COVER SHEET

TITLE: Roles of Zic2 and Zic3 in early development

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ABSTRACT

Roles of *Zic2* and *Zic3* in early development

The Hedgehog (*Hh*) signaling pathway plays a crucial role in modulating embryonic development. Malfunctions in the vertebrate *Hh* pathway involving Sonic Hedgehog Homolog (*Shh*) have been linked to cancers including basal cell carcinoma and developmental disorders like holoprosencephaly. *Zic3,* a zinc-finger transcription factor, is hypothesized to activate *Shh*- mediated *Hh* signaling. This is based on data demonstrating ZIC3 often binds to GLI consensus motif, and *Zic3*-depleted embryos express lower levels of *Shh.* To test this hypothesis, a line of zebrafish embryos carrying a nonsense mutation of *Zic3* was examined for morphology of the forebrain and retina. Unexpectedly, no visible defects were found in embryos homozygous for the mutant allele through five days of development, and an expression assay of three *Hh* pathway genes (*Shh, Hhip,* and *Gli2a)* via *in situ* hybridization in *Zic3* mutants showed normal patterns of *Hh* target expression. These data suggest *Zic3* has redundant functions or gene compensation is occurring, in comparison to *Zic3*-depleted embryos.

In a parallel approach, we are investigating *Alx1*, a candidate target of *Zic2*. *Alx1* is strongly implicated in the development of neural crest (NC) cells, as is the *Zic* gene family. *Zic2* and *Alx1* promote specification of NC cells. Pericytes, a mural cell type important for vascular development, are one of many cell lineages derived from NC cells. *Zic2* mutants exhibit hemorrhage indicative of aberrant vasculogenesis, which may be due to aberrant pericyte formation. To test this hypothesis, immunohistochemistry was used to assay expression of *Pdgfrb*, a gene expressed in pericytes, in *Alx1* morpholino-injected embryos. Analysis of the dorso-medial midrain-hindbrain boundary found smaller quantities of pericytes within *Alx1*- depleted embryos than in controls, suggesting *Alx1* has an impact on migration, differentiation, or a combination of both factors on pericyte development. This study has provided potential insight into *Zic* gene family function in early development and may enhance understanding of diseases associated with it.

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Roles of *Zic2* and *Zic3* in early development

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Abstract

The Hedgehog (*Hh*) signaling pathway plays a crucial role in modulating embryonic development. Malfunctions in the vertebrate *Hh* pathway involving Sonic Hedgehog Homolog (*Shh*) have been linked to cancers including basal cell carcinoma and developmental disorders like holoprosencephaly. *Zic3,* a zinc-finger transcription factor, is hypothesized to activate *Shh*-mediated *Hh* signaling. This is based on data demonstrating ZIC3 often binds to GLI consensus motif, and *Zic3*-depleted embryos express lower levels of *Shh.* To test this hypothesis, a line of zebrafish embryos carrying a nonsense mutation of *Zic3* was examined for morphology of the forebrain and retina. Unexpectedly, no visible defects were found in embryos homozygous for the mutant allele through five days of development, and an expression assay of three *Hh* pathway genes (*Shh, Hhip,* and *Gli2a)* via *in situ* hybridization in *Zic3* mutants showed normal patterns of *Hh* target expression. These data suggest *Zic3* has redundant functions or gene compensation is occurring, in comparison to *Zic3*-depleted embryos.

In a parallel approach, we are investigating *Alx1*, a candidate target of *Zic2*. *Alx1* is strongly implicated in the development of neural crest (NC) cells, as is the *Zic* gene family. *Zic2* and *Alx1* promote specification of NC cells. Pericytes, a mural cell type important for vascular development, are one of many cell lineages derived from NC cells. *Zic2* mutants exhibit hemorrhage indicative of aberrant vasculogenesis, which may be due to aberrant pericyte formation. To test this hypothesis, immunohistochemistry was used to assay expression of *Pdgfrb*, a gene expressed in pericytes, in *Alx1* morpholino-injected embryos. Analysis of the dorso-medial midrain-hindbrain boundary found smaller quantities of pericytes within *Alx1*-depleted embryos than in controls, suggesting *Alx1* has an impact on migration, differentiation, or a combination of both factors on pericyte development. This study has provided potential insight into *Zic* gene family function in early development and may enhance understanding of diseases associated with it.

Introduction

Zic3 as a candidate activator of Sonic Hedgehog Homolog-mediated Hedgehog signaling

The Hedgehog (*Hh)* signaling pathway has been established as an important regulator of embryonic development in both vertebrates and invertebrates. Sonic Hedgehog Homolog (*Shh*) is the best-characterized member of the vertebrate *Hh* pathway and is linked to a variety of diseases. Studies have implied there are numerous regulators of *Shh*, many of which are still unknown (Tucker and Caspary, 2013).

Roles for *Shh* have been well established in a range of processes, such as left-right (L-R) asymmetry and neural patterning (Ingham and McMahon, 2001). Studies have also linked malfunctions in the pathway to cancers, such as basal cell carcinoma, and developmental disorders like holoprosencephaly (Ming *et al.*, 1998). In the last twenty years, the fundamental molecular basis of the pathway has been characterized.

After translation, SHH protein undergoes autocatalytic processing, producing an N-terminal signaling domain to which cholesterol is added. SHH initiates either autocrine or paracrine signaling, the latter of which requires the Dispatched (DISP) protein and can be short or long range. After reaching the target cell, SHH binds to the Patched-1 (PTCH1) receptor, relieving inhibition of Smoothened (SMO) protein. This leads to activation of GLI transcription factors, modulating transcription of target genes. *Hhip,* a target gene of the *Hh* pathway, inhibits *Hh* signaling by binding and sequestering SHH ligand (Ingham and McMahon, 2001). *Zic3* has been proposed to regulate a component of this mechanism (Winata *et al.*, 2013).

Zic3 was classified as a Zinc Finger of the Cerebellum gene due to its conservation of the zinc finger motif found in *Zic1* and because of *Zic3*'s expression in the cerebellum of adult mice (Aruga *et al.*, 1996). It has been identified as a critical component of embryonic development in processes such as ensuring fidelity of embryonic patterning, positioning the primitive streak, and initiating gastrulation (Ware *et al.*, 2006; Sutherland *et al.*, 2013). Another member of the *Zic* family, *Zic2a,* has been identified by our lab as a modulator of *Hh* signaling in the forebrain and

retina (Sanek *et al.*, 2009). In a study by Winata *et al.* (2013), ZIC3 frequently bound to the GLI consensus binding motif in zebrafish embryos when performing a *de novo* motif search using ChIP-Seq, at both 8 and 24 hours post fertilization (hpf). Additionally, the ZIC3 consensus binding motif was near the GLI motif in half of the ZIC3 binding peaks observed. These data suggest competition, or some allosteric regulation, between ZIC3 and GLI for modulating transcription of target genes. There was some conservation of these sites with humans (Winata *et al.*, 2013). Additionally, a *Zic3* microarray expression analysis found four genes integral to the *Hh* pathway differentially expressed in *Zic3*-depleted embryos: *Shh, Shhb, and Gli2a,* differentially expressed at 24 hpf, and *Hhip*, regulated at 8 hpf. Both *Shh* and *Shhb* were upregulated, indicating *Zic3* is an activator of *Shh*-mediated *Hh* signaling. Morphologically, *Zic3* morpholino-depleted embryos exhibited defects in gastrulation and convergent-extension (C-E). A study by Cast *et al.* (2012) found similar gastrulation and C-E defects, as well as L-R asymmetry defects in zebrafish embryos with loss of function *Zic3*. Quinn *et al.* (2012) found evidence suggesting ZIC3 upregulates *Hh* signaling through activation of *Gli3* and *Shh*.

Synthesizing the literature indicates *Zic3* acts as an activator of the *Hh* pathway involving the SHH ligand in a conserved manner. Another *Zic* family member, *Zic2a*, is a known regulator of *Shh*-mediated *Hh* signaling (Sanek *et al.*, 2009). *Zic3* binds to partially conserved GLI consensus motif sites and regulates genes in the *Hh* pathway (Winata *et al.*, 2013). In order to further test the hypothesis put forth by Winata *et al.* that *Zic3* is a candidate activator of the *Shh*-mediated *Hh* pathway, I used a newly derived mutant zebrafish line that carries a disruptive point mutation in the *Zic3* locus. In contrast, Winata *et al.* used morpholino-mediated (MO) knockdown of *Zic3*, which may result in a different degree of developmental disruption than that caused by a chromosomal mutation (Bedell *et al.*, 2011; Rossi *et al.*, 2015; Kok *et al.*, 2015).

Zic2 may regulate neural crest cell development through Alx1

Zic2, another member of the *Zic* gene family, has been implicated in the development of neural crest (NC) cells, a cell type arising from the ectoderm layer that can differentiate into numerous cell types, including melanocytes, craniofacial cartilage and bone, smooth muscle, and glia (Dupin and Sommer, 2012). Defects in neural crest cell development often manifest as craniofacial defects. These defects have been observed in both *Zic2*-depleted and *Zic2* mutant zebrafish models (Teslaa *et al.*, 2013; Roberson et al., in preparation).

RNAseq analysis performed in our lab identified *Alx1* as a candidate target of *Zic2* based on reduced level of it's expression in *Zic2a;Zic2b* compound homozygous embryos (Roberson *et al.*, in preparation). *Alx1* encodes ALX homeobox 1, a transcription factor (McGonnell *et al.*, 2011). Dee *et al.*, (2012) showed craniofacial defects in *Alx1*-depleted embryos that were similar to those observed in *Zic2*-depleted and *Zic2* mutant models. This evidence suggests that *Zic2* is regulates NC cell development through its transcriptional control of *Alx1* expression.

The Grinblat lab has recently generated several *Zic2* mutant compound lines, which carries mutant alleles at both the *Zic2a* and *Zic2b* loci. A proportion of *Zic2a;Zic2b* compound homozygotes exhibit hemorrhage indicative of aberrant vasculogenesis (see Fig. 1), which may be due to aberrant formation of pericytes, a mural cell type derived from NC (Etchever *et al.*, 2001). Pericytes are located in microvessels (arterioles and capillaries) throughout the brain, eye, and kidneys (von Tell, *et al.* 2006). Pericytes, unlike their counterpart mural/smooth muscle cells, are not continuous. They exist as segregated cells embedded in the basement membrane of the vessels (Mandariano, *et al.*, 1993). There are multiple roles for pericytes, including physically supporting vessels, secreting ECM, providing vascular tone, inducing vessel quiescence, supporting the blood brain barrier, promoting contractility to blood vessels, and preventing continued proliferation of endothelial cells once they have covered the vessel (Gaegnel *et al.*, 2009; Armulik *et al.*, 2010; Peppiatt *et al.*, 2006; Benjamin *et al.*, 1998).

During angiogenesis, endothelial cells attract perivascular mural cells via reciprocal signaling. Endothelial cells express Platelet-Derived Growth Factor, PDGF-B, which mural cells

receive via PDGF-Receptor β (PDGF-Rβ), inducing release of angiopoietin1 (Lindahl *et al.*, 1997; Patan *et al.*, 1998). Angiopoietin1 then binds to Tie2 receptors on endothelial cells to promote differentiation (Davis *et al.*, 1996), resulting in two-layered cell formation. Pericytes are highly mobile and migrate along newly formed endothelial tubes (Stratman *et al.*, 2009). Thus, it is plausible that defective pericytes formation is responsible for the hemorrhaging observed in *Zic2* mutant embryos.

Methods

Zic3 mutant zebrafish genotyping assay

Zebrafish were maintained and embryos staged according to established protocols (Kimmel *et al.*, 1995; Westerfield, 2000). A *Zic3* line of zebrafish (sa13365) containing a C>A nonsense mutation in the second exon was obtained from the Sanger Institute Zebrafish Mutation Project. This line is established in the large zebrafish breeding facility the Grinblat lab maintains. This mutant allele encodes a truncated and likely non-functional ZIC3 protein (see Fig. 2). Zebrafish embryos and adults were genotyped to determine the presence of mutant vs wild type alleles of *Zic3* using PCR, restriction enzyme cutting, and MetaPhor gel electrophoresis. Tail clips were obtained from adult fish according to Meeker *et al.* (2007). The forward primer sequence is 5'-TAAATGCGAGTTCGATGGCT-3'. The reverse primer has the sequence 5'-AGAGAGCTGGGATGTGTGTA-3'. The 25 µl PCR reaction contained GoTaq Flexi DNA and 5x Green GoTaq Flexi Buffer (Promega), 25 µM $MgCl_2$, 10 µM dNTPs, 200 nM of each primer, and 1 µl of the cDNA template. Components shared across reactions were initially mixed and then aliquoted to guarantee equivalent reaction conditions. The reaction was run on an Applied Biosystems Veriti Thermal Cycler as follows: denaturation at 95°C for one minute; amplification at 95°C for 20 seconds, 54°C for 30 seconds, and 72°C for 30 seconds (40x). The 30 µl restriction digest reaction used the DdeI restriction enzyme with a recognition sequence of 5'-CTNAG-3' (the proper sequence for a Restriction Fragment Length Polymorphism assay), the PCR reaction,

and CutSmart Buffer (NEB). As before, components shared across reactions were initially mixed and then aliquoted to guarantee equivalent reaction conditions. The reaction was incubated at 37°C for one hour. Only the mutant allele was cut, forming 114 nt and 21 nt bands, while the wild type allele presented with single a 135 nt band.

Morphological assays of Zic3 mutant embryos

Embryos were derived from a cross between heterozygous parents. They were examined for morphology of the forebrain and retina. Any observed developmental defects were tested for linkage to *Zic3* mutation. To do this, embryos were lysed and genotyped as described above.

Gene expression was assayed via *in situ* hybridization (ISH). ISH was performed on 24-hour old embryos using antisense probes for *Shh, Hhip,* and *Gli2a.* Single-color ISH were carried out as previously described (Gillhouse *et al.,* 2004). Although Winata *et al.* (2013) only noted *Hhip* regulation at 8 hpf, the forebrain and retina are not developed at that stage (Kimmel *et al.*, 1995). However, *Hhip* expression is seen in the forebrain at roughly 24 hpf (Hammond and Whitfield, 2009). *Shh,* and *Gli2a* are expressed in the forebrain at 24 hpf (Kur *et al.*, 2011; Karlstrom *et al.,* 1999). *Shh* and *Hhip* produce secreted proteins that diffuse from the forebrain into the developing retina (Sanek *et al.*, 2009). Embryos displaying aberrant expression were genotyped according to the protocol mentioned above.

Pericyte analysis

Tg(*pdgfrb:citrine, acta2:mcherry)*, a double-transgenic line of zebrafish obtained from Nathan Lawson, University of Massachusetts-Amherst, were used to visualize pericyte expression, as *pdgfrb* expression is specific to pericytes (Armulik *et al.*, 2011). 1 nl microinjections according to previous methods (TeSlaa *et al.*, 2013) were performed of a 2:1 morpholino-mix of *Alx1*i1e2, designed to target the intron1–exon2 splice acceptor site 5′-

CACGCCTGAGGAGGTCACAGAAAAT-3' (0.165 mM) and *p53* (0.0825 mM), obtained from Gene Tools.

At five days post-fertilization (dpf), morpholino-injected and control embryos were fixed in 4% formaldehyde in PBS and stained using the following antibodies: Anti-green mouse green fluorescent antibody (1:500 from Millipore) and Alexa Fluor 488 conjugated goat anti-mouse secondary (1:1000). DAPI staining was performed at the time of secondary antibody staining (1:5000 from Invitrogen). Following staining, embryos were mounted with their dorsal side toward the coverslip in VectaShield Antifade Mounting Medium for Fluorescence (Vector Laboratories), and confocal images were taken on with a 40X lens on an Olympus FV1000 with FV10-ASW software (Olympus). The region of interest for pericyte quantification was the dorso-medial midbrain-hindbrain boundary, with pericytes being distinguished from background fluorescence based upon reference images as well as consistent localization through slices. The average number of perictyes was then taken, along with standard deviation.

Results

Zic3 mutants have no morphological defects or aberrant Hh-target expression

No embryos, observed for five days post-fertilization, produced by the *Zic3* heterozygotes displayed any abnormalities (78/78). PCR confirmed there were mutants present among those scored.

Expression of all three genes analyzed, *Shh* (47/47), *Hhip* (37/37), and *Gli2a* (28/31) were normal (See Fig. 3). 3/31 *Gli2a* embryos displayed an aberrant expression pattern (see Fig. 3B), but PCR confirmed this expression was unlinked to *Zic3*.

Alx1-depleted embryos have fewer pericytes in the dorso-medial midbrain-hindbrain

Analysis of embryos at the dorso-medial midbrain-hindbrain boundary showed a significantly lower number of pericytes in Alx1-depleted embryos than in controls (See Fig. 4 and

5). All *Alx1*-depleted embryos appeared to have significantly smaller brains than controls, but this defect was not quantified.

Discussion

Zic3 may have redundant function or undergo gene compensation when nonfunctional

One probable explanation for normal development of *Zic3* mutants is that *Zic3* functions redundantly with other *Zic* gene family members. *Zic* redundancy has been suggested in other vertebrate models (Inoue *et al.*, 2007), and overlapping *Zic* expression patterns have been observed in zebrafish (Grinblat and Sive, 2001). Alternatively, a compensatory mechanism may be activated in *Zic3* mutants, which is not activated by morpholino-mediated knockdown. Rossi *et al.* (2015) has recently shown this to be the case in a line of *egfl7* mutant zebrafish compared to their morphant counterpart. Both possibilities suggest defects seen as a consequence of *Zic3* depletions are not solely due to *Zic3* mutations, but rather reflect functional requirement for several *Zic* genes. Double mutant models including *Zic3* with other *Zics* should be created in order to test these hypotheses.

Alx1 may be involved in differentiation, migration, or both aspects of pericyte development

Although the data are preliminary, the stark difference in the quantity of pericytes between the *Alx1* morphants and controls suggests three possible interpretations. First, *Alx1* could be modulating the expression of genes involved in pericyte differentiation, such as *FoxD3* and *Sox10*, thus directly promoting pericyte formation. The second possibility is that *Alx1* interferes with the proper development of vasculature such that the reciprocal signaling occurring between endothelial cells and pericytes is disrupted, thus causing improper migration of pericytes. Under this scenario, reduction in pericytes in *Alx1* (and *Zic2*) mutant vertebrates should be accompanied by severely disrupted vasculature structure and integrity, perhaps across the entire body. Finally,

it is possible both factors are involved in *Alx1*'s role in pericyte development, with similar implications as the latter interpretation.

In order to determine if there is differentiation failure, *in situ* hybridization with *pdgfrb* probes should be done on *Alx1*MO embryos at 3 dpf to see pericyte development at an earlier stage. There also should be studies conducted to determine if migration is affected using a *fli1a* transgenic, exclusively expressed on endothelial cells (Brown *et al.*, 2000). Finally, experiments should be done to further strengthen the link between *Zic2* and *Alx1*. *Zic2* mutants should undergo the same immunoshistochemistry and confocal imaging protocol as the *Alx1* morphants, to determine if there are similar differences in pericyte numbers. Further, *Alx1* should be overexpressed *Zic2* mutants, to see if it rescues the hemorrhage.

In conclusion, this study has shed light on roles of the *Zic* gene family in early development, specifically with regards to their relationship to the *Hh* pathway as well as pericyte development, and has opened new avenues of research.

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Figures

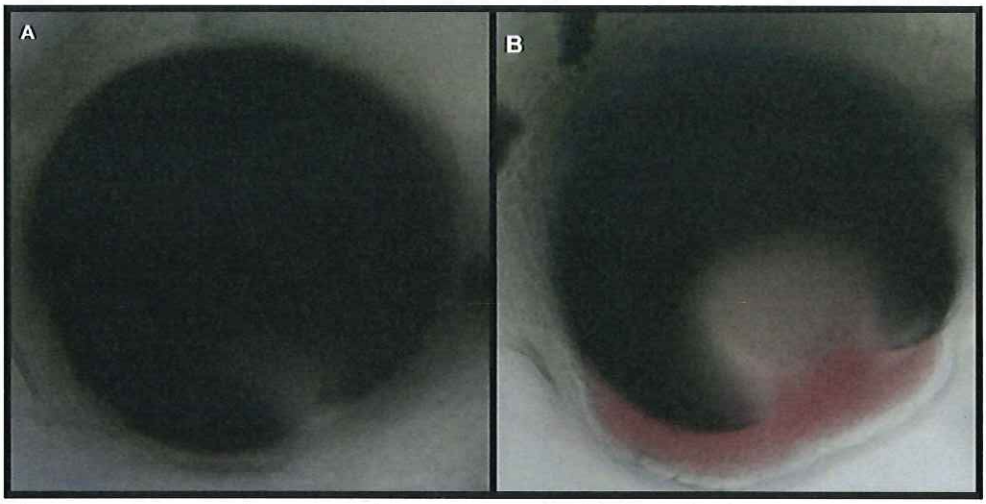

Figure 1: (A-B): *Zic3* compound mutants at 48 hpf **A:** Eye displaying coloboma **B:** Eye displaying coloboma and hemorrhage (cr. Laura Roberson, unpublished)

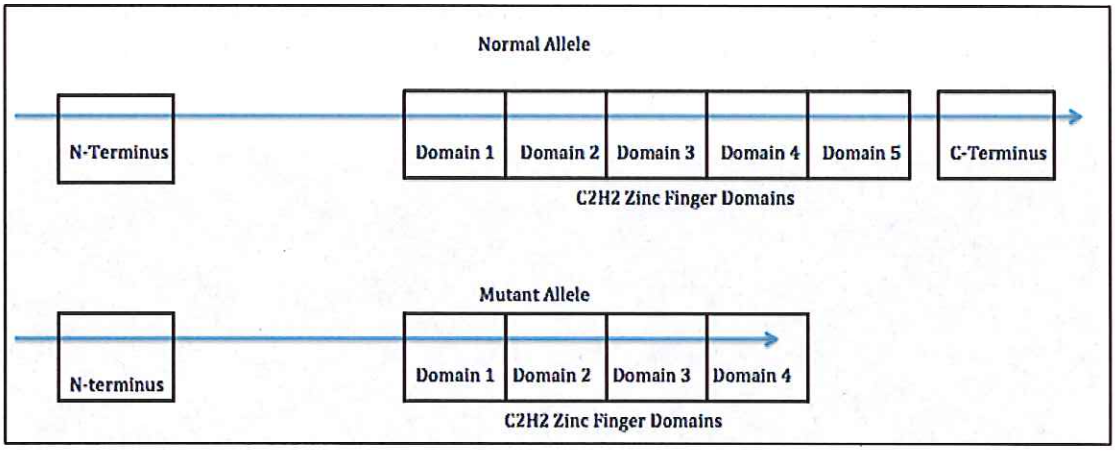

Figure 2: *Zic3* Mutant Allele Schematic

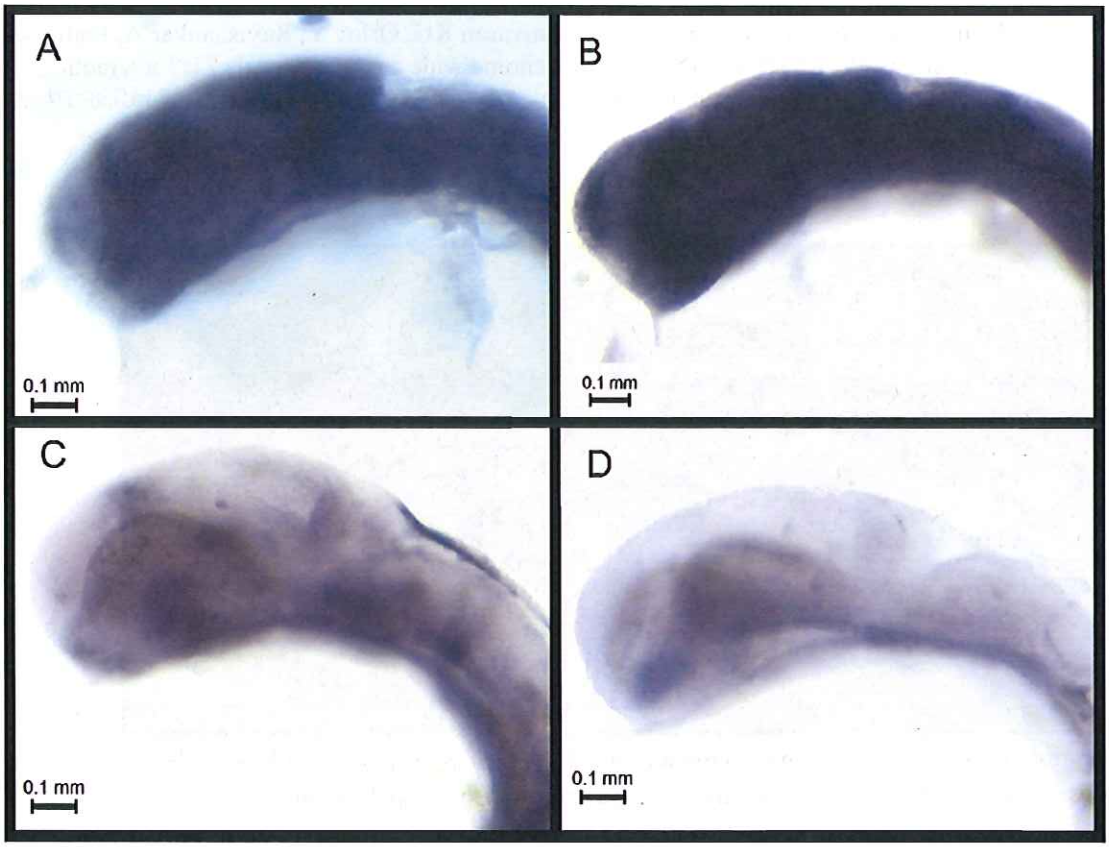

Figure 3: (A-D): mRNA expression of embryos following *in situ* at 24 hpf **A.** *Gli2a* normal expression (28/31) **B.** *Gli2a* aberrant expression, unlinked to *Zic3* (3/31) **C.** *Hhip* normal expression (37/37) **D.** *Shh* normal expression (47/47)

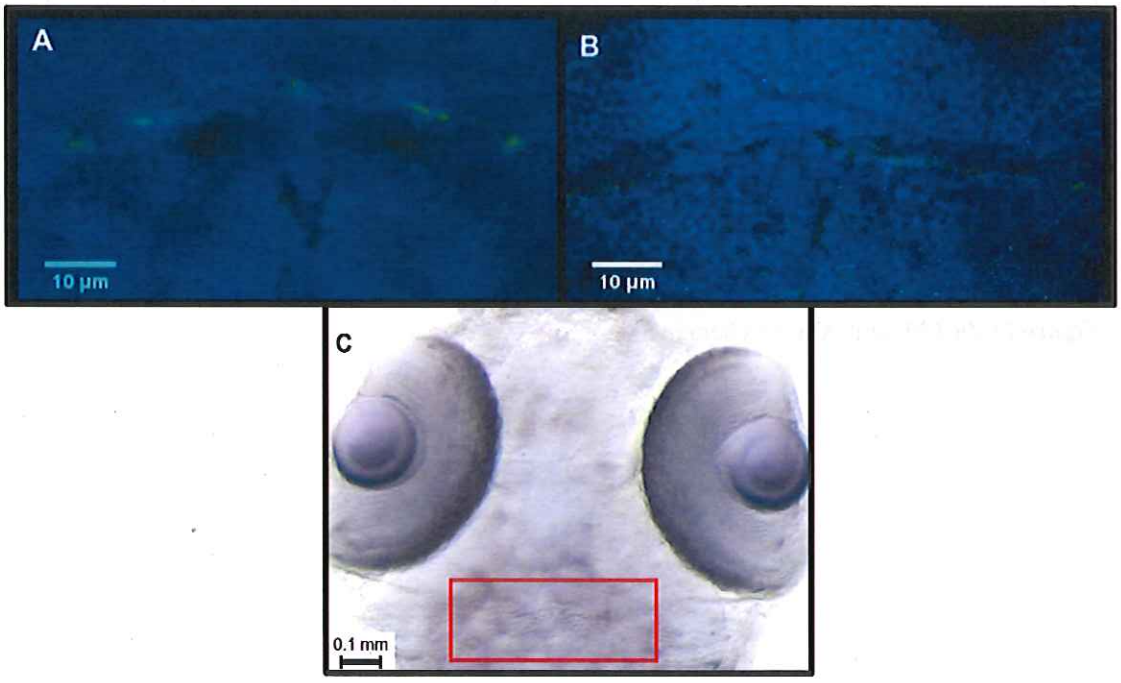

Figure 4: (A-B): Confocal images of Tg*(pdgfrb:citrine; acta2:mcherry)* embryos at 5 dpf with pericytes showing at GFP, in DAPI background **A.** Control embryo **B.** *Alx1*MO-injected embryo **C.** 5 dpf control embryo depicting region of interest (red box): Dorso-Medial Midbrain-Hindbrain Boundary

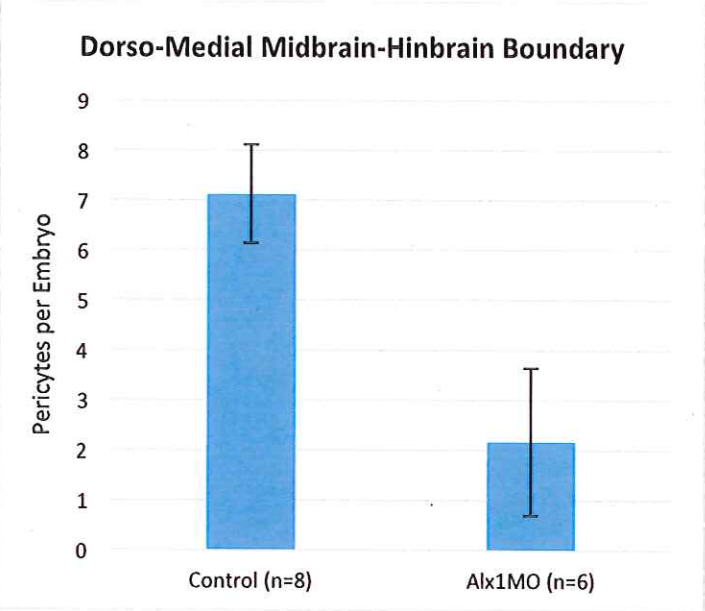

Figure 5: Pericyte quantification at dorso-medial midbrain-hindbrain boundary done from confocal images of Tg*(pdgfrb:citrine; acta2:mcherry)* embryos at 5 dpf with pericytes showing at GFP