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Role of Transient Receptor Potential Channels in Epithelial Morphogenesis in Chick Embryo

Trinity Q Alaka’i Waddell

A thesis submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Role of Transient Receptor Potential Channels in Epithelial Morphogenesis in Chick Embryo

Trinity Q Alaka’i Waddell
Department of Physiology and Developmental Biology, BYU
Master of Science

Transient Receptor Potential channels (TRP) are a superfamily of cationic specific ion channels that are regulated by various stimuli such as temperature, pH, mechanical stress, ligands and ion concentration. The role of TRP channels in disease states such as autosomal dominant polycystic kidney disease, cancer metastasis, and developmental defects lend credence to the belief that they play an important part in epithelial morphogenesis events. The development of somites, neural tube closure and migration of neural crest cells to form things such as the face and heart is a good developmental model for the aforementioned cellular processes. We have shown that TRP channels can be found in the developing ectoderm, hindbrain, and heart and that the inhibition of TRP channels in a developing embryo results in phenotypes suggesting perturbation of cellular remodeling processes. This leads to the question of the specific role of TRP channels in the epithelial mesenchymal transition and remodeling in developing chick embryos.

Keywords: EMT, TRP, development, epithelial morphogenesis, epithelial rearrangement, chick, gallus gallus, neural tube, heart
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INTRODUCTION

Transient Receptor Potential (TRP) channels are cation specific, tetramer forming ion channels with six transmembrane domains that are involved in many cellular systems (Craig Montell, 2005). The first TRP channels were discovered and characterized in *Drosophila* where they helped mediate the function of photoreceptors in response to continuous light stimulation (Minke, 1977; C. Montell, Jones, Hafen, & Rubin, 1985). Since then, they have been found to function by perceiving and responding to hypertonicity in yeast (Zhou et al., 2003), detect and avoid noxious chemicals in nematodes (de Bono, Tobin, Davis, Avery, & Bargmann, 2002), pheromone sensing in mice (Stowers, Holy, Meister, Dulac, & Koentges, 2002), and taste of sweet, bitter and umami (Huang et al., 2019), as well as heat and cold perception in humans (Craig Montell, 2005). It has been made clear that TRP channels function as both whole organism and single cell sensing mediators. For example, as stated above, TRP channels are used to detect specific chemicals by taste or pheromone sensing that enable a whole organism to respond to a stimulus (Huang et al., 2019). On the other hand, TRP channels have been shown to regulate a single cells response to osmolarity (Jiao, Cui, Wang, Li, & Wang, 2017), reactive oxygen species (Taylor-Clark, 2016), and mechanical stress (M. G. Park, Jang, Lee, & Lee, 2017). It is important to note that these channels have been shown to have functions outside of their normal channel activity such as the regulation of ryanodine receptor-mediated calcium release by a subunit of PKD2 (Anyatonwu, Estrada, Tian, Somlo, & Ehrlich, 2007), or the kinase activities of the TRPM family of TRP channels (N. Cai, Lou, Al-Saadi, Tetteh, & Runnels, 2018).

Because of the large variety of stimuli that activate TRP channels and their many cellular functions, they are categorized by homology and not function (Figure 1). The first mammalian
TRP channels discovered are considered the canonical TRP channels and are thus referred to as the TRPC subfamily. The other TRP channel subfamilies were grouped according to homology and named for the first discovered member. Each subfamily is named as follows: TRPV for vanilloid, TRPA for ankyrin, TRPP for polycystin, TRPM for melastatin, TRPML for mucolipin (H. Li, 2017).

The TRPV subfamily consists of six members. TRPV1-4 are mainly found in the skin and ganglia, where they function as sensors for temperature (Zhang, 2015a), pain and itch (Zhang, 2015b). TRPV5 and TRPV6 have the highest calcium selectivity of any of the TRP channels, which is likely connected to their expression in the kidney and gastrointestinal tracts, where they help regulate calcium absorption and homeostasis (Nilius & Owsianik, 2011). The TRPC subfamily consists of 7 subtypes which can be found in the kidneys, heart, vasculature, and lung epithelium regulating many functions, ranging from absorption to contractility to cell cycle progression (Tai, Yang, Liu, & Shao, 2017). The TRPA family is just one protein, TRPA1 which can be found in the dorsal root ganglia and trigeminal ganglia where it regulates pain, itch, and inflammation in skin and viscera in response to noxious chemicals, inflammation and temperature (Bautista, Pellegrino, & Tsunozaki, 2013). The TRPM family consists of 8 different proteins which are varied in function and expression. They can be found in the tongue, heart, pancreatic beta cells, sensory ganglia, immune cells and the kidney (Hantute-Ghesquier, Haustrate, Prevarskaya, & Lehen’kyi, 2018). They play roles in processes such as temperature sensation, taste, insulin release, magnesium homeostasis, ischemic injury and inflammatory responses (Shigeto et al., 2015; Yamamoto & Shimizu, 2016). The TRPML family is not well characterized, but has three members that are involved in lysosomal and endosomal functions (Nilius & Owsianik, 2011). When truncated or not expressed, TRPML1 is the cause of mucolipidosis type IV a lysosomal storage disorder (Dong et al., 2008). TRPP’s consist of 3 or 4
members. There is no consensus on whether to accept PKD1 (Polycystic Kidney Disease 1) as an official member of the TRP family. It is often described as just an associated protein that helps regulate the transport of PKD2, a bona-fide member, to the cell membrane, but cannot function as a channel itself. Otherwise these are found in the kidney regulating cell cycle events (Y. Cai et al., 1999). But there are exceptions; PKD2L1 is expressed in the tongue, where it perceives sour taste (Huang et al., 2019), and PKD2 is expressed in the heart, where it regulates store-operated calcium levels with ryanodine receptors (Anyatonwu et al., 2007).

Since the expression patterns, stimulus, and functions of these channels are so varied they cannot be grouped together simply by function, the following phylogenetic tree in “Figure 1” is provided to show the sequence homology between each family of TRP channels. It was designed using Clustal multiple sequence alignment with a neighbor joining tree and is commiserate with trees provided in the literature (Kumar S., 2018; Saitou N., 1987; Tamura K., 2004).
Figure 1: Chick TRP Superfamily Phylogenetic Tree.
This figure was designed to demonstrate the relationship between the individual proteins in a family and the family groups to the TRP superfamily. It was made using the mRNA sequences of TRP channels found in the model organism chick (*gallus gallus*). In the chick TRPM4 could not be found so the human sequence was used. The TRPV5 sequence was considered to be indistinct from the chick TRPV6 sequence so another human sequence was used. And, finally, like in humans, TRPC2 is a pseudogene which is made clear by the distance in relation between it and the rest of the chick TRPC family.
Despite the classification of TRP channels on the basis of sequence homology, differences in TRP channel regulation, activity, localization, and function are achieved by domain organization and sequence variation (Figure 2). For example, the TRP domain on the C’-terminus near the channel gate of TRPV and TRPM channels (Yue et al., 2015) probably has multiple functions related to channel activation threshold (Garcia-Sanz et al., 2007), but is also known to regulate phosphatidyl inositol biphosphate(PI(4,5)P2) mediated activation of TRP channels (Rohacs, Lopes, Michailidis, & Logothetis, 2005). Another conserved TRP channel domain is the Ankyrin Repeat Domain (ARD), which can be found with 3 or 4 repeats in TRPC channels, 6 repeats in TRPV channels and 14 or 15 repeats in TRPA1 (Nilius & Owsianik, 2011). The ARD is thought to play a role in channel tetramerization, ligand interactions, and protein complex formation that anchors the TRP channel to larger protein complexes in the membrane (Gaudet, 2008). Other domains include the endoplasmic reticulum retention domain which is found in TRPMLs and TRPPs and cause the localization of the protein subunits in the endoplasmic reticulum (Y. Cai et al., 1999). Some TRPMs contain enzyme domains which either hydrolyze ADP to AMP as in the case of TRPM2, or function as kinase domains in TRPM6 and TRPM7 (Hantute-Ghesquier et al., 2018). Calmodulin binding domains can be found in TRPA1, TRPVs, TRPCs, and TRPMs, and phosphorylation sites can be found in TRPCs and TRPVs (Owsianik, D'Hoedt, Voets, & Nilius, 2006). The domain organization of TRP channels is another example of how this superfamily of channels achieves a high degree of variation in both regulation and function.
Figure 2: TRP Channel Structure and Binding Domains.
Representative images showing general structure and varied active and binding domains of specific TRP channels in each subfamily.
Because of the intense structural variety of the TRP superfamily, many of the cellular and molecular roles and functions of TRP channels are still unknown; but there are some consistent functions between this diverse group of ion channels. The obvious, and most commonly shared function between the TRP channels is their ion selectivity. All TRP channels have six transmembrane segments with the fifth and sixth segments determining ion selectivity. Because the transmembrane segments of TRP channels are well conserved, almost all TRP channels are calcium permeable. While a few are highly selective for calcium ions, many only have moderate selectivity of ($P_{Ca}/P_{Na}$ 0.1-20) and are capable of allowing currents of monovalent cations such as sodium (Mulier, Vriens, & Voets, 2017). In contrast, however, TRPM4 and TRPM5 are regulated by intracellular calcium levels, but are selective for monovalent cations and almost impermeable to calcium ions (Launay et al., 2002). Regulation of channels by calcium is another feature shared broadly across the TRP superfamily. Since so many of these channels regulate calcium levels while being regulated by calcium themselves, TRP channels are considered important regulators of this important second messenger. There are varying ways that these channels are regulated by intracellular calcium levels. TRPA, TRPC, TRPV, and TRPM channels all have calmodulin binding domains which, depending on the specific subfamily member and location of expression, can either desensitize the channel to its stimulus or activate the channel (Zhu, 2005). Combining their sensitivity to various stimuli plus their mechanisms for calcium-mediated desensitization suggests that they are involved in fine tuning calcium homeostasis (Raquibil Hasan & Zhang, 2018; Shapovalov, Ritaine, Skryma, & Prevarskaya, 2016). TRPA1 and TRPP2 also have EF hands which are domains that enable the direct binding of calcium to the protein which then regulates its function. The EF hand in TRPP2 is thought to make calcium binding required for channel activation but inhibits the channel function at high calcium levels (Celíc et al., 2012; Petri et al., 2010).
The ability to alter cellular calcium levels lends a large impact to TRP channel activation, allowing it to regulate diverse and fundamental cellular processes, including those that contribute to development and become unregulated in cancer progression (Davis et al., 2014).

Epithelial Remodeling

While there are clearly a wide range of cellular mechanisms that are regulated by TRP channels, the interest of this project is focused on their role in morphogenesis, specifically epithelial-mesenchymal transition (EMT). EMT is the process a cell goes through to convert from being integrated into an epithelial tissue as a polarized, stationary, fixed cell to a non-polarized, migratory, invasive, apoptosis-resistant cell that no longer maintains connections to other cells in the tissue (Kalluri & Weinberg, 2009). EMT is a key process that allows cells to depart from the primary tumor during cancer metastasis and because of this, it has been a target in cancer research studies (Thiery, 2002). The use of a developmental model in this research is appropriate because processes like neural crest cell transition and migration are closely related to cancer metastasis (Gallik et al., 2017). Determining the spatiotemporal localization of TRP channel expression in a developmental model will help clarify their role in disease states that are characterized by dysregulation of epithelial remodeling. Studying the role of TRP channels in developmental events such as neural crest migration and other EMT events in development will give more information about the role of individual channel types in cancer metastasis (Fels, Bulk, Petho, & Schwab, 2018). Because there are 28 known TRP channels, a thorough documentation of their expression was beyond the scope of this project. Therefore, six TRP channels were chosen to be studied based on review of the literature and access to selective pharmacological inhibitors. The channels chosen (TRPC6, TRPV4, TRPM3, TRPM8, TRPA1, and PKD2L1) are known to have roles in cell cycle regulation, migration, and cell survival.
(Vrenken, Jalink, van Leeuwen, & Middelbeek, 2016). Since the structure, stimulus, inhibition, location, and function of the TRP channel superfamily is so varied, more in-depth background, especially regarding their role in epithelial remodeling events, will be given about each of the six TRP channels chosen for this study.

TRPC6

TRPC6 can be found in the brain (albeit at lower concentration than other TRPC’s), cardiac neurons (Calupca, Locknar, & Parsons, 2002), retinal ganglion cells (Warren, Allen, Brown, & Robinson, 2006), olfactory epithelial cells (H. Li, 2017), as well as the kidney, heart, vasculature and lungs (Tai et al., 2017). It is a non-selective cation channel with a selectivity of calcium over sodium ($P_{\text{Ca}}/P_{\text{Na}}$) of 5, making it permeable but not very selective between divalent and monovalent cations (Hofmann et al., 1999). TRPC6 can be directly activated by diacyl glycerol (DAG) or phosphorylation, and has a wide range of cellular and organismal functions (Dietrich & Gudermann, 2014). Langford et al. from our own research group showed that TRPC6 is required for hepatocyte growth factor (HGF)-induced epithelial cell scattering of cultured MDCK cells, a well-established tissue culture model for EMT (Langford, Keyes, & Hansen, 2012). TRPC6 plays a role in metastasis by altering intracellular calcium and thus the expression of things like fibronectin and ZO-1 leading to dysregulation of the cell cycle and increased migration (Song et al., 2013; Yang et al., 2017).

TRPV4

TRPV4 is activated by low osmolarity, mechanical stress, and temperatures greater than 27°C in humans and chick (Raquibul Hasan & Zhang, 2018), and has a selectivity of calcium over sodium ($P_{\text{Ca}}/P_{\text{Na}}$) of about 6, meaning it’s not highly selective but has a higher affinity for
the passage of calcium than sodium (Strotmann, Schultz, & Plant, 2003). While acting as a calcium channel, TRPV4 is also tightly regulated by calcium levels. Calcium’s effects on TRPV4 are such that its presence is required for TRPV4 to be activated but either high levels or continuous exposure to calcium, mediated by calmodulin binding, is also the mechanism of desensitization of the TRPV4 ion channel (Strotmann et al., 2003; Watanabe et al., 2003). Most likely due to the varied functions of TRPV4 and its probable role in epithelial remodeling events, it is the root cause of a few channelopathies (diseases caused by the improper function of a channel), such as Brachyolmia, Charcot-Marie-Tooth disease type 2C, Spinal Muscular Atrophy and Hereditary Motor and Sensory Neuropathy type 2 (Verma, Kumar, & Goswami, 2010).

TRPV4 has also been shown to relocate to the plasma membrane and mediate cellular migration following cytoskeletal rearrangements started by arachidonic acid (Fiorio Pla et al., 2012). Not everyone agrees on the role of TRPV4 in migration and metastasis. While one group found that deletion of TRPV4 resulted in more migration because of disruption of VE-cadherin junctions (Cappelli et al., 2019), another found that calcium influx mediated by TRPV4 activated the AKT pathway resulting in downregulation of E-cadherin and increased migration (W. H. Lee et al., 2017).

TRPM3

Melastatin TRP channel subfamily members TRPM3 and TRPM8 play roles in cancer survival and progression (Hantute-Ghesquier et al., 2018). TRPM3 is a calcium-permeable cation channel with a selectivity of calcium over sodium of anywhere from 0.1 to 10. This range is caused by the varied selectivity of splice variants of TRPM3 (Oberwinkler, Lis, Giehl, Flockerzi, & Philipp, 2005). Because of the range of splice variants of TRPM3, there are likely various stimuli and functions for each. Known stimuli of channel activation vary from osmo-sensation
and homeostasis in the kidney (N. Lee et al., 2003), to activation by sphingosine (Grimm, Kraft, Schultz, & Harteneck, 2005), some steroids (Drews et al., 2014), and noxious heat (Vriens et al., 2011). An example of the variety found in the TRP channels is that a stimulus of noxious heat can cause avoidance of the whole organism mediated by the brain, while another activation by a change in osmolarity on the same channel but in the kidney can help regulate ion gradients. While little is known about the role of TRPM3 in epithelial remodeling events, it has been shown that TRPM3 activity can be regulated by miR-204 to suppress tumor metastasis in head and neck squamous cell carcinoma (Y. Lee et al., 2010).

TRPM8

TRPM8 is calcium permeable. However, a (Pca/Pna) of 1-3, indicates it is not selective for calcium but has a slight preference for calcium over monovalent cations (McKemy, Neuhausser, & Julius, 2002). It is most commonly found in the peripheral nervous system acting as a cold sensor, but has been shown to be expressed in the prostate, vascular smooth muscle, odontoblasts, liver, and urinary tract (Latorre, Brauchi, Madrid, & Orio, 2011). It is well known for its activation by menthol and temperatures below 26°C (Bautista et al., 2007). Like most TRP channels, TRPM8 can be desensitized to its stimulus by high calcium levels, but channel activity is enhanced at low levels of calcium (McKemy et al., 2002). It has been shown that PIP2 is a necessary cofactor to sensitize TRPM8 to activating mechanisms, and that increased calcium activates PLC which then hydrolyzes PIP2 increasing potential desensitization of the channel completing the negative feedback loop (Rohacs et al., 2005). There is new evidence that the process of desensitization of TRPM8 is mediated by G protein-coupled receptors, and specifically the alpha subunit of G proteins binding to it to sensitize it to inhibition (L. Liu et al., 2019). The role of TRPM8 in epithelial remodeling is made apparent in cancer and is wide and
varied. There is evidence that it upregulates EMT in breast cancer cells by activating the AKT pathway (J. Liu et al., 2014), and evidence that it inhibits cell migration by inhibiting Rap1 with its cytosolic tail (Genova et al., 2017). Perturbing TRPM8 function leads to the dysregulation of normal cellular processes in prostate (Genova et al., 2017), pancreatic (Yee, Zhou, & Lee, 2010), lung (Du et al., 2014), breast (J. Liu et al., 2014), bladder (Q. Li, Wang, Yang, Wang, & Li, 2009), melanoma (Guo, Carlson, & Slominski, 2012), and osteosarcoma (Y. Wang et al., 2013) cancer disease states (Hantute-Ghesquier et al., 2018; Yee, 2015).

TRPA1

TRPA1 is unique in that there is only one member of the TRPA family in mammals. It is characterized by its many ankyrin repeat domains and can form both homo- and hetero-tetramers that are permeable to calcium, sodium and potassium with a (Pca/Pna) of about 1 (Sadofsky et al., 2014; Story et al., 2003; Zygmunt & Hogestatt, 2014). The function of this channel is regulated by stimuli such as noxious cold, endogenous and exogenous inflammatory signals, reactive oxygen species, and exogenous irritants that can cause pain or itch (Bautista et al., 2013; Bessac & Jordt, 2008; Taylor-Clark, 2016). TRPA1 contains an EF hand domain which enables the direct binding of calcium (Figure 2), which is believed to directly activate TRPA1 and help mediate the extreme cold sensation (Zurborg, Yurgionas, Jira, Caspani, & Heppenstall, 2007). While the role of calcium in many TRP channels is limited to inhibition at high levels, calcium can activate TRPA1 at low levels via direct binding and inactivate it at high levels with a mechanism probably dependent upon calmodulin binding (R. Hasan, Leeson-Payne, Jaggar, & Zhang, 2017). It is important to note that while the functions of the TRP channels have not all been elucidated due to their variety of stimuli and permeabilities, the complexity of each channel can be further advanced by the formation of hetero-tetramers and complexes like those formed
with TRPA1 and TRPV1 (Weng et al., 2015). The formation of complexes, and the resultant interactions between two similar yet distinct channels, adds a layer of complexity to an already dynamic functionality of TRP channels. TRPA1 plays many roles in cancer, though few roles in EMT-type events have been elucidated. In one such report, TRPA1 mutations were observed in prostate cancer and its activation in cancer-associated fibroblasts results in increased secretion of EMT inducing growth factors (Vancauwenberghe et al., 2017). In another, activation of TRPA1 in lung cancer cells results in cyclooxygenase-2 suppression, resulting in downregulation of growth factor secretion that would normally come from a hypoxic environment (J. Park, Shim, Jin, Rhyu, & Lee, 2016). So far the role of TRPA1 in cell migration has been indirect and usually paired in complex with another protein like TRPM8 or FGFR2 to induce metastasis in lung cancer (Berrout et al., 2017; Du et al., 2014).

PKD2L1

Finally, PKD2L1 is part of the polycystin subgroup of TRP channels, and though it shares about 60% homology with PKD2, not much is known about its molecular role (Delmas, 2005). While PKD2L1 (or TRPP3) normally associates with a PKD1 or PKD1L protein to form a receptor channel, it can form a homo-tetramer that is sensitive to change in pH, cellular swelling, and voltage (T. Shimizu, Janssens, Voets, & Nilius, 2009). PKD2L1 is non selectively calcium permeable with a (Pca/Pna) of 4 (Chen et al., 1999). While PKD2 is important in renal development (Fragiadaki et al., 2017), PKD2L1 seems to have independent roles, such as sour taste perception (Ohishi et al., 2017). The importance of PKD2L1 has recently emerged as it is primarily responsible for ion flux in neuronal and renal primary cilia and can be directly activated by PKA phosphorylation (E. Y. J. Park, Kwak, Ha, & So, 2018). Another recently
elucidated, important function of PKD2L1 is that it helps regulate mitochondrial calcium homeostasis in the heart, resulting in anti-hypertrophic effects on the heart (Lu et al., 2018).

Beyond this, our lab has found evidence that inhibition of TRP channels results in phenotypes suggesting the interference of the proper development of important epithelial remodeling events in the chick embryo.
MATERIALS AND METHODS

Incubation of Chick Embryos

Fertilized chicken eggs were obtained from the following sources; North Carolina State University, Dunlap Hatchery in Caldwell, ID, and LJ Farms in Spanish Fork, UT. They were incubated on their sides at 37°C for 30-38 hours, or to the 7-11 somite stage range. When the embryos had developed to the desired stage, an adapted version of the chick whole-embryo culture method developed by Susan Chapman et al. was used (Chapman, Collignon, Schoenwolf, & Lumsden, 2001).

Explant Preparation

The eggs were cracked in a petri dish so that the embryo would be on the top of the yolk. Depending on the stage of development, varying amounts of thick albumin had to be gently pulled from the top of the embryo using a Kimwipe®. Once the albumin was removed, a previously prepared ring of Whatman no. 2 filter paper, which was cut so that it had a diameter of ~1 inch and a hole in the middle with a diameter of ~0.5 inches, was placed above the embryo to hold it steady. Once done, the vitelline membrane was cut around the edge of the ring to separate the embryo from the rest of the egg. When that was accomplished a second ring was placed ventrally to the first to “sandwich” the embryo between the two rings. The rings were then punctured to ensure the membranes would not slip from between the rings.

Agar-Albumin Culture Dishes

Agar culture dishes were prepared per the protocol in (Darnell & Schoenwolf, 2000). Briefly, 1% w/v of Bacto-agar was mixed with equivalent volumes of boiling saline solution (7.19g NaCl per 1 L distilled autoclaved water) and put in a water bath at 47°C for at least 20
min. Thin albumin was collected from fertilized eggs to the volume of 6 mL per drug treatment plate and placed in the same water bath for at least 20 minutes. While those were warming in the water bath PBS with 0.1% BSA were aliquoted into 15 mL conical vials. For the control plates 1,930 µL PBS/BSA solution was aliquoted then 70 µL of the drug vehicle, DMSO, was suspended in the solution. Finally, 14 µL 100x Penicillin/Streptomycin was added to the solution. This was repeated with each drug treatment with the only variation being the amount of PBS/BSA and drug suspended in DMSO being added. All treatment groups ended with DMSO making up 0.5% or less of the total volume. These solutions were incubated in the same water bath for at least 10 minutes before the prewarmed thin albumin was gently mixed into it. Then 6 ml of the Bacto agar solution was added to each vial to bring the volumes up to 14 ml. Once mixed thoroughly the solution was transferred to 6-well Tissue Culture plates with a volume of 2 ml in each well. These plates were labeled and allowed to solidify for at least 1 hour prior to use. If stored in the 4°C fridge, they were wrapped in foil with a wet paper towel to maintain them, then prewarmed in the incubator before use.

Chemical Inhibitors

Each of the 6 chemical inhibitors used in this study were suspended in DMSO. They were diluted to their determined IC50’s in the protocol mentioned above with the concentration of DMSO never exceeding 0.5%. All inhibitors were purchased from commercial companies (Tocris Scientific: SAR 7334, Phenamil, HC 067047, A 967079; or Alomone Labs: Ononetin, M8-B hydrochloride.) The following “Table 1” includes the drug name, target, structure, and final concentration used.
Table 1: List and Structure of Small Molecule Inhibitors.

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<thead>
<tr>
<th>Drug Name</th>
<th>Acts On</th>
<th>Structure</th>
<th>IC₅₀'s</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAR 7334</td>
<td>TRPC6</td>
<td><img src="image1" alt="SAR 7334 Structure" /></td>
<td>500 nM</td>
</tr>
<tr>
<td>HC 067047</td>
<td>TRPV4</td>
<td><img src="image2" alt="HC 067047 Structure" /></td>
<td>1.1 µM</td>
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<tr>
<td>Ononetin</td>
<td>TRPM3</td>
<td><img src="image3" alt="Ononetin Structure" /></td>
<td>3 µM</td>
</tr>
<tr>
<td>M8 B Hydrochloride</td>
<td>TRPM8</td>
<td><img src="image4" alt="M8 B Hydrochloride Structure" /></td>
<td>500 nM</td>
</tr>
<tr>
<td>A 967079</td>
<td>TRPA1</td>
<td><img src="image5" alt="A 967079 Structure" /></td>
<td>1.5 µM</td>
</tr>
<tr>
<td>Phenamil</td>
<td>PKD2L1 &amp; ENaC’s</td>
<td><img src="image6" alt="Phenamil Structure" /></td>
<td>2 µM</td>
</tr>
</tbody>
</table>
The controls for this process were plates made using 0.5% DMSO, the drug carrier, as the treatment. Once the drug and control plates were fully prepared chick embryos were collected as stated above and gently placed on the agar of each well in the treatment plates. These plates were then incubated in a temperature, humidity, and CO2 controlled tissue culture incubator at 37°C with 5% CO2 for 18-24 hours.

Treatment Collection

Following the incubation period, embryos were washed with 1X PBS then fixed with 2 mL of 4% formaldehyde for one hour at room temperature or overnight at 2-8°C. Once fixed, the embryos were cut out of their vitelline membranes and washed with PBS. Each embryo was imaged three times dorsally and three times ventrally before being moved into 5% sucrose/PBS for 4 hours at room temperature or overnight at 2-8°C. The embryos were then moved to, and stored in, 15% sucrose/PBS at 2-8°C until sectioning.

Cryosectioning

Embryos were prepared for cryosectioning by embedding them in gelatin consisting of 7.5% gelatin (Sigma) and 15% sucrose (both w/v) in PBS. Each embryo, in 15% Sucrose/PBS, was warmed alongside the 7.5% gelatin in a 37°C water-bath for at least 1 hour. Then, each embryo was transferred to a 3 mL aliquot of the gelatin and inverted 4-5 times every 30 minutes for 90 minutes. After the initial 90 minutes, the gelatin was replaced, and the procedure repeated. Once done, the embryos are transferred to molds along with fresh, warm gelatin and are left to solidify for at least 30 minutes at room temperature with the embryo suspended in the middle of the gelatin. The blocks can then be moved forward for cryosectioning or stored in a refrigerator in a box with a wet paper towel for up to 3 days. When preparing for cryosectioning the embryos
location is marked with a marker then the gelatin blocks are flash frozen in liquid nitrogen. The blocks are then sectioned at 12-16 microns per slice with a cryostat. The sections are then mounted, in sequence, on VWR® Superfrost Plus® glass slides.

Immunohistochemistry

Embryo sections of all treatment groups and the control group described in “Chemical Inhibitors”, were rehydrated by placing them in 1X PBS at room temperature for 30 minutes. The gelatin surrounding the embryo sections was removed by placing the PBS with slides in a 37°C water-bath for 5-10 minutes then transferring the slides to pre-warmed PBS for another 5-10 minutes. Finally, the slides were moved into fresh room temperature PBS and they are ready to be stained or stored at 2-8°C.

The following antibodies were used: Laminin (mouse IgG1, Developmental Hybridoma 3H11) to measure morphological changes, HNK1 (mouse IgM, Developmental Hybridoma 3H5) to visualize the migration of neural crest cells, and DAPI to visualize individual cell nuclei and general tissue morphology. Laminin primary antibody was diluted to a 1:500 concentration with antibody buffer (PBS, 0.1% bovine serum albumin (BSA), 0.1% Tween® 20), HNK1 primary antibody was diluted to a 1:100 concentration with the antibody buffer, and DAPI antibody was diluted to 1:250,000 in the PBS wash. Each slide was removed from the PBS and covered with 300 µL of the primary antibody mixture and allowed to incubate for either four hours at room temperature or overnight at 2-8°C. Each slide was then rinsed three times with PBS then the procedure was repeated with the secondary antibodies at a dilution of either 1:500 or 1:1000 for one hour at room temperature. Following application of the secondary antibodies the slides were washed three times in PBS and covered using BWR micro cover glass 24 X 60 mm No. 1 and
several drops of Fluoromount-G. Fluorescent images of the staining were taken at 10X or 20X with an Olympus BX61 microscope.

**In Situ** Hybridization

Embryos were incubated for the total time that the drug treatment embryos would be incubated then collected for **In Situ** Hybridization. To ensure a functional protocol, a control of FGF8 was used and visualized in the AER of limb buds. The embryos were collected and placed directly in ice cold 4% formaldehyde and stored at 2-8°C. Following fixation, the embryos were washed in PTW (0.1% Tween® 20 1X PBS) three times for five minutes each at room temperature. The embryos are then dehydrated in a stepwise manner from 25%, 50%, 75% and 100% methanol and stored at -20°C. Whole-mount **in situ** hybridization was then performed as described by Wilkinson and Nieto (Wilkinson & Nieto, 1993). In short, formaldehyde-fixed embryos of appropriate developmental stages were buffered and exposed to a DIG-labeled anti-sense RNA probe, which bound to its complimentary mRNA transcripts. After removal of the non-specifically binding probe, the embryos were incubated with anti-DIG alkaline-phosphatase (AP) antibody (1/2000; Roche, Indianapolis, IN), followed by a chromogenic AP substrate. Whole-mount embryos stained for specific mRNA transcripts were then prepared for cryosectioning as previously described and sectional imaging on the Olympus BX61 brightfield microscope.

**Synthesis of Probes**

Primers designed with Primer BLAST specific to each *gallus* TRP channel sequence in PubMed’s gene bank were used to PCR amplify templates for probe synthesis. Primers were reconstituted to a 100ng/µL stock solution and 20ng/µL working solutions. PCR (Polymerase
Chain Reaction) conditions were as follows: 94°C for 5 minutes, then 94°C for 1 minute, 55-63°C for 30 seconds and 72°C for 1 minute repeated for 35 cycles, followed by 72°C elongation period for 10 minutes. The annealing temperature was variable and depended on the primers being used. All primers used spanned the 3’ UTR segment of the sequence. The following “Table 2” includes the primer sequences and base pair length of the predicted products.

Table 2: List of Primers Used to Synthesize Probes.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC6</td>
<td>GGAGGACTCTTCCAGTACCTT</td>
<td>CGTCTCTACCATACTACACAGAGGA</td>
<td>613</td>
</tr>
<tr>
<td>PKD2L1</td>
<td>GGGAGCAGGAAAGAAGCTGGGCT</td>
<td>GAAACACAGGGGCCAGAAGTT</td>
<td>861</td>
</tr>
<tr>
<td>TRPA1</td>
<td>TTTGATTCCGATCGCTTTGTT</td>
<td>TAAGCTGCTGCTTTTCTGTCGGT</td>
<td>766</td>
</tr>
<tr>
<td>PED1</td>
<td>GGTCGTTGTCTACAGCTACCG</td>
<td>TTCCAGTGAAACCCCTCAGCAGCCT</td>
<td>970</td>
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<tr>
<td>PKD2</td>
<td>GAGTGGGGACCAAGGACTGA</td>
<td>ACCCAAGACTGACGTTTCC</td>
<td>714</td>
</tr>
<tr>
<td>TRPV6</td>
<td>CAGGAGAGTGCCCGCATGAAC</td>
<td>ACCACGGGCTTATGCTCAAAGT</td>
<td>669</td>
</tr>
<tr>
<td>TRPC1</td>
<td>ACTGGCACACGTAGCAATCTT</td>
<td>TGGCTGCAACCATCCTGTTT</td>
<td>677</td>
</tr>
</tbody>
</table>

Cloning of Products

The PCR products were ligated into either pGEM-T Easy (Promega) vectors or TOPO-II Cloning kits using each kit’s standard protocol and transformed into Select96 Competent Cells (Promega) or competent cells provided by the Suli Lab (BYU Provo, UT). In short, 3 µL PCR product were added to a ligation reaction containing the pGEM-T Easy vector or TOPO-II Plasmid, buffer, and T4 DNA ligase and allowed to incubate at room temperature for one hour. Following incubation, 3 µL of the ligation reaction were added to competent cells and incubated
on ice for 30 minutes. The cells were then heat-shocked at 42°C for 30 seconds and returned to ice for 5 minutes. The cells were carefully transferred to SOC broth and incubated in a shaker at 37°C for 1 hour. After incubation, 100 µL of each culture was plated on LB/X-gal/Ampicillin plates which were incubated overnight at 37°C.

Mini DNA Preparation

White colonies (devoid of beta-galactosidase activity) were labeled and chosen from the LB/X-gal/Ampicillin plates and used to inoculate 5 mL LB/Ampicillin broth. These were placed in a shaker at 37°C for 6 hours then Zymo DNA preparation kits were used to isolate the plasmids following kit instructions. The cells were pelleted then resuspended and lysed. They were then brought to a neutral pH then washed of extra debris. The DNA was then isolated via centrifugation using the spin columns provided in the kit. Once the DNA is cleaned and collected in the spin column it is eluted with an elution buffer or water at a pH of 7.5.

Probe Synthesis

Anti-sense probes were constructed from partial sequences of TRPC6, TRPC1, PKD1, PKD2, PKD2L1, TRPV6, and TRPA1, all including at least 500 base pairs of the coding region along with at least 100 base pairs of the 3’ UTR of each gene. The PCR product inserts that were inserted into the plasmids were cloned out of the plasmids using a PCR of the plasmids with M13 forward and reverse primers. The resultant product size was confirmed with gel electrophoresis and then purified and prepped for submission to be cycle sequenced. Depending on the direction of the insert, either T7 or SP6 Polymerase were used with DiG labeled nucleotides to synthesize an anti-sense probe that would be specific to mRNA sequences of each
gene product. The product was then confirmed with gel electrophoresis, cleaned, concentrated to a working solution, and stored at -20°C.
RESULTS

Expression Patterns

From the beginning of this project a central premise of the work to be performed was to validate TRP channel gene expression in the developing chick (*gallus gallus*) embryo. To do this, known and predicted chick TRP channel sequences were gathered and analyzed. After review of the literature a plan was made to choose 12 of the 28 TRP channels and describe in three different developmental stages in the chick using *in situ* hybridization. Primers were designed using NCBI BLAST Primer Design to make products of about 750 base pairs including the 3’ UTR and at least 100 base pairs beyond it. The first set of primers are shown in “Table 3”.

These primers were used to make PCR (Polymerase Chain Reaction) products using a cDNA library collected from chick embryos at broadly comparable developmental stages by the Stark Lab. If a PCR product was acquired, then they were moved forward to insertion into a plasmid and cloning in competent cells. Subsequent sequencing would validate the cloned genes and help to determine suitability for *in situ* hybridization. Of the original 12 primer sets only TRPC1, TRPC6, TRPV2, TRPV6, PKD2, PKD2L1, and PKD2L2 yielded PCR products. Of those products, only TRPC1, TRPC6, TRPV6, PKD2, and PKD2L2 made it into the plasmids to be cloned. Of those cloned only TRPC1, TRPC6, TRPV6 and PKD2 ended up being suitable as selective probes for *in situ* hybridization. Because of this poor return from 12 primer sets to 4 potential probes, more primers were designed and utilized on two different occasions. The remaining primer sets are shown in “Table 4”.

24
Table 3: List of First Wave of Primers.
This table includes the first set of primers designed and used to attempt to synthesize probes for in situ hybridization.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Forward 5'-3'</th>
<th>Reverse 5'-3'</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV1</td>
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<td>CGCTCTATGAAAGCAAGCGTT</td>
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</tr>
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<td>TRPV2</td>
<td>TCTGGCTACAGCAAAGTGT</td>
<td>AGGTGAAAACACAGTCATTTGAT</td>
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<td>TRPV3</td>
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<td>ACCAGACCAGCATACCTGAAAC</td>
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<td>TRPV4</td>
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<td>TTTCTGCTGGGCTGCTCCTTT</td>
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<td>TRPV6</td>
<td>CTGGAAGATGGCCCATGAAC</td>
<td>CCACAGGCTTTAGCTCAAAGT</td>
<td>669</td>
</tr>
<tr>
<td>TRPA1</td>
<td>TTTGATTCCGATCGCTTGTG</td>
<td>TAAGCTGCTGCTTTTTCTGTT</td>
<td>766</td>
</tr>
<tr>
<td>TRPC1</td>
<td>ACTGGCAACGTAAGCAAATCTT</td>
<td>TGCTGTGCAAACCATCTCCTTT</td>
<td>677</td>
</tr>
<tr>
<td>TRPC6</td>
<td>GGAAGACTCTTTCCAGTACCT</td>
<td>CGTCTCTACCATATACACAGGAGA</td>
<td>613</td>
</tr>
<tr>
<td>TRPP1 (PKD1)</td>
<td>GGTGTCTTGTCAAGCTACCG</td>
<td>TTOCAATGAAACCCTAGCAGACT</td>
<td>970</td>
</tr>
<tr>
<td>TRPP2 (PKD2)</td>
<td>GGAATGAGGAAACAGGGAACGTGA</td>
<td>ACCCCACCACTGAGCTTTCTCT</td>
<td>714</td>
</tr>
<tr>
<td>TRPP3 (PKD2L1)</td>
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Table 4: List of Second and Third Wave of Primers.

Primers designed in the hopes of synthesizing probes for in situ hybridization. These were the second and third wave of primers designed. Some because earlier versions didn’t work, and some because of new interests in TRP channel function.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Forward 5’-3’</th>
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<td>TRPM7</td>
<td>GGTCAACAACTGGGACTCTG3T</td>
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<td>TRPM7.2</td>
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<td>TRPML1</td>
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<td>TRPML1.2</td>
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<tr>
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<tr>
<td>TRPP3.2 (PKD2L1)</td>
<td>CGGAACGAAAGAAGACTGGGCT</td>
<td>GAAACACAGGGGCGAGAAGTT</td>
<td>861</td>
</tr>
</tbody>
</table>
Figure 3 is a representation of the progress made in this descriptive project for each of the TRP channels. As explained in the figure, some channels were not attempted while others made it to the level of PCR products, probe synthesis, or actual staining. In situ hybridization was done with each probe developed, with the most consistent results being obtained using PKD2 and TRPC6 probes, where the most prevalent staining was observed in the ectoderm, brain, otic vesicle, and heart (Figure 4). Figure 4 shows that the methods being used were correct, demonstrated by the FGF8 control, and the location specific staining mentioned above. Besides PKD2 and TRPC6, the other probes yielded inconsistent results and would need follow-up in future studies.
Figure 3: Progress Towards Expression Pattern Clarification.

Progress of each TRP channel in the process of developing a probe and determining mRNA expression patterns using *in situ* hybridization. Green is successful through that point. Blue is successful to that point on the 2nd or 3rd set of primers. Red is mixed results of success.
Figure 4: In Situ Hybridization Staining

TRP Channel Expression: (A, B) Control in situ staining with FGF8 probe. A. Control stain of AER labeled by FGF8. B. Whole mount image of section (A), clear staining of limb buds by FGF8. (C, D). Staining with PKD2 probe: C. Longitudinal section of left embryo in image (D), staining of ectoderm and mesencephalon. D. Whole mount image of embryos stained with PKD2 probe. Specific staining in heart, developing nervous system, and otic vesicle. E. Section of embryo stained with TRPC6 probe. Specific staining in the ectoderm. F. Section of embryo stained with TRPC1 probe. No specific staining found.
I next moved on to the primary focus, to evaluate the function of these TRP channels in development, using pharmacological inhibition and look for observable, consistent morphological changes.

Pharmacological Inhibition

Once it was decided that small molecule drug inhibition of TRP channels should be used to give an idea of the potential function of these channels in epithelial remodeling events, an experimental plan needed to be determined. The plan developed was that embryos would be treated using the agar-albumin culture dish method developed by Darnell and Shoenwolf (Darnell & Schoenwolf, 2000). The embryos would be around the same developmental stages of between 6 and 11 somite stages, which is a time period of significant epithelial remodeling events such as the closing of the neural tube and neural crest cell migration enabling the potential interference of such events with the application of the drugs chosen. The TRP channels studied would need to be chosen based on evidence in the literature and availability of small molecule inhibitors. The treated embryos would then be sectioned and stained with antibodies to clarify phenotypes observed in whole mount and determine phenotypes that couldn’t been seen in whole mount. Based on available small molecule inhibitors and the research summarized in the introduction, TRPC6, TRPV4, TRPA1, TRPM3, TRPM8 and PKD2L1 were chosen to be studied. Since no studies had been done using the inhibitors chosen on chick embryos, or using the agar-albumin culture dish method, a higher concentration was used for each (Table 5).
TRPA1, as discussed in the introduction, is regulated by stimuli such as noxious cold, endogenous and exogenous inflammatory signals, reactive oxygen species, and exogenous irritants that can cause pain or itch (Bautista et al., 2013; Bessac & Jordt, 2008; Taylor-Clark, 2016). It also has been shown to play roles in epithelial remodeling events in the form of cancer progression. The small molecule inhibitor used for its inhibition is called A-967079 and was bought from Tocris Bioscience (Table 1). TRPA1 is unique to the other channels in this study for a number of reasons. The first is that it is the only member of its family making it unique.
amongst all TRP channels. The second is that it shares the lowest homology between the chick protein sequence and human of 62% identity and 78% positive matches (Table 6).

Table 6: Sequence Homology Between Chick TRP’s and Human.
Table of the homology shared between the chick TRP channels (Channel) and the Human TRP channels (Human). The Identity score is based on the percentage of exact matches. The Positives score is based on the percentage of exact and similarly functioning matches. The Gaps score is the percentage of sequence mismatch or disparity in length.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Identity</th>
<th>Positives</th>
<th>Gaps</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPA1</td>
<td>62%</td>
<td>78%</td>
<td>1%</td>
<td>TRPA1 X1</td>
</tr>
<tr>
<td>TRPC6 X1</td>
<td>80%</td>
<td>85%</td>
<td>8%</td>
<td>TRPC6</td>
</tr>
<tr>
<td>TRPM3 X1</td>
<td>87%</td>
<td>91%</td>
<td>3%</td>
<td>TRPM3 X13</td>
</tr>
<tr>
<td>TRPM8</td>
<td>81%</td>
<td>91%</td>
<td>0%</td>
<td>TRPM8</td>
</tr>
<tr>
<td>TRPV4</td>
<td>85%</td>
<td>91%</td>
<td>1%</td>
<td>TRPV4 X1</td>
</tr>
<tr>
<td>PKD2L1</td>
<td>79%</td>
<td>89%</td>
<td>1%</td>
<td>PKD2L1 isoform 2</td>
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</table>

The final reason, which is related to the second, is that the inhibitor A-967079, might not be as potent in the chick model as in human or mice models. Because TRPA1 is associated with inflammation and pain sensation a lot of work has been done to try and inhibit its negative effects. It has been found that while the inhibitor used has potent inhibitory effects on mice and humans, it does not inhibit TRPA1 found in *xenopus tropicalis* (Nakatsuka et al., 2013). They found that normal inhibition of TRPA1 by A967079 was mediated by interaction with an amino
acid pair of serine and threonine. The western clawed frog has an isoleucine and valine in those positions, rendering the function of the inhibitor useless. By using a SMART Protein BLAST, I found that the residues in the chick TRPA1 5th transmembrane segment were isoleucine and alanine, which is closer to the western clawed frog than to the human sequence. This, along with the phenotypic results gathered could mean that the small molecule inhibitor had no effect on the chick TRPA1 channels treated. An electrophysiological study of the channel being treated with the inhibitor would need to be done to clarify its full effect.

The realization that the small molecule inhibitor might not perform its function on chick TRPA1 proteins didn’t come until after all the embryos had been treated. After treatment, whole mount embryos were analyzed, and the number of perceived defects were counted. The

![Figure 5. TRPA1 Defect Percentages.](image)

Rates of observed defects in embryos treated with A967079 a TRPA1 inhibitor. There were no defects observed at a rate of 50% with an N of five, even with a concentration 10 times that provided as the IC50 for humans.
proportion of embryos with specific defects was calculated and compared to those observed in control untreated embryos (Figure 5).

To represent the potential defects observed in the whole mount treatment groups, two representative embryos were sectioned and stained to clarify the morphological effects of the small molecule inhibitor. The whole mount images provided in (Figure 6), represent the sections stained and imaged. In this treatment group the most significant phenotypes were otic vesicles with altered shapes, changes in heart morphology, and embryos that “turned” left instead of right causing the heart to be on the opposite side of the embryo. Upon closer inspection with the sectioning and staining with laminin, DAPI, and hnk1 as a neural crest marker, no significant otic vesicle defects were found. A noticeable defect that couldn’t be observed in whole mount but became visible in histological sections of the hindbrain was that the neural tube was misaligned while still closed by a thin roof plate (Figure 7). The hearts of A967079-treated embryos appear large and/or misshapen (Figure 8). The sections of this embryo reveal an enlarged dorsal aorta. But overall, compared to the other treatment groups, the TRPA1 small molecule inhibitor A967079 did not seem to have a consistent or broad effect on the development of the embryos.
Figure 6: TRPA1 Whole Mount Images.

(A-C) A967079 treated embryo representative of all imaged. (Scale bar = 1mm) Whole mount imaging showed few defects with areas of concern most frequently found in the size of the heart, a potentially open neural tube (C) (which turned out not to be), and the orientation, or direction the head is turned was reversed. The bar in image (A) is representative of “Figure 7” and the bar in image (B) is representative of “Figure 8”. (D-F) control embryo treated with only DMSO representative of the carrier.
Figure 7: TRPA1 Neural Tube Stains.

(A-C) A967079 inhibitor treated embryo. (D-F) Control comparable section. Blue stain is DAPI, Green stain is Laminin, Magenta stain is HNK1. B, the neural tube in the treatment group appears to be misaligned with a pivot point on the right side too high to match the pivot on the left.
Figure 8: TRPA1 Heart Stains.

(A-C) A967079 treated embryo. (D-F) control embryo. B. Dorsal aorta seems to be enlarged compared to the control embryo.
We next examined the effect of small molecule inhibition of TRPC6 on the developing embryo. TRPC6 has been shown to regulate calcium dependent transcription factors such as NFAT downstream of Receptor Tyrosine Kinase pathways (Langford et al., 2012). This, along with other evidence of its role in metastasis, makes TRPC6 a potential player in epithelial remodeling events during development (Song et al., 2013; Yang et al., 2017). The small molecule inhibitor used for TRPC6 was called SAR 7334 (Tocris Bioscience, Table 1). According to the *in-situ* hybridization staining performed earlier (Figure 4), TRPC6 mRNA was localized in the ectoderm, otic vesicle, heart, and potentially in the caudal end of the embryo. Analysis of the whole mount and sectioned embryos treated with SAR 7334 showed deleterious

![TRPC6 Defect Percentages](image)

*Figure 9: TRPC6 Defect Percentages.*

Rates of observed defects in embryos treated with the TRPC6 inhibitor SAR 7334. The observed defects with rates over 50% were in the heart and open neural tubes. Other significant defects were in the size of the otic vesicles and the hindbrain development.
effects that are consistent with the localization as detected by in situ hybridization (Figures 9-10).

All the treated embryos in the TRPC6 treatment group were compared and representative embryos were selected for histological analysis. Figure 10 includes images of one of the representative embryos with lines representing the locations of sections and stains shown in figures 11-14. It also includes a representative control embryo for easy comparison of the phenotypes.

TRPC6 inhibition resulted in phenotypes that are results of faulty epithelial remodeling events such as neural tube closure and heart development. The neural tube in the caudal end of the embryo, where the newest somites had formed, was open in 50% of SAR 7334-treated embryos (Figure 11). While only about 34% of the treated embryos were scored to have large otic vesicles in the whole mount, the sectioned and stained representatives revealed a higher percentage (Figure 12). Another defect is an apparent change in the shape and, less frequently, the size of the heart (Figure 13). Finally, there was some evidence of faulty cellular differentiation or neural crest development in the rostral neural tube (Figure 14). This could be inhibition of migration due to inactive TRPC6 function, which would fit with previous findings in a reductionist tissue culture model from our group, as reported by Langford et al. (2012). Together these phenotypes suggest that TRPC6 plays an important role in epithelial morphogenesis during development. Further study of TRPC6 function in a developing chick embryo could be useful in elucidating the mechanisms of TRP channel involvement in complex cellular processes such as EMT.
Figure 10: TRPC6 Whole Mount Images.

(A-C) TRPC6 inhibitor SAR7334 treated embryo. (Scale bar = 1mm) (D-F) Control embryo. There were several notable defects in the embryos treated with the inhibitor. The head did not complete its rotation in time, an open neural tube, heart and hindbrain malformations (A). Line in image B representative of (Figure 11). Left line in image C representative of (Figure 12). Right line in image C representative of (Figure 13). Line in image A representative of (Figure 14).
Figure 11: TRPC6 Neural Tube Stains.

(A-C) TRPC6 inhibitor treated embryo. (D-F) Control embryo. (B, E) DAPI. (C, F) Laminin. (A-C) Clear open neural tube made clearer by no basal lamina on caudal half of neural tube (C).
Figure 12: TRPC6 Otic Vesicle Stains.

(A-C) Treated embryos. (D-F) Control embryos. (A-C) shows a larger otic vesicle than you would normally see. It also encompasses all of the neural crest cells marked by HNK1 (C). (B, E) DAPI. (C, F) Laminin green, HNK1 magenta.
Figure 13: TRPC6 Heart Stains.

(A-B) Treated embryos. (C-D) Control embryos. Comparable sections with significantly different heart shape. The treated embryos seem to not have the two chambers separating like the control embryo. (B, D) DAPI.
Figure 14: TRPC6 Rostral Neural Tube Stains.

(A, B) Treatment embryo. (C, D) Control embryo. (A, C) DAPI. (B, D) Green Laminin, Magenta HNK1. B, HNK1 migration potentially dysregulated, too much expression in neural tube and not enough ventral migration. D, Imperfect section of representative control with HNK1 migrating ventrally.
TRPV4

TRPV4 is regulated by osmolarity, mechanical stress, and warm temperatures. It regulates, and is regulated by, calcium levels through diverse and inconsistent mechanisms as explained in the introduction. TRPV4, unlike many of the TRP channels, is known to be the cause of a fair number of channelopathies such as Brachyolmia, Charcot-Marie-Tooth disease type 2C, Spinal Muscular Atrophy and Hereditary Motor and Sensory Neuropathy type 2 (Verma et al., 2010). It has been shown in mice that it’s the overexpression of mutant TRPV4 channels that causes the skeletal dysplasia’s that make up the channelopathies determined by TRPV4 (Weinstein, Tompson, Chen, Lee, & Cohn, 2014). Another relevant study was performed using the chick model organism. It was meant to determine if maternal fever associated birth defects were caused by aberrant activation of TRPV4 during development. It found that the activation of TRPV4 in neural crest cells by using ferrous labeled channels excited by radio waves, caused heart and craniofacial defects common in maternal fever associated birth defects (Hutson et al., 2017).

These evidences were sufficient to justify targeting TRPV4 in chick development. While the channelopathies described were a result of aberrant activation of TRPV4, it is not beyond reason to think that inhibition of that same integral protein might result in developmental defects. When the embryos were treated with HC-067047 a few defects were observed (Figure 15).
The heart of treated embryos seemed to be formed differently than those of control embryos. If the embryos were allowed to develop further, like many treatment groups, there could have been more apparent developmental defects in the form of skeletal dysplasia’s in the case of TRPV4. To clarify, the high rate of potential neural tube defects was based on observation of whole mount embryos but was not determined as a definite neural tube defect because of lack of evidence in sections and manual manipulation. It could be that the roof plate was thinner or wider to create the appearance of a neural tube that hadn’t closed, but the selected embryos for sectioning did not show open neural tubes. A representative embryo is shown in (Figure 16). The main focus in this treatment group was the development of the heart. The most apparent defects found in the sections were increased neural crest migration, aberrant lumen formation (Figure 17), improper heart/head turning, and abnormal heart shape (Figure 18).
The observed phenotypes suggest that TRPV4 is required for important events in early development. While there has been some connection of improper TRPV4 activation with developmental defects, evidence presented here suggests that TRPV4 inhibition could also generate developmental defects. Defining this potential connection could have important implications in human health. Specific effects of TRPV4 inactivation on neural crest cell migration to the face and heart should be more thoroughly described, but some evidence to its importance has been shown here.
Figure 16: TRPV4 Whole Mount Images.

(A-C) HC067047 treated embryo representative of all imaged. (Scale bar = 1mm) Whole mount imaging showed few defects, with areas of concern most frequently found in the shape of the heart, a potentially open neural tube (B) (which turned out not to be), and the orientation, or direction the head is turned was reversed. The left bar in image (C) is representative of “Figure 17” and the right bar in image (C) is representative of “Figure 18”. (D-F) control embryo treated with only DMSO representative of the carrier.
Figure 17: TRPV4 Heart Stains.

(A-C) Treatment embryo. (D-F) Control embryo. (B, E) DAPI. (C, F) Green Laminin, Magenta HNK1. Most discernible difference is the extra epithelial mass with a lumen in (B) the treatment group. Second, there seems to be significantly more neural crest cell expression in the heart of the treatment group (C).
Figure 18: TRPV4 Orientation Stains.

(A-C) Treatment embryo. (D-F) Control embryo. (B, E) DAPI. (C, F) Green Laminin, Magenta HNK1. The overall shape of the control heart (D) compared to the treated heart (A) is clear though hard to characterize as a result of the treatment. The dorsal aorta in the control embryo (E) compared to where it should be in the treated embryo (B) shows there might be a defect in proper development.
TRPM3

TRPM3 is a unique TRP channel. There are so many splice variants that the mode of activation and downstream effects of this “single” channel can rival the variety of some families of ion channels. A quick example of this is that the chick TRPM3 channel shares sequence homology most closely with the X13 variant of the human TRPM3’s (Table 6). As mentioned in the introduction, activation of TRPM3 is varied from osmo-sensation to sphingosines, to steroids; but its role in epithelial remodeling events is fairly unknown. This coupled with the availability of a selective small molecule inhibitor, Ononetin, makes TRPM3 a good target for this study.

Upon application of Ononetin to inhibit TRPM3 activation during a short developmental period, the most pronounced defects were observed in the heart, neural tube, and otic vesicles (Figure 19). Embryos were chosen and sectioned based on their representation of the treatment group. An example of the embryos analyzed to make (Figure 19) is found in (Figure 20).

Though analysis of the whole mount embryos suggested there could be significant defects in the heart, upon inspection of the sections it was difficult to determine any clear developmental abnormalities in that region. On the other hand, there were some interesting phenotypes in the roof plate and ectoderm near the otic vesicles (Figure 21), and a caudal open neural tube (Figure 22).

The defects observed in the roof plate might be affiliated with the reported expression patterns of TRPM3 in sensory neurons (Quallo, Alkhatib, Gentry, Andersson, & Bevan, 2017).
But it is interesting to note the similar phenotypes of open caudal neural tubes in a few of the treatment groups.

Figure 19: TRPM3 Defect Percentages
Rates of observed defects in whole mount embryos treated with the small molecule TRPM3 inhibitor Ononetin. Significant observations were the rate of heart defects, specifically the orientation of the heart, some otic vesicle defects, and open neural tubes.
Figure 20: TRPM3 Whole Mount Images.

(A-C) TRPC6 inhibitor SAR7334 treated embryo. (Scale bar = 1mm) (D-F) Control embryo. Defects seen in the Otic vesicle and heart shape (B). Revered orientation, and an open neural tube (C). The bar in image (B) represents (Figure 21). While the bar in image (C) represents (Figure 22).
Figure 21: TRPM3 Roof Plate and Otic Vesicle Stains.

(A-C) TRPM3 inhibitor treated embryo. (D-F) Control embryo. (B, E) DAPI, (C, F) Laminin. (A-C) (D-F). As seen in image (D), this section was cut thicker than the normal 14 microns causing the neural tube and otic vesicles to look denser. (B) Thicker roof plate is found in the treated embryo along with thicker otic vesicles (A) and a separated basal lamina (C).
Figure 22: TRPM3 Neural Tube Stains.

(A-C) TRPM3 inhibitor treated embryo (D-F) Control embryo. (B, E) DAPI, (C, F) Laminin. (A-C) Clear open neural tube made clearer by no basal lamina on caudal half of neural tube (C).
TRPM8

TRPM8, like TRPM3, is a member of the Melastatin family. It is a calcium permeable cold sensor that, like most TRP channels, can be found in a variety of tissues performing unique functions to its’ environment. It is most commonly found in the peripheral nervous system sensing cold. Unlike TRPM3 a lot of evidence has pointed towards TRPM8 playing a role in epithelial remodeling events that happen during cancer progression. One potential player is its regulation by G protein coupled receptors, which makes it akin to the evidence found that TRPC6 plays a role in epithelial remodeling regulated by receptor tyrosine kinases. This background, along with the availability of M8-B, its small molecule inhibitor, made TRPM8 a target in this research.

When treated with M8-B embryos developed interesting defects that could not all be observed in whole mount images. The whole mount analysis is summarized in (Figure 23) and representative embryos were imaged and compared to controls in (Figure 24). It should be noted that heart defects were determined by external appearance only.

Sectioning reinforced the observations made in whole mount and provided more detail as to the nature of the phenotypes. The neural tube near the otic vesicle was notably wide and open, except for a thin roof plate (Figure 25). This could suggest delayed hinge formation of the neural tube or dysregulated epithelialization. To further the apparent disruption of neural tube/otic vesicle formation, a large epithelial mass was found partially connected to the neural tube (Figure 26). This mass developed its own lumen and potentially merged into the otic vesicle, but started with no basal lamina separating it from the neural tube and encroached on its organization when forming its own lamina. Cells in this epithelial ‘cyst’ did not express HNK1 in the same manner observed in otic vesicles suggesting a disruption in epithelial remodeling. The other
defects observed in sections confirmed what was observed in the whole mount embryos; reversed orientation of the heart (Figure 27) and a caudal open neural tube (Figure 28).

Figure 23: TRPM8 Defect Percentages

Rates of observed defects in whole mount embryos treated with the small molecule TRPM8 inhibitor M8-B. Significant observations were the rates of Heart formation and potential neural tube malformations.
Figure 24: TRPM8 Whole Mount Images.

(A-C) Representative treatment embryos. (D-F) Control embryo. Poor head turning and heart development (A). Otic vesicles perturbed along with heart orientation (B). Open neural tube on caudal end (C). Line in Image A represents (Figure 25). Left line in image B represents (Figure 26), right line represents (Figure 27). Line in image C represents (Figure 28).
Figure 25: TRPM8 Otic Vesicle and Rostral Neural Tube Stains.

(A-C) Treated embryo. (D-F) Control embryo. (B, E) DAPI. (C, F) Green Laminin, Magenta HNK1. Unopen roof plate, but very wide neural tube (A). Potential beginnings of an Otic vesicle (B). No neural crest cells in left side lumen suggesting that there is no otic vesicle on that side at this point and that all the epithelial cells are neural tube cells (C).
Figure 26: TRPM8 Otic Vesicle Stains.

(A-D) Treated embryo. (E-G) Control embryo. (A-D, G) Green Laminin, Magenta HNK1. (F) DAPI. (A, B) One section. (C, D) later section. An unusual epithelial mass was observed (A). No basal lamina suggests it’s part of the neural tube (B). New lumen forms (C). Some basal lamina forms with new lumen (D).
Figure 27: TRPM8 Heart Stains.
Figure 28: TRPM8 Neural Tube Stains.

PKD2L1

PKD2L1 is in the Polycystin family of TRP channels. Knowledge of its functions and roles is limited and recent. It can form as a hetero-tetramer with PKD1 or a homo-tetramer sensitive to pH, mechanical stress, and voltage. It shares 60% homology with PKD2 and 79% identity with human PKD2L1 (Table 6). Because of its similarity to PKD2 it is thought that it might be involved in similar processes. So far, the evidence points to it having distinct roles from that of PKD2. For example, while PKD2 functions by regulating calcium flux from the endoplasmic reticulum in cardiomyocytes PKD2L1 regulates the calcium flux in mitochondria with inhibition of both leading to similar negative impacts (Celic et al., 2012; Lu et al., 2018).

Originally, the plan was to inhibit PKD2 in the developing chick embryos because of its role in polycystic kidney disease and because we had some data about its mRNA expression patterns from in situ hybridization. Unfortunately, there are no small molecule inhibitors for PKD2, and since ADPKD (autosomal dominant polycystic kidney disease) is caused by a loss of function mutation there isn’t a lot of work being done in finding one. Because of this, Phenamil, a PKD2L1 inhibitor was chosen for use in the drug treatment study. An important note, and area that requires further research, is that Phenamil also inhibits epithelial sodium channels. This means that any phenotype seen could be caused by inhibition of either channel exclusively or both together. There were plans to clarify the cause of the defects observed but time did not permit it. Further thoughts will be explained in the Discussion section of this Thesis.

Unique to treatment with other TRP inhibitors, PKD2L1 inhibition by Phenamil prevented proper neural tube closure in over 60% of the treated embryos. It also resulted in significant variation in heart size and otic vesicle development (Figure 29).
Representative images of the treated embryos are found in (Figure 30). The open neural tube, like in other treatment groups, was found in the caudal end of the embryos treated. The embryos that had a reversed orientation also seemed to have hindbrain defects with a thicker neural tube upon inspection of stained sections (Figure 31). The stained images of the open neural tube showed a lack of, or reversed, hinge point, resulting in a flayed open neural tube phenotype (Figure 32). These phenotypes suggest that PKD2L1 plays a role in epithelial remodeling events during development.
Figure 30: PKD2L1 Whole Mount Images.

(A-C) Phenamil treated embryo. (Scale bar = 1mm) (D-F) Control embryo. Large heart (A) and reverse orientation (C). Hindbrain/Neural tube (C). Open caudal neural tube (B). Left bar in Image C represents (Figure 31), right bar represents (Figure 32).
Figure 31: PKD2L1 Otic Vesicle and Rostral Neural Tube Stains.

(A-C) Treated embryos. (D-F) Control embryos. (B, E) DAPI. (C, F) Laminin green, HNK1 magenta. Otic vesicles seem to be normally formed with normal HNK1 expression (A-C). But the roof plate of the neural tube is thicker than normal (B). The heart also has reversed orientation (A).
Figure 32: PKD2L1 Neural Tube Stains.
DISCUSSION

Transient Receptor Potential (TRP) Channels are a diverse and dynamic super family of cation specific ion channels. They are known to be players in disease states (Tai et al., 2017; Verma et al., 2010; Yue et al., 2015), sensation (Moran, Xu, & Clapham, 2004; Zhang, 2015a), calcium regulation (Anyatonwu et al., 2007; Owsianik et al., 2006; Zhu, 2005), cancer (Fels et al., 2018; Hantute-Ghesquier et al., 2018; Yee, 2015), and as shown previously in this lab, regulating epithelial mesenchymal transition (Langford et al., 2012).

It was determined that TRPC6 was necessary for epithelial remodeling from epithelial to mesenchymal cells by Langford et al. (2012), leading the lab to develop further interest in the role of TRP channels on tissue remodeling events. While the earlier study was performed in cell lines and designed to explain the mechanism of involvement of TRPC6 in the downstream pathway of receptor tyrosine kinase activation, the onus of this study was to investigate the role of TRP channels in vivo. The hypothesis was that TRP channel inhibition would result in disruption to normal developmental epithelial remodeling events.

While there are known mechanisms regarding the regulation of epithelial morphogenesis events ranging from proliferation, survival, apoptosis, to migration (Vrenken et al., 2016), understanding of the specific effect of TRP channels in a developing embryo is limited. A large purpose of this study was to provide the scientific community with more understanding of the role of TRP channels in tissue rearrangement events in a developmental model to enable more in-depth studies of the roles of specific channels in specific cellular processes. With the new information gathered on the expression patterns and results of pharmacological inhibition of a few TRP channels, further studies could be done using the chick developmental model to clarify the function of TRP channels not only in development but in epithelial remodeling events that
can be observed in disease states such as skeletal dysplasia’s, autosomal dominant polycystic kidney disease, and cancer.

One of the most apparent phenotypes observed upon the inhibition of TRP channels with small molecule inhibitors was an open neural tube in the caudal end of the embryo. This is interesting for a number of reasons. First, it is clear that ion regulation by TRP channels must be important to properly remodel the epithelialized cells in the neural folds to close. There has been recent work done showing the importance of calcium homeostasis on neural tube closure that gives support to the evidence provided in this study that calcium regulation is required for proper neural tube closure (Abdul-Wajid, Morales-Diaz, Khairallah, & Smith, 2015; Zhao, Cao, Hernandez-Ochoa, Schneider, & Reece, 2019). Second, there were similar phenotypes of an open neural tube in the caudal portion of the embryo in over 30% of TRPM8, TRPC6, and PKD2L1 and over 50% in TRPC6 and PKD2L1 treatment groups. This suggests that these TRP channels play similar roles in the developing neural tube that must help regulate a process in apical constriction for formation of the lateral hinge points. Evidence has been found pointing to the role of primary cilium and G protein coupled receptors (GPCRs) in regulating apical constriction (Shimada & Mukhopadhyay, 2017).

Another group of phenotypes frequently observed in the treated embryos were heart related malformation. This was manifest in general heart size, heart shape, and reversed orientation. To be clear, this study was done to identify broad and easily distinguishable defects in development caused by inhibition of TRP channel function. Because of this, a thorough investigation and explanation of the effects of TRP channel inhibition on heart development could not be completed. The clarification of the role of TRP channels in heart development is an interesting topic and merits further investigation where the focus can be given solely to heart morphology and developmental processes. Of the three observed phenotypes the reversed
orientation is the most clear and obvious defect. Normally chick hearts are a single tube in the ventral midline, then as they loop they turn to the right (Manner, 2004), but in the treatment groups over 30% of TRPV4, PKD2L1, TRPM3, and TRPM8 and 50% of TRPM3 embryos developed their heart loop on the left side of the embryo. It would seem that the inhibition of TRP channels has an effect on left-right asymmetry. But, the evidence of the roles of TRP channels in left-right asymmetry are focused on PKD2 and its function on primary cilia in Henson’s node determining fluid flow resulting in establishment of left-right asymmetry (Yoshiba et al., 2012). It has also been shown that in chicks, left-right asymmetry is originally molecularly determined by Hamburger Hamilton stage 4-6 (Monsoro-Burq & Levin, 2018). These are both events that happen well before the stage that these embryos were treated with TRP channel inhibitors at HH stages 9-11. This means that TRP channels do not play a role in determining the molecular pathway that defines asymmetry, but they might be involved in regulation of the final or functional step of heart symmetry that is regulated by right handed pathways such as pitx2 at HH stages 8 and 9 (Monsoro-Burq & Levin, 2018; Ocana et al., 2017). This could mean that some of the embryos treated were in the process of undergoing left-right heart asymmetry when they were treated with the TRP channel inhibitors while others had already undergone some of the morphological changes necessary before the treatment. Another explanation could be that since there is no default in chirality of the developing embryo, and inhibition of the process that defines asymmetry was performed, the expectation would be to observe 50% of the embryos facing each direction. This means that a 50% defect rate is seen as a total block or complete inhibition of the normal pathway. Not a lot is known about the roles of TRP channels in signaling cascades that determine developmental processes, but there is evidence here that more can be discovered with a closer look at TRP channel function in the regulation of heart development.
The other two clear defects observed in the embryos treated with small molecule TRP channel inhibitors were large or misshapen otic vesicles, and a thick roof plate in the hindbrain. While *in situ* staining showed expression patterns of TRPC6 and PKD2 in the otic vesicles and hindbrain, there is not a lot of information on the potential roles they could be playing in these developmental locations. The thick roof plate could be related to similar functions that result in an open neural tube in the caudal end of the embryo, but much more work would need to be done to clarify the reasons why. While it is known that TRP channels enable hearing in drosophila, mammals don’t seem to require them for hearing, but in other inner ear structures such as epithelial cilia and hair cells (Zanini & Gopfert, 2014). That being said, there hasn’t been clarification on the function of TRP channels on hearing in the chick developmental model. It would be interesting to investigate the effect of these phenotypes at a later stage of development to determine if they play a role in auditory transduction.

While there was some variety in the phenotypes observed by the TRP channel inhibited, there seemed to be frequently shared defects between channels that are known for their diverse function. This could be due to a couple of things. The first is that these embryos were cultured in a cell culture incubator that included 5% carbon dioxide. At this early stage of development this level of carbon dioxide is known to decrease survival rates while incubated in the egg. The normal carbon dioxide level under a hen is about 1% and evidence has been given that suggests that about 1.5% carbon dioxide helps growth without any deleterious effects. During later stages of development 5%, while not beneficial, doesn’t seem to harm the developing embryo (Onagbesan et al., 2007). This could suggest that some, if not all, of the phenotypes observed are a result of a hypercapnic environment. If the developmental defects were caused by the level of carbon dioxide present it would follow that the control embryos would have similar phenotypes. Since the control groups had the same phenotypes but to a lesser degree, another hypothesis
could be that the proper function of TRP channels helps alleviate the burden of the hypercapnic environment and that the inhibition of TRP channels exacerbates the mutagenic effects of the environment. While this discovery is not helpful to the methods employed in this study, it does give interesting insight into the potential function of these TRP channels functioning at the interface between organism and environment. It is known that some TRP channels function as sensors for oxygen, nitrogen, and carbonyl levels in both the normal and reactive levels (S. Shimizu, Takahashi, & Mori, 2014). It’s also interesting to note that TRPC6 is known to regulate response to hypoxic conditions (Liao et al., 2012), and TRPA1 is a sensor for carbon dioxide levels (Y. Y. Wang, Chang, & Liman, 2010). If the effects of TRP channel inhibition observed are really the effects of carbon dioxide levels not being regulated by TRP channels, then PKD2L1, TRPM3, TRPV4, and TRPM8 could be added to the list of TRP channels that regulate cellular response to stressful levels of carbon dioxide.

Second, the similar phenotypes between distinct TRP channels could also mean that the dynamic specificity of TRP channel function is developed over time as tissues and environments become more specialized, and that during early development there are more simple and related functions between TRP channels. This harkens back to the many varied roles of a single TRP channel found in different tissues such as TRPM3 regulating ion homeostasis in the kidney and response to noxious temperatures in the peripheral nervous system, or PKD2L1 sensing sour in the tongue to regulating calcium levels and enabling mitochondrial function in the heart. If there are less diverse tissue types to be expressed in and environments to respond to, but similar expression of the channels, there could be more uniform responses to stimuli.

The results of this study show that TRPC6, PKD2L1, and TRPM8 inhibition, followed closely by TRPM3, resulted in the greatest number, and most interesting, developmental defects. This project was meant to be foundational work for more in-depth studies to be built upon. For
future studies, the caudal neural tube defects caused by inhibition of PKD2L1 and TRPC6 should be investigated. As mentioned earlier, there are many roles TRP channels could play in the process of neural tube closure and a closer look at markers for apical constriction and convergent extension could give answers to the broad roles TRP channels fill in this epithelial remodeling event. The left-handed orientation of the hearts treated with TRPM3 could be investigated with calcium imaging techniques in vivo. An asymmetric pattern of calcium flux observed in wild type embryo heart looping with a perturbed calcium flux in TRPM3 inhibited embryos would suggest a necessary role of calcium and TRPM3 in proper lateralization of the heart. Finally, the effects of TRPM8 inhibition on the hindbrain and heart development could be studied in various ways. It would be interesting to determine if TRPM8 inhibition results in dysregulated neuronal cell differentiation which effects neural tube/hindbrain development. As stated earlier, a more thorough investigation with longer incubation periods could be done to clarify the effects of TRPM8 inhibition on the hearts of these embryos.

TRP channels are a diverse and dynamic superfamily of cationic specific channels that play many roles in normal and diseased physiological states. This study has provided more information regarding the roles of a few of these channels on epithelial remodeling events in the developing chick embryo, and a foundation for future investigation into these interesting cellular and organismal regulators using the chick developmental model organism.
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