KRÜPPEL-LIKE TRANSCRIPTION FACTOR 6 & 7 mRNAs (*KLF6 & KLF7*) EXPRESSION IN THE CENTRAL NERVOUS SYSTEM (CNS) OF THE DEVELOPING ZEBRAFISH

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KRÜPPEL-LIKE TRANSCRIPTION FACTOR 6 & 7 mRNAs (KLF6 & KLF7) EXPRESSION IN THE CENTRAL NERVOUS SYSTEM (CNS) OF THE DEVELOPING ZEBRAFISH

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Thesis

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ABSTRACT

Krüppel-like factors (KLFs) are a family of 17 transcriptional regulatory proteins containing evolutionarily conserved zinc finger DNA binding domains. Klf6 and Klf7, two members of this family, have been shown to play pivotal roles in a variety of processes including differentiation, proliferation, and regeneration of neurons in mammal central nervous systems. However, there is little information about *Klf6* and *Klf7* expression and function in non-mammal vertebrates. In order to provide a foundation for future study(s) of these two Klfs function in non-mammal central nervous system (CNS) development, I used in situ hybridization to examine expression patterns of *Klf6* and *Klf7* in the CNS of developing zebrafish. I found that these two Klfs had differential expression patterns in developing zebrafish, with *Klf6* weakly expressed in only a few brain regions of one day old zebrafish, and its expression became undetectable in older (2-3 days) embryos, while there was an increased expression of *Klf7* in the embryonic brain as development proceeded. CNS structures showing apparent *Klf7* expression included the dorsolateral telencephalon, pretectum, lateral thalamus, hypothalamus, dorsal midbrain, cerebellum, dorsolateral medulla oblongata, dorsal spinal cord, retinal ganglion cell layer and inner nuclear layer of the retina.

ACKNOWLEDGEMENTS

I would like to thank Dr. Daniel Goldman (University of Michigan) for providing the zebrafish *Klf6* and *Klf7* cDNAs. This research was supported by NIH EY13879 (Q. Liu). I was supported by teaching assistantships from the University of Akron.

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CHAPTER I

INTRODUCTION

Krüppel-like Factors

Krüppel-like factors (KLFs) are a group of transcription factors that contain zinc-finger DNA binding domains (Moore et al., 2011). There are three zinc-finger DNA binding domains located in the C-terminal region of each KLF that are highly conserved (reviewed by Bieker, 2001; McConnell and Yang, 2010; Moore et al., 2011; Knoedler and Denver, 2014). This region links the zinc-finger tandem with a highly conserved Krüppel-link (Pearson et al., 2007). Each zinc-finger consists of two beta strands and a short alpha helix, and within the ββα motif, there are highly conserved regions of amino acids, known as the C(cysteine)2H (histidine)2 that chemically interacts with the Zn2+ ion (Brayer and Segal, 2008).

KLFs Binding DNA

The specificities of DNA-protein interactions are exceedingly important to transcription factor's function. KLFs are known to bind to the major groove of regulatory regions of their targets' genes (Brayer and Segal, 2008; Razin et al., 2012). Specifically, the three zinc-fingers in the carboxyl end of each KLF interact with GC/CACCC boxes within the DNA promoter (Moore et al., 2011). Although one zinc-finger interacts with only three nucleotides, the presence of three zinc-fingers in a KLF makes the protein-

DNA interaction more specific and stronger (Persikov et al., 2015). The N-terminal domains of KLFs, which are much less conserved, directly interact with coactivators or corepressors, and they are also used for classifying KLFs into different groups (see below) (Moore et al., 2011; Razin et al., 2012; Persikov et al., 2015).

Types of KLFs and Their Functions

So far 17 different types of KLFs have been isolated in vertebrates, and they are classified into three major groups or subfamilies based mainly on their consensus motifs in their N-terminal domains (Bieker, 2001; Moore et al., 2011). KLF3, 8 and 12 belong to group 1, and each contains a PVALS/T N-terminal consensus sequence known to interact with co-repressors of the C-terminal binding proteins (CtBPs). KLF members of group 2, including KLF1, 2, and 4, have an acidic and inhibitory N-terminal domain (AIN) or an acidic domain coupled with a hydrophobic serine-rich domain in their N-termini (e.g. KLF6 and 7). Group 3 KLFs (e.g. KLF9, 10, 11, 13, 14, and 16) contain a consensus SIN3a-interacting domain in their N-termini. The remaining three KLFs, KLF5, 15 and 17, do not belong to any of the above groups, because they do not have identifiable consensus motifs in their N-termini (Moore et al., 2011).

KLFs are widely expressed in all major tissues and organs including nervous tissues, and they play roles in numerous developmental and physiological processes including adipocyte differentiation, angiogenesis, bone formation, heart and lung

development, neurogenesis and regeneration (Bieker, 2001; McConnell and Yang, 2010; Moore et al., 2011; Knoedler and Denver, 2014).

KLF6 and KLF7

KLF6 is a member of the group 2 KLFs (see above). KLF6 mRNA (*Klf6*) expression in the central nervous system (CNS) was examined in embryonic mice (Laub et al., 2001a). The message was detected in all major brain regions including the forebrain, midbrain, hindbrain, and the spinal cord, although only small subsets of cells in each CNS region contained Klf6 message. Klf6 is known to be involved in tumor suppression and pathogenesis of several cancers (reviewed by Andreoli et al., 2010; Moore et al., 2011), and in regeneration of retinal ganglion cell axons in adult zebrafish (Veldman et al., 2007). There is little information on Klf6 function in development of other nervous structures.

Like Klf6, Klf7 is also a member of the group 2 KLFs, and its mRNA (*Klf7*) was detected in the developing mouse CNS (Laub et al., 2001b). *Klf7* was found in all major CNS structures, and its expression domains in each region were wider than that of *Klf6*. A study of *Klf7* mutant embryonic mice showed that there were defects in several brain structures including olfactory bulb, cerebral cortex, hippocampus and retinal axons (Laub et al., 2005). More recently, Klf7 was demonstrated to enhance axon regeneration in rat corticospinal tracts (Blackmore et al., 2012). Furthermore, Klf6 and Klf7 promotes retinal axon regeneration in adult zebrafish (Veldman et al., 2007).

Zebrafish as a Model Organism

Zebrafish has become a preferred model organism for the study of vertebrate development in general, and development of the CNS in particular (Gemberling et al., 2013; Shi et al., 2015; Czopka, 2016). This is mainly due to many experimental advantages that zebrafish offer. Maintenance of adult zebrafish is easy and low-cost. They routinely produce large number of embryos. The embryos are transparent, which makes possible for observation of internal structures in live embryos. Zebrafish embryos develop fast, and most major brain regions are formed by 24 hours post fertilization (hpf). Furthermore, the zebrafish can reach reproductive stage in 2-3 months. The entire zebrafish genome was sequenced, and there are many molecular tools available to study molecular and/or cellular mechanisms underlying developmental processes. Importantly, molecular pathways regulating animal development have been found to be highly conserved, therefore, findings from studying zebrafish can provide us with potential insights into development of other animals including humans (Gemberling et al., 2013; Shi et al., 2015; Czopka, 2016).

There is no published report, to the best of my knowledge, on detailed *Klf6* and *Klf7* expression in embryonic zebrafish. Knowledge of their expression in developing zebrafish will provide the necessary foundation for future studies to determine their respective functions in zebrafish CNS development. My hypothesis is that *Klf6* and *Klf7* have distinct expression patterns in the developing zebrafish CNS, and this study was designed to test this hypothesis.

CHAPTER II

METHODS & MATERIALS

As described in Liu et al. (1999), zebrafish (Danio rerio) embryos were obtained from breeding in house wild type adults. The adult zebrafish were maintained at their optimal growing/living temperature (i.e. 28˚C), and a 14 hours' light/10 hours' dark cycle was used. To prepare zebrafish embryos for whole mount in situ hybridization (WISH), the embryos were raised in 400 ml cups half filled with fish tank water at 28˚C in an incubator. The water was supplemented with 0.003% 1-phenyl-2thiourea (PTU) to inhibit pigmentation formation in the embryos. The embryos were staged in hours post fertilization (hpf). The fish care procedures were based on those described in the Zebrafish Book (Westerfield, 2007). All animal related procedures comply with NIH standards and were approved by the University of Akron Committees on Use and Care of Animals in Research.

Zebrafish embryos (24-72 hpf) were removed from their chorionic membranes and anesthetized in 0.02% tricaine methanesulfonate (Sigma, St. Louis, MO). The embryos were fixed overnight in 4% paraformaldehyde (Fisher Scientifics, Waltham, MA) in 0.1 M phosphate buffered saline (PBS, pH=7.4) at 4˚C. Subsequent to the fixation, the embryos were washed three times, for 10 minutes each in PBS at room temperature on a moving platform. Embryos for whole mount in situ hybridization

(WISH) were then placed in increasing concentrations of methanol and stored in 100% methanol at -20˚C until used. Embryos for in situ hybridization on tissue sectioning (ISHT) were placed in 20% sucrose (in PBS) for 12-24 hours at 4˚C. The following day the embryos were embedded in a mixture (1:1) of 20% sucrose and OCT compound (Sakura, Netherlands) as described (Barthel and Raymond, 1990) to prevent ice crystals from forming during the freezing and cold storage. Molds for the embedding were made with aluminum foil wrapping around a stopper (about 2 cm in diameter) of a glass bottle. The embryos were oriented with their heads pointed to the bottom, embedded, and quickly placed in a mixture of dry ice and 95% ethanol until the embedding medium solidified. The frozen tissue blocks were placed in labeled biopsy bags and stored at -80˚C until cryosectioned. A cryostat (Bright, OTF5100) was used for cutting the tissue blocks at 16 μm. The tissue sections were placed on Fisher superfrost glass slides (Fisher Scientifics, Waltham, MA). The slides were allowed to air-dry for one hour at room temperature prior to storing at -20˚C.

Klf6 and *Klf7* cRNA Probe Synthesis

cDNAs corresponding to the open reading frames of zebrafish *Klf6* (GenBank Accession No. NM_201416) and Klf7 (GenBank Accession No. BC124329), generously provided by Dr. Daniel Goldman from the University of Michigan, were used as templates to generate respective antisense cRNA probes. Detailed information on the cloning (from 3-day post-optic nerve crush adult zebrafish retinal RNA) of the *Klf* cDNAs was described previously (Veldman et al., 2010). The manufacturer's procedure

(Roche, Indianapolis, IN) for making antisense digoxigenin (DIG)-labeled RNA probes was used. For the WISH/ISHT, I used the protocols described in detail in Liu et al. (1999). Briefly, for WISH, the embryos (n=6 for each stage/probe) were rehydrated through descending concentrations of methanol, treated with proteinase K (Roche, Indianapolis, IN) and fixed in 4% paraformaldehyde. After washing in PBST (PBS supplemented with Tween-20), the embryos were incubated in a hybridization solution (without cRNA probes) at the hybridization temperature (58oC) for two hours. This was followed by replacing the hybridization solution with a fresh hybridization solution containing either the *Klf6* cRNA probes (1 µg/ml) and was hybridized overnight. The next morning, the embryos were washed in 2X SCC, followed by rinsing in 50% fermamide in 2X SSC. Immunocytochemical detection of the DIG-labeled cRNA probes was performed by incubating the embryos in a PBST solution containing an alkaline phosphatase coupled anti-DIG Fab fragment antibody (Roche, 1:5000). Visualization of the labeling was achieved by placing the embryos in a solution made from dissolving a 4-nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) tablet (Roche) in 10 ml of distilled water, placed in dark at room temperature. The reaction was stopped when a satisfactory labeling was achieved.

For ISHT, the tissue sections were rehydrated in decreasing concentrations of ethanol followed by treating with proteinase K. The tissue sections were incubated in 0.1 M triethanolamine (Sigma), rinsed in 0.1 M triethanolamine with 0.25% acetic anhydride (Fisher Scientifics), dehydrated in increasing concentrations of ethanol and air-dried. 75 µl hybridization solution (2 µg/ml of klf7 cRNA probes) was applied to the tissue

sections on each slide, and the tissue sections were hybridized overnight at 58oC. The same anti-DIG antibody and NBT/BCIP tablet was used for the immunocytochemical detection and visualization of the labeling (see above for WISH).

Data Analysis

Processed embryos and tissue sections were observed under an Olympus BX51 microscope equipped with Normarski optics, and a SPOT Insight digital camera (Diagnostic Instrument Inc., Sterling Heights, MI). Digital images were adjusted for contrast and sharpness using Adobe Photoshop 6.0 software (San Jose, CA).

CHAPTER III

RESULTS

Klf6 expression in developing zebrafish

Major brain regions, including the spinal cord are visible at 24 hpf (Ross et al., 1992; Kimmel, 1993). Outside of the CNS, *Klf6* expression was restricted to the bilaterally located-olfactory placode (op) located anterodorsal to the telencephalon (tel), and trigeminal ganglion (gv) (Fig. 1A-D), posterior intestinal region near anus and a column of cells between the anal fin and tail trunk (Figure 1F). In the CNS, *Klf6* was detected in two columns of cells located in the lateral region of the medulla oblongata (Fig. 1E), and in the spinal cord (Fig. 1F). There was no apparent *Klf6* expression in older embryos (Figs. 2 and 3), except a weak expression in the trigeminal ganglion of 36 hpf embryos (Fig. 2B).

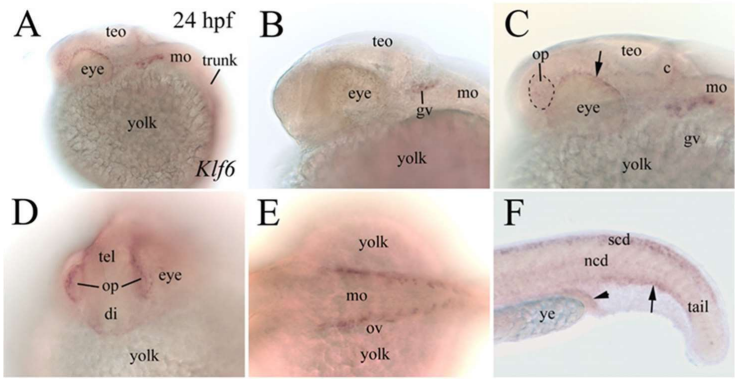

Figure 1. Zebrafish Klf6 expression in 24-hours post fertilization

Klf6 expression in 24 hpf zebrafish. Panels A-C, and F are lateral views, with anterior to the left and dorsal side up. Panel D is a frontal view (dorsal side up), while panel E is a dorsal view (anterior to the left). Panels B and C are higher magnified views of the head region of the embryo shown in panel A. The arrow in panel C points to retinal pigmented epithelium, and the olfactory placode (op) is outlined. The arrowhead in panel F points to stained intestinal region near the anus, while the arrow in F indicates staining between the anal fin and tail trunk. Abbreviations: c, cerebellum; di, diencephalon; gv, Abbreviations: c, cerebellum; di, diencephalon; gV, trigeminal ganglion; mo, medulla oblongata; ncd, notochord; op, olfactory placode; ov, otic vesicle; scd, spinal cord; tel, telencephalon;, teo, optic tectum; ye, yolk extension.

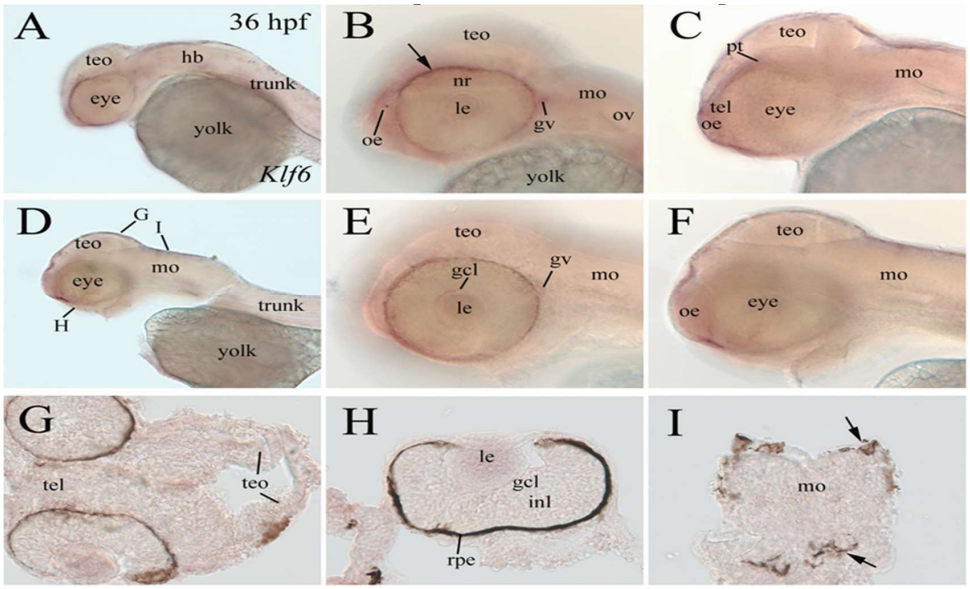

FIGURE 2. Zebrafish Klf6 expression in 36 & 50-hours post fertilization

Klf6 expression in 36 hpf (A-C) and 50 hpf (D-I) zebrafish. Panels A-C, and F are lateral views, with anterior to the left and dorsal side up. Panel D is a frontal view (dorsal side up), while panel E is a dorsal view (anterior to the left). Panels B and C are higher magnified views of the head region of the embryo shown in panel A. The arrow in panel B points to pigmented epithelium. Panels G-I are transverse sections of the head region, with panel H showing a higher magnification of the eye. Arrows in panel I indicate pigmented epithelium. Approximate levels of the sections are indicated in Panel D. Abbreviation: al, anterior lateral line placode; gcl, retinal ganglion cell layer; inl, inner nuclear layer; le, lens; nr, nuclear retina; oe, olfactory epithelium; rpe, retina pigmented epithelium. All other abbreviations are the same as in Fig. 1.

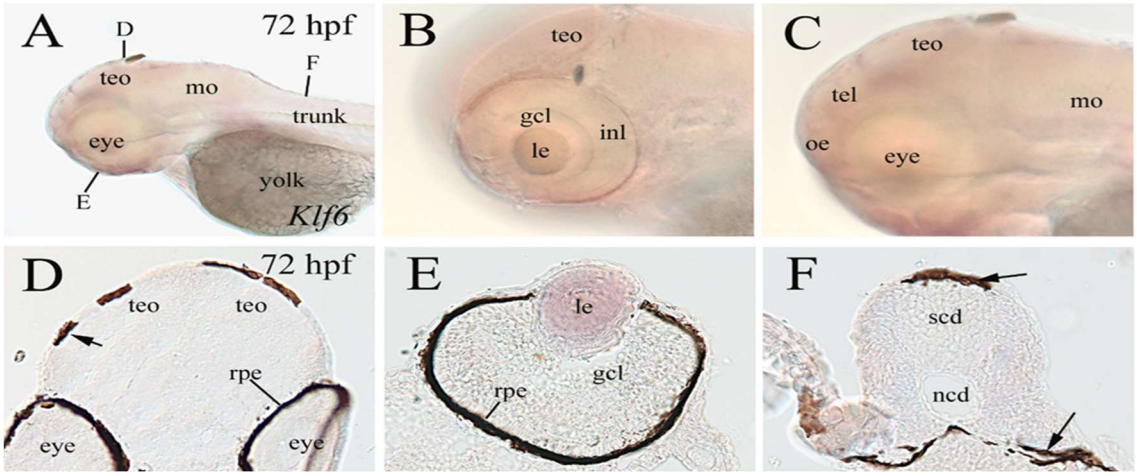

FIGURE 3. Zebrafish Klf6 expression in 72-hours post fertilization. Klf6 expression in 72 hpf zebrafish. Panels A-C are lateral views of a wholemount embryo anterior half and head region, with anterior to the left and dorsal side up. Panels B and C are higher magnifications of the head region shown in panel A, with focusing on the retina (panel B) and brain regions (panel C). Panels D-F are cross sections of embryos showing midbrain (panel D, dorsal up), retina (panel E, dorsal to the left), and trunk (panel F, dorsal up). Levels of the sections are indicated in panel A. Arrows in panels D and F point to pigmented epithelium. All abbreviations are the same as in Figs. 1-3.

***Klf7* expression in developing zebrafish**

In the CNS, Klf7 expression in 24 hpf zebrafish was limited to small regions in the telencephalon, diencephalon and hindbrain (Fig. 4). In the telencephalon, *Klf7*-expressing cells were confined to the most lateral portion the structure (Fig. 4D). In the diencephalon, Klf7 expression was detected in a few cells anterior to the optic tectum (Fig. 4C). *Klf7* expression in hindbrain was confined to the most lateral region of the medulla oblongata (Fig. 4E). *Klf7* expression was also observed throughout the spinal cord (Fig. 4F). Two peripheral nervous structures, the trigeminal ganglion (gV) and anterior lateral line placode (al, Fig. 4B and D), contained *Klf7*-expressing cells. Outside the nervous system, *Klf7* expression was seen in the olfactory placode (Fig. 4A-D), and the intestine near the anal area (Fig. 4F).

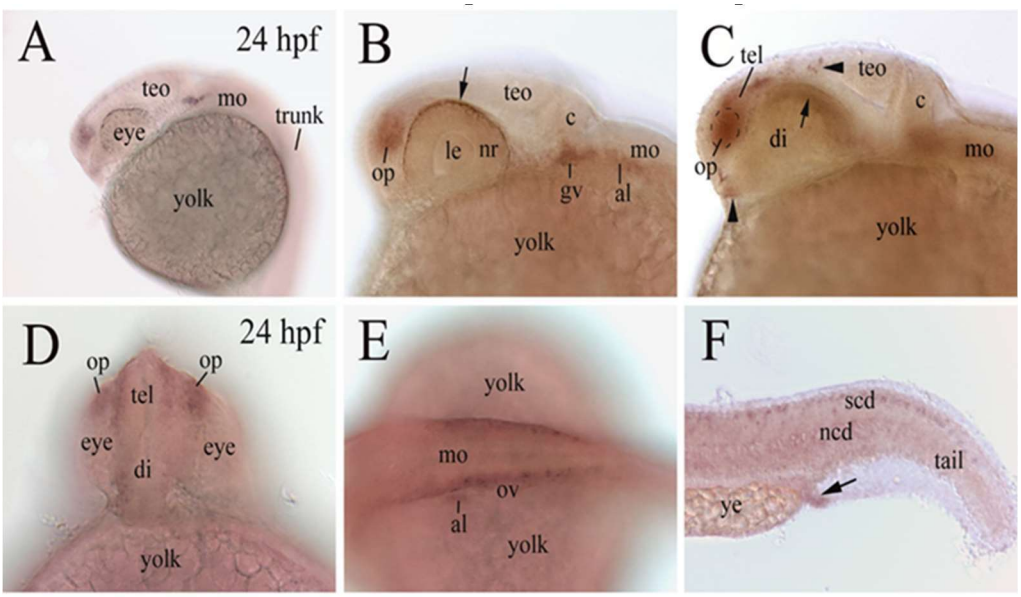

FIGURE 4. Zebrafish Klf7 expression in 24-hours post fertilization
Klf7 expression in 24 hpf zebrafish. Panels A-C, and F are lateral views, with anterior to the left and dorsal side up. Panel D is a frontal view (dorsal side up), while panel E is a dorsal view (anterior to the left). Panels B and C are higher magnified views of the head region of the embryo shown in panel A. The arrow in panels B and C points to retinal pigmented epithelium. Arrowheads in panel C indicate small clusters of Klf7-expressing cells. The arrow in panel F points to Klf7-expressing intestinal cells near the anus. All other abbreviations are the same as in Figs. 1-3.

At 36 hpf, the expression domains as well as expression levels (judging by staining intensity) for *Klf7* increased (Fig. 5). In the CNS, all telencephalic regions, except near the midline, expressed *Klf7* (Fig. 5D). It appeared that both the dorsal telencephalon (known as the pallium) and the ventral telencephalon (i.e. subpallium) showed uniform expression (Fig. 5D). In the diencephalon, *Klf7* expression was seen in both the pretectum (Fig. 5B and C) and hypothalamus (Fig. 5C and D). In the retina, *Klf7* expression was mainly confined to the anterolateral region (Fig. 5B). In the hindbrain, *Klf7* was detected in the ventral portion of the cerebellum, and lateral portion of the medulla oblongata (Fig. 5C and E). *Klf7* continued to be expressed in the spinal cord

(Fig. 5F). *Klf7* expression in the peripheral structures was confined to the trigeminal ganglion and anterior lateral line ganglion (gA, Fig. 5B). The olfactory placode (op) gives rise to the olfactory epithelium (oe), and it continued *Klf7* expression (Fig. 5B-D).

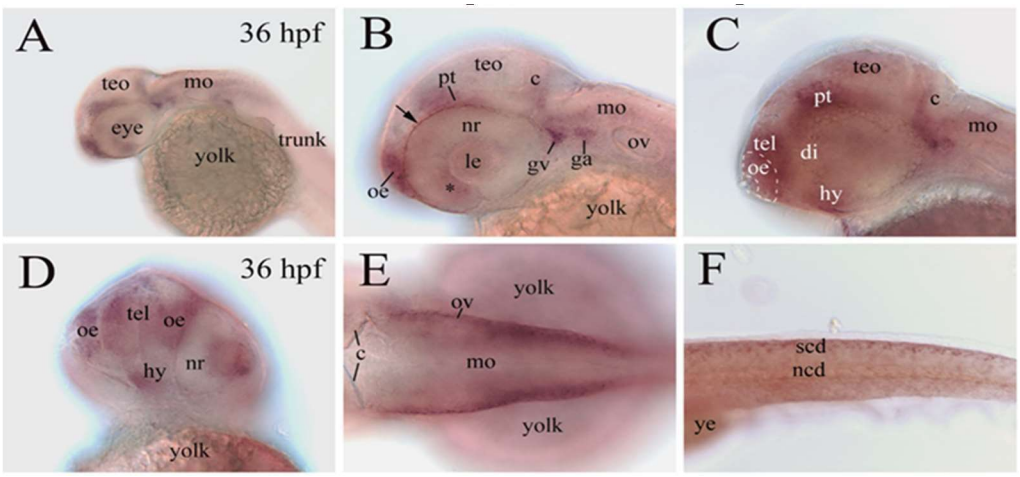

FIGURE 5. Zebrafish Klf7 expression in 36-hours post fertilization
Klf7 expression in 36 hpf zebrafish. Panels A-C and F are lateral views, with anterior to the left and dorsal side up. Panel D is a frontal view (dorsal side up), while panel E is a dorsal view (anterior to the left). Panels B and C are higher magnified views of the head region of the embryo shown in panel A. The arrow in panel B points to pigmented epithelium. Panel D shows a frontal view of the head, while panel E is a dorsal view of the medulla oblongata. Panel F is a lateral view of the posterior trunk region. Abbreviations: al, anterior lateral line placode; ga, anterior lateral line ganglion; hy, hypothalamus; pt, pretectum. All other abbreviations are the same as in Figs. 1-4.

As development proceeded (in 50 hpf and 72 hpf embryos), *Klf7* expression increased throughout all regions of the brain (Figs. 6 and 7). In the telencephalon, *Klf7* was seen throughout the region (Figs 6D and 7D). In 72 hpf embryos, the dorsal telencephalon was more strongly labeled than the ventral telencephalic regions (Fig. 7D). Most cells in the diencephalon (di, Figs. 6D and 7F), pretectum (pt, Figs. 6C and D, 7C and F), optic tectum (TeO, Figs. 6B-D, 7C and F), cerebellum (cl, Figs. 6B-D), and medulla oblongata (Figs. 6F and 7G) were *Klf7*-expressing. In 72 hpf embryos, the

unlabeled regions in the telencephalon (Fig. 7D), diencephalon and optic tectum (Fig. 7F), and medulla oblongata (Fig. 7G) were occupied mainly by developing neural processes (neuropil). In the retina of 50 hpf embryos, *Klf7* expressing cells were confined to the retinal ganglion cell layer (gcl, Fig. 6B and E). This labeling pattern continued in the retina of 72 hpf embryos, except that some cells in the inner portion of the inner nuclear layer (inl, Fig. 7B and E) had become *Klf7* positive. *Klf7* expression in the spinal cord continued in the older embryos, with stronger expression in the cells located in the dorsal and dorsal lateral regions. Similar to the brain regions, the neuropil regions in the spinal cord had no detectable labeling (Fig. 7H). Outside the CNS, *Klf7* expression was found in the trigeminal ganglion, vagal ganglion (gX), and posterior lateral line ganglion (gP) (Figs. 6B and C, 7C).

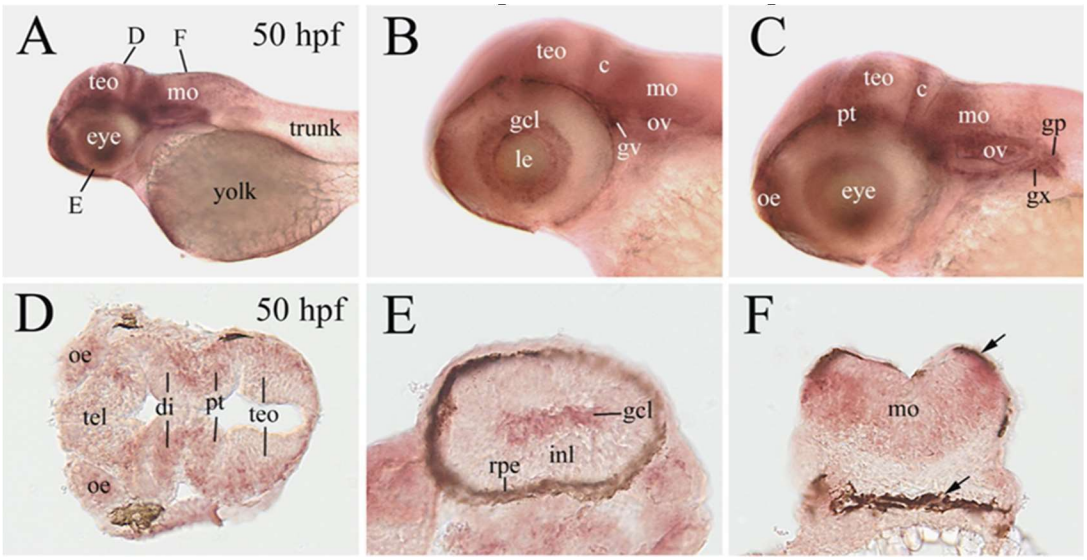

FIGURE 6. Zebrafish Klf7 expression in 50-hours post fertilization
Klf7 expression in 50 hpf zebrafish. Panels A-C are lateral views of a wholemount embryo, with anterior to the left and dorsal side up. Panels B and C are higher magnified

views of the head region of the embryo shown in panel A. Panels D-F are sections (anterior to the left for panel D, dorsal to the left for panel E, and dorsal up for panel F), Levels of the sections are indicated in panel A. Arrows in panel F indicate pigmented epithelium. Abbreviations: gp, posterior lateral line ganglion; gx, vagal ganglion; All other abbreviations are the same as in previous figures.

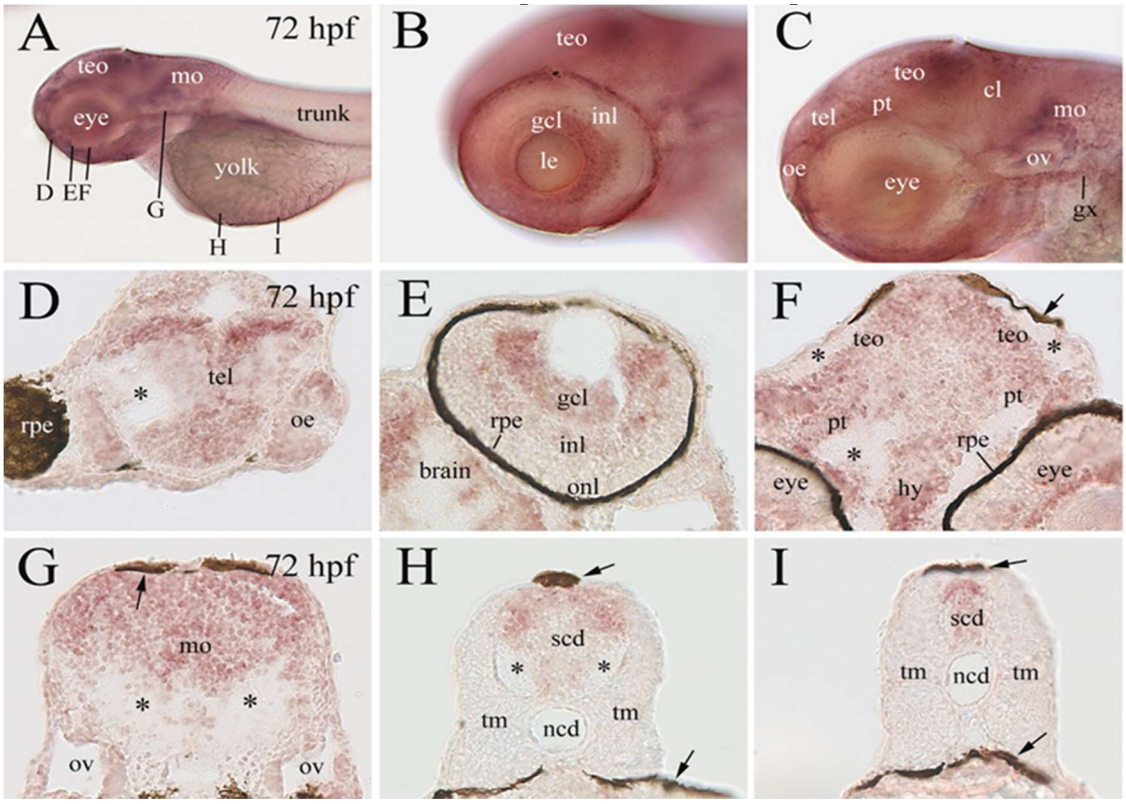

FIGURE 7. Zebrafish Klf7 expression in 72-hours post fertilization

Klf7 expression in 72 hpf zebrafish. Panels A-C are lateral views of a wholemount embryo, with anterior to the left and dorsal side up. Panels B and C are higher magnifications of the head region of the embryo shown in panel A. Panels D-I are cross sections, with levels of each section indicated in panel A. All the section panels have their dorsal sides up, except panel E with its dorsal side to the left. Panels D and E are magnified views of the telencephalon and the retina, respectively. Panel F is a higher magnification of the optic tectum, pretectum and hypothalamus. Panels G and H are magnified views of the hindbrain and anterior spinal cord, respectively. Panel I is a magnified view of the spinal cord from mid-trunk region. The arrows in panels F, G, H and I point pigmented epithelium. Abbreviation: tm, trunk muscles. Asterisks in panels D, F, G and H indicate neuropil regions (cell sparse) of the CNS. All other abbreviations are the same as in previous figures.

CHAPTER IV

DISCUSSION

Dr. Liu's laboratory has successfully used in situ hybridization to study gene expression in both embryonic zebrafish and adult zebrafish for about 20 years. I learned this technique from Dr. Liu, and performed several in situ hybridization experiments on both wholemount zebrafish embryos and adult zebrafish tissue sections, using several different cRNA probes, producing results similar to those obtained by Dr. Liu and Dr. Bhattarai (a fellow graduate student, currently working as a postdoctoral fellow at a medical center in New Jersey). The *Klf6* and *Klf7* probes used in this study were synthesized using cDNAs from Dr. Goldman's laboratory at the University of Michigan. This laboratory published several papers using probes generated from these cDNAs (Veldman et al., 2007, 2010). Additionally, their staining patterns of *Klf6* and *Klf7* on regenerating adult zebrafish retina were similar to that obtained by our laboratory (data not published). Furthermore, using these Klf cRNA probes, we successfully studied *Klf6* and *Klf7* expression in adult zebrafish CNS (Bhattarai et al., 2016), with me as a co-author. Therefore, I am confident that the results I obtained in this study are reliable findings.

Comparative expression of *Klf6*

In the developing mouse, moderate to high expression was mainly found in both neural and non-neural tissues. This expression was specifically localized in the mesenchyme of cells surrounding the neural tube, brain vesicles, uretic and lung buds in young mouse embryos (around E10). As development proceeds (E12-E14), *Klf6* expression in the mouse CNS increased (Laub et al., 2001b). This expression pattern is apparently different from results from this study, in which I found that in the fish CNS, *Klf6* expression is limited to only the hindbrain and spinal cord of 24 hpf embryos (similar to mouse E10, Scholpp & Lumsden, 2010, Kuldeaw and Sugiyama, 2012). Interestingly, *Klf6* expression in the CNS of adult zebrafish is strong and wide (Bhattarai et al., 2016), which is similar to *Klf6* expression (immunocytochemical method) in adult mice (Jeong et al., 2009). The difference in *Klf6* expression between the embryonic zebrafish and mouse embryos maybe species specific, which in turn may reflect different functions of *Klf6* in the CNS development of zebrafish and mouse. Outside the CNS, I found that *Klf6* expression was detected in the olfactory system and trigeminal ganglion, which is similar to its expression in the mouse.

Comparative expression of *Klf7*

Similar to *Klf6*, *Klf7* expression in the young (24 hpf) zebrafish CNS was weak and restricted. But unlike *Klf6*, *Klf7* expression in CNS increased greatly in both expression regions and expression levels in the older embryos. The CNS regions, such as the dorsolateral telencephalon, dorsolateral diencephalon, hypothalamus, retina, dorsal

midbrain, cerebellum and medulla, that express *Klf7* are similar to those in developing mice of similar stages (Laub et al., 2001a). In embryonic mice, *Klf7* expression in the CNS was first detected at E9.5 (approximately equivalent of 18-20 hpf zebrafish, Scholpp & Lumsden, 2010, Kuldeaw and Sugiyama, 2012). The earliest stage of zebrafish I examined was 24 hpf, a few hours older than its equivalent mouse embryos. So, it is possible that *Klf7* expression in zebrafish embryonic CNS starts earlier. Klf7 expression in the mouse brain reaches maximal levels between E11.5-E12 (approximately equivalent of 30-36 hpf zebrafish) and continues to be present in those brain regions in older mice (E15-E18, approximately equivalent of 72 hpf to 6-day post fertilization). I found that in developing zebrafish, Klf7 expression in the CNS becomes much stronger and wider at 36-50 hpf than 24 hpf and continues to stay strong and wide at 72 hpf. These results are similar to those in the developing mice. Furthermore, previous studies in both zebrafish (Mueller and Wullimann, 2003) and mice (Laub et al., 2001a) showed that the lateral and dorsolateral regions of CNS (mantle zone) are occupied mainly by postmitotic cells, while the medial and medioventral (ventricular and subventricular regions) CNS regions contain mainly proliferating cells. Therefore, those *Klf7*-expressing cells in the embryonic zebrafish CNS are likely postmitotic, as shown in the embryonic mice (Laub et al., 2001a). These similarities in *Klf7* expression between zebrafish and mice embryos suggest that *Klf7* play a similar role in the CNS development.

In the mouse, both *Klf6* and *Klf7* show similar developmental expression patterns, except expression domains of Klf7 are wider than that of *Klf6* (see Introduction). This suggest that these two Klfs have similar (overlapping) functions. This idea is somewhat

supported by an in vitro regeneration study in an adult zebrafish retinal preparation in which blocking either Klf6 or Klf7 using antisense morpholino oligonucleotides (MOs) had no significant effect on regeneration of retinal ganglion cell processes, but simultaneously application of both *Klf6* and *Klf7* MOs significantly inhibited the regeneration process (Veldman et al., 2007). On the other hand, *Klf7* mutant show observable defects in the olfactory bulb, cerebral cortex and retinal ganglion cells (see above), suggesting that these two Klfs have distinct functions, as well as overlapping functions. Functions of *Klf6* and *Klf7* can be tested in developing zebrafish using CRISPR technique. Based on their expression patterns in the developing zebrafish CNS, I would predict that Klf7 plays a much more important role in the CNS development than Klf6, although they may have overlapping functions in adult CNS because of their similar expression patterns in the adult zebrafish.

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APPENDIX A

Abbreviations

al	anterior lateral line placode area	mo	medulla oblongata
c	cerebellum	ncd	notochord
di	diencephalon	nr	neural retina
ga	anterior lateral line ganglion	oe	olfactory epithelium
gcl	retinal ganglion cell layer	onl	outer nuclear layer
gp	posterior lateral line ganglion	op	olfactory placode
gv	trigeminal ganglion	ov	otic vesicle
gx	vagal ganglion	pt	pretectum
hb	hindbrain	rpe	retinal pigmented epithelium
hpf	hours post fertilization	scd	spinal cord
hy	hypothalamus	tel	telencephalon
inl	inner nuclear layer	teo	optic tectum
klf	krüppel like factor	tm	trunk muscles
le	lens	ye	yolk extension