Temperature Sensitivity of Neural Tube Defects in *Zoep* Mutants

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Abstract

Neural tube defects (NTD) occur when the flat neural plate epithelium fails to fold into the neural tube, the precursor to the brain and spinal cord. Squint (*Sqt/Ndr1*), a Nodal ligand, and One-eyed pinhead (*Oep*), a component of the Nodal receptor, are required for anterior neural tube closure in zebrafish. The NTD in *sqt* and *Zoep* mutants are incompletely penetrant. The penetrance of several defects in *sqt* mutants increases upon heat or cold shock. In this project, undergraduate students tested whether temperature influences the *Zoep* open neural tube phenotype. Single pairs of adults were spawned at 28.5°C, the normal temperature for zebrafish, and one half of the resulting embryos were moved to 34°C at different developmental time points. Analysis of variance indicated temperature and clutch/genetic background significantly contributed to the penetrance of the open neural tube phenotype. Heat shock affected the embryos only at or before the midblastula stage. Many factors, including temperature changes in the mother, nutrition, and genetic background, contribute to NTD in humans. Thus, *sqt* and *Zoep* mutants may serve as valuable models for studying the interactions between genetics and the environment during neurulation.

Introduction

NEURAL TUBE DEFECTS (NTD) are some of the most common birth defects in the world. In the United States, NTD occurred in ~4.3 in every 10,000 live births during 2006–2008.¹ All NTD are characterized by a failure of the developing neuroepithelium to form a closed neural tube which later gives rise to the brain and spinal cord. NTD are classified by the part of the developing neural tube that fails to close. The two most common NTD are spina bifida, which occurs when the posterior region of the neural tube fails to close, and anencephaly, which occurs when the most anterior region of the neural tube fails to close.^{1,2} Spina bifida can cause mild to severe problems with movement and feeling in the legs. Anencephaly causes severe defects in brain development and is always fatal.³

Human NTD are complex, with multiple genes and environmental factors playing a role.^{2,4–8} This complexity is reflected by the efforts to prevent NTD with folic acid supplementation. In controlled clinical trials, supplementation of the mother's nutrition with folic acid reduced the recurrence of NTD by up to 70%.⁹ Mandatory folic acid supplementation in the grain supply of the United States and other countries has resulted in a 13%–78% reduction in NTD, with

some of the biggest effects occurring in areas with the highest incidence.⁹ Despite folic acid supplementation, NTD persist due to many factors, including failure of the folic acid to reach all pregnant women, folate-resistant NTD, roles for other nutrients, metabolic state of the mother such as the presence of diabetes or obesity, and genetic background.^{2,7,8,10} Thus, a greater understanding of the interactions among genetic and environmental factors affecting neurulation is needed to further reduce the incidence of this congenital defect and improve prenatal care and diagnosis.

The basic mechanisms of neural tube closure or neurulation are well conserved between vertebrate species. In all vertebrates, neurulation occurs by two processes: primary neurulation and secondary neurulation.¹¹ Primary neurulation forms the anterior region of the neural tube that will give rise to the brain and the majority of the spinal cord. It starts with the formation of a flat polarized neuroepithelium that rolls or folds into a tube. NTD are caused by a failure of primary neurulation. Secondary neurulation forms the most posterior part of the trunk neural tube and tail neural tube. It starts with the solid rod of mesenchymal cells that, as development proceeds, transforms into an epithelium that then cavitates to form a tube.

There are some differences between primary neurulation in zebrafish and other vertebrate model systems, such as

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mice, frogs, and chicks.¹¹ In these other vertebrates, the neural plate epithelium folds or rolls to form a tube with a lumen in the middle. In zebrafish, there is an extra step. The neural plate initially folds to form a solid rod of cells. This solid rod subsequently cavitates to form a tube with a lumen.^{12,13} Although the formation of a solid rod of cells is superficially similar to secondary neurulation, careful studies have demonstrated that neurulation in the presumptive brain and anterior spinal cord has the characteristics of primary neurulation.¹¹ First, the neural tube precursors in this region are organized into an epithelium, not a mesenchyme. Second, the neural plate undergoes an ordered process of rolling up to form the closed neural rod, just as in other vertebrates. Finally, the medial–lateral position of cells in the neural plate corresponds to their later dorsal–ventral location in the neural tube: medial cells become the ventral neural tube, whereas lateral cells become dorsal.

Surprisingly, despite the identification of hundreds of mouse mutants with NTD, the formation of a consensus genetic pathway controlling vertebrate primary neurulation is only in its early stages.^{4,14–17} One prominent exception is that mutations in genes encoding components of the Wnt/planar cell polarity pathway disrupt neurulation in humans, frogs, mice, and zebrafish.^{2,18–20} Further, cell adhesion between the neural tube cells is required in all vertebrates, including zebrafish, although the specific proteins that mediate this adhesion appear to vary between species. For instance, in zebrafish and *Xenopus*, misregulation of the adhesion protein N-cadherin causes NTD.^{21–23} In contrast, the neural tube in mouse *ncad* mutants is closed but abnormally shaped, suggesting that other adhesion proteins are sufficient for neurulation.²⁴

Our previous findings demonstrated that, in zebrafish, Nodal signaling is required for neurulation of the region of the neural tube that will give rise to the brain.²³ Nodals are secreted proteins in the transforming growth factor-beta (TGF- β) superfamily. Zebrafish have three Nodal proteins: Squint (Sqt), Cyclops (Cyc), and Southpaw (Spaw).^{25–28} All three zebrafish Nodal ligands signal through a receptor complex containing type I and type II TGF- β receptors and the membrane-associated protein One-eyed pinhead (Oep).^{25–28}

Nodal proteins are required for many events during vertebrate development, including the induction of mesoderm and endoderm, the regulation of left–right asymmetry of visceral organs and the epithalamus, and the formation of the ventral neural tube.^{25–28} Sqt is a very early acting Nodal, and mRNAs encoding Sqt and Oep proteins are supplied maternally and then later transcribed from the zygotic genome. Their “MZ” mutants lack both sources of mRNA, whereas “Z” mutants lack only zygotically supplied transcripts. MZ*oep* and *cyc:sqt* double mutants have the most severe phenotypes, which include an open anterior neural tube, loss of almost all mesodermal, endodermal, and mesodermal tissues, altered left–right brain asymmetry, shortening of the anterior–posterior axis, loss of the ventral-most region of the developing nervous system, and cyclopic eyes.^{23,29–33} *cyc*, *Zoep*, and *Zsqt* mutants have less severe phenotypes, including partial loss of mesodermal and mesendodermal cell types, left–right asymmetry defects, cyclopic eyes, and a curved body axis.^{34–39} All *cyc* mutants have a closed neural tube, whereas *Zsqt* and *Zoep* can have either an open or a closed neural tube.^{23,40}

Interestingly, almost every aspect of the *sqt* mutant phenotype has incomplete penetrance (not all of the homozygous mutants are affected) and variable expressivity (the severity of the phenotype varies among mutant embryos). The variability in *sqt* mutants is due to both environmental and genetic factors.^{23,35,41–45} For instance, many MZ*sqt* mutants have a midline bifurcation (MB) phenotype, where the left and right side of the embryo become separated.⁴⁵ Increased penetrance of the MB phenotype was caused by heat shock (raising the embryos at 34°C instead of 28.5°C) and correlated with decreased non-canonical Wnt signaling, increased canonical Wnt/ β -catenin signaling, and decreased heat shock protein 90 (Hsp90) activity.⁴⁵ Similarly, the penetrance of the cyclopic phenotype was increased by heat shock (34°C) and cold shock (15°C). Decreased penetrance correlated with the presence of Hsp90 activity and with residual activity of Nodal or Activin, a TGF- β protein closely related in sequence and function to Nodal.⁴⁵

We found that *Zoep* mutants also have an incompletely penetrant open neural tube phenotype.^{23,40} As part of an independent undergraduate research project, we tested the hypothesis that the penetrance of the NTD in *Zoep* mutants was affected by temperature. Although other aspects of the *Zoep* mutant phenotype, such as cyclopia, were completely penetrant,³⁴ the percentage of embryos with an open neural tube phenotype varied from 0% to 100% in clutches raised at 28.5°C. Despite this variability, analysis of variance (ANOVA) indicated that temperature and genetic background both significantly contributed to the degree of penetrance of the open neural tube phenotype. The percentage of *Zoep* mutants with NTD increased when embryos were moved to 34°C at or before 4 h postfertilization (hpf), but not when heat shock was initiated at later time points. This timing closely matches what was found for *sqt* mutants^{44,45} and strongly suggests that, during this period, a temperature-sensitive factor interacts with the Sqt/Oep signaling pathway to promote neural tube closure. Many contributors to anterior NTD in zebrafish Nodal signaling mutants mirror the complexity of human NTD, suggesting that *sqt* and *oep* mutants may serve as valuable model systems to give insights into these human birth defects.

Materials and Methods

Fish maintenance

Zebrafish stocks. Zebrafish were maintained at 28.5°C in a 14:10 light:dark cycle in an aquatic facility with a recirculating water system. Embryonic stages were defined morphologically, and *Zoep*^{m134} mutants were identified by their cyclopic phenotype.¹² Clutches of embryos from single pairs of heterozygous adults were spawned in a 28.5°C incubator. To minimize the chance of the same pair being used more than once, fish were selected at random from tanks containing at least five males and five females. In addition, the experiments were done over the course of 4 years, and so used several different generations of parental fish. At selected hpf, one half of each clutch was moved to a 34°C incubator with the same light cycle, whereas the other half remained in the 28.5°C incubator. At ~24 hpf, embryos were divided into those with two normal eyes (WT siblings) and those with one cyclopic eye (*Zoep* mutants), and then fixed in 4% paraformaldehyde and stored at 4°C. After 1–7 days, embryos were moved into 100% methanol and stored at –20°C until analyzed for gene expression.

FIG. 1. Pineal morphology reports anterior neural tube defect in zebrafish embryos. (A) A WT zebrafish embryo has a round-shaped head and a straight body axis. (B) In contrast, a *Zoep* mutant embryo has a pointed head and a curved body axis. (C) In WT embryos, the pineal organ forms a single oval-shaped domain in the center of the dorsal forebrain. (D) In some *Zoep* mutant embryos, the pineal has an oval shape, indicating the neural tube is closed. (E, F) In other *Zoep* mutants, the pineal is elongated or divided into two domains, indicating the anterior neural tube is open. Dorsal views of embryos at ~24 hpf processed for the expression of *otx5* in the pineal organ.

Whole mount *in situ* hybridization. Expression of *orthodenticle homobox 5 (otx5)* in the pineal organ was assayed by whole mount *in situ* hybridization using established methods.^{46–48} Briefly, embryos were incubated with digoxigenin-labeled antisense RNA probe overnight at 70°C in hybridization solution containing 50% formamide. The probe was then detected with an anti-digoxigenin antibody that was covalently linked to the enzyme alkaline phosphatase. The cells containing *otx5* mRNA were visualized by adding the alkaline phosphatase substrates 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), which were transformed into a purple product.

Analysis. Brightfield images of the dorsal side of the embryo heads were taken with a SPOT Insight Fire Wire camera mounted on a Nikon Eclipse 80i microscope. They were then scored on whether they had the normal oval-shaped pineal or an abnormal divided or elongated pineal morphology.²³ ANOVA with JMP 10 software was used to determine whether there was a significant difference in the percentage of embryos with an open neural tube among embryos raised at different temperatures, embryos moved to 34°C at different developmental time points, and embryos from different clutches with different parents.

Results

Penetrance of open neural tube phenotype increases in *Zoep* embryos exposed to 34°C

Although many aspects of the *Zoep* phenotype, such as cyclopia, are present in all mutant embryos, our previous work indicated that the open neural tube phenotype in *Zoep* embryos is incompletely penetrant.⁴⁰ Even siblings from the same clutch could differ in whether they had an open neural tube and in the severity of the neural tube defect. Since environmental temperature can affect the penetrance of the mutant phenotypes in *sqt* mutants,^{44,45} which lack one of the ligands for receptor complex containing the Oep protein, we hypothesized that temperature was also affecting the penetrance of the neural tube phenotype in *Zoep* embryos.

In our first test of this hypothesis, during the summer of 2012, progeny from single pairs of *oep*^{m134}/+ adults were

placed in a 28.5°C incubator within the first few hpf. At different points in development, one half of the progeny was moved to 34°C, a temperature that increased the penetrance of the cyclopic phenotype in *sqt* mutants, whereas the other half remained at 28.5°C.^{44,45} The times embryos were split

FIG. 2. Penetrance of open neural tube phenotype is slightly higher in embryos raised at 34°C and analyzed during the summer of 2012. The percentage (avg ± SD) of *Zoep* embryos in a clutch with an open neural tube phenotype was slightly higher in embryos raised at 34°C than at 28.5°C. The data in this figure are from embryos that were born in the fish facility (~25°C), and so, the penetrance of their neural tube defects may have been affected by cold shock. After being harvested from the fish facility, the embryos in each clutch were split between 28.5°C and 34°C and then maintained at that temperature until ~24 hpf. Analysis of 15 clutches, 7–44 *Zoep* embryos per clutch. Raw data are included in Supplementary Table S1.

TABLE 1. PENETRANCE OF OPEN NEURAL TUBE PHENOTYPE INCREASES WHEN *ZOEP* EMBRYOS ARE EXPOSED TO TEMPERATURES OUTSIDE OF THEIR IDEAL OF 28.5°C

Time embryos were split between 28.5°C and 34°C ^a	Summer 2012		Number of clutches analyzed
	Average percent of embryos with NTD (avg ± SD)		
	28.5°C	34°C	
0–2 hpf	31.3 ± 27.5	24.9 ± 9.5	3
2–4 hpf	17.4 ± 29.6	62.2 ± 15.0	6
4–6 hpf	3.6 ± 5.0	24.9 ± 9.5	2
8+ hpf	20.7 ± 2.9	32.3 ± 20.8	3

^aThese embryos were exposed to ~25°C for the first few hpf. Raw data included in Supplementary Table S1.

hpf, hours postfertilization; NTD, neural tube defects.

occurred between 0 and 2 hpf (cleavage stages), 2 and 4 hpf (early midblastula), 4 and 6 hpf (midblastula to early gastrula), 6 and 8 hpf (early midgastrula), and after 8 hpf (midgastrula onward). The embryos then remained at either 28.5°C or 34°C until ~24 hpf, when they were fixed and processed for expression of the pineal marker *otx5*. Our previous work indicated that the morphology of the pineal organ accurately reports the extent of closure of the anterior neural tube, with an oval pineal morphology indicating a closed neural tube and an elongated or divided pineal morphology indicating an open neural tube (Fig. 1).²³

We found that embryos that moved to 34°C had a significantly greater penetrance of the open neural tube phenotype than embryos raised only at 28.5°C (Fig. 2). However, the pattern of this effect was hard to match to the biology. For instance, the temperature effect was present even when the embryos were moved to the higher temperature after 8 hpf, even though this was after the anterior neural tube had already closed (Table 1 and Supplementary Table S1; Sup-

plementary Data are available online at www.liebertpub.com/zeb). Furthermore, the percentage of embryos with open neural tubes varied greatly among clutches, even when only the embryos maintained at 28.5°C were considered (Fig. 3). Pei *et al.*⁴⁴ reported that the cyclopic phenotype of *sqt* mutants increased when embryos were cold shocked (15°C). Thus, we were concerned that the clutches with high penetrance of NTD might have been cold shocked during their time in the fish facility, which typically has an air temperature of 25°C.

Due to this concern, we changed our experimental design. In our new design, adults were spawned in the 28.5°C incubator instead of the fish facility, ensuring that the embryos would be at 28.5°C from the time of fertilization. The large variation in the penetrance of NTD persisted in the embryos raised at 28.5°C even in this experimental design (Fig. 3 and Supplementary Table S1). Consistent with the results of others, the cyclopic phenotype was completely penetrant; however, there was variation in the shape of the head and in the placement and morphology of the eye (Fig. 4).

To determine the influences on open neural tube penetrance, the data from this new experimental design were analyzed by three-way ANOVA, with temperature, the stage embryos were split between 28.5°C and 34°C, and clutch number/parentage as the variables, and with the 28.5°C and 34°C data from the same clutch paired. This model accounted for a large portion of the variation in the penetrance in the neural tube phenotype, as $R^2=0.78$, meaning 78% of the variation in penetrance of the open neural tube phenotype was explained by considering the three variables. The ANOVA results indicated that temperature ($p=0.016$) and clutch number ($p=0.0081$) had significant contributions to the penetrance of the open neural tube phenotype (Figs. 5 and 6). There was no significant interaction between temperature and stage of treatment when all of the stages were considered together ($p=0.5274$). However, when the stages were considered separately, the difference in penetrance approached significance for embryos divided between 28.5°C and 34°C at

FIG. 3. Variation in penetrance in open neural tube phenotype in clutches raised at 28.5°C. The percentage of *Zoep* embryos within a clutch of siblings with open neural tubes varied from 0% to 100% in different clutches. Note the 2012 data were obtained from fish spawned in the fish facility (~25°C) and, thus, potentially cold shocked during the first few hours of development, while the fish from 2013 to 2015 were spawned in a 28.5°C incubator. The data in this figure are from the control (nonheat shocked) embryos from all of the experiments in this study, with exclusion of one clutch that had only two control embryos. There were 3–22 *Zoep* embryos raised at 28.5°C per clutch. Raw data are included in Supplementary Table S1.

FIG. 4. Variation in eye phenotype among zebrafish embryos from the same clutch. Although all of these *Zoep* sibling embryos have one eye compared to the two eyes in their WT sibling phenotype, the morphology of the head and eye varies. For instance, while the shape of the lens in embryo 5 (e5) is slightly heart-shaped, the lens in e6 is round. The head of e2 has the typical pin head shape that the mutant was named for. In e3, this pinhead shape appears to be tipped on its side, similar to the twisted brain phenotype often seen in *sqt* mutants. One *Zoep* embryo from this clutch is not shown as the eye could not be located, suggesting that it was degraded or severely misplaced. Frontal views of live embryos at ~30 hpf.

0–2 and 2–4 hpf (Fig. 5, Table 2, and Supplementary Table S1). The results were very different when the data from summer 2012 were included, suggesting the colder fish facility temperatures affected neurulation in these embryos (Supplementary Table S2). Together, these data suggest that

the temperature effect largely or completely occurred during the first 4 h of development, corresponding to cleavage stages to midblastula stages.

Discussion

In this study, a team of undergraduate students used classical genetics and statistics to parse out factors contributing to the penetrance of defects in the complex process of neurulation. Three-way ANOVA indicated that genetic background/clutch number and environmental temperature had significant contributions to the penetrance of NTD. Like *Zoep*, the penetrance of the cyclopic and MB phenotypes in *MZsqt* was dependent on temperature and genetic background. Furthermore, the temperature-sensitive period was very similar between the cyclopic and neural tube phenotypes, suggesting the penetrance of these phenotypes was caused by the same underlying mechanism.

Comparison of *sqt* and *Zoep* mutants

In their studies of *MZsqt* embryos, Pei *et al.*^{44,45} found that the penetrance of the cyclopic and MB phenotypes was temperature dependent, but the temperature-sensitive period was different between these two phenotypes. The cyclopic phenotype was affected by a 0.5-h heat shock at 34°C before the midblastula transition, which occurs at the 1024 cell stage (3 hpf) or a 1-h heat treatment between the 1024 cell stage and the onset of gastrulation (3–6 hpf). This is very similar to our finding that moving *Zoep* embryos to 34°C before the sphere stage (4 hpf) caused an increase in the penetrance of the neural tube phenotype that approached significance ($p=0.066$ for movement between 0 and 2 hpf, and $p=0.057$ for movement between 2 and 4 hpf). This raises the possibility that the same underlying defect is responsible for both phenotypes.

The strongest possibility is that higher temperatures are affecting the development of the mesoderm and endodermal tissues. First, the timing of temperature sensitivity closely matches the period when Nodal signaling is acting to induce these tissue layers. Studies with small molecule inhibitors of Nodal signaling indicate that Nodal acts between mid–late gastrula stages.⁴⁹ Furthermore, separation of the eye field to

FIG. 5. Significant increase in penetrance of open neural tube phenotype in *Zoep* embryos raised at 34°C versus 28.5°C during 2013–2015. The percentage of *Zoep* embryos (avg ± SD) in a clutch with an open neural tube phenotype was significantly higher in embryos raised at 34°C than at 28.5°C ($p=0.016$). This graph includes only data from fish spawned in a 28.5°C incubator, and so, there was no cold shock (compare to Fig. 2). At various times postfertilization, the embryos were divided between 28.5°C and 34°C and raised at that temperature until ~24 hpf. Analysis of 37 clutches, 5–30 *Zoep* embryos per clutch. Raw data are included in Supplementary Table S1.

FIG. 6. Significant variation in penetrance of open neural tube in different clutches of *Zoep* siblings. The percentage of embryos with open neural tubes in a clutch of siblings varies from 0% to 100%, whether the embryos are raised at either 28.5°C or 34°C. Analysis of variance indicated that clutch made a significant contribution to the variation in the penetrance of the neural tube phenotype ($p=0.0081$). Each clutch was most likely from a different pair of adults, as the parents were chosen at random from a group (>8) of heterozygous fish. This graph includes only data from fish spawned in a 28.5°C incubator during 2013–2015, and so, the embryos were not exposed to a cold shock. The 2 hpf ranges indicate when the embryos were split between 28.5°C and 34°C. There were 5–30 *Zoep* embryos per clutch. Raw data are included in Supplementary Table S1.

create two eyes as well as closure of the anterior neural tube depend upon the correct development of mesoderm and mesendoderm. For instance, injection of a constitutively activated form of the Nodal receptor subunit Taram-A cell autonomously rescues mesoderm and mesendoderm formation in *MZoep* mutants while also correcting their open neural tube defect.²³ Similarly, anterior prechordal plate mesendoderm/mesoderm is required for separation of the single primordial eye field of vertebrates into the precursors of the two separated eyes.⁵⁰ Induction of prechordal plate mesendoderm requires some of the highest and longest signaling of the mesendoderm/mesodermal tissues in zebra-

fish.^{33,43,51} Since the temperature-sensitive period is shorter for open neural tube phenotypes, a tissue other than prechordal plate is probably also involved.

Temperature effects on human NTD

There is some suggestion that temperature sensitivity is a characteristic of neurulation in many vertebrate species. Exposure to elevated temperatures during development has been associated with increased risk of NTD in humans and mammalian model systems such as mice, hamsters, and even chicks.^{5,52–56} Recent meta-analysis of human studies indicates that maternal fever during the first trimester of pregnancy significantly increases the incidence of several birth defects, including NTD, congenital heart defects, and oral clefts.^{5,6} These studies also provide a potential link between temperature sensitivity and Nodal signaling. Oral clefts in humans are associated with mutations in the Nodal pathway gene *TG* interacting factor (*TGIF*) and are part of a larger syndrome called holoprosencephaly, which occurs when the left and right hemispheres of the brain fail to properly separate.⁵⁷ The cyclopic phenotype in zebrafish with defects in Nodal signaling, including *Zoep* and *sqt* mutants, is also part of a holoprosencephaly disorder. Thus, many aspects of the temperature-sensitive phenotype are shared between zebrafish and mammals, suggesting that zebrafish could serve as a model system for defining the interactions between genetics and environment that contribute to NTD.

The factors causing temperature sensitivity of the neural tube phenotype in humans or zebrafish have not yet been definitively identified. The studies of Pei *et al.*⁴⁴ indicate that the temperature-sensitive factor is unlikely to be *Sqt* itself, as the temperature sensitivity persists in *MZsqt*^{hi975/hi975}

TABLE 2. PENETRANCE OF OPEN NEURAL TUBE PHENOTYPE INCREASES WHEN *ZOEP* EMBRYOS ARE EXPOSED TO HIGH TEMPERATURE BEFORE 4 HPF

2013–2015

Time embryos were split between 28.5°C and 34°C ^a	Average percent of embryos with NTD ^a (avg ± SD)		Number of clutches analyzed	p-Value
	28.5°C	34°C		
0–2 hpf	49.7 ± 42.3	75.3 ± 19.4		
2–4 hpf	31.9 ± 32.9	48.1 ± 34.8	16	0.057
4–6 hpf	22.9 ± 32.2	50.5 ± 12.8	5	0.1483
6–8 hpf	14.1 ± 19.3	4.2 ± 8.4	4	0.5507
8+ hpf	32.0 ± 32.5	42.9 ± 21.3	6	0.4235

^aThese embryos were spawned in a 28.5°C incubator and so, were never cold shocked. Raw data included in Supplementary Table S1.

mutants, which lack all functional Sqt proteins. However, they did find that depletion of Hsp90 family members caused increased penetrance of both the cyclopic phenotype (HSP90a depletion) and MB phenotype (HSP90a and HSP90b depletion) in *MZsqt* embryos.^{44,45} Similarly, targeted deletion of the Hsp70 family members in mice increased the penetrance of the anterior open neural tube defect exencephaly.⁵⁸ Heat shock proteins are upregulated dramatically in response to stress because they act to stabilize unfolded proteins and prevent protein aggregation. This suggests there is an unknown protein or proteins involved in neural tube closure that become unfolded or misfolded upon heat shock, and these proteins could be conserved between zebrafish and mammals.

ANOVA indicated that genetic background had a significant contribution to the variable penetrance of the open neural tube phenotype independently of temperature. The presence of a genetic contribution is also consistent with studies of *MZsqt* mutants. Penetrance of the cyclopic and MB phenotypes was fairly constant upon repeated spawning of the same parental pair of parents.⁴⁵ One possibility is that genetic variations that alter the expression of Wnt pathway genes could influence the penetrance of these phenotypes.⁴⁵ Defects in the Wnt signaling pathway caused increased penetrance of cyclopia (Wnt5b depletion) and the MB phenotypes (Wnt11 depletion) in *MZsqt* embryos. There is also a connection between Wnt and neurulation. Noncanonical Wnt signaling is a key regulator of the convergent extension movements that shape the neural plate, the precursor to the neural tube.⁵⁹ In mice and *Xenopus*, deficient Wnt signaling causes the neural plate to become widened, and consequently, it fails to close.⁶⁰ Mutations in a number of Wnt pathway genes have also been linked to NTD in humans.²⁰ In zebrafish, loss of Wnt signaling causes a widening of the neural tube, but the neural tube is still able to close.^{19,61} Thus, an important next step will be to determine if loss of Wnt signaling increases the penetrance of NTD in zebrafish Nodal signaling mutants.

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Authors' Contributions

Phyo Ma graduated with a B.S. degree in Cell and Molecular Biology from the University of Minnesota Duluth in 2014. Phyo worked as a team with Morgan Swartz to initiate this project, to collect the majority of the data, and to improve the experimental design. She was funded through two UROP fellowships. Phyo presented posters on this work at several local meetings and at the 11th International Meeting on Zebrafish Development and Genetics in Madison, WI, during the summer of 2014. Phyo also generated parts of the first draft of this manuscript and did the research on the connection between hyperthermia and birth defects in mammals.

Morgan (Reno) Swartz graduated from the University of Minnesota Duluth with a B.S. degree in Cell and Molecular Biology in 2013. Morgan worked in concert with Phyo Ma to carry out this project during several semesters of independent research for credit. She presented this work at the University of Minnesota Duluth Research and Artistic Showcase and helped write the initial draft of the manuscript. She is currently in the M.S. program for Physician Assistant Studies at the University of Wisconsin La Crosse.

Lexy Kindt recently completed her Master's degree in the Integrated Biological Sciences program at the University of Minnesota Duluth. She assisted with the statistical analysis of this project and with writing the final draft of the manuscript. Ashley Kangas completed a B.S. degree in Biology at the University of Minnesota Duluth in the Spring of 2015. Her research was funded through UROP fellowships granted in 2014 and 2015. She carried out the final experiments needed to complete the dataset and aided in the analysis of the data and generating the final manuscript. She presented her research at the 2014 and 2015 Undergraduate Symposia at the University of Minnesota Duluth.

Jennifer O. Liang is an Associate Professor in the Department of Biology and part of the Integrated Biosciences Graduate Program. She mentored all of the students working on this project, helped with crossing fish, and wrote the final version of the manuscript.

Disclosure Statement

No competing financial interests exist.

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