Testing if mutated *twist1a* and *twist1b* genes in zebrafish result in open anterior neural tube

Undergraduate Research Project

During development, the process of neurulation forms the neural plate into the neural tube, the precursor to the central nervous system. All vertebrate animals have a central nervous system, consisting of a brain and spinal cord. Abnormal neurulation can result in a neural tube defect (NTD), causing life threatening birth defects. If the neural tube fails to close correctly, the NTD can lead to the following birth defects: anencephaly (anterior to hindbrain neural tube region fails to close), craniorachischisis (spine remains open), or spina bifida (posterior neural tube fails to close) (Copp et al., 2003).

Model organisms have been used to study NTDs and the mechanisms of neural tube closure. Mice and zebrafish are the main genetic vertebrate model organisms, having similar neurulation as in humans. During primary neurulation, dorsal ectoderm cells develop into the epidermis of the skin and the neural plate. During the neural tube folding process, cells change their shape, creating hinges at the midlines and dorsal lateral areas (Barresi and Gilbert, 2020). The medial hinge point (MHP) cells at the neural plate midline, attach to the notochord (Barresi and Gilbert, 2020). The tissue at the two dorsolateral hinge points (DLHP) push the edges of the neural tube towards the midline (Schoenwolf and Smith, 1990). The two epidermis edges come in contact and merge to form the skin over the neural tube (Barresi and Gilbert, 2020). The two edges of the neural tube bind together at the dorsal midline, closing off the neural tube (Schoenwolf and Smith, 1990). This process forms the epidermis layer over the closed neural tube.

The *twist1* gene in mice plays a role during primary neurulation and is necessary for the anterior neural tube to close properly (Chen and Behringer, 1995). In mice, cranial mesoderm tissue must expand for cranial neural tube closure (Copp et al., 2003). The *twist1* gene, expressed in this mesodermal tissue, is required for the mesodermal expansion to occur (Chen and Behringer, 1995). Mutations to the *twist1* gene results in an open anterior neural tube and therefore, anencephaly (Ghouzzi et al., 1996) (Teng et al., 2018).

Zebrafish have two *twist1* genes, *twist1a* and *twist1b*. For my Undergraduate Research Opportunities Project (UROP), I hypothesized the loss of *twist1a* and *twist1b* genes in zebrafish will cause an open anterior neural tube. The neural tube can be visualized from staining and imaging the pineal gland. Using a microscope, a perfect circular pineal organ showed normal anterior neural tube development (Aquilina-Beck et al., 2007). If the pineal was divided or elongated, the neural tube is not closed properly and will result in an NTD. For my project, I gathered data to test my hypothesis. However with the COVID-19 pandemic, I did not get to perform experiments visualizing the pineal organ.

Double mutant zebrafish for *twist1a* and *twist1b* were identified by performing single pair crosses to genotype the adult zebrafish as heterozygotes. In order for the adult zebrafish to be identified as double heterozygotes, a *twist* double mutant phenotype should be expressed in 1/16th of their embryos. The phenotype resulting from the loss of *twist1a* and *twist1b* is a smaller jaw in the zebrafish (Teng et al., 2018). Wildtype zebrafish at eight days post fertilization (dpf) have a well developed jaw structure (Figure 1). In dorsal view, the wildtype jaw protrusion at the anterior end is at a steep angle, forming a distinct arch (Figure 2). The wildtype eyes are also angled towards each other, being in close proximity at the anterior end (Figure 2). The jaw

protrusion of the *twist* double mutant zebrafish at eight dpf is not as angled and appears as a flat arch, not protruding far out as in the wildtype (Figure 2). The amount of space between the double mutant eyes is larger than the wildtype and they are also positioned more parallel to one another (Figure 2).

The *twist* double mutant phenotype in zebrafish larva appeared in three crosses (Table 1). The number of larvae to have the "no jaw" phenotype was very close to the expected 1/16th ratio for a double mutant. To confirm these adult zebrafish are heterozygous for the *twist1a* and *twist1b* gene mutations, fins from the adult zebrafish were clipped and added to polymerase chain reaction (PCR) extraction buffer. These samples were treated with proteinase K to degrade the proteins, boiled to inactivate the proteinase K, and centrifuged to get rid of debris. The supernatant contained the genomic DNA of the adult zebrafish. The samples were used in a PCR reaction recipe and thermocycled. The PCR products would have been run on a gel to confirm this genotype, however with the outbreak of COVID-19, the forward progression of this project has been put on hold until further notice.

Discussion/Future Direction

The PCR genotyping will confirm the potential *twist* double mutant crosses as heterozygous. After PCR, whole mount in situ hybridization (WISH) will image the pineal gland with use of a *otx5* probe and show if the anterior neural tube is tubular (normal) or divided into two pineal domains in their progeny, indicating an open anterior neural tube (mutant) (Aquilina-Beck et al., 2007). This will test my hypothesis if the loss of *twist1a* and *twist1b* genes in zebrafish will result in an open anterior neural tube. Zebrafish and humans have similarities in

the development of the neural tube. If my hypothesis is supported, this could lead to future projects on genetic discoveries of human birth defects.

UROP Experience

My UROP experience was overall very rewarding. I found the UROP application process to be very valuable, as this was my first time writing a research proposal. Through the persistence of setting up countless zebrafish crosses and closely observing the phenotype of each embryo, I have taught myself resilience when working towards research progression. Having patience will be useful in my future career path. I found myself enjoying the "mini victories" of forward movement in my UROP as each week passed. I am grateful for the opportunities and experiences I gained during my project.

I would like to thank and acknowledge Polla Hergert, Dr. Jennifer Liang, students of Dr. Liang's developmental research lab, and the Biology Department at The University of Minnesota Duluth. I appreciate the help during my UROP this semester and making my project a wonderful experience.

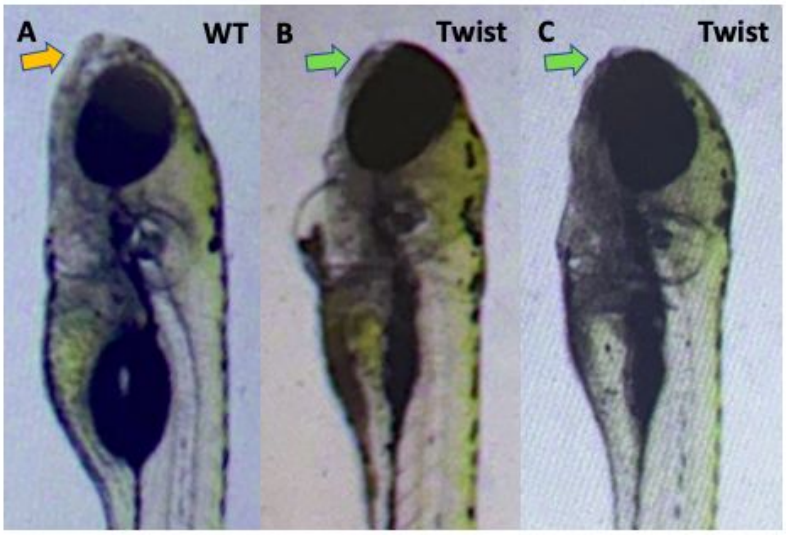

Figure 1. Loss of *twist1a* and *twist1b* gene in zebrafish results in abnormal jaw formation. At eight days post fertilization (dpf), wildtype zebrafish (A) was compared with potential double mutants for the twist gene (B-C). The orange arrow points out normal jaw characteristics from a wildtype zebrafish. The green arrows point to the abnormal jaw phenotype of potential double mutants of the twist genes. All images are anterior at the top and lateral orientation.

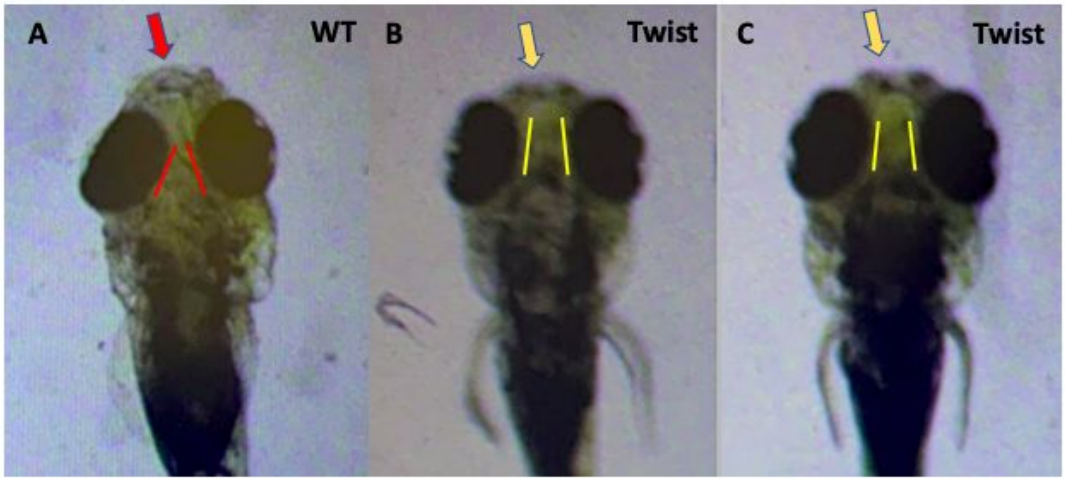

Figure 2. Loss of *twist1a* and *twist1b* gene in zebrafish results in abnormal mouth protrusion and eye slant. The mouth is not at a steep angle for the potential double mutant of the twist gene (yellow arrows, B-C) compared to wildtype zebrafish (red arrow, A) at eight days post fertilization. The eyes on the wildtype zebrafish (red lines, A) are angled towards each other, whereas the loss of *twist1a* and *twist1b* gene (yellow lines, B-C) results in eyes angled more parallel. All images are dorsal view with anterior end at the top.

Table 1. Scoring of zebrafish crosses results in potential double mutant embryos for the *twist1a* and *twist1b* gene in zebrafish. Embryos were scored between six and eight days post fertilization (dpf). The phenotype of each zebrafish was observed and categorized into one of the three groups: jaw (normal developing zebrafish), no jaw (loss of *twist1a* and *twist1b* genes), and weird (zebrafish with abnormal characteristics such as a malformed head).

	Phenotypes of Zebrafish Twist Embryos			
Potential Twist Double Mutant Crosses	**Normal/Jaw (number of embryos)**	**No Jaw (number of embryos)**	**Weird (number of embryos)**	**Double mutant ratio to cross number**
1.	141	10	1	(1/16) = (9.45/151)
2.	95	5	2	(1/16) = (6.38/102)
3.	43	2	2	(1/16) = (2.94/47)

References

Aquilina-Beck A, Ilagan K, Liu Q, Liang JO. "Nodal Signaling Is Required for Closure of the Anterior Neural Tube in Zebrafish | BMC Developmental Biology | Full Text." Accessed April 13, 2020. https://bmcdevbiol.biomedcentral.com/articles/10.1186/1471-213X-7-126.

Barresi MJF, Gilbert SF. 2020. Developmental biology. Twelfth edition. Pages 403-7. New York: Sinauer Associates, an imprint of Oxford University Press.

Chen ZF, Behringer RR. "Twist Is Required in Head Mesenchyme for Cranial Neural Tube Morphogenesis." *Genes & Development* 9, no. 6 (March 15, 1995): 686–99. https://doi.org/10.1101/gad.9.6.686.

Copp AJ, Green NDE, Murdoch JN. "The Genetic Basis of Mammalian Neurulation." *Nature Reviews Genetics* 4, no. 10 (October 2003): 784–93. https://doi.org/10.1038/nrg1181.

Ghouzzi VE, Merrer ML, Perrin-Schmitt F, Lajeunie E, Benit P, Renier D, Burgeois P, Bolcato-Bellemin AL, Munnich A, Bonaventure J. "Mutations of the TWIST Gene in the Saethre-Chotzen Syndrome" 15 (1997): 5.

Schoenwolf GC, Smith JL. Mechanisms of neurulation: traditional viewpoint and recent advances. The Company of Biologists Limited 1990.(Development 109):243–270.

Teng CS, Ting MC, Farmer DT, Brockop M, Maxson RE, Crump JG. "Altered Bone Growth Dynamics Prefigure Craniosynostosis in a Zebrafish Model of Saethre-Chotzen Syndrome." Edited by Richard M White and Marianne E Bronner. *ELife* 7 (October 25, 2018): e37024. https://doi.org/10.7554/eLife.37024.