



# *Sp7/osterix* positively regulates *dlx2b* and *bglap* to affect tooth development and bone mineralization in zebrafish larvae

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Osterix (or Sp7) is an important transcription factor that promotes osteoblast differentiation by modulating the expression of a range of target genes. Although many studies have focused on Osterix/Sp7 regulatory mechanisms, the detailed functions have not been fully elucidated. Toward this end, in this study, we used CRISPR/Cas9 technology to knock out the zebrafish *sp7* gene, and then analyzed its phenotype and biological function. Two knockout *sp7* mutant lines were successfully obtained. The bone mineralization level was significantly reduced in the zebrafish *sp7*<sup>-/-</sup> homozygote, resulting in abnormal tooth development in the larvae. Quantitative real-time polymerase chain reaction showed that loss of *sp7* led to down-regulated expression of the *dlx2b* and *bglap* genes related to tooth development and bone mineralization, respectively. Moreover, cell transfection experiments demonstrated that Sp7 directly regulates the expression of *dlx2b* and *bglap* through Sp7-binding sites on the promoter regions of these two genes. Overall, this study provides new insight into the role of Sp7 in bone mineralization and tooth development.

**Keywords.** *bglap*; bone mineralization; *dlx2b*; *Osterix/sp7*; tooth development

## 1. Introduction

Osterix (or Sp7) is an important zinc finger transcription factor that activates the transcription of downstream target genes through binding DNA, and is thus designated Sp7 protein binding sites by zinc finger domain (Koga *et al.* 2005; Zhang *et al.* 2008). To date, many genes have been identified as downstream targets of Sp7, including *Colla1* (Ortuno *et al.* 2010), *Col5a3* (Yun-Feng *et al.* 2010), *Satb2* (Tang *et al.* 2011), *Mmp13* (Nishimura *et al.* 2012) and sclerostin (Perez-Campo *et al.* 2016). Sp7 is mainly expressed in osteoblasts and plays an important role in the maturation of both osteoblasts and osteocytes (Nakashima *et al.* 2002). Recent studies have also pointed to a role of Sp7 in bone regeneration (Hosoya *et al.* 2013).

The majority of studies focused on the function of Sp7 have used different model organisms to knock out or knock-down the gene with various technologies. Knockout of *Sp7* in mice resulted in severe skeletal loss, and the osteoblasts

could not mature (Nakashima *et al.* 2002). Moreover, *Sp7* mutant mice showed abnormal osteoblast formation and development, which was ultimately lethal at the prenatal stage (Nakashima *et al.* 2002). This study further supported an important role of Sp7 in determining the fate of skeletal development. In addition, the *sp7* gene of Japanese medaka (*Oryzias latipes*) was knocked down via injection with antisense morpholino oligonucleotides (MO) to cause splicing and block translation, resulting in severe bone development abnormalities and a high larval mortality rate (Renn and Winkler 2014). In addition, ossification of more mature bones such as the operculum and cleithrum was delayed but recovered during further development. In the axial skeleton, formation of the neural arches and centra was strongly delayed (Yu *et al.* 2017). Similarly, knockout of *sp7* in zebrafish caused reduced mineralization of the cranial bone and disturbance of the axial bone (Yu *et al.* 2017; Kawai *et al.* 2017). Recent studies also showed that the loss of *sp7* in zebrafish can affect the formation and

differentiation of teeth (Kague *et al.* 2018), suggesting a role of Sp7 in regulating tooth development; however, the precise role and mechanism remain unclear.

Thus, to further explore the role of the zebrafish *sp7* gene in bone mineralization and tooth development, in the present study, we used CRISPR/Cas9 technology to knock out the zebrafish *sp7* gene, which is recent genome editing technology that provides a faster and more convenient method for gene manipulation than conventional mutation generation (Ran *et al.* 2013). The confirmed mutants were analyzed with regard to the effect of *sp7* loss on bone mineralization and tooth development during development. We further conducted cells transfection and rescue experiments to identify the underlying mechanism and identify target genes of Sp7. Together, these results should provide new insights into the role of *sp7* in bone mineralization and tooth development.

## 2. Materials and methods

### 2.1 Fish maintenance

Adult zebrafish of the AB strain were raised in a recirculating water system under a 14-h/10-h light/dark (L/D) cycle at 28°C and fed three times per day. To produce embryos, male and female zebrafish were paired in the evening, and spawning occurred the next day within 1 h after the lights were turned on. The embryos were placed in 10-cm Petri dishes with egg water containing methylene blue (0.3 ppm) and raised in a light-controlled (14-h/10-h L/D) incubator at 28°C.

### 2.2 Design of *sp7* guide RNAs, and generation and genotyping of mutants

*sp7* gRNAs were designed according to the methods described by Hsu *et al.* (2013). The purified gRNA (100 ng/

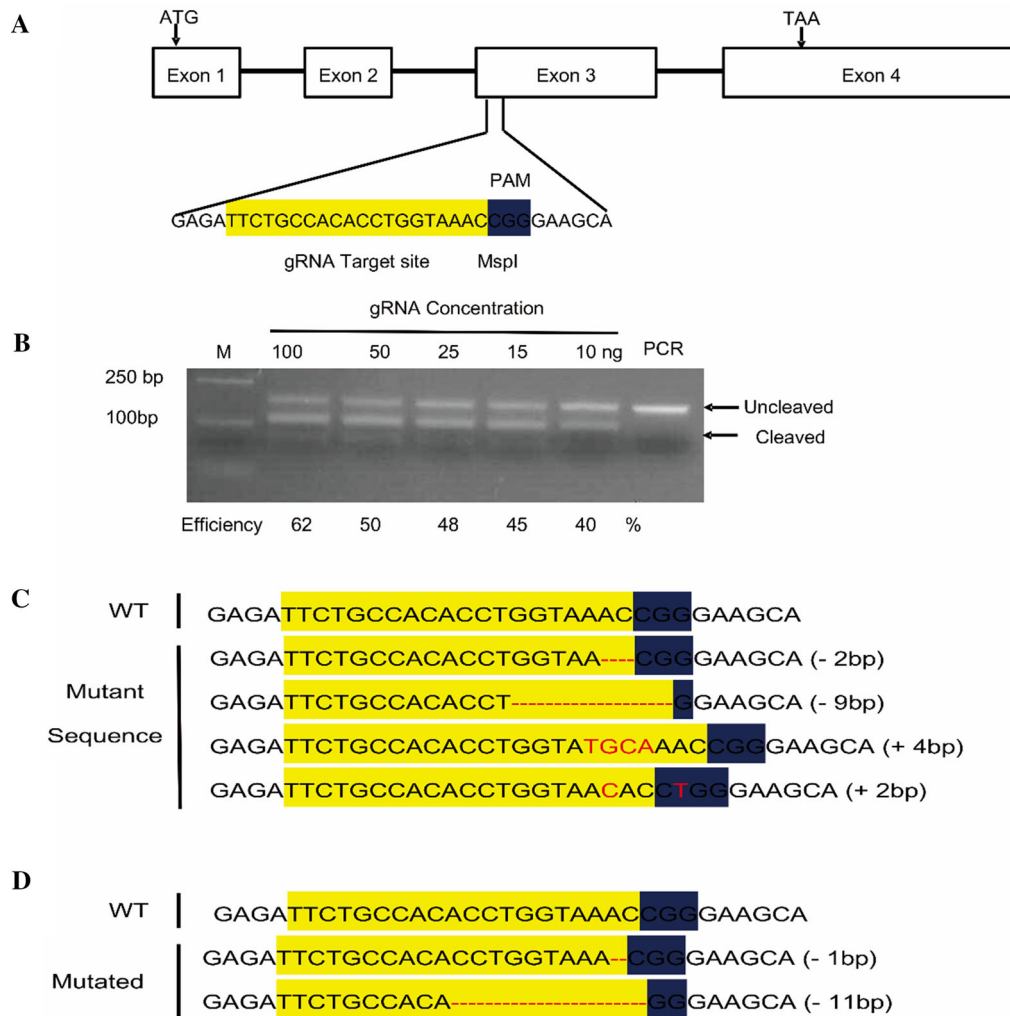
µl each) was co-injected with Cas9 mRNA (300 ng/µl) into zebrafish embryos at the one-cell stage. Adult potential founders were genotyped by fin clipping and then crossed to obtain germ line mosaic F1 embryos. Adult genotyped F1 fish were then outcrossed with wild-type fish to obtain heterozygous F2 offspring. The phenotype of homozygous F3 carriers was then analyzed. For genotyping, larvae or adult fish were anaesthetized with 0.01% or 0.005% ethyl 3-aminobenzoate methanesulfonate (Tricaine; Sigma), respectively. Larvae or clipped caudal fin fragments were lysed individually in 50 µl of 50 mM NaOH and incubated at 95°C for 15 min. The samples were neutralized with 5 µl of 1 M Tris-HCl (pH 8.0). Stained embryos were fixed and washed with 1× phosphate buffered saline with 0.1% (v/v) Tween 20 (PBST) five times before genomic DNA isolation. Individual larvae were lysed with DNA lysis buffer (10 mM Tris-HCl pH 8.2, 50 mM KCl, 0.3% NP 40, 0.3% Tween) and Proteinase K (20 µg/ml; Sigma) at a 49:1 ratio and incubated at 55°C for 60 min and then at 90°C for 10 min. The supernatant contained genomic DNA for analysis. A 147-bp fragment was amplified (using primers GAGGAAA-CACGTTATGGATC and GTTCAGAAGTCATGCTGTAG) and digested with MspI (New England Biolabs). Mutants with undigested fragments were sequenced for confirmation.

### 2.3 Whole-mount skeletal staining

Alizarin red staining was performed on the larvae fish as previously described (Walker and Kimmel 2007). In brief, the zebrafish larvae were collected at 6, 10, and 12 days post-fertilization (dpf) and then fixed in 2% paraformaldehyde overnight. After washing with 10% glycerol/0.5% KOH, the larvae were stained overnight with 0.02% alizarin red stain/10% glycerol/0.5% KOH and then washed overnight twice with 50% glycerol/0.1% KOH. Images were acquired using a stereomicroscope (Leica, M165FC, Germany). Digital images were analyzed to quantify and

**Table 1.** List of primer sequences in this study

Gene name	Forward primer	Reverse primer	Note
<i>dlx2b</i>	TCCTATGGCGCTTATGGAAC	TTTTTGGCTTCCATTCCACC	qRT-PCR
<i>mag</i>	AGCCCAACATTGATGTTCTT	GTGGATTTCTGGGCTCATGT	qRT-PCR
<i>pthlha</i>	ATGCATGATAAAGGCCGAAC	CACCACTGCTGATGCTGACT	qRT-PCR
<i>ercc2</i>	GATGCAAAGGGTCATGGAGT	GCAGTACACGAGCTTGGTGA	qRT-PCR
<i>acvr11</i>	CTTCGACTGCTGCTCACAAAG	GCCAGAAAAGACAGCAGACC	qRT-PCR
<i>ssvb2rs1</i>	TGATGATCAGCGTGGGTTAC	TCCTGCAGTCTGTGTGTGTG	qRT-PCR
<i>smoc2</i>	AATGCAAAGATACGCCCAAG	AGGTTCCATCAGGATTGCAG	qRT-PCR
<i>bglap</i>	CTGCTGCCTGATGACTGTGT	CACGCTTCACAAACACACCT	qRT-PCR
<i>sparc</i>	TGGGAATAAACCCAGTCCAG	CCCTTCTTGCAGTGATGGTT	qRT-PCR
<i>spp1</i>	CACCATCATCCCAGTCCACAG	CAAAGTCTTTTTCGCTCCACA	qRT-PCR
<i>coll1a1a</i>	ACGGCCAGGTCTACAATGAC	TGTGTCTCAGCAGATCACTT	qRT-PCR
<i>coll10a1a</i>	CCGCAGTACCAGCCTTACTC	CTGGCAGACCTTCACCATCT	qRT-PCR
<i>β-actin</i>	ACGAACGACCAACCTAACTCT	TTAGACAACCTACCTCCCTTTGC	qRT-PCR
<i>dlx2b</i>	AAGTCTAGGGAAGTGGGAGG	CATATCCATAGTTGATGGAGG	cDNA clone
<i>bglap</i>	GGCCTCAACCTCTGCTACTGC	TTCTGTGTCAGCTGATCTGTG	cDNA clone



**Figure 1.** Generation of the *sp7* mutant line using CRISPR/Cas9. **(A)** The target site of gRNA selection in *sp7* exons 3; the MspI restriction enzyme cutting site was located in the PAM site to facilitate selection of mutant zebrafish. **(B)** Different concentrations of gRNA were mixed with 300  $\mu\text{g/L}$  Cas9-capped mRNA, and then injected into single-cell zebrafish fertilized eggs. At 24 h post-fertilization, genomic DNA was extracted to check the gRNA efficiency. **(C)** Sequencing results showing different mutants generated by non-homologous recombination repair. **(D)** Two mutant types were ultimately chosen in the F1 generation of zebrafish. Neither mutant encodes Sp7 proteins. The mutant with an 11-bp deletion was chosen for subsequent experiments.

compare the stained area and staining density. Three replicates were performed with 10 fish per replicate.

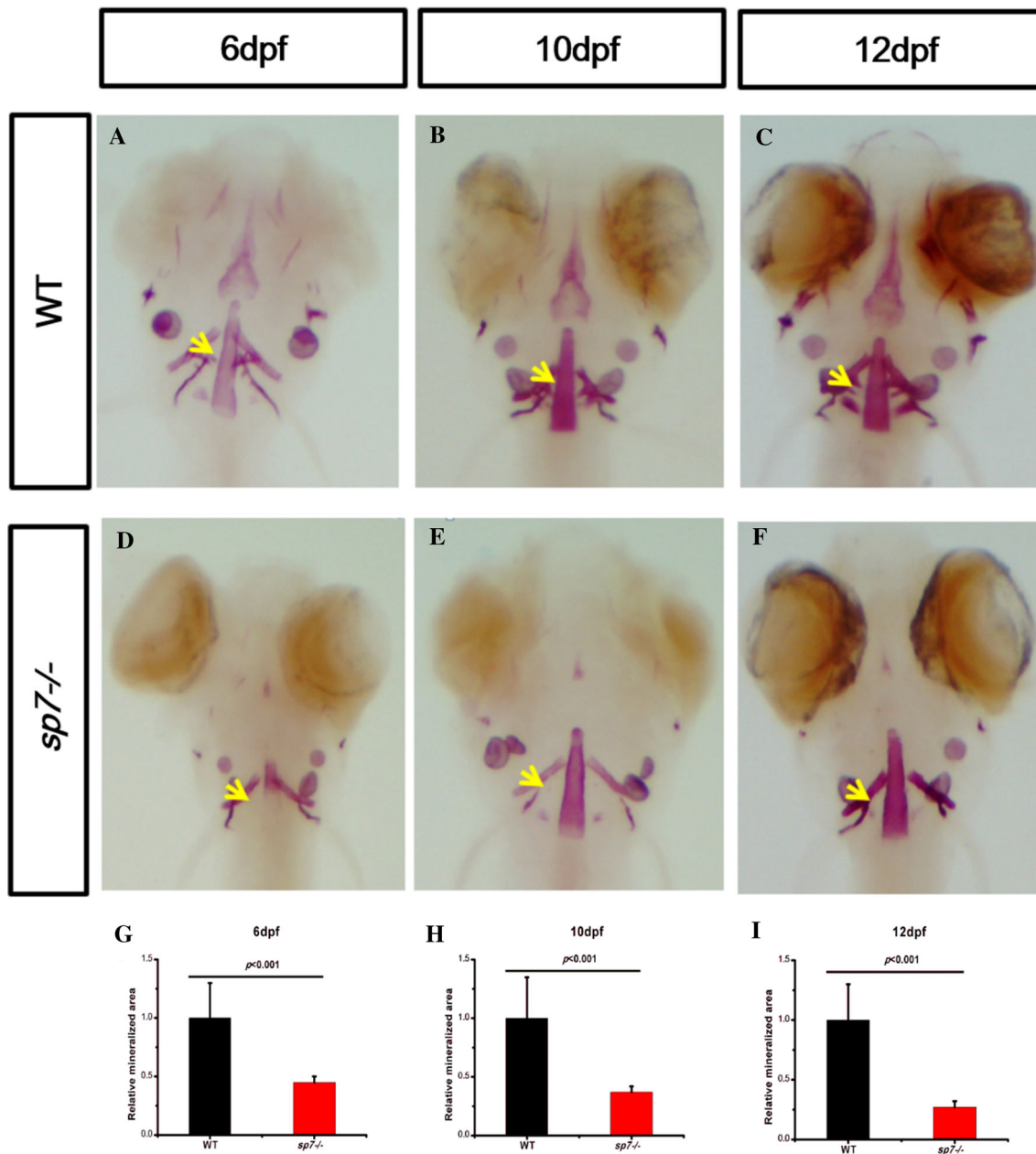
#### 2.4 RNA isolation, cDNA synthesis, and reverse transcription-quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted from more than 25 larvae by TRIzol (Invitrogen) reagent and reverse-transcribed into cDNAs using Superscript III Reverse Transcriptase (Invitrogen). qRT-PCR was then performed on an ABI Step-One Plus instrument using the SYBR (TaKaRa) system with a thermal profile of 40 cycles of 95°C for 10 s and 58°C for 30 s. Each qRT-PCR analysis was performed in triplicate on

three independent biological samples, and  $\beta$ -actin served as the reference gene for relative quantification of expression levels, calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method in Microsoft Office Excel. The qRT-PCR primers were designed based on the exon boundaries using Primer Express 3.0 software, and were checked for self-annealing, hetero-dimers, and hairpin structures with Oligo Analyzer 3.1. The primer sequences are listed in table 1.

#### 2.5 Luciferase activity assay

A segment of the wild type (WT) about 3kb *dlx2b* and *bglap* promoter cloned into the pcDNA4.17 vector

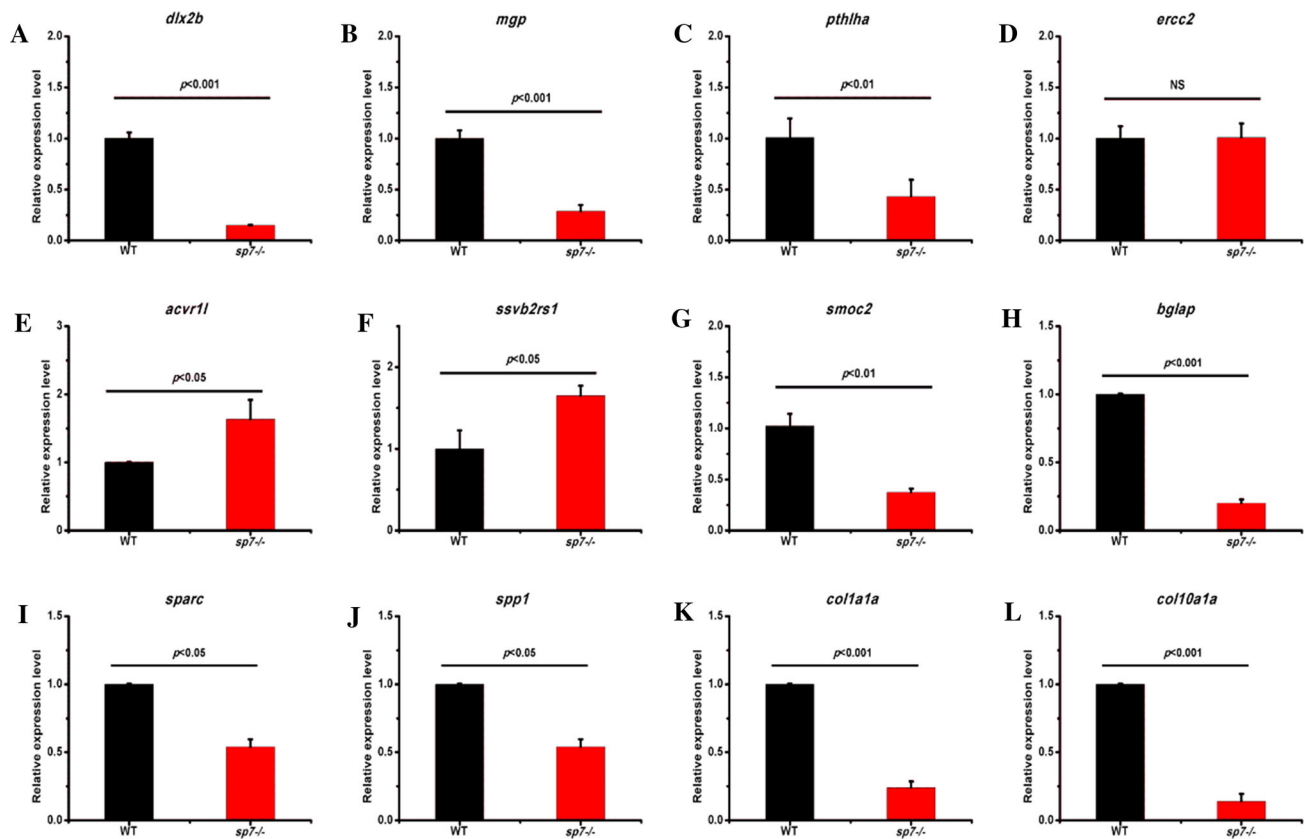


**Figure 2.** Whole-mount skeletal staining showing decreased bone mineralization lack of tooth development in the *sp7* mutant homozygous zebrafish. (A–C) Alizarin Red staining of WT fish at 6, 10, and 12 dpf. (D–F) Alizarin Red staining in *sp7* mutant fish at 6, 10, and 12 dpf. (G–I) Digital image analysis of the stained area and staining density of mineralized tissue via fluorescence intensity. Mean values are plotted ( $n = 10$ ) and Student's t-test was performed to determine statistical significance.

(Promega), and a mutant *dlx2b* and *bglap-luc* was generated by altering the predicted Sp7 binding site via a two-step PCR approach. HEK 293T cells were co-transfected with either a WT or mutant *dlx2b* and *bglap-luc* vector, and added Sp7 expression vector or not, cultured for 24 h, and then assessed to ascertain their exhibited luciferase activity using a dual-luciferase reporter assay system (Promega).

## 2.6 Statistical analysis

Three biological replicates and three technical replicates were performed for each experiment, and all experimental data are presented as mean  $\pm$  standard deviation values. One-way analysis of variance was performed to determine the statistical significance, followed by post-hoc Dunnett's or Turkey's tests to independently compare the effects of *sp7*



**Figure 3.** Tooth development- and bone mineralization-related genes were down-regulated in the *sp7* mutant zebrafish at 6 dpf. (A–G) Tooth development gene expression patterns in the *sp7* mutant zebrafish. (H–L) Bone mineralization gene expression patterns in the *sp7* mutant zebrafish. Student's *t*-test was performed to determine statistical significance.

knockout to wild-type fish. All statistical analyses were conducted using SPSS 16.0 software and values of  $p < 0.05$  were considered to be statistically significant.

### 3. Results

#### 3.1 Generation of *sp7* zebrafish mutants using the CRISPR/CAS9 system

To obtain *sp7* mutants, CRISPR/CAS9 technology was used to knock-out the *sp7* gene in zebrafish. The process of mutation generation and selection is outlined in figure 1. The gRNA target site was exon 3, and the restriction enzyme MspI was chosen to identify the mutation close to the PAM sites. To confirm the activity of the candidate gRNAs generated, different concentrations of gRNA were mixed Cas9, and capped mRNA was injected single-fertilized eggs. The efficiency of the gRNA was 62%, 50%, 48%, 45%, and 40% when mixed at concentrations of 100, 50, 25, 15, and 10 ng, respectively. Uncleaved bands were extracted from the gel and cloned into the pMD-19T vector. The sequencing results demonstrated a difference of mutant type generated by non-homologous recombination repair. Ultimately, two homologous *sp7* mutants were identified in the F2 generation. Bioinformatics

analysis further revealed that these two mutants encode 124- and 45-amino acid truncated proteins, which both result in nonsense mutations. Most of the subsequent experiments were performed using the 11-bp-deletion mutant.

The homozygous *sp7* mutants developed into adults fish, but showed an abnormal body morphology such as a defected gill and crooked spine. Compared with wild-type (WT) zebrafish, the homozygous *sp7* mutant also had a lower body length and body weight (data not shown). Importantly, the homozygous *sp7* mutant was not able to spawn. However, when eggs out from the *sp7* mutant were removed and artificially fertilized with WT sperm, the eggs developed normally. These results suggested that *sp7* likely also plays a role in fertilization.

#### 3.2 Bone mineralization and tooth development were defective in the homozygous *sp7* mutant

Alizarin Red staining performed at 6, 10, and 12 dpf showed that the area of stained bone tissue significantly decreased in the *sp7* mutant compared with WT zebrafish (figure 2). In particular, there was barely any mineralization detected in the craniofacial bones in the mutant, with only a 20% stained area compared with that of WT fish.



### 3.3 Genes related to tooth development and bone mineralization were down-regulated in the *sp7* mutant

As shown in figure 3, compared to the WT fish, the expression levels of the tooth development genes *dlx2b*, *map*, *pthlha*, and *smoc2* significantly decreased in the *sp7* mutant zebrafish; however, the levels of *acvr1l* and *ssvb2rs1* significantly increased, and there was no difference in the expression level of *ercc2*. The expression levels of the bone mineralization genes *bglap*, *sparc*, *spp1*, *colla1a*, and *coll10a1a* significantly decreased in the *sp7* mutant compared with those of WT zebrafish.

### 3.4 *Sp7* positively regulates *dlx2b* and *bglap*

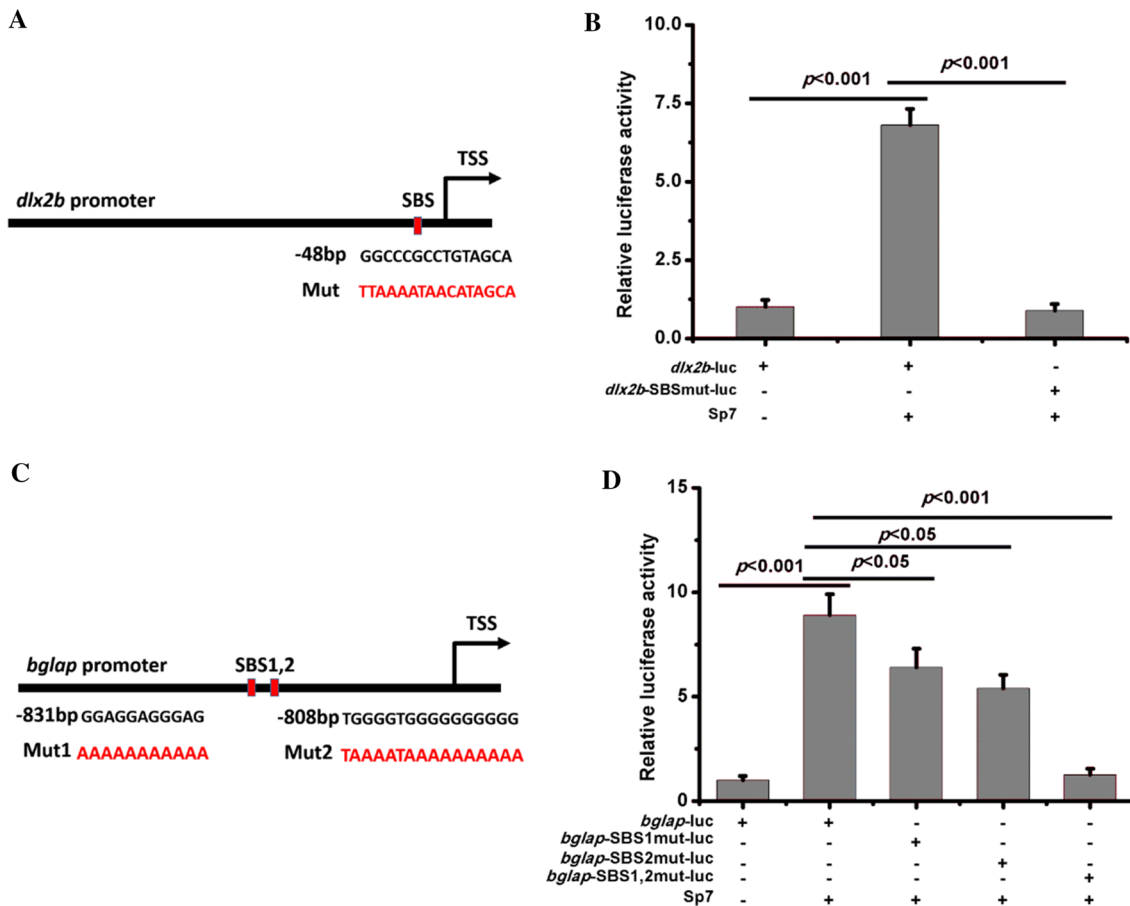
Bioinformatics promoter analysis identified an Sp7-binding site in the 2-kb promoter region of *dlx2b* (figure 4A), which is known to play a role in tooth morphogenesis in mice and zebrafish. Moreover, cell transfection experiments showed that Sp7 significantly up-regulated *dlx2b* expression;

however, when the Sp7-binding site was mutated, this up-regulation was no longer observed (figure 4B).

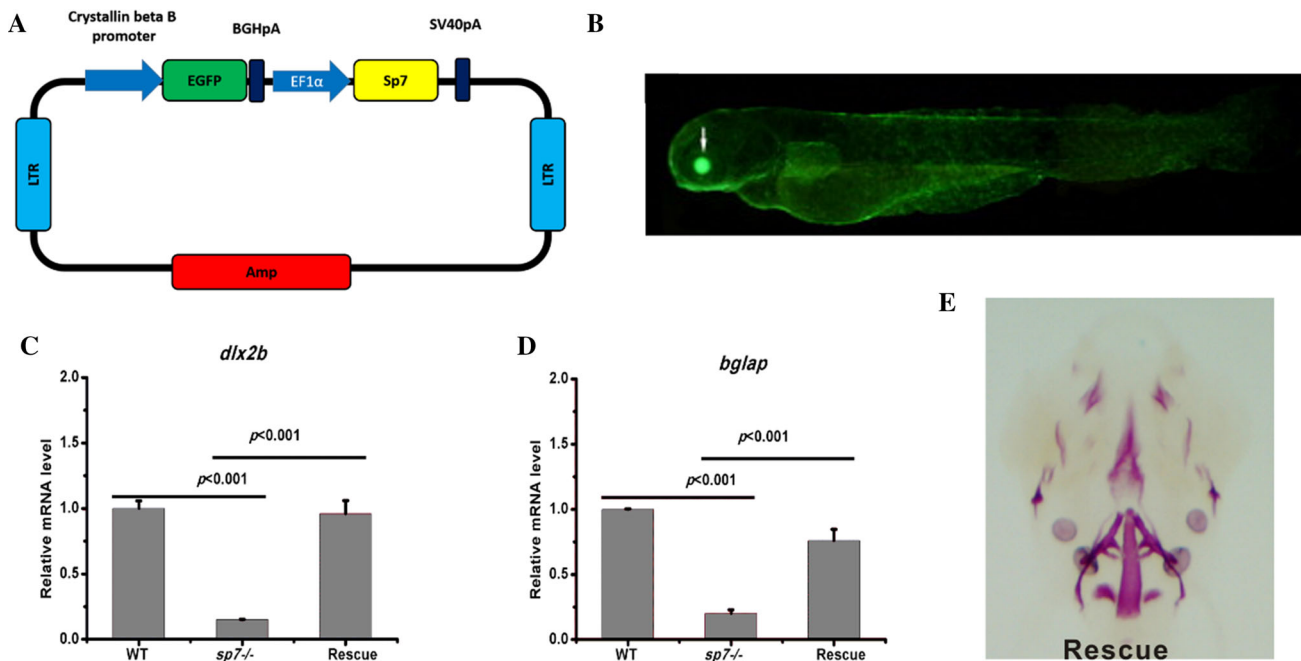
Similarly, two Sp7-binding sites were identified in the promoter region of *bglap* (figure 4C), which encodes osteocalcin secreted by osteoblasts, and is thus implicated in bone mineralization and calcium ion homeostasis. In addition, cell transfection experiments showed that Sp7 up-regulated *bglap* expression, which was not observed with mutation of the two Sp7-binding sites (figure 4D).

### 3.5 Whole-body *sp7* expression rescued tooth development and bone mineralization in *sp7* mutant zebrafish

Transgenic zebrafish were generated that express *sp7* throughout the entire body. The plasmid was generated via a Tol2 backbone, and crystallin beta promoter-driven EGFP was cloned into this vector as a screening tag. EF1 $\alpha$  then triggered *sp7* to express Sp7 in the whole body (figure 5B).



**Figure 4.** Sp7 positively regulates tooth and bone mineralization-genes (*dlx2b* and *bglap*, respectively). (A) Promoter analysis of *dlx2b* identifying one Sp7-binding site. (B) Cell transfection experiment showing that Sp7 regulates *dlx2b* expression via direct interaction with the promoter. (C) Promoter analysis of *bglap* identifying two sp7-binding sites. (D) Cell transfection experiment showing that Sp7 regulates *bglap* by direct interaction with the promoter. SBS: sp7 binding site. Three independent experiments were performed. One-way ANOVA was performed to calculate statistical significance followed by post-hoc Dunnett or Turkey tests.



**Figure 5.** Whole-body expression Sp7 in the *sp7* mutant zebrafish rescued tooth development and bone mineralization. **(A)** Construction of a whole-body expression *sp7* plasmid. **(B)** Whole-body expression of Sp7 in transgenic zebrafish was identified via the naked eye by EGFP fluorescence. **(C)** Whole-body expression of Sp7 in the *sp7* mutant zebrafish rescued *dlx2b* expression. **(D)** Whole-body expression of Sp7 in the *sp7* mutant zebrafish rescued *bglap* expression. **(E)** Whole-body expression of Sp7 in the *sp7* mutant zebrafish rescued the tooth development and bone mineralization phenotype. Three independent experiments were performed. One-way ANOVA was performed to calculate statistical significance followed by *post hoc* Dunnett's or Turkey's tests.

qRT-PCR experiments analysis showed that whole-body *sp7* expression rescued the decrease of *dlx2b* and *bglap* expression from the homozygous mutation. Whole-body Alizarin staining at 12 dpf further demonstrated that whole-body *sp7* expression rescued both bone mineralization and tooth development in the mutant.

#### 4. Discussion

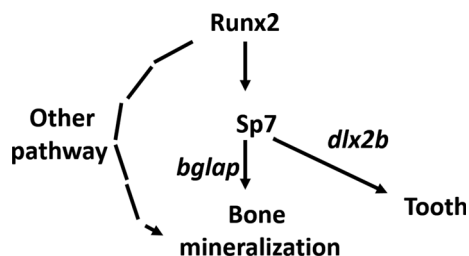
Sp7 is a zinc finger transcription factor that regulates the expression of many genes by binding to corresponding sites on the DNA sequence, and plays an important role in bone development (Koga *et al.* 2005; Nishimura *et al.* 2012). In this study, we used CRISPR/Cas9 technology to successfully construct *sp7* homozygous zebrafish mutants, and confirmed its role in regulating bone development.

*sp7* mutants have been generated in other model organisms. In mice, the loss of *sp7* has a lethal effect before birth due to abnormal development of the bone and osteoblasts (Nakashima *et al.* 2002; Baek *et al.* 2009). Similarly, less than 1% of medaka *sp7* mutants survived beyond 1 month of age, and none survived up to 2 months (Yu *et al.* 2017). By contrast, in zebrafish, all of the homozygous *sp7* mutants could survive to adulthood (Kague *et al.* 2018), confirming previous studies indicating that the function of zebrafish *sp7* slightly differs from that of mammals and medaka.

Establishment of an adult *sp7* mutant also provides the unique opportunity to study the function of Sp7 in adult fish. However, our homozygous *sp7* mutant could not successfully spawn, although *in vitro*-fertilized eggs developed successfully. Thus, the bone damage caused by the loss of *sp7* also appears to affect the ovulation process.

Overall, loss of *sp7* resulted in significant damage to bone mineralization and tooth development. The zebrafish dentition is fully established at around 26 days, comprising 11 teeth positioned on every fifth ceratobranchial (Jackman *et al.* 2004; Wise and Stock 2010; Yuan *et al.* 2017). In the early stage, zebrafish tooth development is similar to those of mammals, and the process of zebrafish bone formation is very similar to that of humans, with the same osteocyte type, bone structure, and extracellular matrix, making it a suitable animal model for human translation (Arnold *et al.* 2008; Slavkin *et al.* 1992). So the zebrafish is a good model to research the tooth pathobiology.

The extremely low degree of bone mineralization in zebrafish *sp7* mutants is consistent with previous results in both zebrafish and medaka (Ortuno *et al.* 2010; Renn and Winkler 2014). Among bone mineralization-related genes, loss of *sp7* decreased the expression levels of *bglap*, *sparac*, *spp1*, *colla1a*, and *coll10a1a* in our results. The latter two genes were previously identified as direct targets of *sp7* (Ortuno *et al.* 2010). *spp1* encodes osteopontin (Reinholt *et al.* 1990), *sparac* encodes osteonectin (Termine *et al.*



**Figure 6.** Model of the how *sp7* regulates tooth development and bone mineralization.

1981), and *bglap* encodes osteocalcin (Weinreb et al. 1990); thus, these three genes play an important regulatory role in bone mineralization, especially *bglap*. The finding that *sp7* can directly regulate *bglap* expression by binding to the *bglap* gene promoter further supports that mineralization of the zebrafish bone is achieved through the interaction of Sp7 with *bglap* (figure 6). However, some of the bones could still be mineralized in the *sp7* mutants, suggesting that there may be additional pathways that also play a role in bone mineralization (figure 6).

The *Dlx* gene family is associated with many developmental processes, such as mandibular and limb development (Verreijdt et al. 2006; Merlo et al. 2000), in which *dlx2* is an important transcription factor in tooth mineralization (Thomas et al. 1995). Previous studies have found that retinoic acid signaling pathway regulates the expression of *dlx2b* in pharyngeal ridge and affects the development of zebrafish teeth (Gibert et al. 2015). And *dlx5* is directed regulated by *sp7* (Lee et al. 2003). In our experiments, we found that Sp7 can positively regulate the development of zebrafish teeth though regulate the expression of *dlx2b*, and Sp7 overexpression can also rescue *dlx2b* expression and tooth loss phenotype, which indicates that Sp7 can regulate tooth development through *dlx2b* (figure 6).

In conclusion, zebrafish Sp7 can directly regulate the expression of the *dlx2b* and *bglap* genes through Sp7-binding sites of the promoter regions. This study thus provides new insights into the role of Sp7 in bone mineralization and tooth development.

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