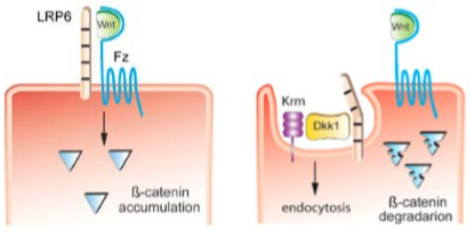
**College of Arts and Sciences**

# Grants for Undergraduate Scholars

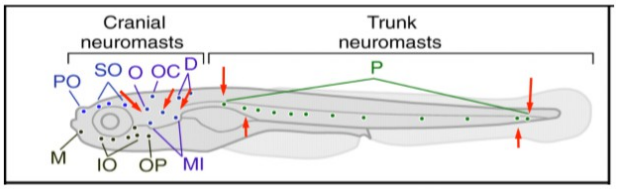
**2013 – 2014**

## Background

The Wnt signaling pathway is responsible for many different developmental processes such as cell proliferation, cell-fate determination, and tissue pattering (Nakamure et al., 2008). Prior studies show that Wnt signaling regulates both development and regeneration of sensory hair cells in the inner ear and lateral line (Wada et al., 2013). Hair cells are sensory receptors found in the inner ear of mammals that are fundamental to our ability to hear and process sound. Hair cells detect the movement of the inner ear fluid, translating mechanical stimulus into neuronal impulse that that are perceived as hearing (Magariños et al., 2012). In mammals, hair cells are found only in the inner ear, which is a hard to reach location inside the cranium, making observation difficult without dissection. In zebrafish, hair cells are found in the lateral line, which is positioned on the anterior and posterior surface of the fish (Aman and Piotrowski, 2011). The development of the lateral line occurs within a few days after fertilization. The speedy development combined with the hair cells' accessible location provides an apt opportunity as the lateral line of the zebrafish can be manipulated readily observed without dissection. The hair cells in the lateral line of zebra fish are clustered into grape like structures called neuromasts. Neuromasts are stereotypic, found in the same position in the lateral line, with the same number of hair cell in a cluster time and time again (Ma et al., 2008). The neuromasts are positioned to respond to change in water movement, which allows the fish to respond to important environmental cues (Aman and Piotrowski, 2011).




Wnt signaling pathway and the role of Krm1 (Niehrs 2006).




Schematic of a larval zebrafish, with red arrows

Here we examine the role of kremen-1 (*Krm1*), a key negative regulator of Wnt signaling, in developmental patterning in the zebrafish lateral line as part of a larger study aimed at understanding Wnt signaling in mechanosensory system development. It is thought that by changing the expression of *Krm1* in the wnt signal pathway, the number of hair cells found in the lateral line can be altered. We predict that underexpression of *Krm1* in early development will activate the wnt signaling pathway, stimulating cell division, and resulting in an increase in the number of hair cells found in the lateral line. An overexpression of *Krm1* in early development is predicted to deactivate the wnt signaling pathway, halting cell division, and resulting in a decrease in the number of hair cells found in the lateral line.

## Methods

For over-expression studies, *Krm1* mRNA was synthesized using the mMessage Machine sp6 kit and purified with Ambion's Megaclear kit. 0-500pg of *Krm1* mRNA (with 2% phenol red) was microinjected into zebrafish embryos at the 1-2 cell stage. For under-

expression studies, *Krm1* morpholino was purchased from GeneTools. 0-500pg of *Krm1* morpholino (with 2% phenol red) was microinjected into zebrafish embryos at the 1-2 cell stage. The transgenic line Brn3c:mGFP was used for all injections, as these fish express membrane-bound GFP in all hair cells, allowing the hair cells to be visualized under a microscope. The developing zebrafish were sacrificed at 3, 4 and 5 days post fertilization (dpf) and hair cell numbers in the lateral line sensory organs (neuromasts) were quantified in three head neuromasts (O1, O2, MI2) and four trunk neuromasts (P1, P2, P11, P12), as shown in Figure 2.

**Results**

*Krm1* over-expression

Figure 3 shows the number of hair cell quantified in each neuromast after injection of *Krm1* mRNA. Four different concentrations were used: 0pg, 100pg, 350pg and 500pg and fish were sacrificed at 5 day post fertilization (dpf). The results show an overall decrease in hair cell numbers found in each neuromast of the zebrafish lateral line with the greatest decrease found at 350pg *Krm1* mRNA and in neuromasts found in the head, O1, O2, and MI2.

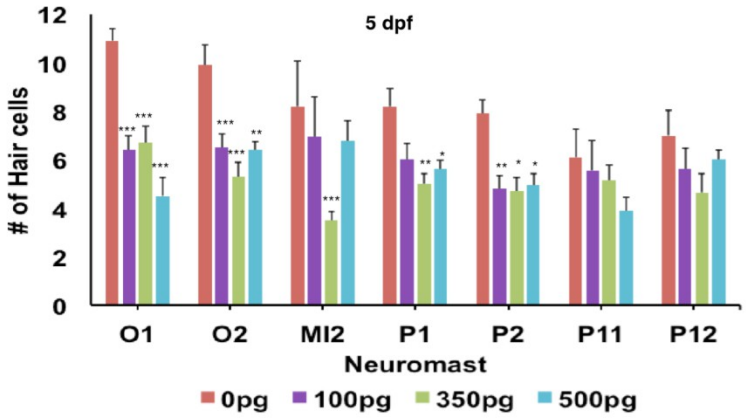



Figure 3. Over-expression of *Krm1* mRNA during early development significantly decrease the number of hair cells per neuromast in the lateral line of larval zebrafish (2-way ANOVA, $p<0.001$). The effect was apparent at all RNA concentrations. Data are presented as mean + 1 s.e.m., n= 9-20 animals per treatment. Controls were injected with 2% phenol red only and were not significant different from uninjected controls ($p=0.7$, data not shown). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 4 show the number of hair cell quantified in each neuromast after injection of *Krm1* mRNA at 350pg at 3 dpf and 4 dpf. Results show that that a 3 and 4 day there is a significant decrease in hair cell number found in each neuromast in the zebrafish lateral line with the greatest decrease found in the head, O1, O2, and MI2, consistent with hair cell counts performed at 5 dpf.

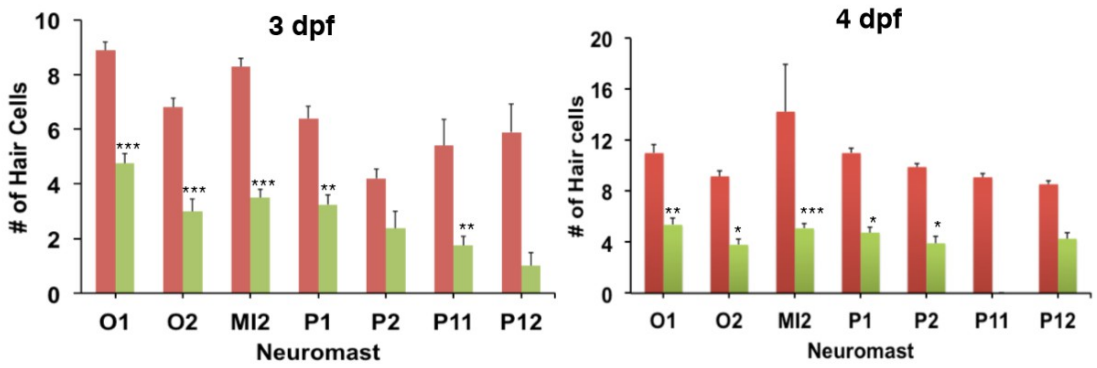
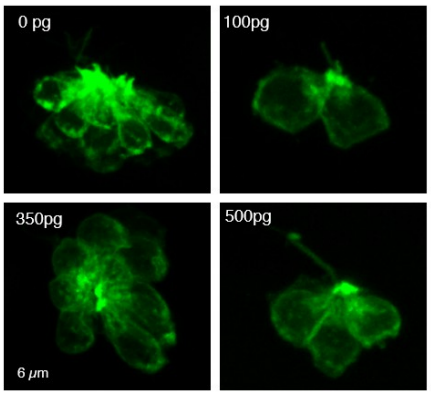
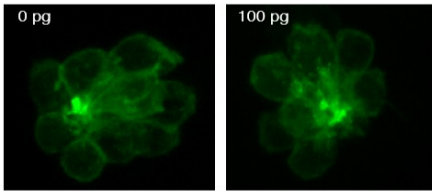



Figure 4. *Krm1* mRNA injection significantly decreased the number of hair cells per neuromast in 3 (left) and 4 (right) dpf larvae ($p<0.001$). Green is 350 pg of *Krm1* mRNA, red is phenol red only. There was a significant effect on both the head and trunk of the animal. Data are presented as mean + 1 s.e.m, n= 9-11 animals per treatment.

Figure 5 shows representative confocal microscopy images of head neuromasts found in the lateral line of the zerbafish that over-expressed *Krm1*. Similar images of tail neuromasts are shown in Figure 6.




Underexpression of Krm 1




Injection of *Krm1* morpholino during hair cell development decreased hair cell number in the larval zebrafish lateral line (data not shown). In contrast, Wada at el. 2013 knocked down dickkofph1b (ddk1b), a

negative regulator of the wnt pathway and saw a significant increase in size. We also injected control sequence morpholino, noticing general toxicity effects that left hair cell number reduced. Due to these control morpholino results we are re-examining Krm 1 morpholino injections using a different morpholino sequence and lower concentration.

**Summary and Future Directions**

Overexpression of *Krm1* during early development results in a significant decrease in the number of hair cell found in head neuromasts, but not neuromasts at the tip of the tail. This is likely due to developmental differences in neuromast formation in different regions of the fish (Nechiporuk and Raible, 2008) These results are consistent with prior research showing that inhibiting Wnt signaling reduces precursor proliferation and hair cell addition in the developing lateral line (Head et al., 2013, Jacques et al., 2014). Our research further defines the mechanism of Wnt inhibition in lateral line development.

Future experiments will re-examine *Krm1* under-expression during early development using a different morpholino. We expect to find an increase in hair cell number per neuromast in these animals.

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