Investigating the roles of E-T coupling and social environment in the stimulus-dependent expression of *parathyroid hormone 2*.

by

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Abstract

In larval zebrafish, *parathyroid hormone 2* (*pth2*) codes for a peptide hormone that is expressed exclusively in cells near the ventral part of the posterior tuberculum, an area of the forebrain involved in sensori-motor control and social behavior. We identified *pth2* in an RNA-seq experiment looking for genes regulated by voltage-gated calcium channel activity. Relative to wild type larvae, *pth2* was down-regulated in larvae where the L-type Cav1.3 channel was inactivated either genetically (*cav1.3a* mutants) or pharmacologically (the Ca^{2+} channel blocker isradipine). Using mRNA *in situ* hybridization on *cav1.3a* mutant larval zebrafish and larvae treated with isradipine, we confirmed that *pth2* expression in zebrafish requires Cav1.3a channel activity. Next, to characterize the regulatory region of the *pth2* gene, we performed a transgenesis experiment using a 2 kbp fragment of the zebrafish genome immediately upstream of the *pth2* coding sequence. This resulted in expression of GFP in cells of the posterior tuberculum and their axons.

Recently, a study investigating gene expression in zebrafish showed that social isolation led to decreased levels of *pth2* expression. We confirmed these results using our stable line of transgenics. Using neomycin and $CuSO_4$, the study also showed that expression of *pth2* was dependent on mechanical stimulation due to the movements of other zebrafish. These findings provided evidence that *pth2* is not expressed when hair cells of the lateral line are killed using ototoxic compounds, but the study did not explore the effects on *pth2* expression when lateral line function is lost genetically. To test this, we performed mRNA *in-situ* hybridization on lateral line mutant zebrafish raised in social or isolated environments. Interestingly, mutants raised socially expressed more *pth2* than wild type fish that were isolated, despite lacking a functional

lateral line. Overall, this research provides further information about the regulation of *pth2* and could help elucidate its role in the social behavior of zebrafish.

Introduction

Tuberoinfundibular peptide of 39 residues (TIP39), also known as parathyroid hormone 2, is a peptide hormone encoded by the *parathyroid hormone 2* (*pth2*) gene. It was originally identified as a selective agonist for parathyroid hormone 2 receptor (PTH2R) in the brain (Usdin et al., 2003). In rodents, it is only expressed in the posterior thalamus and lateral pons, but Pth2-positive neurons have a broad projection pattern matching the distribution of PTH2R (Arpád Dobolyi et al., 2010). TIP39 is involved in emotional processing and has been implicated in behavioral responses to fear, stress, and pain in experiments involving nonfunctional TIP39 and PTH2R in rodents (Coutellier & Usdin, 2011; Arpad Dobolyi et al., 2002). However, it is unknown if it plays a similar role in zebrafish. In larval zebrafish, *pth2* is expressed exclusively in cells near the ventral part of the posterior tuberculum, an area of the forebrain involved in sensori-motor control and social behavior (Bhattacharya et al., 2011). Though *pth2* is only expressed in one small brain region, Parathyroid hormone 2 receptors are found throughout the central nervous system of zebrafish (Bhattacharya et al., 2011). Along with the function of this hormone in zebrafish, there is also a lack of knowledge of the mechanisms controlling expression of *pth2*. Understanding the mechanisms controlling *pth2* expression could provide a more complete picture of its roles in physiology and behavior.

Neuronal activity can regulate the expression of certain target genes. L-type voltage-gated calcium channels (L-VGCCs) open in response to membrane depolarization and have been linked to activity-dependent changes in gene expression (Dolmetsch, 2003). Cav1.3 (Cacna1d) is

a member of the L-VGCC family that is expressed in the brain and acoustico-lateralis sensory systems of zebrafish (Sidi et al., 2004). To determine whether Cav1.3 channels regulate gene expression in zebrafish, we performed RNA-seq experiments comparing transcript abundance between wild type larvae and those where Cav1.3 channels were inactivated either genetically (*cav1.3a* mutants) or pharmacologically (the L-type Ca^{2+} channel blocker isradipine). *pth2* was detected in the dataset of downregulated transcripts (Figure 1).

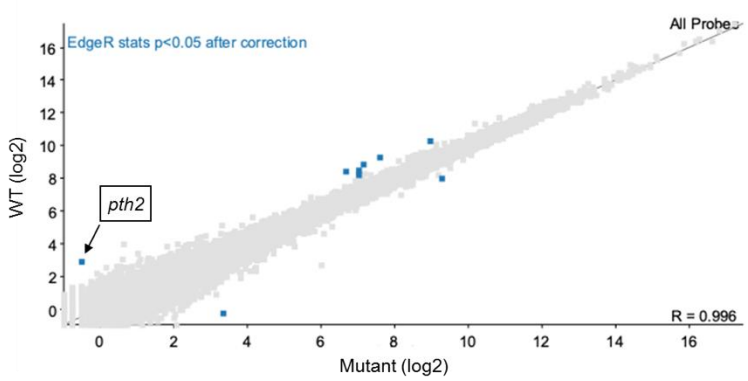

Figure 1. Abundance of mRNA transcripts in wildtype and *cav1.3a* mutant zebrafish. *pth2* was one of several genes downregulated in mutants (Erickson, 2017, unpublished).

In this study, we investigated the role of Cav1.3 channel activity in regulating expression of *pth2*. Using *in situ* hybridization on *cav1.3a* mutant zebrafish larvae and larvae treated with isradipine, we showed that Cav1.3 channel activity is crucial for *pth2* expression. This work led us to consider what DNA regulatory elements are responsible for Ca^{2+}-dependent expression of *pth2*. We performed a transgenesis experiment to characterize the regulatory region of the *pth2* gene, which also allowed us to visualize where *pth2*-positive cells project their axons. We also investigated the effects of social isolation on *pth2* expression levels and the role of the mechanosensory lateral line in that process.

Results

I confirmed the RNA-seq results by using mRNA *in situ* hybridization for *pth2* to show that *pth2* is downregulated in *cav1.3a* mutant larval zebrafish (Figure 2) and larvae treated with isradipine (Figure 3). These results suggest that Ca^{2+}-influx via Cav1.3 channels plays a crucial role in regulating expression of *pth2*. Additionally, I showed that larvae that were washed out of isradipine made a significant recovery in *pth2* expression relative to isradipine-treated larvae that were not washed out (Figure 3).

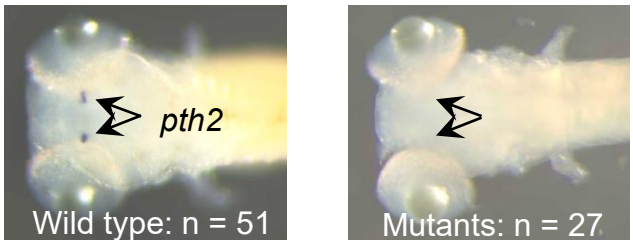

Figure 2. mRNA *in-situ* hybridization for *pth2* on 3 dpf wild type and *cav1.3a* mutant zebrafish larvae. *pth2* transcripts were detected in the brain of wild type larvae (n=51) but were not detected in *cav1.3a* mutants (n=27).

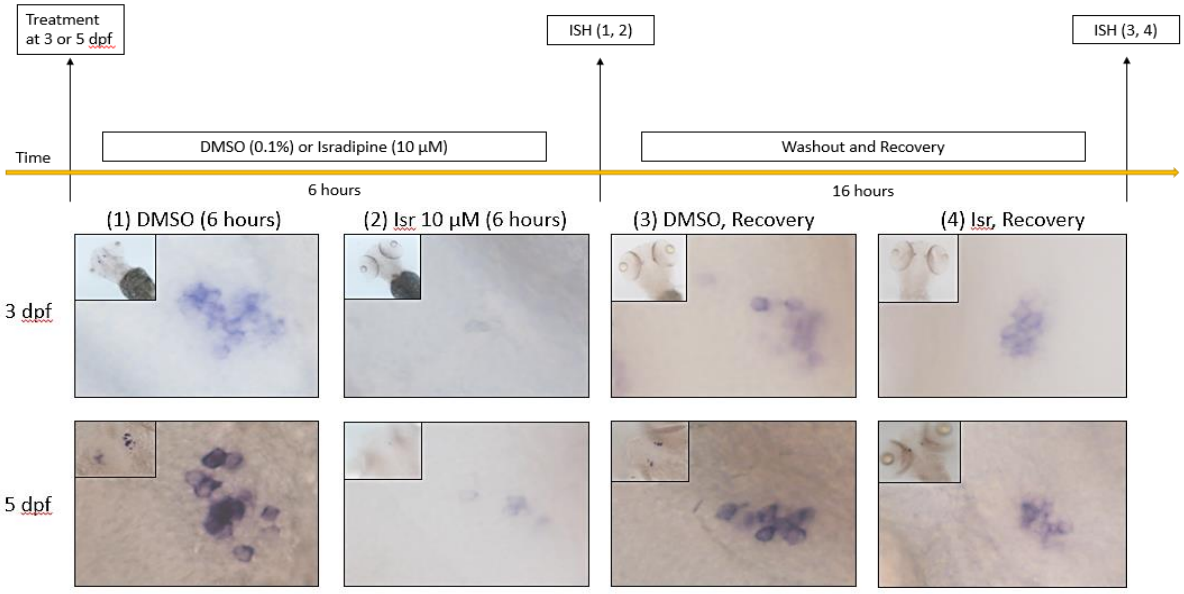

Figure 3. mRNA *in-situ* hybridization for *pth2* on zebrafish larvae treated with isradipine and control groups. Groups 1 and 3 were control groups and Group 2 was treated with isradipine. Group 4 was treated with isradipine, then allowed to recover overnight before being fixed with PFA (Group 1, n = 64; Group 2, n = 47; Group 3, n = 51; Group 4, n = 54).

Given that *pth2* expression is positively regulated by Cav1.3a activity, we hypothesized that a pathway known as excitation-transcriptional (E-T) coupling is acting downstream of Cav1.3a (Figure 4). E-T coupling is a mechanism of gene regulation that involves calcium (Ca^{2+}) entering a cell through an ion channel in response to neural or sensory stimuli (Dolmetsch, 2003). These Ca^{2+} ions interact with signaling proteins to alter patterns of mRNA expression in target cells.

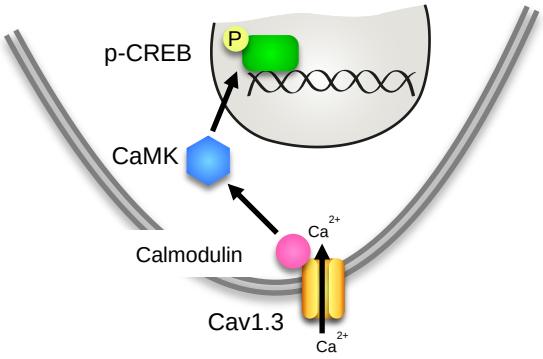

Figure 4. E-T coupling. Stimulus-dependent changes in calcium ion concentration can trigger certain proteins to alter levels of gene transcription.

To better understand how *pth2* expression is regulated, we examined the upstream promoter regions for the *pth2* gene. We found that a 2 kbp fragment of the zebrafish genome upstream of the coding region for *pth2* is evolutionarily conserved across several fish species (Figure 5).

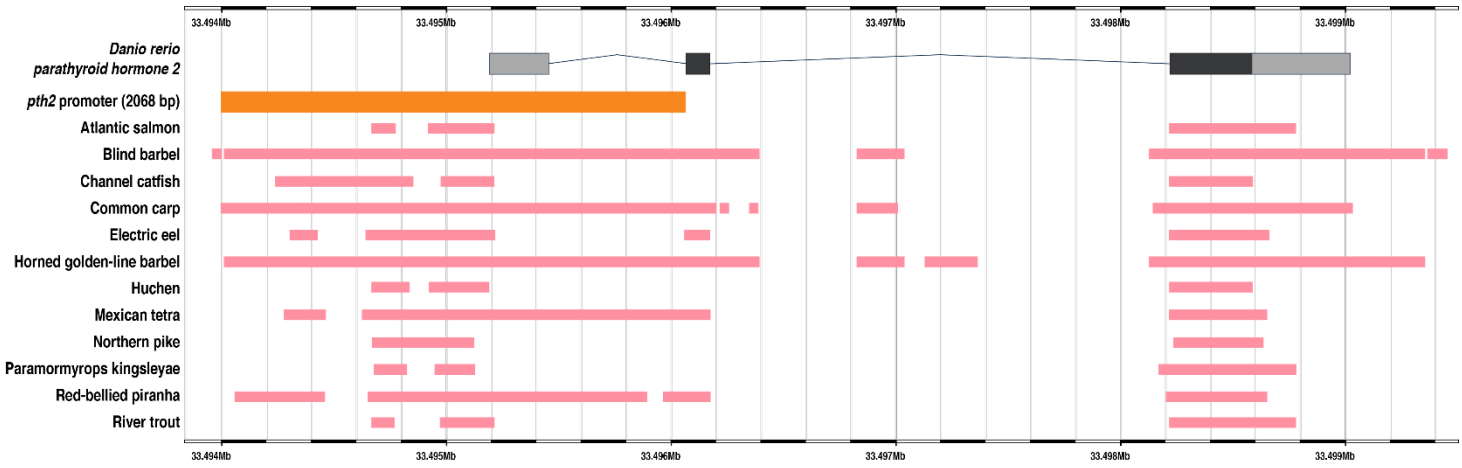

Figure 5. Conserved region in the genomes of several fish species.

The DNA fragment contains three possible cAMP-responsive elements (CREB binding sites), which are found in the promoter region of genes regulated by neuronal activity. With the aim of determining if this fragment acts as the promoter region for *pth2* in zebrafish, we cloned it into a plasmid along with the EGFP reporter gene (Figure 6).

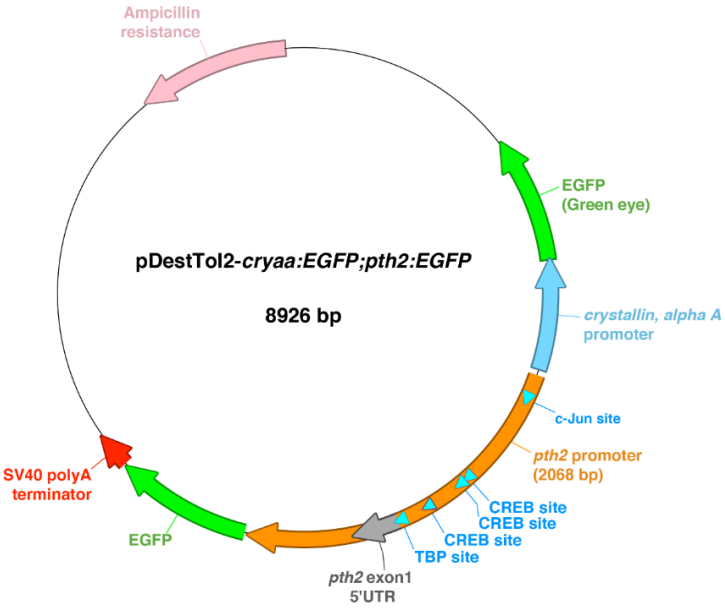

Figure 6. Expression vector with the *pth2* promoter driving expression of GFP.

Expression of GFP was successfully driven by the isolated regulatory elements in cells of the posterior tuberculum and their axons (Figure 7). GFP expression allowed for visualization of the axons of the presumptive *pth2*-expressing cells, which project laterally and toward the anterior ventral part of the forebrain.

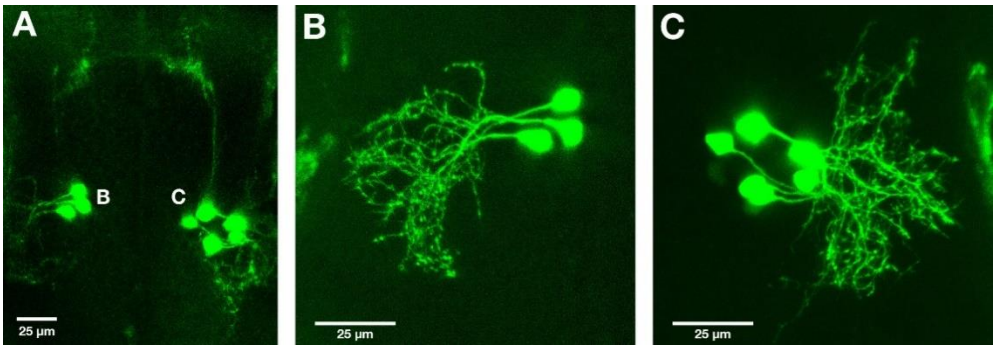

Figure 7. The isolated 2 kbp fragment upstream of the *pth2* coding region drove expression of GFP in cells of the posterior tuberculum and their axons.

Recently, a study investigating gene expression in zebrafish showed that social isolation led to decreased levels of *pth2* transcription (Anneser et al. 2020). In this study, expression of *pth2* was recovered after exposing previously isolated fish to conspecifics for just 30 minutes. Conversely, expression abruptly decreased after isolating fish that were raised socially. To confirm these results, we performed an experiment using our stable line of transgenic zebrafish expressing GFP under control of the presumptive *pth2* promoter. Transgenic fish were raised in social (Group 1) and isolated (Group 2) environments before GFP was imaged, resulting in differential expression of GFP in the two groups (Figure 8).

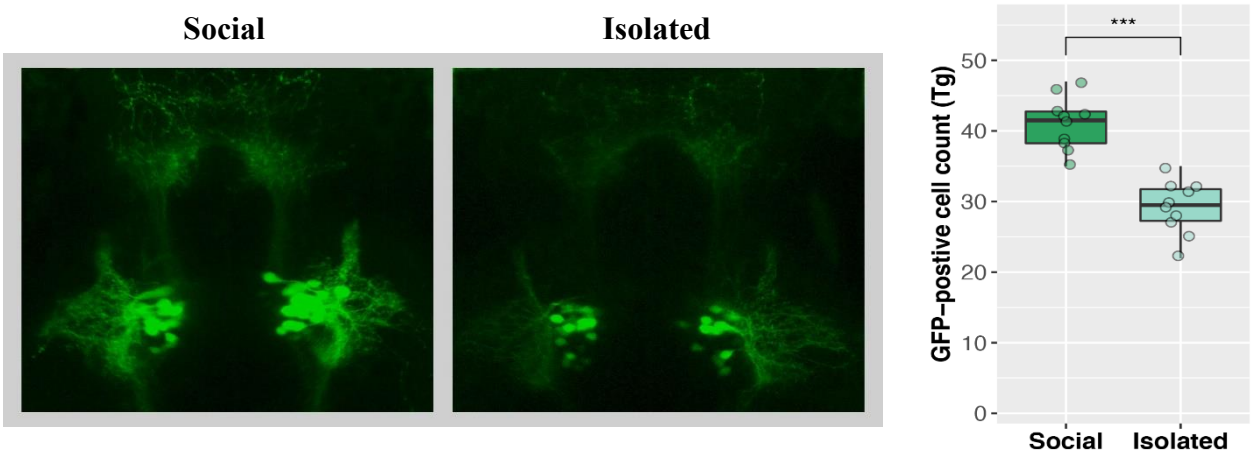

Figure 8: GFP expression was greater in 5 dpf fish raised socially (left) than in isolated fish (right). The graph shows the average numbers of GFP-positive cells. Welch's t-test: t = 6.9893, df = 17.997, p-value = 1.587e-06. 95% confidence interval: 8.322908, 15.477092.

GFP expression was greater in transgenic zebrafish raised socially than in fish raised in isolated environments, although some GFP was detected in isolated fish. To quantify the results, the number of GFP-positive cells in each fish were counted. The average number of GFP-positive cells was significantly higher in socially raised fish than in those that were isolated. These results confirmed that *pth2* expression in zebrafish is dependent on the social context of their environment.

The Anneser et al. study also found that levels of *pth2* transcription were dependent on mechanical stimulation due to the movements of other zebrafish, and not due to chemosensory or visual perception of conspecifics. In support of this finding, the study showed that previously isolated fish in which the lateral line was ablated using neomycin or $CuSO_4$ did not recover expression of *pth2* when exposed to conspecifics for three hours. These findings provided evidence that *pth2* is not expressed when hair cells of the lateral line are killed using ototoxic compounds, but the study did not explore the effects on *pth2* expression when lateral line function is lost genetically.

The lateral line is a sensory system containing hair cells that allows fish to sense movements and vibrations in the surrounding water by converting mechanical stimuli into electrical signals (Mogdans, 2019). In zebrafish, *lhfpl5b* is expressed specifically in hair cells of the lateral line, and codes for a protein that plays an important role in hair cell function (Erickson et al. 2020). A loss-of-function mutation in this gene silences lateral line hair cells, preventing mechano-electrical transduction.

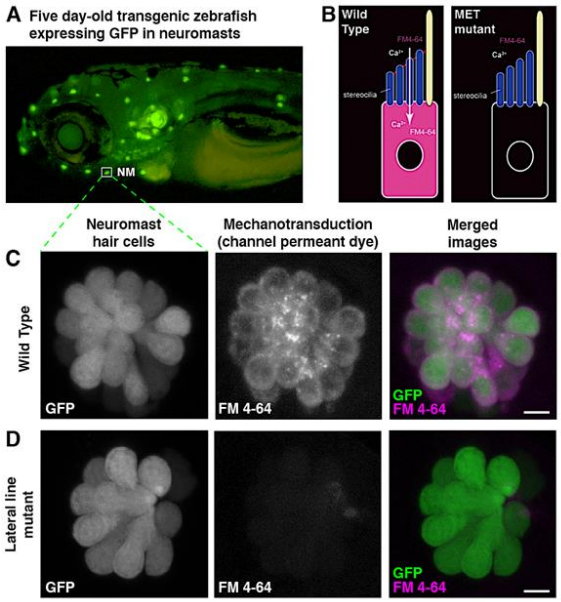

Figure 9: In lateral line mutant fish, the mechano-transduction channel of lateral line hair cells is nonfunctional (Erickson et al. The *lhfpl5* ohnologs *lhfpl5a* and *lhfpl5b* are required for mechanotransduction in distinct populations of sensory hair cells in zebrafish. Front. Mol. Neurosci., 2020).

Anneser et al. proposed that the lateral line is the pathway by which *pth2*-expressing cells receive mechanosensory information. To further test their findings, we performed mRNA *in-situ* hybridization for *pth2* on *lhfpl5b* mutant zebrafish raised in a social environment, mutants raised in isolated environments, and wild type fish raised in social and isolated environments for comparison. The number of *pth2*-positive cells in each fish was counted. We hypothesized that due to their lack of a functional lateral line, *lhfpl5b* mutants would express little to no *pth2*, regardless of whether they were raised socially or isolated. The results showed that socially raised wild type fish expressed *pth2* in the greatest number of cells. However, while isolated fish expressed *pth2* in very few cells, social *lhfpl5b* mutants expressed *pth2* in an intermediate number of cells relative to social wild type fish (Figure 10).

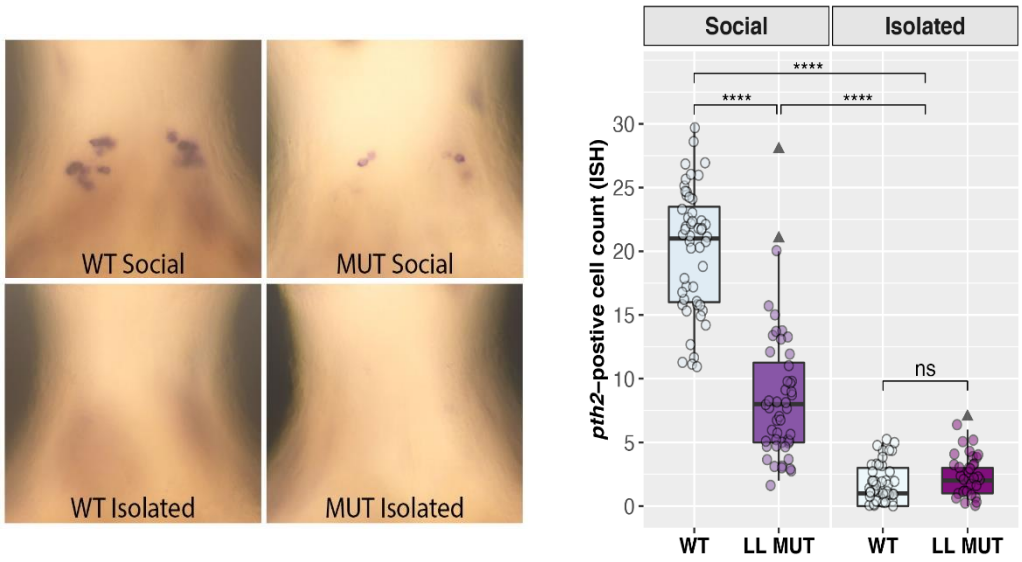

Figure 10: Little to no *pth2* was detected in fish raised in isolated environments. Fewer *pth2*-positive cells were detected in lateral line mutants raised socially than in wild type fish raised socially. Kruskal-Wallis test: p-value = 2.02e-31.

These results confirmed that expression of *pth2* in zebrafish is dependent on both the social context of their environment and on lateral line sensory input.

Discussion

The goal of this research was to gain an understanding of the mechanisms controlling expression of *pth2*. We have shown that *pth2* is downregulated in zebrafish lacking Cav1.3 calcium channel activity. We have characterized the regulatory region of the *pth2* gene. We have also confirmed that expression of *pth2* in zebrafish is dependent on the presence of conspecifics, and that information about the presence of others is partially obtained via the mechanosensory lateral line.

In *cav1.3a* mutant larvae, there was a total loss of *pth2* expression compared to wild type larvae (Figure 2). From this information, it could not be determined whether loss of *pth2* expression was because lack of functional Cav1.3 causes defects in thalamic development, or if it was due to disruption of excitation-transcription coupling, as we hypothesized. Therefore, our next experiment involved the use of the calcium channel blocker isradipine (Figure 3). In this experiment, Group 2 exhibited a substantial decrease in *pth2* expression after treatment with isradipine, relative to the control. Group 4 was also treated with isradipine but was subsequently washed out overnight. After being washed out of isradipine, Group 4 larvae made a significant recovery in *pth2* expression compared to Group 2. These results demonstrate that lack of functional Cav1.3 does not lead to defects in thalamic development. Instead, it can be inferred that E-T coupling is a key process in expression of *pth2*, and Cav1.3 channels are a requirement for that process to occur. In the future, it could be useful to further explore the role of E-T coupling in *pth2* expression. For example, a protein involved in the E-T coupling pathway that could regulate transcription of *pth2* is cAMP-responsive element-binding protein (CREB) - a transcription factor whose activity is regulated by Ca^{2+}-dependent signaling. Increases in intracellular Ca^{2+} due to opening of voltage-gated channels can activate Calmodulin-dependent

kinase (CaMK), leading to phosphorylation of CREB (Barco & Marie, 2011). Once phosphorylated, CREB is more likely to recruit other proteins that can then attract RNA polymerase II to the promoter, increasing levels of transcription. The activity of CREB is one more factor of many that could contribute to the regulation of *pth2* expression. Understanding the influence of CREB and other proteins in the pathway on expression of *pth2* would give us a better idea of how its expression is regulated and could help us draw conclusions about its biological role in zebrafish.

Our transgenesis experiment resulted in expression of GFP in presumptive *pth2*-expressing cells, driven by the isolated regulatory elements (Figure 7). This experiment allowed for the visualization of these cells and the areas of the brain that their axons project to. In the future, this information could be used to help determine the specific roles that *pth2* plays in zebrafish behavior. The stable line of transgenics established in this experiment also opened up the possibility for a wide range of experimental manipulations to better understand how *pth2* is regulated. We used the *pth2* transgenics in our experiment investigating the effects of social isolation on expression of *pth2* (Figure 8). In isolated fish, GFP was expressed in fewer cells than in fish raised socially, although a significant number of GFP-positive cells were still detected. However, using *in situ* hybridization, we detected little to no *pth2* expression in isolated fish (Figure 10). This disparity could simply be attributed to the fact that GFP is easier to detect via confocal microscopy than *in situ*-stained mRNA is using a compound light microscope. Another possible explanation is that post-transcriptional degradation of *pth2* mRNA transcripts occurs at a higher rate than degradation of the mRNA coding for GFP, leading to greater detection of GFP than *pth2* transcripts. This theory could be tested using *in situ* hybridization for *egfp* in the transgenic zebrafish and comparing *egfp* transcript abundance to abundance of *pth2* transcripts.

In our experiment investigating the link between lateral line function and *pth2* expression, we hypothesized that *lhfpl5b* mutants would not be able to sense the presence of others, and therefore would not express *pth2*. However, we found that *lhfpl5b* mutants lacking a functional lateral line expressed some *pth2* when raised in a social environment, although there were less *pth2*-positive cells than in wild type fish (Figure 10). These results do not completely support the previous hypothesis. Instead, they suggest there must be another way that information about the presence of others is conveyed to *pth2*-expressing cells. One possible explanation is that hair cells within the zebrafish ear that are not part of the lateral line system can also transmit sensory information to these cells. *lhfpl5b*, the gene required for lateral line function, is not required for the function of hair cells of the inner ear. These hair cells instead require *lhfpl5a* – a distinct, but related gene – for signal transduction. Future experiments should be performed to investigate whether inner ear hair cells also contribute to *pth2* expression.

Overall, this research has shown that the E-T coupling pathway plays a key role in regulating expression of *pth2*. This means that the TIP39 hormone is produced in direct response to stimuli that trigger neuronal activity. So far, it appears that that stimulus is mechanical perturbation of the water due to the movements of other zebrafish, which is sensed in part by hair cells of the lateral line. This research has demonstrated the usefulness of zebrafish as a model for the study of the *pth2* gene and could help pave the way for future studies into TIP39's roles in physiology and behavior.

Methods

A. Genotyping Calcium Channel Mutants

Tissue samples were obtained from *cacna1da*^{tc323d} fish by excision of a small portion of the caudal fin while the fish were under MS222 anesthesia. DNA was extracted, amplified by PCR, and sequenced (see Table 1 for primers). The chromatogram displaying the sequenced DNA was used to determine which fin-clipped fish were *tc323d* heterozygotes, and which were wild type. To produce mutant and wild type larvae for the calcium channel experiments, *tc323d* heterozygotes were crossed. Wild type and mutant larvae were separated using the tap response. When vibrations are sent through water in a dish due to tapping on the dish, wild type larvae display a startle response. Calcium channel mutants do not respond to the tapping stimulus at all, making it easy to separate them from wild type larvae.

B. Preparation of ISH Probe

The *pth2* gene was amplified by RT-PCR (see Table 1 for primers) and isolated using gel extraction. The gene was cloned into the pCR4-TOPO vector, then transformed into competent bacterial cells. Bacterial minipreps were performed to extract the *pCR4-pth2* plasmid, which was then digested with Not1 restriction enzyme, resulting in a linearized plasmid. The DIG-labeled ISH probe was made using the T3 RNA polymerase promoter from the linearized *pth2* plasmid.

C. Isradipine Treatment

Zebrafish larvae were separated into four groups. Groups 1 and 3 served as controls and were placed in separate 0.1% DMSO solutions. Groups 2 and 4 were placed in separate 10 μM isradipine solutions. Larvae were incubated in their respective solutions for a period of six hours. After the six-hour incubation period, the larvae in Groups 1 and 2 were removed from their

solutions and fixed in 4% paraformaldehyde. The larvae in Groups 3 and 4 were washed out of the isradipine and DMSO solutions and incubated overnight for 16 hours in E3 embryo medium. After incubation, Group 3 and 4 larvae were fixed in 4% paraformaldehyde. Multiple trials were performed using both 3 and 5 dpf (days post fertilization) larvae. After *in situ* hybridization was performed, the larvae were imaged at 40X magnification using an Olympus BH-2 microscope.

D. Creating *pth2* Transgenic Zebrafish

A 2 kbp fragment of the zebrafish genome that is immediately upstream of the *pth2* coding region was amplified by PCR (see Table 1 for primers) with attB4-B1 recombination sites flanking both ends of the sequence. This PCR product was cloned into the pDONR-P4-P1R entry vector and an LR reaction was performed to create the cryGFP-pth2:GFP-pA plasmid containing the 2 kbp sequence along with the EGFP reporter gene. The plasmids were transformed into competent bacterial cells. After cell growth, the plasmids were extracted using bacterial minipreps. Using microinjection, the plasmids (along with transposase RNA) were injected into zebrafish embryos in the 1 to 4-cell stage, establishing a line of *pth2* transgenic zebrafish.

E. Social Isolation Experiment: Lateral Line Mutants

lhfpl5b mutant zebrafish larvae and wild type larvae were separated into four separate groups: social wildtype, social mutants, isolated wildtype, and isolated mutants. The social groups each consisted of thirty larvae, with all thirty being placed in a single dish together. The isolated groups also consisted of thirty larvae each. Isolated larvae were placed in their own individual well of five 6-well dishes. Small strips of paper were placed between individual wells of the 6-well dishes to prevent visual access between wells, and large pieces of paper were placed between each of the dishes for the same reason. Each group was incubated under these

conditions from 2 to 5 dpf, after which they were fixed in 4% PFA. After *in situ* hybridization was performed, the larvae were imaged at 40X magnification using an Olympus BH-2 microscope. Using the images, the results of this experiment were quantified by estimating the number of ISH-stained cells in each of the larvae. For statistical analysis, a Dunn's test was performed after a Kruskal-Wallis test using the R stats package. See Appendix A for details.

F. Social Isolation Experiment: Transgenic Fish

pth2 transgenic zebrafish larvae were identified at 2 dpf by their green eye marker for GFP and separated from nontransgenics. Transgenic larvae were separated into two groups of thirty: social and isolated. The same setup that was used for the lateral line mutant experiment was used for the transgenics, with the social group all in one dish together and the isolated group separated into different wells of five 6-well dishes. Pieces of paper were used to visually isolate the larvae in the isolated group. Each group was incubated under these conditions until 5 dpf, and GFP was imaged using a Zeiss LSM800 confocal. To quantify the results, cell counts were performed using Fiji/ImageJ image processing software. Statistical analysis involved a Welch's t-test, which was performed using the R stats package. See Appendix A for details.

Primer name	Purpose	Sequence
cacna1da-ac123d-F2	To genotype cacna1da^tc323d allele, 203 bp product, sequence with fwd	CAGCCTAATGCCCTGTGTTAG
cacna1da-ac123d-R2		AGTCAGACCTGGAAGGATTTG
pth2_ISH-F	pth2 ISH probe template, 836 bp includes all of CDS plus some 5' and 3' UTR.	CATTGCATGGACGATTTACG
pth2_ISH-R		TGCCATGTCATTCAAAATCC
attB4(-)2kbp_pth2-F	To clone 2068 bp upstream of pth2 ATG; includes attB4F or attB1R sites (underlined) for cloning into Gateway entry vector pDONR-P4-P1R.	GGGGACAACTTTGTATAGAAAAGTTGGTAAAGGACACTCTTTGAGATCCT
attB1(-)2kbp_pth2-R		GGGGACTGCTTTTTTGTACAAACTTGCCTGCAGATGAATAAGTTGAATAATACA

Table 1: Primers for Genotyping Calcium Channel Mutants, Preparation of ISH Probe, and Cloning 2 kbp Upstream Region.

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Appendix A

Social Isolation Experiment: Transgenic Fish

```
#Welch's t-test
pth2_Tg_SIMPLE_t_test <- t.test(social, isolated)
pth2_Tg_SIMPLE_t_test

##
##  Welch Two Sample t-test
##
## data:  social and isolated
## t = 6.9893, df = 17.997, p-value = 1.587e-06
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##   8.322908 15.477092
## sample estimates:
## mean of x mean of y
##      41.0      29.1
```

Social Isolation Experiment: Lateral Line Mutants

Kruskal-Wallis test and effect size:

```
res.kruskal <- pth2_ISH_KW %>% kruskal_test(Cell_count ~ Category)
res.kruskal

## # A tibble: 1 x 6
##   .y.            n statistic    df        p method
## * <chr>      <int>     <dbl> <int>    <dbl> <chr>
## 1 Cell_count   186      146.     3 2.02e-31 Kruskal-Wallis

pth2_ISH_KW %>% kruskal_effsize(Cell_count ~ Category)

## # A tibble: 1 x 5
##   .y.            n effsize method  magnitude
## * <chr>      <int>   <dbl> <chr>   <ord>
## 1 Cell_count   186   0.785 eta2[H] large
```

Dunn post-test pair-wise comparisons:

```
Dpwc_pth2_ISH <- pth2_ISH_KW %>%
  dunn_test(Cell_count ~ Category, p.adjust.method = "bonferroni")
Dpwc_pth2_ISH

## # A tibble: 6 x 9
##   .y.        group1  group2    n1    n2 statistic        p    p.adj p.adj.
```

```
signif
## * <chr>      <chr>   <chr>  <int> <int>     <dbl>    <dbl>    <dbl> <chr>
## 1 Cell_count WT_soc  MUT_s…    51    48    -4.47  7.94e- 6 4.77e- 5 ****
## 2 Cell_count WT_soc  WT_iso    51    49   -10.8   2.70e-27 1.62e-26 ****
## 3 Cell_count WT_soc  MUT_i…    51    38    -9.19  4.00e-20 2.40e-19 ****
## 4 Cell_count MUT_soc WT_iso    48    49    -6.24  4.45e-10 2.67e- 9 ****
## 5 Cell_count MUT_soc MUT_i…    48    38    -4.93  8.18e- 7 4.91e- 6 ****
## 6 Cell_count WT_iso  MUT_i…    49    38     0.906 3.65e- 1 1.00e+ 0 ns
```

Appendix B

mRNA *in situ* Hybridization Protocol:

A. Embryo fixation in 4% paraformaldehyde (PFA)
    a. Make fresh 4% PFA (4 ml total volume). Scale up as necessary.
        i. H_2O: 3.1 ml
        ii. 32% PFA (stored in -20ºC): 500 µl
        iii. 10X PBS: 400 µl
    b. Transfer embryos / larvae to 1.5 ml microfuge tubes, no more than 35 per tube.
    c. Remove excess embryo media.
    d. Add 1 ml of 4% PFA
    e. Fix overnight at 4ºC on rotating platform shaker (located on the floor of the 4ºC fridge).

B. Embryo washes.
    a. After fixation, remove PFA into a used 15 ml conical tube.
        i. PFA is toxic and <u>cannot</u> be poured down the sink
    b. Wash the embryos 5 x 5 minutes in 1 ml PBST on room temperature (RT) rotating platform shaker (white mat).

C. *(Optional for long term storage) Dehydrate in methanol (MeOH)*
    a. Remove last PBST wash and add 1 ml of 100% methanol (MeOH).
    b. Put on RT rotating platform shaker for 10 minutes.
    c. Remove MeOH and replace with 100% MeOH. Store at -20ºC indefinitely.
        i. NOTE: can pause the protocol here indefinitely.
    d. Rehydrate embryos.
        i. Remove 100 % MeOH
        ii. Do a series of 5 minute washes:
            1. 75% methanol/25% PBST
            2. 50% methanol / 50% PBST
            3. 25% methanol / 75% PBST
            4. Lastly, 2x5 min PBST washes.

D. Permeabilization (to allow the probe access to its mRNA targets).
    a. Prepare a fresh batch of 4% PFA following the recipe above (1 ml per in situ tube).
    b. Make a 10 µg / ml solution of Prot K in PBST. Total volume should be 1 ml per tube of embryos you have for in situs.
        i. Locate the 1 mg / ml stock solution (in the -20ºC freezer).
        ii. Dilute it 1:100 in PBST to make a 10 µg / ml working solution
            1. E.g. if you have 6 tubes of embryos, make 6 ml of prot K solution by diluting 60 µl of 1 mg / ml stock into 5940 µl of PBST.
        iii. Remove old PBST from tubes and replace it with 1 ml of prot K solution.

c. Timing of prot K treatment: critical step!!! Perform on room temperature rotating platform shaker (white mat). These times may need to be experimentally optimized depending on the developmental stage, target tissue, and your probe.)
    i. 24 hpf: 10 minutes
    ii. 36 – 48 hpf: 20 minutes
    iii. 3 – 5 dpf: 30 – 45 minutes
d. Remove prot K and add 1 ml 4% PFA. Incubate at RT on rotating platform shaker for 20 min.
e. Remove 4% PFA to a used conical tube
f. Wash 4 x 5 min in PBST to remove residual PFA. Proceed immediately to pre-hybridization.

E. Prehybridization
    a. Turn on the big, circulating water bath set to 69ºC (measures 70ºC on thermometer).
    b. Pre-warm Hyb+tRNA solution in water bath (use floating platform for tubes).
        i. Measure out 700 µl per in situ tube into a 15 ml conical tube. Do not heat the entire tube of hyb+tRNA unless you are using it all.
        ii. NOTE: must be Hyb + tRNA, since the tRNA is used to coat all non-specific binding sites for RNA, thereby preventing non-specific binding of your RNA probe)
    c. Remove PBST from embryos.
    d. Add 700 µl of pre-warmed Hyb + tRNA solution (located in -20ºC).
    e. Incubate tubes at 70ºC for at least 2 hours, up to overnight.
        i. NOTE: Embryos in Hyb can be stored long term at -20ºC if you need to pause the protocol here.

F. Hybridization:
    a. Make 1 ml of your probe + Hyb + tRNA mixture
        i. Quantify your purified probe synthesis reaction using the Nanodrop in S302. Expect something around 50 – 150 ng / µl
        ii. Dilute your probe in Hyb + tRNA so that 1 ml of probe mix contains ~ 200 ng of probe total.
            1. E.g. if your purified probe contains 50 ng / µl, add 4 µl of probe to 1 ml hyb + tRNA.
        iii. Prewarm your probe mixture at 70ºC
    b. Remove the pre-hyb solution to an empty 15- or 50-ml conical tube.
        i. Hyb contains toxic formamide and cannot be poured down the sink.
    c. Add 200 µl of probe + hyb solution to each in situ tube.
        i. Store unused probe+Hyb at -20ºC
    d. Incubate tubes over night at 70ºC.

G. Washes:

a. Prepare your wash solutions. Use 1 ml of each solution per tube per wash step (except Wash 1, 300 µl / tube). Store your wash solutions in 15 ml conical tubes.
    1. NOTE: Use Hyb <u>without</u> tRNA for all wash solutions.
    2. NOTE: SSC is made as a 20X stock. Before starting to prepare your wash solutions, make 50 ml of the following SSC solutions:
        a. 2X SSC + 0.1% Tween-20 (2X SSCT)
            i. 5 ml of 20X SSC
            ii. 44.75 ml molecular biology-grade H2O (found in 1 L bottles on chemical shelf)
            iii. 250 µl 20% Tween-20 (in lab bench drawer)
        b. 0.2 X SSC + 0.1% Tween-20 (0.2X SSCT)
            i. 5 ml of 2X SSC
            ii. 44.75 ml molecular biology-grade H2O
            iii. 250 µl 20% Tween-20 (in lab bench drawer)

- Wash solutions (prewarm to 70 ºC)
    - Wash 1: Hyb (no tRNA).
    - Wash 2: 75 % Hyb / 25% 2X SSCT.
    - Wash 3: 50 % Hyb / 50% 2X SSCT.
    - Wash 4: 25 % Hyb / 75% 2X SSCT.
    - Wash 5: 2x SSCT.
    - Wash 6: 0.2X SSCT.
    - Wash 7: 0.2X SSCT (same as wash 6).
- Wash solutions (room temp)
    - Wash 8: 75 % 0.2X SSCT / 25% PBST.
    - Wash 9: 50 % 0.2X SSCT / 50% PBST.
    - Wash 10: 20 % 0.2X SSCT / 75% PBST.
    - Wash 11: PBST

b. Remove the in situ tubes from the 70ºC water bath. *Very* briefly centrifuge the tubes to collect liquid using the small white centrifuge. Carefully pipet the probe off the embryos and return it into the appropriate probe + Hyb tube.
    i. This probe + Hyb mixture can be reused multiple times, so do not discard.

c. Do the washes using the solutions prepared above:
    i. Wash 1: 5 minutes at 70ºC
    ii. Wash 2: 5 minutes at 70ºC
    iii. Wash 3: 5 minutes at 70ºC
    iv. Wash 4: 5 minutes at 70ºC
    v. Wash 5: 5 minutes at 70ºC
    vi. Wash 6: 30 minutes at 70ºC (high stringency, timing is critical)
    vii. Wash 7: 30 minutes at 70ºC (high stringency, timing is critical)
    viii. Wash 8: 5 minutes at RT
    ix. Wash 9: 5 minutes at RT
    x. Wash 10: 5 minutes at RT
    xi. Wash 11: 5 minutes at RT

H. Anti-DIG detection and labeling:
    a. Blocking
        i. Prepare your Block solution (Make 2 ml per in situ tube):

1. PBST
2. 2% sheep serum (located in -20ºC freezer)
3. 2 mg / ml bovine serum albumin (BSA) (located in 4ºC fridge)
    a. E.g. if you have 4 tubes of in situs, nake 8 ml of Block using 8 ml PBST, 160 µl sheep serum, and 16 mg BSA

ii. Discard the final PBST wash from the embryos and replace with 1 ml of Block.
iii. Incubate at RT for at least 1 hr, or up to overnight at 4ºC, on the rotating platform shaker.

b. Antibody incubation
    i. Using the remaining Block solution, do a 1:5000 dilution of the Roche anti-DIG-AP antibody Fab fragment
        1. E.g. Following the example above, after using 4 ml of Block, you should have 4 ml remaining. Dilute the anti-DIG 1:5000 by adding 0.8 µl to the remaining 4 ml Block
    ii. Discard the old Block and replace with the new Block + anti-DIG
    iii. Incubate at RT for 2-4 hours, or at 4ºC overnight on the rotating platform shaker.
c. Washes to remove excess antibody
    i. Discard the Block + anti-DIG.
    ii. Wash embryos 5 x 15 minutes with PBST at RT on the rotating platform shaker.
        1. You may wash overnight at 4ºC on the rotating platform shaker.

d. Colorization reaction
    i. Color buffer (make fresh every time)
        1. Volumes provided to make 50 ml of Color buffer. Scale up or down depending on the number of tubes you have. You will need 6 ml color buffer per in situ tube.
            a. 100 mM Tris-Hcl, pH 9.5
                i. 5 ml of 1M
            b. 50 mM $MgCl_2$
                i. 2.5 ml of 1M
            c. 100 mM NaCl
                i. 1 ml of 5M
            d. 0.1% Tween-20
                i. 250 µl of 20% Tween-20
            e. Sterile water to 50 ml.
    ii. Wash embryos in 1 ml color buffer 2 x 5 min on RT rotating platform shaker.
    iii. After 2nd wash, transfer embryos to pre-labeled wells of a spot-plate.
    iv. Rinse embryos 2x5 min with 500 µl color buffer in the spot plate.
    v. While doing these washes, make <u>1 ml of detection solution per tube</u> of embryos:
        1. 1 ml color buffer
        2. 4.5 µl NBT 50 mg / ml
        3. 3.5 µl BCIP 50 mg / ml
    vi. After final rinse, replace color buffer with 500 µl detection solution.
    vii. Transfer the spot plate and unused detection solution to a drawer
        1. Keep detection solution and embryos out of light at all times unless viewing.
    viii. To monitor how the coloration reaction is proceeding, carefully transfer the spot plate to the Leica microscope on the side bench and quickly view.
        1. Minimize the amount of time the embryos are in the light, since excess light will turn the embryos pink.
    ix. NOTE: if the coloration reaction is proceeding very slowly, you may need to leave it overnight. To do this, make a 1:10 dilution of your remaining detection solution in color buffer
        1. E.g. Add 100 µl of full-strength detection solution to 900 µl color buffer. Replace the old detection solution with the new dilute solution and leave overnight in the dark, either at RT or at 4ºC.

x. When the embryos have colored to the desired level, stop the coloration reaction by:
    1. Rinsing embryos 2 times with 50 µl color buffer w/o NBT or BCIP.
    2. Rinse twice with water
    3. Wash 3 x 5 min in Stop solution
    4. Wash 2 x 5 min PBST
    5. Store embryos in PBST at 4ºC in the dark before imaging.

Hybridization solution (50 ml)

- make four 50 mL conical tubes at a time (use the entire 100 mL bottle of formamide).
- Label the lids and sides of the conical tubes before starting: one tube "hyb+tRNA", the others simply "hyb)

Ingredient	**Volume**	**Final Concentration**
Water (RNase-free)*	11.24 mL (w/ tRNA+hep) 11.79 mL (w/o tRNA + hep)	
Formamide (deionized)	25 mL	50 %
20X SSC	12.5 mL	5 X
Heparin (50 mg / mL)**	50 µL	50 µg / mL
tRNA (500 mg / mL)**	500 µL	500 µg / mL
Tween-20 (20%)	250 µL	0.1 %
Citric acid (1M)	460 µL	0.092 M

* For the "hyb+tRNA" tube, use 11.24 mL water; for the other tubes, use 11.79 mL

** Add heparin and tRNA only to 1 of the 4 tubes of hyb.

Stop solution (250 ml):

1. 200 ml water
2. 25 ml of 10x PBS
3. 5 ml of 50 mM EDTA pH 8
4. Adjust to pH 5.5 with 1M HCl.
5. Top up to 249 mL
6. Filter with bottle top vacuum filter.
7. Add 1.25 ml 20% Tween-20 and mix.