx1* expression in Zfp462$^{+/-}$ mice. *Pbx1* mRNA level in Zfp462$^{+/-}$ mice significantly decreased compared with that in WT mice ($t(10) = 3.405$, $P < 0.05$;

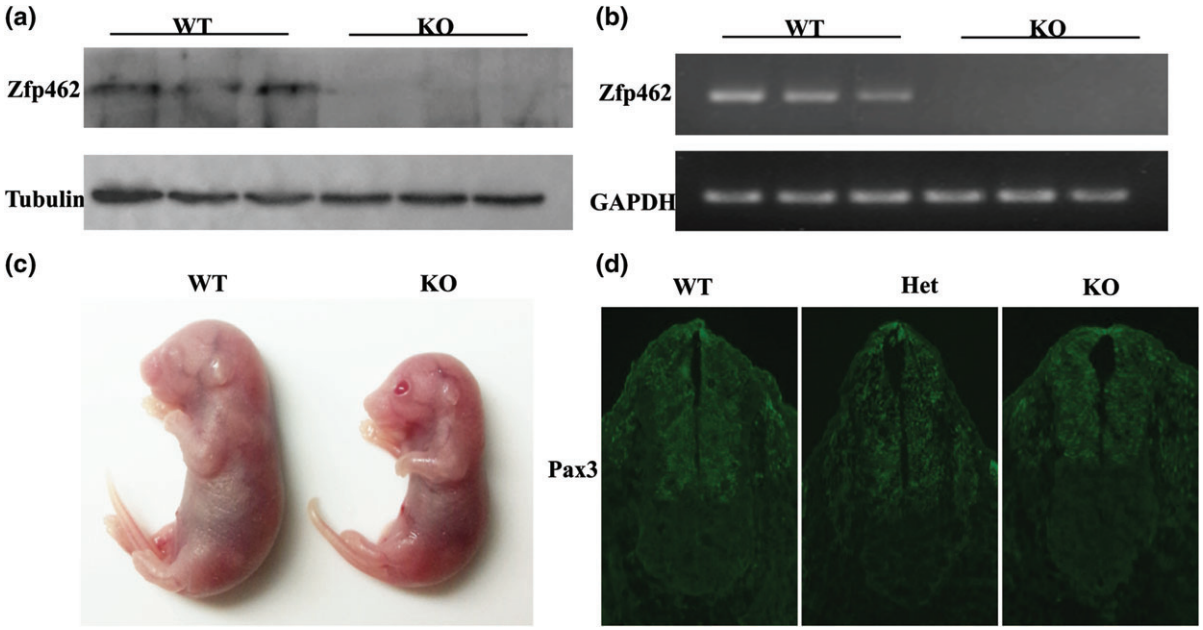

Figure 3: Zfp462 KO resulted in prenatal death in mice. Zfp462 protein expression was examined in Zfp462 KO mice and WT mice at E12.5 by western blot analysis (a). Zfp462 mRNA level was detected in Zfp462 KO brains and WT brains by reverse transcriptional PCR (b). Zfp462 KO embryos at E18.5 were dead with open eyes (c). Embryos from WT, Zfp462$^{+/-}$ and Zfp462 KO mice at E10.5 were cut into sections. Transcriptional factor Pax3 distribution near the neural tube was examined by fluorescent staining (d).

Fig. 5a). Consistent with mRNA expression, Pbx1 protein also markedly reduced in Zfp462$^{+/-}$ mice ($t(8) = 5.599$, $P < 0.05$; Fig. 5b,c). Fluorescent staining further showed the reduction of Pbx1 staining in the cortex in Zfp462$^{+/-}$ mice (Fig. 5d). To investigate whether Zfp462 potentially interacts with Pbx1 *in vivo* and plays a critical role in the brain development, we performed the immunoprecipitation of Pbx1 and Zfp462 from brain tissues. Immunoprecipitation results indicated that Pbx1 interacted with Zfp462 (Fig. 5e). In addition, Pbx1 protein expression had a similar pattern as Zfp462 in the time-course of brain development (Fig. S3). This suggested that Zfp462 might form a complex with Pbx1 and play an important role in the regulation of early brain development.

Since previous studies indicated that Pbx1 was a cofactor of Hoxb8 protein (Knoepfler *et al.* 2001; Neuteboom *et al.* 1995), we examined Hoxb8 expression in Zfp462$^{+/-}$ mice. Similar to Pbx1 expression, *Hoxb8* mRNA significantly decreased in Zfp462$^{+/-}$ mice compared with WT mice ($t(6) = 9.143$, $P < 0.05$; Fig. 5f). In addition, Pbx1 and Meis2 could form a complex with Hox proteins and enhance Hox function by improving the affinity of Hox proteins to specific promoter elements (Nestadt *et al.* 2012; Sagerstrom 2004). However, we did not find significant change with Meis2 in Zfp462$^{+/-}$ mice ($t(6) = 0.4420$, $P > 0.05$; Fig. S4).

Zfp462$^{+/-}$ mice exhibited anxiety-like behaviors with excessive self-grooming

We performed a series of behavioral experiments to characterize the phenotype of Zfp462$^{+/-}$ mice. In the elevated plus maze test, Zfp462$^{+/-}$ mice stayed for a longer time in the closed arm and a shorter time in the open arm than WT mice ($t(30) = 5.115$, $P < 0.05$; Fig. 6a). In the open field test, the total time spent in the inside area significantly decreased in Zfp462$^{+/-}$ mice ($t(30) = 4.339$, $P < 0.05$; Fig. 6b) and center entries also significantly decreased ($t(30) = 5.209$, $P < 0.05$; Fig. 6c) compared with WT mice. In the light–dark box test, light–dark transitions ($t(30) = 2.470$, $P < 0.05$; Fig. 6d) and the time spent in the light compartment ($t(30) = 2.608$, $P < 0.05$; Fig. 6e) significantly reduced in Zfp462$^{+/-}$ mice. In the marble-burying test, the total number of marbles buried in Zfp462$^{+/-}$ group was much more than that in WT group ($t(30) = 2.620$, $P < 0.05$; Fig. 6f). These results clearly indicated that Zfp462$^{+/-}$ mice had anxiety-like behaviors.

More interestingly, Zfp462$^{+/-}$ mice were found to have excessive self-grooming behaviors at 2 months and gradual hair loss in the back as seen in Fig. 4e and Video S1. In the self-grooming test, the grooming time significantly increased in Zfp462$^{+/-}$ mice ($t(30) = 3.518$, $P < 0.05$; Fig. 7a and Video S1); the number of bouts ($t(30) = 2.819$, $P < 0.05$; Fig. 7b), the average time per bout ($t(30) = 5.339$, $P < 0.05$; Fig. 7c) and the percent of interrupted grooming bouts ($t(30) = 2.245$, $P < 0.05$; Fig. 7d) also significantly increased in Zfp462$^{+/-}$ mice. Hair loss in the back was aggravated with age: about 15% and 30% of Zfp462$^{+/-}$ mice had hair loss at the age of 4 and 6 months, respectively. Hair loss was not found in WT littermates in the same cage (Video S1). These data showed that Zfp462$^{+/-}$ mice had hair loss and excessive self-grooming behaviors.

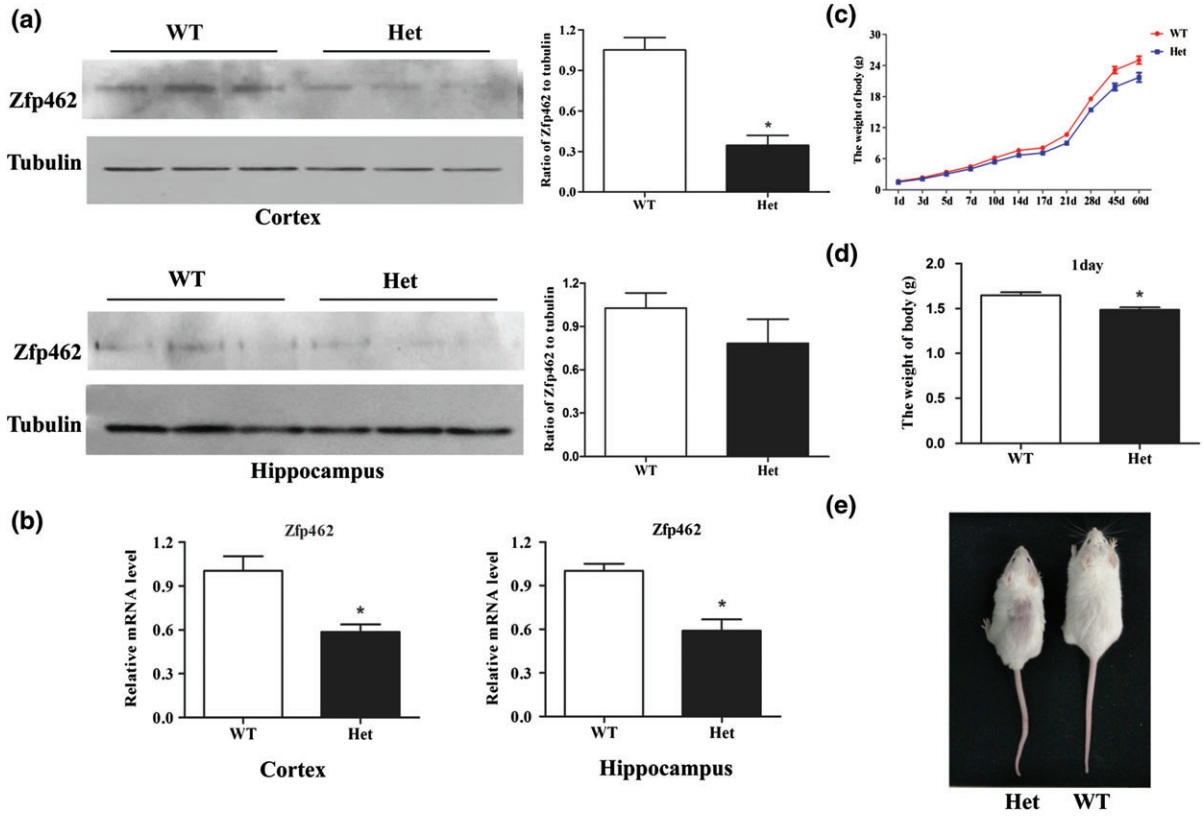

Figure 4: Zfp462$^{+/-}$ mice showed low Zfp462 expression and slow growth. Zfp462 protein expression in the cortex and the hippocampus from WT mice and Zfp462$^{+/-}$ mice was detected by western blot analysis. Tubulin was used as a loading control. Quantitative analysis for Zfp462 protein expression was also performed ($N = 3–4$, a). *Zfp462* mRNA level in the cortex and the hippocampus from WT mice and Zfp462$^{+/-}$ mice was examined by quantitative PCR ($N = 3–4$, b). Body weight was recorded from postnatal 1 day to 2 months and a growth curve of body weight was drawn ($N = 22–31$, c). Body weight was compared between WT mice and Zfp462$^{+/-}$ mice at postnatal 1 day ($N = 22–31$, d). Zfp462$^{+/-}$ mice showed gradual hair loss in the back (e). $^{*}P < 0.05$ vs. WT mice.

To rule out other factors that may affect the behaviors described above, we examined learning and memory ability, movement ability and depression-related tests. In Morris water maze test, no difference was found with the escape latency, the times crossing the platform area and the time spent in different quadrants between WT mice and Zfp462$^{+/-}$ mice ($P > 0.05$; Fig. S6). In the Rotarod test, the latency to fall was not different between the two groups ($t(30) = 0.267$, $P > 0.05$; Fig. S7a). In the forced swimming test and the tail suspension test, there was no difference for immobility time between WT and Zfp462$^{+/-}$ mice ($P > 0.05$; Fig. S7b,c).

IM attenuated anxiety-like and excessive self-grooming behaviors in Zfp462$^{+/-}$ mice

To further show the rescue effect on behaviors in Zfp462$^{+/-}$ mice, we administrated IM, one of the commonly used anti-anxiety medications. After IM treatment for 3 weeks, we found that anxiety-like behaviors and excessive self-grooming behaviors in Zfp462$^{+/-}$ mice were attenuated. In the open field test, IM treatment significantly increased center entries ($F_{1,28} = 15.17$, $P < 0.05$; Fig. 8a) and the total time spent in the inside area in Het + IM mice ($F_{1,28} = 16.70$, $P < 0.05$; Fig. 8b) compared with Het + NS mice. Similarly, in the elevated plus maze test, IM treatment significantly increased the time spent in the open arm in Het + IM mice compared with Het + NS mice ($F_{1,28} = 4.539$, $P < 0.05$; Fig. 8c). In the marble-burying test, IM significantly reduced the total buried marbles in Het + IM mice compared with Het + NS mice ($F_{1,28} = 5.987$, $P < 0.05$; Fig. 8d). Finally, IM treatment also markedly reduced the grooming time in Zfp462$^{+/-}$ mice ($F_{1,28} = 5.249$, $P < 0.05$; Fig. 8e and Video S2). In addition, after IM treatment for 3 weeks, no significant changes were found in hair loss (data not shown) and body weight (Fig. S8) in Zfp462$^{+/-}$ mice.

Discussion

Generalized anxiety disorder (GAD) is a common chronic mental disorder characterized by persistent anxiety and excessive uncontrollable worry accompanying with

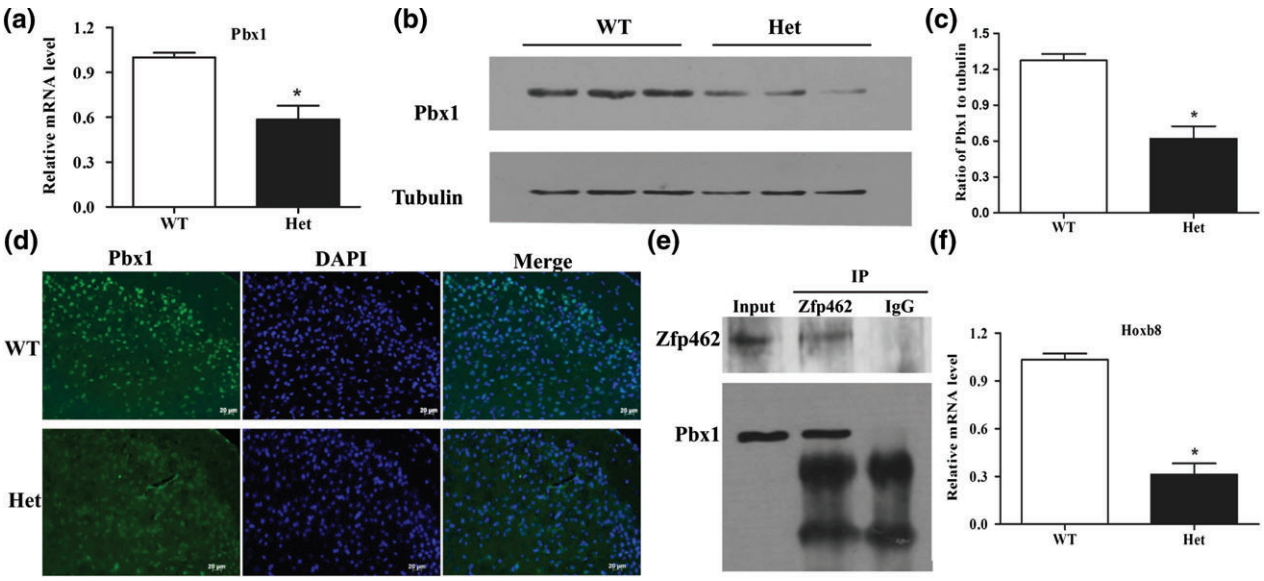

Figure 5: Zfp462$^{+/-}$ mice had lower expression of Pbx1 and Hoxb8. Pbx1 mRNA level in the orbitofrontal cortex from WT mice and Zfp462$^{+/-}$ mice at 2 months was examined by quantitative PCR (a). Zfp462 protein expression in the orbitofrontal cortex from WT mice and Zfp462$^{+/-}$ mice was detected by western blot analysis (b). Quantitative analysis for Pbx1 protein expression was performed (c). Pbx1 expression was detected in the orbitofrontal cortex in WT mice and Zfp462$^{+/-}$ mice by fluorescent staining (d). To show the interaction of Zfp462 and Pbx1, co-immunoprecipitation experiment was carried out (e). *Hoxb8* mRNA level in the cortex from WT mice and Zfp462$^{+/-}$ mice at 2 months was examined by quantitative PCR (f). $^*P < 0.05$ vs. WT mice. $N = 4–5$.

non-specific psychological and physical symptoms (Stein & Sareen 2015). Patients with GAD often complain many symptoms including the difficulty to sleep or concentrate, restlessness, irritability, fatigue/exhaustion, rapid heartbeat, muscle tension and limb numbness. Risk of GAD within life is estimated at 2.6–5.1% (Kamrowska & Gmitrowicz 2016) and the neural mechanisms underlying its symptoms remain unclear. To address the mechanisms of GAD, many animal models of GAD were proposed (Bourin 2015). In this study, we provided a novel genetic mouse model of GAD with anxiety-like behaviors by using a PB transposon system to disrupt *Zfp462* gene. At the same time, we showed that *Zfp462* is an essential protein for mouse survival and embryonic development.

Zfp462 protein is required for mouse embryonic development

Although some studies indicated that Zfp462 is involved in cell survival/pluripotency and early *Xenopus laevis* development (Laurent *et al.* 2009b, Masse *et al.* 2011), the functions of Zfp462 protein in the CNS of rodent animals are still unclear. Among different tissues, the whole brain had the highest level of protein expression (Fig. 1a,b), suggesting the important role of Zfp462 protein in the CNS. The cortex, a critical region associated with many psychiatric disorders, is the most abundant brain region in adult mouse brains (Fig. 1c,d), which is consistent with previous study that Zfp462 is highly expressed at embryonic mouse cerebral cortex (Chang *et al.* 2007). These results indicated that Zfp462 protein might be related to the development of the CNS in mice. Because Zfp462 is expressed in the anterior neuroectoderm and the neural tube through transverse section at E8.25 embryo (Chang *et al.* 2007), therefore, Zfp462 was thought to regulate the development of the neural tube (Chang *et al.* 2007). However, our study showed that Zfp462 KO did not affect neural tube patterning by the staining of patterning markers including Pax3, Arl13b, ISL1/2, Olig2 and Shh (Figs. 3d,S1). Interestingly, Zfp462 KO embryos were found dead with open eyes at E18.5 (Fig. 3c). Therefore, Zfp462 protein is essential for embryonic development as Zfp462 KO caused embryonic lethality in mice. The cause of death in the Zfp462 KO embryos is currently unclear and needs further investigation. In addition, Zfp462$^{+/-}$ mice showed delayed growth and reduced body weight and brain weight (Fig. 4). These findings suggest that Zfp462 protein may regulate embryonic and growth development.

Zfp462$^{+/-}$ mice is a novel genetic mouse model of anxiety disorders

Because Zfp462 KO causes prenatal death in mice, a panel of behavioral tests was performed in Zfp462$^{+/-}$ mice and WT mice. Zfp462$^{+/-}$ mice presented anxiety-like behaviors in anxiety-related tests such as open field test, the elevated plus maze test, the light–dark box test and the marble-burying test (Fig. 6). All these tests supported anxiety-like behaviors in this genetic mouse model. When animals were exposed to different stimuli or stressors (novelty, bright light or elevated space), these behavioral testing paradigms are proposed to model human pathological anxiety and provide a degree of face validity for animal models of anxiety (Ohl 2005; Rodgers

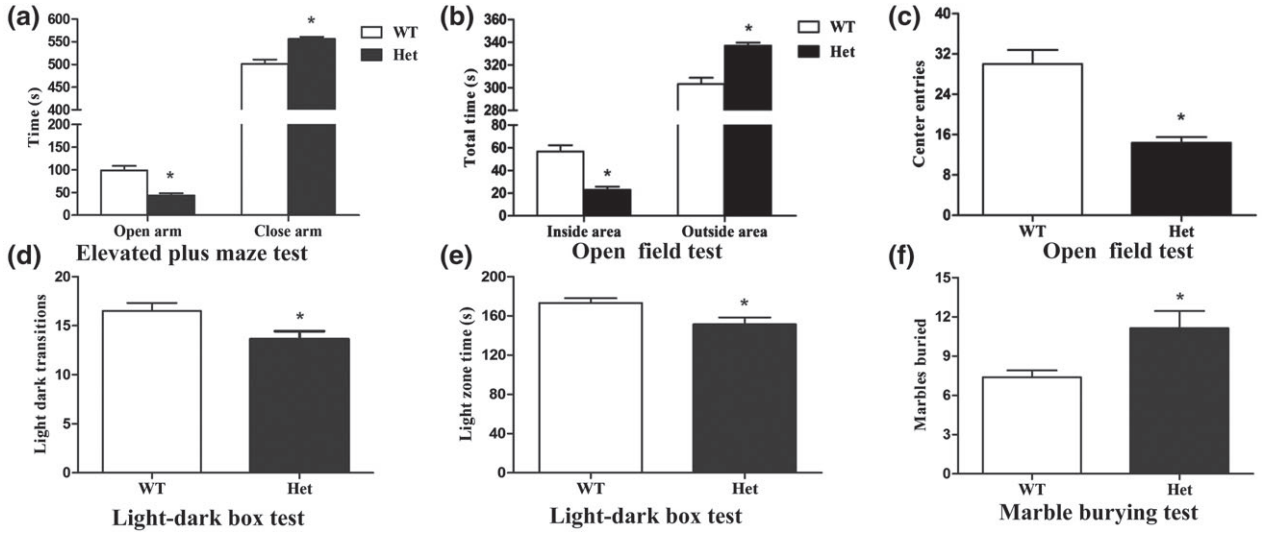

Figure 6: Zfp462$^{+/-}$ mice showed anxiety-like behaviors. Behavioral tests were performed in 2–3-month-old mice. In the elevated plus maze test, the time spent in open arms and closed arms was recorded (a). In the open field test, the total time spent in the inside area was recorded (b); center entries was also recorded (c). In the light–dark box test, the light–dark transitions (d) and the time spent in the light compartment (e) were recorded. In the marble-burying test, the total number of buried marbles was recorded (f). $*P < 0.05$ vs. WT mice. $N = 16–18$.

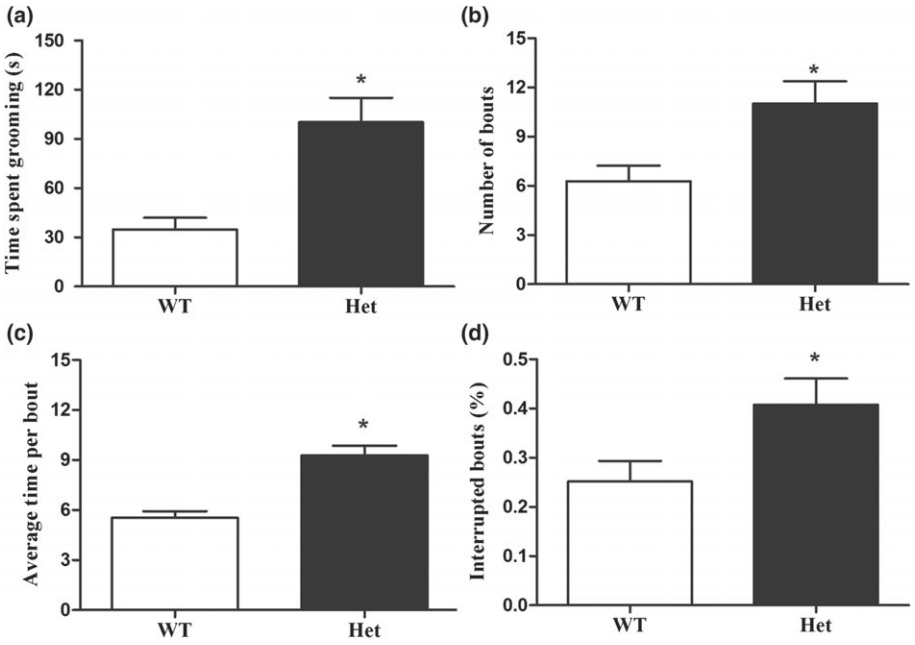

Figure 7: Zfp462$^{+/-}$ mice showed excessive self-grooming behaviors. Self-grooming tests were performed in 2–6-month-old mice. In a 10-min self-grooming test, the grooming time (a), the average grooming time per bout (b), the total number of bouts (c) and the percent of interrupted grooming bouts (d) in Zfp462$^{+/-}$ mice and WT mice were recorded. $*P < 0.05$ vs. WT mice. $N = 16–18$.

et al. 1997). At the same time, these anxiety-like behaviors in Zfp462$^{+/-}$ mice were partially reversed by chronic treatment with IM, a widely used anxiolytic medication (Fig. 8). Behavioral responses to anxiolytics in Zfp462$^{+/-}$ mice mimicked clinical effectiveness of pharmacological agents in patients with GAD, confirming the predictive validity in Zfp462$^{+/-}$ mice. In addition, Zfp462 protein expression decreased in the orbitofrontal cortex and the amygdala (Figs. 4a,S5), two critical brain regions for modulating anxiety-related behaviors (Milad & Rauch 2007; Shin & Liberzon 2010; Sladky *et al*. 2015), but not in the hippocampus. Interestingly, the learning and memory ability, which is related to the functions of the hippocampus, in Zfp462$^{+/-}$ mice was not affected (Fig. S6). Therefore, the decrease of Zfp462 protein in the orbitofrontal cortex and the amygdala is considered as a possible cause of the functional disruption in these areas, providing the evidences of the construct validity for animal models of anxiety. Zfp462$^{+/-}$ mice as a putative animal model of anxiety showed

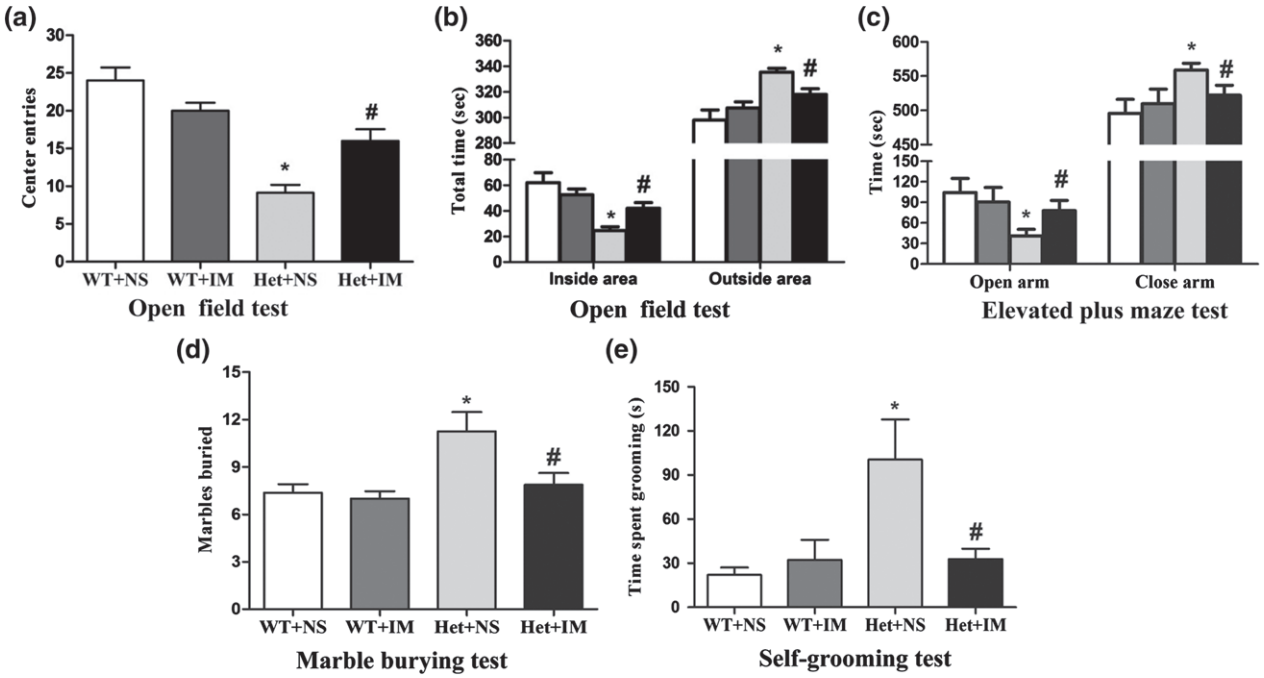

Figure 8: IM attenuated anxiety-like behaviors in Zfp462$^{+/-}$ mice. After 3 weeks of treatment with IM or NS, behavioral tests were performed in Zfp462$^{+/-}$ mice (Het) and WT mice. In the open field test, the center entries (a) and the total time spent in the inside area were recorded (b). In the elevated plus maze test, the time spent in the open arms and the closed arms was recorded (c). In the marble-burying test, the total number of buried marbles (d) was recorded. In self-grooming test, the grooming time in two groups was recorded (e). *$P < 0.05$ vs. WT + NS; #$P < 0.05$ vs. Het + NS. $N = 8$.

its validity on three levels including face validity, construct validity and predictive validity (Bourin 2015). This suggests that Zfp462$^{+/-}$ mice thus provide a useful model for potentially exploring the molecular mechanisms of anxiety disorders and evaluating novel anti-anxiety treatments for its stable phenotypes. In addition, more investigation should be carried out to confirm the association between *ZFP462* gene and anxiety disorders in patients.

More interestingly, Zfp462$^{+/-}$ mice also have hair loss and excessive self-grooming behaviors. This indicated that Zfp462$^{+/-}$ mice have obsessive-compulsive behaviors because excessive self-grooming is considered a feature of some forms of OCD (Bienvenu *et al.* 2009; Greer & Capecchi 2002). The OCD is another chronic, serious and highly prevalent psychiatric disorder that affects between 1.5% and 3% of people all over the world (Ahmari 2016; Stein *et al.* 2000), which is characterized by intrusive disturbing thoughts and repetitive behaviors. Different genetic models of OCD proposed previously were mainly based on excessive self-grooming behaviors (Greer & Capecchi 2002; Shmelkov *et al.* 2010; Welch *et al.* 2007). Compared with these animal models of OCD, Zfp462$^{+/-}$ mice share similar symptoms with Slitrk5 KO mice and Sapap3-mutant mice, which have both anxiety-like behaviors and excessive self-grooming behaviors (Shmelkov *et al.* 2010; Welch *et al.* 2007); however, Zfp462$^{+/-}$ mice had mild symptoms without skin lesions. Contrast with other genetic models of OCD, Hoxb8 KO mice were only reported to have compulsive hair-pulling behaviors and severe skin lesions (Greer & Capecchi 2002). As a partner protein of Zfp462, Hoxb8 expression significantly decreased in Zfp462$^{+/-}$ mice. Compared with Hoxb8 KO mice, Zfp462$^{+/-}$ mice had mild symptoms: (1) excessive self-grooming behaviors were observed at the age of 2 months; (2) hair loss was found at the age of 4 months in 15% Zfp462$^{+/-}$ mice; (3) no skin lesions were found and (4) WT littermates had no hair loss. Importantly, although reduced body weight and hair loss were not reversed by IM treatment for 3 weeks (Fig. S8), but excessive self-grooming behaviors were significantly attenuated (Fig. 8). Therefore, Zfp462$^{+/-}$ mice may be considered a potential mouse model of OCD. However, in current clinical studies, no evidences were found to support the association of *ZFP462* gene with OCD in patients. In addition, a recent review extensively showed that self-grooming in animals is an innate programed behavior that is controlled by a complex neural circuitry and abnormal self-grooming behaviors are observed in many animal models of different neuropsychiatric disorders (Kalueff *et al.* 2016); different disease models are expected to have distinct grooming phenotypes (Kalueff *et al.* 2016). Mice with anxiety-like behaviors display an increase in the amount of time spent grooming with an impaired self-grooming patterning; however, mice with OCD-like phenotype show highly rigid and repetitive grooming patterning (Kalueff *et al.* 2016). Therefore, according to the analysis of grooming microstructure, anxiety-like behavior was further confirmed. Zfp462$^{+/-}$

mice presented more grooming bouts and higher percentage of interrupted grooming than control mice (Fig. 7b,c), indicating an impaired grooming patterning but not a rigid and repetitive grooming patterning in Zfp462$^{+/-}$ mice. These findings showed that excessive self-grooming behaviors in Zfp462$^{+/-}$ mice were a part of symptoms of anxiety-like behaviors.

In addition, we found that Zfp462$^{+/-}$ mice had no deficits in memory and movement (Figs. S6,S7). Also, this model of anxiety did not coexist with depression-related behaviors (Fig. S7). Therefore, these findings ruled out the possibility that anxiety-like behaviors were caused by such factors.

Possible mechanisms underlying the behavioral phenotypes in Zfp462$^{+/-}$ mice

So far, the precise circuits underlying anxiety behaviors have not been established because of its complex network; however, the increasing line of evidences showed that anxiety behaviors are mediated by local and long-range connections between multiple brain areas such as the medial prefrontal cortex, basolateral amygdala, bed nucleus of the stria terminalis and the hypothalamus as outlined in a recent review (Tovote *et al.* 2015). In this study, we found that Zfp462 protein expression decreased in the cortex and the amygdala but not in the hippocampus (Fig. 4a); but how Zfp462 deficiency is linked to anxiety-like behaviors still needs more investigations. As hippocampus is known to be responsible for the functions of the learning and memory (Lazarov & Hollands 2016), stable protein expression of Zfp462 in hippocampus was consistent with normal learning and memory abilities in Zfp462$^{+/-}$ mice (Fig. S6). Therefore, we speculate that the significant decrease of Zfp462 protein expression in the cortex and amygdala is responsible for anxiety-like behaviors in Zfp462$^{+/-}$ mice. As a putative transcriptional factor, Zfp462 deficiency may influence the transcription of downstream targets or destroy the stability of its cofactors, thus disrupting the functions of these anxiety-related nuclei in the cortex and amygdala or the connections with other brain regions. The mRNA expression of Pbx1 and Hoxb8, two partner proteins of Zfp462, also decreased in Zfp462$^{+/-}$ mice, suggesting that Pbx1 and Hoxb8 might be the downstream regulative targets. At the same time, Zfp462 interacts with Pbx1 *in vivo*, which was confirmed by immunoprecipitation (Fig. 5e) and supported the previous study *in vitro* (Laurent *et al.* 2007). Furthermore, Pbx1 forms a complex with Hoxb8 proteins through Pbx1-interaction motif in Hoxb8 protein (Knoepfler *et al.* 2001; Neuteboom *et al.* 1995). Therefore, Zfp462 may form a multiple protein complex with Pbx1 and Hoxb8. Interestingly, Hoxb8 KO mice have severe OCD-like behaviors (Greer & Capecchi 2002). Therefore, the decrease of Hoxb8 and Pbx1 by Zfp462 deficiency may be the cause of anxiety-like behaviors in this animal model.

Conclusions

Zfp462 deficiency caused developmental abnormalities and anxiety-like behaviors with excessive self-grooming in mice. Decreased expression of Zfp462, Pbx1 and Hoxb8 in Zfp462$^{+/-}$ mice may account for anxiety-like behaviors. Zfp462$^{+/-}$ mice provide a useful model of anxiety for determining potential therapeutic targets of anxiety disorders and screening anti-anxiety drugs.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Methods.

Table S1: Primers used in this study.

Figure S1: The neural tube patterning of Zfp462 genotype mice. Embryos were collected, fixed and embedded at E10.5. The distribution of transcriptional factors including Arl13b, ISL1/2, Olig2 and Shh in the neural tube was examined by fluorescent staining.

Figure S2: The brain weight in Zfp462$^{+/-}$ mice and WT mice. Whole brains from Zfp462$^{+/-}$ mice and WT mice at 2 months were weighed by electronic balance. $*P < 0.05$ vs. WT mice. $N = 5–6$.

Figure S3: Time-courses of Pbx1 expressed in mouse brains. Whole mouse brains at different ages (from E7.5 to 3 months) were collected and Pbx1 protein expression was examined by western blot analysis (a). Quantitative analysis for Pbx1 protein expression was performed (b). $N = 3–4$.

Figure S4: Meis2 mRNA level in Zfp462$^{+/-}$ mice and WT mice. Meis2 mRNA level in the cortex from WT mice and Zfp462$^{+/-}$ mice was examined by quantitative PCR. $*P < 0.05$ vs. WT mice. $N = 4–5$.

Figure S5: Zfp462 protein expression was examined in the amygdala of WT mice and Zfp462$^{+/-}$ mice. Zfp462 protein level in the amygdala from WT mice and Zfp462$^{+/-}$ mice was examined by western blot analysis (a). Quantitative analysis for Zfp462 protein expression was performed (b). $*P < 0.05$ vs. WT mice. $N = 3–4$.

Figure S6: Zfp462$^{+/-}$ mice did not show memory deficiency. In the Morris water maze test, the escape latency (a), time spent in target quadrant (b), the times taken to cross the platform area (c) and time spent in the other three quadrants (d–f) were recorded. $N = 16–18$.

Figure S7: Zfp462$^{+/-}$ mice did not exhibit movement deficiency and depression-like behaviors. In the rotarod

test, latency to fall time was recorded (a). The immobility time in the forced swimming test (b) and the tail suspension test (b) was recorded. $N = 16–18$.

Figure S8: The body weight in Zfp462$^{+/-}$ mice and WT mice after IM treatment. After treatment with IM or NS for 3 weeks, the body weight in Zfp462$^{+/-}$ mice and WT mice was weighed by electronic balance. $^{*}P < 0.05$ vs. WT + NS; N.S., not significant. $N = 15–16$.

Video S1: Self-grooming behaviors in WT mice (left cage) and Zfp462$^{+/-}$ mice (right cage).

Video S2: Self-grooming behaviors in Zfp462$^{+/-}$ mice with (right cage) or without (left cage) IM for 3 weeks.