Elucidation of the Evolutionary Origin of the Neural Crest

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Abstract

The evolutionary origin of the neural crest, an embryonic stem cell population unique to vertebrates, has eluded biologists since its discovery. The neural crest is characterized by its epithelial to mesenchymal transition (EMT), migration, and differentiation into stereotyped tissues of the embryo. These processes require an intricate gene regulatory network (GRN) that controls the signaling required for successful neural crest formation and differentiation into target tissue types. It is hypothesized that the neural crest, like other complex tissues, arose from co-option of existing developmental GRNs, but this has not been tested. Here, I will use an invertebrate deuterostome, the sea urchin *L. variegatus*, to look for ancestrally conserved circuits of the neural crest GRN. I hypothesize that genes operating in the neural crest GRN will be found in cells of the *L. variegatus* embryo that undergo similar processes to vertebrate neural crest cells (EMT, migration, etc.), namely primary mesenchyme cells (PMCs), secondary mesenchyme cells (SMCs), pigment cells, and neurons. I have cloned orthologs of vertebrate neural crest genes in the developing embryo of *L. variegatus* including *foxd, phb1, musk, elk3, egr/krox20, and csnrp*. Using RNA *in situ* hybridization, I have found that these genes are expressed in the predicted cell types in sea urchin embryos. Double *in situs* were then performed for *musk / pks* and *foxd / phb1* to demonstrate co-expression of the gene pairs. Both pairs of genes were co-expressed, indicating that they may be part of the same GRNs. If these connections are shared with the neural crest GRN, it will provide evidence that these small GRNs are ancestral to deuterostomes and were co-opted into a single tissue in the vertebrate lineage, which gave rise to the neural crest.

Introduction:

Throughout the history of developmental biology, scientists have failed to discover the evolutionary origin of the vertebrate neural crest since its discovery by His in 1868 (His, 1868). The neural crest is unique to the vertebrate lineage, but the mechanism of its evolution is currently unknown (Douarin & Kalcheim, 1999).

Background on the neural crest

The neural crest, as described by Dauarin & Kalcheim, is a multipotent stem cell population located on the dorsal side of vertebrate embryos on the border between the neural tube and epidermis (Figure 1). Neural crest cells undergo an epithelial to mesenchymal transition (EMT) and migrate to various regions of the embryo, including the heart, nervous system, and craniofacial region. They then differentiate into a wide variety of essential cell types including neurons of the peripheral nervous system, cardiac muscle, the inner ear, melanocytes (Douarin & Kalcheim, 1999). The presence of the neural crest is an important milestone in the road to cephalization, and is thought to be an important factor in the evolutionary transition from filter feeding to active predation (Gans & Northcutt, 1983). The formation and specification of the neural

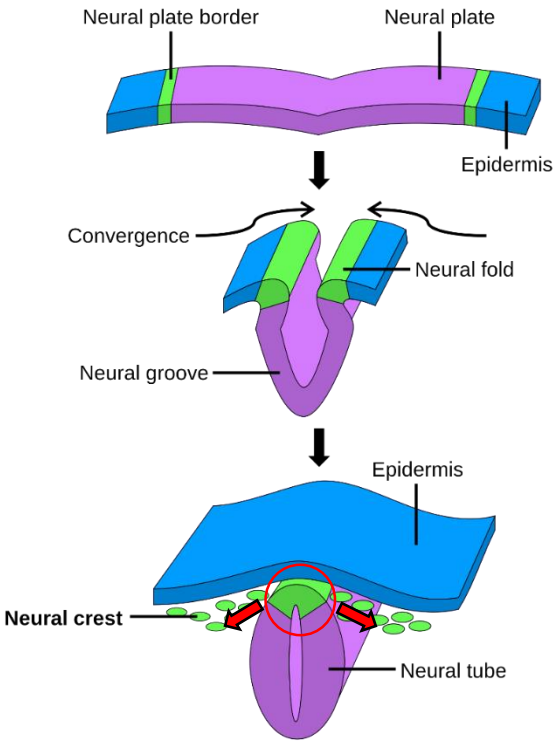


Figure 1: Formation of the neural tube and neural crest from the epidermis in a developing embryo. The red circle and arrows demonstrate neural crest cells migrating from their original location at the dorsal side of the neural tube and differentiating into stereotyped tissues.

crest is controlled by an intricate gene regulatory network (GRN). GRNs are molecular networks in which various regulator molecules control the levels of mRNA and protein in a cell (Davidson, 2006). These regulators can be DNA, RNA, proteins, or combinations of these molecules, which bind to genes and control their expression. Even small disruptions in the neural crest GRN severely impair development of the embryo (Etchevers, Amiel, & Lyonnet, 2006), demonstrating the complexity of the neural crest regulatory network. Based on current knowledge of the mechanisms of genetic evolution, it is highly unlikely that the neural crest evolved in the vertebrate lineage without significant transitory states of some sort (Sauka-Spengler, Meulemans, Jones, & Bronner-Fraser, 2007). Changes in the genetic code accumulate over time through random mutations; thus, the incredibly precise network of control, according to our current understanding, should have taken millions of years and various transition states to evolve (Meulemans & Bronner-Fraser, 2004). However, no "proto-neural crest" tissues have yet been found in lineages outside of vertebrates.

Co-option theory

Since a neural crest cell population has not been found outside of vertebrates, current theory suggest that the neural crest GRN was co-opted from existing gene regulatory circuits (Spirov, Sabirov, & Holloway, 2012). This theory of "co-option" is borrowed from the study of other complex tissue types, such as the four chambered heart, but it has never been tested in the neural crest (Olson, 2006). Perhaps smaller pieces of the neural crest GRN existed in basal animals, where they controlled different developmental processes, and were suddenly co-opted and expressed in the same cells to give rise to a more complex tissue. It is possible that clues to the origin of the neural crest GRN lie in ancient phyla, such as Echinodermata, that share a common ancestor with modern vertebrates.

Why sea urchins?

In order to study the co-option theory, it is important to select a proper model organism. Non-vertebrate, basally branching deuterostomes are good candidates because they share a recent common ancestor with the vertebrate line and are sufficiently ancient to support the amount of time required for the small GRNs to evolve (Figure 2). A recent study identified a cell population in the chordate subphylum *Tunicata* that resembles the neural crest in its gene expression profile. The existence of these tunicate cells indicates that at least parts of the neural crest GRN predated the vertebrate lineage (Abitua, Wagner, Navarrete, & Levine, 2012).

If the neural crest did evolve by co-option, small pieces of the neural crest GRN should exist in the sea urchin *Lytechinus variegatus*, a basally branching echinoderm that has been selected as my model organism. In addition to the advantageous phylogenetic position of sea urchins, *L. variegatus* is also the ultimate deuterostome model for creating developmental GRNs; it is relatively easy to create GRNs in this organism through gene knockdown followed by a search for downstream targets. Furthermore, sea urchin embryos have cell types that undergo the same processes as vertebrate neural crest cells during embryonic development. Primary mesenchymal cells (PMC), secondary mesenchyme cells (SMC), pigment cells, neurons, and ciliary band cells undergo processes similar to neural crest cells, such as epithelial

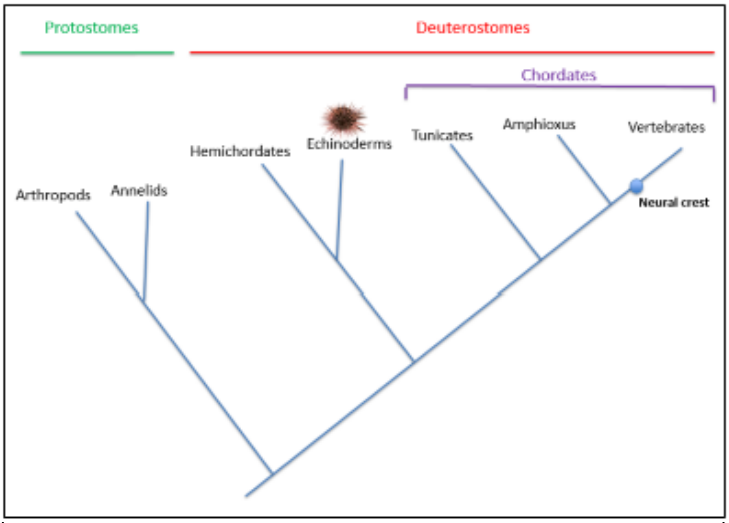
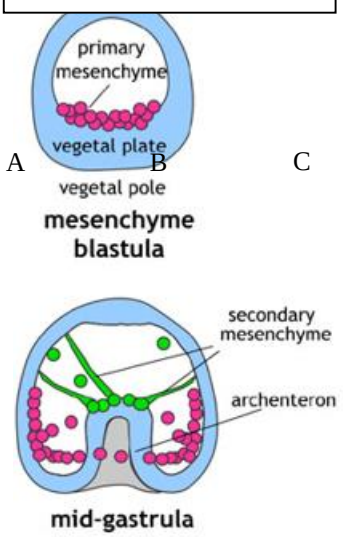


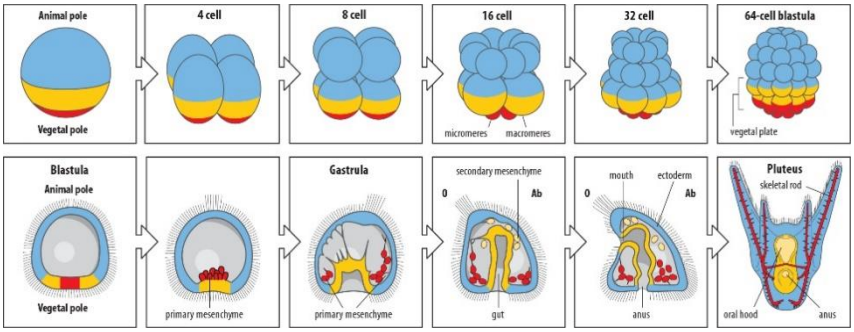
Figure 2: Deuterostome lineage. *L. variegatus*, and echinoderm, is used as the model organism in this study. It is closely related to the vertebrate lineage, making it a good model to use when searching for neural crest orthologs in invertebrates.

Figure 3: Location of primary and secondary mesenchyme cells (Hardin)



to mesenchymal transitions (EMT) and directed cell migration (Figure 3, Figure 4). Embryonic development in sea urchins is relatively simple. The vegetal and animal axes are formed in the single cell zygote and are maintained throughout development (Figure 4, top row). PMC and SMC precursors exist in the blastula stage (Figure 4A). They then undergo EMT and ingress just prior to gastrulation (Figure 4B-C). During late gastrulation, PMCs settle in the vegetal pole in two lateral clusters adjacent to the gut tube, and SMCs migrate throughout the blatoceol (Figure 4D). In this study, I hypothesize that pieces of the neural crest GRN will be found in these cells that share features with neural crest cells in embryos of the *L. variegatus* sea urchin. I hypothesize that pieces of the neural crest GRN will be seen in these cell types. If neural crest genes are found to be expressed in predicted cell types of sea urchin embryos, it suggests that these sub-circuit GRNs are ancient to deuterostomes and it would support the co-option hypothesis.

Figure 4: Development of the sea urchin embryo. Note the position and migration of the PMCs (red) and SMCs (yellow) and gut (orange) (Staveley)



To test the hypothesis, I used the *L. variegatus* developmental transcriptome to first design and create RNA probes for orthologs of neural crest genes found in primary literature and in the published vertebrate neural crest GRN completed by Dr. Marianne Bronner's laboratory (Figure 5) (Betancur et al., 2010). I then used whole mount *in situ* hybridization (ISH) to anneal

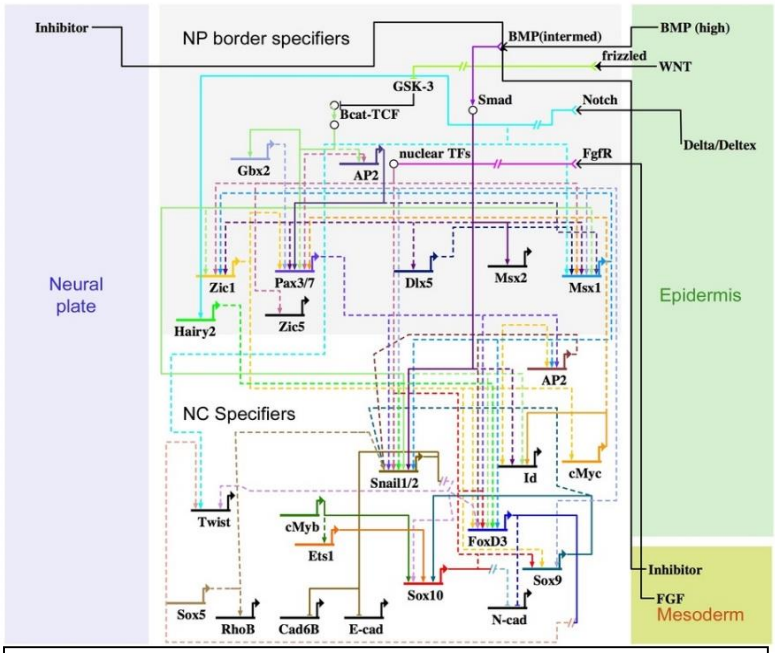


Figure 5: The major components of the vertebrate neural crest GRN. Linkages inferred from knockdown experiments on chick, mouse, zebrafish, lamprey, and Xenopus embryos (Betancur, Bronner-Fraser, & Sauka-Spengler, 2010).

the probes to mRNA of the target genes in *L. variegatus* embryos and expression patterns were visualized in different stages of development. This process demonstrated: a) expression of neural crest gene orthologs in predicted cell types (PMCs, SMCs, etc.) in *L. variegatus* and b) where genes are potentially co-expressed, indicating the possible existence of small GRNs that are shared with the neural crest. Establishing expression of neural crest orthologs in predicted cell types supports the co-option hypothesis. After demonstrating the expression of neural crest genes in these cells, double *in situs* were performed in the second part of this study in an effort to determine if they may connect, providing the basis for building a GRN for the sea urchin. This will allow for comparison to the neural crest GRN in future studies. If connections are conserved between sea urchin tissue and the neural crest, that would provide strong support for the co-option hypothesis (Betancur et al., 2010) (Meulemans & Bronner-Fraser, 2004).

Methods:

Choosing neural crest markers

Using the data published by the Bronner Lab for the neural crest GRN and primary literature research, gene candidates were selected for cloning based on their existence in the vertebrate neural crest. These genes were then searched in the *L. variegatus* transcriptome in order to find orthologous genes existing in our model organism. Depending on the presence of orthologs, the initial candidate pool was narrowed down to the following markers: *foxd* (Betancur et al., 2010), *phb1* (CITATION NEEDED), *csnrp* (Simoes-Costa, Stone, & Bronner, 2015), *musk* (Kwon et al., 2014), *egr/krox20* (Stolt & Wegner, 2015), and *elk3* (Rogers, Phillips, & Bronner, 2013).

Creating probes for vertebrate neural crest markers

Using the sequences for these genes published in the transcriptome, primers were designed and ordered for gene cloning. Upon arrival, polymerase chain reaction (PCR) was used to amplify the genes of interest from cDNA. RNA synthesis protocol was then used to create DIG-labeled RNA probes (RNA with a complementary sequence to the target mRNA) from the amplified cDNA.

Demonstrating expression of neural crest genes in L. variegatus tissues

The RNA probes were applied to *L. variegatus* embryos fixed in PFA at various stages of development, according to the *in situ* hybridization protocol. These stages included early blastula, mesenchyme blastula, gastrula, late gastrula, prism, and pluteus. Embryos were fixed overnight and stored in methanol at -20º C, hybridized overnight with the RNA probe at 65º C, and washed in SSC dilutions. After overnight incubation with Anti-DIG antibody, color reaction was carried out with NBT/BCIP. This technique allowed visualization of gene expression (or

lack thereof) in the sea urchin embryo throughout its developmental stages. After demonstrating the expression of neural crest genes in the sea urchin embryo, the second stage of experiments were initiated.

Demonstrating co-expression of experimental genes

To determine function and connectivity to other neural crest markers, double *in situ* hybridizations were performed. Demonstrating co-expression is a stepping stone for future work that will be able to elucidate connections between these genes and build GRNs. Based on the results from the first part of my experiments, I chose to perform double *in situs* for *foxD* / *phb1* and *musk* / *pks*. *Pks* is a sulfur transferase enzyme known to be expressed in pigment cells. Performing a double stain for *pks* and *musk* confirms expression of *musk* in pigment cells. Double stains for *foxd* and *phb1* demonstrate co-expression of the two genes. New Fluoroscin labeled probes were created using the same process as discussed above for the genes in question. Using fluorescence allowed me to assign colors to the different fluorescence channels in the program ImageJ, which made it possible to demonstrate overlap in expression.

Results:

Part 1

As predicted, the cloned genes were found to be expressed in *L. variegatus* cell types that undergo similar processes to vertebrate neural crest cells. *FoxD* and *phb1* are expressed in the hindgut and *elk3* and *csnrp* are expressed in the PMCs. *egr/krox20* is expressed in neurons. Finally, *musk* is found in pigment cells. All of these cell types were classified as "neural crest-like" because they undergo EMT and exhibit directed cell migration. The results confirmed my

hypothesis that neural crest genes would be found in these cells. The expression patterns are shown in the figures below.

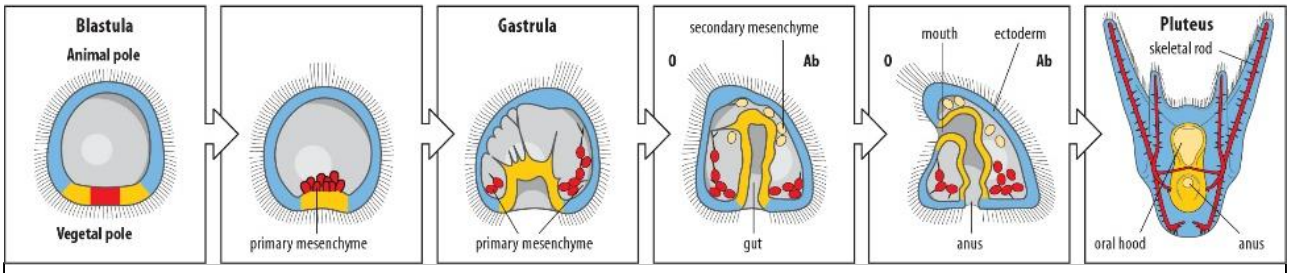


Figure 6: Refer to these figures throughout the results section to orient images to specific stages of development.

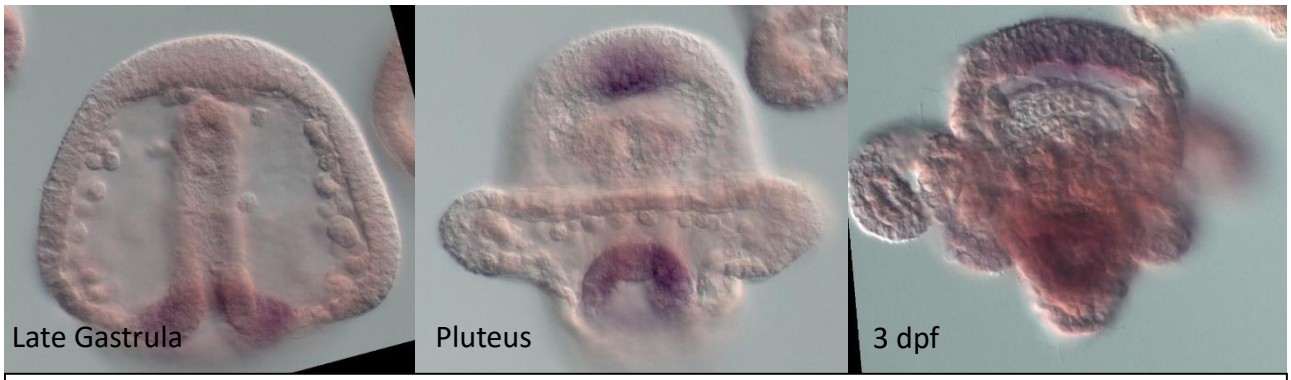


Figure 7: *foxd* – Expressed in the blastopore ring throughout development and in the animal pole at the pluteus stage. May be involved in neural specification in those regions.

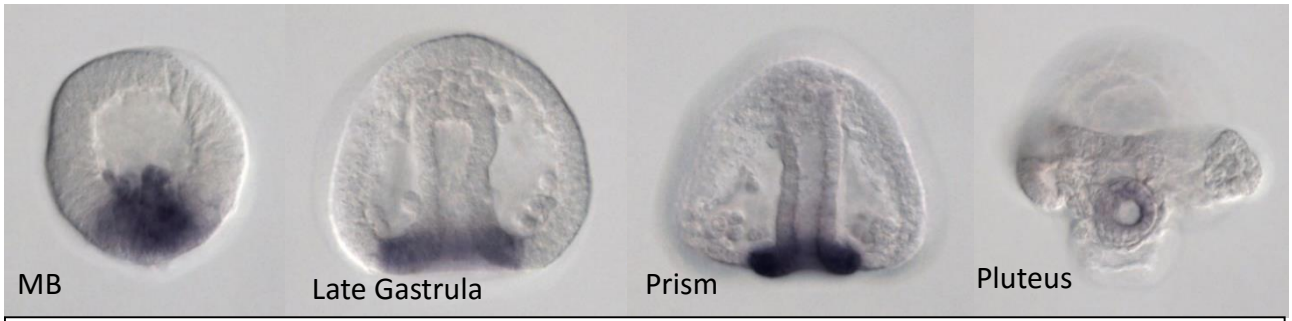


Figure 8: *phb1* – In vertebrates, Phb1 is a transcription factor required for the formation of projections from sensory neurons to the dorsal horn of the spinal cord. The function of Phb1 in sea urchins in unknown, but it may be part of the same GRN as *foxD* because it is expressed in the same cells at the same time.

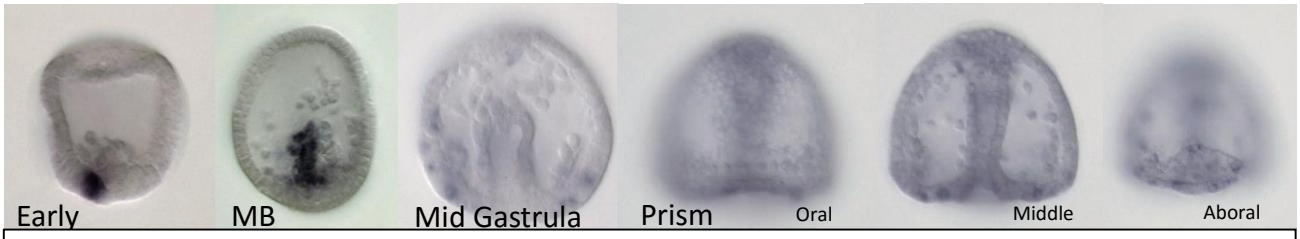


Figure 9: *musk* – kinase necessary for neuromuscular junction formation in vertebrates. In sea urchins, it is expressed in pigment cells and their precursors. This is confirmed by the expression of *musk* in ectodermal cells only on the aboral side of the prism-stage embryo.

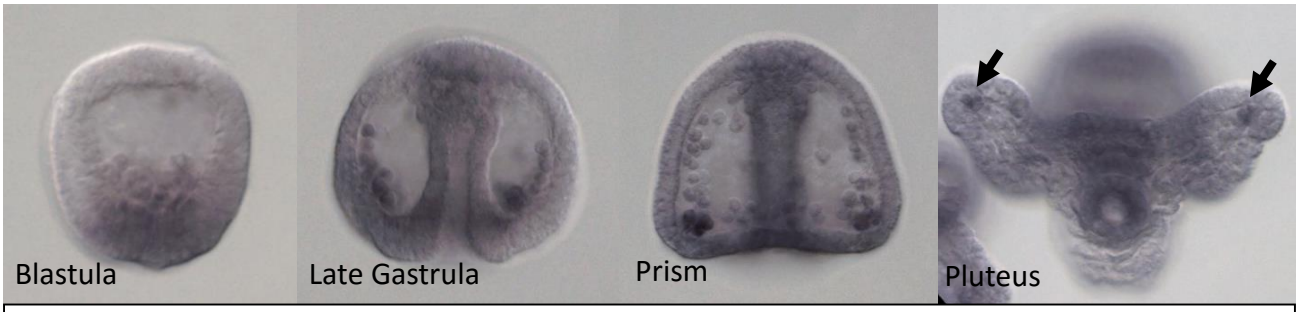


Figure 10: *elk3* – expressed in bilateral PMC clusters that migrate laterally to the tips of the arms at the pluteus stage. Snail, a well-known neural crest marker, is expressed in the same cells and involved in PMC ingression. *Elk3* and Snail may be part of the same GRN in sea urchins.

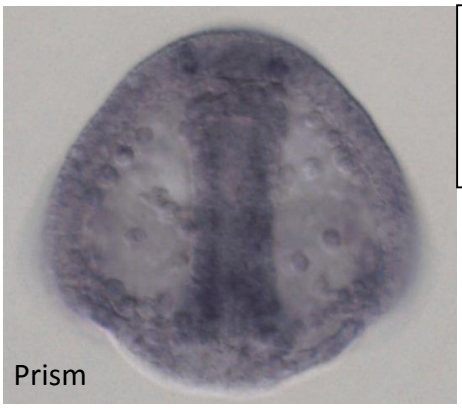


Figure 11: *egr/krox20* – Expressed in vertebrate Schwann cells. Expressed in serotonergic neurons in sea urchins. May be involved in production of myelin like sheaths in invertebrates.

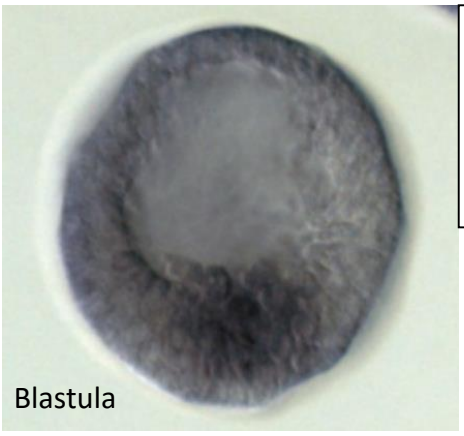


Figure 12: *csnrp* – In vertebrates, *csnrp* is downstream of *wnt* signaling and is sufficient to rescue *wnt* knockdowns. It also activates *foxd*. In sea urchins, it is expressed in the vegetal plate at the blastula stage ony, and may serve to activate *foxd*.

PMCs	SMCs	Neurons	Pigment Cells	Hindgut
mitf	*foxd*	*dlx*	*musk*	*foxd*
elk3	*sip1*	*sip1*		*phb1*
csnrp	*snail*	*ap-2*		
snail		*egr/krox20*		
twist				
ets				
zic				

Figure 13: Expression of vertebrate neural crest genes in the invertebrate sea urchin *L. variegatus*. These genes have been shown to be expressed in predicted cell types. Cells I study in this paper are highlighted in yellow.

Function of studied genes the vertebrate neural crest

Foxd is a transcription factor that is expressed in the vertebrate neural crest and is necessary for neural crest specification. It is highly studied because of its upstream location and the wide variety of genes it controls in the development of the neural crest. It is generally known as an initiator of neural crest development and is involved in neural specification (Betancur et al., 2010).

Phb1 is a transcription factor required for neural crest specification. It is expressed in the neurula stage of development of vertebrate embryos. Neurulation is the developmental process that forms the neural tube and neural crest from the dorsal ectoderm (Figure 1). *Phb1* regulates a subset of genes and stabilizes their fate as neural crest cells. It is also required for the formation of projections from nociceptive sensory neurons to the dorsal horn of the spinal cord in vertebrates (Deichmann et al., 2015).

Musk is expressed in neural crest derived cells in the vertebrate. It is a kinase necessary for neuro/muscular junction formation (Kwon et al., 2014). Additionally, it is involved in the *wnt* signally pathway that controls segmental migration of neural crest cells during development of the embryo. It is required for cells to migrate to their correct somite. *Musk* knockdown experiments in mice have shown developmental defects in which neural crest cells migrate non-segmentally (Banerjee et al., 2011).

Elk3 is expressed in a wide variety of tissue types during vertebrate development. It is highly involved in angiogenesis, but it is also required for neural crest specification. *Elk3* is expressed in head folds, head mesenchyme, intersomitic vessels (adjacent to the neural tube), and migratory cranial neural crest cells. It is an upstream regulator in vertebrates that is necessary for the differentiation from progenitor stem cells into multipotent neural crest cells by activating *foxd* by inhibiting *pax7*, a *foxd* inhibitor. Loss of *elk3* results in severe neural crest migratory defects in development (Rogers et al., 2013).

Egr/krox20 is expressed in Schwann cells in vertebrates. Schwann cells are derived from the neural crest and are thus a vertebrate innovation. These cells produce myelin, a fatty substance that wraps around neurons in segments. Between each segment lies a node of Ranvier, which leave the membrane exposed to the extracellular environment. This arrangement allows much faster signal conduction in nerves based on the principles of capacitance. *Egr/krox20* is necessary for correct myelin formation in Schwann cells (Stolt & Wegner, 2015).

Finally, *csnrp* is a transcription factor known as Axud1 in the vertebrate neural crest. It is a downstream target in the *wnt* signaling pathway, which is activated early in development and is involved in initiating the neural crest program. Interestingly, it is sufficient to rescue *wnt* knockdown in mice. This means that mice genetically engineered to turn off *wnt* are able to

develop normally when treated with ectopic *csnrp*. Furthermore, *csnrp* activates *foxd* in vertebrates, which initiates the neural crest program (Simoes-Costa et al., 2015).

Expression of studied genes in the L. variegatus embryo

The first gene I studied was *foxd*. I first performed *in situs* for early stage embryos, but found no expression in the blastula and early gastrula stages (not pictured). I then performed another round of *in situs* on later stage embryos and found that *foxd* was expressed in the hindgut and blastopore ring starting during mid gastrulation (Figure 7, Late Gastrula). It continued to be expressed in these areas throughout development. *foxd* was also expressed in the animal pole late in development, during the pluteus stage (Figure 7, Pluteus / 3 dpf (days post-fertilization)).

I then conducted *in situs* for *phb1* for early and late stages of development. *Phb1* was expressed in the vegetal plate during the mesenchyme blastula (MB) stage and in the hindgut and blastopore ring during gastrulation. *Phb1* expression decreased during the pluteus stage but was still present (Figure 8). It was thought that *phb1* and *foxd* may co-express during gastrulation, prism, and pluteus stages (Figure 7, 8).

The next gene I studied was *musk*. I performed *in situs* for *musk* and all stages of development. *Musk* was expressed asymmetrically in the ectodermal cells at the early blastula stage (Figure 9, Early). These cells then underwent EMT and ingressed in the mesenchyme blastula (MB) stage and during early gastrulation. *Musk* continued to be expressed asymmetrically in these cells (Figure 9, MB). During mid gastrulation they underwent MET (mesenchyme to epithelial transition), returning to the ectoderm at the prism stage (Figure 9, Mid Gastrula, Prism). At late prism stage, *musk* was expressed in scattered cells only on the aboral ectoderm, indicating expression in pigment cells and their precursors (Figure 9, aboral).

I then performed *in situs* for *elk3* and found expression in the vegetal pole in the early blastula (Figure 10, Blastula). At the gastrula and prism stages, *elk3* was expressed in small subsets of the PMCs located laterally adjacent to the gut tube (Figure 10, Late Gastrula / Prism). These specific cell populations migrated to the tips of the arms at the pluteus stage (Figure 10, Pluteus). Expression of *elk3* was confined to the small PMC subsets throughout development after the blastula stage and during their migration to the tips of the arms.

I performed *in situs* for *egr/krox20* at early stages and found no expression. I then repeated the procedure for later stages and found expression of *egr/krox20* at the prism stage. Expression of *egr/krox20* was localized in two cells in the animal pole known to differentiate into serotonergic neurons (Figure 11) (Stolt & Wegner, 2015). *Egr/krox20* was not expressed in these neurons at the pluteus stage.

The final gene I studied was *csnrp*. I first performed *in situs* in early stage embryos and found that *csnrp* was expressed in the vegetal pole early in development (Figure 12). I then performed *in situs* for *csnrp* at later stages, but did not find any expression in stages after early blastula.

Part 2

Double *in situs* were performed for the gene pair *msuk* / *pks*. *Pks* is a gene known to be expressed in pigment cells. *Musk* and *pks* were shown to co-express in all cells that expressed either gene (Figure 14). Because *pks* is an indicator for pigment cells, this result confirmed the presence of *musk* in pigment cells.

Double *in situs* were also performed for *foxd* / *phb1*. The double *in situs* for *foxd* / *phb1* demonstrated co-expression of the two genes in the hindgut and blastopore ring of the sea urchin embryo (Figure 15), confirming the results from Results, Part 1 (Figure 7, 8).

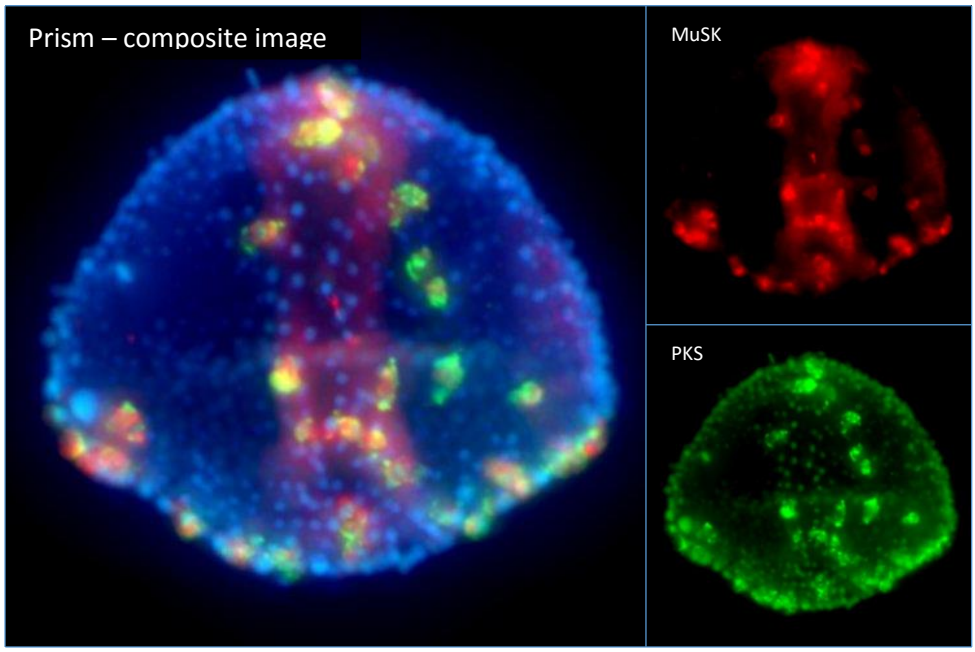


Figure 14: Co-expression of *musk* and *pks*. The composite image (left) demonstrates co-expression of the two genes. Any yellow color in cells indicates that both genes are expressed in that cell. The separated channels can be seen as well (right). This confirms the expression of *musk* in pigment cells.

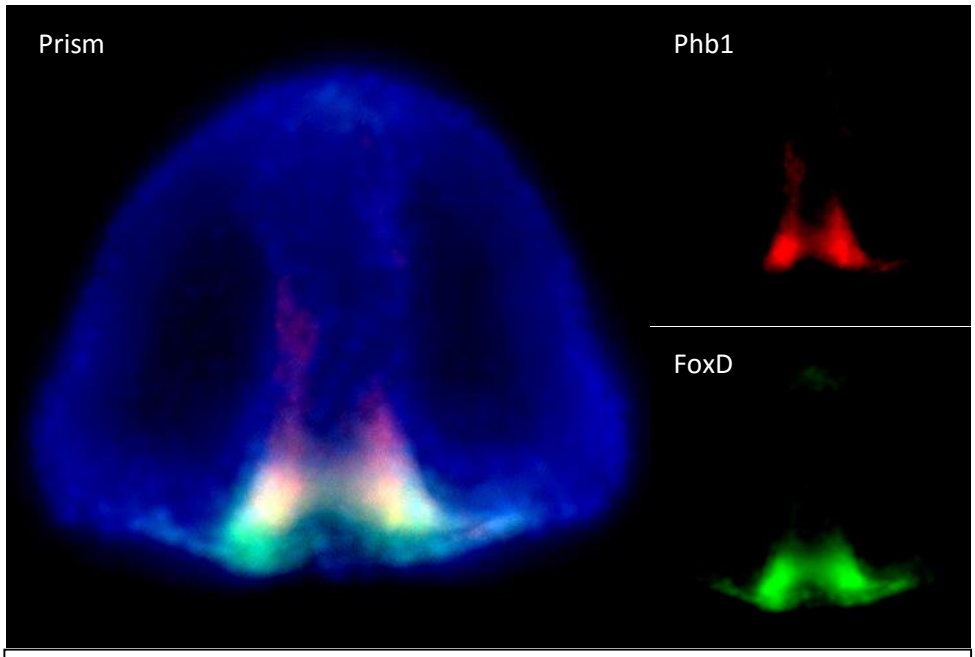


Figure 15: Co-expression of *foxd* and *phb1*. The composite image (left) demonstrates co-expression of the two genes. Any yellow color in cells indicates that both genes are expressed in that cell. The separated channels can be seen as well (right).

Discussion:

Part 1

To address the overall hypothesis of co-option of sub-circuit GRNs giving rise to the neural crest GRN, the first step was finding neural crest genes expressed in the sea urchin embryos. The first stage of experiments involved cloning of sea urchin orthologs of vertebrate neural crest genes and performing *in situs* to visualize expression. All six genes that were cloned and tested demonstrated expression in predicted cell types in sea urchin embryos.

Foxd is an important and highly studied vertebrate neural crest gene because of its central role in neural crest development and the neural crest GRN (Figure 5) (Betancur et al., 2010). *Foxd* was demonstrated to be expressed in the hindgut and blastopore ring in sea urchins (Figure

7). In vertebrates, *foxd* is expressed throughout development and involved in regulating the neural crest program. Sea urchins also express *foxd* throughout development in a single area. While the function of *foxd* in sea urchins is unknown, it may have a similar function as in vertebrates and regulate other genes involved in stereotyped processes (EMT, migration, etc.). Furthermore, the two tissues in which *foxd* is expressed in *L. variegatus* embryos, the hindgut and animal pole domain, are known to be areas of active neurogenesis, it is possible that *foxd* is involved in neural specification, similar to its role in the neural crest.

Phb1 is a recently discovered neural crest gene that is involved in formation of projections from nociceptive sensory neurons to the dorsal horn of the spinal cord in vertebrates, as well as regulation of other neural crest markers (Deichmann et al., 2015). In sea urchins, its ortholog is dorsal root ganglion homeobox gene. It was shown to be expressed in the vegetal pole at the mesenchymal blastula stage of development and in the blastopore ring throughout later stages (Figure 8). The similar function of these orthologs in vertebrates and sea urchins indicates semi-conservation of function, which demonstrates support for the co-option hypothesis.

Csnrp is a transcription factor known as *axud1* in vertebrates. In these species, it is a downstream component of the famous *wnt* signaling pathway. *Wnt* has been shown to be both necessary and sufficient to initiate the neural crest program. Interestingly, knockdown of *axud1* results in downregulation of many neural crest specification genes in vertebrates, which is a similar effect to the knockdown of *wnt* in those species. Axud1 has actually been shown to be sufficient to rescue *wnt* knockdown in mouse trials by activating *foxd* and initiating the neural crest program (discussed above). Because of this effect in vertebrates, the expression pattern of *csnrp* (*axud1* sea urchin ortholog) is especially interesting. It is expressed only during the

blastula stage in the vegetal plate, in the same area as both *foxd* and Phb1 (Figure 9). *Csnrp* may have a similar function in sea urchins to its ortholog in vertebrates, namely expressed early in development to activate *foxd* and initiate migration and differentiation of "neural crest-like" cells in sea urchins (PMCs, SMCs, etc.). This would indicate further conservation of function between the orthologs *csnrp* and *axud1*, lending support for the co-option hypothesis.

Musk is a vertebrate neural crest gene necessary for neuro/muscular junction formation. Interestingly, its sea urchin ortholog seems to have an unrelated function based on its expression pattern. In sea urchins, *musk* is expressed in pigment cells are their precursors only (Figure 10). As can be seen in Figure 4, *musk* expression occurs in the ectoderm in the early blastula stage. These cells ingress proceeding gastrulation, and migrate to the aboral ectoderm during gastrulation, which is indicative of pigment cells. While the function of *musk* in sea urchins is unknown, it could be functioning to control directed cell migration, a role similar to that in neural crest cells. It is also possible that the function of *musk* has changed in the Echinoderm lineage.

Egr is the sea urchin ortholog of *krox20*, a vertebrate gene expressed in Schwann cells, which are derived from the neural crest in these species. It is known that *krox20* is involved in myelin production in vertebrate Schwann cells (Stolt & Wegner, 2015). In sea urchins, its ortholog *egr* is expressed only in serotonergic neurons that appear in the animal pole at the prism stage (Figure 11). Invertebrates do not possess Schwann cells, but they do produce myelin-like sheaths around some specialized neurons. While the function of *egr* in sea urchins is unknown, it is possible that it is involved in production of this "proto-myelin" in the specialized serotonergic neuron precursors in which it is expressed.

Elk3 is another vertebrate neural crest marker with unknown function. In sea urchins, its function is also unknown, but it is expressed in specific bilateral clusters of PMCs that migrate laterally to the tips of the arms at the pluteus stage (Figure 12). Interestingly, it is expressed in the exact same cell clusters as *snail*, an extensively studied neural crest marker (Wu & McClay, 2007). Because of this clear co-expression, it is possible that *elk3* and *snail* are part of the same GRN in sea urchins, which may provide support for the co-option hypothesis once *elk3*'s connection to snail in vertebrates is discovered.

Part 2

The double *in situ* hybridization for *musk* and *pks* confirmed the expression of *musk* in pigment cells (Figure 14). *Pks*, a sulfur transferase known to be expressed in pigment cells, is used as a marker for this cell type. Co-expression of *musk* with *pks* demonstrates *musk* expression in these cells. This finding is important because pigment cells are derived from the neural crest in vertebrates. Furthermore, pigment cells undergo multiple transitions. They begin in the ectoderm, undergo an EMT, then an MET (mesenchymal to epithelial transition), returning to the ectoderm at the prism stage.

The double *in situ* for *foxd* and *phb1* demonstrated co-expression for the genes in the hindgut and blastopore ring (Figure 15). Both genes are expressed in these cell types and their precursors, cells that migrate and undergo both EMT. These cells begin as ectoderm, undergo EMT, and migrate.

Co-expression of two neural crest orthologs in *L. variegatus* indicates that they may be part of the same sub circuit GRN involved in the control of migration and differentiation of PMCs. While it is difficult to draw specific conclusions regarding the function / connections of

these two genes, their co-expression points to evidence for the co-option theory. Demonstrating co-expression is an important stepping stone towards providing irrefutable evidence for co-option.

Conclusions and Future Directions:

The goal of this study was to examine the co-option theory through hypothesis-driven experiments. Here, I have demonstrated both the existence and co-expression of several neural crest gene orthologs in the invertebrate sea urchin *L. variegatus* in an effort to study the validity of the co-option theory. As predicted, *foxd*, *musk*, *phb1*, *egr/krox20*, *elk3*, and *csnrp* were expressed in PMCs, SMCs, pigment cells, and some neurons. The existence of these genes in sea urchin cell types that undergo processes similar to vertebrate neural crest cells supports the co-option theory.

Musk expression in pigment cells was confirmed by its co-expression with a pigment cell marker, *pks*. Furthermore, I demonstrated co-expression of two neural crest orthologs, *foxd* and *phb1*, in the sea urchin hindgut and blastopore ring. While specific connections could not be drawn, these results are a stepping stone for elucidating the GRNs that control the development of these cell types in sea urchins.

Future work should focus in several areas. First, other vertebrate neural crest gene orthologs should be cloned and searched for in sea urchins or other invertebrates closely related to the vertebrate lineage. Next, co-expression of more genes needs to be established, as I have done for *foxd* and *phb1*. These double *in situs* are stepping stones for experiments that can elucidate how co-expressed genes are actually connected in regulatory networks. Finally,

knockdown experiments are needed to establish causal relationships between co-expressed genes. Translation-blocking morpholinos can be injected into single cell *L. variegatus* embryos to knockdown the function of a gene. Then *in situs* can be performed and compared to a control to determine downstream targets. Relationships between genes can then be inferred, which allows for construction of GRNs that can be compared to the existing neural crest GRN. This will ultimately support or disprove the co-option theory, leading to furthered understanding of how the neural crest evolved.

Basic research on the neural crest is important because it leads to further understanding of the genetics and evolution of an important and complex tissue type. Understanding how the neural crest evolved is a necessary foundation for applied research in combating developmental conditions that arise from problems in this tissue.

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