

## Construction and characterization of a *sox9b* transgenic reporter line

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**ABSTRACT** The transcription factor SOX9 is a member of the SRY-related high-mobility-group box (SOX) superfamily of genes. In mammals, *Sox9* plays important roles in many developmental processes including craniofacial, skeletal and heart morphogenesis, retinal and brain development, and gonad differentiation. Human mutations in *SOX9* or the *SOX9* promoter result in campomelic dysplasia, a severe genetic disorder, which disrupts skeletal, craniofacial, cardiac, neural and reproductive development. Due to the duplication of the teleost fish genome, zebrafish (*Danio rerio*) have two *Sox9* genes: *sox9a* and *sox9b*. Loss of *sox9b* in zebrafish results in loss of function phenotypes that are similar to those observed in humans and mice. In order to generate a transgenic *sox9b:EGFP* reporter line, we cloned a 2450 bp fragment of the *sox9b* promoter and fused it to an EGFP reporter. Consistent with reported *sox9b* expression and function, we observed *sox9b:EGFP* in the developing heart, skeletal and craniofacial structures, brain, retina, and ovaries. Our resulting transgenic line is a useful tool for identifying and studying *sox9b* function in development and visualizing a number of zebrafish organs and tissues in which *sox9b* is normally expressed.

**KEY WORDS:** *sox9b*, *Sox9*, *SOX9*, zebrafish, heart, craniofacial, skeletal, ovary, brain, campomelic dysplasia

*SOX9* encodes a transcription factor that is a member of the SRY-related high-mobility-group box (SOX) superfamily of genes. *Sox9* plays critical roles in craniofacial and heart morphogenesis (Hofsteen *et al.*, 2013; Smith *et al.*, 2011; Yan *et al.*, 2005; Yokoi *et al.*, 2009), as well as retinal and brain development (Esain *et al.*, 2010; Scott *et al.*, 2010; Stolt *et al.*, 2003; Yan *et al.*, 2005), and gonad differentiation (Morais da Silva *et al.*, 1996). In humans, mutations that reduce *SOX9* function produce campomelic dysplasia (CD), a severe genetic disorder (Foster *et al.*, 1994; Wagner *et al.*, 1994).

Individuals with CD have numerous skeletal malformations including femoral and tibial bowing, vertebral hypoplasia, cervical spine malformations, as well as underdeveloped shoulder blades and pelvic bones, and 11 pairs of ribs. CD patients also exhibit the Pierre Robin sequence, which is characterized by congenital facial abnormalities, incomplete closure of the roof of the mouth (cleft palate), small jaw/mandible (micrognathia) and posterior positioning of the tongue because of changes in jaw structure (glossoptosis) (Foster *et al.*, 1994; Wagner *et al.*, 1994). In addition, individuals with CD exhibit Tetralogy of Fallot, a set of four

concurrent congenital cardiac abnormalities that include ventricular septal defect, overriding aorta, pulmonary stenosis, and right ventricular hypertrophy (Foster *et al.*, 1994; Wagner *et al.*, 1994). Furthermore, CD is associated with autosomal XY sex reversal (Foster *et al.*, 1994; Wagner *et al.*, 1994), in which genetic males appear, based on primary and secondary sexual characteristics, to develop as females.

Due to the teleost fish genome duplication, zebrafish (*Danio rerio*) have two *Sox9* genes, *sox9a* and *sox9b* (Chiang *et al.*, 2001). *sox9a* and *sox9b* have both overlapping and distinct expression domains as well as shared and divergent functions (Yan *et al.*, 2005). We focused on constructing a tool for specifically visualizing *sox9b* expression, because we are interested in understanding the molecular mechanisms that underlie phenotypes resulting from

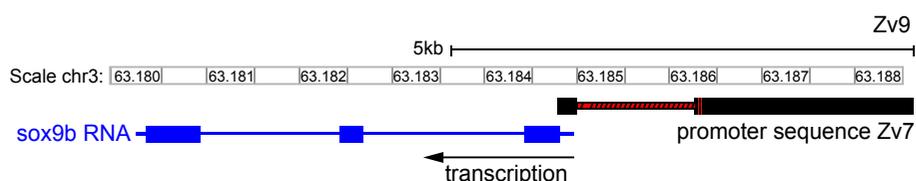
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*Abbreviations used in this paper:* CD, campomelic dysplasia; EGFP, enhanced green fluorescent protein; hpf, hours post fertilization; *sox9b*, sex determining region on the Y chromosome-box 9b, TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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**Fig. 1. Schematic comparing the cloned 2450 *sox9b* promoter sequence in builds Zv7 and Zv9.** BLAT of the cloned 2450 bp *sox9b* promoter sequence in build Zv7 with the sequence in build Zv9. Both a short region adjacent to the transcriptional start site and a ~1.1 kb region align well. However, approximately 1.4 kb has no alignment in build Zv9 and has been replaced by a unique sequence. Solid black bars in the Zv7 promoter sequence schematic indicate areas of sequence alignment. Red regions indicate areas where the sequences in Zv7 and Zv9 do not align.

exposure to the persistent environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Embryonic TCDD exposure produces numerous developmental defects including craniofacial malformations, heart malformations, and impairs regenerative capacity. TCDD exposure also results in the downregulation of *sox9b* in the developing jaw, heart and the regenerating fin, all tissues in which TCDD-induced phenotypes are observed (reviewed in King-Heiden et al., 2012). Thus, indicating that loss of *sox9b* expression is likely an important factor mediating the observed phenotypes. Consistent with this hypothesis and similar to the loss of function

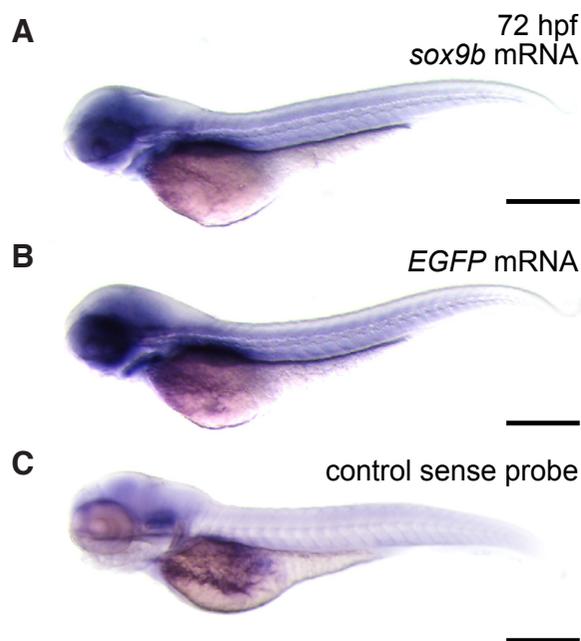
phenotypes observed in humans, loss of *sox9b* in zebrafish results in craniofacial malformations, as well as heart, brain and retinal defects (Esain et al., 2010; Hofsteen et al., 2013; Yan et al., 2005).

We cloned a 2450 bp fragment near the *sox9b* transcriptional start site and fused it to an EGFP reporter to generate a transgenic *sox9b*:EGFP reporter line. Consistent with reported *sox9b* expression and function, we observed *sox9b*:EGFP in developing heart, skeletal and craniofacial structures, brain, retina, and ovaries. Consequently, we have generated a transgenic line that is useful for identifying and studying a number of zebrafish organs and tissues in which *sox9b* is expressed.

## Results and Discussion

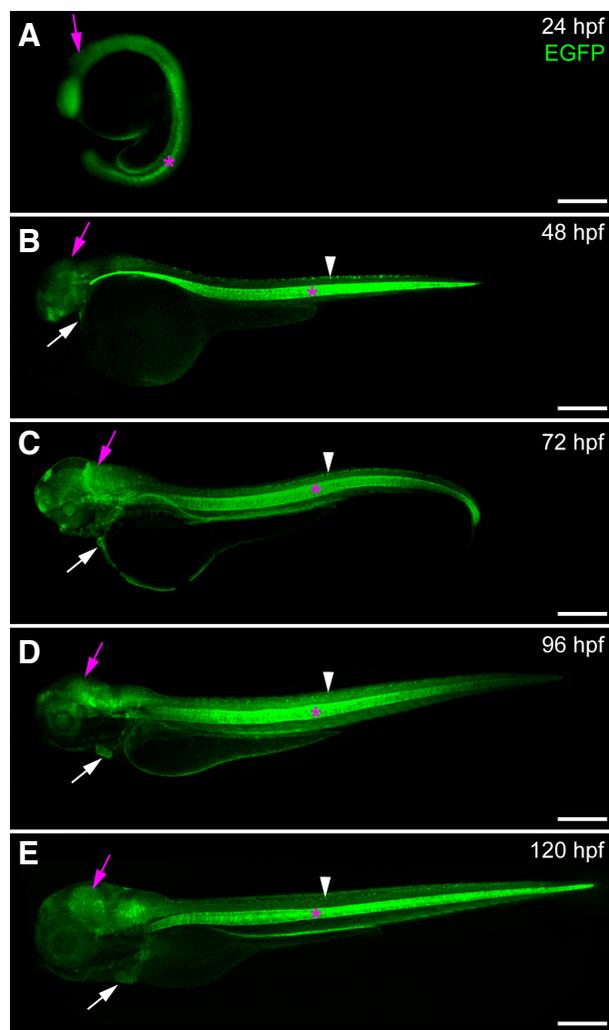
### Cloning and 5' sequencing of the *sox9b* transcriptional start site

This work was initiated using the Zv7 assembly of the zebrafish genome and targeted a region starting ~2500 base pairs upstream of the *sox9b* transcriptional start site. This region was amplified by

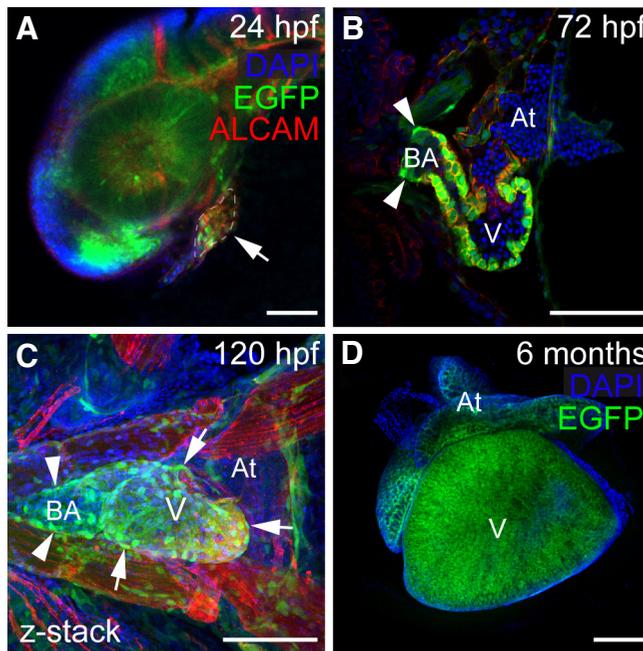


**Fig. 2 (Above). *sox9b* transgenic line recapitulates the endogenous pattern of *sox9b* expression.** (A-C) Lateral views of 72 hpf embryos. To detect *sox9b*, in situ hybridization was performed on whole embryos. (A) *sox9b* mRNA in transgenic embryos. (B) EGFP mRNA in transgenic embryos. (C) A sense probe was used as a control for nonspecific binding. Scale bars, 100 microns.

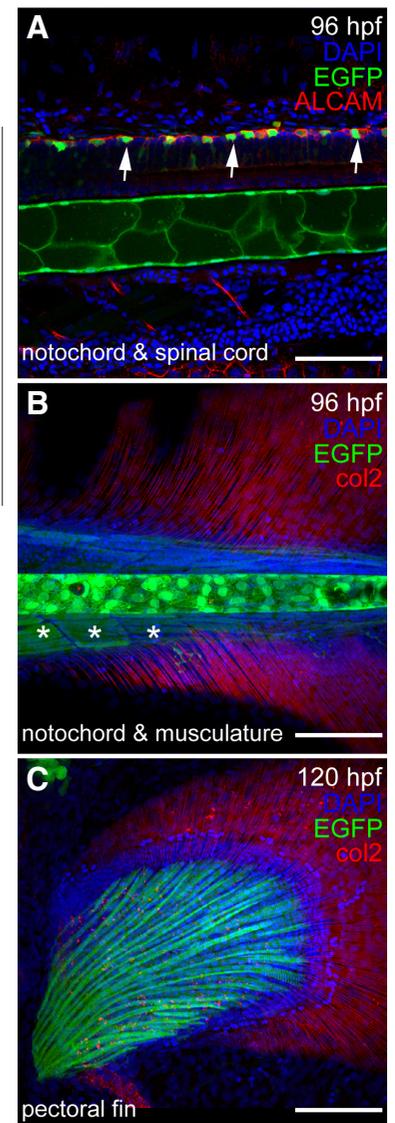
**Fig. 3 (Right). Expression of *sox9b*:EGFP during embryonic and larval development.** (A-E) Lateral views of *sox9b*:EGFP embryos and larvae. (A) Epifluorescent image at 24 hpf. (B-E) Confocal images at 48 hpf (B), 72 hpf (C), 96 hpf (D) and 120 hpf (E). *sox9b*:EGFP expression is detected in the brain (purple arrows), eye, heart (white arrows), jaw, spinal cord (white arrowhead) and notocord (pink asterisks). Scale bars, 100 microns.



**Fig. 4 (Left). Expression of sox9b:EGFP in the heart.** (A-D) Confocal images of sox9b:EGFP expression in embryonic, larval, and adult zebrafish hearts. Cardiomyocytes are marked with activated leukocyte cell adhesion molecule (ALCAM; red in A-C) and nuclei are stained with DAPI (blue); (A) Lateral view of a 24 hpf embryo. The developing heart is outlined and indicated by the arrow. (B,C) Ventral views of a 72 and 96 hpf hearts. Expression of sox9b:EGFP is detected in cardiomyocytes, the bulbus arteriosus (arrowheads in B and C), and in epicardial cells (arrows in C). (D) Ventral view of an adult heart. Expression of sox9b:EGFP in cardiomyocytes continues into adulthood. V is ventricle; At, atrium; BA, bulbus arteriosus. Anterior is to the left in all panels. Scale bars, 50 microns.



**Fig. 5 (Right). Expression of sox9b:EGFP in larval spine, skeleton, and fin.** (A-C) Lateral views of a 96 hpf and 120 larvae. Confocal images of sox9b:EGFP expression (green) in zebrafish spinal cord (A), notochord (A,B), and pectoral fin (C). (A) Neurons in the spinal cord are marked with activated leukocyte cell adhesion molecule (ALCAM; red in A). Expression of sox9b:EGFP is seen in a subset of neurons (arrows) and in the notochord. (B) sox9b:EGFP is also expressed in muscle associated with the spine (asterisks in B) and the developing pectoral fin. Collagen 2 (col2; red in B,C) is expressed in the fins. (A-C) Nuclei are stained with DAPI (blue). Scale bars, 50 microns.



PCR using zebrafish chromosomal DNA as template. According to the Zv7 assembly, our sequence should have a length of 2570 base pairs spanning scaffolds 302.4, 302.5 and 302.6; however, sequencing and alignment with CodonCode Aligner determined that our clone was 2450 base pairs long. The Zv7 build includes ambiguous nucleotides and additional bases, mostly GT repeats, not present in our sequence.

The sequence that we found upstream of *sox9b* varies substantially from build Zv9. Two portions of our cloned sequence, from -1 to -156 and -246 to -1361, produced a strong match with Zv9 (Fig. 1) and previous sequence builds. The remainder of our clone, from -1362 to -2450, matches well in scaffolds 302.5 and 302.6 in build Zv7, but this sequence has been lost in subsequent builds. In build Zv9 a short section of our clone, from -157 to -245, was replaced with a 1378 bp segment not found in our sequence. Given that we are able to produce amplicons anchored within our predicted sequence and within the well-established first exon of *sox9b* using genomic DNA from the AB strain, we conclude that our sequence for this region (Supplementary Fig. S1) and build Zv7 are more accurate for this locus.

TABLE 1

**SOX9B:EGFP EXPRESSION AT DIFFERENT STAGES OF ZEBRAFISH DEVELOPMENT<sup>1</sup>**

sox9b:EGFP expression	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf	5 wk	6 m
Heart	Y	Y	Y	Y	Y	Y	Y
Forebrain	Y	Y	Y	Y	Y	Y	Y
Midbrain	Y	Y	Y	Y	Y	Y	Y
Hindbrain	Y	Y	Y	Y	Y	Y	Y
Olfactory Bulb	-*	Y	Y	Y	Y	NS**	Y
Spinal Cord	Y	Y	Y	Y	Y	Y	Y
Notochord	Y	Y	Y	Y	Y	NS	NS
Eye	Y	Y	Y	Y	Y	NS	Y
Jaw/Jaw cartilages	-	Y	Y	Y	Y	Y	Y
Branchial Arches	NS	Y	Y	Y	Y	Y	Y
Pectoral Fin	-	Y	Y	Y	Y	Y	Y
Vertebrae	-	-	-	-	-	Y	Y
Ovary	-	-	-	-	-	Y	Y
Intestine	NS	NS	NS	NS	NS	Y	Y
Skeletal Muscle	NS	NS	Y	Y	Y	Y	Y
Jaw Muscle	NS	NS	Y	Y	Y	NS	NS
Cranial Nerves	NS	NS	Y	Y	Y	NS	NS

Note: 5-10 embryos were scored for expression at each time point for each organ or structure. \*(-)=organ/structure had not yet formed. \*\*(NS)=Not scored at this time point.

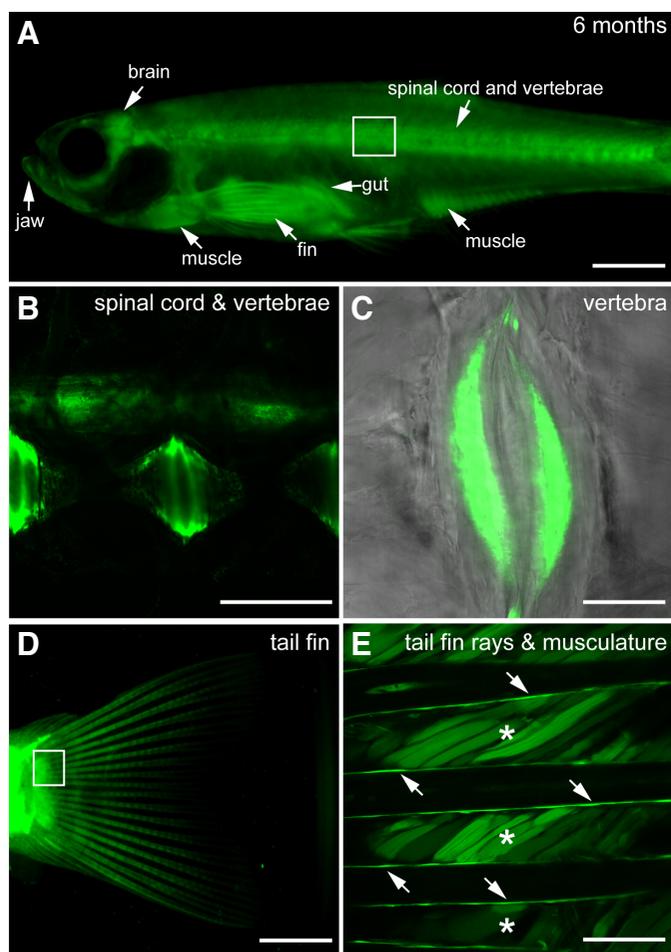
**Creation and confirmation of a sox9b:EGFP reporter**

We used the 2450 bp *sox9b* promoter fragment to create an EGFP reporter plasmid and transgenic zebrafish reporter line. To verify that the *sox9b*:EGFP transgenic line recapitulated the endogenous pattern of *sox9b* expression, we performed *in situ* hybridization and compared expression patterns of *EGFP* mRNA with *sox9b* mRNA (Fig. 2 and data not shown). We found that *EGFP* and *sox9b* mRNA expression patterns were consistent in embryos and larvae during the first five days of development, which indicates that the 2450 bp promoter fragment is sufficient

to drive expression of *EGFP* mRNA in a pattern that resembles endogenous expression of *sox9b* mRNA. Fig. 2 shows the consistent expression patterns in 72 hpf larvae.

#### Expression of *sox9b:EGFP* during early embryonic and larval development

We followed *sox9b:EGFP* expression in developing zebrafish from 24 to 120 hpf (Fig. 3). Expression was detected in tissues previously shown to express *sox9b*, including developing heart (Hofsteen et al., 2013), jaw (van der Velden et al., 2013), brain (Esain et al., 2010), retina (Yokoi et al., 2009), and ovary (Chiang et al., 2001) (see also Table 1). For a more complete characterization of the *sox9b:EGFP* transgenic line, we examined reporter expression in greater detail during cardiac, skeletal, craniofacial, neural, retinal, and ovarian development.



**Fig. 6 (Left).** Expression of *sox9b:EGFP* in adult skeleton and skeletal muscle. (A-E) Lateral views of six month old *sox9b:EGFP* transgenic fish. (B) Area boxed in (A). Expression is detected in the spinal cord and vertebrae. (C) Magnification of the *sox9b:EGFP* expressing growth plates in the vertebrae. (D) Muscle associated with the tail fin and rays within the tail fin express *sox9b:EGFP*. (E) Magnification of the box in (D). Asterisks mark the muscle while arrows point to the rays. Scale bars in (A,D), 150 microns. Scale bars in (B,C,E), 50 microns.

**Fig. 7 (Right).** Expression of *sox9b:EGFP* in larval jaw and associated skeletal muscle. (A-D) Ventral views of 72 hpf larvae stained for synaptic vesicles (SV2; red). *sox9b:EGFP* expression was detected in the perichondrium and in chondrocytes. (B,D) Magnification of regions in the white boxes in panels (A,C) respectively. Expression is associated with the ceratohyal (CH), Meckel's (M), palatoquadrate (PQ), ceratobranchials (not shown) and basihyal (not shown) cartilages. Expression was also detected in muscles associated with the jaw including the intermandibularis anterior (IMA), intermandibularis posterior (IMP), interhyoideus (IH), and sternohyoideus (SH). *sox9b:EGFP* was also observed in the branchial arches (BA). Scale bars, 50 microns.

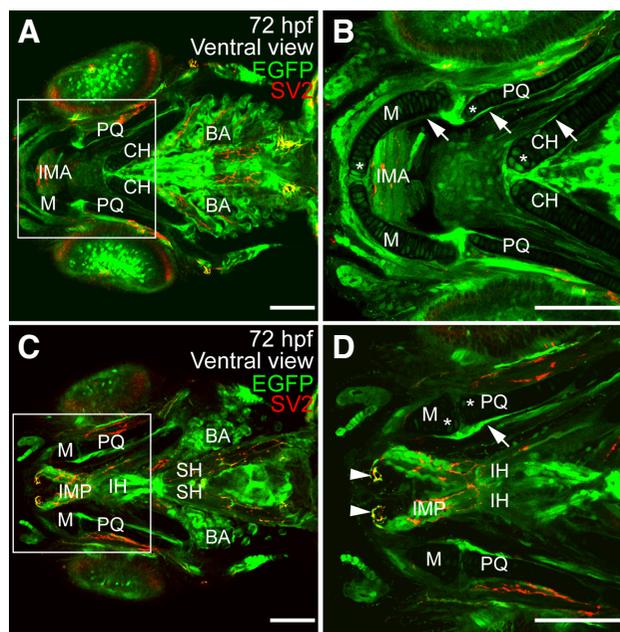
#### Cardiac development

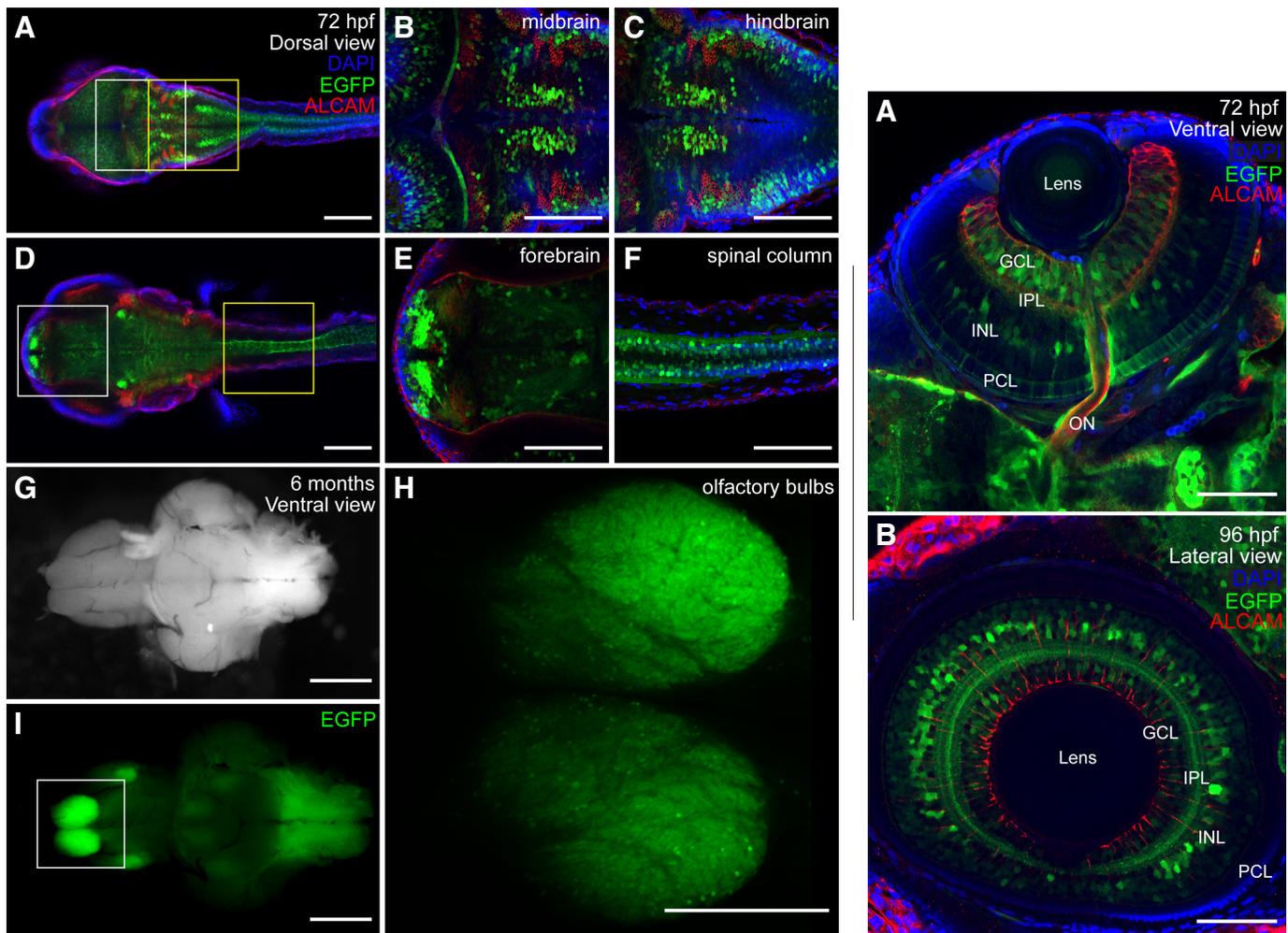
In zebrafish, *sox9b* is necessary for proper development of the proepicardial progenitors cells, the epicardium, and heart valves (Hofsteen et al., 2013). Expression of *sox9b:EGFP* was first observed in the developing zebrafish heart at 24 hpf (Fig. 4A) and *sox9b:EGFP* expression continued to be detected into adulthood (Fig. 4D). We used ALCAM immunostaining to visualize the myocardium and found *sox9b:EGFP* expression in ventricular and atrial cardiomyocytes in both embryonic and adult hearts (Fig. 4B-D). In addition, we observed expression in epicardial cells (arrows in Fig. 4C) and the bulbous arteriosus (Fig. 4B and C).

#### Skeletal and craniofacial development

Humans with CD have numerous skeletal malformations. Consistent with these phenotypes, expression of *sox9b* in zebrafish was detected in the skeleton, fins and jaw during embryonic, larval stages and adult stages. Throughout embryonic and larval development, *sox9b:EGFP* expression was found in the notochord (Fig. 5A and B) and spinal cord (Fig. 5A) as well as the pectoral (Fig. 5C) and tail fins (data not shown). In the adult, *sox9b:EGFP* expression was found in the spinal column (Fig. 6A and B), vertebrae (Fig. 6A-C), and the pectoral and tail fins (Fig. 6D and 6E). In both larvae and adults, *sox9b:EGFP* expression was detected in muscles associated with the spine and fins (Fig. 5A, Fig. 6A and 6E).

In the developing jaw, *sox9b:EGFP* was expressed in the perichondrium, a layer of connective tissue surrounding the





**Fig. 8 (Left). Expression of *sox9b:EGFP* in larval and adult brain. (A-F)** Confocal images showing dorsal views of a 72 hpf larval zebrafish. Larvae were stained for ALCAM (red) and mounted in Vectashield with DAPI (blue). **(B,C)** Magnification of the regions in the white and yellow boxes in (A). **(E,F)** Magnification of the regions in the white and yellow boxes in (D). Expression of *sox9b:EGFP* in the fore-, mid- and hindbrain as well as in the spinal cord. **(G,I)** Bright field images and epifluorescence and ventral views of an adult brain. **(H)** Magnification of olfactory bulb boxed in (I). Scale bars, 50 microns.

**Fig. 9 (Right). Expression of *sox9b:EGFP* in retina. (A,B)** Ventral and lateral views of larval eyes at 72 and 96 hpf stained for ALCAM (red) and mounted in Vectashield with DAPI (blue). **(A)** At 72 hpf, *sox9b:EGFP* expression was observed in a subset of cells in the photoreceptor cell layer (PCL), inner nuclear layer (INL), and ganglion cell layer (GCL) and within the inner plexiform layer (IPL). **(B)** By 96 hpf, *sox9b:EGFP* expression in the PCL was waning, however *sox9b:EGFP* was visible in the INL, IPL, and GCL. Scale bars, 50 microns.

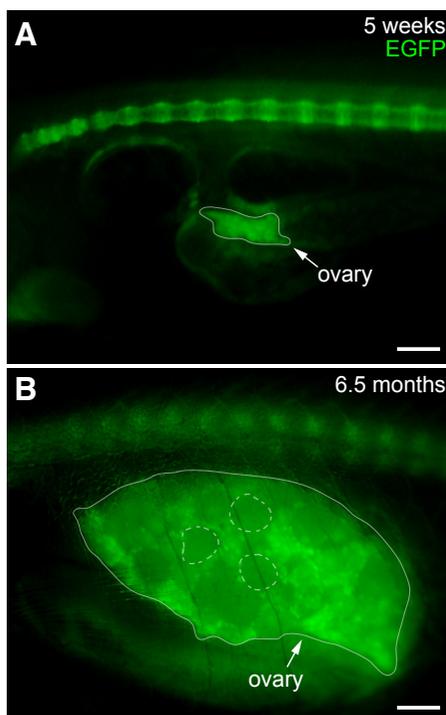
craniofacial cartilages, and in chondrocytes (Fig. 7A-D). During embryonic and larval stages, expression was associated with the ceratohyal, Meckel's, palatoquadrate, ceratobranchials (not shown) and basihyal (not shown) cartilages (Fig. 7A-D). Expression was also detected in muscles associated with the jaw including the intermandibularis anterior (Fig. 7A and B), intermandibularis posterior (Fig. 7C and D), interhyoideus (Fig. 7C and D), and sternohyoideus (Fig. 7C). In addition, *sox9b:EGFP* was observed in the branchial arches at 72, 96, and 120 hpf (Fig. 7A and C).

### Nervous system

*Sox9* plays critical roles during vertebrate neural development. In zebrafish hindbrain, fibroblast growth factor receptor signaling regulates gliogenesis by inducing *sox9a* and *sox9b* expression (Esain *et al.*, 2010). We observed *sox9b:EGFP* expression in the

developing nervous system beginning at 24 hpf and continuing into adulthood (Fig. 2 and Fig. 8). *sox9b:EGFP* expression was detected in the fore-, mid- and hindbrain (Fig. 2 and Fig. 8A-E and G). We also observed expression in the spinal cord (Fig. 8F and Fig. 5A), cranial nerves and lateral line neuromasts (data not shown). *sox9b:EGFP* was observed in what appear to be axon projections (asterisks in Fig. 8E) indicating that *sox9b* may be expressed in neurons in the forebrain.

Signs of CD include underdeveloped or absent olfactory tracts and bulbs (Houston *et al.*, 1983). Consistent with these findings, we found *sox9b:EGFP* expression in the developing olfactory organs (data not shown) and corresponding neural tissue during embryogenesis (Fig. 8D and E), and, in adult fish, strong expression was seen in olfactory epithelium (data not shown) and olfactory bulbs (Fig. 8H and I).



**Fig. 10. Expression of *sox9b*:EGFP in ovary.** Lateral views of juvenile (A) and adult (B) ovary. The ovaries are outlined with a solid line. Examples of mature follicles are outlined with dashed circles. Scale bars, 100 microns.

### Retina

Both *sox9b* and *sox9a* are required for retinal differentiation and regulate the expression of key genes during zebrafish retinogenesis (Yokoi et al., 2009). Consistent with Chiang et al., (2001), we observed *sox9b*:EGFP expression in the retina at 24 hpf when retinal ganglion cells are being generated. At 72 hpf, *sox9b*:EGFP expression was observed in a subset of cells in the photoreceptor cell layer, inner nuclear layer, and ganglion cell layer, as well as projections within the inner plexiform layer (Fig. 9A). By 96 hpf, *sox9b*:EGFP expression in the photoreceptor cell layer was waning, however *sox9b*:EGFP was clearly visible in the inner nuclear layer, inner plexiform layer and ganglion cell layer (Fig. 9B).

### Ovary

In humans, the SRY gene has been identified as the major sex determination gene on the Y chromosome. A peak of Sry transcription factor activity induces *Sox9* expression, leading to the development of primary and secondary male sex characteristics. In zebrafish, the developing ovary expresses *sox9b*, whereas the developing testis expresses *sox9a* (Chiang et al., 2001). Consistent with *in situ* hybridization results (Chiang et al., 2001), we found *sox9b*:EGFP expression in ooplasm of oocytes within the immature ovaries of juvenile zebrafish (Fig. 10A). *sox9b*:EGFP was also found in the mature adult ovary. In adults, the *sox9b*:EGFP signal appeared stronger in the ooplasm of the smaller, less mature perinucleolar oocytes compared to larger, more mature vitellogenic stage follicles (Fig. 10B).

## Conclusions

*Sox9* and the corresponding co-orthologs of *Sox9*, *sox9a* and *sox9b*, are important in a number of developmental processes that are essential for human health. Herein we described construction of a *sox9b* reporter line and provided broad observations about where the reporter is expressed during development. Given that we find *sox9b* expression to be associated with the development of many organs at many different developmental stages, our transgenic reporter line will be useful tool for investigators studying diverse processes in developmental biology as well as genetics and toxicology.

## Materials and Methods

### Zebrafish strains and husbandry

Zebrafish were maintained as described in (Hofstee et al., 2013). The *Tg(-2421/+29sox9b:EGFP<sup>mw2</sup>)* line was generated in casper (*roya9; mitfaw2*) and AB backgrounds (White et al., 2008). The casper line, used for all imaging experiments, is referred to as *sox9b*:EGFP. All procedures involving animals were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison, and adhered to the NIH "Guide for the Care and Use of Laboratory Animals."

### Cloning *sox9b* genomic fragments

Genomic DNA was extracted from zebrafish embryos for use as a PCR template (Xiong et al., 2008). We used the zebrafish genome build Zv7 to design PCR primers. Using the build information, we amplified a 2,450 bp fragment extending in the 5' direction from the base pair immediately adjacent to the transcriptional start site. The 2450 bp PCR product was inserted into the pCR-II TOPO vector for propagation (Invitrogen, Carlsbad, CA). The 2450 *sox9b* clone was inserted upstream of EGFP in the Tol2 transgenesis system as previously described to make reporter constructs (Kwan et al., 2007). Primers used are listed in Supplemental Table S1. All clones were sequenced at the UW Biotechnology Center.

### *sox9b*:EGFP transgenic embryos

The 2450 bp *sox9b* reporter construct (20 ng/μl) was injected into 1-2 cell stage AB or casper embryos and injected embryos were raised to adulthood. Adult founder fish were identified by crossing potential founders to either AB or casper fish and screening offspring for *sox9b*:EGFP expression. *sox9b*:EGFP positive embryos were raised to generate a F1. *sox9b*:EGFP-positive F1 adults were incrossed to generate a homozygous F2 line with a strong *sox9b*:EGFP signal. Images shown are from representative F1 and F2 embryos, juveniles, and adults.

### Whole-mount *in situ* hybridization

Whole mount *in situ* hybridization was performed as previously described (Hofstee et al., 2013). Anti-sense *sox9b* and EGFP RNA probes were synthesized from PCR amplicons made from plasmid DNA (*pCMV-Sport6sox9b*, Open Biosystems, Huntsville, AL) and *p3E-EGFPpA*, (Kwan et al., 2007) as the template. Primers used for *sox9b* were: F', 5'-agc cac acc cct ctt aca gcc a-3'; *sox9b* R', 5'-cga tgt taa tac gac tca cta tag gga gtc agc gtc agg atg cgc t-3'. The resulting *sox9b* probe (537 bp) spans a region encompassing base pairs 2048 to 2584 in the 3' UTR of the *sox9b*-001 cDNA. EGFP F', 5'-gac ggc gac gta aac ggc ca-3'; and EGFP R', 5'-cga tgt taa tac gac tca cta tag ggt tgc tca ggg cgg act ggg t-3'. As a control, a sense EGFP probe was synthesized using the following primers: EGFP F', 5'-cga tgt taa tac gac tca cta tag ggg acg gcg acg taa acg gcc a-3' and EGFP R', 5'-ttg ctc agg gcg gac tgg gt-3'. The resulting EGFP probe (572 bp) spans a region encompassing base pairs 58 to 629 of the

*EGFP* gene. A T7 promoter (bold) was added to the reverse *sox9b* primer, reverse *EGFP* primer and forward control primer for *in vitro* transcription of the RNA probe with T7 RNA polymerase. *In situ* hybridization samples were imaged with an Olympus DP72 digital camera attached to an Olympus SZX16 stereomicroscope.

#### Fluorescent immunohistochemistry and confocal microscopy

Antibody staining was performed as previously described (Hofstee *et al.*, 2013). Primary antibodies were used at the following dilutions in phosphate buffered saline with 4% bovine albumin serum and 0.3% Triton (PBT): activated leukocyte cell adhesion molecule (ALCAM; ZIRC) 1:50, synaptic vesicles (SV2; DSHB) 1:100, and collagen type II (II-II6B3; DSHB) 1:50. Secondary anti-mouse antibodies (Alexa 568; Invitrogen) were used at 1:200 dilution in PBT. Embryos were mounted in Vectashield or Vectashield with DAPI (Vector Laboratories). Confocal images were collected on an Olympus Fluoview FV1000 microscope and were processed using Adobe Photoshop.

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#### Author contributions

JSP performed the fluorescent immunohistochemistry and confocal microscopy, JSP & TRB collected the epifluorescent images, KMX, FRB & AJG cloned the 2450 fragment, & performed sequencing & genotyping. TRB generated the 2450 Casper line. KMX & PH performed *in situ*. JSP, TRB, FRB, WH and REP wrote the manuscript.

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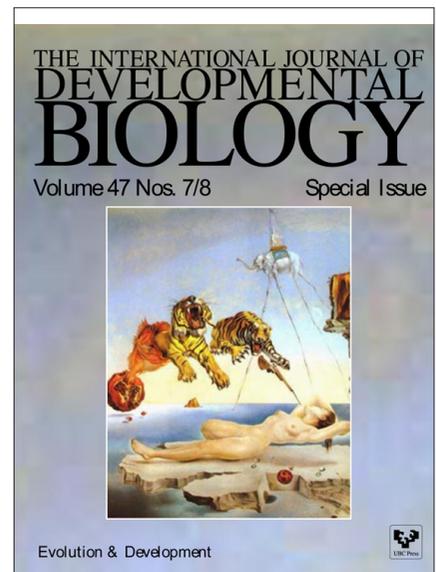
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