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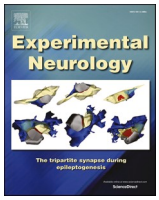

Research paper

FGF binding protein 3 is required for spinal cord motor neuron development and regeneration in zebrafish

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ABSTRACT

Fibroblast growth factor binding protein 3 (Fgfbp3) have been known to be crucial for the process of neural proliferation, differentiation, migration, and adhesion. However, the specific role and the molecular mechanisms of *fgfbp3* in regulating the development of motor neurons remain unclear. In this study, we have investigated the function of *fgfbp3* in morphogenesis and regeneration of motor neuron in zebrafish. Firstly, we found that *fgfbp3* was localized in the motor neurons and loss of *fgfbp3* caused the significant decrease of the length and branching number of the motor neuron axons, which could be partially rescued by *fgfbp3* mRNA injection. Moreover, the *fgfbp3* knockdown (KD) embryos demonstrated similar defects of motor neurons as identified in *fgfbp3* knockout (KO) embryos. Furthermore, we revealed that the locomotion and startle response of *fgfbp3* KO embryos were significantly restricted, which were partially rescued by the *fgfbp3* overexpression. In addition, *fgfbp3* KO remarkably compromised axonal regeneration of motor neurons after injury. Lastly, the malformation of motor neurons in *fgfbp3* KO embryos was rescued by overexpressing *drd1b* or *neurod6a*, respectively, which were screened by transcriptome sequencing. Taken together, our results provide strong cellular and molecular evidence that *fgfbp3* is crucial for the axonal morphogenesis and regeneration of motor neurons in zebrafish.

1. Introduction

Growth factors have crucial cellular functions in the regulation of variety of physiological processes, such as proliferation, differentiation, migration and adhesion in the nervous system, epithelium, and bone. As the representative and conservative family of growth factors, fibroblast growth factors (FGFs), are widely expressed in different organisms taking part in the regeneration and differentiation of many tissues by binding different fibroblast growth factor receptors. FGFs also have the potential effects on tissue damage, repair, and homeostasis maintenance (Moya et al., 2010; Yun et al., 2010). Twenty-two FGFs, containing four intracellular FGFs and eighteen secreted FGFs, have been identified from humans and rodents, which comprise series of structurally similar polypeptide mitogens (Hanneken et al., 1994; Loughnan et al., 1988). Studies have shown that FGFs-mediated signaling pathways are involved in regulating the development of the central and peripheral nervous systems (Alzheimer and Weme, 2003; Yun et al., 2010). It has been reported that FGF1 is mainly expressed in neurons to regulate learning, memory and neuroprotection. While FGF2, FGF3 and FGF8 are involved in mediating neurite sprouting and neurogenesis during development and neuronal regeneration process after spinal cord injury (Goldshmit et al., 2018; Li et al., 2010). It has been shown that chronic exogenous recombinant human FGF9 microinjection in mice could induce depression and anxiety behaviors, but reducing FGF9 expression in the dentate gyrus of the hippocampus with a lentiviral vector could significantly ameliorate spontaneous anxiety behavior (Aurbach et al., 2015).

The mobilization of FGFs is crucial for the bioactivation and function. It is widely believed that the fibroblast growth factor-binding protein (Fgfbp) serves as a carrier protein for this process by reversibly binding FGFs to increasing its bioavailability (Abuharbeid et al., 2006; Taetzsch et al., 2018). Biochemical research uncovered that Fgfbps might have three different binding models to release the FGFs. The first model is that Fgfbps and heparan sulfate bind with FGFs

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asynchronously and Fgfbps can combine near the binding site of heparan sulfate in FGFs to release bioactive FGFs in the extracellular matrix. The second model is that Fgfbps directly bind FGFs before interacting with heparan sulfate proteoglycans (Kamimura et al., 2013; Mongiat et al., 2001; Singhal and Martin, 2011; Taetzsch et al., 2018). The third model is that Fgfbps can cause heparan sulfate proteoglycans degradation by sulfatases, heparanases and other proteases to release the FGFs, and protect them from proteolysis during their migration to FGF receptors (Taetzsch et al., 2018).

The Fgfbps are the family of structurally similar proteins with the FGF and heparin-binding site in C-terminal sequence, which were firstly isolated from human epidermoid carcinoma *in vitro* culture medium (Xie et al., 2006). Three Fgfbps (Fgfbp1, 2 and 3) in humans and zebrafish are different from the rodents who have only two Fgfbps (Fgfbp 1 and 2) (Gibby et al., 2009; Wu et al., 1991). Fgfbp3 is a secretory chaperone protein and plays a role in signal transduction of FGFs regulating many physiological processes. A previous study has demonstrated that human FGFBP3 can bind FGF2 to affect its actions and transient overexpression of human *fgfbp3* in chicken embryos lead to significantly increased vascular permeability, which could be restrained by treating with FGF receptor kinase inhibitor (Zhang et al., 2008). In addition, Fgfbp3 also has a high affinity with FGF19 to modulate glucose and fat metabolism. Knockout of *fgfbp3* in mice can cause a metabolic syndrome by changing lipid metabolism pathways with reduced hepatic triglycerides (Tassi et al., 2018). Moreover, *fgfbp3* is reported to be highly expressed in central nervous systems and is also necessary for the neuronal survival and differentiation of the brain (Dono et al., 1998; Sanchez-Calderon et al., 2007). In mice, the deficiency of *fgfbp3* could induce the anxiety-related behaviors, such as altering the time in the central area of the open-field arena and exhibiting less activity during the light/dark transition test and so on (Yamanaka et al., 2011). Additionally, over-expression of FGF2 in the dorsal root ganglia and spinal cord in mice specifically increased the number of motor neurons and further induced axonal regeneration and growth after nerve injury (Allodi et al., 2014). Similarly, FGF2 could also promote motor neuron axonal arborization and elongation in *in vitro* cultured spinal cord (Allodi et al., 2013). Moreover, a high expression level of *fgfbp3* was also detected in the brain and motor neuron during the early stage of zebrafish embryo development (Li et al., 2018). Therefore, as the chaperone protein of FGF2, whether *fgfbp3* plays a role in the formation and development of spinal cord motor neurons has yet to be investigated.

Motor neurons of the spinal cord in vertebrates have been precisely classified by soma shape and location, axon trajectory, and target muscle innervation (Lewis and Eisen, 2003). In zebrafish, two different kinds of spinal motor neurons, primary motor neurons and secondary motor neurons, have been identified. Furthermore, three distinct primary motor neuron groups, caudal primary motor neurons (CaP), rostral primary motor neurons (RoP) and middle primary motor neurons (MiP), are differentiated according to the axonal trajectory and only three individually primary motor neurons were identified in each spinal segment (Myers, 1985; Myers et al., 1986). CaPs are well-studied because their axons first grow from spinal cord to the target ventral axial muscle (Myers, 1985; Rodino-Klapac and Beattie, 2004). In this study, in a large-scale expression profile detection by *in situ* hybridization, we verified *fgfbp3* expressed in the central nervous system of zebrafish embryos, examined the function of *fgfbp3* during the development of primary motor neurons in the spinal cord by both knockdown and knockout techniques in different transgenic zebrafish, and investigated the possible mechanisms during this process.

2. Materials and methods

2.1. Zebrafish husbandry and strain

The zebrafish embryos and adults were maintained in the zebrafish Center of Nantong University under conditions in accordance with our previous protocols (Wang et al., 2016). The larvae were grown in E3 medium before imaging. Wild type and transgenic zebrafish line, *Tg (mnx1:GFP)^{ml2}* and *Tg(mnx1:GAL4;UAS:nTR-mCherry)*, were used in this study.

Table 1
Summary of primer pairs used for the study.

Primer name	Primer sequence
fgfbp3 ISH-Forward	5'-TCCCTTCTCTTCCTCCTCCA-3′
fgfbp3 ISH-Reverse	5'-ACCCCGTCATCTTCGTCTTT-3'
fgfbp3 sgRNA-Forward	5'-TAATACGACTCACTATAGGCGTGAAAACGC CTGTGAAGTTTTAGAGCTAGAAATAGC-3'
fgfbp3 test-Forward	5'-TTTCTCCTTTCCCTTCTCTTCCT-3'
fgfbp3 test-Reverse	5'-CACATTCTCACTCTTCACCCGAT-3'
fgfbp3 mRNA-Forward	5'-CCGCTCGAGCTGTGTGTGCCCTAGGGTTT-3'
fgfbp3 mRNA-Reverse	5'-GCTCTAGATCACTCTTCACCCGATCCGA-3'
fgfbp3 qRT-PCR-Forward	5'-TCCTGAAAACCCGCCTGTG-3'
fgfbp3 qRT-PCR-Reverse	5'-GGAACATCAGTTTTCCGTTTGCT-3'
neurod6a mRNA-Forward	5'-CGGAATTCCACGAGCTGTTCACCTGCTA-3'
neurod6a mRNA-Reverse	5'-GCTCTAGAAAACCCAGAATCCGAGACGG-3'
neurod6a qRT-PCR-Forward	5'-CAACGCGAGAGAGAGGAACC-3'
neurod6a qRT-PCR-Reverse	5'-GTAATTTTTGGCCAGCCGCA-3'
drd1b mRNA-Forward	5'-CGGAATTCTGACTGCTAACTCGACTCGG-3'
drd1b mRNA-Reverse	5'-GCTCTAGAGGTCCTCAGTGAAGCTTTTGG-3'
drd1b qRT-PCR-Forward	5'-TTCGCCATCTGCGCTCTAAA-3'
drd1b qRT-PCR-Reverse	5'-AACCTACAATCTCCGTGGCG-3'
ef1α qRT-PCR-Forward	5'-CTTCAACGCTCAGGTCATCA-3'
ef1α qRT-PCR-Reverse	5'-CGGTCGATCTTCTCCTTGAG-3'
neurod2 qRT-PCR-Forward	5'-CCTTGGCGACGAGGACTTAG-3'
neurod2 qRT-PCR-Reverse	5'-TTTGGGTCTGTCTCCATCACC-3'
ccdc40 qRT-PCR-Forward	5'-TGGATACCACAGCACTCCCT-3'
ccdc40 qRT-PCR-Reverse	5'-TTCCGGATCCAACACCACAA-3'

2.2. Whole mount in situ hybridization

Digoxigenin-labeled sense and anti-sense RNA probes were synthesized using linearized pGEM-T-easy vector subcloned with *fgfbp3* fragment by *in vitro* transcription with DIG-RNA labeling Kit according to manufacturer's protocol (Roche Applied Science). Whole-mount *in situ* hybridization and sectioning were performed as previously described (Gong et al., 2018). Pictures were taken with an Olympus DP70 camera on an Olympus stereomicroscope MVX10.

2.3. Cell Separation, RNA isolation, RNA sequencing and quantitative RT-PCR

300–400 *Tg(mnx1:GFP)* zebrafish embryos were prepared at 24 hpf and washed three times by PBST followed three washes by Ringer's solution without Ca^{2+}. After being trypsinized by 0.25% trypsin, the reaction was terminated by 10% FBS and filtered by 100 and 40 μm filter membranes. The samples were analyzed using a flow cytometer (BD, Franklin Lakes, NJ). The cells with GPF fluorescence were identified as positive cells and were selected for the RNA extraction and cDNA synthesis.

The brain and spinal cord of wild type and *fgfbp3* adult KO zebrafish were dissected and sampled. Embryos of wild type and *fgfbp3* KO zebrafish at 24, 48, 72 and 96 h post fertilization (hpf) were also sampled and grinded immediately to the TRIzol for later total RNA extraction. The TRIzol reagent was used according to the manufacturer's instructions (Invitrogen, USA). DNase I was then used to remove residual genomic contaminations. The RNA was subsequently quantified by a ND-2000 NanoDrop UV spectrophotometer (nanoDrop Technologies, Inc., USA) and high-quality RNA samples at 48 hpf (OD260/280 ranged 1.8–2.2, RIN $\geq$8.0) were used to construct the sequencing library for the transcriptome sequencing. The final sequencing cDNA libraries were quantified and sequenced using the Illumina HiSeq 4000 with 150 bp pair-end reads produced (Illumina, USA).

2 μg total RNA was reversely transcribed using a reversed first strand cDNA synthesis kit (Fermentas, USA). Quantitative RT-PCR was

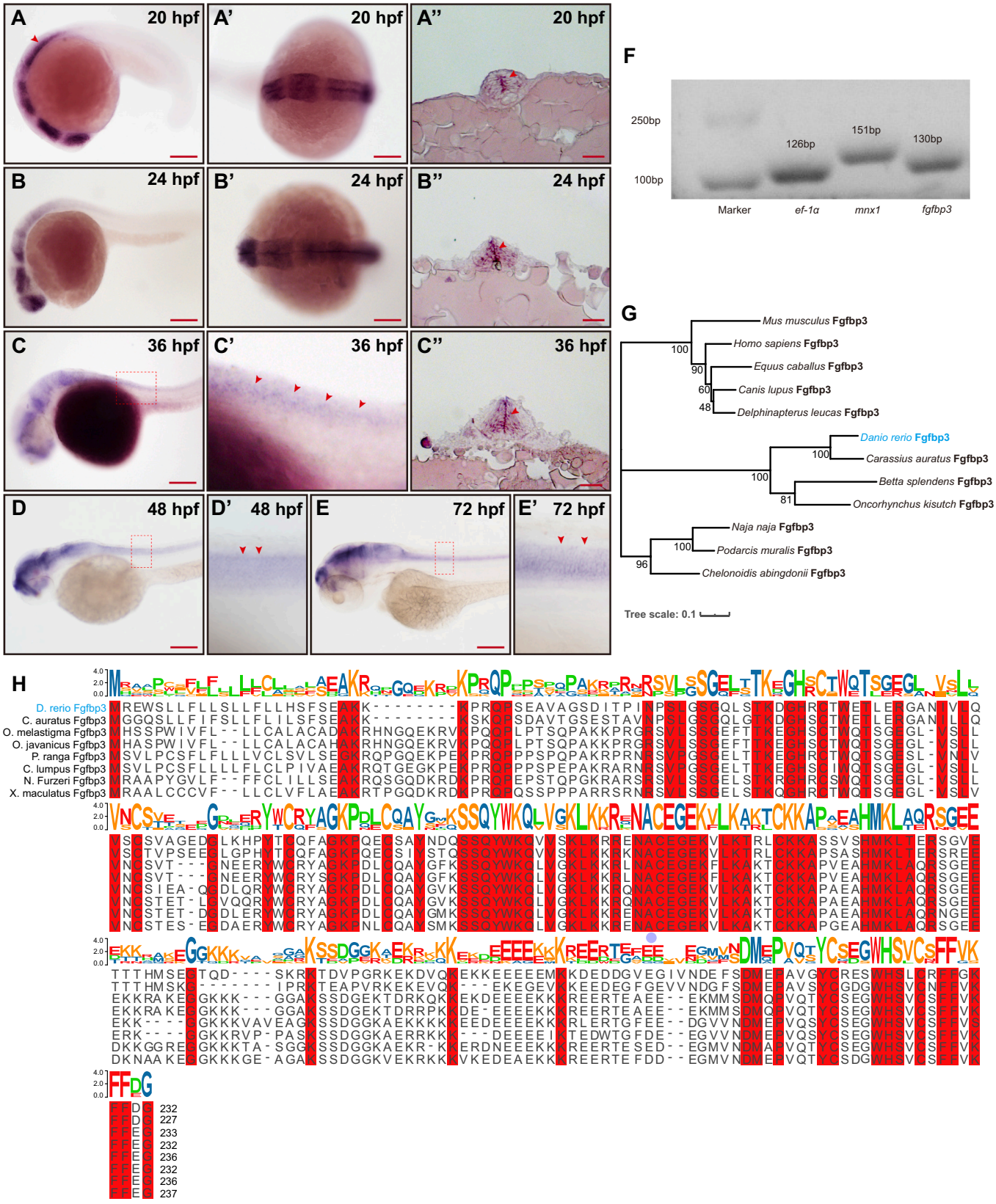

Fig. 1. The expression and sequence analysis of *fgfbp3*. A-E. Localization of *fgfbp3* mRNA by *in situ* hybridization in the zebrafish embryo at different development stages. A' and B': dorsal view of A and B. A", B" and C": The trunk transverse section of the embryos. C'-E': The magnified figure of the region squared in the dashed line of C-E. Scale bar = 200 μm. F. The results of the RT-PCR in the Mnx1-GFP sorted cells. *fgfbp3* is expressed in the selected neuron cells from the *Tg(Mnx1:EGFP)* line, mnx1 gene was used as a control. G. Phylogenetic analysis of Fgfbp3. Neighbor-joining tree was produced with the Mega 5.0 software. H. The alignment of Fgfbp3 amino acid sequences from different species. The identical amino acid residues among all the aligned sequences are shaded in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

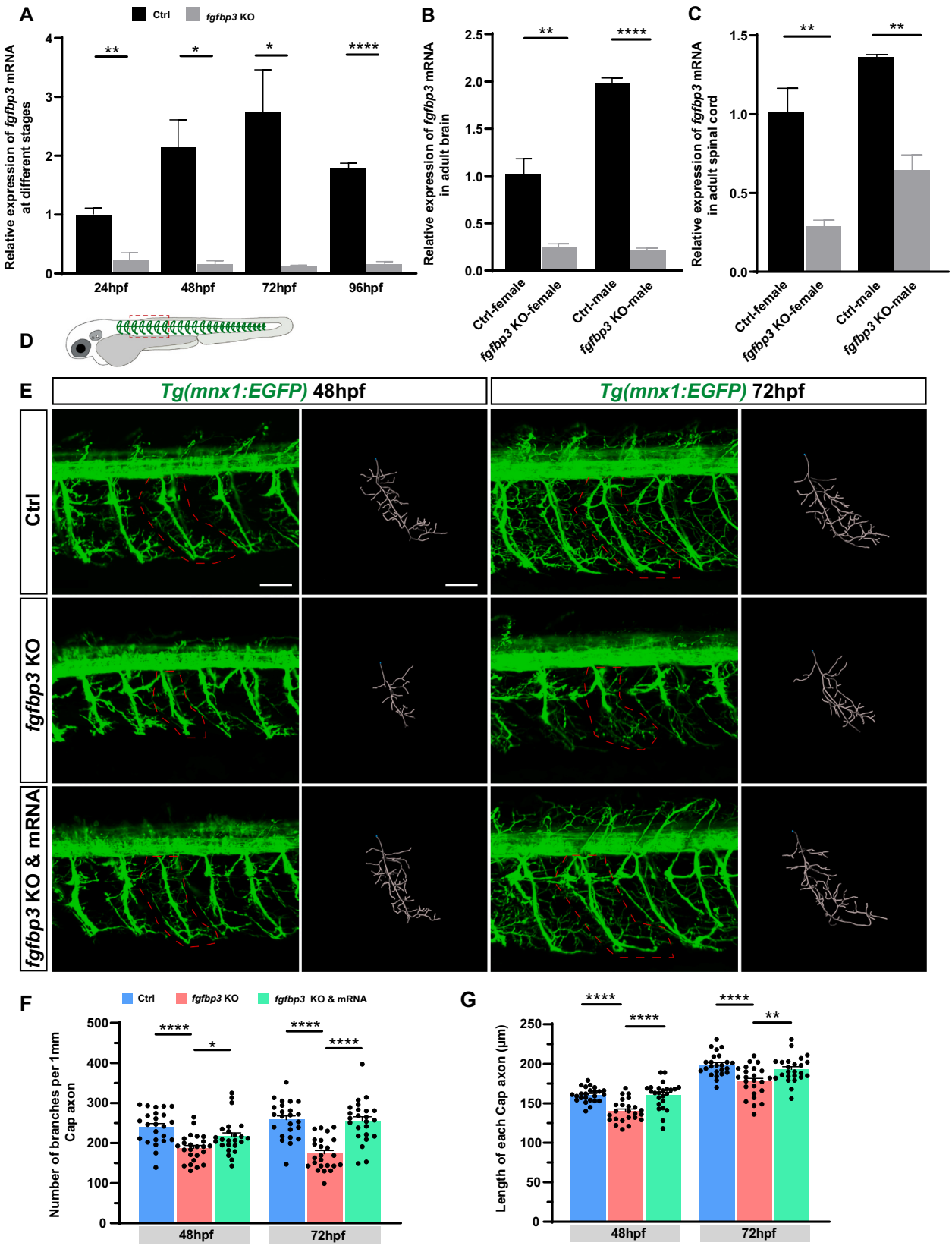

Fig. 2. Knockout of *fgfbp3* caused the defects of primary motor neurons. A-C. The results of the qRT-PCR of *fgfbp3* in the embryo and adult zebrafish. The relative abundances of gene transcripts were shown as mean ± SD ($N = 3$). Scale bar = 50 μm. D. The schematic for the zebrafish with primary motoneurons. The red dash line showed the imaging domain. E. Confocal imaging analysis of primary motor neurons in control, *fgfbp3* KO and rescue zebrafish at 48, and 72 hpf. Caps in red dash line are showed in diagrams. F. The number of branches per CaP axon in three different groups at 48 and 72 hpf. G. The length of CaP axons in three different groups at 48 and 72 hpf. Values with *, **, *** and ****above the bars are significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

performed using the corresponding primers (Table 1) in a 20 μl reaction volume with 10 μl SYBR premix (Takara, Japan) and *elongation factor 1a* (*ef1a*) was used as the internal control. All samples were analyzed in triplicate.

2.4. Morpholino and mRNAs injections

Translation-blocking Morpholino that synthesized by Gene Tools was diluted to concentration of 0.3 mM to inject into one cell stage embryos. The sequence of *fgfbp3* Mo is 5′-ATGGCTGTGTTGATAAA-GAGCATTT-3′. The open reading frame of *fgfbp3* was amplified and cloned into PCS2^{+} vector. After linearized, the 5′-capped *fgfbp3* mRNA was synthesized and purified *in vitro* by the mMESSAGE mMACHIN Kit (Ambion, USA) and RNeasy Mini Kit (Qiagen, Germany), respectively. 100–200 pg of the purified mRNA was injected into one-cell stage embryos.

2.5. sgRNA/ Cas9 mRNA synthesis and injection

The Cas9 mRNA was synthesized by *in vitro* transcription following the previous methods (Gong et al., 2017). *fgfbp3* sgRNAs was generated with the plasmid pT7 by the specific forward primers 5′-TAA-TACGACTCACTATAGGCGTGAAAACGCCTGTGAAGTTTTAGAGCTA-GAAATAGC-3'and a universal reverse primer 5′-AAAAAAAGCACCGACTCGGTGCCAC-3′ (Chang et al., 2013; Qi et al., 2016). Transgenic zebrafish lines were naturally mated to obtain embryos for microinjection. 300 ng/μLCas9 mRNA and 100 ng/μL sgRNA was co-injected into one-cell stage *Tg(mnx1:GFP)^{ml2}* zebrafish embryos. Zebrafish embryos were randomly sampled at 24 hpf for genomic DNA extraction to determine the indel mutations by sequencing.

2.6. Acoustic startle reflex

About 20 larvae were put in a thin layer of culture media in a petri dish that was attached to a mini vibrator. The response of larvae to sound stimulus (a tone burst 9 dB re. m s^{-2}, 600 Hz, for 30 ms) generated by the vibrator was recorded from above by an infrared camera over a 6 s period. The mean moving distance was used to quantify the startle response.

2.7. Microscopy and statistical analysis

After being anesthetized with tricaine, the zebrafish embryos were mounted in 0.7% low melt agarose, and then photographed by NiKon A1R HD25 confocal microscope. *In vivo* time-lapse imaging was taken from 30 hpf or 36 hpf by confocal microscope. To assess the rosette structures, 4–5 z-stacks of confocal images covering the full thickness of the motor neuron were captured and presented as maximum projection in the figures. For the *in situ* hybridization, photographs were taken using an Olympus stereomicroscope MVX10. Statistical analyses were performed by one-way analysis of variance (ANOVA) or student's *t*-test, and P values <0.05 were considered statistically significant.

3. Results

3.1. fgfbp3 is expressed in the central neuron system and is evolutionarily conserved

We performed whole mount *in situ* hybridization with zebrafish embryos by a digoxigenin-labeled *fgfbp3* probe to verify the expression profile of *fgfbp3*. Our results showed that *fgfbp3* is highly expressed in telencephalon, midbrain and medulla oblongata of 20 hpf embryos (Fig. 1 A). From 24 hpf, *fgfbp3* expression level in spinal cord increased markedly (Fig. 1 B-E). The transverse section analysis further confirmed that *fgfbp3* has been highly expressed in the spinal cord (Fig. 1 A", B″ and C″). Moreover, we selected the motor neurons from *Tg(mnx1:GFP)^{ml2}* to further detect the expression of *fgfbp3*. The RT-PCR showed that the expression level of *fgfbp3* was similar to that of mnx1 in the isolated motor neurons (Fig. 1F), which indicated that *fgfbp3* was expressed in zebrafish motor neurons.

In order to understand the evolutionary relationships of zebrafish Fgfbp3 among the vertebrate, the phylogenetic tree of the Fgfbp3 was constructed using Neighbor-joining method. The results showed that the Fgfbp3 from Osteichthyes were clustered in a separate clade from Mammalian Fgfbp3 (Fig. 1G). Moreover, Multiple alignments of Fgfbp3 from different species showed that the zebrafish Fgfbp3 had significantly high amino acid sequence similarities to other Osteichthyes in the FGF2 binding domain and heparin binding domain (Fig. 1H and Supplementary Fig. 1 D). Therefore, the results of phylogenetic tree and Multiple alignments suggested the evolutionary conservatism of Fgfbp3.

3.2. Knockout of fgfbp3 caused the depletion of the fgfbp3 in embryo and adult

In order to investigate the specific function of *fgfbp3* during the embryogenesis, we generated *fgfbp3* knockout zebrafish embryos by CRISPR/Cas9 with target sites near the translation start codon on *Tg (mnx1:GFP)^{ml2}* and *Tg(mnx1:GAL4;UAS:nTR-mCherry)* transgenic line. 5 types of mutations were identified in the F1 offspring by monoclonal sequencing (Supplementary Fig. 1 B). All the lines shared similar primary motor neuron defects (the decreasing of axons length and branching number), which will describe following and the appearance of these zebrafish is normal (Supplementary Fig. 1 E). Subsequently, the 48 bp insertion mutants that caused the early translation termination to lose the FGFs binding domain were used for the following experiments. To verify the efficiency of the knockout, qRT-PCR was performed to detect the expression of *fgfbp3* in different development stages of embryo and adult. The results showed that compared to the control group, the transcripts levels of *fgfbp3* in the *fgfbp3* KO group were significantly lower at all the detection point (Fig. 2A). Similarly, the mRNA levels of *fgfbp3* in the brain and spinal cord of the control adult zebrafish were also remarkably higher than that in the *fgfbp3* KO adult zebrafish (Fig. 2 B, C), which indicated that the deletion mutation indeed alerted the expression profile of *fgfbp3*.

3.3. fgfbp3 is essential for primary motor neurons development

Since studies have shown that *fgfbp3* highly expressed in the spinal cord and motor neurons, we next wanted to further investigate the influence of *fgfbp3* deficiency on primary motor neuron development in the spinal cord. Firstly, we found that in the wild type zebrafish embryos, the axons of Cap extended to the ventral edge of the axial muscles, where it turns dorsally and laterally to project over the lateral surface of the axial muscles. While parts of Cap axons of the *fgfbp3* KO embryos turned dorsally before they grew to the edge of the axial muscles or could not turn normally at 48 hpf (Fig. 2 E). In addition, the number of branches per 1 mm Cap axon in *fgfbp3* KO embryos was remarkably fewer than that in control zebrafish, and the length of Cap axon was significantly shorter in the *fgfbp3* mutants (Fig. 2 F, G). Interestingly, these abnormal phenotypes of motor neurons could not recover when the fish developed to 72 hpf (Fig. 2 E). To further confirm phenotypic specificity, the *in vitro* synthesized *fgfbp3* mRNA containing an intact open reading frame was injected into one cell stage *fgfbp3* KO embryos. The results showed that exogenous *fgfbp3* mRNA partially rescued Cap defects containing the number of branches and the length of axon compared to the control group (Fig. 2 E, F, G), Taken together, these primary defects found in this study were specifically caused by loss function of *fgfbp3*.

Additionally, *fgfbp3* translation blocking morpholino (MO) was also synthesized and injected into the *Tg(mnx1:GFP)^{ml2}* transgenic zebrafish. The phenotype of spinal cord primary motor neuron was detected by confocal imaging before confirming the Mo availability. By co-injecting

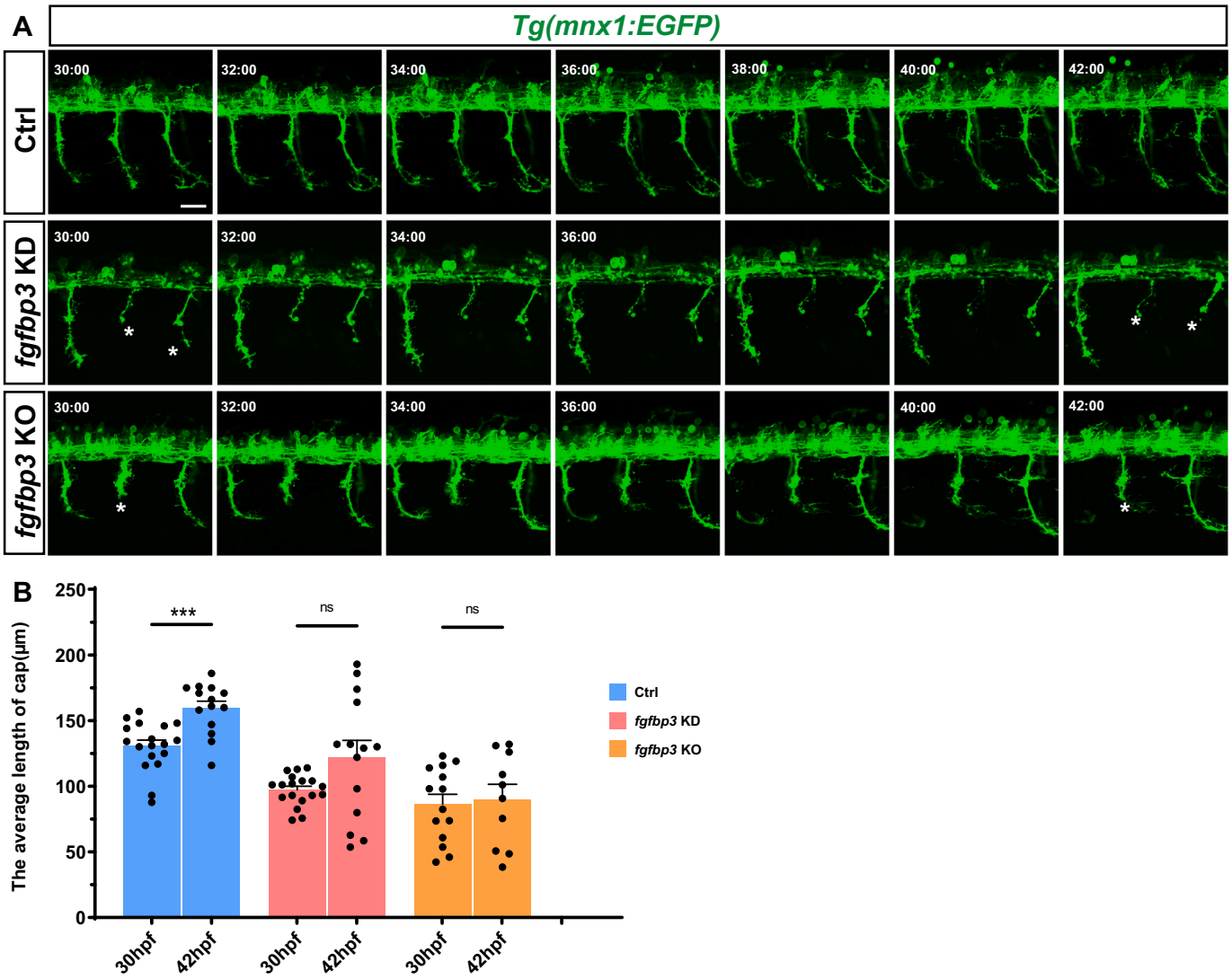

Fig. 3. The deficiency of *fgfbp3* suppressed the growth of Cap axon. A. Time-lapse imaging analysis of the primary motor neuron in control, *fgfbp3* KD and *fgfbp3* KO zebrafish. Asterisks represent truncated axons of Cap. Scale bar = 30 μm. B. The length of CaP axons in three different groups at 30 and 42 hpf. Values with ***above the bars are significantly different ($P < 0.001$).

fgfbp3 MO and a recombinant PCS2^{+} plasmid that was inserted with *fgfbp3* MO binding site and GFP into the AB zebrafish (Supplementary Fig. 2 A). The results showed that compared to the control zebrafish that was only injected with the recombinant construct, the GFP positive signal was rare in the fish co-injected with construct and *fgfbp3* MO, which indicated this MO was highly efficient (Supplementary Fig. 2 B). Moreover, similar to the *fgfbp3* mutant, knocking down of *fgfbp3* also caused remarkable malformation of the motor neuron with short Cap axons and fewer branches (Supplementary Fig. 2 C-E).

3.4. *fgfbp3 deficiency suppressed the growth of Cap axon*

To further investigate the development process of the Caps axons, we performed confocal time-lapse imaging analysis from 30 hpf to 42 hpf with the *Tg(mnx1:GFP)*^{ml2} transgenic line. The results showed that compared to the control embryos, parts of the shorter Cap axons were found in both the *fgfbp3* KD and KO embryos and they were almost with little extension during the whole imaging interval (Fig. 3 and Supplementary Movies1, 2 and 3). In addition, the phenotype of the mutant was also validated with the *Tg(mnx1: GAL4; UAS:nTR-mCherry)* transgenic line whose primary motor neuron was labeled by mCherry. The confocal imaging results showed that the Cap axons of *fgfbp3* KO embryos grew significantly slowly and were significantly slighter than that in the control fish (Supplementary Fig. 3). These malformations of the axons could not recover with the development (Supplementary Fig. 4).

3.5. *The deficiency of fgfbp3 reduced the zebrafish swimming activity*

In order to investigate whether the motor neuron defects caused by the *fgfbp3* deficiency affect the motor ability, we further detected the free-swimming ability of *fgfbp3* KO larvae at 7 dpf. The results showed that the total distance and average speed of *fgfbp3* mutants were significantly decreased compared to control fish (Fig. 4 B, C). The movement trajectory chart also demonstrated that swimming behavior was restrained in the KO group (Fig. 4A). Moreover, a startle response experiment was also performed to verify our results. Similarly, the movement trajectory, swimming distance and the fastest speed of KO group were significantly decreased after stimulus compared to control fish. However, these actions could be rescued by exogenous *fgfbp3* mRNA injection (Fig. 4 D, E, F).

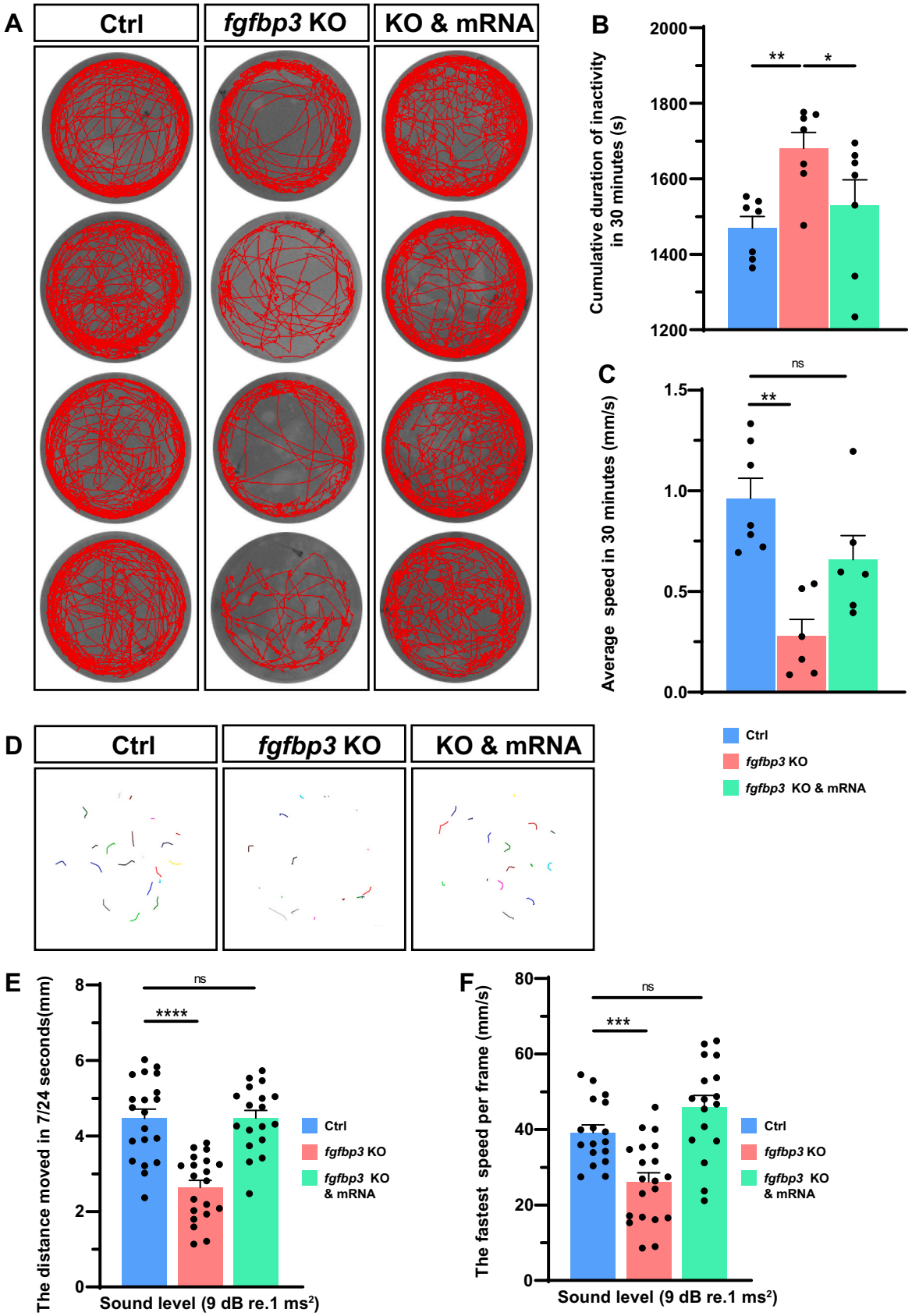

Fig. 4. *fgfbp3* involved in the regulation of zebrafish swimming activity. A. The free-swimming trajectory of the control, *fgfbp3* KO and rescue zebrafish at 7 dpf. B and C. Quantification of the cumulative duration of inactivity and average speed of three different zebrafish groups. D. The swimming trajectory after stimuli of the three different zebrafish groups. E and F. Quantification of swimming distance and the fastest speed of the three different zebrafish groups. Values with *, **, *** and ****above the bars are significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively).

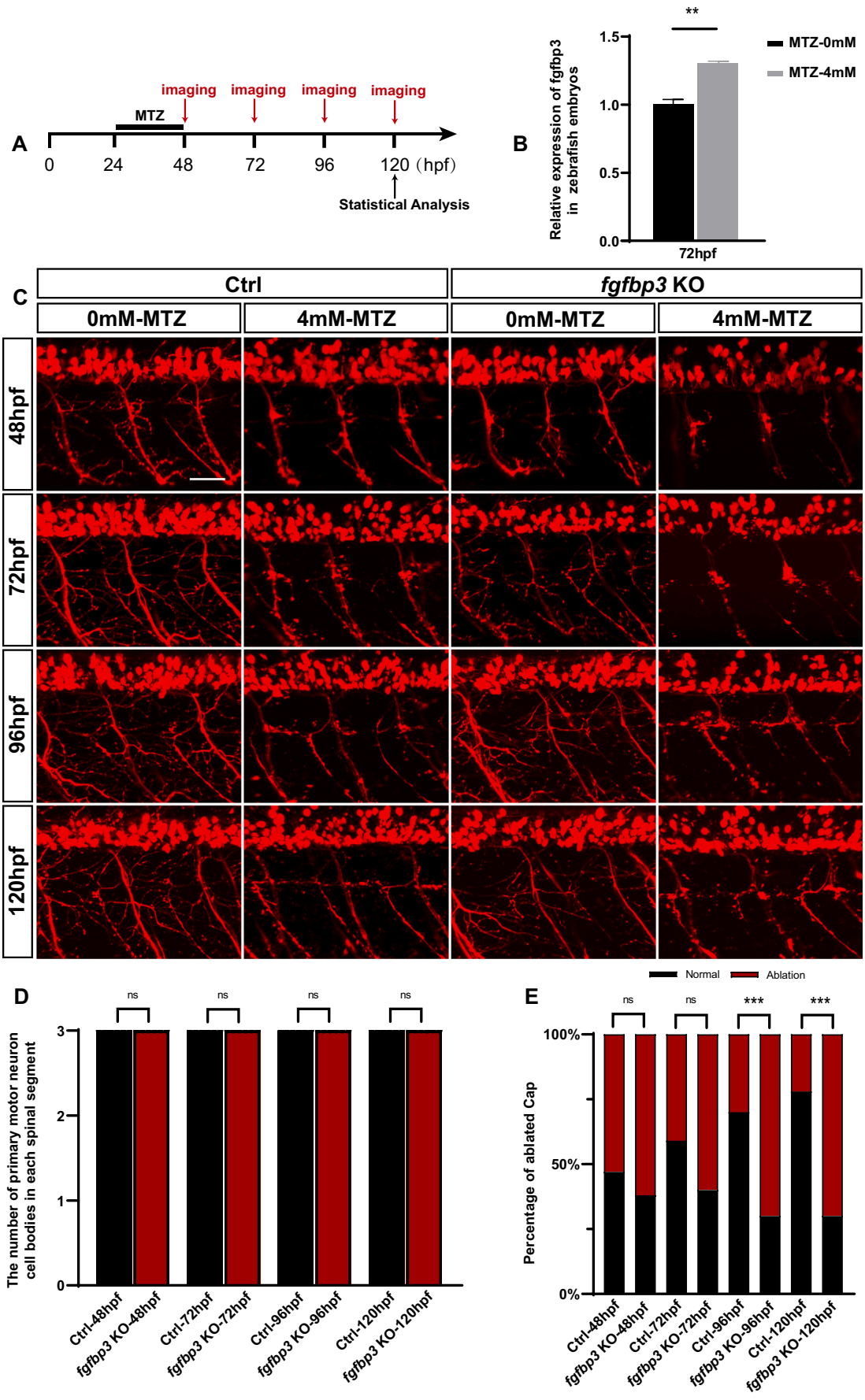

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Fig. 5. Loss of *fgfbp3* suppressed the axon regeneration after drug injury. A: The schematic for the experimental procedure that the *Tg(mnx1: GAL4; UAS:nTR-mcherry)* whose primary motor neuron was labeled by mCherry were treated with MTZ at 24 hpf and then the MTZ was removed after 24 h followed with confocal imaging every 24 h. B. The qRT-PCR analysis of *fgfbp3* expression in the control zebrafish treated with MTZ. C. Confocal imaging analysis of primary motor neurons in control and *fgfbp3* KO zebrafish after treated with MTZ at 48, 72, 96 and 120 hpf. Scale bar = 50 μm. D. Quantification of the number of primary motor neuron cell bodies in each spinal segment in control and *fgfbp3* KO zebrafish after treated with MTZ at different development stage. E. The percentage of the damaged Cap in control and *fgfbp3* KO zebrafish after treated with MTZ at different development stage.

3.6. Loss of fgfbp3 suppressed the axon regeneration

The function of *fgfbp3* in axonal regeneration after drug injury was further investigated by the *Tg(mnx1: GAL4; UAS:nTR-mcherry)* whose motor neuron could be specifically damaged by metronidazole (MTZ). We firstly isolated the motor neurons from *Tg(mnx1: GAL4; UAS:nTR-mcherry)* zebrafish at 24 h post MTZ treatment for the expression analysis of *fgfbp3*, and the PCR results showed that the expression level of *fgfbp3* was significantly increased after MTZ treatment (Fig. 5B). Confocal imaging analysis showed that in both *fgfbp3* mutants and control zebrafish, 4 mM exogenous MTZ degenerated the branches of primary motor neuron axons but not the total neuron. The cell bodies and axons of the primary motor neuron existed with the axons clearly connect with motor neuron cell bodies (Supplementary Fig. 5). Interestingly, we also found that the axons of the primary motor neuron were impaired severely after treatment with MTZ in *fgfbp3* KO embryos compared to the control fish (Fig. 5C). Moreover, the Caps in the control fish were able to partly regenerate new axons and branching at 24 hpf after MTZ treatment, while *fgfbp3* KO fish still maintained fragmented Cap axons, which could not recover until 120 hpf (Fig. 5C).

Moreover, we calculated cell bodies of the primary motor neurons and Cap axons during the time course of injury and regeneration. The results showed that the number of motor neurons cell bodies were not changed. There are three primary motor neurons in each spinal segment in both control and *fgfbp3* mutant embryos treated with MTZ (Fig. 5D and Supplementary Fig. 5). Instead, more than 50% axons of the caudal primary motor neurons (Caps) were injured in the embryos treated with MTZ. The injured Cap axons gradually recovered in the control embryos. While the regeneration of ablated Cap axons was significantly blocked in the *fgfbp3* mutants (Fig. 5 E). These results indicated that the deficiency of *fgfbp3* significantly suppressed the axon regeneration after drug injury.

3.7. Transcriptomic profiling of fgfbp3 KO zebrafish

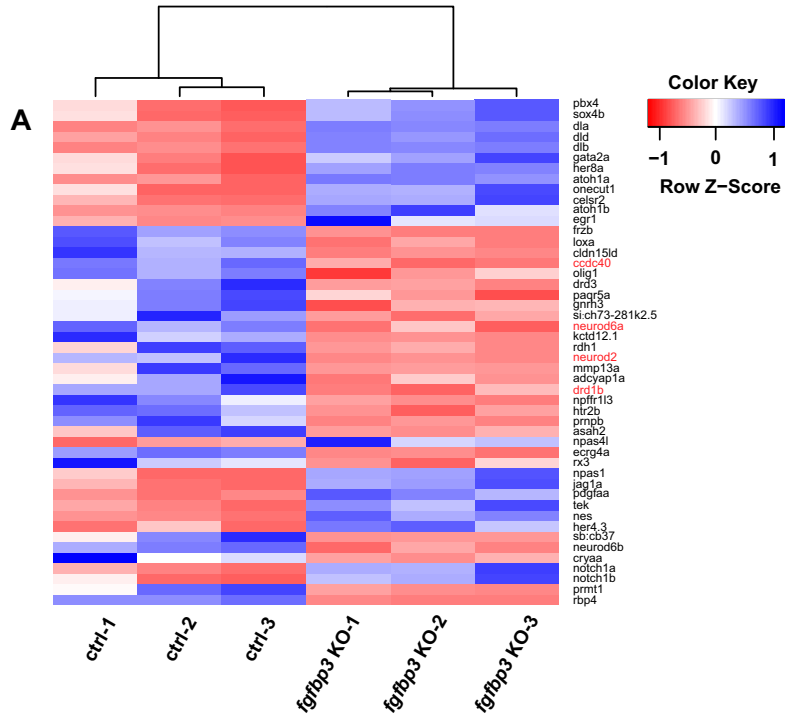
To further explore the mechanism of *fgfbp3* during the development of motor neurons, we performed RNA sequencing (RNA-seq) on wild type and *fgfbp3* KO embryos at 48 hpf. The results displayed that a series of differentially expressed candidate genes that were involved in the neurogenesis and regeneration were identified between *fgfbp3* and wild type zebrafish. These differentially expressed candidate genes contained the *drd1b* and *drd3* that belong to the dopamine receptor, and *neurod6a*, *loxa*, and *ccdc40* belong to the neurogenesis regulatory factor. The expression levels of these genes were significantly suppressed in the *fgfbp3* KO zebrafish (Fig. 6A). We tested the expression profile of four candidate genes; *neurod6a*, *neurod2*, *drd1b*, and *ccd40* in the wild type and the *fgfbp3* KO by qRT-PCR. Similar to RNA-seq data, the expression levels of these genes were significantly decreased in the *fgfbp3* KO at 48 hpf (Fig. 6 B-E). This confirmed the quality of RNA-seq data. In addition, according to the results of RNA-seq and qRT-PCR, we synthesized the *neurod6a* and *drd1b* mRNA to inject into one cell stage *fgfbp3* KO embryos, respectively. The results showed that exogenous *neurod6a* and *drd1b* mRNA could significantly reduce the motor neuronal defects caused by loss function of *fgfbp3*, containing the number of branches and the length of axon (Fig. 6 F, G).

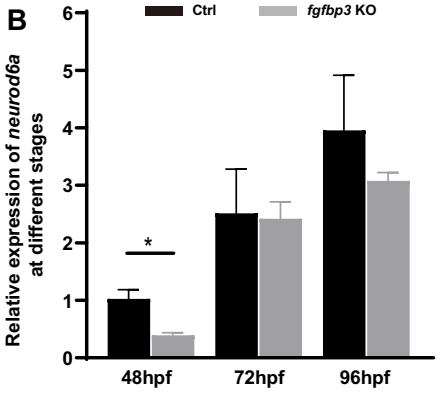
4. Discussion

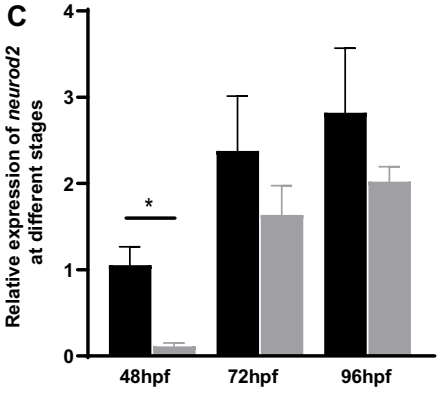
As a highly conserved secretory protein chaperonin, Fgfbp3 could bind different FGFs to be involved in the FGF signaling pathway. Several studies have demonstrated in the central nervous system that *fgfbp3* plays important role in neuronal development and the regulation of emotional disorder (Salyakina et al., 2011; Yamanaka et al., 2011). However, the specific function of *fgfbp3* during the development of peripheral nervous system remained unclear, especially during motor neuron development. In this study, our data reveals the expression of *fgfbp3* as well as Knockdown and Knockout approaches of *fgfbp3* in zebrafish embryos which provides new insights into the functions of *fgfbp3* during the development of primary motor neurons.

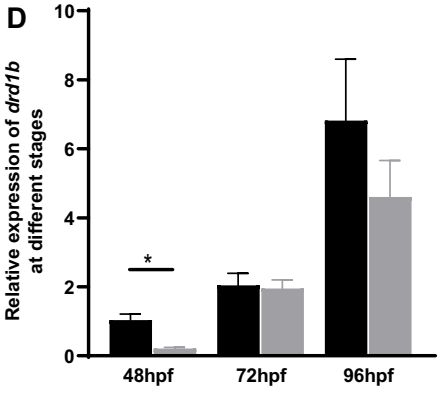
The results of *in situ* hybridization showed *fgfbp3* highly expressed in the brain as well as a positive signal found in the spinal cord, which was verified by the transverse section analysis. This data suggests that *fgfbp3* might be vital for the neuron system development. It has been reported that *fgfbp3* has a high affinity with FGF2, which is one of the neurotrophin that exist in the neurons and glial cells to regulate the function of the nervous system (Ornitz and Itoh, 2001; Sanalkumar et al., 2010). In mice, loss function of FGF2 that secret from NG2 glial cells have been shown to play an important role in mediation of central nervous system homeostasis, significantly caused depressive-like behaviors (Birey et al., 2015). Similarly, Long-term exposure to stress can suppress the expression of FGF2 in the mouse hippocampus to induce depressive behaviors (Cheng et al., 2015). Besides regulating the emotional behavior, FGF2 is known to play a role in neuron axonal branching, growth and neurogenesis (Gyorgyi et al., 2001; Woodbury and Ikezu, 2014). Kefalakes et al., found that culturing astrocytes that lack FGF2 and $SOD1^{G93A}$ motor neurons, a model system to imitate neuropathological and clinical characteristics of amyotrophic lateral sclerosis, remarkably impact the number and maturation of the motor neuron which suggests that astrocytic FGF2 affects motor neurons at a developmental stage (Kefalakes et al., 2019). Moreover, acute focal injection of exogenous FGF2 could not only protect ventral horn neurons from death but also reduce the damage of preganglionic sympathetic motor neurons after experimental contusive spinal cord injury (Teng et al., 1999). These results indicate that FGF2 plays an important role in motor neuron development and regeneration after impairment (Araujo et al., 2017).

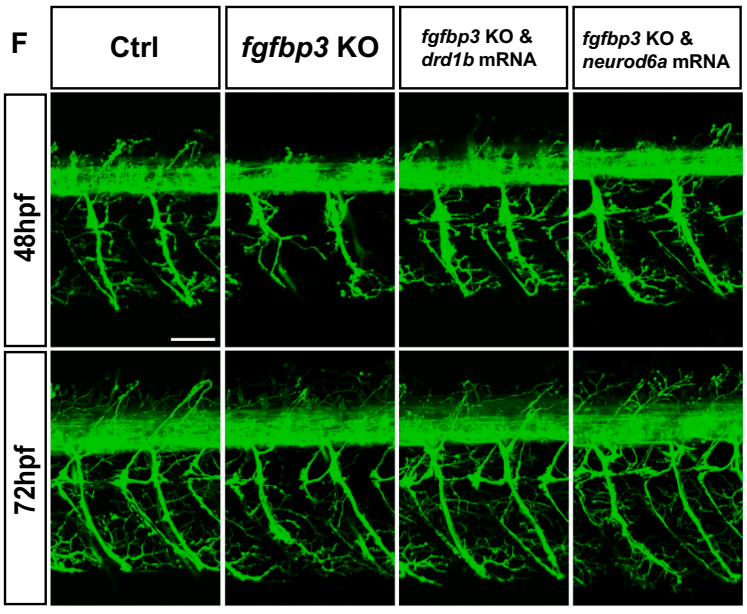
As we know, Fgfbp3 could specifically bind FGF2 whose function might depend on Fgfbp3. Therefore, we hypothesize that *fgfbp3* might also play a role in the development of motor neuron and their regeneration after injuring. In this study, we found that the length of Cap in the *fgfbp3* KO was significantly truncated, while the number of branches per Cap axon was also remarkably decreased after the *fgfbp3* depletion. A similar phenotype is also found in mouse models with FGF2 mutation that the neurite length of primary myenteric plexus cultured *in vitro* is significantly shortened compared with that from the wild type mouse (Hagl et al., 2013). However, the deficient phenotypes of *fgfbp3* mutants were only partially rescued by the overexpression of *fgfbp3* mRNA. This result might be caused by that the exogenous mRNA microinjected hardly mimicked the expression dynamics of endogenous fgfbp3 mRNA. Additionally, the partial rescue of the exogenous RNA in zebrafish was reported in a number of studies (Carlantoni et al., 2021; Matharu et al., 2021; Zhao et al., 2017). Moreover, we also found that compared to the control fish, the primary motor neurons of *fgfbp3* KO zebrafish were not totally regenerated at 3 days after damage termination. These results

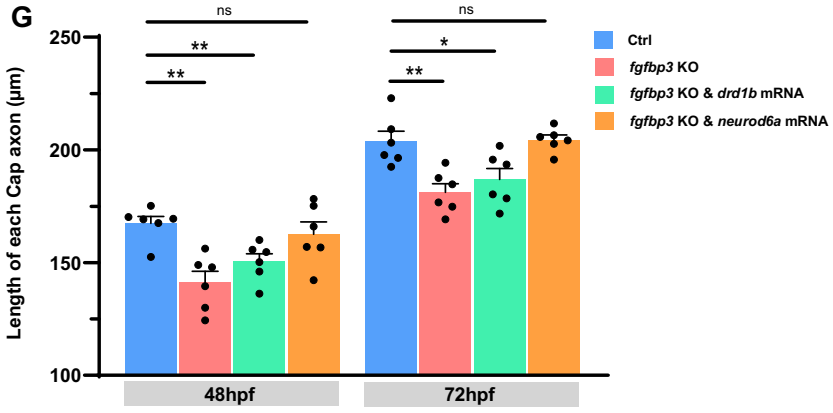












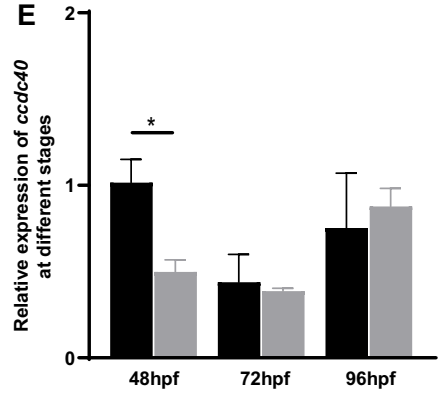


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Fig. 6. The transcriptomic analysis for *fgfbp3* KO and wild type zebrafish. A. The DEGs associated with neurogenesis and regeneration in the transcriptome. *neuroda6a* and *drd1b* that marked in red were used for the followed rescue experiment. B-E. Validation results for the DEGs found in the transcriptomic results. Experimental embryos were sampled at 48, 72 and 96 hpf ($n = 12$). F. Confocal imaging analysis of primary motor neurons in control, *fgfbp3* KO and rescue zebrafish (*drd1b* and *neurod6a* mRNA injected in the *fgfbp3* KO, respectively) at 48, and 72 hpf. Scale bar = 50 μm. G. The length of CaP axons in the four different groups at 30 and 42 hpf. Each bar represents the mean ± SD. Values with *, and **above the bars are significantly different ($P < 0.05$ and $P < 0.01$, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

indicate that *fgfbp3* plays an important role in the development of motor neuron and axon regeneration.

Spinal motor neuron was crucial for a series of locomotion behavior, we further assessed the motor ability of the *fgfbp3* KO by measuring their free swimming and startle response. Our results showed that the average speed of the *fgfbp3* KO was conspicuously decreased compared to control fish. We also noticed after stimulation that the swimming distance and speed were also affected by the loss function of *fgfbp3*, which indicated that swimming ability and startle response were significantly suppressed by the *fgfbp3* deficiency. During development, motor neurons will enter the muscle to form the synaptic connections with the surface of muscle fibers when it departs from the spinal cord. Abnormal development of motor neurons, such as short axons and frequently branching, will lead to slow-twitch muscle fibers disorganized to detach from the vertical somite boundaries or missing, which will further affect the organism's locomotion behavior (Birely et al., 2005). Furthermore, the occurrence of motor behavior is a complex process generated by neuronal networks and the forward locomotion sustentation needs spinal cord motor neurons to output the motor signal (Cappellini et al., 2010). The stop or reset of locomotion upon sudden sensory stimulation rely on fine-tuning of the temporal recruitment of spinal circuits (Fidelin and Wyart, 2014; Roberts et al., 2008). Therefore, the developmental defect of spinal cord motor neuron caused by the *fgfbp3* loss might induce the disorder of motor signal output and the temporal recruitment of spinal circuits to further induce the deficiency of the motor behavior.

As we have shown, many genes are essential for motor neuron development (Gong et al., 2020). In this study, a series of differentially expressed candidate genes containing the *drd1b* and *drd3* that belong to the dopamine receptor, and *neurod6a*, *loxa*, and *ccdc40* that belong to the neurogenesis regulatory factor were identified from the transcriptome sequencing results. Dopamine has been identified as a factor influencing neurogenesis company with dopamine receptors in the developing center nerves system (Popolo et al., 2004). Dopaminergic projections from the brain could promote motor neuron generation in the developing zebrafish spinal cord by activating its receptor. Inhibiting this essential signal during early neurogenesis leads to a long-lasting reduction of motor neuron numbers and impaired motor responses of free-swimming larvae (Reimer et al., 2013). Similarly, the dopamine receptor-Drd1b was also detected in the projection neurons and played important roles in the movement manipulation by interaction with the neurotransmitter (Rivera et al., 2002). On the other hand, Mitochondria is crucial for the neuronal growth cone activity and axonal outgrowth by supplying energy with ATP (Bernstein and Bamburg, 2003). As one Neurogenic transcription factor, Neurod6 can significantly promote the transcription of the mitochondrial-related genes to further accelerate mitochondrial movement and increase mitochondrial mass with cytoskeletal remodeling in developing neurites (Kathleen Baxter et al., 2009). Moreover, overexpression of *neurod6* can activate the expression of glutathione peroxidase and thioredoxin-like in motor neurons to prevent the apoptosis of neurons and promote recovery of motor function after spinal cord motor neuron injury (Jee et al., 2012). Interestingly, we found that the expressions of *drd1b* and *neurod6a* were significantly suppressed by the deletion of *fgfbp3* and overexpression of the two genes remarkably rescued the motor neuron malformation in *fgfbp3* mutants in this study. From our data, it suggests that *fgfbp3* plays a role in motor neuron development by regulating the expressions of a series of target genes such as dopamine receptor and neurogenesis regulatory factor.

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Declaration of Competing Interest

None.

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